A Strategy for Dual Biopolymer Production of P(3HB) and γ-PGA

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Dual Biopolymer Production

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Abstract

BACKGROUND: Production of biopolymers has gained considerable attention because of their biodegradability, biocompatibility, and as suitable replacements for mineral-based polymers. Despite advances in production process, a notable drawback still exists due to high production cost. The aim of this paper is to provide a production strategy for cost reduction. The suggested process may be adopted to other polymers, useable to wide audience in biopolymer research.

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RESULTS: Dual production of two commercially important biopolymers, P(3HB) and γ-PGA, in a single batch from cheap substrates was studied, as proof of concept, for a feasible low cost dual biopolymer production. The dual production from a single batch yielded 1 g/L P(3HB) and 0.4 g/L γ-PGA using *Bacillus subtilis* OK2 (*B. subtilis* OK2). When orange peel was substituted as a cheap carbon source for dual production, coupled pH and dissolved oxygen control proved to be essential to overcome the inhibition imposed by the non-sugar components of the substrate.

The cell lysis and release of P(3HB) granules in the dual production medium can be exploited of as a new approach for separation of this polymer.

CONCLUSION: This proof of concept study provides a new approach from upstream to downstream processing for low cost production of dual biopolymers.

Keywords: Dual production; biopolymers; orange peel; cell self-destruction

INTRODUCTION

The global production of plastics grew from around 1.3 million tonnes (MT) in 1950 to 245 MT in 2006[1] and in 2015 it was estimated to be approximately 300 MT.[2] The fast consumption of plastics and their inefficient recycling has resulted in continuous accumulation of plastics in landfill and marine environment. As an example, in 2012, only 9 per cent of the 32 MT of plastic waste generated in the US was recycled[3] and most of this un-recycled plastic was discharged to dumping sites and to oceans. Plastic pollution can seriously affect living organisms, particularly marine animals, through entanglement, direct ingestion of plastic waste, or intoxication through exposure to chemical contents of plastics and humans through the disruption of the hypothalamic-pituitary-adrenal (HPA) axis or fluctuation in sex hormone levels.[4] Increasing danger of plastic accumulation in recent years highlights the

importance of biodegradable plastics along with the production from alternative raw materials.

Polyhydroxyalkanoates (PHAs) are water insoluble, multifunctional, biodegradable and biocompatible biopolymers, therefore they have been in the researchers' spotlight for some years[5]. PHAs are produced through the fermentation of sugars, lipids, alkanes, alkenes and alkanoic acids in the presence of excess carbon while another essential nutrient, such as nitrogen or phosphorus is limiting.[6,7] Several bacteria [7] including *E. coli, Bacillus sp., Cupriavidus necator, and Pseudomonas putida,* Cyanobacteria[8-10] Archaea[11] and have been reported to be capable of producing PHAs. It is produced in amorphous state and crystallization occurs as soon as it is extracted from cells. PHAs have high crystallinity ranging from 60-80% and high rigidity and is therefore referred to as a semi-crystalline thermoplastic. The mechanical and chemical properties may be adjusted by blending it with other biodegradable polymers.[12] A total of 4,613 papers and 6,844 patents have been published according to Web of Science and Pat-Base ((keywords: Polyhydroxyalkanoate, poly(3-hydroxybutyrate), Fermentation, Metabolic Products)) between the years 2000-2015 on PHAs alone.

Although there is great interest in PHA production, the major bottleneck in its industrial production is the high cost of raw materials, relatively low conversion rates and the downstream processing/operation costs.[6, 7, 13, 14]

One approach is to enhance the profits from the fermentation by increasing the number of valuable products obtained from a single batch; therefore splitting the costs to two, or more products. In this context, 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 a single fermenter compared to multiple fermenters). This bio-refinery approach, however, faces

problems due to the diversity of microorganisms' demands to produce products. Simultaneous or sequential production of two biopolymers with another bio-product has been reported. However, in many cases this has been based on unplanned, *ad-hoc* observation rather than prior deliberate design.[15-18] So, there is great opportunity for structured-investigation, leading to potential economic advantages for bio-industries. Simultaneous production of industrially important biopolymers is an attractive approach for a high-profit bio-refinery.

Poly glutamic acid (PGA) is a naturally occurring, anionic homo-polyamide consisting of D- and L- glutamic acid units connected by amine linkages from α-amino and γ-carboxylic groups.[19] It is produced by *B. subtilis*, *B. licheniformis*, *B. anthracis* and *B. megaterium*[20] as an exo-cellular polymer; either secreted by the cell into the environment or kept in capsules bound to peptidoglycan.[21] Various applications of PGAs exist in the fields of food, agriculture, medical and cosmetics such as thickener, fertiliser, drug carrier, and moisturiser respectively.[19,21] There are two groups of γ-PGA producing bacteria[19], one requiring the addition of L-glutamic acid to the medium for cell growth to initiate γ-PGA production, the other not. For glutamic acid dependent bacteria, the γ-PGA yield increases with increasing L-glutamic acid concentration in the medium, although γ-PGA can be produced at low yields, even in the absence of L-glutamic acid due to the synthesis of L-glutamic acid through the de novo pathway. The interest for PGA, as a biopolymer dates well before PHAs, however there are only 2,423 research articles published and 3,270 patents applied according to Web of Science and Pat-Base between the years 1996 -2015.

