ELUCIDATION OF THE MECHANISM OF ELICITATION IN *PENICILLIUM CHRYSOGENUM*: SYSTEMATIC APPROACH TO STUDY THE EFFECT OF OLIGOSACCHARIDES ON PRODUCTION OF PENICILLIN G

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ABSTRACT

The addition of an oligosaccharide elicitor results in a series of coordinated events leading to stimulation of various morphological and/or physiological responses in *Penicillium chrysogenum*. Whilst elicitation in *P. chrysogenum* has been reported and is now established, no scientific evidence has been put forward to explain the complex phenomenon behind the observations. To expand the potential of elicitation in fungi from laboratory to industrial scale it is essential to establish the potential generic nature of the elicitor's effect and to provide concrete reasons for the changes observed. This work, serves as a starting point for the elucidation of some possible mechanisms.

Mannan oligosaccharides (MO) derived from locust bean gum, and oligomannuronate (OM) from alginate were used in these studies.

The effect of multiple addition of the same elicitor was investigated with a prospect that repeated addition would re-trigger the stimulation resulting in either maintenance of the penicillin G levels or enhancement of the production rate. However repeated addition of the same elicitor did not show any change in the production rate in comparison to single additions. Multiple additions of different elicitor types at different times and concentrations in a 5 L bioreactor showed an increase of 150% and 100% in the penicillin G production rate compared to the control and single elicitor addition cultures.

The importance of structure-activity relationship of oligosaccharides in elicitation was investigated. In this study the reducing end of MO was reduced and the modified MO (MO-R) was used to study the structure-activity relationship. Addition of MO-R resulted in a 65% decrease in the elicitor activity, but did not eliminate the ability of the oligosaccharide to enhance the production of penicillin G when compared to the untreated oligosaccharides.

The effect of single and multiple elicitor addition was also studied at the transcriptional level and showed that the transcript copy number in the elicited cultures was significantly higher ($p < 0.001$) for the three major penicillin G biosynthetic genes (*pcb*AB, *pcb*C and *pen*DE) in comparison to the control cultures.

The effect of elicitor addition on the cytosolic calcium level was investigated. A fluorescent method was developed that provides a dynamic and reliable technique for the analysis of cytosolic calcium changes. The addition of elicitors showed a significant increase in the cytosolic Ca^{2+} compared to control cultures to which no elicitor was added ($p < 0.001$).

The results found in this work aim to bring forward an understanding of the mechanism of action of elicitors in fungal cells.

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AUTHORS DECLARATION

I declare that the present work was carried out in accordance with the Guidelines and Regulations of the University of Westminster. The work is original except where indicated by special reference in the text.

The submission as a whole or part is not substantially the same as any that I previously or am currently making, whether in published or unpublished form, for a degree, diploma or similar qualification at any university or similar institution.

Until the outcome of the current application to the University of Westminster is known, the work will not be submitted for any such qualification at another university or similar institution.

Any views expressed in this work are those of the author and in no way represent those of the University of Westminster.

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LIST OF ABREVIATIONS

$[Ca^{2+}]c$	Cytosolic free calcium
μL	Micro litre
2D	Two-dimensional
3D	Three-dimensional
6-APA	6-aminopenicillanic acid
ACS	Acetyl coenzyme A synthetase
ACV	δ - (L-α-aminoadipyl)-L-cysteinyl-D-valine
ACVS	δ - (L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase
AM	Acetoxymethyl ester
APS	Adenosine-5-phosphosulfate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Ca^{2+}	Calcium ion
CAM	Calmodulin
cAMP	Cyclic adenosine mono-phosphate
cDNA	Complementary DNA
CDW	Cell dry weight
CLSM	Confocal laser scanning microscope/microscopy
CoA	Coenzyme A
C_t	Threshold cycle
Da	Dalton
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOT	Dissolved oxygen tension
DP	Degree of polymerisation
GMA	Glycerol-molasses agar
GOD	Glucose oxidase
GPCR	Green- protein-coupled receptor
GSH	Glutathione
GST	Glutathione-S-transferase
HCl	Hydrogen chloride
HPLC	High pressure liquid chromatography
IAT	Aminopenicillanic acid acyltransferase
IP_3	Inositol triphosphate
IPN	Isopenicillin N
IPNS	Isopenicillin N synthase
K^+	Potassium ions
kDa	kilo Dalton
KOH	Potassium hydroxide
L	Litre
LBG	Locust bean gum
LLD-ACV	δ - (L- α -aminoadipyl)-L-cysteinyl-D-valine

M	Molar
M2K	Mitogen activated protein kinase kinase
M3K	Mitogen activated protein kinase kinase kinase
MAPK	Mitogen activated protein kinase
mL	Milli litre
MO	Mannan oligosaccharides
MO-R	Mannan oligosaccharides-Reduced form
NADPH	Nicotinamide adenine dinucleotide
NRRL	National Center for Agricultural Utilization Research
OG	Oligoguluronate
OM	Oligomannuronate
OPC	Oxopiperidine-2-carboxylic acid
ORF	Open reading frame
PAA	Phenylacetic acid
PAPS	3-phospho-adenosien-5-phosphosulfate
PCL	Phenyl-acetyl coenzyme A ligase
PCR	Polymerase chain reaction
PGM	*Penicillium* growth medium
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMT	Photomultiplier tube
PMT	Photo-multiplier tube
POA	Phenoxyacetic acid
POA	Phenoxyacetic acid
PPM	*Penicillium* production medium
PTS1	Peroxisomal targeting signal 1
QPCR	Real time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
ss DNA	Single stranded DNA
STR	Stirred tank reactor
TLC	Thin layer chromatography
UV	Ultraviolet
v/v	Volume per volume
vvm	Volume of air per volume of medium per minute
w/v	Weight per volume

AIM

The aim of this study was to elucidate the mechanism of elicitation in *P. chrysogenum*.

To address the above aim, the following objectives were considered:

1. To investigate the effects of single addition of oligosaccharide elicitors on the penicillin G production.

2. To study the effects of the oligosaccharides additions on the three major biosynthetic antibiotic genes at the transcriptional level.

3. To investigate the effects of multiple additions of the oligosaccharide elicitors obtained from alginate and locust bean gum on penicillin G and at the transcriptional level of the biosynthetic gene.

4. To initiate and carry out preliminary studies on the possible effects of elicitor addition on the cytosolic calcium ion flux using fluorescent dyes in *P. chrysogenum* P2.

In the following paragraphs the history of elicitation and carbohydrates as elicitors will be briefly reviewed, followed by an overview of the biosynthesis of penicillin G and the three penicillin precursor amino acids. The significance of compartmentalization and the genetic organisation of the genes involved in penicillin biosynthesis will be considered in more detail. Finally the proposed mechanism of elicitation in fungal cultures will be discussed.

Chapter I

Introduction

1.1 INDUSTRIAL AND SCIENTIFIC IMPORTANCE OF β-LACTAM ANTIBIOTICS

The discovery and development of the β-lactam antibiotics, marks one of the most powerful and successful achievements of modern science and technology in the 20th century. The breakthrough in β-lactam antibiotics opened the way for the large scale production of a succession of other important antibiotics and other valuable chemicals (Demain and Elander, 1999). *Penicillium chrysogenum* is an important industrial species due to its ability to produce β-lactam antibiotics. Penicillin was the first important commercial product produced by an aerobic, submerged fermentation at large scale. It represents the world's major biotechnological product with worldwide dosage and sales of approximately 15 billion US dollars or approximately 65% of the total world market for antibiotics (Elander, 2003).

After more than 60 years of research and industrial practice, the production of penicillin G in fed-batch fermentation by *P. chrysogenum* continues to attract research interest (Hegewald *et al*., 2004; Li *et al*., 2005) due to several reasons. One of the major interests is the commercial and therapeutic importance of penicillin and its derivatives. Besides, there are still questions to be answered about the impact of different engineering variables in large scale bioreactors and the complexities of fungal cell growth, which are not yet fully understood (Amanullah *et al*., 2000). Extensive research has generated new information on the mechanisms of cellular reactions and morphological features of the mycelia and their role in the synthesis of the metabolic products (Patnaik, 2001).

Improvement of strains and cultivation procedures to obtain high penicillin yields have been one of the main objectives in industrial antibiotic research. Strain improvement has been achieved by repeated rounds of random mutation and subsequent selective screening (Diez *et al*., 1996). This classical approach is very powerful but has limited mutagenic potential and lacks specificity. Molecular genetics and biochemistry of β-lactam biosynthesis have advanced rapidly over the past few decades. This progress, together with the development of genetic engineering has resulted in a 30,000 fold increase in titre of Fleming's original isolate of *Penicillium notatum* compared to the

currently exploited high-producing strains of *P. chrysogenum* (Peñalva *et al*., 1998). Most of the natural penicillins produced commercially are now chemically modified to semi-synthetic penicillins, such as oxacillin, ampicillin, clavulanic acid, amoxycillin, methicillin, cyclacillin, temocillin and co-amoxiclav (Ball *et al.,* 1980; Rodriguez-Villalobos *et al.,* 2006; Rolinson and Geddes, 2007). This is due to the constant emergence of penicillin-resistant pathogens. Beyond all this the world demand for antibiotic production is ever increasing and one of the ways to supply this demand is to look for novel approaches to increase antibiotic production. One of the possible methods by which the secondary metabolite production can be increased is by elicitation.

1.2 ELICITATION

An elicitor can be defined as a substance which, when introduced in trace amounts to a living cell system as a non-nutrient additive, can stimulate certain morphological and/or physiological effects on the systems. This phenomenon is termed as elicitation. Elicitors are classified as physical or chemical, biotic or abiotic and complex or defined depending on their origin and molecular structure as shown in Table 1.1 (Radman *et al*., 2003a).

1.2.1 Carbohydrates as elicitors

Carbohydrates are the building blocks of many of the structural polymers that give form to living cells and organisms, and they play important roles in the interactions of cells with one another as well as with their environment (Albershiem *et al*., 1992). The traditional view on the biological function of carbohydrates was that they act as energy storage compound (starch and glycogen) or as important constituents of cellular structure (cellulose, peptidoglycan and chitin). However, carbohydrates play a vital role in a vast array of other biological processes. They are vital in their structural role in DNA and RNA, where deoxy-ribose and ribose sugars provide the backbone to which the genetic code is attached (Malik and Ahmad, 2007). The universal free energy molecule ATP is a phosphorylated sugar derivative as are many co-enzymes, which are vital to the functioning of enzymes. Recently the role of carbohydrates in cellular

recognition and in cell-to-cell communication is beginning to be appreciated (Juliano, 1978; Lloyd *et al*., 2007).

Table 1.1 Elicitors of plants and microbial cells (modified Radman *et al*., 2003a)

<table>
<tr><th colspan="6">Elicitors</th><th>Reported effects on *</th></tr>
<tr><td>Physical elicitors</td><td colspan="5">Injury</td><td>P</td></tr>
<tr><td></td><td colspan="2">Abiotic</td><td colspan="3">Metal ions (lanthanum, europium, calcium, silver, cadmium), oxalate</td><td>Pc</td></tr>
<tr><td rowspan="15">Chemical elicitors</td><td rowspan="15">Biotic</td><td>Complex composition</td><td colspan="3">Yeast cell wall, mycelial cell wall, fungal spores</td><td>Pc, F</td></tr>
<tr><td rowspan="14">Defined composition</td><td rowspan="9">Carbohydrates</td><td rowspan="5">Polysaccharides</td><td>Alginate</td><td>Pc, F, B</td></tr>
<tr><td>LBG</td><td>F</td></tr>
<tr><td>Pectin</td><td>Pc, F</td></tr>
<tr><td>Chitosan</td><td>Pc</td></tr>
<tr><td>Guar gum</td><td>Pc</td></tr>
<tr><td rowspan="4">Oligosaccharides</td><td>Mannuronate</td><td>F, B</td></tr>
<tr><td>Guluronate</td><td>F, B</td></tr>
<tr><td>Mannan</td><td>F, B</td></tr>
<tr><td>Galacturonides</td><td>Pc</td></tr>
<tr><td rowspan="2">Proteins</td><td>Peptides</td><td>Glutathione</td><td>Pc</td></tr>
<tr><td>Proteins</td><td>Cellulase, Elicitins, Oligandrin</td><td>Pc</td></tr>
<tr><td>Lipids</td><td></td><td>Lipopolysaccharides</td><td>Pc</td></tr>
<tr><td>Glycoproteins</td><td></td><td>Not characterized</td><td>Pc</td></tr>
<tr><td>Volatiles</td><td></td><td>C6-C10</td><td>Pc</td></tr>
</table>

*** P, plants; Pc, plant cell culture; B, bacterial culture; F, Fungal culture**

From early studies carbohydrates have been implicated in the overproduction of secondary metabolites in plant cell cultures. Early evidence of such signals was obtained during studies of plant-pathogen interactions when oligosaccharides were identified as some of the elicitors in cell-free fungal extracts that could stimulate plants to produce phytoalexins as a defence response to the pathogen (Ayers *et al*., 1976). Since the first

demonstration that linear oligosaccharides of α-1, 4-linked galactosyluronic acid residues could elicit phytoalexin synthesis in soybean (*Glycine max*), oligogalacturonides with a degree of polymerisation (DP) between 8-15 have been reported to exhibit strong biological activity in a number of plants (Côté and Hahn, 1994). Although oligogalacturonides are non-species specific elicitors, the size of the oligomer, its polycationic nature and the specific molecular shape of the chain are important structural features for maximal activity. Sharp and co-workers (1984) investigated the role of specific carbohydrate elicitors on phytoalexin production in soybean cell cultures where they identified 8 distinct oligosaccharides after partial acid hydrolysis of mycelial walls of *Phytophthora megasperma.* Characterization of these oligosaccharides revealed that all but one elicitor-inactive oligosaccharides had 3, 6 -linked glucopyranose residues with the eighth having β-linked glucopyranose residues. The fact that the active and the inactive carbohydrate elicitors show only small structural differences suggests a highly specific recognition of the carbohydrate structure by the elicitor receptors.

The extensive stereochemistry, multiple hydroxyl groups, oxygen atoms and accessible hydrophobic regions characteristic of glycosyl residues make oligosaccharides ideal ligands for precise interactions with recognition sites on proteins. These sites can interact with as many as six glycosyl residues at once. This means that proteins distinguish among a large range of information-carrying oligosaccharides, information being transmitted to cells through oligosaccharide-specific protein receptors (Albershiem *et al*., 1992). The elicitation stimulus leading to rapid changes in the integrity of the plasma membrane has been the subject of intensive studies in plant cell cultures. Several lines of evidence suggest that specific binding sites, linked to a pathway transducing the elicitor stimulus to the nucleus are present on the plant plasma membrane (Benhamou, 1996). Of particular interest has been the identification of specific elicitor–binding proteins that might function as physiological receptors in the signal transduction cascade. The existence of specific high-affinity binding sites has been demonstrated for oligosaccharide, glycopeptides and peptide elicitors, and candidate elicitor- binding proteins have been identified for several of them. The

properties of these binding sites/ proteins are consistent with those expected of physiologically important receptors, although experimental verification of the role of these binding proteins as receptors has not yet been obtained. The purification and characterization of specific elicitor-binding proteins is essential for a detailed understanding of the molecular basis for the signal exchange between hosts and elicitor that leads to activation of host defences (Hahn, 1996).

1.2.1.1 Oligosaccharides

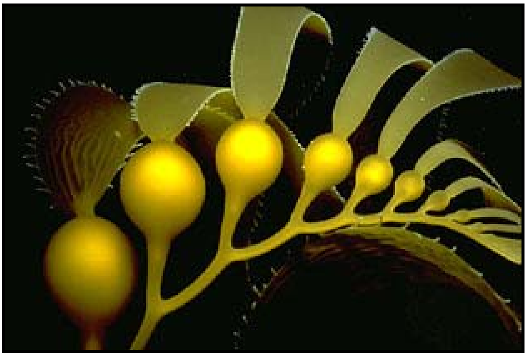
Oligosaccharides are a group of carbohydrates with short chains of 2-20 glycosidically linked monosaccharide residues. Oligosaccharides can exist as both simple oligosaccharides, which on hydrolysis liberate only monosaccharide units and as conjugate oligosaccharides linked to non-saccharides, such as cell surface peptides (Hayes *et al*., 1995) and lipids (D'Souza *et al*., 1992). Their remarkable structural diversity means that carbohydrates can mediate highly specific and complex processes. With only four glycosyl residues, it would be possible to form between 10^4–10^5 different tetrasaccharides with characteristic three-dimensional structures (Davis, 2000). The arrangement of sugar units within naturally occurring carbohydrate polymers is of fundamental importance in determining their properties and complexity. Oligosaccharides and polysaccharides have complex three dimensional shapes due to the variety of attachment points and nature of the monosaccharides. Furthermore, α and β configurations within the same linkages lead to differing secondary structures, for example an α–D-(1, 3) – glucan will have a different structure to a β-D-(1, 3) – glucan.

1.2.1.2 Alginates

Alginates are normally available as monovalent salts of alginic acid extracted from different types of brown seaweed. Alginate bearing weeds are typically found in temperate or cold water. Major commercial sources of alginates are the giant kelps (*Macrocystis pyrifera*) from California, *Ascophyllum nodosum* from the north Atlantic and *Phaeophyceae*, mainly *Laminaria hyperborea* from the British Isles (Figure 1.1). Certain bacteria, including *Azotobacter chrococcum*, *A. vinelandii,* and *Pseudomonas*

aeruginosa also synthesize alginate as extracellular polysaccharide. Alginate was first prepared by Stanford (1886) who established that it was a weak organic acid consisting chiefly of uronic acids. Nelson and Cretcher (1929) isolated D-mannuronic acid and L-guluronic acid from alginate. Haug and Larsen (1962) made precise determination of the uronic acid composition.

Laminaria hyperborea (www.seaweed.ie)



Macrocystis pyrifera (ucsb.piscoweb.org)

Ascophyllum nodosum (www.naturfoto.cz)

Figure 1.1 Different types of brown sea-weed

Alginates are linear unbranched polymers containing β-(1, 4)-linked D-mannuronic acid and α-(1, 4)-linked L-guluronic acid residues (Gacesa, 1988). Although these residues are epimers (D-mannuronic acid residues being enzymatically converted to L-guluronic after polymerization) and only differ at C5, they possess very different conformations; D-mannuronic acid being $^4C_1$ with diequatorial links between them and L-guluronic acid being $^1C_4$ with diaxial links between them. These uronic acids are arranged in block

structures that are mainly homopolymeric, polyguluronate (OG) and polymannuronate (OM) (Figure 1.2) and heteropolymeric blocks with alternating units of mannuronic and guluronic acids (Haug *et al*., 1974). Alginate has its building blocks arranged in a zig-zag pattern. Depending on the weed source and growing condition the ratio of mannuronic and guluronic acid can vary.

Oligomannuronate (OM)

Oligoguluronate (OG)

Figure 1.2 Oligosaccharides derived from alginate by partial hydrolysis

Alginates are used for a variety of application by the food, pharmaceutical and other industries. It is used as a stabilizer for the ice cream industry. Propylene glycol alginate (PGA) is a chemically modified alginate available commercially. PGA with highest clarity and ester content is used in the beer industry as a head retention aid. Lower grade products are typically used in salad dressings, in conjunction with xanthum gum and fruit juice based products as a stabiliser. Another important application of alginate is its use as a matrix for cell immobilization (Mosahebi *et al*., 2001). Through acid hydrolysis biologically active oligosaccharide elicitors can be produced. Recent developments in using alginate as a drug carrier (Tonnesen and Karlsen, 2002), wound dressing (Thomas,

2000) and in transplantation therapy (Fu *et al.,* 2003) have expanded the use of this polymer.

1.2.1.3 Galactomannans

Galactomannans are polysaccharides consisting of a mannose backbone with galactose side groups more specifically, α (1, 4)-linked β-D-mannopyranose backbone with branch points from their 6th position linked to α-D-galactose, i.e., 1, 6-linked α-D-galactopyranose (Figure 1.4) (McCleary, 1979). The exact distribution of the sidechains has been the subject of a lot of study and the best description is probably using Markov chain extension statistics where the chain is built up from one end in a linear fashion and the presence of a galactose sidechain on a residue is determined by the presence or absence of any residues on the preceding two units. Commercially available galactomannans are derived from the seed endosperm of carob (*Ceratonia siliqua*) and guar (*Cyamopsis tetragonolobus*) (Dea and Morrison, 1975) (Figure 1.3).

Ceratonia siliqua

Seeds of *Ceratonia siliqua*

Figure 1.3 Source of galactomannans from carob (www.hort.purdue.edu)

Within the various mannans occurring in nature, wide spectra of structures can be distinguished. The diversity of structure is mainly due to variations in the mannose/galactose ratio and differences in the distribution of galactose units along the mannan backbone (Table 1.2). The galactose content may vary between 10 and 50%. Galactomannans with galactose content higher than 25% are readily soluble in cold

water and yield highly viscous solutions. Galactomannans with galactose content between 18 and 24% are soluble in hot water, but insoluble or only slightly soluble in cold water. These groups of Galactomannans are referred to as the locust bean gums. Locust bean (carob) gum is the refined endosperm of the seed of the carob tree, an evergreen of the legume family (*Ceratonia siliqua*). The tree grows extensively in Spain and is cultivated in many other Mediterranean countries.

Table 1.2 Different Galactomannan substitution levels

Galactomannan substitution levels	
Ivory nut mannan	no galactose
Locust bean gum	1 galactose / 4 mannose
Tara Gum	1 galactose / 3 mannose
Guar Gum	1 galactose / 2 mannose
Fenugreek Gum	1 galactose / 1 mannose

(www. cybercolloids.net/library/carob/structure.php)

Locust bean galactomannans have in general low galactose substitutions, and it is well established in practice that commercial and purified fractions display temperature dependent solubility (Table 1.2). Typically about half of the polysaccharide will dissolve in cold water, and with increasing temperature further portions dissolve until full viscosity is reached. This is referred to as cold water soluble and hot water soluble portions (Pollard *et al*., 2007). Applications of locust bean galactomannans are in paper making and in the textile industry. Galactomannans have also found extensive use in the food industry to enhance texture as thickeners and stabilizers.

Oligosaccharides can be prepared from galactomannans by enzymatic hydrolysis. A range of galactomannan degrading enzymes has been reported in the literature (Dey, 1978); these include α-galactosidase, β-D-mannase and galactomannase. The enzyme β-D-mannase acts by random cleavage of the D-mannan chain, producing a series of manno- and galacto-manno-oligosaccharides (Dekker and Richards, 1976) (Figure 1.4). Elicitation studies in microbial cultures have focused mainly on the use of carbohydrates

as elicitors. However, only few oligosaccharides and polysaccharides have been screened for their elicitation effects. A fast screening method for characterization of a range of carbohydrates is needed for use in microbial cultures as effective and efficient elicitors.

Figure 1.4 Structure of locust bean gum

1.2.2 Elicitation studies in plant systems

Several lines of evidence indicate that all plants, whether they are resistant or susceptible, respond to pathogen attack by the induction of a coordinated resistance strategy ultimately leading to the accumulation of defence gene products (Benhamou, 1996). The term 'Elicitor' was originally used to refer to molecules and other stimuli that induce the synthesis and accumulation of antimicrobial compounds in plant cells, but now commonly used for factors capable of stimulating any type of physiological responses and is not restricted to plant systems (Hahn, 1996). Initial elicitation studies were carried out in plant cell systems and the term Elicitor was coined by Keen (1971) when studying the effects of *Phytophthora megasperma var sojae* (root-rot fungus) culture fluid on soybean phytoalexin production (Keen *et al*., 1971).

Since then numerous reports have accumulated evidence for pathogen-derived compounds that induce defence responses in intact plants and plant cell cultures (Davis and Hahlbrock., 1987; Gundlach *et al*., 1992; Bach *et al*., 1993). The elicitation effects of a variety of compounds acting on an array of plant and plant cell cultures have been

the focus of extensive research over the past decades. Examples of the diverse plant defence mechanisms induced by elicitors include the synthesis and accumulation of antimicrobial phytoalexins (Dixon *et al*., 1983; Ebel, 1986), the induction of cell death (hypersensitive necrosis) (Arlat *et al*., 1994; Gustine *et al*., 1995), the production of glycosyl-hydrolases capable of attacking surface polymer of pathogens (Hahn *et al*., 1993; Messiaen *et al*., 1993), the synthesis of proteins that inhibit degradative enzymes produced by pathogens (Bergmann *et al*., 1994), the production of activated oxygen species (oxidative burst) (Baker *et al*., 1993; Bottin *et al*., 1994) and the modification of plant cell wall by deposition of callose (Grosskopf *et al*.,1991; Kauss *et al*.,1994), hydroxyproline-rich glycoproteins, suberins and/or lignin (Boudart *et al*., 1995; Kogel *et al*., 1988). These elicitors are either oligosaccharides, lipoproteins or glycoproteins. Such biotic elicitors often originate from the pathogen (exogenous elicitors) but in some cases are liberated from the attacked plant by the action of enzymes of the pathogen (endogenous elicitors).

Albersheim and Valent (1978) investigated the interaction of microbial oligosaccharides on plants that activate a variety of plant defence genes. The induction of phytoalexin biosynthesis has gained special importance in biotechnological approaches to improve the production of secondary metabolites. Many of these compounds are of high commercial value as therapeutics or otherwise as biologically active agents. Plant cell cultures are potentially rich sources of valuable pharmaceuticals and other biologically active phytochemicals, but relatively few cultivars and derived cell cultures synthesize secondary metabolites over extended periods and in any amounts suitable for commercial exploitation. However, manipulation of plant cell cultures with elicitors has not only shed light on the different biosynthetic pathways but also increased yields of medically important secondary metabolites. An example is the bio-production of taxol, a diterpenoid found in the bark of Taxus trees. This compound is approved by the Food and Drug administration for the treatment of ovarian and breast cancer. There is a high demand for taxol, but its synthetic production is extremely costly, so biosynthesis in Taxus spp. cell cultures has become the focus of extensive research. Taxus cell suspension could only produce traces of taxanes (Angelova *et al*., 2006; Zhong, 2002)

To improve the taxol production, various kinds of abiotic and biotic elicitors have been introduced which have proven to be very effective for some cultures. Suspension cultured *Taxus chinesis* var *mairei* Y901-L responded to crude elicitors from the fungus *Fusarium oxyspurum* by influencing the general phenylpropanoid pathway and incorporating taxol synthesis. The maximum increase in taxol concentration was 8-fold of the control and there was a similar increase in the amount of phenolics in the culture media. It was found that the active component is the oligosaccharide extracted from the cell wall of *Fusarium oxyspurum* (Yuan *et al*., 2001).

Addition of alginate oligomer to the suspension culture of *Catharanthus roseus* L. or *Wasabia japonica* cells promoted the production of enzymes such as 5'-phosphodiesterase or chitinase respectively. Ajmalicine (a secondary metabolite) production by *C. roseus* CP3 cells was also promoted when alginate oligomer was added to the suspension culture. (Akimoto *et al.,* 1999).

Carbohydrates are not the sole elicitors of plant cell cultures. Other biotic and abiotic elicitors have been developed to improve the yield of plant secondary metabolites such as in the cell cultures of *Taxus chinensis* (Tabata, 2004; Ye *et al*, 2004; Qian *et al*., 2004a, 2004b). A protein such as Nep-1 produced by *Fusarium oxyspurum* elicits ethylene production in leaves of *Nicotiana tabacum* (Jennings *et al*., 2001). Salicylic acid enhances the activation of defence related genes in parsley (Katz *et al*., 2002). Jasmonic acid, methyl jasmonate and its derivatives are a family of important signal transducers and can efficiently stimulate secondary metabolism in plant cells (Nojiri *et al*., 1996; Wang *et al*., 2006). Since the first report regarding the effect of methyl jasmonate on the accumulation of secondary metabolites in plant cell cultures (Gundlach *et al*., 1992), more than 100 plant species have been demonstrated to respond to the addition of methyl jasmonate to the culture medium by accumulating secondary metabolites (Haider *et al*., 2000). To date, most of the effect of jasmonate stimulated secondary metabolite biosynthesis in plant cell cultures have been focussed on the induction of plant defence responses including oxidative and nitric oxide bursts by the addition of jasmonates (Chong *et al*., 2005; Wang and Wu, 2005).

Elicitation studies have shown promise in increasing the yields of the target products and cutting the production costs. From the above studies it became evident that elicitors could play an important role in the enhanced production of commercially important compounds.

1.2.3 Elicitation studies in microbial systems

The use of oligosaccharides and polysaccharides as elicitors to enhance production of plant metabolites has been extended recently to microbial cultures for overproduction of commercially useful by-products such as pigments (Nair *et al*., 2005), antimicrobials (Murphy *et al*., 2007) and enzymes (Petruciolli *et al*., 1999). The increased production through elicitation of the secondary metabolites from plant cell cultures has opened up a new area of research which might have important economical benefits for the bio-pharmaceutical industry. It has been recently established, in the last decade, that some microbial cell cultures show physiological changes when challenged by certain elicitors. Many commercially important secondary metabolites have been over produced by microbial cultures. It is clearly of practical as well as theoretical interest to seek the evidence of enhancement of secondary metabolites in microbial cultures.

Elicitation in fungal cultures was first reported in (1997) by Ariyo and co-workers when the addition of oligosaccharides derived from alginate was found to enhance the penicillin G production in shaken flask cultures of *P. chrysogenum*. Differences in the degree of polymerization of the oligosaccharide elicitors appear to have an effect on the level of elicitation observed. Later studies by the same group (Ariyo *et al*., 1998) showed that mannan oligosaccharides (MO) derived from locust bean gum had an even greater effect on the enhancement of the production of penicillin G by the fungus compared to oligosaccharides derived from alginate. The addition of alginate derived elicitor oligomannuronate (OM) and oligoguluronate (OG) oligosaccharides increased penicillin G yield by 47% and 49%, respectively. The effect of locust bean gum derived mannan oligosaccharides was more pronounced with 69% higher yield than the control cultures (Radman *et al*., 2004a). While there was no noticeable change in the cell dry weight between the control and elicited cultures, changes were observed in the

extracellular concentrations of secreted intermediates of penicillin biosynthesis such as δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) and isopenicillin (IPN) (Tamerler *et al*., 2001).

Recent studies have looked at the relationship of penicillin G production and morphological effects in *P. chrysogenum*. Addition of an oligosaccharide elicitor to the cultures resulted in increases of up to 47% in hyphal tip number concomitant with an increase of up to 120% in penicillin G levels (Radman *et al*., 2004b). It is believed that production of penicillin G occurs at the tips of the hyphae. This increase, together with the possible activation of penicillin G-regulatory genes, can explain the overproduction of secondary metabolites and change in the morphological pattern observed in *P. chrysogenum*. The addition of oligosaccharide elicitor also stimulated the onset of sporulation and spore production in *P. chrysogenum* liquid cultures. Addition of MO to the stirred tank reactor cultures induced the highest concentration of spores followed by OM and OG supplemented cultures. In cultures supplemented with oligosaccharide elicitors, sporulation was induced earlier when compared with control cultures (Radman, 2002). The reasons for this enhanced effect and the differing extent of the effect on the concentration of spores by the oligosaccharide elicitors are yet to be understood. It can also be argued that the time and level of sporulation are related to the defence mechanism of the fungus. Elicited cultures of *P. chrysogenum* showed an increase in the penicillin G production rates and levels of other intermediary metabolites. Increased secondary-metabolite production rates cause a higher rate of nutrient consumption, which, in turn, can induce sporulation as a survival mechanism.

Intracellularly in *P. chrysogenum*, elicitors not only exert an effect on secondary metabolite production but also on other metabolite production such as Reactive oxygen species (ROS). In this case the addition of the mannan oligosaccharide elicitor decreased the production of ROS by up to 54% (Radman *et al*., 2004a). The above finding suggested the possibility of complex, interacting pathways linking the overproduction of penicillin G to a decrease in ROS activity. Similar enhancement in the formation of other metabolites and enzymes with oligosaccharide elicitors was observed in other fungal species/strains. In (1999) Petruciolli and co-workers reported the overproduction

of glucose oxidase (GOD) by the fungus *Penicillium variable*. Large increases (up to 70%) in GOD activity in cultures of *P. variable* were observed when supplemented with alginate, locust bean gum and oligosaccharides, OM and OG. Since the early 1950s glucose oxidase has been widely used in powdered egg manufacture and paper test strips for diabetic patients. A new application for GOD is its use in biosensors. It has been used as an ingredient of toothpaste. This enzyme might also be a good alternative to traditional chemical and physical treatments in food preservation. Significant increases were also observed in catalase levels on the addition of oligosaccharide elicitors in *P. chrysogenum* cultures compared to control cultures (Radman, personal communication). The effect of different elicitors on pigment production has also been reported in different strains of *P. chrysogenum* (Asilonu *et al*., 2000; Nair *et al*., 2005) and in *Monascus* species (Shin *et al*., 1998). Chrysogenin is a yellow pigment produced by strains of *P. chrysogenum* and has not been well characterized since its description in the 1930's (Clutterbuck *et al*., 1932). Chrysogenin production was increased by up to 55% and 27% in two different strains of *P. chrysogenum* on addition of mannan oligosaccharides as elicitors (Nair *et al*., 2005). Monascus pigments have traditionally been used in China and Taiwan for processing alcoholic beverages, red soybean gels, meats, and vegetables (Carels and Shepherd, 1977). An approximate 10-fold increase in pigment production was observed in liquid co- cultures with *S. cerevisiae* (Lim *et al*., 2000).

Although the elicitor effects in plant and fungal cultures are well documented, little is reported on elicitation in bacterial cultures. The effect of oligosaccharides as enhancers of antibiotic production has been investigated in *Streptomyces* species, filamentous bacteria. The addition of the elicitors increased the production of the antibiotic oxytetracycline to a maximum of 119%. Notable effects were also observed in the morphological pattern and at the transcriptional level (Sangworachat, 2006). Similar to the effects in *P. chrysogenum* the addition of oligosaccharides showed a decrease in ROS levels, but were related to an increase in catalase activity (Radman *et al.,* 2006). Recent work on *Bacillus licheniformis* has shown an increase in bacitracin A antibiotic production on addition of oligosaccharide elicitors (Murphy *et al*., 2007). The addition

of the elicitors increased production of the antibiotic to a maximum of 29% in elicited cultures compared to control cultures.

The work carried out so far, on elicitation in liquid cultures of *P. chrysogenum* has provided us with an established system to further investigate the effects of elicitors. As knowledge of the biosynthetic pathway for the production of penicillin G and the morphology are much better established compared to other fungi, this fungus is an ideal model for research.

1.3 OVERVIEW OF PENICILLIN G BIOSYNTHESIS IN *P. chrysogenum*

Penicillins belong to the large and complex family of β-lactam antibiotics, members of which possess one common structural motif, the β-lactam ring (Hoover, 1983). The β-lactam ring enables these antibiotics to selectively inhibit enzymes involved in the cell-wall assembly of bacteria. Disturbance of the subtle balance that exists between wall degrading and synthetic enzyme activities cause reduced growth rates and results eventually in lysis of the bacterial cell. In penicillins, the β-lactam ring is fused with a five-member thiazolidine ring. Hydrophobic penicillins are solely produced by filamentous fungi. In nature, *Penicillium* species produce a mixture of primarily hydrophobic penicillins containing different acyl-side chains. A complex regulatory network, e.g. environmental factors such as pH, carbon source, amino acids, nitrogen source and endogenous metabolic signals controls the biosynthesis of penicillin (Sanchez *et al*., 1988). The penicillin biosynthesis pathway consists of three major enzymes, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS), isopenicillin N synthase (IPNS), and aminopenicillanic acid acyltransferase (IAT), which together catalyze the formation of penicillin from its amino acid precursors.

Although the biosynthetic pathway comprises of only three major enzymes, the biochemical process leading to the formation of hydrophobic penicillins is quite complicated. For instance, ACVS possesses four different catalytic activities and the complete biosynthesis of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) comprises of ten separate steps. Besides, the three essential enzymes, a specific acyl-CoA synthetase involved in side-chain precursor activation and an unknown ligase which activates ACVS by attaching three pantotheine co-factors post-translationally are also involved in penicillin biosynthesis (Bañuelos *et al*., 2000). During penicillin biosynthesis several related metabolites are formed and some of these tend to accumulate in the medium. Bis-LLD-ACV is formed through non-enzymatic oxidation of LLD-ACV. *In vivo,* reduction of bis-LLD-ACV could be carried out by a NADPH-dependent thioredoxin reductase, which has been isolated from *P. chrysogenum* (Gunnarsson *et al*., 2004). Part of α-aminoadipate is lost by the irreversible formation of 6-oxopiperidine-2-carboxylic acid (OPC), the cyclized δ-lactam of α-aminoadipate

(Figure 1.5). The biochemical pathway for the formation of OPC is not understood, but the extent of formation ranges from 6-60% relative to the formation of penicillin (on a molar basis) (Evers *et al*., 2004).

All three penicillin biosynthetic enzymes of *P. chrysogenum* have been purified and characterized extensively (Hillenga, 1999). In the first step of the penicillin biosynthetic pathway (Figure 1.5), LLD-ACV is formed from the three amino acid precursors L-α-aminoadipic acid (an intermediate of the L-lysine biosynthetic pathway), L-cysteine, and L-valine by ACV synthetase (ACVS) (Brakhage, 1998). In the course of this reaction, L-valine is converted to the D isomer. Oxidative ring closure of the linear tripeptide leads to the first β-lactam, isopenicillin N (IPN). This reaction is catalyzed by the IPN synthase (IPNS) (Barredo *et al*., 1989a). In the final step, the L- α-aminoadipic acid side chain is exchanged for a hydrophobic acyl group catalyzed by acyl coenzyme A: 6-aminopenicillanic acid acyltransferase (IAT).

Features of the major enzymes and of the acyl- coenzyme A (acyl-CoA) synthetase are summarized below:

1.3.1 ACV synthetase

The first step in the biosynthesis of penicillins is the condensation of three precursor amino acids, namely L-α- aminoadipate, L- cysteine and L- valine into the tripeptide δ -(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). It is well established that the formation of the two peptide bonds and the epimerization of valine are catalyzed by a single multienzyme of 424 kDa with non-ribosomal peptide synthetase activity termed ACV synthetase (ACVS) (Aharonowitz *et al*., 1993). ACVS is encoded by the *pcb*AB gene that is part of a cluster which includes the other two key enzymes of the penicillin biosynthesis pathway. ACVS from different organisms show remarkable sequence conservation with each other. The central conserved regions with highest sequence conservation are involved in binding and activation of the precursor amino acids. Each region contains adenylate, acyl carrier and condensation modules consisting of conserved sequences arranged in a characteristic order (Smith *et al*., 1990). The

adenylate module contains sequences involved in adenylate formation, ATP binding and a region determining the substrate specificity. The acyl carrier module contains a conserved serine residue that functions as the attachment site for the pantotheine co-factor that is derived from coenzyme-A. ACVS is a typical example of channelled metabolism, integrating adenylation, peptide-bond formation, epimerization and product release by thioesterase activity into a single multi-enzyme complex. The exact mechanism of LLD-ACV biosynthesis is still a subject of discussion (Martin, 1998).

The localization of ACVS has been a matter of debate for some time. Initially, it was described as a membrane associated protein and found to co-sediment with vesicles of either Golgi or vacuolar origin. However, the amino acid sequence of *P. chrysogenum* ACV synthetase contains no recognizable targeting information for the endoplasmic reticulum or the vacuole, and although the protein is hydrophobic in nature, it does not harbour any trans-membrane regions. Localization studies by traditional fractionation experiments were obscured by the fact that ACVS is a highly unstable enzyme and very sensitive to proteolytic degradation. On the basis of improved cell lysis protocols and immuno-gold electron microscopy analysis the subcellular location of this protein was determined. The studies confirmed the ACVS to be a cytosolic enzyme (Figure 1.6). The cytosolic localization is more on par with the pH optimum of this enzyme, as the acidic vacuole would not support activity. Moreover, the vacuole is highly proteolytic which seems contradictory with the protease sensitivity of the multidomain ACVS and the release of a product tripeptide. ACVS is situated at the borderline of primary and secondary metabolism and is strictly controlled at the genetic and enzymatic level. ACVS activity is also modulated via feedback inhibition by bis-LLD-ACV (Theilgaard *et al*., 1997).

1.3.2 Isopenicillin N synthase

IPNS, a nonheme Fe (II)-dependent oxidase, catalyzes the second step in β-lactam biosynthesis, the oxidative cyclization of LLD-ACV to IPN (Figure 1.5). IPNS, a 38 kDa cytosolic enzyme, is the most thoroughly characterized penicillin biosynthetic

Figure 1.5 Schematic pathway for the biosynthesis of penicillin G in *P. chrysogenum.* (Adapted from Hillenga, 1999)

enzyme (Figure 1.6). In a unique enzymatic step, IPNS mediates the transfer of four hydrogen atoms from the ACV tripeptide in a desaturating ring closure with concomitant

reduction of dioxygen to water (Perry *et al*., 1998). The IPNS reaction *in vitro* requires ferrous iron, molecular oxygen as a co-substrate and ascorbate as an electron donor to form the β-lactam and thiazolidine ring of IPN. From X-ray diffraction experiments, using the substrate analogue δ-(L-α-aminoadipyl)-L-cysteinyl-L-S-methyl-cysteine in the crystal it was concluded that closure of the β-lactam ring precedes the closure of the five-membered thiazolidine ring (Burzlaff *et al.,* 1999). IPNS activity strongly depends on the dissolved oxygen concentration and on the presence of Fe^{2+} *in vitro* (Bainbridge *et al*., 1992). IPNS accepts LLD-ACV only in its reduced state, bis-ACV formed by oxidation has to be reduced before it can be converted. In *P. chrysogenum*, a broad-range disulfide reductase belonging to the thioredoxin family of oxidoreductases was found which efficiently reduced bis-ACV to the thiol monomer. When the reductase was coupled to IPNS *in vitro*, it converts bis-ACV to IPN and was therefore suggested to play a role in penicillin biosynthesis. This means that LLD-ACV produced by ACVS can directly be used as the substrate for IPNS. The question whether these two enzymes are organised in a metabolon or large complex is not yet known.

IPNS shows broad substrate specificity in particular with alterations in the L-α-aminoadipic acid moiety and the valine residue of ACV. This finding can be used in creating novel penicillin from ACV analogues although cyclisation of unnatural tripeptides occurs at lower efficiency (Wolfe *et al*., 1984).

1.3.3 Acyl-coenzyme A: isopenicillin acyltransferase

The final step in the biosynthesis of hydrophobic penicillins is mediated by acyl-coenzyme A: isopenicillin N acyltransferase (IAT). The hydrophilic L-α-aminoadipic acid side chain is exchanged for a hydrophobic acyl group, e.g. phenylacetyl in penicillin G (Figure 1.5). IAT shows broad substrate specificity. By addition of appropriate precursor molecules, the fermentation can be directed towards specific penicillin, e.g. phenylacetic acid is added for the production of penicillin G, phenoxyacetic acid is added for production of penicillin V.

IAT is a hetero-dimeric enzyme consisting of a 11 kDa α-subunit and a 29 kDa β-subunit. It is synthesised as a 40 kDa pre-protein from the *pen*DE gene and undergoes autocatalytic processing to form the heterodimer. Both subunits possess a C-terminal PTS1 signal that targets this enzyme to a microbody or peroxisome. Fractionation studies as well as immuno-gold labelling experiments localized IAT to microbodies (Figure 1.6). Consequently the substrate IPN has to enter the microbody before it can be converted. It is not known yet whether this occurs by diffusion over the membrane, or by facilitated or active transport. The side-chain replacement catalyzed by the acyltransferase results in the formation of hydrophobic penicillin, with increased antimicrobial activity as compared to isopenicillin N (Martin *et al*., 1994).

Six different catalytic activities have been assigned to acyltransferase: 1) acyl-CoA: IPN acyltransfer, 2) acyl-CoA: 6-APA acyltransfer, 3) IPN amidohydrolysis, 4) penicillin transacylation, 5) penicillin amidohydrolysis and 6) acyl-CoA hydrolysis. A two step enzymatic process for conversion of IPN to penicillin G by IAT has been proposed but a well-integrated exchange mechanism seems more likely. In the first step, IPN is deacylated to 6-aminopenicillanic acid (6-APA), which in the second step is acylated to penicillin G through addition of a phenylacetyl group from its CoA derivative (Figure 1.5). Thus, two enzymatic functions are required, an isopenicillin-N-amidohydrolase and acyl-CoA: 6-aminopenicillanic acid acyltransferase activity. The cloning and sequencing of the *pen*DE gene encoding IAT revealed that the *P. chrysogenum* enzyme has the required activities (Alvarez *et al*., 1993).

