# Dual biopolymer production and separation from cultures of Bacillus spp.

**Sukan, A.**

# Dual Biopolymer Production and Separation from Cultures of *Bacillus* spp.

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A thesis submitted in partial fulfilment of the requirements of the University of Westminster for the degree of Doctor of Philosophy

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## ABSTRACT

In the search of alternative new materials for biodegradable plastics, biopolymers provide attractive solutions with their vast range of applications. A challenge in industrial production of biopolymers is their high cost, and one approach to minimise the cost is expanding the number of valuable products obtained from a single batch.

The aim of this thesis was the dual production of biopolymers, P(3HB) and γ-PGA from cheap substrates with the view to lay grounds for a feasible, innovative, low cost production process. A common denominator between the two biopolymers focused in this thesis, (P(3HB) and γ-PGA), was that they both could be produced by *Bacillus* sp. One out of five strains screened, *Bacillus subtilis* OK2, was selected and the structures of both biopolymers produced were confirmed.

Subsequently, optimisation of the production medium *via* statistical optimisation tools, and scaling-up of the process from shaken flasks to fermenters were carried out. Statistical design tool Placket Burman (PB), (Design Expert 6.0), was used to determine the effect of medium components on γ-PGA and P(3HB) production and to identify the crucial medium components in production media. The outcome of PB analysis of dual polymer production did not match the PB analysis of single polymer production. Considering the complexity of the dual polymer production mechanism, central composite design was applied after the number of parameters was reduced from five to three. A medium composed of 20 g/L glucose, 1.5 g/L yeast extract, 2.4 g/L citric acid, 32 g/L glutamic acid and 12 g/L ammonium sulphate was identified as the dual polymer production medium. Using an inoculum medium different from the production medium proved to have a positive effect on the production. Consequently, 1 g/L P(3HB) and 0.4 g/L γ-PGA in shaken flasks and 0.6 g/L P(3HB) and 0.2 g/L γ-PGA in single batch fermenters were produced with the strain *Bacillus subtilis* OK2.

Selection of biowaste for the dual production was conducted using four biowastes; rapeseed cake, wheat bran, *Spirulina* powder and orange peel; using four pre-treatment methods, acid treatment, alkaline treatment, water infusion, and microwave exposure. γ-PGA production could not be detected when any of the waste materials was used as a sole medium component. Orange peel using water infusion pre-treatment was found to be the most suitable biowaste for the production of P(3HB). Bioreactor experiments showed that 1.24 g/L P(3HB) could be produced using orange peel as carbon source supplemented with yeast extract and citric acid. Dual polymer production using orange peel as carbon source proved to be more challenging as some of the ingredients in orange peel interfered with the dual production and inhibited production of both polymers. Although the different sugars in orange peel had a positive effect on production, pH control coupled with DOT control proved to be essential to overcome inhibition and 0.2 g/L of each polymer were produced in 79 h.

For the separation of the two polymers from the culture broth, magnetic field, floatation, and sedimentation methods were investigated. Exposure to magnetic field was found to be inhibitory for P(3HB) production. The use of floatation and sedimentation for the online separation of cells with and without polymer to facilitate a recycle strategy exhibited negative results. This was found to be due to cells undergoing cell lyses at the early stages of the fermentation releasing P(3HB) granules into the fermentation medium. The size distribution of these granules was identified. The results elicit the possibility of using cell auto-lysis behaviour for the separation of the two polymers from the culture broth leading to a reduction of costs.

*“In the fields of observation, chance only favours the mind which is prepared.”*

*Louis Pasteur*
*December 7, 1854*

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## List of Abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| $A_p$ | projection area of the particle |
| APS | ammonium per sulphate |
| CA | cellulose acetate |
| CCD | central composite design |
| CD | drag coefficient. |
| cdw | cell dry weight |
| CI | confidence interval |
| cm | centimetre |
| CoA | coenzyme A |
| D | diameter |
| DAF | dissolved air floatation |
| DC | direct current |
| $d_L$ | liquid density |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DOT | dissolved oxygen tension |
| $d_P$ | particle density |
| DP | double/dual polymer |
| DSMR | dry shiitake mushroom residue |
| e-PL | poly-e-lysine |
| EPS | exopolysaccharide |
| FB | buoyancy force |
| FD | drag force |
| FDA | food and drug administration |
| FID | flame ionization detector |

| | |
|---|---|
| FTIR | Fourier transform infrared spectroscopy |
| g | gravitational acceleration/grams |
| G- | Gram-negative |
| G+ | Gram- positive |
| GC | gas chromatography |
| GD | glutamate dehydrogenase |
| GF | gravitational force |
| GOGAT | glutamate 2-oxoglutarate aminotransferase |
| GRAS | generally regarded as safe organism |
| GS | glutamine synthase |
| h | hour |
| H | height |
| HPLC | high performance liquid chromatography |
| kDa | kilo Dalton |
| L | litre |
| LPS | lipopolysaccharide |
| LPSs | lipopolysaccharides |
| LTA | lipoteichoic acid |
| m | metre |
| M | molar |
| Mcl- | medium chain length |
| MF | magnetic field |
| MFG | magnetic field generator |
| MGPR | monosodium glutamate production residues |
| min | minutes |
| ml | millilitre |
| mm | millimetre |
| mT | millitesla |

| | |
|---|---|
| MT | million tons |
| N | normal |
| NADH | nicotinamide adenine dinucleotide reduced form |
| NADPH | nicotinamide adenine dinucleotide phosphate reduced form |
| NB | nutrient broth |
| nm | nano meter |
| NMR | nuclear magnetic resonance |
| $N_{Re}$ | Reynolds number |
| OD | optical density |
| OMW | olive mill wastewater |
| OP | orange peel |
| oop | out of plane |
| P | pump/ product concentration g/L |
| P(3HB) | poly (3-hydroxybtyric acid) |
| PA | polyamide |
| PB | Placket-Burman |
| PBAT | polybutyrate adipate terephthalate |
| PBS | polybutylene succinate |
| PC | polycarbonate |
| PCL | polycaprolactone |
| PET | polyethylene terephthalate |
| PGA | polyglycolic acid |
| PHA | polyhydroxyalkanoate |
| PHAs | polyhydroxyalkanoates |
| PHBV | poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) |
| PHO | poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) |
| PHUE | poly(3-hydroxy-10-undecenoate-co-3-hydroxy-8-nonenoate-co-3-hydroxy-6- heptenoate) |
| PLA | polylactic acid |

| | |
|---|---|
| PTT | polytrimethylene terephthalate |
| PUR | polyurethane |
| Q | specific rate |
| $Q_P$ | specific polymer production rate |
| $Q_S$ | specific sugar consumption rate |
| RI | refractive index |
| rpm | revolutions per minute |
| RS | rapeseed cake |
| S | total carbohydrate (substrate) concentration (g/L); Spirulina |
| Scl- | short chain length |
| SD | standard deviation |
| SDS | sodium dodecyl sulphate |
| SE | standard error |
| SEM | scanning electron microscope |
| SF | shaken flask |
| SMF | static magnetic field |
| SSF | solid state fermentation |
| STR | stirred tank reactor |
| t | time |
| TCA | tri-carboxylic acid |
| TEMED | tetramethylethylenediamine |
| TLC | thin layer chromatography |
| TPE | thermoplastic elastomers |
| UV | ultra violet |
| v | flow rate |
| v/v | volume per volume |
| $V_p$ | volume of the particle |
| $V_S$ | settling velocity |

| | |
|---|---|
| vvm | volume air per volume culture per minute |
| w/v | weight per volume |
| WB | wheat bran |
| X | cell concentration (g/L) |
| Y | yield |
| YE | yeast extract |
| $Z_x$ | distance |
| γ-PGA | gamma-poly glutamic acid |
| μ | Viscosity; specific growth rate |
| μl | microliter |
| ρ | density of the liquid |
| $\rho_p$ | density of the particle |
| 3D | 3 dimensional |

# Chapter 1: Introduction

# 1. INTRODUCTION

## 1.1 Bioplastics an Outlook

Increasing global population and the related demands of the public could exceed the limits of natural resources in the next decades. The total global production of plastics grew from around 1.3 million tonnes (MT) in 1950 to 245 MT in 2006 (Panda *et al.*, 2010). This trend continued with 299 MT of plastics produced in 2013, representing a 4 % increase over 2012 (Gourmelon, 2015). An analysis of plastics consumption on a per capita basis shows that this has now grown to over 100 kg/y in North America and Western Europe. The highest consumption of plastics among different countries is found in USA which is equal to 27.3 MT per year. The world plastics consumption in 2000 was 170 MT (Panda *et al.*, 2010) and in 2015 it is estimated to be approximately 300 MT (Koller and Braunegg, 2015).

Mineral oil (crude oil) is a major unsustainable resource and a significant percentage of it is used to manufacture plastic products. In 2005, 18 million barrels of crude oil-equivalent were used to manufacture 2 million polyethylene terephthalate (PET) bottles. The annual plastics production is estimated to surpass 300 million tons by 2015 worldwide (Arikan and Ozsoy, 2015). Due to excessive fossil fuel consumption, the atmospheric concentrations of $CO_2$ and $CH_4$ have exceeded the natural levels (Eco-cycle reports, 2010). The rapid depletion of crude oil and the mounting adverse effects on the environment of the synthetic products accompanied with their increasing rates of consumption are the driving forces to investigate alternatives to the mineral-based products. The fast consumption of single use mineral plastics and their inefficient recycling are causing continuous accumulation of plastics in nature. As an example, in 2012, only 9 per cent of the 32 million tonnes of plastic waste generated in the US was recycled (Eriksen *et al.*, 2014). Figure 1.1 reveals the levels of plastic debris found in the oceans. The red areas marked in the Figure show regions thought to have 10 kg of plastic debris/ $km^2$.

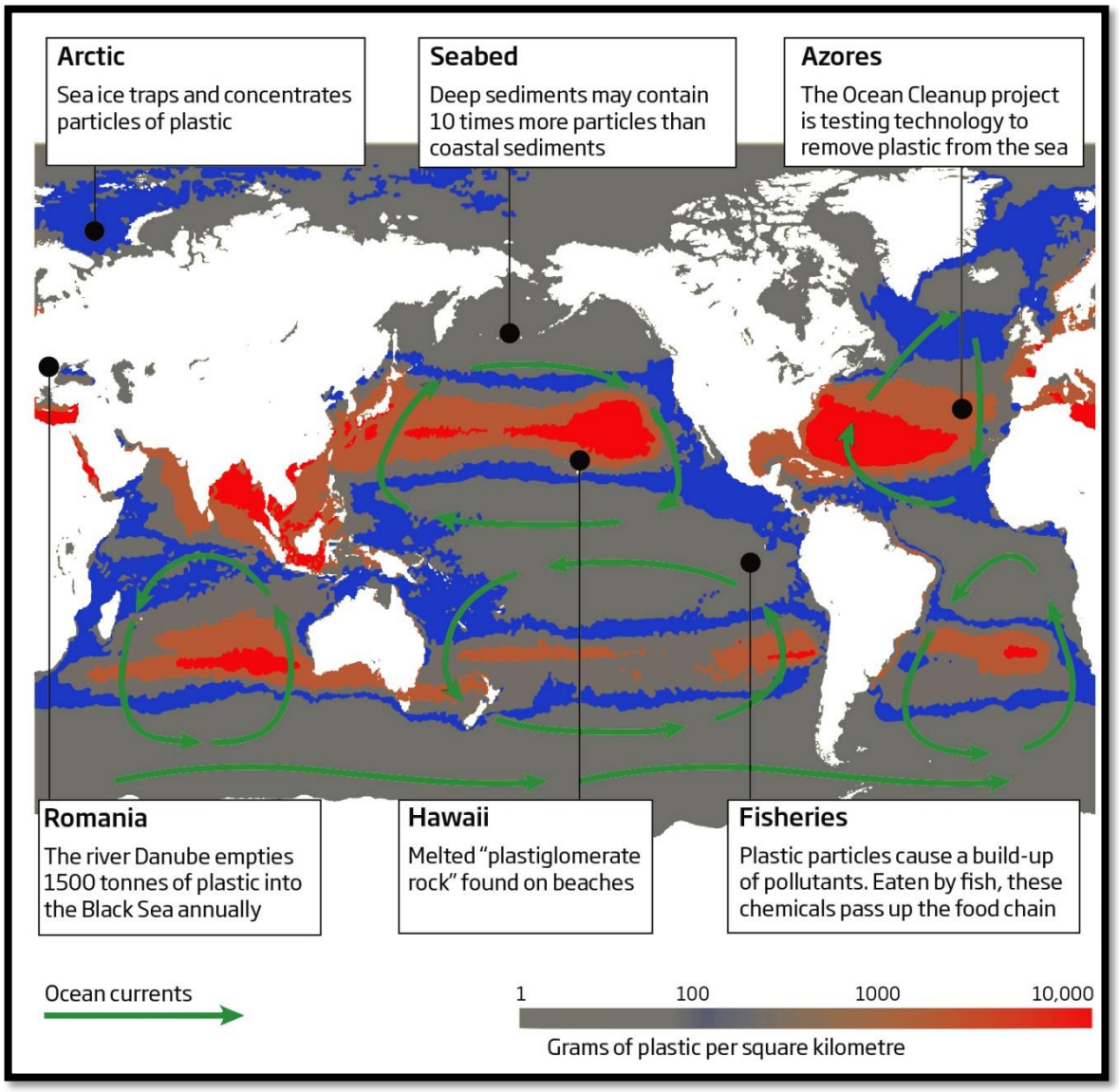



**Figure 1. 1:** Plastic debris found in the oceans (Eriksen *et al.*, 2014, Modified from New Scientist, Reed 2015).

Between the years 2010 and 2011, the total plastic production in Europe reached 58 million tonnes, up by almost 2%. Demand from the processing industry reached 47 million tonnes, increasing by 1.1% and collected post-consumer waste reached 25.1 million tonnes, increasing by 2.4% compared to 2010 values. Out of these, 10.3 million tonnes were disposed of and 14.9 million tonnes were collected for recycling. Although the ratio of recovering increased by 5.7% it is still very low (Plastics Europe, 2012).

Plastic pollution can seriously affect lands, waterways and oceans. Living organisms, particularly marine animals, can be affected through entanglement, direct ingestion of

plastic waste, or intoxication through exposure to chemical contents of plastics that could cause problems in biological functions. Humans can also be affected through the disruption of the thyroid hormone axis or fluctuation in sex hormone levels (Mathieu-Denoncourt *et al.,* 2014). Increasing danger of plastic accumulation in recent years highlights the importance of biodegradable plastics along with the production of polymers from alternative raw materials. Biopolymers are one of these alternatives for the manufacturing of biodegradable plastics, with their vast range of applications, biodegradability and eco-friendly manufacturing processes.

"Polymer" is a word derived from two Greek words, "poly" meaning many and "mer" meaning unit or part. It is a large molecule made of many repeated subunits. Biopolymers are polymers of biological origins. In our everyday life we encounter many biopolymers. As the most abundant energy source, carbohydrates are one of the examples. They are consisted of long repeated sugar units and based on their structure they are also called as polyhydroxy aldehydes. Hair is another example which is constructed from protein filaments. Muscle, cellulose and DNA are some other common examples of biopolymers in our everyday life. The term "biopolymer" is used in several different ways depending on the application. The generally accepted definition covers polymers that belong to the above mentioned categories; they are either renewable,-biodegradable or both. Biopolymers can also be produced by a range of microorganisms and plants. Natural rubber as an example to plant based biopolymers can be extracted or collected from the tree *Ficus elastica* using traditional techniques. Microbial biopolymers are produced either directly via fermentation or by chemical polymerisation of monomers, which are in turn produced through fermentation. Microorganisms producing biopolymers require specific nutrients and controlled environmental conditions (Marjadi and Dharaiya, 2011). It is believed that biopolymers of bacterial origin are produced either as a result of microbial defence mechanism or as storage material. In both cases, the produced biopolymers can be

decomposed by bacteria. These biopolymers have many desirable functionalities and properties, including biocompatibility. Thus they find applications in different sectors including the medical field (Philip *et al*., 2007). Many of the biopolymers are biodegradable but not all biodegradable biopolymers are necessarily produced from biomass. Some of the petroleum-based polymers are also certified as biodegradable so are classified as biopolymers (Matsuura *et al*., 2008).

**Table 1. 1:** Types of Bioplastics by production processes (Modified from Matsuura et al., 2008)

| Source | Process | Examples |
|---|---|---|
| Natural polymers from plants | Extraction/Polymerisation | Cellulose acetate (CA), Starch polymer, rubber |
| Bacteria Polyester | Fermentation | Polyhydroxyalkanoates (PHA) |
| Crude Oil | Chemical Polymerization | Polylacticacid (PLA)<br><br>Polybutylene succinate (PBS)*<br><br>Aliphatic polymers e.g. *<br>Polyglycolic acid (PGA)<br><br>Aliphatic-aromatic-copolymers e.g. *<br>Polycaprolactone (PCL) |
| **made from crude oil but biodegradable.* | | |

Global production of biobased plastics and their respective percentages can be seen in Figure 1.2.

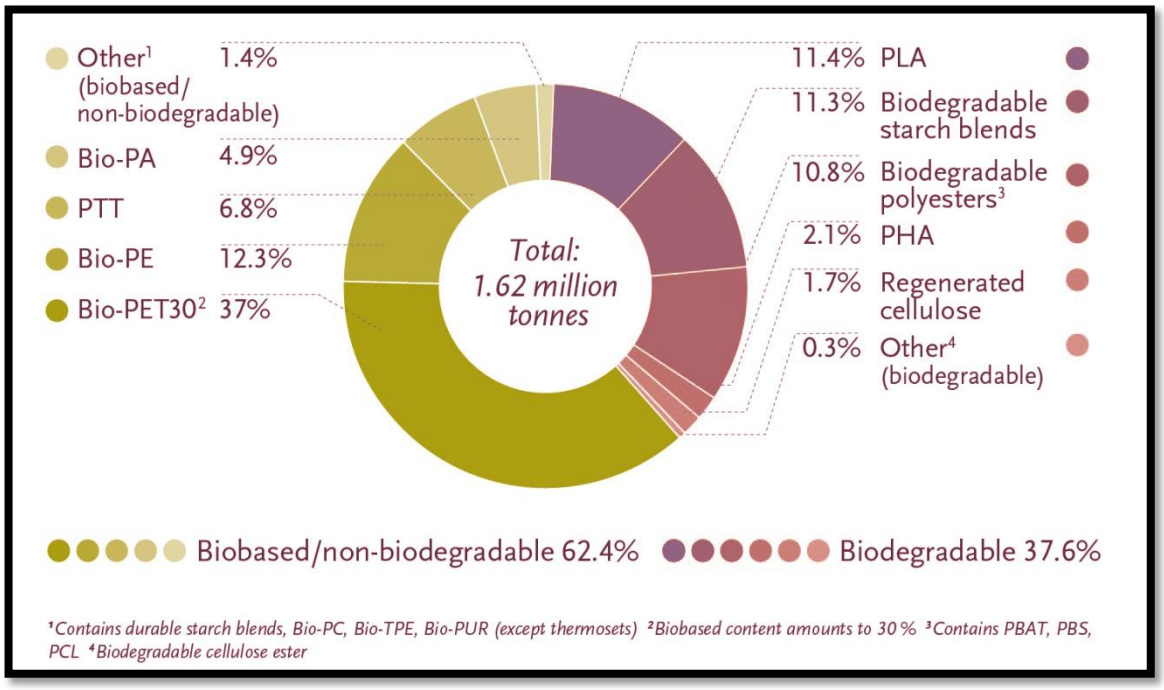



**Figure 1. 2:** Global production of bio-based plastics, PTT: polytrimethylene terephthalate, PA: polyamide, PE: polyethylene, PLA: polylactic acid, PC: polycarbonate, TPE: thermoplastic elastomers, PUR: polyurethane, PBAT: polybutyrate adipate terephthalate, PBS: Polybutylene succinate, PCL: Polycaprolactone, PET: polyethylene terephthalate (Modified from European Bioplastics, 2014).

Some biopolymers are already produced in larger scales by the industry, for example polylactic acid (~185,000 tonnes in 2014) and starch based polymers (~183,000 tonnes in 2014). However, many other classes of biopolymers are still to be produced at commercial scale. For example majority of polyhaydroxyalkanoates (PHAs), with great potential application in variety of bio-sectors with production levels of ~34,000 tonnes in 2014, are still far below the level of other biopolymers (European Bioplastics, 2014).

The limitations to viable commercial processes are mainly due to production costs, including expensive raw materials as carbon substrates, their relatively low conversion rates, methods of isolation for intracellular biopolymers, and use of co-carbon substrates (e.g. co-feeding of butyric acid and valeric acid) for copolymer production (Patel *et al.*, 2005; Keshavarz and Roy, 2010).

Therefore, producing a biopolymer from a renewable feedstock is not always the answer for cheap manufacturing. In a typical bioprocess more than 50% of the cost comes from

downstream processing. Along with a cheap raw material, environment friendly and energy efficient processes need to be developed to be able to compete with petroleum-based polymer industry. Since bioprocess technologies are aiming towards zero-waste policy within the bio-refinery concept, having a stable, optimised production system will provide a big advantage. Therefore research in this area is shaping to find efficient, integrated processes utilising every possible waste stream to form a bio-refinery and produce commodities as economically as possible.

### 1.2 Polyhydroxyalkanoates (PHAs)

Among biopolymers, PHAs have attracted much attention in recent years due to their varied mechanical properties, biocompatibility and biodegradability (Francis, 2011). A total of 4,613 publications and 6,844 patents have been published between the years 2000-2015 on PHAs alone (Research done on Web of Science and Pat-Base with the key words: Polyhydroxyalkanoate, Polyhydroxybutyrate, Fermentation, Metabolic Products). However their high cost of manufacturing is reflected to the product prices, and their low productivity has limited their use to very specialised application such as special medical appliances (hearth valves, stents, wound dressings, drug delivery etc.) (Nigmatullin *et al.*, 2015).

#### 1.2.1 PHAs Production, Types and Characteristics

Polyhydroxyalkanoates (PHAs) are water insoluble and multifunctional biopolymers which are produced by different gram-positive and gram-negative bacteria such as, *Bacillus* spp., *Pseudomonas* spp. (Sanchez *et al.*, 2011), *Azobacterium, Burkholderia* spp. (Di *et.al.*, 2013), *Alcaligenes eutrophus* (Gitel Zon *et al.*, 2001), *Ralstonia eutropha* (Yung *et.al.*, 2011) and there are many recombinant strains of undisclosed nature in Patents (Skraly *et al.*, 2003; Aquin and Vezina, 2000; Glumoff and Hiltunen, 2002 ), as well as *E. coli* (Choi *et al.*, 2005, Yaqiu *et al.*, 2013), *Bacillus* sp. (Qingsheng and Zhen, 2009; Kumari *et al.*, 2009; Huang and Liu, 2013), *Cupriavidus necator* (Sato and Nagaoka, 2008) .

PHAs are endo-cellular polymers and accumulate in the cytoplasm of the cell (Figure 1.3). Although the main producers of PHAs are bacteria, there are other examples. Some studies have shown that blue-green algae can be a good source of PHAs (Yoldaş *et al.*, 2003). There are several examples in literature where PHAs are produced (between 0.7-77 %PHA/cdw) by Cyanobacteria (Yoldaş *et al.*, 2003; Balaji *et al.*, 2013) and Spirulina (Shivastav *et al.*, 2010; Jau *et al.*, 2005). In addition to that, transgenic plants for production of variety of PHAs are another topic of investigation (Snell *et al.*, 2015; Douglas and Proirier, 1997, Nichols *et al.*, 2006; Brumley *et al.*, 2006) (between 0.1- 40 %PHA/dw). Yeast spp. are also reported to be able to accumulate PHAs (Şafak *et al.*, 2002). Apart from the biological material as sources for production, these biopolymers can be chemically synthesised by using ring opening polymerisation of β–lactones however the cost associated with it is high (Jacquel *et al.*, 2008).

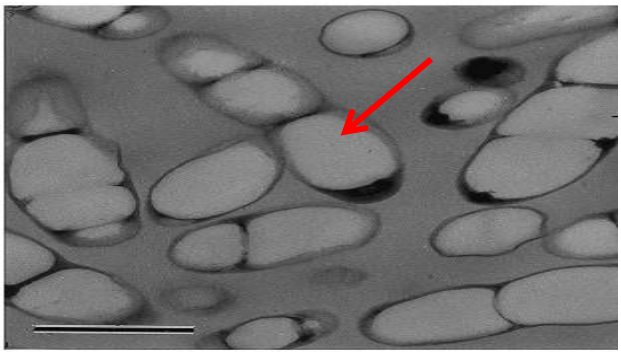


**Figure 1. 3:** SEM image of P(3HB) granules in bacteria, indicated with a red arrow (Sudesh *et al.*, 2000)

The structure of PHAs produced by bacteria can be manipulated by genetic or physiological strategies (Steinbuchel, 1991). Their molecular weight varies between 200-3000 kDa. This is entirely dependent on the polymer producing organism and the conditions it is being cultivated in (Byrom 1994; Lee 1995). The general structure of PHAs is presented in Figure 1.4 and functional groups presented as R can vary from $C_1$ (methyl) to $C_{13}$ (tridecyl) thus the length of these functional groups have a significant effect on physical and chemical properties of the polymer. PHAs are also identified as homo-

polymers and hetero-polymers depending on whether one or more than one type of hydroxyalkanoate is found in the monomer unit.

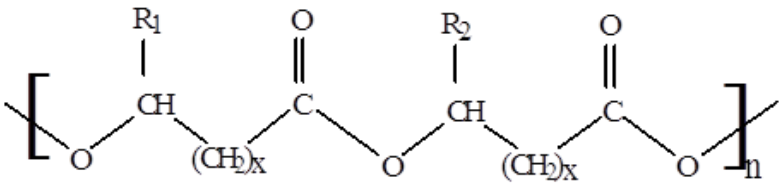


**Figure 1. 4:** The general structure of Polyhydroxyalkanoates (n=100-30000, x=1-4, R: functional group) (Francis 2011)

There are two main groups of PHAs, namely, short chain length (scl)-PHAs and medium chain length (mcl)-PHAs. Scl-PHAs consist of 3-5 carbon atoms and mcl-PHAs contain 6-13 carbon atoms. Several bacteria (e.g. *E. coli, Bacillus sp., Cupriavidus necator, Alcaligenes eutrophus etc.*) have been reported to be capable of producing both scl- and mcl-PHAs (Philip *et al.*, 2007). Today, more than 100 different monomers have been incorporated into mcl-PHAs (Hartmann *et al.*, 2005). There are also a number of patents disclosing different methods of tailoring the PHA polymer (Levin *et al.*, 2013), controlling the sequence (Skraly and Martin, 2000), or the composition of the functional groups (R) (Zhang *et al.*, 2012, Ashby *et al.*, 2015; Xiaowu *et al.*, 2009, Tetsuya and Hiroka, 2008). Scl-PHAs are stiff and brittle whereas mcl-PHAs are more flexible (See crystallinity in Table 1.2). The ratio and the type of functional groups and monomers depend on the medium composition, mainly the carbon source (Francis, 2011). Some of the physical and chemical properties of scl- and mcl-PHAs are compared to poly(propylene) (PP) based on their similar properties and, are given in Table 1.2.

**Table 1. 2:** Physical and chemical properties of scl-PHAs and mcl-PHAs compared to PP. (adapted from Zinn and Hany, 2005).

| | Scl-PHA | Mcl-PHA | Poly(propylene) |
|---|---|---|---|
| ***Crystallinity (%)*** | 40-80 | 20-40 | 70 |
| ***Melting point (°C)*** | 80-180 | 30-80 | 176 |
| ***Density g/cm$^3$*** | 1.25 | 1.05 | 0.91 |
| ***UV resistance*** | Good | Good | Poor |
| ***Solvent resistance*** | Poor | Poor | Good |
| ***Biodegradability*** | Good | Good | None |
| ***Large scale production*** | Partially Available | Partially Available | Available |

Among these types of PHAs a scl-PHA, poly hydroxybutyric acid (P(3HB)), has attracted much attention by researches. It is isotactic polyester and is produced by a variety of bacteria (e.g. *E. coli, Bacilus sp.*, *Cupriavidus necator*). Like other PHAs, P(3HB) is biocompatible and biodegradable. There are reports showing that the polymer enhances bone growth and healing (Misra *et al.*,, 2007; Knowles *et al.*, 1991). Similar to other scl-PHAs, it has high crystallinity ranging from 60-80% and high rigidity therefore referred as semi-crystalline thermoplastic. The polymer can be found in amorphous state in bacteria as it is produced and crystallization occurs as soon as it is extracted from cells. The mechanical and chemical properties may be adjusted by blending with other biodegradable polymers (Misra *et al.*, 2007).

### 1.2.2 Biosynthesis and Metabolism of PHAs

There are four main pathways and five key enzymes involved in PHA production pathways. As shown in Figure 1.5 the starting point for all pathways is Acetyl-CoA which is converted from related carbon sources. Also, PHA production relies on three key metabolic pathways, namely; citric acid (TCA) cycle, fatty acid do novo synthesis pathway and fatty acid β-oxydation cycle. The P(3HB) production from sugars, which is the main

focus of this thesis, involves three enzymes, namely β-Ketothiolase (PhaA), NADPH dependent acetoacetyl-CoA reductase (PhaB) and PHA synthase (PhaC). This mechanism was first demonstrated in cell free extracts of *Bacillus megaterium* KM by Merrick and Doudoroff (1961). The first enzyme, PhaA promotes the condensation of two acetyl coenzyme A (acetyl-CoA) functional groups in a reversible manner into acetoacetyl CoA. The second enzyme, PhaB, reduces acetoacetyl-CoA to R-(-3)-hydroxybutyryl-CoA and last enzyme PhaC polymerases R-(-3)-hydroxybutyryl-CoA to make P(3HB) (Chen, 2010; Anderson and Dawes, 1990; Aldor and Keasling, 2003).

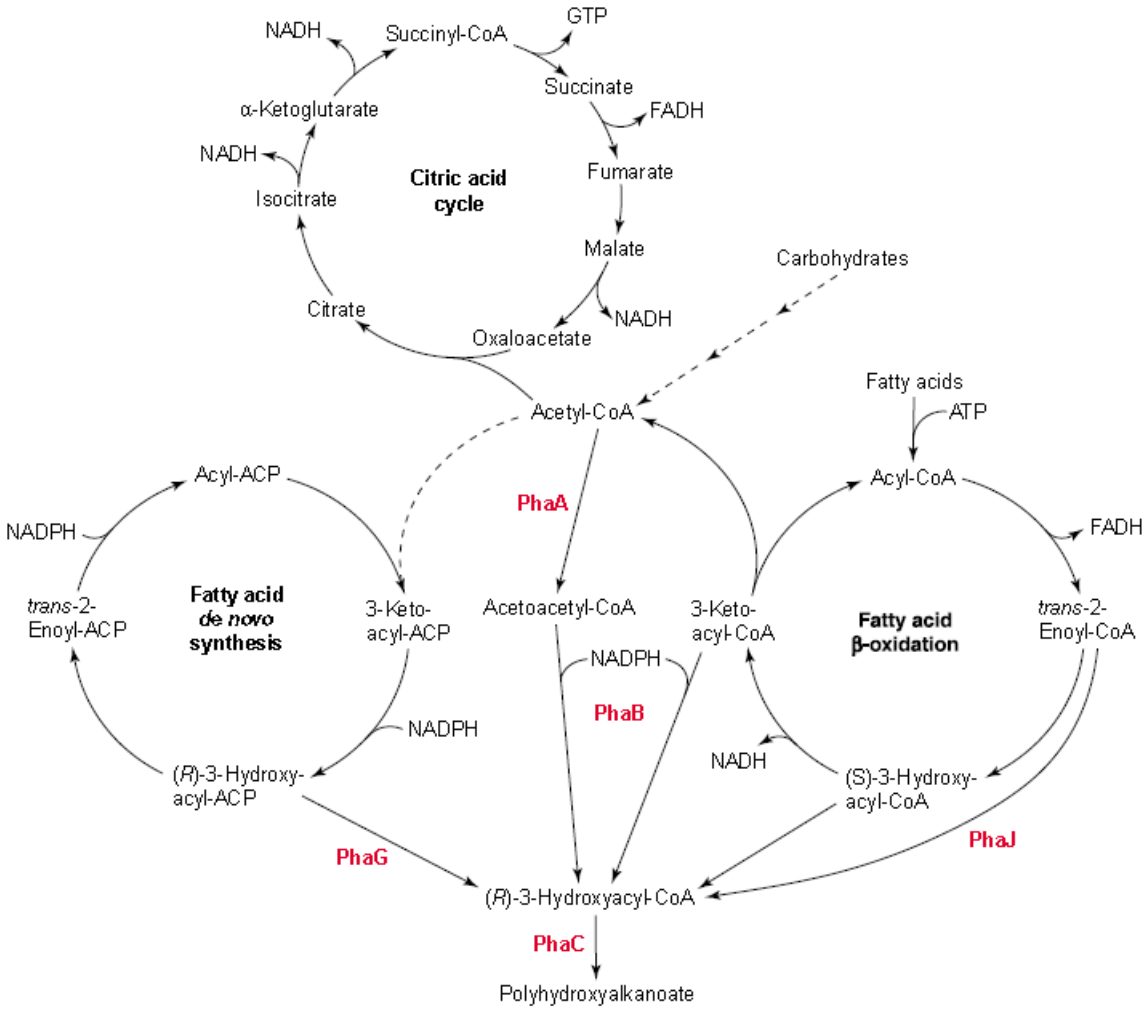



**Figure 1. 5:** Schematic representation of pathways and associated enzymes involved in PHA synthesis (Aldor and Keasling 2003).

Other pathways involving PhaG and PhaJ enzymes, which are 3-Hydroxyacyl-ACP-CoA transferase and (R)-Enoyl-CoA hydratase respectively, are related to other types of PHAs production and mainly are found in *Pseudomonas* sp. (Chen, 2010).

Availability of nutrients and their ratios are the major factors for P(3HB) metabolism. Under balanced growth conditions high levels of CoA is present causing consumption of Acetly-CoA through TCA cycle, inhibiting P(3HB) synthesis pathway. Under unbalanced conditions, such as limitation of a nutrient and high carbon presence, NADH starts building up. The first enzyme in TCA cycle, citrate synthase, has two natural inhibitors or regulators, NADH and citrate. Presence of high levels NADH or citrate inhibits citrate synthase which causes a reduction in the rate of oxidation of acetyl-CoA. In order to regulate excess acetyl-CoA in the cell, it is directed to P(3HB) pathway resulting an accumulation P(3HB). Oxygen is also reported as effecting P(3HB) levels. When oxygen levels are high, CoA concentration increases as acetyl-CoA is consumed through TCA cycle thus decreasing the flow of carbon source to PHA synthase pathway (Doi and Steinbüchel, 2001). The catabolism of P(3HB) is not initiated until all the excess carbon source is depleted (Figure 1.5) (Anderson and Dawes, 1990; Aldor and Keasling, 2003).

PHAs are accumulated within the cell in the form of granules (Rehm, 2003). During the studies on PHA granule formations, it was discovered that the granules are not randomly scattered in the cytoplasm. It was observed that each granule is formed close to the inner membrane and migrate to the centre of the cell or alternatively, emerge right at the centre and continue to grow at central location (Tian *et al.*, 2005) The two models proposed for the formation of PHA granules are schematically presented in Figure 1.6 a and b. It has been reported that these granules contain 97-98% P(3HB) of the dry weight and the remainder is lipids and proteins, 0.5% and 2% respectively.

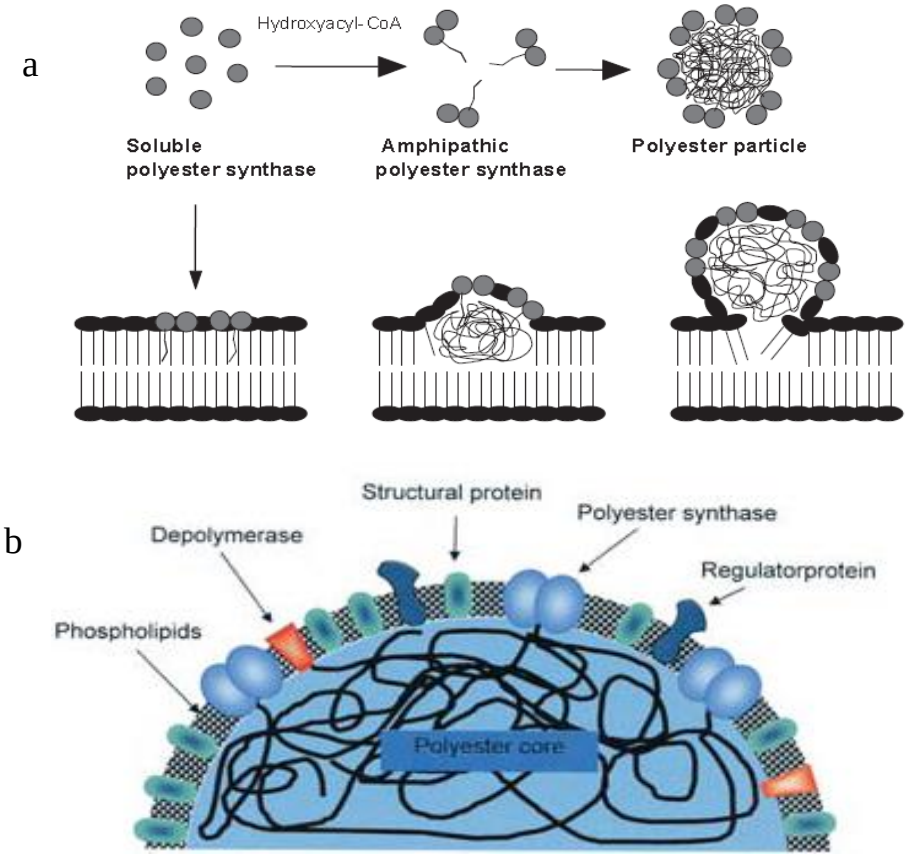



**Figure 1. 6:** Proposed models for PHA granule formation. (a) Formation of the granule from soluble polyester synthases and budding granule from polyester synthase particle embedded in the membrane (Rein, 2007) (b) PHA granule with associated membrane proteins. (Rehm, 2003)

The granules have been reported to have several proteins embedded in phospholipid membrane such as PHA synthase and PHA depolymerase (Doi and Steinbüchel, 2001). The structure of the granule is represented in Figure 1.6 b. It was reported that these granules are spherical in shape and their sizes vary from 200 to 700 nm. They have been observed in cultures of *R. rubrum, B. cereus* and *B. mageterium* (Doi and Steinbüchel, 2001). Status of the polymer in the native P(3HB) granules was first reported by Branard and Sanders (1989) by high resolution $^{13}$C-NMR spectroscopy. They reported that the granules keep the polymer in amorphous state, not crystalline. These authors also proposed that inactivation of the granules are related to their state. At crystalline state, depolymerase enzyme is unable to degrade the polymer.

### 1.2.3 Raw Materials for Production of PHAs

One of the most important factors affecting the overall economics of PHAs production is cost of raw materials. Glucose has been found to be the most efficient substrate for the production of the scl-PHAs however other substrates such as sucrose, glycerol and acetic acid have also been used to produce scl-PHAs. Some organisms can be grown on petroleum-derived carbon sources such as alkenes, alkanes and aldehydes which actually act as precursor substrates for the production of structurally related mcl-PHAs (Francis, 2011). As the process is scaled-up, the raw materials contribute to an increasing fraction of overall manufacturing costs, and these raw material costs are dominated by the carbon source (Castilho *et al.,* 2009). The economic feasibility of bulk PHA production is intrinsically coupled to developing efficient biotechnological processes from inexpensive carbon sources. Therefore, use of waste products as carbon sources presents the advantage of simultaneously enabling a decrease in disposal costs and the production of value-added products (Du *et al.,* 2012).

Many papers have been published so far on the production of PHAs from different raw materials. The suitable waste materials for PHA production can be grouped into six media categories. These are, sugar-based media, starch based media, cellulosic and hemi-cellulosic media, whey-based media and oil- and glycerol-based media. In addition there are studies reporting the use of gaseous carbon sources such as methane (Criddle *et al.,* 2012), volatile organic compounds (Herrema and Kimmel, 2007; Billington *et al.,* 2008) and urban aerobic active sludge (Zhenggui *et al.,* 2012)

The most common, inexpensive carbon source as an industrial/agricultural waste material is molasses, either from sugarcane or beet. As can be seen from Table 1.3, various strains have been evaluated for their capability to produce PHAs from beet molasses, sugar cane and date syrup. The highest production level reported from molasses is 23 g PHA/L culture using *Azotobacter vinelandii* (Page *et al.,* 1992), followed by 22 g/L using *Pseudomonas*

*fluorescens* (Jiang *et al.*, 2008). The research groups mentioned above, achieved these concentrations by submerged fermentation and the PHA reported is P(3HB). From the results reported in literature and shown in Table 1.3, it can be concluded that, within current technology it is not feasible to produce just PHAs from *Bacillus* sp. because of their low production levels (Omar *et al.*, 2001; Khiyami *et al.*, 2011; Yilmaz and Beyatli 2005).

One group of waste materials which can be easily utilized by various microbes is starch based waste material. The results of Haas *et al*. (2008) yielding 94 g/L PHA from *R. eutropha* using potato starch as a substrate as well as production level obtained by Koutinas *et al.* (2007), which was 51.1 g/L from *C. necator*, using wheat, shows the potential of starch based media and importance of utilizing it for high value added materials production.

Cellulose and hemi-cellulose-based waste materials have also been extensively studied. Within this group, bagasse is a waste material from sugar industry. In 2011, Brazil, as a top sugar producer, processed 625 million tons of sugarcane and generally, 280 kg of humid bagasse is generated from 1 ton of sugarcane (Chandel *et al.*, 2011). Silva *et al.*, (2004) studied the utilization of bagasse as a substrate for PHA production using *Burkhalderia sacchari*. They obtained around 2 g/L PHA using bagasse as a cheap waste material. The Figures mentioned above, represent the industrial potential of bagasse for biopolymer industry.

**Table 1. 3:** Types of raw materials used in the production of PHAs

| Type | *Medium* | *Organism* | *PHA concentration (g/L)* | *Gram stain* | *Scale* | *Reference* |
|---|---|---|---|---|---|---|
| **Sugar Based** | Beet molasses | *Bacillus cereus* | 0.16 | + | SF | Yilmaz and Beyatli (2005) |
| | | *Azotobacter vinelandii* | 23.00 | - | 2.5L | Page *et al.* (1992) |
| | | *Recombinant E.coli* | 9.00 | - | 5L | Liu *et al.* (1998) |
| | Sugar cane | *Pseudomonas fluorescens* | 22.00 | - | 5L | Jiang *et al.* (2008) |
| | Date syrup | *Bacillus SA* | 5.80 | + | 1L | Khiyami *et al.* (2011) |
| | | *Bacillus megaterium* | 1.50 | + | 30L | Omar *et al.* (2001) |
| **Starch based** | Soluble starch | *Bacillus cereus* | 0.48 | + | SF | Halami (2008) |
| | | *Azotobacter chroococcum* | 25.00 | - | 1L | Kim (2000) |
| | Potato starch | *Ralstonia eutropha* | 94.00 | - | 3.5L | Haas *et al.* (2008) |
| | Wheat | *C.necator* | 51.10 | - | SF | Koutinas *et al.* (2007) |
| **Cellulose and hemi-cellulose based** | Bagasse | *Burkhalderia sacchari* | 2.73 | - | 10L | Silva *et al.* (2004) |
| | | *Burkhalderia cerpacia* | 2.33 | - | 10L | Silva *et al.* (2004) |
| | | *C.necator* | 3.90 | - | SF | Yu and Stahl (2008) |
| | Wheat Bran | *Halomonas boliviensis* | 4.00 | - | 1.3L | Van-Thuoc *et al.* (2008) |
| | Soy bean | *Recombinant E.coli* | 4.40 | - | SF | Lee (1998) |
| **Whey Based** | Whey | *Recombinant E.coli* | 5.20 | - | SF | Lee *et al.* (1997) |
| | | *Recombinant E.coli* | 32.00 | - | 1L | Kim (2000) |
| | | *Recombinant E.coli* | 21.00 | - | 5L | Nikel *et al.* (2006) |
| | | *Recombinant E.coli* | 96.20 | - | 1.5L | Ahn *et al.* (2000) |
| | | *Recombinant E.coli* | 35.50 | - | 10L | Park *et al.* (2002) |
| | | *Recombinant E.coli* | 20.10 | - | 150L | Park *et al.* (2002) |
| | | *Pseudomonas hydrogenovora* | 1.27 | - | 1.7L | Koller *et al.* (2008) |
| | | *Hydrogenophaga pseudoflava* | 3.00 | - | 10L | Koller *et al.* (2007) |
| | | Osmophilic wild type strain* | 5.50 | | 42L | Koller *et al.* (2005) |
| | | *Haloferaz mediterranei* | 7.20 | - | 220L | Koller (2015) |
| **Oil and glycerol based** | Olive oil mill waste | *Recombinant P.putida* | 0.13 | - | SF | Ribera *et al.* (2001) |
| | Waste Glycerol | *C.necator* | 38.10 | - | 1.5L | Cavalheiro (2009) |
| | | Osmophilic wild type strain* | 16.20 | | 10L | Koller *et al.* (2005) |
| | Soybean oil | *C.necator* | 85-95 | - | 5L | Kahar *et al.* (2004) |
| | Waste frying oil (from rapeseed) | *C.necator* | 1.20 | - | SF | Verlinden *et al.* (2011) |
| | Canola oil | *W. Eutropha* | 18.27 | - | 3L | Cuellar *et al.* (2011) |

**Under characterization*
*SF: shaken flask*

Glycerol, as a side product of biodiesel production, is one of the major substrates of biological processes. Each year 500,000 tons of crude glycerol is produced as a by-product of biodiesel production (Koller and Braunegg, 2015). Cavalheiro (2009) and Koller *et al.*, (2005) successfully utilized this product for PHA production by using the *C.necator*, Cavalheiro obtained 38 g/L PHA. Koller *et al.*, (2005) reached 16 g/L level of production with an osmophilic wild type strain using glycerol as a sole carbon source. Different concentrations of PHAs obtained using oil-based waste materials have been presented in literature, with the highest production by *C. necator* of 85-95 g/L reported by Kahar *et al.*, (2004).

Among the waste raw materials discussed, the most promising one stands out as whey. Whey, due to its high nitrogen content (14-16%N in protein, Robert, 2006) and availability, is an attractive substrate, and is still one of the most studied waste materials. It is the main waste material of cheese and casein production. Within the European Union approximately 40 million tons of cheese whey is produced annually, 13 million of which remains unutilized (Koller *et al.*, 2005). As can be seen from Table 1.3, the majority of the work carried out using whey as a substrate is on recombinant *E.coli* fermentation. In addition, whey was used in the largest reactor scale (220 L). The highest level of production observed was 96.2 g/L PHA by Ahn *et al.*, (2000). Park *et al.*, (2002) investigated production of P(3HB) from whey by fed-batch culture of recombinant *Escherichia coli* in a 30 L fermenter (starting volume 10 L) and scaled up the process to 300 L (working volume 150 L) fermenter. Under high aeration conditions (600 L/h for 30 L fermenter and 9000 L/h for 300 L fermenter, to keep % DOT at 30%) and keeping lactose below 2 g/L, cdw reached to 12 g/L with 9% (w/w) P(3HB) content. They reported that accumulation of P(3HB) could be further increased by increasing lactose concentration. By employing this strategy, they reported 51 g/L cdw with a 70% (w/w) P(3HB) content produced after 26 h of cultivation. When the fermentation was scaled up to

300 L using the same strategy, 30 g/L cdw with 67% (w/w) P(3HB) content was obtained in 20 h.

In addition, there is further research focusing on utilization of waste streams from whey fermentation. A recent study conducted by Koller (2015) was on utilization of spent fermentation broth and cell debris after extraction of the polymer. Cultures of *Haloferaz mediterranei* were tested in repetitive fermentation. The strain used, *Haloferax mediterranei*, an extreme halophile, is of outstanding importance. It is described as robust, genetically stable, has broad substrate spectrum, and has good quality of the produced PHA. In addition it is capable of producing other marketable side products such as bacterioruberins, extracellular polysaccharides, or halocins. Its extremophilic character, manifested by the high salt requirement for growth, enables the exclusive cultivation of this strain even under non-sterile conditions without endangering the fermentation by microbial contamination. In the study reported by Koller, the spent fermentation broth was utilized to replace a considerable part of fresh saline which the organism needs. In addition to that, the cell debris recovered from the extraction process was recycled in consecutive fermentation which demonstrated a capability of replacing 29% of expensive yeast extract needed for each batch. At the end of 65 h fermentation in a 300 L batch fermenter, 7.20 g/L P(3HB-co-3HV) was produced (Koller 2015). Park *et al.* (2002) also reported fermentation in a 300 L fermenter, they obtained 35.5 g/L PHA using whey as a substrate.

There are also studies on the development of other recombinant strains for the production of PHAs on cheap substrates. Strains are developed which can utilize amino acid degradation pathways, using alpha-ketoglutarate, or succinate as substrate (Huisman *et al.*, 2002).

When the strains used for the production of PHA from raw materials are investigated as a whole (Table 1.3), it can be seen that most of the strains used are Gram-negative. Gram-negative bacteria are generally preferred for PHAs production due to their high

productivities and capabilities of utilising a range of carbon sources. In addition *E.coli* as a Gram negative bacterium is widely used for genetic modification and gene transfer applications in order to obtain high producing recombinant strains. The main concern with Gram-negative bacteria however, is the presence of lipopolysaccharide (LPS) endotoxins in the bacteria's outer cell membrane, which may co-purify with crude PHA polymer during the extraction process (Rai *et al.,* 2011). LPS endotoxin is a pyrogen which can elicit a strong inflammatory response (Ray *et al.,* 2013), making the PHA polymer unsuitable for biomedical applications. Removal of LPS endotoxin can be achieved through extensive treatment of PHA polymer with oxidizing agents (i.e., sodium hypochlorite, ozone, hydrogen peroxide), with repeated solvent extractions, or with solvent extraction followed by purification with activated charcoal (Rai *et al.,* 2011; Chen and Wu, 2005; Wampfler et al., 2010). These methods however, increase the overall complexity and cost of PHA production and lead to changes in PHA polymer properties (i.e., reduction in molecular mass and poly-dispersity).

Compared to Gram-negative bacteria, Gram-positive bacteria were mostly found to produce scl-PHA, and in addition, their PHA productivity is low. So, Gram-positive bacteria have yet to be adopted for commercial PHA production.

Despite generally accumulating lower amounts of PHA, Gram-positive bacteria are advantageous over Gram-negative bacteria owing to their lack of LPS which may make them a better source of PHA raw material for biomedical applications (Valappil *et al.*, 2007). However, some Gram-positive bacteria are known to produce lipidated macroamphiphiles including lipoglycans and lipoteichoic acids (LTA), which have immunogenic properties similar to LPS (Ray *et al.,* 2013). *Corynebacterium, Nocardia, Rhodococcus* are the main bacteria genera reported to produce lipoglycans, and LTA production was reported in the genera *Bacillus, Clostridium, and Staphylococcus*. However, some of these strains, which are capable of producing PHAs, lack LTA.

Therefore, further investigation will be required to verify if these substances are present or not in these strains (Tan *et al.,* 2014). At present, the immunogenic effects of lipidated macroamphiphiles in PHA remain unknown. Future *in vitro* or *in vivo* evaluation studies is essential to evaluate the suitability of PHAs, derived from Gram-positive bacteria, for biomedical applications (Ray *et al.,* 2013).

### 1.2.4 Production Strategies

Choice of production process for PHAs depends on various parameters including strain type, carbon source and nitrogen sources and ratios, cultivation time, reactor volume. Optimizing the process to obtain high product yield with high conversion rates is the key in process design. Various types of fermentation strategies used to achieve high yields of PHAs are discussed in detail in this section.

#### 1.2.4.1 Batch Fermentation

Batch fermentation is one of the most popular operation modes due to its flexible nature and ease of operation as well as low operation costs. The critical point in batch production of PHAs is the time of harvesting. After depletion of carbon source, bacteria start degrading the accumulated PHA which results in low productivity. PHA accumulation starts at the beginning of the stationary phase thus the growth phase has to be completed and medium needs to remain as nutrient limiting. In some cases PHA productivity is low, due to low biomass concentration because nitrogen limitation starts before the growth phase is completed (Katırcıoğlu *et al.,* 2004). Valappil and co-workers used batch fermentation for production of P(3HB) using *Bacillus cereus* SPV organism and Kannan and Rehacek medium with glucose as main carbon source. The maximum P(3HB) obtained was 29% P(3HB)/cdw (Valappil *et al.,* 2007). Similarly Kulpreecha and co-workers employed a batch fermentation process in a jar fermenter. Using *Bacillus megaterium* and sugarcane molasses as carbon source they achieved 61% PHA/cdw in a total volume of 7 L

(Kulpreecha *et al*., 2009). On the other hand, using the same carbon source but a different organism, Tripathi and colleagues obtained 76% PHA/cdw with *Pseudomonas aeruginosa* using a medium consisting cane molasses 40 g/L, urea 0.8 g/L and fermentation conditions of temperature 36 °C, pH 7.0 and agitation speed 175 rpm in a 7.5 L batch fermenter (Tripathi *et al.,* 2012). This great difference underlines the importance of the selection of the organism in batch cultures.

#### 1.2.4.2 Fed-Batch Fermentation

Fed-batch fermentation is one of the most suitable strategies for PHAs production. It is mainly preferred due to its ability to reach high cell densities. However, cell growth and polymer accumulation need to be monitored to avoid low cell concentration or inadequate polymer accumulation. For example, in cases where nitrogen is the limiting nutrient the overall PHA production is still considered low mainly because of insufficient biomass for PHA accumulation (Katırcıoğlu *et al*., 2004). Some examples of fed-batch fermentation are given in Table 1.4. It can be seen from the table that using a suitable strain, achieving productivities above one is possible. Although fed-batch culture usually enhances cell density, single stage fed-batch culture under nitrogen limited conditions, may not result in high PHA accumulation. Sun *et al.*, (2007) found that *Pseudomonas putida* KT2440 could only accumulate 27% PHA/cdw when nutrient limited conditions were employed. However, when nonanoic acid limited growth conditions were employed at exponential phase, it produced 70 g/L biomass with 75% PHB/cdw. When culture was fed at different stages of exponential phase it was observed that at higher specific growth rates the overall PHA productivity increased but biomass production decreased yielding less % PHA/cdw (67% PHA/cdw) due to high oxygen demand. Sun *et al.*, (2007) also demonstrated that under their experimental conditions nitrogen limitation was not necessary since it can limit the culture growth in fed-batch cultures.

**Table 1. 4:** Fed batch fermentations conducted with various organisms using different carbon sources and their related productivities, cell dry weights, and polymer productions. P; productivity (g/L.h), CT; cultivation time (h)

| Organism | Carbon source | CT (h) | Fermentation Process Type | PHA (g/L) | cdw (g/L) | P (g/L.h) | References |
|---|---|---|---|---|---|---|---|
| *C.necator* | Glycerol | 60 | Fed-Batch | 52 | 75 | 0.92 | (Tanadchangsaeng and Yu, 2012) |
| *P.putida* | Hydrolysed corn oil | 46 | Fed-Batch | 28 | 103 | 0.61 | (Shang *et al*., 2008) |
| *P.putida* | Oleic acid | 70 | Fed-Batch | 13.52 | 30.22 | 0.19 | (Marsudi *et al*., 2009) |
| *B.megateriu m* | Sugar cane molasses + cane molasses and urea | 24 | Fed-Batch | 30.52 | 72.6 | 1.27 | (Kulpreecha *et al*., 2009) |
| *P.putida* | Glucose + nonanoic acid | 25 | Fed-batch | 14.3-30.1 | 33.6-54.1 | 0.63–1.09 | (Sun *et al*., 2009) |
| *P.putida* | Glucose + nonanoic acid | 48 | Fed-batch | 52.5 | 70 | 1.09 | (Sun *et al*., 2009) |
| *R.eutropha* | Waste potato starch | 70 | Fed-Batch | 94 | 179 | 1.47 | (Haas *et al*., 2008) |
| *R.eutropha* | Glucose | 50 | Fed-Batch | 121 | 164 | 2.42 | (Kim *et al*., 1994a) |
| *A.latus* | Sucrose | 18 | Fed-Batch | 71.5 | 143 | 3.97 | (Yamane *et al*., 1996) |
| *A.latus* | Sucrose | 20 | Fed-Batch | 98.7 | 112 | 4.93 | (Wang and lee 1997) |
| *B.cereus SPV* | Glucose | 72 | Fed-Batch | 0.89 | 3.0 | 0.01 | (Valappil *et al*., 2007) |
| *A. eutrophus* | Glucose + Propionic acid | 46 | Fed-Batch | 117 | 158 | 2.54 | (Kim *et al*., 1994b) |
| *A. eutrophus* | Tapioca | 59 | Fed-Batch | 61.5 | 106 | 1.04 | (Kim *et al*., 1995) |
| *C.necator* | Soybean oil | 96 | Fed-Batch | 95.8 | 126 | 0.99 | (Kahar *et al*., 2004) |
| *H.boliviensis* | Glucose | 33 | Fed-Batch | 35.4 | 44 | 1.07 | (Quillaguaman *et al*., 2005) |
| *H.boliviensis* | Glucose | 40 | Fed-Batch | 42.5 | 62 | 1.06 | (Guzman *et al*., 2009) |
| *P.extorqueus* | Methanol | 170 | Fed-Batch | 149 | 233 | 0.88 | (Suzuki *et al*., 1986) |

Hence, the best approach in fed-batch cultures is to use a feed composed of limiting nutrients at the rate of utilization. This avoids any shift in the metabolism of the organism towards by-products and keeps high the production of the product of interest. In the case of PHAs, feeding the growth limiting component and resulting in its accumulation in the

medium, may result in re-initiation of growth and thus consumption of the accumulated PHA. Therefore, the feeding strategy should be adjusted carefully.

### 1.2.4.3 Continuous Fermentation

Continuous culture, or chemostat, is the third alternative in production of PHAs. This strategy is very much preferred by researchers due to its potential of obtaining high productivities especially when strains with high maximum specific growth rate are used. In studies with continuous culture for PHA production, the PHA content of the cells was found to be growth rate dependent. When dilution rate is increased, while biomass is increasing, the PHA content of cells decreases. Preusting and co-workers demonstrated this concept. When the dilution rate of cultures of *Pseudomonas oleovorans* was increased from 0.09 $h^{-1}$ to 0.46 $h^{-1}$ the PHA content of cells decreased from 40% PHA/cdw to 8% PHA/cdw and the cell density decreased from 2.25 to 1.32 g/L (Preusting *et al.*, 1991). Therefore, PHA content, biomass and productivity in continuous cultures need to be optimized. In order to improve economics of continuous culture fermentations two approaches may be adopted. One, intracellular PHA content can be increase to higher levels or as a second approach; the steady state cell concentration can be increased. In other words the system can be kept at steady state under high cell density conditions. In the second approach, when the culture is switched to nutrient limited conditions to accumulate PHA, other nutrients must be present in sufficient levels to avoid any metabolic shift or cell death. In high cell density cultures it might be necessary to ensure the dissolved oxygen above a required threshold. However, contrary to this, a new concept in continuous PHA production, has been introduced in recent years, the so called dual nutrient limitation.

Dual nutrient limitation concept was first introduced and reported by Egli and Zinn in 2003. The concept is still under discussion amongst researchers. What they claim is in contrast to “law of the minimum” which is known as Liebig’s law where one of the

nutrients in the growth media determines the maximum cell density (Egli and Zinn, 2003). Egli and Zinn (2003), however, claim that the growth of microbial cultures can be limited simultaneously by two or more nutrients (Egli and Zinn 2003; Zinn *et al.*, 2004). In their study, they suggested that, during the growth of bacteria at constant dilution rate as a function of the C:N ratio, there are three distinct regimes. One, a clear carbon limited regime where nitrogen is in excess; two, a transient regime where both carbon and nitrogen concentrations are below the detection limit; this is named as "dual nutrient limited region", and finally a nitrogen limited regime where carbon is in excess. This concept is claimed to be useful in making tailor made PHAs since under dual limiting conditions the composition of the feed is directly reflected to the accumulating PHA (Egli and Zinn 2003). However, since in the claim both nutrients are below detection limits, this does not mean that both are limiting the growth, in the micro scale still one off the nutrients might be limiting the organism and the concept completely depends on the detection limits of the system used.

The most challenging aspect in continuous culture for PHA production is the organism used. Many organisms, specifically *Bacillus sp.*, synthesise PHA in two phases. First, the culture is grown under non-limiting conditions (there is no, or little, PHA production at this stage) until it reaches the stationary phase. Then at the second phase (stationary phase) the organism starts accumulating PHA. However, the specific growth-rate usually is very low at the stationary phase. This limits the use of continuous culture as a tool to control the productivity of these organisms.

#### 1.2.4.4 Multi-Stage and Other Types of Fermentation Systems

Due to the non-growth associated production of PHA, researchers have investigated multi-stage fermentations where in the first stage high cell concentration is achieved under balanced nutrient condition and then at the consecutive stages, the culture is transferred to subsequent fermenters where nutrient limited conditions and various excess carbon sources

are introduced. The most common multi-stage fermenter system is a 2-stage system where the first reactor is dedicated to biomass accumulation and the second to PHA accumulation. For the second stage, some researchers have adopted fed-batch fermentation due to its flexibility and high yields (Chakraborty *et al*., 2012; Jung *et al*., 2001). Other researchers have used continuous fermentation. In this case the multistage cultures allow usage of different dilution rates in different reactors hence achieving higher PHA concentrations and productivities. Using 0.21 $h^{-1}$ for cell growth stage (first fermenter) and 0.16 $h^{-1}$ for the accumulation stage (second fermenter), Jung and co-workers achieved a high overall productivity of 1.06 g/L.h and 63% PHA/cdw using *Pseudomonas oleovorans* (Jung *et al*., 2001). Similarly Atlić and colleagues used a 3-stage fermentation system where the first stage was operated as batch to achieve adequate growth and second stage was operated under 0.139 $h^{-1}$ dilution rate which resulted 1.85 g/L.h productivity using *C. necator* cultures (Atlić *et al*., 2011). A multi-stage study was published by Horvat and co-workers in 2013 using *Pseudomonas oleovorans* (Horvat *et al.*, 2011). In their study, a 5-stage continuous bioreactor cascade was mathematically modelled and optimised for production of P(3HB). The system was modelled based on partial growth associated PHA production under nitrogen limited growth conditions. First stage of the reactor cascade was modelled based on continuous biomass production under balanced nutrient conditions. The second stage was a dual substrate controlled process, discussed in Section 1.2.4.3. Final 3 stages were adjusted such that they maintained continuous excess carbon and deficient nitrogen conditions. The simulated results suggested that the productivity of the proposed system could be increased from 2.14 g/L.h to 9.95 g/L.h if certain conditions were applied (Horvat *et al.*, 2013).

Another interesting approach in the production of PHAs is a two phase chemostat culture. Preusting and co-workers (1993) reported the use of a continuous operation system with a two phase liquid medium under high cell density culture of *Pseudomonas oleovorans*

(Preusting *et al.*, 1993). Using n-octane as carbon source and ammonium as nitrogen source, PHA productivity was reported to be 0.17 g PHA/L.h with a dilution rate of 0.20 $h^{-1}$. In order to increase the productivity, the nitrogen concentration was doubled in the feed which caused an increase in cell density from 2.6 g/L to 5.5 g/L with a productivity of 0.28 g PHA/L.h. High cell density further caused an oxygen limitation in the culture which was reported to be resolved by increasing agitation and aeration. Further optimisation of culture conditions was reported to lead to a cell density of 11.6 g/L and a PHA productivity of 0.58 g/L.h. Overcoming oxygen limitation in the high cell density cultures by adopting different reactor design was the approach used by Tröger and Harvey (2009). In their design, oscillatory baffled reactor (OBR), a type of plug flow reactor, with highly uniform and controllable mixing, was used to enhance gas to liquid mass transfer in the cultures of *Pseudomonas putida*. The work proved that OBR produces higher optical density and cell dry weight than a conventional stirred tank reactor. However, PHA production was only confirmed by florescent microscopy and was not quantified (Tröger and Harvey, 2009).

In a patent filed in 2004, a continuous flow bioreactor system that included a bioreactor, an optional post-bioreactor preparation chamber, a cell sorter, and an optional pre-bioreactor preparation chamber in a closed loop, useful for enriching a heterogeneous cell population growing in the bioreactor with an isolated subpopulation of cells was described by Abu Absi *et al.* (2004). The paper suggested that this system could be used to enhance PHA production, the produced PHA can be stained in the post-bioreactor preparation chamber using Nile red, and cells containing PHA can thus be detected simultaneously.

A two-stage continuous fermentation was described by Fan and Zhao (1995) for the process of producing poly-beta-hydroxy butyrate from methanol. The cells were continuously multiplied in the first tank and the P(3HB) content was increased under nitrogen limitation in the second tank. This two stage continuous fermentation approach

achieved popularity and was adapted by other researchers in the following years (Sun *et al*, 2007; Atlić *et al.*, 2011; Posada *et al.*, 2011).

In another patent a sequencing bioreactor operating in alternating phases of nutrient starvation and carbon starvation was described (Criddle and Pieja, 2009). The aim was to select for PHA-producing microbes. The bioreactor was operated under non-sterile conditions with mixed cultures of methanotrophs. Periodic biomass-wasting (PHA harvesting) was applied at the end of the carbon feed phase, gradually lengthening the time period of carbon starvation phase. This culture strategy was implemented to create a penalty for rapid PHA degradation and incentive for PHA accumulation. The culture was periodically supplemented with fresh inoculum to compensate for the loss of bacterial cultures in PHA harvesting.

### 1.2.5 Global Market and Commercialisation of PHAs

Although some companies produce PHAs in small amounts, the high cost of production has hampered rapid progress for applications. Table 1.5 shows some examples of commercial PHAs. However, despite these attempts the production cost of PHAs cannot compete with those of petrochemical plastics yet. There are several companies active in PHAs business, however the competitiveness and market uncertainty as well as instability of the prices do not allow for SMEs to get into the market and open up new businesses. Currently, 1 kg PHA, under trade name Mirel PHA, is sold for $11 by Metabolix (David Scarlet, personal communication, September 4, 2015). In order to reduce costs, it is important to produce PHA in high yields. Studies are continuing to bring down the cost of the polymer. In 2007, it was suggested that the polymer can become competitive if the price range drops to 2.20 - 0.99 $/kg range or below (Philip *et al*., 2007). However, the fluctuations in oil prices due to discovery of new oil reservoirs make it difficult to provide reliable price estimates.

There are three main cost factors in the production of PHAs. In a case study for production of P(3HB), Zahari and co-workers state that three cost factors besides the production costs contribute to the total cost of the polymer. These are reported as raw materials (29%), equipment depreciation (27%) and extraction and purification (20%) (Zahari *et al.*,2015). Research mainly focuses on two areas which are raw material costs and improvements in the extraction process. Price of PHA per kilo, sold by different companies, is presented in Table 1.6. For PHAs producer, having a low cost recovery step has become a strategic asset to stay competitive. The major step of the separation process is the extraction of PHAs granules. But, in order to get a better recovery, a pre-treatment step could be included to improve the cell disruption and release of PHA granules (Jacquel *et al.,* 2008). With the cost cutting modifications in the production and downstream methods, PHAs have the potential to be the next generation of environmentally friendly materials with broad range of applications.

**Table 1. 5:** Past and present companies in production of PHAs

| Company, Country | Types of PHA | Production scale (t/a) | Current status and remarks |
|---|---|---|---|
| ADM, USA (with Metabolix) | Various PHAs | 50 000 | Ended alliance with Metabolix and stopped production on 2012 due to uncertainty in costs and market |
| BASF, Germany | P(3HB), PHBV | Pilot scale | Shut down in 2005 |
| Biocycles, Brazil | P(3HB) | 100 | Active |
| Biomers, Germany | P(3HB) | N/A | Active |
| Bio-On, Italy | PHA (unclear) | 10 000 | Active |
| Chemie Linz, Austria | P(3HB) | 20–100 | Not active |
| ICI, UK | PHBV | 300 | Not active |
| Jiang Su Nan Tian, China | P(3HB) | Pilot scale | Active |
| Jiangmen Biotech Ctr, China | PHBHHx | N/A | Not active |
| Kaneka, Japan (with P&G) | Various PHAs | 4000 | Active |
| Meredian, USA | Various PHAs | 300000 | Rights acquired from P&G in 2007, active |
| Metabolix, USA | Various PHAs | N/A | Active |
| Biogreen Mitsubishi gas, Japan | P(3HB) | 50 | Active |
| Monsanto, USA | P(3HB), PHBV | Pilot scale | Rights acquired by Metabolix in 2002, Closed down in 2004 |
| Newight Technologies , USA | PHA from greenhouse gasses | 50 | Active since 2003 |
| P&G, USA | Various PHAs | Pilot scale | Rights transferred to Meredian in 2007, Sold the trade name Nodax to Meredian, production stopped |
| Shandong Lukang, China | Various PHAs | Pilot scale | Active |
| Shantou Lianyi Biotech,China | Various PHAs | Pilot scale | Not active |
| Shenzhen O'Bioer, China | Various PHAs | N/A | Active |
| Tepha, USA | P(4HB) | N/A | Active |
| Tianjin Green Bio-Science | P3HB-4HB | 10 000 | Active, signed distribution agreement with Metabolix in 2013 |
| Tianjin Northern Food, China | P(3HB) | Pilot scale | Active |
| Yikeman, Shandong, China | PHA (unclear) | 3000 | Active |
| Zhejiang Tian An, China | PHBV | 2000 | Active |
| Goodfellow, UK | P(3HB)/PHV | N/A | Active |
| SIRIM (Malaysia) | Various PHAs | Pilot scale | Became active in 2011 |
| Telles USA | P(3HB) | 50000 | Joint venture between ADM and Metabolix, Operates under Metabolix since 2012 |

The main method of PHA recovery from the fermentation broth is comprised of a separation operation for polymer containing cells, a cell-disruption step, and an extraction and debris removal stage. Although a number of researchers have worked on downstream processes for biopolymers, innovations and improvements on the separation and purification step have only been incremental.

**Table 1. 6:** Price of commercial PHAs reported up to 2010 (Updated and improved based on the data presented by Jacquel *et al.,* 2008)

| Product Name | Type | Company | Price (per kg) | Country |
|---|---|---|---|---|
| Biomer® | P(3HB) | Biotechnology Co. | €20 (2003) €3-5 (2010) | Germany |
| Biocycle® | P(3HB) | Industrial S/A company | - | Brazil |
| Biogreen® | P(3HB) | Mitsubishi GAS Chemical | €10-12 (2003) €2.5-3 (2010) | Japan |
| - | P(3HB) | Metabolix (BASF, ADM)* | €2.20 (2010)* | USA |
| - | P(3HB) | Jiangsu Nantian Group | - | China |
| - | P(3HO) | Metabolix (BASF, ADM)* | €2.20 (2010)* | USA |
| Biopol ® | P(3HB-co-(3HV) | Metabolix (BASF, ADM)* | €10-12 (2003) € 3-5 (2010)* | - |
| - | P(3HB-co-(3HV) | Industrial S/A company | - | Brazil |
| ENMAT ® | P(3HB-co-(3HV) | Tianan Biologic Material | - | China |
| Nodax | P(3HB)-co-(3HHx) | Procter & Gamble | €2.50 | USA |
| - | P(3HB)-co-(3HHx) | Jiangsu Nantian Group | - | China |
| - | P(3HB)-co-(3HHx) | Lianyi Biotech | >$5 | China |
| **After strategic alliance between ADM and Metabolix* | | | | |

In the race for commercialisation, one of the most important elements to place a company ahead of others is the number of patents it holds. Between the years 2000 to 2015, 3,513 out of 6,844 patent applications have been filed by one company. As understood from the activities and remarks listed the Table 1.5, the most dominant actor in PHAs field is Metabolix Inc from USA. In terms of patent applications Metabolix also by far hold the highest number of applications (857) within 15 years (Figure 1.7).

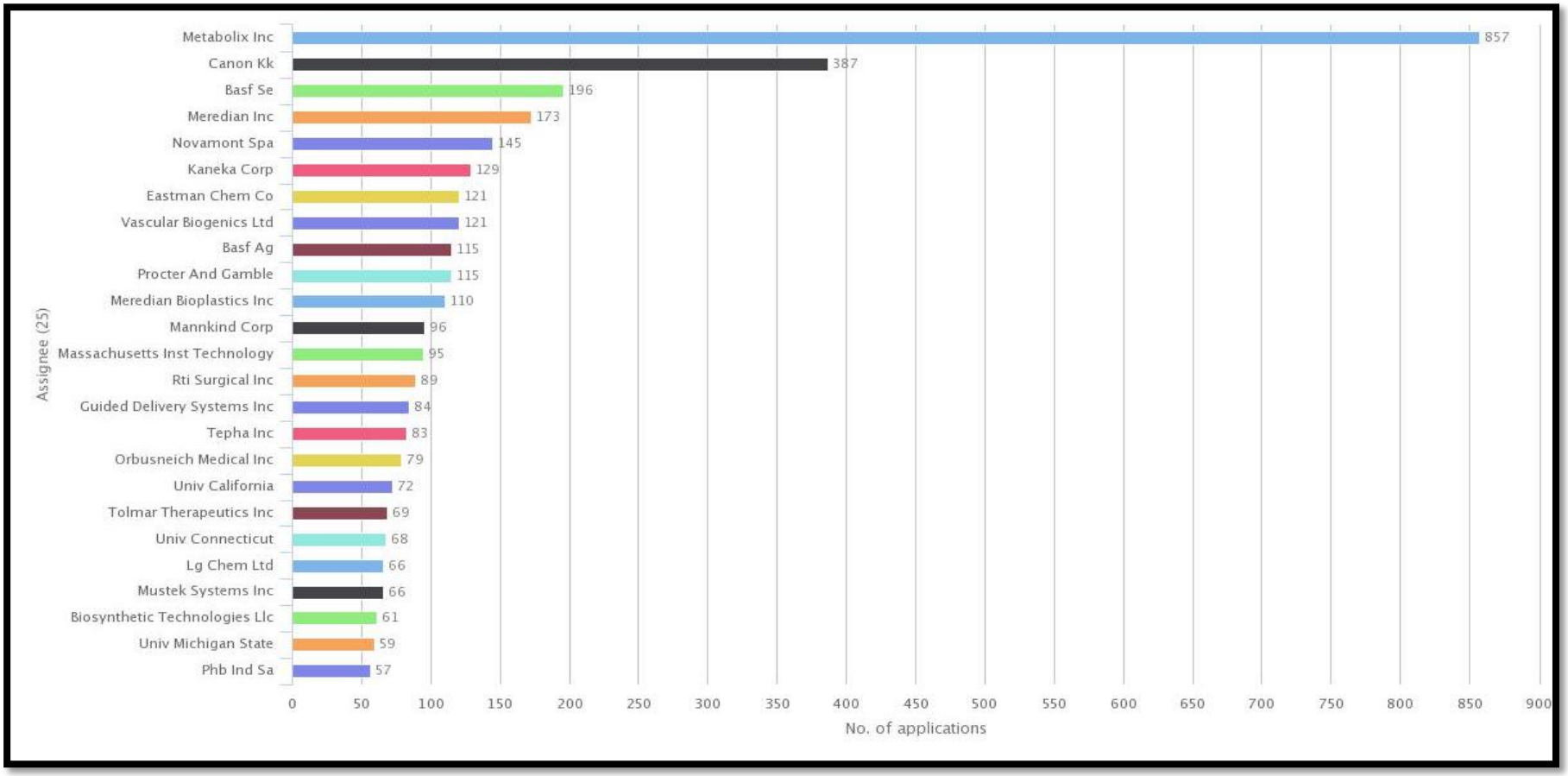



**Figure 1. 7:** Distribution of number of patent applications by companies between the years 2000-2015. (Research done on Pat-Base with the key words: Polyhydroxyalkanoate, Polyhydroxybutyrate, Fermentation, Metabolic Products)

## 1.3 Poly-γ-glutamic Acid (γ-PGA)

The interest for polyglutamic acid (PGA), as a biopolymer date further back than PHAs. However, there are only 2,423 research articles published and 3,270 patents were applied between the years 1996 -2015 (research done on Web of Science and Pat-Base with the key words: Poly-L-Glutamate, Glutamic Acid, Glutamic Acid Homo-Polymer, Poly-Glutamic Acid, Glutamic Acid Polymer, Fermentation) in contrast to 6,844 patents and 4,613 articles published between the years 2000-2015 on PHAs (research done on Web of Science and Pat-Base with the key words: Polyhydroxyalkanoate, Polyhydroxybutyrate, Fermentation, Metabolic Products). Quadrant Drug Delivery Ltd., CP Medical Inc. and Coca-Cola Co. are the top three companies holding the patents on PGA, indicating PGAs' wide range of applications in different sectors.

### 1.3.1 Structure and Properties

Poly amino acids are polyamides composed of amino acid linked with amide bonds. They differ from proteins in several aspects. Proteins are composed of a variety of amino acids, while poly amino acids are composed of only one type of amino acid (at least in the backbone) (Bajaj and Singhal 2011a). In addition amide linkages in proteins are only formed between α-amino and α-carboxylic groups, but in poly amino acids other side chain functions such as γ-carboxylic groups and ε-amino groups are involved (Bajaj and Singhal 2011a). Three types of poly amino acids are found in nature. They are; poly-γ-glutamic acid (γ-PGA), poly-ε-lysine (ε-PL) and cyanophycin.

γ-PGA is a naturally occurring, anionic homo-polyamide that consists of D- and L-glutamic acid units connected by amine linkages from α-amino and γ-carboxylic groups (Figure 1.8)(Shih and Van 2001). It is produced by *B. subtilis*, *B. licheniformis*, *B. anthracis*, and *B. megaterium* (Yao *et al*., 2009) and is an exo-cellular polymer. It

can be secreted by the cell into the environment or can be kept in capsules bound to peptidoglycan (Ogunleye *et al.*, 2015). It is abundantly found in mucilage of natto (a traditional food in Japan made with fermented soybeans) which is a mixture of fructan and polyglutamic acid produced by *Bacillus natto*.

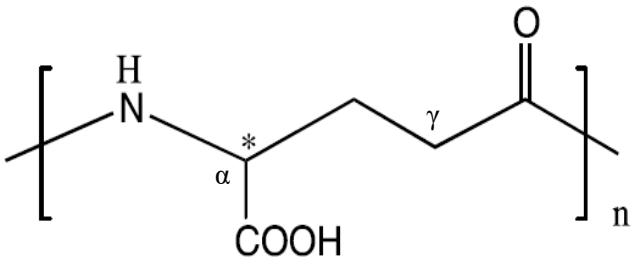



**Figure 1. 8:** Structure of γ-PGA showing α and γ polymerisation binding sites and (*) functional group binding spot (Shih and Van 2001).

Organisms produce γ-PGA for different reasons. The function of γ-PGA produced by the organism depends on whether γ-PGA is peptidoglycan-bound or released. Peptidoglycan-bound γ-PGA helps the organism to confer virulence or to act as a source of glutamate under starvation conditions. If released to the environment, it helps the organism to encounter harsh conditions. In addition, γ-PGA protects the bacterial cells against phage infection and prevents antibodies from gaining access to the bacterium (Ogunleye *et al.*, 2015).

γ-PGA has been known and investigated for more than 70 years. It is highly water soluble, biodegradable, edible and non-toxic to humans and to the environment. It may be separated from other neutral components by electrophoresis and unlike proteins it is resistant to proteases, which cleave only α-amino bonds. Contrary to other proteins, γ-PGA is only stained with methylene blue, but not with Coomassie blue (Candela and Foulet 2006). γ-PGA produced by different *Bacillus* sp. can be found in various sizes from 10 kDa to 1000 kDa (Ashiulchi and Misono 2002). Properties of γ-PGA such as its

water solubility, biodegradability and edible nature makes it attractive for applications in medicine, food, plastics and many other industries (Shih and Van 2001). Some of its key properties can be listed as follows;

- *Water absorption capacity* –It can absorb water approximately 5000 times greater than its own weight.
- *Hydrophilicity* – As the molecular weight decreases, water solubility increases.
- *Viscosity* – As the molecular weight increases, the viscosity increases.
- *Biodegradability* –It can be degraded (it is metabolisable) by microorganisms into water and $CO_2$.

Exploitation of these properties in various industrial fields are summarised in Table 1.7.

**Table 1. 7:** Applications of γ-PGA in various fields (Adapted from Shih and Van 2001 and Ogunleye *et al.,* 2015)

| Field | Application |
|---|---|
| Water and waste water treatment | Metal chelation or absorbent |
| | Bioflocculant |
| | Dye removal |
| Food industry | Thickener |
| | Cryoprotectant |
| | Bitterness relieving agent |
| | Texture enhancer |
| | Animal feed additives |
| | Food supplement/osteoporosis preventing agent |
| | Oil reducing agent |
| Agriculture | Biocontrol agent and/or fertiliser |
| Medical | Drug carrier or slow release material |
| | Curable biological adhesive and hemostatic or medical bonding kit |
| | Suture thread |
| | Tissue engineering (composite with chitosan) |
| | Calcium absorption |
| Cosmetics | Moisturiser |
| Membrane | Absorbent |
| | Enantioselective agent |
| Other | Water absorbent |
| | Dispersant |
| | Biodegradable plastic |
| | Glucose sensor |
| | Influenza virus inhibitor |
| | Antibacterial activity |
| | Gene delivery |

### 1.3.2 Biosynthesis and Metabolism

Extensive research has been carried to find out the metabolic pathway and enzymes related to γ-PGA synthesis in order to enhance the productivity. In bacteria γ-PGA synthesis is carried out in two steps. In the first step the synthesis of D- and L- glutamic acid takes place. In the second step these units join together to form γ-PGA (Bajaj and Singhal 2011a). While there are several interpretations on the mechanism of the synthesis, basically two suggestions, namely, direct and indirect conversion of glutamic acid to its isomers dominate the scene, and are involved in production pathway of poly glutamic acid. However, the enzymes involved in these reactions are not fully revealed (Shih and Van 2001, Ogunleye *et al.* 2015).

There are two types of bacteria which produce γ-PGA. These are glutamic acid dependent and glutamic acid independent organisms. Glutamic acid independent production is a rare case, so most researchers have studied the pathways of glutamic acid dependent organisms. *Bacillus subtilis* IFO 3335 is one these glutamic acid dependent bacteria. The γ-PGA production pathway proposed for this organism can be seen in Figure 1.9. As the Figure shows, γ-PGA is mainly produced from citric acid and ammonium sulphate. It is proposed that L-glutamic acid is produced from citric acid via iso-citric acid and α-ketoglutaric acid pathway in the TCA cycle. Conversion of α-ketoglutaric acid to L-glutamic acid is proposed to occur in two ways. In the presence of extracellular glutamine a pathway involving the enzymes glutamine synthase (GS) and glutamate 2-oxoglutarate aminotransferase (GOGAT) is used. In the absence of extracellular glutamine a pathway involving glutamate dehydrogenase is used.

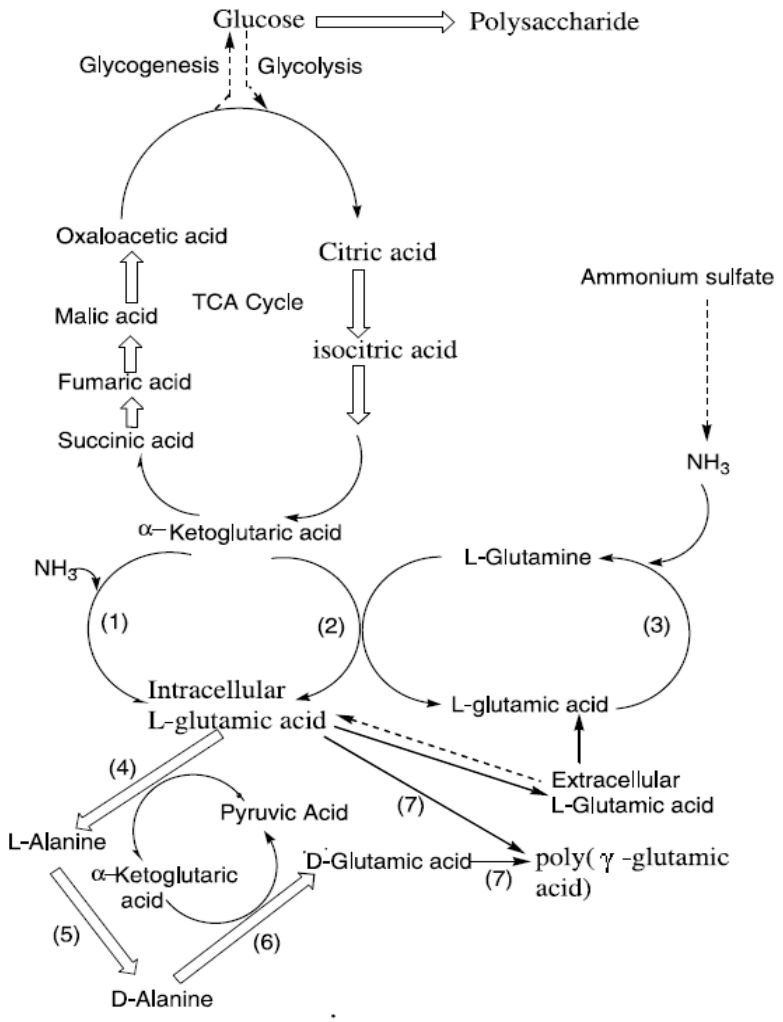



**Figure 1. 9:** The proposed metabolic pathway for *Bacillus subtilis* IFO 3335 (Shih and Van 2001).

### 1.3.3 Production, Potential Feed Stocks and Raw Materials

As discussed previously, several *Bacillus* sp. are capable of producing γ-PGA as an extracellular viscous material or as a capsular component. Extensive amount of work has been carried out to determine the nutritional requirements for cell growth and γ-PGA production. As the industrial application of γ-PGA has increased, the γ-PGA producing organisms have attracted more interest including even revisiting microbes classifiaction criteria. According to Shih and Van (2001) γ-PGA producing bacteria are divided into two main groups. One group requires addition of L-glutamic acid to the medium for cell growth and to initiate γ-PGA production, the other group does not require L-glutamic acid. As reported by Ogunleye *et al.* (2015), the L-glutamic-acid dependent bacteria include strains like; *B. subtilis* (chungkookjang), *B. licheniformis* 9945a, *B. subtilis* CGMCC 0833, *B.licheniformis* NK-03 and *B. subtilis* (natto) ATCC 15245. The non-L-glutamic acid dependent producers include *B. subtilis* C1, *B. amyloliquefaciens* LL3 and *B. subtilis* C10.

For glutamic acid dependent bacteria, the γ-PGA yield increases with increasing L-glutamic acid concentration in the medium. However, these bacteria can produce γ-PGA even in the absence of L-glutamic acid in the medium due to the synthesis of L-glutamic acid through the *de novo* pathway, but the yields tend to be low (Kunioka & Goto, 1994).

The main medium components reported in literature for production of γ-PGA are glutamic acid, citric acid, glycerol /glucose (or any other sugar) and a source of $NH_4^+$ (like ammonium sulphate or ammonium chloride) (Shih and Van 2001; Ogunleye *et al.*, 2015; Bajaj and Singhal 2011a). The L-glutamate independent bacteria are more preferable for industrial γ-PGA production due to their low cost of production and

simple fermentation process, but yields may not be as satisfactory and medium requirements can be expensive (Cao *et al.*, 2011). Although there have been many studies on the production, reports on the production of γ-PGA from agro-industrial waste materials have been limited.

Wang and co-workers studied the production of γ-PGA on solid state fermentation (SSF) in shaken flasks. The medium consisted of perlite, soybean, sweet potato peel and mineral salts using *Bacillus subtilis*. The average γ -PGA yield in SSF, using perlites, soybean curd and sweet potato residues as solid substrates was 3.63%/dw in 250 ml flask. Because of the high viscosity and water-absorbability of γ -PGA, the porosity of SSF became a critical factor. The perlite, an inert and non-nutritive material, was only used as the support giving enough room for oxygen, water, nutrition and heat transfer (Wang *et al.*, 2008).

In another study, large-scale SSF for the production of γ-PGA by *Bacillus amyloliquefaciens* C1 using agro-industrial residue was investigated. Dairy manure compost was selected as an abundant agro-industrial waste material. Due to lack of glutamic acid in the waste material, the medium was supplemented with residues from monosodium glutamate production. There was a lag phase in the fermentation up to 4 days, after which, the exponential growth phase was observed with a rapid increase in the biomass. The number of viable cells reached a maximum of $4.8\times10^9$ CFU/g after 30 days. The increase in γ-PGA production closely followed the growth pattern of the bacterium, which reached a maximum of 0.6% (w/w) after 20 days (Yong *et al.*, 2011).