This study aims to produce two commercially important biopolymers, namely poly(3-hydroxybutyrate) (P(3HB)) and poly-γ-glutamic acid (γ-PGA) from a single organism in a single batch. The strain *B. subtilis* OK2 was used for its ability to produce the two polymers in this proof of concept study, not as a strain with high production levels. Dual production of the two polymers was realised in both shaken flask and fermenter scale using a dual-production

medium and respective culture conditions, optimised prior to this study within our research group.[22] The culture conditions was not tested or change since this was outside the scope of this research. Different inoculum media were tested to promote the dual production. In order to reduce the costs of raw materials for the dual production, the main carbon source was replaced with an agro-industrial waste. In addition, medium-dependent self-disruptive behaviour of *B. subtilis* OK2 was observed and reported. This feature could be exploited in future studies of downstream processing for further cost reduction.

EXPERIMENTAL

Chemicals

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.

Bacterial Strain

The recombinant strain, *B. 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. The strain was modified from a natural isolate of *B. subtilis* natto strain using a plasmid vector, and has been described in detail.[23]

Culture Media and Growth Conditions

Nutrient broth (20 mL) in a 50 mL shake flask was inoculated with a loop-full of *B. 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.

A defined production medium (containing (g/L) 20 glucose, 2.5 yeast extract, 3 KCl, 5 $(NH_4)_2SO_4$, and 1 Soytone (enzymatic digest of soybean meal)) was used for P(3HB) as previously reported [13] with cultivation at 30 ^oC and 200 rpm for 72 h.

For γ-PGA production, an in-house developed medium, containing (g/L) 20 citric acid, 30 mono sodium glutamate, 15 $(NH_4)_2SO_4$, 20 glycerol, 1 K_2HPO_4 and Na_2HPO_4, 0.5 $MgSO_4$, 0.2 $CaCl_2$, 0.05 $FeCl_3$ and 0.02 $MnSO_4$ and Medium E [24] was used for with cultivation at 37 ^oC and 180 rpm for 48 h after inoculation.

Dual polymer production medium optimised prior to this study, composed of (g/L) 32 glutamic acid, 20 glucose, 12 $(NH_4)_2SO_4$, 1.5 yeast extract, 2.4 citric acid was used throughout the experiments with cultivation at 30 ^oC and 200 rpm for 48 h after inoculation.[22]

The bio-waste, orange peel was obtained from Just Ingredients, UK in powder form and pre-treated as described in literature.[5]

Polymers Extraction and Quantification

For the extraction of P(3HB), the cells were harvested by centrifugation (Denley BS400 Centrifuge) at 4,500 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^oC for 1 h. The mixture obtained was centrifuged at 2,200 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.[13]

For the quantification of gP(3HB) content per CDM, a slight modification to the crotonic acid method reported in literature was made.[25] 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 three times, before incubation, half-way through and after incubation. The absorbance of the crotonic acid concentration converted from P(3HB) was

measured at 235 nm (Jenway 6503 UV-vis spectrophotometer) and the concentration was calculated using a calibration curve.

For the extraction of γ-PGA, a predetermined volume of the culture broth (250 µL to 5 mL) was centrifuged at 5,000 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 10,000 g for 4 min and the precipitate was freeze-dried.[26]

For the spectrophotometric measurement the freeze-dried crude γ-PGA sample was re-dissolved in 1mL distilled water and centrifuged (10,000 g) to remove the suspended particles. Absorbance was measured at 216 nm.[27] γ-PGA concentration was determined using a calibration curve.

Other Methods

The fermentations were carried out using Electrolab FerMac310/60 equipped with automated control in 2 L stirred tank reactors (1.5 L working volume) with turbine type impellers. During the fermentation, pH, temperature and dissolved oxygen (%DOT air saturation) were monitored or controlled depending on experimental requirements. Samples were taken against time. The exhaust gas from the fermenter (%CO_2) was analysed using FerMac 368 Gas Analyser, calibrated using a standard gas mixture containing 5.09 % CO_2, 18.28 % oxygen, and 76.63 % nitrogen.

Phenol-sulphuric acid method was applied for the determination of total carbohydrate content.[28] A sample (200 µL) from the fermentations and/or standard solution 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.

Microscopic observation of bacterial cells was carried out without any pre-treatment or staining using Nikon phase contrast microscope, under phase contrast setting, to observe individual cells and to 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 were carried out on a Nano-Sight LM10 and analyses were performed using a beta version of NTA 3.1 software. For the measurements of particle size the culture broth containing granules was centrifuged at 8,900 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.

RESULTS

Testing different inoculum media

Four different media were tested for their effects on growth of the organism. Change in lag phase and adaptation behaviour was observed when the inoculum was grown in a media different than the production medium.

When the inoculum was grown in nutrient broth (NB) and re-inoculated into NB or P(3HB) medium, there was almost no lag phase. However, a shorter lag phase was observed when inoculum was pre-grown in γ-PGA medium compared to the other cases (Figure 1).

Fig. 1 here

Cases starting with inoculum grown in P(3HB) or dual production media are not included in Table 1 (when the inoculum was transferred to dual production medium almost no growth or very poor growth was observed). The inoculum grown on NB combined with other media was included for comparison purposes. Specific growth-rates and doubling times are calculated for each medium, between 2-6 hours according to $t_d=0.693/\mu$. (Table 1).

Table 1 here

At the end of the cultivation period (30 h), the final optical density observed for the culture pre-grown in γ-PGA medium and inoculated to dual production medium was higher than all media tested.

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 (unpublished data). When grown in nutrient broth and P(3HB) production medium, the bacteria exhibited standard rod shape phenotypes while in γ-PGA medium the bacteria formed long chains of filamentous-like structures. Overall, *B. subtilis* OK2 grown in dual production medium using inoculum grown in γ-PGA medium showed better growth and production.

Production in Shaken Flasks

Subsequent to these preliminary investigations, the growth of *B. subtilis* OK2 and production profile of dual production culture was investigated in shaken flasks (Figure 2). The dual production medium was inoculated with 16 h inoculum grown in γ-PGA medium and was incubated for 72 h. An average of 1 g/L P(3HB) and 0.4 g/L γ-PGA were produced, using *B. subtilis* OK2 which is not a high-producing strain.

