WestminsterResearch
http://www.westminster.ac.uk/westminsterresearch

Study of the effects of oligosaccharides in liquid cultures of *Penicillium chrysogenum.*

Romeo Radman

School of Life Sciences

This is an electronic version of a PhD thesis awarded by the University of Westminster. © The Author, 2002.

This is a scanned reproduction of the paper copy held by the University of Westminster library.

The WestminsterResearch online digital archive at the University of Westminster aims to make the research output of the University available to a wider audience. Copyright and Moral Rights remain with the authors and/or copyright owners.
Users are permitted to download and/or print one copy for non-commercial private study or research. Further distribution and any use of material from within this archive for profit-making enterprises or for commercial gain is strictly forbidden.

Whilst further distribution of specific materials from within this archive is forbidden, you may freely distribute the URL of WestminsterResearch: (http://westminsterresearch.wmin.ac.uk/).

In case of abuse or copyright appearing without permission e-mail repository@westminster.ac.uk

' LIF: PHD

Study of the Effects of Oligosaccharides in Liquid Cultures of *Penicillium chrysogenum*

Romeo Radman

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

October 2002

Acknowledgements

I would like to start by thanking my Supervisor, Professor Keshavarz, for his patience, support and dedication throughout my studies. His wisdom I have always relied upon. Also, I would like to thank Professor Bucke for his scientific input and advice.

My thanks also go to the technical staff that have always proved to be very resourceful and most helpful.

To my friends and colleagues I owe many thanks for always being there to help and who have given me immeasurable support over the years. Although many to include, Teresa Saez, Elliot Bland, Nuntaka Sangwarachat, Eva Perez, Sue Sharpe, Fiona and Stephen Bradley, deserve special mention.

Last, to my family I owe much for their love and support that they have shown over the years. For this I am most thankful and grateful.

Abstract

Oligosaccharides and polysaccharides have different effects on the morphology and production of secondary metabolites by *Penicillium chrysogenum* P2 (ATCC 48271). Addition of oligosaccharides, derived from sodium alginate and locust bean gum, to submerged cultures of *P. chrysogenum* P2, at milligram per litre concentration (150 mgL^{-1}), increased secondary metabolite levels and spore production, caused changes in morphology and germination of spores, and affected the production of Reactive Oxygen Species. The source of the oligosaccharides controlled their effects on the cultures.

Oligosaccharides when added to submerged cultures of *P. chrysogenum* P2 increased both penicillin G and extracellular levels of 6-aminopenicillanic acid concentrations. The oligosaccharides had no significant effects on biomass levels. Locust bean gum-derived oligosaccharides (mannan oligosaccharides, DP 5-8), showed the highest levels of enhancement in both penicillin G and 6-aminopenicillanic acid concentrations. Sodium alginate-derived oligosaccharides, (oligoguluronate, DP 7 and oligomannuronate, DP 7), also induced elicitation of penicillin G and 6-aminopenicillanic acid. Oligomannuronate was shown to be more effective than oligoguluronate. In *P. chrysogenum* P2 cultures mannan, oligomannuronate and oligoguluronate oligosaccharides enhanced yields of penicillin G by 101%, 78% and 59%, respectively. Addition of mannan, oligomannuronate and oligoguluronate oligosaccharides enhanced the levels of 6-aminopenicillanic acid by 39%, 26% and 19%, respectively.

The addition of oligosaccharides and polysaccharides to spores of *P. chrysogenum* P2 in liquid medium had varying (inhibitory or stimulatory) effects on germination, germ-tube and clump development.

The addition of oligosaccharides to submerged cultures of *P. chrysogenum* P2 showed effects on clump size and hyphal tip numbers. Mannan oligosaccharides had the greatest effect on morphology followed by oligomannuronate and oligoguluronate oligosaccharides.

Oligosaccharides also speeded-up the sporulation and increased the concentration of spores of *P. chrysogenum* P2 in liquid cultures. Mannan oligosaccharides had the greatest effect followed by oligomannuronate and oligoguluronate oligosaccharides.

8-aminonaphthalene-1,3,6-trisulphonate-tagged oligosaccharide studies showed that the oligosaccharides pass through the cell wall of *P. chrysogenum* P2 suggesting a possible mechanism through modulation of gene function. The elicitation pattern was shown to be similar to untagged oligosaccharides.

Oligosaccharides and polysaccharides were shown to inhibit production of Reactive Oxygen Species in *P. chrysogenum* P2. The highest level of inhibition was elicited by mannan followed by oligomannuronate and oligoguluronate oligosaccharides, and then locust bean gum and alginate.

The results of the study showed the potential of oligosaccharides as elicitors of secondary metabolites in *P. chrysogenum* P2 as a filamentous fungus model. Understanding the elicitation mechanism could provide routes for further exploitation of the potential of filamentous fungi in production of commercial products.

Table of contents

Aim. 1

1. INTRODUCTION **2**

1.1 Elicitation 3
1.1.1 Elicitation in Plants 3
1.1.2 Elicitation in Animal Cell Cultures 6
1.1.3 Elicitation in Bacterial Cultures 7
1.1.4 Elicitation in Fungal Cultures 8

1.2 *Penicillium chrysogenum* 10
1.2.1 Penicillin Biosynthesis in *P. chrysogenum* 12
1.2.1.1 Introduction 12
1.2.1.2 Overview of Synthesis 14
1.2.1.2.1 Biosynthesis of δ-/L-α-aminoadipyl/-L-cysteine (dipeptide AC) 14
1.2.1.2.2 δ-/L-α-aminoadipyl/-L-cysteinyl-D-valine (tripeptide ACV) 14
1.2.1.2.3 Biosynthesis of Isopenicillin N 15
1.2.1.2.4 Benzylpenicillin Biosynthesis 16

1.3 Carbohydrates as Elicitors 18
1.3.1 Oligosaccharides 19
1.3.1.1 Introduction 19
1.3.1.2 Oligosaccharide Building Blocks 20
1.3.1.3 Oligosaccharide Sequences 21
1.3.1.4 Alginates 22
1.3.1.5 Galactomannans 24

1.4 Image Analysis in Relation to Morphological Effects on Production26

1.5 Sporulation and Germination 32

1.6. Transport Mechanisms in Fungi 35
1.6.1 The Cell Wall 35
1.6.2 Transport Across Fungal Cell Membrane 37
1.6.3 The Plasma Membrane 39
1.6.4 Cell Membrane Receptors 40

1.7 Reactive Oxygen Species 42

2. MATERIALS and METHODS........45

2.1 Chemicals 46

2.2 Culture Strain........47

2.3 Media........48
2.3.1 Agar Medium for Strain Maintenance........49
2.3.2 Inoculum Growth Medium........50
2.3.3 Semi-Defied Production Medium........51

2.4 Culture Conditions 52
2.4.1 Penicillin Production in Shaken Flask Cultures........52
2.4.2 Production of Secondary Metabolites in Bioreactor Cultures........52
2.4.3 Addition of Phenylacetic Acid to Penicillin Cultures........53

2.5 Preparation of Oligosaccharides........54
2.5.1 Preparation of Alginate Oligosaccharides by Partial Acid Hydrolysis........54
2.5.2 Preparation of Mannan Oligosaccharides by Enzymatic Hydrolysis of LBG........56
2.5.3 Determination of Oligosaccharide Size by Thin Layer Chromatography of Oligosaccharides........56

2.6 Addition of Oligosaccharides to Production Cultures........59

2.7 Assays........61
2.7.1 Biomass Assay........61
2.7.2 Benzylpenicillin and 6-aminopenicillanic acid 61

2.8 Visualisation of Tagged Oligosaccharides and Penicillin G Production in Shaken Flask Cultures of *P. chrysogenum* P2........63

2.9 Investigation of Reactive Oxygen Species in *P. chrysogenum* P2.....64

2.10 Morphological Studies of *P. chrysogenum* P2 66

2.11 *P. chrysogenum* P2 Germination and Inoculum Morphology Development Studies........67

2.12 Sporulation Studies for *P. chrysogenum* P2 68

3. RESULTS **69**

Introduction to Results 70

3.1 Production and Enhancement 71

3.2 Inoculum Morphology Studies 84

3.3 Morphology Studies of *P. chrysogenum* P2 STR Cultures 88

3.4 Sporulation Studies 97

3.5 ANTS labelled Oligosaccharide Studies 99

3.5 Reactive Oxygen Species 103

4. DISCUSSION **113**

4.1 Effects of Oligosaccharides and Polysaccharides on *P. chrysogenum* P2 – Brief Review of Results 114

4.2 Structure and Size Relationship of Oligosaccharides to Overproduction 116

4.3 Overproduction of Penicillin G and 6-APA in *P. chrysogenum* P2 STR 118

4.4 Morphology Studies 122

4.5 Sporulation in *P. chrysogenum* P2 126

4.6 ROS Production in *P. chrysogenum* P2 128

5. FUTURE WORK **131**

6. CONCLUSION......136

7. REFERENCES......138

List of Figures

Fig 1.1 Schematic pathway for the production of penicillin in *P.* chrysogenum......................17

Fig. 1.2 Schematic representation of acid hydrolysed alginate oligosaccharides.......................23

Fig. 1.3 Schematic representation of mannan oligosaccharide...25

Fig 3.1 Biomass production in Control and Elicited STR fermentations of *P. chrysogenum* P2 ...73

Fig 3.2 Penicillin G and 6-APA concentration in Control and Elicited cultures of *P. chrysogenum* P2 74

Fig 3.3 Biomass production in Control and Elicited STR fermentations of *P. chrysogenum P2* ...76

Fig 3.4 Penicillin G and 6-APA concentration in Control and Elicited cultures of *P. chrysogenum* P2 77

Fig 3.5 Biomass production in Control and Elicited STR fermentations of *P. chrysogenum P2* ...79

Fig 3.6 Penicillin G and 6-APA concentration in Control and Elicited cultures of *P. chrysogenum* P2...80

Fig 3.7 Penicillin G percentage yields of elicitor added STR cultures of *P. chrysogenum* P2 compared to Control..83

Fig 3.8 Effect of oligosaccharides and polysaccharides on germ-tube length of *P. chrysogenum* P2 spores 86

Fig 3.9 Effect of oligosaccharides and polysaccharides on *P. chrysogenum* P2 inoculum morphology..87

Fig 3.10 Differences in hyphal tip numbers in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions 89

Fig 3.11 Differences in hyphal tip numbers in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions 90

Fig 3.12 Differences in hyphal tip numbers in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions..91

Fig 3.13 Differences in clump size in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions 93

Fig 3.14 Differences in clump size in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions 94

Fig 3.15 Differences in clump size in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions..95

Fig 3.16 Spore numbers per millilitre of culture in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions 98

Fig 3.17 Percentage increase in penicillin G production compared to control in shake flasks cultures of *P. chrysogenum* P2 using OG-, OM-, and MO-ANTS moieties as elicitors...........102

Fig 3.18 Reactive oxygen species in fermentations of *P. chrysogenum P2* after 24 hours..........104

Fig 3.19 Reactive oxygen species in fermentations of *P. chrysogenum P2* after 48 hours..........105

Fig 3.20 Reactive oxygen species in fermentations of *P. chrysogenum P2* after 72hours...........106

Fig 3.21 Reactive oxygen species in fermentations of *P. chrysogenum P2* after 96 hours..........107

Fig 3.22 Percentage ROS produced after 24 hrs for *P. chrysogenum* P2 in presence of elicitors.108

Fig 3.23 Percentage ROS produced after 48 hrs for *P. chrysogenum* P2 in presence of elicitors.109

Fig 3.24 Percentage ROS produced after 72 hrs for *P. chrysogenum* P2 in presence of elicitors.110

Fig 3.25 Percentage ROS produced after 96 hrs for *P. chrysogenum* P2 in presence of elicitors.111

Fig 3.26 Average percentage inhibition of ROS produced for *P. chrysogenum* P2 in presence of elicitor..112

List of Tables

Table 2.1 Glycerol-Molasses Agar Medium..49

Table 2.2 *Penicillium* Growth Medium 50

Table 2.3 Penicillin Production Medium 51

Table 2.4 DP of oligosaccharides by TLC analysis 60

Table 2.5 HPLC gradient profile for analysis of penicillin G and 6-APA....................................62

Table 3.1 Production Rate and Specific Productivity for penicillin G production in *P. chrysogenum* P2 fermentations for the control and elicited cultures (71-94 hrs)..........................81

Table 3.2 Production Rate and Specific Productivity for 6-APA production in *P. chrysogenum* P2 fermentations for the control and elicited cultures (71-94 hrs) 82

Table 3.3 Percentage *P. chrysogenum* P2 spore germination for Control and Elicited shaken flask cultures..85

Table 3.4 Percentage differences in number of hyphal tips compared to control and average biomass concentration using CDW for OG, OM, and MO supplemented cultures 92

Table 3.5 Percentage differences in mean clump areas compared to control and average biomass concentration using CDW for OG, OM, and MO supplemented cultures..............................96

List of Pictures

Picture 3.1 Hyphae of *P. chrysogenum* P2 under control conditions (x1000) 100

Picture 3.2 Hyphae of *P. chrysogenum* P2 supplemented with ANTS-OM (x1000) 100

List of Abbreviations

AC - δ-L-α-aminoadipyl-L-cysteine

ACV - δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine

ANTS - 8-aminonaphthalene-1,3,6-trisulphonate

ATCC – American Type Culture Collection

ATP - adenosine triphosphate

6-APA – 6-aminopenicillanic acid

CDW – Cell dry weight

DCF - 2', 7'-Dichlorofluorescein

DCFH-DA - 2', 7'-Dichlorodihydrofluorescein diacetate

DMSO – Dimethyl sulfoxide

DOT – Dissolved oxygen tension

DP – Degree of polymerisation

FACE - Fluorophore-assisted carbohydrate electrophoresis

g – Relative Centrifugal Force

GM – Glycerol-molasses agar medium

GOD – Glucose oxidase

GSH – glutathione

HPLC – High pressure liquid chromatography

IPN – Isopenicillin N

LBG – Locust bean gum

LLD-ACV - δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine

MO – Mannan oligosaccharides

OG – Oligoguluronate

OM – Oligomannuronate

PAA – Phenylacetic acid

PGM – *Penicillium* growth medium

PMT – Photo-multiplier tube

PPM – *Penicillium* production medium

ROS – Reactive oxygen species

RPK – Receptor protein kinase

rpm – Revolutions per minute

SOD – Superoxide dismutase

STR – Stirred tank reactor

TLC – Thin layer chromatography

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 investigate physiology and morphology of the fungus *P. chrysogenum* P2 under the effect of alginate and locust bean gum oligosaccharides in liquid culture.

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

1. To investigate the effects of the oligosaccharides on the production of 6-aminopenicillanic acid by *P. chrysogenum* P2.

2. To study the effects of the oligosaccharides on germination of *P. chrysogenum* P2 spores and the development of morphology and spore formation.

3. To investigate the effects of the oligosaccharides and alginate and locust bean gum on the production of Reactive Oxygen Species in *P. chrysogenum* P2.

4. To initiate and carry out preliminary studies on the possible mechanism(s) of elicitation of antibiotic synthesis by oligosaccharides in *P. chrysogenum* P2.

1. INTRODUCTION

1.1 Elicitation

An elicitor may be defined as a substance, which when introduced to a living cell system, in small quantities, *initiates* or *improves* the biosynthesis of specific compounds. Thus, elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors.

1.1.1 Elicitation in Plants

The initial studies in the field of elicitation were carried out in plant cell systems. Plant cell cultures are potentially rich sources of valuable pharmaceuticals and other biologically active phytochemicals, but relatively few cultures synthesize secondary metabolites over extended periods in amounts comparable to those found in the whole plant. Metabolic pathway engineering of plant cells to improve the yields of valuable secondary compounds generally has not been possible. However, manipulation of plant cell cultures with elicitors not only has shed light on the different biosynthetic pathways but also increased yields of medically important secondary metabolites such as Taxane by *Taxus chinensis* (Srinivasan *et al.*, 1996).

Catharanthus roseus produces a wide range of indole alkaloids as part of its secondary metabolism. The low yield of these valuable indole alkaloids in plants has been the major motivation to produce them by cell and tissue cultures. A number of metabolic manipulations has been carried out in order to further understand their production mechanism. Light is thought to affect both enzyme induction and activity (Shanks and Bhadra, 1998).

Precursor feeding is a method that is used to probe rate-limiting steps by increasing the substrate supply. By identifying precursors, which after feeding enhance the production of alkaloids, the

specific part of the pathway that may have flux limitations can be identified. However, in contrast to precursor feeding studies, addition of fungal elicitors has induced enzyme levels in the indole alkaloid pathway (Whitehead and Threlfall, 1992). The mechanism of fungal elicitation for induction of enzymes in plant systems in response to elicitors is not completely understood. Therefore, well-defined elicitors as compared to crude fungal homogenates are being used for detailed studies. The three elicitors chosen were pectinase, an endopolygalacturonase: chitin, a component of fungal cell walls; and jasmonic acid, a signal transducer in the defence response. Pectinase increased levels of tabersonine approximately 2.5 times the control, but had no significant effect on the other alkaloids (Shanks and Bhadra, 1998). Chitin selectively enhanced ajmalicine levels (by 50%), but no significant effect was observed on the other alkaloids. In contrast, addition of jasmonic acid affected all of the alkaloid levels.

Xyloglucan is a major structural polysaccharide of primary cell walls of higher plants and presumably its main biological role is to contribute to wall strength (Fry, 1989). However, York, (1984) discovered that oligosaccharides derived from xyloglucan may also serve a signalling role. The oligosaccharides were found to inhibit the auxin-promoted growth of etiolated pea stem segments. Fungal derived oligosaccharides activate a number of plant defence mechanisms. Certain fungal oligo-β-glucans can elicit phytoalexin synthesis in higher plants (Albersheim and Valent, 1978). Phytoalexins are anti-microbial compounds synthesised by plants after exposure to micro-organisms.

Carbohydrates are not the sole elicitors of plant cell cultures. Salicylic acid, for example, enhances activation of defence related genes in parsley (Katz *et al.*, 2002). Proteins can act as elicitors too.

Nep-1 produced by *Fusarium oxysporum* elicits ethylene production in leaves of *Nicotiana tabacum* (Jennings *et al.,* 2001).

1.1.2 Elicitation in Animal Cell Cultures

Elicitation in animal cell cultures does not relate to production of secondary metabolites, however it relates to activation of number cell pathways. Pectin-like polysaccharides have been reported to activate immune system complexes by Yamada, (1994).

It has been shown that glucans obtained from fungi enhance the resistance of common carp, *Cyprinus carpio*, against bacterial infections through the activation of the non-specific immune system (Fujiki *et al.*, 1994). This study has demonstrated that sodium alginate isolated from *Undaria pinnatifida* enhanced the resistance of carp to *Edwardsiella tarda* infection. The disease resistance occurred within a few days, earlier than would be expected if the specific immune response was involved. Therefore, it seems likely that the alginates stimulated the non-specific immune system, presumably the reticuloendothelial system (Fujiki *et al.*, 1994).
Other effects on animal cell cultures induced by elicitors include induction of IL-6 in humans and mice, and increased proliferation of antibody producing cells (Skjak-Braek and Espevik, 1996).
Work carried out by Bland *et al.* (2001) has show that carbohydrate elicitors have a significant effect on the stimulation and inhibition of Reactive Oxygen Species (ROS) in neutrophils. This observation was linked to the structure of the oligosaccharide tested.

1.1.3 Elicitation in Bacterial Cultures

Although the effects of bacterial-derived elicitors in plant and animal cell cultures are well documented, little is reported on elicitation in bacterial cultures. Work on *Bifidobacteria* has show that in the presence of alginate elicitors there is an increase in biomass (Akiyama *et al.*, 1992).
The lack of research in this field could be due to the fact that efficient systems are already being used to enhance and overproduce metabolites. Recombinant plasmid technology ensures that bacteria can be used successfully as hosts for the overproduction of a number of metabolites, for example polyhydroxyalkanoates in *Pseudomonas putida* (Solaiman *et al.,* 2001).

1.1.4 Elicitation in Fungal Cultures

In fungal cultures, as in plant cell cultures, elicitors induce a number of secondary metabolite pathways. Most inducible elicitors are derived from sodium alginate and locust bean gum.
Glucose oxidase (GOD) catalyses oxidation of glucose by molecular oxygen and is produced industrially as a by-product of the gluconic acid fermentation from *Apergillus niger* . Since the early 1950s glucose oxidase has been widely used in powdered egg manufacture (Baldwin *et al.*, 1953) and paper test strips for diabetic patients (Hunt, 1956). A new application for GOD is its use in biosensors (Filipiak *et al.*, 1996). Recent studies have shown an increase of 70% in GOD concentration in cultures of *Penicillium variabile* P16 when elicited with alginate (Petruccioli *et al.*, 1999).

The fungus *Monascus purpureus* is well known for its ability to produce eight closely related polyketide pigments ranging in colour from bright yellow to deep red (Jazlova *et al.*, 1996). Some of these pigments have long been used in Asian countries as food colorants and their wide potential applicability is generally recognized (Jazlova *et al.*, 1996). The fermentative production of such pigments can be obtained both in solid state and in submerged cultivations and many studies have aimed to assess the effects of various macro- and micro- elements of production media on pigment synthesis as well as to optimise production (Jazlova *et al.*, 1996). Among the possible approaches to maximize pigment production, strain improvement techniques have been widely used. *Monascus purpureus* pigment production is also enhanced in the presence of calcium alginate (Fenice *et al.*, 2000).

Alginate oligosaccharides have been shown to have significant impact on the production of penicillin G in *P. chrysogenum* (Ariyo *et al.*, 1997). This paper showed for the first time that alginate derived oligosaccharides elicited overproduction of penicillin G in shaken flasks. Differences in the degree of polymerisation appear to have an effect on the level of elicitation observed. Later studies by the same group (Ariyo *et al.*, 1998) showed that this overproduction can also be observed at bioreactor level with a maximum increase of 69% in penicillin G production. Not only is penicillin G production elicited in *P. chrysogenum* but chrysogenin is as well (Asilonu *et al.*, 2000). Chrysogenin is a pigment produced by *P. chrysogenum* and its biological role is not well understood. Other strains of *Penicillium* have shown to elicit secondary metabolites (Ariyo *et al.*, 1997).

The work on elicitation in *P. chrysogenum* has provided us with an established system to further investigate the effects of elicitors. As knowledge of the metabolic pathway for penicillin G production and the *P. chrysogenum* morphology are well established, this fungus is an ideal model for research.

1.2 *Penicillium chrysogenum*

P. chrysogenum is a fungus that grows naturally in a filamentous form, and belongs to the *Penicillium* genus. *Penicillium* consists of long, branched, threadlike filaments of cells called hyphae. These form a tangled mass or tissue aggregation called a mycelium (Peberdy, 1985). The hyphae have cross walls called septa with pores that permit protoplasmic streaming.

Species of *Penicillium* are recognized by their dense brush-like spore-bearing structures. The conidiophores are simple or branched and are terminated by clusters of flask-shaped phialides. The spores (conidia) are produced in chains from the tips of the phialides, with the youngest spore at the base of the chain, and are nearly always green. Branching is an important feature for identifying *Penicillium* species. Some are unbranched and simply bear a cluster of phialides at the top of the stipe. Others may have a cluster of branches, each bearing a cluster of phialides. A third type has branches bearing a second order of branches, bearing in turn a cluster of phialides. These three types of spore bearing systems (penicilli) are called monoverticillate, biverticillate and terverticillate respectively. *Penicillium* is a large genus encountered almost everywhere, and usually the most abundant genus of fungi in soils.

Unlike many other antibiotics in medical use penicillin was not found by mounting large screening programmes but chance played an extraordinary role in its discovery.

The discovery of penicillin at St. Mary's Hospital by Alexander Fleming, after a plate inoculated with staphylococci was accidentally contaminated with *Penicillium notatum* (Fleming, 1929), has lead to a revolution in the treatment of systemic infections. Penicillins have broad clinical utility.

Penicillium chrysogenum is an important industrial organism due to its capacity to produce penicillin, which is still one of the main commercial antibiotics (Brakhage, 1998) constituting more than 60% of the world market of antibiotics (Mendez and Salas, 1998). Due to the development of semisynthetic derivatives with improved efficacy (Nayler, 1991), the efficiency of penicillin production has improved. This has resulted in a 30,000 fold increase in titre of Fleming's original isolate of *P. notatum* compared to currently exploited high-producing strains of *Penicillium chrysogenum* (Peñalva *et al.*, 1998).

1.2.1 Penicillin Biosynthesis in *P. chrysogenum*

1.2.1.1 Introduction

Penicillins are compounds that are mainly derivatives of either the penam (penicillin) ring system or the 3-cephem (cephalosporin) ring system. The differences between individual penicillins lie in the structure of their side chains that vary from single chain carboxylic acids to cyclic phenolic (aromatic) molecules.