In a recent study, γ-PGA production by *Bacillus subtilis* NX-2 was carried out through SSF with dry mushroom residues and monosodium glutamate production residues (MGPR: a substitute of glutamate). Dry shiitake mushroom residue (DSMR) was found

to be the most suitable solid substrate among the ones tested in 500 mL shake flasks containing 10 g of DSMR and 10 g of MGPR. In order to increase the production, industrial waste glycerol was added as a carbon source supplement to the solid-state medium. As a result, γ-PGA production increased by 34.8%. The batch fermentation produced 115.6 g/kg γ-PGA/dry matter (Tang *et al.*, 2015).

Due to the high glutamic acid and/or carbon source demand of the stains, selection of a suitable waste material has been a challenge, and in the studies conducted with waste materials, an additional glutamic acid source has been used to fulfil the needs of the organism.

In Table 1.8 some potential waste materials for production of γ-PGA are listed with their respective glutamic acid contents. Among these waste materials cotton seed flour has the highest glutamic acid content; however the other waste materials also show significant amounts and might prove suitable.

**Table 1. 8:** The glutamic acid contents of various waste materials.

| Waste Material | Glutamic acid Content (w% Dry Base) | Reference |
|---|---|---|
| Canola seed cake | 7.9% | (Bell and Keith 1990) |
| Coffee Pulp[2] | 7.7 %[1] | http://www.trc.zootechnie.fr/node/549 |
| Instant coffee by product[3] | 12.9%[1] | http://www.trc.zootechnie.fr/node/549 |
| Spirulina | 8.3% | http://nutritiondata.self.com/foods-000093000000000000000.html http://nutritiondata.self.com/foods-000093000000000000000.html |
| Orange/tomatoes | 8.0% | http://nutritiondata.self.com/foods-000093000000000000000.html |
| Broccoli Raab (rabe) | 7.9% | http://nutritiondata.self.com/foods-000093000000000000000.html |
| Cotton seed flour | 13.4% | http://nutritiondata.self.com/foods-000093000000000000000.html |
| Cotton seed cake | 1.96% | (Donkoh and Kotoku 2009) |
| Maize | 1.56% | (Donkoh and Kotoku 2009) |
| Maize Bran | 4.14% | (Donkoh and Kotoku 2009) |
| Rice Bran | 3.27% | (Donkoh and Kotoku 2009) |
| Wheat Bran | 3.85% | (Donkoh and Kotoku 2009) |
| Sunflower seed cake | 5.2% | (Mutayoba *et al.*, 2011) |
| Palm kernel cake | 3.2% | http://www.agriculturesource.com/p-palm-kernel-meal-923978.html |
| Brewer's spent grains | 4.64% | (Donkoh and Kotoku 2009) |
| Soy bean meal | 7.5% | (Mutayoba *et al.*, 2011) |
| Pumpkin seed meal | 4.9% | (Mutayoba *et al.*, 2011) |

[1]Percentage in total protein
[2]Contains 11.3% protein based on dry matter
[3]Contains 10.9% protein based on dry matter

### 1.3.4 Fermentative Production of γ-PGA

For commercial application γ-PGA needs to be produced in high yields and in large amounts. To be able to achieve high productivities, the requirements of the organisms need to be considered. Although each organism has its own limits, some can be pushed further to obtain much higher γ-PGA production with simple fermentation techniques or metabolic engineering.

Addition of inducers into the culture medium in small concentrations is one of the techniques to improve both product quantity and quality in γ-PGA fermentation. One of the techniques discussed in literature is the addition of NaCl. Several researchers reported that, addition of NaCl increases both the yield and the molecular weight of the γ-PGA, due to the change in ionic strength of the medium (Birrer *et al.,* 1994; Sung *et al.,* 2005; Ogawa *et al.,* 1997). Besides the effects on the yield and molecular weight, Ogawa and co-workers (1997) reported that addition of 3% NaCl to the medium maintained a stable cultivation and prevented foaming.

Wu and co-workers (2008) investigated production of γ-PGA using *B. subtilis* CGMCC 0833. In their study addition of DMSO or Tween-80 or glycerol to the medium stimulated the conversion of glucose to glutamate as the carbon flux from 2-oxoglutarate to glutamate was enhanced. In a medium composed of glucose, glutamic acid, ammonium sulphate, $K_2HPO_4$, $MgSO_4$ and $MnSO_4$, they reported a production of 34.40 g γ-PGA /L induced with Tween-80, 32.7g γ-PGA /L induced with DMSO and 31.7g γ-PGA /L induced with glycerol in comparison to un-induced conditions, 26.7 γ-PGA /L (Wu *et al.,* 2008).

An increase in the yield of γ-PGA was achieved by the addition of precursors for γ-PGA (Bajaj and Singhal, 2009a). When *B. licheniformis* NCIM 2324 was fed with L-

glutamine (0.07 g/l) and α-ketoglutaric acid (1.46 g/l) in addition to the basal medium, the γ-PGA yield increased from 26.12 g/L to 35.75 g/L.

In another study using *B. subtilis* R23, only α-ketoglutaric acid was seen to improve γ-PGA yield (Bajaj and Singhal, 2009b) while L-glutamine had no effect on the polymer yield. These studies demonstrate that addition of relatively inexpensive metabolic precursors can lead to a reduced cost in γ-PGA production.

Altering the operation conditions as well as operational modes is reported to be an alternative to modifying medium composition. Accumulation of γ-PGA in the medium makes the broth viscous. This affects mass transfer rates especially with respect to gas to liquid mass transfer. Several reports refer to the importance of oxygen transfer for the production of γ-PGA. When Cromwick and co-workers (1996) increased the aeration from 0.5 L/min to 2.0 L/min and agitation from 250 rpm to 800 rpm during production of γ-PGA with *B. licheniformis*, the yield increased significantly from 15 g/L to 23 g/L (Cromwick *et al.*, 1996). Another study conducted with the strain *B. subtilis* BL53 investigating the effect of stirrer speed and $K_La$, showed that an increase in the stirrer speed from 500 rpm to 1000 rpm increased the productivity from 0.3 g/L.h to 0.8 g/L.h and reduced the cultivation time by 50%. However the authors state that continuing the cultivation caused a decrease in γ-PGA concentration due to shear stress (da Silva *et al.*, 2014).

A similar study on the effect of impeller speed was conducted by Korean researchers. *B. subtilis* RKY3 was cultured under different agitation speeds as well as different aeration rates in a 7 L jar fermenter. The findings proposed that the production needed a certain amount of aeration. Under no aeration conditions no PGA was produced. However, when the aeration was increased from 0.5 L/min to 2 L/min by 0.5 increments

the production was observed to increase in all trials, but was slightly lower at 2 L/min. Similarly for agitation, when the speed was increased from 100 rpm to 400 rpm by increments of 100, the best results were obtained at 300 rpm. At 400 rpm the production was similar to 100 rpm but the cdw was higher compared to all other runs (Jeong *et al.*, 2014).

Another group of Korean researchers (Yoon *et al.*, 2000) reported a simple operation mode for production of γ-PGA with high productivity using cultures of *B. licheniformis* ATCC 9945 in a 2.5 L fermenter with a working volume of 950 ml. The medium used was composed of glutamic acid, glycerol, citric acid and $NH_4Cl$ and the fermenter was operated in fed-batch mode. Pulsed feedings of citric acid at a rate of 1.44 g/L.h and L-glutamic acid at a rate of 2.4 g/L.h resulted in the production of 35 g/L γ-PGA. The culture was maintained at pH 6.5 and air saturation was kept at 40%. The pulsed feeding regime started after 23 hours which corresponded to the time when citric acid was depleted. This strategy resulted in a productivity of 1 g/L.h (Yoon *et al.*, 2000). Similarly, Abdel Fattah and co-workers (2007) reported that pulsed feedings of citric acid to the cultures of *B. licheniformis* SAB-26 increased the γ-PGA production from 59.90 g/L to 88 g/L in a medium composed of Casein hydrolysate, $KH_2PO_4$ and $(NH_4)_2SO_4$ (Abdel Fattah *et al.*, 2007).

One of the recent methods, which has started attracting the attention of researchers, is integrated processes. In order to increase productivity and conversion rates, integration of fermentation to a unit operation can lead up to high purity products and yields. Kumar and Pal (2015) reported an integrated process for the production of γ-PGA from renewable carbon sources using continuous membrane integrated hybrid process. The continuous fermentation system integrated with a microfiltration and an ultrafiltration

unit ensured 96% recovery of the unconverted sugars and a yield of 0.6g/g sugar along with high product purity. The productivity achieved was 0.91 g/L.h with an average γ-PGA concentration of 36.5 g/L from the cultures of *B. licheniformis* NCIM 2324 (Kumar and Pal, 2015).

### 1.4 Dual Biopolymer Production

A significant problem to overcome in the industrial production of biopolymers is the high cost of raw material and relatively low product conversion rates (Valappil *et al*., 2008; Castilho *et al*., 2009). One approach to minimise the cost of raw materials is expanding the number of valuable products obtained from a single batch.

Simultaneous production of two or more valuable microbial products through the same process has always been desirable. This is due to the potential reduction in the overall cost and simplicity of operation (obtaining product from single fermenter compared to multiple). This bio-refinery approach, however, faces problems due to the diversity of microorganisms' demands to produce products. Whilst simultaneous or sequential production of biopolymers with another biopolymer or bio-product has been reported, in many cases this has been based on unplanned observation rather than prior deliberate design. In this context, there is great opportunity for investigation, leading to potential economic advantages for bio-industries. Simultaneous production of biopolymers is an attractive approach for a high profit bio-refinery.

#### 1.4.1 Dual Production with PHAs

Due to their vast range of applications and intracellular synthesis PHAs have attracted the attention of researchers for dual production. Intracellular production of at least one product provides the advantage in the separation of two products, the second being extracellular. Several researchers have investigated the potential of PHAs production

along with an extracellular product to exploit this advantage, underlining the scientific importance, but not commercial potential (Table 1.9).

The most common and abundant class of extracellular products in many bacteria is Exopolysaccarides (EPS). Lama *et al.* (1995) studied the effects of growth conditions on PHAs and EPS production in 1 L laboratory fermenter. However, what makes their study unique to other studies published is the production of two different intracellular biopolymers along with an extracellular EPS. The occurrence and characterization of P(3HB) was investigated in *Anabaena cylindrical* strain cultivated under different growth conditions in batch culture. It was reported that using appropriate precursors, the strain could accumulate P(3HB) and a co-polyester, which is a blend of poly hydroxyvalerate (PHV) and P(3HB) as hetero-polymer (P(3HB)+PHV). EPS production was also observed during this process. EPS was partially characterised and the importance of growth conditions was investigated for all products. A production of 0.012 g/L P(3HB)+PHV and 0.325 g/L EPS was reported using the standard medium for cyanobacteria (BG-11 medium), supplemented with acetate in a single-stage batch fermentation. Addition of acetate or glucose to the BG-11 growth medium enhanced P(3HB) biosynthesis, while the addition of citrate caused a strong decrease in P(3HB) content of the cells. For production of copolymer (P(3HB)+PHV), the medium was supported by propionate or valerate as carbon sources to initiate the hetero-polymer production. Addition of these substrates caused a large decrease in cell-yield. The presence of $NaNO_3$ resulted in a 7-fold increase compared to the control, but addition of fatty acids caused a sharp decrease in the production of EPS.

The physico-chemical factors influencing the production of P(3HB) and EPS by a yellow-pigmented *Azotobacter beijerinckii* was investigated (Pal *et al.*, 1999). Under

nitrogen-free conditions P(3HB) accumulation started after 20 hours and reached its maximum at the late exponential phase (around the $30^{th}$ hour) followed by a sharp decline, whereas EPS production was associated with cell growth. According to the report 2.73 g/L P(3HB) and 1.5 g/L EPS were produced from a single-stage batch fermentation. It was indicated that the P(3HB) production was carbon-source dependent while organic nitrogen sources enhanced both P(3HB) and EPS production. Inorganic nitrogen sources however had a negative effect on the production of both classes of biopolymers. In this strain, oxygen limited conditions favoured production of both P(3HB) and EPS, independent of carbon sources.

Quagliano and Miyazaki (1999) investigated production of P(3HB) and EPS with *Azotobacter chroococcum* strain isolated from soil samples. The effects of various carbon sources with and without the addition of ammonium were investigated. The addition of ammonium resulted in a reduction in both P(3HB) and/or EPS production in media containing glucose, fructose or sucrose. Also P(3HB) was accumulated only in cultures grown on glucose and sucrose. However, EPS was secreted copiously in the presence of fructose and glucose. When a complex carbon source, molasses, was used P(3HB) accumulated in the cells while EPS was produced from the start for 24 hours After 48 hours of fermentation, P(3HB) started to decay, but EPS continued to be produced. When sugar cane molasses was used, 2.75 g/L P(3HB) and 1.5 g/L EPS was obtained from the single stage batch fermentation.

Wang and Yu (2007) investigated different concentrations of glucose and $(NH_4)_2SO_4$ in batch cultures of *Ralstonia eutrophia*. Production of EPS occurred along with cell growth, whereas P(3HB) was produced only under nitrogen limited and cell growth limited conditions. Specific P(3HB) production-rate had an exponential correlation with

both specific cell growth-rate and EPS production-rate. Under statistically optimized conditions, using central composite design, the final production level reported was 12 g/L P(3HB) and 0.13 g/L EPS.

**Table 1. 9:** Examples of dual production with PHAs

| Strain | Product A | Product B | Medium | Product A (g/L) | Product B (g/L) | Operation Mode | Cultivation Type | Reference |
|---|---|---|---|---|---|---|---|---|
| *Anabaena cylindrica* | P(3HB)/PHV | EPS | BG11+acetate | 0.01 | 0.32 | Batch | single stage | Lama *et al*., 1995 |
| *Azotobacter beijerinckii* | P(3HB) | EPS | Glucose / Mannitol | 2.73 | 1.50 | Batch | single stage | Pal *et al*., 1999 |
| *Azotobacter chroococcum* | P(3HB) | EPS | Sugar cane molasses | 0.75 | 0.60 | Batch | single stage | Quagliano and Miyazaki, 1999 |
| *Ralstonia eutropha* | P(3HB) | EPS | Glucose / $(NH_4)2SO_4$ | 12.00 | 0.13 | Batch | single stage | Wang and Yu, 2007 |
| *Pseudomonas aeruginosa* | PHA | Rhamnolipid | palm oil | 0.79 | 0.43 | Batch | single stage | Marsudi *et al*., 2008 |
| *Corynebacterium glutamicum* | P(3HB) | Glutamate | Glucose | 7.00 | 18.00 | Batch | 2 stage | Jo *et al.,* 2009 |
| *Pseudomonas putida* | PHO | PHUE * | Octanoic acid / 10-undecenoic acid | 0.15 | 0.65 | Continuous | 2 stage | Hartmann *et al.*, 2010 |
| *Pseudomonas mendocina* | PHA | Alginate oligosaccharides | Glucose | 0.33 | 0.57 | Batch | single stage | Guo *et al.*, 2011 |
| *Haloferax mediterranei* | PHBV** | EPS | Glucose/yeast extract | 12.96 | 1.31 | Fed-Batch | single stage | Koller *et al.*, 2015 |
| *Sinorhizobium meliloti* | P(3HB)/PHV | EPS | Rice bran hydrolysate | 3.60 | 11.80 | Batch | single stage | Devi *et al*., 2012 |
| *Pseudomonas putida* | PHA | Gluconate | Glucose/ ammonium | 0.90 | 4.40 | Batch | single stage | Poblete-Castro *et al*., 2013 |

** Poly(3-hydroxy-10-undecenoate-co-3-hydroxy-8-nonenoate-co-3-hydroxy-6- heptenoate)*
*** Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate)*

Apart from EPSs, researchers have investigated the possibility to produce other industrially valuable products along with biopolymers. Marsudi and co-workers (2008) studied the utilization of palm oil for simultaneous production of PHAs and rhamnolipids by *Pseudomonas aeruginosa* in 3 L fermenter. It was shown that palm oil hydrolysed by lipase into fatty acids and glycerol were favourable carbon sources for production of PHAs and rhamnolipids respectively. *Pseudomonas aeruginosa* utilizes fatty acids through B-oxidation and glycerol *via de novo* fatty acid synthesis pathway. In their study, PHA and rhamnolipid production started after the nitrogen source was exhausted in the medium. Oleic acid and glycerol were used as carbon sources for simultaneous production of PHA and rhamnolipids. Overall, palm oil was the best carbon source for the simultaneous production of PHAs and rhamnolipids in a single stage batch culture and 0.79 g/L (36% PHA/cdw) PHA and 0.43 g/L rhamnolipid was reported to be produced from 7 g/L of palm oil.

Jo *et al.* (2008) manipulated the production of two different products by controlling the concentration of medium ingredients. In their study, P(3HB) and glutamate were produced by a two-stage fermentation of using variable biotin concentration in the *Corynebacterium glutamicum* medium. It was reported that when a low concentration of biotin (0.3 µg/L) was used, glutamate was produced. The production shifted towards P(3HB), by the addition of biotin at a concentration of 9 µg/L. As a final concentration, 7 g/L (36% PHA/cdw) P(3HB) and 18 g/L glutamate were produced using glucose as the carbon source.

While investigating the enhancement of PHA production by *Pseudomonas putida* using in silico-driven metabolic engineering, it was noted that significant amount of gluconate was produced along with PHA (Poblete Castro *et al.*, 2013) in 2 L bioreactor. The double production was not intentionally planned and it was fortuitous. This work is one of the

latest publications reporting double production. Here, 0.9 g/L PHA and 4.4 g/L gluconate were produced from single-stage batch fermentation.

The dual production of two different PHAs from *Pseudomonas putida* was reported using a two-stage chemostat system, (Hartmann *et al.,* 2010). The chemostat system was composed of two laboratory scale (3.7 L) bioreactors connected in series. The main variation from existing two-stage chemostat reactors was the use of different substrates in the two reactors in order to obtain two different biopolymers from a single process. The system had three feed inlets to supply fresh medium and two types of precursors were added into the culture for the production of biopolymers. The main inlet was assigned to the fresh medium which was added continuously to the first stage fermenter. The precursor for the PHA production was octanoic acid for the first fermenter and 10-undercenoic acid for the second fermenter. The precursors were pumped separately into the fermenters. Poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) (PHO) and poly(3-hydroxy-10-undecenoate-co-3-hydroxy-8-nonenoate-co-3-hydroxy-6-heptenoate) (PHUE) were successfully produced at levels of 0.155 g/L and 0.645 g/L respectively from the same strain. The overall polymer content was reported as 53.8% per cell dry weight, and a blend of the two types of PHA polymers with a structural (monomeric) purity of 85-95 mol % was obtained. It was found that the two-stage chemostat was a valuable fermentation system for the production of large amounts of PHA per cell and two different polymers could be produced in the same strain, which are easy to separate with simple solvent technology due to their different chemical properties. This is the only continuous culture study that has been reported in dual bio-polymer production.

One of the recent examples for dual production with PHAs was published by Koller and co-workers (2015). An archaeon *Haloferax mediterranei* strain DSM 1411 was used to study the production of two types of biopolymers. They reported that the production of two polymers compete with each other for the available exogenous carbon source. At the end

of 61.75 h fermentation they achieved 0.21 g/L.h and 0.02 g/L.h productivity for PHBV and an EPS respectively. An interesting point to note was that due to the high salinity conditions of the medium, the process did not require sterilisation which is a great advantage for industrial application.

### 1.4.2 Dual production with other bio-products

Other examples of dual production include EPS and other bio-products (Table 1.10). Production of two different exopolysaccarides by *Pseudomonas* sp. was investigated by Christensen *et al.* (1985) in 5 L fermenters. One of the exopolysaccarides, which was named EPS A, was produced during the exponential phase and contained in its structure glucose, galactose, glucuronic acid and galacturonic acid. The polymer produced viscous solutions, forming gels at high concentrations. The other polymer was called EPS B. It was released at the end of the exponential phase and in the stationary phase, contained N-acetyl glucosamine, 2-keto-3-deoxyoctulosonic acid, an unidentified 6-deoxyhexose and O-acetyl groups. It also produced aqueous solutions of low viscosity. The highest production was 0.10 g/L for EPS A and 0.14 g/L for EPS B using glucose and yeast extract as carbon and nitrogen sources.

Samain and co-workers (1997) studied an EPS, namely EPS 1664, production by a marine bacterium *Alteromonas* sp. in 2L fermenters. Under nitrogen limited conditions, the strain was capable of producing exopolysaccarides; one was secreted into the medium and the other one was cell-membrane-associated. It was shown that the cell-membrane-associated polysaccharide was different from the secreted one and was named as EPS 1664B. The polymer productions started simultaneously after 10 hours following the depletion of nitrogen in the environment. When fed-batch culture was used with a continuous nitrogen supply, keeping nitrogen concentration to a minimum level (state), both polymers were produced in the presence of nitrogen. By using fructose/ammonium chloride medium in a

fed-batch culture, 7.5 g/L of secreted polysaccharide and 5 g/L of the cell-membrane-associated polysaccharide were produced.

Liu *et al.*, (2004) used cheese whey as a substrate to simultaneously produce nisin and lactic acid. Production was optimized using statistically based experimental design, in a whey-based medium using *Lactococcus lactis*. Significant parameters were screened using a Placket-Burman design, and the selected parameters were optimized using a central composite design. As a result 19.3 g/L lactic acid and 92.9 mg/L nisin were produced in a medium containing cheese whey, yeast extract, $MgSO_4$, and $KH_2PO_4$.

**Table 1. 10:** Examples of dual production of biopolymers with other bio-products

| Strain | Prod. A | Prod. B | Medium | Prod. A (g/L) | Prod. B (g/L) | Mode | Stage | Reference |
|---|---|---|---|---|---|---|---|---|
| *Pseudomonas sp.* | EPS A | EPS B | Glucose / Yeast extract | 0.104* | 0.14* | Batch | single stage | Christensen *et al.*, 1985 |
| *Alteromonas sp.* | EPS 1664 | cell-associated EPS (1664B) | Fructose / amonium chloride | 7.50 | 5.00 | Fed-batch | single stage | Samain *et al.*, 1997 |
| *Lactococcus lactis* | Nisin | Lactic acid | Whey | 0.92 | 19.30 | Batch | single stage | Liu *et al.*, 2004 |
| *Bacillus subtilis* | Levan | γ-PGA | Sucrose/ L-glutamate | 5.04 | 6.96 | Batch | single stage | Shih and Yu, 2005 |
| *Bacillus subtilis / Streptomyces albulus* | Levan | Poly-e-lysine | Sucrose | 60.00 | 4.37 | Batch | Sequential 2 stage | Shih *et al.*, 2011 |
| *Bacillus subtilis* | γ-PGA | Fibrinolytic enzyme | Molasses/MSG waste | 103.5 g/kg* | 986 U/g** | Solid Sate F. | Single stage | Zeng *et al.*, 2013 |
| *Pseudomonas aeruginosa* | EPS | Lipase | Sunflower oil/Tween 80 | 0.36* | 2.00 U/ml** | Batch | single stage | Dimitrijevi´c *et al.*, 2011 |

**The highest concentration of each product corresponding to different fermentation times*
***Maximum activity at different fermentation times*

Other examples on dual production involve levan, and lactic acid; the latter being the precursor for Polylactic acid (PLA) production. Shih and Yu (2005) studied simultaneous

production of levan and polyglutamic acid (PGA) from *Bacillus subtilis*, both polymers were produced in a medium containing sucrose and glutamic acid. However, when one of the two substrates was absent, the organism produced only one of the polymers. Levan, 5 g/L, and PGA, 6.9 g/L, were produced in a single batch fermentation by optimising sucrose and glutamic acid concentrations.

Dual production has been observed also in a similar batch in a sequential fermentation. Shih and co-workers (2011) investigated the potential of sequential fermentation for the production of two biopolymers, levan and poly-e-lysine (e-PL) in a 5 L fermenter. *Bacillus subtilis* (natto) was cultivated in sucrose medium to produce levan. The product and the cells were separated and the remaining broth containing small sugar molecules was used for the production of e-PL by *Streptomyces albulus*. Using a two-stage process, 60 g/L levan and 4.37 g/L e-PL were produced in 72 hours. The main novelty in their work was the significant reduction in solvent usage for biopolymer recovery, as well as the use of waste media from the first stage fermentation to produce a second valuable product. Overall, the process is described as green and environmentally friendly with a strong bio-refinery concept.

Zeng and co-workers (2013) investigated the co-production of γ-PGA and fibrinolytic enzyme by *Bacillus subtilis* GXA-28 strain. Different from the other studies reported, they used solid state fermentation on tray with soybean residue, sugar cane molasses and mono sodium glutamate waste liquor as substrate. The strain used was thermophilic (cultivation at 50°C) which allowed production under sterilised or non-sterilised conditions. The authors reported that no visible difference was observed in cell growth and enzyme production between sterilised and non-sterilised fermentation but the γ-PGA maximum production was higher in non-sterilised fermentation. Overall the highest enzyme activity of 986 U/g-substrates after 24 h, and 103.4 g γ-PGA/ kg substrate after 22 hours was reached.

Dimitrijevic *et al.* (2011) investigated a *Pseudomonas aeruginosa* strain capable of simultaneous production of EPS and lipase. An alginate-like EPS, along with lipase, was produced in a single fermentation and the EPS was used to encapsulate lipase in order to investigate the potential of using the system for immobilization applications. Sunflower oil and Tween 80 were used as raw materials and 0.36 g/L EPS and 2 U/ml lipase were produced from a single-stage fermentation. This is the first report that mentions an alginate-type EPS produced by *P. aeruginosa*. It reports, at the same time, a possible application area for EPS, as a support for immobilization of lipase produced by the same strain.

### 1.5 Separation of Dual Products from Fermentation Medium

One of the most significant costs of bioprocesses, which directly affects to the final price of the product, is downstream processing. In recent studies, separation and purification of the product in general was calculated to be $1/5^{th}$ of the whole production costs (Zahari *et al.*,2015). Although this ratio is very much dependent on the nature of the product, complexity of the fermentation broth and number of steps required, it is useful as a rough estimate showing the extent of the costs. As discussed in Section 1.4, dual production of biopolymers offers a great advantage towards reducing the production costs. However, as also presented in Section 1.4.1 (on page 49, Table 1.9), most of the research conducted on dual polymer production has been concentrated on the production of an intracellular and an extracellular product. This is to facilitate easy recovery of the product from the fermentation medium and to separate the two products from each other without introducing an additional step. But on the other hand, since both, the broth and the biomass, contain a valuable product, separation and recovery of both products, with minimum loss, need to be conducted carefully.

Upon completion of PHA production in the culture, separation of cell mass from the liquid phase of the cultivation broth is conducted by well-established techniques like

sedimentation, filtration, centrifugation, or flocculation (Koller *et al.*, 2013). Recovery of PHAs from non-PHA mass requires substantial and often underestimated cost. Hence, product recovery (downstream processing) displays a defining process step in PHA manufacturing (Koller *et al.*, 2013). Recovery and purification of PHA containing cells are comprised of 4 steps. These methods, in the order of process, are;

- The separation of the PHA rich biomass,
- The extraction of the polymer from the separated biomass,
- Concentration and purification of PHA, and
- Precipitation and drying.

In the case of dual production, among the steps listed, separation of biomass is the last step where biomass containing one of the products is in contact with the fermentation broth which contains the other product in dissolved form. Therefore, design of this step is crucial in order to ensure that both products are recovered fully. Among the separation methods mentioned previously (sedimentation, filtration, centrifugation and flocculation), the ones suitable to recover both products present in biomass and liquid fraction, are centrifugation, floatation and sedimentation. Furthermore, the requirement of dry products with strict impurity levels suggests a filter centrifugation (Todaro and Vogel, 2014). To underline the advantages, disadvantages as well as opportunities, this section will focus on solid liquid separation techniques from the point of view of dual polymer separation.

### 1.5.1 Centrifugation

One of the most well established and widely used solid-liquid separation methods is centrifugation. Centrifuges are used in many manufacturing industries to separate suspended solids from liquid utilizing the centrifugal acceleration of the suspended particles directed outward from the axis of rotation. This force initiates the particle movement to the centrifuge periphery where it is trapped or contained by the wall of the

rotating body. Much experience and information related to industrial-scale centrifugation exists within companies manufacturing the centrifugal machinery and these sources should not be overlooked when seeking information.

Centrifugal separation may be applied to mixtures for different reasons. Possible scenarios for centrifugal separations are summarised in Fig 1.10.

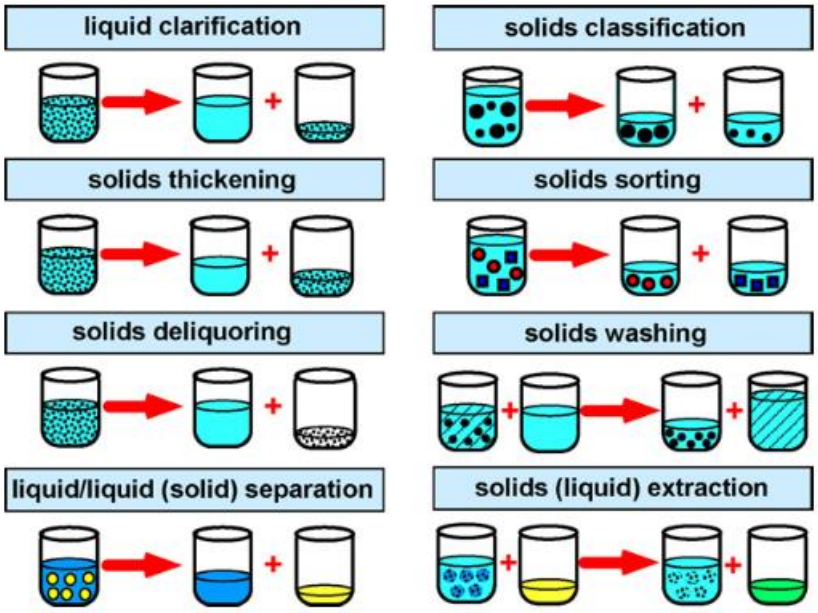



**Figure 1. 10:** Tasks for centrifugal separation (Todaro and Vogel, 2014).

In dual production systems the aim is “solids de-liquoring” and possibly “solids washing” where the solid fraction has to be removed intact from the mixture “free” of the liquid fraction (in which the other product is solubilised), while the liquid fraction should be separated without much loss. This is because the valuable product needs to be further extracted from the liquid. Different types of centrifuges, their particle size range and maximum separation acceleration are given in Table1.11.

**Table 1. 11:** Characteristics of Separator Types (Wang *et al.,* 1979).

| Type | Mode of solids discharge | feed flow rate (L/h) | Feed solids content (% by vol.) | Solids flow rate (L/h) | G-force developed | Consistency of solids |
|---|---|---|---|---|---|---|
| Disk solid bowl | Manual | 20-100,000 | <1 | 0 | <10,000 | Firm paste |
| Solids ejecting radical | Intermittent | 20-100,000 | <25 | <3,000 | <14,500 | Thick flowing slurry |
| Solids ejecting axial | Intermittent | 1,000-150,000 | <15 | <1,000 | <15,000 | Thick flowing slurry |
| Nozzle, pressurized discharge | Continuous | 1,000-180,000 | 4-30 | between 150-40,000 | <15,000 | Thick flowing slurry |
| Peripheral nozzle | Continuous | 300-250,000 | 2-30 | between 3,000-60,000 | <11,000 | Thin slurry |
| Decanter centrifuge | Continuous with scroll | 20-120,000 | 5-50 | <50,000 | <10,000 | Thick slurry to semi solid |
| Tubular bowl | Manual | 20-7,000 | <1 | 0 | <31,000 | Firm paste |
| Multichamber bowl | Manual | 100-20,000 | <5 | 0 | <9,000 | Firm paste |
| Centritech machines | Intermittent | 5-100 | <1 | <15 | <100 | Very thin slurry |
| Inverted solid bowl | Intermittent with scrapper | 10-6,000 | 1-30 | <1,000 | <20,000 | Firm paste |

In the case of double product separation of bacteria from the fermentation broth, considering the bacterial cell size varies between 2-8 µm (Weart *et al*., 2007), the most efficient type of centrifuges are disc, tubular and solid bowl. Among these, disc and tubular centrifuges can be operated in continuous mode but solid bowl cannot. In continuous operation, solids leave the separator with a considerable fraction of liquid. However, in the dual production where there is also a product in liquid fraction, this means loss of the product in the liquid phase. In batch separation (bowl centrifuge) the bowl needs to be emptied manually which might cause a loss in solid matter, and therefore the product itself. In addition, in centrifugal separation the solid material is usually washed in order to minimise impurities carried with fermentation broth. However in the dual production this can cause dilution of the liquid fraction which might cause additional cost in the next steps

of downstream processing. Thus, all of these aspects need to be considered when centrifugation is used to separate two products (solid and liquid) in fermentation broth.

### 1.5.2 Floatation

Floatation of particles is a process for selectively separating hydrophobic particles from hydrophilic. This is used in several processing industries. Historically this was first used in the mining industry, where it was one of the great enabling technologies of the 20$^{th}$ century. The development of froth flotation improved the recovery of valuable minerals, such as copper- and lead-bearing minerals. Along with mechanized mining, it allowed the economic recovery of valuable metals from much lower grade ores than before (Clayton *et al*., 1991).

In literature, the flotation process is generally described using three sub-processes. First an effective collision between a particle and an air bubble has to take place. This process is driven by the hydrodynamics near the rising air bubbles. Then, if there is a net attractive force between the two objects, attachment of the particle to the air bubble can take place (Figure 1.11). Counteracting, when a particle–bubble complex has been formed it may be broken up (Van Hee *et al*., 2006). Flotation is a successful unit operation especially when the density difference ($d_P$ - $d_L$) between the liquid ($d_L$) and the particles ($d_P$) is near to zero. When the particle is attached to the air bubble the apparent density decreases and density difference becomes negative, so according to Stokes Law the "settling velocity" of the particle changes direction (becomes negative) and the particle is able to float. The size and the velocity of the bubbles are parameters defining the efficiency of the separation.

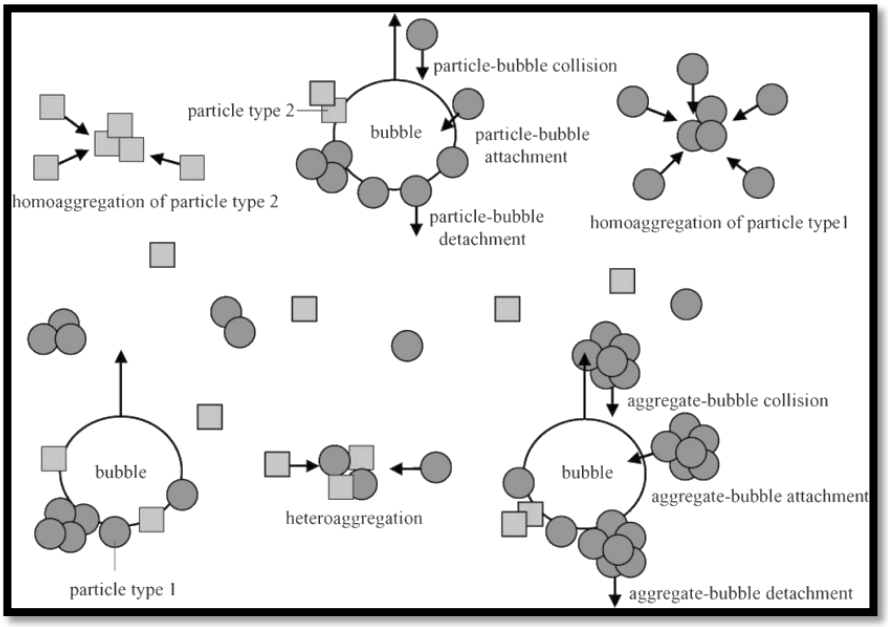



**Figure 1. 11:** Schematic representation of the attachment processes in particle-bubble and particle-particle interactions (Van Hee *et al*., 2006).

Air flotation of particles has been applied in biotechnology for the separation of whole cells from the fermentation medium. In this context different areas have been investigated including the relationship between contact angle of the particle with air-water interface, and cell adhesion to an air–water interface (Chattopadhyay *et al*., 1995) floatability of yeast cells in relation to medium conditions and yeast strain (Palmieri *et al*., 1996; Oliveira *et al*., 2003), and flotation of bacterial cells (Sklodowska *et al*., 1998; Vlaski *et al.*, 1996). In mineral and plastic recovery processes, selective flotation has been successfully applied for particle–particle separation (Shen *et al.*, 2001).

A low-cost alternative for separation of PHAs could be floatation. Ivanov *et al.* (2015) discussed the use of floatation for separation of PHA granules from disrupted cells. As a simple treatment, flotation must be performed with the air bubbles of small diameter and intensive aeration to have the sufficient specific surface area for PHA granule adhesion and a fast foam formation rate for its continuous removal. For the effective batch or continuous flotation, there must be an automatic liquid level and the liquid supply rate control.

Furthermore, the level of foam and the foam removal rate in the flotation tank have to be controlled to ensure proper separation of biomass and PHA granules (Ivanov *et al*., 2015).

Another way for PHA granule separation and concentration is dissolved air flotation (DAF) in batch mode. This method involves supply of compressed air into the tank together with a suspension of bacterial biomass to create dissolved oxygen saturation under high pressure (ei. at 5 atm). This suspension is subsequently fed into the flotation tank at atmospheric pressure. The smallest air bubbles are released in the flotation tank due to the difference in gas solubility at the high and the atmospheric pressures. These small-sized air bubbles adsorb the most hydrophobic particles at their surfaces and float them forming the foam layer. The foam, containing concentrated hydrophobic substances, is removed and collected. Van Hee and co-workers (2006) efficiently applied DAF for separation of PHA granules from disrupted *Pseudomonas putida* cells. They report the bubbles of air have the hydrophobic surface, when the pH of the solution containing PHA granules, is adjusted to about 3.0 - 3.5, the difference in hydrophobicity of the surface of PHA granules and cell walls ensures the preferable adhesion of PHA granules onto the air bubbles and facilitates the formation of granule aggregates. Using this concept, a purity of 86% w/w was obtained in three consecutive batch flotation steps. The efficiency of this flotation is affected by the air bubbles, cells, and PHA granules interactions as well as flow rates of liquid and foam, sizes of the particles, hydrophobicity, and surface charge (zeta potential) which is depended on the pH. In addition theoretical calculations showed that the purity in this process can reach up to 95% w/w (Van Hee *et al*., 2006).

### 1.5.3 Sedimentation

Sedimentation is the tendency for particles in suspension to settle out of the fluid in which they are entrained, and come to rest against a barrier. This is due to their motion through the fluid in response to the forces acting on them: these forces may be due to gravity, buoyancy, centrifugal acceleration, electromagnetism or a combination. Sedimentation is

applied in many chemical engineering operations and processes such as filtration (as a pre-treatment), fluidization, two- phase flow and environmental engineering (McCabe *et al.*, 1993; Senthilkumar *et al.*, 2008).

The rate data of sedimentation process is very important for designing equipment for separating suspended solid particles from liquid or gas stream (Mondal and Majumdar, 2004). Industrial sedimentation operations may be carried out batch- wise or continuously using thickeners. Viscosity of the medium is the main physical property, which affects the sedimentation. Density of particles is again an important factor that affects their settling velocity. As the density of the particles increase, the settling rate is also increase. Batch sedimentation is employed for bio-processing or the process fluids in recycle. Batch sedimentation is mainly used in pharmaceutical industries and continuous sedimentation is used in waste water treatment applications (Senthilkumar *et al*., 2008).

As a particle settles in a fluid, it accelerates until the drag force due to its motion equals the submerged weight of the particle. At this point, the particle will have reached its terminal velocity, $V_p$. If free settling, there are three forces acting upon a particle. These forces are, gravitational force ($F_G$), drag force ($F_D$) and buoyancy force ($F_B$) (Figure 1.12)

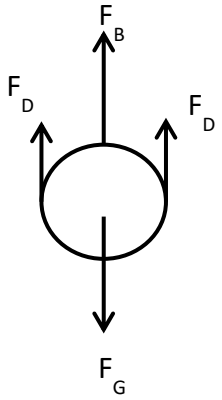



**Figure 1. 12:** Forces acting on a particle in case of free settling.

Sedimentation theory predicts that, in ideal settling, the settling velocity depends on six parameters. The settling velocity is a function of these parameters as given by Stokes law (Steinour, 1944) presented in equation 1.1;

$$Vs = \sqrt{\frac{2(\rho_p - \rho) g\, V_p}{C_D A_p \rho}} \quad (1.1)$$

Where $V_s$ is the settling velocity of the particle, $\rho_p$ is the density of the particle, $\rho$ is the density of the liquid, $V_p$ is the volume of the particle, g is the gravitational acceleration, $A_p$ is the projection area of the particle and $C_D$ is the drag coefficient. $C_D$ is inversely proportional to the Reynolds number ($N_{Re}$) and the relation is given in equation 1.2.

$$C_D = \frac{24}{N_{Re}} \quad (1.2)$$

Where;

$$N_{Re} = \frac{v\rho D}{\mu} \quad (1.3)$$

In the equation 1.3, v is flowrate (m/s) and μ is the liquid viscosity (Pa.s). So, to increase the settling velocity either density of the liquid or diameter of the particle or drag coefficient need to be decreased. Since drag coefficient is inversely proportional to Reynolds number, increasing Reynolds number will decrease the drag coefficient. Since the properties of the liquid is determined by the fermentation broth in the production vessel, the dimensions of the container can only be altered where the liquid is subjected to sedimentation. When the diameter of the particle becomes significant with respect to the diameter of the container, where particle-particle and particle-wall hydrodynamic interaction effects are of the same order, the particles tend to get forced away from the wall through something known, as wall effect (Di-Felice and Parodi 1996). In the case of bacterial separation, this can be observed when the container diameter is very small or the bacterial suspension has a high cell density (concentrated) (Richardson and Zaki 1997). In

order to compensate for this, the flow/settling conditions, the flow regime (laminar or turbulent), the diameter of the particle and the diameter of the container need to be known and selected accordingly. In addition the viscosity of the liquid plays an important role and needs to be considered (Richardson and Zaki 1997; Garside and Al-Dibouni, 1977).

One of the most important parameters determining the settling conditions is the liquid viscosity. As discussed in Section 1.4.1, most of the research conducted on the dual polymer production has been concentrated on the production of one intracellular and one extracellular product in order to facilitate easy recovery of the product from the fermentation medium. As the viscosity of the extracellular products change during the course of fermentation, suitable separation methodologies need to be adopted to cater for this. When the product concentration is high, for example in production of γ-PGA, the fermentation broth can become highly viscous hence affecting the settling velocity. Due to the viscous nature of fermentation broth, sedimentation parameters need to be considered for high velocity solutions. For example, Di-Felice and Parodi (1996) have studied the wall effect on sedimentation velocity of suspended particles in viscous solutions. In their study, wall effect had a considerable influence on settling velocity only for dilute suspensions in viscous solution. In concentrated solutions the wall effect was not observed. Although the effect was clearly demonstrated by their results, the reasons behind this phenomenon have not been discussed by the authors (Di-Felice and Parodi 1996).

Sedimentation serves as one of the alternatives for the separation of cellular material from fermentation broth. Despite the long times required for settling bacterial mass and their separation from the liquid fraction, what gives sedimentation an advantage over centrifugation is the capability of separating particles with different properties at different levels of the sedimentation vessel. In order to obtain distinct zones, settling velocities and settling conditions needs to be identified.

### 1.5.4 Application of Static Magnetic Field in Fermentation

Use of magnetic field in bioprocesses has attracted attention of researchers since early 70's; however, the main focus has been on the use of magnetic beads in fermentation broth for separation. These beads are added directly to the fermenter and the target product (either a molecule or biomass) gets attached onto the beads via different mechanisms such as adsorpsion or covalent bonding and is then separated from the broth by applying a magnetic field. This approach has been utilised in pharmaceutical industry, enzyme separation and cell sorting applications and magnetic particles of different shapes and sizes have been developed as magnetic carriers (Ito et al., 2005).

On the other hand, our current understanding of the impacts of static magnetic field on living cells is still limited (Velizarow, 1999). Although there are different publications reporting the effects of static magnetic fields on different species and mamalian cells (Hong, 1995; Miyakoshi, 2005), there are very few studies discussing the effects on microbial cells. An E. coli mutation assay was used to assess the mutagenic effects of strong static magnetic fields (Zhang et al., 2003). Various mutant strains of E. coli were exposed up to 9 T for 24 h and the frequencies of mutations were then determined. The results for survival or mutation obtained with the wild-type E. coli strain GC4468 and its derivatives defective in DNA repair enzymes or redox-regulating enzymes showed no effect of exposure. On the other hand, the mutation frequency was significantly increased by exposure to a static magnetic field of 9 T (Zhang et al., 2003).

It is suggested that exposure to mild static magnetic field activates ions within the cell membrane, which in turn makes the cell membrane leaky. Galvanovski and Sandblom (1998) proposed that static magnetic field affects calcium signalling pathways, and particularly cytosolic calcium oscillators, which cause Ca2+ release to the medium. Fojt et al., (2004) discussed the effects of static magnetic field on bacteria and argued that the main theories which try to explain the biological effects of electromagnetic fields are based

on the possible effects on the permeability of the ionic channels in the membrane. This can affect ion transport into the cells and result in biological changes in the organisms (Fojt et al., 2004).

Based on the limited literature reporting the possible effects of magnetic field on microbial cell membranes, the utilisation of static magnetic field on PHA producing bacteria was investigated in this thesis. Exploitation of static magnetic field as a possible tool in downstream processing, making cell membrane leaky resulting in the release of PHA granules was investigated.

### 1.6 Aims and Objectives

This thesis focuses on the dual production of two commercially important polymers PHA and γ-PGA. A common denominator between the two types of polymers (PHA and γ-PGA) is that they can both be produced by *Bacillus* sp. The main difference between the two polymers, in terms of production, is that γ-PGA is secreted out of the cell but PHAs are accumulated inside the cell. Using a Gram-positive strain, which does not contain LPS, makes it a better source of PHA for biomedical applications. Since both polymers are valuable, and are produced by the same genus, it may be possible to produce both of them simultaneously in a single fermentation. This can cut down the production costs by up to 50%.

The aim of this project is the dual production of biopolymers, PHA and γ-PGA, from cheap substrates with the view to lay grounds for a feasible, innovative, low cost production process for the two commercially important polymers.

To address the aim, the following objectives were investigated:

- To select a strain which is capable of producing both polymers (PHA and γ-PGA).

- To select an agro-industrial waste suitable for the fermentation process producing both polymers (PHA and γ-PGA).
- To optimize medium components via statistical optimisation tools and the culture conditions.
- To produce both polymers (PHA and γ-PGA) in a single fermentation process.
- To select and propose a separation method to recover the two polymers cheaply.

For the novel aspects of this thesis; dual production of the two commercially important polymers (PHA and γ-PGA together), from a single batch was not studied before as presented in Section 1.4. The utilisation of an agro-industrial waste material for dual production is an additional asset in terms of cost reduction in the process. Furthermore, an area which has been neglected by researchers, studies on economic separation of two products cheaply are presented in this thesis with some findings which will benefit other researchers in their future work.

# Chapter 2: Materials and Methods

## 2.0 MATERIALS AND METHODS

### 2.1 Materials

All materials used in this study were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK) unless otherwise stated. All qualitative and quantitative assays were carried out using analytical grade reagents.

High pressure liquid chromatography (HPLC) and gas chromatography (GC) assays were carried out using HPLC and GC grade chemicals and 18.2mΩ water.

### 2.2 Cultures

#### 2.2.1 Bacterial Strains

The recombinant strain, *Bacillus subtilis* OK2 was obtained from the University of Westminster, London, UK, culture collection, with the courtesy of Prof. Fujio Kawamura, Department of Life Sciences, Rikkyo University.

*Bacillus 46.2, Bacillus 2d.1, Bacillus 159 and Bacillus 20.4* were kindly obtained from Ege University, Engineering Faculty, Department of Bioengineering culture collection.

#### 2.2.2 Maintenance of Cultures

Stock cultures were grown at 30°C in nutrient broth and were maintained at 4°C on nutrient agar slants. Vials were frozen in 20% glycerol and kept at -18°C.

### 2.3 Culture Media and Growth Conditions

#### 2.3.1 Inoculum Preparation

Nutrient broth (20 ml) in a 50ml shake flask was inoculated with a loop full of *Bacillus subtilis* OK2 from nutrient agar slant and was incubated for 6 h at 30 $^{o}$C. This culture (5%

v/v) was transferred into 50 ml of respective production media and was incubated for approximately 17 h at 30 $^{o}$C prior to utilisation as inoculum in each experiment.

### 2.3.2 Fermentation

#### P(3HB) Production

A defined culture medium for P(3HB) production was used as previously reported by Valappil *et al.* (2008). The medium contained; 20 g/L glucose, 2.5 g/L yeast extract, 3 g/L KCl, 5 g/L $(NH_4)_2SO_4$, and 1g/L Soytone (enzymatic digest of soybean meal). The culture was incubated at 30$^{o}$C and 200 rpm for 72 h.

#### γ-PGA Production

A medium containing 20 g/L citric acid, 30 g/L mono sodium glutamate, 15 g/L $(NH_4)_2SO_4$, 20 g/L glycerol, 1 g/L $K_2HPO_4$ and $Na_2HPO_4$, 0.5 g/L $MgSO_4$, 0.2 g/L $CaCl_2$, 0.05 g/L $FeCl_3$ and 0.02 g/L $MnSO_4$d, Medium E (Leonard *et al.,* 1958) with some modifications, developed in-house was used for γ-PGA production.

The culture was incubated at 37 $^{o}$C and 180 rpm for 48 h after inoculation.

#### Double Polymer Production

Double polymer production medium optimised during the study was used throughout the experiments. The medium was composed of 32 g/L glutamic acid, 20 g/L glucose, 12 g/L $(NH_4)_2SO_4$, 1.5 g/L yeast extract, 2.4 g/L citric acid. The culture was incubated at 30 $^{o}$C and 200 rpm for 48-72 h after inoculation, depending on the experiment.

## 2.4 Equipment

### 2.4.1 Fermentation Equipment

The fermenter was Electrolab FerMac310/60 equipped with automated control. The experiments were carried out in a 0.5 L or 2 L batch reactors equipped with turbine type impellors. During the fermentation, pH, temperature and dissolved oxygen (%DOT air

saturation) were monitored or controlled depending on the experiment requirements. Samples were taken against time. The culture optical density at 600 nm, P(3HB) and γ-PGA concentrations, cell dry weight (cdw) and total carbohydrate content were determined by using the samples taken from the fermenter at intervals. The exhaust gas from the fermenter (%$CO_2$) was analysed using FerMac 368 Gas Analyser. The gas analyser was calibrated using a standard gas mixture containing 5.09 % $CO_2$, 18.28 % oxygen, and 76.63 % nitrogen.

### 2.4.2 Other Equipment

Nitrogen content was determined using Gerhardt Kjeldahl apparatus. The GC used was Thermo Fisher Corporation; Milan, Italy; freeze dryer was Thermo Savant ModulyoD. Nanoparticle analysis was carried out by NanoSight LM10, NanoSight Ltd., UK. The samples were centrifuged in Denley BS400 Centrifuge (4490 g). The balances used were a VWR and a Sartorius-steadim analytical balance. pH meter used was Metler-Toledo S230 pH/conductivity meter. Spectrophotometric measurements were taken using Jenway 6503 UV-vis spectrophotometer. Cultures were incubated in Inova 4430 orbital incubator shaker. Microscopic observations were carried out using a Nikon phase contrast microscope.

## 2.5 Waste materials

### 2.5.1 Types of Waste Materials

Four different agro-industrial waste materials namely, rapeseed cake (RS), orange peel (OP), wheat bran (WB), and *Spirulina* powder (S) were used. All the waste materials were obtained from local sources and were used in dried powder form. Powdered orange peel was purchased from Amazon UK in dried powder form.

### 2.5.2 Pre-treatment of the Waste Materials

Four different pre-treatment methods were tested. As the first step in pre-treatment operations, all waste materials were suspended in water at a concentration of 10 g/L.

Following the pre-treatments, all the media were filtered, pH adjusted to pH7 and sterilised at 110°C for 10 min prior to inoculation.

#### Mild Acid Treatment

Waste materials were subjected to mild acid conditions (Coded as A), by adjusting the pH value to pH 3.5 using concentrated sulphuric acid and were incubated at 70°C and 100 rpm for 2 h.

#### Mild Alkaline Treatment

Waste materials were subjected to mild alkaline conditions (Coded as B), by adjusting the pH value to pH 10.5 using 4M sodium hydroxide, and were incubated at 70 °C and 100 rpm for 2 h.

#### Water Suspension Treatment

Waste materials were suspended in water (Coded as C). The pH was not controlled. The samples were incubated at 70°C and 100 rpm for 2 h.

#### Microwave Treatment

Microwave treatment of the waste was performed at 800W for 2 min (Coded as D). The pH was not controlled prior to or during the treatment.

## 2.6 Analytical Methods

### 2.5.1 P(3HB)

#### Extraction of P(3HB)

For the extraction of P(3HB), the cells were harvested by centrifugation at 6000 rpm (4490 g) for 10 min and then were freeze dried. P(3HB) was extracted by treating 1 g of the freeze-dried cells with a mixture containing 50 ml of chloroform and 50 ml of a 30% sodium hypochlorite solution in an orbital shaker at 100 rpm and 37$^{o}$C for 1 hour. The mixture obtained was centrifuged at 4000 rpm (2220 g) for 10 min which resulted in three separate phases. The P(3HB) was recovered from the bottom phase, i.e. that of chloroform, by precipitation using 10 volumes of ice-cold methanol (Valappil et al., 2008)

#### Crotonic Acid Method for P(3HB) Determination

For the identification of the P(3HB) content, a slight modification was made to the crotonic acid method of Law and Slepecky (1961). The chloroform phase (100 µl) obtained from the extraction stage was transferred into a clean tube, air dried and mixed with 5 ml concentrated sulphuric acid. The tube was incubated in an 80 °C water bath for 1 h. It was mixed vigorously, but intermittently before incubation, half-way through and after incubation. The absorbance of the crotonic acid concentration converted from P(3HB) was measured at 235 nm and the concentration was calculated using a calibration curve (Appendix, Section 8.1). This method was used to quantify the amount of P(3HB) produced throughout this thesis.

#### Sudan Black Test for P(3HB)

*Bacillus* species were grown on nutrient agar plates as a single colony. After 48 h of incubation, approximately 8 ml Sudan black solution (0.2% Sudan black B in 96% ethanol) was added to the plates and the plates were incubated for 10 min. Subsequently, the dye

was decanted and plates were gently rinsed with 10 ml 100% ethanol. Inclusions dyed in the middle of the colonies were visualised by naked eye (Ghate *et al*., 2011)

**P(3HB) Determination by Gas Chromatography (GC)**

Freeze-dried biomass was ground to a powder and put into a gas-tight screw-capped tube. Acidified methanol (2 ml, 3% v/v sulphuric acid, 2.5 g/L methyl-benzoate) and chloroform (2 ml) were added. The methylbenzoate was used as an internal standard to improve accuracy. The tube was closed and kept at 90-100°C for 5 h. After rapid cooling, 4 ml distilled water was added to achieve a good phase separation. The sample was vortexed for 10 seconds and the chloroform-phase was then collected from the bottom and filtered through a PTFE-filter and 1 µl was injected into a gas chromatograph (GC) equipped with flame ionization Detector and Agilent DB-WAX capillary column 30 m × 0.25 mm × 0.25 µm, with gas flow at 1.5 ml/ min, helium (He) as carrier gas and nitrogen ($N_2$) as make-up gas. Column temperature range was 100-220°C, temperature gradient was 15°C/ min at 250°C inlet temperature, 300°C detector temperature and Split ratio of 20. (Braunegg *et al*., 1978)

A calibration curve was drawn using pure P(3HB) from solvent extraction. The P(3HB) content was determined from the peak-areas of the methyl-3-hydroxybutyrate and the standards (Appendix, Section 8.2) although this method was only used for monomer composition determination in this thesis.

### 2.6.2 γ-PGA

#### Extraction of γ-PGA

For the extraction of γ-PGA, the culture broth (as required from 250 µl to 5 ml) was centrifuged at 6000 rpm (4990 g) for 10 min. The supernatant was separated and mixed with 4 volumes of cold methanol and left overnight at 4°C for precipitation. After

precipitation, the crude γ-PGA was collected by centrifuging at 10000 g for 4 min and precipitate was freeze dried (Kambourova *et al*., 2000).

## Quantification of γ-PGA

### *Gravimetric Determination of γ-PGA*

For gravimetric determination 250 µl of cell-free supernatant was transferred to a pre-weighed Eppendorf tube, extracted, and freeze-dried as described previously (Section 2.6.2 under extraction of γ-PGA). The tube was weighed again and the difference in weight was recorded as crude γ-PGA.

### *Spectrophotometric Determination of γ-PGA*

For spectrophotometric measurement 250 µl of cell free supernatant was transferred to an Eppendorf tube, extracted and freeze-dried as described previously (Section 2.6.2 under extraction of γ-PGA). Dried sample was re-dissolved in 1ml distilled water and centrifuged (10000 g) to remove the suspended particles. Absorbance was measured at 216 nm (Zeng et al 2012). γ-PGA concentration was determined using a calibration curve (Appendix, Section 8.3).

The correlation between the gravimetric and spectrophotometric determinations of γ-PGA is presented in Figure 2.1. The $R^2$ value was found to be 0.88 showing that the two methods are in good correlation. However due to higher accuracy and ease of use of the spectrophotometric method, later experiments were analysed spectrophotometrically. This has been specified in the results section as appropriate.

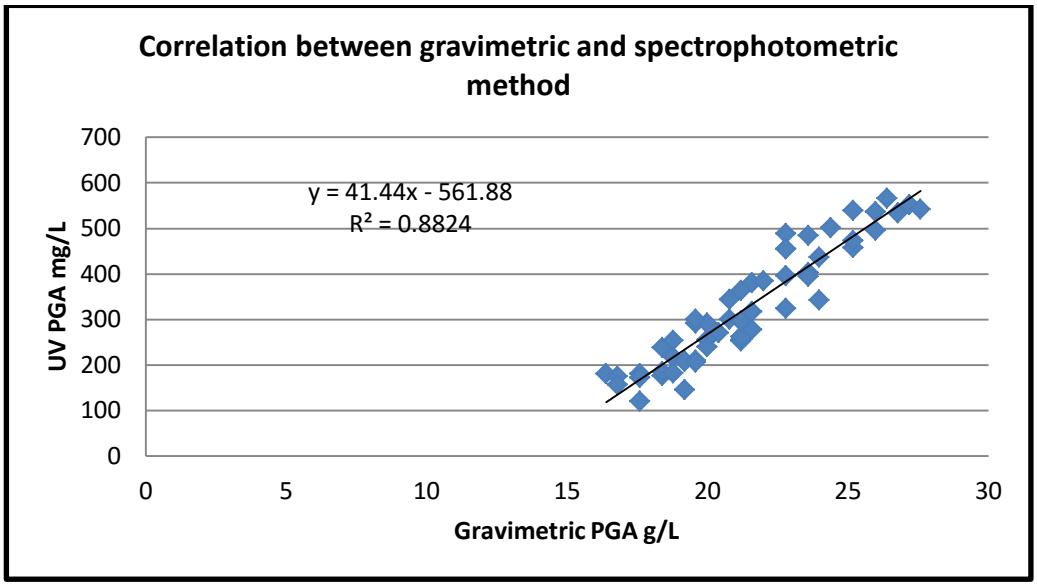



**Figure 2. 1:** Correlation between gravimetric and spectrophotometric methods

### Hydrolysis and Thin Layer Chromatography (TLC)

Dried crude γ-PGA sample was re-dissolved in distilled water (0.5-1% w/v) and mixed with equal volume of 6 M HCl. The mixture was incubated at 100°C overnight for the hydrolysis, and neutralized with 6M NaOH after the incubation (Kambourova *et al*., 2000). The hydrolysed and neutralized γ-PGA was subjected to TLC analysis in order to confirm the amino acid composition. For the mobile phase a mixture of butanol-acetic acid- water (12:3:5) was used. After the treatment with mobile phase, the silica plates were sprayed with 0.2% ninhydrin in acetone and heated to 110 $^{o}$C for visualization.

### SDS-PAGE

The precipitated polymer was freeze dried and re-suspended in deionised water. Purified γ-PGA (10 µl) was mixed with 2x Laemmli buffer and analysed on a 5% resolving gel and with no stacking gel. The current was set at 1 mA per lane for 1 hour. The gel was fixed with 60% ethanol (5 min) and was thoroughly washed with distilled water (2 min) to remove residual SDS. After equilibration with 3% acetic acid (5 min) the gel was stained with methylene blue dye (15 min) and de-stained with deionised water for visualization

(overnight) (Yamaguchi *et al.*, 1996). The buffers used for SDS-page are reported in Appendix, Section 8.4.

#### Glutamic Acid Assay

For glutamic acid determination, 50 µl of sample mixed with 450 µl of distilled water and 500 µl of ninhydrin reagent (Appendix, Section 8.5). The reaction mixture was boiled for 20 minutes and was diluted four fold with a mixture of 1:1 propanol and water. Absorbance was read at 590 nm against blank where 50 µl of 80% ethanol replaced the sample (Sadasivam and Manickam, 2005). Glutamic acid concentration was calculated according to the standard prepared with glutamic acid solution with varying concentrations between 10-100 mg/L. The standard curve is presented in Appendix, Section 8.5.

### 2.6.3 Other Methods

#### Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used primarily to identify and confirm the chemical structure of the polymers; P(3HB) and γ-PGA. For this a sample of the polymers produced in this study and a sample of the commercially purchased polymers (from sigma-as controls) were placed directly onto the diamond crystal, and infrared absorption spectra were recorded over the wavelength region of 4000-500 $cm^{-1}$ using a Perkin Elmer System 2000 FTIR spectrophotometer. All spectra were collated based on 64 scans, 2 $cm^{-1}$ resolution, and compared.

#### RPM to g calculation

The calculations for RPM to g was carried out using the following equation;

$$\text{G-force} = 1.12 \times R \times (RPM/1000)^2$$

Where R is the max radius of the centrifuge used in mm.

## Biomass Determination

### *Cell dry weight (cdw)*

Samples (5- 10 ml depending on the experiment) taken from the fermentation broth were centrifuged at 6000 rpm (4990 g) to separate the cells. The cell pellet was washed with distilled water and freeze-dried before weight measurement.

### *Optical Density (OD)*

Optical density of cultures was measured at 600 nm against water as blank.

## Total Carbohydrate Analysis

Phenol-sulphuric acid method was applied for the determination of total carbohydrate content (Dubois et al., 1956). A sample (200 µl) from the fermentations and/or standard solutions was mixed with 200 µl of 5% phenol solution and 1 ml of concentrated sulphuric acid was added slowly. Following 10 min of incubation, the reactants were vortexed and further incubated for 30 min at room temperature. Colour development was measured using a spectrophotometer at 490 nm and the total carbohydrate concentration was determined using a standard curve.

## Total Nitrogen Analysis

Total nitrogen analysis was carried out using the Kjeldahl method (1883) with slight modification at Ege University, Department of Bioengineering, Izmir, Turkey. Dried orange peel (1g samples) was mixed with 10 ml of sulphuric acid and one salt (NaCl) tablet enhanced with Selenium. The pH was measured and recorded. The mixture was treated for 3 h at 415 °C and after cooling down, 50 ml of distilled water was added and the liquid was connected to distillation unit. The solutions used for distillation in the distillation apparatus were 40% NaOH and 3% $H_3BO_3$. The distilled solution was titrated to its original pH of the initial sample with 0.1 N HCl and the volume of acid consumed

was used to calculate nitrogen content. The nitrogen content is calculated using equation 2.1.

$$\frac{(Sample\ [ml] - Blank\ [ml]) \times N\ of\ acid\ \times 1.4007}{weight\ of\ sample\ [g]} \qquad (2.1)$$

*Sample: ml of acid consumed in titration for the sample*
*Blank: ml of acid consumed in titration for the blank*
*N of acid: Normality of the acid used for titration.*

### Determination of Sugar Type and Content

The analysis of the sugar type and amount present in orange peel was carried out at Ege University Pharmacological Research and Development Centre (ARGEFAR), Izmir, Turkey using HPLC. The HPLC system was equipped with $NH_2$-Inertsil HPLC column (4.6x250mm, 5µm) or Nucleogel Sugar 810 Ca300/7.8 column as required. RI detector was used in all runs and mobile phase was Acetonitrile/water (75:25).

### Microscopic Observations

Microscopic observations of bacterial cells were carried out without any pre-treatment or staining. Culture samples (5-10 µl) were taken from the fermentation broth, placed on a microscope slide and covered with a cover slide. Observations were carried out under the phase contrast settings of the microscope, to observe individual cells and monitor P(3HB) accumulation exploiting the different refractive properties of the P(3HB) granules, the cytoplasm and the cell membrane.

### P(3HB) Granule Size Measurements

P(3HB) granule size measurements was carried out on a Nano-Sight LM10. All sample preparation and measurements analysis was performed by Nano-Sight using a beta version

of NTA 3.1 software. For the measurements of particle size the culture broth containing granules was centrifuged at 8000 rpm (8882 g) for 10 min. Supernatant was diluted 10 times and loaded to the counting chamber. Three consecutive videos, each 166 s long, were recorded and analysed in batch mode to ensure statistical invariance.

### 2.7 Calculations

#### 2.7.1 Yields

Based on standard fermentation profiles, the cell yields ($Y_{x/s}$) as biomass (g/L) per substrate (g total carbohydrate/L), product yields for P(3HB) ($Y_{pP(3HB)/s}$) and γ-PGA ($Y_{pPGA/s}$) were determined as cumulative values, using the total amount of total carbohydrate consumed up to the time under observation, using the equations 2.2 to 2.4

*X: g cdw/L*
*P: Product concentration g/L*
*S: Total carbohydrate (substrate) concentration g/L*
*t: time*
*Y: yield*

**Cumulative Yields**

$$Y_{x/s} = x(t) / (s(0)-s(t)) \quad \text{.....2.2}$$

$$Y_{pP(3HB)/s} = P_{P(3HB)}(t) / (s(0)-s(t)) \quad \text{.....2.3}$$

$$Y_{pPGA/s} = P_{PGA}(t) / (s(0)-s(t)) \quad \text{.....2.4}$$

Similarly the instantaneous yields were determined using the derivatives of the metabolic functions; dX(t), dS(t), $dP_{P(3HB)}(t)$, $dP_{PGA}(t)$, obtained from the fermentation profiles using equations 2.5 to 2.7.

**Instantaneous Yields**

$$Y_{x/s} = dX(t) / dS(t) \quad \text{.....2.5}$$

$$Y_{PP(3HB)/s} = dP_{P(3HB)}(t) / ds(t) \quad \text{.....2.6}$$

$$Y_{PPGA/s} = dP_{PGA}(t) / ds(t) \quad \text{.....2.7}$$

### 2.7.2 Specific Rates

The specific growth rate, total carbohydrate consumption rate and product formation rate were calculated using equations 2.8 to 2.10.

*X: g cdw/L*
*P: Product concentration (g/L)*
*S: Total carbohydrate (substrate) concentration (g/L)*
*t: time (h)*
*Q: specific rate*

**Specific Growth rate**

$\mu = 1/X * dX/dt$ ..……2.8

**Specific Polymer Production Rate**

$Q_p = 1/X * dP/dt$ ……2.9

**Specific Total carbohydrate consumption Rate**

$Q_s = 1/X * dS/dt$ ……..……2.10

Calculations carried out using the experimental data are given in detail in section 3.5.2

### 2.7.3 Statistical Analysis

Data required statistical analysis were analysed by using one way analysis of variance (ANOVA) coupled with Tukey test. P values of $< 0.05$ were considered significant. Statistical calculations were performed using the statistical package for the social sciences (SPSS) version 12.0 statistical software. Significance and related information for the data sets analysed are given in the caption of each relevant figure.

## 2.8 Experimental Design

Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. The response can be represented graphically, either in the three-dimensional space or as contour plots that help visualize the shape of the response surface.

A popular and economical approach, within response surface methods, giving information only on the effects of single factors is the Plackett–Burman (PB) method. PB designs are used for screening experiments because in a PB design the main effects are, in general, heavily confounded with two-factor interactions.

Central composite design (CCD), however, is an experimental design which is used for constructing a quadratic model. This method uses coded values and performs linear regression and iterative approaches (Anderson and Whitcomb, 2007).

A desirable design of experiments should exhibit a distribution of points throughout the region of interest to provide as much information as possible. In the case of problems with a large number of design variables, the experiments may be time-consuming even with the use of CCD. However CCD provides opportunity for visualisation of interactions between parameters tested as well as predicting the response for the given vales of selected independent parameters.

One of the major drawbacks of the response surface models is that, the area under investigation can only be approximated by a polynomial model. This is intentionally set in such a way to allow keeping the experiments simple, focused, and affordable (Anderson and Whitcomb, 2005). However, biological systems do not necessarily fit to quadratic models and the approximation sometimes becomes a restriction which should be taken into consideration. In this thesis, Placket-Burman Design followed by CCD was used to optimise the medium compositions.

### 2.8.1 Placket Burman Design (PB)

As a first step in the optimisation using shaken flasks, a Plackett-Burman screening design (PB) was used to analyse the main medium constituents influencing growth and production using Design-Expert 6.0 (trial version). The PB was set up using required factors, with two coded levels (-1 and +1) and was run to evaluate the linear effects. The results were fitted with a first-order model.

### 2.8.2 Central Composite Design (CCD)

A central composite design (CCD) was applied using Design-Expert 6.0 (trial version), at five levels in order to explore the effect of variables on the response within the region of investigation. Experiments were performed according to experimental design matrix presented separately before each individual experiment and responses are presented under the relevant results sections.

The matrix for three variables was varied at five levels (-α, -1, 0, +1, +α). The higher level of variable was designated as ‘‘+1’’, the lower level was designated as ‘‘-1’’, centre point was designated as “0” and star points (the extreme ends of the screening levels) were designated as “-α” and “+α”. In the optimization process the response can be related to the chosen variables by linear or quadratic models. A quadratic model is given as;

$$y = \beta_0 + \sum_{i=1}^{a} \beta_{ii} X_i + \sum_{i=1}^{a} \beta_{ii} X_i^2 + \sum_{i=1}^{a} \sum_{j=i+1}^{a} \beta_{ij} X_i X_j + e \qquad (2.11)$$

Where $y$ is the response, $\beta_0$ the constant coefficient, $X_i$ ($i$ = 1–a) are non-coded variables, $\beta_i$ are the linear, and $\beta_{ii}$ are the quadratic, and $\beta_{ij}$ are the second-order interaction coefficients. The value “a” varied in each designed matrix and was indicated in relevant results sections. The “α” value was selected as 1.6817 (rotatable design) in all experiments. The parameters throughout the study were coded only with letters rather than $X_i$ for

simplicity. Data were processed for Equation 2.11 using Design-Expert 6.0 program including ANOVA to obtain the interaction between the process variables and the response. The quality of the fit of the polynomial model was expressed by the coefficient of determination $R^2$.

### 2.9 Experiment-Set-up

#### 2.9.1 Magnetic Field Experiment Setup

The setup shown in Figure 2.2 was specially designed to create a magnetic field to be linked to a fermenter. The cells cultured in the fermenter (1) were pumped via a peristaltic pump (2) into the tubular container (3) surrounded by a magnetic field generator (MFG) creating a magnetic field via the DC generator (4). Co-current magnetic field (10 mT) exposure was used with a flow-rate of 12.5 ml/min through the exposure pipe. Circulation rate was 32 min with a flow-rate of 12.5 ml/min. The flow rate calculation was based on the calibration curve in Appendix, Section 8.6.

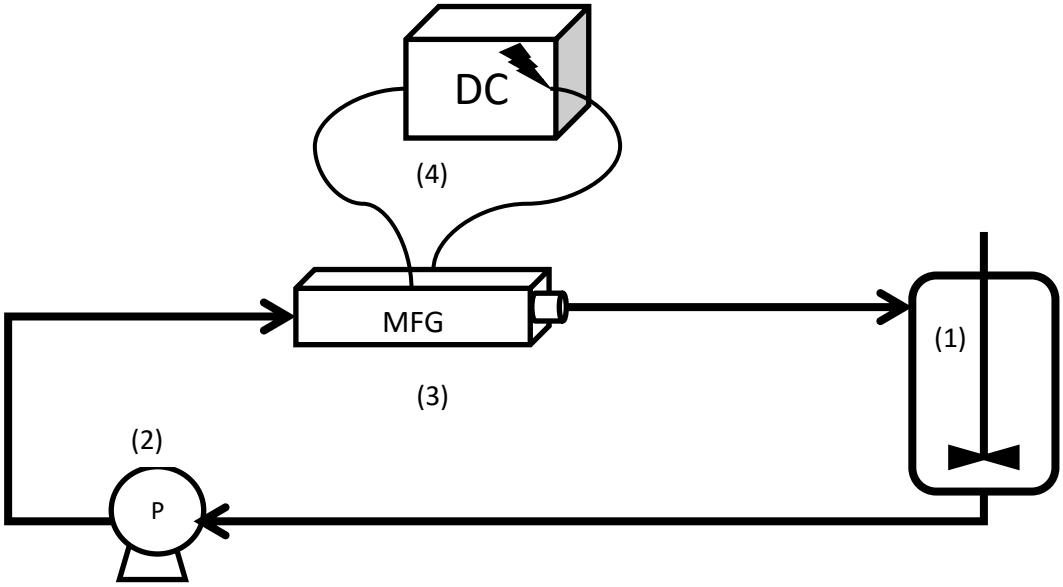



**Figure 2. 2:** Experiment setup for magnetic field study

The test (MF exposed) and the control (MF not exposed) fermentations were set up, and 7 samples were taken over 72 hours from each fermenter. The optical density ($OD_{600}$), cell

dry weights (cdw) and P(3HB) concentrations were determined as described previously in Section 2.6.

### 2.9.2 Foaming and sedimentation column design

A special column (Figure 2.3) with sample ports at various heights was custom designed to determine the behaviour of polymer-containing and polymer-free cells during foam aided and gravitational separation. Samples were taken from the foam and liquid fractions and analysed for P(3HB) content and cdw. This column was used both as floatation and a sedimentation column in the respective experiments described in relevant sections.

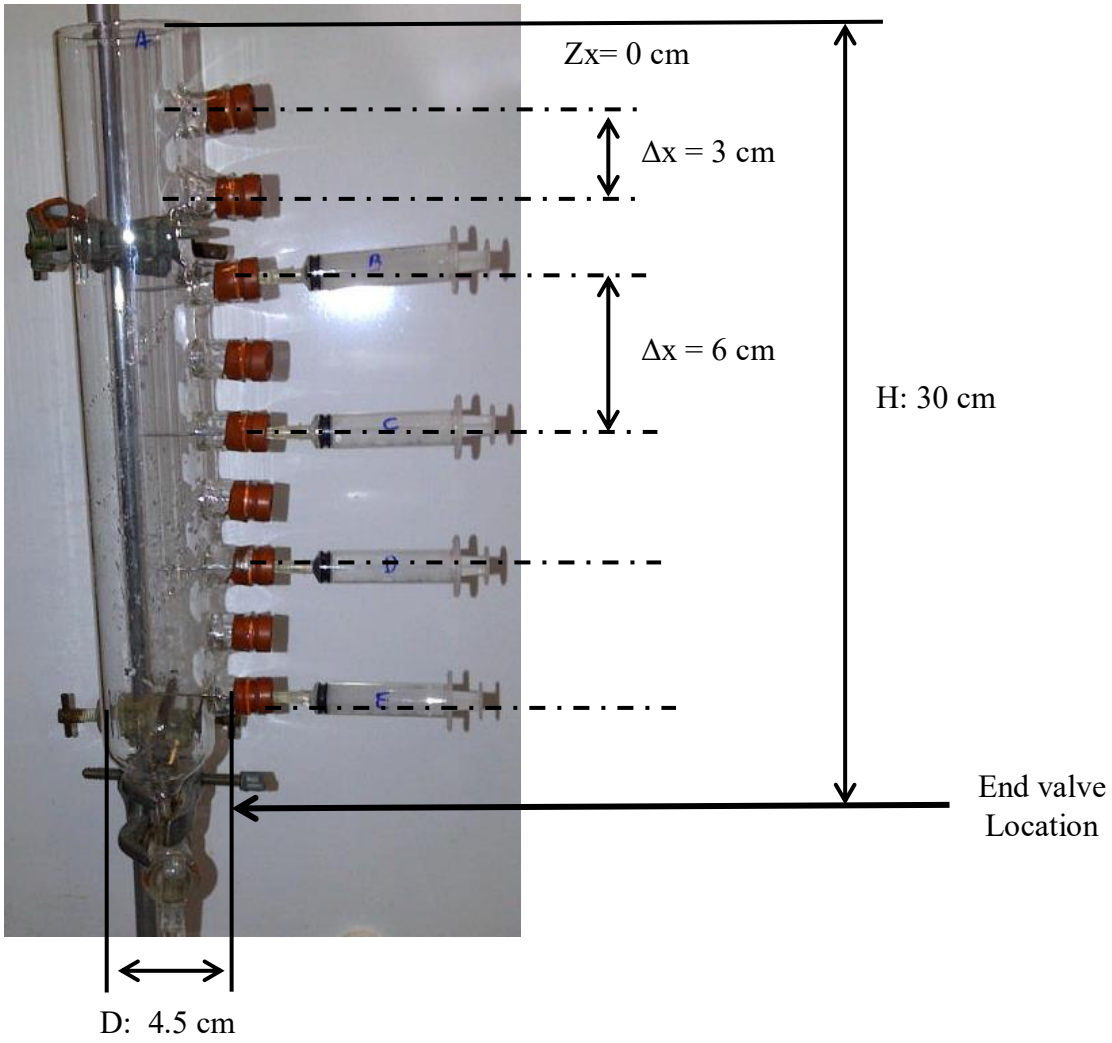



**Figure 2. 3:** In-house designed floatation column

### 2.9.3 Sedimentation setup

Preliminary sedimentation tests were carried out in the column setup shown in Figure 2.4, to determine the sedimentation behaviour of polymer-containing and polymer-free cells during gravitational sedimentation. The system was set up in order to investigate the potential of sedimentation as an on-line method for separation. The preliminary setup used had 10 cm diameter with 10 cm total height. The working height used was 7 cm and samples were taken from three different zones. These were; top zone (1 cm from the top of the liquid), middle zone (3.5 cm from the top of the liquid) and bottom zone (6.5 cm from the top of the liquid).

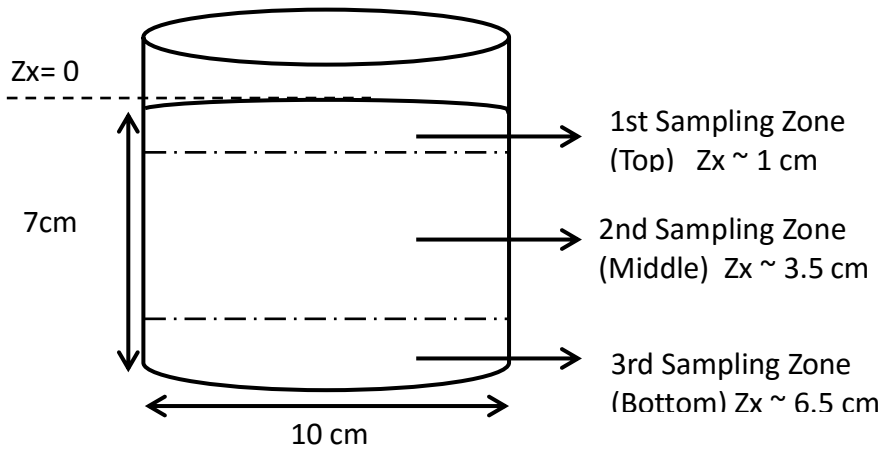



**Figure 2. 4:** Preliminary sedimentation column setup

To further develop a suitable sedimentation device for separation of the above mentioned cells, a taller sedimentation column was used with multiple ports at various heights (Figure 2.3) to determine the %P(3HB)/cdw more accurately and to assess the settling of the cells more precisely. The aim was to determine the minimum settling time required for the concentration of P(3HB)-rich cells as well as to identify their exact location in the column to identify the correct connection point to the fermenter. Samples were taken from the sampling ports at different heights and analysed for P(3HB) content and cdw. The column diameter was 4.5 cm and total height was 30 cm. There were 9 sampling ports (3 cm apart

from each other) located at the side of the column for easy access and sampling. At the bottom of the column (30 cm from the top) there was a valve for controlled discharge. In order to sample from distinct zones, sample locations were selected 6 cm apart.

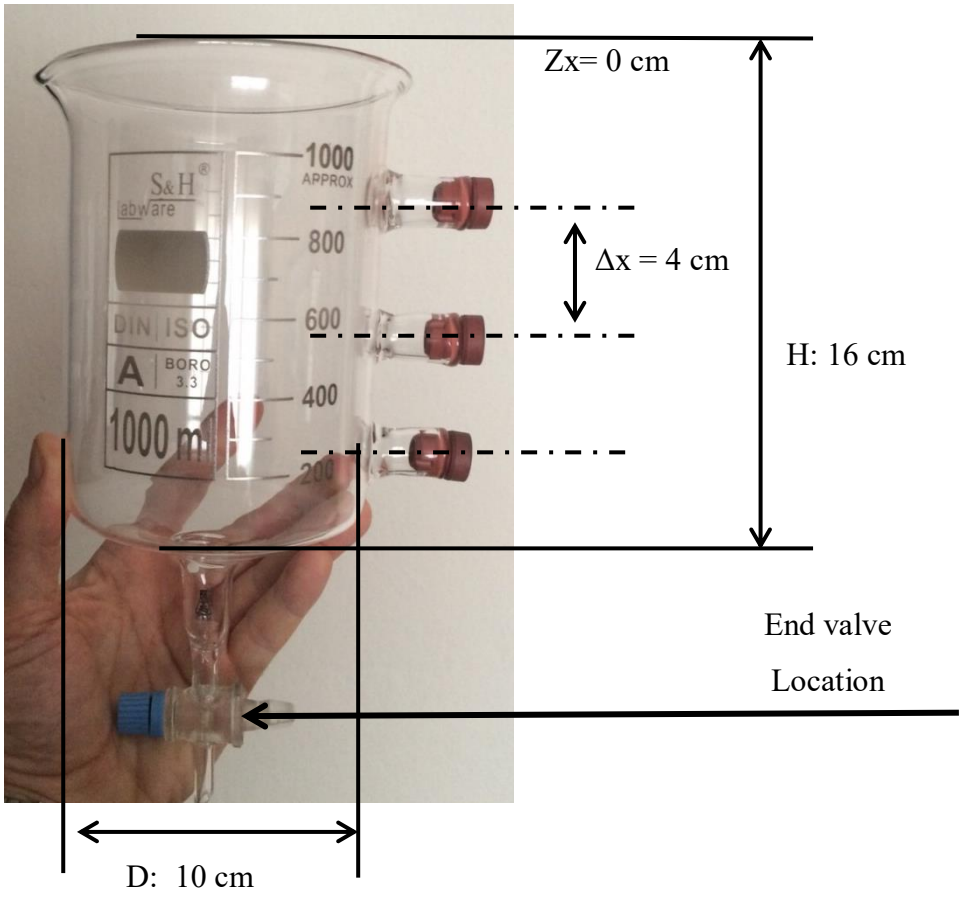



**Figure 2. 5:** In-house designed sedimentation vessel

However, due to the smaller cross-sectional area of the column, laminar flow conditions could not be established and a higher turbulence was observed. Therefore a larger diameter sedimentation vessel (Figure 2.5) was designed. The vessel used had 10 cm diameter with 16 cm total height. The working height was 15 cm. There were 3 sampling ports (4 cm apart from each other) located at the side of the vessel for easy access and sampling. At the bottom of the column (Figure 2.5) there was a valve for controlled discharge. Samples were taken from the top (4 cm from the top of the liquid), medium (8 cm from the top of the liquid) and bottom (12 cm from the top of the liquid) levels and analysed for P(3HB) content of the cells and their cdw.

# Chapter 3: Results

# 3. RESULTS

The focus of this thesis is the dual production of two commercially important polymers, P(3HB) and γ-PGA. Results obtained throughout the study are presented in this chapter. The studies include identification of a suitable strain and selection of a biowaste for the dual production of P(3HB) and γ-PGA, followed by the optimisation of production in shaken flasks, and scaling up to production in laboratory-scale fermenters. A table summarising the fermenter runs conducted is given in appendix, Section 8.8. Various separation methods were investigated to facilitate downstream processing.

The results chapter is divided into seven sections. **Section 3.1** describes the different strains tested for their capability to produce both polymers. The products obtained from the selected strain are confirmed using several different methods and these results are also presented in this section.

In the second part (**Section 3.2**) batch fermentation profiles, using defined production media obtained from literature, of the selected strain for P(3HB) and γ-PGA are presented.

In **Section 3.3** the first step of optimisation studies, the initial media screening using Placket-Burman (PB) method, is demonstrated. In order to identify the important medium components, P(3HB) production medium and γ-PGA production medium were screened separately. The results of the experiments, designed to identify the effects of the selected defined medium components for P(3HB) production, are also given in this section. This is followed by a final screening for the dual production of both polymers. The outcome of this section is the identification of key medium components for the dual production and this data was used for the optimisation studies using central composite design (CCD).

**Section 3.4** contains the results for CCD optimisation study for the dual polymer production. The difficulty of employing the CCD optimisation method to a dual production system is demonstrated leading to the re-design of the CCD by simplification of some variables. The results obtained through this approach are presented together with

their interpretations. Analysis of each individual medium component is included in the CCD matrix and completed. Identification of their respective independent effects on P(3HB) and γ-PGA productions are also included. This section also presents the results of the studies on the effect of inoculum medium. The optimised double polymer (DP) medium and the inoculum conditions identified for the efficient production are used throughout the next sections.

**Section 3.5** presents the results for dual polymer production using the optimised DP medium and inoculum conditions presented in the previous section. Double polymer production profiles in shaken flasks and in stirred tank bioreactors are shown. In addition, process parameters such as, specific growth rate, doubling time, yields and specific rates are calculated and presented in this section.

In **Section 3.6**, the results of utilisation of different biowastes are reported. Investigations on four different biowastes and four different pre-treatment methods, are shown with respect to their suitability for the production of P(3HB) and γ-PGA. The results of the experiments on the selection and characterisation of the selected biowaste, orange peel, are reported. As a final part of this section, results of the experiments on the use of orange peel as carbon source for the production of P(3HB) and double polymer are presented.

The last section of this chapter (**Section 3.7**) contains results of the studies for the recovery of double polymer. The results of several different approaches such as, the application of static magnetic field the utilisation of floatation, and sedimentation are presented.

## 3.1 Screening of Different Strains and Product Confirmation

Since the aim of the project is dual production of the two polymers, P(3HB) and γ-PGA, five strains were compared with respect to their polymer production capabilities.

### 3.1.1 Screening for γ-PGA and P(3HB)

Five bacterial strains obtained from culture collections (University of Westminster culture collection and Ege University bacterial culture collection) were first tested for their γ-PGA production capabilities. Cultures were grown in γ-PGA production medium available within the research group (Section 2.2). Since γ-PGA is an extracellular polymer, its extraction was carried out from the culture supernatant as described in Section 2.5.2. The concentration of precipitate obtained from the extraction step was determined using gravimetric method (Table 3.1). Presence of γ-PGA was tested using TLC by confirming the only amino acid present in the precipitate was glutamic acid (TLC plate is presented in Appendix, Section 8.7). This precipitate was defined as crude γ-PGA. Although all of the strains were able to produce γ-PGA, *Bacillus* OK2 and *Bacillus* 2d.1 yielded higher titres.

**Table 3. 1:** Screening of different strain for γ-PGA production. Data represents the main value of duplicate runs.

| Name | Crude γ-PGA (g/L) | Presence of γ-PGA |
|---|---|---|
| *Bacillus subtilis* OK2 | 4.00 | + |
| *Bacillus* 46.2 | 3.70 | + |
| *Bacillus* 2d.1 | 4.58 | + |
| *Bacillus* 159 | 2.72 | + |
| *Bacillus* 20.4 | 3.70 | + |

Subsequently the same strains were tested for their P(3HB) production capabilities. Since P(3HB) is an intracellular polymer, cell dry weights (cdw) were determined (Table 3.2).

**Table 3. 2:** Screening of different strains for P(3HB) production

| Strain | cdw in P(3HB) medium (g/L) | P(3HB) |
|---|---|---|
| *Bacillus* OK2 | 2.4 | + |
| *Bacillus* 46.2 | 0.3 | - |
| *Bacillus* 2d.1 | 0.9 | - |
| *Bacillus* 159 | 2.0 | + |
| *Bacillus* 20.4 | 0.5 | - |

The existence of P(3HB) was confirmed using Sudan Black B method (Section 2.5.1) where the dark stains were clearly visible and located in the middle of the single colonies on the agar plates, distributed homogeneously in all colonies on the plate. Although some black spots were observed in other strains such as *Bacillus* 2d.1 too, since these spots were not at the centre of the colony and they were not visible in every colony on the plate so they were not considered as P(3HB) inclusions (Ghate *et al.*, 2011; Flora *et al.*, 2010). Homogeneously spread stained spots at the centre of the colonies could only be observed in *Bacillus subtilis OK2* and *Bacillus 159* strains (Figure 3.1). Further discussion on the stained inclusions is presented in Section 4.1.