Fig. 2 here

A discrepancy was observed between optical density and CDM measurements. After 24 h of fermentation, the optical density of the culture started to decline. This decline was not observed in CDM measurements.

Production in Fermenters

Dual production production was tested in 2 L bioreactor (working volume of 1.5 L). Several oxygen regimes were investigated and results obtained are given in Table 2.

Table 2 here

Comparing the reactor performances 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 CDM is obtained. Although poor oxygen transfer rates in flasks is generally agreed, 50 mL working volume in 250 mL Erlenmeyer flasks provided sufficient oxygenation to enable good levels of dual production. In addition, rigorous agitation of the culture for long periods provoked extensive foaming due to other extracellular products.

Based on the importance of % DOT level and bearing in mind the foaming tendency at high stirrer speeds, a regime was adopted for the *B. subtilis* OK2 strain, where % DOT was maintained at 25% air saturation for the first 24 h and then dropped to 10% air saturation. This aeration strategy provided the culture with sufficient dissolved oxygen and enabled control of foaming. The titres obtained for the two polymers were 0.49 g/L and 0.18 g/L P(3HB) and γ-PGA respectively (Figure 3).

Fig. 3 here

Production of dual biopolymers using orange peel as carbon source

Orange peel was tested as a carbon substitute for the dual production. 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. All other medium components and their concentrations were kept the same as the cultivation conditions identified in the previous experiments. The initial fermentation was carried for 54 h during which, 76 mg/L of γ-PGA and 34 mg/L of P(3HB) were obtained. The growth and production profiles were different than the results observed in dual production mediua fermentation. In this fermentation, a rapid increase was observed in the culture pH and after 25 h, the pH exceeded 7.5 which made the conditions in the fermenter unfavourable for bacterial growth and P(3HB) production (figure not shown). Subsequently, the fermentation was repeated under pH controlled conditions (Figure 4); under this condition, approximately 0.2 g/L of each polymer was obtained after 79 h.

Fig. 4 here

Investigation on self-destruction and P(3HB) Granules

The self-destruction behaviour of *B. subtilis* OK2 was first observed in fermentations with dual production medium. Disintegrated cells released polymer granules into the medium. The phenomenon was further tested in fermentations with different medium compositions, in order to identify if it was organism-specific or triggered by dual production medium composition. Microscopic investigation of cultures in P(3HB) medium showed very robust cells even after 72 h of cultivation (Figure 5b and 5d). The P(3HB) accumulation was microscopically visible throughout the time period and the accumulated P(3HB) was metabolised towards the end of the fermentation; hence leading to a slight reduction in cell size.

Fig. 5 here

Microscopic observations revealed that sporulation and auto-lysis occurred only in the dual production medium. The cells were robust after 24 h of cultivation (Figure 5a), but not after 72 h (Figure 5c), indicating that the behaviour was not organism specific.

Small spherical vesicle-like structures were observed to in the culture broth in addition to spores. The liquid broth containing these granules was treated with 30% NaOH and chloroform mixture. When the chloroform phase was subjected to crotonic acid treatment it was found to contain P(3HB), validating the composition of the granules. In order to identify the size distribution of the granules, samples from a 48 h culture were centrifuged at 8,900 g for 10 min. The supernatant containing suspended granules were measured using NTA3.1 software (Figure 6a and 6b).

Fig. 6 here

The diameter of majority of the particles was between 100 nm to 300 nm (Figure 6a). Particles up to 200 nm in size had a wide distribution of intensities, while larger particles

(>300nm), predominantly showed intensities above 0.6 (Figure 6b), indicating the presence of higher amounts of refractive material (most likely P(3HB) in this case).

DISCUSSION

Inoculum Studies

The duration of the fermentation process is one the variables determining the productivity of an industrial microbial batch culture and the length of the lag phase has a significant contribution in this. Since growing the inoculum in a medium different to the production environment usually results in a longer lag phase, preparing a pre-inoculum to adapt the culture to production conditions is usually implemented. The major reason that *B. subtilis* OK2 grown in dual production medium using inoculum from γ-PGA medium showed better growth and production is possibly due to the fact that the lack of certain nutrients (e.g. glucose) or presence of high stress factors (e.g. glutamic acid) [29,30] imposed upon the culture by the γ-PGA medium were rapidly reversed with the carbon-rich composition of the dual production medium (containing 20 g/L glucose). Transferring the culture from high-stress conditions into moderate stress conditions may be stimulating the metabolism leading to higher polymer production, hence increasing the productivity. Moreover, the high glutamic acid content present in γ-PGA and dual production media possibly creates a stress factor leading to a longer adaptation phase when transferred from NB medium. Researchers have demonstrated that high glutamic acid creates high-stress conditions leading to sporulation in *Bacillus* cultures. [29,30] Thick but long filamentous structures were observed microscopically in dual production medium inoculated with a culture grown on γ-PGA production medium. Formation of long chains of cells in *Bacillus* culture has been identified as a major indication of stress.[31] These findings indicate that the bacteria were under stress in γ-PGA medium and were unable to grow in an effective manner. It should be noted that the time of sampling may have contributed to these observations, since the culture entered the exponential phase after

15 h in γ-PGA medium while the commencement of the exponential phase in other cultures took about 5 h.

We are not aware of any literature so far reporting effect of different inoculum media on polymer production.