1.3.4 Acyl-CoA synthetase: precursor activation

In penicillin biosynthesis, the aminoadipyl side-chain of IPN is commonly exchanged with a phenylacetic acid (PAA) or phenoxyacetic acid (POA) side chain, yielding penicillin G and penicillin V, respectively, by the microbody-located IAT. Prior to this reaction, PAA and POA have to be activated to their CoA thioesters. Theoretically this activation can be carried out by an enzyme displaying either acetyl-coenzyme A synthetase (ACS) activity, phenyl-acetyl-coenzyme A ligase (PCL) activity, or alternatively via a glutathione-dependent pathway involving glutathione-S-transferase

(GST) activity. This phenomenon has not been well studied, but current view considers the last option unlikely (Evers *et al*., 2004).

A gene encoding a cytosolic ACS of *P. chrysogenum* has been identified and isolated. Disruption of this gene did not result in a decrease in penicillin production, meaning that ACS cannot be solely responsible for activation of precursors. In another study a PCL of *P. chrysogenum* containing a C-terminal peroxisomal targeting signal (PTS1) was identified. This suggests a peroxisomal location of the activating enzyme which would seem advantageous as it is then in the same compartment as IAT and the peroxisomal concentrations of activated precursors would be higher than in the cytosol. In addition PAA and POA are more likely to easily diffuse across the peroxisomal membrane than their activated counterparts thereby providing a means of retention. Overproduction of PCL, on the other hand does not appear to result in higher penicillin production either. However, overproduction of a presumably cytosolic located heterologus PCL from *Pseudomonas putida* U increased the penicillin production by 100% whereas overproduction of the homologous peroxisomal PCL of *P. chrysogenum* did not affect penicillin production. These results give an ambiguous view on the role of the peroxisomal PCL (Minambres *et al*., 1996).

1.4 SYNTHESIS OF Β-LACTAM PRECURSORS

P. chrysogenum obtains the penicillin precursor amino acids, L-α-aminoadipic acid, L-cysteine and L-valine through *de novo* synthesis. L-α-aminoadipic acid is a key intermediate in the lysine biosynthetic pathway of fungi. L-α-aminoadipic acid is recycled in hydrophobic penicillin biosynthesis although not with 100% efficiency. L-cysteine and L-valine are end-products of biosynthetic pathways and common protein constituents.

1.4.1 L-α-aminoadipic acid

L-α-aminoadipate is an intermediate of the L-lysine biosynthesis pathway. The intracellular level of L-α-aminoadipate can be a limiting factor in the overall penicillin synthesis rate. The addition of L-α-aminoadipate to the growth medium enhances the β-lactam production (Lopez-Nieto *et al*., 1985). Therefore, the lysine biosynthetic pathway is extremely important for and interconnected with the β-lactam pathway. L-lysine biosynthesis starts with the condensation of acetyl-CoA and α-ketoglutarate into homocitrate by homocitrate synthase. This enzymatic reaction was until recently believed to take place in the mitochondria of *P. chrysogenum*, however a localization study using a green fluorescent protein-fusion of homocitrate synthase indicated that this protein is located in the cytosol. The first half of the L-lysine biosynthesis is completed by the conversion of homocitrate to L-α-aminoadipic acid through the intermediates *cis*-homoaconitate, homoisocitrate and α-ketoadipic acid. L-α-aminoadipic acid is converted to lysine via α-aminoadipate-6-semialdehyde and saccharopine. The lysine pool has a great influence on penicillin production since lysine is a potent inhibitor of penicillin biosynthesis. Lysine decreases the biosynthesis of penicillin by inhibiting and repressing homocitrate synthase, thereby depriving the cell of α-aminoadipic acid, a precursor for penicillin synthesis (Bañuelos *et al*., 2002).

1.4.2 L-cysteine

The synthesis of cysteine in *P. chrysogenum* is dependent on the active uptake of sulphate from the exterior of the cell. The sulphate assimilation pathway catalyses the reduction of sulphate via sulphite to sulphide and subsequently sulphide is converted into cysteine. The reduction of sulphate into sulphite is catalysed by three enzymes: ATP sulphurase converts inorganic sulphate into adenosine-5-phosphosulphate (APS) which is then activated into 3-phospho-adenosine-5-phosphosulphate (PAPS) by APS-kinase and reduced to sulphite by PAPS reductase (Foster *et al.*, 1994). Sulphite is reduced to sulphide by sulphite reductase (Marzluf, 1993). The location of enzymes involved in the reduction of sulphate has not been described. Sulphide is the basis for biosynthesis of L-cysteine, which occurs via two different pathways in β-lactam producing fungi: the transsulphuration and the sulphhydrylation pathway. L-cysteine, synthesized via the transsulphuration pathway used for penicillin pathway in *P. chrysogenum*, is formed by cleavage of L-cystathionine derived from the intermediate L-homocysteine, which is formed from L-methionine or O-acetyl-L-homoserine (Nuesch *et al.*, 1987).

1.4.3 L-valine

The biosynthesis of valine starts with the condensation of pyruvate with hydroxyethyl thiamine pyrophosphate into α-acetolactate by acetohydroxy acid synthase. The conversion of α-acetolactate into L-valine is catalysed by three enzymes. Acetohydroxy acid isomeroreductase converts α-acetolactate into dihydroxyisovalerate. Dihydroxy acid dehydrase converts dihydroxyisovalerate into α-ketoisovalerate which in turn is converted to L-valine by the branched chain amino acid glutamate transaminase. All four enzymes are thought to be located inside the mitochondrial matrix. Consequently valine has to be translocated to the cytosol to become available for ACVS (Evers *et al.*, 2004).

1.5 COMPARTMENTALIZATION OF PENICILLIN G BIOSYNTHESIS

Biosynthesis of the penicillin precursor amino acids proceeds in different cellular compartments. Cell fractionation, electron microscopy and molecular genetic studies have shown a distinct spatial organization of the penicillin biosynthetic pathway in *P. chrysogenum*. Cell fractionation studies in *P. chrysogenum* suggested that ACVS is attached to vacuoles, but more recent studies and the biochemical properties of ACVS, *i.e.* optimal pH, co-factor requirement and protease sensitivity, indicate that ACVS is a free cytosolic enzyme (Van der Lende *et al.*, 2002). Like ACVS, IPNS is localized in the cytosol. Electron microscope studies in combination with immunological detection methods demonstrated that acyltransferase is located in a microbody. This observation is substantiated by genetic studies indicating the location of the *P. chrysogenum* acyltransferase in the microbody (Figure 1.6) (Müller *et al.*, 1992)

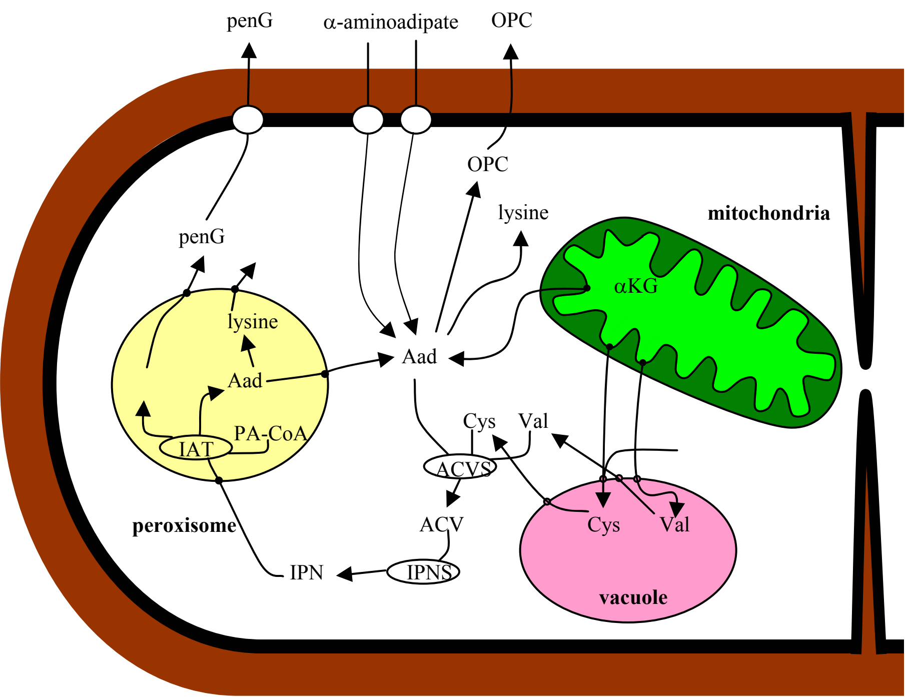


Figure 1.6 Localization of the penicillin G biosynthesis (Modified from Evers *et al.*, 2004) Aad: α-aminoadipate; Cys: Cysteine; Val: Valine; αKG: α-ketoglutarate

The distinct subcellular organization of penicillin biosynthesis enables the spatial separation, regulation and optimization of the processes involved. The penicillin yield of

industrial strains implies a substantial drain on precursors and co-factors such as CoA, ATP and NADPH. The cellular localization and pool sizes of these compounds are important determinants in penicillin biosynthesis. Compartmentalization facilitates channelling of the co-factors, precursors and intermediates involved to the sites of biosynthesis implying transport of these compounds through the cell and across organellar membranes. Besides the microbody, organelles like mitochondria and vacuoles are of specific importance in penicillin biosynthesis. Like the plant vacuole, the fungal vacuole is involved in a variety of cellular processes and fulfils several general functions. The fungal vacuole amongst others is involved in the active and stringent control over the cytosolic concentration of many different constituents. The bulk of free basic and neutral amino acids and a substantial part of the acidic amino acids are localized in the vacuole (Kubicek and Kubicek, 1992). Studies of precursor amino acid pools utilized in penicillin biosynthesis indicated a high turnover of the vacuolar pools. L-α-aminoadipic acid and cysteine are toxic at moderate concentrations; their cytosolic levels are therefore regulated tightly through storage in the vacuole and controlled release from this organelle (Lendenfeld *et al*., 1993). Transport in and out of the vacuole involves H^+-amino acid antiport systems present in the vacuolar membrane. Like glutathione, LLD-ACV might at elevated cytosolic levels be stored in the vacuole.

Microbodies (peroxisomes) are indispensable organelles that can be found in practically all eukaryotic cells. Although their morphology is relatively simple their physiological properties are remarkably complex. The organelles are involved in pathways of primary, intermediary and secondary metabolism. They may be regarded as organelles in which specific metabolic conversions take place mostly by non-membrane bound enzymes. A major advantage of the presence of a peroxisomal permeability barrier is that it permits the cells to precisely adjust the levels of different intermediates of primary metabolism required for specific metabolic pathways. The role of microbodies with respect to penicillin biosynthesis became clear, when IAT was shown to be located in this organelle. When the putative targeting signal was removed, the enzyme was not directed to the microbody but instead localized in the vacuole and surrounding cytosol. Under these conditions, penicillin production ceased although the enzyme was expressed in

vivo and active in vitro (De Lucas *et al*., 1997)**.** One of the possible explanations could be that another essential enzymatic step, precursor activation by PCL might occur only inside microbodies and that these activated precursors are now sequestered from IAT inside the microbody. On the other hand in a mutant strain of *Aspergillus nidulans* lacking functional peroxisomes, penicillin production still occurs with the peroxisomal enzymes mislocalized to the cytosol. Although this suggests that peroxisomes are not essential for penicillin production, a positive correlation between penicillin yield and peroxisomes numbers has been implicated. The exact reason for this correlation is not known, but this may relate to an increase in the amount of enzymes of the biosynthetic pathway (Müller *et al*., 1991).

1.6 GENETIC ORGANIZATION OF THE PENICILLIN G BIOSYNTHETIC GENES

Genes for the biosynthesis of secondary metabolites are usually arranged in clusters together with genes for resistance to the toxic action of secondary metabolites on the producer organisms and sometimes with genes for biosynthesis of antibiotic precursors (Martin, 2000). β-lactam biosynthetic genes from several prokaryotes and fungi have been cloned and their genomic arrangement has been determined (Figure 1.7). In both *P. chrysogenum* and *A. nidulans,* the three genes encoding ACVS, IPNS, and IAT, named *pcb*AB, *pcb*C and *pen*DE, respectively are tightly clustered in a 15 kb DNA region. The conserved arrangement of penicillin biosynthesis genes in different species is undoubtedly important for coordinated regulation of expression of the three genes.

1.6.1 *pcb*AB

Penicillin biosynthesis begins with the non-ribosomal condensation of three precursor amino acids by a single multienzyme, the ACVS, that functions in a similar manner to the peptide synthetases that mediate the non-ribosomal synthesis of peptide antibiotics. The gene coding for ACVS was designated *pcb*AB and was first cloned in *P. chrysogenum* (Diez *et al*., 1990) by complementation of mutants in penicillin biosynthesis and by transcriptional mapping of the *Penicillium* genome. Transcriptional mapping studies revealed a long transcript of about 11.5 kb. An open reading frame was

found containing 11.4 bp that encodes a protein of 3792 amino acids with a deduced molecular weight of 426 Da. No introns were found in the *pcb*AB gene. The *Penicillium* genes *pcb*C and *pcb*AB, are immediately adjacent to each other and are divergently transcribed, with a 1.2 kb intergenic region serving as a regulated promoter for both genes (Renno *et al*., 1992).

1.6.2 *pcb*C

The first *pcb*C (encoding IPNS) cloned gene was from *Acremonium chrysogenum*. Since then, other IPNS structural genes have been cloned from nine other β-lactam producing organisms, including the *pcb*C genes from *P. chrysogenum* and *A. nidulans*. The *pcb*C gene of *P. chrysogenum* encodes a protein of 331 amino acids with a deduced molecular mass of 37.9 Da. The *pcb*C genes do not contain introns. The *pcb*C sequence from different microorganisms showed identity percentage greater than 60%, even between *pcb*C genes of prokaryotic and eukaryotic origins (Gutiérrez *et al*., 1999a). The IPNS proteins from different origins are very similar (Martin and Gutiérrez, 1995). This similarity is scattered through out the protein than confined to a few well defined regions.

1.6.3 *pen*DE

The *pen*DE gene of *P. chrysogenum* was found to reside downstream from the *pcb*C gene and encoded a protein with a deduced molecular mass of 38,943 Da (Barredo *et al*., 1989b). Interestingly, the N-terminal amino acid sequence deduced from the purified protein (Alvarez *et al*., 1987) match with a region located within the gene, not with the amino terminal sequence of the ORF suggesting a processing of the protein. Three introns were present in the amino-terminal half of the gene. Characterization of the IAT enzyme showed that it consists of two subunits of approximate molecular weights, 11 kDa and 29 kDa where the amino-terminal sequence of the smaller subunit correspond to that predicted for the amino-terminal sequence of the *P. chrysogenum pen*DE ORF (Martín and Gutiérrez, 1992,1995).

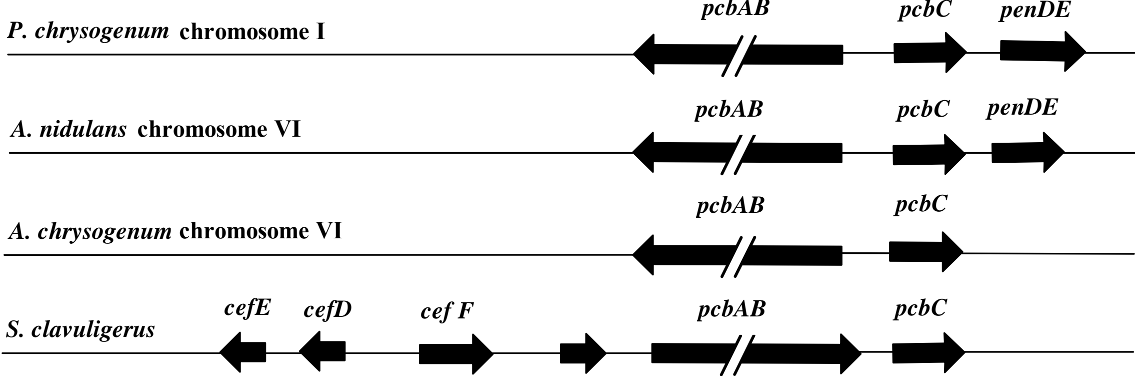


Figure 1.7 Representative β-lactam biosynthetic gene clusters of bacteria and fungi aligned at the start sites of *pcb*C genes. (Modified from Aharonowitz *et al*., 1992)

1.7 MOLECULAR CONTROL OF EXPRESSION OF PENICILLIN BIOSYNTHETIC GENES

Investigations of the expression of penicillin biosynthesis genes were performed mainly with the *Escherichia coli* reporter genes *lacZ* and *uidA*, encoding β-galactosidase (β-Gal) and β-glucuronidase (β-Glu), respectively (Brakhage *et al*., 1992). These studies led to the finding that the promoter strengths of penicillin biosynthesis genes are rather different. It was shown that in *P. chrysogenum*, *pen*DE had lower expression than *pcb*C and three fold-higher expression than *pcb*AB. On the basis of reporter gene fusions studies, it became evident that the expression of *pcb*AB was much weaker than that of *pcb*C (Feng *et al*., 1994). The intergenic regions between *pcb*AB and *pcb*C thus seem to contain the information required for the significant difference in expression levels between *pcb*AB and *pcb*C. The low expression of *pcb*AB is, at least in wild-type strains, rate limiting for penicillin production, because over expression of *pcb*AB led to drastically increased production of penicillin (Kennedy and Turner, 1996) while similar over expression of *pcb*C and *pen*DE did not (Fernández-Cañón and Peñalva, 1995).

It seems rational to assume that the expression of penicillin biosynthesis is coordinated to ensure the synthesis of penicillin by the concomitant appearance of all gene products. Biosynthesis could be expressed simultaneously; i.e., the genes could be activated by the same regulatory factors or could be sequentially induced. The expression of the

penicillin biosynthesis genes is most probably coordinated, supported by the appearance of all three penicillin biosynthetic gene transcripts in *P. chrysogenum* at the same time during a fermentation run (Renno *et al*., 1992). However some observations have shown coordinated expression of genes to be more complicated. Differential regulation of *pcb*AB, *pcb*C and *pen*DE in *A. nidulans* has been observed in response to exogenous signals. The expression of *A. nidulans pcb*AB and *pcb*C genes was significantly repressed by L-lysine. In the fermentation medium with glucose as the carbon source, only *pcb*C was significantly repressed by glucose. In fermentation medium, *pcb*AB and *pcb*C gene fusions were expressed for up to 68 and 46h, whereas the *pen*DE expression was detected for only about 24 h.

In contrast to the other penicillin biosynthesis genes, the *pen*DE genes of both *A. nidulans* and *P. chrysogenum* encode three introns. In theory, introns could play a role in regulating *pen*DE expression.

Previous studies of the formation of secondary metabolites in batch fermentations led to the definition of two phases: the growth phase (trophophase) and the period of secondary-metabolite production (idiophase). However, when lactose was used as the sole carbon source, there was no sharp separation of the trophophase and idiophase with respect to penicillin production or the expression of penicillin biosynthesis genes in *A. nidulans* (Brakhage *et al.,* 1992). For some secondary metabolites, it seems likely that their production in a clear-cut idiophase reflects the inhibiting effects of certain compounds in the medium rather than an intrinsic temporal delay in the product formation pattern. This view is consistent with the results of Renno *et al*., (1992) who showed that in wild-type *P. chrysogenum* (NRRL 1951) and also in the high penicillin producing strain P2, the highest steady-state level of mRNAs of all penicillin biosynthesis genes was observed during maximal growth in both shake flasks and 2-litre fermenters. However, some observations imply that in *P. chrysogenum* there might be a temporal expression of β-lactam biosynthesis genes. *pcb*C steady-state mRNA levels increased with the age of the culture, indicating preferential transcription of the gene at late growth times.

Carbon source regulation seems to act at several points of the penicillin biosynthesis: (i) flux of L-α-aminoadipic acid (Hönlinger and Kubicek, 1989), (ii) activation of side chain precursors, (iii) transcription of penicillin biosynthesis genes and (iv) post transcriptional regulation of penicillin biosynthesis genes (Brakhage, 1998). Glucose represses the formation of ACVS and IPNS in *P. chrysogenum*. This problem has been partially overcome by feeding sub-repressing doses of glucose and by using lactose as the carbon source. Since in general the fungus grows better with glucose than with lactose, the production of penicillin appears to be favoured by suboptimal growth conditions. An involvement of cyclic AMP (cAMP) in carbon source regulation is controversial. In *P. chrysogenum*, cAMP levels were high during growth on lactose and decreased markedly when glucose was added (Kozma *et al*., 1993).

Penicillin production is subject to pH regulation mediated by transcriptional factor PacC (Hillenga *et al*., 1995). It acts as a wide-domain regulator via repression at neutral and acidic pH values and induction at alkaline pH values. PACC binds to the intergenic region between the *pcb*AB and *pcb*C genes and enhances transcription of the *pcb*C gene at alkaline pH. Unlike the mechanism operative in *A. nidulans*, alkaline pH does not override the negative effects of carbon catabolite repression in *P. chrysogenum*. The reason for the pH-mediated regulation of penicillin biosynthesis is unclear.

A major constraint determining the productivity of aerobic fermentations and more profoundly aerobic fungal processes in submerged cultures is the provision of adequate dissolved oxygen (% DOT). Oxygen is needed for growth and maintenance (respiration) and by oxidative enzymes involved in penicillin biosynthesis. The effects of oxygen on penicillin productivity are dependent on degree, duration and timing of its limitation (Rollins *et al.,* 1991).

Besides PACC, PENR1, a CCAAT box-binding protein is involved in the repression, induction and expression of *pcb*AB, *pcb*C and *pen*DE respectively. Three recessive *trans*-acting mutations have been characterized in *A. nidulans* and correspond probably to positively acting regulatory genes. Computer analysis showed that DNA elements with a high degree of sequence identity to the *A. nidulans* PENR1 site reside within the

intergenic regions of both *P. chrysogenum* and *A. chrysogenum* and the *pen*DE promoter region of *P. chrysogenum*. Nitrogen metabolite repression is generally regarded as a wide-domain regulatory system, which operates to ensure that a constant nitrogen supply is readily available for growth in response to widely variable or rapidly changing environments. Recently, it was demonstrated that in *P. chrysogenum*, ammonium directly influenced the expression of penicillin biosynthetic genes mediated by the nitrogen regulatory protein NRE. It was shown that the expression of both the *pcb*AB and *pcb*C genes was repressed by the addition of 40 mM ammonium chloride to lactose grown culture (Feng *et al*., 1994).

Because penicillins are synthesized from amino acid precursors, it was conceivable that amino acids play a role in the regulation of their biosynthesis. This was supported by the observation that in both *A. nidulans* and *P. chrysogenum* the addition of L-lysine to fermentation medium led to reduced penicillin titers (Brakhage *et al*., 1992). Since L-α-aminoadipic acid is a branch point between L-lysine and the penicillin biosynthetic pathways, L-lysine inhibition of penicillin biosynthesis was suggested to operate at one or more steps of the L-lysine pathway. This was based on the notion that L-lysine caused feedback inhibition of homocitrate synthase activity in *P. chrysogenum* (Brakhage, 1998). Homocitrate synthase catalyzes the initial reaction of L-lysine biosynthesis. In *P. chrysogenum* 75% of this activity is in the cytoplasm and 25% is in mitochondria, hence compartmentalization might be important. α-aminoadipate reductase is also inhibited by L-lysine and the inhibition occurs at physiological concentrations (Jaklitsch *et al*., 1987). It seems very likely that the L-α-aminoadipic acid pool available for penicillin production is reduced by L-lysine through feedback inhibition and through repression of several L-lysine biosynthetic genes and enzymes. Differential effects due to various amino acids on the expression of the penicillin biosynthesis genes *pcb*AB and *pcb*C and penicillin production were measured in *A. nidulans*. *pcb*AB expression seemed to be more subject to regulation by external amino acids than did *pcb*C expression (Then Bergh and Brakhage, 1998).

Discrepancies observed between the expression of structural genes and enzyme specific activities of the corresponding proteins suggested that besides transcriptional regulation,

post-transcriptional regulation of penicillin biosynthesis genes occurs. Comparison of the genetic organization of penicillin biosynthesis in industrial *P. chrysogenum* strains and low-producing wild-type strains revealed significant modifications. The important genetic features of *P. chrysogenum* strains are amplification of structural genes and massive increase in steady-state levels mRNA levels.

To summarize the research on the regulation of biosynthesis of β-lactam antibiotics, the β-lactam biosynthetic genes are transcribed from specific promoters and regulated transcriptionally and very probably post-transcriptionally. It can be assumed that there are other regulatory circuits which remain yet to be discovered.

1.8 Real time PCR for mRNA quantitation

Many cellular decisions concerning survival, growth and differentiation are reflected in altered patterns of gene expression and the ability to quantitate transcription levels of specific genes has always been central to any research into gene function (Zamorano *et al*., 1996). Four methods are in comon use for the quantification of transcription: northern blotting, *in situ* hybridisation, RNAse protection assays and the reverse transcription polymerase chain reaction (RT-PCR). The main limitation of the first three techniques is their comparatively low sensitivity (Melton *et al*., 1984).

Real-time PCR (QPCR) quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferable alternative to other forms of quantitative RT-PCR that detects the amount of final amplified product at the end-point. Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the end-point detection. In comparison to conventional RT-PCR, real-time PCR also offers a much wider dynamic range of up to 10^7-fold (compared to 10^3-fold in conventional RT-PCR). Dynamic range of any assay determines how much target concentration can vary and still be quantified. A wide dynamic range means that a wide range of ratios of target and normaliser can be assayed with equal sensitivity and specificity. It follows that the broader the dynamic range, the more accurate the quantitation (Dudarewicz *et al*., 2005).

The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Higuchi *et al.,* 1993). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product.

There are three main fluorescence-monitoring systems for DNA amplification: (1) Hydrolysis probes (2) Hybridising probes; and (3) DNA-binding agents (Pandey *et al*., 2003). The system used in the following studies is based on the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I). It does not bind to ssDNA. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Wittwer *et al*.,1997).

The threshold cycle or the C_t value is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. A fixed fluorescence threshold is set significantly above the baseline. The threshold cycle is when the system begins to detect the increase in the signal associated with an exponential increase of PCR product during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end-point). The slope of the log-linear phase is a reflection of the amplification efficiency. The efficiency of the reaction can be calculated by the formula (Labrenz *et al*., 2004):

$$\text{Exponential amplification} = 10^{(-1/\text{slope})}$$

$$\text{Efficiency} = [10^{(-1/\text{slope})}]-1$$

The efficiency of the PCR should be 90 - 100% (– 3.6 > slope > – -3.1). A number of variables can affect the efficiency of the PCR (Bustin, 2004; Wong and Medrano, 2005) which includes length of the amplicon, secondary structure and primer quality. Although valid data can be obtained that fall outside of the efficiency range, the QPCR should be further optimised or alternative amplicons designed.

For the slope to be an indicator of real amplification (rather than signal drift), there has to be an inflection point. This is the point on the sigmoidal curve when the log-linear phase begins. The important parameter for quantitation is the C_t (Higuchi *et al.*, 1993). The higher the initial amount of DNA, sooner the accumulated product is detected in the PCR process, lower the C_t value. (Gibson *et al.*, 1996). Some software allows determination of the C_t value by a mathematical analysis of the sigmoidal curve. This provides better run-to-run reproducibility. A C_t value of 40 or higher means no amplification and this value cannot be included in the calculations. Besides being used for quantitation, the C_t value can be used for qualitative analysis as a pass/fail measure.

The quantification strategy is the principal marker in gene quantification. Generally, two strategies can be performed in QPCR. The levels of expressed genes may be measured by absolute or relative quantitative QPCR. Absolute quantitation uses serially diluted standards of known concentrations to generate a standard curve. The standard curve produces a linear relationship between C_t and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their C_t values (Heid *et al.*, 1996). Relative quantification measures the relative change in mRNA expression levels based on either an external standard or a reference sample, also known as a calibrator (Livak and Schmittgen, 2001). The reliability of an absolute QPCR assay depends on the condition of 'identical' amplification efficiencies for both the native target and the calibration curve in the following kinetic PCR. In addition, the concentration of serial dilution should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay. Relative quantification is easier to perform than absolute quantification because a calibration curve is not necessary. It is based on the expression levels of a target gene versus a housekeeping gene (reference or control

gene) and in theory is adequate for most purposes to investigate physiological changes in gene expression levels. The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple QPCR experiments. There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantitation assays. Depending on the method employed, these can yield different results and thus discrepant measures of standard error (Liu and Saint, 2002; Muller *et al*., 2002).

1.9 PROPOSED MECHANISM OF ELICITATION IN FUNGI

Intensive research has been dedicated to establish the mechanisms of elicitation in plants, but no scientific evidence has been put forward to explain the mechanisms in fungal cells (Pitta-Alvarez *et al*., 2000; Vasconsuelo and Boland, 2007). Research in both systems has been based mainly on the biotic and particularly on carbohydrate elicitors. A recent advance, in the understanding of the mechanisms underlying the expression of plant disease resistance, has allowed the following course of events through (Benhamou, 1996):

1) The perception of a signal by the cell. Signalling pathways are initiated when a signal binds its receptor.
2) The intracellular transduction of this recognition signal. Ligand binding to the receptor induces assembly of signalling pathway components.
3) The synthesis of defence molecules. Generation of secondary messengers which carries signal to cell interior.
4) Transport of defence molecules to strategic sites. Phosphorylation/dephosphorylation cycles under control of the signalling pathway activate/deactivate additional pathway components.
5) Enzyme cascades amplify the signal converting molecules to their active forms.

The multi-component defence response has shown to culminate in a number of physical and biochemical changes in the host. In the absence of detailed experimental data and based on the information obtained from the investigation of the mechanism of action of carbohydrate elicitors in plant systems, a hypothetical model for the proposed mechanism of action of oligosaccharide elicitors in fungi is postulated (Figure 1.8). This model is based on the defence mechanism model (signalling cascade model) in plants (Ebel and Mithofer, 1998) and constructed from evidence in literature.

1.9.1 Signal perception

Only oligosaccharides of a specific size range are found to exert elicitation. This suggests the receptor has a well defined shape akin to that of mammalian receptors (Petruccioli *et al*., 1999). The effective concentrations at which the elicitation occurs gives an indication as to which type of mechanism is most possible. At very low elicitor concentration, the interaction is most likely to be at the membrane surface level. The cytoplasmic membrane of eukaryotic cells is a fluid mosaic of phospholipids and proteins. Signal perception is the first step of the elicitor signal transduction cascade and for example, recognition of different stimuli is central to the ability of plants to respond through membrane depolarization, activation of kinases, generation of reactive oxygen species, ion fluxes and cytoplasm acidification (Benhamou, 1996). A small degree of receptor occupancy can generate a large increase in secondary messenger levels, which in turn generates an even larger physiologic response. The signal is amplified at every step of the signal transduction process (Figure 1.8). The ability of receptors to transmit signals can be increased or decreased by phosphorylation of the receptor, leading to sensitisation or desensitisation of cells to various signals (Gual *et al*., 2005). Three classes of receptors are involved in signal transduction:

1) Receptors that penetrate the plasma membrane and have intrinsic enzymatic activity
2) Receptors that are coupled to G-proteins
3) Intracellular receptors.

Although numerous elicitor binding sites have been identified localised in the plant plasma membrane, very little has been known in fungal cells. Undoubtedly, this primary event in elicitor signal transduction should be an important subject of future investigations.

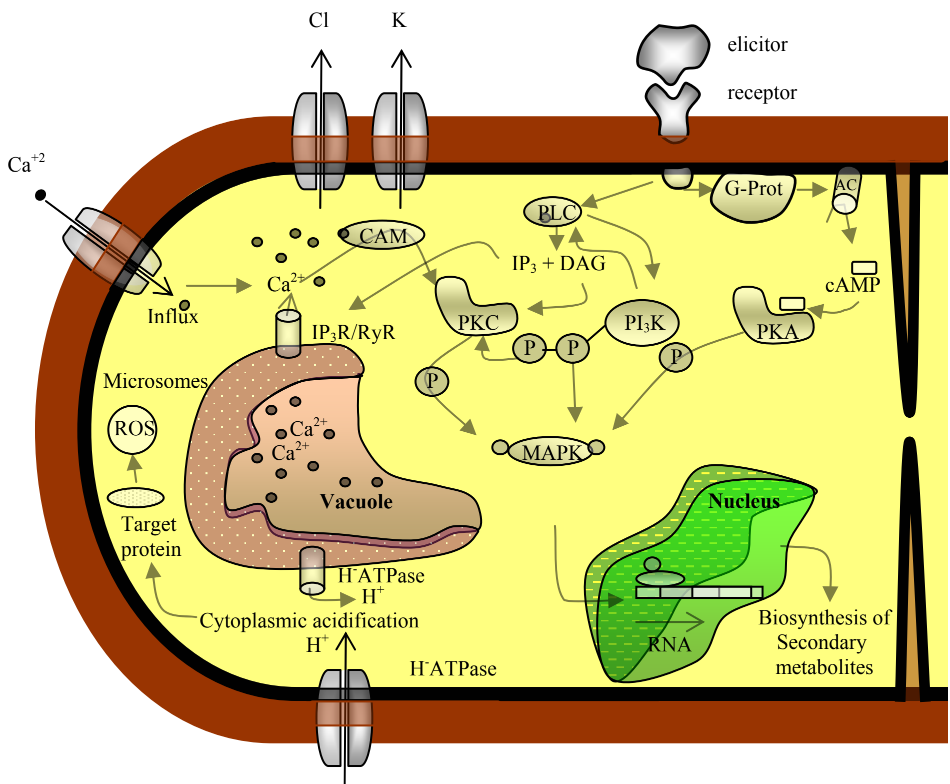


Figure 1.8 Hypothetical mechanism of elicitation in fungi

1.9.2 Intracellular signalling systems involved in elicitation

Diverse intracellular signalling systems are believed to be involved in elicitation. A variety of stimuli reach the cell at the same time causing different classes of receptors to promote or inhibit production of many of the same secondary messengers. Additionally, the same cellular response may be induced by multiple signal pathways (Figure 1.8). Interaction of different signalling pathways permits the fine-tuning of cellular activities (Bruce *et al*., 2003). G-proteins have been identified in many eukaryotic systems including fungi (Trinci *et al*., 1990). G-proteins have been involved in various cellular processes linked to growth, hormone signalling, and development and defence responses. There are evidences confirming that the PLC/IP_3-DAG/PKC pathway occurs in plants and other eukaryotic cells and plays a role in the responses of cells to elicitors (Shigaki and Bhattacharyya, 2000; Vasconsuelo *et al*., 2003; Wang, 1999). Accordingly, a number of elicitors have been found to activate phospholipase C (PLC) in plants causing polyphosphoinositide turnover and production of the secondary messenger's

inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 together with DAG, is a messenger molecule used in signal transduction in biological cells. Its main functions are to mobilize Ca^{2+} from storage organelles, which can bind to calmodulin or activate protein kinases and to regulate cell proliferation and other cellular reactions (Vasconsuelo and Boland, 2007). Studies in the cascade of events produced in plants by elicitors showed that the rapid influx of Ca^{2+} ions and efflux of K^+ and Cl^- occur prior to production of ROS, activation of kinases, gene transcription, and phytoalexin production (Nurnberger *et al*., 1994). Fungal vacuoles appear to be the major storage organelles for Ca^{2+} and several other divalent cations such as Mn^{2+} and Zn^{2+}. Cornelius and co-workers (1989) demonstrated that IP_3 stimulates Ca^{2+} release from vacuoles of *Neurospora crassa*. IP_3 also promotes Ca^{2+} influx from the exterior (Berridge and Irvine, 1989). The Ca^{2+} liberated into the cytoplasm binds to calmodulin (CAM) and triggers a cascade of protein phosphorylation that results in the activation of transcription of defence genes. Calmodulins have been identified in many fungi and yeasts (Hubbard *et al*., 1982; Trinci *et al*., 1990). DAG activates protein kinase C (PKC) which in turn phosphorylates certain proteins. The existence of multiple subspecies of PKC has been demonstrated in *Neurospora crassa* (Favre and Turian, 1987) and *Saccharomyces cerevisiae* (Ogita *et al*., 1990). In parallel to this, other molecules can also be involved in the transduction of the signal, such as cAMP. The cAMP system has been reported in filamentous fungi (Gadd, 1994). A variation in the levels of reactive oxygen species (ROS) constitutes another physiological change observed in fungal cells when challenged with elicitors and might also be involved in the transduction of the signal (Radman, 2002).

Successful signal transduction demands the creation of highly branched, interconnected chemical networks. One of the most frequently employed organic switches is the addition and removal of a phosphate group to a protein. Enzymes that cause the phosphorylation of other proteins are known as kinases. Removal of the phosphate group is accomplished by protein phosphatases. Protein kinases represent one of the largest families of regulatory enzymes (Martin *et al*., 2003).

1.9.2.1 cAMP-dependent protein kinase (PKA)

cAMP-dependent protein kinase, also known as protein kinase A (PKA) refers to a family of enzymes whose activity is dependent on the level of cyclic AMP (cAMP) in the cell. Each PKA is a holoenzyme that consists of two regulatory and two catalytic subunits (Dickman and Yarden, 1999). Under low levels of cAMP, the holoenzyme remains intact and is catalytically inactive. When the concentration of cAMP rises, cAMP binds to the two binding sites on the regulatory subunits, which then undergo a conformational change that releases the catalytic subunits. The free catalytic subunits can then catalyze the transfer of ATP terminal phosphates to protein substrates at serine, or threonine residues. This phosphorylation usually results in a change in activity of the substrate. Since PKAs are present in a variety of cells and act on different substrates, PKA and cAMP regulation are involved in many different pathways. In addition, the effects of PKA phosphorylation are generally transient because protein phosphatases quickly dephosphorylate PKA targets (Fimia and Sassone-Corsi, 2001; Schwartz, 2001). The role of the cAMP signalling pathway in yeast, through PKA, is of nutrient sensing and regulation of diverse biological processes including growth, metabolism, stress resistance and entry into either meiosis or pseudohyphal differentiation (D'Souza and Heitman, 2001). It therefore controls energy metabolism and growth. Filamentation, conidation, morphogenesis, mating and stress tolerance in *N. crassa* are controlled by cAMP signalling.

1.9.2.2 Protein kinase C (PKC)

PKC is a family of multifunctional protein serine/threonine kinases that express a pseudosubstrate site and a membrane interaction phosphatidylserine binding site and differ in structure, function and cofactor requirements. They are divided into three subfamilies: conventional, novel, and atypical based on their second messenger requirements. Conventional PKCs require Ca^{2+}, diacylglycerol (DAG), and a phospholipid such as phosphatidylcholine for activation. Novel PKCs require DAG, but do not require Ca^{2+} for activation. Thus, conventional and novel PKCs are activated through the same signal transduction pathway as phospholipase C. On the other hand,

Atypical PKCs require neither Ca^{2+} nor diacylglycerol for activation (Parker, 1992; Nishizuka, 1995). Several components of the PKC family of proteins have been described in *N. crassa* (Favre and Turian, 1987). In the absence of stimulation, protein kinase C is present as a soluble cytosolic protein that is catalytically inactive.

1.9.2.3 Mitogen activated protein kinase (MAPK)

Recognition of elicitation stimulus leads to the activation of specific genes. MAPK cascades are major components of downstream receptor/sensors that transduce external signals into intracellular responses in all eukaryotes (Figure 1.8). Mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Pearson *et al.*, 2001). Plant MAPKs are activated by a variety of biotic and abiotic stimuli, including pathogen attack, wounding, temperature, drought, salinity, osmolarity, UV irradiation, ozone and reactive oxygen species. This activation affects other pathways or specific genes (Stratmann and Ryan, 1997). The application of new biochemical and molecular tools, as well as animal studies, suggest that there exists more complexity in the action of MAPKs in elicitation. This deduced from the fact that a given elicitor generally not only activates a single but several MAPKs. Extracellular stimuli lead to activation of a MAP kinase via a signalling cascade (MAPK cascade) composed of MAP kinase, MAP kinase kinase (M2K) and MAP kinase kinase kinase (M3K). M3K that is activated by extracellular stimuli phosphorylates a M2K on its serine and threonine residues, and then this M2K activates a MAP kinase through phosphorylation on its serine and tyrosine residues. This MAP kinase signaling cascade has been evolutionarily well-conserved from yeast to mammals (Vasconsuelo and Boland 2007).

The MAPK cascade results in translocation of a transcriptional activator from the cytoplasm into the nucleus, where it activates transcription of MAPK-dependent genes. Sometimes this activation also involves induction at transcription, translational or posttranslational levels. A given elicitor might activate a number of MAPK pathways, and different stimuli can activate the same pathway. MAPK activation generally affects

specific gene expression. It has been observed in the final steps of the addition of chitosan in suspension cultures of *Rubia tinctorum*, signalling are mediated by translocation of MAPK to the nucleus resulting in the stimulation of anthraquinone synthesis. In turn, MAPK phosphorylates transcription factors, which results in increased expression of genes coding for enzymes, which play a critical role in the biosynthetic pathway of secondary metabolites including phytoalexin (Vasconsuelo, 2005). In *R. tinctorum* infected with *P. aphanidermatum* that contains chitosan, it has been shown that the increase in anthraquinone levels is preceded by an increase in isochorismate synthase transcript and activity levels (Van Tegelen *et al*., 1999).

There is an interaction between the cAMP and MAPK signalling cascades in the regulation of fungal development (D'Souza and Heitman, 2001). As an example, the transition between the yeast and filamentous morphology in *S. cerevisiae* is regulated by a MAPK cascade, that also regulates mating of haploid cells, and a nutrient-sensing GPCR that produces signalling via cAMP and PKA (Pan *et al*., 2000).

1.10 ROLE OF CALCIUM AND OTHER IONS IN SIGNAL TRANSDUCTION

A change in cytoplasmic Ca^{2+} induced by a stimulus is the result of a change in the rate of uptake or release of Ca^{2+} across the cell membrane, or across the membranes of an intracellular organelle.

Intracellular Ca^{2+} pools within the cells is present in the form of:

1) Ca^{2+} bound to the outer surface of the cell
2) free Ca^{2+} in the cytoplasm and in organelles
3) Ca^{2+} bound to small molecules, proteins, nucleic acids and phospholipids; and
4) Crystalline deposits of calcium phosphate and occasionally calcium oxalate, as well as structural complexes of Ca^{2+}.

The exchange of external Ca^{2+} with internal pools of Ca^{2+} can be quite fast. For example, Ca^{2+} bound to molecules in the cytoplasm exchanges rapidly with free Ca^{2+}, which in turn exchanges with the external Ca^{2+} in a few minutes. However, Ca^{2+}within organelles or crystalline Ca^{2+}deposits may take many minutes or even hours to exchange with external Ca^{2+}, particularly in resting cells.

Meaurement of Ca^{2+} influx or efflux can provide four pieces of evidence in establishing a regulatory role for intracellular Ca^{2+}:

1) a change in Ca^{2+} flux initiated by the primary stimulus provides evidence for an 'active' role for Ca^{2+}
2) estimation of the size of intracellular Ca^{2+} pools and their alteration to physiological stimuli, can help in the identification of their physiological function
3) measurement of fluxes of Ca^{2+} across individual membranes enable the mechanism of Ca^{2+} uptake or release to be elucidated; and
4) uni- and bidirectional Ca^{2+} pumps or Ca^{2+} channels can be identified.

One of the physiological changes induced by the presence of elicitors in the medium is a change in ion fluxes (K^+, Cl^- and Ca^{2+}) through the cell membrane (Mathieu *et al.*,

1991). Ion channels can be regulated by a variety of secondary messengers including Ca^{2+}, pH changes and cAMP. They can also initialise specific cellular responses through their linkage to signal transduction cascades. Ion channels propagate signals by modulating membrane potential or by directly affecting cellular ion composition. An influx of Ca^{2+} to the cytoplasm from the extracellular environment and intracellular reservoirs was observed in several studies (Mithofer *et al*., 2001; Castañeda and Perez, 1996; Gelli *et al*., 1997). The presence of elicitors has also been reported to cause stimulation of K^+ and Cl^- efflux (Bach *et al*., 1993; Blatt *et al*., 1999; Ivashikina *et al*., 2001; Zimmermann *et al*., 1999).

Membranes contain many open K^+ channels but few open Na^+ or Ca^{2+} channels. As a result, the major ionic movement across the plasma membrane is that of K^+ from the inside outward, leaving an excess of negative charge on the inside. In this way, resting K^+ channels play the dominant role in generating the electric potential across the plasma membrane of animal cells. However, in plant and fungal cells the membrane potential is generated by transport of H^+ ions out of the cell by an ATP-powered proton pump. ATP-powered pumps are ATPases that use the energy of ATP hydrolysis to move ions or small molecules across the membrane against a chemical concentration gradient or electric potential. Ion pumps transport ions against their concentration gradients.

Potassium is required for processes such as turgor maintenance, charge balancing and protein synthesis. Transport of potassium across the plasma membrane of fungi has been described in detail (Rest *et al.,* 1995). Small fluxes of K^+ can lead to large variations in membrane potential (Rodriguez-Navarro, 2000).