Penicillins, produced by various species of *Penicillium*, especially *P. chrysogenum*, as well as other species of fungi, all possess the same bicyclic ring system, consisting of a β-lactam ring cis-fused to a thiazolidine ring (Kurylowicz *et al.*, 1987).

Over one hundred penicillins, differing only in the nature of the N-acyl side chain, can be produced by fermentation. Aliphatic or aryl-substituted aliphatic carboxylic acids constitute suitable side-chain precursors (Behrerns, 1949) for penicillin production. In the absence of a suitable side-chain precursor, fermentations of *P. chrysogenum* lead to the formation of isopenicillin N (Cole, 1966).

Other examples of natural penicillins include penicillin X, pentenylpenicillin (penicillin F), and heptylpenicillin (penicillin K). As a result of plasmid-born β-lactamases the anti-microbial efficacy of most of the natural penicillins declined and production of commercially semi-synthetic penicillins has been implemented. These include oxacillin and ampicillin (Rolinson, 1979).

Current research in β-lactam is focusing on a greater understanding of metabolic regulation, metabolic engineering and detailed genetic structure of the genes responsible for the enzymatic synthesis of the now vast array of β-lactam antibiotic molecules. Research on the mechanisms of action of the β-lactams has now reached a new level having well defined targets. Penicillin-binding proteins and their associated genes have been identified and are undoubtedly linked to both biosynthetic and lytic events (Demain and Elander, 1999).

1.2.1.2 Overview of Synthesis

Biosynthesis of penicillin by *P. chrysogenum* involves the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (tripeptide ACV) as intracellular precursor. This intermediate is formed from L-α-aminoadipic acid, L-cysteine and L-valine (Fig. 1.1). The terminal reaction in the sequence is a transacylation between isopenicillin N and an activated side-chain acid to produce penicillin (Elander, 1980; Demain, 1983).

1.2.1.2.1 Biosynthesis of δ-/L-α-aminoadipyl/-L-cysteine (dipeptide AC)

The initial reaction of benzylpenicillin biosynthesis is the condensation of L-α-aminoadipic acid and L-cysteine to form the dipeptide AC. Direct evidence for the incorporation of the cysteine molecule into the dipeptide AC was obtained by isotopic studies.

This stage of biosynthesis is catalysed by the enzyme δ-L-α-aminoadipyl-L-cysteine synthetase (Lara *et al.*, 1982).

The reaction is based on the consumption of ATP needed to activate amino acids for non-ribosomal biosynthesis of peptides.

1.2.1.2.2 δ-/L-α-aminoadipyl/-L-cysteinyl-D-valine (tripeptide ACV)

To the dipeptide AC, L-valine is added to form the tripeptide ACV (Fig. 1.1) The L-α-aminoadipic acid is involved in the initial peptide-forming steps of tripeptide ACV. Lysine is a

potential inhibitor of L-α-aminoadipic acid and penicillin biosynthesis (Demain and Masurekar, 1974). The fungal biosynthetic pathway to lysine involves L-α-aminoadipic acid as intermediate. The L-α-aminoadipic acid not only reverses the inhibitory effect of lysine, but also stimulates penicillin biosynthesis (Somerson *et al.*, 1961).

The L-valine is converted to the D-form during activation and is then added to the tripeptide ACV. The use of ^{14}C-labeled valine provides direct evidence for the incorporation of intact L-valine into penicillin. Although the valine constituent of the tripeptide ACV is of D-configuration, the stimulation of synthesis of penicillin by the L-form and the inhibitory effect of D-valine showed L-valine to be the actual precursor (Demain, 1983).

1.2.1.2.3 Biosynthesis of Isopenicillin N

In the pathway of biosynthesis of β-lactam antibiotics the tripeptide ACV is converted by isopenicillin N synthetase to β-lactam-thiazolidine (Fig. 1.1). This enzyme requires molecular oxygen, cofactors and ferrous ions (White *et al.*, 1982). The cyclization of tripeptide ACV to isopenicillin N by a cell-free extract from *P. chrysogenu*m was described by Abraham *et al.*, (1981). The formation of ACV and IPN has been reported to be repressed by glucose (Hönlinger and Kubieck, 1989).

1.2.1.2.4 Benzylpenicillin Biosynthesis

The terminal reaction of benzylpenicillin biosynthesis is an exchange of the L-α-aminoadipic acid of isopenicillin N for phenylacetate from phenylacetyl:coenzyme A. This acyltransfer reaction is catalysed by enzymes of a single chain exchange mechanism composed of phenylacetyl: coenzyme A ligase, phenylacetyl:coenzyme A hydrolase and phenylacetyl-transferase.

Phenylacetyl:coenzyme A ligase in benzylpenicillin producing strain of *P. chrysogenum* was described by Brunner and Rohr, (1975). The enzyme appears in the initial stages of benzylpenicillin production and then increases during the stage of rapid production of the antibiotic.

The final step in benzylpenicillin biosynthesis involves the direct N-acylation of 6-aminopencillanic acid by the coenzyme A activated side-chain precursor (Fig. 1.1). The reaction occurs directly (with a lower rate) or as a two-step process to form 6-APA as an intermediate which is then acylated to form penicillin G. Phenylacetyl:coenzyme A hydrolase activity was described in extracts of high benzylpenicillin yielding mutants of *P. chrysogenum* by Spencer, (1975).

The penicillin acyltransferases are enzymes usually present in penicillin producing stains that are capable of transferring the side-chain from phenylacetyl:coenzyme A to 6-aminopenicillanic acid. Acyltransferase activity was shown in extracts of different stain of *P. chrysogenum* by Spencer and Maung, (1970).

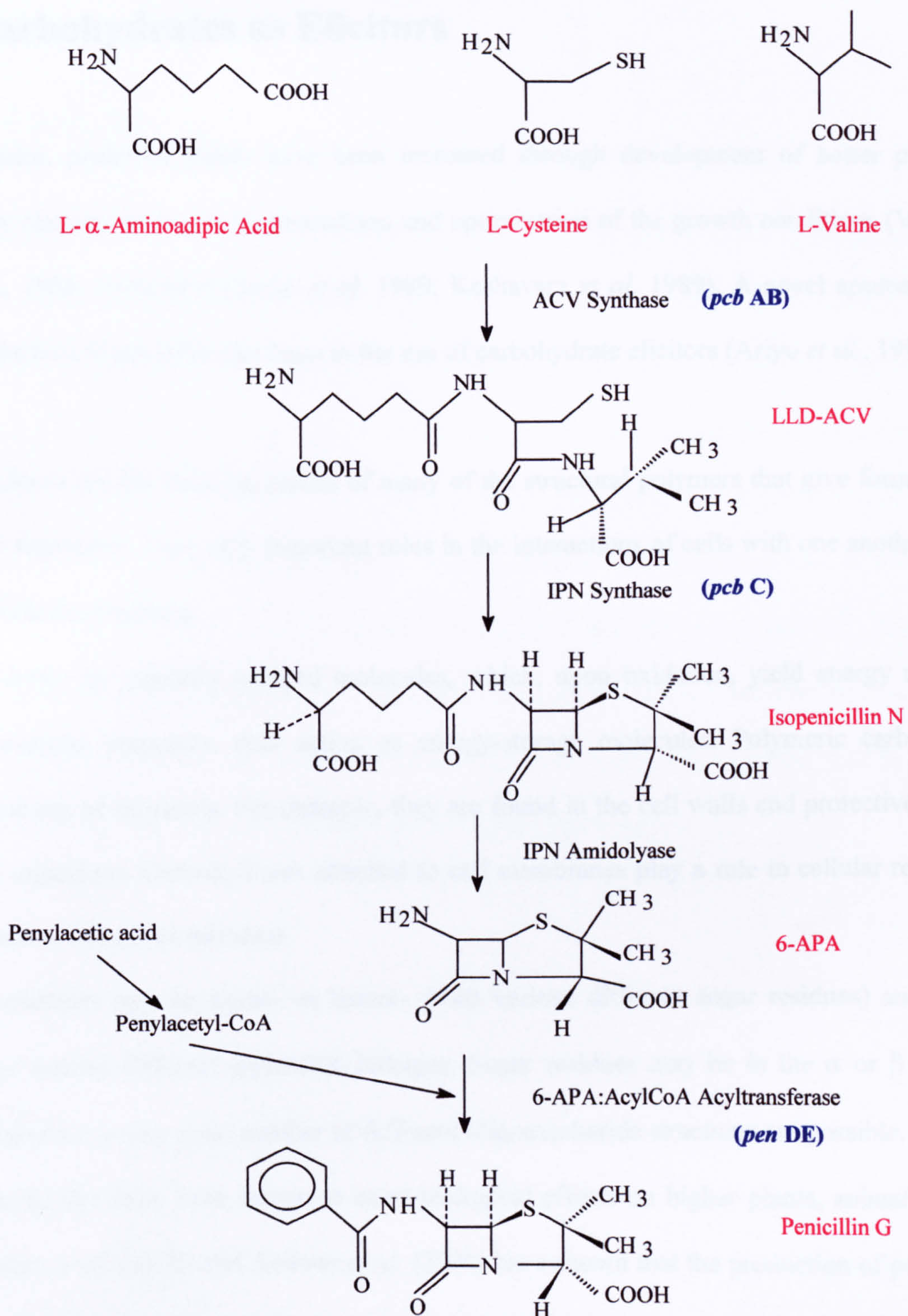


Fig 1.1 Schematic pathway for the production of penicillin in *P. chrysogenum* (genes regulating enzymes shown in blue).

1.3 Carbohydrates as Elicitors

For decades, penicillin yields have been increased through development of better production strains by classical mutagenesis procedures and optimization of the growth conditions (Vanek and Hostalek, 1986; Fernández-Canón *et al.* 1989; Keshavarz *et al.* 1989). A novel approach to the overproduction of penicillin has been in the use of carbohydrate elicitors (Ariyo *et al.*, 1997).

Carbohydrates are the building blocks of many of the structural polymers that give form to living cells and organisms. They play important roles in the interactions of cells with one another as well as with their environment.

Carbohydrates are partially reduced molecules, which, upon oxidation, yield energy needed to drive metabolic processes, thus acting as energy-storage molecules. Polymeric carbohydrates serve a variety of functions. For example, they are found in the cell walls and protective coatings of many organisms. Carbohydrates attached to cell membranes play a role in cellular recognition and in cell-to-cell communication.

Oligosaccharides may be homo- or hetero- (with various different sugar residues) and may be linked by various different glycosidic linkages. Sugar residues may be in the α or β anomeric form. Therefore, a very great number of different oligosaccharide structures are possible.

Oligosaccharides have been shown to exert biological effects on higher plants, animal cells and fungi. Ariyo *et al.* (1998) and Asilonu *et al.* (2000) have shown that the production of penicillin is significantly enhanced when alginate- and Locust Bean Gum (LBG)- derived oligosaccharides are supplemented as elicitors to *P. chrysogenum* cultures.

1.3.1 Oligosaccharides

1.3.1.1 Introduction

Oligosaccharides are a group of carbohydrates with short chains of 2-20 glycosidically linked monosaccharide residues. The monosaccharides can be liberated by hydrolysis. Simple oligosaccharides are classified as di-, tri-, saccharides according to the number of monosaccharide molecules produced on hydrolysis; this number is also referred to as the degree of polymerisation (DP) of the oligomer. Oligosaccharides having a degree of polymerisation between 2-3 are sweet tasting, soluble in water and are classified as sugars, whereas higher members are devoid of taste. Oligosaccharides can exist as both simple oligosaccharides, that on hydrolysis liberate only monosaccharide units, and conjugate oligosaccharides linked to non-saccharides, such as cell surface peptides (Hayes *et al.*, 1995) and lipids (D'Souza *et al.*, 1992). Hydrolysis of conjugate oligosaccharides liberates both monosaccharides and the aglycons.

1.3.1.2 Oligosaccharide Building Blocks

The monosaccharide units in oligosaccharides are mostly hexose (6-carbon) sugars with pyranose (6-membered ring) structures. An example of this type of sugar is β-D-glucopyranose (D-glucose), which is the most common building block of natural carbohydrate polymers. Oligosaccharide building blocks may also include monosaccharides with furanose (5-membered ring) structures. The disaccharide sucrose consists of a D-glucose residue in pyranose form linked α-1, 2 to D-fructose in furanose form.

The oligosaccharides used in this study oligo (β-D-mannose), oligo (β -D- mannuronate) and oligo (α-L-guluronate) are hexoses. The two latter oligosaccharides are composed of modified monosaccharide units in which the C-6, CH_2OH group has been oxidised to COO^- (carboxylate) groups.

1.3.1.3 Oligosaccharide Sequences

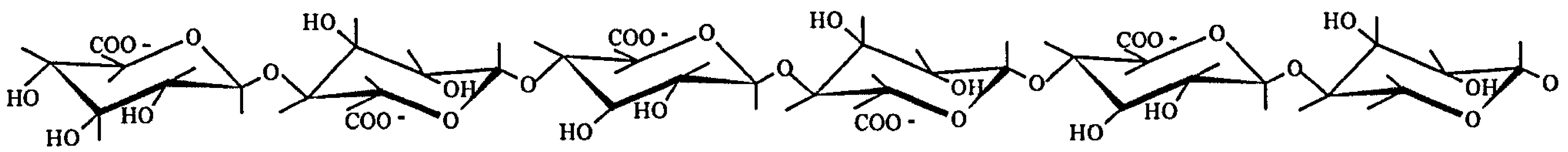
The arrangement of sugar units within naturally occurring carbohydrate polymers is of fundamental importance in determining their properties and complexity. The sequences of sugar units that occur in carbohydrate polymers can be classified into three main groups, these are periodic, interrupted and aperiodic sequences (Rees, 1977). The simple periodic sequence is characterised by the arrangement of monosaccharide units with a single type of linkage in a repeating pattern along the carbohydrate chain, the oligo (β -D-mannose) chain is of this type. However, more complex carbohydrate structures exist with interrupted periodic sequences which may contain different types of linkages. A typical example of a carbohydrate polymer that has both repeating and interrupted periodic sequences is the alginate polysaccharide representation β - 1,4 linked mannuronate and α-1, 4-linked guluronate.

Many carbohydrate structures also exist with aperiodic sequences, variations in linkage position and sometimes variations in D and L configuration. Branched structures are also frequently encountered, particularly among conjugate oligosaccharides. These structures are found as cell constituents in every living organism, where they function as receptors in immune systems as cell surfaces receptors for hormones and neurotransmitters, and as recognition molecules involved in the processes of cell sorting during tissue development. Carbohydrate attached to immunoglobulins is typical of this type of structure.

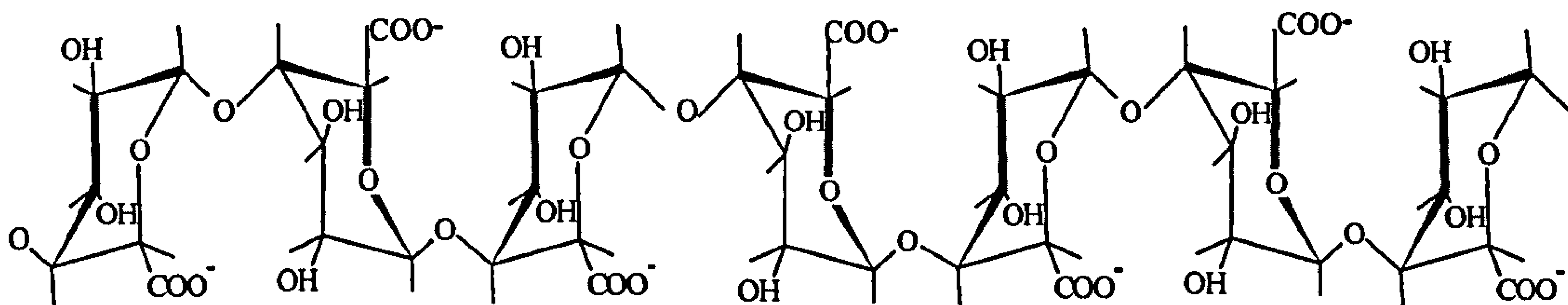
1.3.1.4 Alginates

Alginates are normally available as monovalent salts of alginic acid extracted from different types of brown seaweed. Certain bacteria, including *Azotobacter chrococcum, A. vinelandii, Pseudomonas aeruginosa* also synthesize alginate as an 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. Haug *et al.*, (1974) made precise determination of the uronic acid composition. Alginate is a linear polysaccharide comprised of 1,4 linked β-D-mannuronate and α-L- guluronate. These uronic acids are arranged in block structures that are mainly homopolymeric (polyguluronate and polymannuronate) with alternating units of mannuronic and guluronic acid. Alginate has its building blocks arranged in a zig-zag pattern.

Alginate is used for a variety of applications by the food, pharmaceutical and other industries (Gacesa, 1988). Alginates are mainly used as gelling and thickening agents in a wide variety of applications. Another important commercial application of alginate is its use as a matrix for cell immobilization (Bucke, 1987). Through acid hydrolysis biologically active oligosaccharides can be produced (Fig. 1.2) (See Materials and Methods section).



Oligomannuronate



Oligoguluronate

Fig. 1.2 Schematic representation of acid hydrolysed alginate oligosaccharides.

1.3.1.5 Galactomannans

Commercially available galactomannans are derived from the seed endosperm of carob (*Ceratonia siliqua*) and guar (*Cyamopsis tetragonolobus*) (Dea and Morrison, 1975).

Galactomannans have a backbone of β-1,4-linked D-mannopyranosyl residues. Some mannopyranosyl moieties in the backbone are substituted with single unit α-1,6-linked D-galactopyranosyl residues.

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. 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 and are obtained from carob seeds (*C. siliqua*). The locust bean gums interact strongly with polysaccharides such as agar, kappa-carrageenan and xanthan gum to form strong gels at low concentrations in water (Dea and Morrison, 1975). This property of locust bean gums has been exploited commercially. The galactomannans have also found extensive use as thickeners and stabilizer for the food industry.

Oligosaccharides can be prepared from galactomannan by enzymatic hydrolysis (Fig. 1.3). A range of galactomannan enzymes have been reported in literature (Dey, 1978); these include α-galactosidase, β-D-mannanase and galactomannanase. The enzyme β-D-mannanase acts by

random cleavage of the D-mannan chain, producing a series of manno- and galacto-manno-oligosaccharides (Dekker and Richards, 1976).

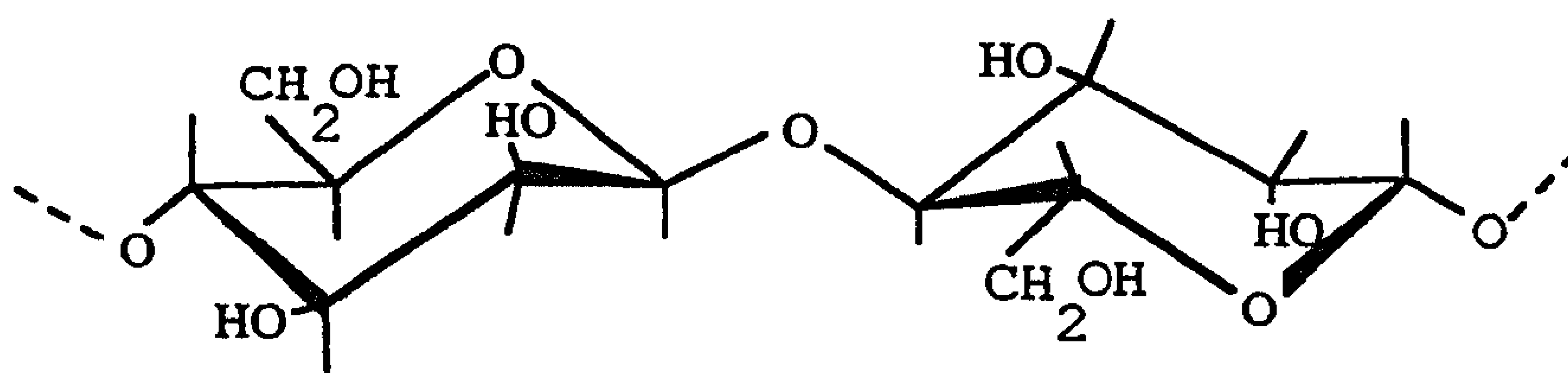


Fig. 1.3 Schematic representation of mannan oligosaccharide

1.4 Image Analysis in Relation to Morphological Effects on Production

Although filamentous microorganisms 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 to quantify. This lack of appropriate tools can hinder the optimisation and control of the fermentation.

Filamentous microorganisms such as fungi and *Streptomycetes*, are widely used for the production of secondary metabolites at the industrial scale. Because these metabolites are an offshoot of the main metabolic pathways, particular physiological conditions are required for their production. In spite of the apparent complexity of the interaction of microorganisms with their environment, medium and process development as well as strain improvement were often done on a trial and error basis. For instance, the initial strain improvement program for *P. chrysogenum* was mainly based on the mutation-selection approach (Herbash *et. al.*, 1984).

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 enhanced microscopy was used in the detection of luminescence-marked bacterial cells *in situ* in environmental samples (Rattray *et al.,* 1994). Such a technique can facilitate risk assessment of genetically engineered microorganisms introduced into the environment. Image analysis in the food industry has been used in developing an effective cleaning procedure for removing biofilms from food-contact surfaces (Wirtanen *et al.*, 1995).

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 mycelia masses referred to as pellets. The particular form exhibited is determined not only by the genotype of the strain, but also by the nature of the inoculum, as well as, the chemical, physical and culture conditions (Suijdam *et al.*, 1980; Schügerl *et al.*, 1997 and Papagianni, 1999). These morphological growth forms can have a significant effect on the rheology of the fermentation broth and thus the bioreactor performance. Pelleted morphologies usually result in Newtonian broths and better mass transfer rates (in the broth) compared to viscous and often pseudoplastic filamentous cultures (Suijdam *et al.*, 1980). However, autolysis inside the pellets, due to oxygen limitation, results in a large portion of fungal mass being metabolically inactive (Suijdam *et al.*, 1980). In *Aspergillus oryaze* this pelleted morphology is desirable for the production of citric acid. However, the filamentous morphology is of interest in *P. chrysogenum* fermentations (Amanullah *et al.,* 1999). 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 (Papagianni *et al.*, 1999) and penicillin fermentations (Calam, 1987). 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.

Inoculum quality, size, type or age, is of prime importance in determining the outcome of filamentous fermentations. Studies have shown that pellets are formed at inoculum levels below

10^6 spores mL^{-1}, while at higher inoculum levels dispersed growth prevails (Vecht-Lifshitz *et al.*, 1990). Studies with *P. chysogenum* have also shown that with a production culture started with an inoculum seeded with 10^3 spores mL^{-1} the pellets grew larger, while with 10^4 spores mL^{-1} in the inoculum the tiny pellets remained small but became more numerous (Calam, 1987). Application of image analysis in a number of studies demonstrates quantitatively the transition from the pelleted to free filamentous form by changing inoculum levels (Tucker and Thomas, 1992). Therefore, morphological development in filamentous fermentations can be manipulated with inoculum size.

Image analysis systems are also being used in characterising the germination process of spores and viability testing (Paul *et al.*, 1993).

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. The morphology of mycelial fungi in liquid culture affects culture rheology and this in turn may affect product yield. Wiebe and Trinci (1991), with the aid of image analysis, looked at the effects of dilution rate on the morphology of *Fusarium graminearum* C106 in continuous culture. *F. graminearum* C106 is a colonial variant which arose spontaneously in a prolonged continuous culture of *F. graminearum* A3/5. The appearance of C106 colonial variants in Quorn mycoprotein fermentations resulted in premature termination of the continuous culture with a consequent loss of productivity (Cunliffe *et al.*, 1990). This work showed that the main hyphal length and the hyphal growth-unit of *F. graminearum* C106 decreased when the dilution rate increased. But at lower dilution rates hyphal growth unit stayed constant. For *F. graminearum*

A3/5, however, hyphal length, hyphal growth unit and hyphal growth volume increased with increased dilution rate.

New developments have lead to manufacturing of on-line image analysis systems that have been used to fully characterise the relationship between morphology and production in submerged cultures. Treskatis (1996) used such a system to look at the changes in morphology during the course of a typical fermentation of *Steptomyces tendae*. *S. tendae* produces nikkomycin. Nikkomycin Z is an antifungal drug that competitively inhibits chitin synthetase in the fungal cell wall. It was shown that with time the morphology changed from rough pellet structures in the exponential phase to smooth pellets in the late exponential phase. At this stage the available phosphate was exhausted and the production of nikkomycin had begun. There were morphological changes at this point where it was found that the majority of the cells were now smooth.