Oxygen dependency and dual production

During the shake flask productions with the dual production medium using the inoculum pre-grown in γ-PGA medium the CDM and optical density measurements were not correlated. Although this could be explained with accumulation of P(3HB) causing an apparent stability/increase in CDM, when the polymer weight was subtracted from CDM measurements it still did not correlate with OD_{600} measurements. When CDM was plotted against OD_{600} data, two distinctive regions corresponding to growth and polymer accumulation stages were observed. This finding is in agreement with recent reports in literature.[32,33] In these studies, it is stated that there is a need for two different optical density vs CDM calibration curves since the correlations for growth and production regions are different.

In addition, during the production in the flask, a decrease in both product concentrations was observed at around 55h. Although the reason behind this observation was not clear, this phenomenon was present in all repeats of the experiment, and does not appear to be an error in measurements. One of the possibilities could be due to the disruption of the cells. When the cells burst some of the enzymes secreted to the medium could cause breakdown of products. The disruption behaviour is discussed later in this section.

As shown in results section, during the production in shaken flasks and bench top fermenters a large oxygen dependency of the production was observed. This finding is supported by a number of studies on γ-PGA production using *Bacillus* sp., where cell growth, carbon utilisation and γ-PGA[34, 35] and P(3HB) [36, 37] yields have been correlated with stirrer speed and

air flow rates, and thus the availability of oxygen up to an optimum level such as 60% air saturation. Further increases had either no or detrimental effects due to the oxidative and shear stresses under high agitation speeds[38].

The common denominator of all these findings is the significance of dissolved oxygen levels in the culture medium which was verified in this study. Using this knowledge and the foaming tendency of the culture the adapted aeration strategy developed as provided good results as shown previously, and dual production from a single fermentation was achieved. Currently, most of the work reported in literature is on the dual production of PHAs and extracellular polymeric substances (EPS)[39], but no study has been carried out on production of PHAs and γ-PGA. This is the first report, with the best of our knowledge, showing the dual production of P(3HB) and γ-PGA in a single batch.

Since the raw material cost is considered a major parameter for the feasibility of industrial production of biopolymers, the main carbon source was replaced by an agro-industrial bio-waste to improve production costs. In a previous study[5] it was reported that orange peel, infused in water, is a very suitable raw material for the production of P(3HB) as replacement for glucose in the medium. Based on this finding, orange peel was tested as a carbon substitute for the dual production. Initial experiments in use of orange peel as a sole carbon source resulted with a rapid change in pH. This may be due to the ingredients of the orange peel, the only items different in the media composition of the fermentations. Apart from variety of sugars, orange peel contains nitrogen, ash, phenols, tannins, cellulose, pectin, hemicellulose, and lignin.[40] The amino acids composition of orange peel has been reported as alanine, γ-aminobutyric acid, asparagine, aspartic acid, glutamic acid, leucine, phenylalanine, proline, serine and valine.[41] An increase of pH in γ-PGA fermentation has been reported to be due to ammonification of organic nitrogenous compounds such as amino acids.[42] Although the dual production medium contains glutamic acid and yeast extract, which itself is rich in

amino acids, the presence of additional glutamic acid possibly creates a buffering effect thus preventing excessive pH increase in dual production using glucose as carbon source. Therefore, the rich amino acid content of orange peel in this case may be the reason for the increase in pH.

In the medium containing orange peel infusion, a longer lag phase, compared to the dual production medium fermentation was observed. This was expected as phenols and tannins present in orange peel may cause a longer lag phase for the organism. The phenolic compounds present in bitter and sweet orange peel have been investigated[43] to be 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). These major phenolic contents may be potential inhibitors for the process.

Although, both polymers were produced at levels lower than those obtained in optimised dual production medium, it was demonstrated that it is possible to produce both polymers using orange peel as a cheap carbon source in a single fermentation. Further investigation is needed in order to increase the productivity using this agro-industrial waste as a substrate.

Studies on the dual production of biopolymers using bio-wastes are very limited in literature. Production of P(3HB) and EPS was studied[44] culturing *Azotobacter chroococcum* strain isolated from soil samples using molasses as a complex carbon source. Also, in a recent example of dual production, rice bran hydrolysate was used as a carbon source for the production of P(3HB) and Polyhydroxyvalerate (PHV) as well as EPS by *Sinorhizobium meliloti* in a single stage fermentation conducted in the shaken flasks[16]. To the best of our knowledge, this is the first report on production of dual biopolymer in a fermenter using a biowaste, orange peel in this case.

Self-destruction Behaviour and P(3HB) granules

Another new concept that is introduced with this study is the exploitation of the self-destruction behaviour of the organism for facilitating the separation of the polymer from the

culture medium. A link between the self-destruction behaviour of *Bacillus* cells with sporulation has been reported in literature.[45,46] The auto-lysis behaviour is triggered with the induction of sporulation. It has been reported[47] that glucose is required for sporulation in a medium promoting sporulation of *Bacillus larvae*, a poorly sporulating strain. They also reported that yeast extract (1.5 to 2.25 %) was required for growth and had to be balanced for sporulation. They suggested that failure to sporulate at high yeast extract concentrations was probably due to excessive acid accumulation in the medium.

In this study, the glucose concentrations of dual production and P(3HB) media were the same, but yeast extract concentration was lower in the dual production medium compared to P(3HB) medium. In addition, P(3HB) medium contained Soytone (enzymatic digest of soybean meal) as an additional nitrogen source. High levels of nitrogen source in the P(3HB) medium (more yeast extract and soytone) could be the reason for the late or even non-sporulating behaviour of the organism in the P(3HB) medium[47].