Calcium plays a universal role as an intracellular signal (second messenger) in eukaryotes including filamentous fungi and is able to transduce a wide range of chemical or physical signals through different intracellular responses. Calcium-mediated signal transduction typically involves transient increases in cytosolic free calcium ($[Ca^{2+}]_c$). This small increase in $[Ca^{2+}]_c$ concentration would activate anion channels, which would lead to membrane depolarisation and would trigger the opening of voltage-gated Ca^{2+}

channels, resulting in a greater elevation of the intracellular Ca^{2+} concentration (Zimmermann *et al*., 1999).

The $[Ca^{2+}]_c$ increase may simply act as a switch in signalling or alternatively, the unique signature of the $[Ca^{2+}]_c$ change may encode specificity in the signalling response. In filamentous fungi, ($[Ca^{2+}]_c$) signalling has been implicated in numerous process including: hyphal tip growth, hyphal branching, cell cycle, dimorphism, cytoplasmic movement, chitin synthesis and sporulation (Knight *et al.,* 1993). Ca^{2+} plays a crucial role mediating elicitor actions, Ca^{2+} spiking being one of the earliest events that regulates almost all pathways involved in the elicitation process. It is recognized that elicitor –induced calcium influx is an early response of plant cells, generally resulting in changes from the ($[Ca^{2+}]_c$ resting level of 50-100 nM to 1-5 μM, within 5 min after elicitor treatment. This fact raises several questions how different elicitors can develop distinct responses mediated by the same single messenger. Several studies have demonstrated that ($[Ca^{2+}]_c$) signals triggered by different stimuli differ in amplitude, frequency, duration and intracellular localization. Elicitation studies in *Nicotiana plumbaginifolia* cultures reported a biphasic calcium increase on addition of oligosaccharides elicitor, the first peak induced by the influx of extracellular Ca^{2+} whereas the second pulse was caused by PLC activation and IP_3-dependent ($[Ca^{2+}]_c$) release from intracellular calcium stores (Lecourieux *et al*., 2002).

1.11 Image Analysis: To study intracellular calcium and morphological changes

Confocal laser scanning microscopy (CLSM) is a technique for obtaining high-resolution optical images. The principle of confocal microscopy was originally patented by Marvin Minsky in 1957, but it took another thirty years and the development of lasers for CLSM to become a standard technique towards the end of the 1980s (US patent 3013467). Following refinements of the prototype models, pioneering confocal images of fixed biological samples were published to show the tremendous promise of confocal laser scanning microscopy (CLSM) for providing non-invasive, high-resolution optical sections and three-dimensional (3D) reconstructions of these sections (Czymmek *et al.,* 1994; Gilroy, 1997; Pawley, 1995). The key features of confocal microscopy is its ability to produce in-focus images of thick specimens, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically-complex objects (Ferrando and Spiess, 2000).

In a confocal laser scanning microscope, a laser beam passes through a light source aperture and then is focused by an objective lens into a small focal volume within a fluorescent specimen. A mixture of emitted fluorescent light as well as reflected laser light from the illuminated spot is then recollected by the objective lens. A beam splitter separates the light mixture by allowing only the laser light to pass through and reflect the fluorescent light into the detection apparatus. After passing a *pinhole*, the fluorescent light is detected by a photo detection device (photomultiplier tube), transforming the light signal into an electrical one that is recorded by a computer. The detector aperture obstructs the light that is not coming from the focal point. The out of focus light is suppressed: most of the light returning is blocked by the pinhole, resulting in sharper images than those from conventional fluorescence microscopy techniques, and permits one to obtain images of various z axis planes (also known as z stacks) of the sample (Figure 1.9). The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the

plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. The beam is scanned across the sample in the horizontal plane by using one or more oscillating mirrors. This scanning method usually has low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage. The computer can generate a three-dimensional picture of a specimen by assembling a stack of these two-dimensional images from successive focal planes (Matsumoto, 1993).

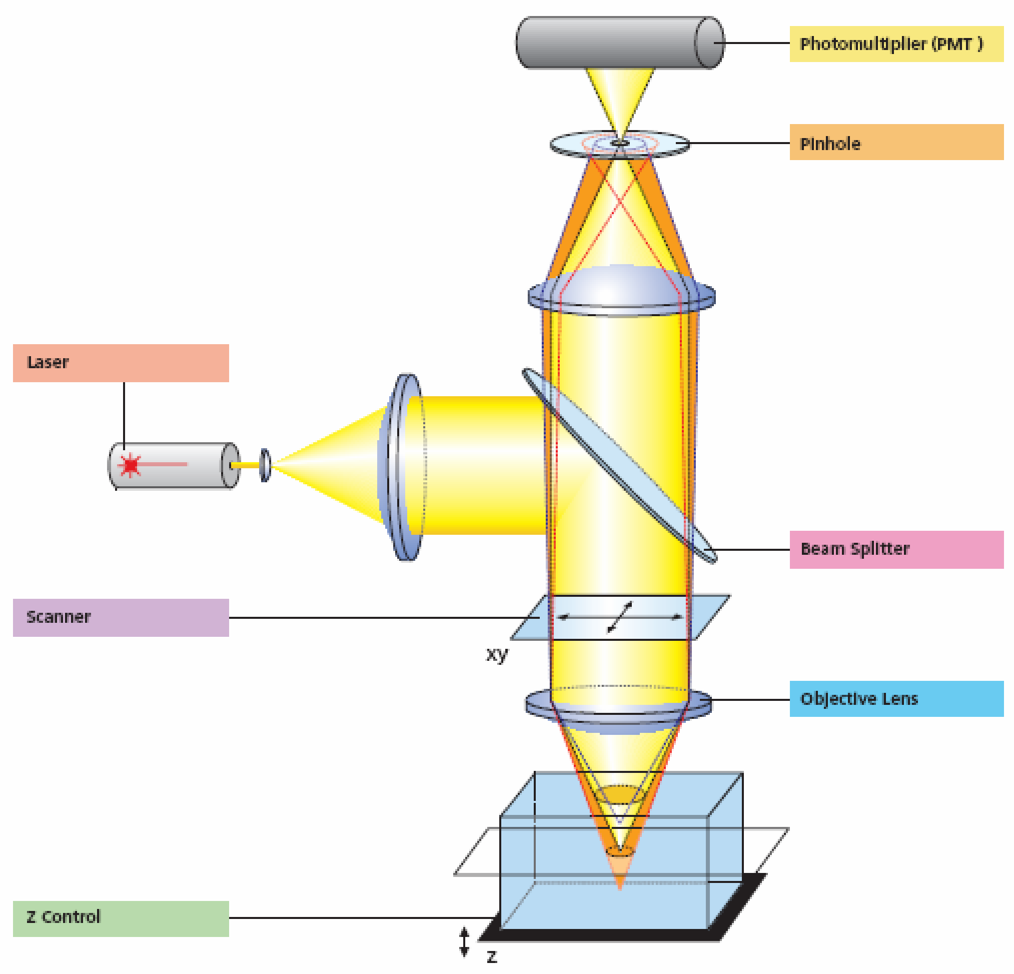


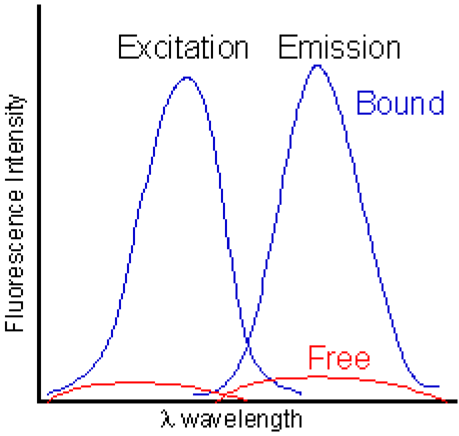
Figure 1.9 Schematic representation of the principle of CSLM (www.zeiss.de)

Confocal microscopy also provides a substantial improvement in lateral resolution and the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation. Because CLSM depends on fluorescence, a sample usually needs to be treated with fluorescent dyes to make objects visible (Fellers and Davidson, 2007). However, the actual dye concentrations can be low to minimize the disturbance of biological systems: some instruments can track single fluorescent molecules. Also, transgenic techniques can create organisms that produce their own luminescent or fluorescent chimeric molecules (such as aequorin technology). At the beginning of this decade changes in intracellular calcium concentrations began to be analyzed by *in vivo* confocal microscopy (Cornell-Bell *et al*., 1990; Gehring *et al*., 1990; Hernandez-Cruz *et al*., 1990; Niggli and Lederer, 1990; Williams, 1990; Williams *et al*., 1990). Since then, literally hundreds of confocal studies have analyzed calcium dynamics in eukaryotes ranging from protists, fungi, and plants (Hepler and Gunning, 1998; Inoue *et al*., 1998; Klauke and Plattner, 1997; Viarengo *et al*., 1996) to animals.

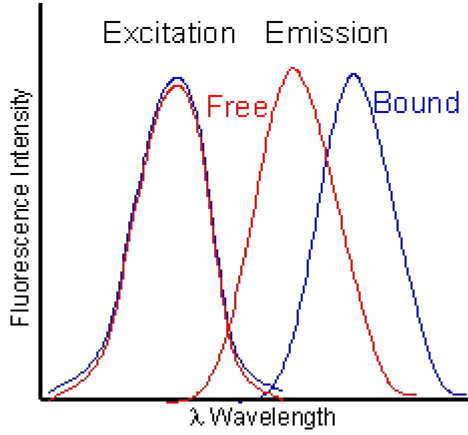
1.12 DETECTION OF INTRACELLULAR CALCIUM FLUX USING FLUORESCENT DYES

The divalent cation, calcium, serves as a mediator for a large number of intracellular biological responses. The advent of specific fluorogenic compounds that specifically bind to calcium has allowed investigators to study changes in Ca^{2+} under a variety of conditions. Calcium ion probes are probably the most widely used intracellular indicators. These indicators have evolved largely through the efforts of Roger Tsien and colleagues (Cobbold and Lee, 1991). Calcium ion detection is most often accomplished by using a dye that has a recognition portion as well as a region that confers fluorescence.

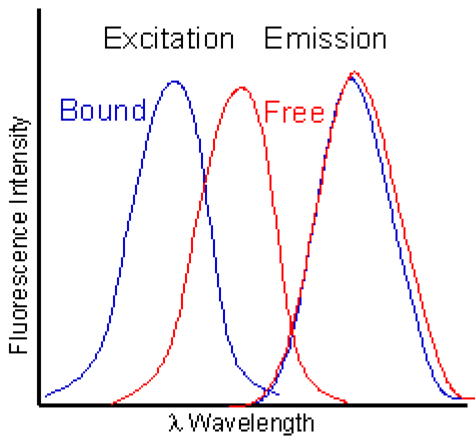
Calcium indicator dyes can be categorized into two groups; the first are the dyes that increase their fluorescence in the presence of calcium (non-ratiometric dyes). The calcium indicator dyes, calcium orange, Fluo-3 and Fluo-4 are representative of these dyes that increase their fluorescence in the presence of calcium ion (Ca^{2+}) without changing wavelengths (Figure 1.10.A). The second group are the ratiometric dyes that have different excitation or emission wavelengths in the presence of calcium than they have in its absence (Figure 1.10.B and 1.10.C). The calcium indicator dyes, Fura-2 and Indo-1 are representative of ratiometric dyes.e.g. Fura-2, upon binding Ca^{2+}, exhibits a shift in its excitation peak from 338 nm to 366 nm (Haughland, 2002), making Fura-2 the dye of choice for microscopy, where it is easier to change excitation wavelengths than emission.



A) Intensity shift when bound/unbound to calcium: non-ratiometric measurements



B) Emission spectral shift: ratiometric measurements



C) Excitation spectral shift: ratiometric measurements

Figure 1.10 Ratiometric and Non-ratiometric dyes

There are advantages and disadvantages to each of the two groups of calcium indicators. The ratiometric dyes, Indo-1 and Fura-2, are excited by UV-light, which can result in high background fluorescence from the microplate or cause cellular photo-damage due to its higher energy. Ratiometric determinations also require rapid filter switching by the microplate reader, particularly when intracellular calcium levels are changing rapidly. Optimally both wavelengths are being measured simultaneously, but this is not always possible. Photo-bleaching of the compound itself may present a problem if only one filter set is used, but is corrected for if ratiometric determinations are being made. Additionally, ratiometric analysis can also correct for dye leakage. Direct measurement

dyes have been developed for excitation in the visible wavelength range resulting in less phototoxicity. In general, direct measurement indicators have a greater fluorescence response to calcium than the ratiometric dyes. This may allow for the use of lower intracellular concentrations of indicator dye reducing any cytotoxic effects of the dye (Haughland, 2002).

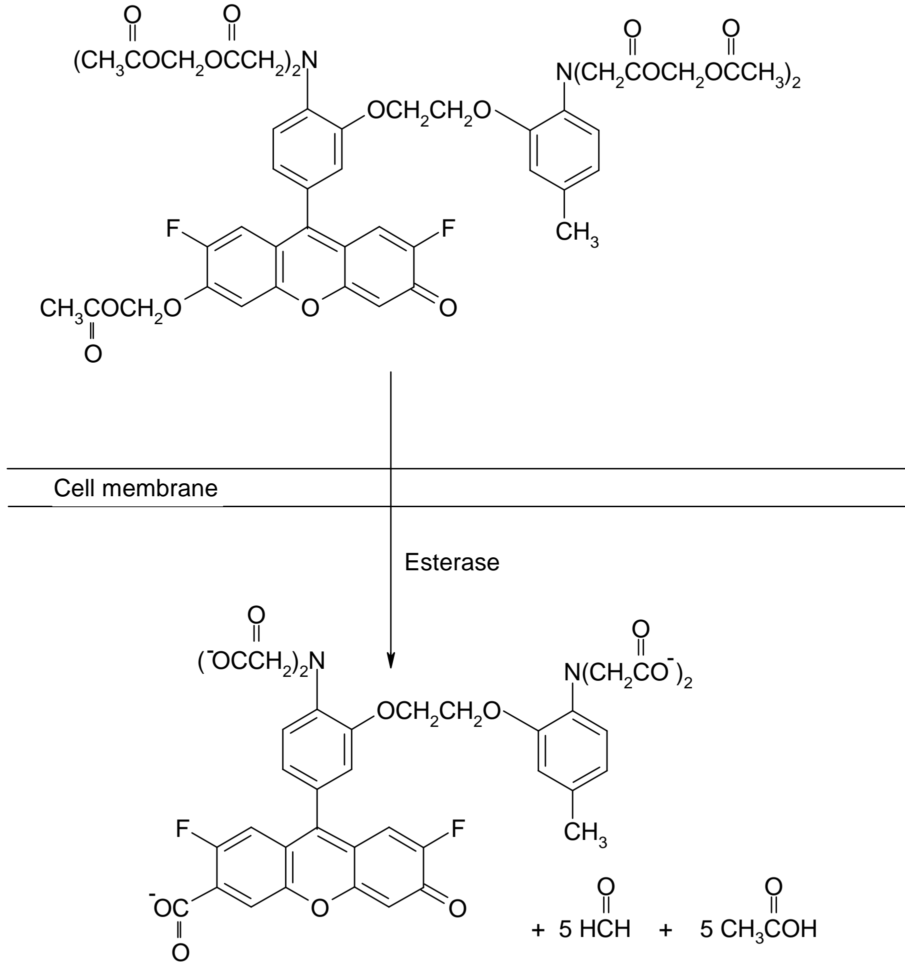


Figure 1.11 Schematic diagram of the processes involved in loading cells using membrane-permeant acetoxymethyl (AM) ester derivatives of fluorescent indicators, in this case Fluo-4.

Loading techniques can be divided into two basic groups: the treatment of individual cells with microinjection or bulk loading where large numbers of cells are treated

simultaneously. There are several different bulk loading procedures applicable to large populations; the most commonly used is acetoxymethyl (AM) ester loading. This non-invasive technique uses compounds where the carboxylate groups of the indicator dyes have been derivatized as acetoxymethyl esters resulting in a nonpolar compound that is permeable to cell membranes. Once inside the cell, these compounds are hydrolyzed by intracellular esterases (Figure 1.11). Once acted upon by esterases, the resultant activated indicator is now a polar molecule that is no longer capable of freely diffusing through the cell membrane, essentially trapping the compound inside the cell. AM Ester cell loading, while the easiest and most straightforward method to bulk load cells, is not without problems. It is assumed that the calcium indicator dye distributes evenly throughout the cell cytosol and is equally responsive to intracellular calcium concentrations. Unfortunately, AM esters and their hydrolysis products can accumulate in any membrane-enclosed organelle. In some instances, these molecules, particularly the polyanionic-hydrolyzed moieties, can be partitioned by active transport systems. Cells with low levels of intracellular esterase activity may not completely activate the indicator dye. Leakage of the active indicator dye from loaded cells can also occur. It must be noted that complete hydrolysis results in the formation of cytotoxic compound by-products, such as a formaldehyde and acetic acid (Hibbs, 2004).

CHAPTER II

MATERIALS AND METHODS

2.1 Culture and Strains

Penicillium chrysogenum P2 strain (ATCC 48271) was used for the study of penicillin G production. *P. chrysogenum* P2 is an improved strain which produces higher concentration of penicillin G than the wild type strain. This strain was obtained from American Type Culture Collection (ATCC), Rockville, Maryland USA.

2.2 Chemicals and Reagents

All chemicals used in this work were obtained from VWR LTD, UK unless stated otherwise. High Pressure Liquid Chromatography (HPLC) assays were performed using HPLC grade solvents and water, while other (e.g. thin layer chromatography) quantitative and qualitative assays were carried out using analytical grade reagents. General-purpose reagents were used for media preparation.

For molecular biology studies, RNeasy Plant Mini Kit, for total RNA isolation, RNase-Free DNase enzyme for removal of contaminating DNA and QIAquick Gel Extraction Kit was obtained from Qiagen, UK. Improm-II^{TM} Reverse Transcription System and PCR master mix were obtained from Promega, UK; the primers were purchased from Invitrogen, UK. The Riboladder Long (RNA Step ladder) and Hyperladder V (DNA step ladder) were purchased from Bioline, UK. SYBR Green Jumpstart Taq ready mix for Quantitative Real time PCR was obtained from Sigma.

For Calcium flux studies, Fluo–4, AM; Celltrace calcein redorange, AM; Calcium ionophore A23187 (calcimycin) were obtained from Fisher Scientific, UK. DMSO was obtained from Sigma.

2.3 Preparation of oligosaccharides

2.3.1 Preparation of Alginate oligosaccharides by partial acid hydrolysis

Materials

Sodium alginate
Hydrochloric acid
Sodium hydroxide
Sodium chloride

Method

Two oligosaccharide fractions were prepared from sodium alginate by partial acid hydrolysis according to the method described by Asilonu and co-workers (2000). Ten grams of sodium alginate was gradually dissolved in 500 mL of distilled water by heating and stirring. On complete dissolution, 500 mL of warm 0.6 M hydrochloric acid was gradually added to the dissolved sodium alginate to make a final solution of 0.3 M HCl. This was to ensure homogeneous depolymerisation. The solution was then refluxed at 100°C for 6 h to undergo hydrolysis. After refluxing, the solution was rapidly cooled to room temperature to stop the hydrolysis, and centrifuged at 3000 rpm (30 *g*) for 30 minutes. The supernatant was discarded and the pellet was collected, rinsed with, and re-dissolved in approximately 300 mL of distilled water. 0.3 M sodium hydroxide solution was added until all solid particles in the solution were brought to complete dissolution. The volume of the neutralised, yellow solution was measured and sodium chloride was added to make a final concentration of 0.5% (w/v). An equal volume of absolute ethanol (99%) was added to the solution and allowed to stand overnight. The precipitate and the supernatant were separated by centrifugation [3000 rpm (30 *g*) for 30 minutes] to ethanol and solid fractions.

The ethanol fraction was discarded. The precipitate left after removal of the ethanol fraction was rinsed and re-dissolved in approximately 200 mL of distilled water and the

pH was adjusted to 2.85 using 0.3 M HCl. It was then centrifuged into two separate fractions: the insoluble (solid) and soluble (liquid) fractions. The soluble fraction was concentrated by vacuum evaporation before it was frozen and freeze-dried to a powder designated as oligomannuronate (OM). The insoluble fraction was also freeze-dried to a powder designated as oligoguluronate (OG). The hydrolysis products were analysed by thin-layer chromatography (TLC, section 2.3.4).

2.3.2 Preparation of mannan oligosaccharides by enzymatic hydrolysis of locust bean gum

Materials

Locust Bean Gum (Sigma)
Gammanase® (Novozyme Ltd., Denmark)

Method

Mannan oligosaccharides were prepared from Locust Bean Gum (LBG) by enzymatic hydrolysis with Gammanase enzyme mixture. 2% (w/v) LBG in distilled water was hydrolysed using 0.2% (v/v) of Gammanase enzyme at 80°C. The reaction was carried out for 5 minutes and quenched by heating to 100°C. The mixture was cooled to room temperature. The cold water soluble fraction in the supernatant was collected and frozen at –80°C. The frozen sample was then freeze dried. The hydrolysis products were analysed by TLC and designated the abbreviation MO (mannan oligosaccharides, section 2.5).

2.3.3 Preparation of reducing–end derivatives of mannan oligosaccharides (MO-R) by reduction

The reducing-end derivatives of mannan oligosaccharides were kindly prepared and provided by Dr. Gopalkrishna Lakkireddy for the study of the biological activity of the reducing-end derivatized mannan oligosaccharides in *P. chrysogenum.*

Materials

Mannan oligosaccharides
Sodium hydroxide
Sodium borohydride
Methanol

Method

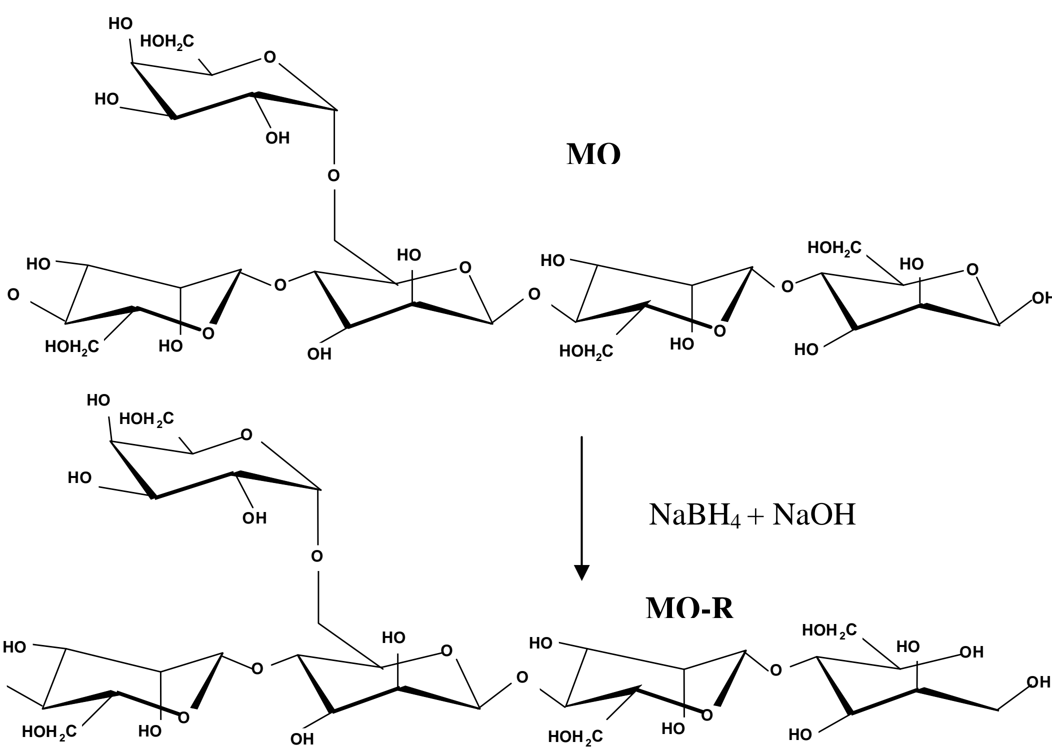


Figure 2.1 Reduction of mannan oligosaccharides (MO) to its reducing–end derivatives (MO-R)

Reducing end derivatives of mannan oligosaccharides (MO-R) were generated by chemical reduction of MO. 75 mg of MO was dissolved in 7.5 ml of double distilled water and the pH was adjusted to 10 using 0.1 M NaOH. Equal volume of freshly prepared 2M $NaBH_4$ in 0.1 M NaOH was added and the mixture was incubated under reflux at 45°C for 16 h. The cooled solution was neutralized using 50% acetic acid and remaining borate ions were removed by repeated wash with 9:1 methanol: 50% acetic acid mixture (Figure 2.1). Final wash was done with methanol. The final pellet was dried under a stream of air at 25°C. Presence of carbohydrate in the solution was confirmed by phenol-sulphuric acid assay and complete reduction of the reducing end was confirmed by testing with DNSA (dinitro-salicylic acid).

2.3.4 Detection of oligosaccharides by thin layer chromatography (TLC)

Materials

Silica gel chromatography plates (Merck, Germany)
Ethanol
Butan-1-ol
Naphthoresorcinol (Sigma)
Diphenylamine (Sigma)
Sulphuric acid
Maltotriose (Sigma)
Maltopentose (Sigma)
Maltohexaose (Sigma)
Maltohepatose (Sigma)

Methods

The DP and the purity of the oligosaccharides derived from sodium alginate and LBG were determined by TLC. The DP was determined for partial acid hydrolysed samples of

sodium alginate oligosaccharides (OM and OG) and enzyme hydrolysed LBG derived oligosaccharides (MO).

TLC of all purified oligosaccharides was carried out on glass backed silica gel 60 plates. The adsorbent thickness of the plates was one millimetre. The chromatography solvent contained: water, 20%; ethanol, 30% and butan-1-ol 50%. The solvent (150 mL) was prepared in a five-litre (25 x 8 x 25 cm) TLC tank and left closed for 1 hour to saturate the tank before use. Equal volumes (l5 μL) of oligosaccharides and known standards (1 g L^{-1}) were applied on the TLC plates at horizontal l5 mm intervals and air-dried. After drying, the plates were placed in the solvent and allowed to run for 7 h. Plates were then removed from the solvent, air dried and sprayed with identifying reagent. The identifying reagent contained a mixture of 0.2% naphthoresorcinol (w/v) and 0.4% diphenylamine (w/v) in ethanol (96 mL) and four millilitre of concentrated sulphuric acid (added before use). Sprayed plates were allowed to dry for 5 minutes and then were placed in a 100°C oven for 10 to 15 minutes to develop the characteristic TLC spots used for the identification of the oligosaccharides.

2.4 MEDIA

2.4.1 Agar Medium for Strain Maintenance

Glycerol-molasses agar medium (GMA) was used for sporulation and propagation of *P. chrysogenum* strains. The pH of GMA was adjusted to 6.5 with 3M KOH before sterilizing at 121°C for 15 minutes. GMA contained the following concentrations of ingredients in grams per litre (g L^{-1}) of distilled water:

Table 2.1 Composition of glycerol-molasses agar medium

Ingredients	Concentration (g L^{-1})
Agar No. 3 (Oxoid)	15.000
Copper II sulphate pentahydrate ($CuSO_4.5H_2O$)	0.010
Glycerol	7.500
Potassium dihydrogen phosphate (KH_2PO_4)	0.060
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.050
Sodium chloride (NaCl)	10.000
Iron II sulphate heptahydrate ($FeSO_4.7H_2O$) (Sigma)	0.003
Bacteriological peptone (Oxoid)	5.000
Molasses (Applefords Foods,UK)	2.500
Yeast extract (Oxoid)	1.000

The agar medium was inoculated with the spore suspension from the working glycerol stock stored at -20°C and then incubated in a 26°C incubator for a week. The fully sporulated fungal culture was kept at 4°C in airtight containers. For inoculation, the spores were recovered by shaking with 5 mL of sterile 0.01% (w/v) Tween 80 in water with glassbeads. The concentration of spores in the suspension was calculated by counting with a haemocytometer.

2.4.2 Inoculum Growth Medium

Penicillium Growth Medium (PGM) was based on a modified medium developed by Jarvis and Johnson (1947), and used for the growth of *P. chrysogenum* P2. Salts, carbon and nitrogen sources were sterilised separately. Salts and nitrogen were sterilised at 121°C for 15 minutes. Carbon sources were sterilised at 115°C for 15 minutes. The iron source was filter-sterilised to minimise oxidation. The medium was adjusted to pH 6.5 with 3 M KOH before autoclaving. PGM contained the following ingredients in g L^{-1} of distilled water:

Table 2.2 Composition of *Penicillium* growth medium

Ingredients	Concentration (g L^{-1})
Ammonium sulphate ($(NH_4)_2SO_4$)	13.00
Calcium chloride ($CaCl_2.2H_2O$)	0.05
Copper II sulphate pentahydrate ($CuSO_4.5H_2O$)	0.01
Ethylene diaminetetraacetic acid (EDTA)(Sigma)	0.55
Iron II sulphate heptahydrate ($FeSO_4.7H_2O$)	0.25
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.25
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.02
Potassium dihydrogen phosphate (KH_2PO_4)	3.00
Sodium sulphate (Na_2SO_4) anhydrous	0.50
Zinc sulphate ($ZnSO_4.7H_2O$)	0.02
Lactose (Sigma)	10.00
Mycological peptone (Oxoid)	5.00
Sucrose	20.00

2.4.3 Semi-defined Penicillin Production Medium

The semi-defined penicillin production medium (PPM), used for the production of penicillin in shaken flask and bioreactor studies had the same composition as PGM except for lactose, mycological peptone, and sucrose. PPM was prepared following the same method as for preparation of PGM. PPM contained the following ingredients in g L^{-1} of distilled water.

Table 2.3 Composition of semi-defined penicillin production medium

Ingredients	Concentration (g L^{-1})
Ammonium sulphate ($(NH_4)_2SO_4$)	13.00
Calcium chloride ($CaCl_2.2H_2O$)	0.05
Copper II sulphate pentahydrate ($CuSO_4.5H_2O$)	0.01
Ethylene diaminetetraacetic acid (EDTA)	0.55
Iron II sulphate heptahydrate ($FeSO_4.7H_2O$) (Sigma)	0.25
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.25
Manganese sulphate monohydrate ($MnSO_4.H_2O$)	0.02
Potassium dihydrogen phosphate (KH_2PO_4)	3.00
Sodium sulphate (Na_2SO_4) anhydrous	0.50
Zinc sulphate ($ZnSO_4.7H_2O$)	0.02
Lactose (Sigma)	50.00
Mycological peptone (Oxoid)	1.00
Sucrose	10.00

2.5 CULTURE CONDITIONS

P. chrysogenum inocula for shaken flask and bioreactor studies were initially grown in shaken flasks containing sterile PGM. Inoculum medium was inoculated with a spore suspension to give a final concentration of 10^6 spores mL^{-1}. For each set of fermentation, the same spore inoculum stock was used. The shaken flask cultures of *P. chrysogenum* inocula were incubated in an orbital shaker at 26°C at a speed of 200 rpm for 48 h. Absence of contamination and morphology of the culture were assessed under the microscope before inoculation in the production medium.

2.5.1 Penicillin G production in shaken flask cultures

After 48 h of growth in PGM, 10% of inocula were transferred into sterile PPM in 500 mL shaken flasks and then incubated as above. To maintain the pH, 10 g L^{-1} of $CaCO_3$ was added to the PPM media as a buffering agent. The culture broth was assayed for penicillin G and carbohydrate utilisation over 120 h unless indicated otherwise. Biomass determination was carried out at the end of the fermentation.

2.5.2 Penicillin G production in bioreactor cultures

2 L and 5 L STR (Stirred Tank reactors) were used for bioreactor studies. The 2L fermenter vessels (2 L STR, FerMac360, Electrolab Ltd., UK) had an internal diameter of 12 cm and shaft length of 24 cm. Two impellers were used for agitation with 6 blades (diameter 5.5 cm and height 1.6 cm). The 5L fermenter vessels (5 L STR, FerMac, Electrolab Ltd., UK) had an internal diameter of 16 cm and shaft length of 31.5 cm. Two impellers were also used for agitation with 6 blades (diameter 8.4 cm and height 1.6 cm). No baffles were used. The bioreactors were sterilised at 121°C for 45 minutes while containing only the salts from the production medium. Carbon and nitrogen sources sterilised separately and filter sterilised iron source were added to the bioreactors aseptically to make a total medium volume of 1.35 L for 2 L bioreactor and 3.6 L for 5 L bioreactor.

For each set of experiments, three bioreactors were run in parallel with equal working volumes for the control and elicited fermentations. The pH was automatically maintained between 6.45 and 6.55 using sterile 2 M ammonium hydroxide and 2 M sulphuric acid. The airflow rate and temperature were kept at 1.0 vvm and 26°C respectively. The dissolved oxygen tension (DOT) was controlled and maintained above 30% air saturation with the stirrer speed ranging from 250 to 600 rpm. There were no significant differences in the agitation rates between the control and elicited fermenter.

2.5.3 Preparation of the stock solution of phenylacetic acid

A stock solution of phenylacetic acid (PAA), the precursor for penicillin G, was prepared by dissolving l00 g of PAA in a litre of warm distilled water in a fume cupboard. Drop-wise addition of 10 M sodium hydroxide helped the dissolution of PAA in water. The pH of the stock solution was adjusted to neutral with KOH/H_2SO_4. The stock solution was sterilised at 115°C for 15 minutes.

2.5.4 Addition of phenylacetic acid (PAA)

PAA was added for penicillin G production to both control and elicited cultures after 24 h of growth in PPM. In both shaken flask and bioreactor cultures, 1.0 g L^{-1} of PAA was initially added after 24 h and was maintained between 0.5 - 1.5 g L^{-1} by intermittent additions where necessary. PAA was monitored using the HPLC method. The details are as mentioned in 2.6.3.

2.5.5 Addition of oligosaccharide elicitors

Oligosaccharide elicitors were dissolved in distilled water at the required concentrations and sterilised at 115°C for 15 min. No elicitor was added to the control culture. Single elicitor additions of mannan oligosaccharide at 150 mg L^{-1} were added 48 h after inoculation to the production media. For the multiple elicitor addition studies, an experimental setup was designed (Table 2.4) to optimise the type, concentration and

time of addition of the second elicitor, with MO at a concentration of 150 mg L^{-1} at 48 h as the first elicitor.

Table 2.4. Experimental setup designed to optimize the type, concentration and time of addition of the second elicitor.

Flask No	Elicitor I	Concentration (mg L^{-1})	Addition time (h)	Elicitor II	Concentration (mg L^{-1})	Addition time (h)
1 control	-	-	-	-	-	-
2	MO	150	48	-	-	-
3	MO	150	48	MO	75	72
4	MO	150	48	MO	75	96
5	MO	150	48	MO	150	72
6	MO	150	48	MO	150	96
7	MO	150	48	OM	75	72
8	MO	150	48	OM	75	96
9	MO	150	48	OM	150	72
10	MO	150	48	OM	150	96

2.6 ASSAYS

2.6.1 Biomass Assay

Materials

Whatman filter paper No.1
Filtration Unit
Distilled water

Method

Biomass production was measured as cell dry weight (CDW) per litre of culture broth. CDW was determined by filtration of 10 mL culture sample on pre-weighed filter paper (Whatman No. 1) and washing thoroughly with 20 mL of distilled water. The mycelia on the filter papers were weighed after drying to constant weight and the CDW determined by difference between the total weight and that of the filter paper alone. Biomass assays were carried out in triplicate.

2.6.2 Total carbohydrate assay

Materials

Phenol crystals (Sigma)
Sulphuric acid
HPLC grade water

Method

Total carbohydrate content of fermentation broth was determined by phenol sulphuric acid assay as described by Chaplin and Kennedy (1994). Phenol sulphuric acid assay was carried out in glass test tubes by adding 200 µL of 5% phenol solution to 200 µL of standard, samples or blank (water) and mixing by gentle shaking in a fume cupboard. Phenol solution was made in HPLC grade water. Concentrated sulphuric acid (1.0 mL)

was then rapidly added to the surface of the solutions without touching the sides of the tubes and allowed to stand undisturbed for 10 minutes. After 10 minutes, the tubes were mixed thoroughly and allowed to stand for a further 30 minutes. The absorbance of the reaction mixtures was determined at 490 nm. Carbohydrate concentration of the samples was determined by using glucose standard curve in the range of 10 – 100 μg mL^{-1}.

2.6.3 HPLC assay for detection of penicillin G and phenylacetic acid

Materials

Penicillin G standard (Sigma)
Phenylacetic acid
Potassium dihydrogen orthophosphate
Dipotassium hydrogen orthophosphate
HPLC grade water
Acetonitrile

Method

The concentrations of penicillin G and phenylacetic acid in culture broth samples were determined by HPLC (Dionex HPLC system (Chromelon)). Broth samples were centrifuged at 10,000 rpm (75 *g*) for five minutes. The supernatants were collected using a 1 mL sterile syringe and filtered once through 0.2 μm Dynaguard syringe filters. Samples were then analysed by a gradient HPLC method (Adlard *et al*., 1991) (Table 2.5) using a 5 μm C8 column and guard (Phenomenex, UK). Guard columns were used to ensure quality of results is maintained through protection of the column.

The mobile phases used were HPLC grade acetonitrile and 0.01 M potassium dihydrogen orthophosphate buffer made using HPLC grade water. The buffer pH was adjusted to 5.5 with 0.01 M di-Potassium hydrogen orthophosphate buffer. Each broth sample was analysed in triplicate. The column was washed thoroughly with the buffer and HPLC grade water for 30 minutes prior to and after sample analysis. The unknown

concentration of penicillin G in culture broth sample was determined by using penicillin standard curve in the range of 0.125 – 1 g L^{-1} (Appendix. 4.)

Table 2.5 HPLC gradient profile for analysis of penicillin G and PAA

Sector	Time (min)	Acetonitrile (%)	KH_2P0_4/K_2HP0_4 buffer (%)
Equilibrium	5	0	100
1	10	0	100
2	15	20	80
3	5	0	100

2.7 MOLECULAR BIOLOGY STUDIES

2.7.1 Total RNA isolation

Materials

RNeasy Plant Mini Kit (Qiagen, UK Ltd)

Method

Total RNA was isolated from the fungal cultures using the RNeasy Plant Mini Kit from Qiagen UK. The RNeasy procedure represents a novel technology for RNA isolation. It combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica gel membrane. Biological samples are first lysed and homogenised in the presence of a highly denaturing guanidine isothiocyanate (GITC)- containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. A short (2 min) incubation at 56°C is imperative in order to ensure complete lysis of the cells. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy mini column where the total RNA binds to the membrane and contaminants are efficiently washed away. High quality RNA is then eluted in 30 µl, or more of nuclease free water. The manufacturer's protocol for total RNA isolation from fungal cultures was followed. A further on-column DNase digestion with RNase-Free DNase set was used to remove any residual trace of DNA which would be detrimental for further studies including Real Time- PCR. The detailed protocol is as mentioned in the Appendix.1.

The total RNA isolated from samples was stored at -80°C. Under these conditions, no degradation of RNA was detected after 1 year. All samples demonstrated high purity and concentrations as measured by the A_{260}/A_{280} ratio, generally yielding ratios around 1.8 - 2.0. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per mL. The RNA integrity of the samples was also determined by agarose gel electrophoresis run along

with the standard molecular weight markers. The ribosomal bands, 28S and 18S should appear as sharp bands on the ethidium bromide stained gel.

2.7.2 Complementary DNA (cDNA) synthesis

Materials

ImProm-II^{TM} Reverse Transcription System (Promega, UK Ltd

Method

ImProm-II^{TM} Reverse Transcription System from Promega, UK Ltd was used for cDNA synthesis. The ImProm-II^{TM} Reverse Transcription System is a convenient kit that includes a reverse transcriptase and an optimized set of reagents designed for efficient synthesis of first-strand cDNA in preparation for PCR amplification. The optimized reaction buffer and powerful reverse transcriptase provided in the ImProm-II Reverse Transcription System enable robust, full-length cDNA synthesis.

Reverse transcription reactions of up to 1µg of total RNA were performed in 20µl reactions comprised of components of the ImProm-II^{TM} Reverse Transcription System. The experimental total RNA was quantified spectrophotometrically at A_{260} using Eppendorf BioPhotometer and later combined with random primers. The primer/template mix is then thermally denatured at 70°C for 5 minutes and chilled on ice. A reverse transcription reaction mix is assembled on ice to include nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor. As a final step, the template-primer combination is added to the reaction mix on ice. Following an initial annealing at 25°C for 5 minutes, the reaction is incubated at 42°C for up to one hour. Then the reaction is incubated at 70°C for 15 minutes to thermally inactivate the enzyme. Because no cleanup or dilution is necessary following the cDNA synthesis, the product may be directly added to amplification reactions. The detailed method is as mentioned in the Appendix.2.

2.7.3 Primer designing

The primers for the gene of interests were designed using the Primer3 software (Rozen and Skaletsky, 2000). The genes of interest were primarily the three major penicillin biosynthetic genes namely, *pcb*AB, *pcb*C and *pen*DE. 18S rRNA was used as an internal control for normalisation in QPCR conditions as it is less prone to biological variation and should be constitutively expressed under all conditions (Suzuki *et al*., 2000).

Table 2.6 Primer design for the penicillin G biosynthetic genes

Primer name	Sequence	GC%	Tm °C	Product size (bp)
***pcb*AB Forward**	5'atgtgcaggccaaggtaaac3'	50.0	60.0	162
***pcb*AB Reverse**	5'ccagttcagtctggtgctca3'	55.0	60.0	
***pcb*C Forward**	5'acggcaccaaattgagtttc3'	45.0	60.0	170
***pcb*C Reverse**	5'ggtgatgtgtgccatgtagc3'	55.0	60.0	
***pen*DE Forward**	5'acgaagaagacggacgaaga3'	50.0	60.0	151
***pen*DE Reverse**	5'tgcgggtattaagcatgaca3'	60.1	60.1	
18S rRNA Forward	5'cgacttcaggaaggggtgta3'	55.0	60.1	234
18S rRNA Reverse	5'cttggatgtggtagccgtt3'	50.0	59.99	

The primers for the above genes were designed such that the T_m (melting temperature) of the primers was 60°C and the final product size would be between 150-250 bp (Table 2.6). The final concentration of the working stock of each primer sequence was kept at 10 µM. The working stock was used for further reactions and the Master stock stored at -20°C.

2.7.4 Polymerase Chain Reaction (PCR)

2.7.4.1 Conventional PCR

Materials

PCR master mix (Promega, UK Ltd)

Method

In order to confirm if the primers designed gave individual products of desired molecular weights, the cDNA obtained from Reverse transcription reaction was used directly as a template and amplified using PCR (Perkin Elmer Thermal Cycler), employing PCR master mix from Promega. The components for a PCR reaction were mixed as follows for a 25 µl reaction volume:

Table 2.7 Composition for the conventional PCR

Component	Volume (µl)
2X PCR master mix	12.5
Upstream primer	2.5
Downstream primer	2.5
cDNA template	2.5
Nuclease free water	5.0
Total volume	**25.0**

The following profile (Table 2.7) was used for the amplification of the template DNA. The product sizes of the four genes of interest are shown in Table 2.5. The PCR products were visualized on a 3.5% agarose gel prepared in TBE buffer. Hyper ladder V from Bioline was used as the DNA marker.

Table 2.8 Conventional PCR profile

Temperature	Time	Condition	Cycles
94˚C	2 min	Denaturation	1
94˚C	1 min	Denaturation	
60˚C	1 min	Annealing	30
72˚C	2 min	Extension	
72˚C	5 min	Final extension	1
4˚C	No limit	Soaking	

2.7.4.2 Real Time PCR

Material

SYBR Green Jumpstart Taq ReadyMix (Sigma, UK Ltd).

Method

Quantitative PCR conditions

PCR experiments were conducted in the ABI Prism 7000 Sequence Detection system (Applied Biosytems) using SYBR Green Jumpstart Taq ReadyMix (2X) contains: 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 7mM $MgCl_2$, 0.4 mM each dNTP (dATP, dCTP, dGTP, dTTP), stabilizers, 0.05 unit/µL Taq DNA Polymerase, Jumpstart Taq antibody, and SYBR Green I). All reactions were performed in 25 µl reaction volumes, that contained 1X SYBR Green Jumpstart Taq ReadyMix, 1X internal reference dye, specific primers, template cDNA and made up to final reaction volume using nuclease free water. ROX was used as a convenient passive reference dye for normalizing signals due to non-PCR related fluorescence fluctuations that occur either between wells or over time.

Table 2.9 Composition for Real time PCR

Component	Volume (µl)
SYBR Green Jumpstart Taq ready mix	12.5
Forward primer	2.0
Reverse primer	2.0
Template	2.0
ROX	0.25
dH_2O	6.25
Total	**25.0 µl**

PCR profile:

The following profile was used for the amplification of the template DNA.