The relationship between morphology and citric acid production was investigated in submerged fermentation of *A. niger* (Paul *et al.*, 1999). In citric acid fermentation the morphology of *A. niger* varies between filamentous and pelleted forms with an intermediate aggregate (but still dispersed) morphology (clumps) (Tucker *et al.*, 1992; Paul and Thomas, 1998). The work by Paul *et al.* (1999) gives a quantitative description (using image analysis) of how the morphology of *A. niger* inocula affects the subsequent fermentation morphology and its relation to citric acid production. They concluded that smaller pellets (1.5 mm) gave a higher citric acid yield than larger pellets (3.0 mm). Surprisingly their study suggested that filamentous morphology gave a higher citric acid yield compared to pelleted morphologies, although previous studies had shown the opposite (Kisser *et al.*, 1980; Gomez *et al.*, 1988). It is interesting to note that the citric acid titre between the small pellets and filamentous morphologies was similar for most of the fermentations suggesting that the small pellets were too small for significant diffusion limitations to have

occurred. This study (Paul *et al.,* 1999) shows the importance of image analysis for providing a quantitative understanding of the effects of morphology on production.

Recent studies using image analysis have allowed for new avenues to be explored. Image analysis was used to investigate inoculum concentration and morphology changes on protease secretion (Papagianni and Moo-Young, 2002). Image analysis has also proved useful in strain selections of *Metarrhizium anisopliae* (Yang *et al.*, 2001) and *Penicillium* sp. (Dorge *et al.,* 2000).

While the overall morphology of pelleted and filamentous forms is important, the relation of hypha, in particular hyphal tip, with biochemical mechanisms has not been well established and thus better understanding of hyphal function is essential.

Hypha grow by deposition of cell wall material at the apical tip, resulting in the characteristic cylindrical shape of the hypha. Formation of hyphae is the normal mode of development in the mycelial fungi. The ability to control wall synthesis in filamentous fungi is of major importance in many industrial processes but in particular where the mycelial biomass is to be used for human or animal feeding purposes. Electron microscope studies have shown that a young hypha consists of 3 relatively distinct zones: an apical zone; subapical zone; and a zone of vacuolation. Early work by Katz *et al.* (1972) on *A. nidulans* proposed a model for exponential growth and branch initiation based on observations of *A. nidulans* grown in shaken flask cultures.

They concluded that:

- The rate of extension at the growing points of short hyphae is proportional to both the hyphal length and the specific growth rate, i.e. short hyphae grow exponentially.
- Each growing point can extend at a rate characteristic of the organism and independent of the mass specific growth rate.

- A new growing point is formed when the capacity of the hypha to extend exceeds that of the existing points.

It was therefore concluded that the branching frequency would be proportional to specific growth rate.

Morphology of the hyphae can also be studied in detail using image analysis. Hyphal extension in *A. oryzae* and *A. niger* in relation to glucose concentration has been investigated (Müller *et al.,* 2000). The growth kinetics of the single hyphae of *A. oryzae* has been determined on-line with the aid of image analysis (Christiansen *et al.,* 1999). The study showed that the number of branches formed on a hypha is proportional to the length of the hypha that exceeds a certain minimum length required supporting the growth of a new branch.

The effects of elicitors on the morphology of *P. chrysogenum* have not been reported before and this together with studies into the uptake of oligosaccharides by *P. chrysogenum* should bring the first steps in an approach to the elucidation of the elicitation mechanism.

1.5 Sporulation and Germination

The characteristic asexual spore in higher fungi is the conidium. Conidia are classically defined as specialised, non-motile, asexual propagules that are usually formed from the side or tip of sporogenous cells and do not develop by progressive cytoplasmic cleavage (Aleopoulos and Mims, 1979). There are a huge variety of morphologically distinct conidial types produced by the higher fungi ranging from simple spheres to spirally curved and star-shaped structures (Cole, 1986). Conidial development is the result of an intricate series of tightly regulated biochemical events. Under normal conditions, one of the requirements for conidiation in ascomycetes is that cells undergo a defined period of vegetative growth to acquire developmental competence before switching from relatively undifferentiated vegetative growth to conodiphore development (Axelrod *et al.,* 1973). The mechanisms that control the acquisition of developmental competence and the switch from undifferentiated vegetative growth to connidiphore development are not fully understood, but genetically regulated (Pastushok and Axelrod, 1976; Timberlake and Marshall, 1988; Chang and Timberlake, 1992).

Studies have shown that sporulation is influenced by environmental factors that include nutrition (Smith, 1978). Skromne *et al.*, (1995) examined the effects of glucose and nitrogen limitation on sporulation of *A. nidulans* and the expression of brlA gene which plays a fundamental role in the switch from vegetative growth to sporulation. The study showed that glucose starvation induced brlA gene rapidly and resulted in spore formation. Nitrogen showed a similar pattern however spore formation was much reduced. Atmospheric factors such as carbon dioxide, temperature, relative humidity and light are important too (Barnett and Lilly, 1955; Taber, 1966; Sekiguchi *et al.,* 1975; Manachere, 1980; Mooney and Yager, 1990). Nutrient stress by high calcium ion

concentration in culture medium is known to influence conidiation in ascomycetes (Hadley and Harrold, 1958; Righelato *et al.*, 1968; Ugalde and Pitt, 1983; Adams *et al.*, 1998).

In favourable conditions fungal spores go through two stages: swelling and the emergence of germ-tubes (which describes germination). The germ-tube is the slender hypha that emerges from a germinating spore. Nutrient reserves within a spore are limited, so a germ-tube must quickly reach an environment having nutrients and other favourable factors for growth. To achieve this germ-tubes employ a remarkable number of sensory responses based on their environmental requirements. The following table shows some of the research work carried out to investigate these sensory responses.

Investigation	Reference	Micro-organism
Germ-tubes growing away from each other	Robinson (1980)	*Geotrichum candidum*
Growth towards amino acids	Manavathu and Thomas (1985)	*Achlya ambisexulalis*
Growth towards volatile compounds	Carlile and Matthews (1988)	*Chaetomium globosum*
Growth towards oxygen	Robinson (1973)	*Geotrichum candidum*
Growth away from oxygen	Carlile and Tew (1988)	*Phytophthtora citricola*
Growth away from light	Carlile (1970)	*Physarum polycephalum*

Recent studies have investigated the environmental factors that can affect germination in *Penicillium.* Inanova and Marfenina (2001) studied the effects of sucrose concentration, temperature and pH on the germination of *Penicillium* spores. The spore germination rate was found to reach a maximum at different values of the environmental factors studied. A similar study included the effects of water activity on the germination of *P. chrysogenum* spores. Sautour *et al.*, (2001) looked at the combined effect of temperature, water activity and pH on the germination of spores. Higher rates of spore germination were associated with a high level of water activity. A significant positive interaction between temperature and water activity was observed. Under these specific conditions, pH did not have a significant effect on the germination of *P. chrysogenum* spores.

Kimura *et al.* (2002) investigated the relationship of conidial enzymes xylanase and pectinase on spore germination and germ-tube development in *P. expansum*. Use of xylan or pectin as the sole carbon source did not initiate germination. The type of carbon source or enzyme levels did not matter with regards to germination, however, it was found that the enzyme levels did affect the elongation of germ-tubes.

1.6. Transport Mechanisms in Fungi

1.6.1 The Cell Wall

Cell walls in fungi include very different structures ranging from the thin, more or less unstructured cell wall in the growth zone, to the thick, strongly structured cell wall that covers older parts of a hypha. The volume of the cell wall may vary between 15 and 40% of the total hyphal volume (Clipson *et al.*, 1989). Both the volume and the composition of the cell wall can change as a response to the environment, for example, as a response to different sugars (Gooday and Trinci, 1980) or to toxic heavy metals (Collins and Storzky, 1989).

The cell wall counteracts the internal osmotic pressure. The cell wall is not a size exclusion barrier for low molecular weight solutes. Concerning membrane transport, two effects of the cell wall are of importance: the functional groups of the cell wall determine, together with those of the plasma membrane, the local concentration of ions near the transport systems; additionally, cations are concentrated whereas anions are repulsed, and the water that is bound to the cell wall acts selectively allowing ions to move only by diffusion (Grignon, 1991). It is this layer, and not the plasma membrane, that is the main permeability barrier for small, lipophilic molecules.

Protons, or the proton gradient, are the most important ions within the bioenergetic network of a hypha. As in plants, the fungal cell wall with its functional groups is expected to enable a hypha to maintain a pH within the cell wall that is 1 to 2 units lower than the bulk pH (Grignon, 1991). A collapse of the proton gradient can be expected only if the bulk pH is strongly buffered (Slavik and Kotyk, 1984).

Plant and animal cells detect many different stimuli, transduce the information into appropriate intracellular components, and respond by altering their metabolism, growth and development. Several types of intracellular components have been identified that function in the network of eukaryotic signal transduction pathways, including protein kinases, protein phosphatases, lipases, nucleotide exchange factors, ion channels, G proteins, lipid kinases and transcription factors (Dickson, 1995).

The order and function of many proteins within several animal-signalling pathways, from the perception of the stimulus to the activation of gene transcription, have been determined (Duffy and Perrimon, 1994).

By contrast, understanding of signalling events in plants is much more basic. Although multiple plant genes have been identified that are homologous to genes involved in animal signal transduction, their function in plants is largely unknown. Many signals are initially perceived by transmembrane receptors, a large number of which function by activation of an intrinsic protein kinase domain.

These types of receptors are known as receptor protein kinases (RPKs) and can be subdivided into two families: those that autophosphorylate on tyrosine residues and those that autophosphorylate on serine (Lemmon, 1994).

As the plant cell model is the closest model for further understating of the fungal receptor system, this in itself illustrates the difficulties in understanding the fungal transport and signalling system.

1.6.2 Transport Across Fungal Cell Membrane

The maintenance of chemical and electrical gradients across membranes is one of the main characteristics of living cells. Transport proteins embedded in the plasma membrane build up and maintain these gradients. Important tasks, such as signal transduction, uptake of nutrients, and homeostasis of the cell are facilitated by membrane transport.

The properties of plasma membrane transport are closely associated with the environment and with the ecological niche of filamentous fungi. The kind and the amount of available nutrients result in special adaptations of membrane transport (Wainwright, 1988; Jennings, 1990), and the characteristics of plasma membrane transport determine at least in part the competitive ability of a fungus in a specific environment (Jennings, 1995).

Most of the biotechnological processes with fungi aim at the overproduction of a specific metabolite. Membrane transport is involved in such a process in three ways: uptake of nutrients, uptake of precursors, and the excretion of product(s). The uptake of a precursor is limiting and it depends on its uptake mechanism. For instance, phenylacetic acid is taken up by diffusion through the lipid bilayer. However, it has a toxic effect as it dissipates the trans-membrane pH gradient (White *et al.,* 1999) and results in the inhibition of adenosine triphosphate (ATP) formation during electron transport (Eriksen *et al.,* 1998).

Membrane transport exerts its most important influence on product formation via the excretion mechanism(s). Four different mechanisms are possible: diffusion through the lipid bilayer,

diffusion mediated by a uniport, a symport mechanism, and an antiport mechanism (Konings *et al.*, 1992).

The latter three of these transport modes involve a transport protein. In these cases, regulation is important. Regulation can be specific (exerted by the transported molecule) or nonspecific (via the proton gradient or the membrane potential). In all of these cases, the yield may be influenced by the mechanism of excretion.

The nonuniform distribution of solutes within cells is one of the main characteristics of living cells. The most important devices responsible for the nonuniform distribution of ions are the various active transport systems in the plasma membrane and in the intracellular membranes. In general, protons, calcium, and sodium are transported out of the cytoplasm into subcellular compartments or into the extracellular medium whereas potassium and other nutrients are transported into the cytoplasm. Amino acids, magnesium, calcium, and phosphate are further transported into and stored in vacuoles (Klionsky *et al.*, 1990) or in the case of calcium in vesicles of the endoplasmatic reticulum (Takeuchi *et al.*, 1988). Transport of protons into vacuoles contributes to the homeostatsis of the cytoplasmic pH (Klionsky *et al.*, 1990).

Transport systems are not distributed equally along a hypha (Belozerskaya and Potapova, 1993). A region of about 200 μm behind the tip of the plasma membrane seems to be void of the H^+-ATPase, and the number of transport systems for nutrient uptake (e.g. amino acids) seems to be higher. The consequences are that toward the hyphal tip a gradient develops: the membrane potential decreases toward the tip, there is a positive inward current (uptake of amino acids) and the pH on the outer surface increase because of the proton influx via proton symports. The protons

needed for nutrient uptake at the hyphal tip are supplied by the H^+-ATPases. It was estimated that about one tenth of the protons pumped out flow along the hypha toward the tip (Takeuchi, 1988). Intracellularly, two gradients toward the tip were found: a gradient of vesicles of the endoplasmatic reticulum and, together with it, a gradient of calcium toward the hyphal tip (Takeuchi, 1988) and a decreasing potassium concentration toward the tip (Jennings, 1979).

1.6.3 The Plasma Membrane

An important task of the plasma membrane is the uptake of nutrients. In addition to this, the overall homeostasis of the intracellular environment is an key task of the plasma membrane. The plasma membrane is also involved in signal transduction. Very few details are known about signal transduction in filamentous fungi (Gadd, 1995).

The composition of the plasma membrane is best known in *Neurospora crassa*. The lipid content of fungal plasma membrane ranges from 30 to 50% (Weete, 1980). In *N. crassa*, the main components of the plasma membrane are phosthatidylcholine (46%), phosphatidylethanolamine (39%), and phosphatidylinositol (15%) (Losel, 1990). The plasma membrane is, in addition to the endoplasmatic reticulum, the only membrane in *N. crassa* that contains a high proportion of sterols, mainly ergosterol (up to 22 %) (Losel, 1990).

The transport systems are not distributed evenly along a hypha. Slayman (1992) has shown that the H^+-ATPases occupy about one third of the area of plasma membrane.

1.6.4 Cell Membrane Receptors

The outer surface of cells is covered with various carbohydrates, which are covalently linked either to a lipid or to a protein. Such glycolipids and glycoproteins are integral parts of cell membranes in most organisms and take part in interactions between cells or between cells and external molecules (Hughes, 1975).

Receptor functions have often been attributed to carbohydrates. Evidence that the receptors of several biologically active substances, such as thyrotropin and related glycoprotein hormones (Fishman, 1976), are glycolipids or glycoproteins has been shown.

The structural complexity and the small amounts of each specific carbohydrate-containing molecular species occurring in cell membranes have made the isolation and analysis of these molecules very difficult.

1.7 Reactive Oxygen Species

It has been shown that various species of active oxygen, which are produced from dioxygen, i.e. the superoxide ion, $O_2^{\cdot-}$, the hydroxyl radical $^{\cdot}OH$, and hydrogen peroxide, H_2O_2, are deleterious to cellular growth and development of many, if not all, living organisms (Cross *et al.*, 1998). Such active oxygen species, also termed 'Reactive Oxygen Intermediates, ROI'(Govrin and Levine, 2000), 'Reactive Oxygen Species (ROS)' (Emri *et al.*, 1999), or 'active oxygen' (Mallakin *et al.*, 1999), can disrupt cellular membranes, induce oxidative changes in DNA, and disrupt cellular metabolism (Halliwell, 1998).

ROS including superoxide anion, hydrogen peroxide and hydroxyl radicals are continuously generated in aerobic micro-organisms. The decomposition of hydrogen peroxide is of primary importance to be avoided as it causes severe oxidative injuries. This is due to the fact that this decomposition gives rise to one of the most reactive oxygen metabolites, the hydroxyl radicals, in the Fenton and Haber-Weiss reactions (Aust *et al.*, 1985).

Biological damage caused by oxygen radicals includes oxidation of membrane fatty acids, resulting in lipid peroxidation, oxidation of proteins and DNA damage (Farr and Kogoma, 1991 and Wang and Schellhorn, 1995). Because of this micro-organisms have developed methods to prevent and repair oxidative damage. The cell's defensive repertoire against ROS includes enzymatic defence responses, such as production of superoxide dismutase (SOD) and catalase (Wang and Schellhorn, 1995); and non-enzymatic antioxidants, such as biosynthesis of ascorbate and tocopherols (Halliwell, 1999 and Sigler *et al.*, 1999). ROS have been extensively studied in bacteria (Demple, 1996) and yeasts, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces*

pombe and *Candida albicans* (Flattery-O'Brian *et al.*, 1993; Lee *et al.*, 1995 and Jamieson *et al.*, 1996).

In animal cell systems, the role of ROS in neutrophils has been investigated (Bland *et al.*, 2001). Neutrophils are the most abundant of all the circulating immune cells (40-75%). They are the first line of defence against invading micro-organisms (Edwards, 1994). Neutrophils are capable of producing a wide range of substances that are potentially harmful to micro-organisms and these are divided into two groups: a) substances produced by oxygen dependent mechanisms or b) substances produced by oxygen independent mechanisms (Kuby, 1997). The oxygen dependent mechanisms are the most well researched, these mechanisms produce toxic and oxidising agents, such as $O_2^{\bullet}$, H_2O_2, HOCl, NO, NO_2, NH_2Cl, whilst the oxygen independent mechanisms use toxic peptides and hydrolytic enzymes. The production of ROS by neutrophils is a vital step in the killing of foreign organisms.

Polysaccharides have been shown to have a varied effect on neutrophils, for example, zymosan β-glucans have been shown to prime neutrophils by binding to a lectin receptor site on complement receptor 3 (Vetvicka *et al.*, 1996). This activates neutrophils to engulf and destroy target cells that inactivate bound complement 3b. On the other hand, it has also been shown that N-acetyl-D-glucosamine and mannose in combination can inhibit the production of superoxide from neutrophils (Zhang and Petty, 1994).

Research in filamentous fungi is limited to studies on *Aspergillus* sp. and *Neurospora* sp. (Munkres, 1992 and Kawasaki *et al.*, 1997). In *Penicillium* sp. very little is published with regards to ROS production and regulation. Emir *et al.* (1999) investigated the effects of menadione on oxidative stress in *P. chrysogenum*. Detailed studies in *Penicillium* sp. have been limited to the effects of glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) in the elimination of hydrogen

peroxide. GSH has been proven to play a crucial role in some other important biochemical processes as well, including a great number of bioreductive reactions, transports and the detoxification of different xenobiotics (Penninckx and Elskens, 1993). Moreover, GSH is structurally analogous to δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, which is an important intermediate of the penicillin G biosynthesis in *P. chrysogenum* P2 and, as a result, GSH may suppress penicillin production by inhibiting δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthase and isopenicillin N synthase activities (Ramos *et al.,* 1985 and Sanchez *et al.,* 1988).

2. MATERIALS and METHODS

2.1 Chemicals

All chemicals used in assays were obtained from BDH Limited, Poole, United Kingdom 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, hydrolysis, purification reactions, image analysis and dye tagging methods.

2.2 Culture Strain

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

2.3 Media

Different media were used for fermentations according to the requirements of the studies:

a) *Penicillium* Growth Medium (PGM) – this medium was prepared in several parts. The inorganic salts, carbon source (sucrose and lactose), nitrogen source (mycological peptone and ammonium sulphate) and $FeS0_4.7H_2O$ were each separately dissolved in distilled water and the pH was individually adjusted to 6.5 with 3M KOH before autoclaving. The inorganic salts solution was autoclaved at 121 ^0C for 15 minutes. The carbon and nitrogen sources were autoclaved at 115 ^0C for 15 minutes. All components were then aseptically added together after sterilisation and mixed before fermentation. The $FeSO_4.7H_2O$ solution was filter sterilised (using a 0.02 μm-syringe filter) before addition to the rest of the medium, prior to inoculation.

b) Penicillin Production Medium (PPM) – This medium was prepared as above. The only exception was in the concentration of the carbon and nitrogen sources. The details are given in Section 2.3.3.

2.3.1 Agar Medium for Strain Maintenance

Glycerol-molasses agar medium (GM) was used for sporulation and propagation of *P. chrysogenum* P2 strains. The pH of GM was adjusted to 6.5 with 3M KOH before they were autoclaved at 121 ^{0}C for 15 minutes. GM (Table 2.1) contained the following concentrations of ingredients in grams per litre (gL^{-1}) of distilled water:

Ingredients	Concentration (gL^{-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 ($FeSO_4.7H_2O$)	0.003
Bacteriological peptone	5.000
Molasses	2.500
Yeast extract	1.000

Table 2.1 Glycerol-Molasses Agar Medium

2.3.2 Inoculum Growth Medium

Penicillium Growth Medium (PGM) was based on a modified medium developed by Jarvis and Johnson (1947), and was used for the growth of *P. chrysogenum* P2. The medium was adjusted to pH 6.5 with 3M KOH before autoclaving. PGM contained the following ingredients in gL^{-1} of distilled water (Table 2.2):

Ingredients	Concentration (gL^{-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 ($FeSO_4.7H_2O$)	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	10.00
Mycological peptone	5.00
Sucrose	20.00

Table 2.2 *Penicillium* Growth Medium

2.3.3 Semi-Defied 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 contained the following ingredients in gL^{-1} of distilled water (Table 2.3):

Ingredients	Concentration (gL^{-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 ($FeSO_4.7H_2O$)	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	50.00
Mycological peptone	1.00
Sucrose	10.00

Table 2.3 Penicillin Production Medium

2.4 Culture Conditions

P. chrysogenum P2 inocula for shaken flask and bioreactor studies were grown in 100 mL lots of sterile PGM in 500 mL flasks. The shaken flask cultures of *P. chrysogenum* P2 inocula were incubated in an orbital shaker at 26 ^{0}C at a speed of 200 rpm with 2 cm throw for 48 hours.

2.4.1 Penicillin Production in Shaken Flask Cultures

After 48 hours of growth in PGM, 10.0 mL of inocula were transferred into 90 mL of sterile PPM in 500 mL flasks and then incubated as above. The shaken flask cultures were assayed for penicillin G over 120 hours unless indicated otherwise. Biomass determination was carried out at the end of the fermentation (144 hours).

2.4.2 Production of Secondary Metabolites in Bioreactor Cultures

Two litre controlled bioreactors with the same parameters were used for all bioreactor studies. The bioreactors were sterilised at 121 ^{0}C for 40 minutes while containing only the salts for production medium. Separately sterilised carbon and nitrogen sources and filter sterilised $FeSO_4.7H_2O$ were added to the bioreactors aseptically to make a total medium volume of 1.35 L before inoculation with 150 mL of 48-hour inoculum.

Fermentations were carried out using two or three bioreactors in parallel with equal working volumes of 1.50 L for the control and test fermentations. The cultures pH of all fermentations was automatically maintained between 6.5 and 6.7 with sterile 2M ammonium hydroxide and sulphuric acid. PPM was used for penicillin production and the dissolved oxygen tension (DOT) was automatically controlled above 30 percent air saturation with stirrer speed ranging from 250 to 600 rpm. There were no significant differences in the agitation rates between the control and elicited fermenters. The airflow rate and temperature were kept at 1.0 vvm and 26 ^{0}C respectively.

The 2 L fermenter vessels (LH Fermentations Ltd., Bucks, UK) used had an internal diameter of 12 cm, a shaft length of 17.5 cm to which two impellers were attached. The distance of the top impeller, with a diameter of 5 cm, was 11 cm from the top of the shaft. This impeller was 4 cm apart from the bottom impeller, which had a diameter of 3 cm. This impeller was placed 2 cm above the line sparger. Each impeller had 5 tips. No baffles were used for fermentations.

2.4.3 Addition of Phenylacetic Acid to Penicillin Cultures

A stock solution of phenylacetic acid (PAA), the precursor for penicillin G, was prepared by dissolving 100 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 NaOH. The stock solution was sterilised by autoclaving at 115 ^{0}C for 15 minutes and added to both control and test cultures after 24 hours of growth in PPM. To shaken flask cultures, PAA was added at concentration of 1.0 gL^{-1}. In bioreactor cultures, 1.0 gL^{-1} of PAA was initially added after 24 hours and was maintained between 0.5 to 1.5 gL^{-1} by intermittent additions where necessary. PAA was monitored using the HPLC method outlined in Section 2.7.

2.5 Preparation of Oligosaccharides

2.5.1 Preparation of Alginate Oligosaccharides by Partial Acid Hydrolysis

Three oligosaccharide fractions were prepared from sodium alginate by partial acid hydrolysis according to the method described by Asilonu *et al.* (2000)(Fig. 2.1). 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 ^{0}C for six hours 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 retentate was collected, rinsed with, and re-dissolved in approximately 300 mL of distilled water. Sodium hydroxide solution (0.3 M) 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 0.50% (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, its pH 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).

2.5.2 Preparation of Mannan Oligosaccharides by Enzymatic Hydrolysis of Locust Bean Gum

Mannan oligosaccharides were prepared from LBG by enzymatic hydrolysis with Gamanase enzyme mixture (Novozyme Ltd, Denmark) (Fig. 2.1). LBG (1.0 g) was dissolved in 50 mL distilled water and heated to 80 ^{0}C and 0.1 mL of Gamanase enzyme was added. The reaction was carried out for 5 minutes and quenched by heating to 100 ^{0}C. The mixture was cooled to room temperature and frozen at –70 ^{0}C. The frozen sample was then freeze dried. The hydrolysis products were analysed by TLC and designated the abbreviation MO (mannan oligosaccharides).