Concomitant with the cell disruption, small spherical vesicle-like structures were observed to in the culture broth in addition to spores. The vesicles exhibited Brownian motion and are described as native P(3HB) granules that form a stable suspension in liquid after centrifugation.[48] Based on the literature, the P(3HB) granules are spherical in shape and their sizes vary from 200 to 700 nm.[49] The biosynthesis of microbial PHA granules has been studied[50] to be water-insoluble cytoplasmic nano-sized inclusions formed with an amorphous polyester core and polyester synthase covalently attached to the surface, surrounded by a phospholipid membrane and sizes vary from 200 to 700nm. It was still unclear whether larger granules occurred due to fusion events or whether simple increase in size took place based on continuous polymerization. Similar results for the PHA granules have been obtained from *Bacillus* sp. NQ-11/A2, reporting spherical (0.51 ± 0.074 μm in diameter) PHA granules[51].

The release of PHA granules into the culture broth in fermentations using dual production medium may lead to simplification of downstream processes. This study demonstrates that granule formation is dependent on the composition of the culture medium, indicating that media conditions may be tailored to trigger the release of polymer-containing vesicles to facilitate the separation of the polymer from the culture broth, in turn positively affecting downstream processing costs in industrial production.

Overall, this presented study identified the optimum culture conditions and suggest a production strategy for dual production of two commercially important biopolymers, P(3HB) and γ-PGA. The results provide insight into the complexity of the dual production mechanism, and the effects of culture conditions in stirred-tank reactors. The opportunities and challenges associated with the use of bio-wastes, specifically orange peel, for the simultaneous production of P(3HB) and γ-PGA are highlighted. Furthermore, 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.

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Figure Captions

Fig. 1 Growth curves of *B.subtilis* OK2 grown in different fermentation media using inoculum pre-grown in nutrient broth (NB), and γ-PGA medium

Fig. 2 Fermentation profile for growth, P(3HB) and γ-PGA production, and total carbohydrate consumption in flasks for 72 h. Conditions; 30° C, 200 rpm

Fig. 3 Fermentation profile (76h) for (a) growth, dual 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, and 1 vvm. Points represent average of triplicate measurements

Fig. 4 Profile for (a) growth, dual production, total carbohydrate consumption (b) %DOT and pH, using orange peel as carbon source, at constant %DOT at 25% saturation by varying stirrer speed between 250-500 rpm, and pH at 6.5, 30°C and 1 vvm. Points represent average of triplicate measurements

Fig. 5 Microscopic observation of growth and P(3HB) accumulation of *Bacillus subtilis* OK2 in dual production medium after (a) 24 h and (c) 72 h and in P(3HB) production medium after (b) 24 h and (d) 72 h. Enlarged sections can be seen under insets of each picture. 400x magnification in phase contrast mode

Fig. 6 Distribution of (a) size of granules and concentration of each size cluster, (b) intensity of the granules in the culture supernatant. After centrifugation (8,900 g, 10 min), 10x diluted. Merged result of three replicates

Table 1: Specific growth-rates and doubling times of *B. subtilis* OK2 in different media between 2-6 hours of cultivation

Inoculum grown in	Medium	μ (h^{-1})	t_d (h)
NB	NB	0.535	1.30
NB	P(3HB)	0.600	1.16
NB	γ-PGA	0.135	5.13
NB	DP	0.034	20.39
γ-PGA	DP	0.232	2.99

Table 2: Comparison of dual polymer titres and CDM levels in 24 h cultures in shaken flasks (SF) and at different %DOT levels in stirred tank reactors, all values are in g/L

	Time	% DOT	CDM	P(3HB)	γ-PGA
SF	24 h	-	2.68	0.114	0.25
Ferm-1	24 h	Not controlled (Constant agitation at 250 rpm) 0 % DOT after 24 h	1.98	0.021	0.010
Ferm-2	24 h	Controlled at 25 %	3.12	0.051	0.329
Ferm-3	24 h	Controlled at 10%	7.40	0.056	0.031
Ferm-4	24 h	Variable (Constant agitation at 400 rpm, DOT fluctuated between 80-30%) 80 %	3.81	0.068	0.042