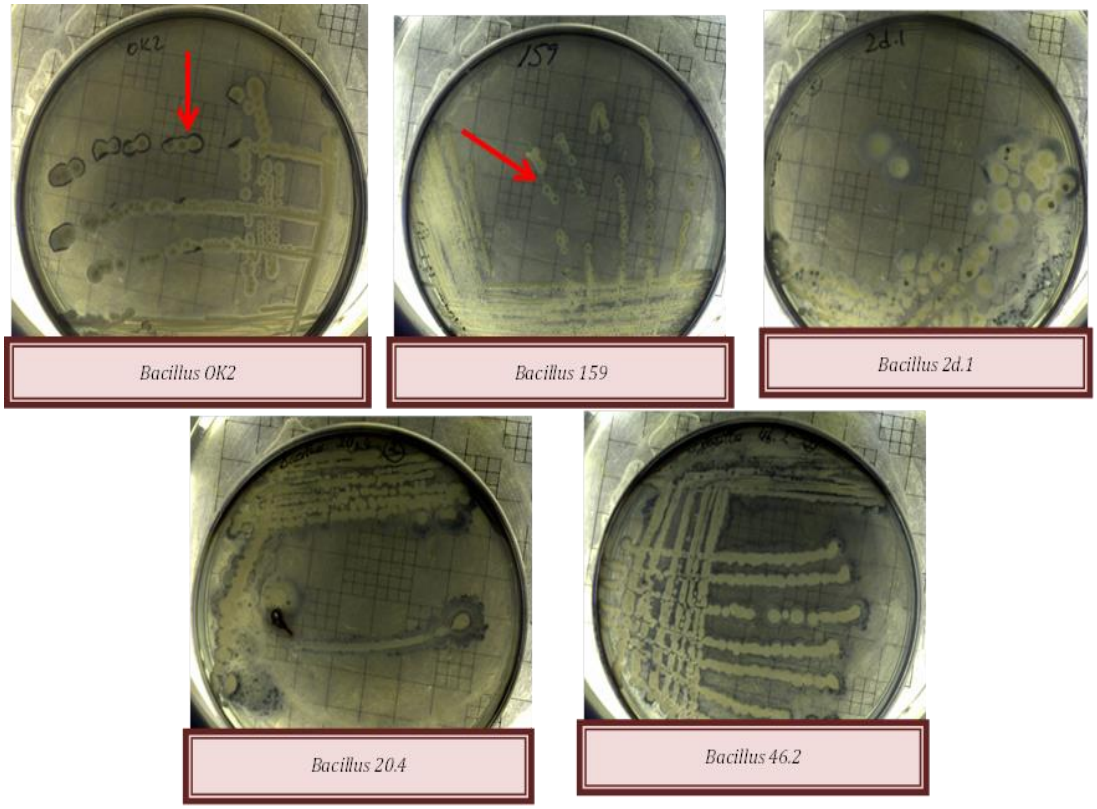



**Figure 3. 1:** Cultures stained with Sudan Black B for confirmation of P(3HB) presence.

*Bacillus subtilis* OK2 and *Bacillus 159* were the only two strains that produced both of the polymers, therefore P(3HB) production was quantified for these strains using crotonic acid method (Section 2.5.1). P(3HB) production of *Bacillus subtilis* OK2 strain was found to be higher compared to *Bacillus* 159. After 72 h of growth *Bacillus subtilis* OK2 produced 37.3 mg/L P(3HB) whereas *Bacillus* 159 produced 0.87 mg/L (Figure 3.2). Since OK2's γ-PGA production was also one of the highest among the tested strains (Table 3.1), *Bacillus subtilis* OK2 strain was chosen and used in all experiments from this point onwards.

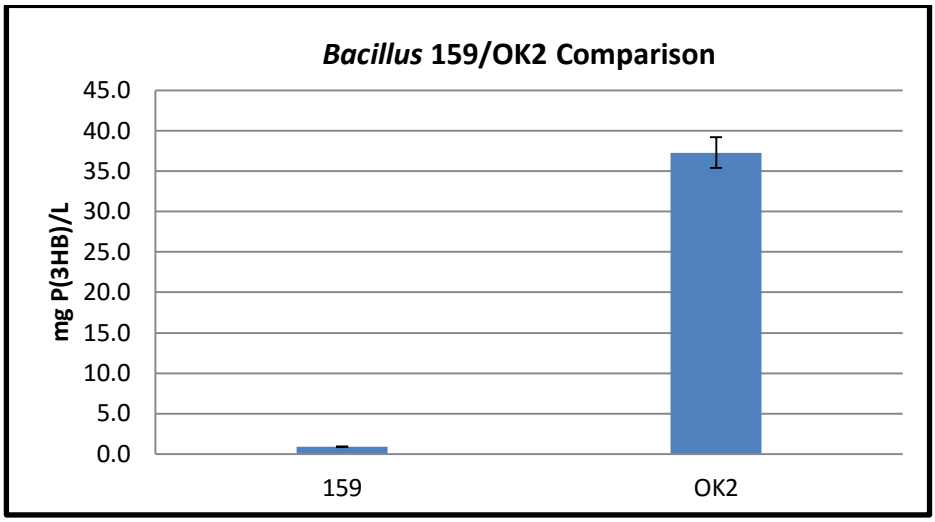



**Figure 3. 2:** *Bacillus subtilis* OK2 and *Bacillus* 159 comparison for P(3HB) production. The Figure represents triplicate measurements from two different experiments.

### 3.1.2 Product Confirmation for P(3HB) and γ-PGA

Both polymers were produced in 250 mL shaken flasks, with 50ml working volume using *Bacillus subtilis* OK2 in their subsequent production media, then extracted and purified (Figure 3.3) in order to confirm the molecular structure of the polymers. FTIR scan was performed as described in Section 2.5.3. P(3HB) produced by *Bacillus subtilis* OK2 was compared to the standard purchased from Sigma and found to be an exact match (Figure 3.4).

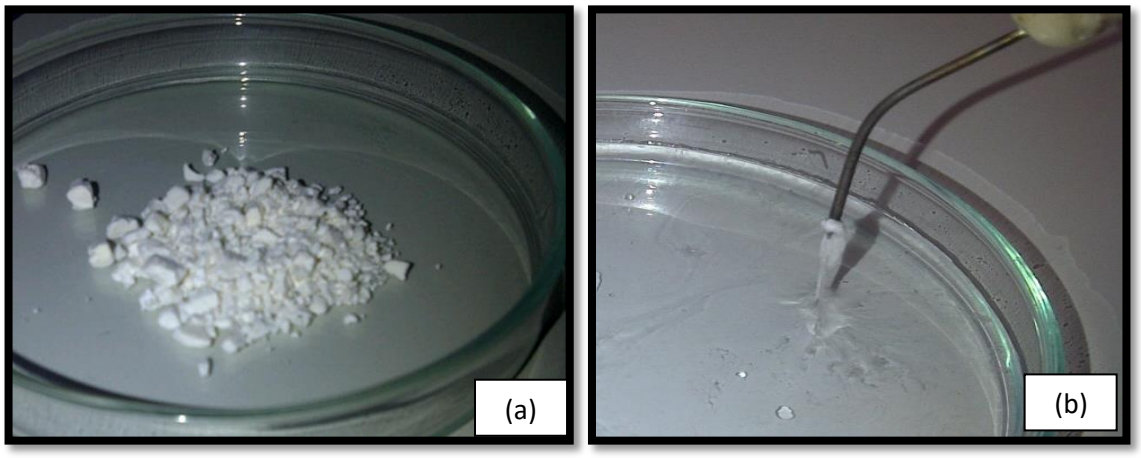



**Figure 3. 3:** Polymers obtained γ-PGA (a), P(3HB) (b)

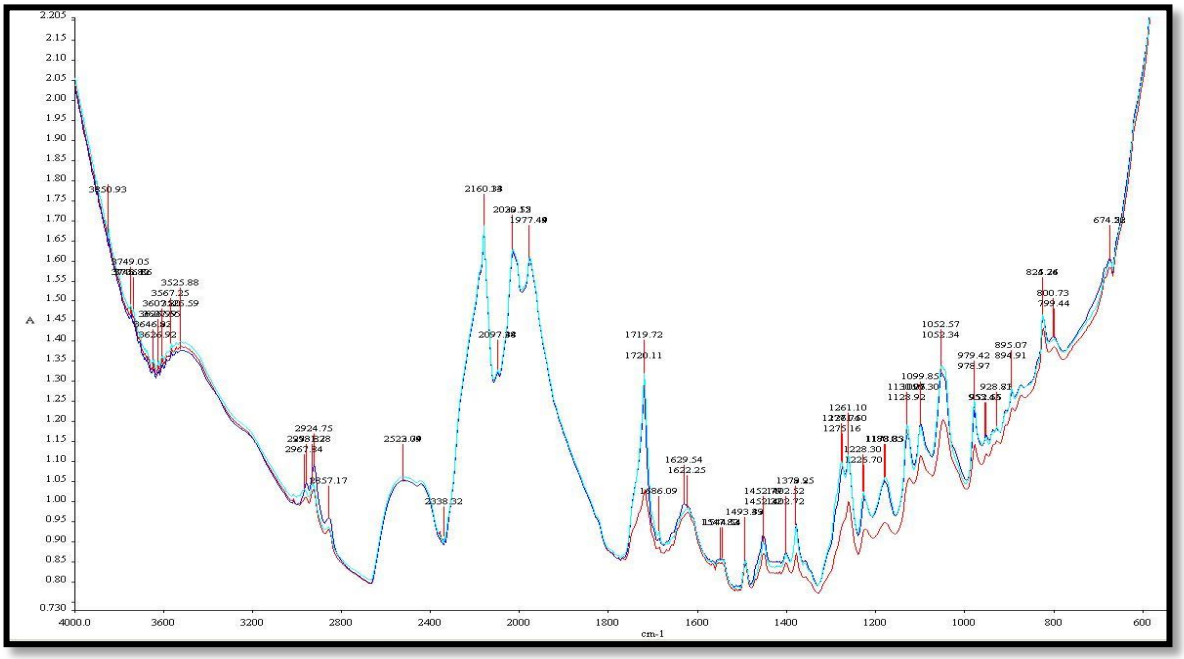



**Figure 3. 4:** Confirmation of type of P(3HB) produced by *Bacillus subtilis* OK2 using FTIR. Light blue profile: P(3HB) standard from Sigma, Red profile: P(3HB) produced by *Bacillus subtilis* OK2.

Subsequently, γ-PGA produced by *Bacillus subtilis* OK2 was tested using FTIR against standard γ-PGA purchased from Sigma. The profile is presented in Figure 3.5. Although the profiles were not an exact match the functional group peaks were present in both. This confirmed that the product produced was γ-PGA. Differences in peaks and possible reasons for the mismatches are discussed in Section 4.1. Further confirmation of the γ-PGA was carried out using SDS-Page as described in Section 2.6.2 with a standard purchased from Sigma (Figure 3.6). The analysis confirmed the product was a shorter chain length γ-PGA compared to the standard from Sigma (mw ≥1,000,000). This explained the mismatch observed in the FTIR profile. Further discussion on FTIR profile and SDS-page is presented in Section 4.1.

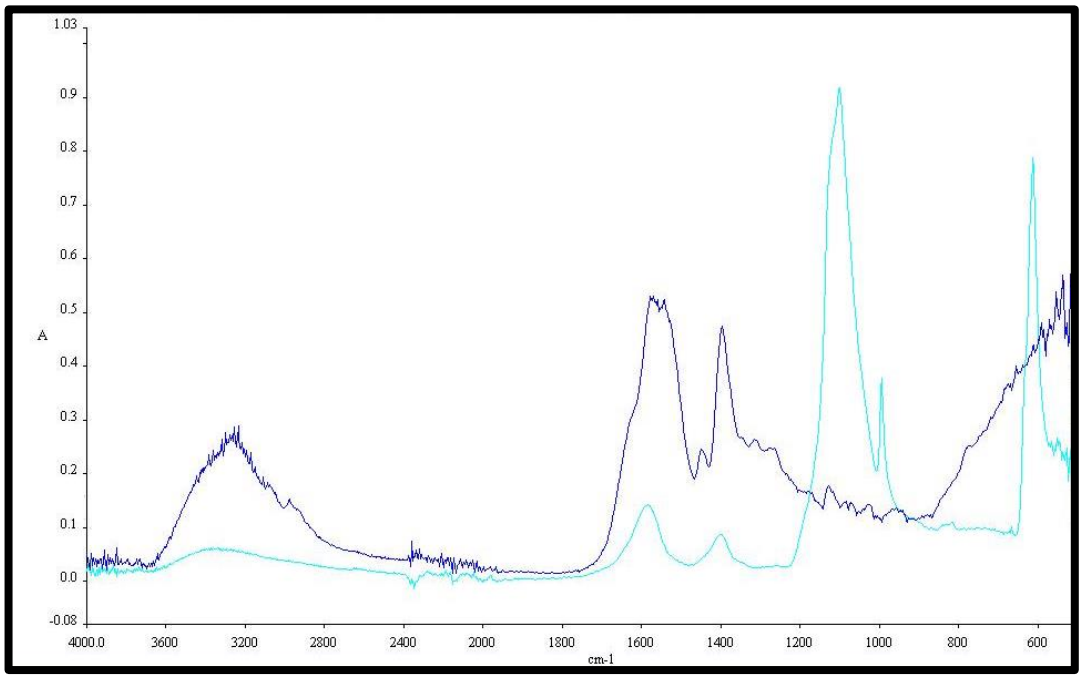



**Figure 3. 5:** Confirmation of type of γ-PGA using FTIR. Dark blue, standard γ-PGA; light blue, γ-PGA produced by *Bacillus subtilis* OK2.

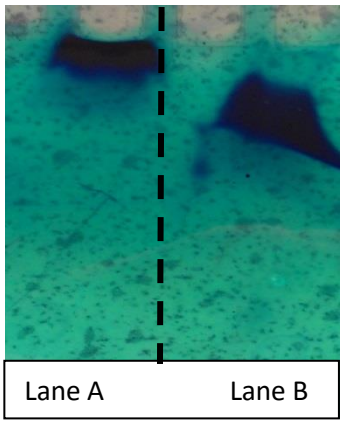



**Figure 3. 6:** Confirmation of type of γ-PGA using SDS-Page. Lane A, Standard γ-PGA; Lane B, γ-PGA produced by *Bacillus subtilis* OK2

The monomer composition of P(3HB) produced by *Bacillus subtilis* OK2 was confirmed by GC-FID analysis as described in Section 2.5.1. As seen in Figure 3.7, the only monomer found to be present was hydroxyl butyric acid which was converted to methyl-hydroxy butyric acid after methanolysis.

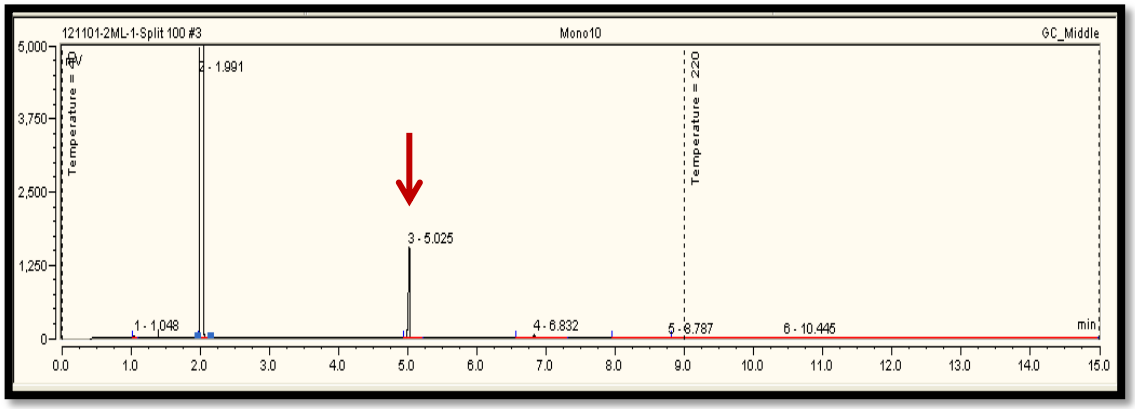



**Figure 3.7:** GC-FID profile of methanolised P(3HB) produced by *Bacillus subtilis* OK2. Arrow shows methyl-hydroxy butyric peak observed

## 3.2 Batch Fermentation Profile for γ-PGA and P(3HB)

Batch fermentations were conducted in 0.5 L fermenters with 0.4 L working volume for productions of γ-PGA and P(3HB) using *Bacillus subtilis* OK2 to determine its growth and product formation profiles.

### 3.2.1 Growth of *Bacillus subtilis* OK2 and γ-PGA Fermentation Profile

Fermentation profile for γ-PGA fermentation is presented in Figure 3.8. The fermentation was carried out using γ-PGA medium described in methods section (Section 2.3.2). γ-PGA formation is observed to be growth associated. The γ-PGA concentration increased with increasing culture optical density. Glutamic acid concentration was observed to be constant until 53 h and an increase was observed afterwards. Following the increase, the concentration stayed around the same level till the end of the fermentation (from 72 h to 90 h). This is further discussed in Section 4.1. Since, glutamic acid was observed not to be consumed; it was not monitored in the experiments conducted throughout this work, from this point onwards. After 50$^{th}$ h of the fermentation a slight increase in total carbohydrate concentration was observed. This could be due to exopolysaccharide (EPS) produced during the fermentation. The investigation of EPS was outside the scope of this work, therefore was not characterised. Further discussion for the fermentation profile is presented in Section 4.1.

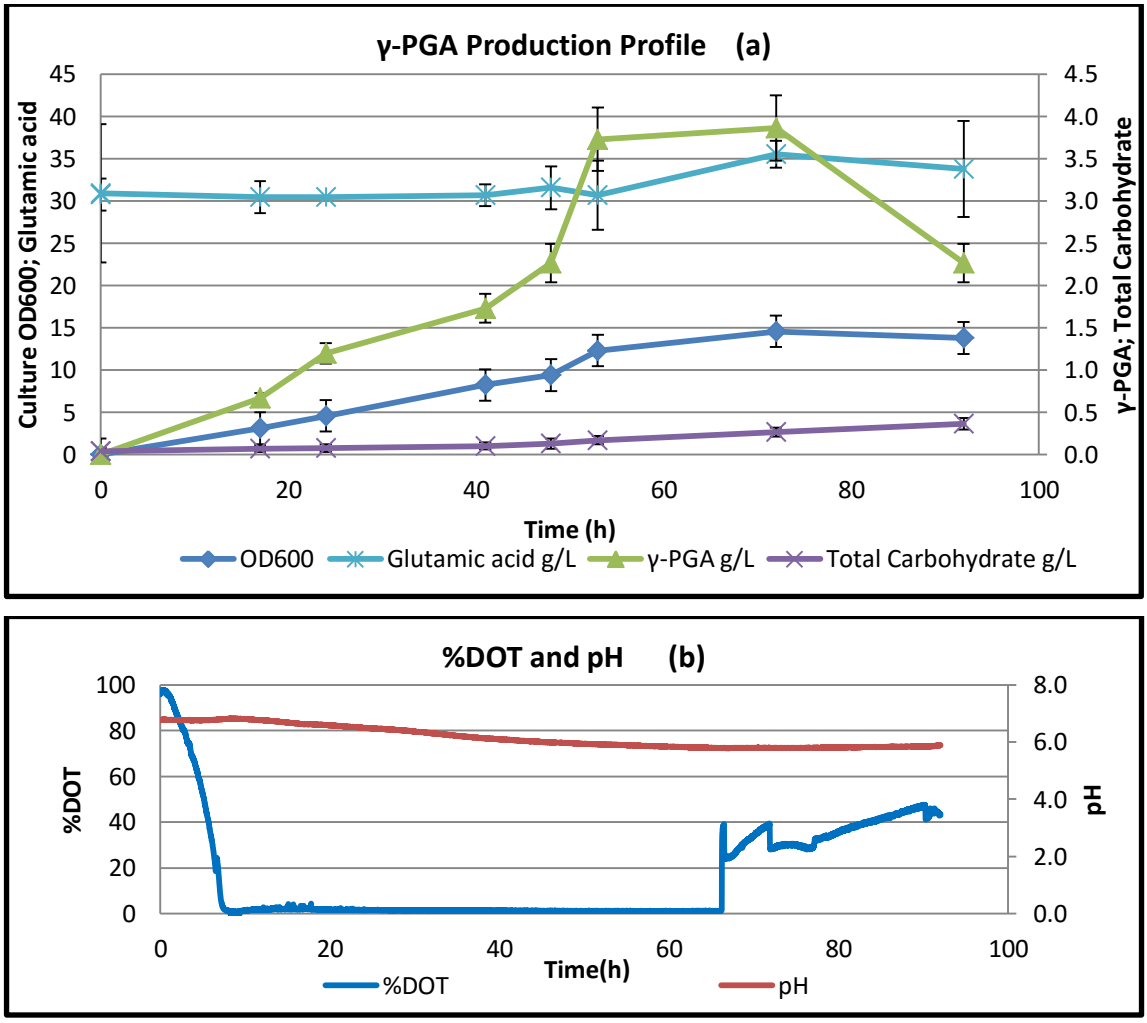



**Figure 3.8:** Profile for growth of *Bacillus subtilis* OK2, γ-PGA production, change in glutamic acid and total carbohydrate concentrations (a), % DOT and pH profiles (b) at 37°C, 300 rpm, 1 vvm

### 3.2.2 Growth of *Bacillus subtilis* OK2 and P(3HB) Fermentation Profile

Fermentation profile for P(3HB) production is presented in Figure 3.9. The fermentation was carried out using PHA medium described in methods section (Section 2.3.2). P(3HB) formation is commenced after 20 h which corresponds to the stationary growth phase. This confirmed by $OD_{600}$ values levelling out and the increase in the % DOT level. P(3HB) accumulation was observed mainly during stationary phase. The total carbohydrate concentration decreased until the 20$^{th}$ h, but increased briefly before decreasing again to

the end with a concentration of 10 g/L at 70 h when the fermentation was terminated. The fermentation profile is further discussed in Section 4.1.

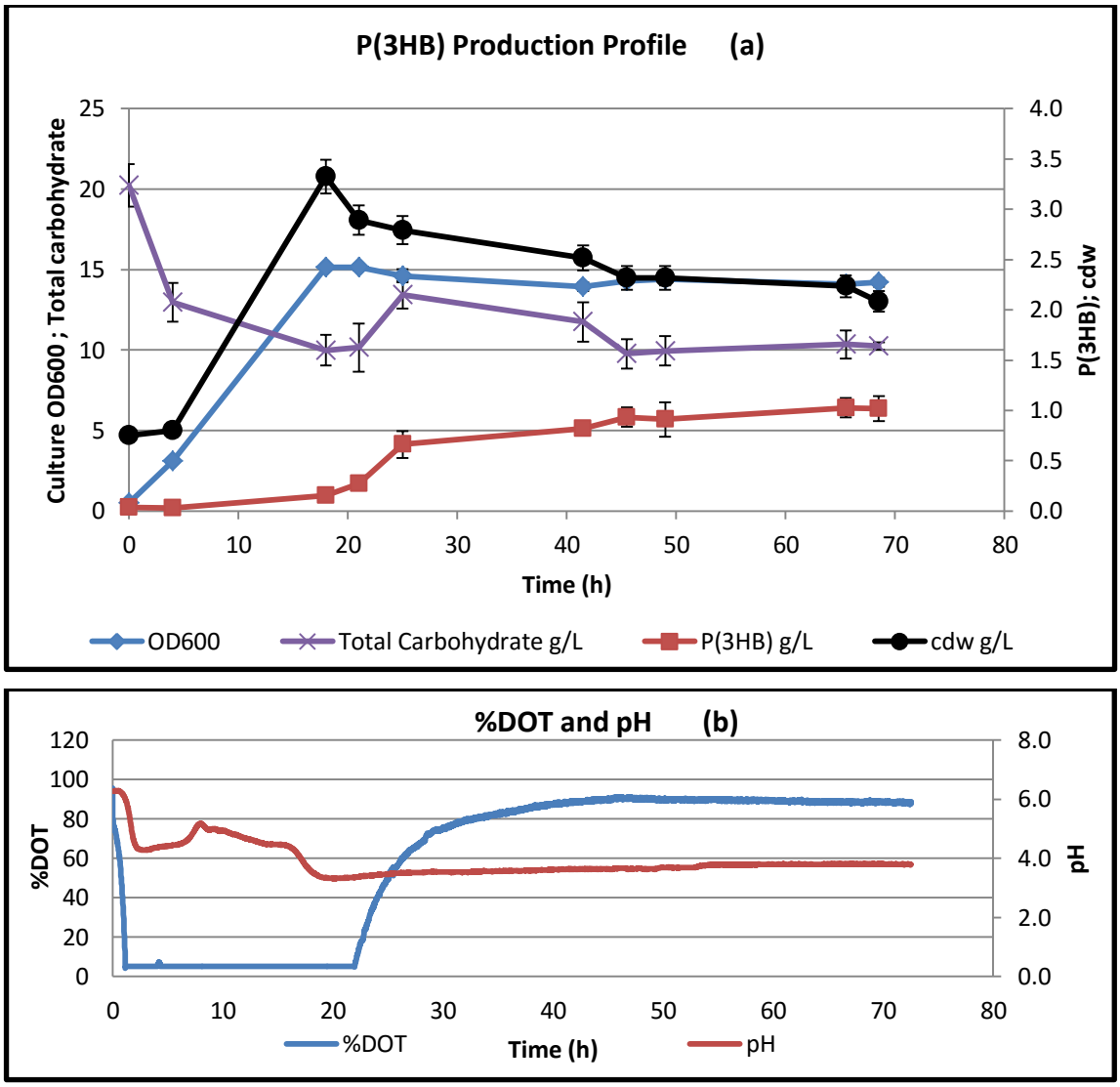



**Figure 3.9:** Profile for growth of Bacillus subtilis OK2, P(3HB) production, change in total carbohydrate concentration (a), % DOT and pH profiles (b) at 37°C, 400 rpm, 1 vvm

## 3.3 Screening of Defined Media Using Placket Burman (PB)

Linear effects were identified by testing each component in two levels and fitting it to a first order model.

### 3.3.1 Production of γ-PGA and P(3HB)

γ-PGA production medium containing glutamate, citric acid, ammonium sulphate, potassium phosphate di-basic, magnesium sulphate, sodium phosphate di-basic, ferric chloride, manganese sulphate, calcium chloride and glycerol were subjected to PB analysis. The range was adjusted in two levels, zero and actual concentration in the recipe. Perturbation chart obtained from the PB analysis (Figure 3.10) showed that glutamate, citric acid and ammonium sulphate have positive effects while manganese sulphate, sodium phosphate di-basic, magnesium sulphate and calcium chloride have negative effects on γ-PGA production. No significant effect was observed for potassium phosphate di-basic, ferric chloride and glycerol (Figure 3.10).

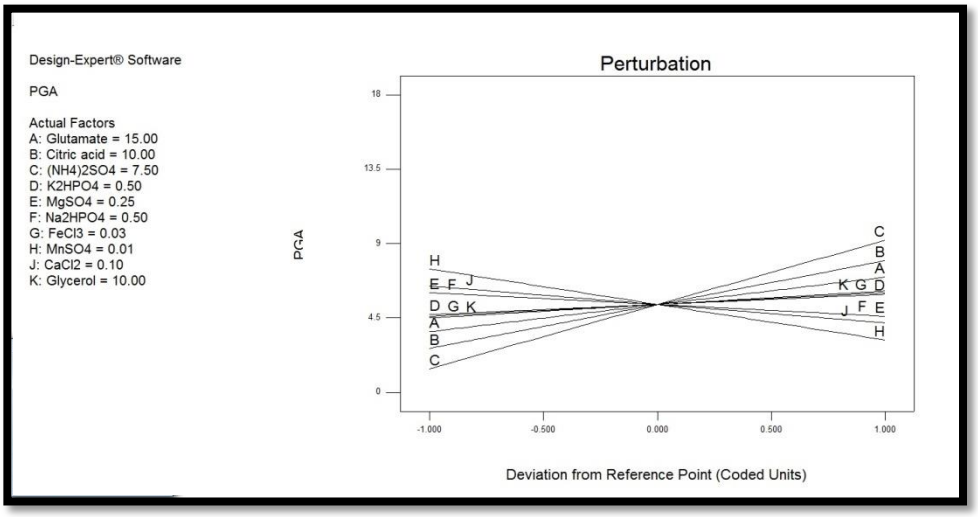



**Figure 3. 10:** γ-PGA Medium Composition Screening by Placket Burman Design

Subsequently, P(3HB) production medium containing glucose, yeast extract, potassium chloride, ammonium sulphate and Soytone (enzymatic digest of soybean meal) were subjected to PB analysis. The range was again adjusted in two levels, zero and actual concentration in the recipe. Perturbation chart obtained from PB analysis showed that glucose and yeast extract have positive effects while potassium chloride, ammonium sulphate and soytone have negative effects on P(3HB) production (Figure 3.11).

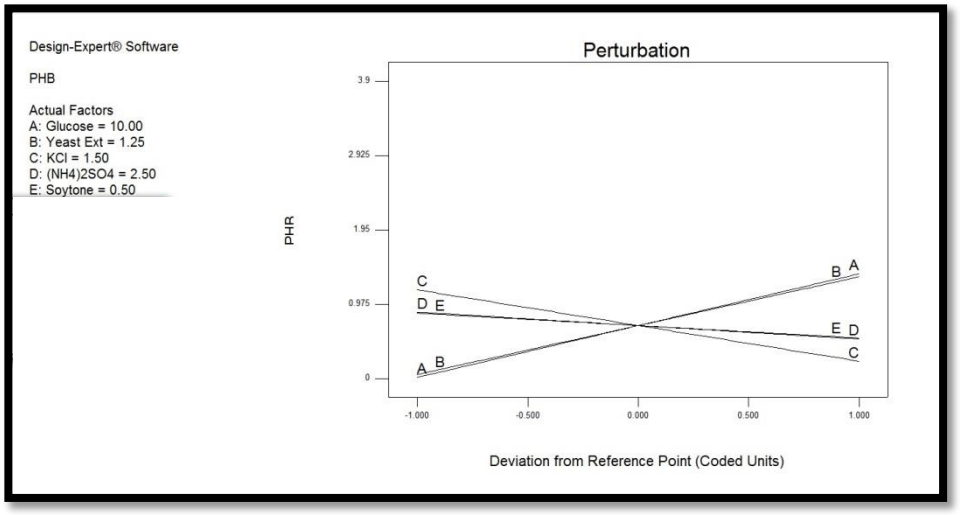



**Figure 3. 11:** P(3HB) Medium Composition Screening by Placket Burman Design

### 3.3.2 Effects of Defined Medium Components in P(3HB) Production

Since PB analysis suggested pronounced effects for the two components glucose and yeast extract, their individual and combined effects were further investigated with respect to their effects on cdw and P(3HB) concentration. When glucose and yeast extract were tested in their original concentration, 20 g/L and 2.5 g/L respectively, their combined effect gave a higher yield of cdw and P(3HB) concentration (Figure 3.12).

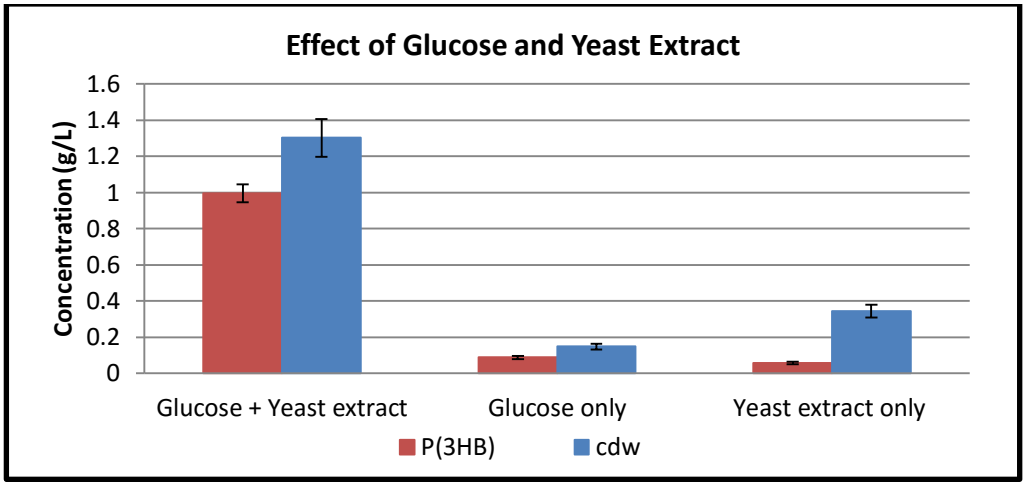



**Figure 3.12:** Effect of glucose (20 g/L) and yeast extract (2.5 g/L) on P(3HB) production. Triplicate measurements in two different experiments

It is well established that in many strains, P(3HB) production occurs under nutrient limited conditions. Preliminary results showed that *Bacillus subtilis* OK2 produces P(3HB) under nitrogen limited conditions. In the literature it has been suggested that, addition to the culture of a limiting nutrient, such as nitrogen or phosphorous, after the commencement of P(3HB) production in very small amounts may improve P(3HB) accumulation (Page, 1992). In order to test this concept yeast extract in concentrations ranging from 0 to 50 mg/L was added to P(3HB) production medium after 24 h and 48 h of growth. Addition of 6.25 mg/L of yeast extract to 48 h culture resulted to 33% increase in P(3HB) accumulation (Figure 3.13).

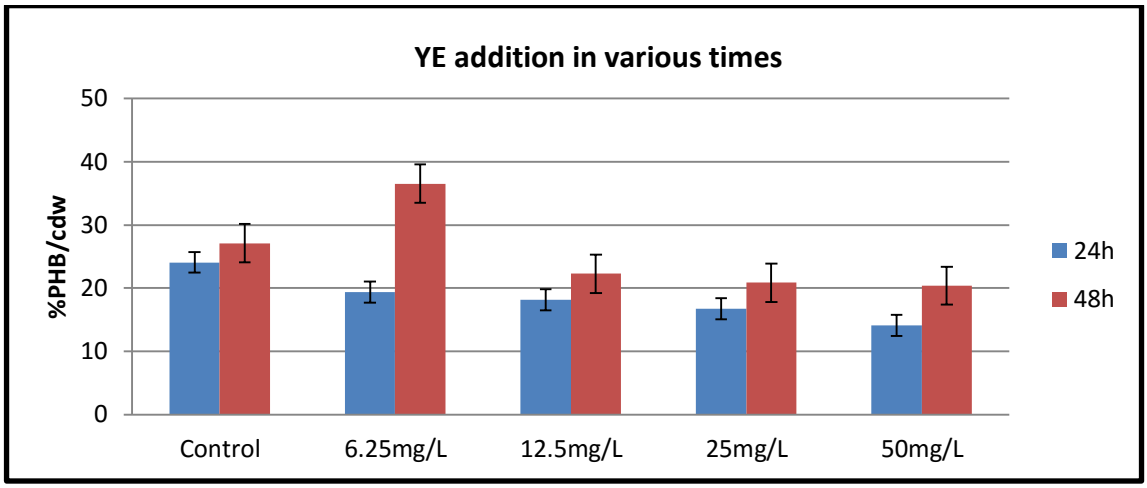



**Figure 3.13:** Effect of addition time of yeast extract on P3(HB) production. Triplicate measurements in two different experiments. Statistical analysis given in Section 8.9.1, all levels found significant at the level $p<0.05$ compared to the control.

As discussed in Section 1.2.2, one of the pathways for P(3HB) production starts at the beginning of TCA cycle. Under suitable conditions Acetyl-CoA is directed to PHA production pathway. According to literature, citrate synthase enzyme has a significant effect on PHA production (Anderson and Dawes 1990, Kessler and Witholt 2001). Kessler and Witholt reported that high concentration of NADH and NADPH inhibits the citrate synthase activity, enhancing P(3HB) production. Citrate is also an inhibitor of the first

enzyme in the TCA cycle, citrate synthase. Addition of citrate in concentrations ranging between 0 to 5 g/L was tested for its effects on P(3HB) production. An increase in P(3HB) concentration was observed in presence of citrate (Figure 3.14). A further increase in concentration of the added citrate resulted in the inhibition of growth of the culture.

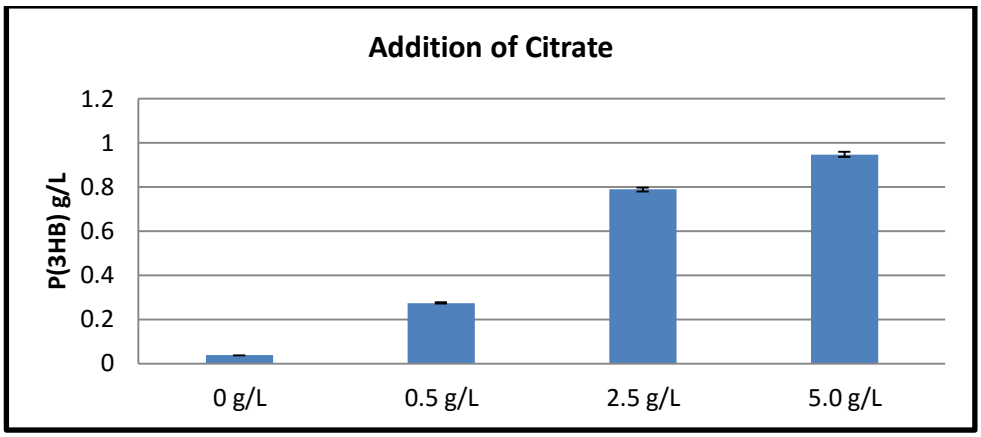



**Figure 3. 14:** Effect of Citrate concentration on P(3HB) production. Triplicate measurements in two different experiments.

### 3.3.3 Production of Double Polymer

Based on the results obtained from individual PB analyses for the production of each polymer, a new PB analysis was designed to further investigate the effects of medium components in the case of dual polymer production.

Medium components selected in the previous experiments; glucose, yeast extract, glutamic acid, ammonium sulphate and citric acid, were subjected to PB analysis. The range was again adjusted in two levels; zero and actual concentration in the recipes apart from citrate due to its high concentration in γ-PGA recipe. The maximum concentration of citrate was kept at 2.4 g/L as the determined optimum concentration. The perturbation chart obtained from PB analysis for each polymer individually showed that glucose, yeast extract and glutamic acid have positive effects whereas citric acid has negative effect on P(3HB) production. The effect of ammonium sulphate was not significant (Figure 3.15).

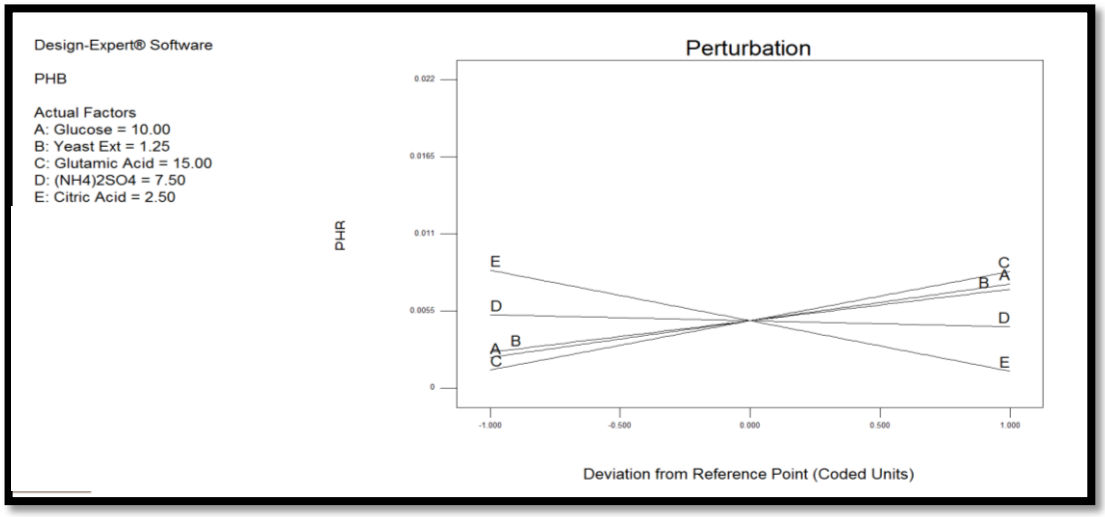



**Figure 3. 15:** Double Polymer Medium Composition Screening by Placket Burman Design for the production of P(3HB)

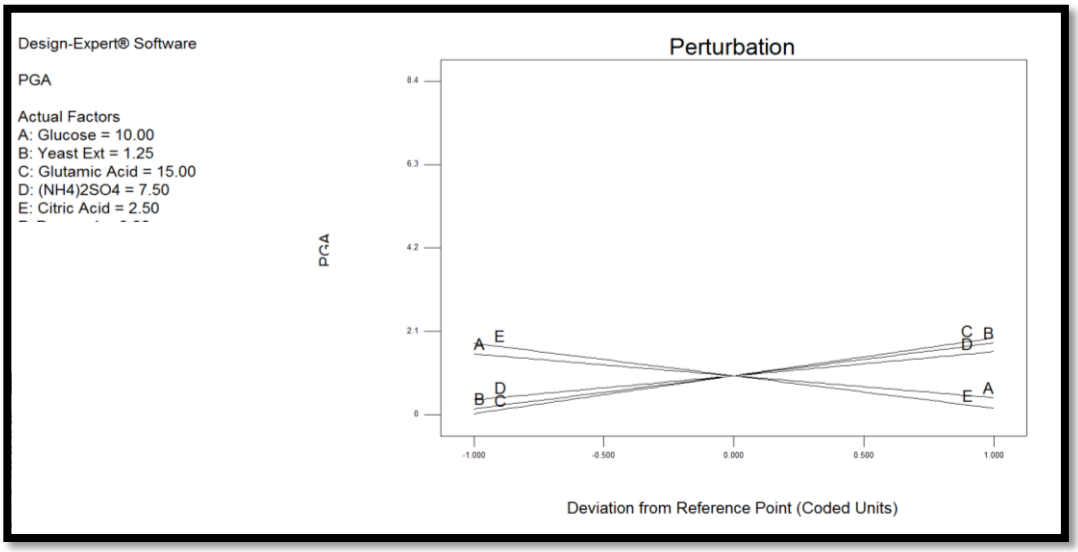



**Figure 3. 16:** Double Polymer Medium Composition Screening by Placket Burman Design for the production of γ-PGA

For γ-PGA production, yeast extract, ammonium sulphate and glutamic acid had positive effects whereas glucose and citric acid had negative effects (Figure 3.16).

According to the results of PB charts, 4 parameters, glucose, yeast extract, glutamic acid and ammonium sulphate were selected for further optimization studies of double polymer production.

### 3.4 Optimisation of Double Polymer Production

#### 3.4.1 Central Composite Design (CCD) for Double Polymer Production

The four parameters identified through PB analysis were subjected to central composite design to optimize the dual production of P(3HB) and γ-PGA as well as to investigate the interaction between the parameters. The total number of experiments with four variables in five levels was 27. Twenty seven experiments were augmented with four replications at the centre points to evaluate the pure error. The experiments were conducted in shake flasks at 30°C and 200 rpm. The P(3HB) and γ-PGA concentrations were taken as the responses. Experiments were performed according to experimental design matrix given in Table 3.3, within the ranges indicated and analysed as described in Section 2.7.2. The levels selected for each parameter was based on the maximum levels investigated in previous experiments (PB design) and literature.

**Table 3. 3:** Experimental design matrix showing five levels for CCD (α=1.69)

| Parameter (g/L) | Code | -α | -1 | 0 | +1 | +α |
|---|---|---|---|---|---|---|
| **Glucose** | A | 5.00 | 8.75 | 12.50 | 16.25 | 20.00 |
| **Yeast Extract** | B | 1.00 | 1.75 | 2.50 | 3.25 | 4.00 |
| **$(NH_4)_2SO_4$** | C | 1.00 | 4.50 | 8.00 | 11.50 | 15.00 |
| **Glutamic Acid** | D | 1.00 | 8.25 | 15.50 | 22.75 | 30.00 |

Based on the data obtained from the experiment an ANOVA table was generated by the program to evaluate the best fitting model for the data set individually for each polymer. ANOVA table for γ-PGA produced is presented in Table 3.4. The p-value for the best fitting model was found to be significant however two of the variables screened (glucose and yeast extract) were not significant, meaning their contribution to the γ-PGA production is negligible. This is partially in correlation with the result obtained from PB study. This is also discussed in detail in Section 4.2. $R^2$ value was found to be 0.83 suggesting that the model was in good correlation with the data.

**Table 3. 4:** ANOVA table for γ-PGA production in double polymer medium with four variables

| | Sum of Squares | Degrees of Freedom | Mean Square | F Value | p-value |
|---|---|---|---|---|---|
| **Model** | 162.53 | 14 | 11.60 | 4.33 | 0.0076 |
| **A-Glucose** | 0.58 | 1 | 0.58 | 0.22 | 0.6500 |
| **B-Yeast Ext** | 1.18 | 1 | 1.18 | 0.44 | 0.5187 |
| **C-$(NH_4)_2SO_4$** | 35.20 | 1 | 35.20 | 13.13 | 0.0035 |
| **D-Glutamic Acid** | 106.96 | 1 | 106.96 | 39.90 | < 0.0001 |
| **AB** | 2.15 | 1 | 2.15 | 0.80 | 0.3880 |
| **AC** | 0.16 | 1 | 0.16 | 0.06 | 0.8111 |
| **AD** | 3.48 | 1 | 3.48 | 1.30 | 0.2765 |
| **BC** | 1.13 | 1 | 1.13 | 0.42 | 0.5270 |
| **BD** | 1.13 | 1 | 1.13 | 0.42 | 0.5270 |
| **CD** | 3.48 | 1 | 3.48 | 1.30 | 0.2765 |
| **$A^2$** | 1.36 | 1 | 1.36 | 0.51 | 0.4895 |
| **$B^2$** | 1.67 | 1 | 1.67 | 0.63 | 0.4441 |
| **$C^2$** | 1.95 | 1 | 1.95 | 0.73 | 0.4098 |
| **$D^2$** | 0.15 | 1 | 0.15 | 0.06 | 0.8122 |
| **Residual** | 32.17 | 12 | 2.68 | | |
| **Lack of Fit** | 28.96 | 10 | 2.89 | 1.80 | 0.4090 |
| **Pure Error** | 3.21 | 2 | 1.60 | | |
| **Std. Error** | 1.63 | | | **$R^2$** | 0.834 |

3D graphs representing these findings as well as the interactions between the variables for γ-PGA production are shown in Figure 3.17.

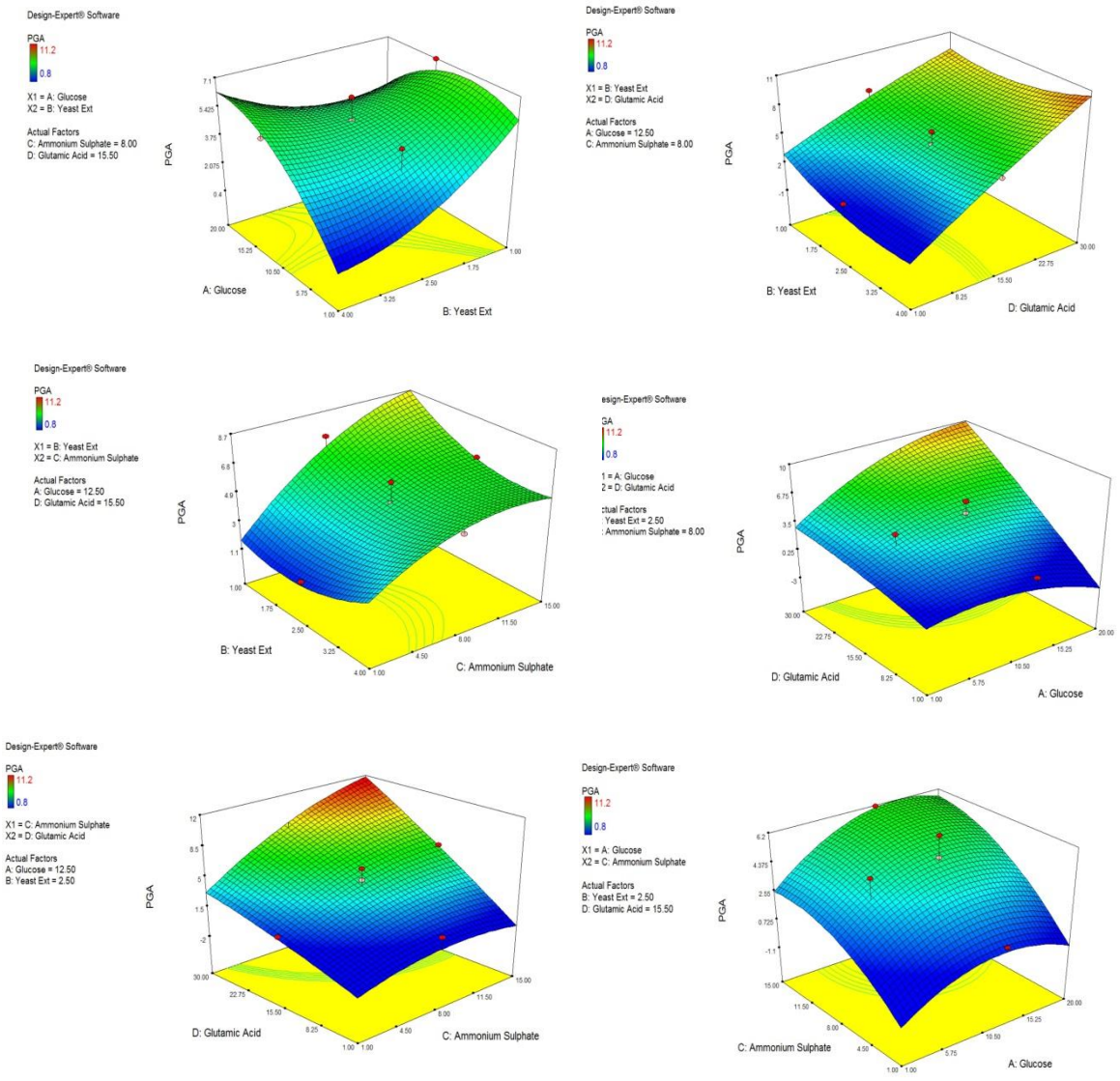



**Figure 3.17:** 3D graphs showing the interactions amongst the variables screened for γ-PGA production.

Similarly ANOVA table generated for P(3HB) production is presented in Table 3.5. Contrary to γ-PGA data, the best fitting model for the data set obtained for P(3HB) production did not fit. The p-value for the best fitting model was found not to be significant and none of the parameters screened were significant except for yeast extract. $R^2$ value was also found to be 0.65 representing a poor fit.

**Table 3. 5:** ANOVA table for P(3HB) production in double polymer medium with four variables

| | Sum of Squares | Degrees of Freedom | Mean Square | F Value | p-value |
|---|---|---|---|---|---|
| **Model** | 2093.24 | 14 | 149.52 | 1.56 | 0.2214 |
| **A-Glucose** | 33.29 | 1 | 33.29 | 0.35 | 0.5659 |
| **B-Yeast Ext** | 478.86 | 1 | 478.86 | 5.01 | 0.0449 |
| **C-$(NH_4)_2SO_4$** | 35.98 | 1 | 35.98 | 0.38 | 0.5509 |
| **D-Glutamic Acid** | 677.35 | 1 | 677.35 | 7.09 | 0.0207 |
| **AB** | 19.74 | 1 | 19.74 | 0.21 | 0.6575 |
| **AC** | 0.09 | 1 | 0.096 | 0.001 | 0.9752 |
| **AD** | 3.47 | 1 | 3.47 | 0.036 | 0.8521 |
| **BC** | 8.40 | 1 | 8.40 | 0.088 | 0.7719 |
| **BD** | 98.58 | 1 | 98.58 | 1.03 | 0.3298 |
| **CD** | 448.90 | 1 | 448.90 | 4.70 | 0.0510 |
| **$A^2$** | 15.02 | 1 | 15.02 | 0.16 | 0.6987 |
| **$B^2$** | 249.98 | 1 | 249.98 | 2.62 | 0.1317 |
| **$C^2$** | 0.82 | 1 | 0.82 | 0.0085 | 0.9278 |
| **$D^2$** | 61.01 | 1 | 61.01 | 0.64 | 0.4398 |
| **Residual** | 1146.50 | 12 | 95.54 | | |
| **Lack of Fit** | 1105.78 | 10 | 110.58 | 5.43 | 0.1654 |
| **Pure Error** | 40.73 | 2 | 20.36 | | |
| **Std. Error** | 9.77 | | | **$R^2$** | 0.646 |

3D graphs generated based on the non-fitting model for P(3HB) production are shown in Figure 3.18. As seen clearly from the 3D graphs, no optimum point can be identified neither can trends be interpreted.

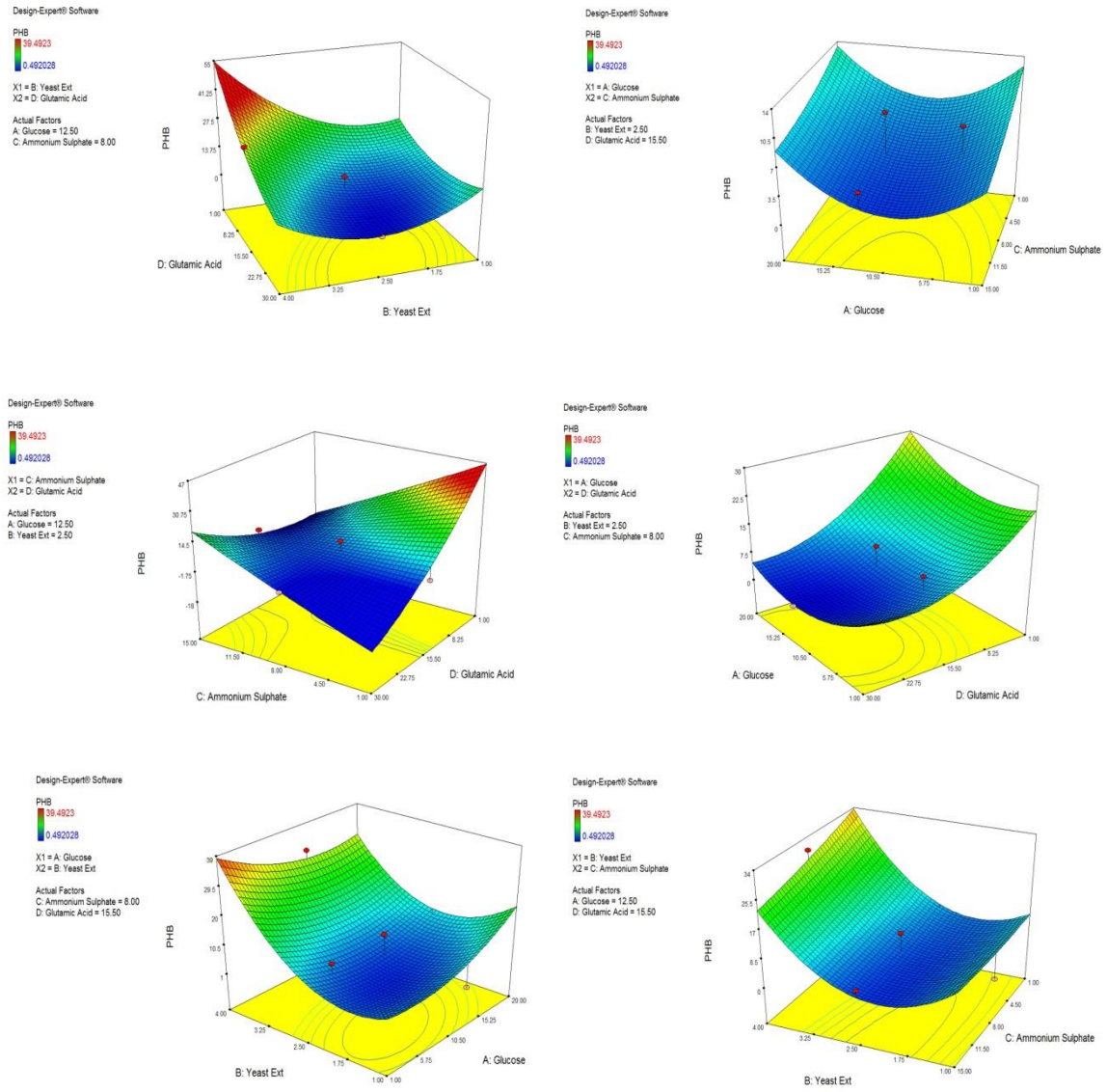



**Figure 3. 18:** 3D graphs showing the interactions amongst the variables screened for P(3HB) production.

It was concluded that double polymer medium and production mechanism were too complex to be modelled using Design Expert and CCD, therefore could not be directly optimised using parameters suggested by PB. A more complex and integrated approach was needed in order to reduce the parameters and to enable the use of CCD for optimization of dual production of P(3HB) and γ-PGA. Therefore, experiments were conducted on simplification of parameters, and simplified CCD is presented in Section 3.4.2.

### 3.4.2 Simplifying Variables and Optimization of Double Polymer Production

Based on the previous experiments (Section 3.3.2) and literature (Akaraonye *et al.*, 2010; Zafar *et al.*, 2012), C/N ratio is known to have a crucial importance in P(3HB) production and so is considered to be an important parameter for dual polymer production. The complexity of the double polymer production metabolism was demonstrated in previous section (CCD for DP, Section 3.4.1). In order to keep the important parameters constant for at least one of the polymers it was necessary to keep fixed the ratio of C/N. Different yeast extract compositions were tested in a model DP medium where all other medium components (Glucose: 20 g/L; Glutamic Acid: 20 g/L; Ammonium Sulphate: 7.5 g/L; Citric acid: 1.5 g/L) were kept constant so that the effect of further addition/supplementation of organic nitrogen source (yeast extract) could be determined.

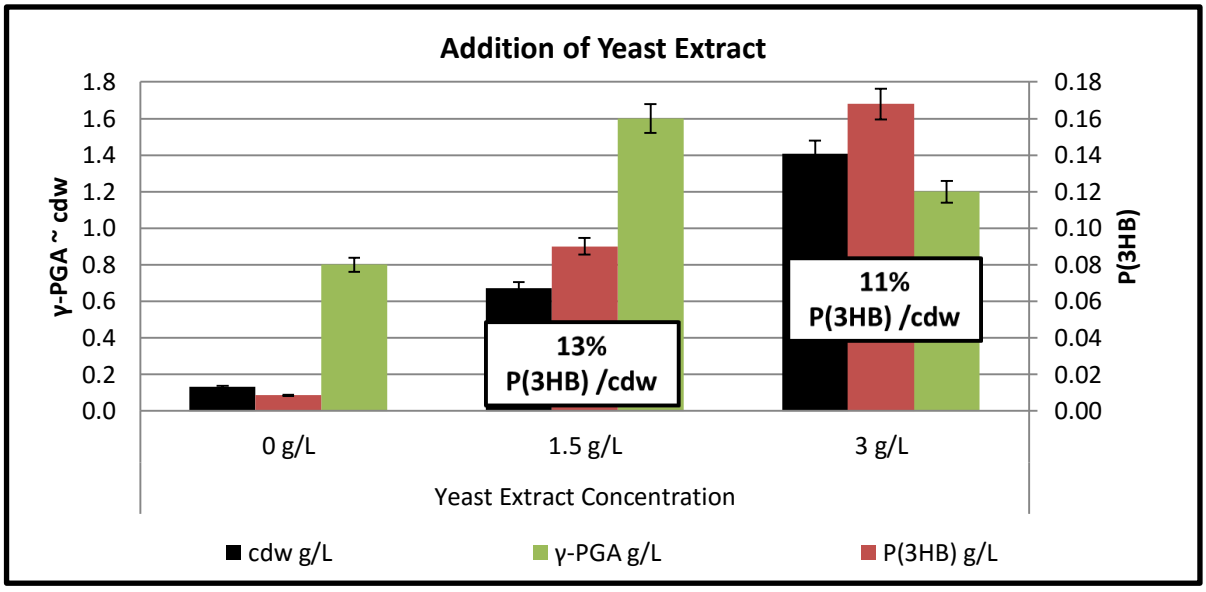



**Figure 3. 19:** Effect of Yeast Extract Addition in dual polymer production under constant glucose (20 g/L), glutamic acid (20 g/L), ammonium sulphate (7.5 g/L) and citric acid (1.5 g/L). Statistical analysis given in Section 8.9.2, all levels found significant at the level $p<0.05$ compared to the control.

Although P(3HB) concentration was found to be higher using 3 g/L yeast extract, the percentage P(3HB)/cdw (13%) was higher for 1.5 g/L yeast extract, in addition γ-PGA production was found to be higher for 1.5 g/L yeast extract (Figure 3.19). Therefore, from this point onwards glucose and yeast extract levels were kept constant at 20 g/L and 1.5 g/L respectively.

Subsequently a new CCD was designed under the simplified conditions. The positive effect of citric acid on production of P(3HB) was shown in previous experiments (Section 3.3.2) and was already demonstrated in literature (Kessler and Witholt, 2001, Anderson and Dawes, 1990, Aldor and Keasling, 2003). Although, PB screening study for double polymer production suggested that the effect of citric acid is negative (Section 3.3.3), it was decided to include it to the design matrix due to the positive effects found in this study and reported in the literature. The concentration of citric acid was decided to be kept a low level (0-3 g/L) in order to avoid the growth inhibition effect, which has been explained in section 3.3.2.

The total number of experiments with three variables in five levels was 18. Eighteen experiments were augmented with four replications at the centre points to evaluate the pure error. The experiments were conducted in shake flasks at 30°C and 200 rpm. The P(3HB) and γ-PGA concentrations were taken as the responses. Experiments were performed according to experimental design matrix given in Table 3.6, within the ranges indicated and analysed as described in Section 2.7.2.

**Table 3. 6:** Experimental design matrix showing five levels for CCD (α=1.69)

| Parameter (g/L) | Code | -α | -1 | 0 | +1 | +α |
|---|---|---|---|---|---|---|
| **Citric Acid** | A | 0.00 | 0.61 | 1.50 | 2.39 | 3.00 |
| **Glutamic Acid** | B | 0.00 | 8.11 | 20.00 | 31.89 | 40.00 |
| **$(NH_4)_2SO_4$** | C | 0.00 | 3.04 | 7.50 | 11.96 | 15.00 |

ANOVA table generated by the program for γ-PGA production is presented in Table 3.7. The p-value for the best fitting model was found to be significant as well as two of the variables screened (glutamic acid and ammonium sulphate). However, citric acid was not significant. The reasons for this are discussed in detail in Section 4.2. $R^2$ value was found to be 0.89, which was a similar value to the one obtained from the previous CCD, suggesting that the model was in good correlation with the data. The model obtained from the data is presented in equation 3.1.

**Table 3. 7:** ANOVA table for γ-PGA production in double polymer medium with three variables

| | **Sum of Squares** | **Degrees of Freedom** | **Mean Square** | **F Value** | **p-value** |
|---|---|---|---|---|---|
| **Model** | 94.645 | 9 | 10.5161 | 9.35 | 0.0008 |
| **A-Citric Acid** | 0.0049 | 1 | 0.00497 | 0.004 | 0.9483 |
| **B-Glutamic acid** | 23.469 | 1 | 23.4693 | 20.88 | 0.0010 |
| **C-$(NH_4)_2SO_4$** | 41.296 | 1 | 41.2968 | 36.74 | 0.0001 |
| **AB** | 0.2222 | 1 | 0.22222 | 0.20 | 0.6661 |
| **AC** | 0.1422 | 1 | 0.14222 | 0.13 | 0.7295 |
| **BC** | 15.308 | 1 | 15.3089 | 13.62 | 0.0042 |
| **$A^2$** | 0.0192 | 1 | 0.01928 | 0.02 | 0.8984 |
| **$B^2$** | 9.5581 | 1 | 9.55811 | 8.50 | 0.0154 |
| **$C^2$** | 5.8590 | 1 | 5.8590 | 5.21 | 0.0456 |
| **Residual** | 11.2416 | 10 | 1.12416 | | |
| **Lack of Fit** | 6.12157 | 5 | 1.22431 | 1.20 | 0.4247 |
| **Pure Error** | 5.12 | 5 | 1,024 | | |
| **Std. Error** | 1.06 | | | **$R^2$** | 0.894 |

$$\boldsymbol{PGA(g/L) = 2.027 + (0.019 * A) + (1.311 * B) + (1.739 * C) + (0.167 * AB) - (0.133 * AC) + (1.383 * BC) + (0.037 * A^2) + (0.814 * B^2) + (0.638 * C^2)} \quad (3.1)$$

3D graphs representing these findings as well as the interactions between the variables for γ-PGA production are shown in Figure 3.20. Interaction between ammonium sulphate and glutamic acid can be clearly seen in the Figure. However, as indicated in ANOVA table, citric acid did not have any significant effect on γ-PGA production.

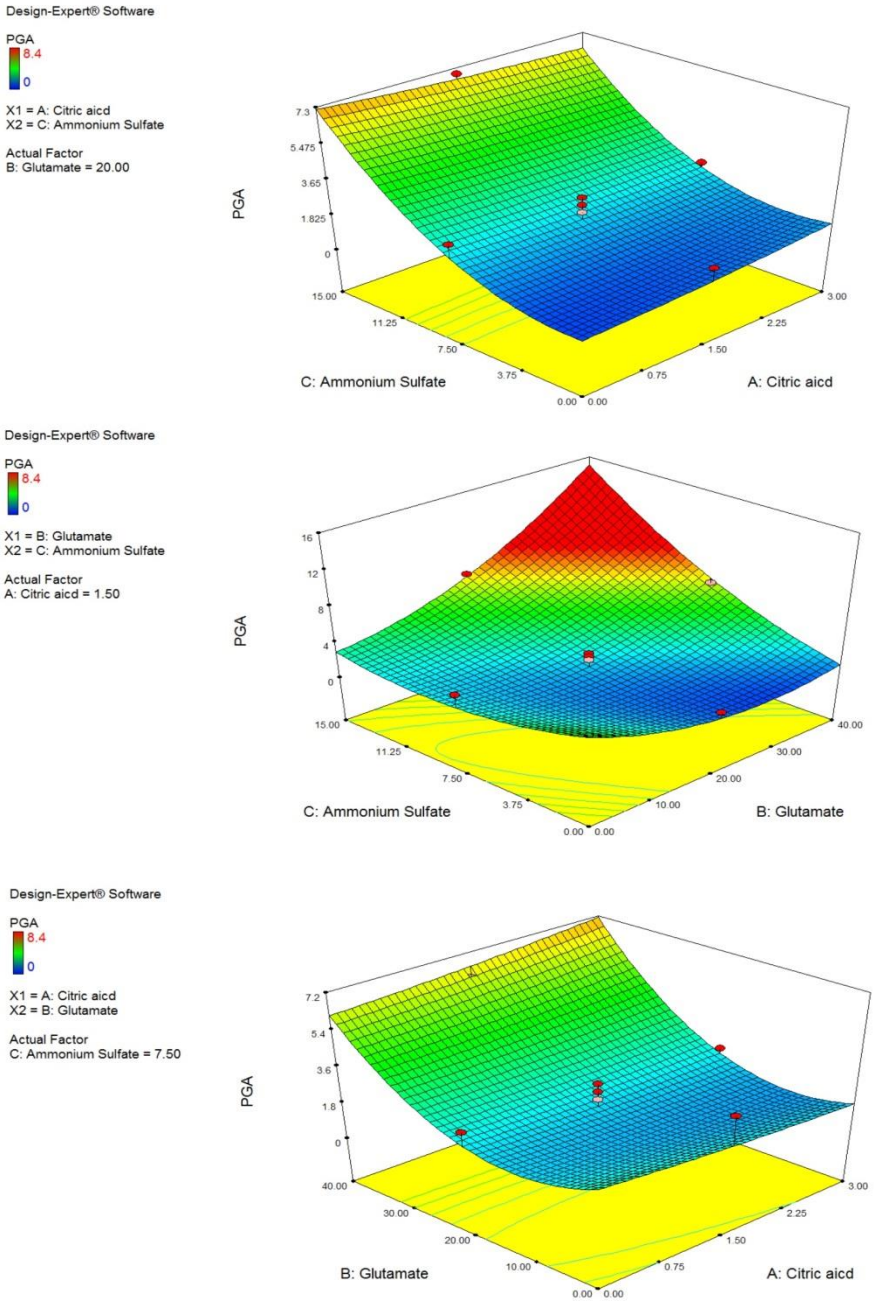



**Figure 3. 20:** 3D graphs showing the interactions amongst the variables screened for γ-PGA production.

Furthermore, ANOVA table generated for P(3HB) production to evaluate the best fitting model for the data set is presented in Table 3.8. The p-value for the best fitting model was found to be significant as well as for the two of the variables screened (citric acid and ammonium sulphate). However, glutamic acid was found not to be significant which is in disagreement with the previous result obtained from double polymer PB screening. This might be due to the simplification of the parameters and keeping ratio of C/N constant. This is further discussed in Section 4.2. $R^2$ value was found to be 0.91, which was much higher compared to the one obtained from the previous CCD, suggesting that the model was in much better correlation with the data. The model obtained from the data for P(3HB) production is given in equation 3.2.

3D graphs representing these findings and the interactions between the variables for P(3HB) production are shown in Figure 3.21. Interaction between ammonium sulphate and citric acid can be clearly seen in the Figure however, as indicated in ANOVA table, glutamic acid did not have any significant effect on P(3HB) production. This is also further discussed in Section 4.2

**Table 3. 8:** ANOVA table for P(3HB) production in double polymer medium with three variables

| | Sum of Squares | Degrees of Freedom | Mean Square | F Value | p-value |
|---|---|---|---|---|---|
| **Model** | 0.0448 | 9 | 0.0049 | 11.79 | 0.0003 |
| **A-Citric Acid** | 00195 | 1 | 0.0195 | 46.18 | < 0.0001 |
| **B-Glutamic Acid** | 0.0013 | 1 | 0.0013 | 3.10 | 0.1086 |
| **C-$(NH_4)_2SO_4$** | 0.0117 | 1 | 0.0117 | 27.80 | 0.0004 |
| **AB** | 0.0001 | 1 | 0.0001 | 0.25 | 0.6294 |
| **AC** | 0.0058 | 1 | 0.0058 | 13.86 | 0.0040 |
| **BC** | 0.0004 | 1 | 0.0004 | 1.11 | 0.3169 |
| **$A^2$** | 0.0007 | 1 | 0.0007 | 1.63 | 0.2304 |
| **$B^2$** | 0.0026 | 1 | 0.0026 | 6.24 | 0.0315 |
| **$C^2$** | 0.0019 | 1 | 0.0019 | 4.67 | 0.0559 |
| **Residual** | 0.0042 | 10 | 0.0004 | | |
| **Lack of Fit** | 0.0030 | 5 | 0.0006 | 2.66 | 0.1530 |
| **Pure Error** | 0.0011 | 5 | 0.0002 | | |
| **Std. Error** | 0.02 | | | **$R^2$** | 0.914 |

$$\boldsymbol{PHB(g/L) = 0.106 + (0.038 * A) - (0.010 * B) + (0.029 * C) - (0.004 * A} \quad (3.2)$$

$$\boldsymbol{(0.027 * AC) - (0.008 * BC) - (0.007 * A^2) + (0.014 * B^2) - (0.012 * C^2)}$$

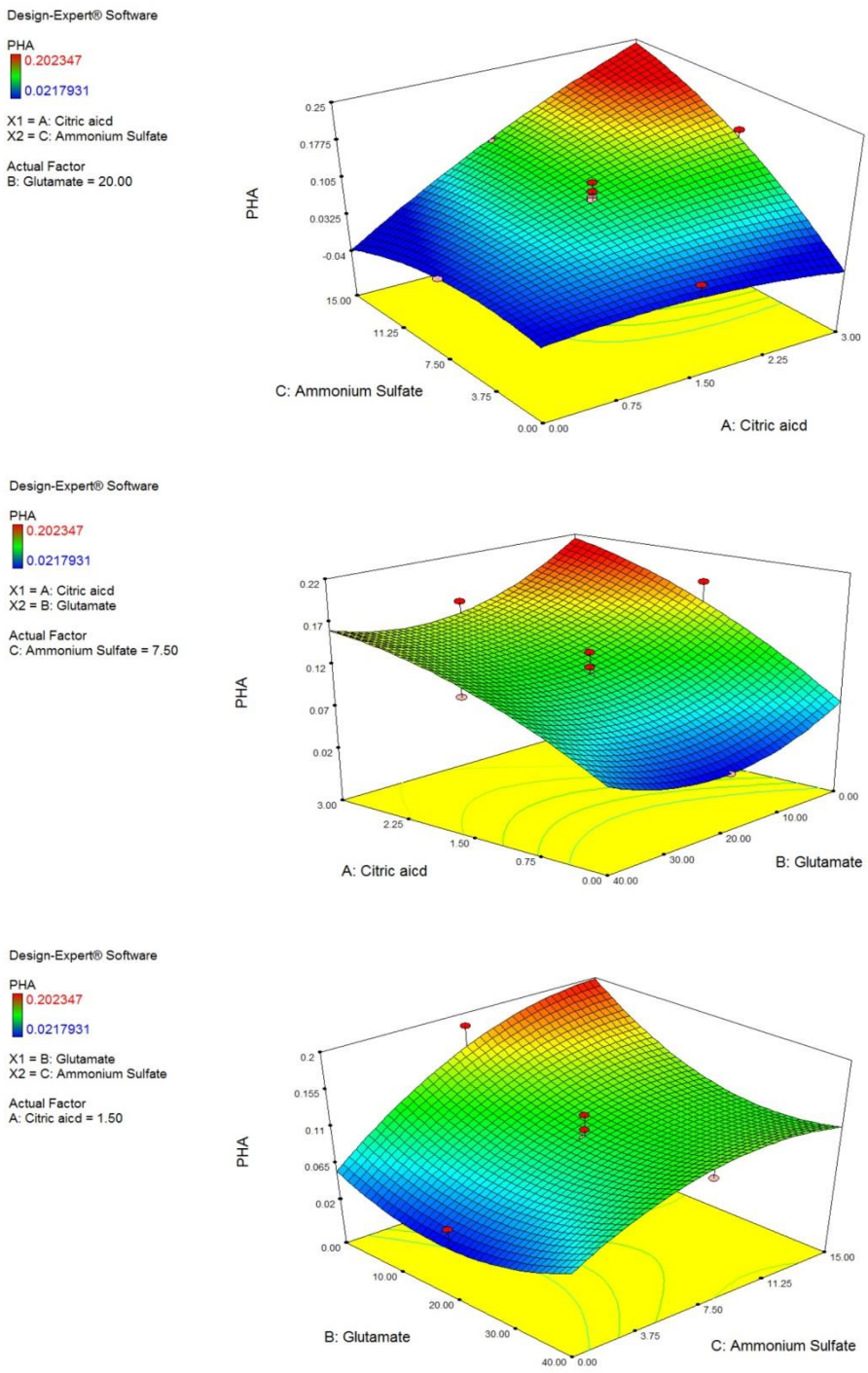



**Figure 3. 21:** 3D graphs showing the interactions amongst the variables screened for P(3HB) production.

As a result of CCD analysis and based on the models generated, an optimum point was calculated and suggested by the program for the highest production of P(3HB) and γ-PGA within 95% confidence level. The medium composition is presented in Table 3.9. According to the suggestion the optimum medium for double polymer production should

contain citric acid, Glutamic acid, and ammonium sulphate at levels of 2.4 g/L, 32 g/L and 12 g/L respectively.

**Table 3. 9:** Optimized medium compositions and production prediction with confidence ranges

| **Citric acid (g/L)** | **Glutamic acid (g/L)** | **$(NH_4)_2SO_4$ (g/L)** | **P(3HB)/ γ-PGA (g/L)** | **Desirability** |
|---|---|---|---|---|
| 2.39 | 31.98 | 11.96 | 0.174/8.01 | 0.895 |
| **Response** | **Prediction** | **SE Mean** | **95% CI low** | **95% CI high** |
| P(3HB) (g/L) | 0.174 | 0.017 | 0.14 | 0.21 |
| γ-PGA (g/L) | 8.01 | 0.87 | 6.07 | 9.93 |

### 3.4.3 Analysis of the effects of the individual media components based on CCD

CCD analyses provided a holistic picture of the integrated effects. Individual responses, for each parameter, are only shown based on the model generated and sometimes can be misleading. In order to observe the actual individual effects of each medium component, the data gathered for CCD was further analysed. Keeping two out of three parameters constant, the effects of increasing the concentrations of individual medium components on production of two polymers were investigated. Figures 3.22, 3.23 and 3.24 show the effect of different concentrations of citric acid, glutamic acid and ammonium sulphate on P(3HB), γ-PGA and cdw respectively.

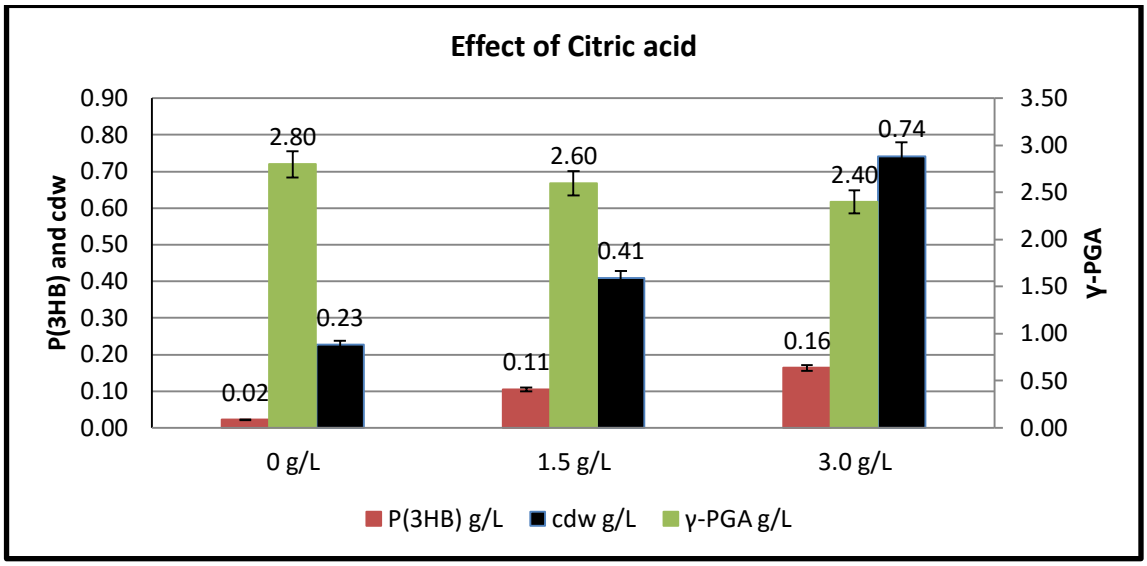



**Figure 3. 22:** Effect of Citric Acid on growth and double polymer production. Other medium components; glucose 20 g/L, yeast extract 1.5 g/L glutamic acid 20 g/L, ammonium sulphate 7.5 g/L. Statistical analysis given in Section 8.9.3, all levels found significant at the level $p<0.05$ compared to the control (0 g/L).

When individually evaluated citric acid addition improved P(3HB) production and slightly decreased γ-PGA production (Figure 3.22). However, when dual polymer production is taken into consideration it can be seen that citric acid addition has a positive effect. γ-PGA concentration decreased by 14% when 3 g/L citric acid was added to the medium compared to the production in the medium with no citric acid. This negative effect can be neglected since 3 g/L citric acid increased P(3HB) production eight times compared to the production with no citric acid.

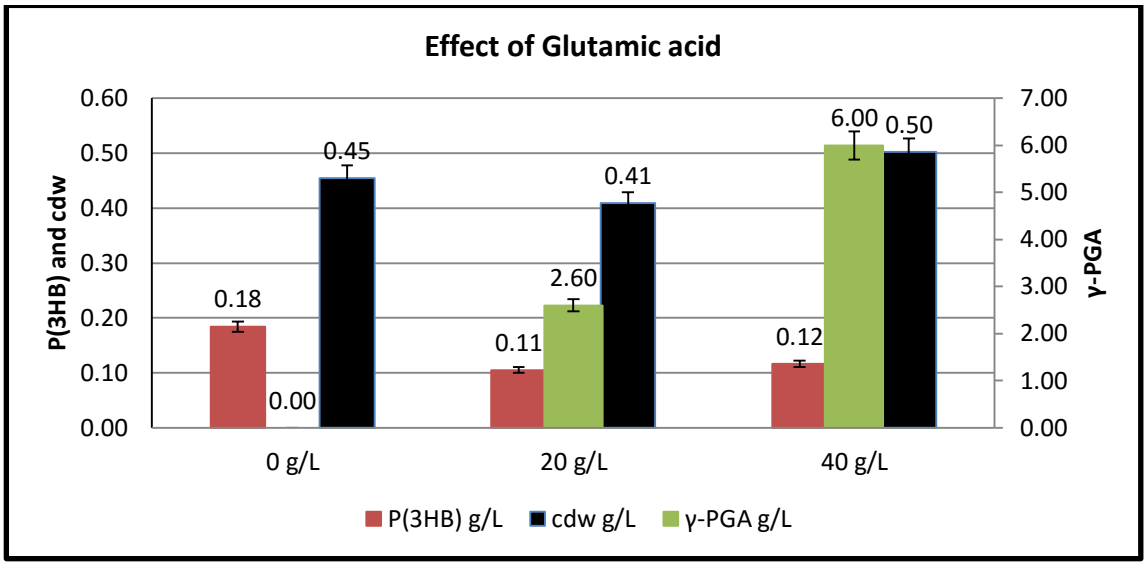



**Figure 3. 23:** Effect of glutamic acid on growth and double polymer production. Other medium components; glucose 20 g/L, yeast extract 1.5 g/L citric acid 1.5 g/L, ammonium sulphate 7.5 g/L Statistical analysis given in Section 8.9.4, all levels found significant at the level $p<0.05$ compared to the control (0 g/L).

Similarly, it was observed that glutamic acid is definitely essential for γ-PGA production (Figure 3.23). When there was no glutamic acid present in the medium, γ-PGA was not produced at all whereas when there was 20 g/L, 2.6 g/L γ-PGA was produced. It can be seen also that presence of glutamic acid decreased P(3HB) production by 38% in the medium containing 40 g/L glutamic acid compared to the medium with no glutamic acid. Further addition of glutamic acid improved γ-PGA production, but P(3HB) level remained almost constant. The combined effect on double polymer production is more complex and further discussed in Section 4.2.

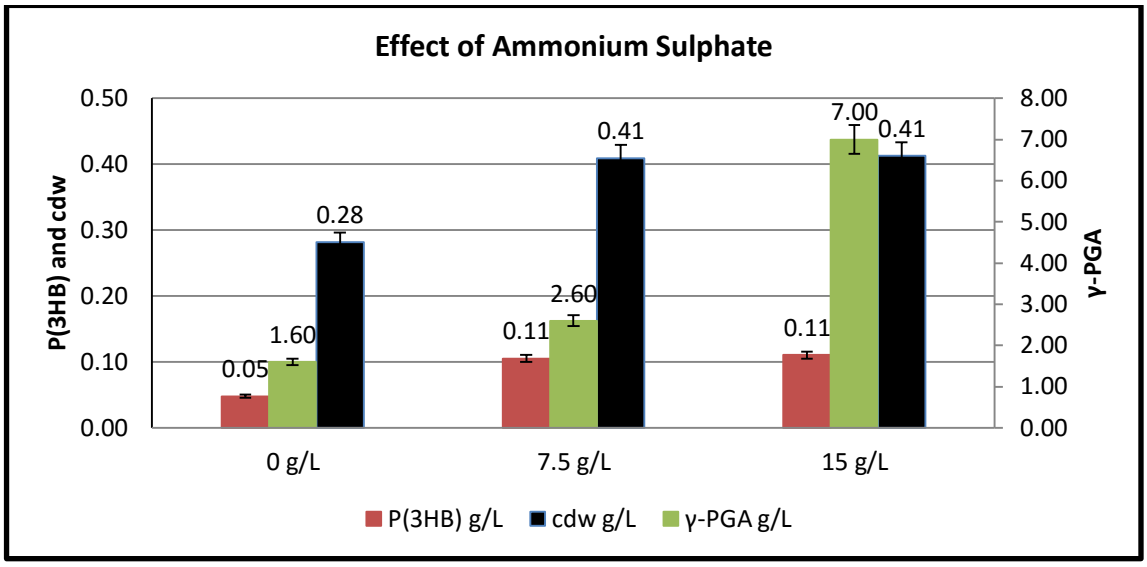



**Figure 3. 24:** Effect of ammonium sulphate on growth and double polymer production. Other medium components; glucose 20 g/L, yeast extract 1.5 g/L glutamic acid 20 g/L, citric acid 1.5 g/L. Statistical analysis given in Section 8.9.5, all levels found significant at the level $p<0.05$ compared to the control (0 g/L).

Ammonium sulphate also contributed to the production of both polymers. From the Figure 3.24 it can be seen that the presence of ammonium sulphate has doubled the P(3HB) production and γ-PGA production. The effect was much pronounced on γ-PGA than for P(3HB). Although it is not a definite requirement for double production, presence enhances the levels produced.

Consequently, the optimized dual polymer production medium composition suggested by the program as; 20 g/L glucose, 1.5 g/L yeast extract, 32 g/L glutamic acid, 12 g/L ammonium sulphate and 2.4 g/L citric acid was selected and used in all experiments from this point onwards and referred to as double polymer (DP) medium.

### 3.4.4 Effect of Inoculum conditions

Productivity in batch cultures depends on how fast the organism grows and produces the desired product. The inoculum medium (pre-culture) also has an effect on the subsequent growth profile in the production medium. During the optimisation studies for dual polymer medium, it was observed that the lag phase in DP medium was longer than expected (20 h in DP medium compared to 4 h in P(3HB) medium) in the shake flask cultures, and in fact inoculum medium had a noticeable effect on growth and polymer production of the final culture. Four different media were tested for their effects on growth of the organism. When inoculum was grown in nutrient broth (NB) and re-inoculated into NB or P(3HB) medium, there was almost no lag phase (Figure 3.25 a-b). However, when NB grown inoculum was transferred into γ-PGA or DP media there was a considerable lag phase, in fact very little growth on DP medium was observed (Figure 3.25 c-d). The curves of best fit for the growth curves are presented in Figure 3.25 and the actual growth profile comparisons are given in Figure 3.26.