Table 2.10 Real time PCR profile

Component	Temperature (°C)	Time
Initial Step	50	2 min
Activation of Taq polymerase	95	10 min
40 cycles	95	15 s
	60	1 min

2.7.5 Gel extraction

Material

QIAquick Gel Extraction Kit, Qiagen UK Ltd

Method

The QIAquick Gel Extraction Kit was used to extract and purify DNA from agarose gels for the absolute quantification of the gene of

interest. The protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer.

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely-designed silica-gel membrane. Special buffers provided with each kit are optimized for efficient recovery of DNA. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water. The manufacturer's protocol for DNA purification and extraction from agarose gel was followed.

The DNA extracted for each gene was quantified spectrophotometrically at A_{260} using Eppendorf BioPhotometer. An absorbance of 1 unit at 260 nm corresponds to 50 µg of DNA per mL. A calibration curve (gene copy number versus C_t) was plotted using serially diluted concentrations of each gene of interest extracted. The standard curve produces a linear relationship between C_t and initial amounts of total RNA or cDNA, allowing the determination of the concentration of unknowns based on their C_t values. To enumerate gene copy numbers of unknown samples, one calibration curve was routinely run with each sample set and compared with previous curves to check for consistency between the runs.

2.8 STUDIES ON FLUXES OF IONS THROUGH THE CELL MEMBRANE OF *P. chrysogenum*

2.8.1 Detection of the change in concentration of cytosolic calcium

Materials

Fluo-4, AM, 1mM solution in DMSO (Fisher)
Celltrace calcein redorange AM (Fisher)
Calcium ionophore A23187 (calcimycin) (Fisher)
DMSO (Sigma)

Method

The change in the concentration of free cytosolic calcium in the fungal cells incubated in the presence or absence of oligosaccharides was measured and recorded using the FLUOstar Optima Plate Reader and the Confocal Scanning Laser Microscopy.

2.8.2 Detection of changes in calcium concentration using FLUOstar Optima plate reader

P. chrysogenum inocula for shaken flask were grown in 500 mL flasks containing 100 mL sterile PGM. The cultures were incubated in an orbital shaker at 26°C at a speed of 200 rpm with 2cm throw. After 48 h, 10 mL of inocula were transferred into 90 mL of sterile PPM in 500 mL flasks and then incubated as above. PAA was added after 24 h of growth in PPM.

After 48 h of incubation, 2.0 mL of the culture broth was centrifuged. The pellet was resuspended and incubated in 20 mL PPM to attain equilibration. Single use aliquots (5 mL) were incubated with 5 µL mL^{-1} of Fluo-4, AM (1mM) for 45 minutes in the dark at 37°C. To remove the excess AM ester the aliquots were washed three times with PPM and then plated in a 12 well micro plate. The fluorescence of the dyes was read at a specific excitation and emission wavelength using the FLUOstar programme.

The assay was done using the following parameters: Excitation filter was 485 nm, emission filter was 520 nm, injection speed was 200 µL sec^{-1}, dispense depth were in accordance with the volume in the well and gain is adjusted. Before elicitor injection 5 measurements (1 per 6.2 seconds) were done to establish the baseline (Kinetic window I). 100 µL of the elicitor (MO at 150 mg L^{-1}) was transferred by the auto injector to the wells. While the elicitor was injected no measurements were taken. After the elicitor was injected measurements were resumed simultaneously (1 per 6.2 seconds) for 30 intervals (Kinetic window II). Immediately after elicitor addition, mixing was performed by the system for 4 seconds (Figure 2.2). This procedure described above was then repeated for the rest of the wells.

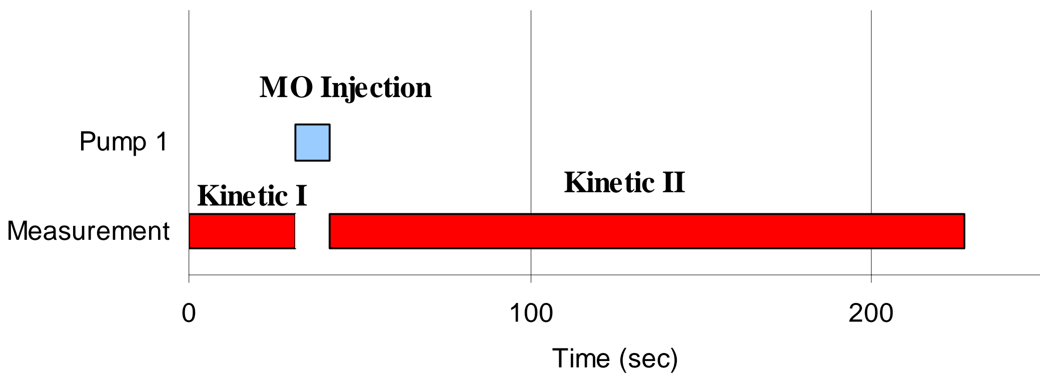


Figure 2.2 Schematic representation of the kinetic window for the microplate reader

2.8.3 Preparation of fungal cells for Confocal Laser Scanning Microscopy

Refer to section 2.8.2 for the culture growing conditions.

5 mL aliquots of culture broth each were incubated with 5 µl mL^{-1} of Fluo-4, AM (1mM) and Celltrace calcein redorange (1mM) for 45 minutes in the dark at 37°. Slides were prepared for each sample and mounted for observation under the CLSM. Images were taken by Leica SP2 TCS confocal microscopy and the Leica software version 2.61 was used for analysis. The following parameters were adopted for the capturing of the images (Kerrigan *et al*., 2006)

- 512 x 512 bit
- Grey resolution of 8 bit
- Pinhole: 1.00 airy unit
- Single line averaging
- Beam splitter 488/543/633
- Laser speed 400 Hz

The different dyes used in the experiment have different properties. These dyes are summarised in Table 2.11. Fluo-4 is a high affinity calcium indicator. Celltrace calcein red-orange is well-retained by live cells that possess intact plasma membranes, and consequently it is a useful cell tracer and indicator of cell viability. For the production of three-dimensional images and movies of cell, z-stack series of image slices were taken at 1 µm intervals in depth. These series were then reassembled using Imaris version 5.5 into three dimensional (3D) images. At the wavelengths stated in table, Fluo-4, AM emission is calcium sensitive and can therefore be used as a measure of cytsolic calcium dynamics.

Table 2.11 Dyes used in confocal microscopy for imaging and analysis of cytosolic calcium flux

Conditions	Fluo-4, AM	Celltrace Calcein red-orange
Maximum excitation (nm)	485 nm	520 nm
Maximum emission (nm)	570 nm	590 nm
Light source	Ar	He-Ne
Use	High affinity calcium indicator	Cell tracer and indicator of cell viability
Structure		

2.9 Statistical analysis

All statistical analysis was performed using Graph Pad InStat 3 software (Graph Pad Software, San Diego, California, USA). Pair-wise multiple comparisons between the conditions studied were made using one-way Analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparison test. *P* values < 0.05 were considered significant.

Chapter III

Results

3.1 PRODUCTION AND ENHANCEMENT STUDIES

The addition of oligosaccharide elicitors to *P. chrysogenum* cultures has proven in the past to increase the production of secondary metabolites as compared to control fermentations to which no elicitors were added (Ariyo *et al.,* 1998, Nair *et al.,* 2005).

The results presented in this section compare the production of biomass, penicillin G, and the carbohydrate consumption in shaken flasks and stirred tank reactors (STR). All fermentations were carried out in triplicate and the standard error has been applied to all graphs. Two oligosaccharides have been used in this study: MO and OM. They have shown to enhance penicillin G production. There was no significant difference in biomass production using the elicitors compared to the control cultures to which no elicitor was added.

Concentration of penicillin G has been shown to increase in the presence of single addition of MO when compared to control, but the concentration declines during the latter part of the fermentation. Therefore multiple addition of elicitor was applied in order to study the effect of elicitors to sustain the production level. It was observed that penicillin G level increased further in the presence of the multiple additions of the two different elicitors (MO and OM) when compared to the control (no elicitor added) and single addition (MO).

3.1.1 Single elicitor addition studies

3.1.1.1 Production of penicillin G

Figure 3.1 shows the production of the secondary metabolite penicillin G in *P. chrysogenum* P2. The addition of elicitors to *P. chrysogenum* cultures caused an increase in the concentration of penicillin over the control cultures to which no elicitor was added. The maximum penicillin G concentrations were 0.59 g L^{-1} and 0.97 g L^{-1} at 96 h ($p < 0.0011$) for control and single elicitor added culture respectively.

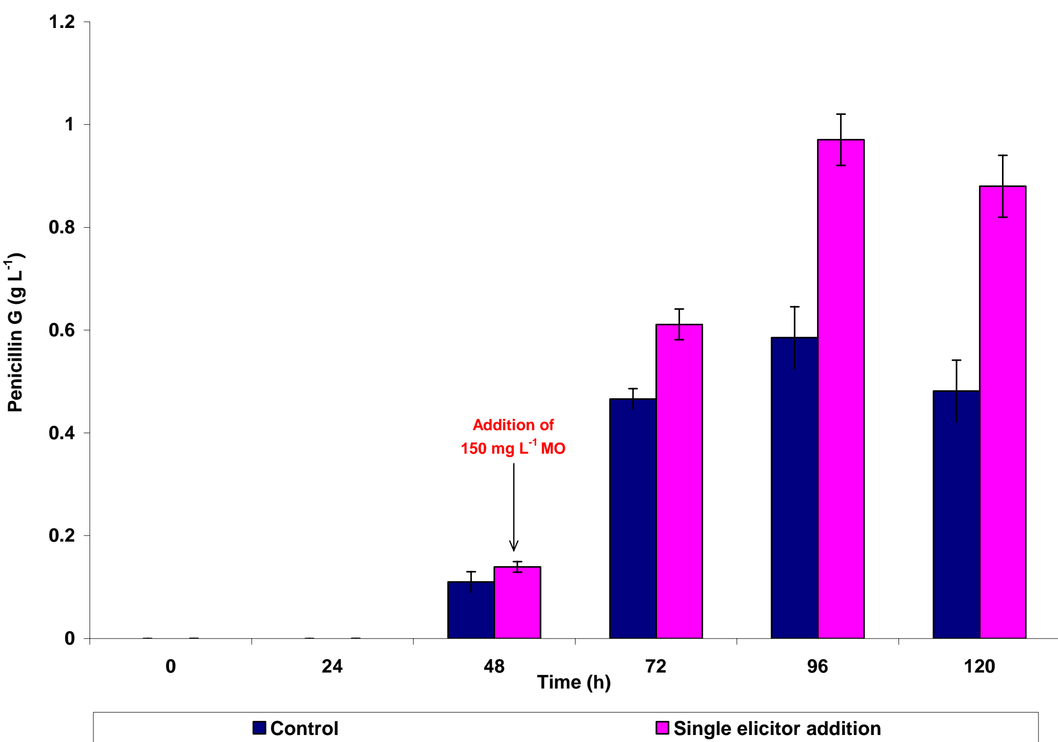


Figure 3.1 Penicillin G concentration in control (no elicitor added) and single elicitor (150 mg L^{-1} of MO at 48 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

The increase in penicillin G production in the single elicitor added cultures compared to control cultures was 66% at 96 h, subsequent to which there was a gradual decrease in the penicillin G concentration in both control and elicitor added culture.

The penicillin G production rate was calculated as the difference between initial and maximum concentration penicillin G produced (Table 3.1). The rate of penicillin G production was 100% higher in the single elicitor added culture compared to the control cultures. All analysis for these results was carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.05$).

Table 3.1 Production-rate for penicillin G production in *P. chrysogenum* P2 fermentations for the control and elicited cultures (48-96 h)

	Elicitor	Penicillin G production rate (g h^{-1})
Control	-	0.01
Elicited	MO	0.02

3.1.1.2. Biomass production

Figure 3.2 compares the biomass concentrations in cell dry weight (CDW) at 120 h between the control (no elicitor added) and single elicitor (150 mg L^{-1} of MO at 48 h) added cultures of *P. chrysogenum* P2. No notable difference was observed in biomass concentration between the control and single elicitor added cultures ($p > 0.05$)

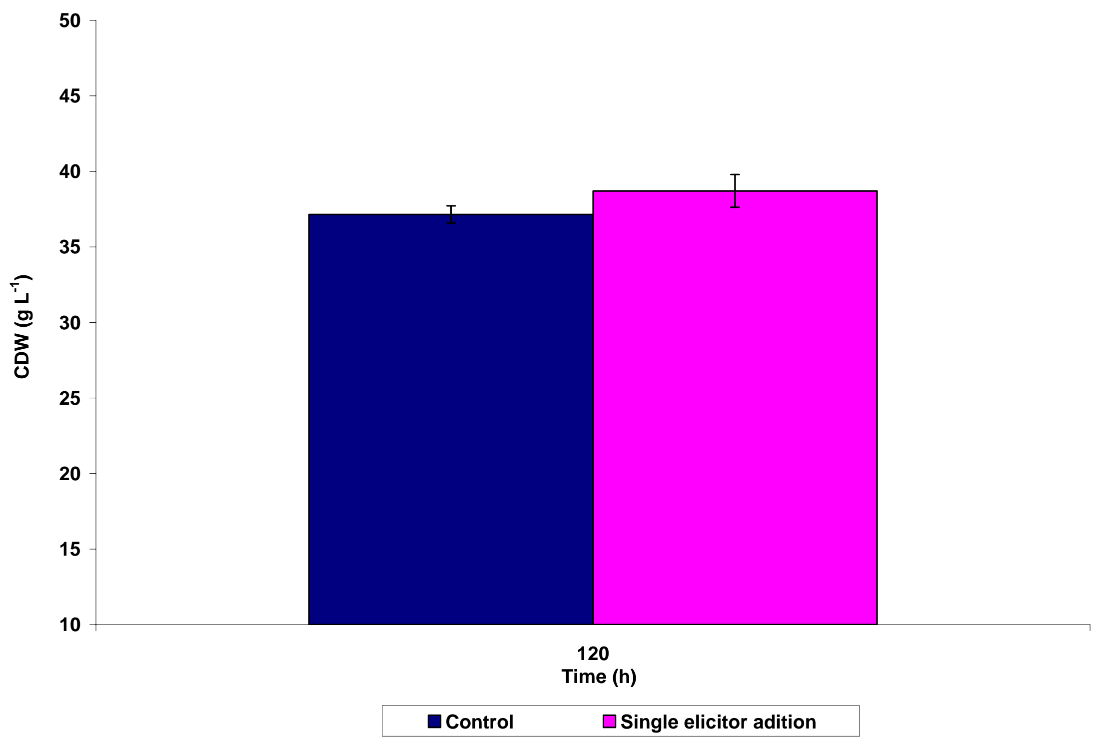


Figure 3.2 Final biomass production in control (no elicitor added) and single elicitor (150 mg L^{-1} of MO added at 48 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

3.1.1.3. Consumption of carbohydrates

Figure 3.3 shows the carbohydrate concentration at different stages of the fermentation between control (no elicitor added) and single elicitor (150 mg L^{-1} of MO at 48 h) added cultures of *P. chrysogenum* P2.

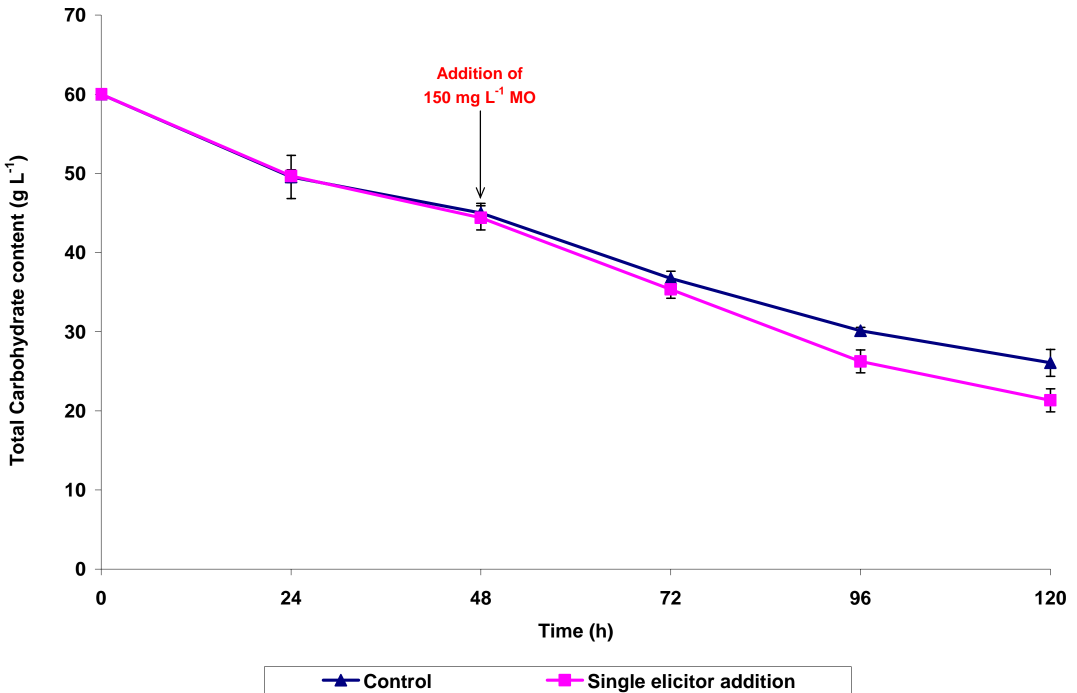


Figure 3.3 Total carbohydrate consumption in control (no elicitor added) and single elicitor (150 mg L^{-1} of MO added at 48 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

Consumption of carbohydrates was calculated as the difference between initial and residual concentration of carbohydrates (Table 3.2). There was a 27% increase, a significant difference ($p < 0.05$) in the rate of carbohydrate consumption in the elicited cultures compared to the control cultures. All analyses for these results were carried out in triplicate.

Table 3.2 Carbohydrate consumption rate in *P. chrysogenum* P2 fermentations for the control and elicited cultures (48-96 h)

	Elicitor	Carbohydrate consumption rate (g h^{-1})
Control	-	0.30
Elicited	MO	0.38

3.1.2 Addition of reduced mannan oligosaccharide (MO-R)

Oligosaccharides have many biological activities but little is known about the structure-function relationship that leads to their activity. In the following study the reducing end of the oligosaccharide elicitor (MO) was modified by sodium borohydride. This treatment reduces, but does not eliminate, the ability of the oligosaccharides to enhance the production of penicillin G when compared to the untreated oligosaccharides. For the sake of convenience the untreated oligosaccharide elicitor is denoted (MO) and the treated oligosaccharide is denoted (MO-R).

3.1.2.1 Production of penicillin G

Figure 3.4 shows the production of the secondary metabolite, penicillin G in *P. chrysogenum* P2 cultures. The addition of elicitors (both treated and untreated) to penicillin fermentations caused an increase in the concentration of penicillin over the control fermentation (no elicitor was added). The maximum penicillin G concentrations were 0.61 g L^{-1}, 1.10 g L^{-1} and 0.76 g L^{-1} at 120 h for the control, untreated and treated MO added culture respectively.

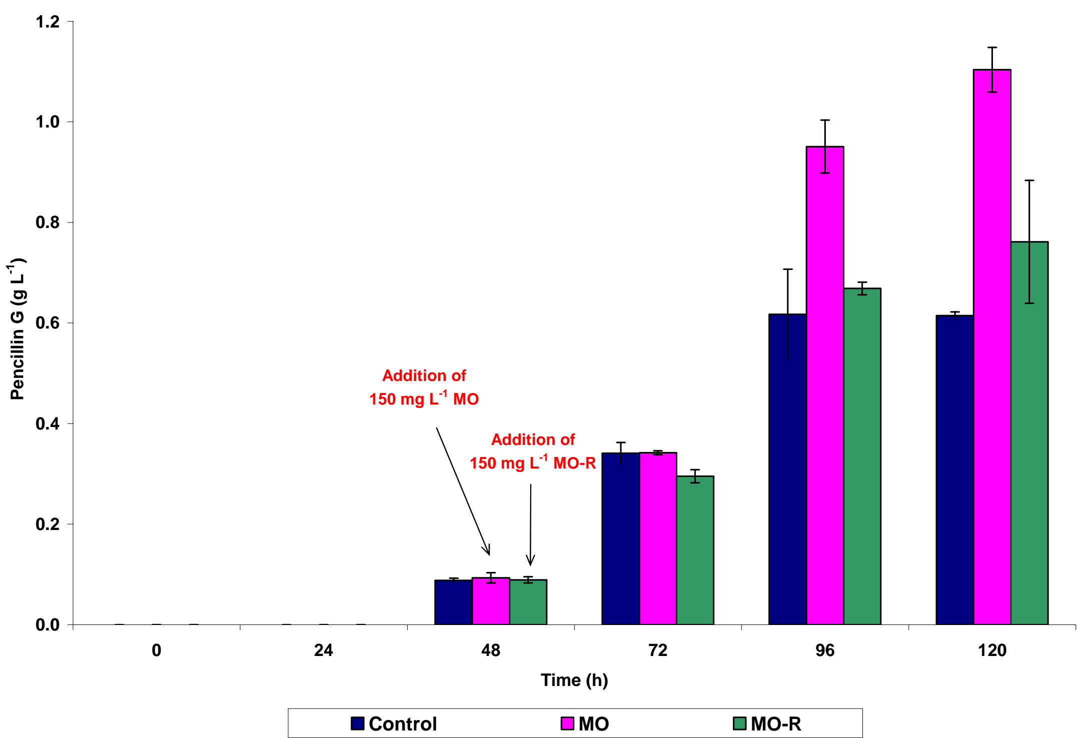


Figure 3.4 Penicillin G concentration in control (no elicitor added), MO (150 mg L^{-1} of MO at 48 h) and MO-R (150 mg L^{-1} of reduced MO at 48 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

The penicillin G production rate was calculated as the difference between initial and maximum concentration penicillin G produced. The rates of penicillin G production are 100% and 29% higher in the untreated and treated elicitor added cultures compared to the control culture (Table 3.3). There was a significant difference in the penicillin G levels between the MO added cultures compared to MO-R added and control cultures ($p < 0.01$).

Table 3.3 Production rate for penicillin G production in *P. chrysogenum* P2 fermentations for the control and elicited cultures (48-120 h)

	Elicitor	Penicillin G production rate (g h^{-1})
Control	-	0.007
Elicited	MO	0.014
Elicited	MO-R	0.009

3.1.2.2. Biomass production

Figure 3.5 compares the final biomass concentration in cell dry weight (CDW) between shaken flask fermentations of the control and elicitor added cultures. The elicitors used, untreated (MO) and treated (MO-R), did not elicit a significant increase in the biomass compared to the control ($p > 0.05$). The final biomass at the harvest period (120 h) was 38.2 g L^{-1}, 38.7 g L^{-1} and 38.2 g L^{-1} for the control, untreated and treated elicitor added cultures respectively.

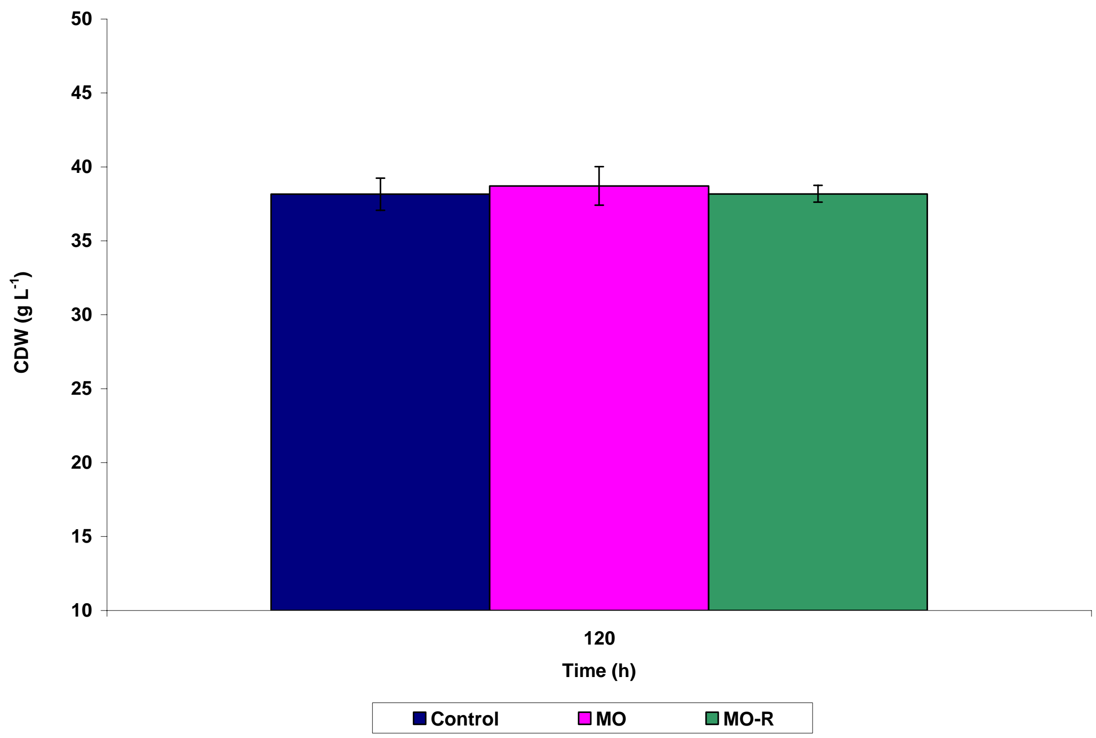


Figure 3.5 Biomass concentrations in control (no elicitor); MO (150 mg L^{-1} of MO at 48 h) and MO-R (150 mg L^{-1} of reduced MO at 48 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

3.1.2.3. Consumption of carbohydrates

Figure 3.6 shows the carbohydrate concentration at different stages of the fermentation between control (no elicitor added), untreated (MO) and treated (MO-R) elicitor added cultures of *P.chrysogenum*. The rate of carbohydrate consumption was measured as shown in Table 3.4.

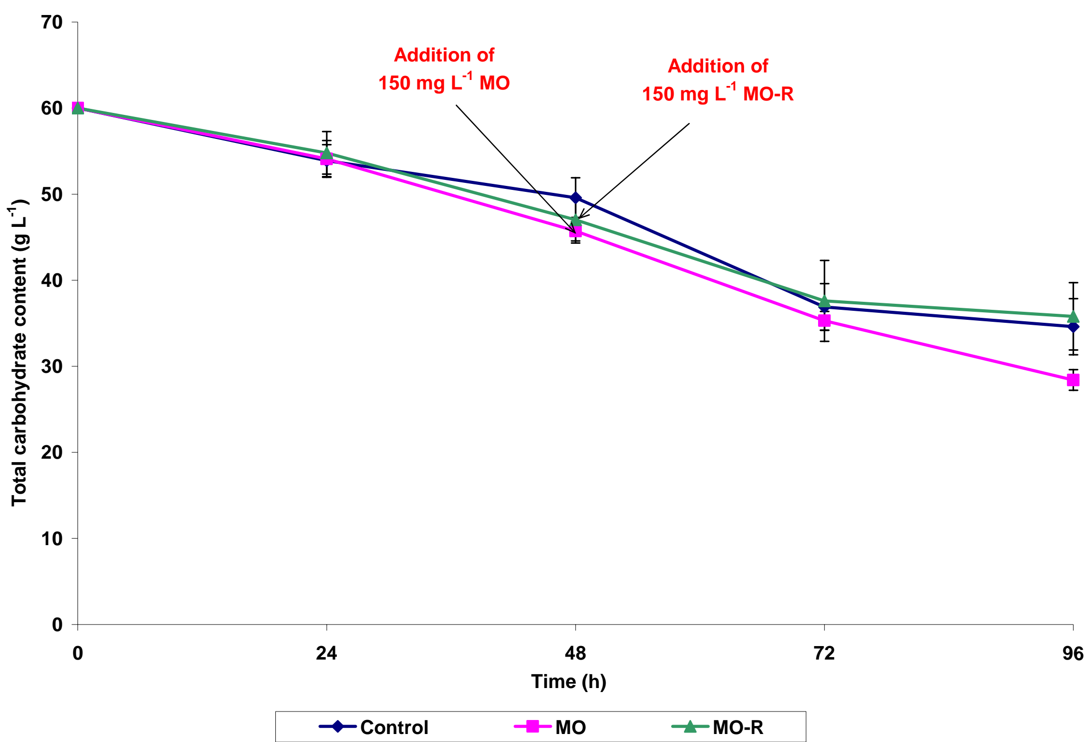


Figure 3.6 Carbohydrate consumption in control (no elicitor added), MO (150 mg L^{-1} of MO at 48 h) and MO-R (150 mg L^{-1} of reduced MO at 48 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

The rate of carbohydrate consumption was found to be 24% higher in MO added cultures compared to the control cultures ($p < 0.05$). There was no significant difference in the rate carbohydrate consumption found between the reduced-MO (MO-R) added and control cultures ($p > 0.05$).

Table 3.4 Carbohydrate consumption rate for penicillin G production in *P. chrysogenum* P2 fermentations for the control and elicited cultures (48-120 h)

	Elicitor	Carbohydrate consumption rate (g h^{-1})
Control	-	0.265
Elicited	MO	0.329
Elicited	MO-R	0.252

3.1.3 Multiple elicitor addition studies

The studies carried out in this section were aimed to investigate the effect of repeated addition of elicitors to the culture. An experimental set up as shown in Table 2.4 was designed to optimize the type, concentration and time of addition of the second elicitor while adding MO at 48 h with 150 mg L^{-1} as the first elicitor.

3.1.3.1 Optimisation studies: Shaken flask studies

Based on the experimental set up as shown in Table 2.4, a series of shaken flasks experiments were carried out and the results are as shown in Figure 3.7.

3.1.3.1.1 Production of penicillin G

The concentrations of penicillin G in fermentation samples from the control and elicited cultures are shown in Figure 3.7. At 48 h, before the first elicitor addition there were no significant differences in the penicillin G level in all the shaken flasks ($p > 0.05$).The addition of different concentration and types of elicitors to the shaken flask cultures at different addition time caused an increase in the concentration of penicillin over the control fermentation (no elicitor was added). The maximum penicillin G concentration obtained was 1.70 g L^{-1} (Flask No 8) compared to 1.27 g L^{-1} in single elicitor added cultures (Flask No 2) and 0.98 g L^{-1} for control cultures at 144 h ($p < 0.001$). No

significant differences in the final biomass CDW were observed at the harvest of the shaken flask cultures ($p > 0.05$).

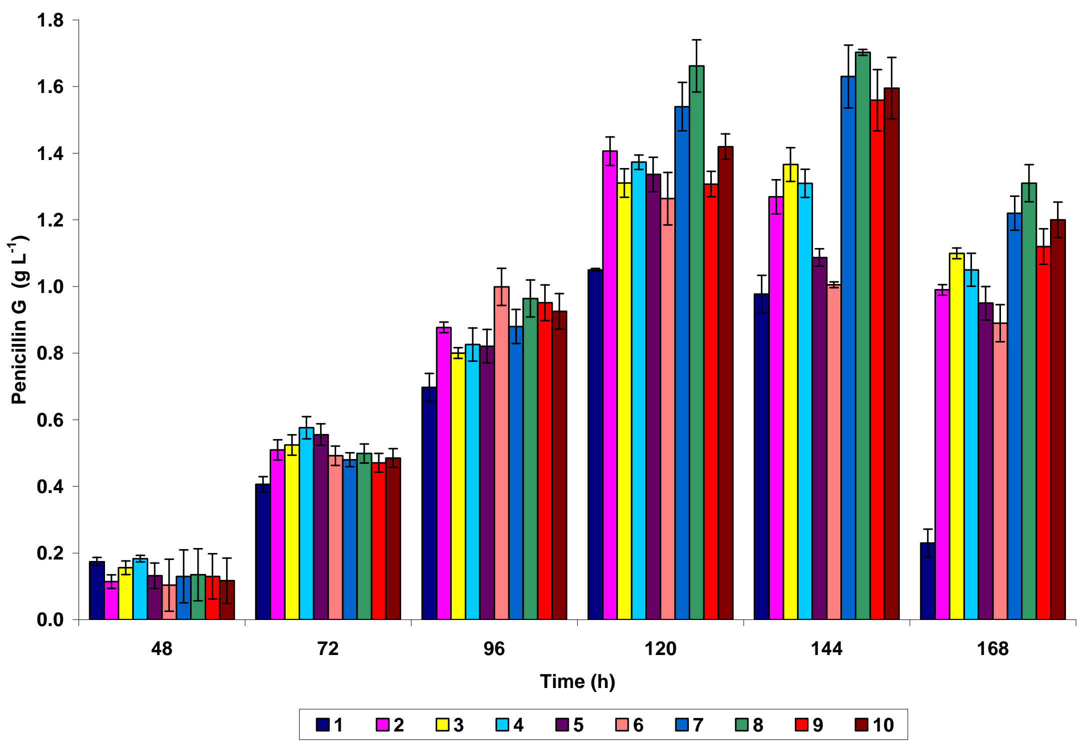


Figure 3.7 Penicillin G concentration in control and elicited shaken flask cultures of *P. chrysogenum* P2 with different concentration and types of elicitors added at different addition time. Standard error between triplicate samples was lower than 1% in all cases. ■ **Control (no elicitor added); ■ 150 mg L^{-1} of MO at 48 h; ■ 150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of MO at 72 h; ■ 150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of MO at 96 h; ■ 150 mg L^{-1} of MO at 48 h followed by 150 mg L^{-1} of MO at 72 h; ■ 150 mg L^{-1} of MO at 48 h followed by 150 mg L^{-1} of MO at 96 h; ■ 150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 72 h; ■ 150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h; ■ 150 mg L^{-1} of MO at 48 h followed by 150 mg L^{-1} of OM at 72 h; ■ 150 mg L^{-1} of MO at 48 h followed by 150 mg L^{-1} of OM at 96 h.**

On the basis of the results obtained from the optimisation studies (Figure 3.7) for the addition of the second elicitor, 75 mg L^{-1} OM at 96 h was chosen as the optimal condition for further enhancement in penicillin G production. Based on this information three systematic sets of experiments were carried out in shaken flasks (3.1.3.2), 2 L

bioreactors (3.1.3.3) and 5 L bioreactors (3.1.3.4) with the following experimental set up (Table 3.5):

Table 3.5: Experimental setup to compare the effect of multiple elicitor addition to single elicitor addition and control cultures in *P. chrysogenum* cultures

Flask No	Elicitor I	Concentration (mg L^{-1})	Addition time (h)	Elicitor II	Concentration (mg L^{-1})	Addition time (h)
Control	-	-	-	-	-	-
Single Addition	MO	150	48	-	-	-
Multiple addition	MO	150	48	OM	75	96

3.1.3.2 Shaken flask studies

3.1.3.2.1 Production of penicillin G

Figure 3.8 shows the penicillin G concentration in fermentation samples from the control and elicited shaken flask cultures. The addition of single and multiple elicitors caused a significant increase in the concentration of penicillin over the control fermentation (no elicitor added) ($p < 0.001$). The maximum penicillin G concentrations were 0.97 g L^{-1}, 96 h (single elicitor addition), 1.25 g L^{-1}, 144 h(multiple elicitor addition) compared to 0.59 g L^{-1}, 96 h (control cultures).

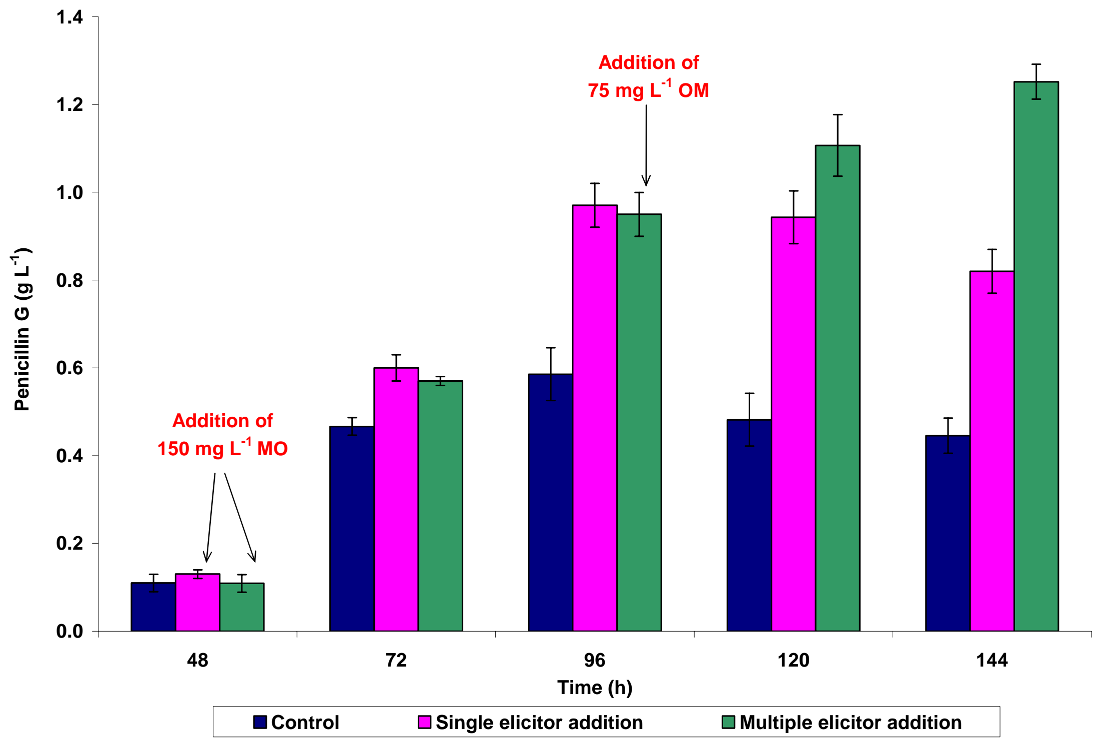


Figure 3.8 Penicillin G concentration in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

The rate of penicillin G production was 133% higher in the single elicitor added culture compared to the control culture before the addition of the second elicitor. The rate of penicillin G production in multiple elicitor added cultures was 300% and 71% higher compared to the control and single elicitor addition respectively (Table 3.6). All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.05$).

Table 3.6 Penicillin G production rate in the shaken flask cultures for the control and elicited (48-144 h)

	Elicitor	**Penicillin G production rate (g h^{-1})**
Control	-	0.003
Single elicitor addition	MO	0.007
Multiple elicitor addition	MO, OM	0.012

3.1.3.2.2 Biomass production

Figure 3.9 compares the final biomass concentration in cell dry weight (CDW) between shaken flask fermentations of the control and elicited cultures. The single addition and multiple addition of elicitors, does not elicit notable increase in biomass concentration compared to the control ($p > 0.05$). The final biomass at the harvest period (144 h) was 37 g L^{-1}, 39 g L^{-1} and 39 g L^{-1} CDW for the control, single addition and multiple additions of elicitor respectively.

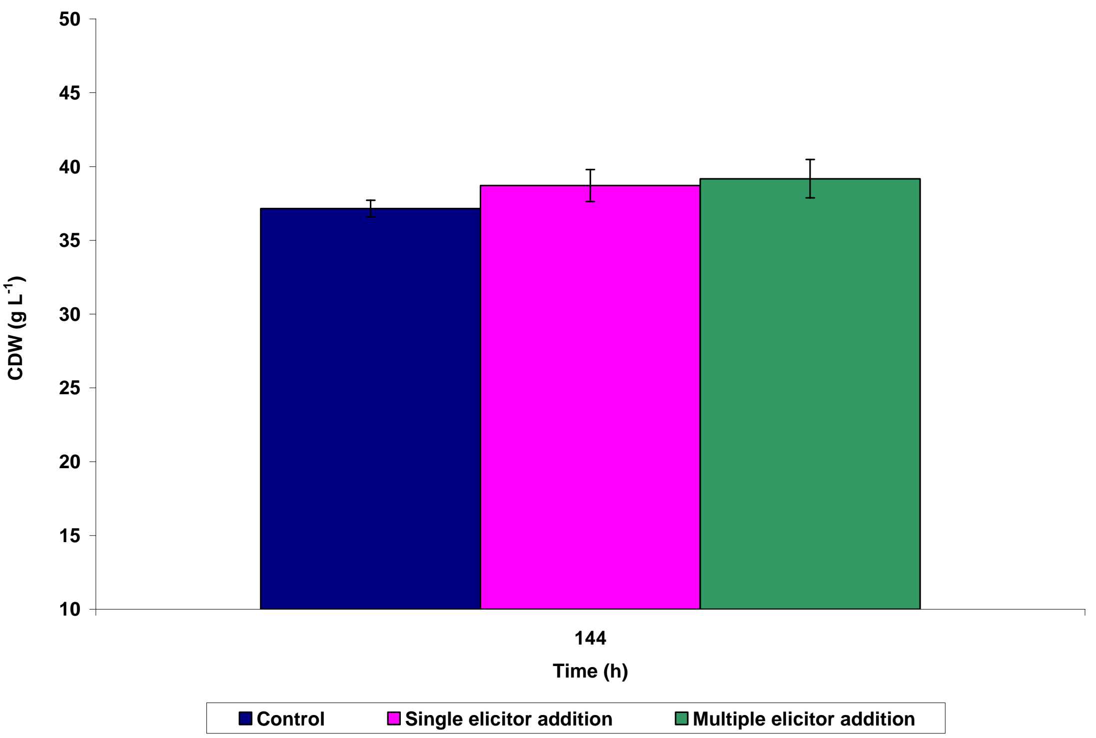


Figure 3.9 Biomass concentrations in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

3.1.3.2.3 Consumption of carbohydrates

Figure 3.10 shows the concentration of total carbohydrates at different stages of the fermentation between control and elicited cultures (single and multiple elicitor addition). On the addition of the elicitors to the culture there was an increase in carbohydrate consumption. The rate of carbohydrate consumption was compared between the control and elicited cultures and shown in Table 3.7.

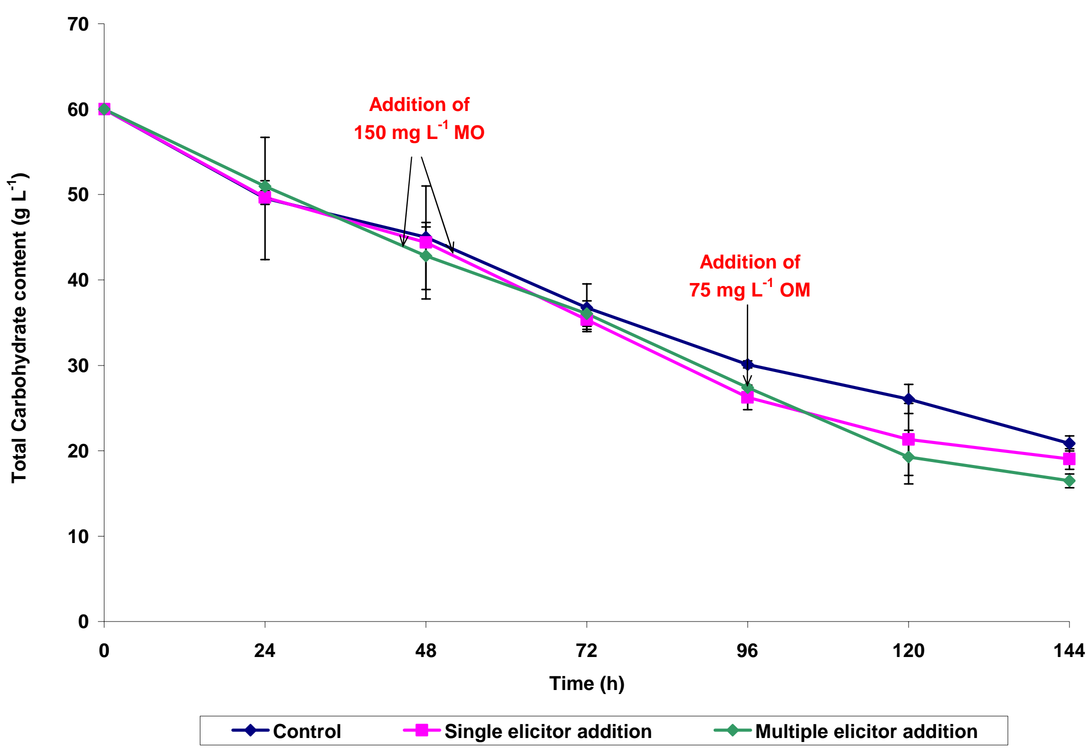


Figure 3.10 Total carbohydrate consumption in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added shaken flask cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

Consumption of carbohydrates was calculated as the difference between initial and residual concentration of carbohydrates. The rate of carbohydrate consumption is 4% and 8% higher in the single and multiple elicitor added culture compared to the control

cultures. All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.05$).