Figure 1

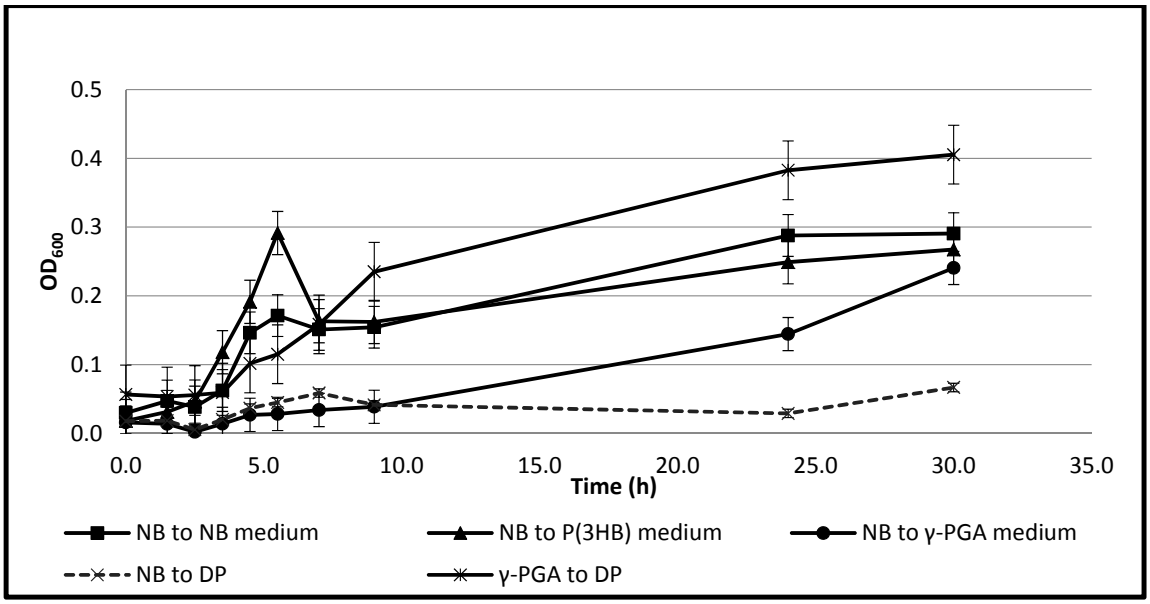
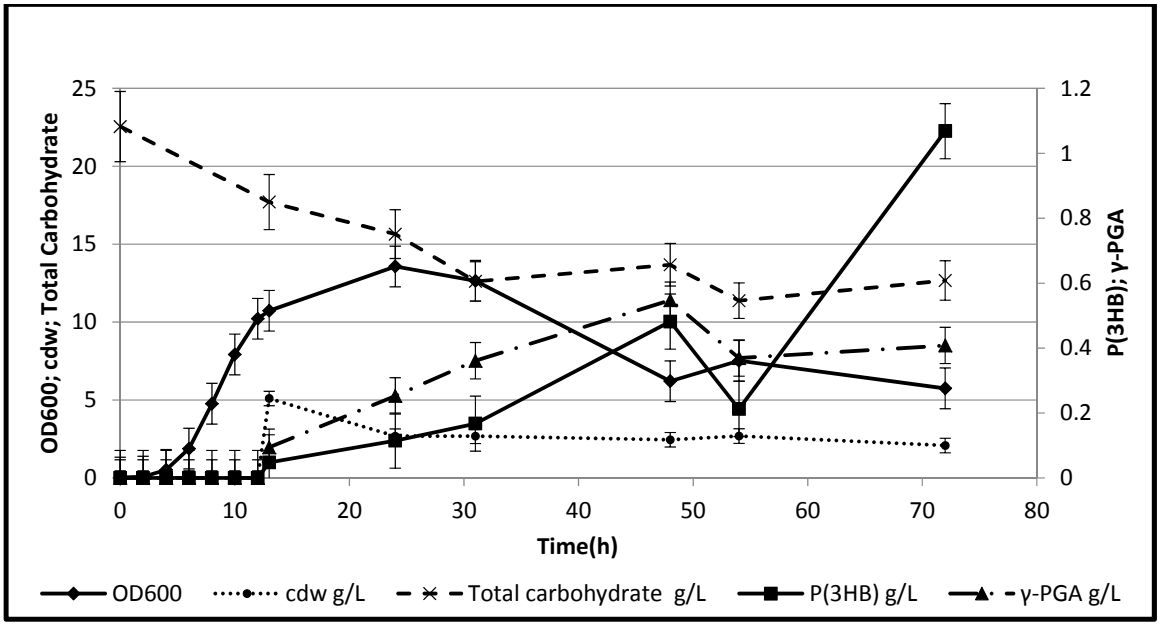


Fig. 1 Growth curves of *B. subtilis* OK2 grown in different fermentation media using inoculum pre-grown in nutrient broth (NB), and γ-PGA medium



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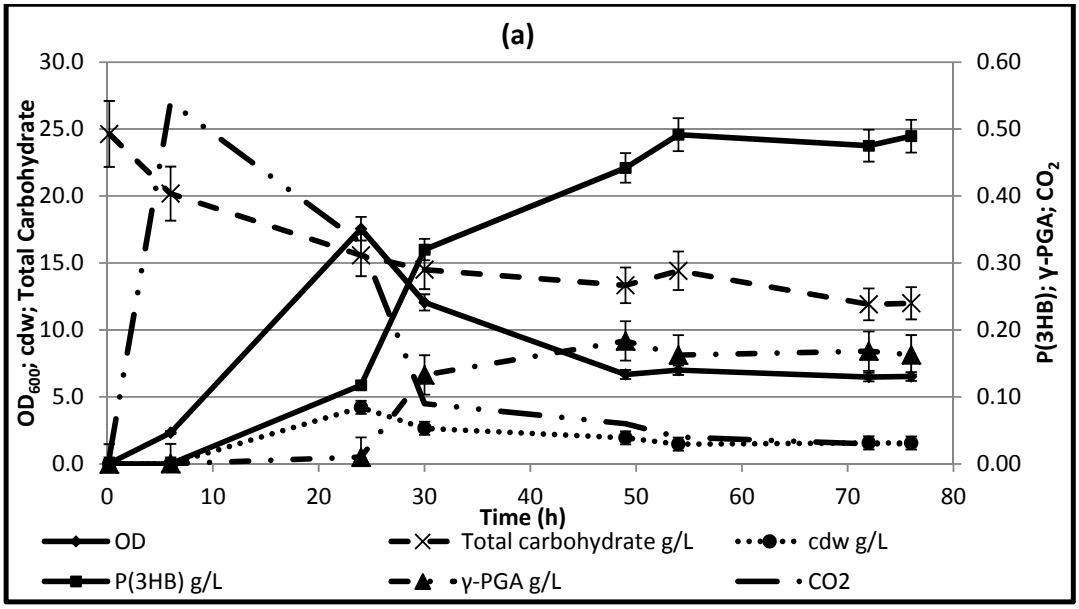