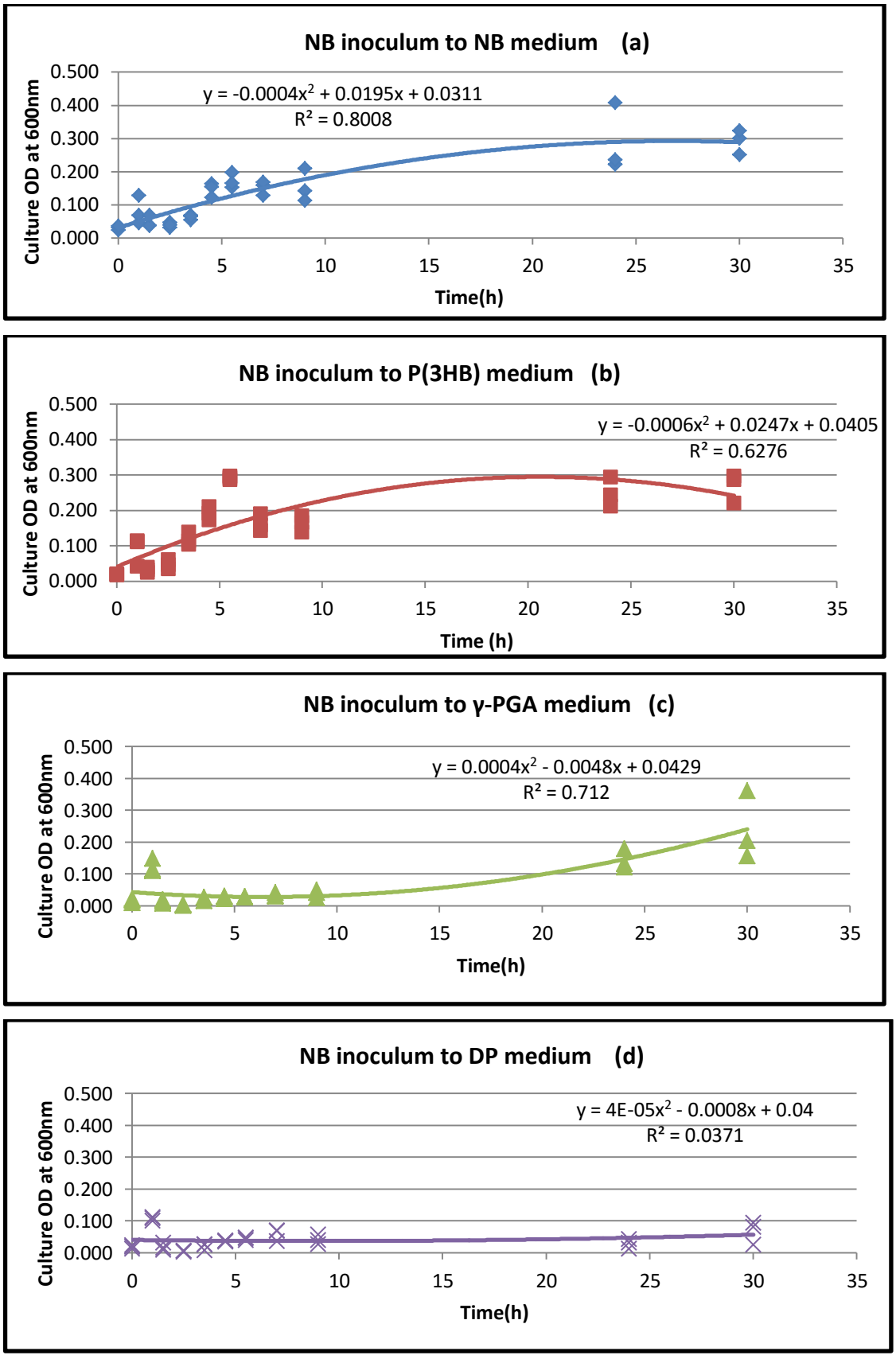



**Figure 3. 25:** Growth curves of *B.subtilis* OK2 grown on (a) nutrient broth, (b) P(3HB) medium, (c) γ-PGA medium (d) double polymer-DP medium using inoculum grown in nutrient broth

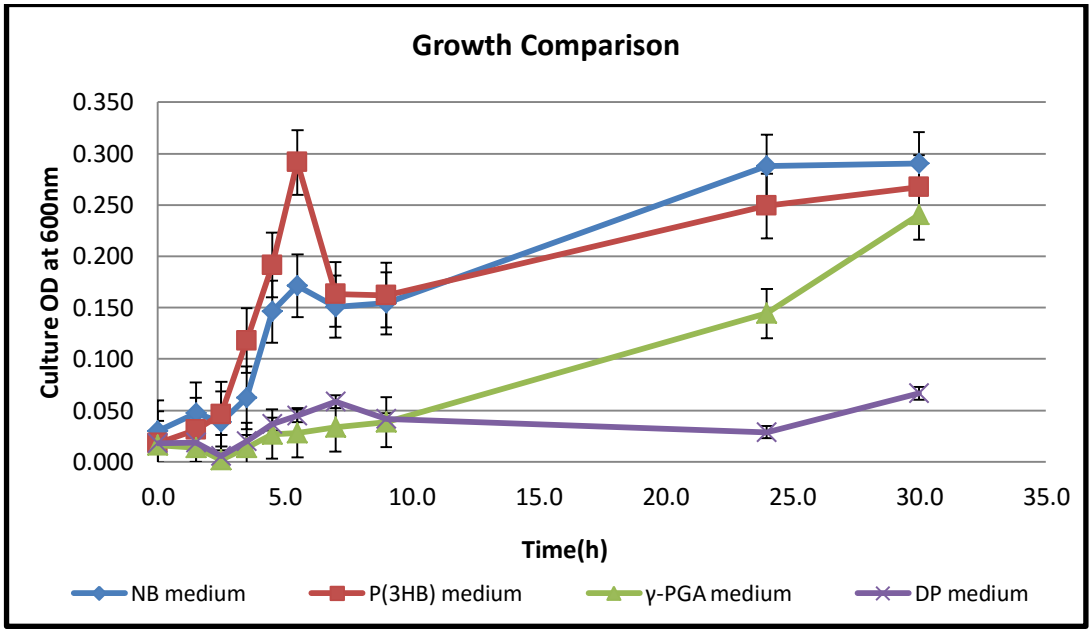



**Figure 3. 26:** Growth curves of *B.subtilis* OK2 grown in different fermentation media using inoculum pre-grown in nutrient broth. Duplicate measurements in three experiments.

**Table 3. 10:** Specific growth rate and double time of *B. subtilis* OK2 in different media

| Medium | μ ($h^{-1}$) | $t_d$ (h) |
|---|---|---|
| NB | 0.535 | 1.30 |
| P(3HB) | 0.600 | 1.16 |
| γ-PGA | 0.135 | 5.13 |
| DP | 0.034 | 20.39 |

The specific growth rates and doubling times in each medium is presented in Table 3.10. As shown by the doubling times the growth in DP medium from NB inoculum was very weak and had long lag phase. Therefore, different inoculum media were considered in

order to decrease the lag phase and increase the productivity. The 16 h cultures grown in P(3HB) and γ-PGA media were inoculated into DP medium (Figure 3.27).

A better growth and double polymer production was observed when inocula were grown in γ-PGA medium. Due to better growth and dual production, the inoculum from γ-PGA medium to DP medium was further investigated.

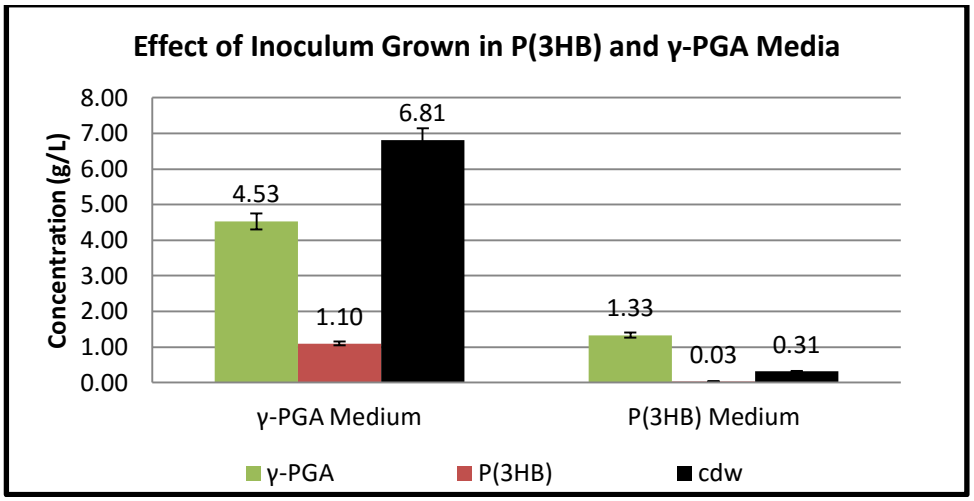



**Figure 3. 27:** Double polymer production in DP medium with inocula grown in P(3HB) and γ-PGA media. Triplicate measurements in two different experiments.

The growth curve comparison of cultures is presented in Figure 3.28. It can be seen that culture inoculated from γ-PGA medium into DP medium has shown a better growth and higher $OD_{600}$ than even the control which is represented by NB to NB curve. The modelled growth curve from γ-PGA to DP can be seen in Figure 3.29.

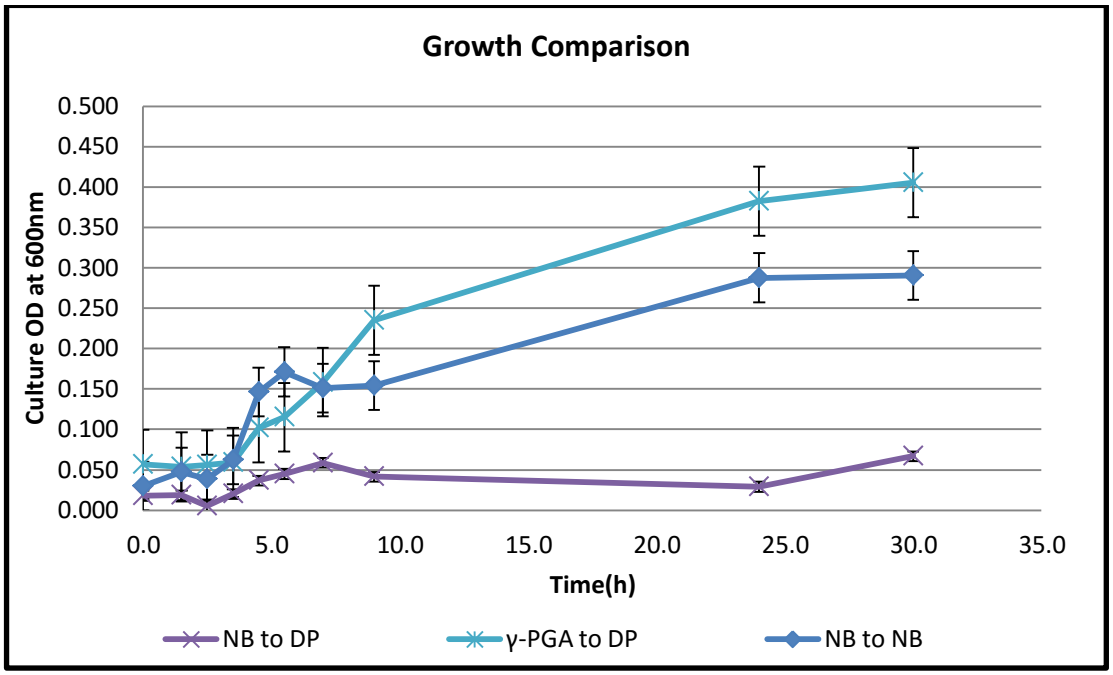



**Figure 3. 28:** Growth curve of *B.subtilis* OK2 grown on double polymer medium with inoculum grown on nutrient broth and γ-PGA medium

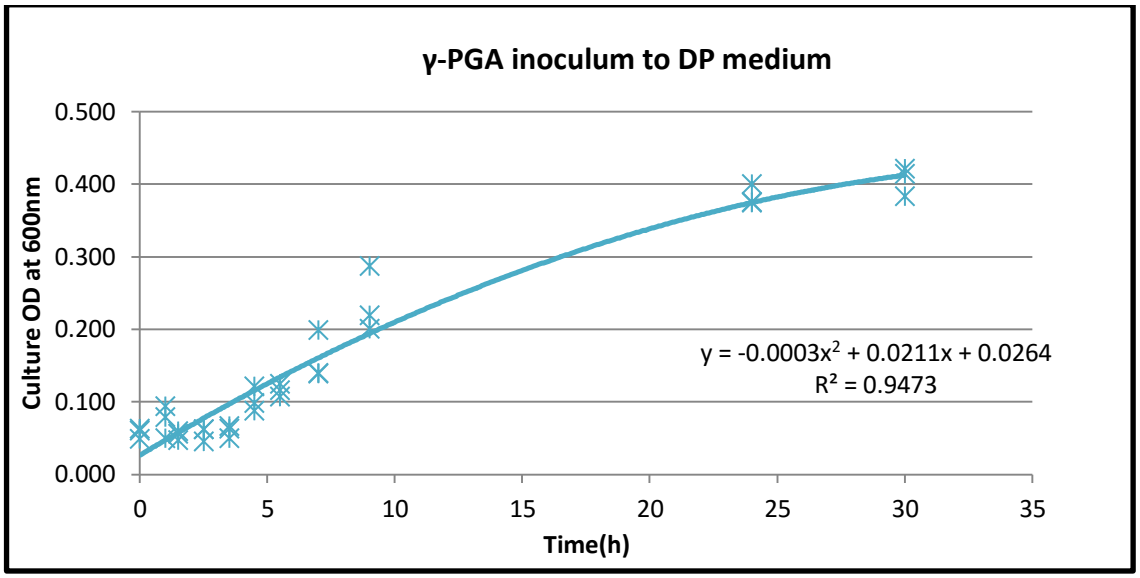



**Figure 3. 29:** Growth curve of *B. subtilis* OK2 grown on double polymer-DP medium using inoculum grown in γ-PGA medium.

To elucidate the effect of different inocula on cell growth and morphology, the cultures were investigated under the microscope after the 6 h of cultivation.

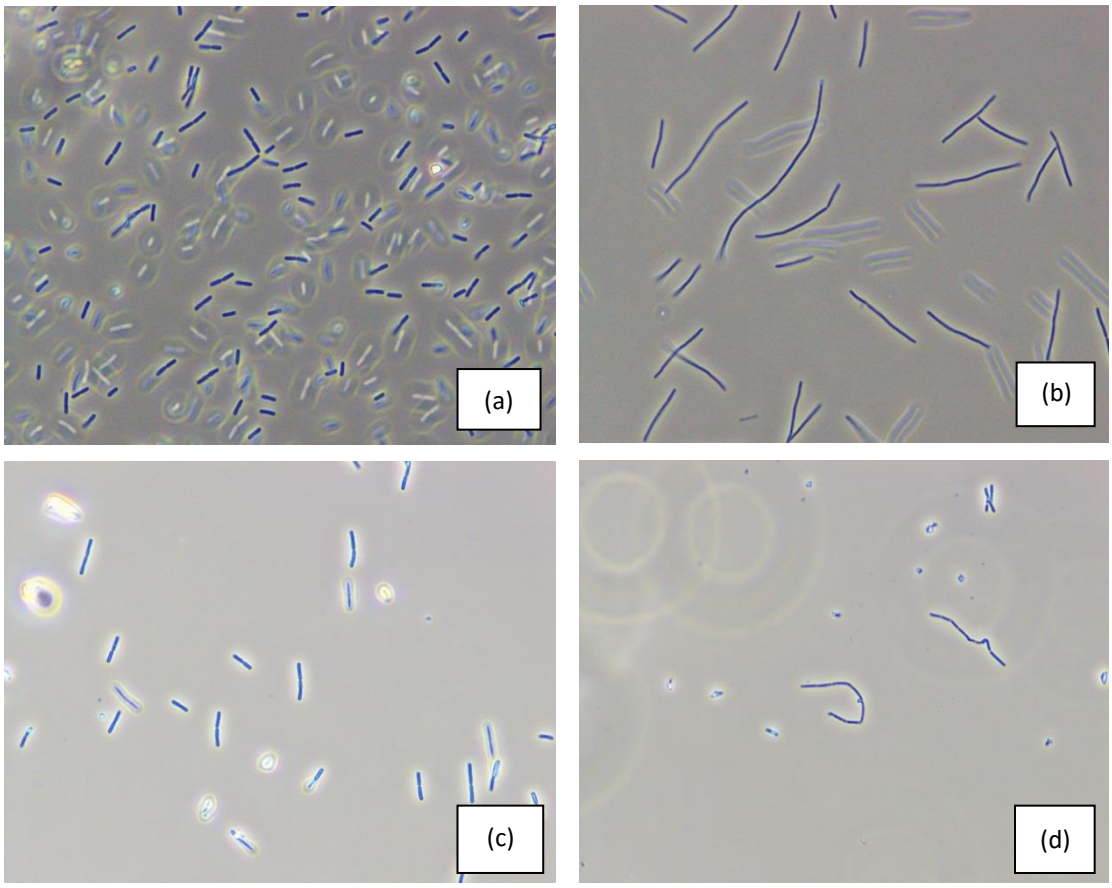



**Figure 3. 30:** Microscopic observation of *B. subtilis* OK2 growth in different media after 6 h. (a) NB medium, (b) DP medium inoculated from culture grown in γ-PGA medium, (c) P(3HB) medium, (d) γ-PGA medium. (400x magnification, phase contrast mode)

Figure 3.30 shows that the culture morphology is very different in different media. The culture grown in NB medium was dense and the bacteria looked short and in singular form (Figure 3.30a). This was the same in the culture grown in P(3HB) medium (Figure 3.30c). The culture grown in γ-PGA medium did not develop well and concentration of cells was low (Figure 3.30d). In DP medium inoculated with culture grown in γ-PGA medium bacteria again formed long chain structures but was thinner and more in number. The reasons for this behaviour are further discussed in Section 4.2. Based on the findings presented, from this point onwards all DP production experiments were carried out using inocula grown in γ-PGA medium.

### 3.5 Double Polymer Production

#### 3.5.1 Production Profile in Shaken Flasks

In the previous section the medium composition was optimized, and the effects of independent components were studied. In addition, the growth of the organism under different inoculum conditions was investigated. Using this data, the growth of *Bacillus subtilis* OK2 and production profile of double polymer was investigated in shaken flasks. Results obtained from triplicate runs are shown in Figure 3.31. The optimized DP medium was inoculated with 16h inoculum grown in γ-PGA medium and was incubated for 72 h at 30° C and 200 rpm in an orbital shaker.

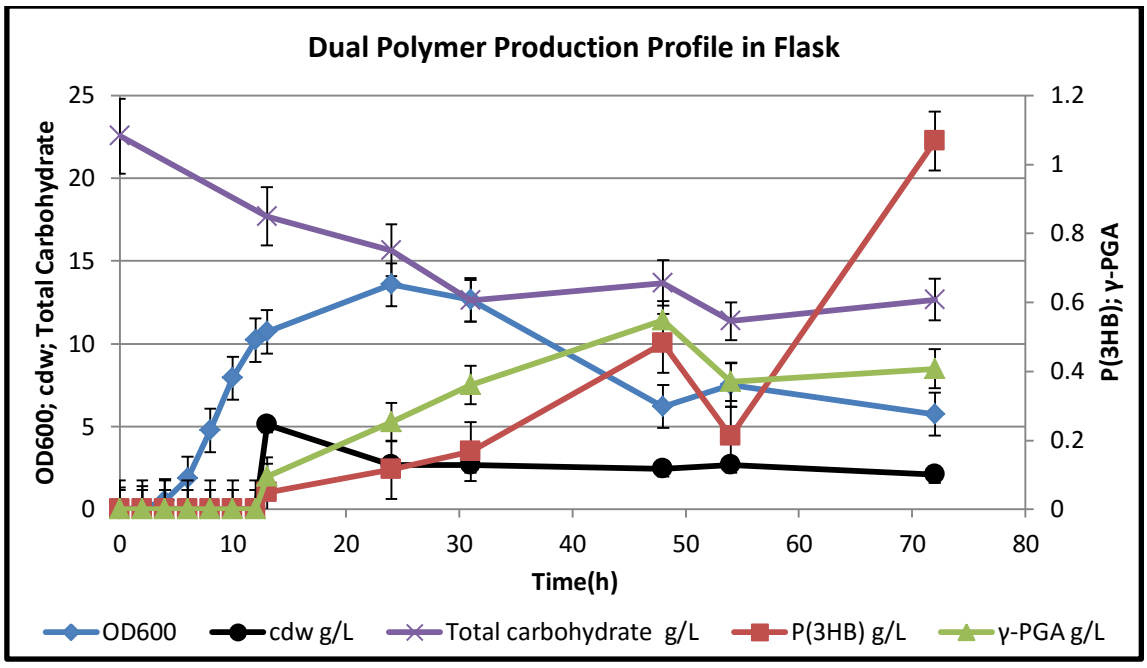



**Figure 3. 31:** Fermentation profile for, growth, P(3HB) and γ-PGA production, and total carbohydrate consumption in flasks for 72 h. Conditions; 30°C 200 rpm, average of triplicate experiments

The culture growth started after 4 h and the exponential phase continued until around 12 h. The culture optical density started to decline after 24 h but this trend did not reflect in cdw. Both polymer productions started at around similar time (after 13 h) however the trends were different. γ-PGA production started faster and reached to maximum concentration after 48 h whereas P(3HB) production was slower (between 13 h – 30 h) but accelerated

after 30 h. Total carbohydrate concentration dropped from at around 22.2 g/L to 12.3 g/L. By 72 h approximately half of the carbon source was still present in the culture broth, 1g/L P(3HB), and 0.4 g/L γ-PGA was produced. The microscopic observations of different phases of the fermentation are shown in Figure 3.32.

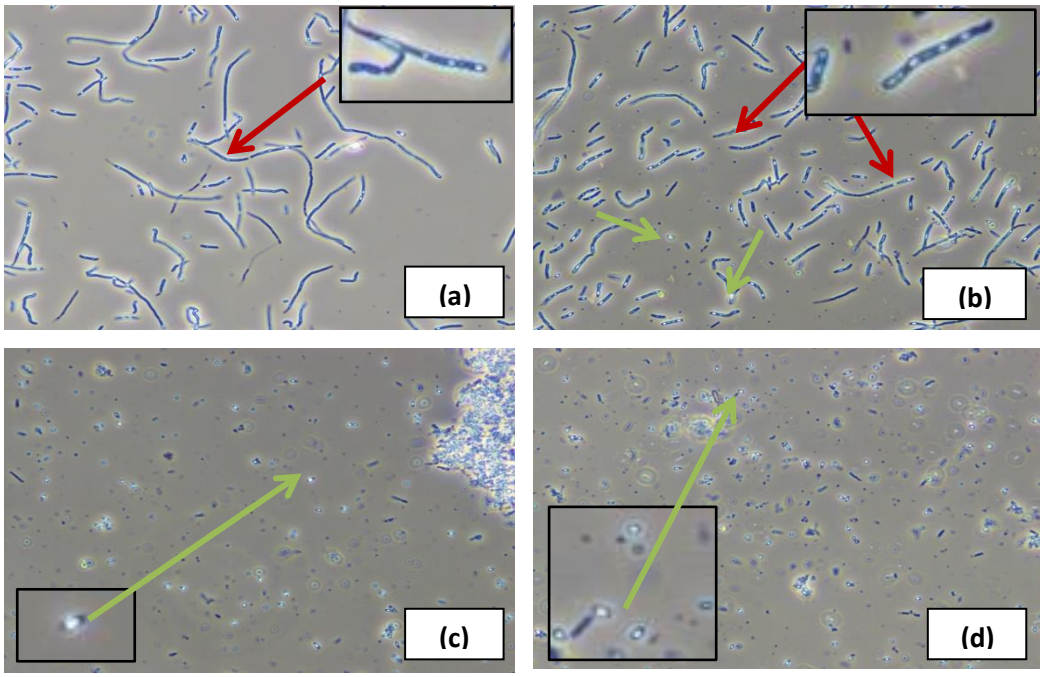



**Figure 3. 32:** Microscopic observation of *Bacillus subtilis* OK2 at different phases of double polymer fermentation in flasks (a) 24 h, (b) 30 h, (c) 48 h, (d) 72 h. (400x magnification, phase contrast mode)

Start of the accumulation of P(3HB) as granules can be observed in Figures 3.32a and 3.32b (indicated by red arrows). It appears that that the decline in culture $OD_{600}$ was due to the cell lysis starting after 30 h. Start of the decrease in cell size and release of endospores can be observed in Figure 3.32b (indicated with green arrows). These microscopic observations of *Bacillus subtilis* undergoing cell lysis have also been observed and defined by Muchova *et al*., (2011). In addition, after 48 h clumps of cell debris were visible under microscope (Figure 3.32c, indicated with a green arrow).

The growth and production profiles were plotted and respective correlations were obtained. Curves of best fit for P(3HB) and γ-PGA production, total carbohydrate consumption and $OD_{600}$, are presented in Figure 3.33 and 3.34. Respective correlations, equations and

functions were obtained based on data (fermentation data on page 125, Figure 3.31). Using the functions obtained from the fermentation profile (Figure 3.31), productivity, yield and specific rates could be derived. This provided a better insight into the double polymer production profile.

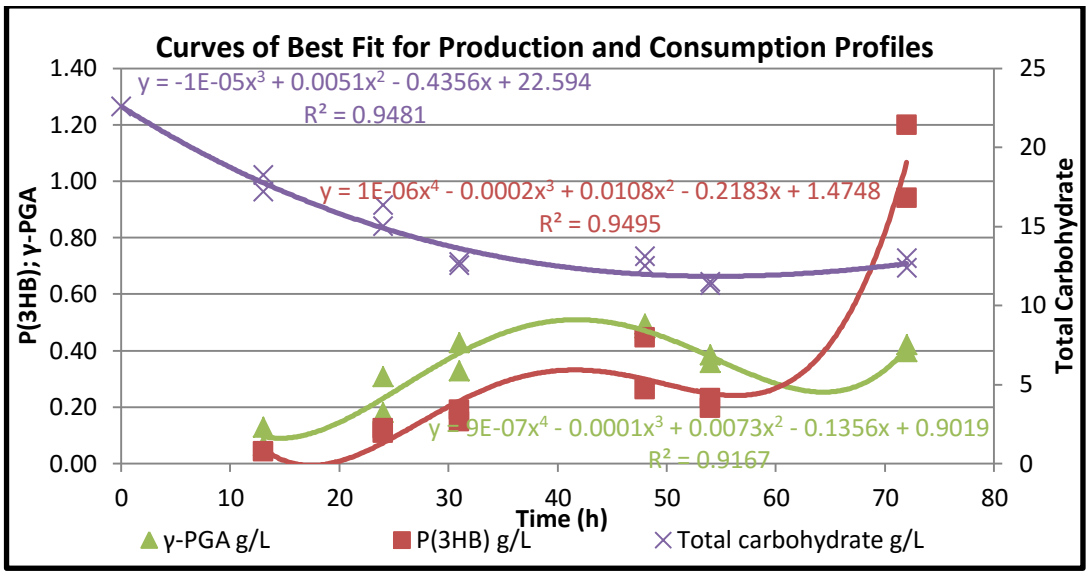



**Figure 3. 33:** Curves of best fit for γ-PGA and P(3HB) productions and total carbohydrate consumption.

The curves of best fit are useful to predict the trends of the culture and interpolate the points between samples. However, they must not be used to extrapolate outside the range of investigation since it might cause misleading interpretations of the culture behaviour. The functions presented are valid between 0 to 72 h of cultivation and must be used and interpreted within these limits.

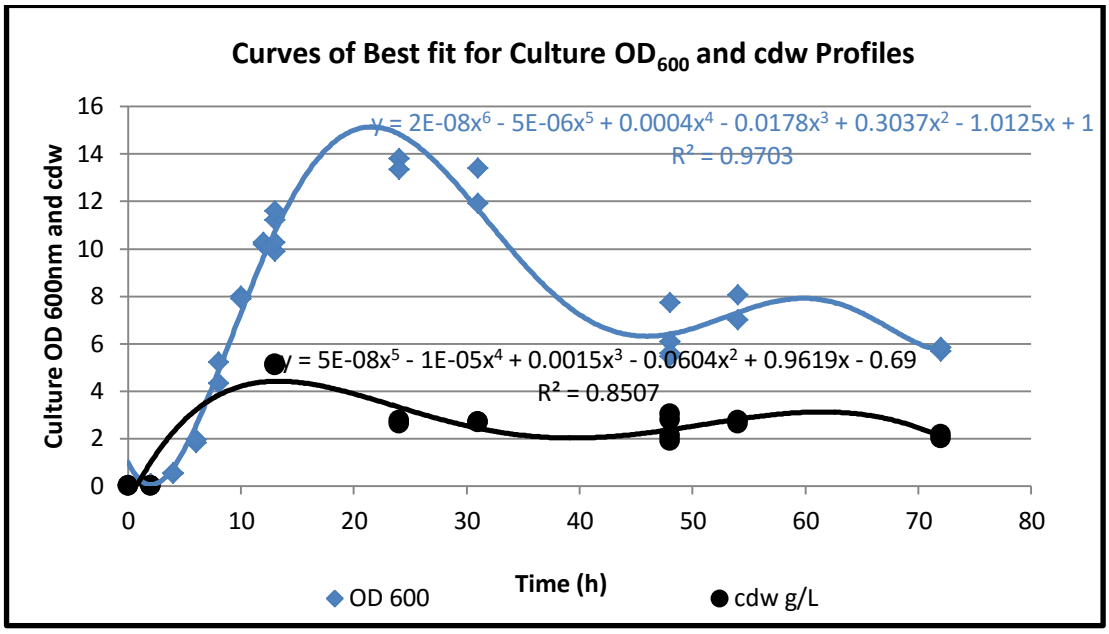



**Figure 3.34:** Curves of best fit for culture growth $OD_{600}$ measurements and cdw (g/L).

When culture optical density profile and cdw profile are compared a distinct discrepancy is observed between the two. In double polymer production fermentations, the $OD_{600}$ profile obtained was not parallel to the cdw profile. In the P(3HB) fermentation using P(3HB) medium (on page 97, Figure 3.9a) this discrepancy was not as clear as presented here (Figure3.34). This phenomenon has been observed by other researchers as well and reported in literature (Martinez *et al.*, 2015; Le Meur *et al.*, 2013). In the works published by Martinez *et al.* (2015) and Le Meur *et al.* (2013), it was reported that two separate calibration curves for the optical density vs cdw are needed since the correlations for growth region and production region are different. There could be a number of reasons for the discrepancy. Intracellular accumulation of the polymer in the form of granules within the cell creates heterogeneities in the cytoplasm leading to a different refractive index. This phenomenon makes the granules visible under phase contrast microscope (Figure 3.35). Although this phenomenon seems like it might interfere with the $OD_{600}$ measurements, the $OD_{600}$ measurements are correlated in P(3HB) fermentations using P(3HB) medium.

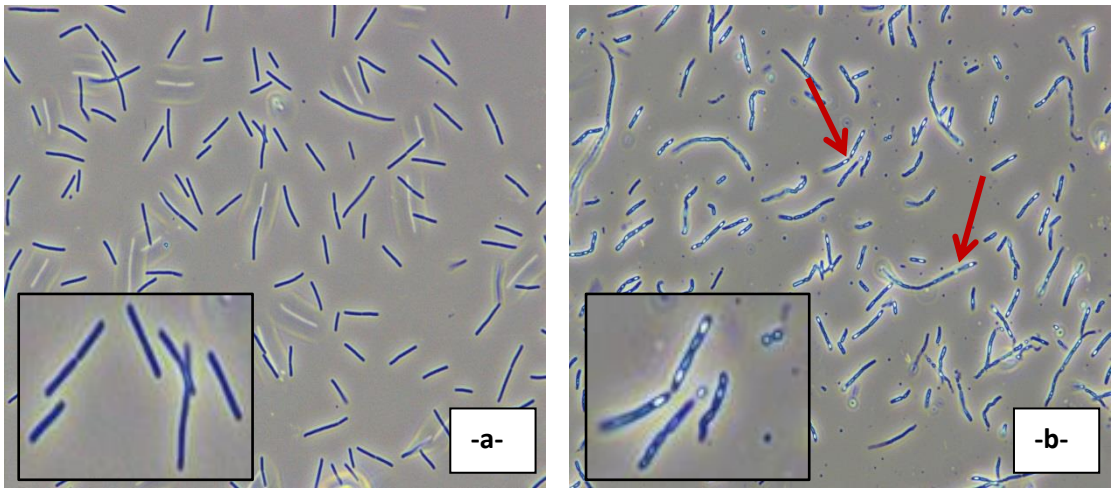



**Figure 3. 35:** Microscopic observation of cell (a) not containing polymer (b) containing polymer. Polymer inclusions are indicated by arrows and enlarged in the corner insets (at 400x, phase contrast mode).

The reason might be that the cell lyses cannot be directly measured by cdw, since samples taken contain cell debris of the dead cells and live cells together. But the lyses can be clearly observed under microscope (Figure 3.32c and d) along with spore formation. Sporulation-related cell lysis in P(3HB) medium occurs at much later phases after 200 h (related observations are presented on page 186, Figure 3.87) than in the DP medium. This minimises the interference in the measurement during the course of fermentation in P(3HB) medium. These arguments are further discussed in Section 4.3.

The correlation between culture $OD_{600}$ and cdw in DP medium was calculated for the two different phases; growth phase and P(3HB) accumulation phase (Figure 3.36). It is clearly visible that the correlation between $OD_{600}$ and cdw is linear with $R^2$ = 0.93 during the growth phase (Figure 3.37), while for the production phase, the linear equation is not a good fit ($R^2$ = 0.36). Consequently it was concluded that the correlation of $OD_{600}$ vs cdw was different for polymer containing and non-containing cells.

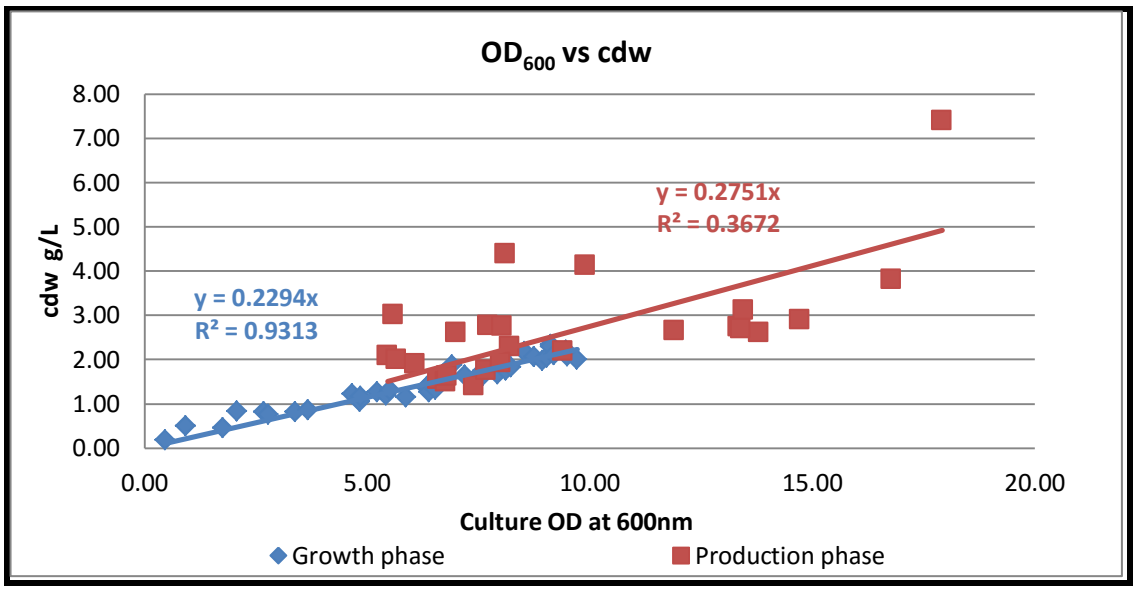



**Figure 3. 36:** $OD_{600}$ vs cdw in different phases of fermentation in DP medium.

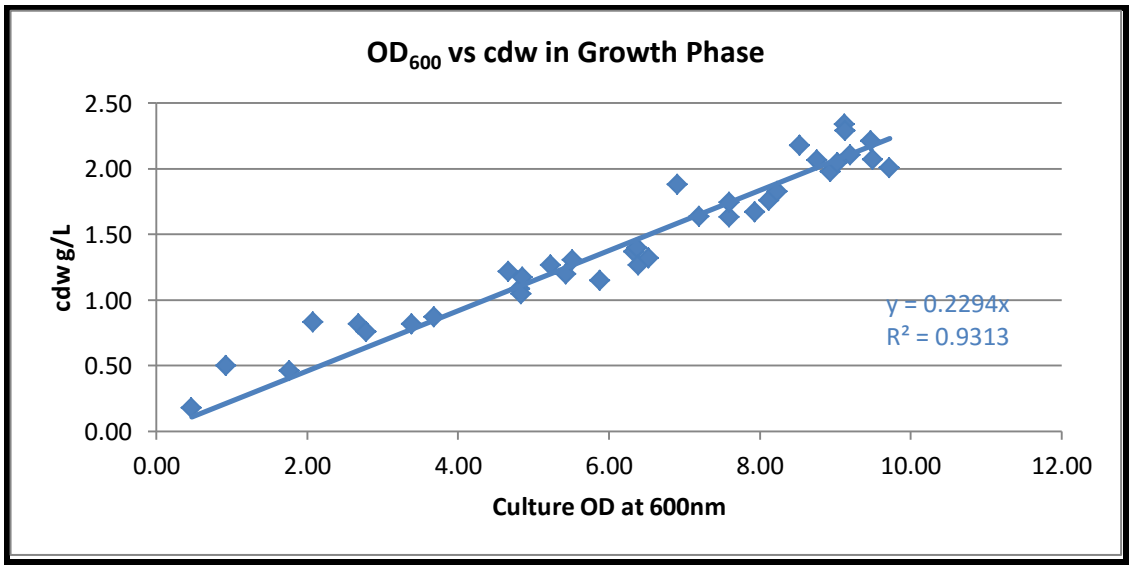



**Figure 3.37:** Conversion of $OD_{600}$ into cdw based on non-polymer containing cell data.

To be able to calculate the yield and specific rates, a normalized cell dry weight profile was obtained using $OD_{600}$ vs cdw correlation data presented in Figure 3.37. Normalised cdw data and curves of best fit are presented in Figure 3.38.

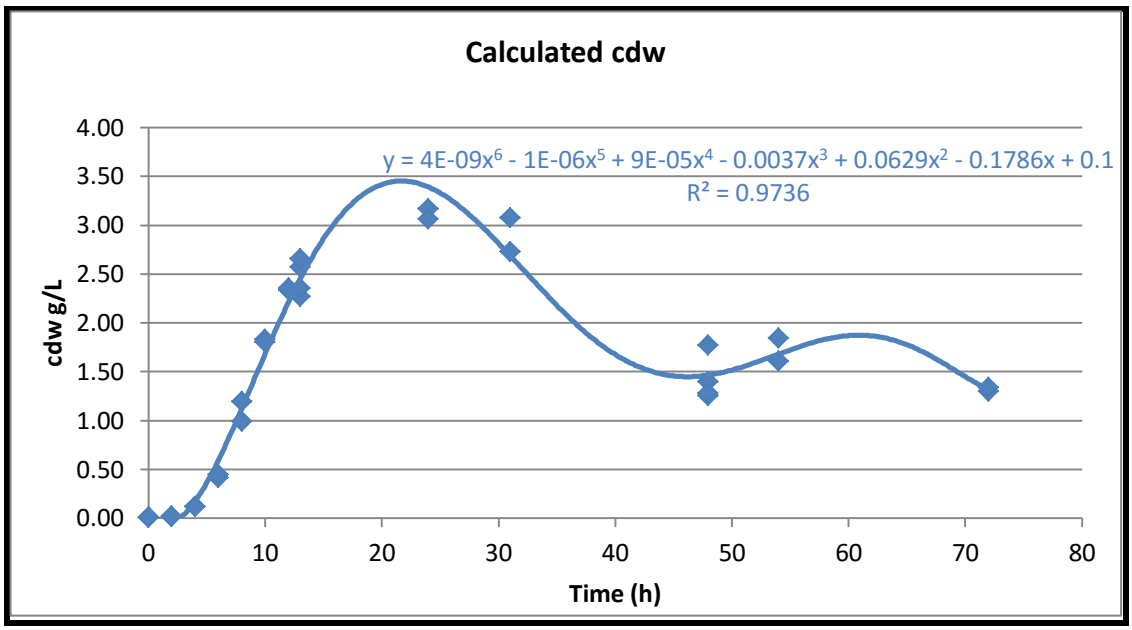



**Figure 3. 38:** Normalised cell dry weight profile and curve of best fit with the respective function.

Using the normalised growth curve, maximum specific growth rate and doubling time were calculated as 0.32 $h^{-1}$ and 2.47 h respectively (Figure 3.39).

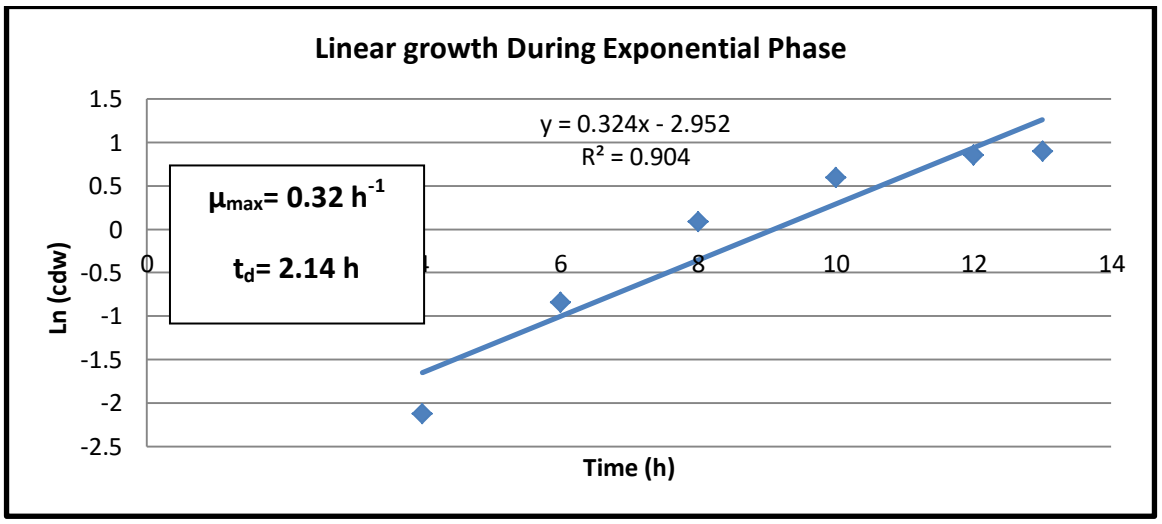



**Figure 3. 39:** Linear growth during exponential phase showing an average specific growth rate of 0.32 $h^{-1}$ and an average double time of 2.14 h.

### 3.5.2 Calculation of Yields and Specific Rates Equations

Based on the curves of best fit and their respective equations, it was possible to calculate some of the important process parameters. Using the equations and their derivatives cumulative and instantaneous yields and specifics rates were calculated as presented below.

*X: g cdw/L*
*P: Product concentration g/L*
*S: Total carbohydrate (substrate) concentration g/L*
*t: time (h)*
*Y: yield*
*Q: specific rate*

**Cumulative Yields**

$Y_{x/s} = x(t) / (s(0)-s(t))$

$Y_{p1/s} = P_1(t) / (s(0)-s(t))$

$Y_{p2/s} = P_2(t) / (s(0)-s(t))$

**Instantaneous Yields**

$Y_{x/s} = dx(t) / ds(t)$

$Y_{P(3HB)/s} = dP_{P(3HB)}(t) / ds(t)$

$Y_{\gamma\text{-PGA/s}} = dP_{\gamma\text{-PGA}}(t) / ds(t)$

**Specific Rates**

**Specific Growth rate**

$\mu = 1/x * dx/dt$

**Specific Polymer Production Rate**

$Q_p = 1/x * dP/dt$

**Specific Sugar Consumption Rate**

$Q_s = 1/x * dS/dt$

**Functions and Derivatives**

**Cells**

$X(t) = 4* 10^{-9} t^6 – 1*10^{-6} t^5 + 9* 10^{-5} t^4 – 0.0037 t^3 + 0.0629 t^2 – 0.1786 t + 0.1$

$dx/dt = 24* 10^{-9} t^5 – 5*10^{-6} t^4 + 36* 10^{-5} t^3 – 0.0111 t^2 + 0.1258 t – 0.1786$

**P(3HB)**

$P_{P(3HB)}(t) = 1* 10^{-6} t^4 – 0.0002 t^3 + 0.0108 t^2 – 0.2183 t + 1.4748$

$d P_{P(3HB)}/dt = 4* 10^{-6} t^3 – 0.0006 t^2 + 0.0216 t – 0.2183$

**γ-PGA**

$$P_{PGA}(t)= 9*10^{-7}\, t^4 - 0.0001\, t^3 + 0.0073\, t^2 - 0.1356\, t + 0.9019$$

$$d\, P_{PGA}/dt= 36*10^{-7}\, t^3 - 0.0003\, t^2 + 0.0146\, t - 0.1356$$

**Total carbohydrate (Substrate)**

$$S(t)= -1*10^{-5}\, t^3 + 0.0051\, t^2 - 0.4356\, t + 22.594$$

$$dS/dt= -3*10^{-5}\, t^2 + 0.0102\, t - 0.4356$$

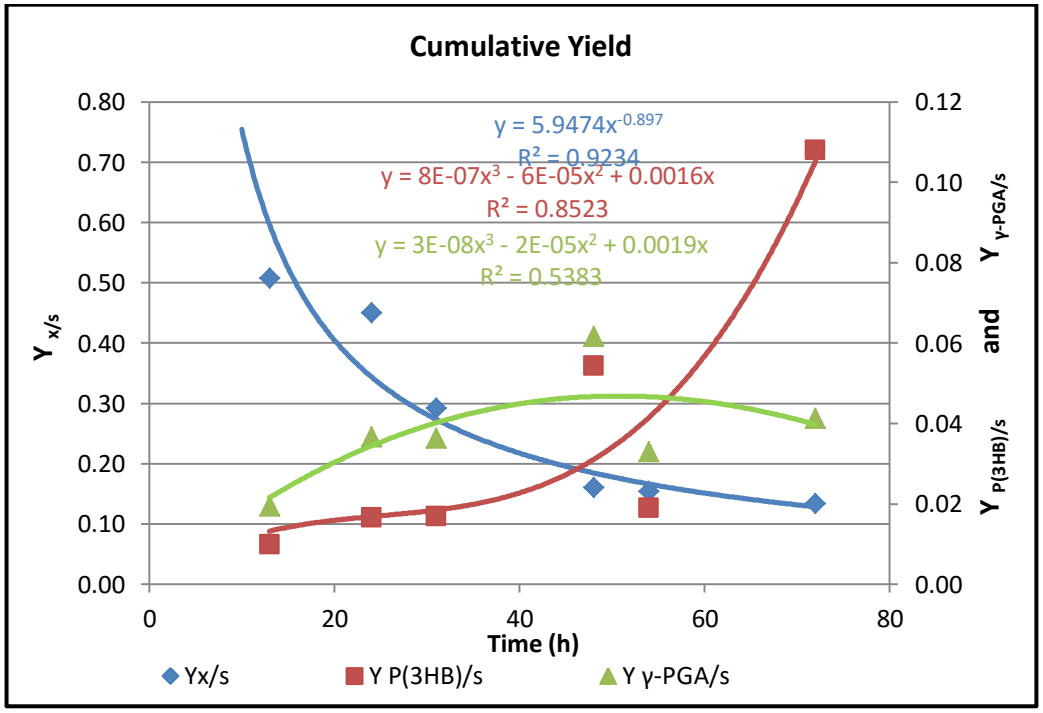



**Figure 3. 40:** Cumulative yields for biomass, P(3HB) and γ-PGA production in flasks

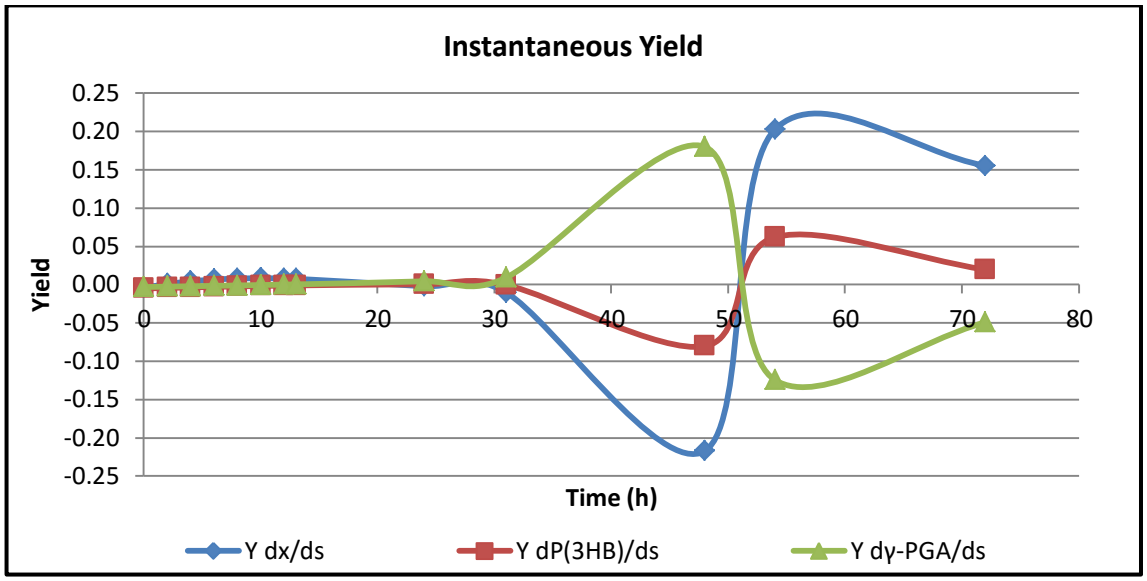



**Figure 3. 41:** Instantaneous yields for double polymer production in flasks

When cumulative and instantaneous yields per unit substrate consumed are studied closely, it is observed that there are two important milestones in the course of the fermentation with respect to the sugar consumption metabolism of the culture, after 30 h and 50 h. The metabolism shifts from cell growth to γ-PGA production around 30 h and there is a second shift from γ-PGA to P(3HB) production at around 50 h (Figures 3.40 and 3.41) . This is in correlation with the production profiles of individual polymers (Figures 3.8 and 3.9). Since γ-PGA is a growth associated product and P(3HB) is a secondary metabolite this shift is expected.

Graphs for specific rates indicate that the growth rate is at its highest in the first 20 h and later on it levels off and this curve is in complete agreement with specific carbohydrate consumption rate. Specific P(3HB) production starts after the growth has levelled off and reaches its maximum at around 50 h. This is confirmed with the specific sugar consumption curve which reflects that sugar is being utilised for P(3HB) production and the cells are accumulating the polymer (Figure 3.42).

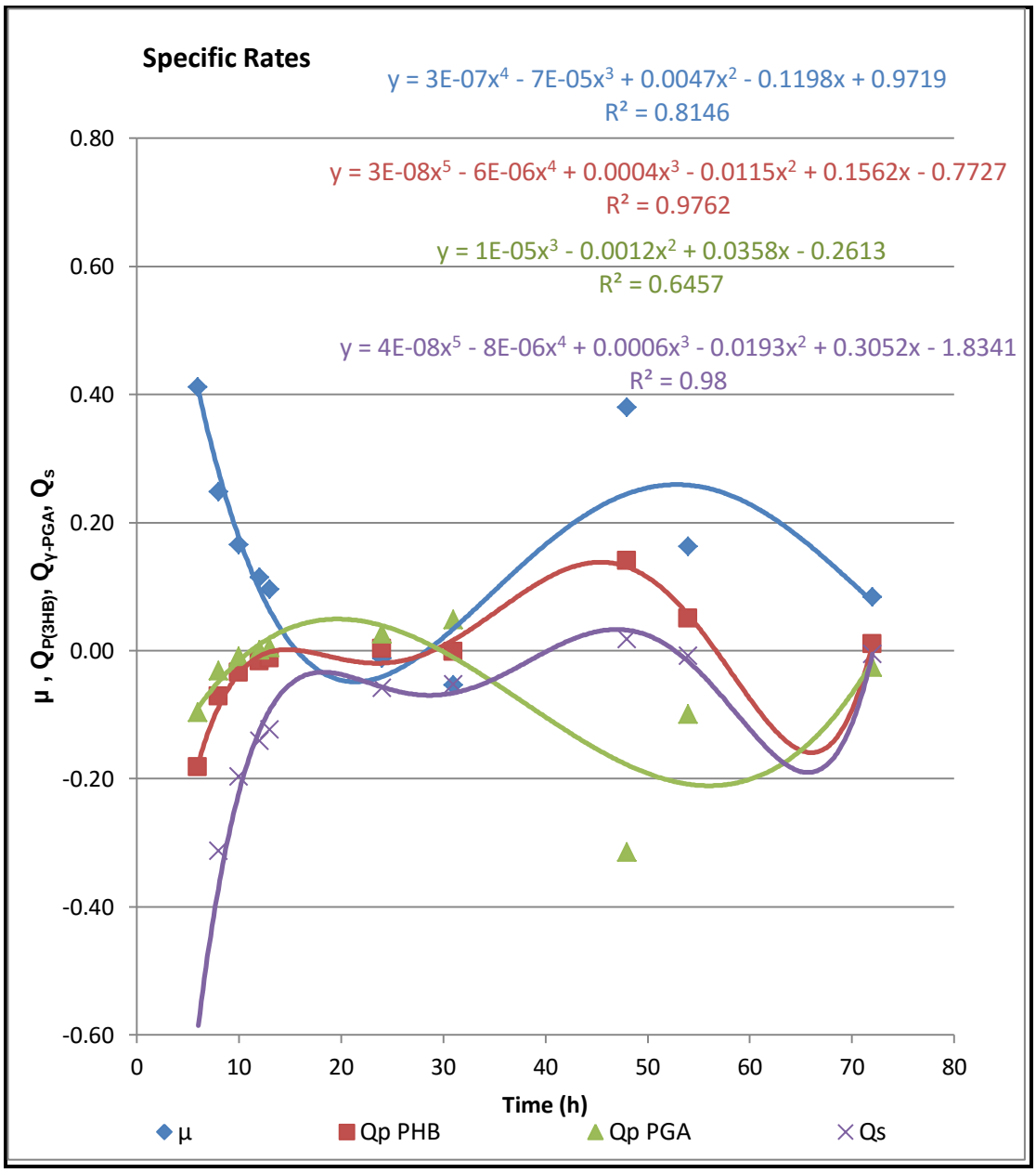



**Figure 3. 42:** Specific rates under double polymer production in shaken flasks

### 3.5.3 Double Polymer Production in Reactors

Double polymer production was tested in 2 L bioreactor with a working volume of 1.5 L. At 250 rpm stirrer speed the reactor became oxygen limited at the very beginning (after 5 h) This prevented the production of both polymers, particularly P(3HB) (Figure 3.43). In addition, pH of the culture dropped below 5.5 after 12 h and remained below this value for the duration of the fermentation. Increase in the $CO_2$ level after 7 h indicated the start of growth and metabolic activity.

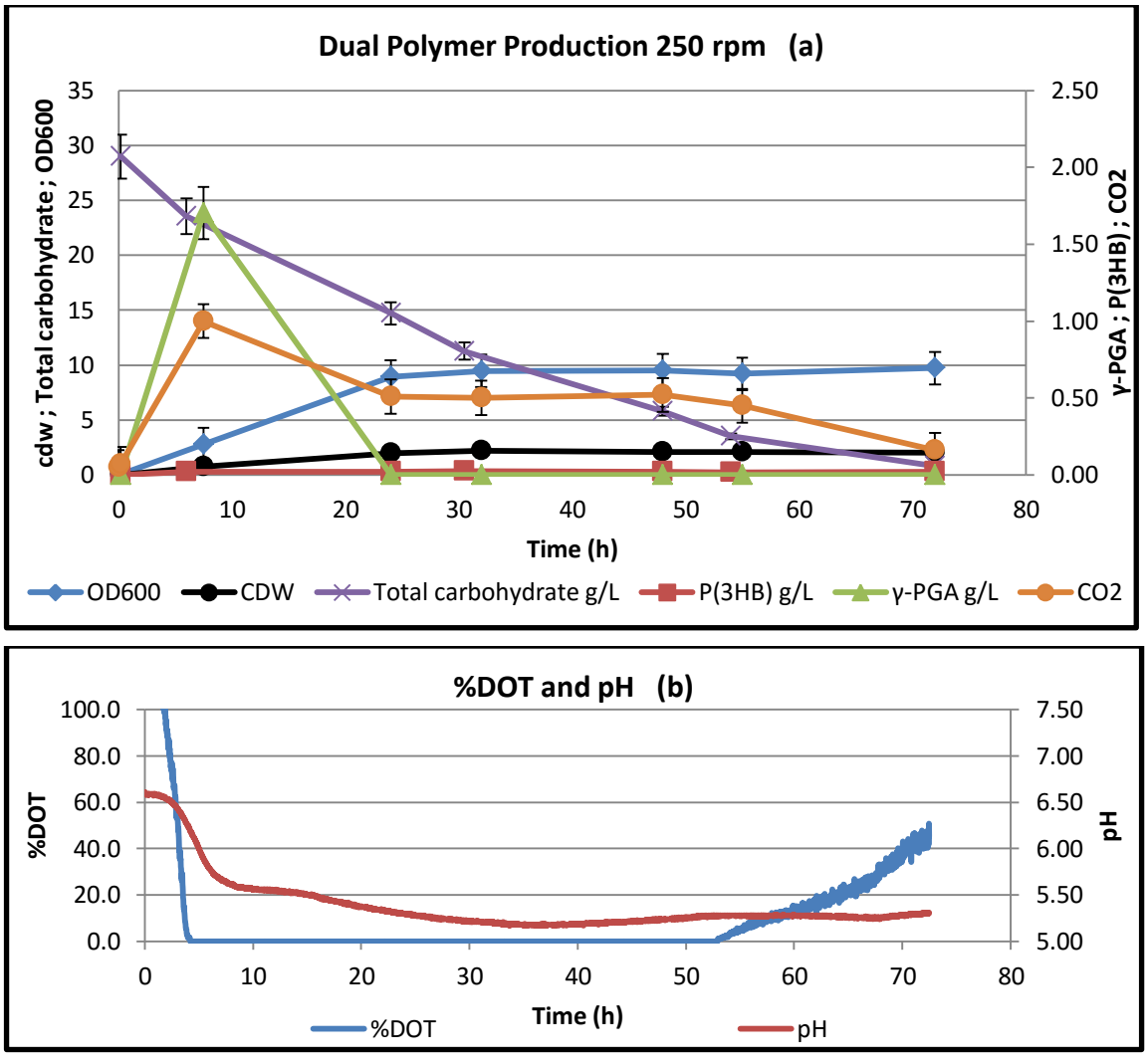



**Figure 3. 43:** Profile of (a) growth, double polymer production, total carbohydrate consumption (b) %DOT and pH, with constant stirrer speed at 250 rpm, 30°C, 1 vvm. Points represent average of triplicate measurements.

In the second experiment carried out in the bioreactor, the stirrer speed was increased to 400 rpm which enabled the % DOT to stay above 30%. However, after 24 h the culture

foamed extensively and the foaming could not be controlled. Therefore fermentation had to be terminated. It was observed that, within the first 24 h, there was noticeable production of both of the polymers proving the importance of % DOT for the production of the double polymer (Figure 3.44). The increase in $CO_2$ level after 6 h was less compared to the previous fermentation. Also, pH of the culture did not fall below 5.5 this time, the minimum pH observed was 5.6 after 6 h and later on it started increasing and more or less stabilised around pH 7 (Figure 3.44b).

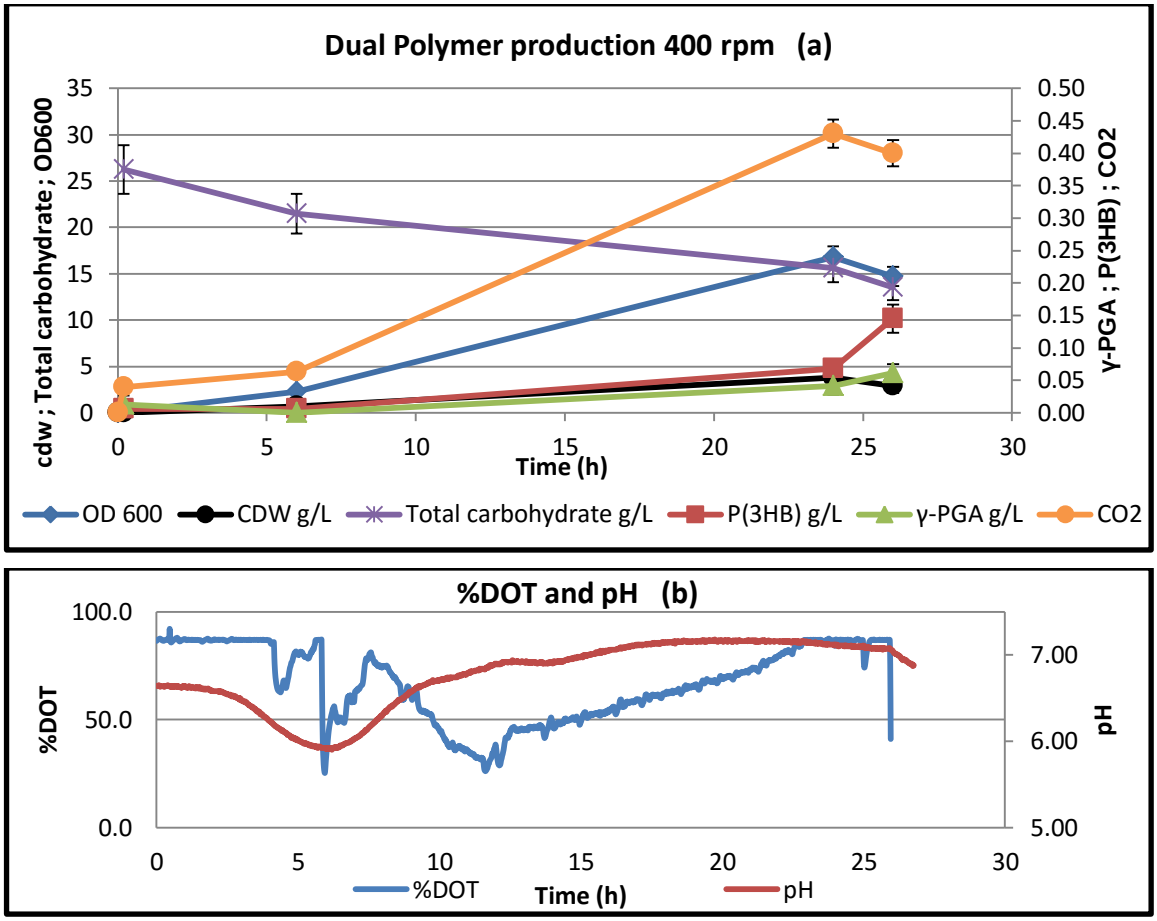



**Figure 3. 44:** Profile of (a) growth, double polymer production, total carbohydrate consumption (b) %DOT and pH, with constant stirrer speed at 400 rpm, 30°C, 1 vvm. Points represent average of triplicate measurements. Terminated due to excessive foaming

Consequently, a third fermentation was conducted where the % DOT was maintained at 25 % saturation by varying the stirrer speed between 250-400 rpm. Excessive foaming was again observed therefore fermentation had to be terminated after 24 h. However the yield

of γ-PGA was higher in this run. Since only the first 24 h could be observed, the effect on P(3HB) production was not determined fully (Figure 3.45). Increase in the $CO_2$ level was more consistent and the maximum level reached was lower compared to the first run (constant agitation at 250 rpm, on page 136, Figure 3.43). In this experiment pH again followed a similar trend to the previous experiment (Figure 3.44b) and did not exceed pH 7 (Figure 3.44b).

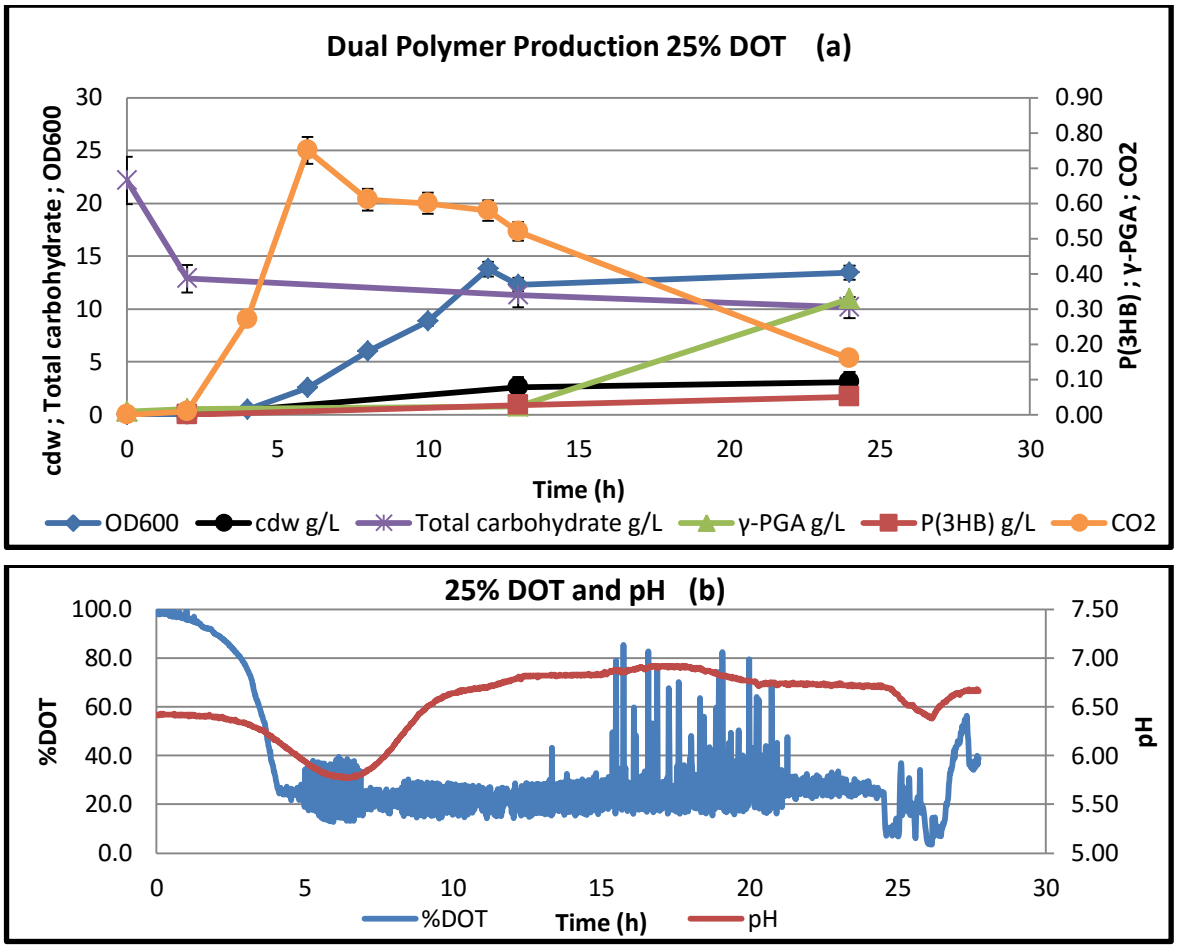



**Figure 3. 45:** Profile of (a) growth, double polymer production, total carbohydrate consumption (b) % DOT and pH, where DOT was controlled at 25% saturation by varying stirrer speed between 250-400 rpm, 30°C, 1 vvm. Points represent average of triplicate measurements

In order to prevent excessive foaming, maintaining DOT at 10% saturation was tried (Figure 3.46). Foaming was observed again after 24 h therefore, silicone-based anti-foam was added (1 ppm) to suppress foaming. This was the first run which anti-foam was used to control foaming. This was continued in subsequent runs where foaming was observed

and anti-foam addition was necessary using automated foam control. At the end of 48 h due to the decline in the culture optical density, cell dry weight and increasing % DOT, the fermentation was stopped, the final titres achieved were 0.25 g/L γ-PGA and 0.09 g/L P(3HB) which were unsatisfactory. The sudden decrease in the $CO_2$ level after 8 h was thought to be due to foam formation and anti-foam addition, which is further discussed in Section 4.3.

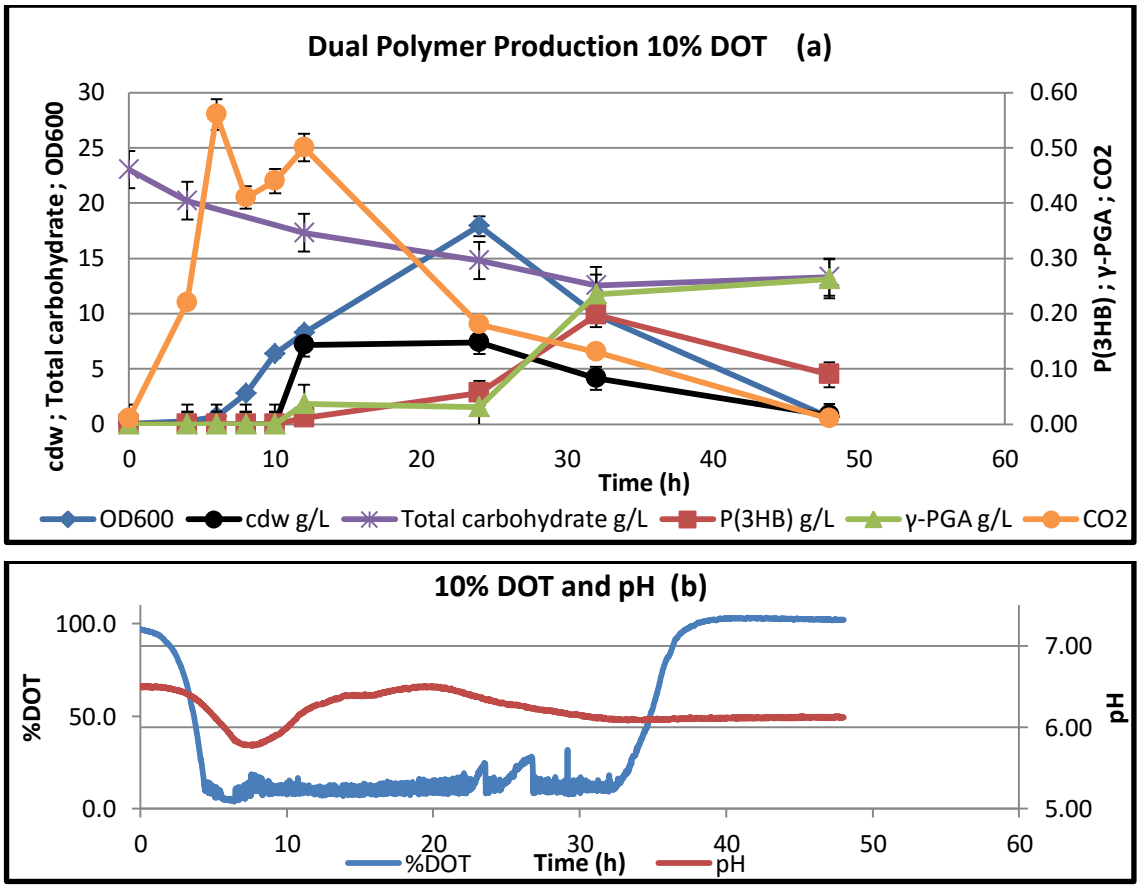



**Figure 3. 46:** Profile of (a) growth, double polymer production (b) %DOT and pH, where DOT was controlled at 10% saturation by varying stirrer speed between 250-400 rpm until 8 h and increased to 300-400 rpm after that, 30°C, 1 vvm, Points represent average of triplicate measurements.

These results demonstrated the importance of the oxygen level in the fermenter. Polymer concentrations and cdw values at the end of 24 h of cultivation are compared for the runs conducted up to this point and are presented in Figure 3.47. γ-PGA concentration obtained in the run conducted with constant % DOT saturation at 25% was found to be highest. Low

P(3HB) concentration at 24 h could be misleading due to the late commencement of P(3HB) production in the course of the fermentation.

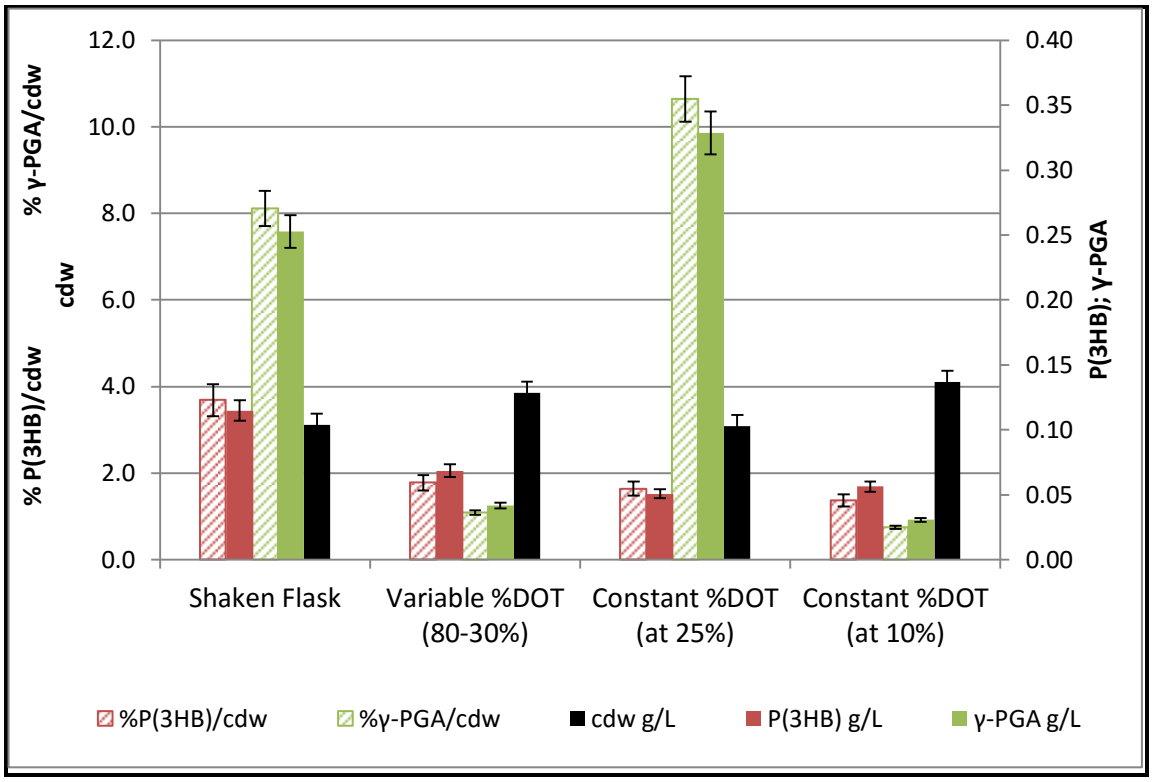



**Figure 3. 47:** Comparison of double polymer titres and cdw levels in 24 h cultures at different %DOT levels with shaken flask experiment.

When the reactor performances were compared with the flask productions, it appears that the cellular metabolism favours polymer production in the flasks while under the controlled conditions in the fermenter higher cdw is obtained. Although poor oxygen transfer rates in flasks is general perception, 50 ml working volume in 250 ml Erlenmeyer flasks provided sufficient oxygenation to enable good levels of dual polymer production. Based on these findings and bearing in mind the importance of % DOT level as well as the foaming tendencies of the culture, a regime was adopted where % DOT was maintained at 25% air saturation for the first 24 h and then dropped to 10% air saturation (Figure 3.48).

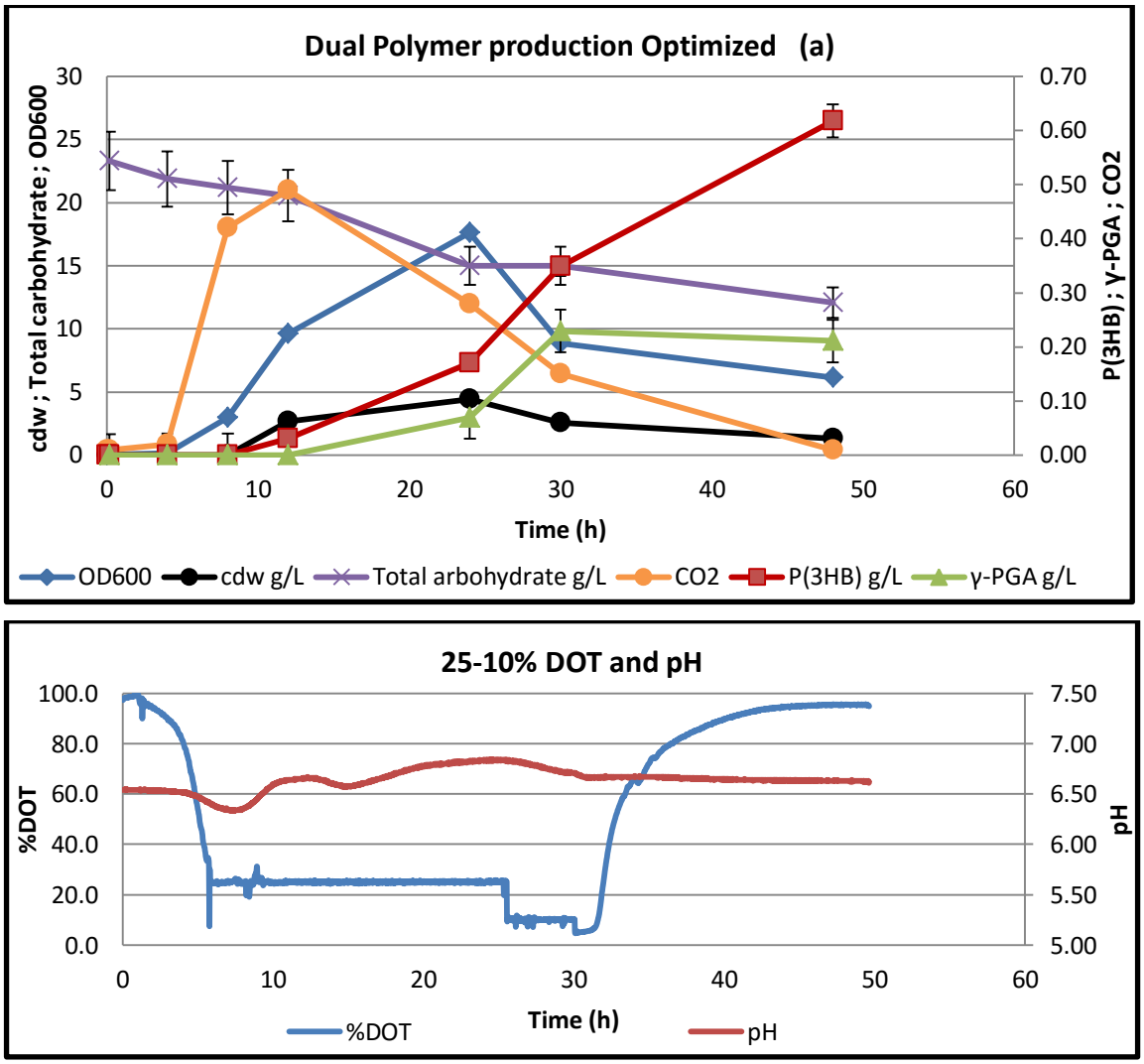



**Figure 3. 48:** Profile for (a) growth, double polymer production, total carbohydrate consumption (b) % DOT and pH, where DOT was controlled at 25-10% saturation by varying stirrer speed between 250-500 rpm, 30°C, 1 vvm. Points represent average of triplicate measurements

Maintaining the DOT at 25% during the first 24 h and then decreasing it down to 10% air saturation provided the culture with sufficient dissolved oxygen and enabled the foaming tendencies to be controlled successfully. The titres obtained for the two polymers were 0.620 g/L and 0.210 g/L P(3HB) and γ-PGA respectively.

Based on the results obtained in this fermentation, curves of best fit for growth, total carbohydrate consumption and polymer production profiles were generated. Curves of best fit for the normalised cdw representing growth profile is presented in Figure 3.49, production and consumption curves are presented in Figure 3.50.

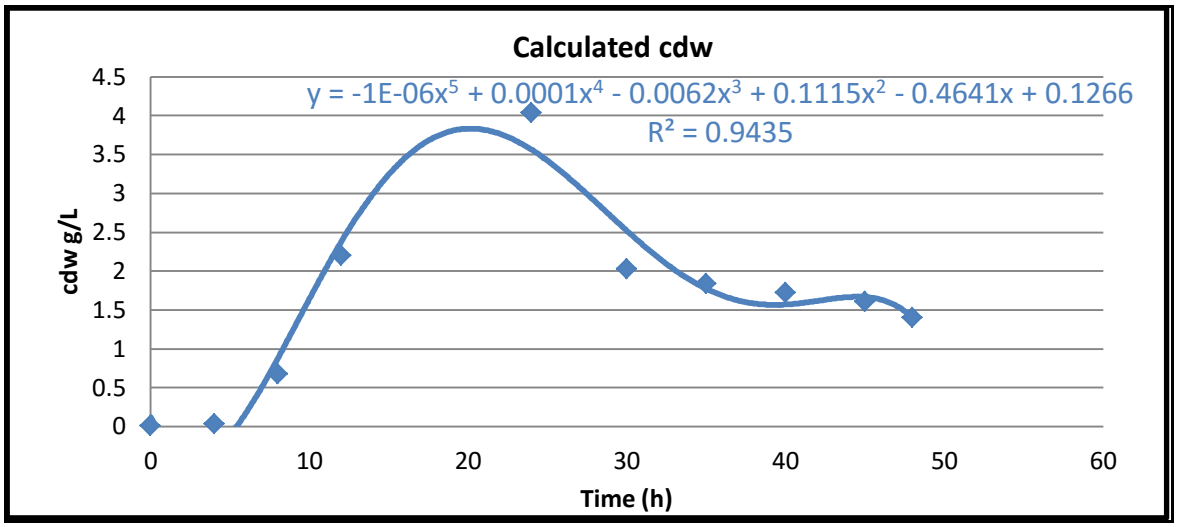



**Figure 3. 49:** Normalised cell dry weight profile for optimized dual polymer fermentation

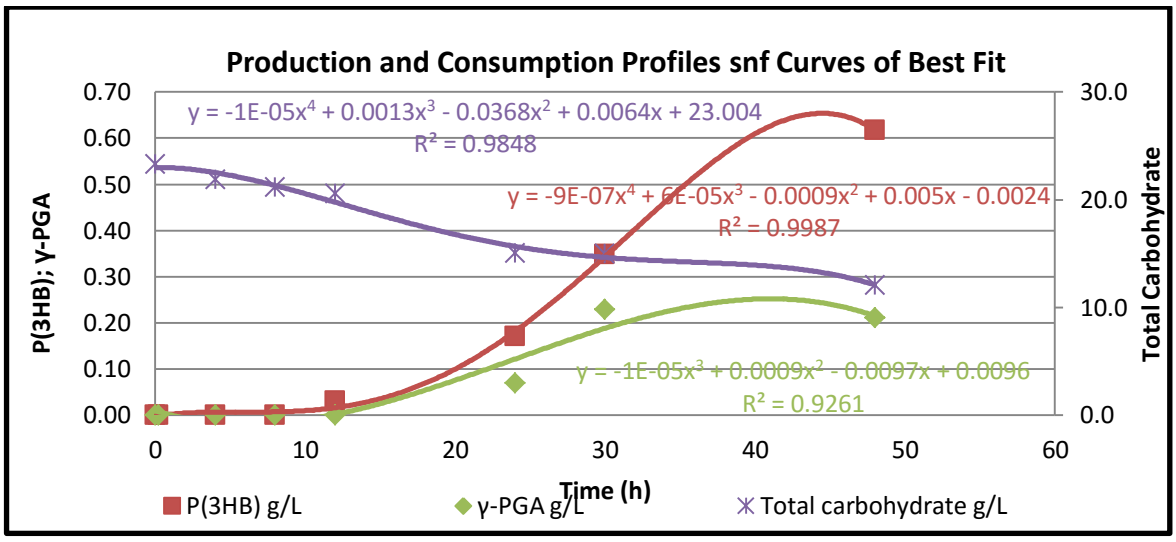



**Figure 3. 50:** Curves of best fit for polymer production and sugar consumption profiles for optimized dual polymer fermentation.

Using the equations of the curves of best fit, specific rates were calculated in a similar way as presented in Section 3.5.2. Calculated specific rates were plotted against time and presented in Figure 3.51. Polymer productions and total carbohydrate consumption profiles are similar to those obtained from flask productions. However, P(3HB) production profile, which has been observed to be oxygen dependent, was found to be different. The P(3HB) production commenced earlier in the reactor compared to the flask due to better controlled oxygen transfer rates.

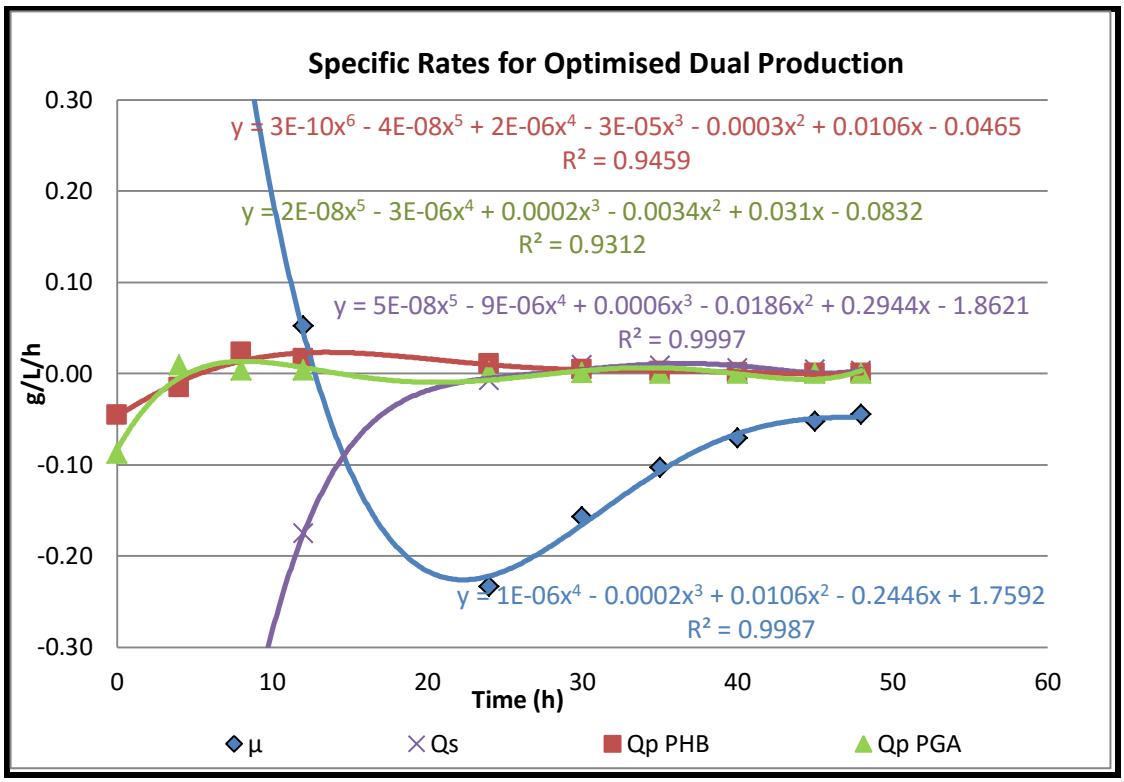



**Figure 3. 51:** Specific rates for optimized dual polymer fermentation.

The main difference between the reactor and the flask runs is the improved culture homogeneity in the bioreactors due to the controlled environment. According to the specific production curves obtained in the reactor, γ-PGA starts to be produced earlier in the course of the fermentation and the specific production rate remains more or less constant. Similarly P(3HB) production commences earlier compared to the flask fermentations (Figure 3.42) and again the specific production rate remains constant throughout the fermentation.

In the light of the previous experiments a final run was carried out in order to see the time course of the fermentation for 76 h and to observe the changes in the levels of both polymers within the reactor throughout the course of the fermentation. The conditions were kept exactly the same as the optimised dual polymer production but duration was 76 h.

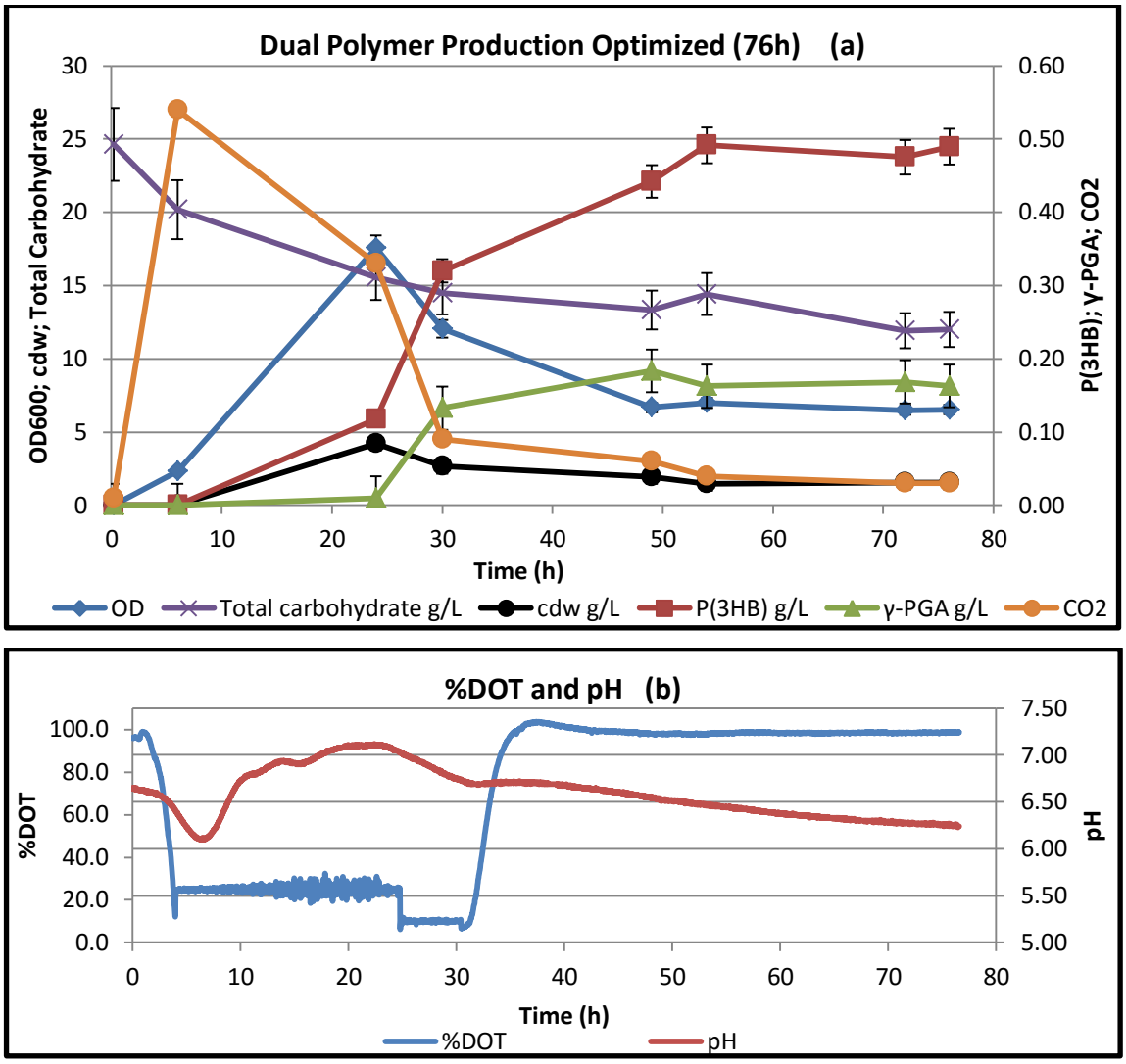



**Figure 3. 52:** 76 h fermentation profile for (a) growth, double polymer production, total carbohydrate consumption (b) % DOT and pH, where DOT was controlled at 25-10% saturation by varying stirrer speed between 250-500 rpm, 30°C, 1 vvm. Points represent average of triplicate measurements.