Table 3.7 Total carbohydrate consumption rates in *P. chrysogenum* P2 fermentations for the control and elicitor added cultures (48-144 h)

	Elicitor	**Total carbohydrate consumption rate ($g\ h^{-1}$)**
Control	-	0.25
Single elicitor addition	MO	0.26
Multiple elicitor addition	MO, OM	0.27

3.1.3.3 Bioreactor studies (2 L)

3.1.3.3.1 Production of penicillin G

Figure 3.11 shows the penicillin G concentration in 2 L bioreactor cultures of *P. chrysogenum* P2. The addition of single and multiple elicitors caused an increase in the concentration of penicillin over the control cultures (no elicitor added). The maximum penicillin G concentrations were 0.49 g L^{-1} (single elicitor addition) and 0.67 g L^{-1} (multiple elicitor addition) compared to 0.28 g L^{-1} at 120 h for the control cultures ($p < 0.001$).

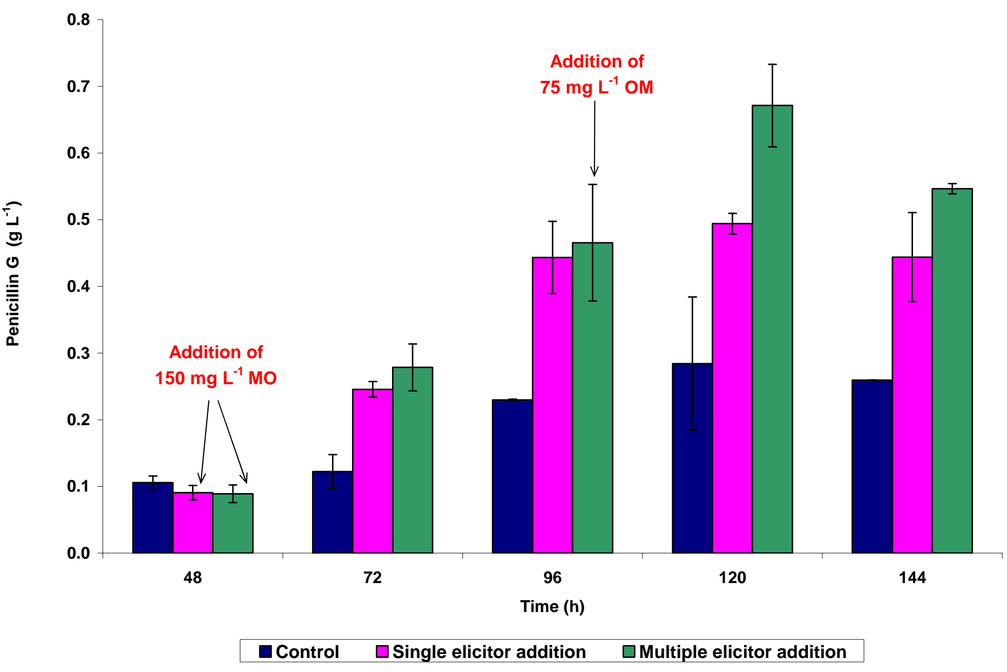


Figure 3.11 Penicillin G concentration in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added 2 L STR cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

The penicillin G production rate was calculated as the difference between initial and maximum concentration penicillin G. The rate of penicillin G production was 200%

higher in the single elicitor added culture compared to the control cultures. The rate of penicillin G production in multiple elicitor added cultures was 300% and 33% higher compared to the control and single elicitor addition respectively (Table 3.8). All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.01$).

The specific productivity was 121% higher in the single elicitor added culture compared to the control culture. The specific productivity in multiple elicitor added cultures was 218% and 43% higher compared to the control and single elicitor addition respectively. All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.01$).

Table 3.8 Production rate and Specific productivity of penicillin G fermentations for the control and elicited cultures (48-120 h)

	Penicillin G Production Rate (g h^{-1})	**Penicillin G Specific Productivity (mg g^{-1} h^{-1})**
Control	0.002	0.178
Single elicitor addition	0.006	0.395
Multiple elicitor addition	0.008	0.566

3.1.3.3.2 Biomass production

Figure 3.12 compares the biomass concentration in cell dry weight (CDW) between control and elicited STR fermentations (single addition and multiple additions).The single addition and multiple addition of elicitors, do not elicit a notable increase in biomass compared to the control. No notable difference was observed in biomass concentration between the three fermenters ($p > 0.05$).

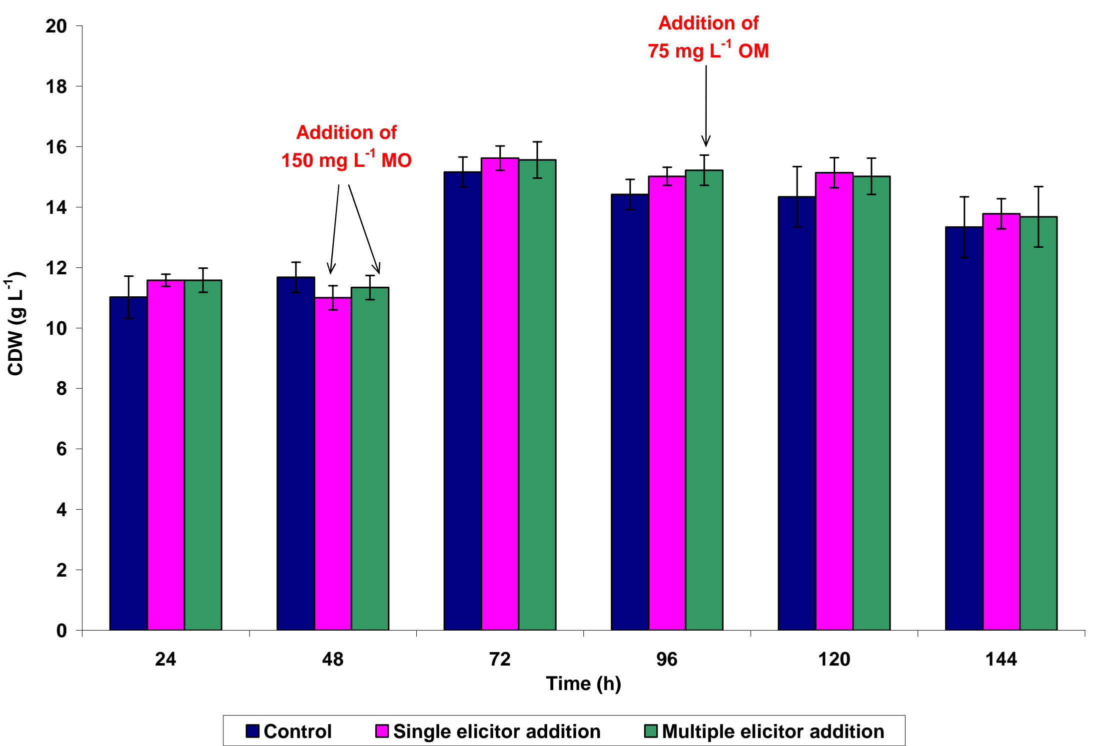


Figure 3.12 Biomass concentrations in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added 2 L STR cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

3.1.3.4 Bioreactor studies (5 L)

3.1.3.4.1 Production of penicillin G

Figure 3.13 shows penicillin G production in 5 L cultures of *P. chrysogenum* P2. The addition of single and multiple additions of elicitors caused an increase in the concentration of penicillin over the control fermentation (no elicitor added). The maximum penicillin G concentrations were 1.57 g L^{-1} (single elicitor addition) and 1.92 g L^{-1} (multiple elicitor addition) compared to 0.85 g L^{-1} for the control cultures at 120 h ($p < 0.001$).

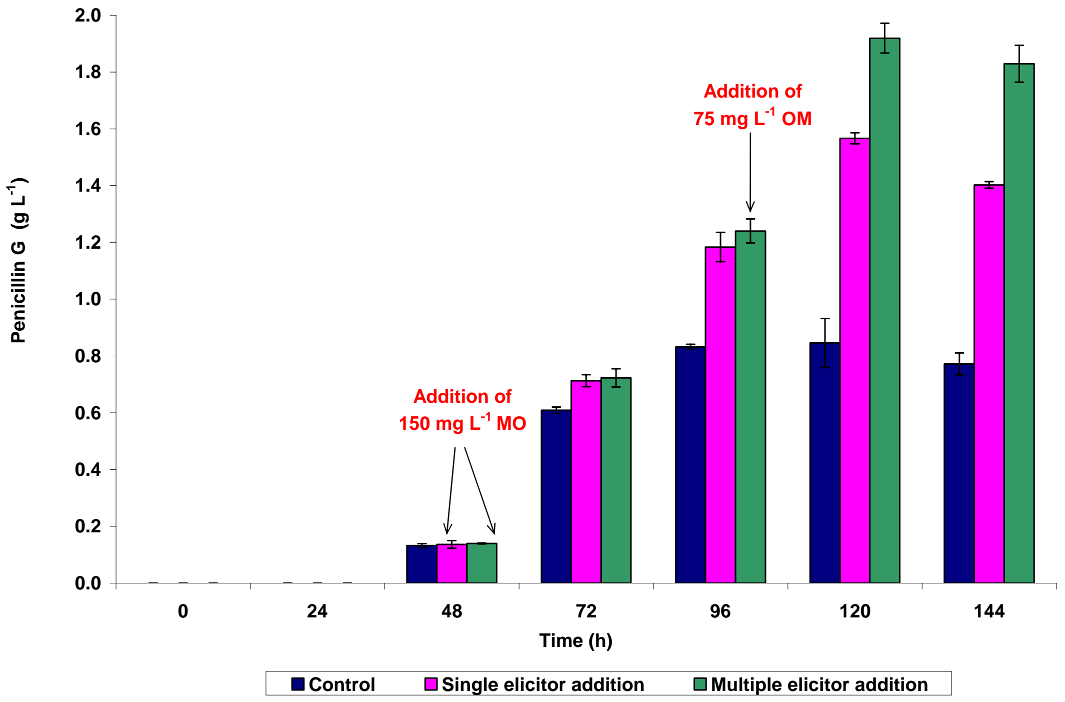


Figure 3.13 Penicillin G concentration in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added 5 L STR cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

The penicillin G production rate was calculated as the difference between initial and maximum concentration penicillin G. The rate of penicillin G production was 100%

higher in the single elicitor added culture compared to the control cultures. The rate of penicillin G production in multiple elicitor added cultures was 150% and 50% higher compared to the control and single elicitor addition respectively (Table 3.9). All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.05$).

The specific productivity was 100% higher in the single elicitor added culture compared to the control culture. The specific productivity in multiple elicitor added cultures was 150% and 50% higher compared to the control and single elicitor addition respectively. All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.05$).

Table 3.9 Production rate and Specific productivity of penicillin G in fermentations for the control and elicited cultures (48-120 h)

	Penicillin G Production Rate (g h^{-1})	**Penicillin G Specific Productivity (mg g^{-1} h^{-1})**
Control	0.010	0.6
Single elicitor addition	0.020	1.2
Multiple elicitor addition	0.025	1.5

3.1.3.4.2 Biomass production

Figure 3.14 compares the biomass concentration in cell dry weight (CDW) between the control and elicited STR fermentations (single elicitor addition and multiple elicitor additions).The single addition and multiple addition of elicitors, do not elicit a notable increase in biomass concentration compared to the control. No notable difference was observed in biomass concentration between the three fermenters ($p > 0.05$).

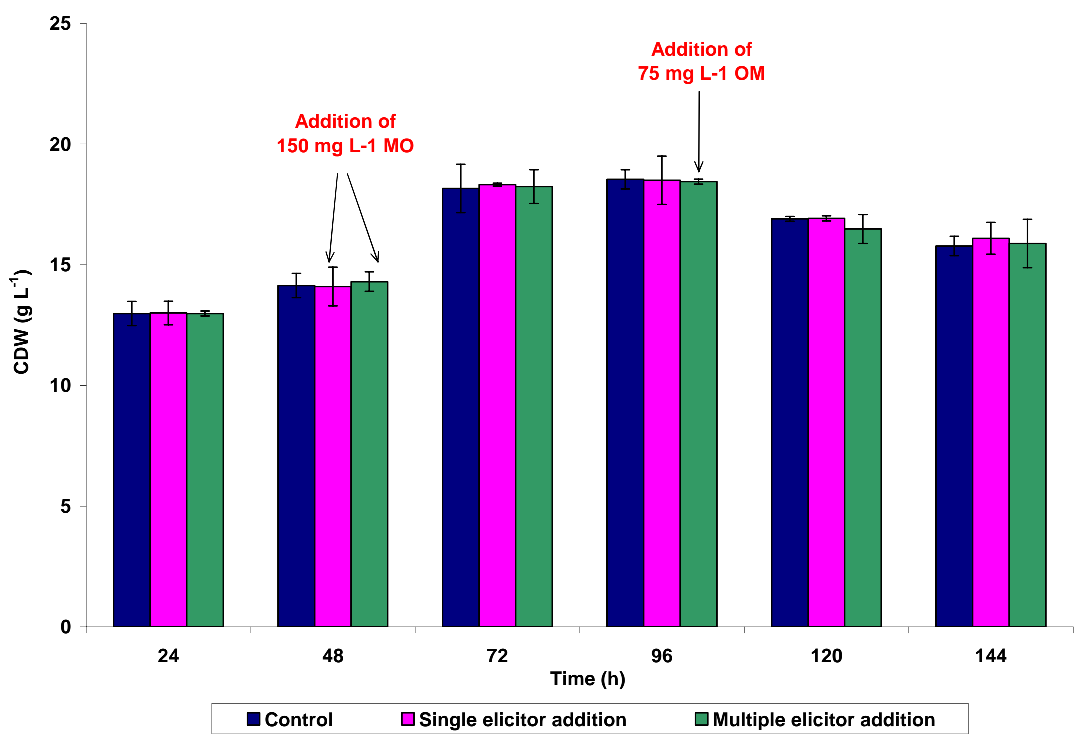


Figure 3.14 Biomass concentrations in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added 5 L STR cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

3.1.3.4.3 Consumption of carbohydrates

Figure 3.15 shows the concentration of carbohydrates at different stages of the fermentation between the control and elicited STR fermentations (single elicitor addition and multiple elicitor additions).

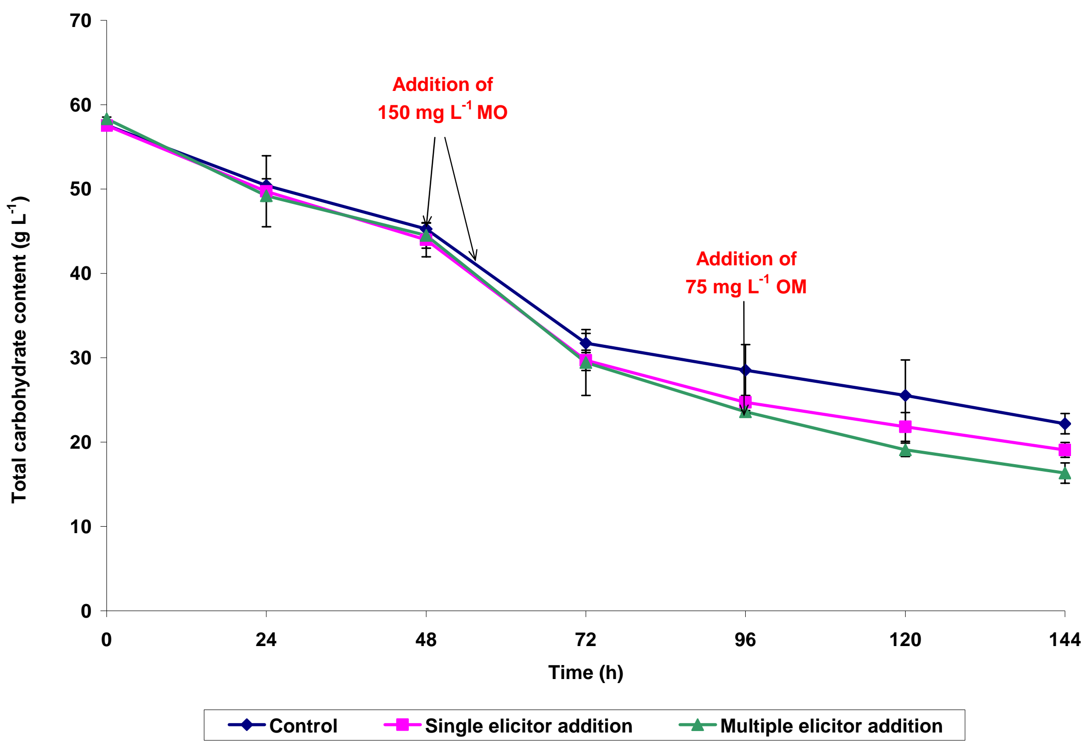


Figure 3.15 Carbohydrate consumption in control (no elicitor added), single elicitor (150 mg L^{-1} of MO at 48 h) and multiple elicitor (150 mg L^{-1} of MO at 48 h followed by 75 mg L^{-1} of OM at 96 h) added 5 L STR cultures of *P. chrysogenum* P2. Error bars indicate standard deviation between triplicate samples.

Consumption of carbohydrates (Table 3.10) was calculated as the difference between initial and residual concentration of carbohydrates. The rate of carbohydrate consumption is 15% and 30% higher in the single and multiple elicitor added culture compared to the control cultures. All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($p < 0.05$).

Table 3.10 Total carbohydrate consumption rates in *P. chrysogenum* P2 fermentations for the control and elicited cultures (48-120 h)

	Elicitor	Total carbohydrate consumption rate ($g\ h^{-1}$)
Control	-	0.27
Single elicitor addition	MO	0.31
Multiple elicitor addition	MO, OM	0.35

3.2 MOLECULAR BIOLOGY STUDIES

The results presented in this section compares the transcript copy number of the major biosynthetic genes of the penicillin G production for the control and elicitor added cultures in shaken flasks and stirred tank reactors (STR). All the analyses were carried out in triplicate and the standard error was applied to all graphs. The biosynthetic genes used in this study were *pcb*AB (ACV synthetase), *pcb*C (Isopenicillin N synthase) and *pen*DE (Acyl-coenzyme A: isopenicillin acyltransferase). An increase in the transcript copy number of the biosynthetic genes was observed, correlating with the enhancement of penicillin G production on addition of the elicitors. The transcript copy number of the genes increased in the presence of the single addition of MO compared to the control, but the increase was more evident in the presence of multiple additions of elicitors MO and OM.

3.2.1 Total RNA isolation

Total RNA was used as the starting material for the quantification of mRNA concentration corresponding to the genes of interest. RNA was isolated from pelleted cells of *P. chrysogenum* cultures using the RNeasy Plant Mini Kit following the protocol (2.7.1). The integrity of the samples were confirmed by the presence of the two 28S and 18S ribosomal RNA (rRNA) and the smear represented the mRNA. 200 bp DNA step ladder (Lane 7) was used as a marker (Figure 3.16).

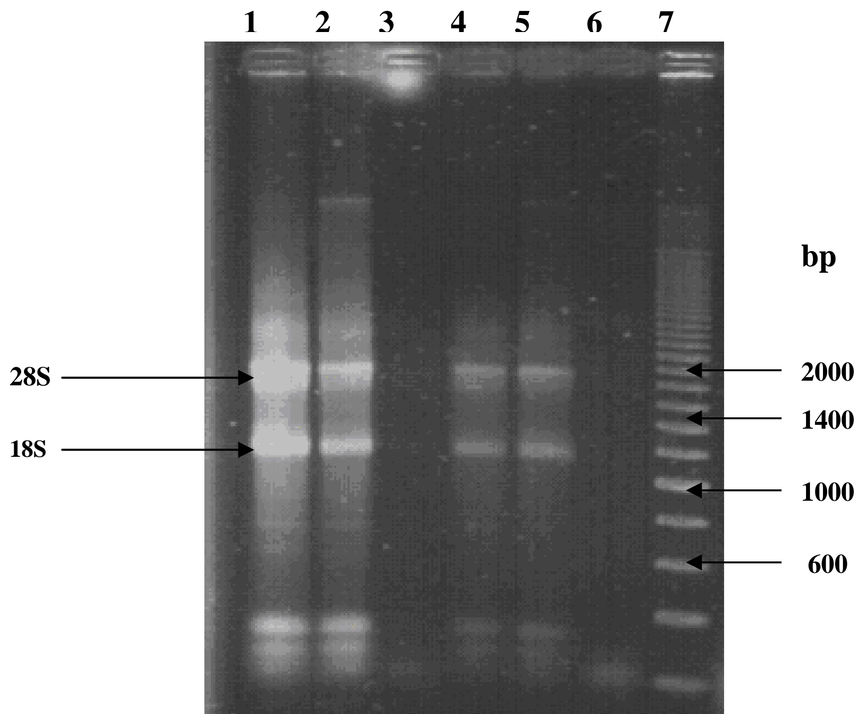


Figure 3.16 Agarose gel (0.8%) stained with ethidium bromide showing total RNA preparation from a 48 h culture of *P. chrysogenum* P2 (ATCC 48271). Lane 1-2: Total RNA first eluate, Lane 4-5: Total RNA second eluate and Lane 7: 200 bp DNA step ladder.

3.2.2 Conventional Polymerase Chain Reaction (PCR)

After the extraction, the total RNA was quantified using Eppendorf BioPhotometer. Then 1 µg of the total RNA was combined with random primers and used for synthesis of full length first-strand cDNA in preparation for PCR amplification. Primers were designed using the Primer3 programme for the amplification of the major penicillin biosynthetic genes *pcb*AB*, pcb*C*, pen*DE and the normalizing gene 18S rRNA and β-actin. These primers were first tested using conventional PCR prior to Real-Time PCR. Amplification products (Figure 3.17) for each gene of interest had the expected product size as shown in Table 2.6.

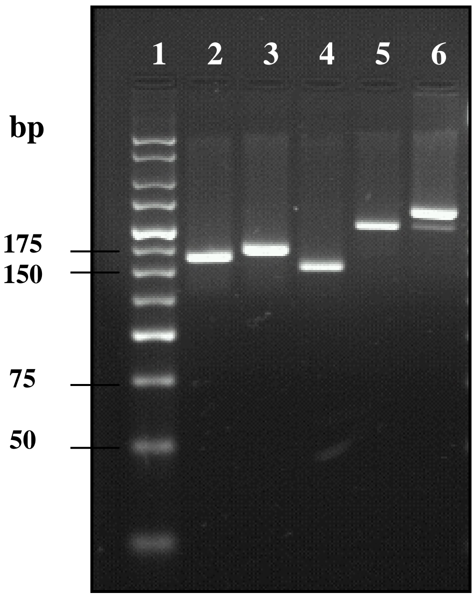


Figure 3.17 Agarose gel (3.5%) stained with ethidium bromide showing the PCR products for the penicillin G biosynthetic genes of *P. chrysogenum* P2 (ATCC 48271) Lane 1: 25 bp DNA step ladder, Lane 2: 162 bp *pcb*AB gene fragment, Lane 3: 170 bp *pcb*C gene fragment, Lane 4: 151 bp *pen*DE gene fragment, Lane 5: 205 bp *β-actin* gene fragment and Lane 6: 234 bp *18S rRNA* gene fragment

This confirmed that the designed primer and the programme for the PCR amplification were optimal.

3.2.3 Real Time PCR (QPCR)

3.2.3.1 Amplification and Dissociation plot for *pcb*AB gene

Figure 3.18 shows the amplification curve for the *pcb*AB gene which was employed to determine the C_t value. High mRNA concentration is associated with a low C_t value. The dissociation plot confirms that only a single product with identical T_m is produced and there is no primer-dimer formation. Negative control without any template and RNA-control were employed to set the threshold and check for DNA contamination in the original RNA extraction respectively. All the samples were analysed in triplicates.

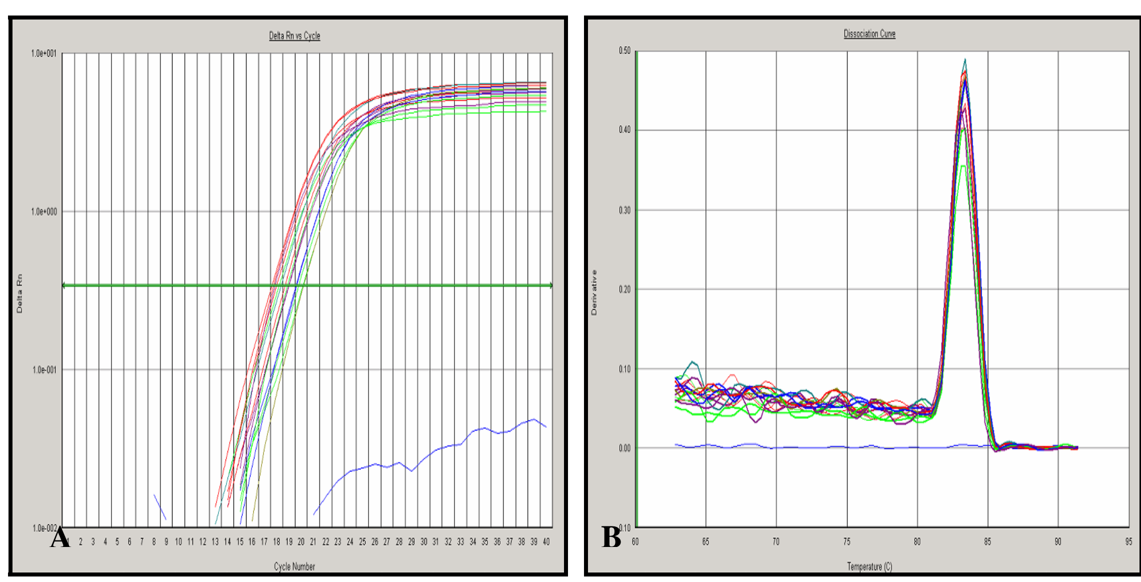


Figure 3.18 The amplification curve (A) and dissociation plot (B) for the mRNA corresponding to the *pcb*AB gene using the ABI Prism 7000 Sequence Detection system

3.2.3.2 Amplification and Dissociation plot for *pcb*C gene

Figure 3.19 shows the amplification curve for the *pcb*C which was employed to determine the C_t value. The amplification plot demonstrates the cycle number at which the amplification begins which corresponds to the concentration of the starting material.

The threshold to calculate the C_t values for the gene of interest were set above the negative control automatically. The dissociation plot confirms that only a single product with identical T_m is produced and there is no primer-dimer formation.. The samples were analysed in triplicate.

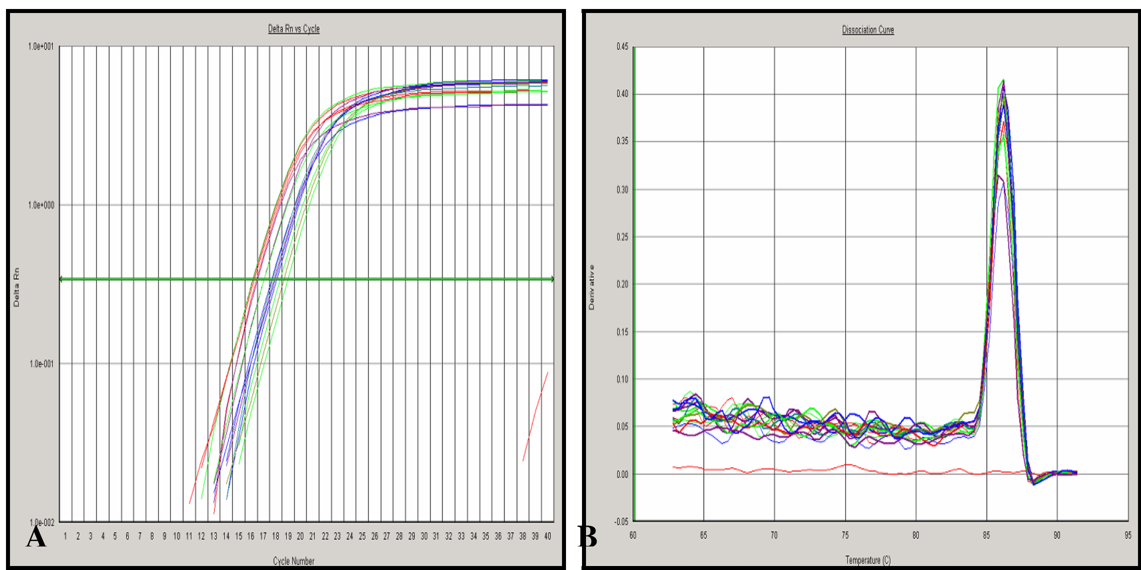


Figure 3.19 The amplification curve (A) and dissociation plot (B) for the mRNA corresponding to the *pcb*C gene using the ABI Prism 7000 Sequence Detection system

3.2.3.3 Amplification and Dissociation plot for *pen*DE gene

Figure3.20 shows the amplification curve and dissociation plot for the *pen*DE gene which was employed to determine the C_t value. High mRNA concentration is associated with a low C_t value. It is apparent from the dissociation plot that the melting temperature of the amplicon occurs at 84.5°C. No contaminating products were found present in this reaction as it would show up as an additional peak separate from the desired amplicon peak. All the samples were analysed in triplicate.

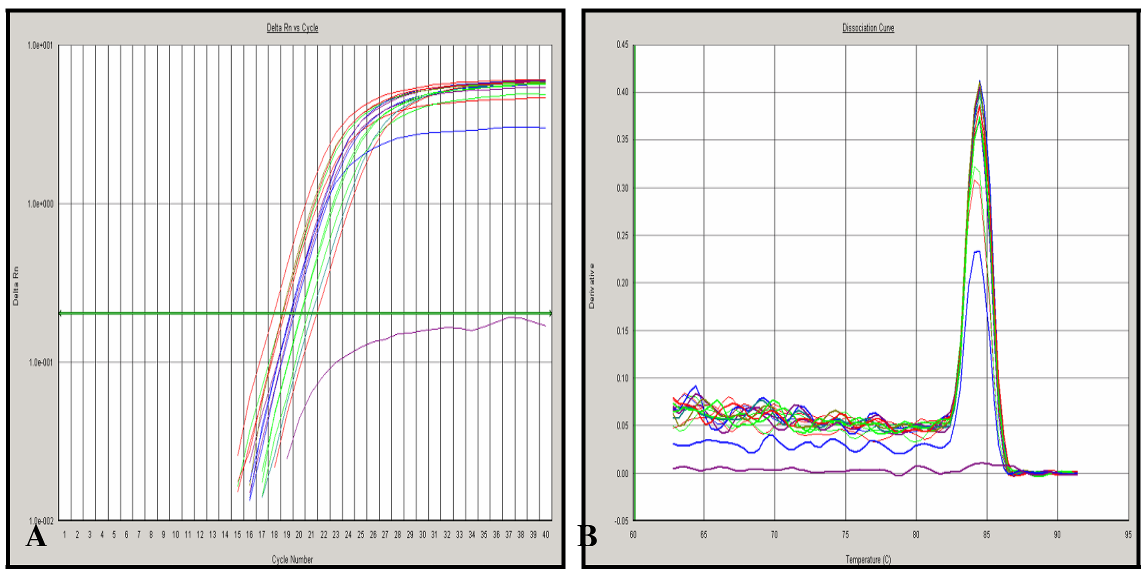


Figure 3.20 The amplification curve (A) and dissociation plot (B) for the mRNA corresponding to the *pen*DE gene using the ABI Prism 7000 Sequence Detection system

3.2.3.4 Amplification and Dissociation plot for 18S rRNA gene

Figure 3.21 shows the amplification curve for the 18S rRNA which was employed as an internal control (normalising gene) for relative quantification. The T_m of the amplicon starts at the point of inflection of the dissociation curve profile. The inflection point occurs at 86°C for the 18S rRNA. The dissociation plot confirms that only a single product with identical T_m is produced and there is no primer-dimer formation. Each sample was analysed in triplicate.

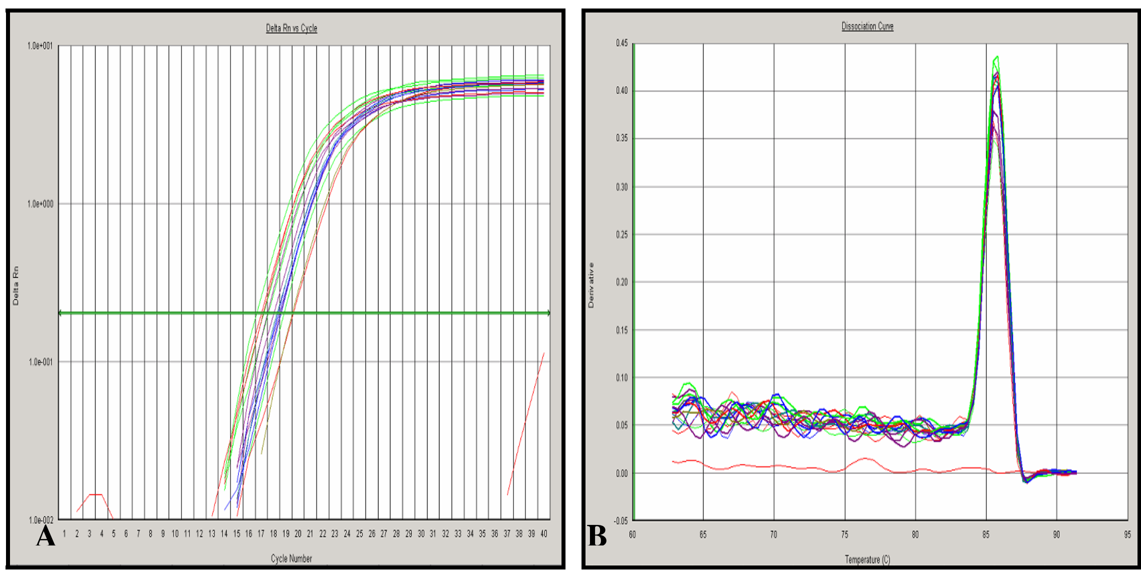


Figure 3.21 The amplification curve (A) and dissociation plot (B) for the mRNA corresponding to the 18 S rRNA gene using the ABI Prism 7000 Sequence Detection system (Applied Biosytems).

3.2.3.5 Standard calibration curve for Absolute quantification

A linear relationship was obtained by plotting the threshold cycle against the logarithm of known amount of the initial template (Figure 3.22). The equation of the line that best fits the data was determined by regression analysis. The slope was calculated for each set to estimate the efficiency of the real time PCR (Table 3.11). The copy numbers of the mRNA corresponding to the gene of interest were calculated using the following equation assuming that each genome had only one gene of interest and that molecular weight of 1 bp was 660 g mol^{-1}: number of copies per microliter = (6×10^{23})(DNA concentration)/molecular weight of one genome, where 6×10^{23} is the number of copies per mole, the DNA concentration is given in grams per microliter, and the molecular weight of one genome is given in grams per mole. The technique was optimized by using triplicate reactions and running independently diluted standard curves repeatedly under identical conditions. To enumerate transcript copy numbers of unknown samples,

one calibration curve was routinely run with each sample set and compared with previous curves to check for consistency between the runs.

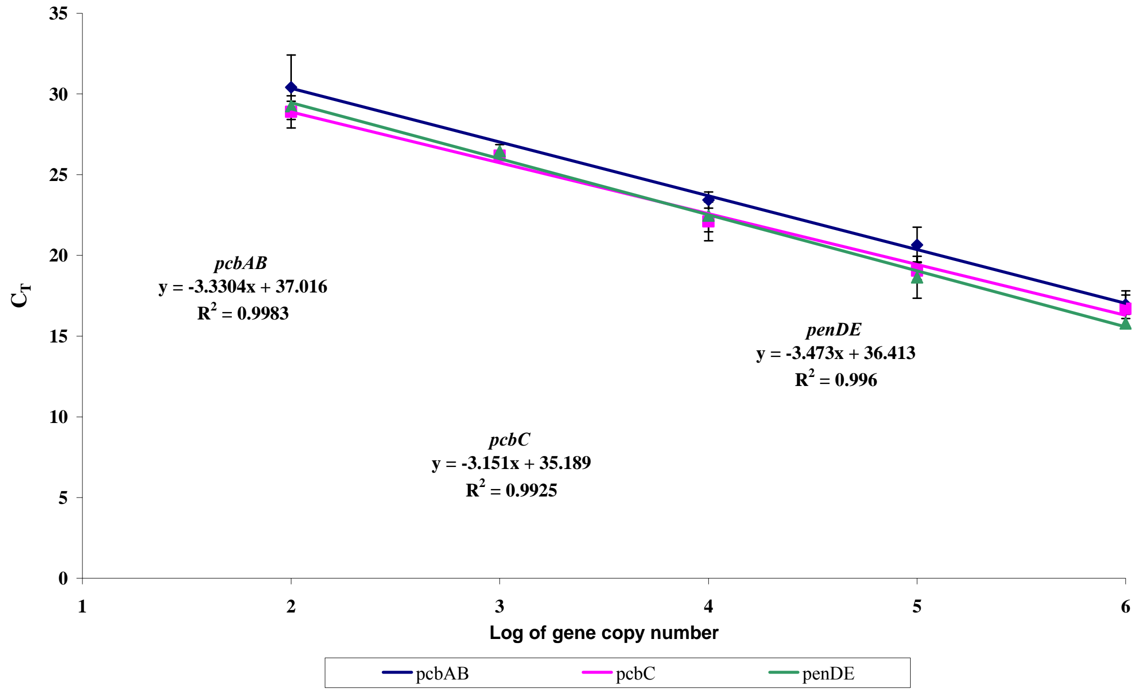


Figure 3.22 Standard curves obtained from 10-fold serial dilution of the three major penicillin G biosynthetic genes. C_T values are the averages of three repetitions. Bars represent standard errors. Correlation coefficient (R^2) values, slope and efficiency are shown in Table 3.11.

Table 3.11 Correlation coefficient (R^2), slope and efficiency of standard curves obtained from 10-fold serial dilutions of the genes of interest from *P. chrysogenum* P2 (ATCC 48271).

Gene	R^2	Slope	Efficiency* (%)
***pcb*AB**	0.9983	-3.3304	99.65
***pcb*C**	0.9925	-3.151	107.66
***pen*DE**	0.996	-3.473	94.06

*** Efficiency was estimated by the formula E = [($10^{-1/slope}$) -1]** (Labrenz *et al.*, 2004)

3.2.4 Multiple elicitor addition studies

3.2.4.1 Shaken flask studies

A) *pcb*AB transcript copy number

In the control cultures the maximum *pcb*AB gene transcription was detected at 48 h (Figure 3.23). A steep decline in transcription was observed from 48 h to 96 h. For both single and multiple elicitor addition cultures, the transcriptional level of *pcb*AB gene was high at 72 h. Transcript levels in these cultures decreased after 72 h. But in the multiple addition cultures after the addition of the second elicitor the transcription level increased. For *pcb*AB gene, the transcript copy number at 72 h in the both the elicited cultures was 19 $x10^6$ compared to 6 $x10^6$ in the control cultures.

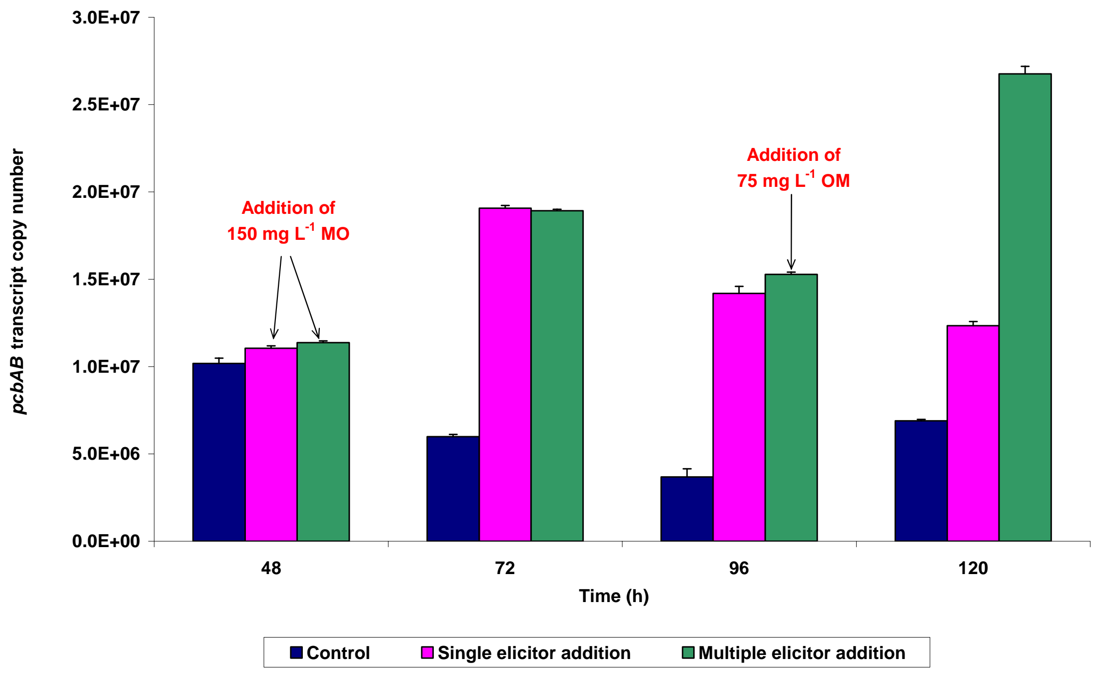


Figure 3.23 Transcript copy number of penicillin G biosynthetic gene *pcb*AB during the course of cultivation in shaken flask cultures. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

Increases of 216%, in *pcb*AB gene transcription were observed after the addition of the first elicitor compared to the control cultures at 72 h ($p < 0.001$ for both conditions). After the addition of the second elicitor the transcript copy number of *pcb*AB gene increased to 26 x10^6 in the multiple elicitor culture compared to 12 x10^6 in the single addition cultures, an increase of 116% ($p < 0.001$).

B) *pcb*C transcript copy number

In the control cultures the maximum *pcb*C gene transcription was detected at 48 h (Figure 3.24). A steep decline in transcription was observed from 48 h to 96 h. For both single and multiple elicitor added cultures, the transcriptional level of *pcb*C gene was high at 72 h compared to the control. Transcript levels in these cultures decreased after 72 h.

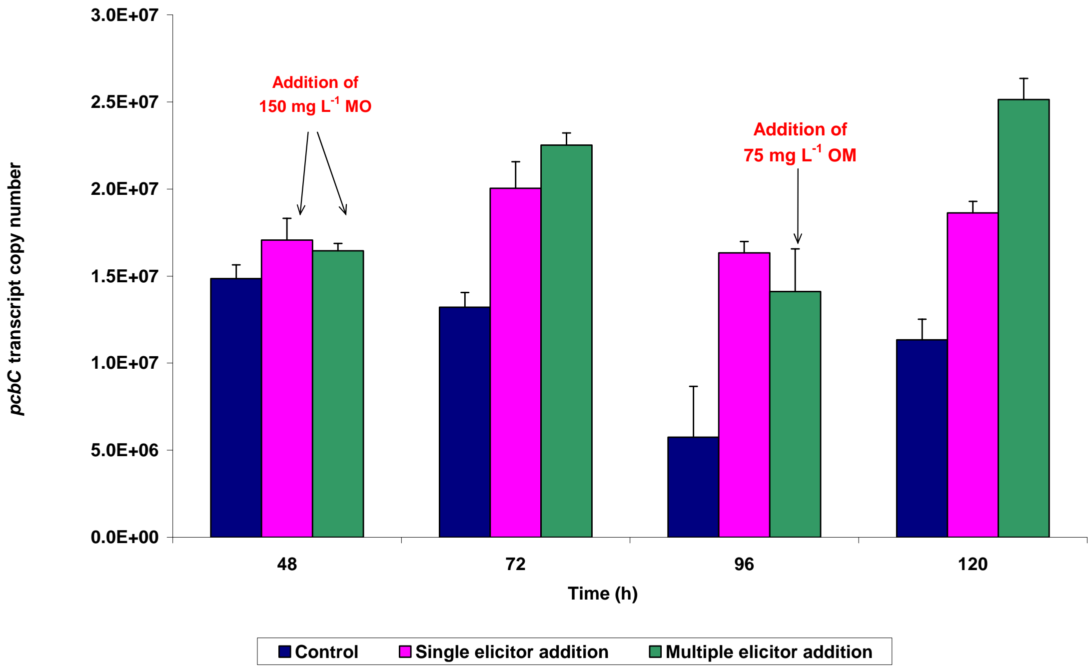


Figure 3.24 Transcript copy number of penicillin G biosynthetic gene *pcb*C during the course of cultivation in shaken flask cultures. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

But in the multiple elicitor cultures after the addition of the second elicitor the transcription level increased. For *pcb*C gene, the transcript copy number at 72 h in the elicited cultures was 21 $x10^6$ compared to 13 $x10^6$ in the control cultures, an increase of 61% ($p < 0.001$). After the addition of the second elicitor the transcript copy number of *pcb*AB gene increased to 25 $x10^6$ in the multiple elicitor culture compared to 18 $x10^6$ in the single addition cultures, an increase of 39% ($p < 0.001$)

C) *pen*DE transcript copy number

In the control cultures the maximum *pen*DE gene transcription was detected at 48 h (Figure 3.25). A steep decline in transcription was observed from 48 to 96 h.