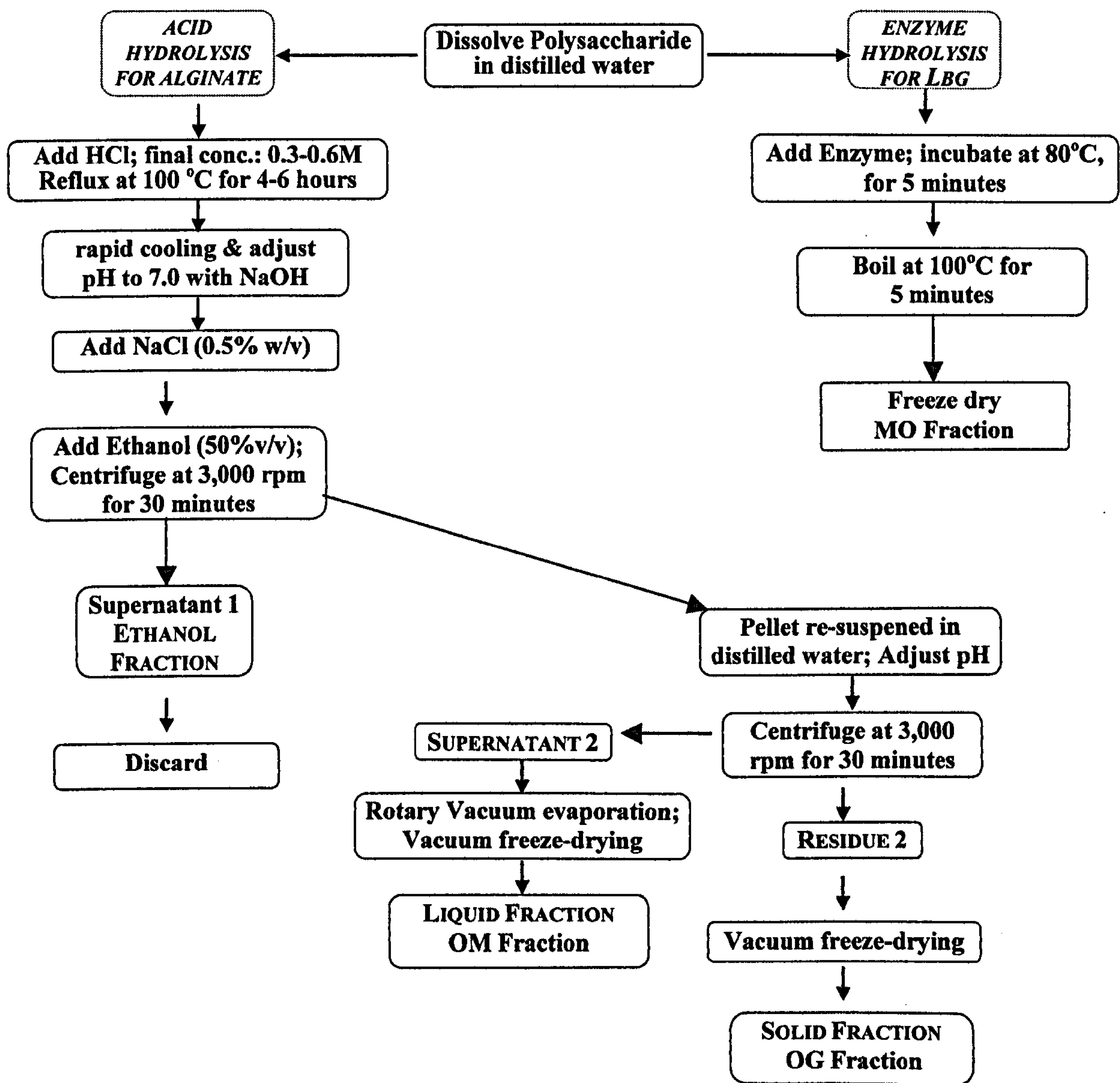


Fig. 2.1 Flow chart for production of oligosaccharides from polysaccharides.

2.5.3 Determination of Oligosaccharide Size by Thin Layer Chromatography of Oligosaccharides

The DP and the purity of the oligosaccharides derived from sodium alginate or LBG were determined by TLC. The DP was determined for partial acid hydrolysis 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 (15 μL) of oligosaccharides and known standards (1 gL^{-1}) were applied on the TLC plates at horizontal 15 mm intervals and air-dried. After drying, the plates were placed in the solvent and allowed to run for 7 hours. 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 ^{0}C oven for 10 to 15 minutes to develop the characteristic TLC spots used for the identification of the oligosaccharides.

2.6 Addition of Oligosaccharides to Production Cultures

The initial work on the use of carbohydrate elicitors in the overproduction of secondary metabolites in P. *chrysogenum* was carried out by Ariyo *et al.*, (1997, 1998) and by Asilonu *et al.*, (2000). The time of addition plays a crucial rule in the effects of oligosaccharides on the overproduction of penicillin G in this system. Work by Ariyo *et al.*, (1997) showed that the addition of oligosaccharides (OM, OG and MO) had their greatest effect on overproduction at 48 hours. This has also been shown to be the case in plant cell cultures (Marero *et al.*, 1997).

The concentration of elicitors added is also of great importance. For elicitation to be a viable process the concentration of elicitor added must be in mgL^{-1} for them not to be considered a carbon source, and thus the overproduction effect will be attributed to an increase in the available carbon. For this, extensive studies were carried out by Ariyo *et al.*, (1997) in *P. chrysogenum* P2 and Pertuccioli *et al.*, (1999) in *P. variabile* P16 systems. Work on penicillin G and GOD production showed that concentrations of between 50 – 200 mgL^{-1} had overproduction effects in both systems.

Based on the work mentioned above for this study the time of addition was at 48 hours and the concentration of elicitor added was 150 mgL^{-1}.

The DP for the oligosaccharides is given bellow:

Oligosaccharide	DP
OG	7
OM	7
MO	5-8

Table 2.4 DP of oligosaccharides by TLC analysis

The effects of all added oligosaccharides to the cultures at microgram per millilitres concentrations were studied on the following:

- Cell biomass
- Penicillin G and 6-APA concentrations
- Spore germination
- Morphology of *P. chrysogenum* P2
- Sporulation of liquid culture
- Visualisation of oligosaccharides in *P. chrysogenum* P2
- Reactive Oxygen Species

2.7 Assays

2.7.1 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.

2.7.2 Benzylpenicillin and 6-aminopenicillanic acid

The concentrations of penicillin G and 6-APA in culture broth samples were determined by HPLC (Perkin Elmer Series 4). 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.

6-APA standard was kindly provided by Dr Peter Clark (ACS Dobfar UK Ltd.).

Samples were then analysed by a gradient HPLC method (Adlard *et al.*, 1991) using a Spherisorb 5µm 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 0.03 M potassium dihydrogen orthophosphate buffer made using HPLC grade water. The buffer pH was adjusted to 5.5 with

0.03 M dipotassium 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 and after sample analysis.

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

Table 2.5 HPLC gradient profile for analysis of penicillin G and 6-APA.

2.8 Visualisation of Tagged Oligosaccharides and Penicillin G Production in Shaken Flask Cultures of *P. chrysogenum* P2

Fluorophore-assisted carbohydrate electrophoresis (FACE) technique was used to label oligosaccharides according to the method of Kumar *et al.* (1996). Oligosaccharides were labelled by adding 5 μL of 0.15 M 8-aminonaphthalene-1,3,6-trisulphonate (ANTS) in 15% (v/v) acetic acid and 5 μL of 1 M sodium cyanoborohydride in DMSO to excess oligosaccharide (1000 μg). The oligosaccharide-ANTS mixture was incubated at 37 ^{0}C for 16 hours. ANTS-labelled oligosaccharides were isolated by electrophoresis on 20% polyacrylamide gel that ran at constant current of 20 mA for 2 hours. The ANTS-labelled oligosaccharide portion was cut out of the gel, freeze-dried and stored in the dark at –70 ^{0}C until use.

To visualise the location of ANTS-labelled oligosaccharide in mycelia of *P. chrysogenum* P2 strain, the ANTS-labelled oligosaccharide was re-dissolved in a millilitre of sterile distilled water and added to a 48-hour culture of *P. chrysogenum* P2 to a final concentration of 150 mgL^{-1}. Samples were taken from the flasks at the point of addition and observed under long UV microscope (x400 and x1000) and pictures were taken as to ascertain the location of the ANTS-labelled oligosaccharide with relation to mycelia. Samples were then taken at 24 hours intervals for 120 hours and were analysed for penicilin G.

2.9 Investigation of Reactive Oxygen Species in *P. chrysogenum* P2

The method used for the identification of Reactive Oxygen Species (ROS) was developed from that described by Bland *et al.* (2001). This modified method was used to investigate the effects of alginate, LBG and their oligosaccharides on the production of ROS by *P. chrysogenum* P2.

2', 7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma, UK) was used as an indicator of cell activation of ROS. It was stored in an opaque airtight container at –20 ^{0}C.

DCFH-DA is a non water-soluble powder, thus 0.001 g of it was initially dissolved in 1 mL of ethanol (HPLC grade) and then transferred to 500 mL of PPM salt solution (made in HPLC grade water). The final concentration of DCFH-DA was 10 μg mL^{-1}.

Oligosaccharides and polysaccharides were weighed to a concentration of 0.F15 % (w/v) and were dissolved in PPM salt solution (made in HPLC grade water).

The test was performed in a Dynatech 'white clear bottom' microplate (to minimise 'cross-talk' between wells). All solutions were made up fresh on the day of the experiment. Positive [2', 7'-Dichlorofluorescein (DCF)] and negative (PPM salt solution) controls had no polysaccharide present.

Each well on the microplate contained 130 μL of oligosaccharide or polysaccharide (0.15% w/v) solution (control was PPM salt solution), 130 μL of *P. chrysogenum* P2 suspension, and 130 μL DCFH-DA solution using a multi-channel automatic pipette.

Fluorescence was recorded every 5 minutes for 60 minutes on a Packard Fluorocount microplate fluorometer at 485 nm excitation and 530 nm emission. The gain was set at 1 and the photo-multiplier tube (PMT) was set at 1100. No maximum was set for any well (readings were taken from the centre of the wells to avoid any edge effects). All work was carried out in triplicate.

2.10 Morphological Studies of *P. chrysogenum* P2

Studies on the morphological changes of *P. chrysogenum* P2 clump size and number of tips were undertaken using Leica image analysis system.

Samples from STR were used for these studies. Broth samples were gently shaken to ensure hyphal entanglement was minimised and diluted to 1 gL^{-1} (Parker *et al.*, 1989). One hundred microliters of sample were evenly spread on a glass slide. The slides were covered with 1 percentage methylene blue for 1 min to stain the fungus. The slides were then washed with distilled water and left to dry.

The different morphologies were investigated using Leica image analysis software linked to a light microscope. Observations were made at all magnifications and x100 and x200 were considered the most appropriate for analysis. The software was calibrated at these magnifications using a micrometer slide.

An average of 100 pellets were analysed for each sample for area of clumps and number of hyphal tips. All work was carried out in triplicate.

2.11 *P. chrysogenum* P2 Germination and Inoculum Morphology Development Studies

The effect of oligosaccharides on the germination of *P. chrysogenum* P2 spores was investigated. Shaken flask experiments were carried out in these studies. Ninety-nine millilitres PGM was inoculated with 1 mL of 1x 10^7 mL^{-1} spore suspension. Control and elicitation experiments were undertaken. For elicitation studies OM, MO, OG, LGB and Alginate were used. Samples taken at two-hour intervals for 24 hours were analysed for germination and development of hyphae/clumps. An average of 100 spores were assayed for germination or hyphae development for each sample. All work was carried out in triplicate.

2.12 Sporulation Studies for *P. chrysogenum* P2

For this study, samples were taken every 24 hours from STR from the control and elicited (OM, MO and OG) cultures. Samples were taken up to 144 hours.

These samples were thoroughly mixed and a dilution of 1:10 was made of all samples after which the spore count was determined. Spores were counted using a haemocytometer.

3. RESULTS

Introduction to Results

In this chapter the results of this study are presented. They highlight the effects of oligosaccharides and in some cases polysaccharides on penicillin G and 6-APA production as well as their effect on morphology and sporulation. Elicitors were added to a concentration of 150 mgL^{-1} at 48 hours. Oligosaccharide elicitors have a DP of 7 for OG and OM and a DP of between 5-8 for MO.

The chapter is divided into subsections each presenting a particular research aspect. It is divided as follows:

- **Production studies** – the effect of oligosaccharide elicitors on production of biomass, penicillin G and 6-APA

- **Inoculum morphology studies** – the effect of oligosaccharides and polysaccharides on spore germination and inoculum development in *P. chrysogenum* P2

- **Morphology studies** – the effect of oligosaccharides on the pattern of hyphal development

- **Sporulation studies** – the effect of oligosaccharides on sporulation of *P. chrysogenum* P2

- **ANTS labelled oligosaccharide studies** – observations of oligosaccharides in relation to *P. chrysogenum* P2 using ANTS

- **Reactive oxygen species** – the effect of oligosaccharides and polysaccharides on production of reactive oxygen species in *P. chrysogenum* P2

3.1 Production and Enhancement

The results in this section relate to the elicitation of biomass, penicillin G, 6-APA in stirred tank reactors. All fermentations were carried out in triplicate and the standard error was applied to all graphs. Three oligosaccharides have been used separately in this study: OM, OG and MO. They have all shown enhancement of penicillin G and 6-APA production. There was no significant difference in biomass production using the elicitors.

Concentrations of both penicillin G and 6-APA have been shown to increase in the presence of OG when compared to control. For penicillin G and 6-APA a maximum increase of 52% and 19% was observed respectively.

Concentrations of both penicillin G and 6-APA have been shown to increase in the presence of OM when compared to control. For penicillin G and 6-APA a maximum increase of 70% and 26% was observed, respectively.

Concentrations of both penicillin G and 6-APA have been shown to increase in the presence of MO when compared to control. For penicillin G and 6-APA a maximum increase of 128% and 39% was observed, respectively.

3.1.1 Oligoguluronate Elicitation

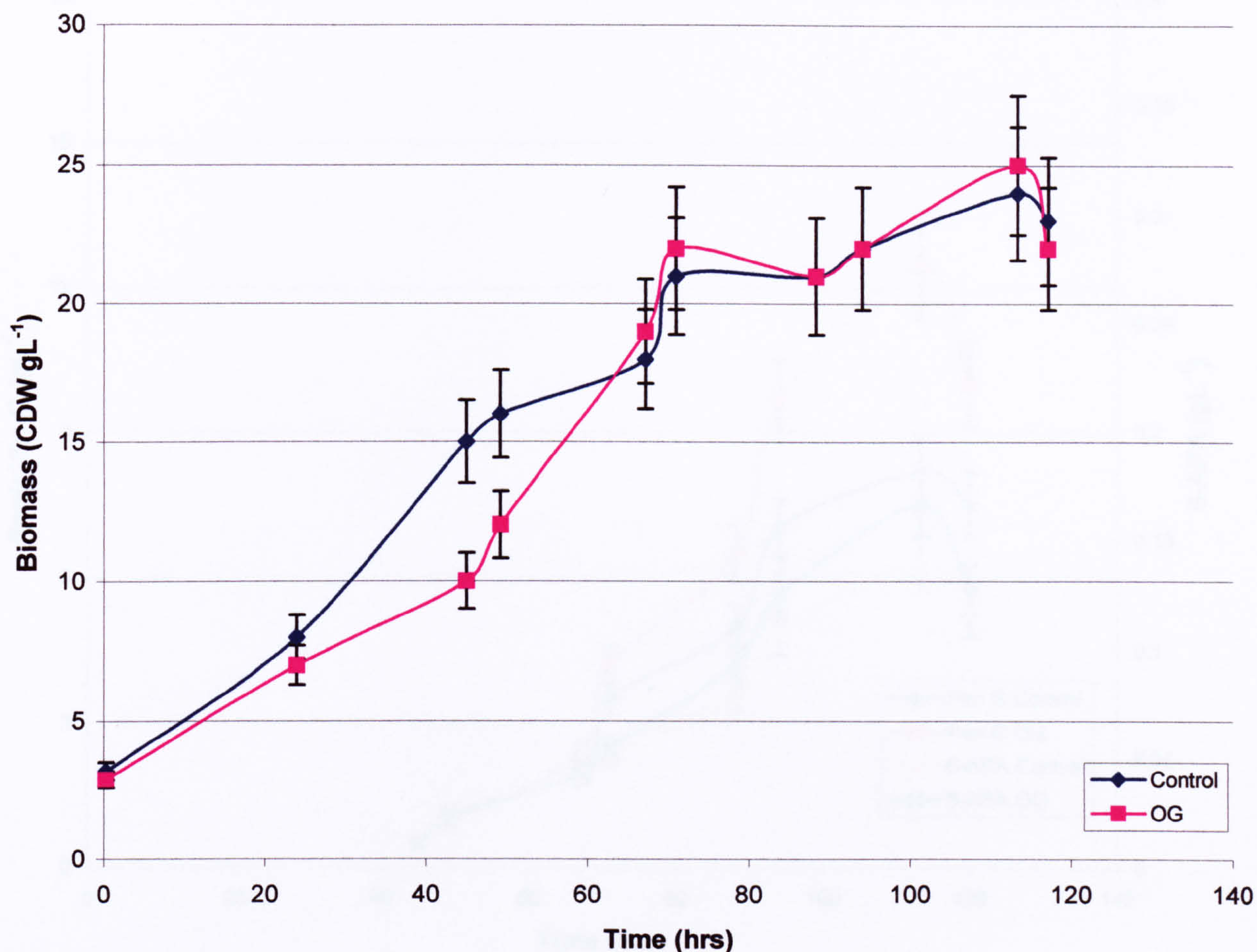


Fig 3.1 Biomass production in Control and Elicited STR fermentations of *P. chrysogenum* P2

Figure 3.1 compares the biomass concentration in cell dry weight (CDW) between STR fermentations of control and elicited cultures. The elicitor used, OG, does not elicit a notable increase in biomass compared to the control. The maximum biomass concentrations were 24 gL^{-1} and 25 gL^{-1} after 113 hrs for control and elicited cultures, respectively.

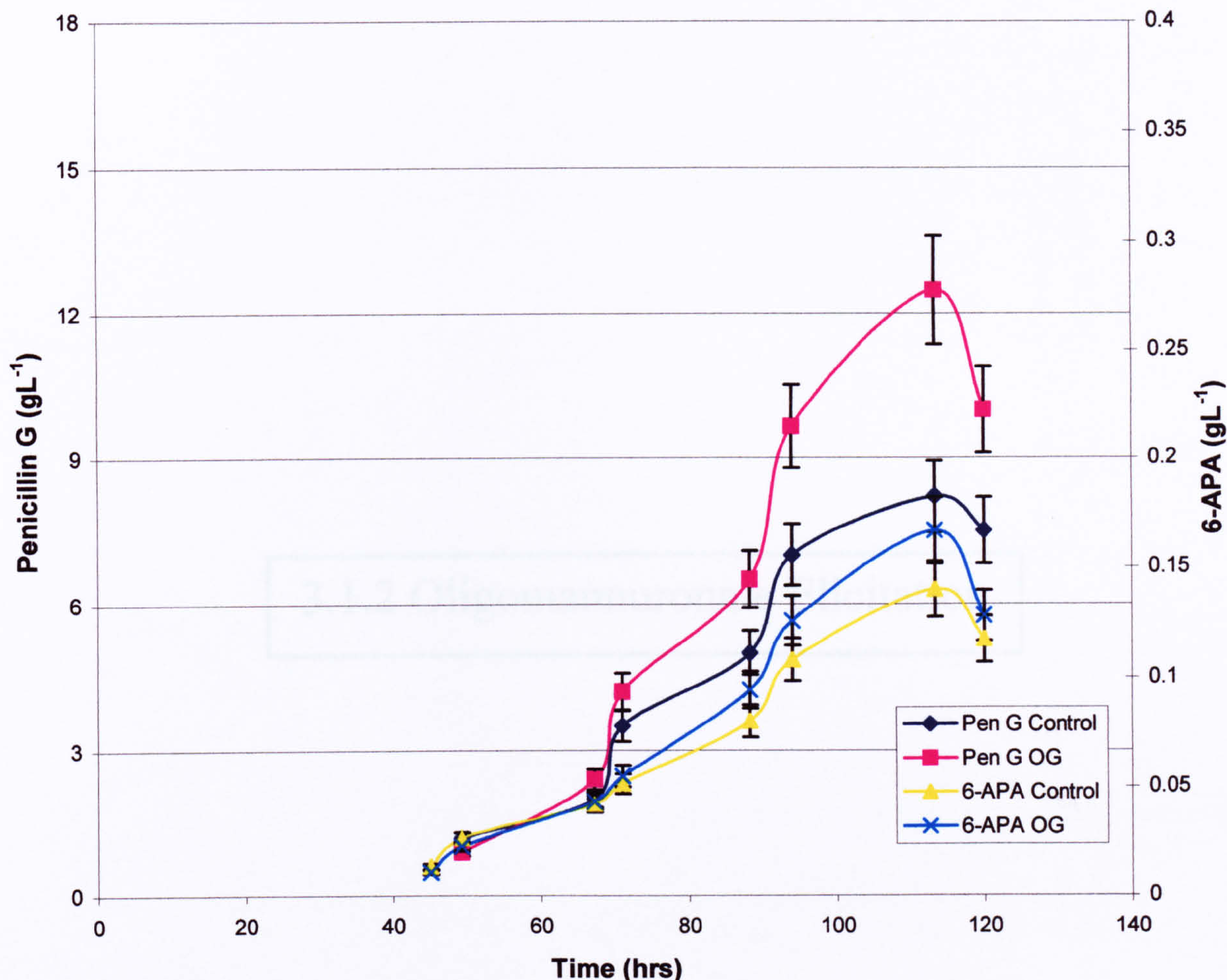


Fig 3.2 Penicillin G and 6-APA concentration in Control and Elicited cultures of *P. chrysogenum* P2

Figure 3.2 shows the production of two secondary metabolites of *P. chrysogenum* P2. Levels of both penicillin G and 6-APA have been shown to increase in the presence of OG when compared to control. For penicillin G and 6-APA a maximum increase of 52% and 19% was observed, respectively, after 113 hrs.

3.1.2 Oligomannuronate Elicitation

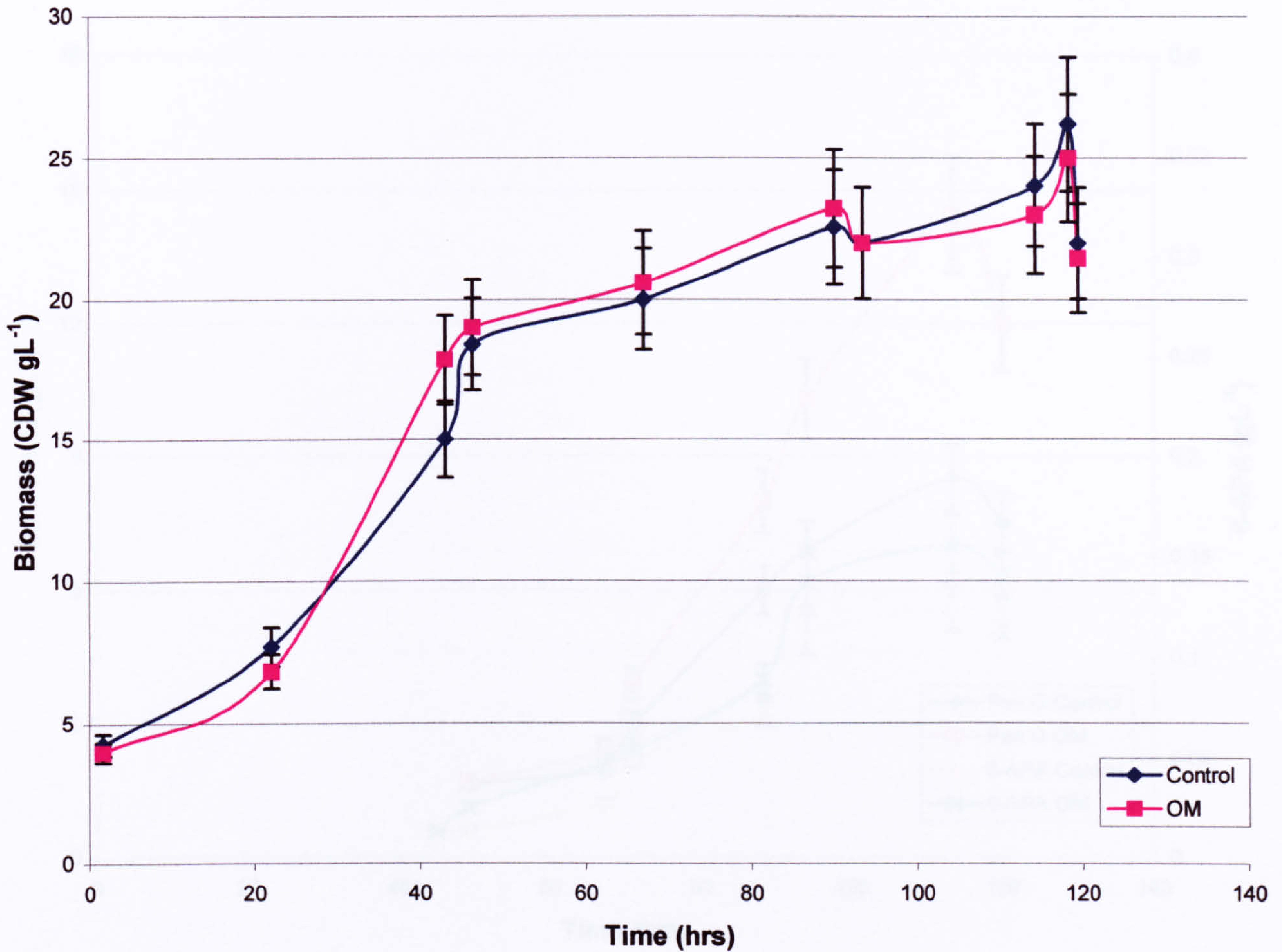


Fig 3.3 Biomass production in Control and Elicited STR fermentations of *P. chrysogenum P2*

Figure 3.3 compares the biomass concentration in CDW between STR fermentations of control and elicited cultures. The elicitor used, OM, does not elicit a notable increase in biomass compared to the control. The maximum biomass concentration was 26 gL^{-1} and 25 gL^{-1} after 113 hrs for control and elicited cultures, respectively.