Production of both polymers levelled off after 54 h for a period of 24 h. The highest concentration of P(3HB) was observed at 54 h while for γ-PGA it was 50 h. The concentrations did not change after this point until the end of 76 h for both polymers. Since the levels of polymers did not change after 54/50 h, this profile indicated that the optimum time for terminating the process of double polymer production is between 50 to 54 h (Figure 3.52).

### 3.6 Utilisation of Different Biowaste Materials

Since one of the aims of this project was the development of a feasible, low cost biopolymer production process, possible utilisation of cheap substrates were investigated using four different agro-industrial biowaste materials.

#### 3.6.1 Growth and Production using Biowaste

Four different biowaste materials namely, rapeseed cake (RS), wheat bran (WB), *Spirulina* powder (S) and orange peel (OP) were selected based on their favourable compositions and previously reported data, and were tested for their capability to produce both P(3HB) and γ-PGA. Four different pre-treatment methods were applied to find the most efficient method for the extraction of the essential nutrients for P(3HB) and γ-PGA production, as described in Section 2.4. The treatment methods were; acid treatment (A), alkaline treatment (B), water infusion (C) and microwave exposure (D). γ-PGA production could not be observed in any of the cultures where these waste materials were used as the sole medium component. This was due to the lack of glutamic acid, therefore at this stage only P(3HB) production capabilities were compared. Each biowaste was used without supplementation of any other nutrient.

Figures 3.53 to 3.56 present sugar content, cell dry weight and P(3HB) production for each waste material and for the pre-treatment method applied. Amongst the waste materials tested, highest sugar content was found in orange peel (Figure 3.53). All pre-treatment methods applied to orange peel yielded approximately the same sugar concentration.

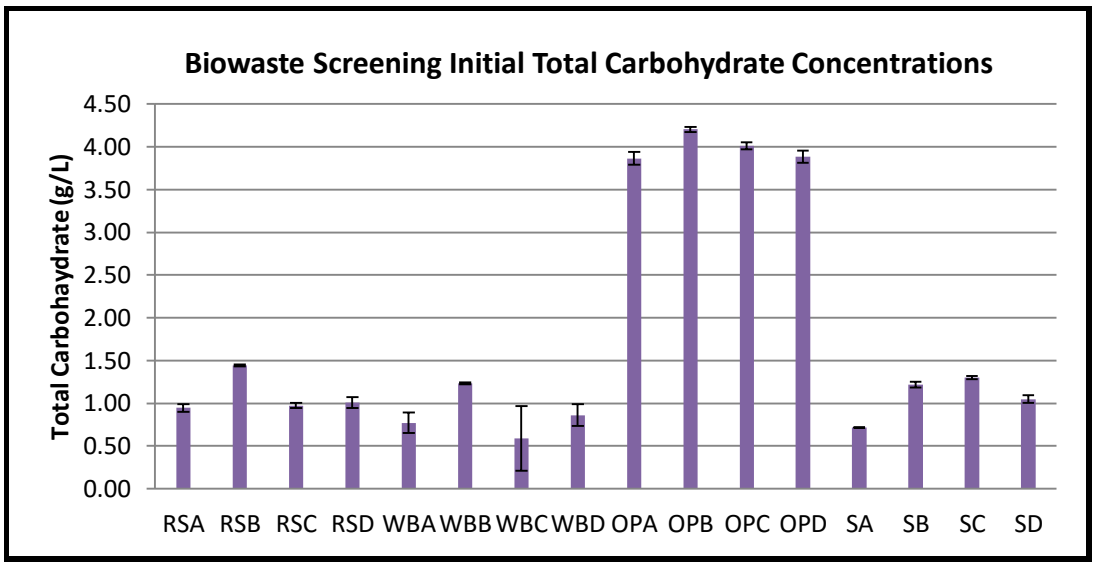



**Figure 3. 53:** Comparison of total carbohydrate contents of different biowastes. RS-rapeseed cake, WB-wheat bran, OP-orange peel, S- Spirulina powder; A-acid treatment, B- alkaline treatment, C-water infusion, D-microwave treatment, Figure represents duplicate measurements from two different experiments

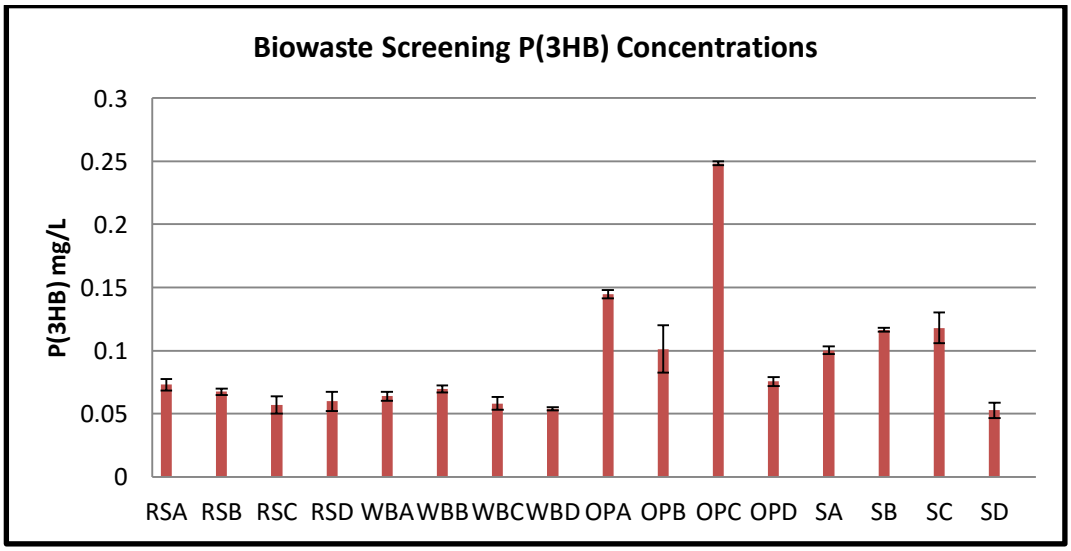



**Figure 3. 54:** Comparison of P(3HB) concentration using different biowastes. . RS-rapeseed cake, WB-wheat bran, OP-orange peel, S- Spirulina powder; A-acid treatment, B- alkaline treatment, C-water infusion, D-microwave treatment, Figure represents duplicate measurements from two different experiments

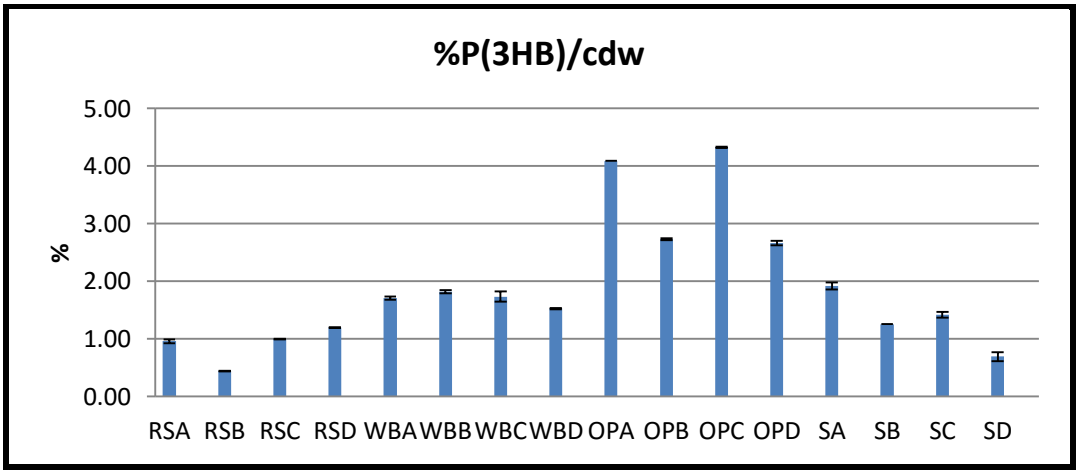



**Figure 3. 55:** Comparison of P(3HB) yields using different biowastes. RS-rapeseed cake, WB-wheat bran, OP-orange peel, S- Spirulina powder; A-acid treatment, B-alkaline treatment, C-water infusion, D-microwave treatment, Figure represents duplicate measurements from two different experiments

The highest P(3HB) content was found in orange peel treated with water infusion method (C), followed by mild acid treatment (A). This trend was also observed in %P(3HB)/cdw values (Figure 3.54 and 3.55).

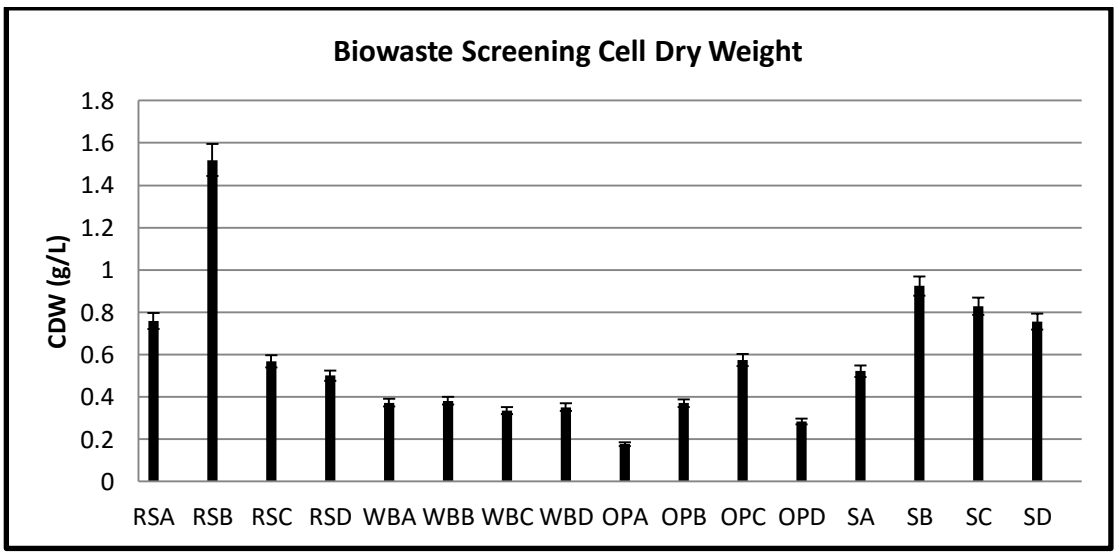



**Figure 3. 56:** Comparison of cell dry weight using different biowastes. . RS-rapeseed cake, WB-wheat bran, OP-orange peel, S- Spirulina powder; A-acid treatment, B- alkaline treatment, C-water infusion, D-microwave treatment, average of duplicate experiment.

The highest cdw was obtained from rapeseed cake (Figure 3.56), followed by *Spirulina* both treated with mild alkaline.

Each waste material was also evaluated individually and the effect of pre-treatments was compared. The level of P(3HB) production was very similar in all pre-treatment methods for rape seed cake. The highest production was obtained with acid treatment (0.73 mg/L) followed by microwave treatment (0.60 mg/L). The highest cdw of all was with rapeseed cake using alkaline treatment.

P(3HB) production levels using wheat bran was similar to the ones obtained with rapeseed cake. The highest production from wheat bran was using alkaline treatment (0.7 mg/L) followed by acid treatment (0.64 mg/L). The biomass levels obtained with all treatments using wheat bran was the lowest of all biowastes used.

The average P(3HB) levels using Spirulina power as nutrient source was higher than RS and WB biowastes. Apart from microwave treatment all experiments resulted more than 1 mg/L. In addition cdw obtained was similar to RS. For Spirulina powder, amongst all treatment methods water infusion gave the best P(3HB) production (1.18 mg/L) followed by alkaline treatment (1.17 mg/L).

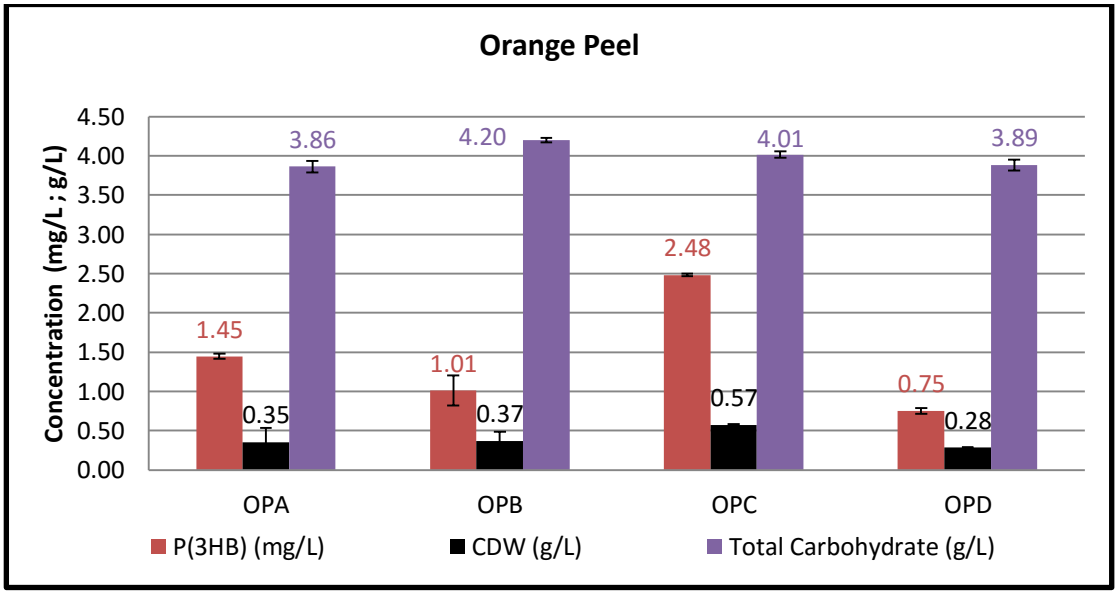



**Figure 3. 57:** Growth and P(3HB) production using orange peel (OP) as substrate. A: acid treatment, B: alkaline treatment, C: water infusion, D: microwave treatment, The Figure represents duplicate measurements from two different experiments. Statistical analysis given in Section 8.9.6, all levels are significant at the level $p<0.05$ compared to OPC.

Amongst the waste materials orange peel showed highest total carbohydrate content of all treatments used. The highest total carbohydrate obtained was using alkaline treatment (4.20 g/L) followed by water infusion (4.01 g/L). For OP treated with water infusion method showed the highest P(3HB) production (2.48 mg/L) this is followed by acid treatment (1.45 mg/L) (Figure 3.57). Although alkaline treatment showed the highest total carbohydrate content, P(3HB) production was on the third place. The effects of treatment methods on biowastes and polymer production are further discussed in Section 4.4.

Based on the P(3HB) results, orange peel treated with water infusion method, was selected, and further experiments continued using orange peel treated with water infusion as substrate.

### 3.6.2 Total Nitrogen and Carbohydrate Content of Pre-treated Orange Peel

Since orange peel medium was selected as a potential substrate, total nitrogen and carbohydrate contents of orange peel treated with water infusion method were investigated. Since *Bacillus subtilis* OK2 produces P(3HB) under nitrogen limited conditions, nitrogen content of the substrate has an important role. Nitrogen content of dried orange peel and yeast extract was measured with Kjedahl apparatus and compared. Findings are presented in Table 3.11.

**Table 3. 11:** Nitrogen content of orange peel compared to yeast extract.

| Sample (1 g) | Nitrogen Percentage (%) |
|---|---|
| Dry orange peel | 0.89 |
| Yeast extract | 10.25 |

Nitrogen percentage in 1g of dried orange peel was found to be almost 9 fold less compared to 1g yeast extract. Although P(3HB) produced by using orange peel was one of the highest amongst the biowaste used in this project, this result showed that nitrogen is needed to be supplemented to orange peel in order to get a desired ratio of C:N and obtained higher titres of P(3HB).

Subsequently, the total carbohydrate content of the water infusion pre-treated orange peel was investigated. Different concentrations of orange peel powder were pre-treated by water infusion method and the total carbohydrate contents were measured. The total carbohydrate content vs orange peel concentration graph obtained is presented in Figure 3.58. It was found that the total carbohydrate content released into the solution after the treatment was approximately half of the mass treated.

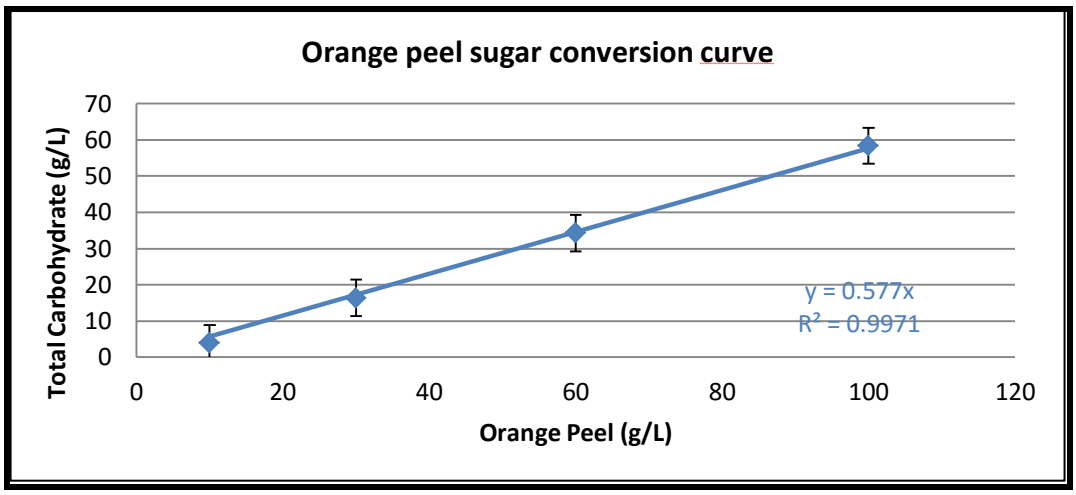



**Figure 3. 58:** Total carbohydrate content of orange peel infusion, Figure represents triplicate measurements from two different experiments

After the total sugar content determination, the types of sugars present in orange peel were investigated. HPLC analysis showed four major peaks and three were identified to be different sugars; namely, glucose, fructose and sucrose (Figure 3.59). Percentage distribution of these sugars was measured to be 42%, 23% and 35% of sucrose, glucose and fructose, respectively. The fourth peak, which is the second one on the left after the solvent peak (Figure 3.59), was not identified. However, the amount was lower compared to the ones identified.

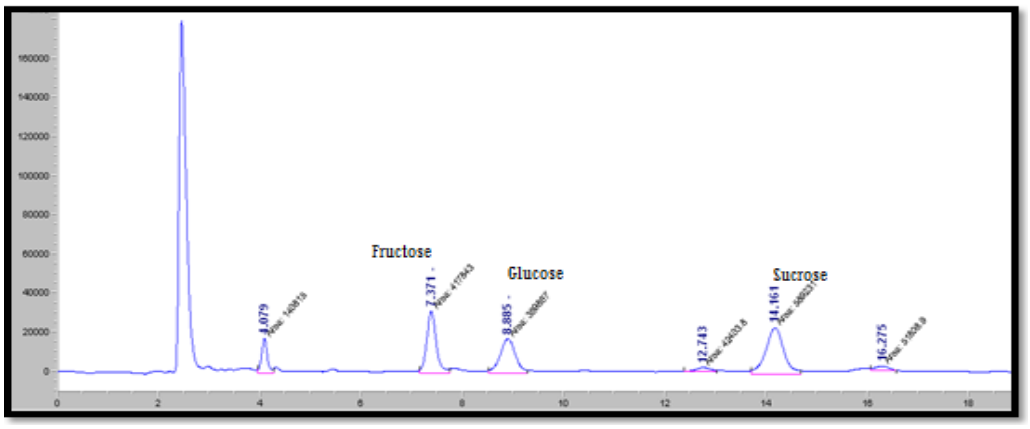



**Figure 3. 59:** Sugar composition of orange peel infusion

### 3.6.3 Pre-treated Orange Peel as Carbon Source for Polymer Production

Orange peel treated with water infused method was tested at different concentrations of orange peel for the production of P(3HB). Levels of cdw and P(3HB) concentrations obtained from different orange peel concentrations (as sole medium component) are presented in Figure 3.60.

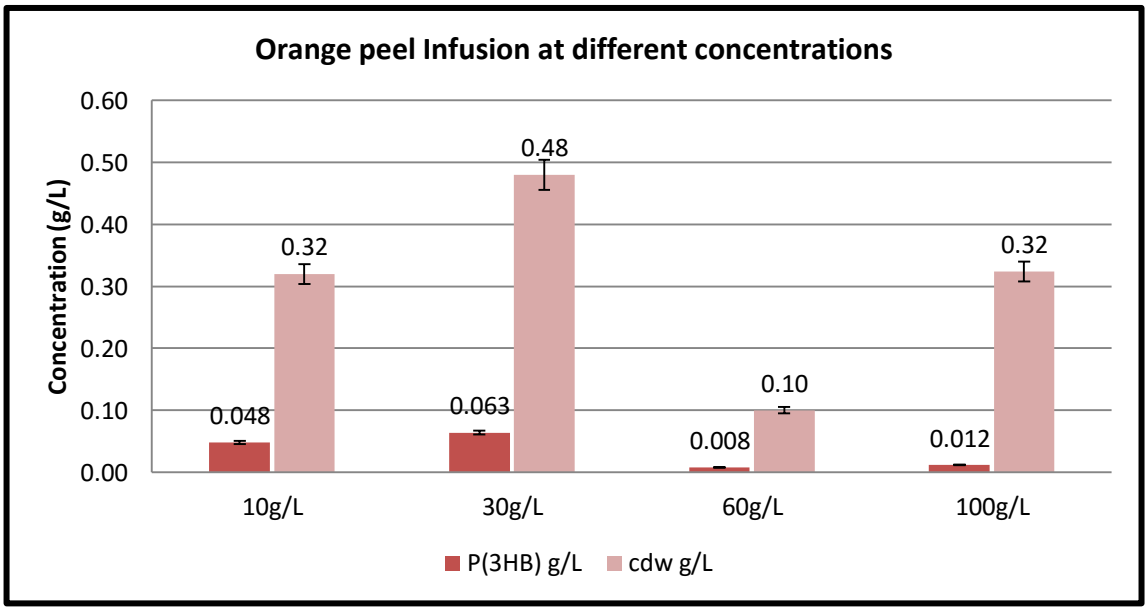



**Figure 3. 60:** Cell growth and P(3HB) Production at different initial orange peel concentrations, Figure represents triplicate measurements from two different experiments

The medium obtained from treating 30 g/L orange peel gave the highest cdw and P(3HB) yield. Total carbohydrate and nitrogen concentrations in 30 g/L of orange peel infusion were; 17 g/L and 2.25 g/L respectively. No γ-PGA was produced using this medium indicating that orange peel needed to be supplemented by other nutrients in order to produce both γ-PGA and P(3HB).

### 3.6.4 CCD for P(3HB) with Orange Peel Infusion as Carbon Source

Since a good P(3HB) production was observed with orange peel as carbon source, it was considered worthwhile to investigate P(3HB) production further. Thus, a new CCD was designed in order to determine the effect of citric acid and yeast extract additions on P(3HB) production since these two chemicals were found to be significant for P(3HB) production (Section 3.3). Orange peel infusion was substituted with glucose. The total number of experiments with three variables (orange peel, yeast extract and citric acid) was 18. Eighteen experiments were augmented with four replicates at the centre points to evaluate the pure error. The experiments were conducted in 250 ml shake flasks with 50 ml working volume at 30°C and 200 rpm. P(3HB) concentration was taken as the response. Experiments were performed according to experimental design matrix given on Table 3.12, within the ranges indicated and analysed as described in Section 2.7.2.

**Table 3. 12:** Experimental design matrix showing five levels of CCD for P(3HB) production using orange peel infusion as carbon source (α=1.69)

| Parameter (g/L) | Code | -α | -1 | 0 | +1 | +α |
|---|---|---|---|---|---|---|
| **Orange Peel (OP)** | A | 30.00 | 40.57 | 55.00 | 69.43 | 80.00 |
| **Yeast Extract (YE)** | B | 1.00 | 1.63 | 2.50 | 3.37 | 4.00 |
| **Citric acid** | C | 0.00 | 0.42 | 1.00 | 1.58 | 2.00 |

Based on the data obtained from the experiments an ANOVA table was generated by the program to evaluate the best fitting model for the data set for P(3HB). ANOVA table is presented in Table 3.13. The p-value for the best fitting model was found to be significant as well as all the variables screened. $R^2$ value was found to be 0.954 suggesting that the model was in very good correlation with the data.

**Table 3. 13:** ANOVA table for CCD using orange peel as carbon source

| | Sum of Squares | Degrees of Freedom | Mean Square | F Value | p-value |
|---|---|---|---|---|---|
| **Model** | 0.86 | 9 | 0.043 | 18.447 | 0.0002 |
| **A-OP** | 0.19 | 1 | 0.088 | 37.555 | 0.0003 |
| **B-YE** | 0.36 | 1 | 0.163 | 69.908 | < 0.0001 |
| **C-Cit** | 0.12 | 1 | 0.056 | 23.822 | 0.0012 |
| **AB** | 0.01 | 1 | 0.004 | 1.617 | 0.2393 |
| **AC** | 0.06 | 1 | 0.029 | 12.386 | 0.0079 |
| **BC** | 0.01 | 1 | 0.004 | 1.843 | 0.2116 |
| **$A^2$** | 0.03 | 1 | 0.015 | 6.599 | 0.0332 |
| **$B^2$** | 0.08 | 1 | 0.034 | 14.674 | 0.0050 |
| **$C^2$** | 003 | 1 | 0.012 | 5.308 | 0.0502 |
| **Residual** | 0.04 | 8 | 0.002 | | |
| **Lack of Fit** | 0.03 | 5 | 0.002 | 0.926 | 0.5627 |
| **Pure Error** | 0.02 | 3 | 0.002 | | |
| **Std. Error** | 0.072 | | | **$R^2$** | 0.954 |

The model obtained from the analysis is given in equation 3.3

$$\boldsymbol{PHB(g/L) = 0.12 + (0.08 * A) - (1.11 * B) + (0.063 * C) - (0.022 * AB) + (0.06 * AC) - (0.023 * BC) + (0.03 * A^2) + (0.049 * B^2) + (0.03 * C^2)} \quad (3.3)$$

3D graphs generated based on the model are presented in Figure 3.61. As can be observed from the plots as well, all variables showed an interaction with each other. The most striking interaction was observed between orange peel and citrate.

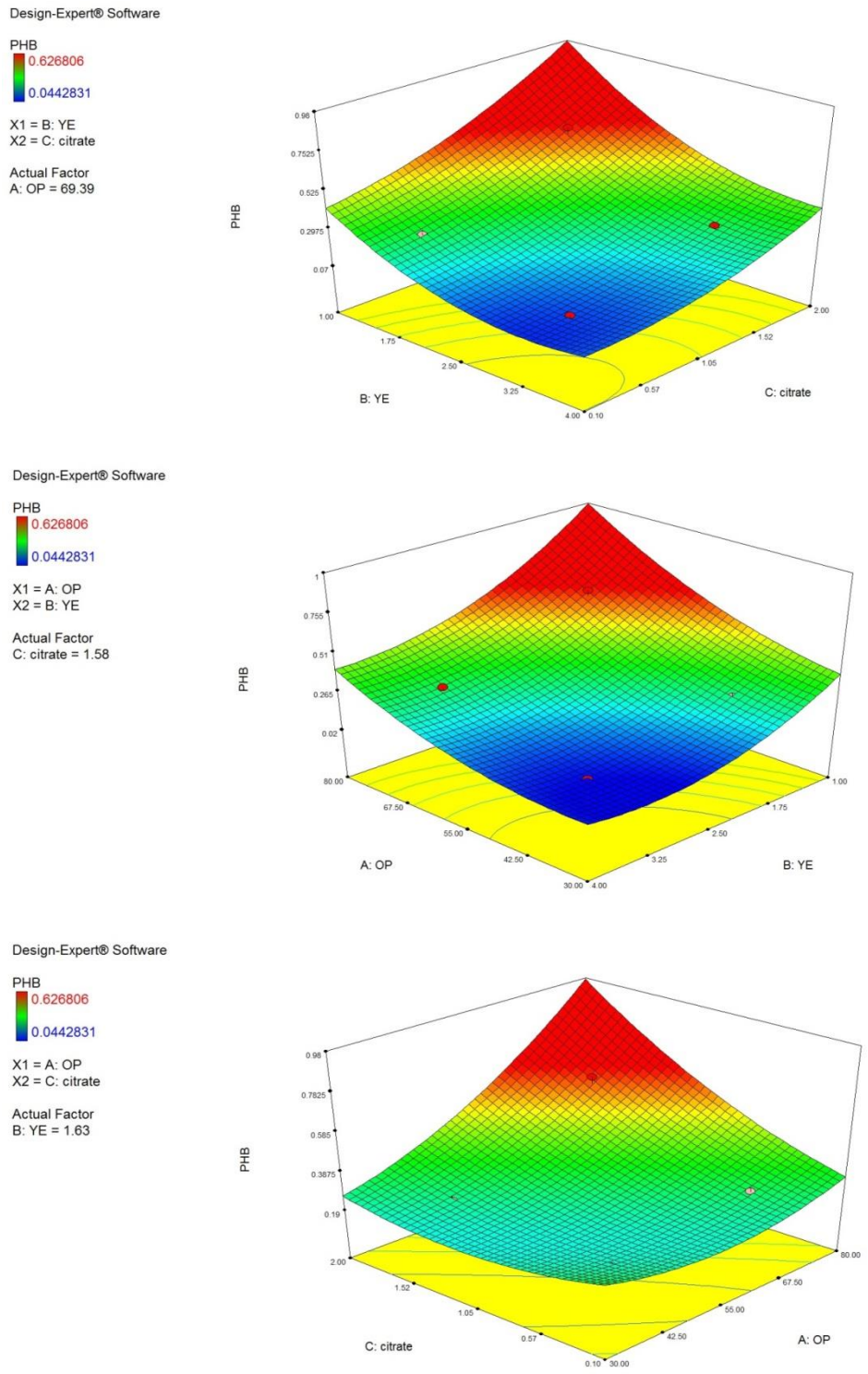



**Figure 3. 61:** 3D graphs showing the interactions amongst the variables screened for P(3HB) production using orange peel as carbon source.

Based on the results obtained from CCD the optimised levels for P(3HB) production with their confidence levels are presented in Table 3.14. The optimised medium component

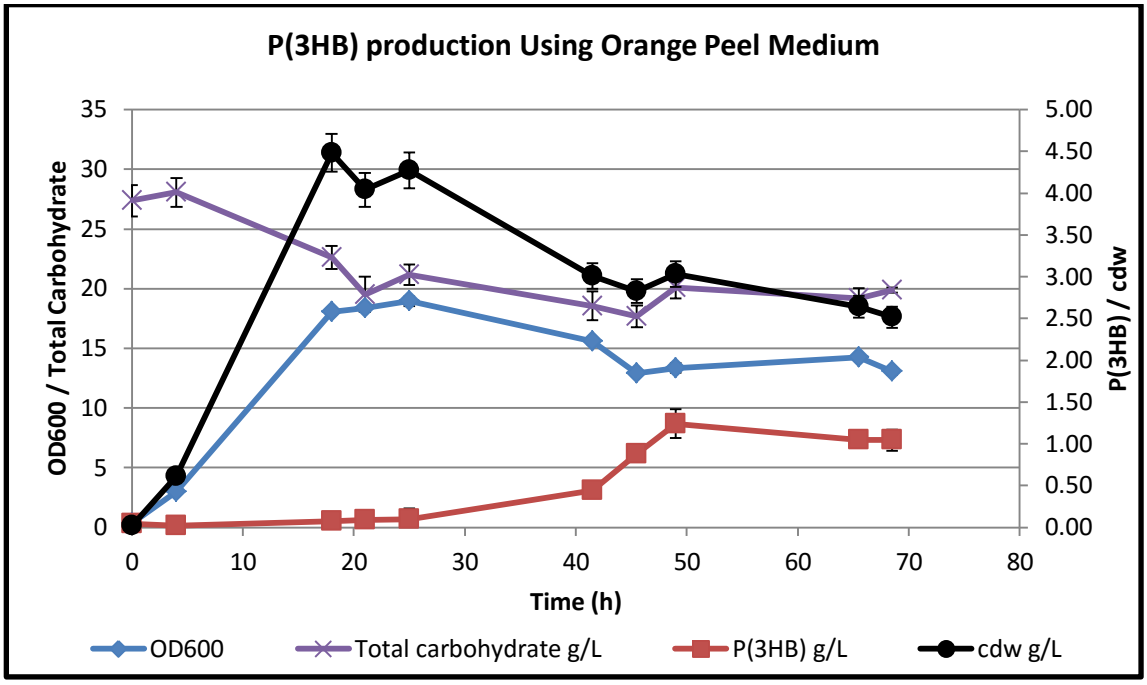
values were found to be 69.4, 1.63 and 1.58 g/L for orange peel infusion, yeast extract and citrate respectively.

**Table 3. 14:** Optimized medium compositions and production prediction with confidence levels

| Number | OP (g/L) | YE (g/L) | Citrate (g/L) | P(3HB) (g/L) |
|---|---|---|---|---|
| 1 | 69.39 | 1.63 | 1.58 | 0.589 |
| **Response** | **Prediction** | **SE Mean** | **95% CI low** | **95% CI high** |
| P(3HB) (g/L) | 0.589 | 0.039 | 0.498 | 0.697 |

The model was validated in shaken flask runs using the levels suggested by the program and production was found to be within the confidence levels. Using these optimised levels, the production was scaled-up in a 0.5 L STR with 0.4 L working volume. The growth and the production profiles for P(3HB) production using orange peel infusion as carbon source were studied. The fermentation profile is given in Figure 3.62.

At the end of 72 h approximately 1 g/L P(3HB) was obtained although highest production was after 50 h with 1.24 g/L P(3HB). Oxygen profile showed a similar trend to P(3HB) production using standard medium using glucose (on page 97, Figure 3.9), however the pH was constant until 50 h and then slightly increased from pH 6 to pH 7 within the next 20 h (Figure 3.62b). When the optical density and cdw values were compared with P(3HB) production with glucose medium (on page 97, Figure 3.9) it was observed that in orange peel medium, $OD_{600}$ is 8.5% lower and cdw is 19.5% higher than those obtained in glucose medium. These results indicated that orange peel is a good replacement for glucose for P(3HB) production. Further comparison and discussion is presented in Section 4.4



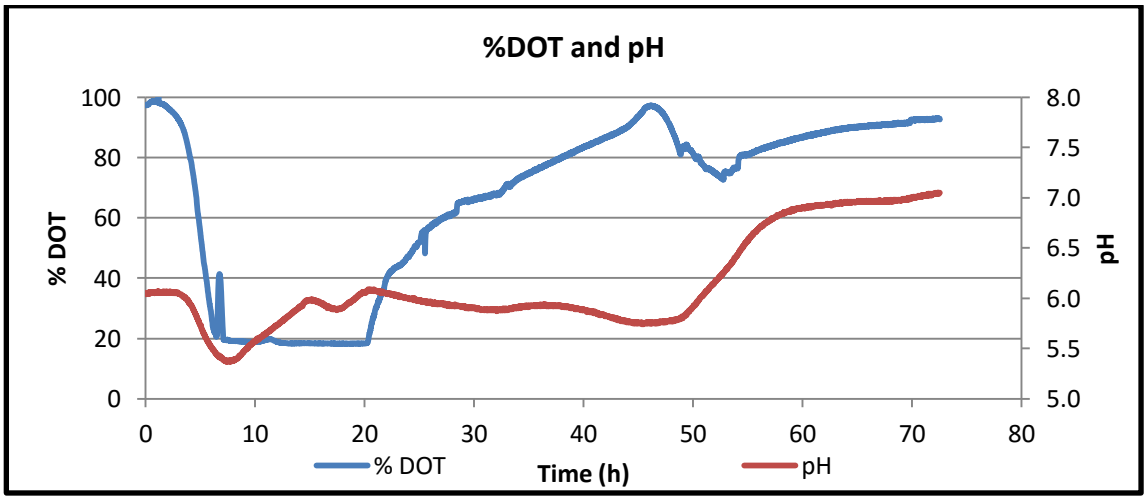




**Figure 3. 62:** Profile for (a) growth of *Bacillus subtilis* OK2 and P(3HB) production (b) %DOT and pH, using orange peel CCD optimised levels at 400 rpm, 30°C, 1 vvm. Points represent average of triplicate measurements.

### 3.6.5 Double Polymer Production with Pre-treated Orange Peel Infusion Medium

The data obtained for the behaviour of the organism in the orange peel infusion medium and in the dual production medium was combined in order to have a system for the production of double polymer from the pre-treated agricultural biowaste, orange peel. As discussed in Section 3.6.3, orange peel infusion by itself gave the best P(3HB) production with 17 g/L sugar content. Similarly, the optimum point obtained from CCD suggested that yeast extract and citric acid addition improves P(3HB) production (Section 3.6.4).

Results in Section 3.5.1 showed that significant quantities of both polymers could be obtained using CCD by keeping the C/N ratio constant. Thus, it was decided to replace the carbon source, which was 20 g/L glucose in optimised DP medium, with the equivalent orange peel infusion solution.

The total sugar content of orange peel infusion was adjusted to 20 g/L prior to sterilisation (110°C, 10 min) and added to the medium separately. Since the best conditions defined in reactor studies (Section 3.5.3) was keeping DOT at 25%, the same conditions were maintained in the experiments using orange peel infusion as glucose replacement.

The profiles, of the first orange peel fermentation for production of dual polymer can be seen in Figure 3.63. The fermentation was carried for 54 h and only small amounts of both polymers, 76 mg/L and 34 mg/L of γ-PGA and P(3HB) respectively, were obtained. A longer lag phase, compared to the optimised dual polymer fermentation (on page 141, Figure 3.48) was observed along with a rapid increase in pH after 12 h (Figure 3.63b). This rapid increase trend continued and after 25 h the pH exceeded the value of 7.5 which made the conditions in the fermenter unfavourable for bacterial growth and P(3HB) production. In addition, the $CO_2$ level dropped sharply after 25 h, indicating that there was a decline in metabolic activity. This has followed by a decline in culture $OD_{600}$ and cdw. After 54 h, no improvement was observed and the run was terminated.

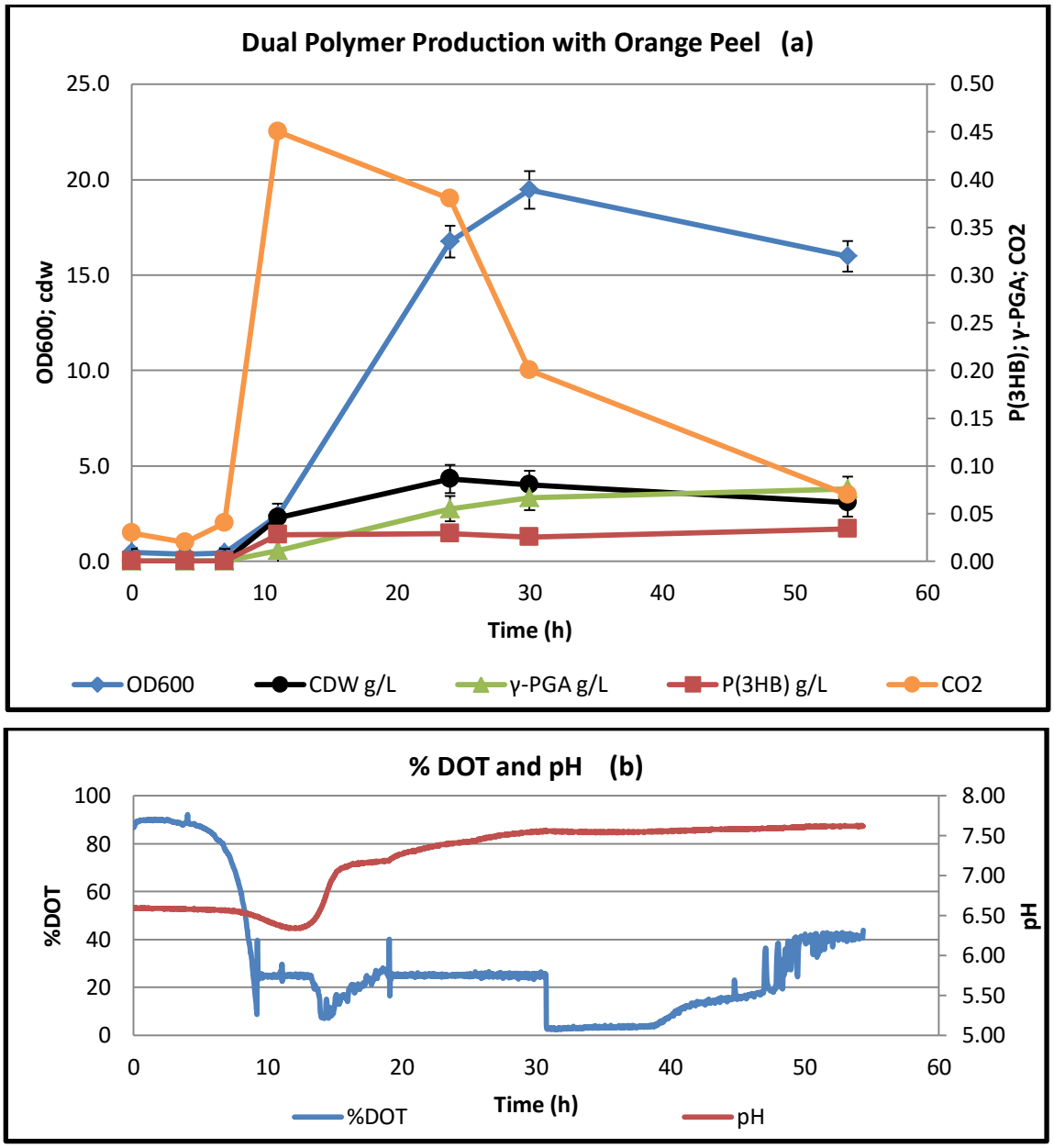



**Figure 3. 63:** Profile for (a) growth, double polymer production, total carbohydrate consumption (b) %DOT and pH, using orange peel as glucose replacement and constant DOT, at 25%, 30°C, 1vvm. Points represent average of triplicate measurements.

Subsequently, another fermenter was set to repeat the run. A similar trend to the previous run was observed (Figure 3.63) in terms of the longer lag phase and pH profile in the first 24 h after which the pH value exceeded pH 7.5. Therefore, after 24 h pH control was introduced to keep pH at 6.6 (Figure 3.64), which was the average pH value in the optimised double polymer fermentation (Figure 3.48).

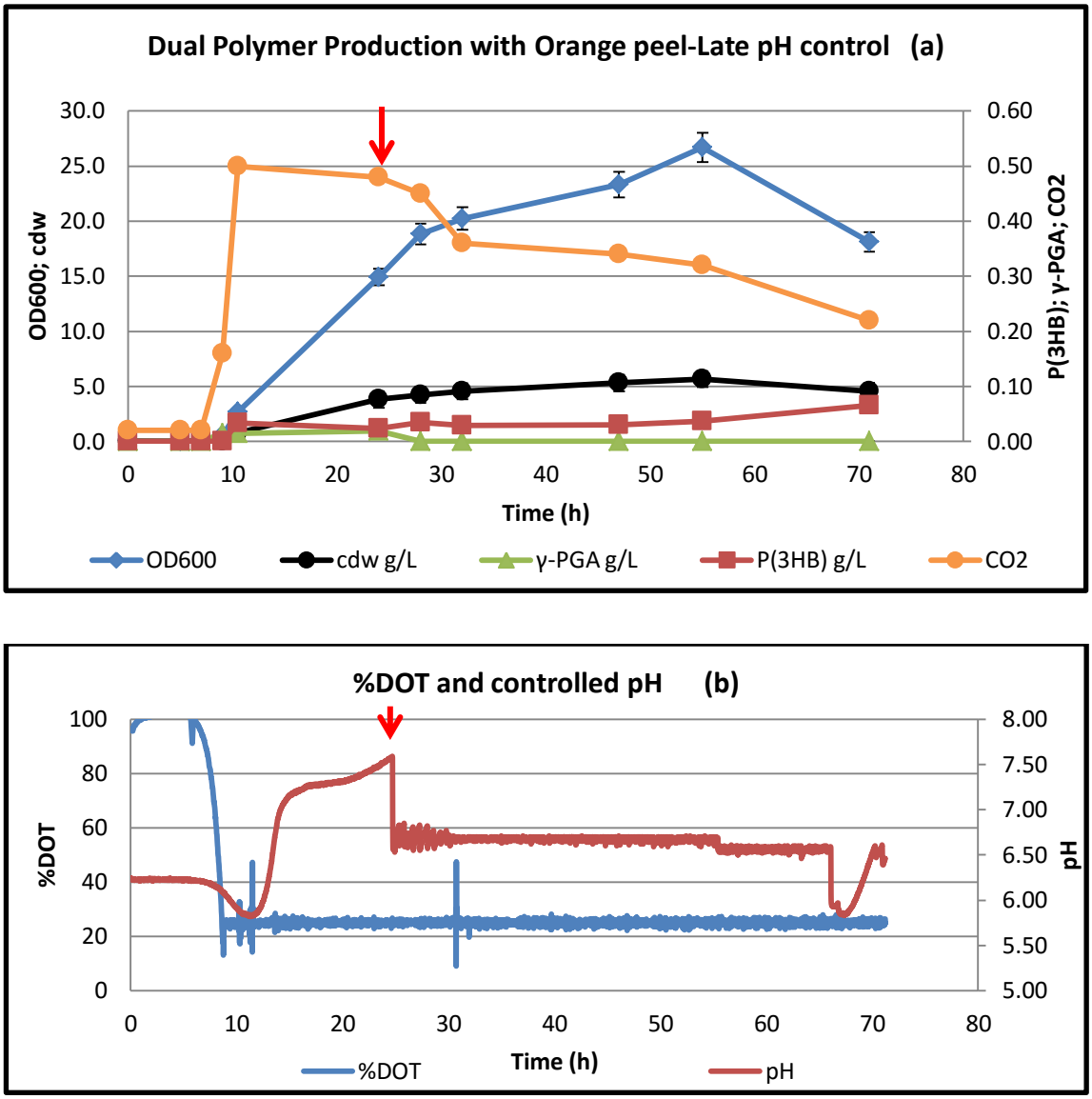



**Figure 3. 64:** Profile for (a) growth, double polymer production, total carbohydrate consumption (b) %DOT and pH, using orange peel as glucose replacement and constant DOT, at 25%, and constant pH at 6.6 after 24 h, 30°C, 1vvm. Points represent average of triplicate measurements

At the end of the 72 h fermentation, a slight increase in P(3HB) concentration (65 mg/L) was observed however γ-PGA production ceased after the pH control was introduced. Following the results obtained from the pH-controlled fermentation, a defined medium to mimic the mixed sugar composition of orange peel infusion was designed, in order to see if the mixed sugar composition in the orange peel infusion has any specific effect on the production of both polymers. Using the compositions reported in section 3.6.2, a sugar composition was designed. The total sugar concentration, 20 g/L, was divided amongst 3

dominant sugars, of orange peel infusion, sucrose, fructose and glucose, according to their original ratios of 42%, 35% and 23%, respectively. The final composition of the medium was calculated as; 8.4 g/L sucrose, 7 g/L fructose, 4.6 g/L glucose, 1.5 g/L yeast extract, 32 g/L glutamic acid, 12 g/L ammonium sulphate and 2.4 g/L citric acid.

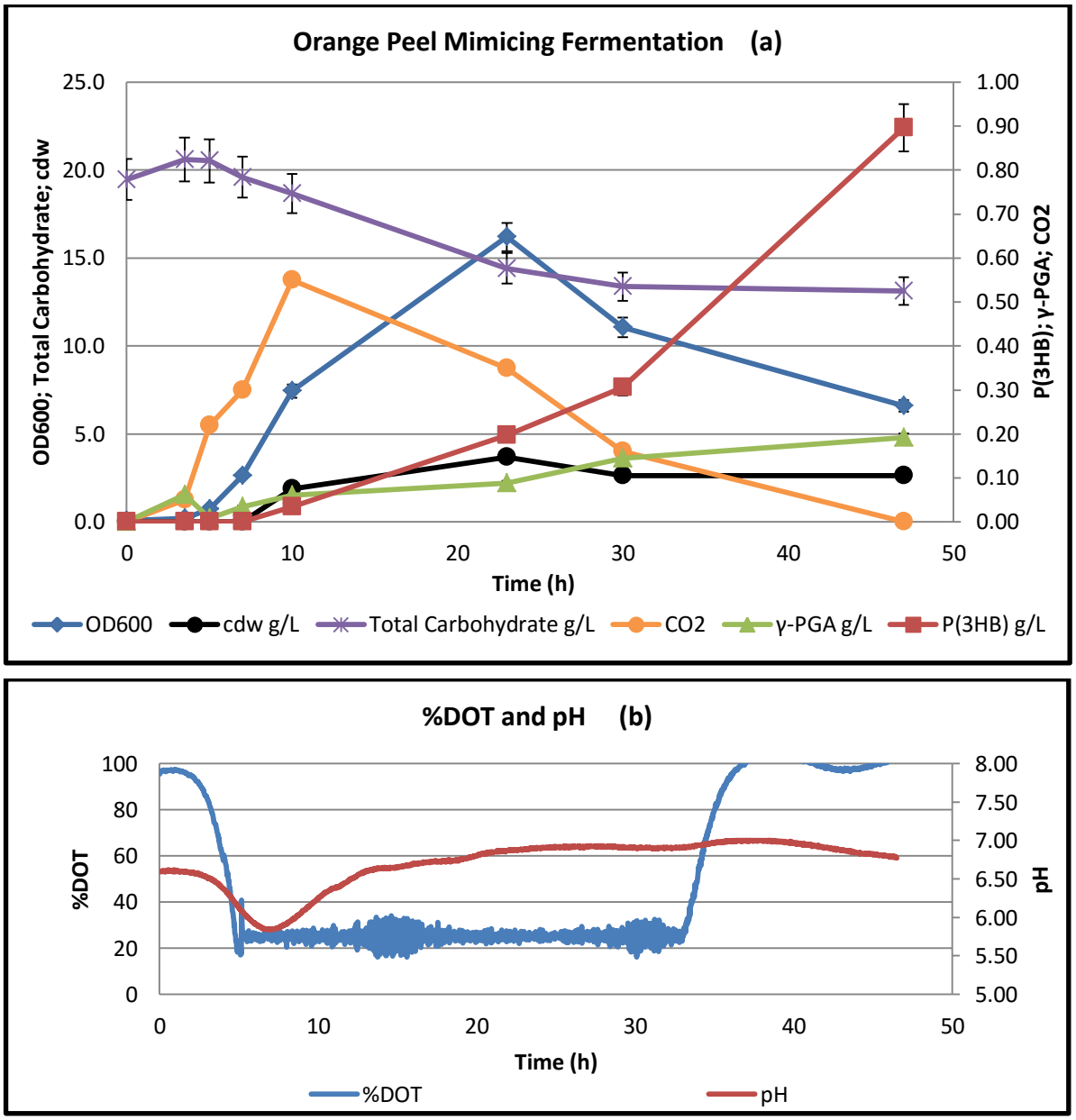



**Figure 3. 65:** Profile for (a) growth, double polymer production, total carbohydrate consumption (b) %DOT and pH, mimicking orange peel infusion sugar composition, constant DOT, at 25%, 30°C, 1 vvm. Points represent average of triplicate measurements.

The profile obtained from the orange peel mimicking fermentation can be seen in Figure 3.65. A similar trend to the optimised double polymer production (on page 141, Figure 3.48) was observed. The lag phase was shorter than orange peel infusion fermentation and significant amount of both polymers, 0.20 g/L γ-PGA and 0.9 g/L P(3HB) were produced at the end of the 48 h fermentation. Although the sharp, early decrease in pH was similar to the orange peel infusion fermentation, the value did not exceed pH 7.0 at a later stage during the course of fermentation (Figure 3.64b).

Considering the importance of pH and % DOT for the productivity of orange peel medium fermentations, it was decided to perform an additional run where pH and oxygen levels were kept constant at pH 6.5 and DOT at 25%. The run was conducted keeping the pH and % DOT constant using DP medium but replacing glucose with orange peel (at 20 g/L). All other ingredients in DP medium (glutamic acid, yeast extract, citric acid, ammonium sulphate) were also present in the levels previously indicated.

Figure 3.66 shows the fermentation profile under controlled pH and %DOT conditions. Comparing the profile and the amounts obtained with the previous orange peel runs, it was seen that pH and % DOT control together enhance the production. Although this result was not as good as the one obtained with the glucose-only medium (on page 141, Figure 3.48) and orange peel mimicking (Figure 3.65) fermentations, approximately 0.2 g/L of each polymer was obtained at the end of 79 h. This may be due to the presence of some inhibitors in orange peel, like phenolic compounds (naringin, neohesperidin, narirutin, and hesperidin (Sawalha *et al.,* 2009)), however it was demonstrated that it is possible to produce both polymers in a single run using orange peel as a carbon source. Further investigation is needed in order to increase the productivity using this agro-industrial waste as a substrate.

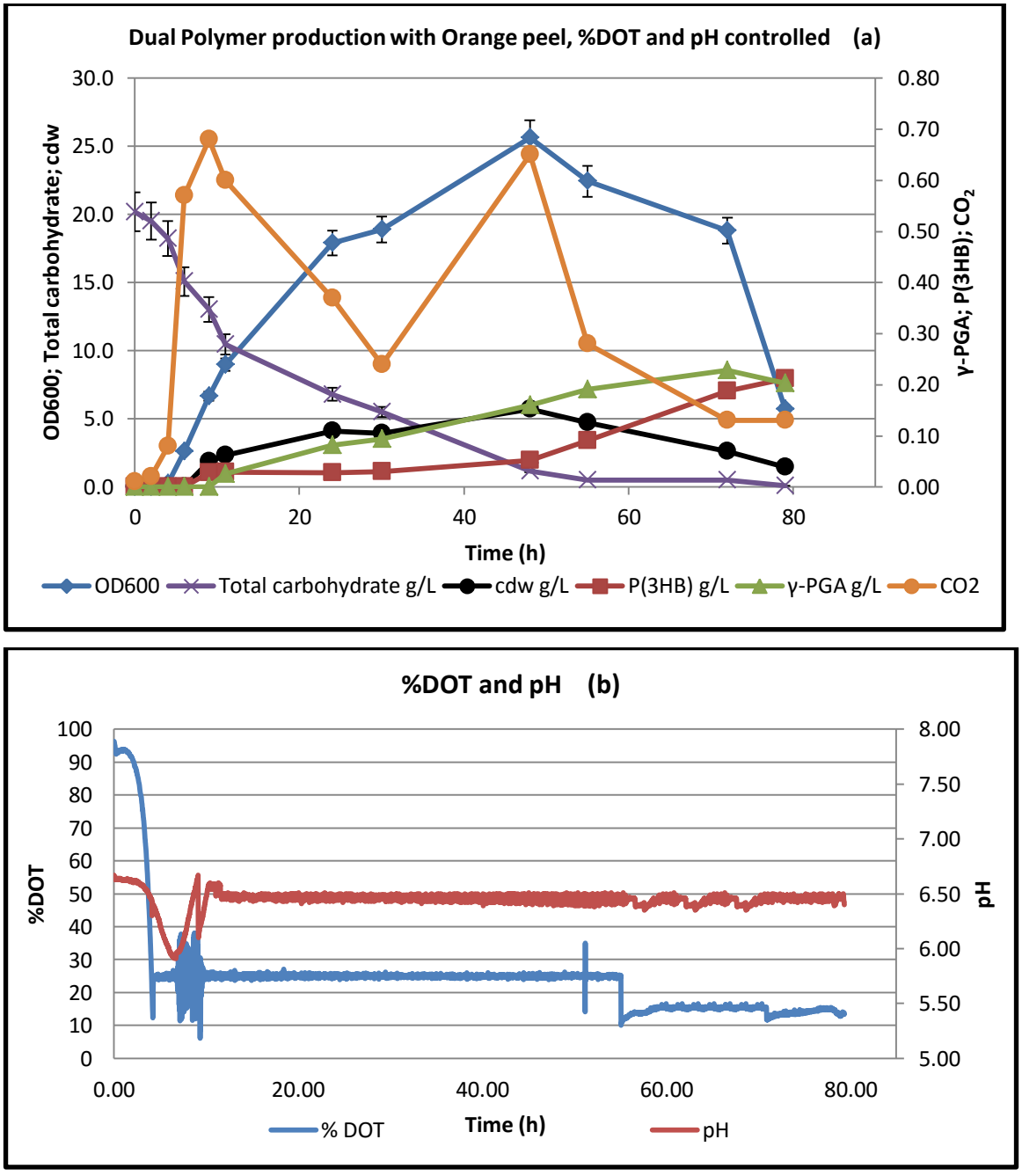



**Figure 3. 66:** Profile for (a) growth, double polymer production, total carbohydrate consumption (b) %DOT and pH, using orange peel as carbon source, at constant DOT at 25% and pH at 6.5, 30°C, 1 vvm. Points represent average of triplicate measurements.

### 3.7 Studies on Recovery of Double Polymers

Downstream processing is a significant cost-factor in the economics of large scale biopolymer productions. This is particularly important in dual polymer systems where one of the products is intracellular and the other is extracellular. In this section of results several concepts were tested in search of a feasible and novel system in order to propose a simple and potentially economic separation operation for the dual-product system.

#### 3.7.1 Static Magnetic Field for Recovery

Several researchers (Galvanovski and Sandblom 1998 ; Fojt *et al.*, 2004) have reported that exposure of mild static magnetic field activates ions within the cell membrane making the cell membrane more leaky. It was argued that if *Bacillus subtilis* OK2 is exposed to a magnetic field, P(3HB) granules might leak outside the cell to facilitate the downstream processing of P(3HB) separation from the cells. This concept was tested using a magnetic field generator applying 10mT magnetic field in a co-current manner to the circulating culture of a batch fermentation (0.5 L STR, 0.4 L working volume) with a flow-rate of 12.5 mL/min (the system described in Section 2.9.1, Figure 2.1). A photograph of the set-up can be seen in Figure 3.67.

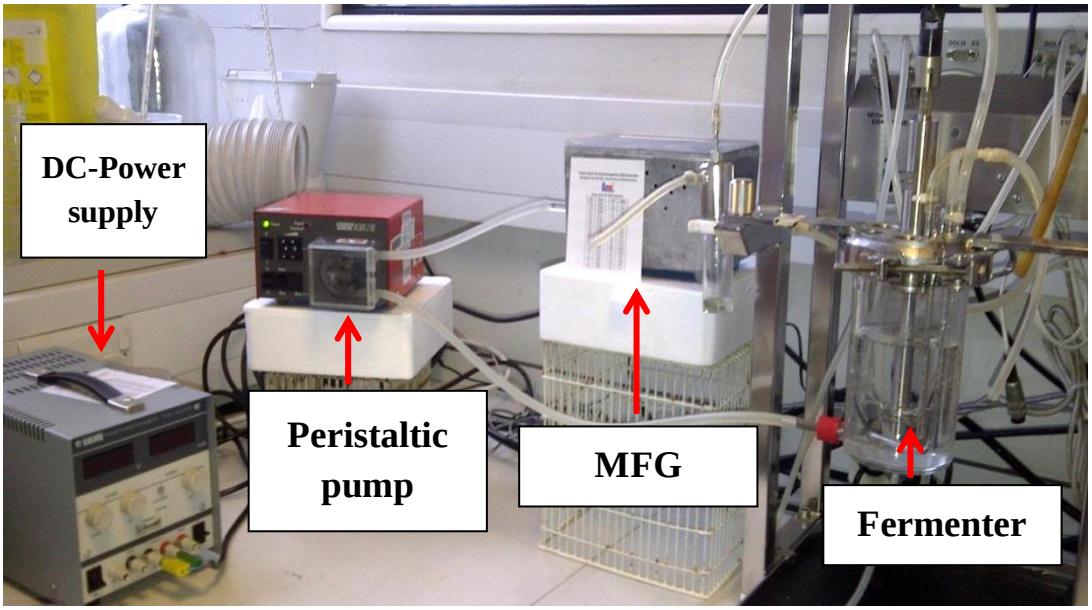



**Figure 3. 67:** Magnetic field generator (MFG) linked to the fermenter.

Results of the test and the control runs of the two parallel 72 h fermentations are given in Figures 3.68 and 3.69. Approximately 1.5 g/L of P(3HB) was produced in the control run but no or very little P(3HB) was detected in the test run exposed to the magnetic field. Similarly in the control run more than 3 g/L cdw was obtained, but in the test run cdw was around 2.5 g/L. These findings suggested that the application of the magnetic field could affect both the cells and their P(3HB) production.

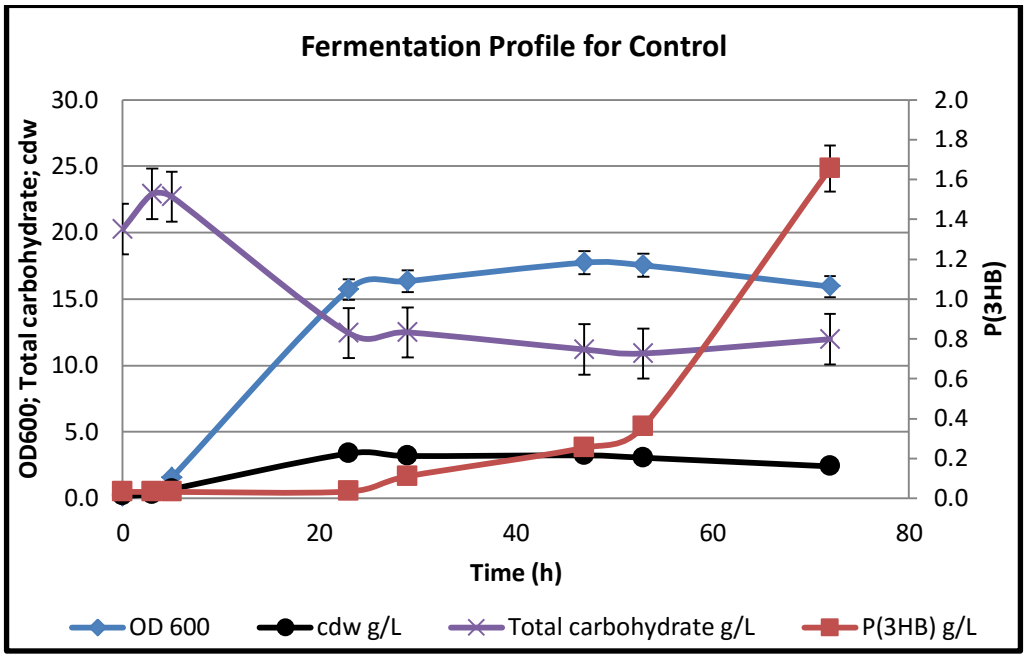



**Figure 3. 68:** Fermentation profile for control run, 300 rpm, 1 vvm, 30°C. Points represent average of duplicate measurements.

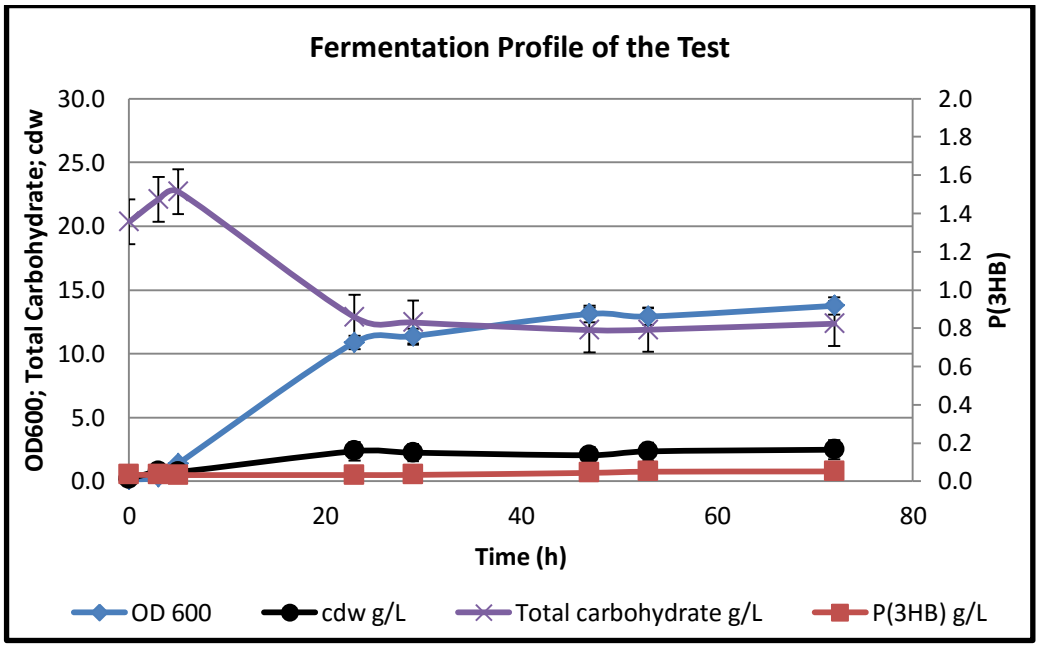



**Figure 3. 69:** Fermentation profile for test run, 300 rpm, 1vvm, 30°C, 10 mT co-current magnetic, Q: 12.5 ml/min, circulation time was 32 min. Points represent average of duplicate measurements.

Furthermore, the supernatants of the last four samples of both control and test were analysed for their P(3HB) contents. No difference between the test and the control runs

could be observed with respect to the amount of P(3HB) detected in the supernatants, indicating that P(3HB) did not leak out of the cells (Table 3.15.)

**Table 3. 15:** Values for P(3HB) detected in cells and the supernatants of the control and the test runs

| Type | Sample No | Time (h) | Control P(3HB) (g/L) | Test P(3HB) (g/L) |
|---|---|---|---|---|
| **Cells** | 0 | 0 | 0.03 | 0.03 |
| | 1 | 3 | 0.03 | 0.03 |
| | 2 | 5 | 0.03 | 0.03 |
| | 3 | 23 | 0.03 | 0.03 |
| | 4 | 29 | 0.11 | 0.03 |
| | 5 | 47 | 0.25 | 0.04 |
| | 6 | 53 | 0.36 | 0.05 |
| | 7 | 72 | 1.65 | 0.05 |
| **Supernatant** | 4 | 29 | 0.03 | 0.03 |
| | 5 | 47 | 0.02 | 0.03 |
| | 6 | 53 | 0.03 | 0.02 |
| | 7 | 72 | 0.04 | 0.04 |

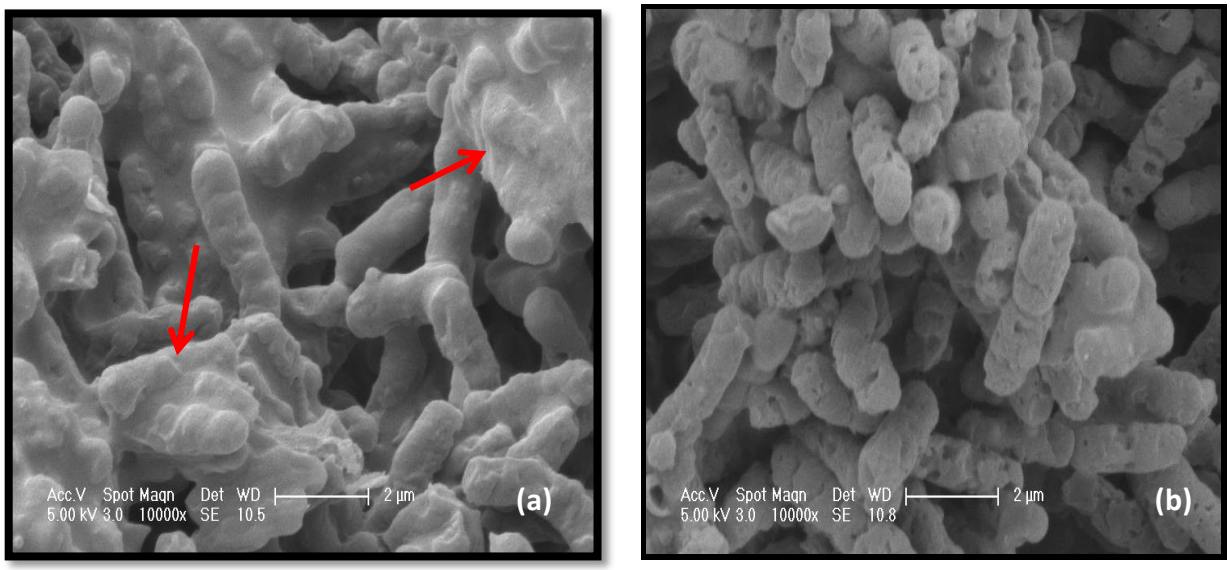



**Figure 3. 70:** SEM images (a) test - MF exposed (b) control – No MF exposure, EPS formed is indicated by red arrows.

SEM images taken after the exposure revealed that the culture exposed the magnetic field resulted in clustering of the cells and production of some kind of exopolysaccharide

(Figure 3.70). In the light of these findings, it was concluded that magnetic field exposure prevented P(3HB) production and the topic was not pursued any further.

### 3.7.2 **Floatation for Recovery**

Floatation is a widely used separation methods especially in bioleaching. It is especially beneficial for removing hydrophobic material from hydrophilic material such as cells from a certain area or fermentation broth without causing any damage. Foaming is a widespread problem in fermentation. As reported in previous sections dual polymer production medium optimised in this study has a high tendency to foam and created problems in some of the runs, as reported in Section 3.5.3. In 1958 Boyles and Lincoln reported the use of floatation for separation of vegetative cells and spores. Exploiting the foaming tendency observed in double polymer production to the benefit of the process through foaming / defoaming chambers was tested for its capability of separating the polymer containing cells from the polymer free cells.

Using a custom designed foaming column described in Section 2.9.2, the 48 h culture was filled into the column linked to an air pump and vacuum chamber (Figure 3.71).

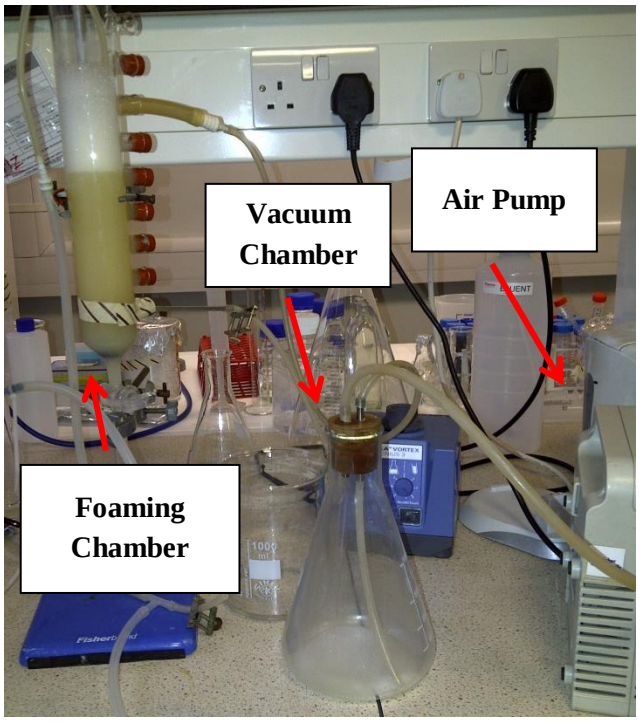



**Figure 3. 71:** Floatation column linked to an air pump and vacuum chamber.

Air was sparged from the bottom of the column using two different sparger types, namely, single orifice (consisted of a single hole of 3 mm inner diameter) and multi-orifice (fish tank type sparger) to produce foams with different sizes (Figure 3.72). The single-orifice sparger produced less stable, larger diameter bubbles, while the multi-orifice sparger produced smaller diameter, more stable bubbles.

Foam was collected into the vacuum chamber from the top and was converted into liquid form, with the help of the vacuum. Samples were taken at the beginning and at the end of the process from the culture broth and the foam were collected throughout the process. All samples were analysed for their cdw and P(3HB) contents (Table 3.16).

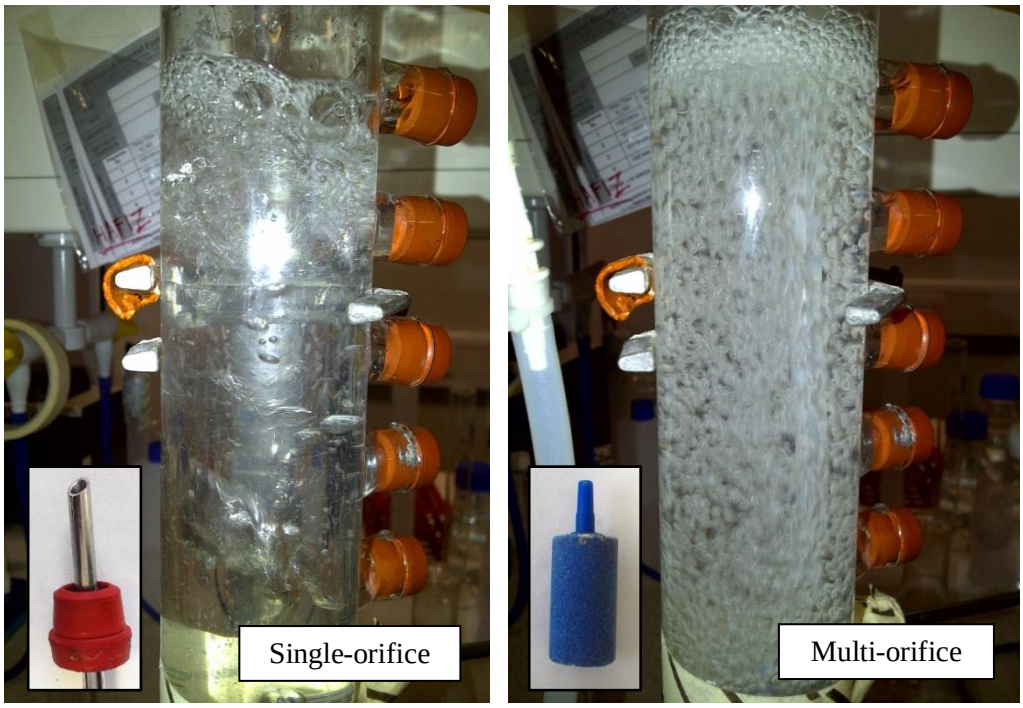



**Figure 3. 72:** Comparison of bubble sizes created by single and multi-orifice spargers

The samples from the foam and liquid fractions were also studied under the microscope and compared with the original culture (Figure 3.73)

**Table 3. 16:** P(3HB), cdw and %P(3HB)/cdw values obtained from floatation experiments

| Type | Place | Time (h) | P(3HB) g/L | cdw g/L | %P(3HB)/cdw |
|---|---|---|---|---|---|
| **Single orifice** | **Liquid** | **Pre Foaming** | 0.67 | 2.07 | 32.11 |
| | | **Post Foaming** | 0.59 | 0.89 | 66.79 |
| | **Foam** | **0.5** | 1.08 | 6.36 | 16.95 |
| | | **1** | 0.95 | 5.54 | 17.14 |
| | | **1.5** | 0.67 | 3.51 | 19.02 |
| **Multi orifice** | **Liquid** | **Pre Foaming** | 1.23 | 2.58 | 47.75 |
| | | **Post Foaming** | 1.16 | 0.635 | 182.34 |
| | **Foam** | **0.5** | 4.21 | 9.84 | 42.90 |

Results presented in Table 3.16 show that a large fraction of cells were carried with the foam by both of the spargers tested. 57% of cells with the single orifice and 75% cells with

multi-orifice sparger were collected in the foam fraction. This was expected since the multi-orifice system contained smaller but a higher number of bubbles providing a larger surface area and more stable foam for cell adsorption. However when the P(3HB) concentration in the foam and liquid fractions (containing less number of cells) were compared it was found that %P(3HB)/cdw was higher in the liquid fraction left after foaming. In addition the P(3HB) content of liquid left after foaming was found to be higher than that of the pre-culture. This indicated that the cells with a higher P(3HB) content were left in the liquid fraction. The P(3HB) containing cells that were removed by the foam were calculated to be 0.074 and 0.075 g/L in the columns with the single and the multi- orifice spargers, respectively, confirming this finding. When the pre and post-foaming liquids were studied with respect to P(3HB) concentrations, the post-foaming liquid fraction showed a considerably higher % P(3HB)/cdw in both single and multi-orifice sparger systems; 66.79 and 182.34 % P(3HB)/cdw respectively. This finding could not be explained with the existing data and literature. Microscopic observations of the culture before (Figure 3.73a) and after (Figure 3.73b and c) foaming has also revealed that multi-orifice sparger has carried most of the cells to the foam fraction. The difference is cell concentrations can be observed in the Figures 3.73b and 3.73c. Post foaming appearance of the culture under microscope revealed some spherical particles in the culture and the broth was dominated with these spherical particles rather than cells. Since the liquid fraction contained more polymer than the foam, it indicated that these spherical particles might be polymer granules released from the cells. This is further discussed in Section 4.5

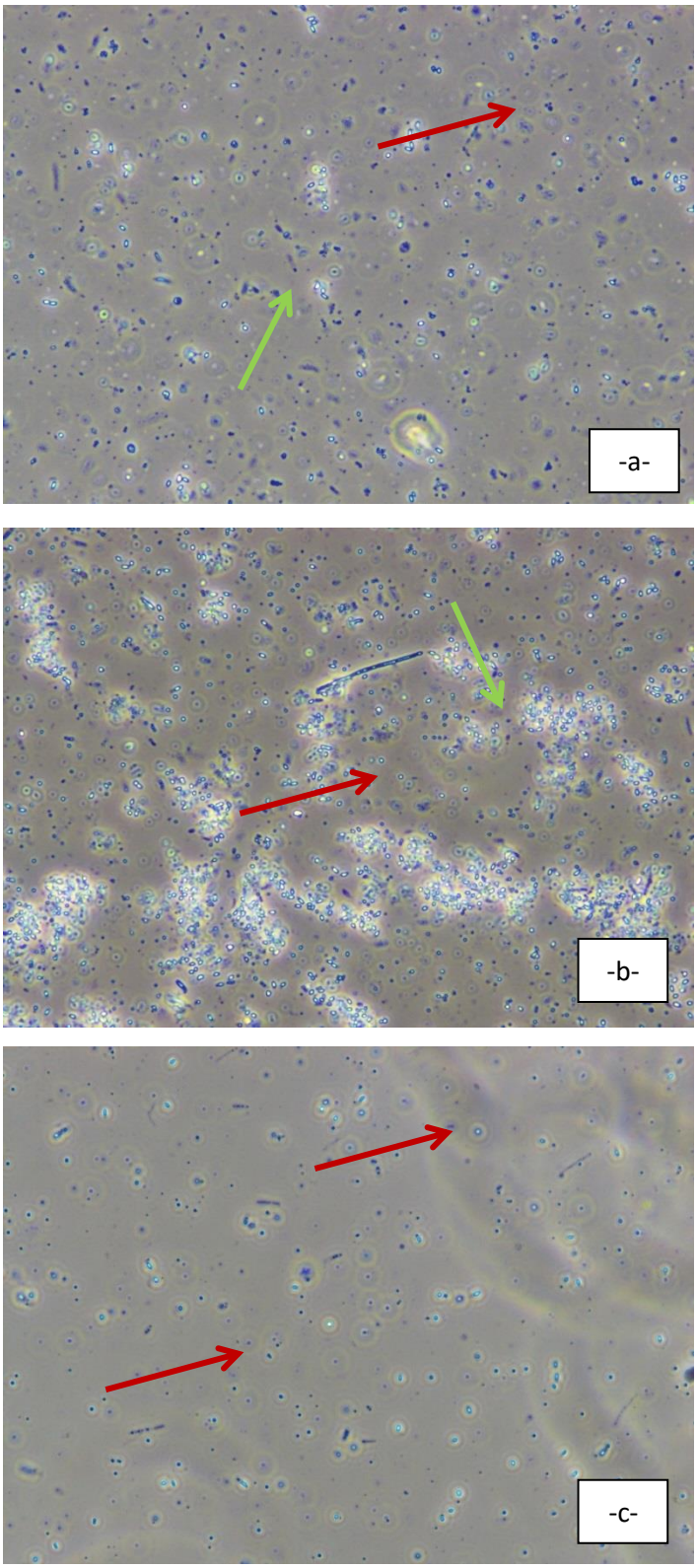



**Figure 3. 73:** Microscopic investigation of the foam (b) and liquid fractions (c) after exposure to 30 min foaming as compared to the original 48 h culture (a) in a multi-orifice sparger foaming column. Spread granules are indicated with red arrows and spores are indicated with green arrows. 400x magnification, phase contrast mode.

### 3.7.3 **Sedimentation for Recovery**

Results reported in Section 3.7.2 showed that foaming preferentially removed non/less pregnant cells indicating that polymer containing cells have a higher density than polymer-free cells. Horowitz and Sanders (1994) reported that the density of the polymer granules varies between 1.17-1.18 g/cm$^3$. The separation behaviour of the cells with higher P(3HB) content was also observed in the preliminary experiment. The observed settling behaviour of the culture was investigated using the set-up described in Figure 3.74. Three distinct zones were observed after 4 h. Analysis of the samples taken from middle and bottom zones showed a higher P(3HB) containing cell concentration in the middle zone (Table 3.17). Top zone contained almost no cells and therefore was not included in analysis.

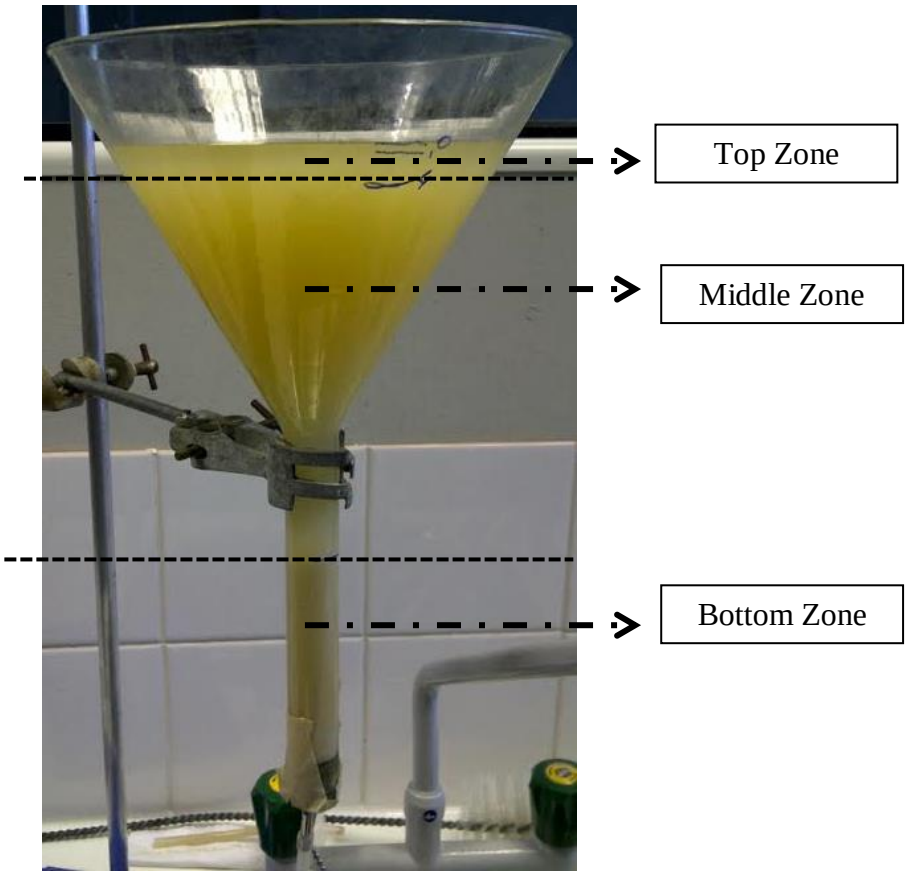



**Figure 3. 74:** Experimental set-up for preliminary sedimentation tests, after 4 h

**Table 3. 17:** Preliminary results for sedimentation of cells by position

| Zone | cdw (g/L) | %P(3HB)/cdw |
|---|---|---|
| **Bottom** | 41,72 | 3,21 |
| **Middle** | 1,25 | 7,63 |

The %P(3HB)/cdw measured was found to be more than double in the middle zone compared to the bottom section. This results showed that cells containing higher P(3HB) favoured the middle zone after sedimentation.

Subsequently, this behaviour of polymer containing cells was further investigated using the beaker set-up shown in Figure 2.3. An 48 h culture with a cdw of 2.76 g/L containing 37.3 % P(3HB)/cdw, was filled into the beaker and samples were taken using a syringe after 2 h, 4 h and 6 h from different zones and were analysed for their cdw and P(3HB) content. The results shown in Figure 3.75 and 3.76 exhibit that cells with higher P(3HB) content start to accumulate in the middle zone and this separation is best observed in 4 h. After 6 h % P(3HB) in top and middle zones start to equalise with respect to %P(3HB) content.