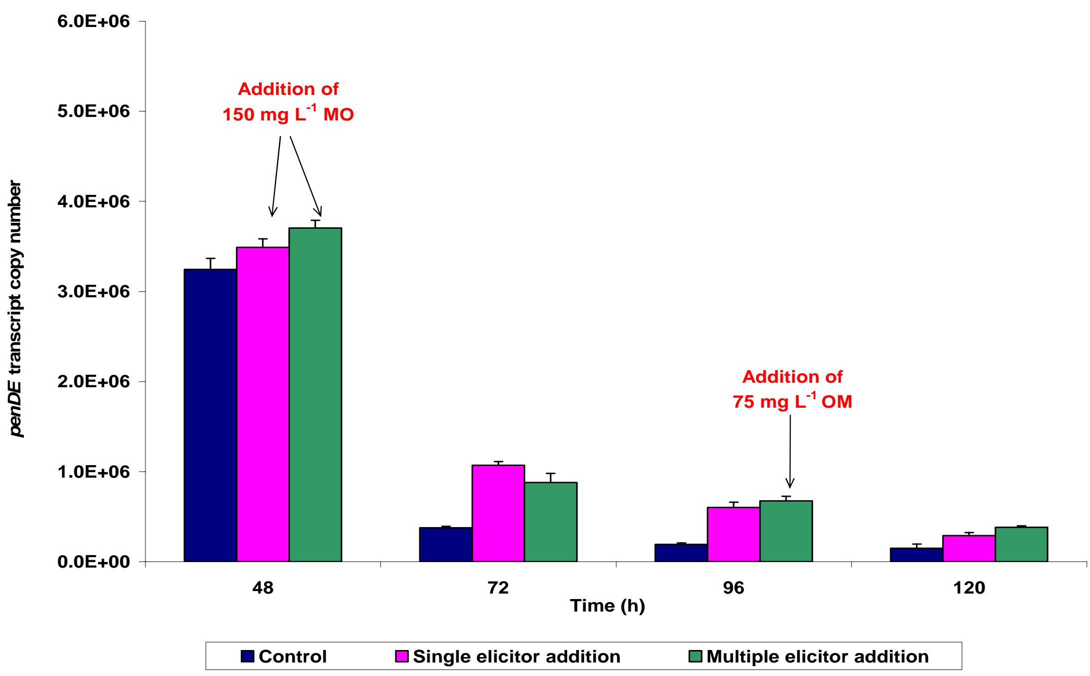


Figure 3.25 Transcript copy number of penicillin G biosynthetic gene *pen*DE during the course of cultivation in shaken flask cultures. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

Higher penDE transcription was observed in elicited cultures (9×10^5) at 72 h compared to the control cultures (3×10^5), an increase of 200% ($p < 0.001$). But in the multiple elicited cultures after the addition of the second elicitor at 96 h the final gene copy number increased to 4×10^5 compared to 3×10^5 in the single elicited cultures, an increase of 33% ($p < 0.05$).

3.2.4.2 Bioreactor studies (2 L)

A) *pcb*AB transcript copy number

In the control cultures the maximum *pcb*AB gene transcription was detected at 72 h (Figure 3.26). A steep decline in transcription was observed from 72 to 120 h in the control cultures. For both the elicited cultures, the transcriptional level of *pcb*AB gene was highest at 72 h compared to the control. Transcript levels in these cultures decreased after 72 h. But in the multiple elicitor added cultures after the addition of the second elicitor at 96 h the transcription level increased.

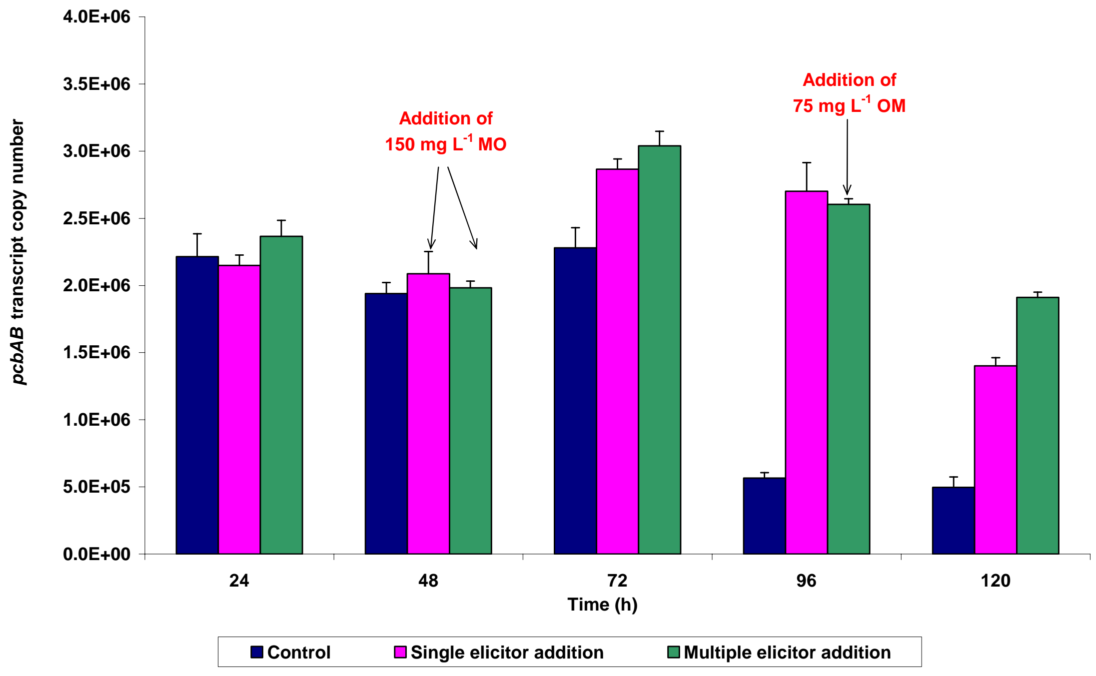


Figure 3.26 Transcript copy number of penicillin G biosynthetic gene *pcb*AB during the course of cultivation in 2 L STR. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

For *pcb*AB gene, the transcript copy number at 72 h in the elicited cultures was 2.9 $x10^6$ compared to 2.2 $x10^6$ in the control cultures, an increase of 29% ($p < 0.001$). After the

addition of the second elicitor the transcript copy number of *pcb*AB gene was 1.9 x10^6 in the multiple elicitor culture compared to 1.4 x10^6 in the single elicitor cultures, an increase of 36% ($p < 0.001$).

B) *pcb*C transcript copy number

In the control cultures the maximum *pcb*C gene transcription was detected at 72 h (Figure 3.27). A gradual decline in transcription was observed from 72 to 120 h. For both the elicited cultures, the transcriptional level of *pcb*C gene was high at 96 h compared to the control. Transcript levels in the elicited cultures decreased after 96 h. But in the multiple elicitor cultures after the addition of the second elicitor the transcription level increased compared to the single elicitor culture.

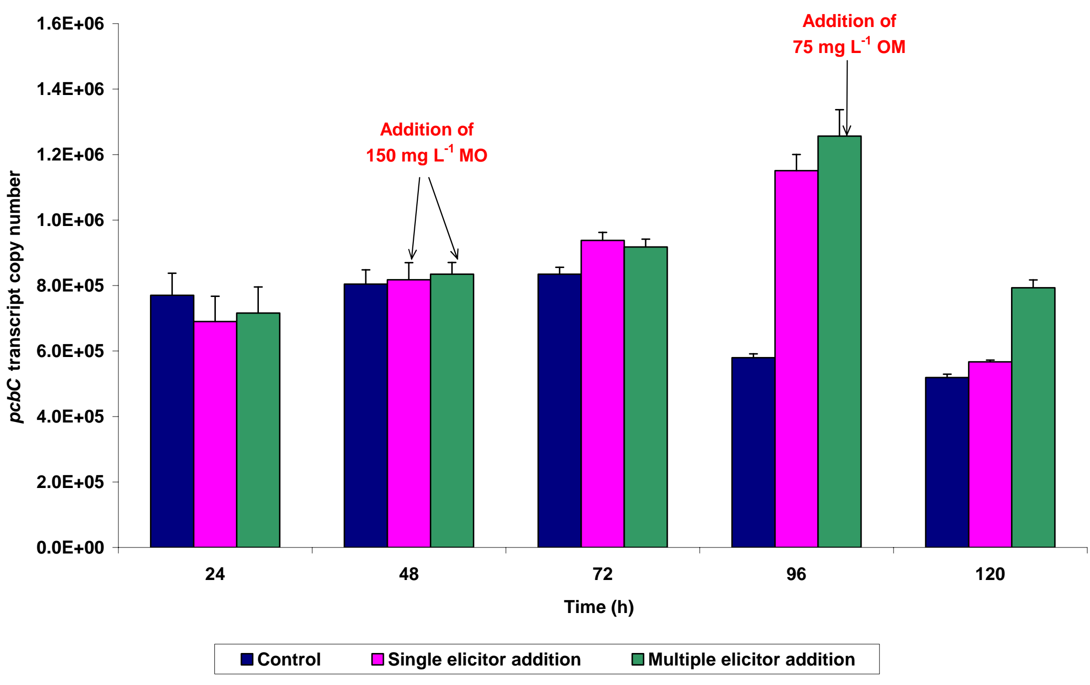


Figure 3.27 Transcript copy number of penicillin G biosynthetic gene *pcb*C during the course of cultivation in 2 L STR. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

For *pcb*C gene, the transcript copy number at 96 h in the elicited cultures was 12 $x10^5$ compared to 5.8 $x10^5$ in the control cultures, an increase of 107%, ($p < 0.001$). After the addition of the second elicitor, the transcript copy number of *pcb*AB gene was 25 $x10^5$ in the multiple elicitor culture compared to 18 $x10^5$ in the single elicitor cultures, an increase of 39% ($p <0.001$).

C) *pen*DE transcript copy number

The maximum *pen*DE gene transcription in all the cultures was detected at 24 h (Figure 3.28). A steep decline in transcription was observed from 24 to 96 h. The transcript levels for the penDE gene was higher for both the elicited cultures compared to the control cultures at 72 h.

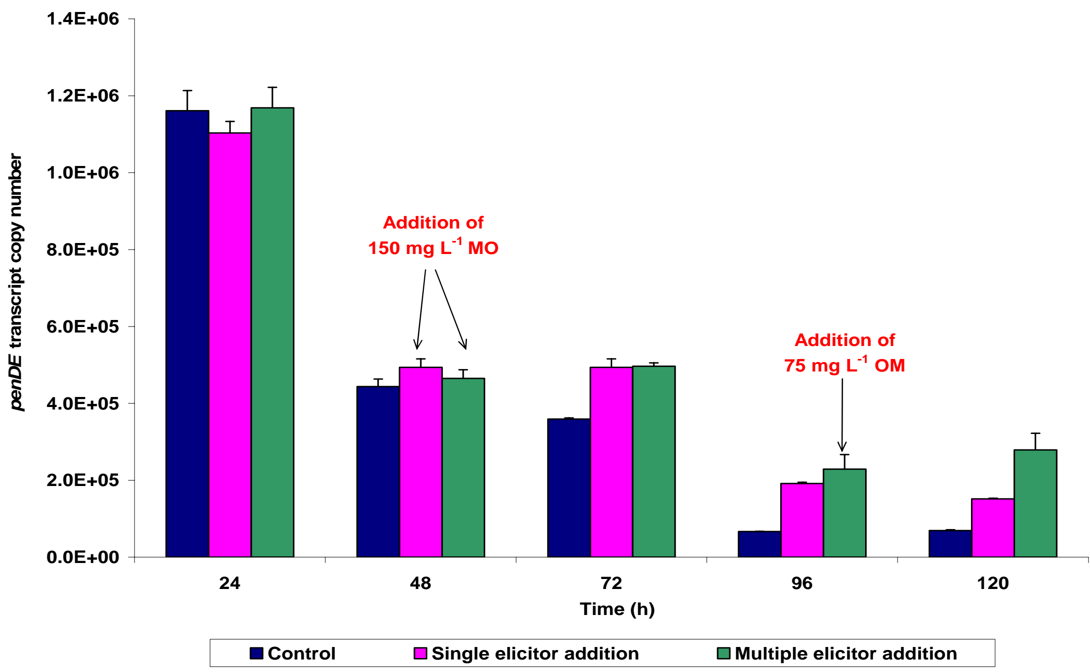


Figure 3.28 Transcript copy number of penicillin G biosynthetic gene *pen*DE during the course of cultivation. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h).

In the multiple elicitor cultures after the addition of the second elicitor the transcription level increased compared to the single elicitor cultures. For *pen*DE gene, the transcript copy number at 72 h in the elicited cultures was 4.9×10^5 compared to 3.6×10^5 in the control cultures, an increase of 36% ($p < 0.001$). After the addition of the second elicitor the transcript copy number of *pen*DE gene was 2.7×10^5 in the multiple elicitor culture compared to 1.5×10^5 in the single elicitor culture, an increase of 80% ($p < 0.001$).

3.2.4.3 Bioreactor studies (5 L)

A) *pcb*AB transcript copy number

In the control cultures the maximum *pcb*AB gene transcription was detected at 48 h (Figure 3.29). A steep decline in transcription was observed from 48 to 196 h. In both elicited cultures, the transcriptional level of *pcb*AB gene was highest at 96 h compared to the control. Transcript levels in these cultures decreased after 96 h. But in the multiple elicited cultures after the addition of the second elicitor the transcription level increased compared to the single elicitor addition cultures.

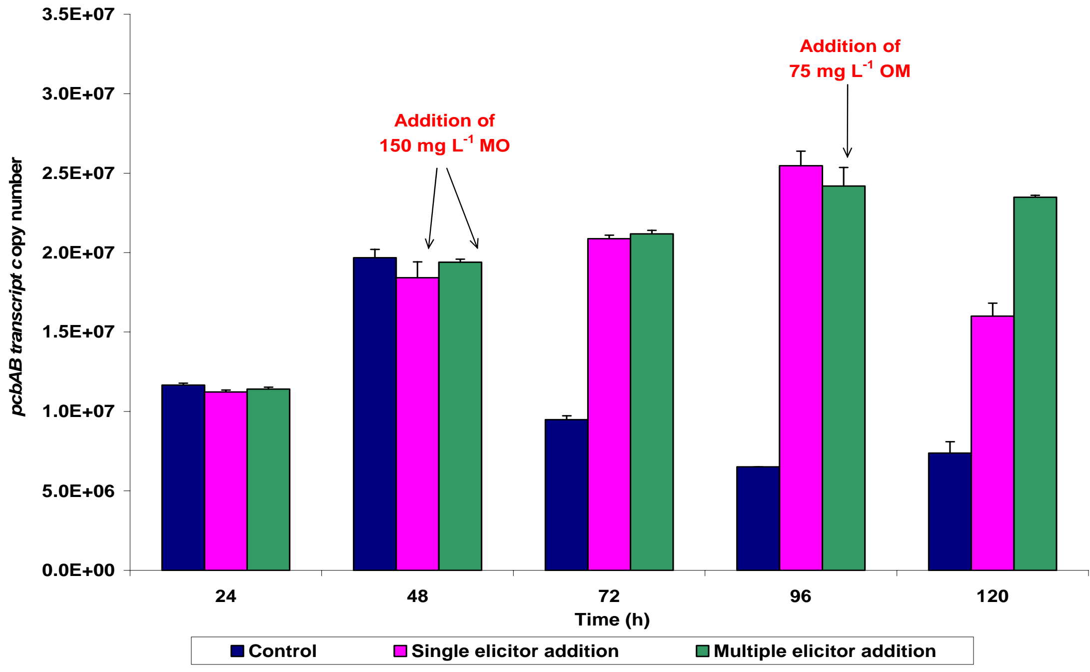


Figure 3.29 Transcript copy number of penicillin G biosynthetic gene *pcb*AB during the course of cultivation in 5 L STR. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

For the *pcb*AB gene, the transcript copy number at 96 h in the single and multiple elicitor added cultures was 25 $x10^6$ compared to 6 $x10^6$ in the control cultures. After the

addition of the second elicitor the transcript copy number of *pcb*AB gene was 23 x10^6 in the multiple addition culture compared to 16 x10^6 in the single elicitor added cultures. Increases of 291% in *pcb*AB gene transcription were observed from the single elicitor cultures over the control cultures at 72 h ($p < 0.001$). After the second addition the *pcb*AB gene transcription increased to 47% in multiple addition cultures compared to the single addition cultures.

B) *pcb*C transcript copy number

In the control cultures the maximum *pcb*C gene transcription was detected at 48 h (Figure 3.30). A gradual decrease in transcription was observed from 48 to 120 h. The transcriptional level of *pcb*C gene was high in both elicited cultures at 72 h compared to the control cultures. Transcript levels in these cultures decreased after 72 h. But in the multiple elicitor cultures after the addition of the second elicitor the transcription level increased. For *pcbC* gene, the transcript copy number at 72 h in both the elicited cultures was 17 x10^6 compared to 5.9 x10^6 in the control cultures, an increase 201% ($p < 0.001$). After the addition of the second elicitor the transcript copy number of *pcb*AB gene was 11.8 x10^6 in the multiple elicitor cultures compared to 5.7 x10^6 in the single elicitor cultures, an increase of 106% ($p < 0.001$).

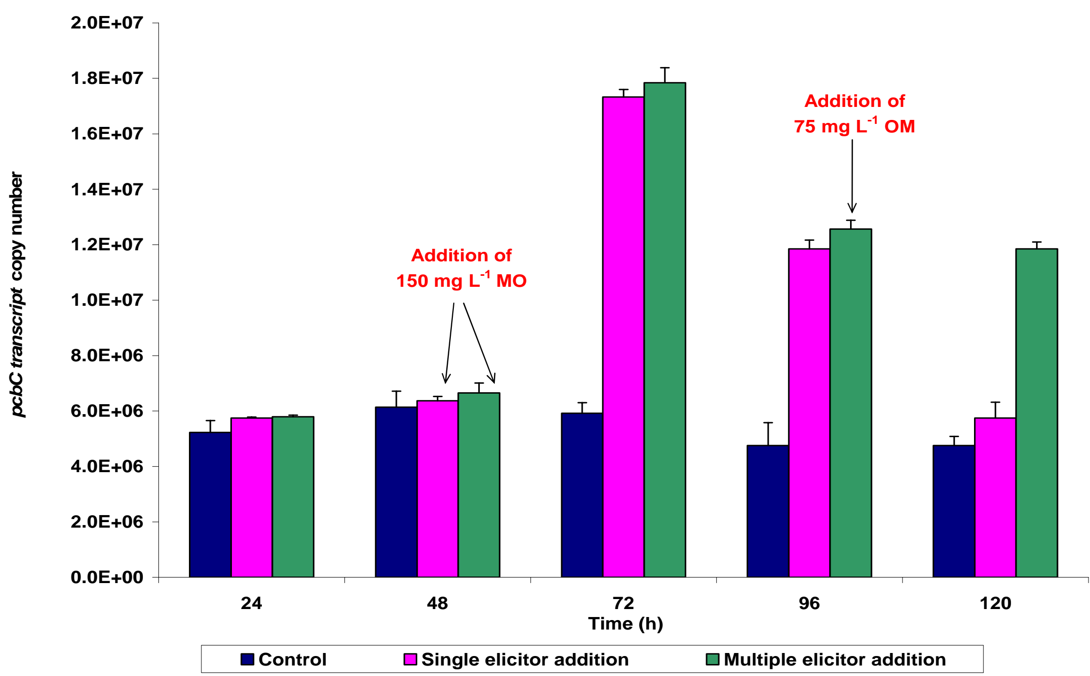


Figure 3.30 Transcript copy number of penicillin G biosynthetic gene *pcb*C during the course of cultivation in 5 L STR. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition culture (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h). Experiments were carried out in triplicate.

C) *pen*DE transcript copy number

The maximum *penDE* gene transcription in all the cultures was detected at 24 h (Figure 3.31). A steep decline in transcription was observed from 24 to 120 h. At 72 h after the addition of the first elicitor, the transcriptional level of *penDE* gene was higher 9.8 $x10^5$ for both the elicited cultures compared to the control cultures7.1 $x10^5$, an increase of 38% ($p < 0.001$). In the multiple elicitor cultures, after the addition of the second elicitor the transcription level increased. After the addition of the second elicitor the transcript copy number for *penDE* was 3.4 $x10^5$ in the multiple elicitor culture compared to 2.2 $x10^5$ in the single elicitor cultures, an increase of 50% ($p < 0.001$).

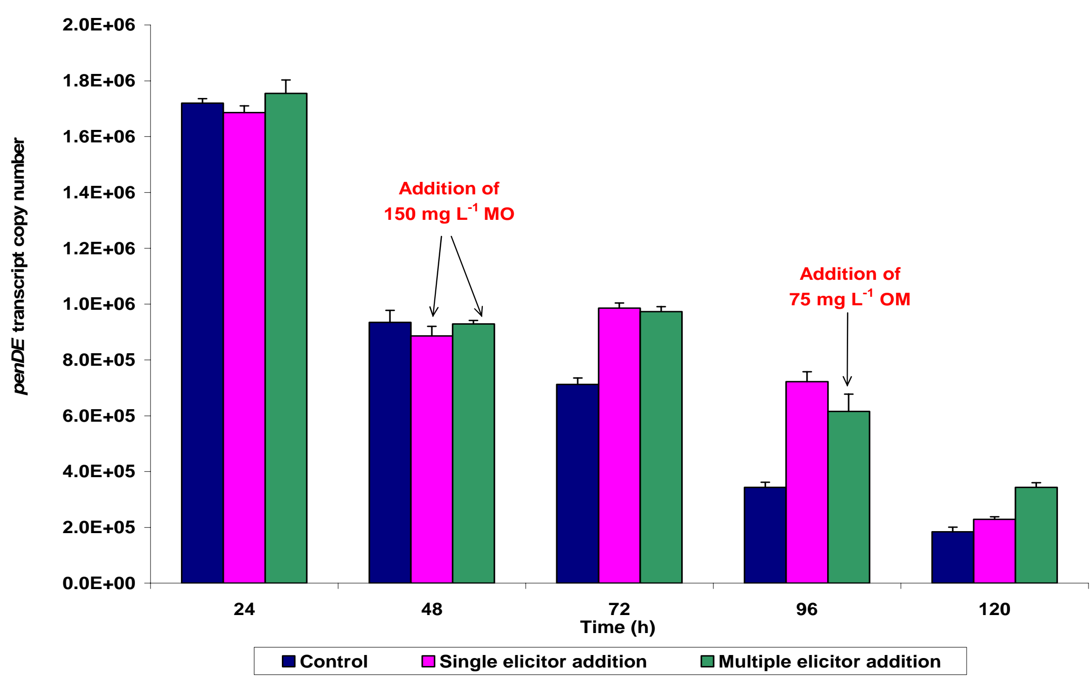


Figure 3.31 Transcript copy number of penicillin G biosynthetic gene *pen*DE during the course of cultivation in 5 L STR. Control (no elicitor added); single elicitor addition (150 mg L^{-1} of MO added at 48 h); multiple elicitor addition (150 mg L^{-1} of MO added at 48 h, followed by 75 mg L^{-1} of OM added at 96 h).

3.3 Ion flux studies

Studies on the cascade of events produced in plant cells by elicitors showed that a rapid flux in ions occur prior to the activation of the cell signalling system. Cytosolic calcium concentration is one of the most important signals within the cell. A change in cytosolic Ca^{2+} induced by a stimulus is the result of a change in the rate of uptake or release of Ca^{2+} across the cell membrane, or across the membranes of an intracellular organelle.

This section shows the results obtained in the study of the effect of elicitors on the cytosolic flux of calcium ions in *P. chrysogenum* P2 (ATCC 48271). The experiments were performed using fluorescent dyes. Two dyes were employed in this study, Fluo-4 AM, a fluorescent calcium indicator, which has an impressive array of applications for imaging Ca^{2+} fluxes that support cellular signal transduction and the transmission and propagation of impulses in excitable cells and CellTrace calcein red-orange AM, a cell-permeant dye, used to determine cell viability in most eukaryotic cells.

The dyes were successfully loaded in the cells following method development for dye loading technique. The method development included optimising the dye loading condition which included optimisation of the pH of the loading buffer. The fluorescence was then measured using confocal scanning laser microscopy (CSLM) and fluorescent plate readers.

3.3.1 Effect of different pH buffer on the uptake of the dyes in *P. chrysogenum* cultures

Mycelium of *P. chrysogenum*, grown for 48 h in PGM followed by 48 h in PPM, was harvested by centrifugation and washed in sterile PPM. Mycelium at a concentration of 10 g L^{-1} was incubated in sterile PPM with pH ranging from 4-9 for 15 min.

Samples were then taken at identical time periods and loaded with both the dyes and incubated for 45 min and later observed under CSLM at the similar conditions (Figure 3.32). Each sample was prepared and observed in triplicates. Error bars indicate variation between all readings.

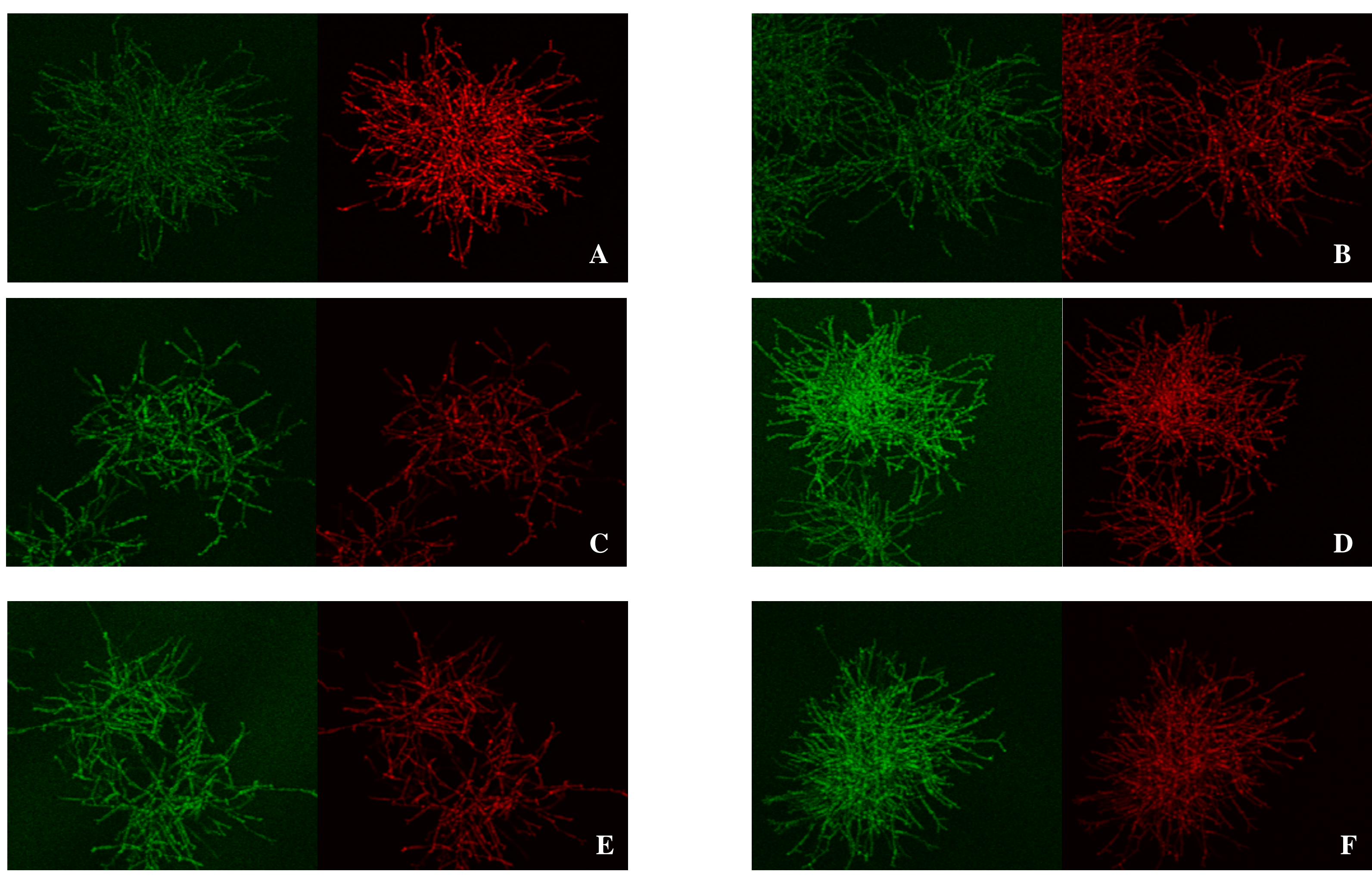


Figure 3.32 Effect of different pH on uptake of Fluo-4 (green) and Calcein (red) dyes in *P. chrysogenum* P2 (ATCC 48271) cultures. (A) pH 4.0 (B) pH 6.0 (C) pH 6.5 (D) pH 7.0 (E) pH 8.0 (F) pH 9.0

Figure 3.33 shows the quantitative analysis on the dye uptake in different pH conditions. The mean fluorescence intensities from each sample were analysed by measuring different region of interests from the sample culture observed in Figure 3.32. The graph shows that the optimum pH for the loading of the CellTrace calcein redorange dye is pH 4.0 and for Fluo-4, AM is pH 7.0. The average represents the efficiency of dye loading conditions where maximum amount of both dyes are loaded in the cell. Therefore pH 7.0 was selected as the effective pH condition for dual loading of the dyes.

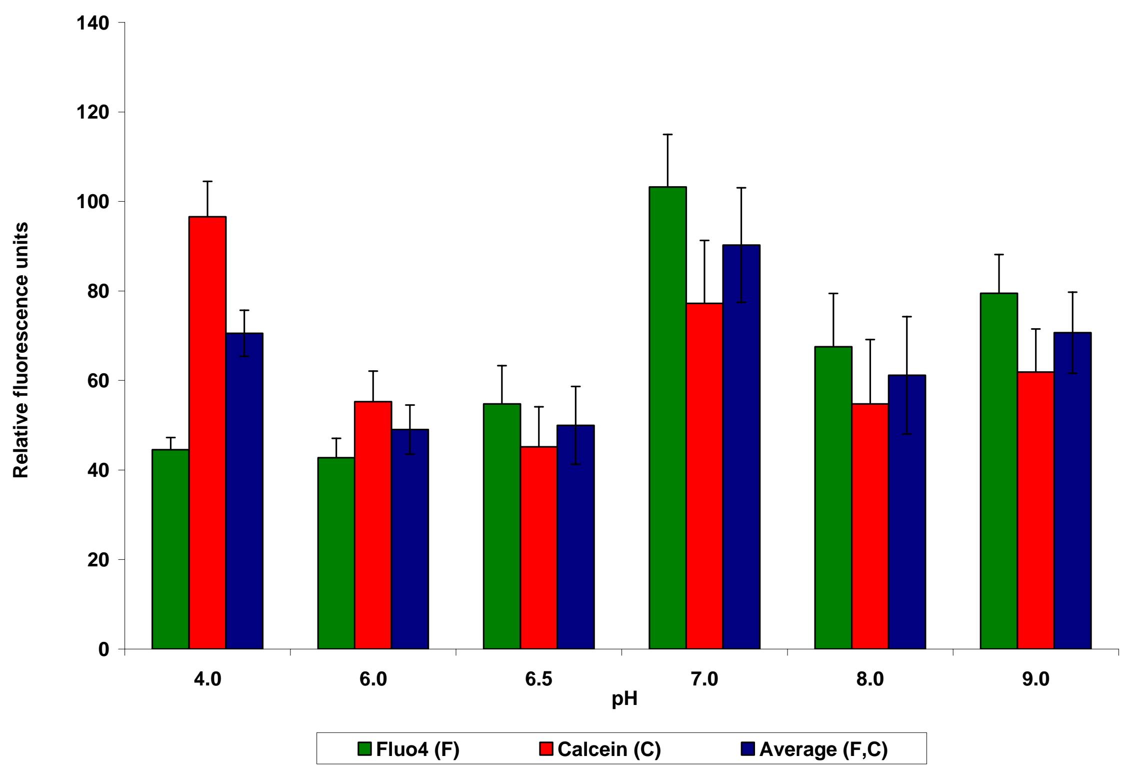


Figure 3.33 Effect of different pH buffers on dye uptake in *P. chrysogenum* P2 (ATCC 48271) cultures using Leica software.

3.3.2 Effect of elicitor addition on the calcium ion flux using fluorescent plate reader

Figure 3.34 compares the responses in relative fluorescence units obtained on the addition of MO, 150 mg L^{-1}, elicitor and water for control. The difference in fluorescence units though very low in terms of percentage increase, is very significant ($p < 0.0001$) in fluorescence units.

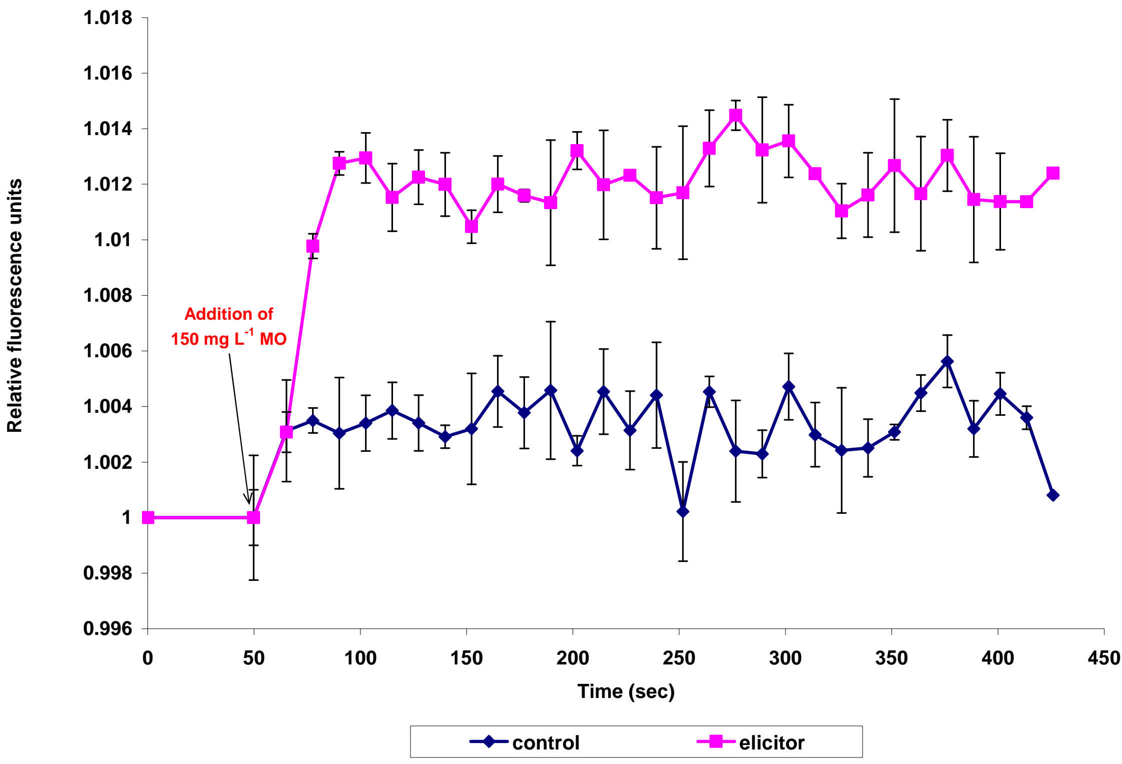
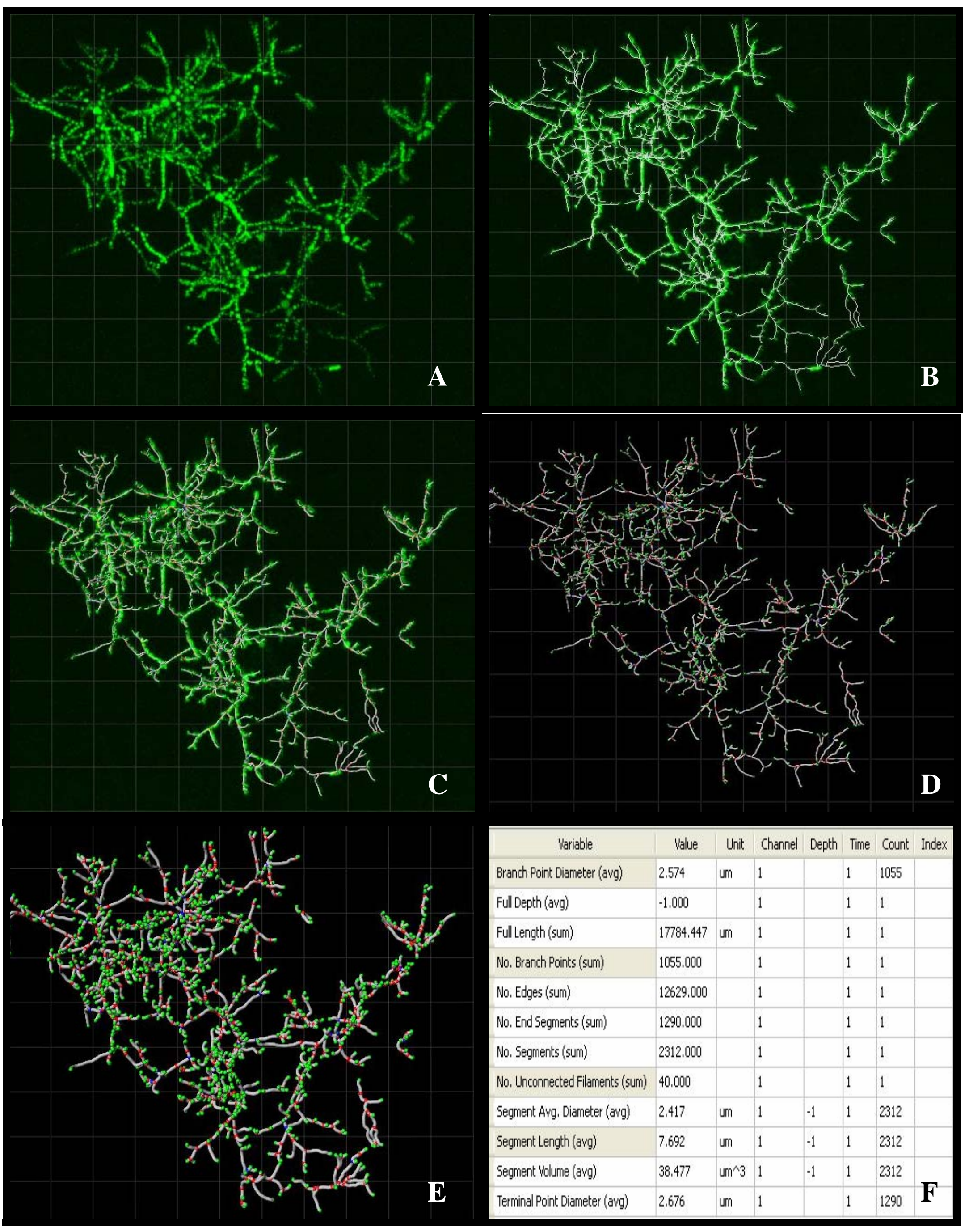


Figure 3.34 Effect of elicitor addition on the cytosolic calcium level in 48 h old cultures of *P. chrysogenum*. The change in flux is measured as relative fluorescence units by using fluorescent plate reader.

3.3.3 Application of Imaris Filament tracer software to study morphological image analysis.



Variable	Value	Unit	Channel	Depth	Time	Count	Index
Branch Point Diameter (avg)	2.574	um	1		1	1055	
Full Depth (avg)	-1.000		1		1	1	
Full Length (sum)	17784.447	um	1		1	1	
No. Branch Points (sum)	1055.000		1		1	1	
No. Edges (sum)	12629.000		1		1	1	
No. End Segments (sum)	1290.000		1		1	1	
No. Segments (sum)	2312.000		1		1	1	
No. Unconnected Filaments (sum)	40.000		1		1	1	
Segment Avg. Diameter (avg)	2.417	um	1	-1	1	2312	
Segment Length (avg)	7.692	um	1	-1	1	2312	
Segment Volume (avg)	38.477	um^3	1	-1	1	2312	
Terminal Point Diameter (avg)	2.676	um	1		1	1290	

Figure 3.35 Step by step process of morphological analysis using Filament Tracker software from Imaris. (A-E are the steps to obtain a 3D- structure, F is the morphological analysis).

Figure 3.35 shows the 3-D projections and Z-projections of confocal stacks and quantifications of average pixel intensities performed using Filament Tracer (Imaris 5.5) software from Bitplane AG, UK. As shown in the Figure 3.35, volume images are converted into a table that retains the topological information of the filament and calculates essential information including the length and diameter of the segments, and the number of branch points. Previous studies on morphological changes using 2D-software for image analysis has showed that in the presence of elicitors the number of hyphal tips increased compared to control. 3-D volume rendering would not be possible using conventional bright field microscopy. Application of the 3D-software would confirm and verify these results.

3.4 Conclusion to the results

Although elicitation in *P. chrysogenum* cultures is now well established, the mechanisms by which this process is induced have not yet been investigated. This work serves as a starting point for the elucidation of some possible mechanisms. Earlier studies with elicitation in *P. chrysogenum* using oligosaccharide elicitors have shown varying effects on the production of penicillin G (Ariyo *et al.,* 1997, 1998). After extensive studies MO was chosen for enhancing the penicillin G production and was added at 48 h in the production medium at 150 mg L^{-1} (Radman, 2002).

In this work, the effect of single elicitor addition (MO) to *P. chrysogenum* cultures showed a significant increase of 66% in the penicillin G level between, 72 h and 96 h compared to the control culture where no elicitor was supplemented ($p < 0.05$). But the production rate in single elicitor added culture was found to decrease gradually, 9% slower compared to the control cultures which showed a steep decrease as the fermentation proceeded. Similar pattern was observed when the culture was analysed for the three major penicillin biosynthetic genes using Real time PCR assay. It was found that the transcript level of the genes from single elicitor added cultures was significantly higher for the three major biosynthetic genes compared to the control cultures ($p < 0.001$).

The importance of structure-activity relationship of mannan oligosaccharides in the elicitation was investigated. In this study the reducing end of MO was reduced and the subsequently modified MO (MO-R) was used to study the structure-activity relationship in comparison to MO. Addition of MO-R resulted in a 65% decrease in the elicitor activity, but did not eliminate the ability of the oligosaccharide to enhance the production of penicillin G when compared to the untreated oligosaccharides.

The effect of multiple addition of the same elicitor was investigated with a prospect that repeated addition would re-trigger the stimulation resulting in either maintenance of the penicillin G levels or enhancement of the production rate. However, repeated addition of the same elicitor did not show any change in the production rate in comparison to single

additions. Multiple additions of different elicitor types at different time and concentration compared to the single addition were investigated and an optimisation study was carried out. This study showed that addition of a different elicitor, at different time and concentration, further enhanced the production level of the penicillin G. The optimisation studies showed that addition of 75 mg L^{-1} of OM at 96 h was the best condition for further enhancement of the penicillin G production rate. The effect of single and multiple elicitor addition was studied at the transcriptional level and was found that the transcript copy number of the elicited cultures was significantly higher ($p < 0.001$) for the three major penicillin G biosynthetic genes in comparison to the control cultures. A significant increase in the transcript copy was also observed number in the multiple elicitor added cultures compared to single elicitor added culture.

The effect of elicitor addition on the cytosolic calcium level was investigated. This study was performed using fluorescent dyes which specifically bind to cytosolic Ca^{2+}. The result was analysed using Confocal Scanning Laser Microscopy (CSLM) and FLUOstar Optima plate readers. The fluorescent dyes were successfully loaded in the cell after optimising the dye loading conditions. The addition of elicitors showed a small but significant increase in the cytosolic Ca^{2+} compared to control cultures to which no elicitor was added ($p < 0.001$). The fluorescent dye-loaded cultures were observed under CSLM using the Filament Tracer rendering 3-D volume to the images. This provided essential mycelial morphology information including the length and diameter of the segments and number of branch points.

CHAPTER IV

DISCUSSION

4.1 PRODUCTION AND ENHANCEMENT STUDIES

4.1.1 Single elicitor addition studies

The effect of oligosaccharide elicitor addition was first reported in plant cultures where the addition of oligosaccharide elicitors resulted in overproduction of secondary metabolites (Keen *et al.,* 1971). Elicitor concentration plays a very important role in the intensity response of the elicitation process. There are two types of dose-response curves known for the effects of biotic elicitors in plants: one which corresponds to a typical saturation profile where overdosage of the elicitor will not affect cell viability and the second type showing a sharp optimum (Dixon *et al*., 1981). Further increase in the concentration of the elicitors above the optimal level had either a negative effect or no effect on the secondary metabolite production. Namdeo and co-workers (2002) reported higher accumulation of ajmalicine in *C. roseus* cultures when treated with different concentrations of elicitor extracts of *T. viride, A. niger and F. moniliforme*. Ajmalicine accumulation was higher in cells elicited with higher concentration (5%) of elicitor extracts as compared to lower concentration (0.5%). However, increasing the concentration further to 10% adversely affected the accumulation of ajmalicine (Namdeo *et al.,* 2002). These results are also supported by the findings of Nef-Campa and co-workers (1994) and Rijhwani and Shanks (1998). High dosage of elicitor has been reported to induce hypersensitive response leading to cell death, whereas, an optimum level was required for induction (Mukandan and Hjorosto, 1990).