Penicillin G and 6-APA production in Control and OM Elicited STR fermentations of *P. chrysogenum* P2

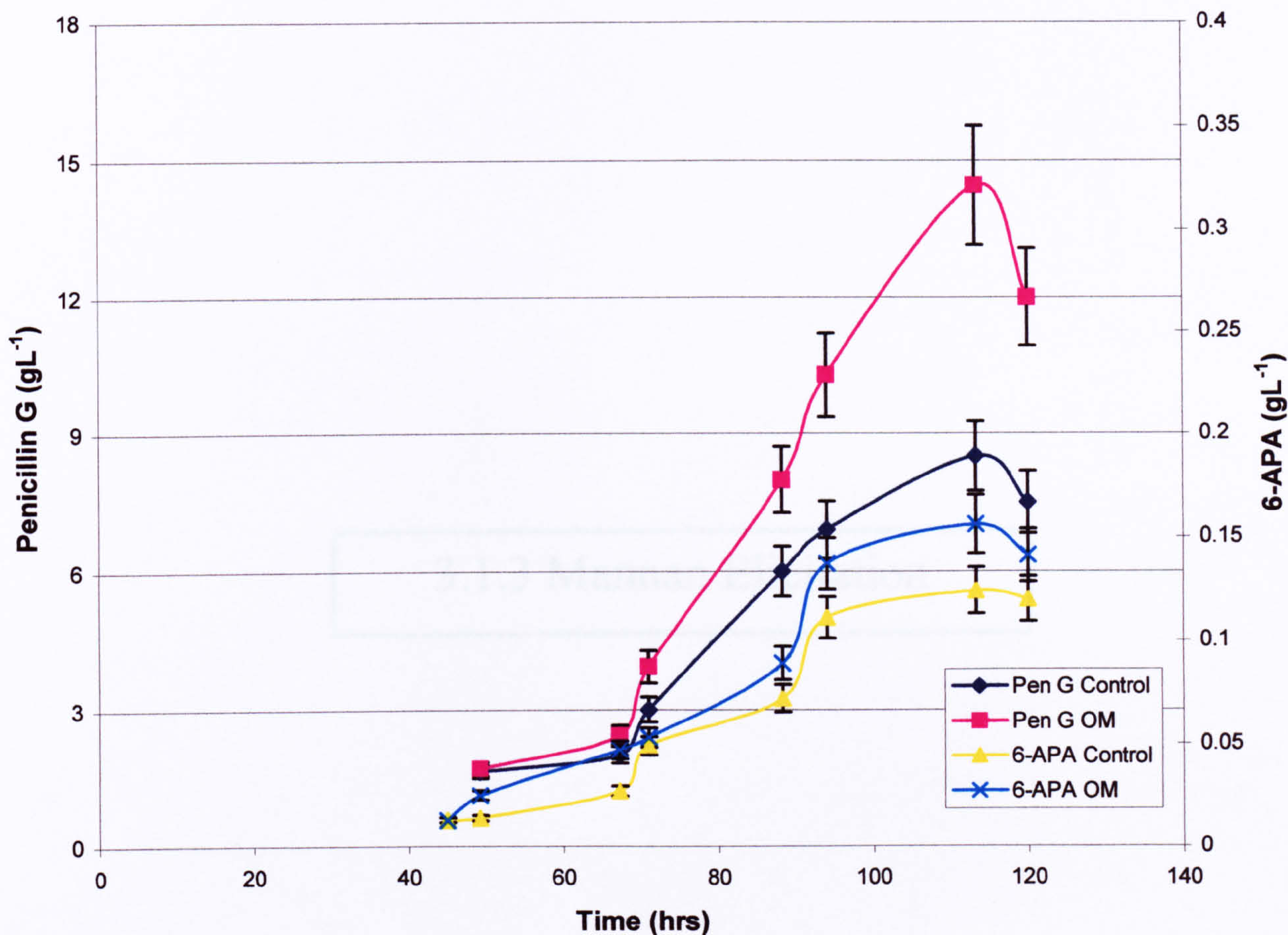
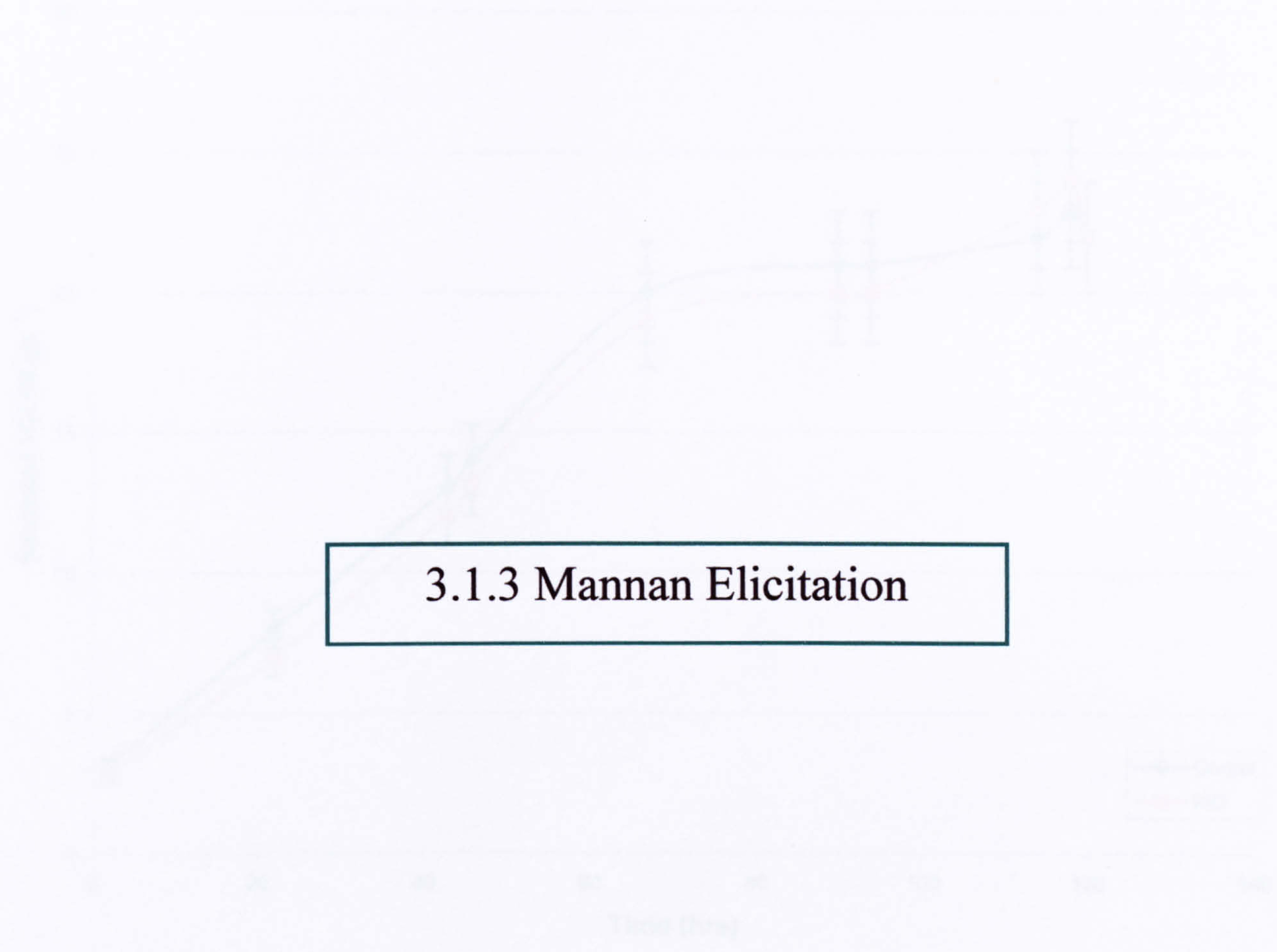


Fig 3.4 Penicillin G and 6-APA concentration in Control and Elicited cultures of *P. chrysogenum* P2

Figure 3.4 shows the production of two secondary metabolites of *P. chrysogenum* P2. Levels of both penicillin G and 6-APA have been shown to increase in the presence of OM when compared to control. For penicillin G and 6-APA a maximum increase of 71% and a 26% was observed, respectively, after 113 hrs.



3.1.3 Mannan Elicitation

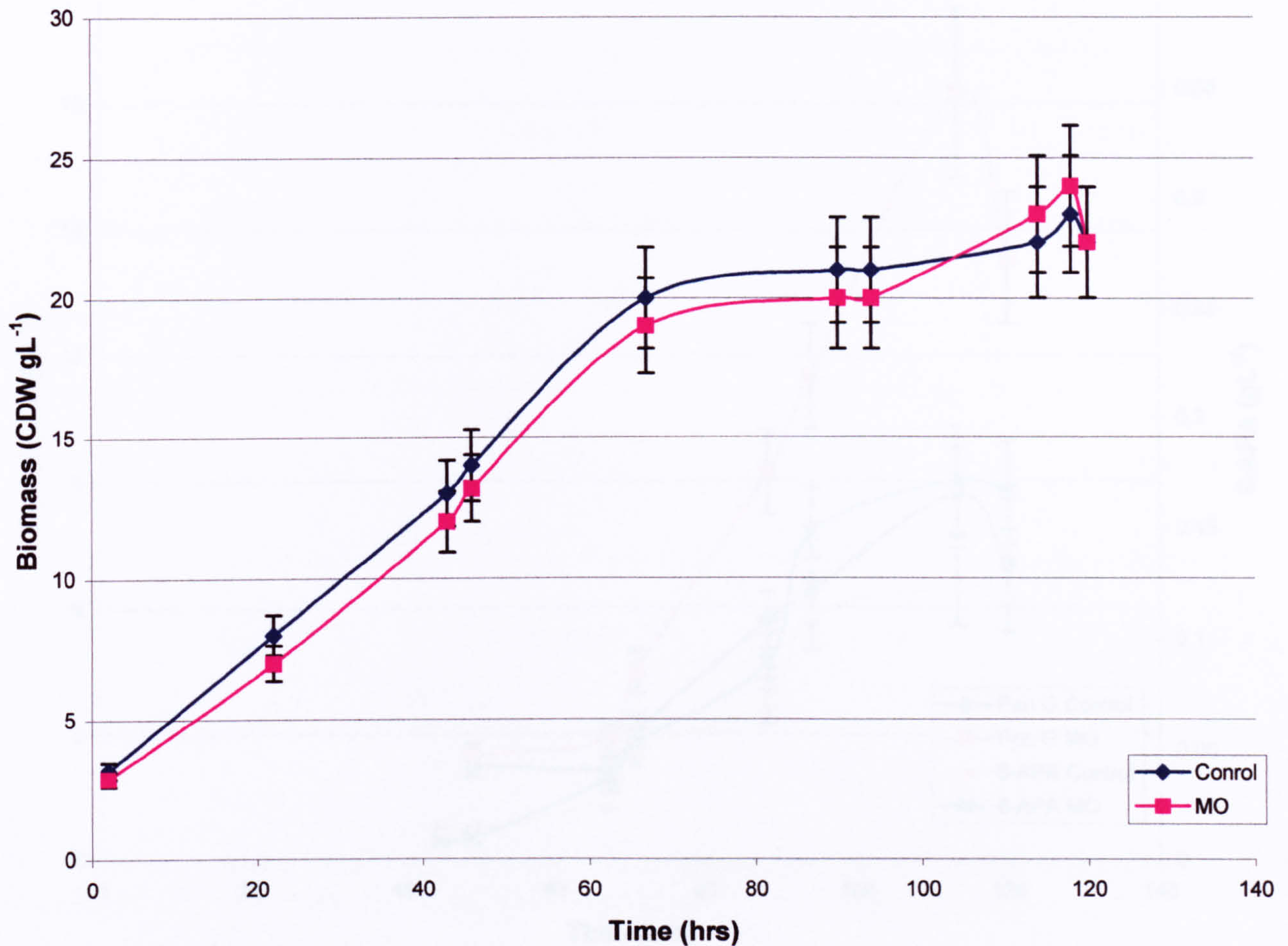


Fig 3.5 Biomass production in Control and Elicited STR fermentations of *P. chrysogenum P2*

Figure 3.5 compares the biomass concentration in CDW between STR fermentations of control and elicited cultures. The elicitor used, MO, does not elicit a notable increase in biomass compared to the control. The maximum biomass was 24 gL^{-1} and 23 gL^{-1} after 118 hrs for control and elicited cultures, respectively.

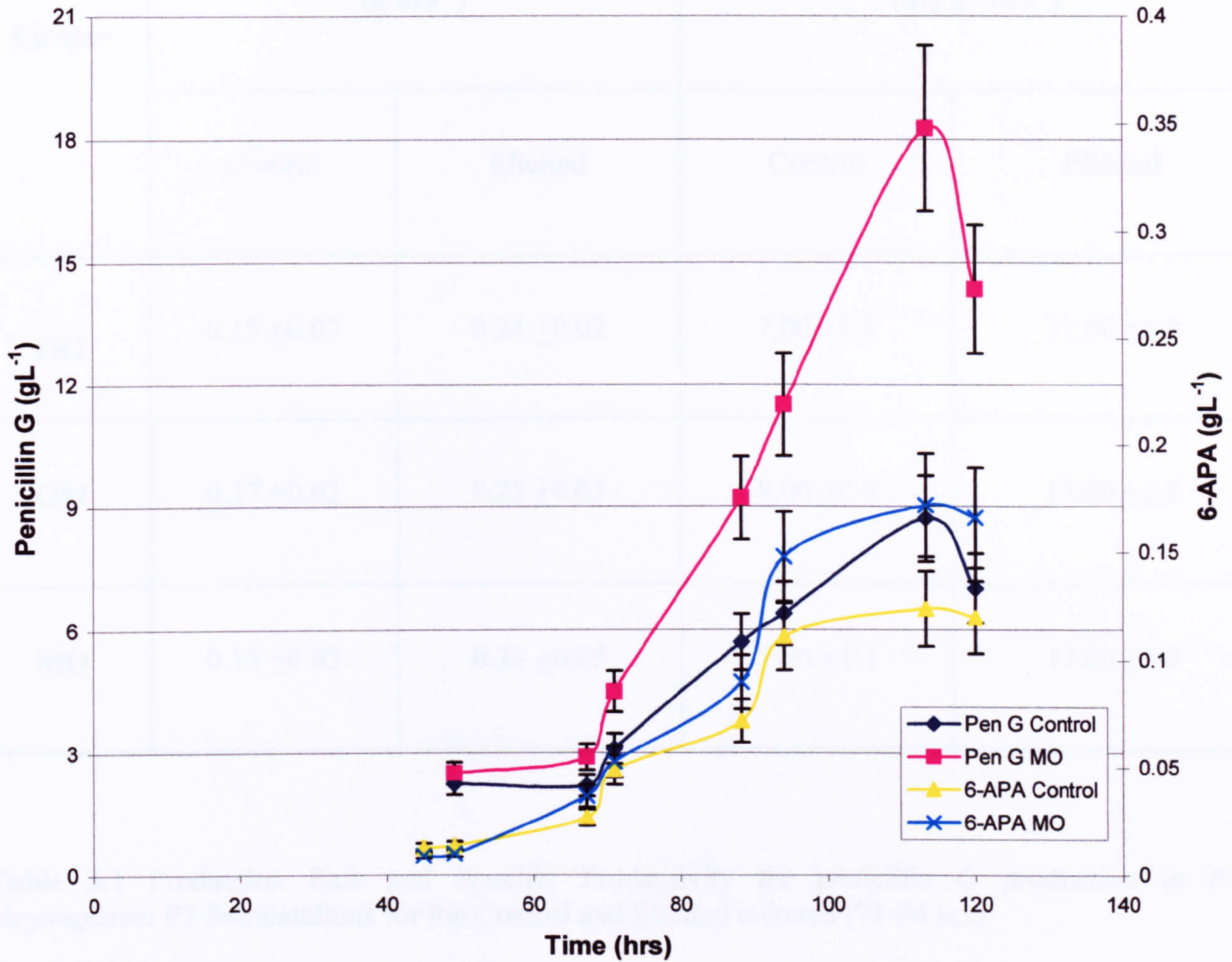


Fig 3.6 Penicillin G and 6-APA concentration in Control and Elicited cultures of *P. chrysogenum* P2

Figure 3.6 shows the production of two secondary metabolites of *P. chrysogenum* P2. Levels of both penicillin G and 6-APA have been shown to increase in the presence of MO when compared to control. For penicillin G and 6-APA a maximum increase of 128% and 39% was observed, respectively, after 113 hrs.

Elicitor	Penicillin G Production Rate ($g\ hrs^{-1}$)		Penicillin G Specific Productivity ($mg\ g^{-1}\ hrs^{-1}$)	
	Control	Elicited	Control	Elicited
OG	0.15 $\pm$0.03	0.24 $\pm$0.02	7.00 $\pm$1.2	11.00 $\pm$1.3
OM	0.17 $\pm$0.02	0.28 $\pm$0.02	8.00 $\pm$0.9	13.00 $\pm$1.2
MO	0.15 $\pm$0.03	0.30 $\pm$0.03	7.00 $\pm$1.1	15.00 $\pm$1.3

Table 3.1 Production Rate and Specific Productivity for penicillin G production in *P. chrysogenum* P2 fermentations for the Control and Elicited cultures (71-94 hrs)

The above results are the averages of three fermentation runs. The highest production rate compared to control can be observed in the MO supplemented cultures, with the lowest in OG supplemented culture. The specific productivity is the highest in MO supplemented cultures and the lowest in OG supplemented cultures.

Elicitor	6-APA Production Rate (mg hrs^{-1})		6-APA Specific Productivity (mg g^{-1} hrs^{-1})	
	Control	Elicited	Control	Elicited
OG	2.50 ±0.3	3.10 ±0.0003	0.124 ±0.005	0.143 ±0.009
OM	2.70 ±0.3	3.70 ±0.0005	0.125 ±0.002	0.168 ±0.012
MO	2.80±0.4	5.20 ±0.0002	0.136 ±0.004	0.264 ±0.009

Table 3.2 Production Rate and Specific Productivity for 6-APA production in *P. chrysogenum* P2 fermentations for the Control and Elicited cultures (71-94 hrs)

The above results are the averages of three fermentation runs. The highest production rate compared to control can be observed in the MO supplemented cultures, with the lowest in OG supplemented culture. The specific productivity is the highest in MO supplemented cultures and the lowest in OM supplemented cultures.

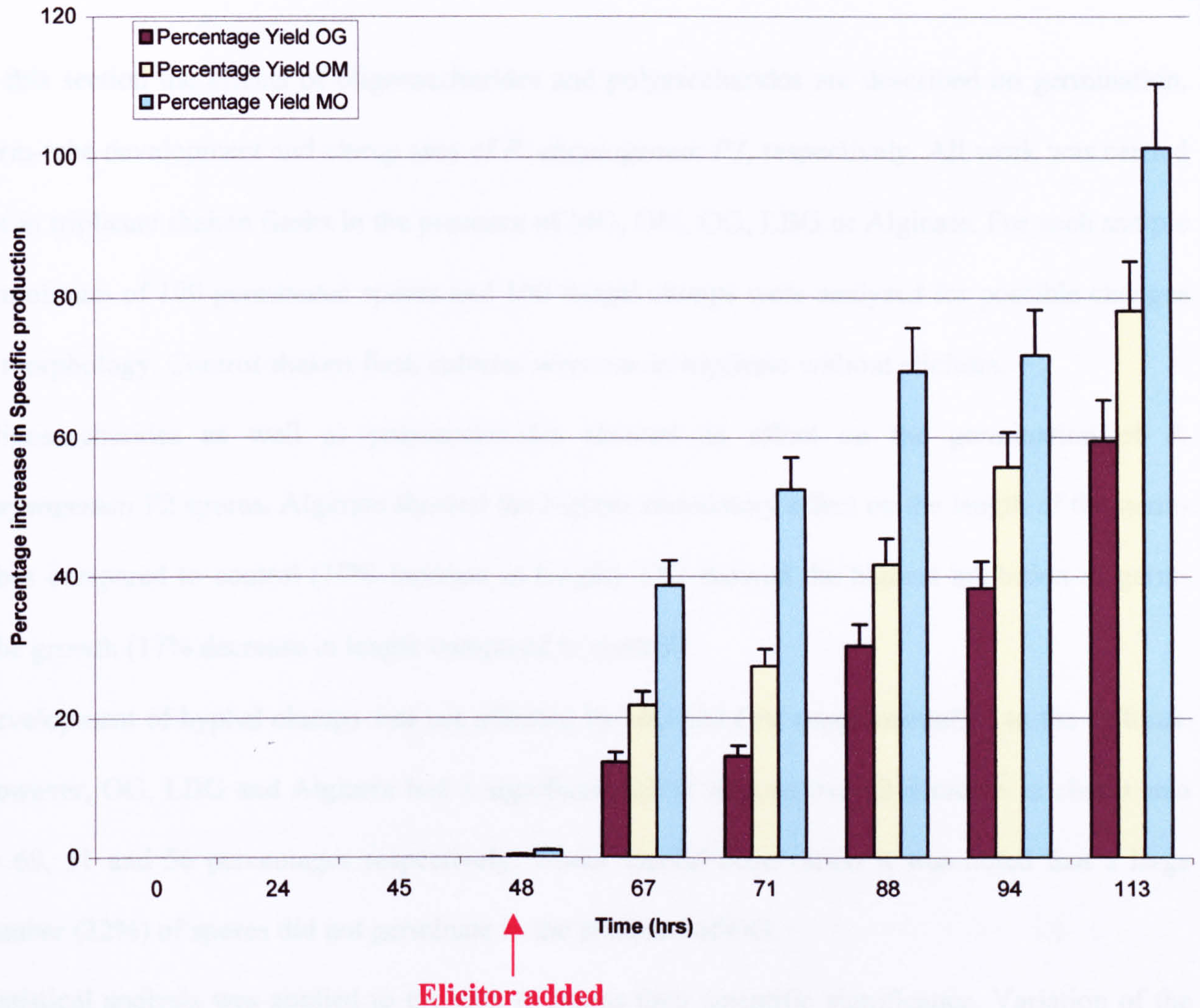


Fig 3.7 Penicillin G percentage yields of elicitor added STR cultures of *P. chrysogenum* P2 compared to Control

Figure 3.7 shows the differences in percentage yields (based on CDW) of penicillin G produced when elicited with OG, OM and MO elicitors. MO shows the highest level of elicitation, with OM and OG having a lesser impact.

3.2 Inoculum Morphology Studies

In this section the effects of oligosaccharides and polysaccharides are described on germination, germ-tube development and clump area of *P. chrysogenum P2*, respectively. All work was carried out in triplicate shaken flasks in the presence of MO, OM, OG, LBG or Alginate. For each sample a minimum of 100 germinated spores and 100 fungal clumps were analysed for possible changes in morphology. Control shaken flask cultures were run in triplicate without elicitors.

Oligosaccharides as well as polysaccharides showed an effect on the germination of *P. chrysogenum* P2 spores. Alginate showed the highest stimulatory effect on the length of the germ-tubes compared to control (17% increase in length). OG showed the highest inhibition of germ-tube growth (17% decrease in length compared to control).

Development of hyphal clumps was not affected by MO and OM supplementation to the cultures. However, OG, LBG and Alginate had a significant effect with an overall decrease in clump area by 69, 51 and 56 percentages respectively. Under careful observation it was noted that a large number (32%) of spores did not germinate in the presence of OG.