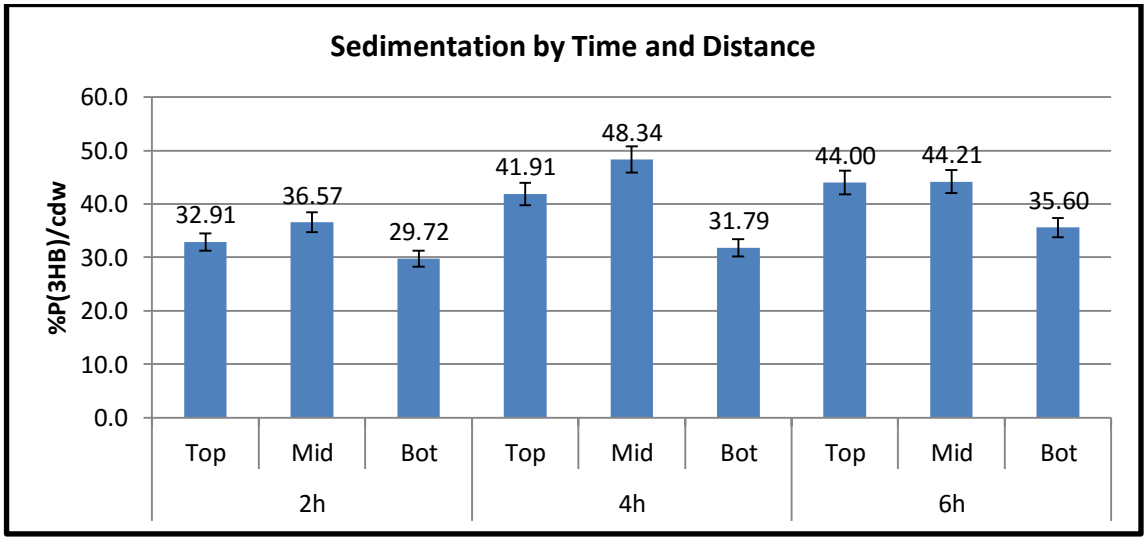



**Figure 3. 75**: %P(3HB)/cdw values at different hours and zones during sedimentation.

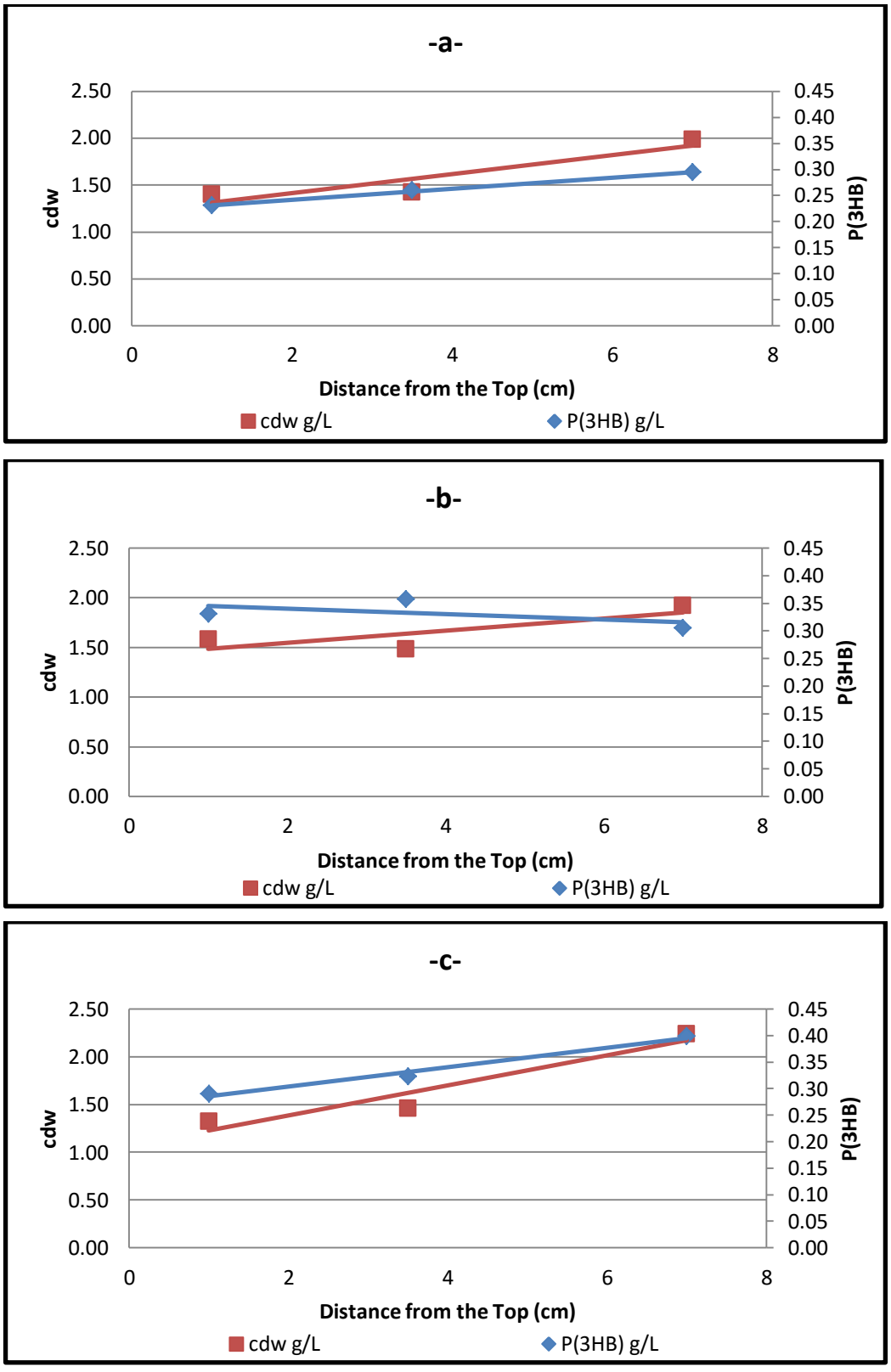
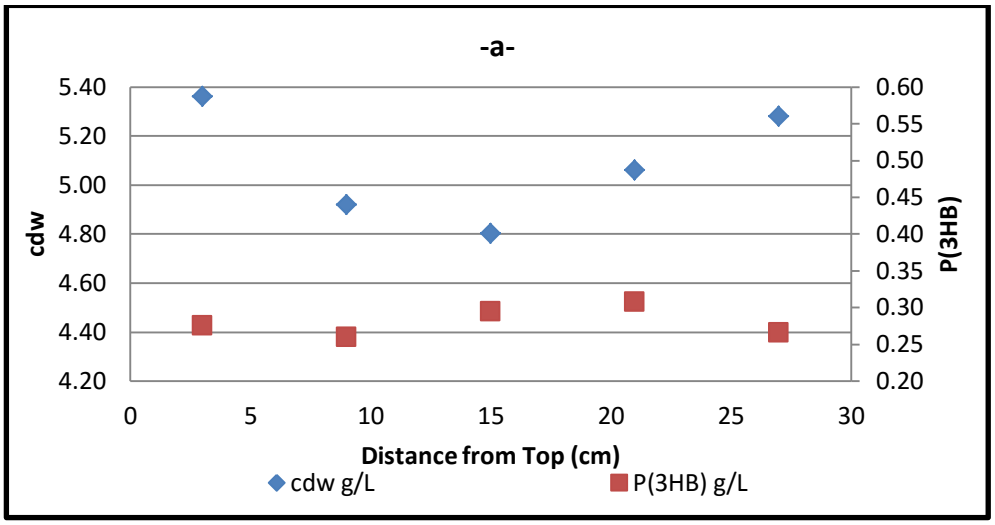
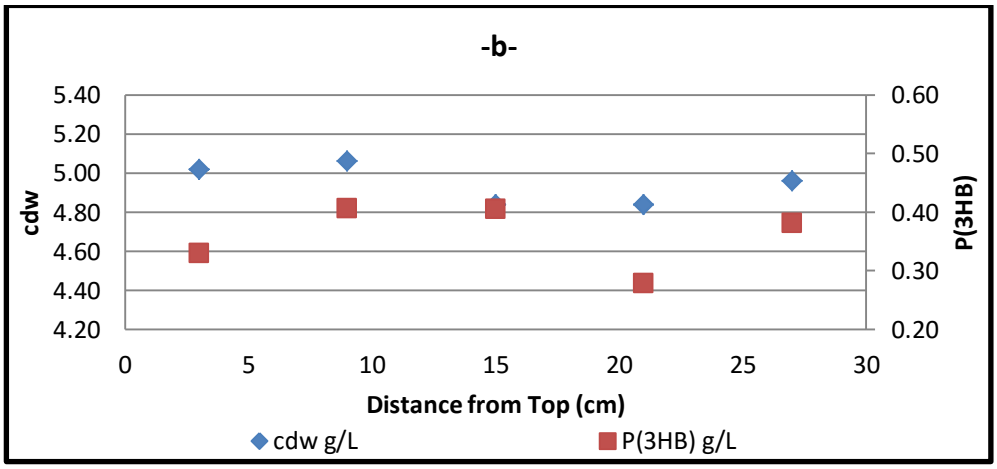



**Figure 3. 76:** Change in P(3HB) concentration and cdw after 2 h (a), 4h (b) and 6 h (c) of sedimentation.

An interesting phenomenon was observed. When the average % P(3HB)/cdw values were calculated for 2 h, 4 h and 6 h, 33.06; 40.68; and 41.27 % P(3HB)/cdw. This increase in polymer percentage could not be explained with existing data and literature. Further discussion about this behaviour is included in Section 4.5.2.

Based on the observation that cells containing more polymer accumulate in the middle section of the tank within 4 h, it was considered that this could be used as an a potential method for online separation linking a separating tank to the fermenter to selectively separate and remove high polymer containing cells and recirculate the cells harbouring less polymer back into the fermenter. Thus, the sedimentation tank was redesigned to enable a good separation of different zones in a time period as short as possible. As described in Section 2.9.2 a new taller sedimentation column was designed with multiple ports (Figure 2.3) to determine the % P(3HB)/cdw more accurately, and to assess the settling of the cells more precisely. The aim was to determine the minimum settling time required for the concentration of P(3HB)-rich cells as well as to identify their exact location in the column to identify the correct connection point to the fermenter. In the designed experiment, a 48 h old culture was added to the sedimentation column and samples were taken from the points 3 cm, 9 cm, 15cm, 21cm and 27 cm from the top of the 30 cm column. Samples were analysed for P(3HB) content and cdw and results are given for different times of sampling, namely 2 h, 2.5 h, 3 h, 3.5 h, and 4 h in Figures 3.77 a-e respectively.



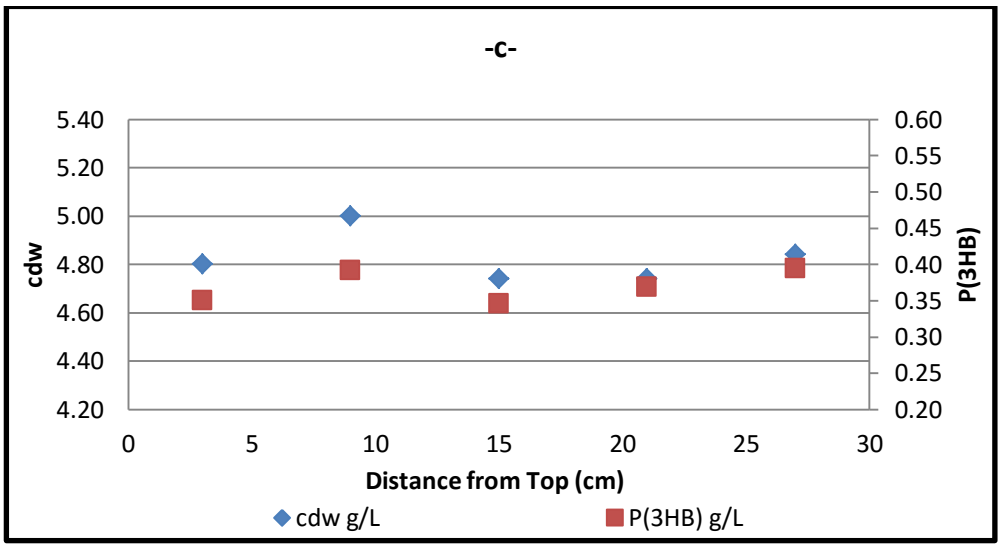
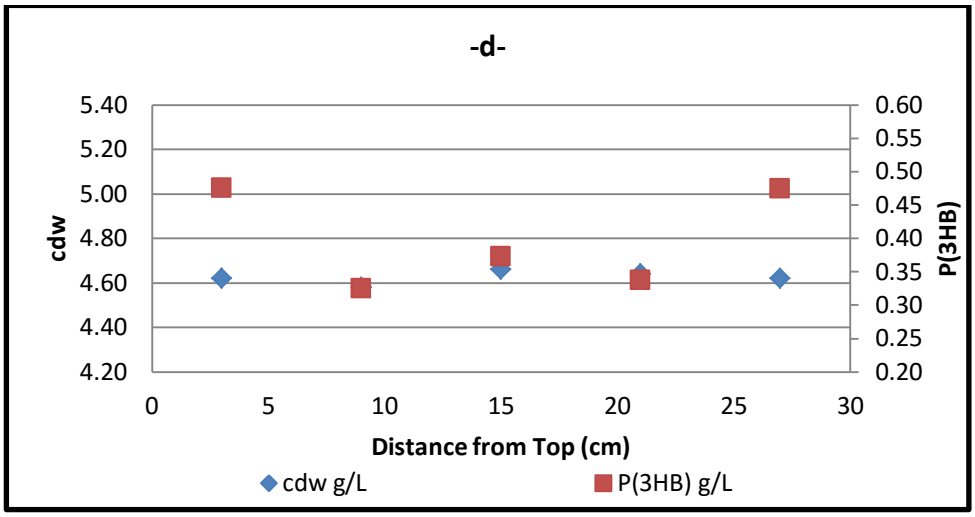




**Figure 3. 77:** Change in P(3HB) concentration and cdw using taller sedimentation column, after 2 h (a), after 2.5 h (b), after 3 h (c), after 3.5 h (d) and after 4 h (e).



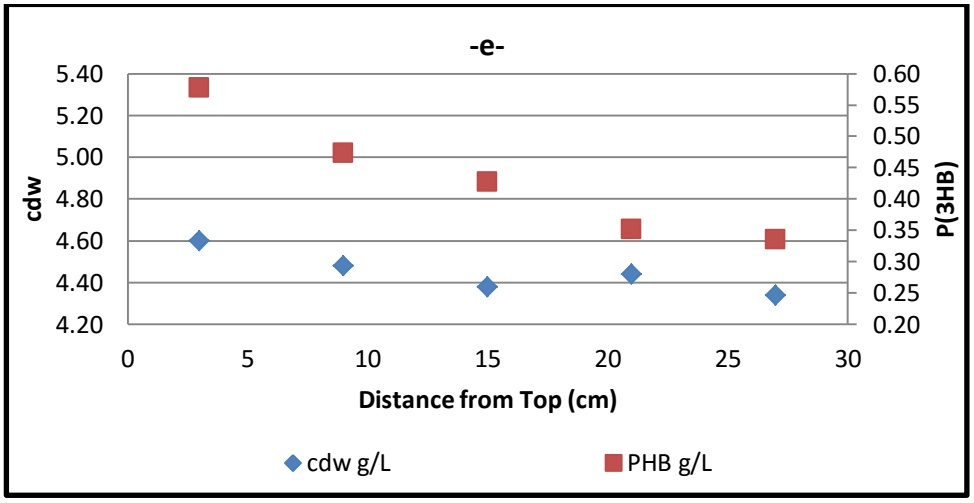




**Figure 3.77(cont.):** Change in P(3HB) concentration and cdw using taller sedimentation column, after 2 h (a), after 2.5 h (b), after 3 h (c), after 3.5 h (d) and after 4 h (e).

Figure 3.77 a shows that after 2 h the separation has already started in terms of cdw but P(3HB) contents are more or less the same. As time passes P(3HB)-rich cells move up and gradually form a gradient after 4 h, where the number of cell harbouring polymers is higher at the top of the column and lower at the bottom while the cdw values are approximately the same. However, the readings taken at different time intervals from the middle points of the column exhibited fluctuations, preventing an accurate calculation of the setting velocity.

Figure 3.78 shows the %P(3HB)/cdw measurements, where a gradual distribution of polymer-containing cells and the change in the percentage polymer by time can be seen.

However, in agreement with the observations of the previous experiment, (where the average %P(3HB)/cdw values were calculated for 2 h, 2.5 h, 3 h, 3.5 h and 4 h); %P(3HB)/cdw of 5.5; 7.29; 7.66; 8.58 and 9.69 were obtained. This, again, shows an increase in the average percentages along the column. Further discussion about this behaviour is included in Section 4.5.2.

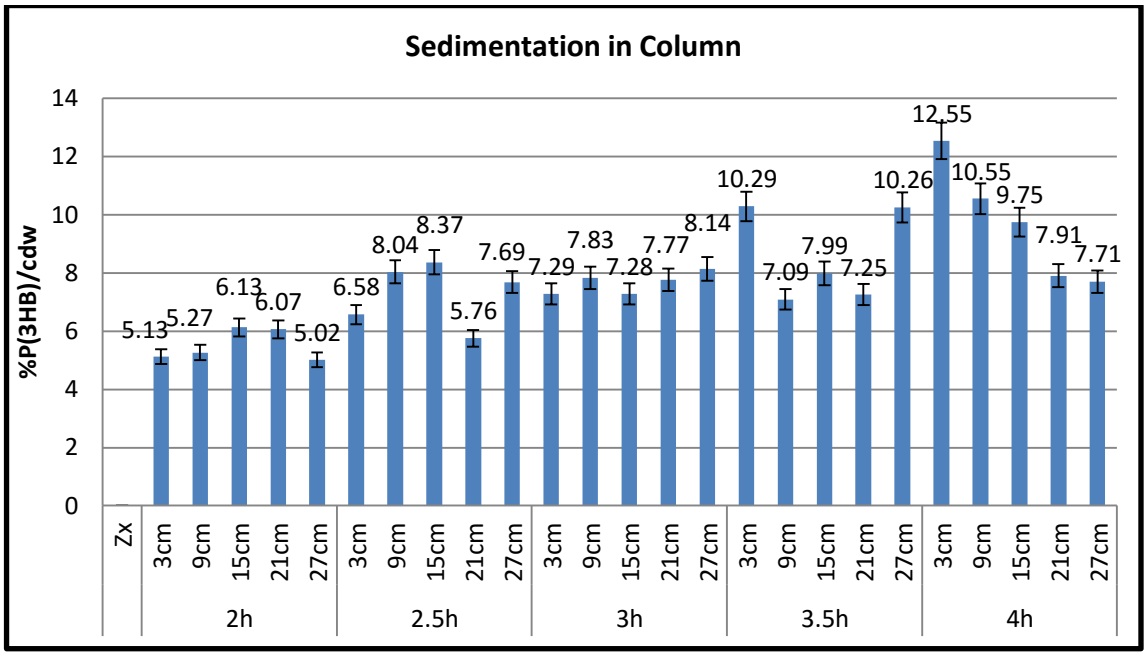



**Figure 3. 78:** Change in %P(3HB)/cdw under higher settling velocity conditions

In the set up used for the second sedimentation experiment, the height of the column was taller to allow an adequate distance between the sampling ports and for different zones to be observed clearly in order to identify better the exact separation locations and sedimentation behaviour. The volume of the column was kept the same as the beaker and the diameter of the column was halved, to prevent a large portion of the culture from being exposed to oxygen-limited conditions for at least 3 to 4 h while settling in the column. However, due to the smaller cross-sectional area of the column laminar flow conditions could not be established during circulation. Removal of samples caused further disturbance in the column, delaying the settling process. In addition small cross-sectional area accentuated the wall effect (Section 1.5.3), further delaying the sedimentation process

and the establishment of the equilibrium. In addition, samples (5 ml each) taken at close intervals decreased the total volume of the liquid considerably, creating a confusion about the liquid height readings.

Consequently a new sedimentation vessel was designed with a larger diameter (10 cm) and side sampling ports 4.5 cm apart from each other, rather than 3 cm, to prevent disturbances during sampling. The new sedimentation experiment was conducted again using a 48 h culture and the designed sedimentation tank described in Section 2.9.3. The results obtained with the larger sedimentation vessel are presented in Figure 3.79. The results obtained were similar to the beaker test (Figure 3.74). The polymer seemed to accumulate in the middle part again after 4 h.

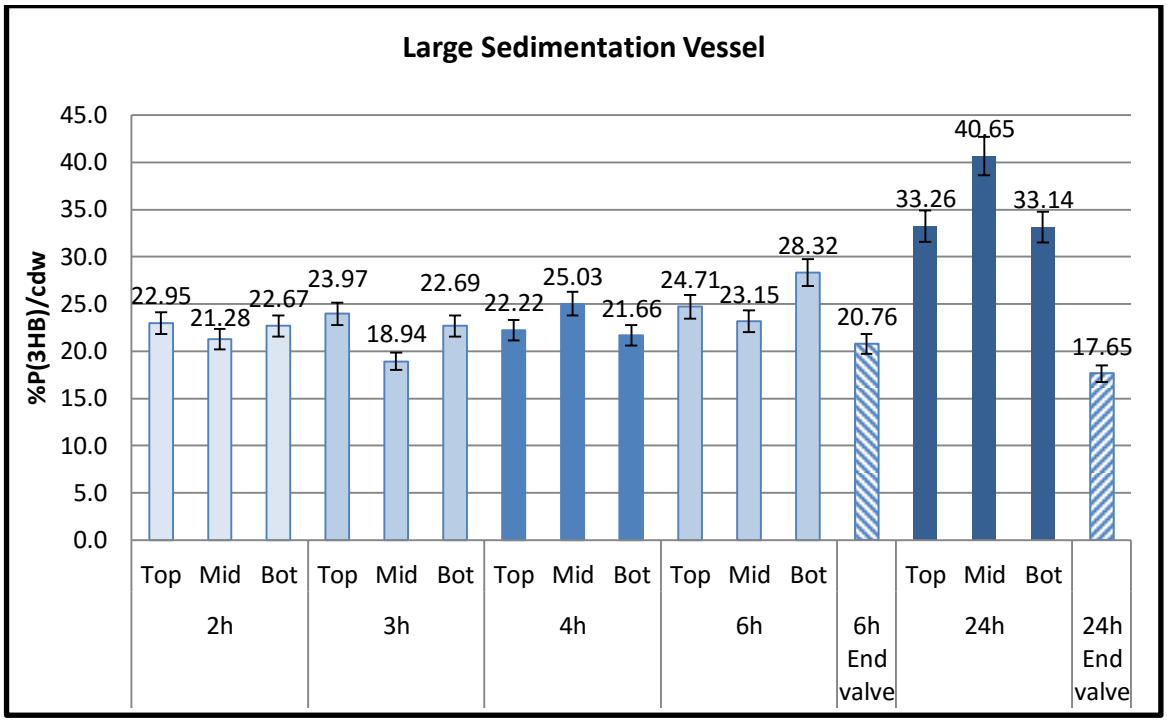



**Figure 3. 79:** Change in %P(3HB)/cdw under low settling velocity conditions

A final sample was taken from the end valve (Figure 2.5) of the sedimentation tank at 24 h. The apparent increase in %P(3HB)/cdw at all points after 24 h and the decrease of cdw throughout the remainder of the vessel was due to the accumulation of cell debris at the bottom of the tank. The sample from the "valve" contained 17 % P(3HB)/cdw with a cdw

value of 14.14 g/L. The cdw measured was the highest of all sample points (Figure 3.79). This could be better observed when the P(3HB) concentration and cdw measurements taken at various points (top, middle, bottom and valve) are plotted against time. Figure 3.80 a and b demonstrate that cells migrate towards the bottom (valve) while the concentration of P(3HB) becomes more or less equal at all other points.

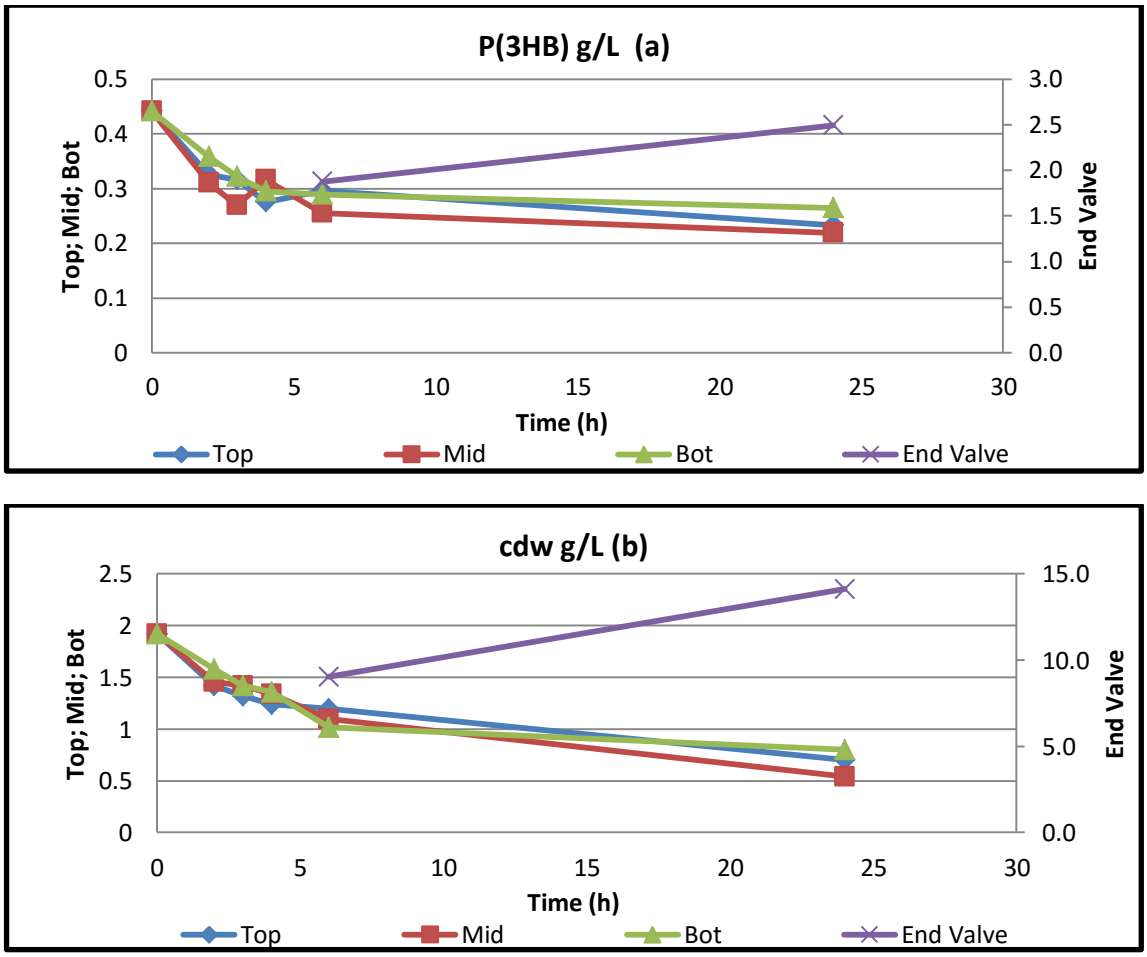



**Figure 3. 80:** Change in (a) P(3HB) concentration (g/L) and (b) cdw (g/L) at different locations of the sedimentation vessel in 24 h.

The average %P(3HB)/cdw was again observed to be increasing with time. It was found to be 22.3% P(3HB)/cdw after 2 h; 21.87% P(3HB)/cdw after 3 h; 22.97% P(3HB)/cdw after 4h; 25.39% P(3HB)/cdw after 6 h and 35.68% P(3HB)/cdw after 24 h. If the "valve" samples were included in the calculations, average %P(3HB)/cdw after 6 h sample goes

down to 24.23 and the average of 24 h sample goes down to 31.17 %P(3HB)/cdw. The accumulation of the cell debris at the end valve was a concern since the bottom part of the sedimentation vessel was thought to be the most suitable point for the recirculation into the fermenter. The apparent increase of %P(3HB)/cdw observed in all sedimentation experiments was due to the accumulation of the cellular material at the very bottom, labelled as end valve in the last experiment. The samples obtained from all levels showed a higher %P(3HB)/cdw value compared to the end valve, suggesting some cells to be floating and a higher proportion were settling.

When samples taken were centrifuged at 8000 rpm (8882 g) for 10 min and studied under microscope to compare with the un-centrifuged sample, small circular structures, resembling vesicles, were spotted (Figure 3.81). These vesicular structures remained suspended in the sedimentation vessel. It was not possible to precipitate them even by centrifugation at 8000 rpm (8882 g).

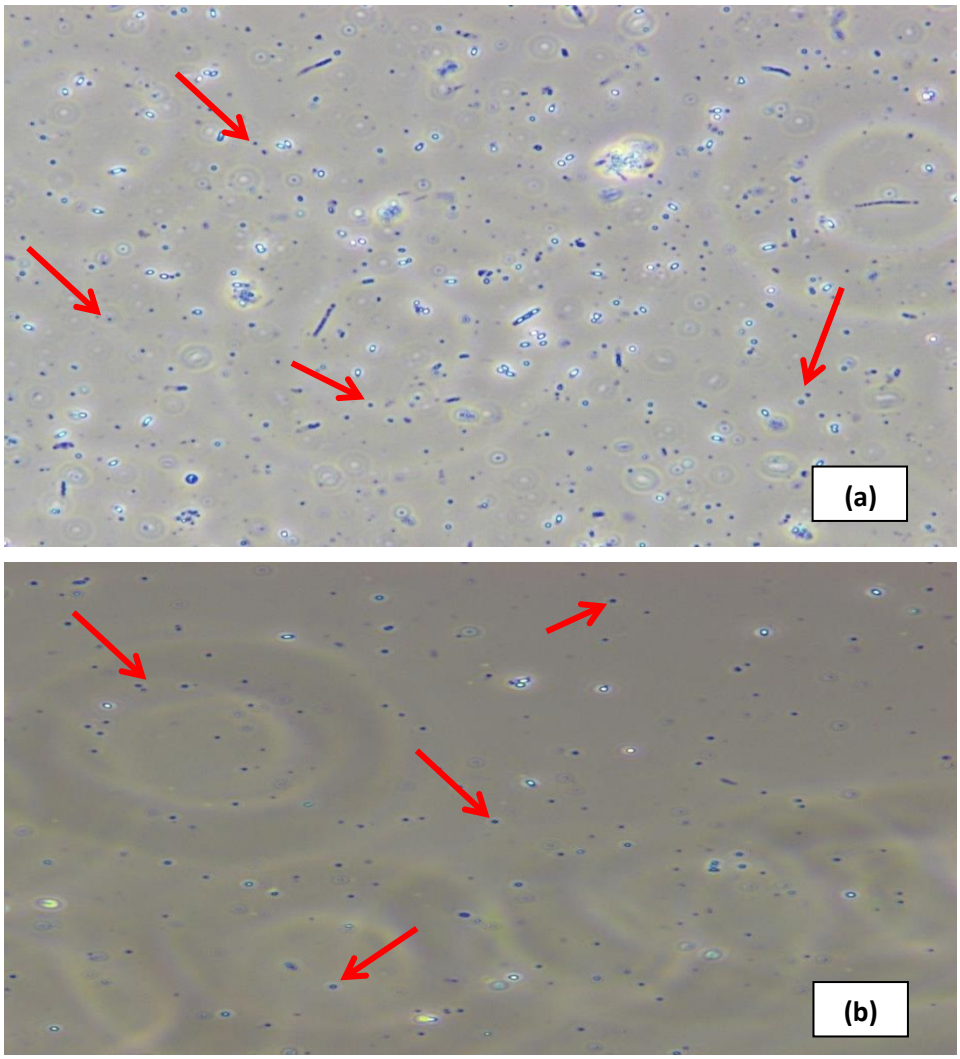



**Figure 3. 81:** Appearance of the culture before (a) and after (b) centrifugation (8000 rpm (8882 g) 10 min) (400x magnification) Red arrows indicate spherical, vesicle type structures having a different refractive index than water.

Consequently online separation of P(3HB) rich cells via sedimentation proved to be unfeasible. It was decided to further investigate of the granules observed under microscope.

### 3.7.4 Investigation of P(3HB) Granules

The vesicles observed in Section 3.7.3 were exhibiting Brownian motions. When the liquid broth containing these granules was treated with 30% NaOH and chloroform mixture, crotonic acid analysis showed that the chloroform phase contained P(3HB). This confirmed that the granules contained polymers. Lundgren *et. al.* (1964), identified these vesicles as native P(3HB) granules and reported that such granules form a stable suspension in liquid

after centrifugation and exhibit Brownian motion behaviour (Lundgren *et. al.* 1964). Based on the literature, the P(3HB) granules are spherical in shape and their sizes vary from 200 to 700 nm (Rein, 2007). In order to confirm the size variation, the size distribution of the granules observed in Section 3.7.3 samples from a 48 h culture were taken and centrifuged at 8000 rpm (8882 g) for 10 min. The obtained supernatant containing suspended granules were measured using NTA3.1 software. The results obtained are shown in Figure 3.82 and 3.83.

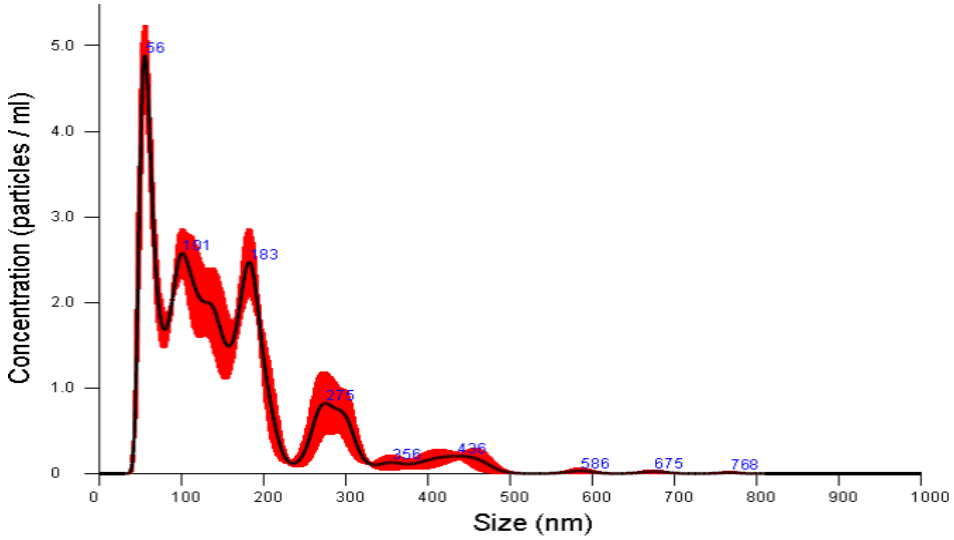



**Figure 3. 82:** Size distribution of granules observed and concentration of each size cluster in the culture supernatant after centrifugation (8000 rpm (8882 g) 10 min), 10x diluted. Merged result of three replicates.

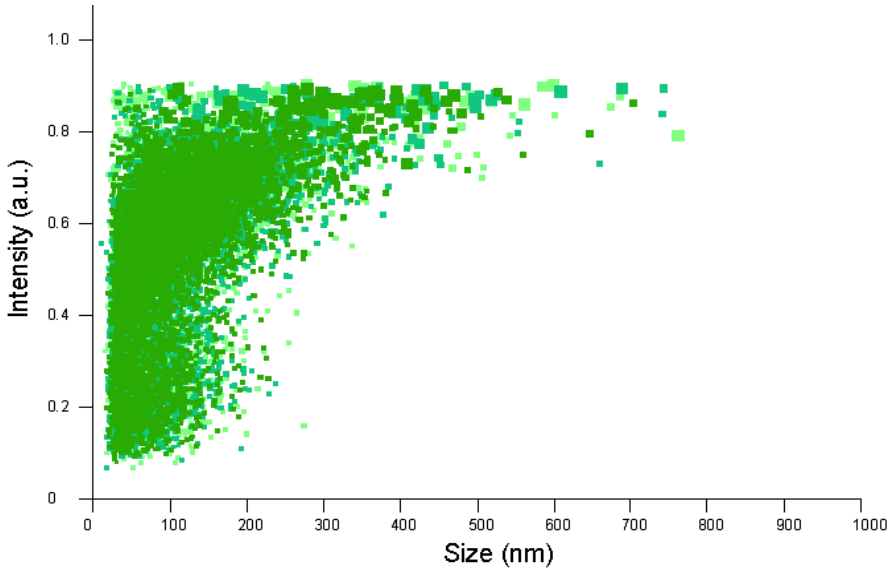



**Figure 3. 83:** Size distribution and intensity of the granules in the culture supernatant after centrifugation (8000 rpm (8882 g) 10 min), 10x diluted. Merged result of three replicates

Size distribution analysis showed that the particles sizes vary between 50 nm to 800 nm. The majority of the particles were between the ranges of 100 to 300 nm. The particle spectral intensity vs size distribution graph presented in Figure 3.83 indicated that up till 200 nm particles were present in various intensities where after 300 nm most of the particles had intensity above 0.6. This analysis suggests that there were significant number of particles present in sample with either no or a very small amount of refractive material (most likely P(3HB) in this case). Whereas, as the size gets bigger the intensity becomes stronger suggesting that the granule contains more polymer and more is compact.

Defining the spherical granules obtained from 48 h culture as native P(3HB) granules suggested that, at a certain point of their maturation, the cells disintegrate and native P(3HB) granules disperse into the environment. This suggested that the culture used for sedimentation and floatation experiments contained disintegrated cells and cell debris. This also explains the apparent increase observed in %P(3HB)/cdw in the sedimentation in Section 3.7.3. Furthermore, the fluctuations observed in sedimentation measurements could also be explained by this phenomenon. The cell debris settling to the bottom end of the sedimentation vessel explains the low P(3HB) content and high cdw measured in the "end valve" sample. In addition, studying the previous microscopic observations again (Figure 3.32c and 3.32d) confirmed that cellular disintegration took place in the fermenter, in cultures grown on DP medium, not in the sedimentation vessel.

This self-destructive behaviour of the organism was further tested under PHA medium conditions, to identify if this behaviour was organism-specific or was triggered by DP medium composition. Samples taken from *Bacillus subtilis* OK2 cultures in PHA medium were observed at different times between 24 h to 200 h under microscope (Figure 3.84).

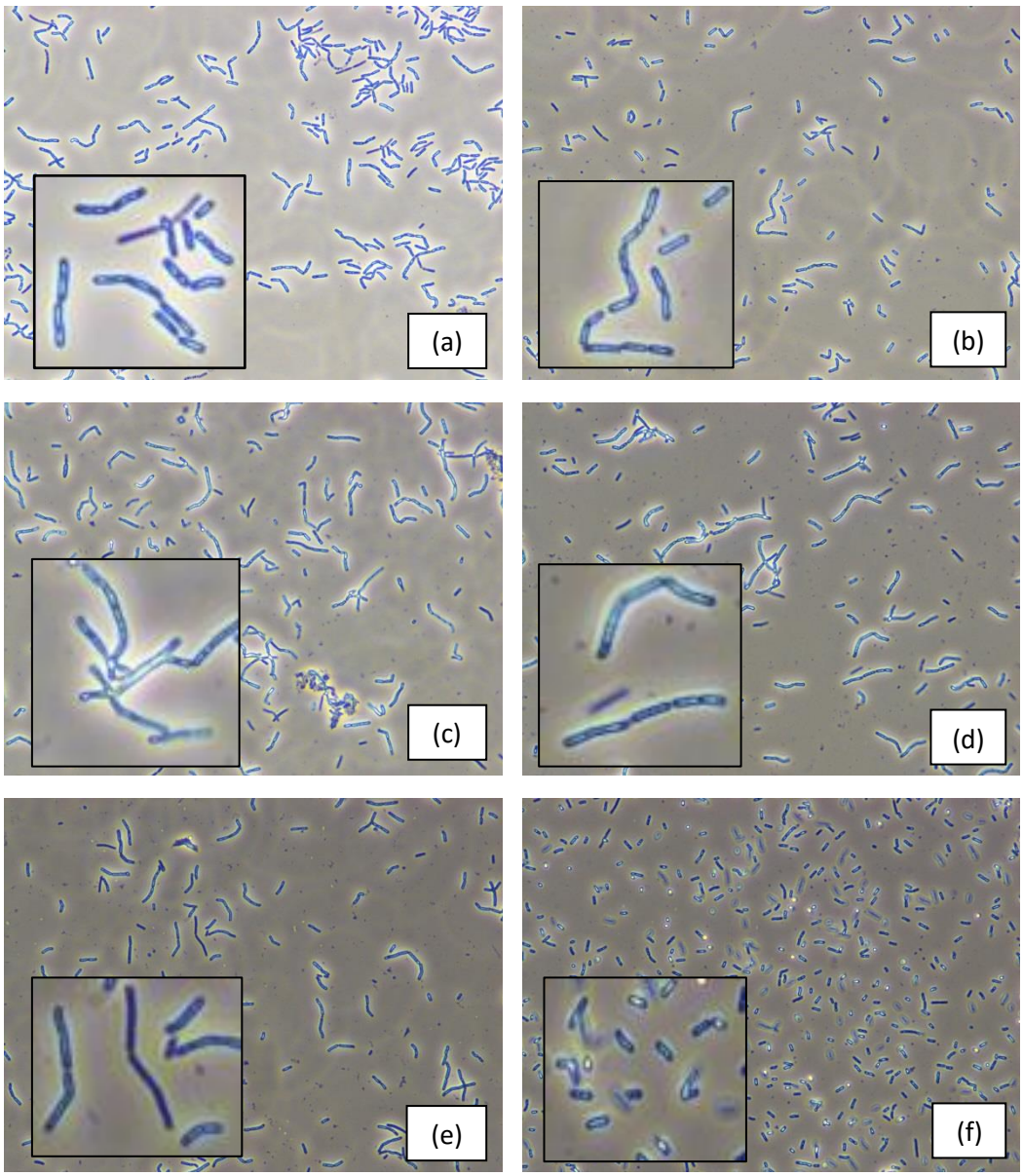



**Figure 3. 84:** Microscopic observation of growth and P(3HB) accumulation of *Bacillus subtilis* OK2 during 200 h; (a) after 24 h, (b) after 48 h, (c) after 72 h, (d) after 168 h, (e) after 180 h and (f) after 200 h. Enlarged sections can be seen under insets of each picture. 400x magnification in phase contrast mode

Microscopic investigation of cultures in PHA medium showed very robust cells even after 180 h of cultivation. The P(3HB) accumulation was microscopically visible throughout the time period and the accumulated P(3HB) was metabolised towards the end of the fermentation (Figure 3.84e) hence leading to a reduction in cell size. The cell size was

noticeably smaller after 200 h and spore formation was observed. This showed that the disintegration behaviour is triggered in the double polymer medium only. Disintegrated cells release polymer granules into the medium. This was in fact observed in the 76-h fermentation (on page 144, Figure 3.52).The release of PHA granules into the culture broth in fermentations using double polymer medium may lead to simplification of downstream processes as it increases the stability of the polymer even under unfavourable conditions.

# Chapter 4: Discussion

# 4.0 DISCUSSION

## 4.1 Production of Intracellular and Extracellular Polymers

Finding a strain capable of producing two polymers, P(3HB) and γ-PGA was one of the aims of this thesis. Five different *Bacillus* strains were selected for screening. The screening was restricted to *Bacillus* strains. This was due to two reasons. One reason was the fact that *Bacillus subtilis* strains are classified as GRAS (generally regarded as safe organisms) by Food and Drug Administration (FDA) (Singh *et al.,* 2009). They are used in many industrial applications–including production of recombinant proteins, amino acids, and fine chemicals. The second reason was that, as a gram positive organism, *Bacillus* sp. do not contain Lipopolysaccharides (LPSs). As explained in Section 1.2.3 LPSs are released into the environment after cell disruption and are usually extracted along with PHAs. LPS endotoxin is a pyrogen which can elicit a strong inflammatory response (Ray *et al.,* 2013) making the PHA polymer unsuitable for biomedical applications. Therefore, *Bacillus* sp. are regarded as a good candidates for PHAs production (Singh *et al.,* 2009).

Screening experiments demonstrated in Section 3.1.1 showed promise. For the production of γ-PGA all five strains screened gave positive results; indicated by precipitate weights ranging between 4.58 g/L to 2.72 g/L. The crude precipitate was measured using gravimetric method. This was considered as an indirect representation of γ-PGA production because some medium components were found to be precipitating as well, however, using crude precipitate of γ-PGA for quantification and comparison purposes is an accepted method, and is described in detail in literature by Kunioka and Goto (1994) and Shih and Van (2001). Further purification steps are needed to obtain pure γ-PGA.

Similarly Sudan black test was used for P(3HB) screening and two strains, namely, *Bacillus subtillis* OK2 and *Bacillus 159,* were identified to be able to produce P(3HB). Although other stains in Figure 3.1 exhibited some dark spots on the petri dish they were not considered as polymer inclusions, based on the Figures presented by Ghate *et al.*

(2011) and Flora *et al.* (2010) stating that single colonies containing P(3HB) should have a dark spot in the middle of the colony. Spiekermann *et al.* (1999) reported that during the treatment of the plates with Sudan black B, use of solvents (such as 100% ethanol) would cause some cells to burst. This could explain why the stained area is just in the middle of the colony. During the destaining procedure some cells could have died and the dye would be removed during the process.

Sudan black B dye has affinity to triglycerides and lipids as well as P(3HB). Therefore, although the location of the stained spot is discriminating, it should be carefully observed and evaluated. For example, as can be seen for *Bacillus* 20.4, the stain also dyed the area around the colonies. This indicates that there might be a triglyceride-type product secreted from the cells. In addition, strain 2d.1 shows some dark black spots randomly scattered on the colonies. These spots are not homogeneously spread in all colonies and may be some type of lipid inclusions secreted by the cells or released due to bursting of the cells due to the destaining procedure. Moreover, the intensity of the colour indicates that the dyed spots in strain 2d.1 are outside the colony. The dyed inclusions observed in *B. subtilis* OK2 and *Bacillus 159* were relatively lighter in colour compared to strain 2d.1 confirming that the dyed components were inside the cells.

In addition, some of the random spots observed in other plates could be due to inadequate de-staining procedure. The location and appearance of the stains observed in strains other than OK2 and 159, do not fit with P(3HB) staining description. Therefore, the strains, apart from *B. subtilis* OK2 and *Bacillus* 159, were kept outside the scope of this study. Further comparison and confirmation of P(3HB) production yields were conducted using the crotonic acid method described in Section 2.0. This showed *B. subtilis* OK2 to be suitable for production of both polymers.

However, further assessment of the quality and confirmation of the type of product was essential. FTIR scans conducted for both isolated polymers confirmed their structure. The

profiles obtained for the extracted P(3HB) matched the commercial P(3HB) purchased from Sigma as shown in Figure 3.3b. The FTIR scan presented in Figure 3.4 was a perfect overlap and GC-FID chromatogram obtained with the produced P(3HB) confirmed the single monomer present, as 3-hydroxybutyrate, so the polymer was identified and confirmed as P(3HB) homo-polymer.

On the other hand, FTIR scan for γ-PGA was not an identical match. The commercial γ-PGA and γ-PGA produced by *B. subtilis* OK2 showed similar bands. Ho *et al.* (2006) reported FTIR spectra for H, Na+, $Mg^{2+}$ and $NH4^{+}$ forms of the polymer. Using the reported absorption peaks, it was possible to identify the observed peaks. The spectra (Figure 3.4) showed a characteristic amide (C-N) absorption at about 1600 $cm^{-1}$ and a weaker carbonyl (C=O) absorption at around 1400 $cm^{-1}$. The strong hydroxyl absorption peaks could also be observed at around 3400 $cm^{-1}$. This was characteristic of OH stretching from the bound hydroxyl group. The small peaks in the range of 2000 $cm^{-1}$ to 2400 $cm^{-1}$ were indicative of N-H stretching. The peak observed at the lower end of the spectra at around 600 $cm^{-1}$, was identified as N-H oop (out of plane) bending. The peaks described up till this point in correlation with the commercial sample. The strong absorption peaks observed in the range from 1085 $cm^{-1}$ to 1165 $cm^{-1}$ are characteristic of C-N groups. However, although this peak was dominant in the sample produced it was not clearly visible in the commercial sample.

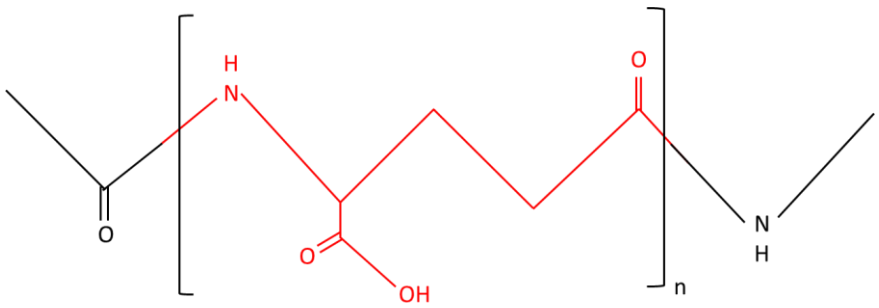



**Figure 4. 1**: γ-PGA repeating unit in relation to respective monomers connected.

Figure 4.1 shows that γ -PGA repeating unit forms C-N bonds within the molecule. Based on the absorption value for the functional groups (C-N absorption at about 1600 $cm^{-1}$, a weaker C=O absorption at around 1400 $cm^{-1}$, strong O-H absorption at around 3400 $cm^{-1}$, N-H stretching from 2000 $cm^{-1}$ to 2400 $cm^{-1)}$ reported by Ho *et al.* (2006) the extra peak characteristic to C-N bonds and other major functional group peaks were identified. FTIR spectra suggested that the polymer produced by *B. subtilis* OK2 is γ-PGA. However, the intensity of the N-H oop (out of plane) bending peak observed suggests that the molecule is likely to be a low molecular weight γ-PGA since the polymer ends with a $NH_2$ ending (Figure 4.1).

This was tested by SDS-page and results presented in Figure 3.6 confirmed that the γ-PGA produced by *B. subtilis* OK2 had a lower molecular weight compared to the γ-PGA standard obtained from Sigma (mw ≥1,000,000). Some of the discrepancy found in FTIR scans can also be due to the impurities precipitated during purification steps of the γ-PGA.

### 4.1.1 Production Profiles

When the production profile of γ-PGA in *B. subtilis* OK2 was investigated, it was observed that the product is growth-associated. As the cell density increased the γ-PGA concentration increased (Figure 3.8a). The viscosity of the culture broth did not increase to the extent described in literature. This is probably due to the low molecular weight nature of the polymer produced (Shih and Van 2001), which was already confirmed by SDS-page and FTIR scan.

In addition the level of production was much lower compared to the values reported. Up to this date, the highest reported production by *Bacillus* sp. is at the level of 101.1 g/L (Huang *et al.,* 2011). In our experiments, the highest γ-PGA production was obtained after 54 h of cultivation. However, achieving high polymer concentrations was not the aim of this project.

One of the interesting findings of this study was the levels of glutamic acid which did not change during the fermentation. Glutamic acid-dependent bacteria consume glutamic acid during the production of γ-PGA and the yield increases with an increase in L-glutamic acid concentration in the medium (Baja and Singhal 2011). However, as explained in Section 1.3.2, glutamic acid-dependent bacteria are capable of both using extracellular glutamic acid as well as synthesizing intracellular glutamic acid. As seen in Figure 1.9 there are two suggested alternative pathways of production. It is stated that some bacteria use L-glutamic acid obtained through the *de novo* pathway (Buescher and Margaritis, 2007), but the γ-PGA yields tend to be low (Kunioka & Goto, 1994, Ogunleye *et al.*, 2015). Based on this information it may be argued that *B. subtilis* OK2 may be producing some amounts of intracellular glutamic acid as well as partially consuming some from the medium. In view of the low levels of production, this may explain the slight fluctuation in glutamic acid content. However, glutamic acid requirement of the strain was also confirmed (Section 3.4.3, Figure 3.23). In the absence of glutamic acid, no γ-PGA was produced by *B. subtilis* OK2. Richard and Margaritis, (2003) presented experimental results of a batch fermentation kinetics of γ-PGA production. In their work, glutamic acid concentration changed slightly, dropped from 30 g/L to 25 g/L, and increasing after 10 h of fermentation. The increase in glutamic acid concentration was also observed in this thesis (Section 3.2.1, Figure 3.8). Their findings were in line with the reports of Kunioka and Goto (1994). They argued that the reasons for the fluctuations in glutamic acid concentration in the medium is due to the accumulation of polyglutamyl hydrolase enzymes in the fermentation broth causing partial degradation of γ-PGA and consequently the release of glutamic acid to the medium (Richard and Margaritis, 2003). This explains the decrease observed in γ-PGA levels toward the end of fermentation at 72 h (Section 3.2.1, Figure 3.8), and also for the increase observed after 53 h during γ-PGA fermentation (Section 3.2.1, Figure 3.8) in this thesis.

Another interesting point was the increase in total carbohydrate concentration. By the end of the 54 h the total carbohydrate concentration was 0.17g/L and at the end of fermentation (after 90 h) it was measured to be 0.37g/L. This indicated the production of exopolysaccharides (EPS) along with γ-PGA. Production of EPS during γ-PGA fermentation has been reported by Kunioka and Goto (1994). They demonstrated that the presence of an additional carbon source such as glucose triggers the production of EPS whereas if no additional carbon source is present apart from citric acid, EPS production will not be triggered. On the other hand, Du *et al.* (2005) reported that replacing glucose with glycerol as a carbon source resulted in no EPS formation in the cultures of *Bacillus licheniformis*. Kunioka and Goto (1994) used *Bacillus subtilis* IFO3335 stain, which is one of the main strains producing γ-PGA, isolated from traditional Japanese food, natto (fermented Japanese soybeans). They reported that glutamic acid was not consumed during the fermentation. The strain used in this thesis (*B. subtilis* OK2) is a native γ-PGA producer from a natto (fermented Japanese soybeans) source and was genetically engineered to produce P(3HB). This may explain the γ-PGA production profile observed in this study.

A number of researchers (Baja and Singhal 2011; Huang *et al.*, 2011; Wang *et al.*, 2008) have reported a rise in culture pH along with γ-PGA production. This increase has been suggested to be in response to ammonification due to degradation of organic nitrogenous compounds such as amino acids (Wang *et al.*, 2008). However, the pH profile obtained in this study (Figure3.8b) did not follow this trend. The culture pH, which was adjusted to pH 6.8 at the beginning of the fermentation, stayed approximately constant for the first 30 h and started to drop after this period. By the end of 90 h it dropped to pH 5.8. The γ-PGA production medium contains glutamic acid and ammonium sulphate as nitrogen sources. In the fermentation profile presented (Figure3.8a) glutamic acid was found not to be consumed, so no organic nitrogen (glutamic acid in this case) went through

ammonification resulting in pH increase. On the other hand, the decrease observed in pH after 30 h can be explained by the change in $NH_4^+$ concentration. In an aqueous solution, pH increases as $NH_4^+$ concentration is increased, and when $NH_4^+$ concentration decreases, a decrease in pH is observed (Serezli and Tabak, 2013). Therefore, the decrease in pH can be explained by the consumption of $NH_4^+$ which was added to the medium in the form of ammonium sulphate.

The P(3HB) production profile obtained in this study is presented in Figure 3.9. As expected, the profile confirmed that P(3HB) production is non-growth associated. The production starts at the end of the exponential phase (18 h) and slowly continues throughout the course of the fermentation. At the end of 70 h, 1.02 g of P(3HB) was produced which corresponded to 45%PHB/cdw from glucose. This value is well in agreement with literature. Wu *et al.* (2001) produced 35%PHB/cdw using molasses as carbon source from *Bacillus JMa5* strain. Similarly, Shamala *et al.* (2003) reported 32.1% P(3HB)/cdw using sucrose as carbon source from *Bacillus brevis*. Borah and co-workers (2002) reported 69.4%P(3HB)/cdw from *Bacillus mycotes* culture using sucrose as carbon source. Tajima *et al.* (2003) stated 32.9%PHB/cdw from *Bacillus* sp. INT005 culture using glucose as carbon source. Total carbohydrate concentration profile followed a decreasing pattern until the end of 20 h and showed a sudden increase followed by a slow decrease. Only half of the carbon source was utilised by the end of 72 h. The changes in total carbohydrate concentration could be explained by the production of EPS. Lee and co-workers (1997) studied production of EPS from *Bacillus polymyxa*. They also observed that the carbon source used was not consumed totally and remained constant at 10g/L after 35 h in a 48 h fermentation. In addition they reported that EPS production is growth-associated and was slowed down/stopped in stationary phase (Lee *at al.*, 1997). Wang and Yu (2007) investigated the effect of glucose and $(NH_4)_2SO_4$ in batch cultures of *Ralstonia eutrophia*. Production of EPS occurred along with cell growth, whereas P(3HB) was

produced only under nitrogen-limited and cell growth-limited conditions. They stated that glucose was completely utilised by the end of 48 h fermentation. However, the analytical method for the measurement of glucose used in their study was glucose-specific whereas in this thesis, total carbohydrate analysis was conducted. In Figure 3.9 total carbohydrate concentration shows a sharp decline between 0 to 20 h. A rapid increase was observed after 23 h which corresponded to the end of exponential and beginning of the stationary phases. Since EPS production is growth-associated, it slows down or stops during the stationary phase. As the total carbohydrate assay was used, the estimation of EPS production in the first 20 h of fermentation may have been disguised by the rapid consumption of glucose in the medium. When the glucose consumption rate decreased at the beginning of the stationary phase, the last stage of EPS production may have become detectable. This was reflected in an increase in the detected total carbohydrate concentration. Since EPS production stops after the culture enters the stationary phase, the slow decrease in total carbohydrate concentration reflected carbohydrate consumption for P(3HB) production. In short, the total carbohydrate curve presented in the Figure 3.9 is possibly a combined curve of EPS production and carbon consumption, explaining the remaining unutilised carbohydrate after 72 h.

### 4.1.2 Effects of medium components

Determining the composition of the medium is crucial in optimisation studies of bioprocesses. An ideal medium needs to supply sufficient nutrients to microbe under study to enable production of the desired product. However, the medium components need to consist of only the essentials to make it as economic as possible. Statistical screening tool Placket-Burman (PB) in the software package Design Expert 6.0 is one of the many statistical tools available to help screening the essential medium ingredients for a target product. PB screening results shown in Section 3.3.1 for both γ-PGA and P(3HB) production, have identified the significant components for production. According to γ-PGA

results (Figure 3.10) glutamate, citric acid and ammonium sulphate have positive effects while manganese sulphate, sodium phosphate di-basic, manganese sulphate and calcium chloride have negative effects on γ-PGA production. Up to this date, all the studies conducted with different *Bacillus* sp. using statistical screening methods have reported glutamate or glutamic acid as a significant necessary component (Soliman *et al.*, 2005; Xiong *et al.*, 2005; Kunioka and Goto, 1994; Jian *et al.*, 2005; *Du et al.*, 2005; Yao *et al.*, 2010; Jeong *et al.*, 2010; Shih *et al.*, 2002). However, among the three positively affective components, glutamic acid was the least important. Although in the previous batch fermentation presented in Section 3.2.1, glutamic acid was found not to be utilised by the organism, PB screening results clearly demonstrated that it has a positive effect. The role of glutamic acid is further discussed in detail later in Section 4.2.1.

The second most important ingredient was found to be citric acid. Several researchers claim that the main carbon flux towards γ-PGA formation is from citric acid (Shih *et al*, 2002; Soliman *et al.*, 2005; Kunioka and Goto, 1994; Yong *et al.*, 2011). Yong *et al.*, (2011) demonstrated that supplementing citric acid to the medium increased the production of γ-PGA to 1.17% w/w, which was the highest titre reported in their experiments where they tested other single components using cultures of *Bacillus amyloliquefaciens* C1. Gotto and Kunioka (1992) reported that citric acid was the best additional carbon source for production. Shih *et al.*, (2002) identified citric acid as one of the most important parameters along with glutamic acid and glycerol, and used citric acid in further optimisation of the production. On the other hand, Xu and co-workers (2005) reported that the strain *Bacillus subtilis* NX-2 could not produce any γ-PGA using citric acid as carbon source.

PB screening results identified ammonium sulphate as the most important medium component. Effect of ammonium sulphate or ammonia in general was highlighted by various researchers (Soliman *et al.*, 2005; Kunioka and Goto, 1994; Jian *et al.*, 2005; Gotto

and Kunioka, 1992). Amongst other variables reported in literature, $MgSO_4$ was found to have a negative effect on production, whereas glycerol and $K_2HPO_4$ were found to be positively affecting the production. In the PB screening conducted in this thesis, although $MgSO_4$ had a negative effect, the effects of glycerol and $K_2HPO_4$ were found to be negligible. Therefore the findings were partially contrary to the results obtained in literature (Shih *et al.,* 2002; Du *et al.,* 2005; Jeong *et al.,* 2010 Xiong *et al.,* 2005; Soliman *et al.,* 2005). This could be due to the special characteristics of the stain *Bacillus subtilis* OK2, which is a genetically engineered strain.

Parallel to the overall results obtained here, Jian and co-workers (2005) identified citric acid, glutamate and $NH_4NO_3$ as the most important variables for γ-PGA production and used those for further optimisation. Kunioka and Goto (1994) investigated γ-PGA production and reported that using citric acid, glutamate, and ammonium sulphate resulted in high γ-PGA production with no by-product production, using *Bacillus subtilis* IFO3335.

A similar experiment was conducted for selecting the significant medium components for P(3HB) production. PB analysis in this thesis showed that glucose and yeast extract have positive effects whereas potassium chloride, ammonium sulphate and Soytone (enzymatic digest of soybean meal) have negative effects on P(3HB) production (Figure 3.11). This result is also in agreement with results reported in literature (Akaraonye *et al.,* 2010, Valappil *et al.,* 2008). It is well known that PHAs are produced under unbalanced culture medium conditions (Akaraonye *et al.,* 2010) such as high carbon and low nitrogen or phosphate concentrations. In this study P(3HB) was produced under conditions of limited nitrogen and excess carbon source. PB design showed that nitrogen and carbon are definitely essential for PHA production by *B. subtilis* OK2. Soytone, is a commercial enzymatic digest of soybean meal and provides an additional nitrogen source. Addition of soytone (adding more nitrogen) into the medium disturbs the C:N balance hence negatively affects the production of P(3HB). Yeast extract is a rich, complex nutrient and is more

favourable for cell growth due to its trace elements and its growth stimulating effect is well established (Ameyama and Matsushita, 1984; Jongejan and Duine, 2012). When the biomass concentration is increased in the culture it provides the opportunity for enhanced P(3HB) production. This was demonstrated in the work of Zafar *et al.*, (2012). When different nitrogen sources were tested using glucose as carbon source, the highest cdw and P(3HB) content was obtained using urea (another complex nitrogen source) followed by simpler nitrogen sources, $(NH_4)_2SO_4$, $NH_4Cl$ and $NH_4NO_3$ in the order of importance.

These pronounced effects of carbon and complex nitrogen sources were further demonstrated in this study by investigating the effects of each component and their combined effects. The results presented in Figure 3.12 confirm the effect of yeast extract as an important component for cell production. The combined effect also gave the best results for P(3HB) production, indicating that the effect of complex nitrogen source is not just limited to increasing cdw. Page (1992) conducted a study on the effects of complex nitrogen sources in media containing sugars using *Azotobacter vinelandii*. His results are in parallel with the findings of this thesis. They reported that increased yields were observed in the media containing pure or unrefined sugars with the addition of complex nitrogen sources.

The effects of the addition of complex nitrogen sources at different times of cultivation and in different amounts were investigated in this study (Section 3.3.2). Addition of 0.625 mg (0.125%) yeast extract after 48 h of cultivation increased the P(3HB) content by 33%. The addition of 0.05% to 0.2% complex nitrogen source (either peptone no3 or yeast extract) resulted in up to 25-fold increase in P(3HB) content. The possible reason for the effect of the addition of yeast extract observed in this thesis after 48 h of cultivation may simply be due to the phase the culture was in. After 24 h, the culture was either at the end of the exponential phase or at the very beginning of the stationary phase and if extra nitrogen source is introduced into the medium, the metabolism is switched towards biomass

production, leading to a small decrease in %P(3HB)/cdw as the amount of nitrogen added increases. After 48 h the culture was definitely at mid or late stationary phase, so the addition of the complex nitrogen source mimics the first starvation stages therefore forces the organism to continue accumulating the polymer.

Lee and Chang (1994) investigated the effects of complex nitrogen source on the production of P(3HB) by recombinant *E.coli*. They reported that P(3HB) production was enhanced when the defined medium was supplemented with various complex nitrogen sources. In their study, nitrogen was supplemented during the active P(3HB) formation phase (after 12-24 h). They concluded that yeast extract as well as fish peptone and peptone no3 increased the yield and organic nitrogenous compounds were responsible for the increase in P(3HB) yield (Lee and Chang, 1994). These findings support the data presented in this study.

Yeast Extract is the water-soluble portion of autolyzed yeast. It is prepared and standardized for bacteriological use and cell cultures through a carefully controlled autolysis induced by heating to around 50°C, to preserve naturally occurring vitamin B complexes, which are claimed to be responsible for its growth-inducing characteristics (Li *et al.,* 2011; Chan-u-tit *et al.*, 2013).

The composition of yeast extract has been studied by early researchers who have reported it as a stimulator of bacterial growth (Grant and Pramer, 1962; Chen *et al.*, 2007; Li, 2007; Li *et al.*, 2011). Based on the literature data the typical composition of yeast extract differs according to the manufacturers, but an average composition can be stated as 8 to 12 % total nitrogen corresponding to a protein content of 50 to 75 %, and amino nitrogen content of 3.0 to 5.2 % expressed on dry matter basis. The total nitrogen content (10.26 %) determined on this thesis falls within this specified range. Yeast Extract also consists of 11-17 % (w/w) ash (Oliveira and Oliva Neto, 2011; Chan-u-tit *et al*., 2013). The ash contains several trace elements such as; Al, Ba, Cd, Co, Cr, Fe, Ga, Mg, Mn, Mo, Ni, Pb,

Cu, Sn, Sr, Ti, V and Zn. Amongst these trace elements, the ones that have the highest percentage are; Mg, Fe, Zn, Cu, and V (Grant and Pramer, 1962).

As discussed in Section 1.2.2, PHA is also produced via alternative pathways. One of those pathways starts from Acetyl-CoA just before the TCA cycle. Acetyl-CoA, as the last intermediate compound before TCA cycle, acts as a junction for PHA accumulation or growth. The first enzyme in TCA cycle, (citrate synthase), has two natural inhibitors or regulators; NADH and citrate. Citrate synthase has a significant effect on PHA production (Anderson and Dawes 1990, Kessler and Witholt 2001). Kessler and Witholt (2001) reported that high concentration of NADH and NADPH inhibits citrate synthase activity, enhancing P(3HB) production. This was discussed in more detail in Section 1.2.2. The results reported in Section 3.3.2, demonstrate the effect of citrate, which is another citrate synthase inhibitor, on P(3HB) production by *B. subtilis* OK2. According to the results of this thesis, citrate has a positive effect on production. This result is in agreement with literature data (Kessler and Witholt, 2001, Anderson and Dawes, 1990, Aldor and Keasling, 2003) where citrate addition into the medium was shown to promote PHA production by blocking the citric acid cycle by directing the acetyl-CoA generated from glucose, towards the PHA production pathway. However, addition of citrate in excess blocks the citric acid cycle completely and stops cellular growth (Kessler and Witholt, 2001, Anderson and Dawes, 1990, Aldor and Keasling, 2003). Figure 3.14, shows that increased concentration of citric acid increased the amount of P(3HB) produced up to a point, further addition of citrate inhibited the growth as mentioned in Section 3.3.2. The possibility of blocking citrate synthase enzyme using citrate has been discussed in literature, but its effects on production of P(3HB) and cell growth has not been demonstrated as clearly as in this study.

The important parameters for the dual production of the two polymers, γ-PGA and P(3HB), were also investigated in this study. The significant parameters obtained from

previous PB designs were tested by another PB design. Differing from single product production, in dual production, glutamic acid was found to be significant for P(3HB) production along with glucose and yeast extract. Citric acid was replaced with yeast extract (Figure 3.15 and 3.16) as a positive medium component for γ-PGA production. Contrary to results obtained from single-product runs, citric acid was found to be negatively affecting the production of both polymers. This could be due to the concentration ranges tested. Since the critical concentrations of the selected components were different for each polymer, it was difficult to determine a single concentration range for the dual polymer PB design. For γ-PGA production, citric acid levels tested were 10 times higher than the levels effective for P(3HB) production. Due to the inhibitory effect of citric acid on growth in the presence of glucose, the levels could not be increased further. As expected, glucose was found to be negatively affecting γ-PGA production in dual production medium.

The positive effect of yeast extract on γ-PGA can be explained by the complex nature of the nutrients which yeast extract contains, as stated previously (page 200) (Li *et al.*, 2011; Chan-u-tit *et al*., 2013).

Although the main reason for the selection of yeast extract was due to its nitrogen content, the other trace elements and nutrients such as vitamin B complexes in yeast extract had probably positive influence on production of P(3HB) and this was discussed earlier (please note comments on pages 199 and 200 of this section).

The effect of yeast extract on growth of bacterial as well as insect cell cultures has been reported too (Ameyama and Matsushita, 1984; Eriksson and Häggström, 2005; Jongejan and Duine, 2012).

Within our knowledge, dual polymer production has not been studied for γ-PGA and P(3HB) together, and in this detail before. The only PB design study for dual production with a polymer and a secondary product was conducted by Liu *et al.* (2004) for the simultaneous production of nisin and lactic acid using cheese whey as a substrate.

Production was optimized using statistically based experimental design, in a whey-based medium using *Lactococcus lactis*. Significant parameters were screened using a PB design, however individual products and their requirements were not discussed since it was not the scope of their study. Comparison of single polymer production results with dual polymer production profiles is first reported in this thesis.

The discrepancy between the results obtained with the dual and single polymer production systems is possibly due to the complexity of dual production mechanism. A system forcing the organism to produce two different products which are competing for the nutrients available has to be designed carefully to obtain balanced results. Consideration of the requirements for the simultaneous production of both polymers while obtaining sufficient growth to provide enough biocatalyst (the cell which is also the product since P(3HB) is intracellular) is critical. Within this complex system of numerous interdependent variables, the statistical tool which was developed based on some simplifications and assumptions prove to be insufficient yielding some misleading results.

### 4.2 Efficiency of CCD for Optimisation of Dual Production

Central composite design (CCD) is an experimental design, used in response surface methodology. It is used for forming a quadratic model without requiring the use of a full-factorial design. It uses coded values and performs linear regression and iterative methods to obtain results (Anderson and Whitcomb, 2007). In this study, the parameters obtained from the screening study for dual polymer production were used in a CCD experiment to optimise the dual production. Four medium components, glucose, yeast extract, ammonium sulphate and glutamic acid, were tested in five levels. The results obtained for each polymer individually in double polymer production medium are given in Section 3.4.1. ANOVA results for γ-PGA (Table 3.4) showed the model generated for production was significant ($P<0.05$), with an $R^2$ value of 0.83. However, two variables (glucose and yeast extract) out of the four variables gave insignificant p-values. As discussed previously in

Section 4.1, glucose and yeast extract were found to be effective variables for P(3HB) production. However, although yeast extract was found to be effective for both polymers, CCD results showed the opposite. ANOVA table for P(3HB) production (Table 3.5) showed the model generated using the data did not fit the data itself in a satisfactory manner. The p-value obtained was greater than 0.2 which indicated that the confidence level is less than 80%. In addition the $R^2$ value was calculated as 0.63. Similar to γ-PGA ANOVA table, only two out of four variables were found to be significant ($p<0.05$) for P(3HB) production. These were yeast extract (as expected) and glutamic acid. The reason why glutamic acid was found to be significant is not clear, however since the model itself was not significant, assessing variables individually was pointless. This contradiction between two polymer productions can also be seen in 3D surfaces generated by the design expert software (Figure 3.17 and 3.18). For γ-PGA, the surfaces are seen to be converging to an optimum, indicated by a plateau region. A negative point was seen in the graphs obtained with P(3HB) production data, as if the production was optimised in a negative manner. In addition, the trends observed in the graphs were not suited for interpretation. This is due to the complexity of the system. From the P(3HB) production point of view, changing glucose and yeast extract concentrations caused different C:N ratios for each experimental run. Changes in ammonium sulphate and glutamic acid concentrations, also contributed to nitrogen levels in the medium. These possibly have added more complexity to the system leading to a situation which is impossible to be explained and interpreted with a simple quadratic model.

Wang and Yu (2007) investigated the dual production of P(3HB) and EPS in batch cultures of *Ralstonia eutrophia* using response surface methodology. From the response surface point of view the difference between their study and the response surface evaluated in this thesis is the number of parameters investigated. Although they managed to get a significant correlation, only two parameters were involved in their model. In the results

presented in Section 3.4, two out of four parameters were shown to be significant for both polymers. This highlights a different aspect of optimisation for dual polymer production.

In the dual production system, four parameters were identified as crucial for the respective polymer productions in PB design. This may be too many for the system to recognise the significant effects and evaluate these effects using a simplified approach to fit a quadratic model. Such a thorough evaluation of the dual production system has not been reported in literature before, not as an experimental observation nor as CCD modelling.

Using the data obtained and evaluating the negative results of CCD optimisation, further simplification of the process variables was carried out. Based on the previous experiments and literature data (Egli and Zinn 2003; Zinn *et al.,* 2004; Akaraonye *et al.,* 2010), C:N ratio is known to have a crucial importance in P(3HB) production and was therefore taken to be an important parameter for dual production. The results of the experiments carried out to fix the C:N ratio were presented in Section 3.4.2. The parameters used for PB screening experiments were revised based on the results obtained from the individual polymer production experiments. The five key medium components which were highlighted as important namely, glucose and yeast extract for P(3HB); glutamic acid, ammonium sulphate and citric acid for γ-PGA, were all included and evaluated together in simplified experiments. Yeast extract was selected as a variable due to its complex nature as demonstrated in Section 3.3.2 and discussed in Section 4.1. The effect of yeast extract concentration was tested by keeping all four medium components at their original levels used for PB design and varying the yeast extract level. As shown in Figure 3.19, P(3HB) concentration as well as cdw increased with increasing yeast extract concentration. This could also be partially observed in CCD presented in Figure 3.18. Although the model, overall, was not significant, yeast extract itself was calculated as significant for the model generated and the 3D graph presented showed an increase with increasing yeast extract concentration under the conditions tested. An interesting finding was the effect of yeast

extract on γ-PGA production. It can be seen that yeast extract addition had a positive effect on the production of γ-PGA in double polymer medium. Evaluating the effect of a single nutrient composition prior to statistical optimisation strategy has also been applied by Jain *et al*., (2005) for the production of γ-PGA. In their study, the effects of the addition of different nitrogen sources on γ-PGA production were presented. Yeast extract, as one of the nitrogen sources tested, showed a positive effect on production, however inorganic sources were found to be more efficient. Peng and co-workers (2015) have also demonstrated the positive effect of yeast extract on γ-PGA production. Contrary to the study of Jain *et al.* (2005), Peng *et al.,* (2015) reported that organic nitrogen sources such as yeast extract or peptone enhance the production of γ-PGA in the cultures of *Bacillus methylotrophicus* more than inorganic nitrogen sources.

The results of yeast extract on P(3HB) production and its coupled effects with glucose were demonstrated in Section 3.3.2 and discussed in Section 4.1. Glucose together with yeast extract are important in P(3HB) metabolism and they are the two key medium components. Therefore, the possibility to obtain a significant quadratic model with statistical optimisation methodology by setting the levels of glucose and yeast extract at a constant value in the medium was tested in order to evaluate the effects of other medium components on P(3HB) production. Hence, glucose and yeast extract concentrations were fixed, based on the experiments carried out and reported in Section 3.4.2, and a further optimisation using CCD with a reduced number of variables was carried out.

One of the major drawbacks of the response surface models is that the area under investigation can only be approximated by a polynomial model. This is intentionally set in such way to allow keeping the experiments simple, focused, and affordable (Anderson and Whitcomb, 2005). However, biological systems do not necessarily fit to quadratic models and the approximation becomes a restriction.

In the results reported in Section 3.4.2, the simplified parameters, citric acid, glutamic acid and ammonium sulphate, were tested at five levels. The design matrix given on Table 3.6 summarises the design surface. In the following sub-sections (4.2.1 and 4.2.2), the effects of these parameters on γ-PGA and P(3HB) production are discussed respectively.

### 4.2.1 γ-PGA production

ANOVA table generated for γ-PGA production, using the data obtained, was presented in Table 3.7. As can be observed, the model was significant with a p-value less than 0.001. The significant parameters, which were glutamic acid and ammonium sulphate, were the same as the ones obtained from PB screening test for dual polymer production. The $R^2$ value obtained from the model was 0.89, which highlighted the accuracy of the model generated. The model generated, presented as equation 3.1, shows the influence of each parameter on the end production. However, the production metabolism is much more complex than to be explained as a simple second degree polynomial. Thus, it is possible to define the role of each component and its relevance with respect to other components through a linear correlation.

In the model (equation 3.1, page 112) parameter A for γ-PGA production, is citric acid and it has the smallest coefficient (+0.019) indicating that the effect of citric acid on γ-PGA production is relatively small but yet positive (since the coefficient is positive). This is an expected result since the effect of citric acid on γ-PGA was shown to be positive on single polymer PB screening experiment (Figure 3.10). Studies conducted on the metabolism of γ-PGA production suggest that citric acid can indirectly contribute to the production. As discussed earlier in Section 4.1, the main carbon flux towards γ-PGA formation is claimed to be from citric acid (Shih *et al,* 2002; Soliman *et al.,* 2005; Kunioka and Goto, 1994). However, due to citric acid's inhibitory effect on the P(3HB) production pathway, the levels of citric acid screened were limited to the range between 0-3g/L for the dual production CCD. The low level of citric acid present in the medium might be the reason

for the small coefficient of citric acid in the model and its high p-value. In the various γ-PGA production media reported in literature, citric acid level varies between 10-20 g/L. It serves as a carbon source in the medium along with a supplementary source such as glycerol. Consequently, a low level of citric acid was selected to promote P(3HB) production, acknowledging the fact that it will lower the γ-PGA levels produced. Strategically P(3HB) is a higher value product compared to γ-PGA, hence improving its yield will be more beneficial to overall process costs.

The parameter B for γ-PGA production was glutamic acid. It showed a higher positive coefficient (+1.311) compared to variable A. This was an expected result since glutamic acid is essential for production of γ-PGA in glutamic acid dependent bacteria. The high coefficient of glutamic acid indicated its importance, compared to citric acid. Previous studies conducted for γ-PGA production have also demonstrated the importance of glutamic acid (Jian *et al.,* 2005; *Du et al.*, 2005; Yao *et al.,* 2010; Jeong *et al.,* 2010; Shih *et al,* 2002). Shi et al., (2006) studied the effects of medium components and optimised the γ-PGA production with *Bacillus subtilis* ZJU-7 using response surface methodology. In the quadratic model they obtained, the coefficient of glutamic acid was positive, but the lowest of all screened. They also stated that the inclusion of glutamic acid in the design was as precursor for γ-PGA production. Since the glutamic acid was found not to be "consumed" in previous experiments, the small but positive effect of glutamic acid obtained in CCD can be explained as a result of this inducer effect.

The final parameter for γ-PGA production was ammonium sulphate, which was parameter C. The coefficient of parameter C was calculated as +1.739. It was the highest of all the three showing the greatest effect. In the work of Soliman *et al.*, (2005), ammonium was reported as having the second highest positive coefficient of all the variables screened with *Bacillus* sp. SAB-26. Ammonium was also reported in their work as the best nitrogen source for the production of γ-PGA. In the work of Jian *et al.,* (2005), ammonium nitrate

was found to be a good source of nitrogen and was included in the CCD along with glutamic acid and citric acid. The model calculated resulted in a significant positive coefficient. When the 3D graphs obtained from the model generated are studied, the effects can be seen very clearly (Figure 3.20). One of the most interesting findings of the 3D graphs was the interaction revealed between glutamic acid and ammonium sulphate. Ammonium sulphate concentration had almost no effect on production at the low levels of glutamic acid, but the effect changed when glutamic acid concentration was increased (Figure 3.20). At high levels of glutamic acid, increasing concentrations of ammonium sulphate showed positive effects on γ-PGA. In other words, at low levels of ammonium sulphate, glutamic acid had no or negative effect on γ-PGA production, while at high levels of ammonium sulphate increasing concentrations of glutamic acid had a positive effect on production of γ-PGA. Another yet less significant interaction was also observed between ammonium sulphate and citric acid. At low levels of ammonium sulphate, increasing concentrations of citric acid had a positive effect on production, however this effect turned into negative when the ammonium sulphate concentration was high.

These interactions can be explained based on the metabolic pathway of the production (Figure 1.9). As explained in Section 1.3.2, γ-PGA is mainly produced from citric acid and ammonium sulphate. It is proposed that L-glutamic acid is produced from citric acid *via* iso-citric acid and α-ketoglutaric acid in the TCA cycle and ammonium sulphate contributes to the production of intracellular L-glutamine acid production *via* glutamine synthase enzyme (Shih and Van 2001). In order to induce the glutamine synthase, extracellular glutamic acid is essential. Thus, the interaction between ammonium sulphate and glutamic acid may be explained through the glutamine synthase path. If glutamic acid is not present the path is not activated, thus addition of ammonium sulphate would not affect γ-PGA production but when glutamic acid is present, the pathway is active and further addition of ammonium sulphate to the medium increases the γ-PGA production.

Furthermore, the interaction between ammonium sulphate and citric acid can be explained via α-ketoglutaric acid contribution to γ-PGA production. α-ketoglutaric acid is derived from citric acid in the medium. At high levels of ammonium, α-ketoglutaric acid is not used for production, but at low levels of ammonia, glutamine synthase path does not work so α-ketoglutaric acid is directed to produce γ-PGA (Figure 4.2).

### 4.2.2 P(3HB) production

ANOVA table generated for P(3HB) production, based on the data obtained, is presented in Table 3.8. The model's p-value was calculated to be 0.0003 showing its high significance with a confidence level higher than 99%. The parameters analysed, citric acid, glutamic acid and ammonium sulphate, were all calculated to be significant apart from glutamic acid. The fact that glutamic acid was not significant in P(3HB) production was an expected response since it was added into the design matrix due to its role on γ-PGA production. The $R^2$ value obtained for the model generated was calculated to be 0.914 showing that the model was in good correlation with the experimental data. The second order polynomial model generated is presented in Section 3.4.2 as equation 3.2.

Parameter B, glutamic acid, in P(3HB) production, showed the smallest coefficient with the only negative value. This indicated that the presence of glutamic acid negatively affected P(3HB) production. This result was contrary to the one obtained with PB screening where glutamic acid was found to be positively affecting the production. This might be because of the fixed glucose and yeast extract levels in the design of the experiment as explained previously (page 206). Since the C:N ratio was already fixed based on their combined effect for the best P(3HB) production, further addition of glutamic acid, an amino acid which is another form of nitrogen source , changed the C:N ratio hence disrupting the equilibrium. In the PB screening experiment, all parameters were changed at the same time, therefore the effect of glutamic acid may have appeared as positive due to lack of other nitrogen sources. Moreover, glutamic acid on its own may not

have been sufficient as a nitrogen source for P(3HB) production since yeast extract provides other essential nutrients for bacterial growth such as vitamin B complexes and trace elements, Mg, Fe, Zn, Cu, and V, as discussed in Section 4.1.

Parameter C for P(3HB) production, was ammonium sulphate, with a coefficient of +0.029, had a p-value of 0.0004. This indicated the positive effect of ammonium sulphate on P(3HB) production. Beaulieu *et al.* (1995) investigated the effects of ammonium salts on the production of P(3HB). They reported that ammonium concentrations ranging between 0.5 to 1.5 g/L had no significant effect on production, however the type of ammonium salt was important and ammonium sulphate was found to be the best of all types tested (Beaulieu *et al.,* 1995). In a similar study Grothe and co-workers (1999) investigated the effects of different nitrogen sources on P(3HB) production by *Alcaligenes latus* under fixed C:N ratios. They reported that the best nitrogen source for the production was ammonium sulphate and yields were more sensitive to changes in concentration (Grothe *et al.,* 1999).

When the effect of citric acid, parameter A for P(3HB) production, was evaluated the coefficient was calculated to be +0.038. This was the highest amongst all variables tested. As explained in Section 1.2.2 and discussed previously in Section 3.3.2, the addition of citric acid has positive effect on P(3HB) production. Under constant C:N ratio conditions, the addition of small amounts of citric acid improved overall production. When the 3D graphs plotted using the model generated are studied, a clear interaction between ammonium sulphate and citric acid can be observed (Figure 3.21). At low citric acid levels, addition of ammonium sulphate did not affect the P(3HB) production. However, when citric acid levels were increased, addition of ammonium sulphate positively affected the production.

The combined effect of ammonium sulphate and citric acid on P(3HB) production was further evaluated within the context of dual biopolymer production. The fact that the

addition of ammonium sulphate only shows a positive effect in presence of citric acid, indicates an interdependent relation. As discussed previously, internal glutamic acid which forms the main component of γ-PGA is synthesised through two different pathways. In the presence of ammonium sulphate and citric acid, L-glutamine pathway is initiated with the contribution of extracellular glutamic acid. This pathway uses nitrogen from ammonium sulphate and carbon from glucose or citric acid (*via* TCA cycle) (Kunioka and Goto, 1994; Shih and Van 2001; Yao *et al.,* 2010). In the presence of citric acid, the first enzyme in the TCA cycle, citrate synthase, is blocked. This prevents the conversion of Acetyl-CoA and its utilisation through the TCA cycle. Hence it is directed to PHA synthase pathway. Meanwhile citric acid present in the medium may be utilised in TCA cycle to form α-ketoglutaric acid. Further addition of both ammonium sulphate and citric acid may, in fact, keep this pathway active and direct all the glucose to PHA synthase pathway, enhancing the production of γ-PGA but also creating a pseudo positive effect for P(3HB) production. A schematic diagram showing this interdependency is presented in Figure 4.2.

The fact that the effect of citric acid on γ-PGA is not visible in 3D graphs may be due to the small amounts present in the medium, resulting in a very small contribution to the final γ-PGA. Yao *et al.,* (2010) investigated the contribution of carbon source *via* TCA cycle to the final γ-PGA production, using $^{13}C$ labelled glucose. They reported that, although glucose was found to affect positively the amount of γ-PGA produced, the amount of $^{13}C$ traced in the final product was very small (6-9%). They also observed a large amount of unutilised glutamic acid left in the medium. Their findings are in line with this study, supporting the proposed role of citric acid and ammonium sulphate on P(3HB) and γ-PGA production.

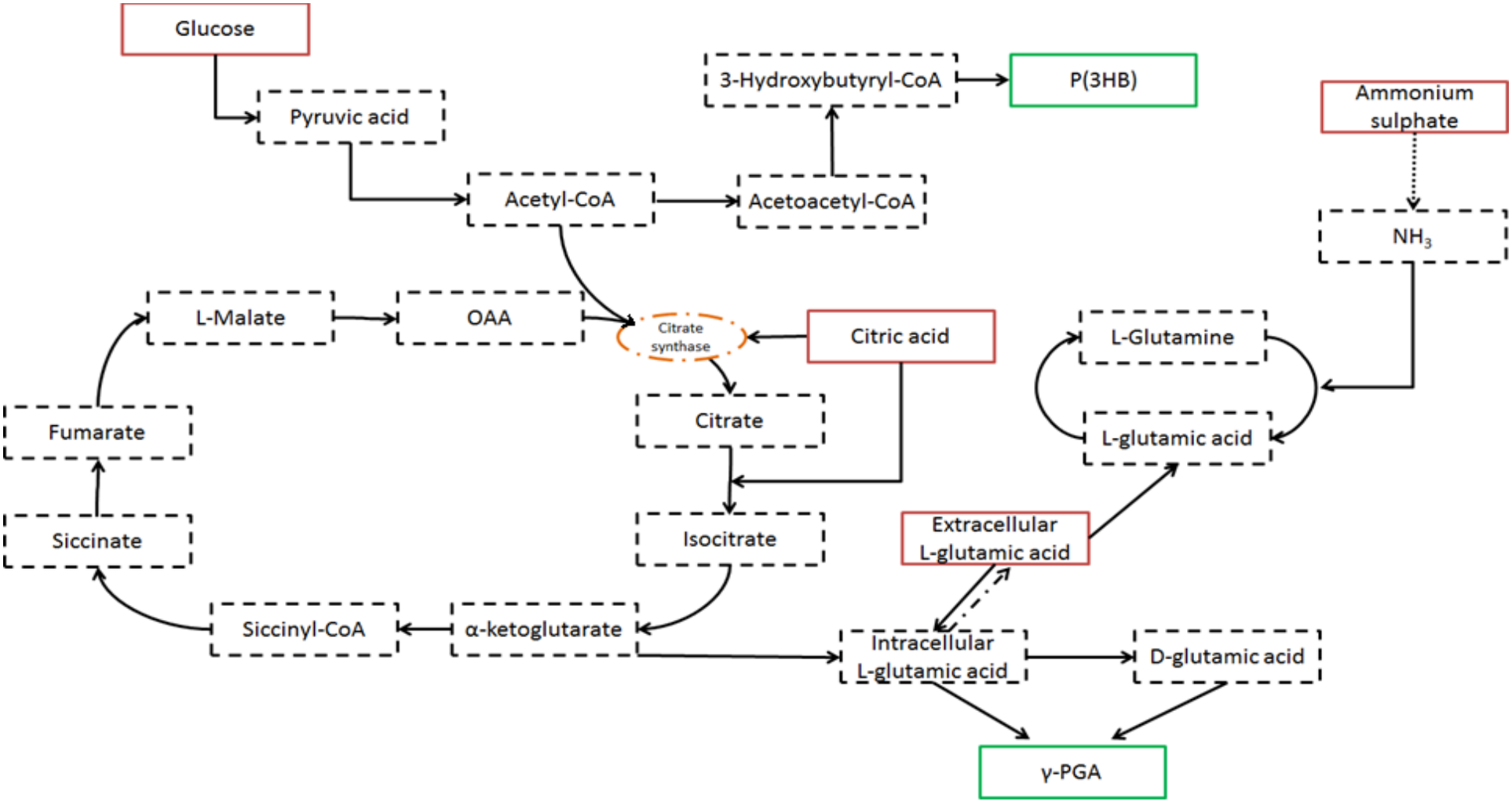



**Figure 4. 2:** A schematic representation of the suggested merged pathways for production of P(3HB) and γ-PGA, drawn based on pathways reported by Shih and Van (2001) and Aldor and Keasling (2003). Red boxes indicate components present in the medium, green boxes indicate products; dotted boxes indicate intermediate products and orange circle represent a key enzyme, OAA: oxaloacetic acid. CoA: Co-enzyme A.

### 4.2.3 Inoculum media for dual production

Time is an important element in commercial bioprocesses. Productivity of a microbial culture depends on how fast the organism grows and produces the desired product in high concentrations in batch cultures. During the optimisation studies for dual polymer medium, it was observed that inoculum medium had a noticeable effect on growth and polymer production of the final culture. In addition, the long duration of the lag phase in shaken flasks was a concern. The experiments conducted with different seed cultures grown on different media are presented in Section 3.4.4. The differences in lag phases with different media can clearly be observed in Figure 3.25. The longest lag phase was observed in double polymer medium inoculated with a culture grown in nutrient broth. The culture required more than 24.h to reach a considerable optical density.

A common practice to minimize the lag phase in fermentation technology is to grow the inoculum culture in the same (or similar) medium as the growth and production medium/a. However, a different approach was required to reduce the lag phase in double polymer medium, since inocula grown in nutrient broth led to a long lag phase. A relatively long lag phase was observed for *B. subtilis* OK2 in the double polymer medium compared to other media used. The difference in growth observed between double polymer medium and other media used was also demonstrated through doubling times and specific growth rates, presented in Table 3.10. The growth in double polymer medium was slower compared to the other media used. The doubling times and specific growth rates calculated in the experiment presented in Section 3.4.4 found to be higher than previously calculated ones. This was due to different aeration and mixing conditions adopted in the experiment. The growth rate experiment (Section 3.4.4) was conducted in 96-well plates was replicated with two independent experiments. Since the

96-well plate was incubated without any mixing the growth was slower than in shaken flasks. Therefore, the doubling times and specific growth rates calculated were higher than the ones calculated in shaken flasks. The growth rate experiment was conducted to see the effect of different media on growth compared to each other. So the results calculated in the growth rate experiment were not compared with the results calculated in shaken flask experiments.