Therefore assuming similar dose responses in fungi, it is important to find the appropriate concentration of the oligosaccharides for the best enhancement. Further increase in the elicitor concentration above the optimal level have shown no significant effect on secondary metabolite production, probably due to the saturation of the elicitor binding sites. The elicitor effect is also correlated to mechanisms of hormone action reported in animal cells and an important aspect of negative regulation in hormone action is that cell surface receptors are internalized. This could also be true in the case of the effect of increasing concentration of elicitors in microbial cultures.

The time and the type of elicitor addition also plays a crucial role on the effect of elicitation. Elicitors added outside the time window would not have the desired responses as most of the elicitor stimulation effects are found to be part of defence mechanisms. In general, the elicitor is in contact with the system until harvest, but the time required for maximum secondary metabolite accumulation is a characteristic of each species and normally preceded by an increase in activity of the metabolic enzymes involved (Vasconsuelo and Boland, 2007). This suggests that cells had to reach certain physiological and biochemical competence before they could respond to external stimuli. Treatment of *R. tinctorum* cell cultures with *Pythium aphanidermatum* leads to a doubling of anthraquinone content which is preceded by a large rise in isochorismate synthase activity (Van Tegelen *et al*., 1999). In the case of *P. chrysogenum*, the penicillin production occurs at 48 h after the inoculation in the production media, hence addition of the elicitor early would not seem appropriate for the desired effect (secondary metabolite production). The role of the type (structure) and activity of the elicitor on the effect of elicitation will be discussed in detail in the next section 4.1.2.

The addition of different oligosaccharide elicitors to cultures of *P. chrysogenum* has been proven in the past to increase the production of secondary metabolites as compared to control fermentations to which no elicitors were added. The use of oligosaccharides for overproduction of penicillin G in fermentation of *P. chrysogenum* was the first reported effect of elicitation in fungi (Ariyo, 1995). In the present study, on the addition of the 150 mg L^{-1} of elicitor (MO), the penicillin G production rate increased compared to the control cultures. The induction of higher production rates implies that the oligosaccharides had a considerable effect on the penicillin G production pathway of the cells. A similar pattern was also observed in the rate of carbohydrate consumption. The rate of carbohydrate consumption was found to be higher in the single elicitor added cultures compared to control cultures. There was no notable difference in biomass between the control and elicited cultures. It can be speculated that as the concentration of the elicitor added was very low, it could not act as an utilisable carbon source, as at harvest there was still sufficient carbon source available and the elicitation effect was not directed towards higher biomass production. Sixty grams per litre of carbon sources were added to each fermenter (lactose, 50 g and sucrose, 10 g) resulting in a biomass growth of between

37-39 g L^{-1}. The addition of 150 mg L^{-1} carbohydrate elicitor cannot have a notable impact on the *P. chrysogenum* P2 biomass in terms of carbon balance.

These findings clearly show that the elicited cultures had a higher metabolic activity compared to the control cultures. The reasons for this are not clear but it can be speculated that the addition of elicitors somehow facilitates or activates particular biochemical pathways such as secondary metabolite production, production of precursor molecules, cell maintenance, development and transport of excess secondary metabolites. The increased metabolic activity is not concomitant with enhanced cell dry weight as there was no significant increase in the biomass. Similar results are observed in various plant cell cultures such as *Saussurea medusa* which produces valuable secondary metabolites such as jaceosidin and hispidulin on the addition of silver nitrate and glutathione (GSH) as elicitors (Zhao *et al.,* 2005). The activities of phenylalanine ammonia lyase were transiently increased after treatment with methyl jasmonate, which suggests that this elicitor modifies jaceosidin and hispidulin production by regulating the phenylpropanoid pathway (Fu *et al.,* 2006).

4.1.2 Single addition of reduced mannan oligosaccharides (MO-R)

Investigation of structure-activity relationship of oligosaccharides has shown that elicitor effectiveness depends on the structure of the oligosaccharide and presence of some functional groups or residues (Sharp *et al.,* 1984; McDougall and Fry, 1989; Cheong *et al.,* 1991). Both the reducing and non-reducing ends play a major role in specific structure-receptor binding and activity. The reducing end of a carbohydrate molecule is responsible for most of the responses in a chemical reaction and biological effects of the reducing end have been investigated in the past for different plant systems (Smith *et al.,* 1994 and Spiro *et al.,* 1998).

The importance of structure-activity relationship of oligosaccharides for maximal elicitor activity is exemplified by the significance of the non-reducing end of the branched trisaccharide of hepta-[β]-glucoside elicitor of phytoalexin accumulation in soybean microsomal membranes. Substitution of either the non-reducing terminal backbone glucosyl residue or the side-chain glucosyl residue closest to the non-reducing end with glucosaminyl or N-acetylglucosaminyl residues reduced the elicitor activity between 10 to 10,000-fold. Elicitor activity was also reported to be

further reduced by 1000-fold if the two side-chain glucosyl residues were attached to adjacent backbone glucosyl residues rather than to glucosyl residues separated by an unbranched residue (Cheong *et al.,* 1991). The conversion of the two non-reducing terminal glucose residues to glucosamine reduced the activity of the oligosaccharides on phytoalexin elicitation (Sharp *et al.,* 1984). Modification of the acidic groups of galactouronic acid by methylesterification abolishes the negative charge thereby reducing the elicitor activity of the oligosaccharide. Spiro and co-workers (1998) showed that the reducing end of the oligogalacturonides is important in the elicitation process in tobacco tissue culture. The reducing end C-1 of di- and trigalacturonides was shown to be required for induction of proteinase inhibitors in tomato plants (Moloshok *et al.,* 1992). The nature of the modification of the reducing end of a chitosan-derived octasaccharide does affect its ability to induce phytoalexin accumulation in pea; the methyl glycoside derivative retains full activity and the methoxyphenyl glycoside derivative is inactive (Hadwiger *et al.,* 1994). Menard and co-workers (2004) showed that a minimum chain length is vital for biological activity of unsulphated as well as sulphated laminarin and that the sulphate residues are essential and cannot be replaced by other anionic groups. Aldington and co-workers (1991) reported that the activity of galacturonic acid oligosaccharides is also dependent on the integrity of their acid groups.

In this study the reducing end of the mannan oligosaccharide was reduced and the the modified MO was used to study the structure-activity relationship. Addition of the treated oligosaccharide elictor MO-R with a modified reducing end resulted in a decrease in the elicitor activity. The treatment significantly reduced, but did not eliminate the ability of the oligosaccharides to enhance the production of penicillin G when compared to the untreated oligosaccharides. The rate of penicillin G production was 92% and 27% higher in the untreated and treated elicitor supplemented cultures compared to the control cultures ($p < 0.001$). The untreated elicitor MO addition had a significant effect, 25% increase ($p < 0.001$) on the rate of carbohydrate consumption by the end of the fermentation compared to control cultures. Importantly there was no significant difference in the carbohydrate consumption found between MO-R and control cultures. There was no significant differences in the biomass between the control and elicited cultures. This clearly shows that elicitation by oligosaccharides is a complex phenomenon and the results presented in

this thesis support the hypothesis that the reducing end plays a very crucial part in the structure-activity of the oligosaccharide elicitor. But the small increase in the penicillin G production in modified elicitor-added cultures shows that modification of the reducing end does not completely inactivate the elicitor activity, suggesting that it is not the only factor contributing to the phenomenon of elicitation. Probably the non-reducing end of the oligosaccharide might also play a role in the binding and activity of the elicitor.

This study suggests and supports the hypothesis that the biological activity of oligosaccharides could be achieved by multiple steps and one of them is by the cell receptors identifying oligosaccharides using the reducing end. Several early studies in plant cell cultures have indicated the presence of oligosaccharide binding sites present on cell surface. In particular, binding studies carried out with partially purified acid – or enzyme-released elicitor-active glucan from *P. sojae* mycelial walls demonstrated the the presence of high-affinity glucan binding sites on soybean root plasma membranes (Yoshikawa and Sugimoto, 1993). It is possible that the reducing end could react with the polar amino acids such as lysine or arginine, on the cell surface leading to a cell signal cascade. These results clearly suggest that the reducing end of the oligosaccharide elicitor could play a crucial role in the identification of the elicitor molecule by the cells and to trigger a metabolic cascade in a 'hormone-like' manner, as suggested in the proposed mechanism of elicitation in Section 1.9.

4.1.3 Multiple elicitor addition studies

In this study repeated addition of MO was designed along with another oligosaccharide elicitor derived from sodium alginate (OM) to investigate if repeated contact of the cells with the same or different elicitor would produce an increase in penicillin G concentration or sustain the production, compared to single addition and control cultures. Repeated addition of the same elicitor failed to show any significant increase in the penicillin G production compared to single addition and control cultures.

This correlates with the hypothesis observed in plant cells where cells respond to stimulation by elicitors along with rapid, transient alkalization of the growth

medium, but behave refractory to second treatment with the same stimulus (Felix *et al.,* 1998). Investigation of this phenomenon in plant cell culture has established that repeated addition of elicitor causes desensitization of cells in a time and concentration-dependent manner. It was observed that the elicitor stimulates the alkalization of the medium which results in a competition for the high affinity binding sites on the cell surface. Desensitization was not associated with increased inactivation of the stimulus or with a disappearance of high-affinity binding sites from the cell surface and thus appears to be caused by an intermediate step in signal transduction. Chemoperception systems in microbes and animals are often desensitized by the continuous presence of the stimulus, allowing an increase in the dynamic range of the sensory system (Dusenbery, 1992). In another experiment, cells reacted with a transient alkalinization of the growth medium when treated with chitin fragments, but did not respond when treated with a second dose of chitin fragments, although they still reacted to xylanase (Felix *et al.,* 1993) or ergosterol (Granado *et al.,* 1995). Reciprocally, when cells were treated with ergosterol, they were refractory to further stimulation with ergosterol but still responded to chitin fragments and xylanase (Granado *et al.,* 1995). These observations indicate that the different chemoperception systems are desensitized in an independent manner. The authors tested two hypotheses to account for the phenomenon of desensitization, first hypothesis was that desensitization might be connected to an increased ability of the cells to modify or inactivate the ligand and the second hypothesis was that the receptor itself might be altered in its affinity to the ligand, or that it might be inactivated or internalized. But neither of the two hypotheses was conclusively proven. Inducible and specific ion fluxes on plasma membranes represent very early events during elicitation of plant cells. The effect of *Phytophthora sojae*-derived β-glucan elicitors with different degrees of polymerization on the plasma membrane potential has been reported with transient depolarization of the plasma membrane. The elicitor concentration necessary for half-maximal depolarization closely resembled the corresponding binding affinities of soybean root membranes toward the respective β-glucans. Upon repeated elicitor treatment, the root cells responded partially refractory, suggesting a complex responsiveness of the system.

It was therefore my interest to study the effect of the addition of a different elicitor at different concentrations and addition times during the *P. chrysogenum* fermentation.

It was clearly observed, from the shaken flask optimization studies that the best choice of the second elicitor for enhancement of penicillin G production was OM and the condition of addition was optimized at 96 h and 75 mg L^{-1}. The results obtained from the shaken flask cultures and subsequently from STR cultures have shown significant increase in the penicillin G production rate and specific production rate. The increase in penicillin G production rate was 64% and 111% in shaken flask cultures, in comparison to 84% and 125% in STR for both the single and multiple elicited cultures respectively compared to the control cultures. A similar pattern of increase in the rate of carbohydrate consumption as reported previously in the single elicitor addition culture was observed when multiple elicitors were added to cultures compared to the control cultures. The rate of carbohydrate consumption in shaken flask cultures was 9% and 5% higher in the multiple and single elicitor added cultures respectively compared to the control cultures at the end of the fermentation run. The rate of carbohydrate consumption in 5 L STR was 30% and 15% higher in the multiple elicitor added and single elicitor added cultures respectively compared to the control cultures at end of the fermentation. There was no notable difference observed in biomass between the control and elicited cultures. The above results clearly show an increase in penicillin G production and carbohydrate consumption in cells grown under controlled conditions. The results also clearly indicate that the energy obtained from increasing carbohydrate consumption is channeled for increasing metabolic activities which in this study includes increasing secondary metabolite production.

This is the first report of repeated addition of elicitors leading to significant enhancement of a secondary metabolite production in *P. chrysogenum* cultures. It should also be noted that the increase in penicillin G production described here equals many years of laborious strain improvement and media optimization procedures. Clearly, a directed application of elicitation can greatly contribute to future improvements in penicillin production rates in *P. chrysogenum* which are crucial given the economic benefits and importance in general health care. This work can have an impact at a larger scale; the selection of this model system was used as a control for future application in different microbial systems.

4.2 MOLECULAR BIOLOGY STUDIES

4.2.1 Single and Multiple elicitor addition studies

Genes for the biosynthesis of secondary metabolites are usually arranged in clusters together with genes for resistance to the toxic action of secondary metabolites on the producer organisms. They are sometimes clustered with genes for biosynthesis of antibiotic precursors. The penicillin biosynthetic pathway has been studied in great detail and has been reviewed by several authors. The three major enzymatic steps in the biosynthetic pathway are catalysed by the enzymes ACVS, IPNS and IAT which are encoded by the structural genes denoted *pcb*AB, *pcb*C and *pen*DE. They are located in a large region on chromosome I (Fierro *et al.,* 1993). Transcription of *pen*DE is under control of an independent promoter. *pcb*AB and *pcb*C are divergently transcribed from a bidirectional promoter and show a similar pattern of temporal expression and regulation which suggests their coordinated expression. These promoter regions are under the control of a variety of regulatory mechanisms (Diez *et al.,* 1990).

Penalva *et al.,* (1998) reported that the combined over-expression of the *pcb*AB gene and the *pcb*C gene resulted in an improved penicillin production, compared to transformed strains over-expressing the genes alone. The expressions of these genes are subject to sophisticated controls by both nutritional and developmental factors. Penicillin biosynthesis in *P. chrysogenum* is strongly regulated by glucose. High glucose concentrations prevent the formation of ACVS and repress IPNS formation and only to a low extent IAT synthesis. Gutierrez and co-workers (1999b) reported that in lactose grown cultures of *P. chrysogenum* the onset of transcription of the *pcb*AB gene occurs early at 24 h, with significant increase from 24-48 h and then a constant steady state; the transcript levels of *pcb*C gene peaked at 48 h and then decreased slightly whereas the transcript levels of *pen*DE gene were low at 24 h and increased continuously up to 96 h. In this study it was observed that in the production medium which contained both sucrose and lactose, *pcb*AB and *pcb*C transcripts levels peaked at around the 72-96 h and then decreased slightly. In contrast the *pen*DE transcripts were detected at high levels in the beginning of the growth phase and the decreased gradually as the fermentation carried on. This supports the

observation in (1992) by Renno and co-workers and also indicates the tight transcriptional carbon regulation in *P.chrysogenum* cultures. Previous studies have reported a similar pattern of increase in the levels of intermediatory products and precursors such as IPN (Tamerler *et al.,* 2001) and 6-APA (Radman, 2002) which might suggest a relation with the enhanced biosynthetic gene copy number. Studies to analyse promoters of the penicillin biosynthesis gene have been reported for *P. chrysogenum* and *A. nidulans.* The promoter strengths of β-lactam biosynthetic genes were reported to be rather different and it was observed that the expression of *pcb*AB was much weaker compared to that of *pcb*C (Feng *et al.,* 1994); while it was shown that *pen*DE has lower expression than *pcb*C in *A. nidulans* (Brakhage *et al.,* 1992).

In this study, QPCR analysis suggests a direct correlation between the enhancement of penicillin G production and the transcription of its biosynthetic genes. Here, a quantitative relation between the increases in the transcript copy number to the product formation in a fungal culture is reported for the first time. It is suggested that the increase is due to the addition of oligosaccharide elicitors. Overall, there was a consistent and significant increase in the transcript copy number of the major biosynthetic genes in the elicited cultures compared to the control cultures. Within the elicited cultures, the multiple elicitor added cultures showed a significant increase at the transcriptional level for the three penicillin biosynthetic genes compared to the single elicitor added cultures and control cultures after the addition of the second elicitor. These results correspond to the penicillin G production levels observed in shaken flask and STR cultures on addition of multiple and single elicitors. The percentage increases in the transcript copy number were reported to be higher for the initial two penicillin biosynthetic genes (*pcb*AB and *pcb*C), correlating to their role as the rate limiting step for penicillin G production (Gunnarsson *et al.,* 2004). The results therefore clearly show the role of the elicitors at the transcriptional level of the biosynthetic genes of penicillin G.

There could be a number of reasons why the oligosaccharides elicitors instigate the overproduction of penicillin G in *P. chrysogenum.* The induced syntheses of penicillin G by elicitors suggest that these elicitors may function as activators of a stress signal or internal defence mechanism in this system. Van der Holst and co-

workers (2001) presented a compelling argument for a receptor based oligosaccharide induced mechanism for elicitation. This work is based on the hypothetical model based on plant elicitation where oligosaccharides are perceived by membrane-bound receptors. Upon binding, these membrane bound receptors get activated thereby transmitting signals through signal transduction which includes protein phosphorylation via a kinase that is either part of the cell membrane receptor itself or is involved in a complex with the membrane receptor. It seems plausible to speculate that the mechanism of elicitation in *P. chrysogenum* occurs according to the model suggested in section 1.9 where the elicitor initiates a chain of signals, possibly triggering a series of events inside the cell including transcriptional activation by the MAP kinases.

4.3 ION FLUX STUDIES: CYTOSOLIC CA^{2+} FLUX

Interest in measuring Ca^{2+} concentration has burgeoned with the discovery of numerous complex cellular processes that either regulate or respond to the level of cytosolic calcium ($[Ca^{2+}]c$). Calcium plays a pivotal role in eukaryotic cell signalling and is involved in the regulation of a wide range of biological activities. Changes in $[Ca^{2+}]c$ have been reported in response to various signals, including hormones, light, abiotic stress and microbial elicitors (Sanders *et al.,* 1999; Reddy, 2001; Rudd and Franklin-Tong, 2001; White and Broadley, 2003). How cells encode/decode the Ca^{2+} signals produced by a plethora of biotic and abiotic stimuli is one of the most fascinating questions in cell biology. In filamentous fungi, Ca^{2+} signalling has been implicated in numerous processes including hyphal tip growth (Jackson and Heath, 1993); hyphal branching (Robson *et al.,* 1991); sporulation (Roncal *et al.,*, 1993) and chitin synthesis (Martinez-Cadina and Ruiz-Herrera, 1987). Calcium signalling, however, is little understood in filamentous fungi largely because easy and routine methods for calcium measurement in living hyphae have been unavailable previously. At rest, eukaryotic cells maintain a very low $[Ca^{2+}]c$, (100-200 nM) despite the high Ca^{2+} concentration present in the extracellular environment or in intracellular Ca^{2+} stores. This calcium homeostasis generates a Ca^{2+} concentration gradient exploited by cells to transmit information. Indeed $[Ca^{2+}]c$ can be changed transiently in response to a stimulus, through a set of channels and pumps allowing Ca^{2+} fluxes across membranes. Studies on both animal and plant cells provide

evidence that the spatial and temporal changes in $[Ca^{2+}]c$ caused by a given signal contribute to the specificity of the biological outcome (Knight, 2000; Ng and McAinsh, 2003; Hetherington and Brownlee, 2004).

Transient changes are detected by intracellular Ca^{2+} sensors which are able to bind to Ca^{2+} and interact under physiological conditions triggering a signal. The direct demonstration of signal-response coupling via Ca^{2+} requires the measurement of $[Ca^{2+}]c$ in living cells. One approach to this is to image $[Ca^{2+}]c$ dynamics in cells loaded with Ca^{2+}-sensitive fluorescent dyes. One of the earlier events observed when plant cells were put into contact with elicitors was the elicitor-induced calcium influx generally resulting in changes from the Ca^{2+} resting level of 50 -100 nM to 1-5 μM, within 5 min after elicitor treatment (White and Broadley, 2003). The aim of the experiments discussed below was to investigate the possibility of a similar occurrence in fungal elicitation. The present study tests the applicability of using Ca^{2+}- sensitive dye to quantify and image cytosolic calcium $[Ca^{2+}]c$ in *P. chrysogenum* cultures chosen for their potential as an experimental system to study Ca^{2+}signalling. Ca^{2+} sensitive fluoroprobe, Fluo-4, AM has been employed to determine the effect of oligosaccharide elicitor addition on $[Ca^{2+}]c$ and to characterise the mechanisms involved in the response for fungal cells therefore providing quantitative, spatially and temporally resolved measurements for $[Ca^{2+}]c$ concentration as they undergo dynamic changes within the living cells.

In general the use of Ca^{2+} dyes for imaging and measuring ion concentrations in filamentous fungi is fraught with problems especially those associated with the introduction of the dye into the cell and secondly preventing it from becoming sequestered within the organelles (Read *et al.,* 1992; Parton *et al.,* 1997; Parton and Read, 1999). From the results, section 3.3.1, it is clearly demonstrated that it is possible to load the fluorescent dyes consistently into the fungal hyphae and that the introduced dyes can be imaged easily during hyphal growth by CSLM. Neither dye loading nor the confocal imaging disrupted the viability of the cells. Unfortunately the imaging of the $[Ca^{2+}]$ c flux could not be measured by CSLM in the living hyphae because the fungal cells were not immobilized to any support matrix and on the addition of the elicitor the cells moved out of focus. Experiments using low melting agarose to immobilize the fungal cultures showed consistent loading of the

fungal hyphae but repeated efforts to capture live imaging of the change in $[Ca^{2+}]c$ flux were unsuccessful. There are various reasons which prevent the imaging of the $[Ca^{2+}]c$ flux change. One is that the immobilized cells were not uniform and therefore it was difficult to obtain clear and homogenous representative image. Similar experimental design was setup to measure the $[Ca^{2+}]c$ flux using the fluorescent plate reader, which showed small but significant increase ($p < 0.0001$) in the relative fluorescence units on addition of the elicitor compared to control cultures (water addition). Previous studies have reported a significant decrease in the extracellular Ca^{2+} levels on the addition of oligosaccharide elicitor (Iturbe, 2005). The following study also reported that the addition of extracellular calcium in the buffer facilitated the efflux of K^{+} ions. Calcium ions could act at the channel proteins themselves, by binding to specific domains or through more indirect mechanism like protein phosphorylation that has been reported to occur prior to potassium flux. Calcium influx is a prerequisite to protein kinase activation and oxidative burst (Blatt *et al.,* 1999). This suggests a distinct calcium mediated signal transduction pathway involved in the mechanism of elicitation. This has been reported by others in different other cell systems.

4.4 APPLICATION OF FILAMENT TRACER FOR IMAGE ANALYSIS

Although filamentous micro-organisms such as fungi and *Streptomyces* sps are widely used in industrial fermentation processes, their growth and differentiation are not yet fully understood, because their biomass has a complex structure, and therefore difficult to describe and quantify. This lack of appropriate tools can hinder the optimisation and control of the fermentation. Characterisation of mycelia morphology is important for physiological studies of filamentous fungi. When grown in submerged culture, filamentous fungi exhibit different morphological forms, ranging from free mycelia trees to densely interwoven mycelial masses referred to as pellets. These morphological growth forms can have a significant effect on the rheology of the fermentation broth and thus the bioreactor performance. The extensive morphological differentiation exhibited by filamentous organisms is often associated with their involvement in chemical differentiation. A close relationship between a particular morphological form and increased process productivities is characteristic for a number of industrially important fermentations e.g. citric acid and

penicillin fermentations. The relationship between fungal morphology and process productivities has attracted interest from both academia and industry and attempts have been made to manipulate morphology to achieve maximal performance.

In recent years there has been a rapid development in fully automated systems for image analysis. Expertise in different research areas has contributed to the development of image analysis systems. Image analysis has been found to be a powerful tool in the investigation of mycelial morphology. This tool can be used to better understand the relationship between morphology and secondary metabolite production. Filament Tracer is the leading software product for the automatic detection of neurons in microscopic images. In this work a novel application of the fluorescent dye loaded fungal cell images were analysed to investigate the mycelial morphology. Studies on the effect of elicitor addition in cultures of *P. chrysogenum* have shown increases of up to 47% in hyphal tip number concomitant with an increase of up to 120% in penicillin G levels (Radman *et al*., 2004b). It is believed that production of penicillin G occurs at the tips of the hyphae. These studies were performed using conventional bright field microscopy which does not provide 3-D volume to images in order to obtain an absolute quantification of the morphological changes. Future application of this software to investigate the mycelial morphology could strengthen and verify these results.

CHAPTER V

CONCLUSION

This work has brought together the results of the effects of oligosaccharides on penicillin G production and changes at molecular level in liquid cultures of *P. chrysogenum*. The novel findings of this project contribute in the unravelling of the overall mechanism of elicitation in fungi. This has, potentially, significant impact on the industrial production of commercially useful microbial by-products.

The addition of the modified form of the oligosaccharides, with a reduced reducing end illustrated the relevance of the structure-activity of the oligosaccharide elicitor, MO in elicitation of penicillin G. The study showed that the activity of the elicitor is not only affected by the reducing end but also by the non-reducing end. The study also speculates the possible role of receptor binding proteins which could be identified by the elicitors. It also suggests that the reducing end of the oligosaccharide elicitor could play a crucial role in the identification of the elicitor molecule by the cells and triggering a metabolic cascade.

Repeated addition of the same elicitor did not show any change in penicillin G levels and production rate in comparison to single elicitor additions. This result suggested the desensitisation of the cultures in repeated contact with the same elicitor. This further supports the involvement of receptor binding sites in elicitation. Multiple additions of different elicitor types at different time and concentration compared to the single addition showed further enhancement in the penicillin G levels (from 1.57 g L^{-1} to 1.92 g L^{-1}) and production rate (from 100% to 150%). A deeper understanding of this phenomenon must await characterization of the receptor involved and the identification of the elements downstream in the signal chain.

The effect of single and multiple elicitor addition was studied at the transcriptional level and was found that the transcript copy number of the elicited cultures was significantly higher ($p < 0.001$) for the three major penicillin G biosynthetic genes *pcb*AB, *pcb*C and *pen*DE in comparison to the control cultures. This shows the possible role of elicitors in triggering signals which result in a series of events inside the cell including specific gene transcriptional activation by the MAP kinases.

An attempt to study the involvement of the change in cytosolic calcium ions on the addition of elicitors was reported in this work. A fluorescent method was developed for the analysis of cytosolic calcium changes in fungi. The fluorescent dyes were successfully loaded in the microbial cell after optimising the dye loading conditions. The addition of elicitors showed significant increase in the cytosolic Ca^{2+} compared to the control cultures to which no elicitor was added ($p < 0.0001$).

The results found in this work, together with previous reports on the effects of oligosaccharide addition to fungal cultures of different sp., brings forward the understanding of the mechanism of the action of elicitors in fungal cells.

CHAPTER VI

FUTURE WORK

The phenomenon of elicitation is very complex. The addition of an elicitor results in a series of coordinated events leading to stimulation of various morphological and /or physiological responses. Several models have been proposed to explain the biochemical basis of the elicitation phenomenon. The mechanism of elicitation in microbial culture is based on the hypothetic model of elicitation in plant culture. Incorporating all the past and current studies on elicitation in microbial cultures a picture of events can be speculated as shown in Figure 6.1

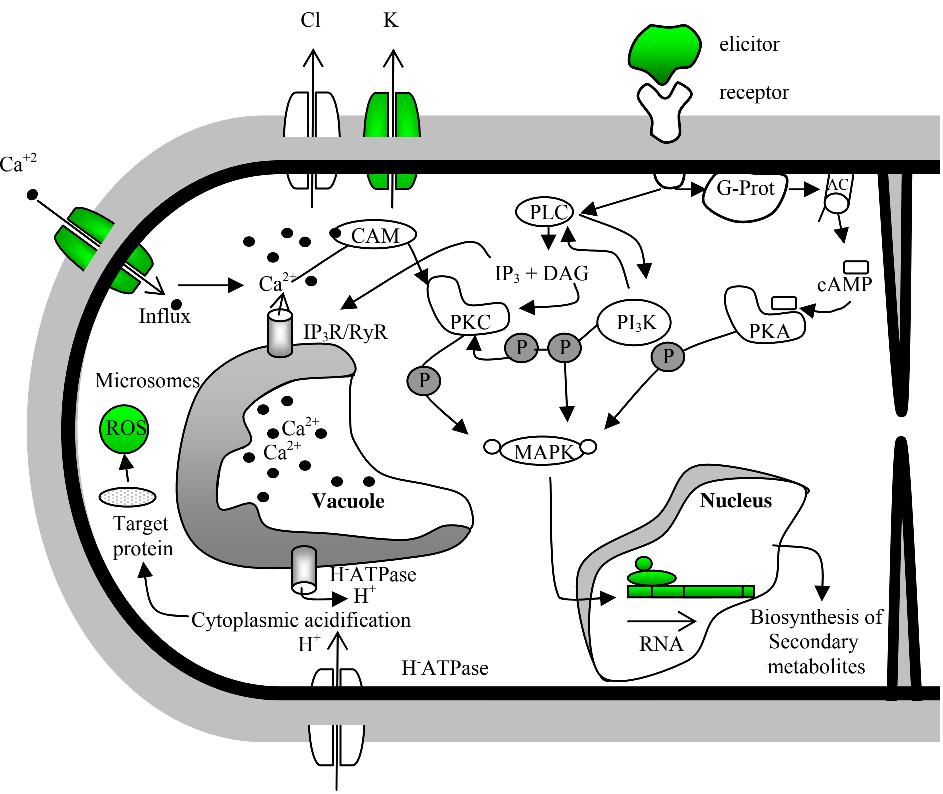


Figure 6.1 Achievements in the elucidation of the mechanism(s) of elicitation

The areas marked in green show the achievements in the elucidation of the mechanism of elicitation. Further studies in understanding the mechanism of elicitation would require, essentially, the identification of specific elicitor-binding receptors and the signal pathway involved in the transduction of the stimulus in response to elicitation.

Another important study would be to investigate the possible role of NADPH-dependent disulphide reductase system in elicitation. There are two NADPH-dependent disulphide reductase system characterized in *P. chrysogenum* at the

protein and gene level, Thioredoxin disulphide reductase (TrxAB) and Glutathione (GSH) disulphide reductase (Cohen *et al.,* 1994).

Once ACV is synthesized in the cell, it is capable of dimerising to bis-ACV, which withdraws it from further processing by IPNS (Figure 1.5). Moreover, bis-ACV inhibits the activity of ACVS (Theilgaard *et al.*, 1997). ACV may also form mixed disulphides with other thiol-containing compounds e.g., GSH. Re-entering of bis-ACV in penicillin biosynthesis is possible by the action of a NADPH-dependent disulphide reductase system (Figure 6.2).

Glutathione has been proved to play a crucial role in many important biochemical processes including protection of cells against oxidative stress (Emri *et al.,* 1997); number of bioreductive reactions, and detoxification of different xenobiotics. GSH is structurally analogous to ACV tripeptide. As a result GSH may suppress penicillin production by inhibiting ACVS and IPNS activities in *P. chrysogenum.* On the other hand, GSH may also contribute to the penicillin biosynthesis at very different points; namely GSH may serve as an important S-source in the biosynthetic process, may take part in the activation of the penicillin side chain precursors (phenyl) and (phenoxy) acetic acids and may maintain a suitable reduced milieu for the biosynthetic enzymes, e.g., for IPNS. In the idiophase of *P. chrysogenum* fermentations, when the productivity is highly dependent on the satisfactory dissolved oxygen concentration, reactive oxygen species may influence the GSH concentrations and hence the β-lactam biosynthesis itself.

To maintain metabolic viability and vitality of microbial strains used in industrial fermentations, the study of cellular responses to metabolic stresses is a very crucial subject for microbial technologists. These microbes are frequently confronted with environmental stresses occurring before, during, and after the fermentation process. Common stresses such as pH and temperature shocks, oxidative stress, osmotic stress and fermentation products toxicity may result in the impairment of the microbial growth, metabolic activity and seriously affect the profit of the industries. GSH is present in high concentration in most living cells from microorganisms to humans.

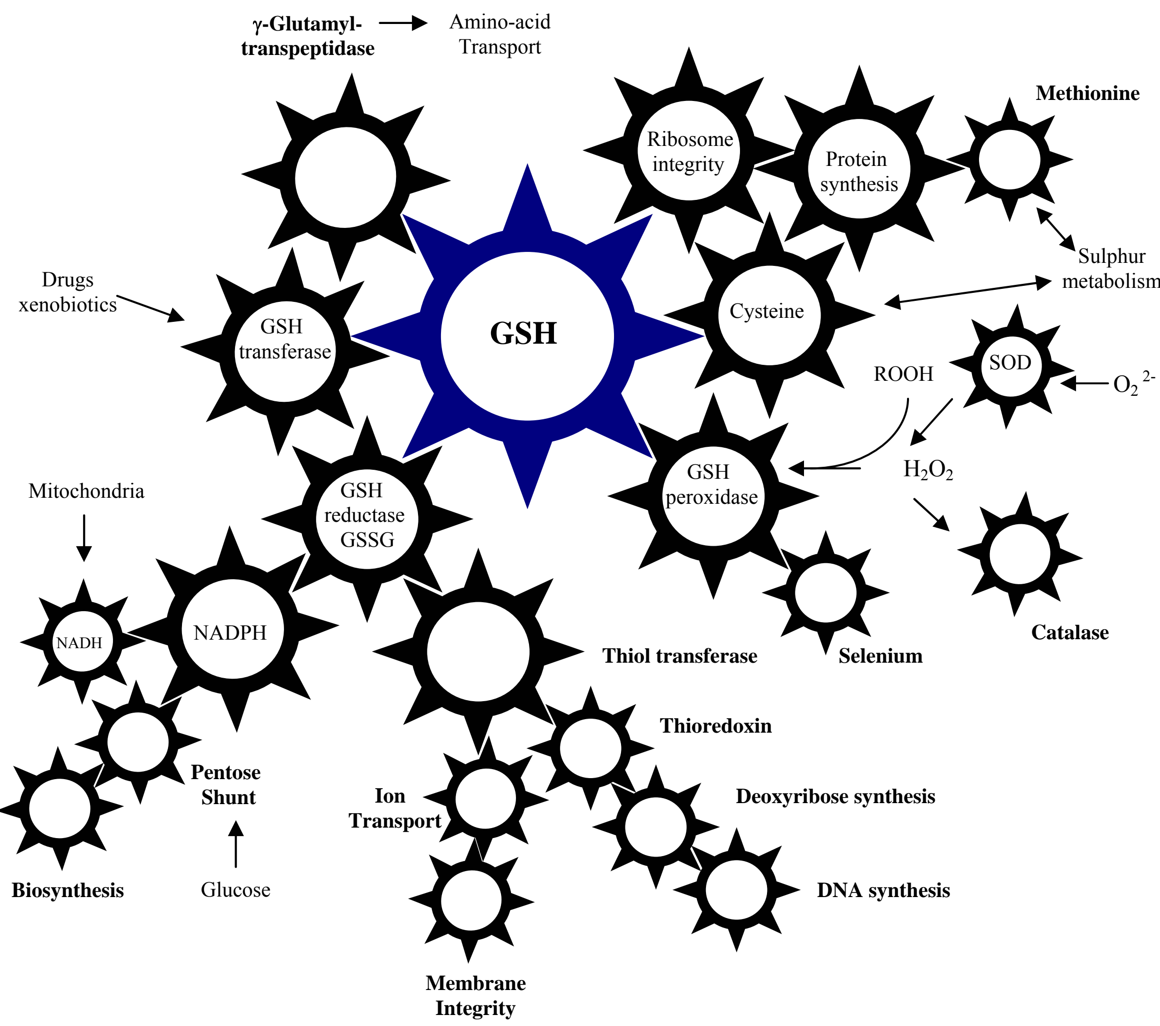


Figure 6.2. The interrelationship of GSH with cellular biochemical systems (Adapted from Mitchell, 1988)

Emri and co-workers (1997) reported that in addition to the GSH-dependent elimination of the peroxides a high catalase activity was present in *P. chrysogenum* cultures. The high peroxidase and catalase activities explain the remarkable resistance of *P. chrysogenum* to the oxidative stress caused by high concentrations of peroxides which were associated with significant increase in GSH disulphide reductase activities. It is tempting to speculate that the decrease in reactive oxygen species reported by Radman and co-workers (2006) in *P. chrysogenum* and *Streptomyces* sps. and the significant increase in the catalase activities reported in *P .chrysogenum* species (Radman, personal communication) and *Streptomyces* sps (Sangworachat, 2007) on addition of oligosaccharide elicitor could be related to the increased GSH disulphide reductase activity. The antibiotic biosynthetic pathway in

P .chrysogenum requires a crucial step of oxidative cyclisation of the IPN, which takes place under reduced condition.

It has been repeatedly shown or suggested that penicillin biosynthesis and GSH metabolism are interconnected (Henriksen *et al.,* 1997, Brakhage, 1998, Emri *et al.,* 1997, 1998). GSH-S-transferase, γ –glutamyl transpeptidase and GSH disulphide reductase are induced, GSH pool is depleted and the oxidized GSH concentration has been found to be increased when *P. chrysogenum* is grown in the presence of glutamate as N-source or when lactose or carbon-starvation conditions were used instead of growth on glucose (Emri *et al.,* 1997, 1998).

6.1 Investigation of the effect of elicitor addition on the LLD-ACV: bis-ACV ratio

The initial steps in the biosynthesis of penicillin G, involve the condensation of α-aminoadipic acid, cysteine and valine to form the tripeptide δ - (L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) and then the cyclisation of LLD-ACV to form isopenicillin N (IPN). LLD-ACV can exist in two states, either as the reduced monomeric form, which possess a free cysteine thiol, or as the oxidized disulfide (bis-ACV) dimer. Significantly only the reduced form of LLD-ACV is converted *in vivo* to IPN by IPN synthase (IPNS). Bis-ACV inhibits the activity of ACVS (Theilgaard *et al.,* 1997) and prevents from further processing from IPNS (Figure 1.5).

It is important to investigate whether the thioredoxin-thioredoxin reductase system (TR) together with the glutaredoxin reductase system (GR) would be able to preserve the reductive environment and maintain the LLD-ACV: bisACV ratio during the production phase of penicillin G. It could prove to be significant between the control and elicited cultures of *P. chrysogenum*. This can be studied by measuring the ratio of LLD ACV: bisACV, using HPLC method (Theilgaard and Nielsen, 1999) during fermentation of *P. chrysogenum* cultures.

6.2 Investigation of the effect of elicitor addition on the disulphide reductase systems (GR and TR) and superoxide dismutase (SOD) in *P. chrysogenum*

LLD-ACV synthesised in the cytosol is capable of dimerising to bis-ACV preventing it from further action by the IPNS. Re-entering of bis-ACV back to penicillin G pathway is possible by the action of a NADPH-dependent TR or GR system. Reactive oxygen species (ROS) are continuously generated in aerobic cultures. The decomposition of the H_2O_2, $OH^{\bullet}$ and $O_2^{\bullet -}$ is of primary importance to avoid severe oxidative injuries to the cells. Glutathione (GSH) plays a crucial role in many important biochemical processes including protection of cells against oxidative stress and a number of bioreductive reactions (Emri *et al.,* 1997). GSH may also serve as to maintain a suitable reduced milieu for the biosynthetic enzymes. SOD, catalase and GR system are interrelated with the GSH (Figure 6.2).

As reported in previous studies, there is a decrease in the ROS level (Radman *et al.,* 2004b) and a concominant increase in catalase levels (Radman, personal communication) on the addition of oligosaccharide elicitors to the *P. chrysogenum* cultures. Therefore investigation of the levels of SOD, glutathione reductase and thioredoxin reductase would give an insight on the effect of elicitor addition at the metabolic level in elicitor and control cultures. These assays can be performed using specific enzyme assay kits available in the market.

6.3 Investigation of the effect of elicitor addition on the phosphorylation of cytosolic proteins

Protein phosphorylation plays a central role for chemosensory transduction in prokaryotes and eukaryotes. Changes in protein phosphorylation have been linked to elicitation in plants. Continuous and rapid reversible-phosphorylation of specific protein substrates is an essential component of signal transduction in elicitor stimulated plant cells, converting receptor signals into cell-specific responses. Activation of protein kinases has been reported in several cases involving protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (Felix *et al.,* 1991; Kelly *et al.,* 1995; Yang *et al.,* 1997; Droillard *et al.,* 2000; Romeis, 2001; Agrawal *et al.,* 2002).

A pulse labelling technique (Felix *et al.,* 1991) can be applied to study the *in vivo* protein phosphorylation in fungal cells based on their highly efficient phosphate transport system. An effect on the phosphorylation of proteins would suggest the signal transduction mechanism leading to elicitation in fungal cells.

6.4 Investigation of the effect of elicitor addition on cytosolic Ca^{2+} ion flux

The experiments on cytosolic Ca^{2+} ion flux or dynamics did not provide definitive results on the involvement of cytosolic Ca^{2+} cascades in elicitation. The method developed to assess cytosolic Ca^{2+} dynamics between control and elicited cultures might need refinement. Further investigations would include an appropriate control to check whether the data obtained are based on the use free dyes are reliable. This would include a dextran-conjugated form of the dye used for the experiment or a

cytoplasmically targeted ion-sensitive recombinant probe, neither of which would be taken up by the organelles.

Alternatively, employing aequorin, a Ca^{2+} regulated photoprotein, as probe could prove useful in monitoring cytosolic free Ca^{2+}. Aequorin has been used successfully in different types of eukaryotic and prokaryotic cells. Its use has been greatly expanded by the availability of aequorin cDNA and the recombinant apoaequorin can be reconstituted into the active photoprotein by simple addition of coelenterazine, the prosthetic group, into the medium.

6.5 Investigation of the presence of specific elicitor binding receptor proteins

Signal perception is the first step of the elicitor signal transduction cascade. Various studies in plant cell culture have identified specific elicitor binding receptor proteins present on the cell surface. Identification of these binding proteins is crucial to study the mechanism of signal transduction.

This can be investigated by labelling the oligosaccharide elicitors which would provide information on the existence and involvement of receptor for these elicitors. This will also give an indication whether the binding proteins are specific or generic to the type of oligosaccharides and whether oligosaccharides derived from different sources have similar or different binding capacity. This will give an insight to the pathway of signal transduction and mechanism of action of elicitation in fungal cells.

6.6 Investigation of the effect of elicitor addition on the transmembrane potential.

Elicitation studies in plant cell cultures have reported plasma membrane depolarization in response to various physiological stimuli. Several processes could lead to plasma membrane depolarization, such as inhibition of proton pumps, activation or modulation of anion, Ca^{2+}, K^+ or nonselective channels. An anion channel could play a major role in elicitor-induced plasma membrane depolarization and elicitor signal transduction. Opening of such channel might allow Cl^- efflux and Ca^{2+} influx which in turn could drive K^+ efflux for repolarization of the membrane potential (Figure 6.1).

The membrane potential measurements studies can be performed using an electrical set up as described by Felle *et al.,* 1995 or alternatively using the MilliCell-ERS (Millipore). A much faster method for measuring membrane potential is the application of voltage-sensitive dyes on the FLIPR (Fluorescent Imaging Plate reader system) using the membrane potential assay kits (Molecular Devices, Sunnyvale, USA).