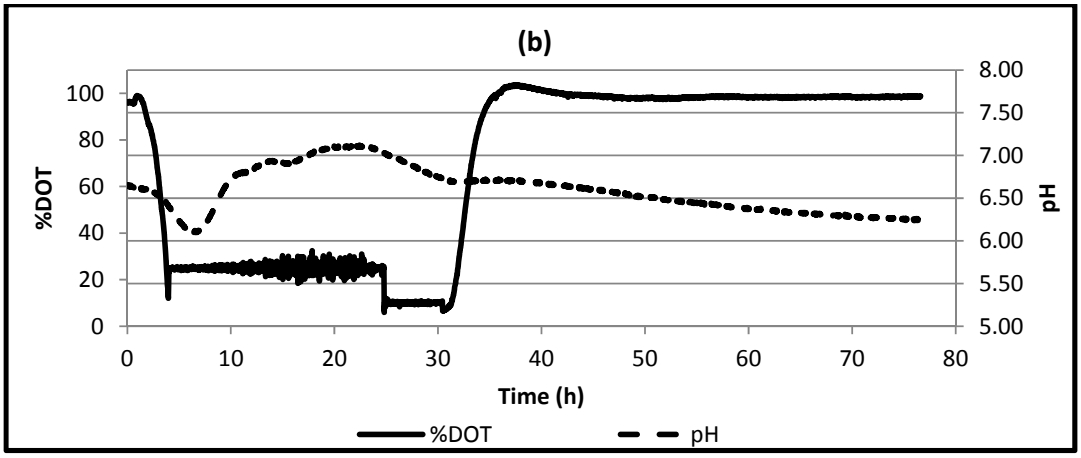

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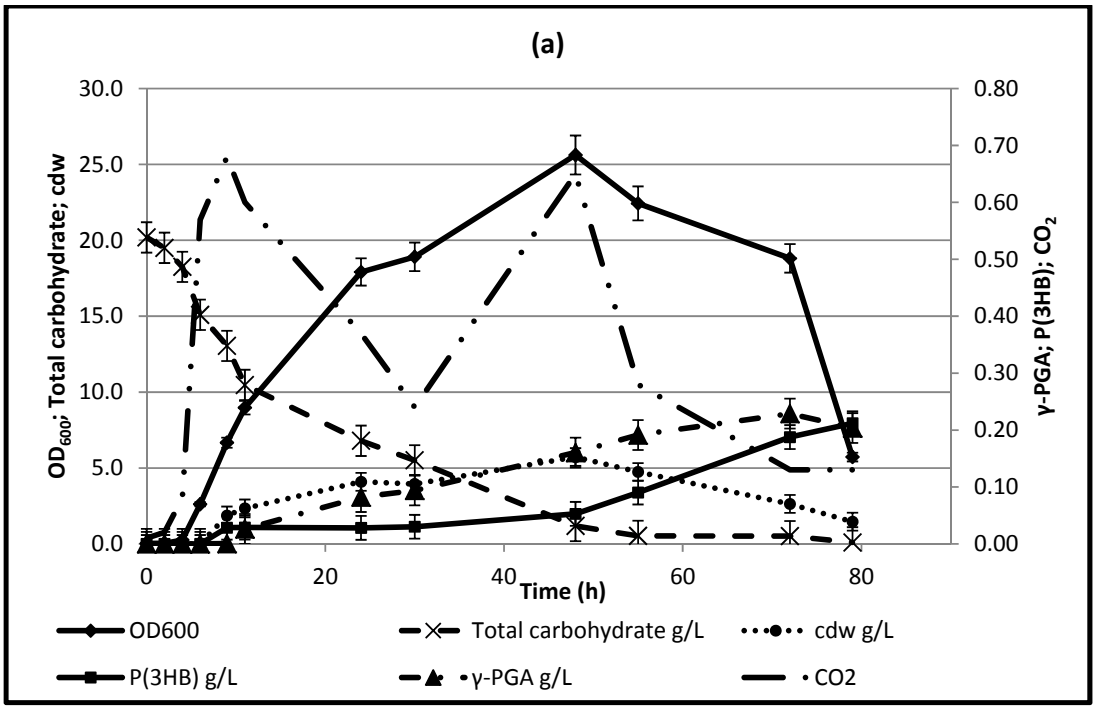