Statistical analysis was applied to the data to assess their scientific significance. Variation of the triplicate samples was investigated using the analysis of variance (ANOVA) method. For all samples the variance ratio was less than 1. It was concluded that there was no significant difference between triplicate samples.

Culture	Time (hrs)			
	0	**12**	**22**	**48**
MO	0	22	98	Clumps
OM	0	21	98	Clumps
OG	0	10	68	Clumps
LBG	0	19	98	Clumps
Alginate	0	37	93	Clumps
Control	0	28	98	Clumps

Table 3.3 Percentage *P. chrysogenum* P2 spore germination for Control and Elicited shaken flask cultures

Table 3.3 shows the effect of oligosaccharides and polysaccharides on germination of *P. chrysogenum* P2 spores over time. OG was shown to be inhibitory on the germination of the spores (10%) after 12 hours while alginate stimulated germination of spores (37%) over the same time period, compared to control.

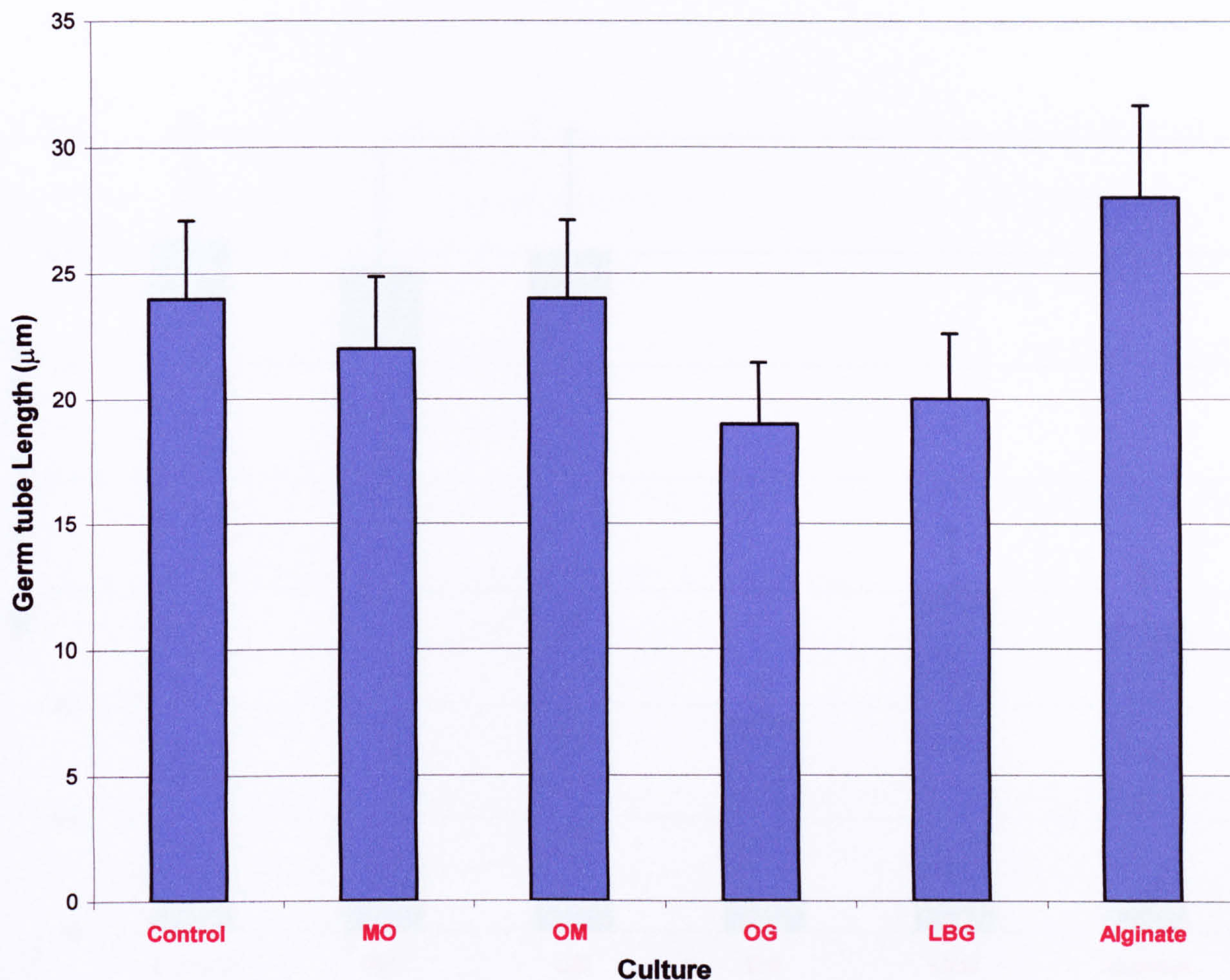


Fig 3.8 Effect of oligosaccharides and polysaccharides on germ-tube length of *P. chrysogenum* P2 spores

Figure 3.8 shows the effect of oligosaccharides and polysaccharides on germ-tube length of *P. chrysogenum* P2 spores. Alginate shows the highest stimulatory effect on germ-tubes compared to control (17% increase in length). OG shows the highest inhibition of germ-tube growth (17% decrease in length compared to control).

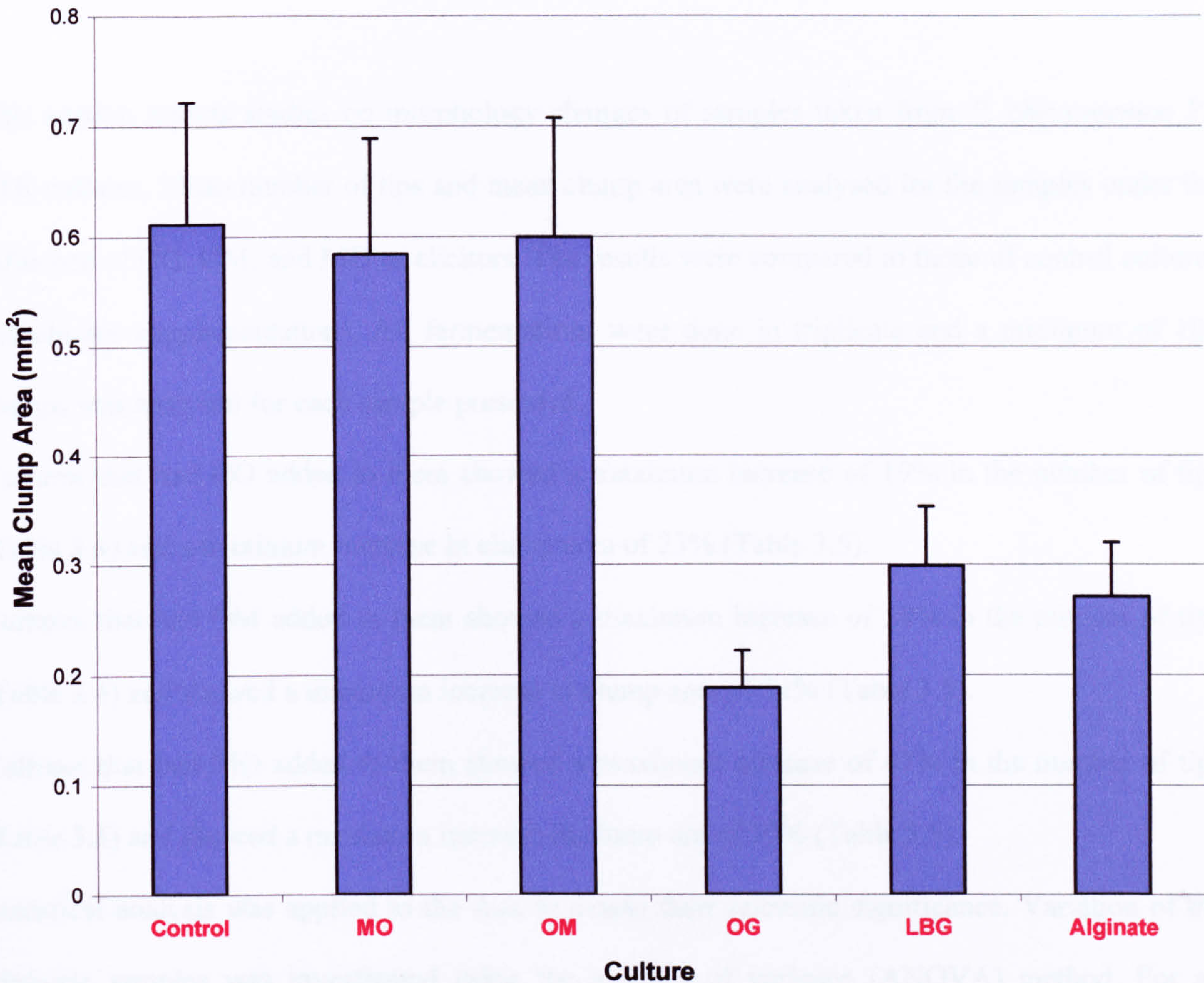


Fig 3.9 Effect of oligosaccharides and polysaccharides on *P. chrysogenum* P2 inoculum morphology

Figure 3.9 shows the effect of oligosaccharides and polysaccharides on the development of hyphal clumps of *P. chrysogenum* P2. MO and OM showed no effect on the development of hyphal clumps. However, OG, LBG and alginate have a significant effect with an overall decrease in clump area by 69, 51 and 56 percentiles respectively. Under careful observation it was noted that a large number of spores did not sporulate in the presence of OG.

3.3 Morphology Studies of *P. chrysogenum* P2 STR Cultures

This section reports studies on morphology changes of samples taken from *P. chrysogenum* P2 STR cultures. Mean number of tips and mean clump area were analysed for the samples under the influence of OG, OM, and MO as elicitors. The results were compared to those of control cultures (no elicitor supplementation). All fermentations were done in triplicate and a minimum of 100 clumps was analysed for each sample presented.

Cultures that had OG added to them showed a maximum increase of 19% in the number of tips (Table 3.4) and a maximum increase in clump area of 23% (Table 3.5).

Cultures that had OM added to them showed a maximum increase of 29% in the number of tips (Table 3.4) and showed a maximum increase in clump area of 31% (Table 3.5).

Cultures that had MO added to them showed a maximum increase of 47% in the number of tips (Table 3.4) and showed a maximum increase in clump are of 59% (Table 3.5).

Statistical analysis was applied to the data to assess their scientific significance. Variation of the triplicate samples was investigated using the analysis of variance (ANOVA) method. For all samples the variance ratio was less than 1. It was concluded that there was no significant difference between triplicate samples.

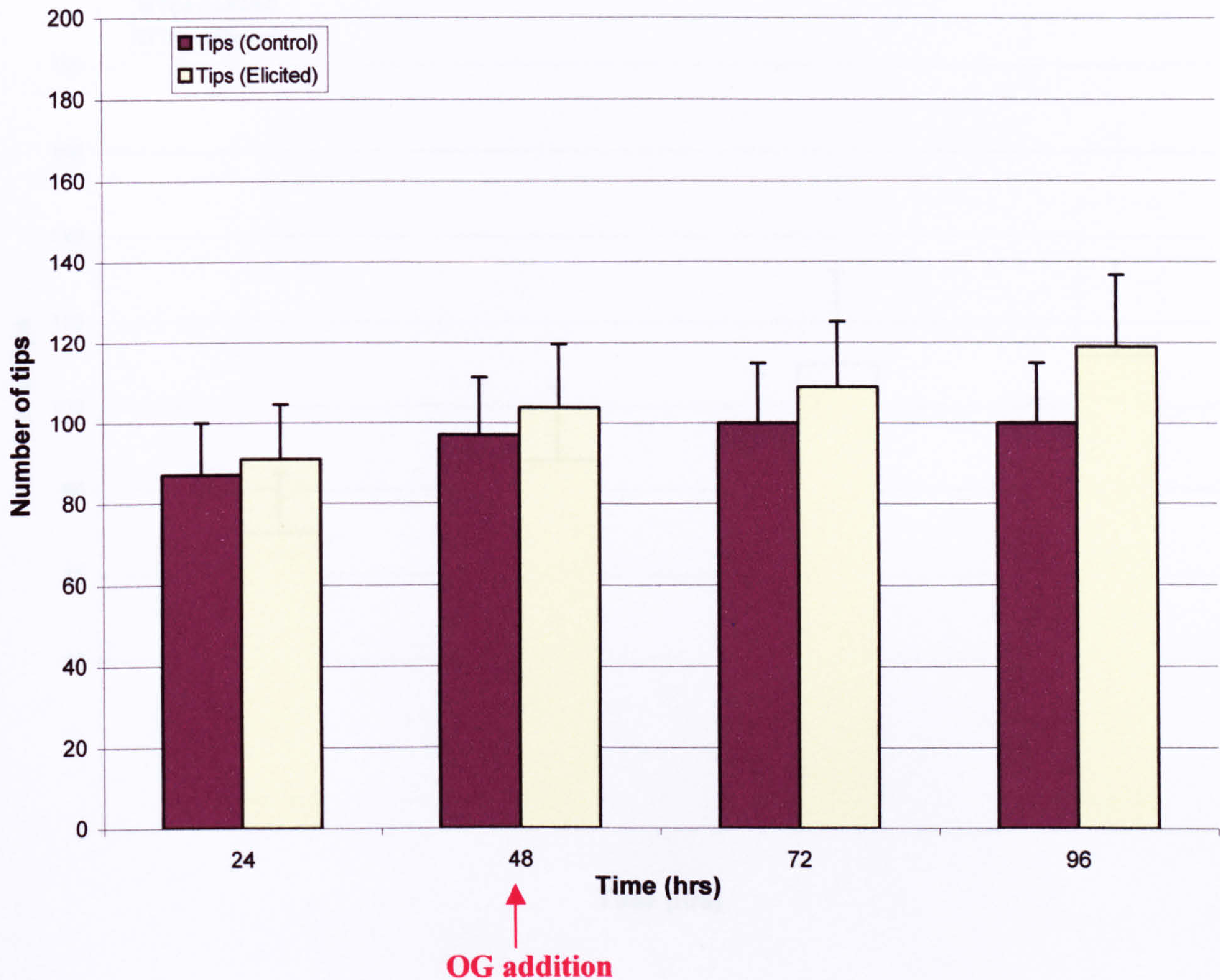


Fig 3.10 Differences in hyphal tip numbers in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.10 shows the differences in the number of hyphal tips of *P. chrysogenum* P2 clumps under control and elicited conditions. Cultures that had OG added to them showed a maximum increase of 19% (Table 3.4).

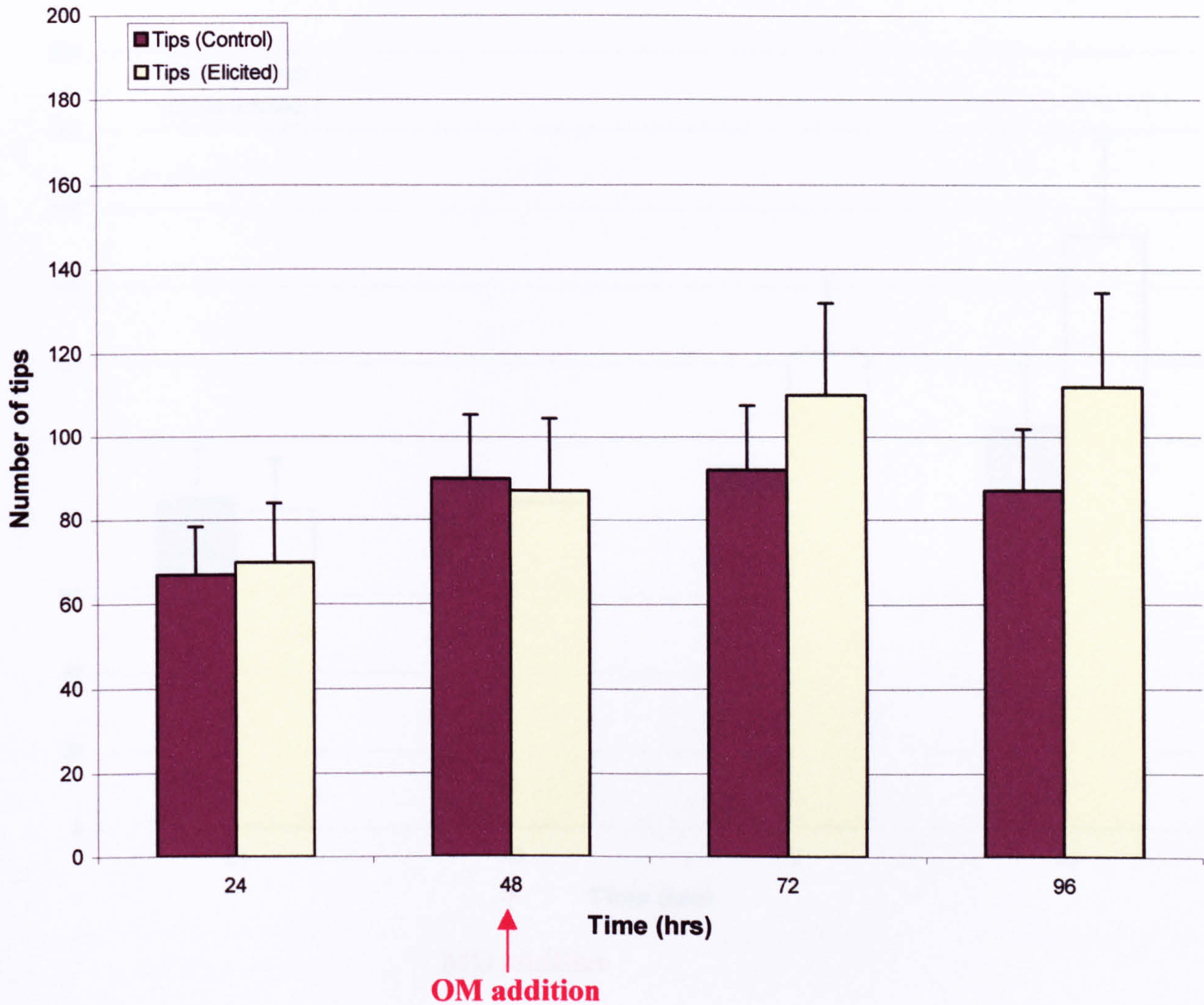


Fig 3.11 Differences in hyphal tip numbers in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.11 shows the differences in the number of hyphal tips of *P. chrysogenum* P2 clumps under control and elicited conditions. Cultures that had OM added to them showed a maximum increase of 29% (Table 3.4).

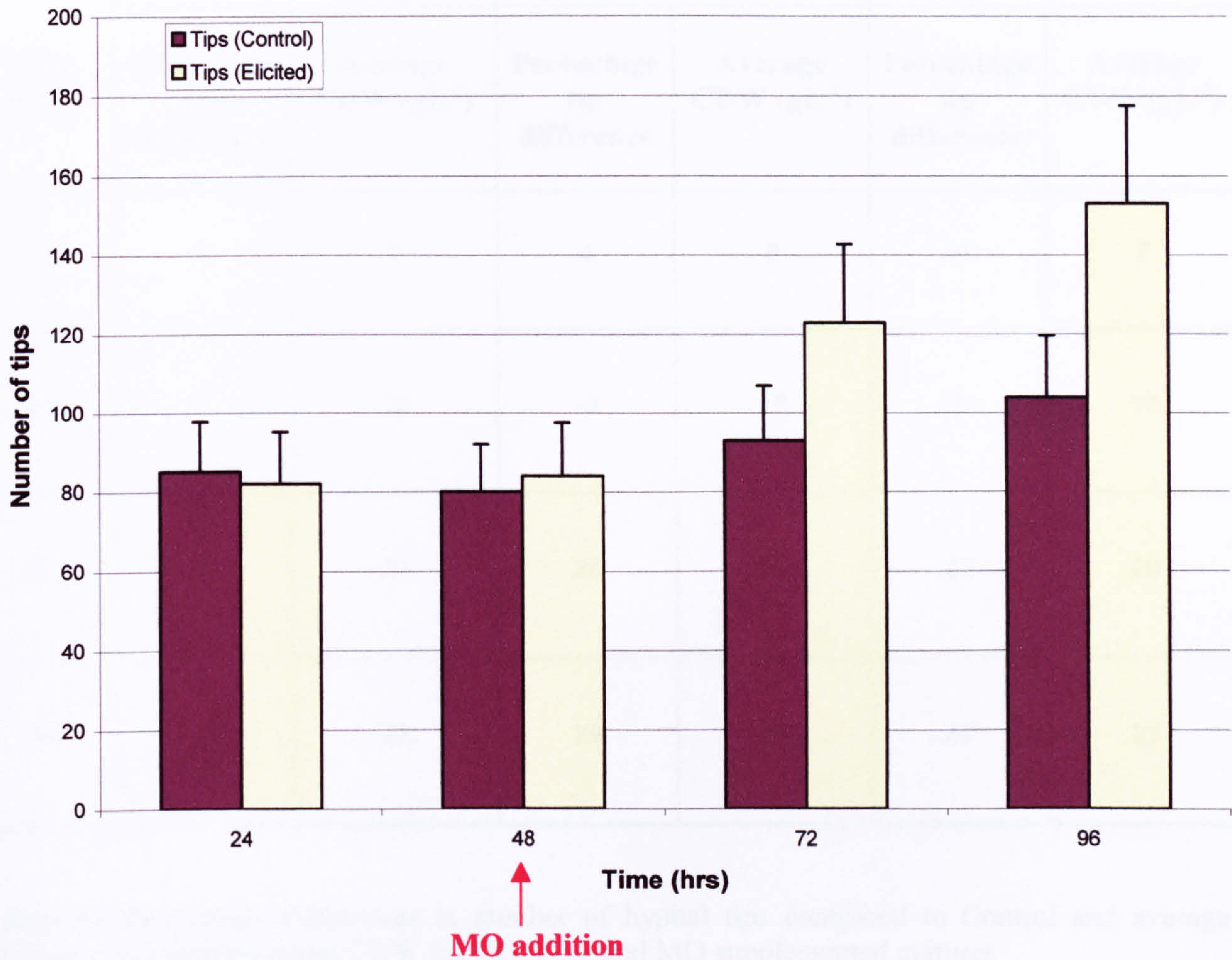


Fig 3.12 Differences in hyphal tip numbers in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.12 shows the differences in the number of hyphal tips of *P. chrysogenum* P2 clumps under control and elicited conditions. Cultures that had MO added to them showed a maximum increase of 47% (Table 3.4).

Time (hrs)	OG		OM		MO	
	Percentage tip Difference	**Average CDW (gL^{-1})**	**Percentage tip difference**	**Average CDW (gL^{-1})**	**Percentage tip difference**	**Average CWD (gL^{-1})**
24	5	7	4	8	-4	7
48	7	12	-3	15	5	13
72	9	21	20	21	32	20
96	19	22	29	23	47	23

Table 3.4 Percentage differences in number of hyphal tips compared to Control and average biomass concentration using CDW for OG, OM, and MO supplemented cultures

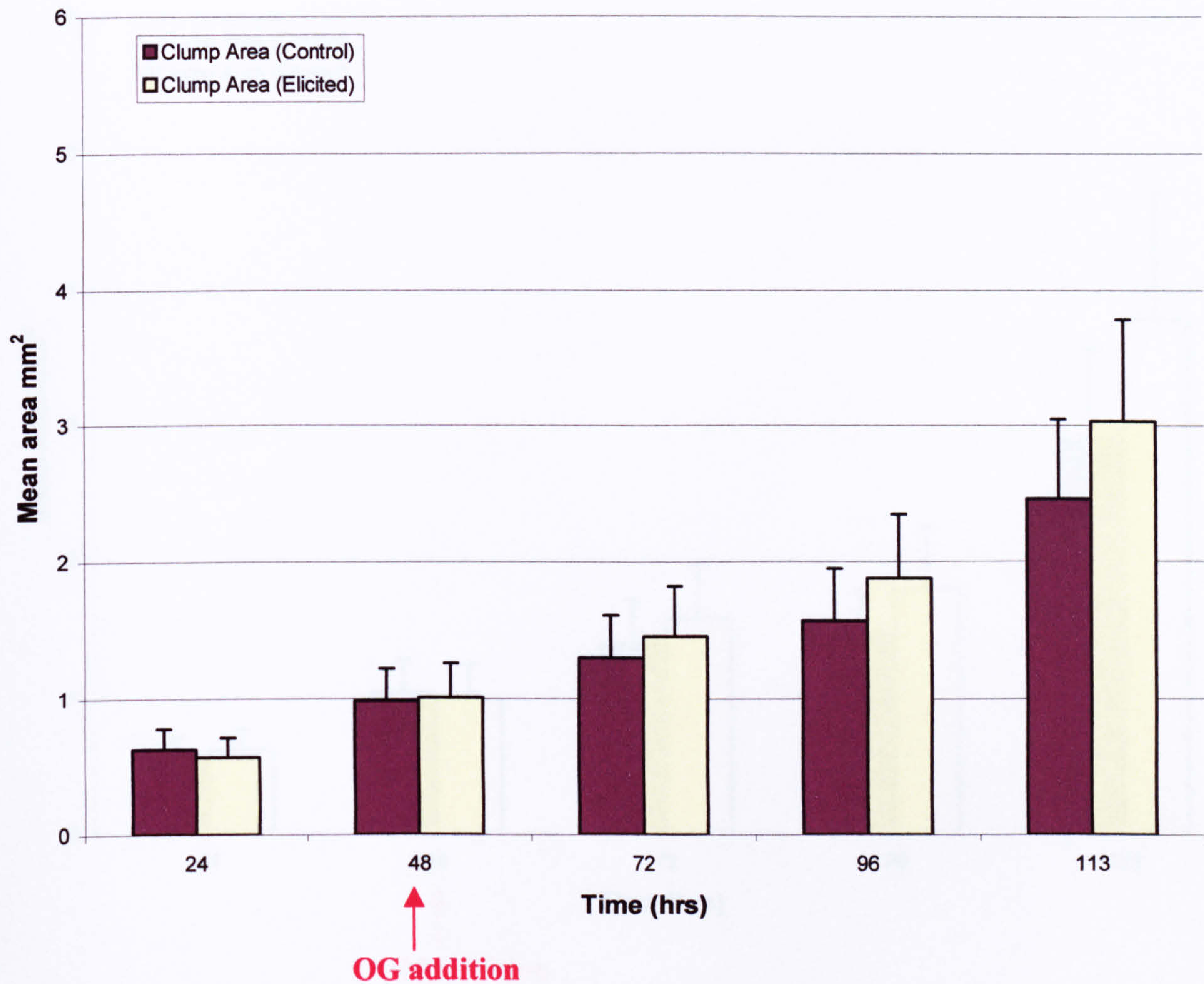


Fig 3.13 Differences in clump size in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.13 shows the differences in mean area of *P. chrysogenum* P2 clumps under control and elicited conditions. Cultures that had OG added to them showed a maximum increase in area of 23% (Table 3.5).