The double polymer medium developed in optimisation studies was inoculated with cultures grown in respective single polymer media. The culture inoculated with culture grown on γ-PGA production medium showed a much better performance compared to the culture inoculated with inoculum grown in P(3HB) production medium (Figure 3.27). The biggest advantage of using inocula from single polymer production media rather than adjusting the bacteria to double polymer medium, and subsequent inoculation was the shorter time. Adoption of double polymer medium resulted in a lag phase of more than 24 h (as presented in Figure 3.25d) however in P(3HB) and γ-PGA production media, the culture entered the exponential phase after 5 h and 15 h of incubation respectively. Also, the culture inoculated in P(3HB) production medium yielded less polymer compared to the one inoculated from γ-PGA medium. When the inoculum grown in γ-PGA medium was inoculated into double polymer medium and the growth curve was compared to the growth curve obtained with culture grown in nutrient broth, it was observed that the maximum optical density was 38% higher. Moreover, the phase contrast microscopic images taken from cultures grown in different media were compared. In nutrient broth and P(3HB) production medium the bacteria exhibited standard rod shape phenotype (Figure 3.30a and Figure 3.30c) while in γ-PGA medium the bacteria showed stress signs such as forming long chains of filamentous structures. Formation of long chains in *Bacillus* has been identified as a major stress

factor by Gerth and co-workers (1998) previously. In γ-PGA medium it was clear that the bacteria were under stress and were unable to grow in a healthy manner (Figure 3.30d). This may also be due to the time of the sampling since in γ-PGA medium, the culture went into the exponential phase after 15 h while the commencement of the exponential phase in other cultures only took about 5 h. In contrast, in the culture of double polymer medium inoculated with a culture grown on γ-PGA production medium, the culture grew, but was still under stress as indicated by thick but long filamentous structures observed microscopically.

The reason that *B. subtilis* OK2 grown in double polymer medium using inoculum grown in γ-PGA medium showed a better growth and production performance may be due to the fact that the lack of certain nutrients or presence of high stress factors imposed on the culture by the γ-PGA medium were rapidly reversed with the carbon-rich nature of the double polymer medium (contains 20 g/L glucose). Transferring the bacteria from high-stress conditions into moderate stress conditions may be stimulating the metabolism leading to higher polymer production, hence increasing the productivity. There is no such study presented in literature so far reporting effect of different inoculum media on polymer production.

### 4.3 Defining the Double Polymer Production Mechanism

In fermentation, time course of any production is very important. Following the optimisation studies and establishment of inoculum conditions, time course of double polymer production in shaken flasks was investigated. Changes in product concentration, growth and total carbohydrate concentrations in shaken flasks are presented (Figure 3.31) in Section 3.5.1, where an average of 1 g/L P(3HB) and 0.4 g/L γ-PGA were produced, using a strain which was not a high-producing strain.

As described in Section 1.4.1, most of the recent works carried out by other researchers has been on the dual production of PHAs and EPS. No work has been carried out on production of PHA and γ-PGA. As an example for the levels produced in batch fermentations so far, the highest production of PHAs with EPS reported was by Wang and Yu (2007). Under statistically optimized conditions, using central composite design, they reported a final production level of 12 g/L P(3HB) and 0.13 g/L EPS. Although the P(3HB) concentration reported was high, the second product concentration was lower than the levels reported in this thesis. Since the strain was used as a proof of concept in this thesis, the ratio of the two products was taken into consideration in efforts to increase the production of both products.

Under fed-batch culture conditions, the highest production reported up to date was by Koller *et al.* (2015). They reported that the production of two polymers compete with each other for the available exogenous carbon source. At the end of 61.75 h fermentation they achieved 12.96 g/L and 1.31 g/L concentrations of PHBV and an EPS respectively. A similar work to this study yet different, was reported by Jo *et al.* (2008). In their study, P(3HB) and glutamate (sodium salt of glutamic acid) were produced by a two-stage fermentation utilising variable biotin concentration by *Corynebacterium glutamicum*. Glutamic acid is the monomer of γ-PGA therefore, what they report should not be considered as dual production of biopolymers. In their study, the production was controlled by varying biotin concentration. When a low concentration of biotin (0.3 µg/L) was used, glutamate was produced. The production shifted towards P(3HB) production, by the addition of biotin at a concentration of 9 µg/L. As a final concentration, 7 g/L (36% PHA/CDW) P(3HB) and 18 g/L glutamate were produced using glucose as the carbon source.

### 4.3.1 Changes in optical density

One of the most important aspects observed in this thesis, in double production, was the change in optical density. After 24 h of fermentation, the optical density of the culture started to decline. This decline was not observed in cdw measurements, possibly due to the accumulation of P(3HB) after 24 h, as will be discussed in Section 4.3.2.

Microscopic observations of the culture at different times of cultivation suggested that this decrease in culture optical density was due to cell lysis. Similar microscopic observations have been reported in literature. Branda and co-workers (2001) studied fruiting body formation by *Bacillus subtilis*. They reported that, formation of the *B. subtilis* fruiting bodies followed a distinctive developmental pathway with marked spatial organization. The cultures, grown without any agitation at 25°C, initially contained only planktonic cells that were highly motile (12 h of cultivation). After 12–24 h of incubation, the population density in the medium reached about 3 x $10^7$ colony forming units per ml (cfu/ml). By 36–48 h, the cell density in the liquid phase of the culture dropped sharply to around 3 x$10^5$ cfu /ml. They also observed that the cells at the air–medium interface became non-motile and formed long chains that were aligned and bound together and after 60 h some cells within the chains began to sporulate, and by 96 h more than 50% of the viable cells within the pellicle were spores. They also mentioned that these spores clustered due to a type of EPS present in the medium. Figure 4.3 shows the comparison between the observations of Brenda *et al.* (2001) and the findings of this thesis. As can be observed, in the first 6 h of cultivation in DP medium, cells exhibited chain forms, which was different from the observation of Brenda *et al.* (2001) as they used a different medium. After 24 h the number of long filamentous structures increased resembling those reported by Brenda et al. (2001) and

after 48 h clusters were visible under microscope similar to ones observed after 96 h by Brenda *et al.* (2001). The difference in the occurrence of these morphological changes between their work and the results presented in this thesis is probably due to the culture conditions. Their work was conducted under static conditions whereas the observations in this thesis were under agitated conditions in shaken flasks.

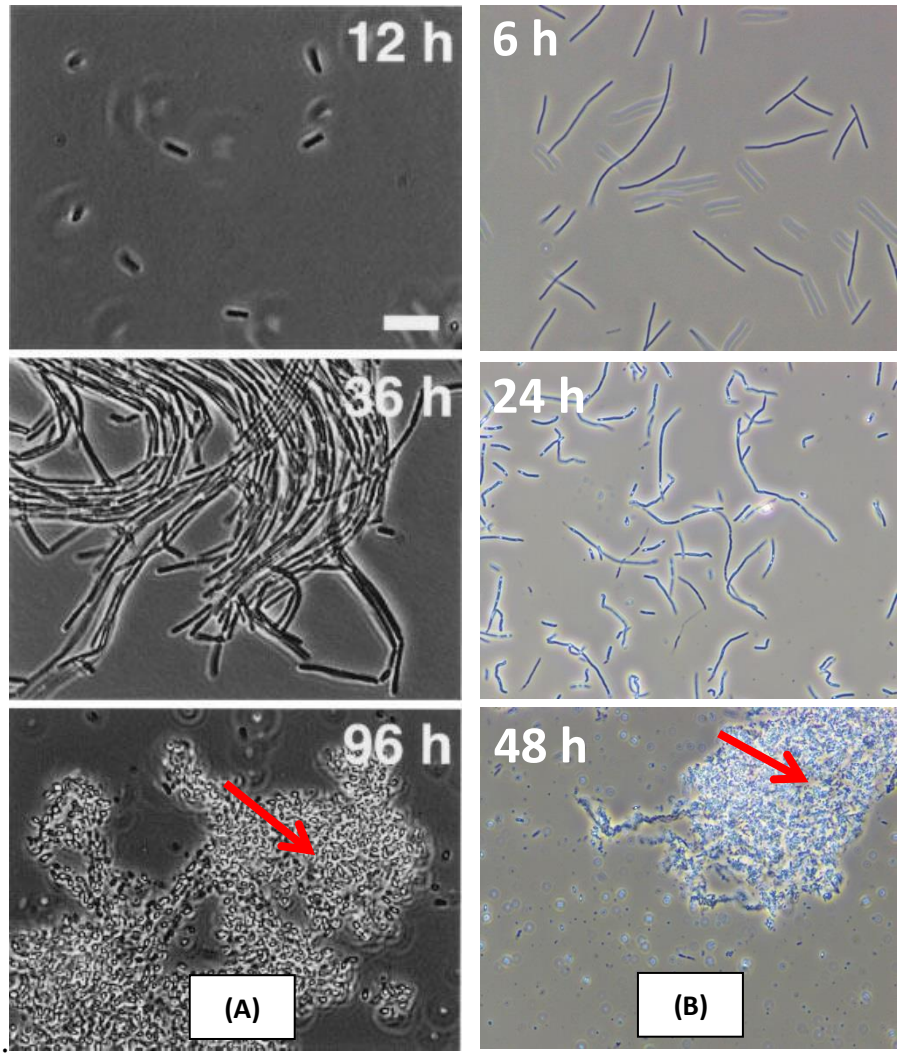



**Figure 4. 3:** Comparison of the microscopic observations of; (A) Branda *et al.*, 2001 (magnification 600x) and (B) this study (magnification 400x) at different times of cultivation. Arrows indicate the spore/cell clusters formed.

These findings lead to the conclusion that the bacterium undergoes sporulation and spores form clusters after 48 h of cultivation. Muchová *et al.*, (2011) observed the

changes in *Bacillus subtilis* cells when treated with lysozyme enzyme and reported similar observations upon enzymatic degradation of the cell wall leading to the dispersion of intracellular organelles into the surroundings. In addition several researchers (Perego and Hoch, 1988; Hageman et al., 1984) studied the correlation of sporulation and protease production behaviour and suggested that protease production might be regulated by the same mechanisms that control spore formation. Since lysozyme is also a type of protease, this supports the idea that during sporulation lysozyme might be secreted leading to cell disruption.

### 4.3.2 Curves of best fit

In order to analyse better the fermentation profile, best fitting curves were drawn on plotted sample points and best fitting equations were obtained (Figures 3.33 and 3.34). These curves of best fit are useful in order to predict the trends of the culture and to interpolate the points between samples, but must not be used to extrapolate outside the rage of investigation since it might cause misleading interpretations of the culture behaviour. The functions presented are valid between 0 to 72 h of cultivation and must be used and interpreted within these limits only.

For production curves, P(3HB) and γ-PGA productions were fitted to $4^{th}$ order polynomial and sugar consumption was fitted to a $3^{rd}$ order polynomial. Curves of best fit for cdw and optical density are given in Figure 3.34. An immediate discrepancy was observed between cdw curve and $OD_{600}$ measurements. The profile for optical density did not match with cdw. Although this could be explained with accumulation of P(3HB) causing a pseudo stability/increase in cdw, when the polymer weight was subtracted from cdw measurements it still did not correlate with $OD_{600}$ measurements. When cdw vs $OD_{600}$ graph was plotted two distinctive regions were observed (Figure 3.36). The

correlations between cdw and optical density were different at growth and polymer accumulation stages. This phenomenon was observed by other researchers as well, and was recently reported in literature (Martinez *et al.,* 2015; Le Meur *et al.,* 2013). In these studies, it is stated that there is a need for two different optical density vs cdw calibration curves since the correlations for growth and production regions are different.

Le Meur *et al.,* (2013) compared P4HB production in different *E. coli* recombinants and identified the best *E. coli* strain regarding cell growth and P4HB accumulation. The effect of growth conditions in batch culture was also studied. For evaluation of the growth they suggested to use two different cdw vs optical density calibration curves since $OD_{600}$ did not match with cdw measurements. Similarly, Martinez *et al.,* (2015) reported the feasibility of producing PHAs by feeding a culture of *Cupriavidus necator* with a pre-treated olive mill wastewater (OMW) at shaken flask scale. In their work they report the use of two different cdw vs optical density curves for accurate measurements. However, none of these researchers have suggested any reasons for the discrepancy. There could be a number of reasons behind the discrepancy. Intracellular accumulation of the polymer in the form of granules within the cell creates heterogeneities in the cytoplasm leading to a different refractive index. The difference in the refractive indices of the cytoplasm and amorphous polymer is what makes the polymer accumulation observable under phase contrast microscope. The other reason might be due to the cell lyses or sporulation which was discussed above and shown in Figure 4.3. The cell lysis or sporulation cannot be directly measured since centrifugation and gravitational measurement takes cell debris, spores and live cells into account together. However, as discussed, it can be clearly observed under the microscope (Figure 4.3). The cells undergo these changes (sporulation and lysis) at

much later phases in P(3HB) medium (200 h , Figure 3.87) than in the DP medium. This also explains why the discrepancy was not clearly observed in P(3HB) production medium. For the reasons stated, cdw and optical density correlation is calculated based on growth phase as also suggested by other researchers (Martinez *et al.,* 2015; Le Meur *et al.,* 2013).

Based on this correlation, the cdw curve of best fit was obtained and was used in calculations of yield and specific rates. In this thesis, two different yields are calculated; namely cumulative and instantaneous yields. The equations used for the calculations are given in Section 3.5.2. The cumulative yield was calculated based on the sample points with the assumption that the changes in between the points are linear (Figure 3.40). However, from the curves of best fit obtained, the changes were observed to be nonlinear. Therefore, using the equations obtained, instantaneous yields were calculated (Figure 3.41). Both of these graphs provided an insight to the process of double polymer production. According to cumulative yield data γ-PGA production dominates production at first and P(3HB) production starts later. Although this was not clearly observed with the curves of best fit, yield data facilitated this observation. The switch of metabolism from γ-PGA to P(3HB) was observed at around the 50 h of fermentation. When the instantaneous yields were investigated, the same trend was observed, however the metabolism switch was identified more precisely after the 51 h of the fermentation. The P(3HB) yields go parallel with cell growth according to the instantaneous yield graph obtained. This trend is expected since P(3HB) accumulation in cells will also be reflected as an increase in the cell dry weight. These findings are in agreement with the production profiles of individual polymers (Figures 3.8 and 3.9). Since γ-PGA is a growth-associated product and P(3HB) is a secondary metabolite, this observed shift in

metabolism from γ-PGA to P(3HB) is expected. The highest yields obtained was calculated as 0.11 for P(3HB) production at 72 h, and 0.06 for γ-PGA production at 48 h. The specific growth rate of this culture at exponential phase was calculated to be 0.28 $h^{-1}$. When the specific production rates were considered, a profile similar to the yields was observed. The specific growth rate was found to be highest around the beginning of the fermentation, as expected, where the culture is in exponential phase.

Specific total carbohydrate consumption rate was in total agreement with specific growth rate. Specific P(3HB) production rate started to increase after 30 h following the decline in γ-PGA production. There was an increase in total carbohydrate consumption observed at around 50 h which corresponded to the highest point of specific P(3HB) production. This was also corresponded the time in instantaneous yield graphs where the switch of metabolism from γ-PGA to P(3HB) was observed.

All the data obtained from double polymer production in shaken flasks show that the production of the two polymers showed a similar trend. γ-PGA production is triggered at the early stages of the growth and continues until around mid-stationary phase. Whereas P(3HB) production commences later at the beginning of stationary phase and continues to increase until the end of the fermentation. This data provides valuable information for the scale up of the system as well as for the selection of a fermentation mode. In case of continuous fermentation, the culture needs to be kept at such a point that both growth and production rates are at maximum values. In the case of double production, both products of interests (γ-PGA and P(3HB)) should be at their maximum levels to provide a high overall productivity. However, as discussed previously, γ-PGA is a growth associated product and P(3HB) is non-growth associated (production starts at stationary phase). As also can be seen on the specific rates graph (Figure 3.42), a

single point where both products are at their maximum specific rate does not exist. Keeping the culture at a high growth rate and producing both polymers at high levels simultaneously is not possible due to different times of production. However, since this was a shaken flask data it was repeated with the production profile data obtained at a larger scale in the fermenters (Section 3.5.3, Figures 3.50 and 3.51).

### 4.3.3 Production in fermenters

When double polymer production was carried out in 2 L fermenters, no polymer production was observed as can be seen in the profile presented in Figure 3.43, and the optical density measurement of growth was lower compared to those obtained in shaken flasks. Cell growth reached to a value of 10 and did not change through the course of 72 h. Contrary to the previous observations, total carbohydrate assay showed that total carbohydrate was completely consumed after 72 h. Carbon dioxide data obtained through the course of fermentation showed a constant $CO_2$ emission from the fermenter until the end (55 h), indicating that the metabolic activity was continuing. There was a small γ-PGA production observed at the beginning although it subsequently diminished. This experiment was conducted at 250 rpm agitation and one of the important observations was the change in dissolved oxygen concentration. After 5 h of the fermentation %DOT dropped to zero and stayed zero until the end of 54 h. This indicated that the oxygen supplied to the fermenter was being consumed rapidly due to the growth of the organism.

The effect of oxygen on the production of both PHA and γ-PGA was reported in literature by several researchers. Cormwick *et al.*, (1996) studied the effect of aeration on γ-PGA production by *Bacillus licheniformis* in a batch fermenter. They reported that increases in stirrer speed and air flow rates, and thus in the availability of oxygen, had

significant effects on cell growth and carbon utilisation as well as on γ-PGA yields. According to their findings, increasing the agitation speed from 250 rpm to 800 rpm doubled the cdw and γ-PGA production. They also mentioned that a very high aeration mode decreased the molecular weight of γ-PGA. Ogawa *et al.,* (1997) studied the production of γ-PGA by *Bacillus subtilis* (natto) in jar fermenters. They reported that an increase in agitation from 300 rpm to 400 rpm doubled the γ-PGA production but a further increase had no effect.

Similarly for P(3HB) production, Kulpreecha *et al.* (2009) studied the effect of culture dissolved oxygen (DO) percentage on the production of P(3HB) by *Bacillus megaterium*. They reported that 60% air saturation appeared to be the optimal DO percentage for growth and P(3HB) synthesis. At a DO level of 40%, the cultivation time was longer; hence, productivity decreased. They suggested that this might be caused by insufficient oxygen in the culture broth. However, further increase in DO levels (80%) significantly reduced the bacterial growth and reduced the P(3HB) yield. The reason for this reduction was reported to be due to the oxidative and shear stresses that resulted from the high agitation speeds. Lopez *et al.,* (2012) observed that oxygen supply at a level of 20 %DOT in batch cultures of *Bacillus megaterium* resulted in 60% P(3HB)/cdw yield. Faccin *et al.* (2013) studied the influence of oxygen transfer rate on the accumulation of P(3HB) by *Bacillus megaterium*. They reported that very high DO values can negatively affect the production and cell growth. However, they also underlined that extremely low oxygen concentrations (like zero in this case) can negatively affect production due to the induction of spore formation at the early stages of the culture or can cause imbalance in bacterial metabolism since oxygen is involved

in TCA cycle. The common denominator of all these findings is the significance of dissolved oxygen levels in the culture medium.

Based on these arguments in literature, several approaches to increase the %DOT in the fermenter were attempted. When the culture was kept at constant agitation (400 rpm) an increase was observed in the production of both polymers. The %DOT did not drop below 30% and was maintained at above 50% most of the time. However, due to aggressive mixing, excessive foaming was observed after 24 h and foaming could not be controlled. Also, after 24 h, the optical density ($OD_{600}$) of the culture exceeded 15 which was slightly above the value obtained in shaken flasks at the same time of cultivation ($OD_{600}$ 13.57 after 24 h in shaken flask).

As far as the pH profile is concerned, in the run conducted with 250 rpm agitation, the pH value dropped to 5.5 from 6.6 but in 400 rpm it dropped to pH 5.9 within the first 5 h and then rapidly increased to pH 7. The pH started decreasing again just before the termination of the run.

Following these findings, two consecutive experiments were carried out with varying %DOT in the culture. DOT was kept constant at 10% and 25 % air saturation by varying the stirrer speed between 250 – 400 rpm, and P(3HB) and γ-PGA productions were observed in both runs (Figure 3.45 and 3.46). When the profiles of the oxygen limited (250 rpm) and 25% constant DOT runs were compared, a similar start in the first 12 h was observed. However, under oxygen-limited conditions (250 rpm) the $CO_2$ level peaked to 1 % after 7.5 h while it was peaked to 0.75 % after 6.5 h in 25 % constant DOT fermentation. Both levelled out between 0.5 % and 0.6 % following the peaks. The stripping of $CO_2$ from the culture under higher agitation conditions was more consistent, while in less agitated cultures $CO_2$ was captured in the air bubbles.

This could explain the difference in $CO_2$ levels observed in the two fermentations. Similarly, the sudden drop of % $CO_2$ observed at 8 h of fermentation with constant 10 % DOT (Figure 3.45) is due to the change in mixing regime. The stirrer speed range was readjusted from 250 – 400 rpm to 300-400 rpm stirrer speed fluctuation to maintain a more constant %DOT level consequently a drop in $CO_2$ level was observed. The pH profile at the region where the sudden drop occurred was not different from other fermentations conducted. In addition DOT level at the region where $CO_2$ drop obsered was constant. This also confirmed that the change in the outlet $CO_2$ was not an outcome of phsiological response of the organisim but a physical phenomenon.

The profile obtained in the run with constant agitation at 250 rpm (oxygen-limited conditions) showed that in the first 12 h the culture could not fully complete its exponential growth phase. The optical densities of both runs at DOT of 10% and 25% (both fermenters were kept at the stated percentages of DOT by varying stirrer speed between 250-400rpm) reached higher values compared to the run with constant 250 rpm. Within the first 24 h, the optical density values were 13.46 ($OD_{600}$) and 17.9 (OD600) in constant 25% DOT and 10% DOT fermentations respectively, whereas it was 8.94 ($OD_{600}$) in the constant 250 rpm run (oxygen-limited conditions). This explained why the culture in the constant 250 rpm run (oxygen limited conditions) did not produce any polymer. According to these findings it was suggested that, DOT is an important factor for the culture to complete its growth phase and enter the phase where it produces P(3HB), otherwise P(3HB) production will not be initiated.

Foaming was observed in both constant DOT fermentations, but could be controlled in 10 % DOT fermentation. Foaming was probably due to the level of surface active agents produced throughout the course of the fermentation. Furthermore, it is reported

in literature that excessive foaming could be caused by γ-PGA accumulation in the medium (Ogawa *et al.,* 1997; Rowe *et al.,* 2003). Therefore, production of γ-PGA in both cases can be another reason for excessive foaming.

At 10% constant DOT, total carbohydrate concentration dropped to half of the initial concentration at the end of 48 h. As discussed previously (Section 4.1 and 4.2) the remaining carbohydrate detected in the culture broth could be attributed to the presence of EPS in the medium. EPS production capability of the organism was observed in γ-PGA production (Figure 3.8) but was not further investigated since it is outside the scope of this thesis.

The pH profile for 10% constant DOT fermentation followed a similar trend to the constant 400 rpm fermentation. One of the main differences between 10% DOT and 25% DOT fermentations was the maximum value that pH reached during the fermentation. In 25% DOT fermentation the pH started at 6.5 and was almost 7 by the end of 15 h and it started to decline after that point, whereas in 10% DOT fermentation it went up from 5.8 (the lowest point reached after 6 h) to pH 6.5 and started to drop again after 20 h. These fluctuations in the pH are most probably due to the metabolites produced in the fermentation. As discussed previously the increase in pH was suggested to be due to ammonification of degradation of organic nitrogenous compounds such as amino acids (Wang *et al.,* 2008). The double polymer production medium contains yeast extract and ammonium sulphate. In aqueous solution, when $NH_4^+$ concentration is increased the pH increases and when $NH_4^+$ concentration decreases, pH decreases (Serezli and Tabak, 2013). In all the fermentations conducted with different DOT values, the medium was DP. Therefore, based on these remarks it can be suggested that the oxygen in the medium has a strong effect on the consumption of nitrogen in the

medium. In all runs, the pH drop-point was observed at after 6 h of fermentation and only in runs with excess oxygen, it started to increase again. The maximum pH value reached, seemed to be correlated with the level of dissolved oxygen present in the medium. At 400 rpm constant agitation, pH exceeded 7 and similarly at 25% DOT run pH increased to almost 7. However in 10% DOT run, it only could go up to pH 6.5. In order to understand the effect of oxygen on pH, the relationship between oxygen and metabolism should be studied. Acetyl-CoA is a key compound in the cell metabolism, and it is oxidized *via* TCA cycle, being either dissimilated to generate biologically useful energy or assimilated for cell growth. The oxidation *via* TCA cycle predominates under balanced growth conditions, with NADH being generated and used in biosynthesis or energy generation. When biosynthesis decreases due to lack of a nutrient, the TCA cycle activity decreases due to the high NADH concentration, resulting in a decrease of acetyl-CoA oxidation *via* TCA cycle. Additionally, the TCA cycle is endergonic (endothermic) when the oxygen is absent or limited, the reducing power (e.g., NADPH) generated is not oxidized *via* electron transport phosphorylation. Therefore, low oxygen supply can reduce the TCA cycle activity; due to both thermodynamic reasons and metabolic control by reducing power (Faccin *et al.*, 2013). This reduction in TCA activity hinders the utilisation of available nutrients. Knowing that the utilisation of nitrogen source causes an increase in pH, it can be deduced that ammonium sulphate and yeast extract, as organic nitrogen sources, do not get utilised completely under oxygen limited conditions and therefore pH does not increase during the course of fermentation under the oxygen limited conditions (constant agitation 250 rpm). The low level of optical density reached also confirms that cell growth is not attained at its full potential. In addition, PHAs are produced under nutrient limited conditions, for the organism used in this work (*B. subtilis* OK2) the limiting nutrient is

nitrogen. If nitrogen is not depleted, then P(3HB) production would not be triggered. But based on the literature findings, excess oxygen can also cause decrease in P(3HB) production (Kulpreecha *et al.*, 2009). Based on this reasoning, a new DOT control regime was tested and as presented in Figure 3.48 and Figure 3.52, the mixed control regime (25% DOT in first 24 h and 10% afterwards) resulted in successful double polymer production. It was also shown in Figure 3.52 that the product stability is maintained after the production reached its maximum.

When the curves of best fit are compared with the ones obtained from the shaken flask experiments, it can be concluded that better controlled environmental conditions resulted in improved culture homogeneity. In Figure 3.51 it was observed that the γ-PGA production still began earlier, but the difference between the commencements of both polymers reduced significantly. The production of both polymers appears to be more concomitant in the fermenter as compared to the shaken flask. Koller *et al.* (2015) reported that in the case of dual production of polymers, two polymers compete with each other for the available exogenous carbon source. This is of course expected. The polymers reported in their study were PHBV and an EPS respectively and the production profiles of these polymers were reported to be parallel to each other, similar to the results obtained in this thesis.

The observed differences between shaken flask and fermenter production profiles might be explained through the culture homogeneity, improved environmental conditions and availability of excess oxygen in the culture even towards the end of the fermentation.

## 4.4 Waste Materials as Carbon Source for Single and Double Production of Polymers

Four different agro-industrial waste materials namely, rapeseed cake (RS), orange peel (OP), wheat bran (WB), Spirulina powder (S), and 4 different pre-treatment methods were tested for their suitability for production of γ-PGA and P(3HB). No additional nutrients were added into the media prepared by the waste materials. This was done deliberately to investigate the yields obtained by the inherent components of the waste materials as the sole ingredients for P(3HB) production. Since γ-PGA production could not be observed in any of the cultures where these waste materials were used as the sole medium component due to the lack of glutamic acid, initially only P(3HB) production capabilities were compared. Due to the low nutrient content of the waste material, the P(3HB) values obtained were low (Figure 3.54). The two top best productions were obtained when orange peel was treated only with distilled water (2.5 mg P(3HB)) and with acid treatment (1.4 mg P(3HB)). Water and alkaline treatments of Spirulina were also high in comparison to the other waste materials (1.2 and 1.16 respectively). %P(3HB)/cdw values presented in Figure 3.55 also confirmed that highest P(3HB) accumulation among all bio-wastes tested was with orange peel.

Anderson and Dawes (1990), and Kessler and Witholt (2001) reported that citric acid promotes PHA formation. This concept is also discussed in detail in Section 4.1 and its effect on P(3HB) production with *B. subtilis* OK2 is demonstrated in Section 3.3.2. The first question was on the citric acid content of orange peel and its possible role on P(3HB) production. Ersus and Cam (2007) investigated the organic acid content of *Citrus aurantium* peels using HPLC. The predominant organic acids in freshly prepared peel samples were found to be oxalic acid (257.5 mg /100g peel), ascorbic acid (117.6

mg /100g peel) and quinic acid (98.5 mg /100g peel), but malic acid and citric acid were not detected. This indicated that, promotion of P(3HB) production is affected not by citric acid but by some other ingredient present in orange peel. When different pre-treatments were compared, orange peel treated by water showed the highest P(3HB) production, although %P(3HB)/dcw values for acid and water treatments were very close to each other. According to Ververis *et al.* (2007) orange peel contains 13.6% cellulose, 6.1% hemicellulose and 2.1% lignin within its dry weight. As a pre-treatment method, hot water treatment mainly removes hemicelluloses. Liquid soluble oligosaccharides are dissolved and separated from insoluble cellulosic and lignin fractions of solid agricultural material. In mild acid treatment, all of the hemi-cellulosic content dissolves in water and the treatment helps hydrolysing lignocellulose content to fermentable sugars. Mild alkaline treatment mainly removes lignin and part of the hemicellulose from the solid content. NaOH treatment has been referred to as the most effective delignification method for agricultural wastes (Taherzadeh and Karimi, 2008) and is also known to be an effective method in breaking ester bonds between lignin, hemicellulose and cellulose. Release of lignin into the liquid medium can be considered as one of the reasons why alkaline treatment does not promote P(3HB) production. Phenolic compounds from lignin structures interfere with the production. Adeboye *et al*. (2014) reported the nature of phenolic compounds present in lignocellulose hydrolysates that exhibit toxicity towards *Saccharomyces cerevisiae*. Among the pre-treated orange peel media, microwave treatment showed the lowest P(3HB) production. Microwave treatment mainly affects lignin and lignocellulose contents (Taherzadeh and Karimi, 2008). The cellulose component of lignocellulose can be degraded into fragile fibres and low molecular weight oligosaccharides. However this is only observed in the presence of lignin. The fact that orange peel contains only 2% lignin may explain why

microwave treatment was not as effective as other treatments. In addition, microwave treatment is more effective in air than in solution (Taherzadeh and Karimi, 2008).

In addition to P(3HB) data, the total carbohydrate (Figure 3.53) content and the cdw (Figure 3.56) were measured. Among all the waste materials tested, the highest sugar content was observed with the orange peel, indicating that orange peel is a very rich carbon source. All pre-treated orange peel media showed low cdw. This suggests that the composition of ingredients in the orange peel does not support high cell growth. However, when cdw data was compared, the highest cdw was obtained in alkaline treated rapeseed cake. Radwan and Lu (1976) studied the solubility of proteins of rapeseed cake in aqueous solutions and reported that the solubility increases by increasing temperature and pH. This suggests that a higher amount of protein is solubilised when rape seed cake is treated in alkaline environment at high temperatures. Since nitrogen is one of the main building blocks of cell growth, insufficient nitrogen content may have reflected in low cdw, this can explain the high cdw content obtained in RSB.

There are only a few reports in literature on utilisation of orange peel as carbon source for P(3HB) production. In a patent, a new pre-treatment method is described for citrus residues to enable their utilisation as a carbon source for PHA production (de Aragao *et al.,* 2008). The invention involves the use of citric residues generated by fruit juice industry to produce citric molasses to be utilised as fermentation medium. However the pre-treatment described, involving a series of physical and chemical processes, is costly compared to the pre-treatment method applied in this work. In another patent published and protected in India, Kanekar *et al.,* (2007) described the production of a co-polymer PHB-co-PHV using orange and banana peel power as substrates by *Bacillus cereus.* In

their invention, they describe that orange peel alone as substrate does not produce any polymer however, when the orange peel is supplemented with glucose then the production is higher than the medium containing only glucose. Therefore, they suggest that orange peel can be used to enhance PHA production rather than a carbon source. Kulkarni *et al.,* (2015) studied production of PHA, where agro-wastes like fruit peels, bagasse and de-oiled cakes were screened as a sole source of carbon. The strain, *Halomonas campisalis* MCM B-1027 was used for the production of PHA. They reported that banana peel, orange peel and bagasse are the best agro-industrial waste materials for the production of PHAs. 1% (v/v) aqueous extract of bagasse was found to be the optimum carbon source with 47% PHA production on dry cell weight basis. For the PHA production with orange peel extract (extract was: 5%w/v orange peel in water) 1% (v/v) of extract was added to production medium as carbon source. Production medium was composed of 0.1% (w/v) yeast extract, 4.5% (w/v) sodium chloride and trace element solution consisting of g/l calcium chloride, 0.13, magnesium sulphate, 0.38, potassium chloride, 0.75 and sodium bromide, 0.2. They reported that the organism produced 42% PHA within 48 h. Among all this work published in literature none of them presented the potential of orange peel as pure carbon source without any addition of extra nutrients. Although orange peel by itself is not enough for reaching high titres, it has the potential to be used as sole carbon source. A novelty of the work in this thesis compared to other work published, is the use of orange peel as a sole carbon source using a simple and cheap extraction/pre-treatment method.

As described earlier (Section 3.6.2) orange peel containing 0.58 g total carbohydrate (42%, 23% and 35% of sucrose, glucose and fructose, respectively), and 0.0089 g nitrogen was a very suitable carbon source for the production of P(3HB). The nitrogen content of orange peel was almost nine fold less than the nitrogen content measured for

yeast extract (102.5 mg/g). Since P(3HB) is produced under nitrogen limited conditions, the high carbon source and low nitrogen content could explain the suitability of orange peel for the P(3HB) production.

Santi and co-workers (2015) studied the dilute acid hydrolysis of orange peel for ethanol production. The chemical composition of the dry orange peel powder was reported as; (mg/g): glucose, 65.1; fructose, 69.1; xylose, 14.2; galactose, 60.1; arabinose, 60.0; total nitrogen, 8.9; ash, 74.3; total phenols, 11.8; total tannins, 6.5; cellulose, 260.4; pectin, 160.6; hemicellulose, 118.8; Klason lignin, 18.5; acid-soluble lignin, 0.9 (Santi *et al.*, 2015). As can be seen from the reported content, the values obtained in this thesis were very similar to the ones reported by Santi *et al.* (2015). However the pre-treatment method used in this thesis was hot-water infusion method whereas Santi *et al.* (2015) applied acid hydrolysis. As discussed previously in acid treatment, all of the hemi-cellulosic content dissolves in water and the treatment helps hydrolysing lignocellulose content to fermentable sugars. The hot water treatment mainly removes hemicelluloses. Liquid soluble oligosaccharides are dissolved and separated from insoluble cellulosic and lignin fractions of solid agricultural material (Taherzadeh and Karimi, 2008) Therefor, probably not all of the substances reported by Santi et al. (2015) are available in the pre-treated orange medium presented in this thesis.

### 4.4.1 Optimisation of P(3HB) production using orange peel as carbon source

Based on the good results observed with orange peel as carbon source, a new CCD was designed in order to determine the effect of citric acid and yeast extract additions on P(3HB) production since these two chemicals were found to be significant for P(3HB)

production (Section 3.3). As was mentioned in Section 3.6.4, the CCD was designed with given parameters in Table 3.13.

As a result of the analysis, a model was obtained (Equation 3.3) and levels of each parameter were optimized. As shown previously in Table 3.13, the model was found to be significant with all three parameters tested ($p<0.05$). The $R^2$ of the model obtained was 0.954. As can be seen from the model (Equation 3.3) the coefficients of A and C are positive indicating that the contribution of those parameters are positive for P(3HB) production, which in this case are Orange peel and citrate level. Whereas, variable B, which is yeast extract in this model, has a negative coefficient indicating that, contribution of B is negatively affecting the P(3HB) production.

It is well established that PHAs are produced under limiting nutrient conditions and in the presence of excess carbon source (Akaraonye *et al.*, 2010). In this case the limiting nutrient was nitrogen which was supplied to the medium as yeast extract in low concentrations (1 – 4 g/L) compared to carbon source (30 – 80 g/L). The model shows that in the presence of low nitrogen source, more P(3HB) will be produced. This result is in agreement with literature data (Akaraonye *et al.*, 2010, Valappil *et al.*, 2008). On the other hand quadratic effect of nitrogen source showed a positive effect on P(3HB) production (coefficient of B). Although, the contribution was significantly lower compared to the linear effect, this still indicates that a small amount of nitrogen actually promotes P(3HB) production. This argument was validated with preliminary experiments presented in this study (Section 3.3.2) as well as previous studies reported by other researchers. Effect of nitrogen source on P(3HB) production has already been discussed in Sections 4.1 and 4.2.

The other parameter, A, carries a positive coefficient. This indicates that excess carbon needs to be present in the medium for high P(3HB) production. Again this statement is very well in agreement with the values stated by other researchers (Valappil *et al.,* 2008, Akaraonye *et al.,* 2010, Keshavarz and Roy, 2010).

The last parameter investigated was citrate (Parameter C). The parameter C had a positive coefficient indicating that the presence of citrate affects the P(3HB) production positively. This was explained by several researchers (Kessler and Witholt, 2001, Anderson and Dawes, 1990, Aldor and Keasling, 2003) through the production pathways of PHAs. This has already been discussed in Section 4.1.

The model can also be interpreted considering the values of the coefficients. Comparing the coefficients of each variable, it can be seen that the highest one is 1.11 (coefficient of B). This indicates that the negative contribution of the yeast extract is more important compared to the other parameters (A and C); a small change in this variable will make a bigger difference with respect to the other parameters. The second most important variable is orange peel (variable A). The positive contribution of the carbon source is more significant compared to citrate (variable C). When point prediction is carried out in order to maximize the P(3HB) production within the current culture conditions, the levels of orange peel, yeast extract and citrate were found to be 69.39 g/L, 1.63 g/L, 1.58 g/L respectively (Table 3.14).

According to the optimum point predicted by the program, 0.589 g P(3HB) /L culture broth (30% P(3HB)/cdw) was achievable using the suggested levels. The predicted point and the confidence levels are presented in Table 3.14. The values obtained were well correlated with literature. Yields of 58.6% PHA/cdw (Reddy *et al.,* 2008) and 32.9%PHB/ cdw (Tajima *et al.,* 2003) were obtained using glucose as carbon source

and *Bacillus megaterium,* and *Bacillus* sp. Thirumala *et al.,* (2010) investigated the effects of different sugars on P(3HB) production using *Bacillus* sp. They reported yields ranging between 24% P(3HB)/ cdw (using mannitol as carbon source) and 70% P(3HB)/ cdw (using glucose as carbons source).

Only few studies have been conducted using waste materials for PHA production by *Bacillus* sp. The main waste materials tested were sugar-based materials like beet or cane molasses and date syrup. The yields varied from 5.8g P(3HB)/L to 0.16g P(3HB)/L (Yilmaz and Beyatli, 2005, Khiyami *et al.,* 2011, Omar *et al.,* 2001). Although, Kulkarni *et al.* (2015) did not use *Bacillus* sp for the production of PHA, with the strain *Halomonas campisalis* MCM B-1027 they reported a production of 42% PHA/cdw (0.7 g/L cdw obtained) within 48 h of cultivation using orange peel extract as substrate. In this context, the difference of their work and the work described in this section of the thesis is the medium composition. The medium presented here only contains orange peel, yeast extract and citric acid. But their medium contains; orange peel extract, yeast extract, sodium chloride, calcium chloride, magnesium sulphate, potassium chloride, and sodium bromide. In addition, the total PHA produced was higher in this thesis (0.59 g/L P(3HB), compared to the work presented by Kulkarni *et al.* (2015) (0.33 g/L PHB-co-PHV).

Using these optimised levels, the system was tested in a STR in order to study growth and the production of P(3HB) using orange peel infusion as carbon source. The fermentation profile is given in Figure 3.62. The P(3HB) obtained from the orange peel fermentation (1.24 g P(3HB) /L) was higher than the control fermentation (1.02 g P(3HB)/L) (Figure 3.9) showing that the orange peel is a good replacement for glucose, it even promoted the production of P(3HB).

So far, the best P(3HB) production level reported with *Bacillus* sp. is by Khiyami *et al.* (2011). This group achieved a yield of 68 g P(3HB)/L by using 15% v/v date syrup as carbon source with *Bacillus* SA, whereas other studies with *Bacillus* sp. have resulted in much lower yields. Halami (2008) investigated batch fermentation using starch as raw material and reported a production of 0.48 g/L. Similarly Omar *et al.*, (2001) reported 1.5 g/L production using 2% w/v date syrup as carbon source. Although the production in control fermentation (Figure 3.9) was lower, the % P(3HB) per cell dry weight was higher (49% P(3HB)/cdw). This could be due to the earlier commencement of P(3HB) production which was after 20 h, while it was after 40 h with the orange peel medium.

Another reason for this difference could be due to differences in the pH profiles of the respective fermentations. The pH profile of the orange peel fermentation indicated that citric acid in the medium also acted as a buffer keeping the pH more or less stable around pH 6 (Figure 3.62b), while in the control fermentation, the pH dropped to pH 4 around the 20 h when the culture went into the stationary phase (Figure 3.9b). The rise in P(3HB) concentration coincided with the pH profile, suggesting that this pH drop could have inhibited sporulation as well as cellular utilisation of the stored P(3HB) (Philip *et al.*, 2009, Nakata 1963).

The total carbohydrate assay showed a similar profile for both fermentations (orange peel and control). Some peaks were observed in both total sugar profiles indicating the possible presence of other EPSs being simultaneously produced during the fermentation. These peaks could be clearly observed in the standard fermentation, around after 20 h whereas in the orange peel fermentation there were two peaks around 25 h and 45 h coinciding with the interval when P(3HB) production starts. It could be seen that the sugar was not used completely during the fermentation. This could be

because of the depletion of nitrogen source in the fermenter. This observation is also reported by Omar *et al.* (2001), showing that the carbon source (sucrose their study) was not consumed completely.

### 4.4.2 Dual production using orange peel as carbon source

The experience gained in previous experiments and knowledge obtained on production of both polymers as well as organism's behaviour in the orange peel medium were combined in order to obtain a strategy for dual production with orange peel (Section 3.6.5). However, the experiments based on replacing carbon source in optimised DP medium with orange peel infusion gave some unexpected results.

As presented in Figure 3.63a the amount of both polymers produced was low. When the fermentation profile was compared with the optimised dual production in DP medium (Figure 3.48), it was observed that there was a longer lag phase in the orange peel medium. This was expected since orange peel infusion medium is a complex medium composed of many ingredients as stated previously (page 235). Phenols and tannins present in orange peel could have caused a longer lag phase for the organism. Sawalha *et al.* (2009) studied the phenolic compounds present in bitter and sweet orange peel and found that, naringin (5.1 ± 0.4 mg/g), neohesperidin (7.9 ± 0.8 mg/g), narirutin (26.9 ± 2.1 mg/g) and hesperidin (35.2 ± 3.6 mg/g) are present as major phenolic compounds in bitter and sweet orange peels which can be potential inhibitors. However, the lag phase observed was only 3 h longer (7 h compared to 4 h in the DP medium) Final optical density and cdw measurements at the same time period of the fermentation were similar in orange peel and DP media. The optical density was slightly higher in orange peel compared to the DP medium.

Another interesting difference observed was the change in pH. The usual trend observed in all DP fermentation with glucose was a sudden drop coinciding to the exponential phase and a slow and steady increase in the pH following the commencement of the stationary phase. This increase usually continued until the end of 24 h of fermentation (generally until about pH 7) and started to decline again after that. In orange peel fermentation the pH followed a similar trend in the first 24 h but subsequently, instead of slowly declining, it continued increasing and after 27 h of fermentation, it exceeded pH 7.5.

Stain *et al.,* (2015) reported that apart from variety of sugars, orange peel contains nitrogen, ash, phenols, tannins, cellulose, pectin, hemicellulose, and lignin. As stated previously, Wang *et al.,* (2008) reported that the increase in pH in γ-PGA fermentation occurs due to ammonification of degradation of organic nitrogenous compounds such as amino acids. The dual production medium contains glutamic acid and yeast extract. Although, yeast extract by itself is rich in amino acids, the additional glutamic acid and yeast extract did not cause an excessive pH increase in optimised dual production (Figure 3.48b and 3.52b).

Townsley *et al.* (1953) investigated the amino acid composition of orange peel. They reported that the main amino acids found in the peel are alanine, γ-aminobutyric acid, asparagine, aspartic acid, glutamic acid, leucine, phenylalanine, proline, serine and valine. Therefore, the rich amino acid content of orange peel in this case may be the reason for the increase in pH.

When the pH was controlled after the first 24 h of orange peel fermentation, it was observed that P(3HB) production slightly increased but was not yet as expected. The culture pH increased above pH 7.5 within first 24 h and no γ-PGA was produced.

When a simulated medium consisting of a mixture of sugars at the same ratios as orange peel was formulated, 30% more P(3HB) and approximately same amount of γ-PGA was obtained. In addition, pH did not rise above pH 7 and followed the exactly same profile as it did in the DP medium fermentation. Therefore, it was suggested that it was not the sugars causing the pH increase and preventing production of both polymers.

Subsequent experiments conducted by controlling the pH provided promising results. Although, both polymers were produced at levels lower than those obtained in optimised DP medium, it was demonstrated that it is possible to produce both polymers using orange peel as carbon source in single fermentation.

Studies on the dual production of biopolymers using bio-wastes are very limited in literature. Quagliano and Miyazaki (1999) investigated production of P(3HB) and EPS with *Azotobacter chroococcum* strain isolated from soil samples, using a complex carbon source, molasses. P(3HB) accumulated in the cells while EPS was produced from the start for 24 h. After 48 h of fermentation, P(3HB) started to degrade, but EPS continued to be produced. When sugar cane molasses was used, 2.75 g/L P(3HB) and 1.5 g/L EPS was obtained from the single stage fermentation in the shaken flasks.

A recent and successful dual production example is by Devi *et al.,* (2012). They used rice bran hydrolysate as carbon source for the production of P(3HB) and PHV as well as EPS in a single stage fermentation conducted in the shaken flasks. P(3HB) and PHV were accumulated inside the cells in varying compositions and EPS was secreted outside by *Sinorhizobium meliloti*. At the end of 72 h fermentation, they reported a production of 3.6 g/L P(3HB)/PHV mixture and 11.8 g/L EPS.

The production of dual biopolymer in a fermenter using a biowaste such as orange peel has never been reported before in literature.

### 4.5 Separation of Biopolymers from Culture Broth

Being able to produce two different biopolymers from a single batch of fermentation is a desirable and attractive method. However, as explained in Section 1.5, there are some challenges associated with it. Since both, the broth and the biomass, contain a valuable product, separation and recovery of both products, with minimum loss needs to be conducted carefully. In order to address these challenges, three different approaches have been tested in this thesis. Those are exposure of the culture to magnetic field, separation by floatation and separation by sedimentation.

#### 4.5.1 Separation using static magnetic field

The interaction of static magnetic field (SMF) with living organisms is a rapidly growing field of investigation. However, despite the increasing number of studies on the effects of the interaction of SMFs with living organisms, many gaps still remain. In literature researchers have claimed that exposure of mild static magnetic field activates ions within the cell membrane making the cell membrane more leaky. Galvanovski and Sandblom (1998) suggested that the static magnetic field effects calcium signalling pathways and particularly cytosolic calcium oscillators which cause $Ca^{2+}$ release to the medium.

Fojt *et al.,* (2004) discussed the effects of magnetic field on bacteria and argued that the main theories that try to explain the biological effects of electromagnetic fields are based on the possible effects on the permeability of the ionic channels in the membrane. This can affect ion transport into the cells and result in biological changes in the organisms (Fojt *et al.,* 2004).

This concept was tested in the set up shown in Figure 3.67. From the results presented in Figures 3.68 and 3.69, an interesting observation was made on the difference in

optical density and cdw measurements of the control and the test cultures. It could be seen on Figure 3.68 that the culture exposed to the static magnetic field attained a lower optical density compared to the control (at 23 h: $OD_{600}$ test was 10.88; $OD_{600}$ control was 15.72).

Kohno and co-workers (2000) studied the effect of static magnetic fields on bacteria *Streptococus mutans*, *Staphylococcus aureus* and *E. coli*. They reported that the exposure to magnetic field (0 to180 mT) caused strength-dependent decreases in the growth rate and number of bacteria for *S. mutans* and *S. aureus* when cultured under anaerobic conditions, but that their growth was not inhibited under aerobic conditions. *E. coli* cultures were not affected by magnetic field (Kohno *et al.,* 2000). Strašák *et al.,* (2002) studied the effects of low-frequency magnetic fields on *E. coli*. They reported that when the bacterium was exposed to a magnetic field of between 2.7-10 mT the growth curve of the exposed culture was lower than the control. The ability of bacteria to form colonies decreased with increasing magnetic field intensity and with increasing time of exposure. They concluded that the effect of magnetic field was bactericidal. The question of how the magnetic field can kill or negatively affect bacterial growth is still unsolved. The main theories that try to explain the biological effects of electromagnetic field are based on the possible effects on the permeability of the ionic channels in the membrane which can lead to problems with ion transport into the cells and this can result in biological changes in the organisms. The other possible effects are the formation of free radicals due to magnetic field exposure (Fojt *et al.*, 2004).

In the culture exposed to magnetic field almost no P(3HB) was detected in the cells, and since the polymer was expected to leak outside the cells, the supernatant was also tested for the P(3HB). However, again almost no P(3HB) was detected in culture supernatant.

Therefore it was concluded that, exposure to magnetic field inhibited polymer production. However, SEM images of the bacteria revealed that the magnetic field exposure has caused or induced the production of some type of extracellular matrix. This matrix is usually referred to as biofilms. Biofilms are characterized by two features: the cells within the aggregates are non-motile, and they produce an extracellular polysaccharide (EPS). The Gram positive soil bacterium *Bacillus subtilis* is a model organism for the study of biofilm formation. *B. subtilis* biofilms are manifested either as floating pellicles or as colonies with complex architecture (Guttenplan *et al.,* 2010). Therefore it can be concluded that static magnetic field has initiated the biofilm formation and aggregation of *B. subtilis* OK2. Since this was out of the scope if this study and P(3HB) formation was inhibited, the topic was not pursued any further.

### 4.5.2 Separation using floatation and sedimentation

As explained in Section 1.5 separation of the product from fermentation broth contributes significantly towards the manufacturing costs in bioprocesses. Upon completion of P(3HB) production in the culture, separation of cell mass from the liquid phase (the cultivation broth) is conducted by well-established techniques like sedimentation, filtration, centrifugation, or flocculation. Since in the dual production, the cell mass and the culture liquid both contain valuable products, the choice of separation methodology needs to take this into consideration. In Section 3.7.2 and 3.7.3, application of floatation and sedimentation for the separation of cells (hence the P(3HB)) from the culture broth (containing γ-PGA) were investigated.

Zhang *et al.* (2015) studied foam separation as a novel method for integration of fermentation and cell separation. They suggested foam separation as a convenient strategy for cell recycle. This was one of the ideas behind the application of foam

separation to double polymer fermentation. When cells accumulate the polymer, their shape and properties undergo some changes. As demonstrated, the polymer content can constitute 30% of the bacterial mass in this thesis. From the examples in literature, this can go up to 90% (Magdouli *et al.,* 2015). The polymeric mass takes up most of the space in the cell cytoplasm. Horowitz and Sanders (1994) reported that the density of the polymer inclusion varies between 1.17-1.18 g/cm$^3$. This could be used as a potential for separation of polymer containing cells from polymer-free cells.

Compared to classic separation technologies, such as extraction, ion-exchange adsorption, evaporation and membrane separation, foam separation has many advantages. It avoids the loss of biomass due to organic solvent toxicity. In the foam separation, the rising bubbles contribute also to the homogenization of the fermentation broth. In most aerobic fermentations, the bubbles provide oxygen for cells (Zhang *et al.*, 2015). As early as 1958, Boyles and Lincoln reported the use of floatation for separation of vegetative cells and spores exploiting size differences. The apparatus they used was an early but similar version of what was used in this thesis. The high foaming tendency of the fermentation broth, which was a challenge in Section 3.5.3, was exploited as a cost-benefit for the process through the use of foam separation. The successful application of the foam separation process in this thesis would have led to a system for fermentation coupled with foam separation similar to Zang's method (Zhang *et al.,* 2015). Results presented in Section 3.7.2 demonstrated the effect of single and multi-orifice spargers on the separation of polymer-containing and non-polymer containing cells. However, the results were negative. When the cdw and P(3HB) contents were analysed (Table 3.16) it was observed that the liquid phase contained more %P(3HB)/cdw compared to the foam which was the opposite of what expected. Non-polymer containing mass was carried out by the foam.

The effect was enhanced with the use of multi-orifice sparger. This finding regarding the sparger type was in line with the findings of Boyles and Lincoln (1958). Post foaming appearance of the culture under microscope (Figure 3.73) revealed some spherical particles in the culture and the broth was dominated with these spherical particles rather than cells. Since the liquid fraction contained more polymer than the foam fraction, it indicated that these spherical particles might be polymer inclusions released from the cells thus implying that the sparging might have caused some damage to the cells. Further investigation of this phenomenon revealed that the cells were actually disintegrating in the fermenter, before they were subjected to separation. At later stages of the project when these spherical particles were further investigated, it was found that they were native P(3HB) granules(Section 3.7.4). This finding subsequently explained the results obtained in the floatation experiments.

When the cells disintegrate, cell debris scatters around along with spores formed. The microscopic observations showed that floatation selectively removed cell debris and spores from the culture supernatant leaving P(3HB) granules in the broth. Van Hee *et al.* (2006) studied the selective dissolved-air flotation for the separation of medium-chain-length PHA inclusion bodies (or granules) from *Pseudomonas putida* cell debris. They used microscopic observation to ascertain the aggregation behaviour, and the flotation behaviour was determined as a function of pH and aluminium chloride concentration. They stated that addition of aluminium chloride was due to its cationic multivalent species that can reduce electrostatic interactions. They reported that both *P. putida* cell debris and PHA granules have an iso-electric point of approximately pH 3.5. Selective aggregation and as a result selective flotation of PHA granules were observed near this pH (Van Hee *et al.,* 2006).

Reducing the pH or addition of $AlCl_3$ to the fermentation broth was not considered in this thesis due to the nature of the dual production system. Fermentation broth was at pH 6 at the end of the fermentation when the broth was subjected to floatation. Any further reduction in pH would cause the γ-PGA to precipitate from the solution along with P(3HB) granules. This was undesirable; the co-precipitation of both products complicated the separation. Also, the addition of $AlCl_3$ would create a problem with respect to product purity threatening PHAs medical application potential. Van Hee *et al.* (2006) also worked with mcl-PHAs which they reported as having lower densities (1g/cm$^3$) compared to P(3HB) (1.17-1.18 g/cm$^3$ - Horowitz and Sanders, 1994).

The negative results obtained with floatation, lead to the continuation of the search for a suitable separation method for polymer-containing and non-containing cells. Preliminary experiments (Figure 3.74) for sedimentation showed three distinctive regions appearing within 4 h. The cdw and P(3HB) content analysis showed that there was considerable cellular mass located at the middle section with a higher polymer content compared to the bottom section. This behaviour was further confirmed using a beaker set up as described in Figure 2.3. Results from the beaker set-up showed that the separation of the cells was feasible again in 4 h and more polymer-containing cells were accumulating at the middle section. These preliminary results were encouraging to pursue the topic further. In search of a system to be linked to the fermenter in order to separate cells, and recycle them, more elaborate set-ups were investigated.

An online separation system was designed to be linked to the fermenter, to exploit the sedimentation behaviour. The idea was to find the location in the separation chamber where the highest polymer containing bacteria gather so that they could be withdrawn from the system whereas the ones which did not contain enough polymer or were at the

earlier stages of the polymer accumulation could be recycled into the fermenter. Therefore, determination of the settling time and the exact location for high polymer containing cells for the removal from the system was crucial. As discussed in Section 2.9.2 and explained in detail in Section 3.7.3, a taller sedimentation column with several side ports was used for the next sedimentation experiment. Since the aim of the experiment was to link the sedimentation column to the fermenter, the separation was intended to be done in a volume as small as possible. Although the aim was to minimise the settling time, the volume of the culture broth to be kept outside the fermenter and recycled back could have become an issue due to the lack of mixing and aeration. Keeping a large volume of the culture broth outside the fermenter for sedimentation would create oxygen limited conditions and change in the culture temperature, and these would negatively affect the production. As stated in Section 3.7.3, the total volume of the first two set ups was 500ml. Sampling at every half an hour between 2 to 4 h from five different locations showed that the separation was possible at 4 h (Figure 3.77). Also, due to the fluctuations in the samples taken at different intervals, it was not possible to calculate the settling time. The fact that the zones were not very clear until about 4 h, made it impossible to have an efficient integrated fermenter-separator system. It was argued that due to the smaller cross-sectional area of the column, laminar flow conditions could not be established. Removal of samples caused further disturbance in the column, delaying the settling process. In addition, samples (5ml each) taken at close intervals decreased the total volume of the liquid considerably, creating an inaccuracy about the liquid height readings.

Moreover, the small cross-sectional area accentuated the wall effect and further delaying the sedimentation process and the establishment of equilibrium.

When the diameter of the particles become significant with respect to the diameter of the container, (in the case of bacterial separation, when the container diameter is very small or the culture is very dense), the particles are exposed to wall effects (Di-Felice and Parodi, 1996; Garside and Al-Dibouni, 1977; Richardson and Zaki, 1997). To test these arguments, a final sedimentation experiment was carried out in a larger sedimentation tank described in Section 2.9.3. The results obtained were similar to the beaker experiment but were not sufficient to enable the tank to be connected to the fermenter. The differences between the layers were not sharp enough to distinguish the zones.

None of the designs tested for sedimentation could improve the separation nor shorten the time required for the sedimentation. Over all, despite the previous findings, the sedimentation experiments yielded negative results with respect to on-line separation of polymer containing cells. However, in all experiments an unexpected increase in the average %P(3HB)/cdw values was observed (Section 3.7.3). Since the auto-lysis behaviour was not known at the time of the sedimentation experiments it was not possible to explain the uncertain behaviour and inconsistent results. This can be explained through the later experiments through the existence of the native P(3HB) granules.

It was observed that the bottom layer in the preliminary experiment (Figure 3.74) was in fact cell debris. When the samples and sedimentation zones were investigated in all setups it was found that cell debris was settling to the bottom of the sedimentation columns or vessels and the P(3HB) granules were floating. The bottom part was accumulating at the location which was labelled as “end valve”. The high cellular mass measured at the end valve location was also presented in Figure 3.80b. As discussed

with the floatation section, the negative results obtained for the sedimentation experiment were also due to the auto-lysis behaviour. In all set-ups it was tried to separate the polymer containing cells from cells which do not contain polymer. Since it was found that the cells were disintegrating during the fermentation, the entities settling were the cell debris and P(3HB) granules. These granules were found to be quite persistent in the fermentation broth (Figure 3.81).

Rehm (2003) studied the biosynthesis of microbial PHA granules. He described the granules as water-insoluble cytoplasmic nano-sized inclusions formed with an amorphous polyester core and polyester synthase covalently attached to the surface. The PHA granules are surrounded by a phospholipid membrane and sizes vary from 200 to 700nm. He also reported that it was still unclear whether larger granules occurred due to fusion events or whether simple increase in size took place based on continuous polymerization (Rehm, 2003).

Prabhu *et al.* (2010) reported similar results for the PHA granules obtained from *Bacillus* sp. NQ-11/A2. They indicated that PHA granules were spherical and intact in shape. These granules were 0.51 ± 0.074 µm is size. There were three to nine granules in each cell. These granules were either single or in budding groups (Prabhu *et al.*, 2010). All these observations and findings from the literature confirmed the existence of P(3HB) granules.

Characterisation of the granules was presented at the end of the results chapter (Section 3.7.4). Lundgren *et. al.* (1964), studied the P(3HB) granules and their characteristics. Similar to the findings of this thesis, they reported that the granules exhibit Brownian motion in liquid culture visible under microscope. Size distribution analysis showed that

the particle sizes vary between 50 nm to 800 nm. The majority of the particles are between the ranges of 100 to 300 nm (Figures 3.82 and 3.83).

Furthermore, a link between the autolysis behaviour of *Bacillus* cells with sporulation has been reported (Brenda *et al.* 2001; Muchová *et al.*, 2011), and also discussed in this thesis in Section 4.3. Brenda *et al.*, (2001) reported that the auto-lysis behaviour is triggered with the induction of sporulation. In the microscopic observations presented in Figure 3.32 and 3.84, it was seen that the sporulation and auto-lysis behaviour only happen in the DP medium. When the culture was grown in PHA medium, the individual cells were observed even after 200 h. Also, sporulation in the DP medium was induced at much earlier stages of culturing compared to PHA medium. Figure 4.4 compares the cultures at the same time of incubation in PHA and DP medium. The spores formed can be seen in DP medium after 24 h (bright oval inclusions shown with red arrows) but these cannot be observed in PHA medium at the same time of incubation. This also suggests that in DP medium, P(3HB) accumulation and sporulation happens simultaneously.

Dingman and Stahly (1983) reported that glucose was required for sporulation and suggested a new medium promoting sporulation of *Bacillus larvae*, a poorly sporulating strain. More than $10^7$ spores per ml of culture were formed when the glucose concentration in the medium was between 16.7 and 55 mM (the highest concentration tested). Heat-resistant spore counts corresponded with direct microscopic spore counts, showing about 17 times higher spore formation. They also reported that yeast extract was required for growth and had to be balanced for sporulation. Efficient sporulation ($>10^7$ spores per ml of culture) occurred only in the presence of 1.5 to 2.25% yeast extract. They suggested that failure to sporulate at high yeast extract concentrations was

probably due to excessive acid accumulation in the medium. The addition of 9 mM of sodium pyruvate and 0.03 M tris-maleate gave the best sporulation results.

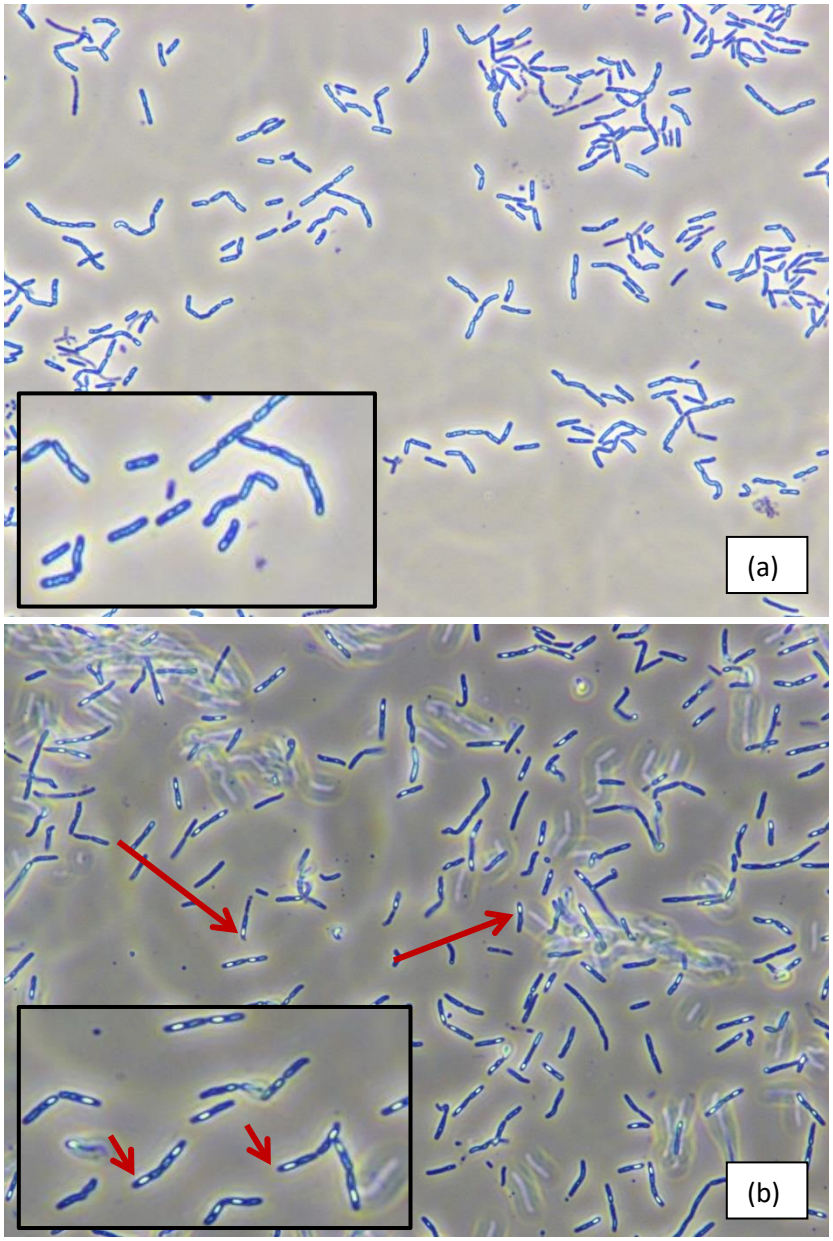



**Figure 4. 4:** Microscopic observations of 24 h cultures of *Bacillus subtilis* OK2 in (a) PHA medium and (b) DP medium. Spores formed in DP medium are shown with red arrows.

In this thesis, the glucose concentrations of DP and PHA media were the same, but yeast extract concentration was lower in the DP medium compared to PHA medium. In addition, PHA medium contained Soytone (enzymatic digest of soybean meal) as an

additional nitrogen source. Based on the findings of Dingman and Stahly (1983), presence of high levels of nitrogen source in the PHA medium (more yeast extract and soytone) could be the reason for the late or even non-sporulating behaviour of the organism in the PHA medium.

In addition, the clusters observed in Figure 3.32 and discussed on the Figure 4.3 were not visible in PHA medium. Bajaj and Singhal (2011b) studied flocculation properties of γ-PGA produced from *Bacillus subtilis* isolate. They reported that the γ-PGA produced by the organism had a molecular weight of $6.2 \times 10^6$ Da and showed excellent flocculation activity in the kaolin suspension. This could suggest that maybe γ-PGA in the medium is actually contributing the cluster formation of the spores. The contribution of the EPSs to spore cluster formation has been suggested already in literature (Brenda *et al.* 2001) but up to date there have been no reports on such effect of γ-PGA.

As can be seen from very limited literature, the formation of P(3HB) granules and their exploitation as a method of polymer separation from the culture broth is a new and exciting route. Fermentation conditions triggering the early onset of cell auto-lysis will prove to be a useful tool in dual product separation.

# Chapter 5: Conclusion

## 5.0 CONCLUSION

The aim of this thesis was the dual production of biopolymers, PHA and γ-PGA from cheap substrates with the view to lay grounds for a feasible, innovative, low cost production process for the two commercially important polymers.

Two strains were identified to be suitable for the dual biopolymer production and further comparison showed that *Bacillus subtilis* OK2 was the most suitable one for the purpose. The structures of both biopolymers were confirmed using various analytical methods. It was found that the intracellular polymer produced by *Bacillus subtilis* OK2 is a homo-polymer, P(3HB), and extracellular polymer is γ-PGA with a lower molecular weight compared to the standard γ-PGA obtained from Sigma (mw ≥1,000,000).

Using statistical design tools the important medium components for the dual production by *Bacillus subtilis* OK2 were identified to be glucose and yeast extract for P(3HB) and citric acid, ammonium sulphate and glutamic acid for γ-PGA. Further investigation on effects of yeast extract and citric acid demonstrated that both medium components enhance P(3HB) production. Up to 5 g/L citric acid promoted P(3HB) production and levels above 5 g/L inhibited cell growth. The possibility of using citric acid for enhancing production has been discussed in literature, but its effect on P(3HB) and cell growth has not been demonstrated as clearly as in this study. The role of yeast extract was found to be not just limited to increasing cdw, but its addition to the culture medium after 48 h of cultivation further promoted P(3HB) accumulation. This is the first time where an exact time is reported for the addition of complex nitrogen sources to enhance PHA production.

The CCD used for medium optimisation was found unsuitable for the optimisation of dual production of P(3HB) and γ-PGA system. Based on these observations it is concluded that current statistical optimisation tools are insufficient to optimise and

model the dual production. The mechanism for the simultaneous production of the two polymers is far more complex than to be modelled using simple quadratic equations, and requires more comprehensive tools. However, simplification of the variables for the dual production enabled the use of CCD for optimisation of the dual production, indicating that CCD can work on simpler models successfully.

Medium composed of 20 g/L glucose, 1.5 g/L yeast extract, 2.4 g/L citric acid, 32 g/L glutamic acid and 12 g/L ammonium sulphate was identified as dual polymer production medium. Such a thorough evaluation of the dual production system has not been reported in literature, neither as experimental observation nor as CCD modelling. In addition, the knowledge obtained from the dual production of the two polymers of interest resulted in the suggestion of a possible integration of the two metabolic pathways showing the interdependencies of the two polymers.

Furthermore, investigation of inoculum conditions showed that using an inoculum medium different from the production medium can have a positive effect on the production. The use of culture grown in γ-PGA medium as inoculum for the dual polymer production can notably decrease the lag phase and improve the production. There is no such study presented in literature so far reporting effect of different inoculum media on polymer production.

As a result of optimisation and inoculum condition studies it was concluded that the production of dual biopolymers, P(3HB) and γ-PGA, from a single batch is possible. Following the method presented, 1 g/L P(3HB) and 0.4 g/L γ-PGA was produced in single batch with the strain *Bacillus subtilis* OK2 in shaken flasks.

Scaling up of dual polymer production experiments showed that the production is oxygen dependent. When DOT falls below 10%, P(3HB) and γ-PGA are not produced. The culture is very susceptible to foaming therefore foam control must be applied. The

minimum anti-foam addition to control foaming can be achieved using a dissolved oxygen control strategy of maintaining 25% DOT during the first 24 h followed by dropping the level of DOT to 10%. Contrary to shaken flask studies, the production of the two polymers occurred simultaneously. It was concluded that using the optimised dual polymer medium and the defined inoculum conditions, along with the dissolve oxygen maintenance strategy, it was possible to produce 0.6 g/L P(3HB) and 0.2 g/L γ-PGA in a 48 h batch fermentation.

Different bio-wastes and pre-treatment methods were tested in order to reduce the costs of dual polymer production. Among the four different bio-wastes and four pre-treatment methods investigated, orange peel pre-treated with water infusion method, was found the be the most suitable bio-waste for the production of P(3HB). γ-PGA production was not detected with these bio-wastes as the sole medium component. The sugar composition of orange peel infusion was identified to consist of 58% water soluble carbohydrates (mainly glucose, fructose and sucrose) and 0.89% total nitrogen. It was concluded that it is possible to produce 1.24 g/L P(3HB) using orange peel, supplemented with citric acid and yeast extract in a 72 h batch fermentation.

Dual polymer production using orange peel as carbon source proved to be more challenging as some of the ingredients in orange peel interfered with the dual production and inhibited production of both polymers. Although it was found that the different sugars in orange peel had a positive effect on production, pH control coupled with dissolved oxygen control proved to be essential to overcome the inhibition, when orange peel was used as carbon source. Adopting the optimised conditions obtained for the double polymer production in dual polymer medium, and maintaining the pH at 6.5, it was possible to produce 0.2 g/L of each polymer in single batch fermentation in 79 h

using orange peel. The dual production of biopolymers in a fermenter using orange peel has never been reported before in literature.

For the separation of the two polymers from the culture broth, magnetic field, floatation and sedimentation methods were investigated. Exposure to magnetic field was found to be inhibitory for P(3HB) production. No target polymer was detected in the cell or in the supernatant. However, SEM images revealed that EPS production was triggered under static magnetic field. Subjecting the culture broth to sedimentation or floatation at the end of the dual polymer fermentation, yielded unexpected results which were explained by the presence of the aforementioned granules. The sizes of these granules were found to be between 50 to 800 nm. When the granule intensity data was evaluated together with size data, it was found that the granules above 200 nm in diameter, contained significant amounts of polymer. The granules were observed to be released into the culture broth upon initiation of cell lysis. In addition the cell lysis behaviour of *Bacillus subtilis* OK2 was found to be a medium-dependent response. The cell lysis and release of granules occurred only in the optimised dual production medium. A relation between cell lysis and sporulation was noted and the time of cell lysis was found to be dependent on the time of the induction of sporulation. The exploitation of P(3HB) granules as a method of polymer separation from the culture broth is suggested as a new approach. Fermentation conditions triggering the early onset of cell auto-lysis can be a useful tool in dual product separation that can reduce the downstream processing costs.

In conclusion, the data presented in this study, provides insight into the complexity of the dual polymer production mechanism, as well as on the effects of medium components in the dual production. It also highlights the opportunities and challenges associated with the use of bio-wastes for the simultaneous production of P(3HB) and γ-PGA. In addition, the results elicit the possibility of using cell auto-lysis behaviour and

P(3HB) granules for the separation of the two polymers from the culture broth leading to a reduction of costs.

In conclusion, the new findings of this study highlights:

1- A better understanding of the effect of medium components on the dual polymer production and its mechanism for the target polymers

2- Opportunities and challenges in using biowastes for the dual biopolymer production

3- Use of orange peel as a potential carbon source for the biopolymers production

4- As a longer term objective, using cell auto-lysis behaviour and P(3HB) granules for online product separation.

# Chapter 6: Future work

## 6.0 FUTURE WORK

This project has provided novel findings in biopolymer production leading to further research opportunities for potential industrial applications. In this context, a number of questions and potential areas of further study on dual production of biopolymers has been identified. These may be grouped under six main headings;

- Strain focused studies,
- Studied on improvement of statistical optimisation tools,
- Studies on metabolic engineering and proteomics,
- Studies on fermentation strategies,
- Strategies on dual production purely from biowastes,
- Studies on separation strategies.

This project proved that, using appropriate isolates, it is possible to produce double polymers (P(3HB) and γ-PGA) from cheap agricultural biowaste. However, for industrial application further studies are needed to obtain robust high producer strains preferably with low oxygen demand. This could be achieved through an extensive strain selection and identification programme. Genetic engineering approaches, where successful, will provide more sophisticated product opportunities, but the stability and robustness of the designed strains, as well as their fulfilling environmental regulations are important points to consider.

In selecting the strains, prior to any optimisation or process scale-up, their lipoglycan and lipoteichoic acid content must be investigated. These are components with immunogenic properties similar to LPS and thus extra separation may be required which might affect the economics of the process.

The use of statistical optimisation tools and their limitations for dual production processes have been demonstrated in this work. In order to fully utilise statistical tools certain simplification methods were adopted. Although the medium was optimised according to the conditions given by the program (Design Expert 6.0), further investigation of the fixed components (glucose and yeast extract), and their interactions with other medium components (glutamic acid, ammonium sulphate and citric acid) may lead to improved productivities. The limitations of the statistical optimisation tools, as mentioned earlier, is a serious drawback. However, the interactions between each medium component revealed by the statistical tools provide a better picture of culture metabolism. The findings of this project clearly indicated the need for better and more comprehensive software that can overcome these limitations. Modelling the production with quadratic equations is not suitable for dual production. Models with two dependent variables should be adopted and used for modelling of this process. This requires a multidisciplinary approach, where the biochemical engineers using the program would provide necessary information to software engineers in order to produce a new improved version of the statistical optimisation tools.

While the metabolic pathways leading to the production of the two biopolymers is known, manipulation of the pathways through metabolic engineering may provide higher productivities. In this context, proteomics studies can provide the necessary details for subsequent pathway manipulation. For example, identification of proteins that are expressed when the culture is grown in double polymer medium and comparison with the proteins in single polymer production systems. As seen earlier in the case of fermentation with citric acid as an ingredient, mapping the pathways for dual polymer production and identifying the key enzymes involved in the pathways may help to further increase the production. Also, since glutamic acid was found not to be

consumed, the exact role of glutamic acid in *Bacillus subtilis* OK2 strain for γ-PGA production and for dual polymer production should be investigated.

Changing the medium in which the inoculum was grown showed a significant decrease in lag phase and an increase in total biomass. In addition, the time of incubation of the inoculum medium deserves further elaboration.

Bioreactor studies showed that double polymer production with the chosen strain was oxygen dependant. In the absence of oxygen no polymer was produced. This was only observed in dual production medium. Oxygen, as one of the electron accepters in TCA cycle, has a role in metabolic pathways. Through proteomics studies, its effect on protein, and enzymes expressions could be determined. This need to be investigated together with other medium components and their interactive role should be revealed.

Addition of inducers into the culture medium, as a fermentation strategy, needs to be investigated. In this work, the role of addition of complex nitrogen source on the production of P(3HB) was demonstrated along with the effect of citric acid addition. However, their effects on the dual polymer production are yet to be investigated. Citric acid addition into the culture medium promoted P(3HB) production. Based on the results obtained, its addition after a certain time of cultivation, may also have a positive effect on further utilisation of residual carbon source. This needs to be investigated. In addition, the effects of the inducers summarised in Section 1.3.4 for γ-PGA production can be explored in dual polymer production.

Throughout the project, batch fermentation mode was used for the production of the two polymers. Based on the data obtained on the effects of medium components, fed-batch operation mode can be an option to increase productivity. It was found that almost half of the carbon source was not utilised at the end of the fermentation. Addition of small

quantities of complex nitrogen sources and citric acid may initiate utilisation of the remaining carbon source, and increase productivity. According to the findings in this work, the addition should be between 24 to 48 h of fermentation.

When the yield and specific rate curves were investigated it was observed that the production of P(3HB) started after the exponential phase. Therefore, continuous production does not look like a viable option. However, in specific production curves, a point at which specific growth rate and specific production rates for both polymers are positive can be observed. At that point the values are very small showing that a continuous culture with low dilution rate could be operated. This needs to be tested to see if the productivity can match the costs associated with it. A recycle process could also be considered.

Results presented in this thesis showed that orange peel is a rich biowaste suitable as a replacement for conventional carbon sources. Use of orange peel for P(3HB) and dual polymer production was demonstrated. P(3HB) production was optimised and conducted in small-scale controlled fermenters. Since the production level was satisfactory for the studied strain, scale-up of the system should be conducted and the system should be tested at pilot scale. The strain used in this study was for the investigation of the potential for dual production of the target biopolymers; it was not a high producer for P(3HB). So for future studies, it can be replaced with a suitable industrial strain (if available) to investigate the process at high production levels. In addition, alkaline treated rapeseed cake was found to enhance biomass production. A strategy to use alkaline treated rapeseed cake together with orange peel can be adapted in order to construct a medium solely composed of biowastes. Orange peel maybe used as carbon source to trigger PHA biosynthesis, and treated rapeseed cake can be used to

boost growth and biomass formation. The pre-treatment parameters for both orange peel water infusion (temperature, biowaste amount, duration), and rapeseed cake alkaline treatment (temperature, pH, biowaste amount, duration) need to be optimised. Also, optimum ratio of orange peel to rapeseed cake needs to be determined. The dual production levels using orange peel were below the expected values. It was suggested that some ingredients in orange peel are inhibiting the productions of both polymers and instigate a gradual increase in pH. Although this was partially overcame by pH and oxygen control, further investigation is needed to find out what ingredient in orange peel is interfering with the production and causing pH increase.

Application of magnetic field to *Bacillus subtilis* OK2 culture was investigated as a separation strategy but did not give the expected results. However, the magnetic field generator constructed in this study may be used for future studies to evaluate the effects of magnetic field on bacterial or fungal cultures. The easy connection to any fermenter gives opportunities to adjust the magnitude, the exposure time and the direction of the magnetic field. In addition, SEM images taken in this study showed that exposure to static magnetic field triggered EPS production of *Bacillus subtilis* OK2. This behaviour needs to be investigated. The type of EPS, and the amount produced should be measured. In addition, EPS is known as one of the building blocks of biofilm formation. Therefore, effect of magnetic field on biofilm formation and biofilm control can be another topic of investigation. Overall, effect of low static magnetic field on production of industrially important metabolites is an area which should not be neglected.

The negative results obtained in floatation and sedimentation experiments confirmed that the P(3HB) granules in the broth and cell lysis behaviour of the organism were responsible for this outcome. The cell lysis was found to be dependent on the medium

in which the organism is cultured. The medium component or components responsible for triggering this behaviour need to be investigated. The relationship of these granules to the sporulation could be explored and included in the attempts to find reasons for the cell lysis. Investigation of the time lapse of protein expressions from the beginning of spore formation until the start of cell lysis may give insights into the whole process. Determination of the reasons for cell lysis and the conditions for the induction of cell lysis may help researchers to propose a self-disruption recipe which may lead to a new approach in cell disruption methods as well as contribute to a significant reduction in downstream processing costs. In addition, the granules, identified in this study, need to be characterised. The stability of the granules in fermentation broth, and the state of the polymer inside the granules should be determined. Finally, the granules suspended in the fermentation broth maybe separated using floatation techniques as proposed in the literature and as shown in this study. A possible online granule separator linked to the fermenter maybe designed to separate the granules and recycle the cells which are not lysed yet in order to increase the cell lysis efficiency.

# Chapter 7: References

## 7.0 REFERENCES


Abdel Fattah, Y.R., Soliman, N.A., Berekaa, M.M., 2007. Application of Box-Behnken design for optimization of poly-c-glutamic acid production by *Bacillus licheniformis* SAB-26. Research Journal of Microbiology, 2 (9), 664–670.

Abu Absi N. R. , Kacmar J. A. , Srienc F., Zamamiri A. 2004. High Throughput Bioreactor. University of Minnesota assignee. Patent US2004214314A. 28 Oct.

Adeboye, P. T., Bettiga, M., and Olsson, L. 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express, 4(1), 46.

Ahn, W.S., Park, S.J., Lee, S.Y., 2000. Production of poly(3-hydroxybutyrate) by fedbatch culture of recombinant *Escherichia coli* with a highly concentrated whey solution. Applied and Environmental Microbiology, 66, 3624–3627

Akaraonye, E., Keshavarz, T., Roy, I., 2010. Production of polyhydroxyalkanoates: the future green materials of choice. Journal of chemical technology and biotechnology 85 (6), 732-743.

Albuquerque, M., Eiroa, M., Torres, C., Nunes, B., Reis, M., 2007, Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. Journal of biotechnology, 130, 411-421.

Aldor, I. S., Keasling, J. D., 2003, Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. Current Opinion in Biotechnology 14 (5), 475-483.

Ameyama, M., and Matsushita, K. 1984. Vitamins and Hormones, 46, 229-267.

Anderson, A. J., Dawes, E. A., 1990, Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological reviews 54 (4), 450-472.

Anderson, M. J., and Whitcomb, P. J., 2007. DOE simplified: Practical tools for effective experimentation 2nd edition, Taylor & Francis group, CRC Press

Anderson, M. J., Whitcomb, P. J. 2005.RSM simplified: optimizing processes using response surface methods for design of experiments. Productivity press,

Aquin S., Vezina L. P. 2000 Method For Producing Polyhydroxyalkanoates In Recombinant Organisms. Ca minister agriculture and food assignee. Patent US6492134B. 15 Mar.

Aragao, G. M. F., Netto, W. S., Ienczak, J. L., Fiorese, M. L., Dalcaton, F., Schmidt, F., Deucher, R., Vecchi, C., Rodrigues, R. C., 2008. Preparation of PHA (Polyhydroxyalkanoates) From a Citric Residue. Google Patents

Arikan, E. B., and Ozsoy, H. D. 2015. A Review: Investigation of Bioplastics. Journal of Civil Engineering and Architecture ,(9) 188-192

Ashby R. D. , Solaiman D.K. Y. , Strahan G.D., 2015. Production Of Tunable Polyhydroxyalkanoate Biopolymers Using Glycerol And Levulinic Acid. Secretary of Agriculture as US gov assignee. Patent US8980593B. 17 March.

Ashiuchi, M. and Misono, H. 2002, Biochemistry and molecular genetics of poly-γ-glutamate synthesis. Applied microbiology and biotechnology, 59.1: 9-14

Atlić, A., Koller, M., Scherzer, D., Kutschera, C., Grillo-Fernandes, E., Horvat, P., Chiellini, E. and Braunegg, G. 2011. Continuous production of poly ([R]-3-hydroxybutyrate) by *Cupriavidus necator* in a multistage bioreactor cascade. Applied microbiology and biotechnology, 91, 295-304.

Bajaj, I. and Singhal, R. 2009a Enhanced production of poly(γ-glutamic acid) from *Bacillus licheniformis* NCIM 2324 by using metabolic precursors. Applied Biochemistry and Biotechnology, 159(1), pp.133-141.

Bajaj, I. and Singhal, R. 2009b Sequential optimization approach for enhanced production of poly(γ-glutamic acid) from newly isolated *Bacillus subtilis.* Food Technology and Biotechnology, 47(3), pp.313-322.

Bajaj, Ishwar, and Singhal, Rekha. 2011a Poly (glutamic acid) an emerging biopolymer of commercial interest. Bioresource technology 102.10 : 5551-5561.

Bajaj, I. B., and Singhal, R. S. 2011b. Flocculation properties of poly (γ-glutamic acid) produced from *Bacillus subtilis* isolate. Food and Bioprocess Technology,4(5), 745-752.

Balaji, S., Kadiyala G., and Bhaskaran M.. 2013. A review on production of poly-β-hydroxybutyrates from cyanobacteria for the production of bio plastics Algal Research. 2, 278-285.

Beaulieu, M., Beaulieu, Y., Melinard, J., Pandian, S., and Goulet, J. 1995. Influence of Ammonium Salts and Cane Molasses on Growth *of Alcaligenes eutrophus* and Production of Polyhydroxybutyrate. Applied and Environmental Microbiology, 61(1), 165-169.

Bell J. M., and Keith M. O., 1990. A survey of variation in the chemical composition of commercial canola meal produced in western Canadian crushing plants. Canadian Journal of Animal Science 71:469-480.

Billington S.L , Christian S. J , Criddle C. S , Frank C.W , Morse M.C. , Pieja A. J., 2008 Bacterial Poly(Hydroxy Alkanoate) Polymer And Natural Fiber Composites. The Board Of Trustees Of The Leland Stanford Junior University assignee. Patent US2008160567A. 03 July.

Birrer, G.A., Cromwick, A.M., Gross, R.A., 1994. Poly(glutamic acid) formation by Bacillus licheniformis 9945a: physiological and biochemical studies. International Journal of Biology and Macromolecules. 16, 265–275.

Borah. B., Thakur, P.S. and Nigam A., 2002. The influence of nutritional and environmental conditions on the accumulation of poly-beta-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. J Applied Microbiology 92:776-783

Boyles, W. A., and Lincoln R. E., 1958, Separation and concentration of bacterial spores and vegetative cells by foam flotation. Applied microbiology 6(5) 327

Branard, G. N., Sanders, J. K. M. 1989 The poly-β-hydroxybutyrate granule in vivo. A new insight based on NMR spectroscopy of whole cells. Journal of biological chemistry, 264, 3286-3291.

Branda, S. S., González-Pastor, J. E., Ben-Yehuda, S., Losick, R., and Kolter, R. 2001. Fruiting body formation by *Bacillus subtilis*. Proceedings of the National Academy of Sciences, 98(20), 11621-11626

Braunegg G, Sonnleitner B, Lafferty RM, 1978, A rapid gas chromatographic method for the determination of poly(b-hydroxybutyric acid) in microbial biomass. Europian Journal of Applied Microbiology and Biotechnology 6:29–37

Brumley S. M., Brumbley S. , Prunell M. P. Petrasovits L. , Nielsen L., Mcqualter R., Chong B.. 2006.Transgenic Plants Used As A Bioreactor System Ugar Research Australia Ltd , Queensland Univ assignee. Patent US2006259998A. 16 Nov.

Brunner, K. H., and Hemfort, H. 1988. Centrifugal separation in biotechnological processes. Downstream Processes: Equipment and Techniques. Advances in Biotechnological Processes, 8, 1.

Buescher, J. M. and Margaritis, A. M. 2007. Microbial biosynthesis of polyglutamic acid biopolymer and applications in the biopharmaceutical, biomedical and food industries. Critical Reviews Biotechnology 27, 1– 19

Byrom, D. 1994 Polyhydroxyalkanoate, In: Mobley DP, editor, Plastics from microbes: microbial synthesis of polymers and polymer precursors, Hanser, Munich, pp 5–33

Candela, T., Fouet, A.. 2006, Poly-gamma-glutamate in bacteria. Molecular microbiology, 60.5: 1091-1098.

Castilho L. R., Mitchell D. A., Freire D. M. G., 2009, Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Biosource Technology 100:5996-6009.