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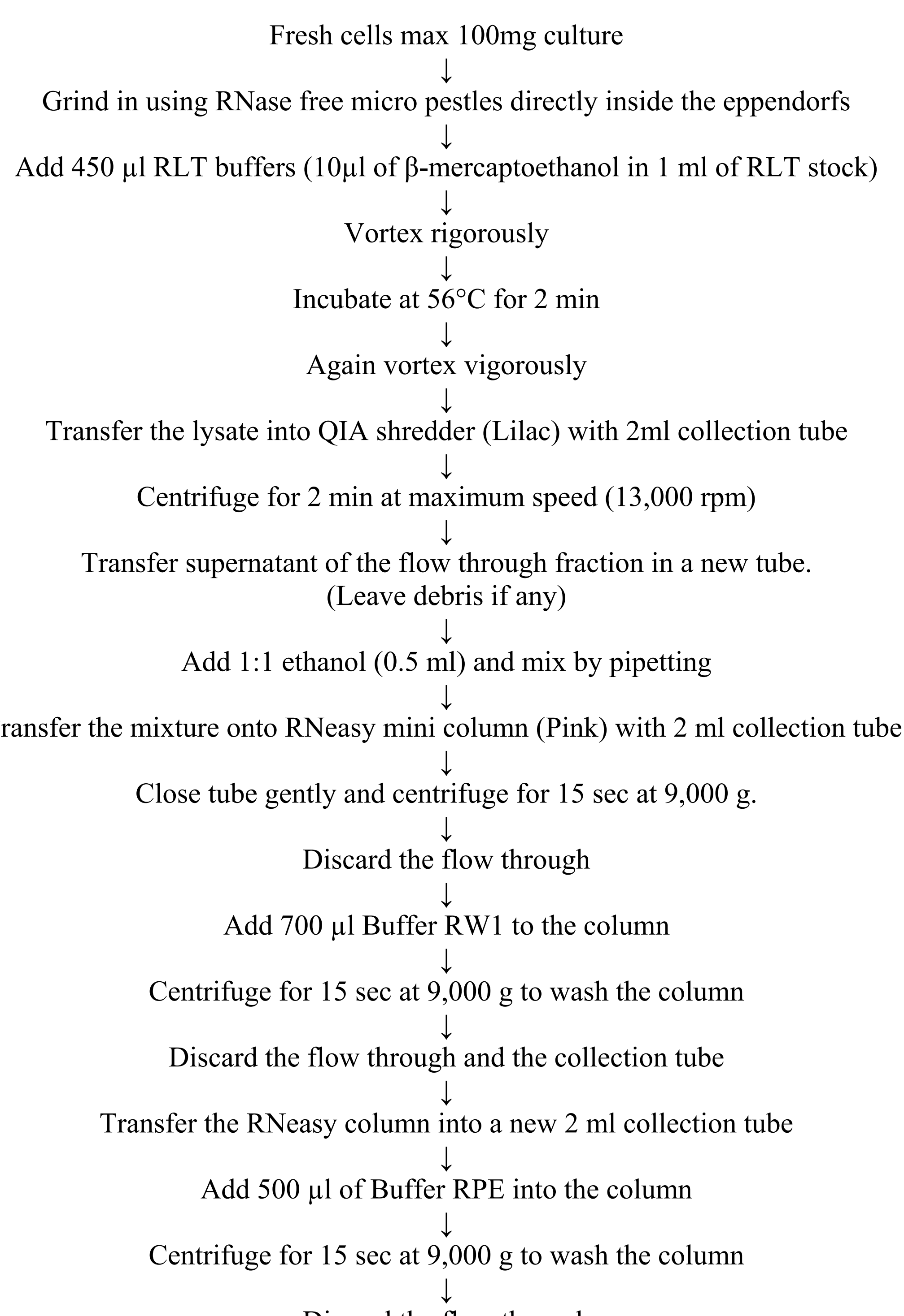
APPENDIX

Appendix 1

Total RNA isolation: Protocol from Qiagen RNeasy kit



↓
Add another 500 µl Buffer RPE to the column
↓
Centrifuge for 2 min at 9,000 g to dry the column
↓
Discard the flow through and the tube
↓
Centrifuge again at 13,000 g for 2 min to eliminate any ethanol carryover using a new collection tube
↓
Transfer the RNeasy column to a new 1.5 ml collection tube
↓
Elute with 25 µl of RNase free water in to the collection tube by centrifuging at 9,000g for 1 min
↓
Repeat again with another 25 µl of RNase free water in a new tube
↓
Then load the sample (10 µL + 2 µL loading dye in 1% agarose gel made in TBE buffer

Appendix 2

cDNA synthesis using ImProm-II Reverse Transcription System

Denaturation

Sterile eppendorfs are initially placed on ice
↓
Thaw the experimental RNA on ice and return any unused portion to the freezer
↓
On ice, combine the experimental RNA (up to 1 µg) and the cDNA primers (random) in nuclease free water for a final volume of 5 µl per RT reaction
↓
Multiply the volumes to accommodate multiple reactions
↓
Close each tube of RNA tightly.
Place the tubes into a preheated 70°C water bath for 5 minutes.
↓
Immediately chill in ice water for at least 5 minutes.
Spin each tube for 10 s in a microcentrifuge to collect the condensate and maintain the original volume.
↓

Keep the tubes closed and on ice until the RT mix is added.

Reverse Transcription

Prepare the RT mix by combining the following components
in a sterile 1.5 ml tube on ice. Vortex gently to mix.

↓

Experimental Reaction

Nuclease free water	6.7µl
5X Reaction buffer	4.0µl
$MgCl_2$, 25mM	1.8µl
dNTP mix, 10mM	1.0µl
Recombinant RNasin	0.5µl
Improm-II RT	1.0µl
Final volume	15 µl

Aliquot 15 µl of the RT mix to each reaction tube on ice

↓

Add 5µl of RNA and primer mix to each reaction, giving a final reaction volume of 20 µl per tube.

↓

Anneal

Place the tubes in a controlled temperature water bath at 25 °C and incubate 5 minutes.

↓

Extend

Incubate the tubes at 42°C in a water bath for up to one hour.

↓

Inactivate

Incubate the reaction tubes at 70°C for 15 minutes, to thermally inactivate the enzyme.

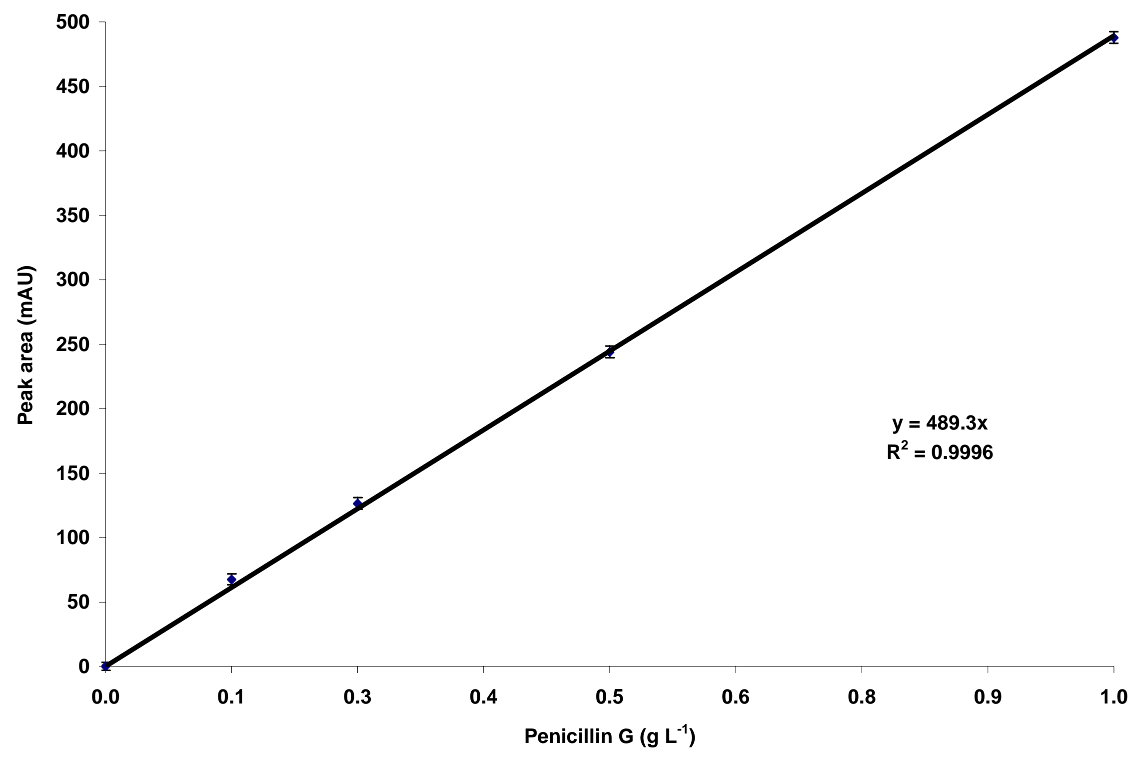
Appendix 3

Composition of 5x TBE Buffer (Tris-Borate-EDTA)

Component	Concentration (g L^{-1})
Tris Base	54.0
Boric acid	27.5
EDTA	4.5

Appendix 4

Standard Penicillin G curve



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Towards unravelling the elicitation mechanism in cultures of *Penicillium chrysogenum*: Chrysogenin Elicitation

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1. Introduction

Elicitors are substances that increase concentration of by-products when added to cultures as non-nutrient additives. Elicitors are classified as physical or chemical, biotic or abiotic and complex or defined depending on their origin and molecular structure. Filamentous microbes such as fungi and *Streptomycetes*, are widely used for production of secondary metabolites at the industrial scale. The effect of oligosaccharide elicitors as enhancers of secondary metabolite production has been studied in plant cell systems and recently in fungal cultures (Radman *et at*., 2003, p. 91). *Penicillium chrysogenum* has been used over the years as a model for better understanding of secondary metabolite production and its relationship to the microbe's physiological characteristics. Few studies have looked at this relationship when oligosaccharide elicitors are used, the most potent of which has been shown to be Mannan oligosaccharides derived from locust bean gum (Ariyo *et al.,* 1998, p. 165). Elicitation studies on *P. chrysogenum* for the enhancement of penicillin G, as a model secondary metabolite, have shown the antibiotic concentration increases considerably in the presences of Mannan oligosaccharides (Ariyo *et al.,* 1998, p. 165; Tamerler *et al.,* 2001, p. 53; Radman *et al*., 2004a, p. 147). A good knowledge of elicitation process mechanism(s) is required to exploit elicitation at industrial scale. Given the complexity of the process due to the many factors involved, unravelling of the mechanism is not an easy task. However, elicitation has provided a new path for the understanding of these complex interactions. Our recent studies have looked at the relationship of penicillin G production and its effects on morphology and Reactive Oxygen Species (ROS) production (Radman *et al*., 2004a, p. 147; Radman *et al*., 2004b, p. 40). Addition of oligosaccharide elicitor to cultures of *P. chrysogenum* results in increases of up to 47% in hyphal tip number (Radman *et al*., 2004b, p. 40) concomitant with an increase of up to 120% in penicillin G levels. Intracellulary in *P. chrysogenum*, elicitors not only exert an effect on secondary metabolite production but also on other metabolites production such as ROS. In the latter case in the presence of oligosaccharide elicitors a decrease in cellular ROS by up to 54% was observed (Radman *et al*., 2004a, p. 147).
The amount of chrysogenin, a pigment produced by the archetypal *P. chrysogenum* NRRL1951, was also enhanced after the addition of oligosaccharides derived from

sodium alginate and pectin (Asilonu *et al.,* 2000, p. 931). Chrysogenin is a yellow pigment produced by strains of *P. chrysogenum* that has not been well characterised since its description in 1930's. To understand further the mechanism of action of oligosaccharide elicitors this study investigates elicitation of chrysogenin in a different *P. chrysogenum* strain, MUCL 30168, in the presence of different elicitor, Mannan oligosaccharides.

2. Materials and Methods

2.1 Locust Bean Gum oligosaccharides

Enzymatic hydrolysis of locust bean gum (LBG) with Gamanase enzyme mixture (Novozyme Ltd, Denmark) resulted in the production of Mannan oligosaccharides (MO). Gamanase enzyme (0.1 mL) was added to 50 mL of distilled water in which 1.0 g LBG was dissolved and heated to 80 ^{0}C. The reaction was quenched by heating to 100 ^{0}C after 5 minutes. The mixture was cooled to room temperature then frozen at –70 ^{0}C and the frozen sample was then freeze dried.

2.2 Microorganism and Media

Penicillium chrysogenum MUCL 30168 used in this study was a gift from the culture collection of Université Catholique de Louvain, Belgium.
Two culture media were used in this study: *Penicillium* Growth Medium (PGM) and Penicillin Production Medium (PPM). For germination and inoculum development PGM was used in shaken flasks (SF) and it contained (gL^{-1}): $(NH_4)_2SO_4$, 13.00; $CaCl_2.2H_2O$, 0.05; $CuSO_4.5H_2O$, 0.01; EDTA, 0.55; $FeSO_4.7H_2O$, 0.25; $MgSO_4.7H_2O$, 0.25; $MnSO_4.H_2O$, 0.02; KH_2PO_4, 3.00; Na_2SO_4, 0.50; $ZnSO_4.7H_2O$, 0.02; Lactose, 10.00; Mycological peptone, 5.00 and Sucrose, 20.00. The pH of the medium was adjusted to 6.5 with KOH prior to inoculation. For penicillin G production PGM was used in stirred tank reactors (STR) and it contained (gL^{-1}): $(NH_4)_2SO_4$, 13.00; $CaCl_2.2H_2O$, 0.05; $CuSO_4.5H_2O$, 0.01; EDTA, 0.55; $FeSO_4.7H_2O$, 0.25; $MgSO_4.7H_2O$, 0.25; $MnSO_4.H_2O$, 0.02; KH_2PO_4, 3.00; Na_2SO_4, 0.50; $ZnSO_4.7H_2O$, 0.02; Lactose, 50.00; Mycological peptone, 1.00 and Sucrose, 10.00. Medium pH was adjusted to 6.5 with KOH prior to inoculation. For shaken flask studies calcium carbonate was added to a final concentration of 10 gL^{-1}.

2.3 Culture Conditions

2.3.1 Shaken flask studies

For shaken flasks studies *P. chrysogenum* MUCL 30168 inoculum was prepared in 100 mL lots of sterile PGM in 500 mL flasks. The shaken flask cultures were incubated in an orbital shaker at 26 ^{0}C at a speed of 200 rpm with 2 cm throw for 48 hours before their aseptic transfer to PPM medium for chrysogenin production. Sterile PPM (200 mL lots) in 1 L flasks were used for shaken flask studies.

2.3.2 Bioreactor studies

For bioreactor studies *P. chrysogenum* MUCL 30168 inoculum was prepared in 100 mL lots of sterile PGM in 500 mL flasks. The shaken flask cultures were incubated in an orbital shaker at 26 ^{0}C at a speed of 200 rpm with 2 cm throw for 48 hours before their aseptic transfer to the fermenter. Two stirred tank bioreactors (2 L STR, FerMac360, Electrolab Ltd., UK) with the same parameters were used for bioreactor studies. Sterilisation of bioreactors was at 121 ^{0}C for 40 minutes while containing only the salts solution of the production medium. Carbon and nitrogen sources were sterilised separately and $FeSO_4.7H_2O$ was filter sterilised. These were added to the bioreactors aseptically to make a total medium volume of 1.35 L before inoculation with 150 mL of the 48-hour shaken flask cultures (fermentation working volume of 1.5L).

Control (no oligosaccharide supplement) and test fermentations were carried out in parallel using the same inoculum and the same batch of medium. The pH of the fermentations was automatically maintained between 6.5 and 6.7 using sterile 2M ammonium hydroxide and sulphuric acid. Dissolved oxygen tension was controlled automatically above 30 percent air saturation with stirrer speeds ranging from 250 to 600 rpm. The changes in the agitation rates for the control and the elicited fermentations were similar. The temperature and airflow-rate were kept at 26 ^{0}C and l.0 vvm respectively.

2.4 Addition of oligosaccharides

For both shaken flask and bioreactor studies the time of addition was at 48 hours after inoculation and the concentration of the oligosaccharide elicitor added was 150 mgL^{-1}.

2.5 Analysis of the sample for growth, total carbohydrate concentration and chrysogenin levels

Biomass production was measured as cell dry weight (CDW) per litre of culture broth. For shaken flask studies total biomass was obtained at the end of experiment.

For analysis of chrysogenin levels samples of filtered broth (1mL) were added to 9 mL of ethanol and centrifuged at 2100 g for 10 minutes to remove precipitate. The supernatant was measured at 410 nm. Supernatants having absorbencies greater than 0.6 were diluted with ethanol before further spectrophotometric measurements were carried out. The zero reference used was PPM sampled from shaken flasks/bioreactors before inoculation. The reference sample was treated as the sterile broth samples before use.

Total carbohydrate content of fermentation broth was determined by phenol sulphuric acid assay as described by Chaplin and Kennedy (1994). Phenol sulphuric acid assay was carried out in glass test tubes by adding 200 μL of 5% phenol solution to 200 μL of standard, samples or blank (water) and mixing by gentle shaking in a fume cupboard. Phenol solution was made in HPLC grade water. Concentrated sulphuric acid (1.0 mL) was then rapidly added to the surface of the solutions without touching the sides of the tubes and allowed to stand undisturbed for 10 minutes. After 10 minutes, the tubes were mixed thoroughly and allowed to stand for a further 30 minutes. The absorbance of the reaction mixtures was determined at 490 nm using a Perkin Elmer Lambda 35 UV/VIS

spectrophotometer. Carbohydrate concentration of the samples was determined by using glucose standard curve in the range of 10- 100 µg/mL.

3. Results and Discussion

As mentioned in the previous section, the operating conditions were held the same for both fermentations in the bioreactors. The stirrer speed was automatically increased (Fig. 1) to keep the %DOT above 30% at all times. Under these conditions, the %DOT profile was similar in both fermentations. Mannan oligosaccharides had notable effect on the production of chrysogenin in both shaken flask and bioreactor cultures. In shaken flasks peak elicitation was observed at 96 hours with a maximum increase in chrysogenin level (as absorbance reading at 410 nm) of 17%, compared to the control. Although a growth profile could not be obtained for shaken flask studies due to sample size limitations, the total biomass difference at the end of fermentations between the control and the elicited cultures varied within 10% of the mean. Elicitor-supplemented cultures of *P. chrysogenum* MUCL 30168 were also shown to have an enhanced metabolic activity. The maximum rate of carbon utilisation was 66% higher in Mannan oligosaccharide supplemented cultures compared to the control (data not shown).
In bioreactor cultures the highest elicitation occurred earlier, at 72 hours, and the percentage increase in chrysogenin compared to the control level was much higher (55%). Moreover, the overproduction of chrysogenin was sustained for up to 120 hours compared to 96 hours in shaken flasks.
For bioreactors the maximum rate of carbon utilisation was 50% higher in Mannan oligosaccharide supplemented cultures compared to the control. Although this is lower than in shaken flasks, the overall rates of carbon utilisation were higher for bioreactor cultures. This is due to better mixing and aeration in bioreactors. There are no significant differences in biomass concentrations between the control and the elicited

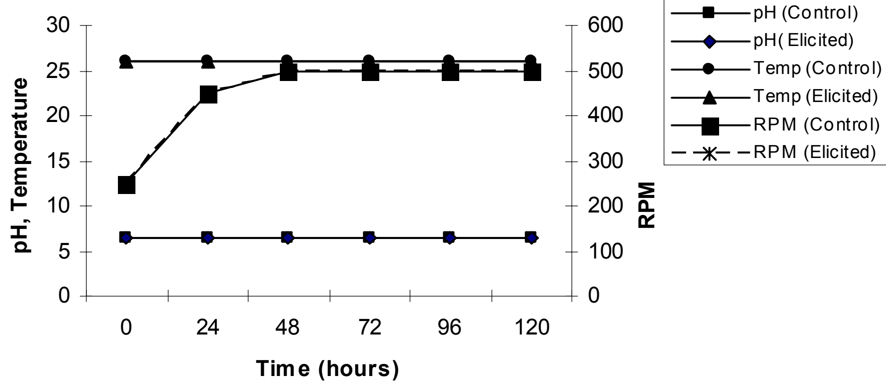

Figure 1. Graph showing the different parameters maintained during the 2L fermentation of *P. chrysogenum* MUCL 30168

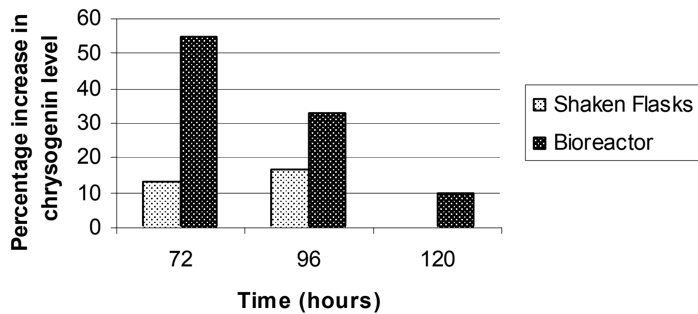

Figure 2. Percentage increase in chrysogenin levels (absorbance at 410 nm) in the elicited cultures of *P. chrysogenum* MULC 30168 compared to the control.

cultures indicating that the changes in metabolic activity (higher carbohydrate consumption in the supplemented cultures) have culminated in increases in chrysogenin level.

All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant (*P<0.05*, Anova, SPSS12, SPSS Inc., USA).

The biological function of chrysogenin is not known and the mechanism that leads to the overproduction of the pigment can only be speculated. It is plausible, as previously hypothesised (Radman *et al*., 2003, p. 91) that a cascade mechanism could be at play that leads to a transduction mechanism taking place at cellular receptor level.

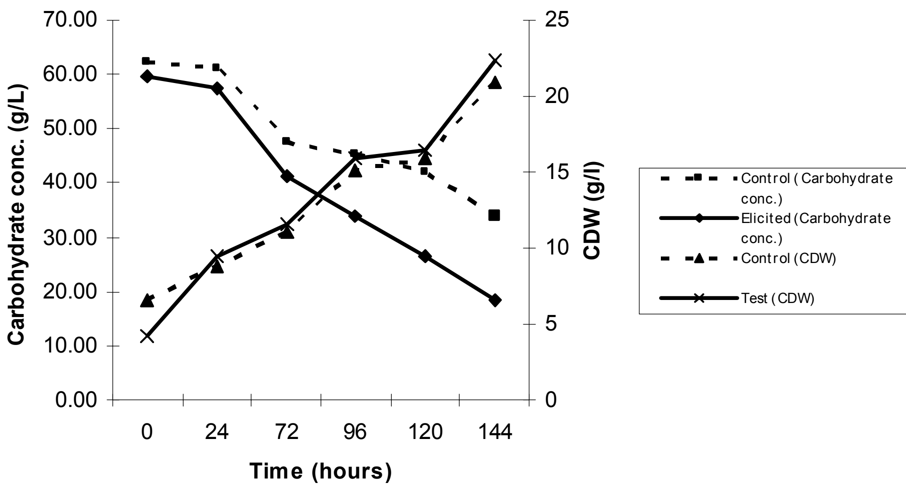

Figure 3. Dry cell weight and carbohydrate assay for 2L bioreactor cultures of *P. chrysogenum* MUCL 30168.

This study shows that although the strain used is different from that previously reported, chrysogenin levels can still be enhanced by the use of elicitors.
If indeed elicitation can be demonstrated to be generic in character then this can have implication for both research and industry. For research it will allow to further investigation into the elucidation of the mechanism of elicitation. For industry it will allow for a seamless transfer and implementation of elicitation strategies from public research to industrial sensitive application.

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ELICITOR EFFECTS ON CHRYSOGENIN PRODUCTION IN LIQUID CULTURES OF *Penicillium chrysogenum* USING MANNAN OLIGOSACCHARIDES

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Effect of mannan oligosaccharides preparation (MO) as elicitor on chrysogenin pigment production in liquid cultures of two different strains of *Penicillium chrysogenum* species was investigated. Addition of MO to the liquid cultures at 48 h after inoculation enhanced chrysogenin production in comparison to control cultures (no elicitor added). Chrysogenin production was increased by up to 55% and 27% in *P. chrysogenum* cultures of MUCL 30168 and NRRL 1951 respectively.

INTRODUCTION

The fungal kingdom offers enormous biodiversity, with around 70,000 known species, and an estimated 1-5 million species in total. Most of these are filamentous fungi with great metabolic complexity. In particular, they are known for their production of enzymes and secondary metabolites, many of which have been exploited (Turner, 2000). Elicitors are substances that increase concentration of metabolites when added to cultures as non-nutrient additives. This phenomenon is termed as "elicitation". Elicitors are classified as physical or chemical, biotic or abiotic and complex or defined depending on their origin and molecular structure. Filamentous microbes such as fungi and *Streptomyces* are widely used for production of secondary metabolites at the industrial scale. The effect of oligosaccharide elicitors as enhancers of secondary metabolite production has been studied in plant cell systems and recently in fungal cultures (Radman *et at*., 2003, p. 91). *P. chrysogenum* has been used over the years as a model for better understanding of secondary metabolite production and its relationship to the microbe's physiological characteristics. Few studies have looked at this relationship when oligosaccharide elicitors are used, the most potent of which has been shown to be mannan oligosaccharides preparation derived from locust bean gum (Ariyo *et al.,* 1998, p. 165). Elicitation studies on *P. chrysogenum* for the enhancement of penicillin G, as a model secondary metabolite, have shown considerable increases in the antibiotic concentration in presence of mannan oligosaccharides (Ariyo *et al.,* 1998, p. 165; Tamerler *et al.,* 2001, p. 53; Radman *et al*., 2004a, p. 147). Recently we have studied the relationship between penicillin G production and morphology of the fungus (Radman *et al*., 2004b, p 229). Addition of oligosaccharide elicitors to cultures of *P. chrysogenum* results in an increase of up to 47% in hyphal tip number concomitant with an increase of up to 120% in penicillin G levels. Intracellularly, in *P. chrysogenum*, elicitors not only exert an effect on secondary metabolite production but also on other metabolites production such as ROS. In the latter case in the presence of oligosaccharide elicitors a decrease in cellular ROS by up to 54% was observed (Radman *et al*., 2004a, p. 147). It has also been suggested that the elicitors increase the transcriptional level of the three penicillin biosynthetic genes, namely *pcb*AB, *pcb*C, and *pen*DE (Gang *et al*., 2001).

The effect of elicitors on pigment production has also been reported in *P. chrysogenum* [NRRL 1951 (Asilonu *et al*., 2000) and MUCL 30168 (Nair *et al*., 2005)] and in *Monascus* species (Shin *et al*., 1998). Elicitation in pigment production has also been found in plant cell culture systems (Hirner *et al*., 2001, p. 315).

Chrysogenin is a yellow pigment produced by strains of *P. chrysogenum* and has not been well characterized since its description in the 1930's (Clutterbuck *et al*., 1932). However, the producer of the pigment, *P. chrysogenum* is a well-studied fungus, thus a good model to study pigment enhancement by oligosaccharides in

fungal cultures. This study for the first time investigates the generic effect of mannan oligosaccharides preparation as elicitor in the production of chrysogenin from different strains of *P. chrysogenum*.

MATERIALS AND METHODS

Culture

P. chrysogenum MUCL 30168 and NRRL 1951 (Rockville, MD) was used for the study of chrysogenin pigment production. *P. chrysogenum* MUCL 30168 used in this study was kindly donated by the Université Catholique de Louvain, Belgium.

Media

Two culture media were used in this study: Growth Medium (GM) and Production Medium (PM). For germination and inoculum development GM was used in shaken flasks (SF) and it contained (g L^{-1}): $(NH_4)_2SO_4$, 13.00; $CaCl_2.2H_2O$, 0.05; $CuSO_4.5H_2O$, 0.01; EDTA, 0.55; $FeSO_4.7H_2O$, 0.25; $MgSO_4.7H_2O$, 0.25; $MnSO_4.H_2O$, 0.02; KH_2PO_4, 3.00; Na_2SO_4, 0.50; $ZnSO_4.7H_2O$, 0.02; Lactose, 10.00; Mycological peptone, 5.00 and Sucrose, 20.00. The pH of the medium was adjusted to 6.5 with 2M KOH prior to inoculation. For chrysogenin production PM was used in stirred tank reactors (STR) and it contained (g L^{-1}): $(NH_4)_2SO_4$, 13.00; $CaCl_2.2H_2O$, 0.05; $CuSO_4.5H_2O$, 0.01; EDTA, 0.55; $FeSO_4.7H_2O$, 0.25; $MgSO_4.7H_2O$, 0.25; $MnSO_4.H_2O$, 0.02; KH_2PO_4, 3.00; Na_2SO_4, 0.50; $ZnSO_4.7H_2O$, 0.02; Lactose, 50.00; Mycological peptone, 1.00 and Sucrose, 10.00. The pH of the medium was adjusted to 6.5 with 2M KOH prior to inoculation. For shaken flask studies calcium carbonate was added to a final concentration of 10 g L^{-1}.

Preparation of mannan oligosaccharides

Enzymatic hydrolysis of locust bean gum (LBG) with Gammanase enzyme mixture (Novozyme Ltd, Denmark) resulted in the production of mannan oligosaccharides preparation (MO). Gammanase enzyme (0.1 mL) was added to 50 mL of distilled water in which 1.0 g LBG was dissolved and heated to 80 ^{0}C. The reaction was quenched by heating to 100 ^{0}C after 5 minutes. The mixture was cooled to room temperature then frozen at –70 ^{0}C and the frozen sample was then freeze dried. Thin layer chromatography was used to assign the degree of polymerization (5-8).

Culture conditions

The inoculum was prepared in 100 mL sterile GM in 500 mL flasks. The shaken flask cultures were incubated in an orbital shaker at 26 ^{0}C at a speed of 200 rpm with 2 cm throw for 48 hours before aseptically transferring to the fermenter. Two stirred tank bioreactors (2 L STR, FerMac360, Electrolab Ltd., UK) with the same parameters were used for bioreactor studies. The 2 L STR used had an internal diameter of 10 cm, a shaft length of 20 cm to which two impellers were attached. No baffles were used for fermentations. Sterilisation of bioreactors was at 121 ^{0}C for 40 minutes while containing only the salts solution of the production medium. Carbon and nitrogen sources were sterilised separately and $FeSO_4.7H_2O$ was filter sterilised. These were added to the bioreactors aseptically to make a total medium volume of 1.35 L before inoculation with 150 mL of the 48-hour shaken flask cultures (fermentation working volume of 1.5L).

Control (no oligosaccharide supplement) and test (oligosaccharide supplement) fermentations were carried out in parallel using the same inoculum and the same batch of medium. The pH of the fermentations was automatically maintained between 6.45 and 6.55 using sterile 2M ammonium hydroxide and sulphuric acid. Dissolved oxygen tension was controlled automatically above 30 percent air saturation with stirrer speeds ranging from 250 to 600 rpm. The changes in the agitation rates for the control and the elicited fermentations were similar. The temperature and airflow-rate were kept at 26 ^{0}C and l.0 vvm, respectively.

Addition of oligosaccharides

The time of addition of the MO elicitor was at 48 hours after inoculation and the concentration of the MO added to the cultures was 150 mg L^{-1}.

Biomass Assay

Biomass production was measured as cell dry weight (CDW) per litre of culture broth. CDW was determined by filtration of 10 mL culture sample on pre-weighed filter paper (Whatman No. 1) and washing thoroughly with 20 mL of distilled water. The mycelia on the filter papers were weighed after drying to constant weight and the CDW determined by difference between the total weight and that of the filter paper alone. Biomass assays were carried out in triplicate.

Total Carbohydrate assay

Total carbohydrate content of fermentation broth was determined by phenol sulphuric acid assay as described by Chaplin and Kennedy (1994). Phenol sulphuric acid assay was carried out in glass test tubes by adding 200 µL of 5% phenol solution to 200 µL of standard, samples or blank (water) and mixing by gentle shaking in a fume cupboard. Phenol solution was made in HPLC grade water. Concentrated sulphuric acid (1.0 mL) was then rapidly added to the surface of the solutions without touching the sides of the tubes and allowed to stand undisturbed for 10 minutes. After 10 minutes, the tubes were mixed thoroughly and allowed to stand for a further 30 minutes. The absorbance of the reaction mixtures was determined at 490 nm using a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. Carbohydrate concentration of the samples was determined by using glucose standard curve in the range of 10-100 µg/mL.

Chrysogenin assay

Blank as zero reference was PPM diluted if necessary with ethanol (in accordance with OD of the test samples). For analysis of chrysogenin levels, fermenter samples (1 mL filtered broth) were added to 9 mL of ethanol and centrifuged at 2100 g for 10 minutes to remove precipitate. The supernatant was measured spectrophotometrically at 410 nm. Supernatants having absorbencies greater than 0.6 were diluted with ethanol before further spectrophotometric measurements.

All analyses for these results were carried out in triplicate and the overall difference between the control and the elicited cultures was statistically significant ($P<0.05$, ANOVA, SPSS12, SPSS Inc., USA).

RESULTS AND DISCUSSION

MO elicitor had notable effect on the production of chrysogenin in bioreactor cultures of MUCL 30168. The peak elicitation was observed at 72 hours. The percentage increase in chrysogenin compared to the control was 55%. The chrysogenin production rate was 36% higher and the specific productivity was 50 % higher than the control. The overproduction of chrysogenin was sustained for up to 120 hours (Figure 1).

The maximum rate of carbon utilisation was 43% higher in mannan oligosaccharides supplemented cultures compared to the control. There were no significant differences in biomass concentrations or in the growth rate between the control and the elicited cultures (Table 2). This indicates that the changes in metabolic activity (higher carbohydrate consumption in the supplemented cultures) favoured increased chrysogenin levels (Figure 2).

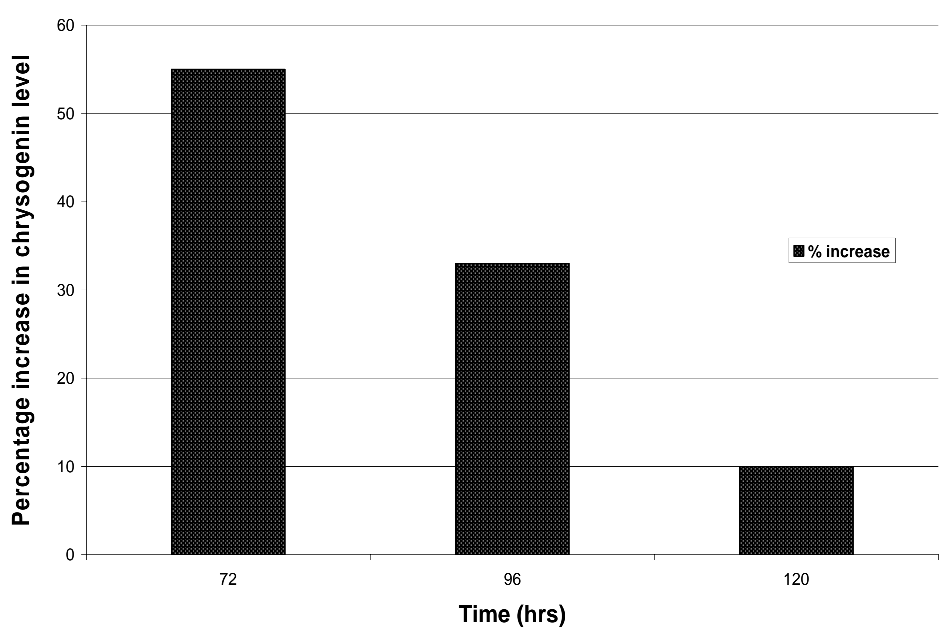

Figure 1: Percentage increase in chrysogenin levels in the elicited cultures of *P. chrysogenum* MUCL 30168 compared to the control in 2 L STR, supplemented with 150 mg L^{-1} at 48 hrs. (*P<0.05*, ANOVA, SPSS12, SPSS Inc., USA)

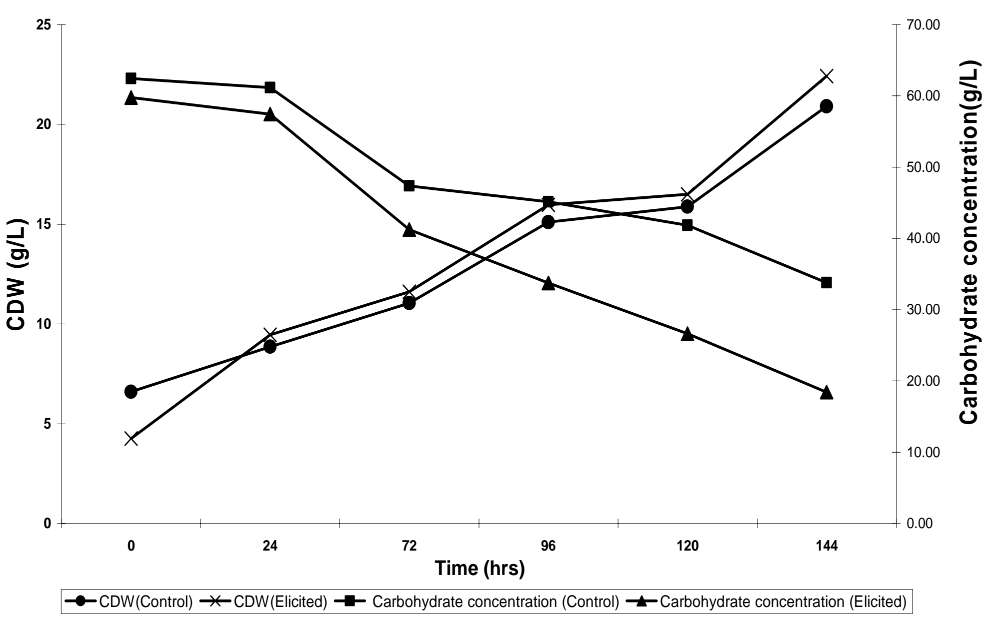

Figure 2: Cell dry weight (CDW) and total carbohydrate consumption for control and elicited cultures of *P. chrysogenum* MUCL 30168 in 2 L STR, supplemented with 150 mg L^{-1} at 48 hrs. (*P<0.05*, ANOVA, SPSS12, SPSS Inc., USA).

Elicitation was also observed in *P. chrysogenum* NRRL 1951. For this strain the maximum elicitation was noted at 120 hours with an increase of over 25% compared to control. The chrysogenin production rate of the elicited culture was 27% higher and specific productivity 22% higher than the control (Figure 3).

The maximum rate of carbohydrate consumption was 11% higher in elicitor supplemented cultures compared to control (Figure 4). There were no significant differences in biomass concentration or growth rate between the control and elicited cultures (Table 2).

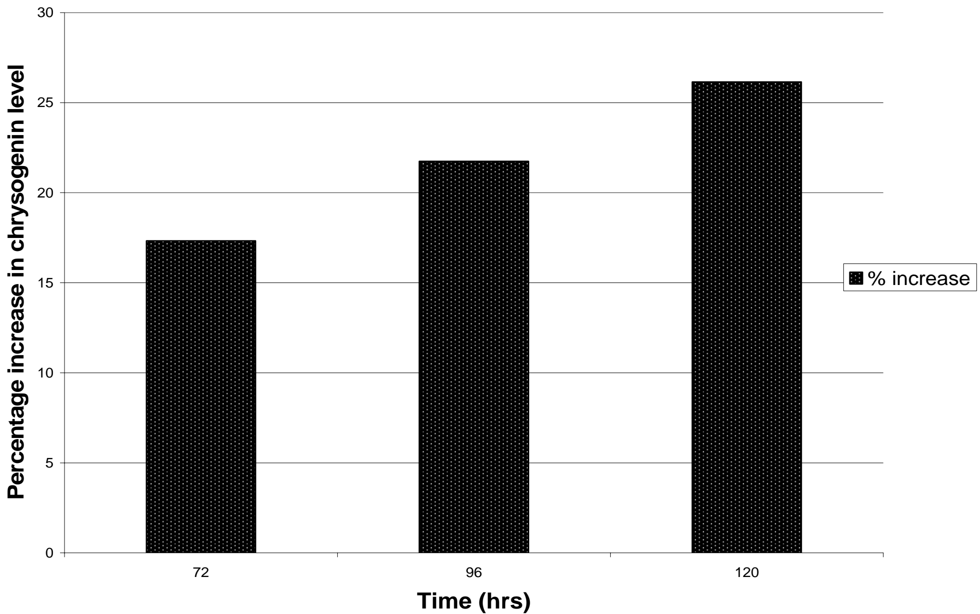

Figure 3: Percentage increase in chrysogenin levels in the elicited cultures of *P. chrysogenum* NRRL 1951 compared to the control in 2 L STR, supplemented with 150 mg L^{-1} at 48 hrs. (*P<0.05*, ANOVA, SPSS12, SPSS Inc., USA)

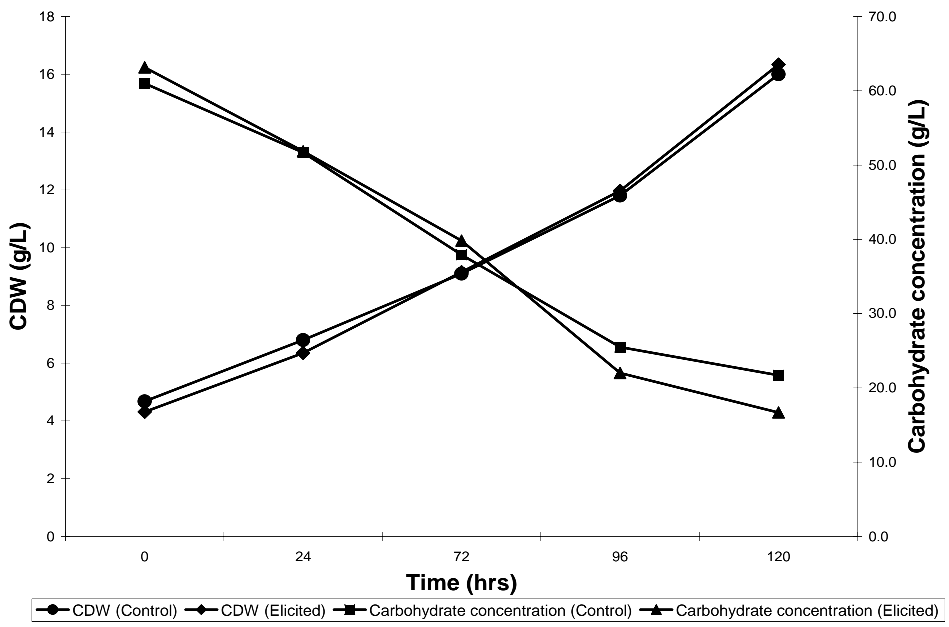

Figure 4: Cell dry weight (CDW) and total carbohydrate consumption for control and elicited cultures of *P. chrysogenum* NRRL 1951 in 2 L STR, supplemented with 150 mg L^{-1} at 48 hrs. (*P<0.05*, ANOVA, SPSS12, SPSS Inc., USA)

Table 1: Production Rate and Specific Productivity for chrysogenin production in two different strains of *P. chrysogenum* fermentations for the Control and Elicited cultures (24-96 hrs)

Culture	Chrysogenin Production rate (abs hrs^{-1})		Chrysogenin specific productivity (abs $g^{-1}hrs^{-1}$)	
	Control	**Elicited**	**Control**	**Elicited**
***P.chrysogenum* MUCL 30168**	0.03	0.04	2.00×10^{-3}	3.00×10^{-3}
***P. chrysogenum* NRRL 1951**	0.06	0.07	9.00×10^{-3}	11.00×10^{-3}

Table 2: Growth rate of the two different strains of *P. chrysogenum* fermentations for the Control and Elicited cultures (24-96 hrs)

Culture	Growth Rate (g hrs^{-1})	
	Control	**Elicited**
***P.chrysogenum* MUCL 30168**	0.11	0.11
***P. chrysogenum* NRRL 1951**	0.07	0.08

Table 3: Carbohydrate consumption rate of the two different strains of *P. chrysogenum* fermentations for the Control and Elicited cultures (24-96 hrs)

Culture	Carbohydrate Consumption Rate (g hrs^{-1})	
	Control	**Elicited**
***P.chrysogenum* MUCL 30168**	0.23	0.33
***P. chrysogenum* NRRL 1951**	0.35	0.39

The above findings show the generic effect of mannan oligosaccharides as elicitors of chrysogenin. The specific productivity and the percentage of elicitation were different for the two strains. The two strains had different growth rate and different metabolic activity. In the elicited cultures of NRRL 1951 the rate of carbohydrate consumption was 20% higher than the elicited cultures of MUCL 30168, however, the growth rate was 36% lower. This indicated the difference in metabolic activity of the two strains which had impact on the production of chrysogenin. The rate of specific productivity varied; with the elicited culture of NRRL 1951 producing almost 267% more than the elicited cultures of MUCL 30168. Because of the limited information on the pigment chrysogenin and its metabolic pathway, very little can be explained about the mechanism by which the elicitor affects its overproduction. Recent studies in various other systems such as *Monascus* species have reported elicitation in the pigment production when co-cultured along with yeasts (Shin., *et al* 1998). The yeast cell wall has high percentage of mannan (Clinton, 1970, p. 1197) which could correlate with the results obtained.

This study together with previous studies on the overproduction of secondary metabolites by mannan oligosaccharides (Ariyo *et al.,* 1998, p. 165; Tamerler *et al*., 2001, p. 53; Radman *et al*., 2004a, p.147) highlights for the first time the generic effect of this elicitor. This perhaps shows that the mechanism of action of mannan oligosaccharides preparation in fungi is well conserved and further studies are needed to understand this effect. If indeed the use of mannan oligosaccharides is generic on the enhancement of secondary metabolites, this could have a significant impact on possible industrial applications.

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