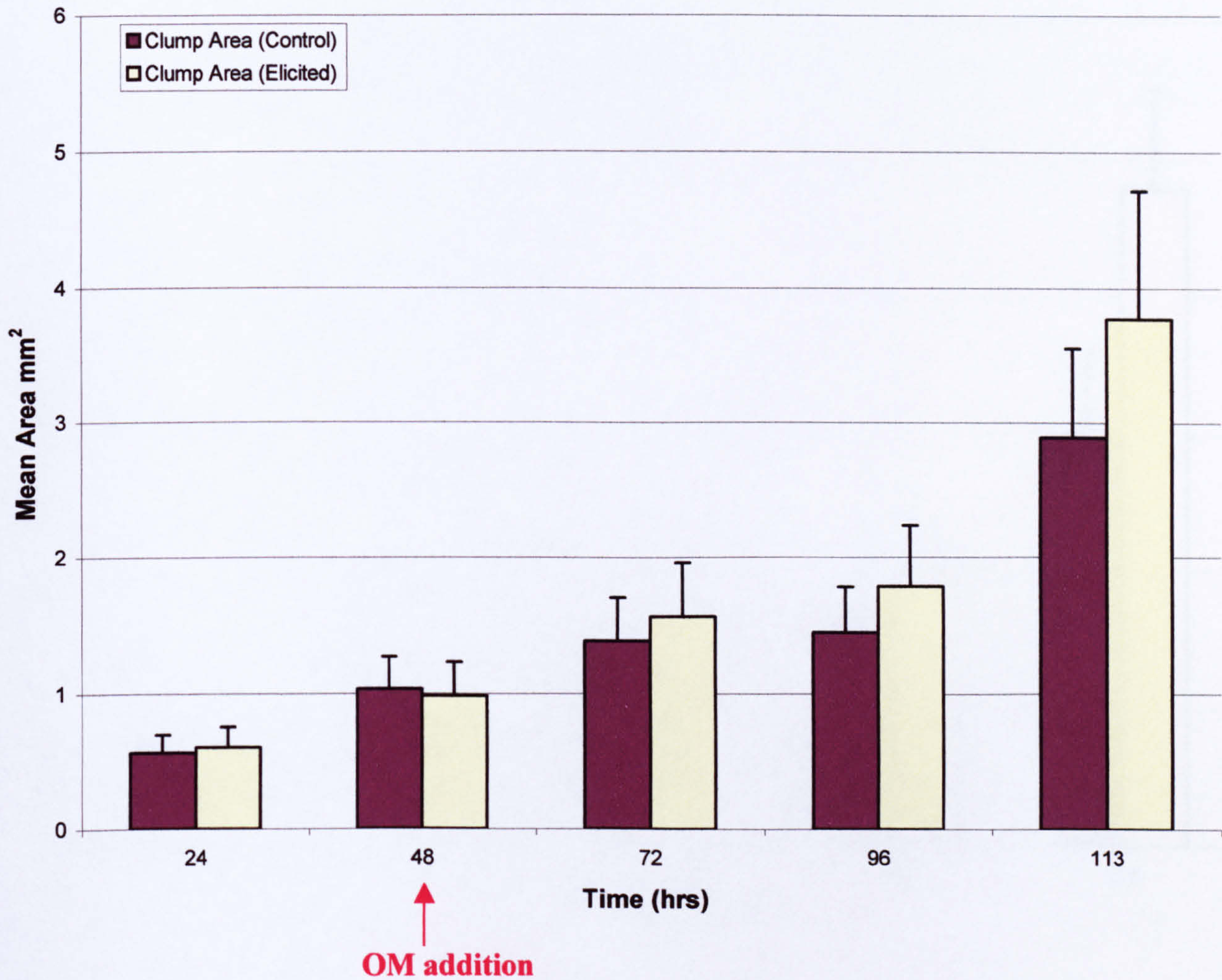


Fig 3.14 Differences in clump size in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.14 shows the differences in mean area of *P. chrysogenum* P2 clumps under control and elicited conditions. Cultures that had OM added to them showed a maximum increase in area of 31% (Table 3.5).

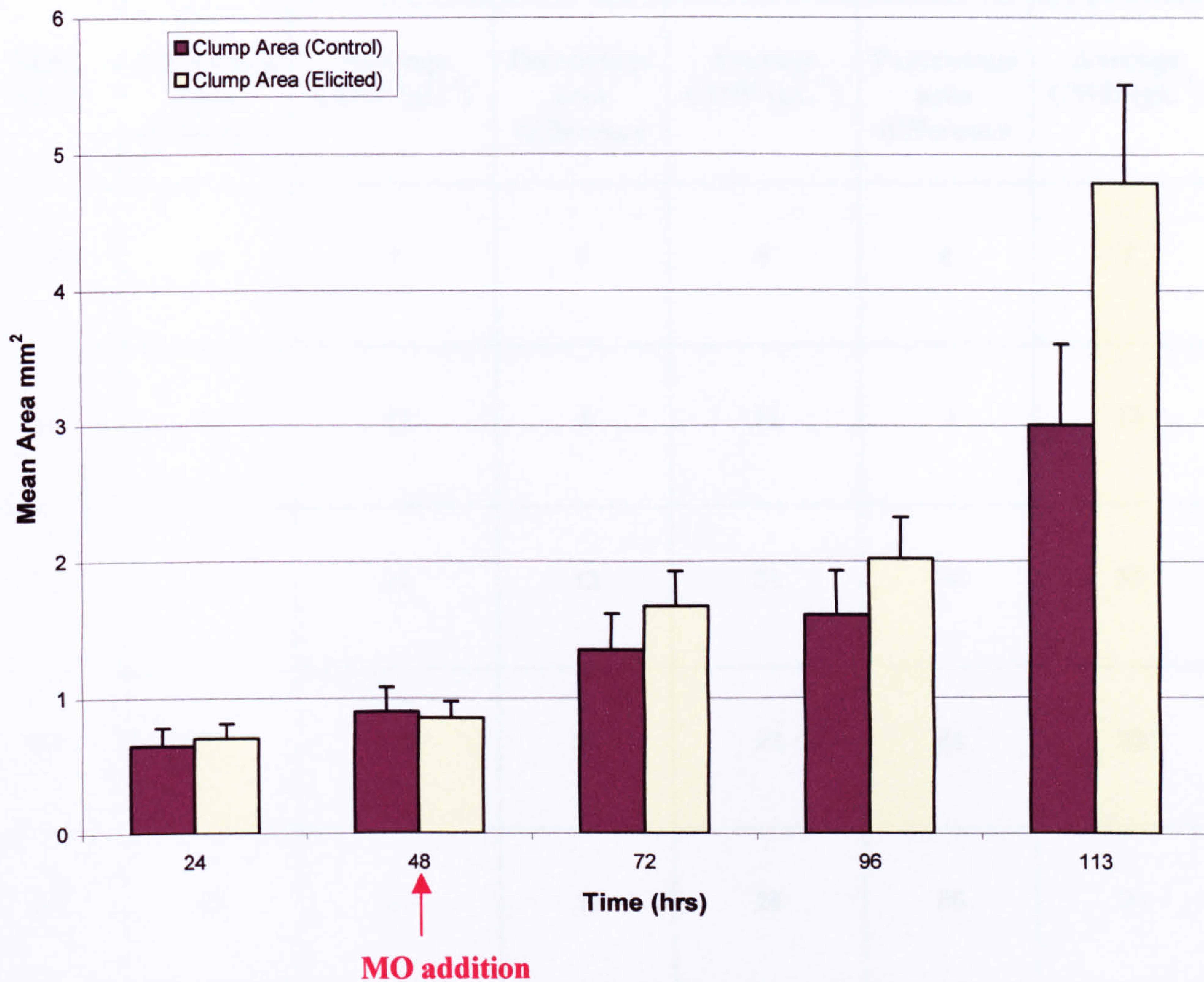


Fig 3.15 Differences in clump size in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.15 shows the differences in mean area of *P. chrysogenum* P2 clumps under control and elicited conditions. Cultures that had MO added to them showed a maximum increase of 59% (Table 3.5).

Time (hrs)	OG		OM		MO	
	Percentage area difference	Average CDW (gL^{-1})	Percentage area difference	Average CDW (gL^{-1})	Percentage area difference	Average CWD (gL^{-1})
24	-9	7	7	8	8	7
48	2	12	-5	15	-5	13
72	12	21	13	21	24	20
96	20	22	23	23	26	23
113	23	24	31	25	59	24

Table 3.5 Percentage differences in mean clump areas compared to Control and average biomass concentration using CDW for OG, OM, and MO supplemented cultures

3.4 Sporulation Studies

This section investigates the effect of elicitors (OG, OM, and MO) on sporulation. All data presented was carried out based on triplicate fermentations.

In cultures where elicitors were added the onset of sporulation was earlier (approximately 120 hrs) than the control (approximately 144 hrs).

This study showed that elicitors have a significant impact on the spore production when added to *P. chrysogenum* P2 cultures (compared to control). Spore concentration was at its highest when MO was supplemented to the cultures.

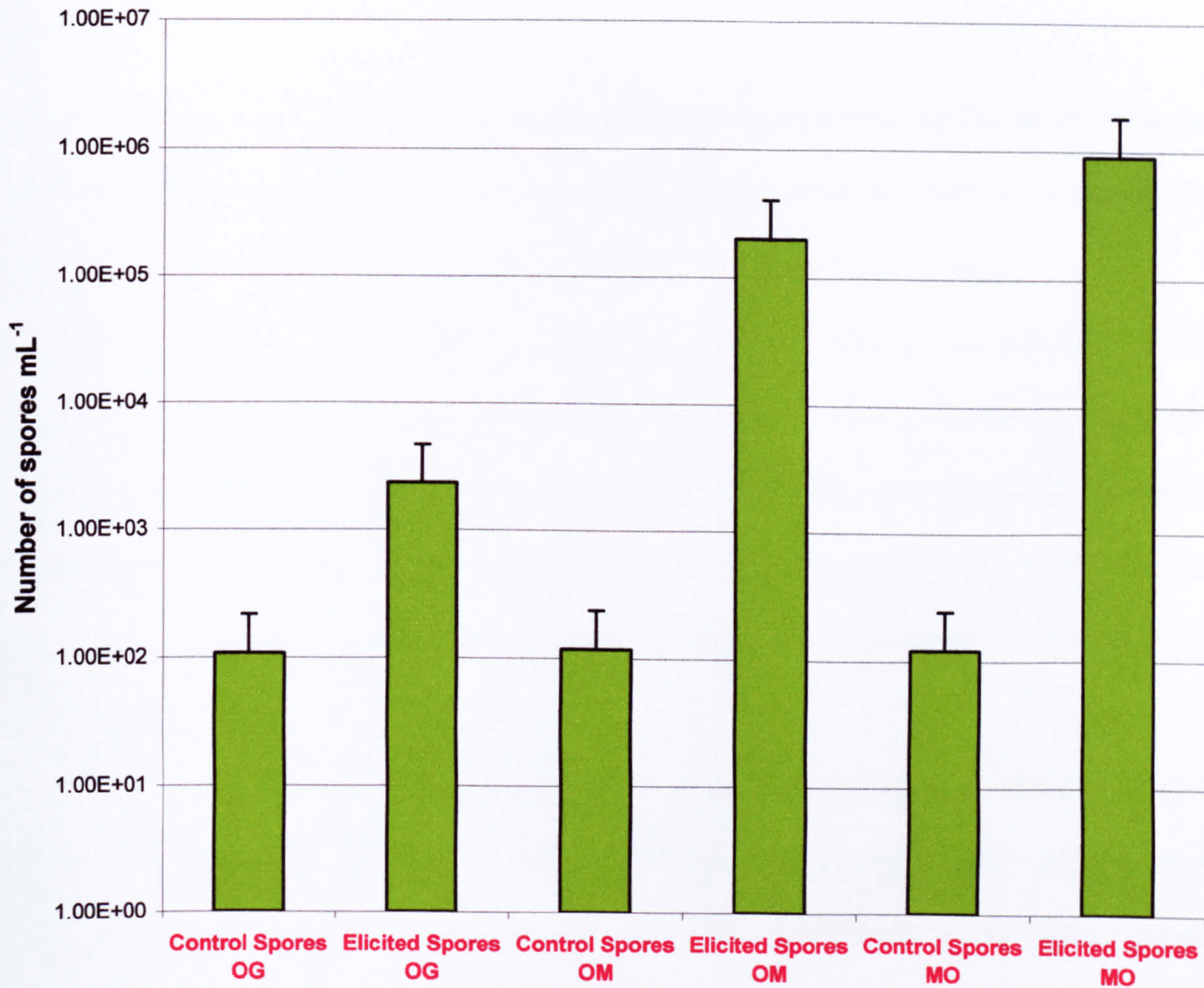


Fig 3.16 Spore numbers per millilitre of culture in STR fermentation of *P. chrysogenum* P2 under Control and Elicited conditions

Figure 3.16 shows the effect of OG, OM, and MO on sporulation in STR cultures of *P. chrysogenum* P2. MO has the greatest effect followed by OM and OG respectively.

3.5 ANTS labelled Oligosaccharide Studies

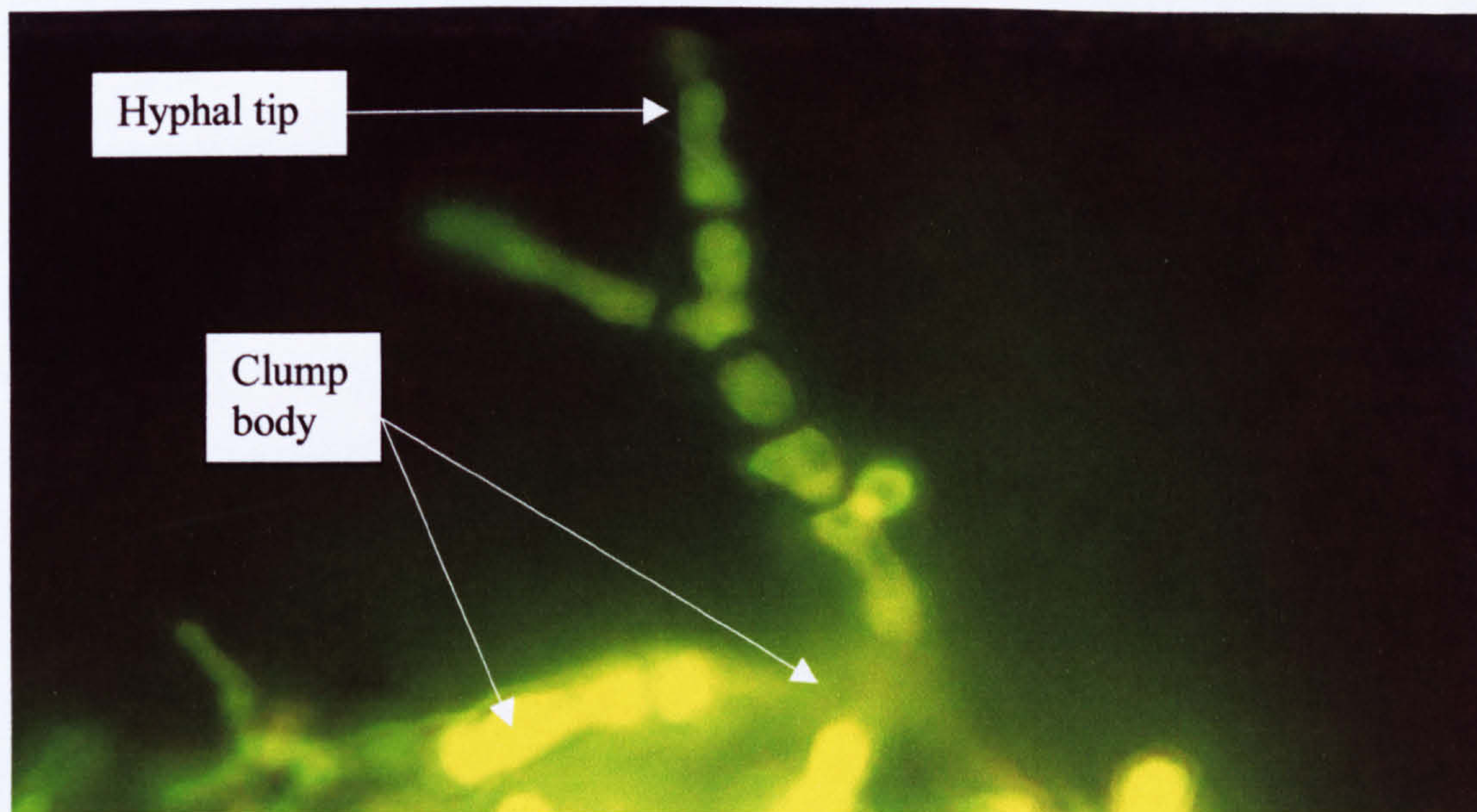
This section investigates the effects of oligosaccharides labelled with ANTS, on *P. chrysogenum* P2 and penicillin G production. ANTS derivatives of OG, OM, and MO were studied for the production of penicillin G. The work was carried out in triplicate shaken flasks.

The pictures presented were taken immediately after the addition (10 minutes) of ANTS-oligosaccharide moieties to the shaken flasks. For best representation of findings pictures at 1000 magnification have been presented. There were no visible differences between the fluorescence of OM-ANTS and MO-ANTS samples when observed under the microscope. However, the OG-ANTS addition resulted in a visibly fainter fluorescence. The control samples also showed faint background fluorescence.

When the effects of ANTS-oligosaccharides were studied under careful microscopic observation, it was noted that the cell wall of the hyphal tips could not be seen (very faint). This indicated that the ANTS-complex had passed through the cell wall. Also, central parts of the hyphae appeared to be more fluorescent than the tips.

Penicillin G levels were also investigated on the samples taken from the above-mentioned flasks. There was a similar pattern of elicitation to those shown in previous studies (Fig. 3.7). MO showed the highest enhancement with a maximum of 82% when compared to control. OM and OG caused lower enhancements of penicillin G production with 61% and 49% maximum elicitation, respectively, when compared to control.

Picture 3.1 Hyphae of *P. chrysogenum* P2 under control conditions (x1000)



Picture 3.2 Hyphae of *P. chrysogenum* P2 supplemented with ANTS-OM (x1000)

3.4.1 Penicillin G production in the presence of ANTS-oligosaccharide complex

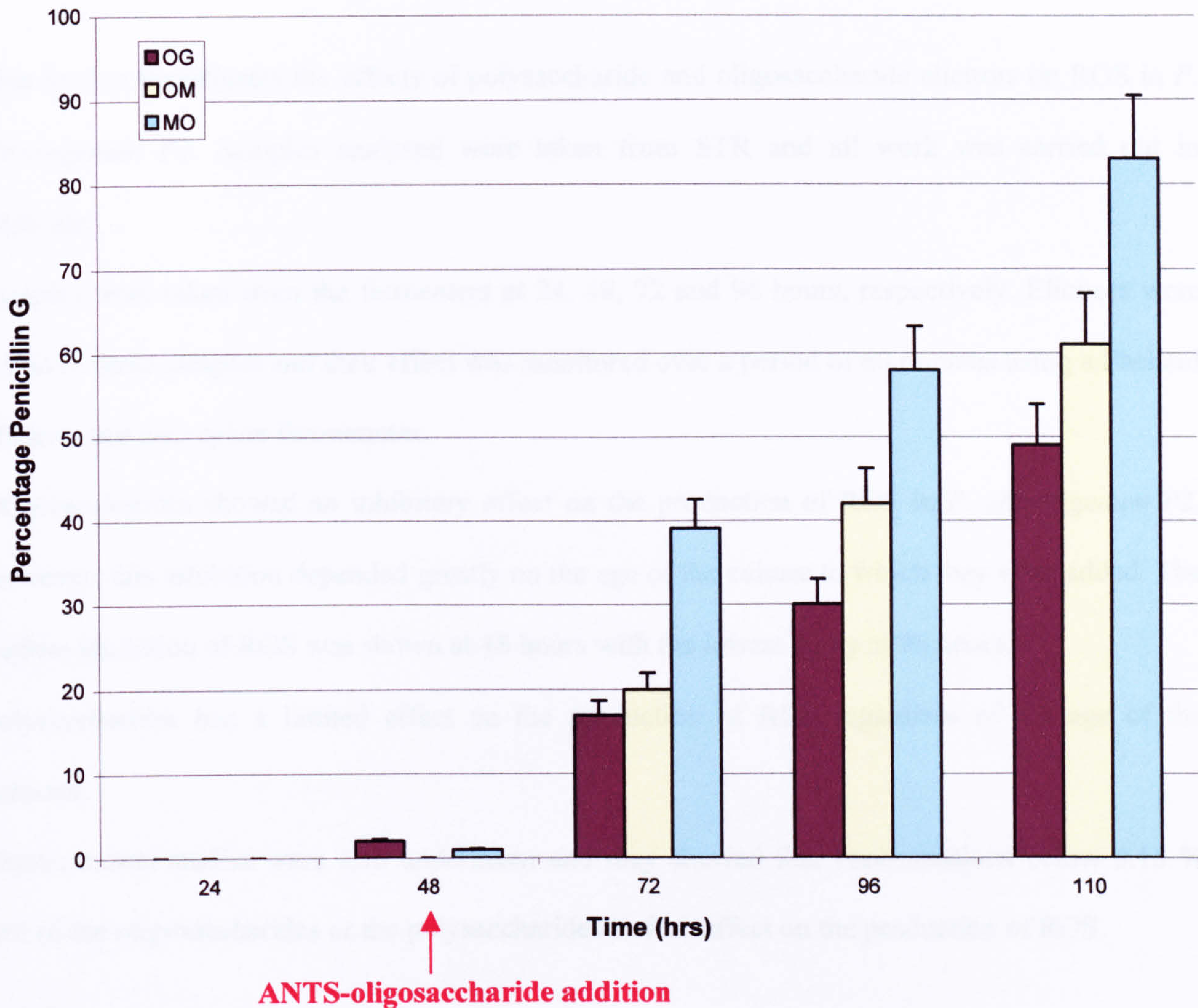


Fig 3.17 Percentage increase in penicillin G production compared to control in shake flasks cultures of *P. chrysogenum* P2 using OG-, OM-, and MO-ANTS moieties as elicitors

Figure 3.17 illustrates the effect on penicillin G of ANTS-oligosaccharide moieties when added to shake flask cultures of *P. chrysogenum* P2. The ANTS-oligosaccharide moiety displays the same elicitation pattern as seen by the individual non-labelled elicitors (Fig. 3.7).

3.5 Reactive Oxygen Species

This section investigates the effects of polysaccharide and oligosaccharide elicitors on ROS in *P. chrysogenum P2.* Samples analysed were taken from STR and all work was carried out in triplicate.

Samples were taken from the fermenters at 24, 48, 72 and 96 hours, respectively. Elicitors were added to these samples and their effect was monitored over a period of 60 minutes using a Packard Fluorocount microplate fluorometer.

Oligosaccharides showed an inhibitory effect on the production of ROS in *P. chrysogenum* P2. However, this inhibition depended greatly on the age of the culture to which they were added. The highest inhibition of ROS was shown at 48 hours with the lowest being at 96 hours.