Cavalheiro, J.M.B.T., Almeida, M.C.M.D., Grandfils, C., Fonseca, M.M.R., 2009. Poly(3-hydroxybutyrate) production by C*upriavidus necator* using waste glycerol. Process Biochemistry, 44(5), 509-515.

Chakraborty, P., Kasiviswanathan M., and William R. G.. 2012. PHA productivity and yield of *Ralstonia eutropha* when intermittently or continuously fed a mixture of short chain fatty acids. Biomedical Research International 2012

Chandel, A. K., da Silva, S. S., Carvalho, W., and Singh, O. V. 2012. Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products. Journal of chemical technology and biotechnology, 87(1), 11-20.

Chan-u-tit, P., Laopaiboon, L., Jaisil, P., and Laopaiboon, P. 2013. High level ethanol production by nitrogen and osmoprotectant supplementation under very high gravity fermentation conditions. Energies, 6(2), 884-899.

Chattopadhyay, D., Rathman, J. F., and Chalmers, J. J. 1995. The protective effect of specific medium additives with respect to bubble rupture.Biotechnology and bioengineering, 45(6), 473-480.

Chen J., Y. Zhang, G. C. Du, Z. Z. Hua, and Y. Zhu, 2007.Biodegradation of polyvinyl alcohol by a mixed microbial culture, Enzyme and Microbial Technology, vol. 40, no. 7, pp. 1686–1691,

Chen, G. Q. 2010. Plastics completely synthesized by bacteria: polyhydroxyalkanoates. In Plastics from bacteria Springer Berlin Heidelberg.Chen, G. Q. pp. 17-37.

Wu, Q. 2005. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials, 26(33), 6565-6578.

Chen, Guo-Qiang. 2009. A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. Chemical Society Reviews 38.8 2434-2446.

Choi B. H., Jeong Y.A. , Jung I. L. , Kim I. G., Kim K. C.,, Park H.K. , 2005. Recombinant E*scherichia coli* and overproduction method of polyhydroxybutyrate using the strain. Korea atomic energy research institute assignee. Patent US2005227340A. 13 Oct.

Christensen, B. E., Kjosbakken, J., and Smidsrød, O. 1985, Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas sp*. strain NCMB 2021. Applied and environmental microbiology, 50(4), 837-845.

Clayton, R., Jameson, G. J., and Manlapig, E. V. 1991. The development and application of the Jameson cell. Minerals Engineering, 4(7), 925-933.

Criddle C.S. , Hopkins G. D , Sundstrom E.R , Wu W. M. 2012.High Solids Fermentation for synthesis of polyhydroxyalkanoates from gas substrates. The board of trustees of the leland stanford junior university assignee. Patent US2012028321A. 02 Feb.

Criddle C. S .and Pieja A. J. 2009. Use of selection pressures to enable microbial biosynthesis of polyhydroxyalkanoates from anaerobic degradation products. Board of trustees of leland stanford junior univ assignee. Patent US2009317879A. 24 Dec.

Cromwick, A. M., Birrer, G. A., and Gross, R. A. 1996. Effects of pH and aeration on γ-poly (glutamic acid) formation by *Bacillus licheniformis* in controlled batch fermentor cultures. Biotechnology and Bioengineering, 50(2), 222-227.

Cuellar M. R. L., Flores A. J., Rodriguez J. N G., Guevara F. P., 2011, Production of polyhydroxyalkanoates with canola oil as carbon source. International journal of biological macromolecules, 48, 74-80.

da Silva, S. B., Cantarelli V.V., Ayub M.A. Za., 2014, Production and optimization of poly-γ-glutamic acid by *Bacillus subtilis* BL53 isolated from the Amazonian environment. Bioprocess and biosystems engineering 37.3 469-479.

Devi, E. S., Vijayendra, S. V. N., and Shamala, T. R. 2012. Exploration of rice bran, an agro-industry residue, for the production of intra-and extra-cellular polymers by *Sinorhizobium meliloti* MTCC 100. Biocatalysis and Agricultural Biotechnology, 1(1), 80-84.

Di Felice, R., and Parodi, E. 1996. Wall effects on the sedimentation velocity of suspensions in viscous flow. AIChE journal, 42(4), 927-931.

Di , C. F. X. , Feng J. , Gu Y. , Guan H. , L. Yizhen , S. C. , Wang K. , Wang S. , Zhang X., 2013. *Burkholderia* sp. and method for fermentation synthesis of PHA by adopting the same. Nankai Univ , Univ Nankai, assignee. Patent CN103232955A. 27 Mar.2013.

Dimitrijević, A., Veličković, D., Rikalović, M., Avramović, N., Milosavic, N., Jankov, R., and Karadžić, I. 2011, Simultaneous production of exopolysaccharide and lipase from extremophylic *Pseudomonas aeruginosa* san-ai strain: A novel approach for lipase immobilization and purification. Carbohydrate Polymers, 83(3), 1397-1401.

Dingman, D. W., and Stahly, D. P. 1983. Medium promoting sporulation of *Bacillus larvae* and metabolism of medium components. Applied and environmental microbiology, 46(4), 860-869.

Doi, Y., and Steinbüchel, A. 2001. Biopolymers, vol. 3a. Polyesters I: biological systems and biotechnological production.

Donkoh A., and Kotoku V. A., 2009. Nutritive value of feedstuff for poultry in Ghana: chemical composition, apparent metabolizable energy and ileal amino acid digestibility. Livestock Research for Rural Development 21, Retrieved October 18, 2011, from http://www.lrrd.org/lrrd21/3/donk21032.htm.

Douglas D. Edward , Pr. Y., 1997. Processes For Producing Polyhydroxybutyrate And Related Polyhydroxyalkanoates In The Plastids Of Higher Plants. Michigan State University East Lansing assignee. Patent US5610041A 11 Mar. 1997.

Du C., Sabirova J., Soetaert W., Lin S. K. C., 2012,Polyhydroxyalkanoates Production from Low-cost Sustaniable Raw materials. Current Chemical Biology 6 14-25

Du, G., Yang, G., Qu, Y., Chen, J., and Lun, S. 2005. Effects of glycerol on the production of poly (γ-glutamic acid) by *Bacillus licheniformis.* Process Biochemistry, 40(6), 2143-2147.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P., Smith, F., 1956, Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28, 350-356.

Eco-cycle, 2010, Environmental facts and Figures to inspire action toward zero waste. http://www.ecocycle.org/files/pdfs/Eco-CycleEnvironmentalFacts.pdf (accessed November 25, 2012).

Egli, T., and Zinn M.. 2003. The concept of multiple-nutrient-limited growth of microorganisms and its application in biotechnological processes. Biotechnology advances 22.1 35-43.

Eriksen, M., Lebreton, L. C., Carson, H. S., Thiel, M., Moore, C. J., Borerro, J. C., Galgani, F., Ryan, G. P., and Reisser, J. 2014. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PloS one,9(12), e111913.

Eriksson, U., and Häggström, L. 2005. Yeast extract from Express Five serum-free medium contains factors at about 35 kDa, essential for growth of *Trichoplusia ni* insect cells. Biotechnology letters, 27(20), 1623-1627.

Ersus, S., and Cam, M. 2007. Determination of organic acids, total phenolic content, and antioxidant capacity of sour *Citrus aurantium* fruits. Chemistry of Natural Compounds, 43(5), 607-609.

European Bioplastics, 2014. Institute for Bioplastics and Biocomposites, Nova-Institute. Production Capacity. Accessed May 1, 2015. http://en.european-bioplastics.org/market/market-development/production-capacity/.

Faccin, D. J. L., Rech, R., Secchi, A. R., Cardozo, N. S. M., and Ayub, M. A. Z. 2013. Influence of oxygen transfer rate on the accumulation of poly (3-hydroxybutyrate) by *Bacillus megaterium*. Process Biochemistry, 48(3), 420-425.

Fan C. and Zhao S. 1995. Preparation Of Betal-Polyhydroxybutyrate. Chengdu Inst Of Biology Chinese Academy Of Sciences assignee. Patent CN1104683A. 05 Jul. 1995.

Flora, G. D., Bhatt, K., and Tuteja, U. 2010. Optimization of culture conditions for polyhydroxybutyrate production from isolated *Bacillus* species. Journal of Cell Tissue Resources, 102, 2235-2242.

Fojt, L., Strašák, L., Vetterl, V., and Šmarda, J. 2004. Comparison of the low-frequency magnetic field effects on bacteria Escherichia coli, *Leclercia adecarboxylata* and Staphylococcus aureus. Bioelectrochemistry, 63(1), 337-341.

Francis, L. 2011. Biosynthesis of polyhydroxyalkanoates and their medical applications. University of Westminster, PhD Thesis.

Galvanovskis, J., and Sandblom, J. 1998. Periodic forcing of intracellular calcium oscillators Theoretical studies of the effects of low frequency fields on the magnitude of oscillations. Bioelectrochemistry and bioenergetics, 46(2), 161-174.

Garside, J., and Al-Dibouni, M. R. 1977. Velocity-voidage relationships for fluidization and sedimentation in solid-liquid systems. Industrial and engineering chemistry process design and development, 16(2), 206-214.

Gerth, U., Krüger, E., Derré, I., Msadek, T., and Hecker, M. 1998. Stress induction of the *Bacillus subtilis* clpP gene encoding a homologue of the proteolytic component of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. Molecular microbiology, 28(4), 787-802.

Ghate, B., Prashant, P., Kulkarni, C., Mungi, D. D., Patel, T. S., 2011, P(3HB) production using novel agro-industrial sources from different *Bacillus* species. International Journal of Pharma and Bio Sciences 2(3), 242-249.

Gitel Zon I I., Kalacheva G. S., Kuznetsov B. N., Shabanov V F., Volova T G., 2001. Method for Preparing polymer of ss-hydroxy- butyric Acid. Institut Biofiziki so ran assignee. Patent RU2207375C. 27 Oct. 2001.

Glumoff T. , Hiltunen K. 2002. Method to control cellular (3r)-hydroxyacyl-coa esters, precursor molecules for polyhydroxyalkanoate synthesis in genetically modified organisms. Oulun yliopisto assignee. Patent US2002173014A. 01 Feb. 2002.

Goto, A., and Kunioka M. 1992. Biosynthesis and hydrolysis of poly (γ-glutamic acid) from *Bacillus subtilis* IF03335. Bioscience, biotechnology, and biochemistry 56.7 1031-1035.

Gourmelon G., 2015 Global Plastic Production Rises, Recycling Lags. World watch institute, http://www.worldwatch.org/global-plastic-production-rises-recycling-lags-0 , Accessed May 2015.

Grant, C. L., and Pramer, D. 1962. Minor element composition of yeast extract.Journal of bacteriology, 84(4), 869.

Grothe, E.,. Moo-Young M, and Chisti Y., 1999. Fermentation optimization for the production of poly (β-hydroxybutyric acid) microbial thermoplastic. Enzyme and Microbial Technology 25.1 132-141.

Guo, W., Song, C., Kong, M., Geng, W., Wang, Y., and Wang, S. 2011, Simultaneous production and characterization of medium-chain-length polyhydroxyalkanoates and alginate oligosaccharides by *Pseudomonas mendocina* NK-01. Applied microbiology and biotechnology, 92(4), 791-801.

Guttenplan, S. B., Blair, K. M., and Kearns, D. B. 2010. The EpsE flagellar clutch is bifunctional and synergizes with EPS biosynthesis to promote *Bacillus subtilis* biofilm formation. PLoS genetics, 6(12), e1001243.

Guzmán, G., Van-Thuoc, D., Martín, J., Hatti-Kaul, R., Quillaguamán, J. 2009. A process for the production of ectoine and poly(3-hydroxybutyrate) by *Halomonas boliviensis*. Applied Microbiology Biotechnology. 84:1069–1077

Haas R., Jin B., Zepf F.T., 2008. Production of Poly(3-hydroxybtyrate) from waste potato starch. Bioscience Biotechnology Biochemistry, ,72, 253-256.

Hageman, J. H., Shankweiler, G. W., Wall, P. R., Franich, K., McCowan, G. W., Cauble, S. M., ... and Quinones, C. 1984. Single, chemically defined sporulation medium for *Bacillus subtilis*: growth, sporulation, and extracellular protease production. Journal of bacteriology, 160(1), 438-441.

Halami, P.M., 2008. Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. World Jourbnal of Microbiology. Biotechnology. 4, 805–812

Hartmann R., Hany R., Pletscher E., Ritter A., Witholt B., 2005. Tailor-made olefinic medium-chain-length poly [(R)-3-Hydroxyalkanoates] by Pseudomonas putida GPo1: Batch Versus Chemostat production. Biotechnology and Bioengineering 93/4:737-746

Hartmann, R., Hany, R., Witholt, B., and Zinn, M. 2010. Simultaneous biosynthesis of two copolymers in *Pseudomonas putida* GPo1 using a two-stage continuous culture system. Biomacromolecules, 11(6), 1488-1493.

Herrema M. D., Kimmel K. 2007. Method For The Production Of Polyhydroxyalkanoic Acid. Hedrrema Kimmelllc assignee. Patent US2007202581A. 30 Aug. 2007.

Hong, F. T. 1995. Magnetic field effects on biomolecules, cells, and living organisms. Biosystems, 36(3), 187-229.

Horowitz, D. M., and Sanders, J. K. 1994. Amorphous, biomimetic granules of polyhydroxybutyrate: preparation, characterization, and biological implications. Journal of the American Chemical Society, 116(7), 2695-2702.

Horvat, P., Špoljarić, I. V., Lopar, M., Atlić, A., Koller, M., and Braunegg, G. 2013. Mathematical modelling and process optimization of a continuous 5-stage bioreactor cascade for production of poly [-(R)-3-hydroxybutyrate] by *Cupriavidus necator*. Bioprocess and biosystems engineering, 36(9), 1235-1250.

Huang S. , Liu Y. 2013. *Bacillus Shackletonii* And Application Thereof In Fermentation Production Of Polyhydroxybutyrate. South China Univ of technology assignee. Patent CN103421714A. 04 Dec. 2013.

Huang, J., Du, Y., Xu, G., Zhang, H., Zhu, F., Huang, L., and Xu, Z. 2011. High yield and cost-effective production of poly (γ-glutamic acid) with *Bacillus subtilis.* Engineering in Life Sciences, 11(3), 291-297.

Huisman G. , Martin D. 2002 , Peoples Oliver Biological Systems For Manufacture Of Polyhydroxyalkanoate Polymers Containing 4-Hydroxyacids. Metabolix Inc assignee. Patent US2002187530A. 12 Dec. 2002.

Ito, A., Shinkai, M., Honda, H., & Kobayashi, T. 2005. Medical application of functionalized magnetic nanoparticles. Journal of bioscience and bioengineering, 100(1), 1-11.

Ivanov, V., Stabnikov, V., Ahmed, Z., Dobrenko, S., and Saliuk, A. 2015. Production and applications of crude polyhydroxyalkanoate-containing bioplastic from the organic fraction of municipal solid waste. International Journal of Environmental Science and Technology, 12(2), 725-738.

Jacquel, N., Lo, C. W., Wei, Y. H., Wu, H. S., and Wang, S. S. 2008. Isolation and purification of bacterial poly (3-hydroxyalkanoates). Biochemical Engineering Journal, 39(1), 15-27.

Jau, M. H., Yew, S. P., Toh, P. S., Chong, A. S., Chu, W. L., Phang, S. M., .and Sudesh, K. 2005. Biosynthesis and mobilization of poly (3-hydroxybutyrate)P (3HB) by *Spirulina platensis*. International journal of biological macromolecules, 36(3), 144-151.

Jeong, G. T., Kim, J. N., Ryu, H. W., and Wee, Y. J. 2014. Improved production of poly (γ-glutamic acid) by *Bacillus subtilis* RKY3 and its recovery from viscous fermentation broth as a biodegradable polymer. Journal of Chemical Technology and Biotechnology, 89(5), 728-734.

Jeong, J. H., Kim, J. N., Wee, Y. J., and Ryu, H. W. 2010. The statistically optimized production of poly (γ-glutamic acid) by batch fermentation of a newly isolated *Bacillus subtilis* RKY3. Bioresource technology, 101(12), 4533-4539.

Jian Y. , Loosdrecht Van M. Cornelis M. , Muyzer G., Kleerebezem R., Johnson K. 2009. Process For Selecting Polyhydroxyalkanoate (Pha) Producing Micro-Organisms. Delft Univ Technology assignee. Patent WO09153303A2. 23 Dec. 2009.

Jian, X., Chen S.and Yu Z.2005., Optimization of process parameters for poly γ-glutamate production under solid state fermentation from *Bacillus subtilis* CCTCC202048. Process Biochemistry 40.9 3075-3081.

Jiang, Y., Song, X., Gong, L., Li, P., Dai, C., Shao, W., 2008. High poly(bhydroxybutyrate) production by *Pseudomonas fluorescens* A2a5 from inexpensive substrates. Enzyme Microbial. Technology. 42, 167–172

Jo, S. J., Leong, C. R., Matsumoto, K. I., and Taguchi, S. 2009, Dual production of poly (3-hydroxybutyrate) and glutamate using variable biotin concentrations in

*Corynebacterium glutamicum*. Journal of bioscience and bioengineering, 107(4), 409-411.

Jongejan, J. A., and Duine, J. A. (Eds.). 2012. PQQ and quinoproteins: proceedings of the First International Symposium on PQQ and Quinoproteins, Delft, the Netherlands, 1988. Springer Science and Business Media.

Jose San, 2012 Global Plastics Consumption, California (PRWEB) February 14, 2012, http://www.prweb.com/releases/plastics_bioplastics/engineered_plastics/prweb9194821.htm , Accessed May 2015.

Jung, K., Hazenberg, W., Prieto, M. and Witholt, B. 2001 Two-stage continuous process development for the production of medium-chain-length poly(3-hydroxyalkanoates). Biotechnology Bioengineering 72:19-24

Kahar, P., Tsuge, T., Taguchi, K., Doi, Y., 2004. High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. Polymer. Degradation and Stability. 83, 79–86

Kambourova, M., Tangney, M., and Priest, F. G. 2001. Regulation of polyglutamic acid synthesis by glutamate in *Bacillus licheniformis and Bacillus subtilis*. Applied and environmental microbiology 67(2), 1004-1007.

Kanekar, P.P., Nilegaonkar, S.S., Sarnaik, S. S., Pnraj, M., and Jog J. P. 2007. A process for production of a co-polymer PHB-co-PHV by *Bacillus cereus*. Indian Patent No 244785

Kaparaju, P., Serrano, M., Thomsen, A. B., Kongjan, P., and Angelidaki, I. 2009, Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept. Bioresource Technology, 100(9), 2562-2568.

Katırcıoğlu, H., Aslim, B., Yüksekdağ, Z.N., Mercan, N. and Beyatli, Y. 2003 Production of poly-beta-hydroxybutyrate (PHB) and differentiation of putative *Bacillus* mutant strains by SDS-PAGE of total cell protein. African Journal of Biotechnology 2:147–149

Keshavarz, T., and Roy, I., 2010, Polyhydroxyalkanoates: bioplastics with a green agenda. Current opinion in microbiology 13 (3), 321-326.

Kessler, B., Witholt, B., 2001, Factors involved in the regulatory network of polyhydroxyalkanoate metabolism. Journal of biotechnology 86 (2), 97-104.

Khiyami, M. A., Al-Fadual, S. M., & Bahklia, A. H. 2011. Polyhydroxyalkanoates production via *Bacillus* plastic composite support (PCS) biofilm and date palm syrup. Journal of Medicinal Plants Resources, 5, 3312-3320.

Kim, B., Lee, S., Lee, S., Chang, H., Chang, Y. and Woo, S. 1994a. Production of polyhydroxybutyrate by fed batch with glucose concentration control in *Ralstonia eutropha*. Biotechnology and. Bioengeering, 43, 892-898.

Kim, B.S., Lee, S.C., Lee, S.Y., Chang, H.N., Chang, Y.K. and Woo, S.I. 1994b Production of poly(3-hydroxybutyric-co-hydroxyvaleric acid) by fed-batch culture of *Alcaligenes eutrophus* with substrate control using on-line glucose analyzer. Enzyme Microbial Technology. 16:556-561

Kim, B.S., 2000. Production of poly(3-hydroxybutyrate) from inexpensive substrates. Enzyme Microbial. Technology. 27, 774–777

Kim, B. S.,Chang, H. N. 1995. Control of glucose feeding using exit gas data and its application to the production of PHB from tapioca hydrolysate *byAlcaligenes eutrophus*. Biotechnology techniques, 9(5), 311-314.

Kjeldahl, J., 1883, A new method for the determination of nitrogen in organic matter. Analytical. Chemistry, 22, 366.

Knowles, J.C. and Hastings, G.W. 1991. In vitro degradation of a PHB/PHV copolymer and a new technique for monitoring early surface changes. Biomaterials, 12 (2): 210-214

Kohno, M., Yamazaki, M., Kimura, I., and Wada, M. 2000. Effect of static magnetic fields on bacteria: *Streptococcus mutans, Staphylococcus aureus, and Escherichia coli.* Pathophysiology, 7(2), 143-148.

Koller M., Chiellini E.,Braunegg G. 2015. Study on the Production and Re-Use of Poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) and Extracellular Polysaccharide by the *Archaeon Haloferax mediterranei* strain DSM 1411. Chemical and Biochemical Engineering Quarterly, 29(2).

Koller, M., and Braunegg, G. 2015. Biomediated production of structurally diverse poly (hydroxyalkanoates) from surplus streams of the animal processing industry. Polymer, 60.

Koller, M., Bona, R., Braunegg, G., Hermann, C., Horvat, P., Kroutil, M., 2005. Production of polyhydroxyalkanoates from agricultural waste and surplus materials. Biomacromolecules 6, 561–565.

Koller, M., Bona, R., Chiellini, E., Fernandes, E.G., Horvat, P., Kutschera, C., Hesse, P., Braunegg, G., 2008. Polyhydroxyalkanoate production rom whey by *Pseudomonas hydrogenovora*. Bioresource. Technology. 99, 4854–4863

Koller, M., Hesse, P., Bona, R., Kutschera, C., Atlić, A., and Braunegg, G. 2007. Potential of Various Archae-and Eubacterial Strains as Industrial Polyhydroxyalkanoate Producers from Whey. Macromolecular bioscience, 7(2), 218-226.

Koller, M., Niebelschütz, H., and Braunegg, G. 2013. Strategies for recovery and purification of poly [(R)-3-hydroxyalkanoates](PHA) biopolyesters from surrounding biomass. Engineering in Life Sciences, 13(6), 549-562.

Koller, M.. 2015. Recycling of Waste Streams of the Biotechnological Poly (hydroxyalkanoate) Production by *Haloferax mediterranei* on Whey. International Journal of Polymer Science 2015

Koutinas, A. A., Xu, Y., Wang, R., and Webb, C. 2007. Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. Enzyme and Microbial technology, 40(5), 1035-1044.

Kulkarni, S. O., Kanekar, P. P., Jog, J. P., Sarnaik, S. S., and Nilegaonkar, S. S. 2015. Production of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) by *Halomonas campisalis* MCM B-1027 using agro-wastes. International journal of biological macromolecules, 72, 784-789.

Kulpreecha, S., Boonruangthavorn, A., Meksiriporn, B. and Thongchul, N. 2009. Inexpensive fed-batch cultivation for high poly (3-hydroxybutyrate) production by a new isolate of Bacillus megaterium Journal of bioscience and bioengineering, 107, 240-245.

Kumar, A. S., and Mody, K., 2009. Microbial exopolysaccharides: variety and potential applications. Microbial production of biopolymers and polymer precursors. Applications and Perspectives, 229-253.

Kumar, R. and Parimal P. 2015. Fermentative production of poly (γ-glutamic acid) from renewable carbon source and downstream purification through a continuous membrane-integrated hybrid process. Bioresource technology 177 141-148.

Kumari K. Siddappa L. , Tumkur R. S.. 2009. A Process For The Preparation Of Polyhydroxyalkanoates From Unhydrolysed Starch Using A Novel Strain Of *Bacillus* Sp (Mtcc 5150). Council Of Scientific And Industrial Research assignee. Patent IN00774D2005A. 19 Jun. 2009.

Kunioka, M., and Goto A., 1994. Biosynthesis of poly (γ-glutamic acid) from L-glutamic acid, citric acid, and ammonium sulfate in *Bacillus subtilis* IFO3335. Applied Microbiology and Biotechnology 40.6 867-872.

Lama, L., Nicolaus, B., Calandrelli, V., Manca, M. C., Romano, I., and Gambacorta, A. 1995, Effect of growth conditions on endo-and exopolymer biosynthesis in *Anabaena cylindrical*, 10 C. Phytochemistry, 42(3), 655-659.

Law, J. H., Slepecky, 1961, R. A., Assay of poly-β-hydroxybutyric acid. Journal of bacteriology, 82, 33-36.

Lee, I. Y., Seo, W. T., Kim, G. J., Kim, M. K., Ahn, S. G., Kwon, G. S., and Park, Y. H. 1997. Optimization of fermentation conditions for production of exopolysaccharide by *Bacillus polymyxa.* Bioprocess Engineering, 16(2), 71-75.

Lee, S. Y., and Chang, H. N. 1994. Effect of complex nitrogen source on the synthesis and accumulation of poly (3-hydroxybutyric acid) by recombinant

*Escherichia coli* in flask and fed-batch cultures. Journal of environmental polymer degradation, 2(3), 169-176.

Lee, S.Y. 1995. Bacterial Polydroxyalkanoates. Biotechnology and Bioengineer 49:1-14

Lee, S.Y., 1998. Poly(3-hydroxybutyrate) production from xylose by recombinant *Escherichia coli.* Bioprocess Eng. 18, 397–399

Lee, S.Y., Middelberg, A.P.J., Lee, Y.K., 1997. Poly(3-hydroxybutyrate) production from whey using recombinant *Escherichia coli*. Biotechnology. Letters. 19, 1033–1035

Leonard, C. G., Housewright, R. D., & Thorne, C. B. 1958. Effects of some metallic ions on glutamyl polypeptide synthesis by *Bacillus subtilis*. Journal of bacteriology, 76(5), 499-503.

Levin D. B., Sharma P. 2013. Microbial Production Of Polyhydroxyalkanoates. University Of Manitoba assignee. Patent US2013288323A. 31 Oct. 2013.

Li Y., 2007. Principles and Technology of Fermentation Engineering, Higher Education Press, Beijing, China,

Li, M., Liao, X., Zhang, D., Du, G., and Chen, J. 2011. Yeast extract promotes cell growth and induces production of polyvinyl alcohol-degrading enzymes. Enzyme research, 2011.

Liu, C., Liu, Y., Liao, W., Wen, Z., and Chen, S. 2004, Simultaneous production of nisin and lactic acid from cheese whey. In Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO (pp. 627-638). Humana Press.

Liu, F., Li, W., Ridgway, D., Gu, T., Shen, Z., 1998. Production of poly-bhydroxybutyrate on molasses by recombinant *Escherichia coli*. Biotechnology. Letter. 20, 345–348

Lopez, J. A., Naranjo, J. M., Higuita, J. C., Cubitto, M. A., Cardona, C. A., and Villar, M. A. 2012. Biosynthesis of PHB from a new isolated *Bacillus megaterium* strain: outlook on future developments with endospore forming bacteria. Biotechnology and bioprocess engineering, 17(2), 250-258.

Lundgren, D. G., R. M. Pfister, and J. M. Merrick. 1964. Structure of poly-β-hydroxybutyric acid granules. Journal of general Microbiology 34.3 441-446.

Magdouli, S., Brar, S. K., Blais, J. F., and Tyagi, R. D. 2015. How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production?. Biomass and Bioenergy, 74, 268-279.

Marjadi D. and Dharaiya N., 2011. Bioplastic: A better Alternative for sustainable future. Search and Research 2:159-163.

Marsudi, S., Tan, I. K. P., Gan, S. N. and Ramachandran, K. 2009. Production of medium chain length polyhydroxyalkanoates from oleic acid using *pseudomonas putida* pga1 by fed batch culture. Makara, 11, pp. 1-4.

Marsudi, S., Unno, H., and Hori, K. 2008, Palm oil utilization for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by *Pseudomonas aeruginosa*. Applied microbiology and biotechnology, 78(6), 955-961.

Martinez, G. A., Bertin, L., Scoma, A., Rebecchi, S., Braunegg, G., & Fava, F. (2015). Production of polyhydroxyalkanoates from dephenolised and fermented olive mill wastewaters by employing a pure culture of *Cupriavidus necator*. Biochemical Engineering Journal, 97, 92-100.

Mathieu-Denoncourt, J., Wallace, S. J., de Solla, S. R., and Langlois, V. S. 2014. Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. General and comparative endocrinology.

Matsuura E., Ye Y., He X., 2008, Sustainability Opportunities and Chalanges of Bioplastics. Master Thesis, School of Engineering, Blekinge Institute of Technology. Karlskrona, Sweden

McCabe, W. L., Smith, J. C., and Harriott, P. 1993. Unit operations of chemical engineering (Vol. 5, p. 154). New York: McGraw-Hill.

Merrick J. M. and Doudoroff M., 1961 Enzymatic synthesis of poly-β-hydroxybutyric acid in bacteria, Nature 189, 890-892.

Mingfeng C., Weitao G.Li L., Cunjiang S., Hui X., Wenbin G., Yinghong J., Shufang W., 2011, Glutamic acid independent production of poly-γ-glutamic acid by *Bacillus amyloliquefaciens* LL3 and cloning of pgsBCA genes, Bioresource Technology, 102, (5), 4251-4257, ISSN 0960-8524.

Misra, S.K., Watts, P.C.P., Valappil, S.P., Silva, S.R.P., Roy, I., Boccaccini, A.R. 2007. Poly(3-hydroxybutyrateJ/Bioglass composite films containing carbon nanotubes. Nanotechnology.18:075701

Miyakoshi, J. 2005. Effects of static magnetic fields at the cellular level. Progress in biophysics and molecular biology, 87(2), 213-223.

Muchová, K., Wilkinson, A. J., and Barák, I. 2011. Changes of lipid domains in *Bacillus subtilis* cells with disrupted cell wall peptidoglycan. FEMS microbiology letters, 325(1), 92-98..

Mutayoba S. K., Dierenfeld E., Mercedes V. A., Frances Y., knight C.D., 2011. Determination of chemical composition and ant-nutritive components for Tanzanian locally available poultry feed ingredients. International Journal of Poultry Science 10/5:350-357.

Nichols S. E , William D H. , Briggs K. , Matthias L., Jian G D. , Kanwarpal D. , Kristen K. B. , Li C., Chun P. L. 2006 , Production Of Polyhydroxyalkanoate

In Plants. Pioneer Hi Bred Int assignee. Patent US2006252139A. 09 Nov. 2006.

Nigmatullin, R., Thomas, P., Lukasiewicz, B., Puthussery, H., and Roy, I. 2015. Polyhydroxyalkanoates, a family of natural polymers, and their applications in drug delivery. Journal of Chemical Technology and Biotechnology.

Nikel, P.I., Almeida, A., Melillo, E.C., Galvagno, M.A., Pettinari, M.J., 2006. New recombinant *Escherichia coli* strain tailored for the production of poly(3-hydroxybutyrate) from agro-industrial by-products. Applied and Environmental Microbiology. 72, 3949–3954

Ogawa, Y., Yamaguchi, F., Yuasa, K., and Tahara, Y. 1997. Efficient production of γ-polyglutamic acid by *Bacillus subtilis* (natto) in jar fermenters.Bioscience, biotechnology, and biochemistry, 61(10), 1684-1687.

Ogunleye, A., Bhat, A., Irorere, V. U., Hill, D., Williams, C., and Radecka, I. 2015. Poly-γ-glutamic acid: production, properties and applications. Microbiology, 161(1), 1-17.

Oliveira, A. M., and Oliva Neto, P. D. 2011. Improvement in RNA extraction from *S. cerevisie* by optimization in the autolysis and NH3 hydrolysis. Brazilian Archives of Biology and Technology, 54(5), 1007-1018.

Oliveira, K. F., Souza, C. S., Kilikian, B. V., and Laluce, C. 2003. Yeast flotation viewed as the result of the interplay of supernatant composition and cell-wall hydrophobicity. Colloids and Surfaces B: Biointerfaces, 29(4), 309-319.

Omar, S., Rayes, A., Eqaab, A., VOß, I., Steinbüchel, A., 2001, Optimization of cell growth and P3HB accumulation on date syrup by a *Bacillus megaterium* strain. Biotechnology letters 23 (14), 1119-1123.

Page, W.J., Manchak, J., Rudy, B., 1992. Formation of poly(hydroxybutyrate-cohydroxyvalerate) by *Azotobacter vinelandii* UWD. Applied Environmental Microbiology. 58,2866–2873

Page, W.J., 1992. Production of poly-β-hydroxybutyrate *by Azotobacter vinelandii* UWD in media containing sugars and complex nitrogen sources. Applied microbiology and biotechnology, 38 (1), 117-121.

Pal, S., Manna, A., and Paul, A. K. 1999, Production of poly (β-hydroxybutyric acid) and exopolysaccharide by *Azotobacter beijerinckii* WDN-01. World Journal of Microbiology and Biotechnology, 15(1), 11-16.

Palmieri, M. C., Greenhalf, W., and Laluce, C. 1996. Efficient flotation of yeast cells grown in batch culture. Biotechnology and bioengineering, 50(3), 248-256.

Panda, A. K., Singh, R. K., and Mishra, D. K. 2010. Thermolysis of waste plastics to liquid fuel: A suitable method for plastic waste management and manufacture of value added products—A world prospective. Renewable and Sustainable Energy Reviews, 14(1), 233-248.

Park, S.J., Park, J.P., Lee, S.Y., 2002. Production of poly(3-hydroxybutyrate) from whey by fed-batch culture of recombinant *Escherichia coli* in a pilot-scale fermenter. Biotechnology Letters. 24, 185–189

Patel, M., Marscheider-Weidemann, F., Schleich, J., Hüsing, B., and Angerer, G. 2005. Techno-economic feasibility of large-scale production of bio-based polymers in Europe. IPTS Technical Report EUR 22103 EN, Seville.

Peng, Y., Jiang, B., Zhang, T., Mu, W., Miao, M., and Hua, Y. 2015. High-level production of poly (γ-glutamic acid) by a newly isolated glutamate-independent strain, *Bacillus methylotrophicus*. Process Biochemistry, 50(3), 329-335.

Perego, M., and Hoch, J. A. 1988. Sequence analysis and regulation of the hpr locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. Journal of Bacteriology, 170(6), 2560-2567.

Philip S., Keshavarz T., Roy I., 2007. Polyhydroxyalkanoates: biodegradable polymers with range of applications. Journal of Chemical Technology and Biotechnology 82:233-247.

Plastics Europe, 2012, An analysis of European plastics production, demand and waste data for Association of Plastics Manufacturers, http://www.plasticseurope.org/Document/plastics-the-facts-2012-9894.aspx Accessed May 2015.

Poblete-Castro, I., Binger, D., Rodrigues, A., Becker, J., Martins dos Santos, V. A., and Wittmann, C. 2013. In-silico driven metabolic engineering of *Pseudomonas putida* for enhanced production of poly-hydroxyalkanoates.Metabolic engineering. 15, 113-123.

Posada, J. A., Naranjo, J. M., López, J. A., Higuita, J. C., and Cardona, C. A. 2011. Design and analysis of poly-3-hydroxybutyrate production processes from crude glycerol. Process biochemistry, 46(1), 310-317.

Prabhu, N. N., Santimano, M. C., Mavinkurve, S., Bhosle, S. N., and Garg, S. 2010. Native granule associated short chain length polyhydroxyalkanoate synthase from a marine derived *Bacillus* sp. NQ-11/A2. Antonie van Leeuwenhoek, 97(1), 41-50.

Preusting, H., Kingma, J., and Witholt, B. 1991. Physiology and polyester formation of *Pseudomonas oleovorans* in continuous two-liquid-phase cultures.Enzyme and microbial technology, 13(10), 770-780.

Qingsheng Q. , Zhen K. 2009. Method For Producing Polyhydroxyalkanoate Using Engineering Strain. Shandong Univ assignee. Patent CN101363034A. 11 Feb. 2009.

Quagliano, J. C., and Miyazaki, S. S. 1999, Biosynthesis of poly-β-hydroxybutyrate and exopolysaccharides on *Azotobacter chroococcum* strain 6B utilizing simple

and complex carbon sources. Applied biochemistry and biotechnology, 82(3), 199-208.

Quillaguaman, J., Hashim, S., Bento, F., Mattiasson, B. and Hatti-Kaul, R. 2005. Poly(β-hydroxybutyrate) production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as substrate. Journal of Applied Microbiology. 99:151-157

Rai, R., Keshavarz, T., Roether, J. A., Boccaccini, A. R., and Roy, I. 2011. Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. Materials Science and Engineering: R: Reports, 72(3), 29-47.

Ray, A., Cot, M., Puzo, G., Gilleron, M., and Nigou, J. 2013. Bacterial cell wall macroamphiphiles: pathogen-/microbe-associated molecular patterns detected by mammalian innate immune system. Biochimie, 95(1), 33-42.

Reed, C. 2015. Plastic Age: How it's reshaping rocks, oceans and life. New Scientist. 28 Jan 2015. Issue 3006.

Rehm, B.H. 2003. Polyester synthases: natural catalysts for plastics. Biochemistry Journal. 376:15–33

Rein, B.H.A. .2007. Biogenesis of Microbial Polyhydroxyalkanoate Granules: a Platform Technology for the Production of Tailor-made Bioparticles Current Issues on Molecular Biology. 9:41-62

Ribera, R.G., Monteoliva-Sanchez, M., Ramos-Cormenzana, A., 2001. Production of polyhydroxyalkanoates by *Pseudomonas putida* KT2442 harboring pSK2665 in wastewater from olive oil mills (alpechín). Electronic Journal of Biotechnology. 4, 116–119

Richard, A., and Margaritis, A. 2003. Rheology, oxygen transfer, and molecular weight characteristics of poly (glutamic acid) fermentation by *Bacillus subtilis*.Biotechnology and bioengineering, 82(3), 299-305.

Richardson, J. F., and Zaki, W. N. 1997. Sedimentation and fluidisation: Part I.Chemical Engineering Research and Design, 75, S82-S100.

Robert, C. 2006. Comprehensive review of scientific literature pertaining to nitrogen protein conversion factors. Bulletin-International Dairy Federation, (405), 1-12.

Rowe, G. E., Margaritis, A., and Wei, N. 2003. Specific Oxygen Uptake Rate Variations during Batch Fermentation of *Bacillus thuringiensis* Subspecies kurstaki HD-1. Biotechnology progress, 19(5), 1439-1443.

Sadasivam, S., and Manickam, A. 2005. Biochemical methods. New Age International.

Samain, E., Miles, M., Bozzi, L., Dubreucq, G., and Rinaudo, M. 1997, Simultaneous production of two different gel-forming exopolysaccharides by an

Alteromonas strain originating from deep sea hydrothermal vents.Carbohydrate polymers, 34(4), 235-241.

Sanchez, C. C., Isabel F. E., Jose L.G L., and Maria A.P J., 2011. System For Improving The Production Of Polyhydroxyalkanoates (Bioplastic) By Fermentation From Glycerol Using A Genetically Modified Strain Of *Pseudomonas Putida*. Consejo Superior Investigacion, assignee. Patent WO13072541A1. 16 Nov. 2011

Santi, G., Jasiulewicz, J., Crognale, S., D'Annibale, A., Petruccioli, M., and Moresi, M. 2015. High Solid Loading in Dilute Acid Hydrolysis of Orange Peel Waste Improves Ethanol Production. BioEnergy Research, 1-11.

Sato S. , Nagaoka T. 2008. Method For Producing Polyhydroxyalkanoate. Kaneka Corp assignee. Patent JP2008086238A. 17 Apr. 2008.

Sawalha, S. M., Arráez-Román, D., Segura-Carretero, A., & Fernández-Gutiérrez, A. 2009. Quantification of main phenolic compounds in sweet and bitter orange peel using CE–MS/MS. Food Chemistry, 116(2), 567-574.

Senthilkumar, K., Sivakumar, V., and Kannadasan, T. 2008.. Experimental Investigation on Relationship between Sedimentation Rate Constants of Solid Materials. Modern Applied Science, 2(5), p80.

Serezli, R., and Tabak, A. 2013. Ünye Bentoniti ile Sulu Ortamdan Amonyum ($NH_4$) Adsorbsiyonu. Ekoloji, 22(87), 35-42.

Shamala, T.R., Chandrashekar, A., Vijayendra, S.V. and Sharma, L. 2003. Identification of polyhydroxyalkanoate (PHA)-producing *Bacillus* spp. using the polymerase chain reaction (PCR). Journal of Applied Microbiology. 94:369-374

Shang, L., Jiang, M., Yun, Z., Yan, H. Q. and Chang, H. N. 2008. Mass production of medium-chain-length poly (3-hydroxyalkanoates) from hydrolyzed corn oil by fed-batch culture of *Pseudomonas putida.* World Journal of Microbiology and Biotechnology, 24, 2783-2787.

Shen, H., Forssberg, E., and Pugh, R. J. 2001. Selective flotation separation of plastics by particle control. Resources, Conservation and Recycling, 33(1), 37-50.

Shi, F., Xu, Z., and Cen, P. 2006. Optimization of γ-polyglutamic acid production by *Bacillus subtilis* ZJU-7 using a surface-response methodology. Biotechnology and Bioprocess Engineering, 11(3), 251-257.

Shih, I. L., Wang, T. C., Chou, S. Z., and Lee, G. D. 2011, Sequential production of two biopolymers-levan and poly-ε-lysine by microbial fermentation. Bioresource Technology, 102(4), 3966-3969.

Shih, I. L., Y. T. Van, and Y. N. Chang. 2002. Application of statistical experimental methods to optimize production of poly (γ-glutamic acid) by *Bacillus*

*licheniformis* CCRC 12826. Enzyme and Microbial Technology 31.3 213-220.

Shih, L., and Yu, Y. T. 2005, Simultaneous and selective production of levan and poly (γ-glutamic acid) by *Bacillus subtilis.* Biotechnology letters, 27(2), 103-106.

Shih, Lung and Van, Yi-Tsong. 2001, The production of poly-(γ-glutamic acid) from microorganisms and its various applications. Bioresource Technology, 79.3: 207-225.

Shrivastav, A., Sanjiv K. M., and Sandhya M., 2010. Polyhydroxyalkanoate (PHA) synthesis by *Spirulina subsalsa* from Gujarat coast of India. International journal of biological macromolecules 46.2 255-260.

Silva, L.F., Taciro, M.K., 2004. Ramos, M.E.M., Carter, J.M., Pradella, J.G.C., Gomez, J.G.C., 2004. Poly-3-hydroxybutyrate (P3HB) production by bacteria from xylose, glucose and sugarcane bagasse hydrolysate. Journal of. Indian Microbiology and Biotechnology. 31, 245–254.

Sklodowska, A., and Matlakowska, R. 1998. Relative surface charge, hydrohobicity of bacterial cells and their affinity to substrate during copper bioleaching from post-flotation wastes. Biotechnology letters, 20(3), 229-233.

Skraly F. , Gjalt W H., Oliver P P. 2003. A Transgenic Microbial Polyhydroxyalkanoate Producers. Metabolix Inc assignee. Patent US2003228669A. 13 Jun 2003.

Skraly F. A , Martin D. P 2000. Production of Block Copolymers of Polyhydroxyalkanoates In Biological Systems. Metabolix Inc assignee. Patent WO0006762A1. 10 Feb. 2000.

Snell, K. D., Singh, V., and Brumbley, S. M. 2015. Production of novel biopolymers in plants: recent technological advances and future prospects. Current opinion in biotechnology, 32, 68-75.

Soliman, N. A., Mahmoud M. B., and Yasser R. A. 2005. Polyglutamic acid (PGA) production by Bacillus sp. SAB-26: application of Plackett–Burman experimental design to evaluate culture requirements. Applied microbiology and biotechnology 69.3 259-267.

Spiekermann, P., Rehm, B. H., Kalscheuer, R., Baumeister, D., and Steinbüchel, A. 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Archives of microbiology, 171(2), 73-80.

Steinbüchel, A. 1991. Polyhydroxyalkanoic acids, In: Byron D, editor, Biomaterials: Novel-Materials from Biological Sources. Stockton Press, New York, pp 124-213

Steinour, H. H., 1944.. Rate of sedimentation. Nonflocculated suspensions of uniform spheres. Industrial and Engineering Chemistry, 36(7), 618-624.

Strašák, L., Vetterl, V., and Šmarda, J., 2002. Effects of low-frequency magnetic fields on bacteria Escherichia coli. Bioelectrochemistry, 55(1), 161-164.

Sudesh, K., Abe, H. and Doi, Y. 2000. Synthesis, structure and properties of Polyhydroxyalkanoates: Biological polyesters. Journal of Progress in Polymer Science 25:1503-1555

Sun, Z., Ramsay, J. A., Guay, M., and Ramsay, B. A., 2007. Carbon-limited fed-batch production of medium-chain-length polyhydroxyalkanoates from nonanoic acid by *Pseudomonas putida* KT2440. Applied microbiology and biotechnology, 74(1), 69-77.

Sung, M.H., Park, C., Kim, C.-J., Poo, H., Soda, K. and Ashiuchi, M., 2005. Natural and edible biopolymer poly-γ-glutamic acid: synthesis, production and applications. Chemical Reactions, 5, pp.352-366.

Suzuki, T., Tsuneo Y., and Shoichi S., 1986. Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of methylotroph. Applied microbiology and biotechnology 23.5 322-329.

Şafak, S., Mercan, N., Aslim, B., and Beyatli, Y. 2002. A study on the production of poly-β-hydroxybutyrate by some eukaryotic microorganisms.Turkish. Electronic. Journal of Biotechnology, 1, 11-17.

Taherzadeh, M. J., Karimi, K., 2008, Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. International Journal of Molecular Sciences 9 (9), 1621-1651.

Tajima, K., Igari, T., Nishimura, D., Nakamura, M., Satoh, Y. and Munekata, M. 2003. Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil. Journal of Bioscience and Bioengineering 95:77-81

Tan, G. Y. A., Chen, C. L., Li, L., Ge, L., Wang, L., Razaad, I. M. N.,and Wang, J. Y., 2014. Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. Polymers, 6(3), 706-754.

Tanadchangsaeng, N. and Yu, J. 2012. Microbial synthesis of polyhydroxybutyrate from glycerol: Gluconeogenesis, molecular weight and material properties of biopolyester. Biotechnology and bioengineering.

Tang, B., Xu, H., Xu, Z., Xu, C., Xu, Z., Lei, P., and Feng, X. 2015. Conversion of agroindustrial residues for high poly (γ-glutamic acid) production by *Bacillus subtilis* NX-2 via solid-state fermentation. Bioresource Technology.

Tetsuya F. and Hiroka N. 2008. Process For Producing Polyhydroxyalkanoate. Kanekacorp. Assignee. Patent WO08090873A1. 31 Jul. 2008.

Tian, J., Sinskey, A.J. and Stubbe, J. (2005) Kinetic studies of polyhydroxybutyrate granue formation in Wautersia eutropha H16 by transmission electron microscopy. Journal of Bacteriology. 187:3814-3824

Todaro, C. M., and Vogel, H. C. (Eds.). 2014. Fermentation and biochemical engineering handbook. William Andrew.

Townsley, P. M., Joslyn, M. A., and Smit, C. J. B., 1953. The Amino Acids In Various Tissues of Citrus Fruits and In Orange Protein, .Journal of Food Science, 18(1-6), 522-531.

Tripathi, A. D., Yadav, A., Jha, A. and Srivastava, S. 2012. Utilizing of Sugar Refinery Waste (Cane Molasses) for Production of Bio-Plastic Under Submerged Fermentation Process. Journal of Polymers and the Environment, 1-8.

Tröger, C. N., and Adam P. H.. 2009. The production of polyhydroxyalkanoates using an oscillatory baffled bioreactor. Chemical Product and Process Modeling 4.5

Valappil, S. P., Rai, R., Bucke, C., Roy, I., 2008, Polyhydroxyalkanoate biosynthesis in *Bacillus cereus* SPV under varied limiting conditions and an insight into the biosynthetic genes involved. Journal of applied microbiology, 104, 1624-1635.

Valappil, S.P., Peiris, D., Langley, G.J., Herniman, J.M., Boccaccini, A.R., Bucke, C. and Roy, I. 2007. Polyhydroxyalkanoates (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterised *Bacillus* spp. Journal of Biotechnology 127:475-487

van Hee, P., Elumbaring, A. C., van der Lans, R. G., and Van der Wielen, L. A. 2006. Selective recovery of polyhydroxyalkanoate inclusion bodies from fermentation broth by dissolved-air flotation. Journal of colloid and interface science, 297(2), 595-606.

Van-Thuoc D, Quillaguamn J, Mamo G, Mattiasson B. 2008, Utilization of agricultural residues for poly(3- ydroxybutyrate) production by *Halomonas boliviensis* LC1. Journal of Applied Microbiology 104: 420-8.

Verlinden R. A. J., Hill D. J., Kenward M. A., Williams C. D., 2011, Piotrowska-Seget Z., Radecka I. K.. Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidis necator*.AMB Express, 1:11

Velizarov, S. 1999. Electric and magnetic fields in microbial biotechnology: possibilities, limitations, and perspectives. Electro and Magnetobiology, 18(2), 185-212.

Ververis, C., Georghiou, K., Danielidis, D., Hatzinikolaou, D.G., Santas, Corleti, V., 2007, Cellulose, hemicelluloses, lignin and ash content of some organic materials and their suitability for use as paper pulp supplements. Bioresource Technology 98(2), 296-301.

Vlaski A, van Breemen A, Alaerts G. 1996. Optimisation of coagulation conditions for the removal if cyanobacteria by dissolved air flotation or sedimentation. Journal of Water SRT-Aqua 45: 253–261.

Wampfler, B., Ramsauer, T., Rezzonico, S., Hischier, R., Kohling, R., Thony-Meyer, L., and Zinn, M. 2010. Isolation and purification of medium chain length poly (3-hydroxyalkanoates)(mcl-PHA) for medical applications using nonchlorinated solvents. Biomacromolecules, 11(10), 2716-2723.

Wang, D. I., Cooney, C. L., Demain, A. L., Dunnill, P., Humphrey, A. E., and Lilly, M. D. 1979. Fermentation and enzyme technology. New York: Wiley.

Wang, F. and Lee, S.Y. 1997. Poly(3-hydroxybutyrate) production with high productivity and high polymer content by a fed-batch culture of *Alcaligenes latus* under nitrogen limitation. Applied and Environmental Microbiology 63:3703–3706

Wang, J., and Yu, H. Q. 2007. Biosynthesis of polyhydroxybutyrate (PHB) and extracellular polymeric substances (EPS) by *Ralstonia eutropha* ATCC 17699 in batch cultures. Applied microbiology and biotechnology, 75(4), 871-878.

Wang, Q., Chen, S., Zhang, J., Sun, M., Liu, Z., and Yu, Z. 2008. Co-producing lipopeptides and poly-γ-glutamic acid by solid-state fermentation of *Bacillus subtilis* using soybean and sweet potato residues and its biocontrol and fertilizer synergistic effects. Bioresource technology, 99(8), 3318-3323.

Weart, R. B., Lee, A. H., Chien, A. C., Haeusser, D. P., Hill, N. S., and Levin, P. A. 2007. A metabolic sensor governing cell size in bacteria. Cell, 130(2), 335-347.

Wu, Q., Huang, H., Hu, G.H., Chen, J., Ho, K.P. and Chen, G.Q. (2001). Production of poly-3-hydroxybutyrate by *Bacillu*s sp. JMa5 cultivated in molasses media. Antonie van Leeuwenhoek. 80:111-118

Xiaowu L. , Liu X. , Guoqiang C., Chen G.2009. Method For Increasing Content Of 3-Hydroxy Valeric Acid Monomer Of Polyhydroxyalkanoate Prepared By Fermentation. Shantou University assignee. Patent CN101565722A. 28 Oct. 2009.

Xiong, C., Shouwen, C., Ming, S., and Ziniu, Y. 2005. Medium optimization by response surface methodology for poly-γ-glutamic acid production using dairy manure as the basis of a solid substrate. Applied microbiology and biotechnology, 69(4), 390-396.

Xu, H., Jiang, M., Li, H., Lu, D., and Ouyang, P. 2005. Efficient production of poly (γ-glutamic acid) by newly isolated *Bacillus subtilis* NX-2. Process Biochemistry, 40(2), 519-523.

Yamane, T., Fukunaga, M. and Lee, Y.W. 1996. Increased PHB productivity by high cell- density fed-batch culture of *Alcaligenes latus*, a growth-associated PHB producer. Biotechnology and Bioengineering 50:197-202

Yamaguchi, F., Ogawa, Y., Kikuchi, M., Yuasa, K., & Motai, H. 1996. Detection of γ-polyglutamic acid (γ-PGA) by SDS-PAGE. Bioscience, biotechnology, and biochemistry, 60(2), 255-258.

Yao, J., Xu, H., Shi, N., Cao, X., Feng, X., Li, S., and Ouyang, P. 2010. Analysis of carbon metabolism and improvement of γ-polyglutamic acid production from *Bacillus subtilis* NX-2. Applied biochemistry and biotechnology,160(8), 2332-2341.

Yaqiu W., Tian J., Qian X., Liu L., Li Y., Li J, Huang J., Chi S. 2013. Recombinant Mutant Strain Capable Of Producing Polyhydroxyalkanoate. China Agricultural Univ Assignee. Patent CN103275 915A. 04 Sep. 2013.

Yilmaz, M., Beyatli, Y., 2005, Poly-b-hydroxybutyrate (PHB) production by *a Bacillus cereus* M5 strain in sugarbeet molasses. Zuckerindustrie 130 (2), 109-112.

Yoldaş, M. A., Katırcıoğlu, H., and Beyatlı, Y. 2003. Bazı Mavi-Yeşil Alglerin (Cyanophyta-Cyanobacteria) Poli-β-hidroksibütirat (PHB) Üretimi ve Antimikrobiyal Aktivitelerinin İncelenmesi. Ege Üniverisitesi Su Ürünleri Dergisi, 20, 3-4.

Yong, X., Cui, Y., Chen, L., Ran, W., Shen, Q., and Yang, X. 2011. Dynamics of bacterial communities during solid-state fermentation using agro-industrial wastes to produce poly-γ-glutamic acid, revealed by real-time PCR and denaturing gradient gel electrophoresis (DGGE). Applied microbiology and biotechnology, 92(4), 717-725.

Yoon, H.Y., Do, J.H., Lee, S.Y., Chang, H.N., 2000. Production of poly-glutamic acid by fed-batch culture of *Bacillus licheniformis*. Biotechnology. Letters. 22, 585–588.

Yu, J., Stahl, H., 2008. Microbial utilization and biopolyester synthesis of bagasse hydrolysates. Bioresource. Technology. 99, 8042–8048

Yung, C. C., Lan C. W., 2011. Method For Synthesizing Polyhydroxyalkanoate Using A Microorganism. Yuan Ze University assignee. Patent US2013122559A. 15. Nov. 2011.

Zafar, M., Kumar, S., Dhiman, A. K., and Park, H. S. 2014. Maintenance-energy-dependent dynamics of growth and poly (3-hydroxybutyrate)[P (3HB)] production by *Azohydromonas lata* MTCC 2311 using simple and renewable carbon substrates. Brazilian Journal of Chemical Engineering, 31(2), 313-323.

Zafar, M., Kumar, S., Kumar, S., and Dhiman, A. K. 2012. Optimization of polyhydroxybutyrate (PHB) production by *Azohydromonas lata* MTCC 2311 by using genetic algorithm based on artificial neural network and response surface methodology. Biocatalysis and Agricultural Biotechnology, 1(1), 70-79.

Zahari, M. A. K. M., Ariffin, H., Mokhtar, M. N., Salihon, J., Shirai, Y., and Hassan, M. A. 2015. Case study for a palm biomass biorefinery utilizing renewable non-food sugars from oil palm frond for the production of poly (3-hydroxybutyrate) bioplastic. Journal of Cleaner Production, 87, 284-290.

Zeng, W., Chen, G., Zhang, Y., Wu, K., and Liang, Z. 2012. Studies on the UV spectrum of poly (γ-glutamic acid) based on development of a simple quantitative method. International journal of biological macromolecules, 51(1), 83-90.

Zeng, W., Li, W., Shu, L., Yi, J., Chen, G., and Liang, Z. 2013. Non-sterilized fermentative co-production of poly (γ-glutamic acid) and fibrinolytic enzyme by a thermophilic *Bacillus subtilis* GXA-28. Bioresource technology, 142, 697-700.

Zhang B. , Daidi F. , Fan D. , Huang J.o , Jin Z., Longan S., Yanhui Z., Zhang Y. , Zhihua J., 2012. Method For Synthesizing Benzene-Containing Polyhydroxyalkanoate By Mixed Fermentation Of High-Density Microbes Under Nitrogen Restriction. Ningbo Inst Of Technology Zhejiang Univ assignee. Patent CN102517369A. 27 Jun. 2012.

Zhang, D., Dong, K., Xu, D., Zheng, H., Wu, Z., and Xu, X. 2015. Process improvement for fermentation coupling with foam separation: a convenient strategy for cell recycle. Asia-Pacific Journal of Chemical Engineering.10 (3), 466–475.

Zhang, Q. M., Tokiwa, M., Doi, T., Nakahara, T., Chang, P. W., Nakamura and Yonei, S. 2003. Strong static magnetic field and the induction of mutations through elevated production of reactive oxygen species in Escherichia coli soxR. International journal of radiation biology, 79(4), 281-286.

Zhenggui L. , Yuanpeng W. , Wang Y. , Wang H. , Sun H. , Qingbiao L. , Ning H. , Liu Z.i , Li Q. , Hong S. , He N., 2012. Method For Synthesizing Polyhydroxyalkanoate By Using Residual Sludge Broth As Substrate. Xiamen University assignee. Patent CN102505025A. 20 Jun. 2012.

Zinn, M. and Hany, R. 2005. Tailored Material Properties of Polyhydroxyalkanoates through Biosynthesis and Chemical Modification. Advance Engineering Materials 7(5), 408-411.

Zinn, M., Witholt B., and Egli T., 2004. Dual nutrient limited growth: models, experimental observations, and applications. Journal of biotechnology113.1, 263-279.

# Chapter 8: Appendix

## 8.0 APPENDIX

### 8.1 P(3HB) UV measurement Calibration Curve

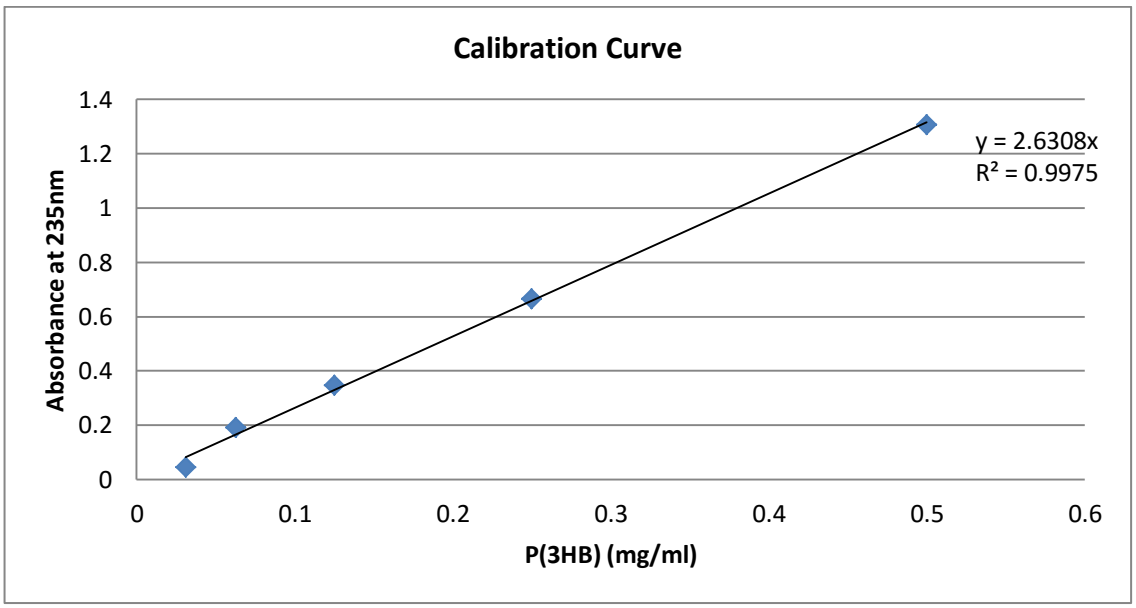



**Figure 8.1:** Calibration curve for P(3HB), crotonic acid method

### 8.2 P(3HB) GC measurement Calibration Curve

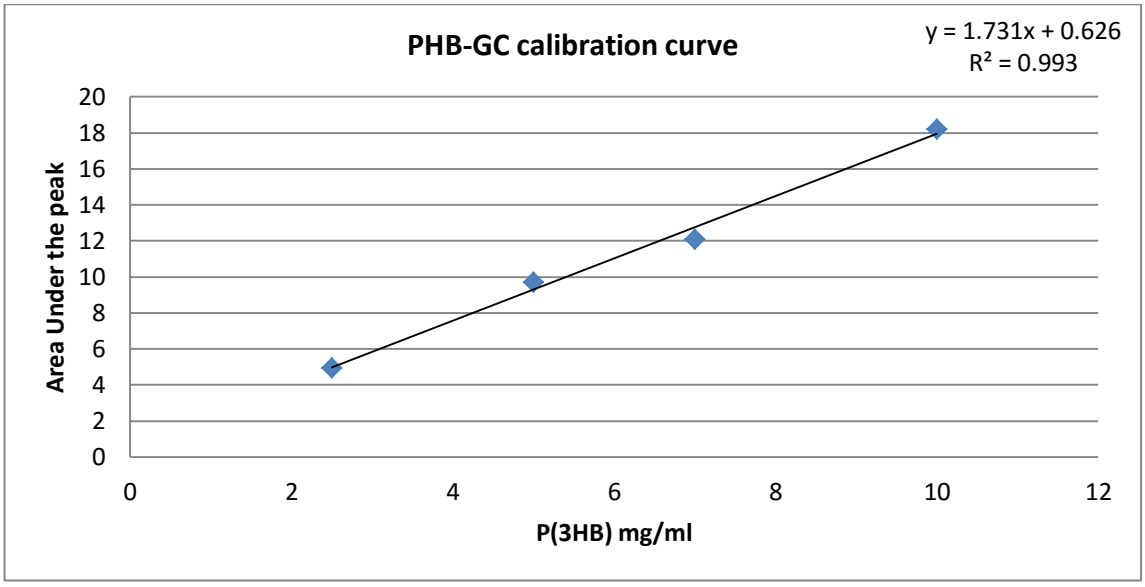



**Figure 8.2:** Calibration curve for P(3HB), GC method

### 8.3 γ-PGA UV method Calibration Curve

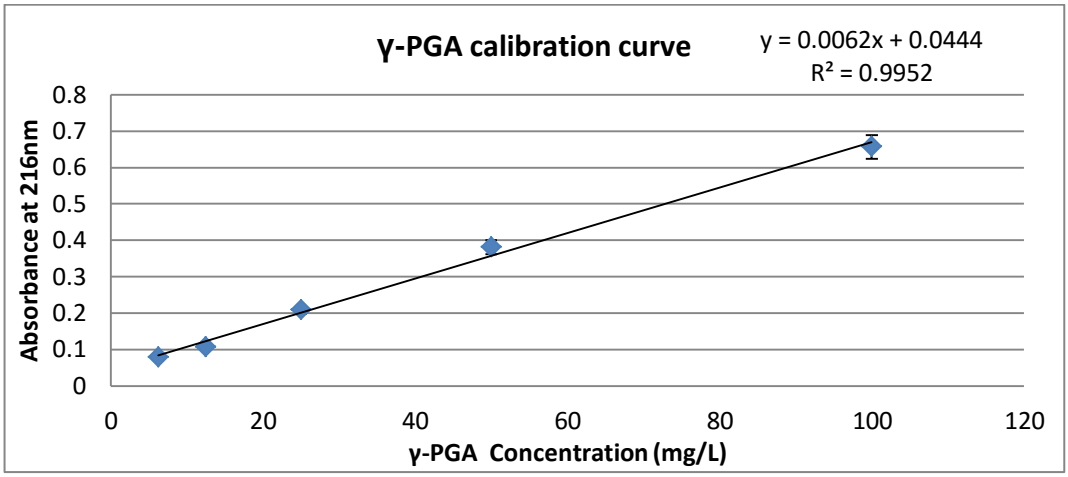



**Figure 8.3:** γ-PGA UV method calibration curve

### 8.4 Buffers needed for SDS-Page

Buffers used in SDS-page conducted are given below.

**Table 8.1:** Concentrations for the ingredients of 10x running buffer stock solution

| Reagent | Amount (g/L) |
|---|---|
| Tris base | 30.2 |
| Glycine | 144.0 |
| SDS | 10.0 |

**Table 8.2:** Concentrations for the ingredients of 5% resolving gel. Final volume for 1 gel should be 7.5 mL

| Reagent | Volume (ml) |
|---|---|
| Acrylamide | 0.875 |
| Resolving gel buffer (1M tris HCL pH 8.8) | 1.82 |
| $H_2O$ | 4.725 |
| SDS | 0.070 |
| APS | 0.060 |
| TEMED | 0.012 |

## 8.5 Glutamic Acid Assay

### 8.5.1 Calibration Curve

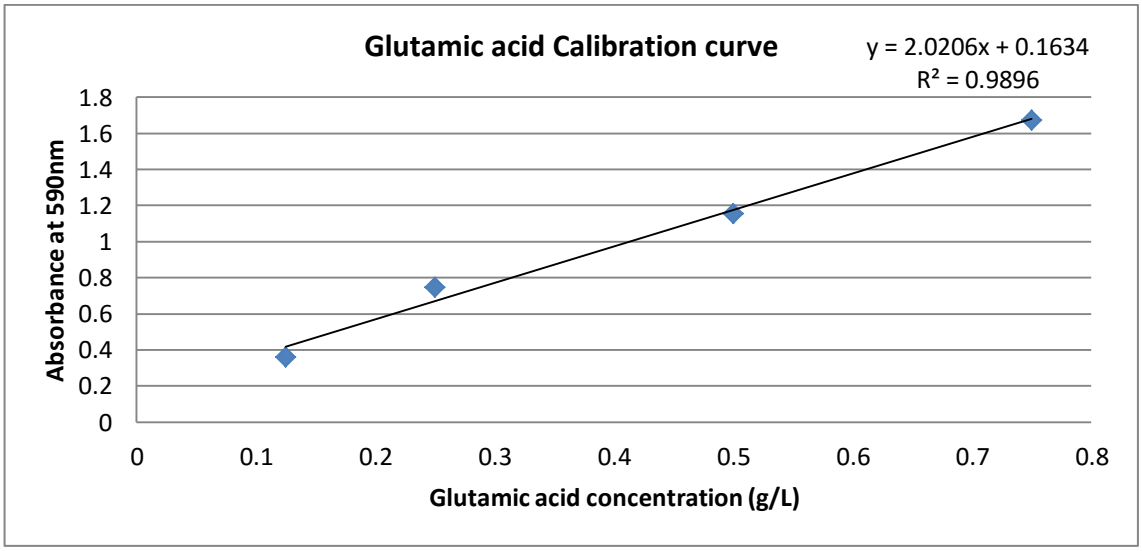



**Figure 8.4:** Glutamic acid calibration curve for spectrophotometric determination

### 8.5.2 Reagents for Glutamic acid assay

**Table 8.3:** Amounts needed for the preparation of ninhydrin reagent needed for spectrophotometric glutamic acid assay

| **Chemical** | **Amount** |
|---|---|
| Stannous chloride ($SnCl_2.2H_2O$) | 0.8 g |
| Ninhydrin | 20 g |
| Citrate buffer (pH 5.0) | 0.2M |
| 2-methoxyethanol | 500ml |

**Procedure**

Dissolve 0.8g Stannous chloride in 500ml citrate buffer. Add this solution to 20g ninhydrin dissolved in 500ml 2-methoxyethanol. Store at -20C and protect from light.

## 8.6 Flow Rate Calculation Curve for Peristaltic Pump

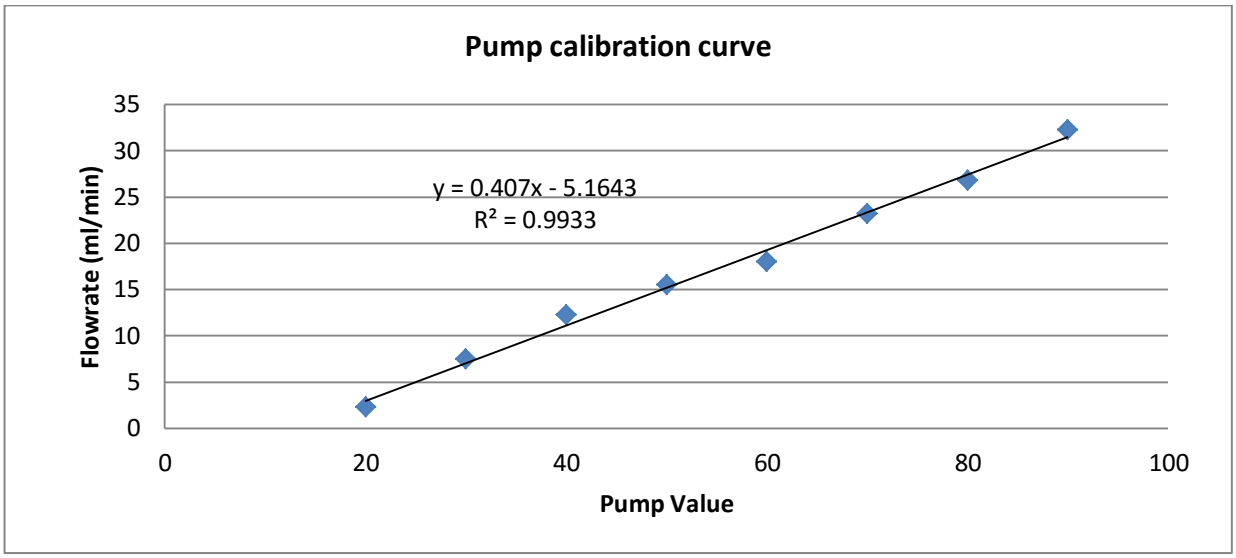



**Figure 8.5:** Flow rate calibration curve used for the peristaltic pump attached to the MFG.

## 8.7 TLC plate for glutamic acid assay of γ-PGA

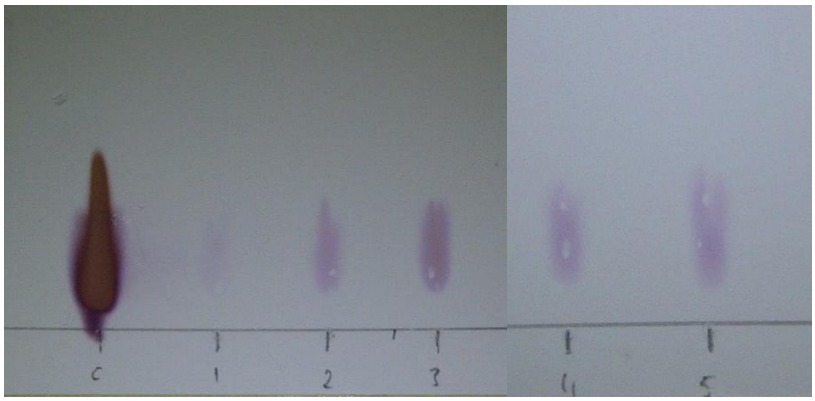


**Figure 8.6:** TLC plate showing glutamic acid obtained from acid hydrolysis of γ-PGA obtained from strains screened for γ-PGA production. Lane C: control (glutamic acid only), lane 1: acid hydrolysed γ-PGA from Bacillus subtilis OK2, lane 2: acid hydrolysed γ-PGA from Bacillus 46.2, lane 3: acid hydrolysed γ-PGA from Bacillus 2.d1, lane 4: acid hydrolysed γ-PGA from Bacillus 159 and lane 5: acid hydrolysed γ-PGA from Bacillus 20.4.

### 8.8 Compiled Table of Runs

**Table 8. 4:** Compiled table of runs conducted throughout the thesis. OP: orange peel, DP: dual polymer, DOT: dissolve oxygen tension, OD: optical density, cdw: cell dry weight, ferm: fermentation, prod: production

| Run Name | Figure No | Reactor size (L) | Working volume (L) | Ferm. Time (h) | rpm | Medium | Carbon source | Sugar Consumed (g/L) | pH range | min % DOT | max % $CO_2$ | max cdw (g/L) | max $OD_{600}$ | max P(3HB) (g/L) | max γ-PGA (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| γ-PGA ferm. | 3.8 | 0.5 | 0.4 | 92 | 300 | PGA | Glycerol/citric acid | 0.4* | 6.7-5.8 | 1% | - | - | 14.60 | - | 3.86 |
| P(3HB) ferm. | 3.9 | 0.5 | 0.4 | 72 | 400 | PHA | Glucose | 9.8 | 6.4-3.4 | 5% | - | 3.3 | 15.13 | 1.02 | - |
| Flask DP prod. | 3.31 | 0.25** | 0.05 | 72 | - | DP | Glucose | 9.8 | - | - | - | 5.1 | 13.57 | 1.06 | 0.55 |
| Constant 250 rpm DP prod. | 3.43 | 2 | 1.5 | 72 | 250 | DP | Glucose | 28.2 | 6.6-5.2 | 0% | 1 | 2.2 | 9.72 | 0.02 | 1.7*** |
| Constant 400 rpm DP prod. | 3.44 | 2 | 1.5 | 26 | 400 | DP | Glucose | 12.7 | 5.9-7.2 | 30% | 0.43 | 3.8 | 16.78 | 0.15 | 0.06 |
| Constant 25% DOT DP prod. | 3.45 | 2 | 1.5 | 24 | 250-400 | DP | Glucose | 12.0 | 5.8-6.9 | 25% | 0.75 | 3.1 | 13.80 | 0.05 | 0.33 |
| Constant 10% DOT DP prod. | 3.46 | 2 | 1.5 | 48 | 250-400 | DP | Glucose | 9.7 | 5.8-6.5 | 10% | 0.56 | 7.4 | 17.92 | 0.20 | 0.26 |
| 25- 10% DOT DP prod. | 3.48 | 2 | 1.5 | 48 | 250-500 | DP | Glucose | 11.2 | 6.3-6.8 | 10% | 0.49 | 4.4 | 17.60 | 0.62 | 0.23 |
| 25- 10% DOT DP prod. repeat | 3.52 | 2 | 1.5 | 76 | 250-500 | DP | Glucose | 12.6 | 6.1-7.1 | 10% | 0.54 | 4.2 | 17.56 | 0.49 | 0.19 |

* Only exopolysaccharide produced was detected by total carbohydrate analysis
** shaken flask run
***measured with gravimetric γ-PGA method

**Table 8. 4 (cont.):** Compiled table of runs conducted throughout the thesis. OP: orange peel, DP: dual polymer, DOT: dissolve oxygen tension, OD: optical density, cdw: cell dry weight, ferm: fermentation, prod: production

| Run Name | Figure No | Reactor size (L) | Working volume (L) | Ferm. Time (h) | rpm | Medium | Carbon source | Sugar Consumed (g/L) | pH range | min % DOT | max % $CO_2$ | max cdw (g/L) | max $OD_{600}$ | max P(3HB) (g/L) | max γ-PGA (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| OP P(3HB) ferm. | 3,65 | 0,5 | 0,4 | 72 | 400 | Optimised OP | Orange peel infusion | 8,3 | 5,5-7,0 | 20% | - | 4,5 | 18,95 | 1,24 | - |
| OP-DP prod. | 3,66 | 2 | 1,5 | 54 | 250-500 | DP | Orange peel infusion | - | 6,3-7,6 | 3% | 0,45 | 4,3 | 19,46 | 0,03 | 0,08 |
| OP-DP prod. late pH control | 3,67 | 2 | 1,5 | 72 | 250-500 | DP | Orange peel infusion | - | 5,8-7,5 | 20% | 0,5 | 5,7 | 26,68 | 0,07 | 0,03 |
| OP mimicking ferm. | 3,68 | 2 | 1,5 | 48 | 250-500 | DP | Glucose/ fructose/ Sucrose | 7,5 | 5,8-7,0 | 25% | 0,55 | 3,7 | 16,20 | 0,89 | 0,19 |
| OP pH-%DOT control ferm. | 3,69 | 2 | 1,5 | 79 | 250-500 | DP | Orange peel infusion | 20,1 | 6,0-6,6 | 10% | 0,68 | 5,7 | 25,60 | 0,21 | 0,22 |

## 8.9 Statistical Analysis by SPSS

### 8.9.1 Yeast Extract addition experiment

| Code | Parameter |
|---|---|
| 0.0 | Control |
| 1.0 | 0.625 mg/L |
| 2.0 | 1.25 mg/L |
| 3.0 | 2.5 mg/L |
| 4.0 | 5.0 mg/L |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 24 h | Between Groups | 163.872 | 4 | 40.968 | 22.849 | .000 |
| | Within Groups | 17.930 | 10 | 1.793 | | |
| | Total | 181.802 | 14 | | | |
| 48 h | Between Groups | 547.710 | 4 | 136.928 | 43.436 | .000 |
| | Within Groups | 31.524 | 10 | 3.152 | | |
| | Total | 579.234 | 14 | | | |

### Post Hoc Tests

**Multiple Comparisons**

Tukey HSD

| Dependent Variable | (I) V1 | (J) V1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| 24 h | .0 | 1.0 | 4.734908477353610* | 1.093312724678315 | .010 | 1.136726495135889 | 8.333090459571330 |
| | | 2.0 | 5.904942307367513* | 1.093312724678315 | .002 | 2.306760325149792 | 9.503124289585234 |
| | | 3.0 | 7.339116823024298* | 1.093312724678315 | .000 | 3.740934840806578 | 10.937298805242019 |
| | | 4.0 | 10.004350176541404* | 1.093312724678315 | .000 | 6.406168194323684 | 13.602532158759125 |
| 48 h | .0 | 1.0 | -9.416731372841756* | 1.449684712735841 | .001 | -14.187762840345574 | -4.645699905337938 |
| | | 2.0 | 4.846624221528039* | 1.449684712735841 | .046 | .075592754024220 | 9.617655689031857 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 3.0 | 6.253169144442087* | 1.449684 71273584 1 | .010 | 1.48213767 6938268 | 11.0242006 11945904 |
| | 4.0 | 6.746466227746229* | 1.449684 71273584 1 | .006 | 1.97543476 0242410 | 11.5174976 95250047 |

*. The mean difference is significant at the 0.05 level.

### 8.9.2 Yeast Extract Concentration

| Code | Parameter |
|---|---|
| 0.0 | 0 g/L |
| 1.0 | 1.5 g/L |
| 2.0 | 3 g/L |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| cdw g/L | Between Groups | 2.465 | 2 | 1.233 | 552.575 | .000 |
| | Within Groups | .013 | 6 | .002 | | |
| | Total | 2.478 | 8 | | | |
| γ-PGA g/L | Between Groups | .960 | 2 | .480 | 85.147 | .000 |
| | Within Groups | .034 | 6 | .006 | | |
| | Total | .994 | 8 | | | |
| P(3HB) g/L | Between Groups | .038 | 2 | .019 | 570.830 | .000 |
| | Within Groups | .000 | 6 | .000 | | |
| | Total | .038 | 8 | | | |
| %PHB/cdw | Between Groups | 78.845 | 2 | 39.422 | 92.750 | .000 |
| | Within Groups | 2.550 | 6 | .425 | | |
| | Total | 81.395 | 8 | | | |

## Post Hoc Tests

**Multiple Comparisons**

Tukey HSD

| Dependent Variable | (I) V1 | (J) V1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound | 95% Confidence Interval Upper Bound |
|---|---|---|---|---|---|---|---|
| cdw g/L | .0 | 1.0 | -.541000* | .038562 | .000 | -.65932 | -.42268 |
| | | 2.0 | -1.277000* | .038562 | .000 | -1.39532 | -1.15868 |
| | 1.0 | .0 | .541000* | .038562 | .000 | .42268 | .65932 |
| | | 2.0 | -.736000* | .038562 | .000 | -.85432 | -.61768 |
| | 2.0 | .0 | 1.277000* | .038562 | .000 | 1.15868 | 1.39532 |
| | | 1.0 | .736000* | .038562 | .000 | .61768 | .85432 |
| γ-PGA g/L | .0 | 1.0 | -.800000* | .061304 | .000 | -.98810 | -.61190 |
| | | 2.0 | -.400000* | .061304 | .002 | -.58810 | -.21190 |
| | 1.0 | .0 | .800000* | .061304 | .000 | .61190 | .98810 |
| | | 2.0 | .400000* | .061304 | .002 | .21190 | .58810 |
| | 2.0 | .0 | .400000* | .061304 | .002 | .21190 | .58810 |
| | | 1.0 | -.400000* | .061304 | .002 | -.58810 | -.21190 |
| P(3HB) g/L | .0 | 1.0 | -.081544* | .004720 | .000 | -.09603 | -.06706 |
| | | 2.0 | -.159467* | .004720 | .000 | -.17395 | -.14498 |
| | 1.0 | .0 | .081544* | .004720 | .000 | .06706 | .09603 |
| | | 2.0 | -.077923* | .004720 | .000 | -.09241 | -.06344 |
| | 2.0 | .0 | .159467* | .004720 | .000 | .14498 | .17395 |
| | | 1.0 | .077923* | .004720 | .000 | .06344 | .09241 |

*. The mean difference is significant at the 0.05 level.

### 8.9.3 Effect of Citric acid Concentration

| Code | Parameter |
|---|---|
| 0.0 | 0 g/L |
| 1.0 | 1.5 g/L |
| 2.0 | 3.0 g/L |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| cdw g/L | Between Groups | .410 | 2 | .205 | 159.875 | .000 |
| | Within Groups | .008 | 6 | .001 | | |
| | Total | .417 | 8 | | | |
| γ-PGA g/L | Between Groups | .240 | 2 | .120 | 3.228 | .112 |
| | Within Groups | .223 | 6 | .037 | | |
| | Total | .463 | 8 | | | |
| P(3HB) g/L | Between Groups | .031 | 2 | .015 | 242.714 | .000 |
| | Within Groups | .000 | 6 | .000 | | |
| | Total | .031 | 8 | | | |

**Post Hoc Tests**

**Multiple Comparisons**

Tukey HSD

| Dependent Variable | (I) V1 | (J) V1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound | 95% Confidence Interval Upper Bound |
|---|---|---|---|---|---|---|---|
| cdw g/L | .0 | 1.0 | -.182000* | .029231 | .002 | -.27169 | -.09231 |
| | | 2.0 | -.515333* | .029231 | .000 | -.60502 | -.42565 |
| γ-PGA g/L | .0 | 1.0 | .200000 | .157427 | .460 | -.28303 | .68303 |
| | | 2.0 | .400000 | .157427 | .097 | -.08303 | .88303 |
| P(3HB) g/L | .0 | 1.0 | -.083435* | .006474 | .000 | -.10330 | -.06357 |
| | | 2.0 | -.141909* | .006474 | .000 | -.16177 | -.12204 |

*. The mean difference is significant at the 0.05 level.

### 8.9.4 Effect of Glutamic acid Concentration

| Code | Parameter |
|---|---|
| 0.0 | 0 g/L |
| 1.0 | 20 g/L |
| 2.0 | 40 g/L |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| cdw g/L | Between Groups | .013 | 2 | .007 | 4.895 | .055 |
| | Within Groups | .008 | 6 | .001 | | |
| | Total | .021 | 8 | | | |
| γ-PGA g/L | Between Groups | 54.320 | 2 | 27.160 | 297.737 | .000 |
| | Within Groups | .547 | 6 | .091 | | |
| | Total | 54.867 | 8 | | | |
| P(3HB) g/L | Between Groups | .011 | 2 | .005 | 43.837 | .000 |
| | Within Groups | .001 | 6 | .000 | | |
| | Total | .012 | 8 | | | |

### Post Hoc Tests

**Multiple Comparisons**

Tukey HSD

| Dependent Variable | (I) V1 | (J) V1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound | 95% Confidence Interval Upper Bound |
|---|---|---|---|---|---|---|---|
| cdw g/L | .0 | 1.0 | .046000 | .029832 | .338 | -.04553 | .13753 |
| | | 2.0 | -.047333 | .029832 | .321 | -.13887 | .04420 |
| γ-PGA g/L | .0 | 1.0 | -2.600000* | .246605 | .000 | -3.35665 | -1.84335 |
| | | 2.0 | -6.000000* | .246605 | .000 | -6.75665 | -5.24335 |
| P(3HB) g/L | .0 | 1.0 | .079190* | .009138 | .000 | .05115 | .10723 |
| | | 2.0 | .067660* | .009138 | .001 | .03962 | .09570 |

*. The mean difference is significant at the 0.05 level.

### 8.9.5 Effect of Ammonium sulphate Concentration

| Code | Parameter |
|---|---|
| 0.0 | 0 g/L |
| 1.0 | 7.5 g/L |
| 2.0 | 15 g/L |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| cdw g/L | Between Groups | .033 | 2 | .017 | 33.122 | .001 |
| | Within Groups | .003 | 6 | .001 | | |
| | Total | .036 | 8 | | | |
| γ-PGA g/L | Between Groups | 49.520 | 2 | 24.760 | 353.795 | .000 |
| | Within Groups | .420 | 6 | .070 | | |
| | Total | 49.940 | 8 | | | |
| P(3HB) g/L | Between Groups | .007 | 2 | .004 | 117.136 | .000 |
| | Within Groups | .000 | 6 | .000 | | |
| | Total | .007 | 8 | | | |

### Post Hoc Tests

**Multiple Comparisons**

Tukey HSD

| Dependent Variable | (I) V1 | (J) V1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound | 95% Confidence Interval Upper Bound |
|---|---|---|---|---|---|---|---|
| cdw g/L | .0 | 1.0 | -.126667* | .018261 | .001 | -.18270 | -.07064 |
| | | 2.0 | -.130667* | .018261 | .001 | -.18670 | -.07464 |
| γ-PGA g/L | .0 | 1.0 | -1.000000* | .216000 | .009 | -1.66275 | -.33725 |
| | | 2.0 | -5.400000* | .216000 | .000 | -6.06275 | -4.73725 |
| P(3HB) g/L | .0 | 1.0 | -.057207* | .004522 | .000 | -.07108 | -.04333 |
| | | 2.0 | -.062338* | .004522 | .000 | -.07621 | -.04846 |

*. The mean difference is significant at the 0.05 level.

### 8.9.6 Production of P(3HB) Using Orange peel

| Code | Parameter |
|---|---|
| 1.0 | OPA |
| 2.0 | OPB |
| 0.0 | OPC |
| 3.0 | OPD |

**ANOVA**

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| CDW (g/L) | Between Groups | .140 | 3 | .047 | 167.049 | .000 |
| | Within Groups | .002 | 8 | .000 | | |
| | Total | .142 | 11 | | | |
| Total Carbohydrate (g/L) | Between Groups | .217 | 3 | .072 | 2.376 | .146 |
| | Within Groups | .244 | 8 | .030 | | |
| | Total | .461 | 11 | | | |
| P(3HB) (mg/L) | Between Groups | 5.224 | 3 | 1.741 | 451.088 | .000 |
| | Within Groups | .031 | 8 | .004 | | |
| | Total | 5.255 | 11 | | | |
| %PHB/cdw | Between Groups | 6.982 | 3 | 2.327 | 99.917 | .000 |
| | Within Groups | .186 | 8 | .023 | | |
| | Total | 7.168 | 11 | | | |

**Post Hoc Tests**

**Multiple Comparisons**

Tukey HSD

| Dependent Variable | (I) V1 | (J) V1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval Lower Bound | 95% Confidence Interval Upper Bound |
|---|---|---|---|---|---|---|---|
| CDW (g/L) | .0 | 1.0 | .219500* | .013630 | .000 | .17585 | .26315 |
| | | 2.0 | .202500* | .013630 | .000 | .15885 | .24615 |
| | | 3.0 | .290000* | .013630 | .000 | .24635 | .33365 |
| Total Carbohydrate (g/L) | .0 | 1.0 | .148649 | .142572 | .731 | -.30792 | .60521 |
| | | 2.0 | -.189189 | .142572 | .573 | -.64575 | .26738 |
| | | 3.0 | .128378 | .142572 | .805 | -.32819 | .58494 |
| P(3HB) (mg/L) | .0 | 1.0 | 1.035807* | .050731 | .000 | .87335 | 1.19826 |
| | | 2.0 | 1.471035* | .050731 | .000 | 1.30858 | 1.63349 |
| | | 3.0 | 1.729512* | .050731 | .000 | 1.56705 | 1.89197 |
| %PHB/cdw | .0 | 1.0 | .240319 | .124610 | .289 | -.15873 | .63936 |
| | | 2.0 | 1.600903* | .124610 | .000 | 1.20186 | 1.99995 |
| | | 3.0 | 1.669903* | .124610 | .000 | 1.27086 | 2.06895 |

*. The mean difference is significant at the 0.05 level.