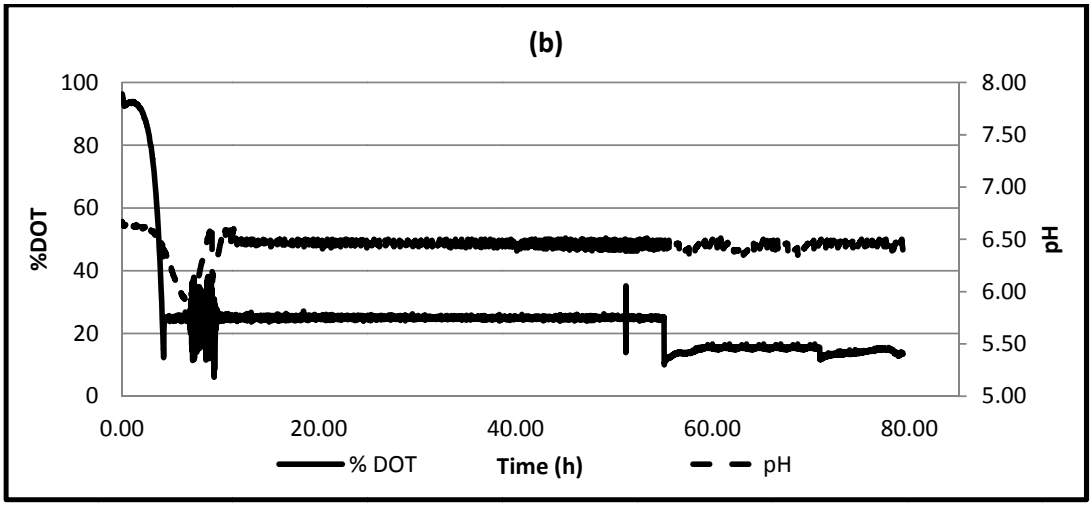

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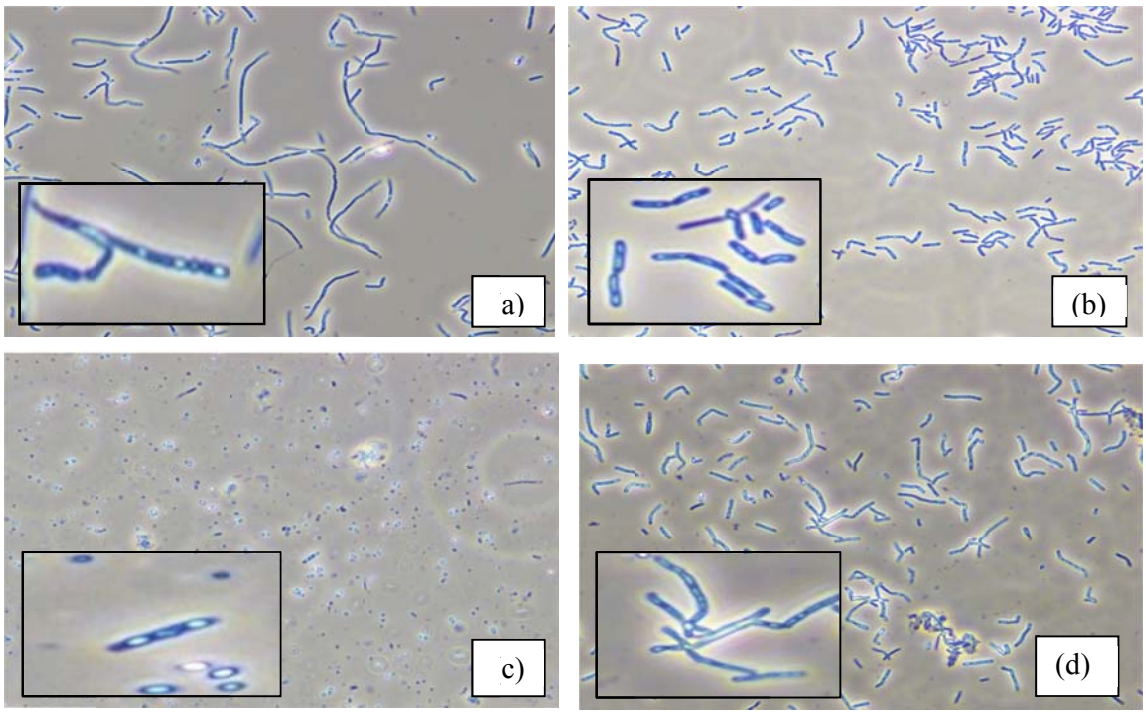

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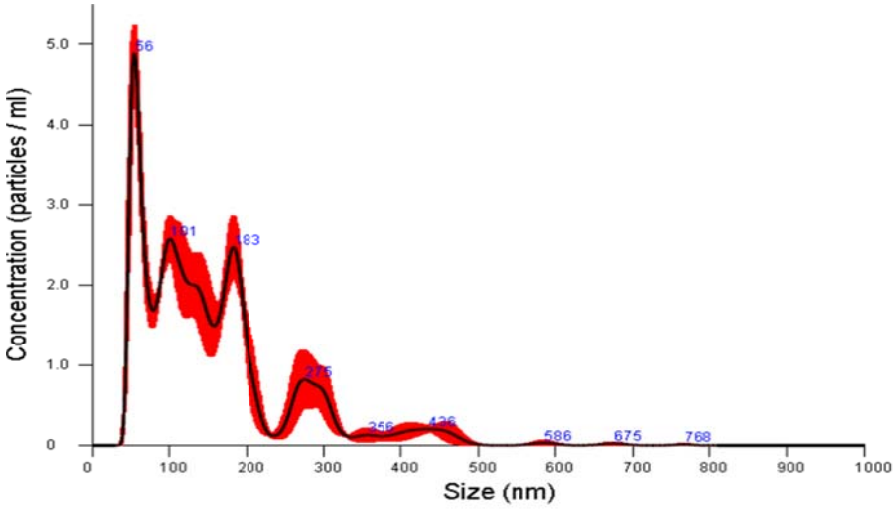
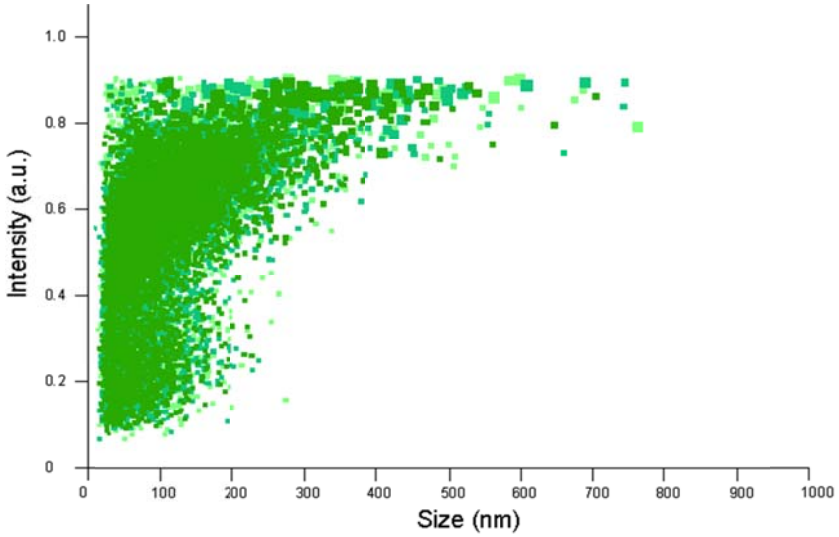


(a)



(b)

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