Polysaccharides had a limited effect on the production of ROS regardless of the age of the cultures.

Concentration studies were also undertaken and they showed that concentrations below 0.15 % w/v of the oligosaccharides or the polysaccharides had no effect on the production of ROS.

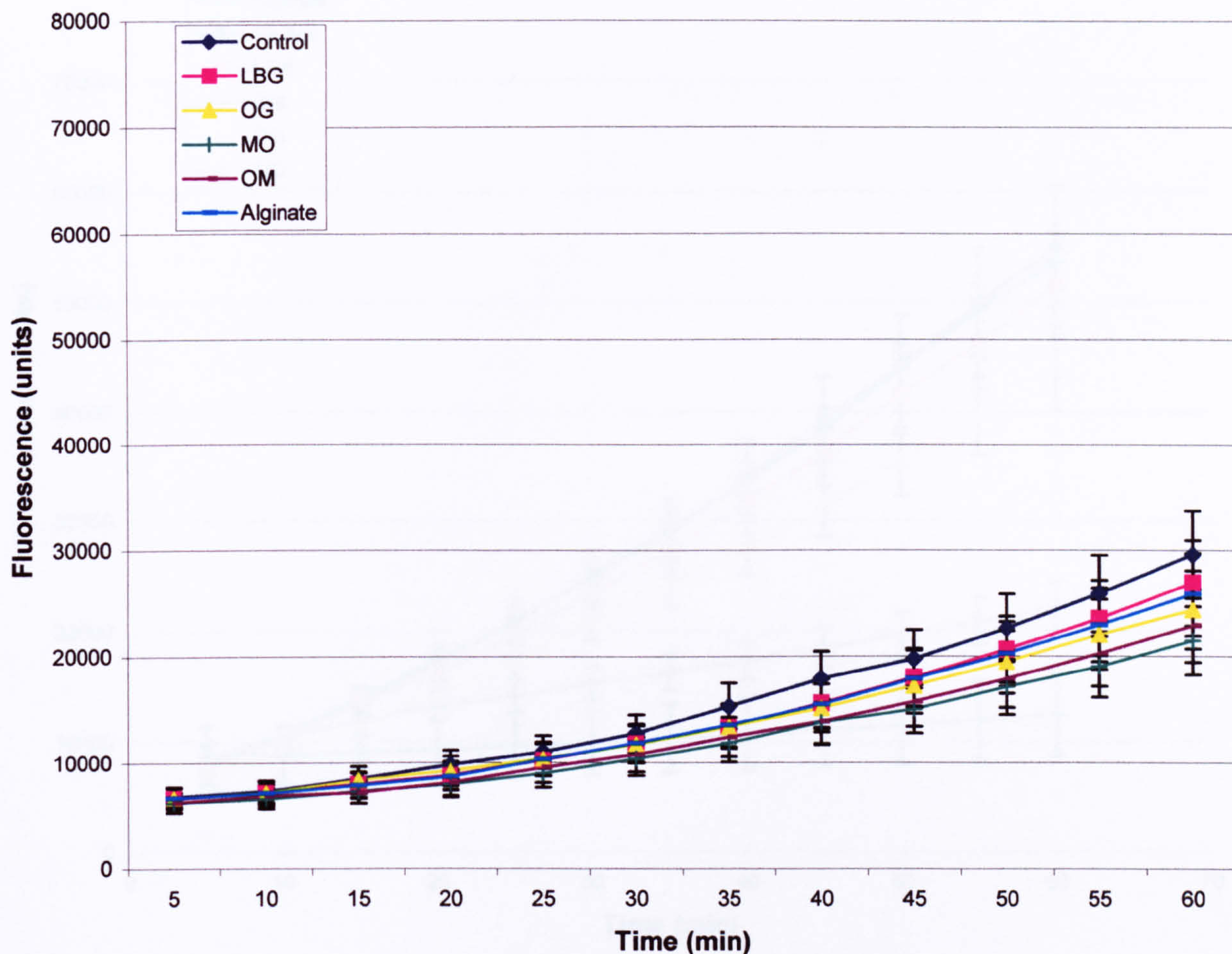


Fig 3.18 Reactive oxygen species in fermentations of *P. chrysogenum* P2 after 24 hours

Figure 3.18 shows the production of ROS when polysaccharides and oligosaccharides were added to *P. chrysogenum* P2 after 24 hrs of growth. MO appears to be more inhibitory whereas alginate is least inhibitory to ROS production.

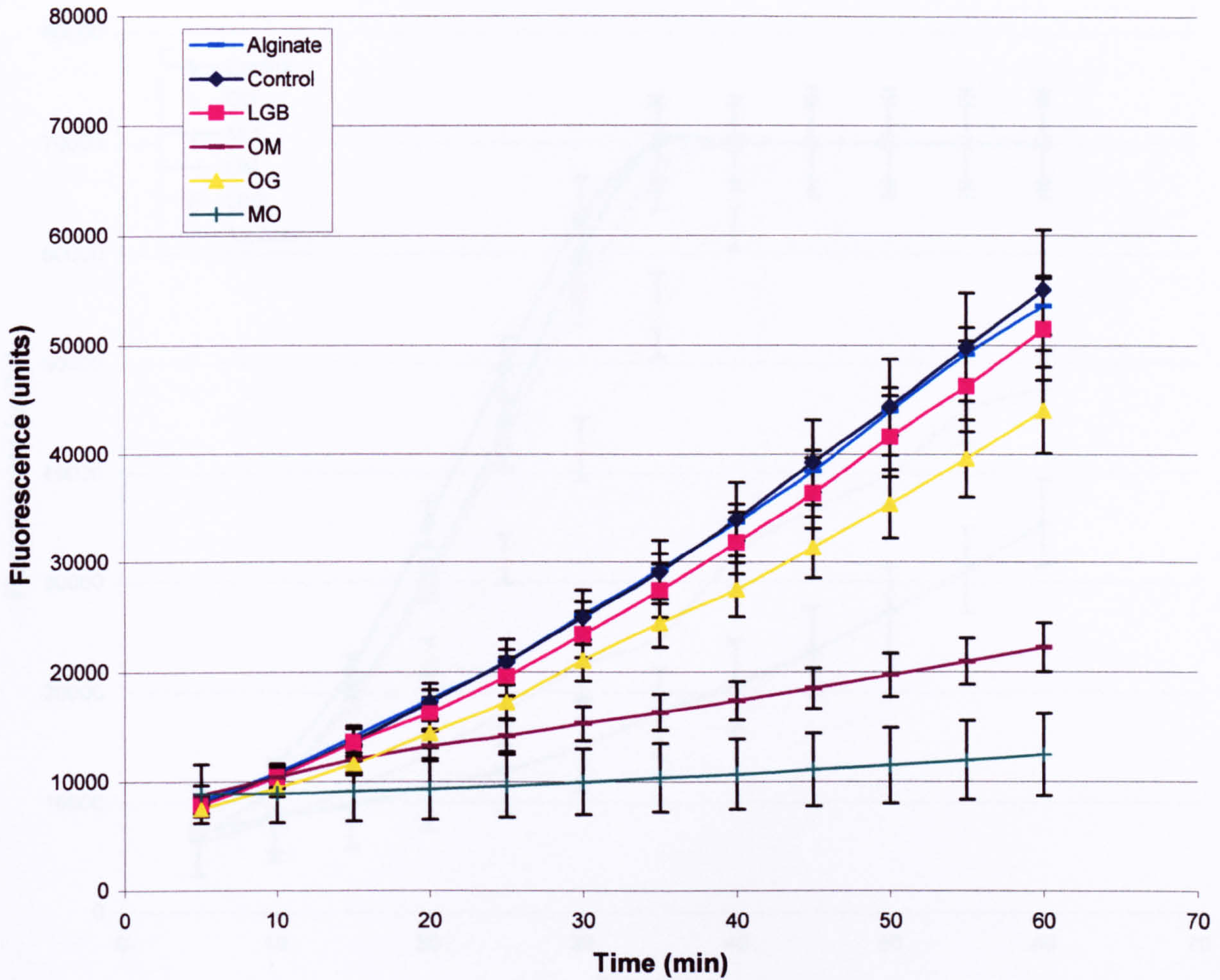


Fig 3.19 Reactive oxygen species in fermentations of *P. chrysogenum* P2 after 48 hours

Figure 3.19 shows the production of ROS when polysaccharides and oligosaccharides were added to *P. chrysogenum* P2 after 48 hrs of growth. MO appears to be more inhibitory whereas alginate is least inhibitory to ROS production.

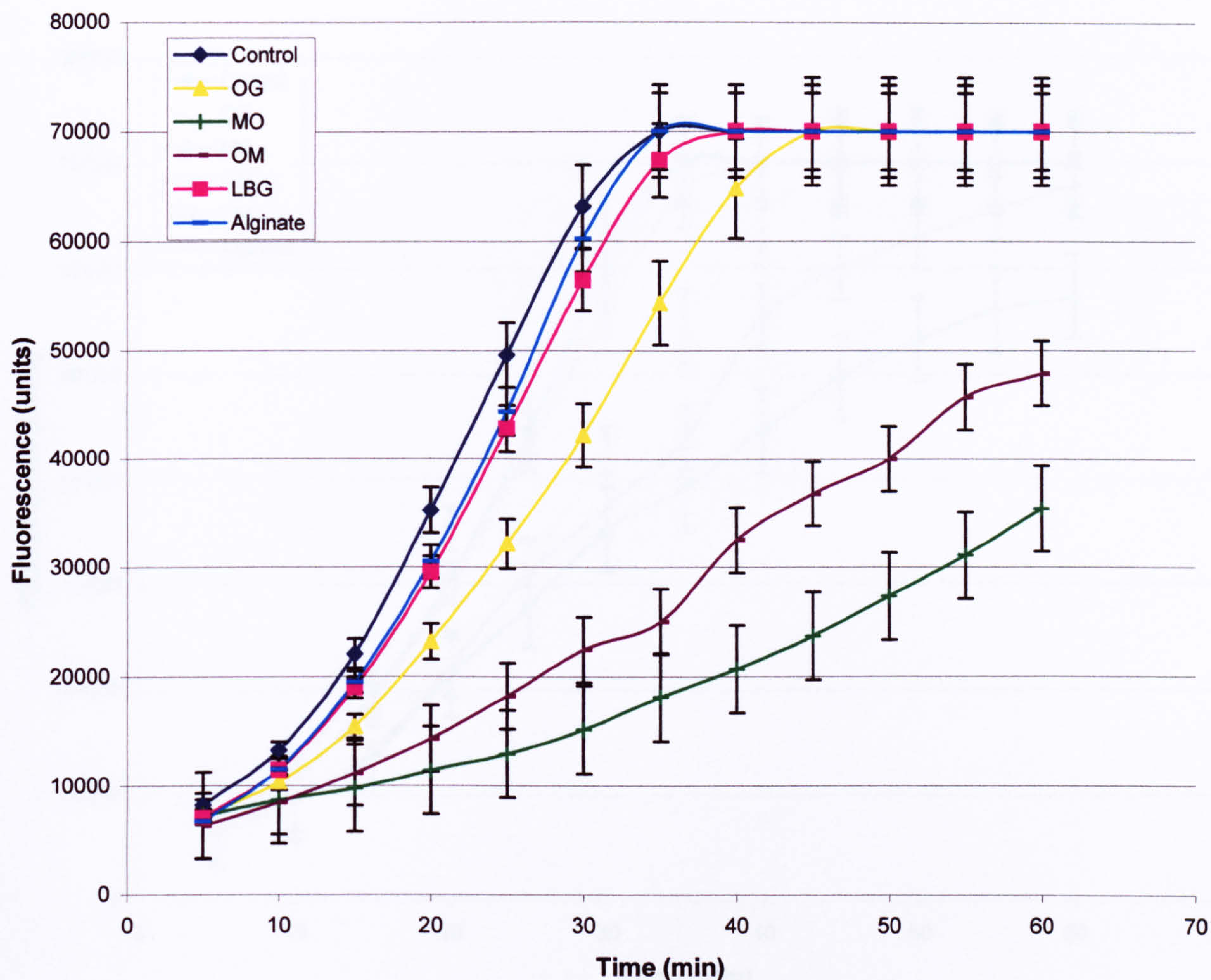


Fig 3.20 Reactive oxygen species in fermentations of *P. chrysogenum* P2 after 72hours

Figure 3.20 shows the production of ROS when polysaccharides and oligosaccharides are added to *P. chrysogenum* P2 after 72 hrs of growth. MO appears to be more inhibitory whereas alginate is least inhibitory to ROS production.

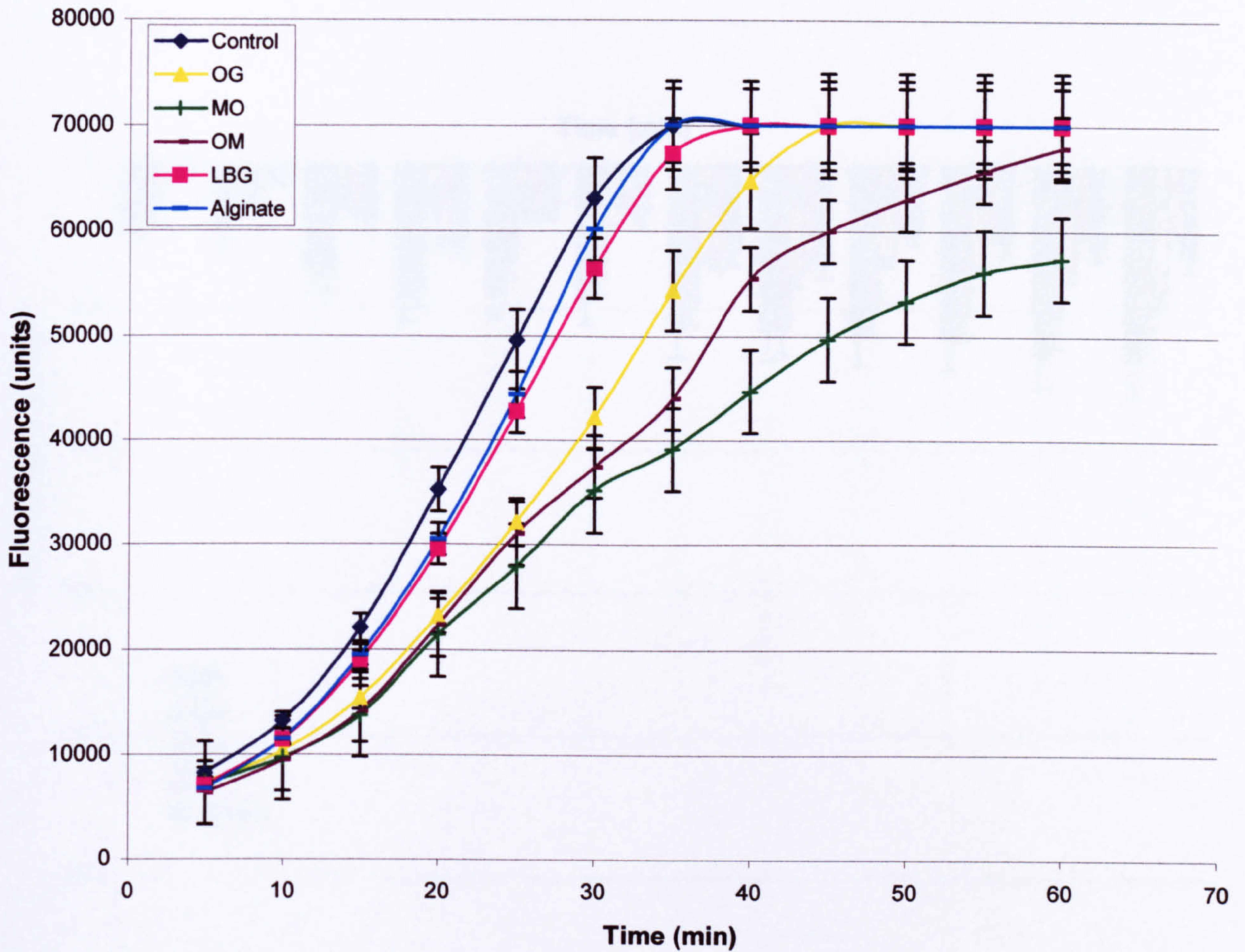


Fig 3.21 Reactive oxygen species in fermentations of *P. chrysogenum* P2 after 96 hours

Figure 3.12 shows the production of ROS when polysaccharides and oligosaccharides were added to *P. chrysogenum* P2 after 96 hrs of growth. MO appears to be more inhibitory whereas alginate is least inhibitory to ROS production.

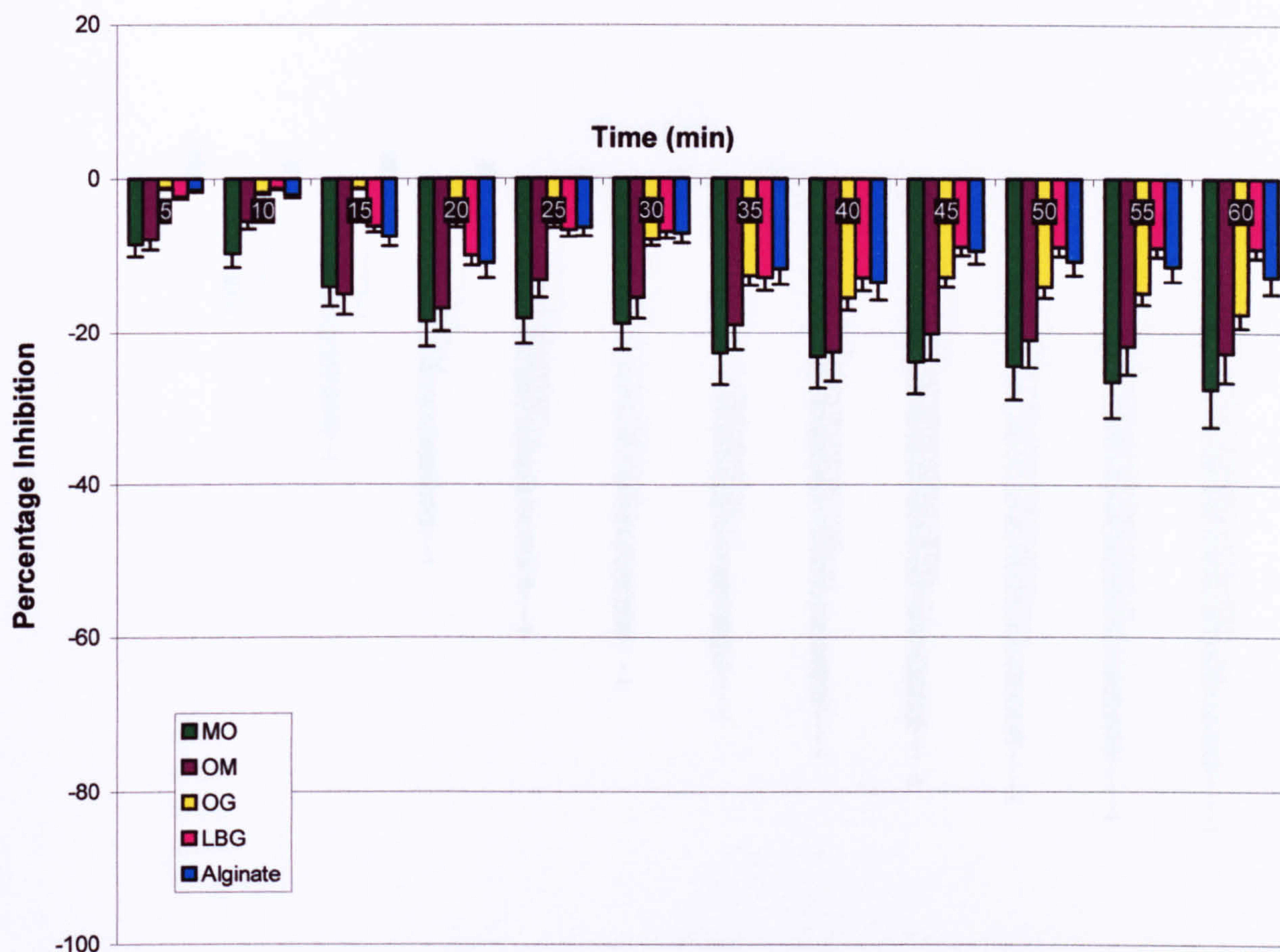


Fig 3.22 Percentage ROS produced after 24 hrs for *P. chrysogenum* P2 in presence of elicitors

Figure 3.22 shows the percentage inhibition of ROS compared to control when oligosaccharides and polysaccharides were added to *P. chrysogenum* P2 after 24 hrs. The highest inhibition was shown by MO and OM, 26% and 23% respectively, compared to control.

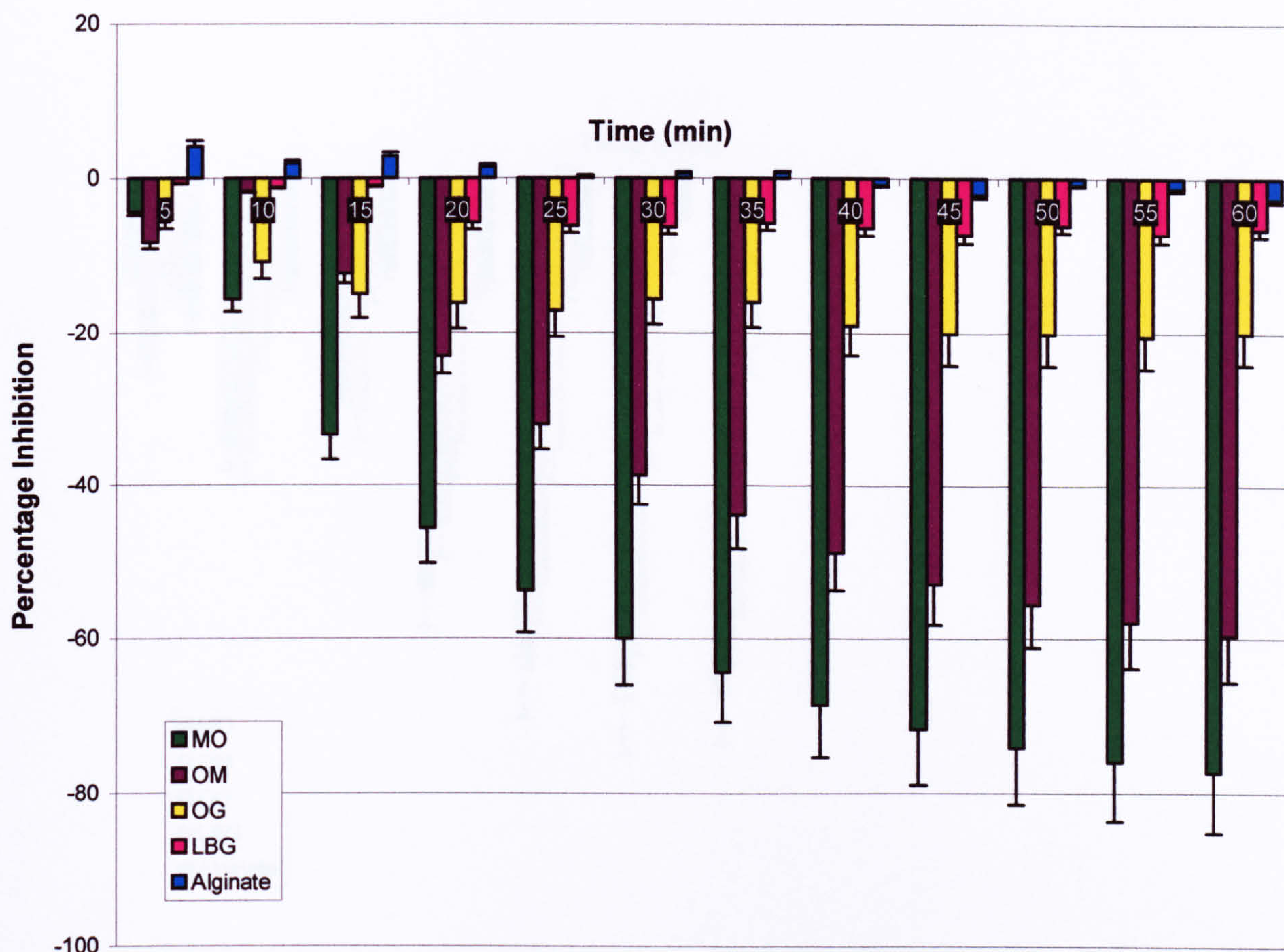


Fig 3.23 Percentage ROS produced after 48 hrs for *P. chrysogenum* P2 in presence of elicitors

Figure 3.23 shows the percentage inhibition of ROS compared to control when oligosaccharides and polysaccharides were added to *P. chrysogenum* P2 after 48 hrs. Inhibition of ROS is at its maximum, with the highest effect shown by MO an OM, 76% and 58% respectively, compared to control. Polysaccharides show the least inhibition with small stimulatory effect by alginate (3%) and with a maximum percentage inhibition by LBG of only 7%, compared to control.

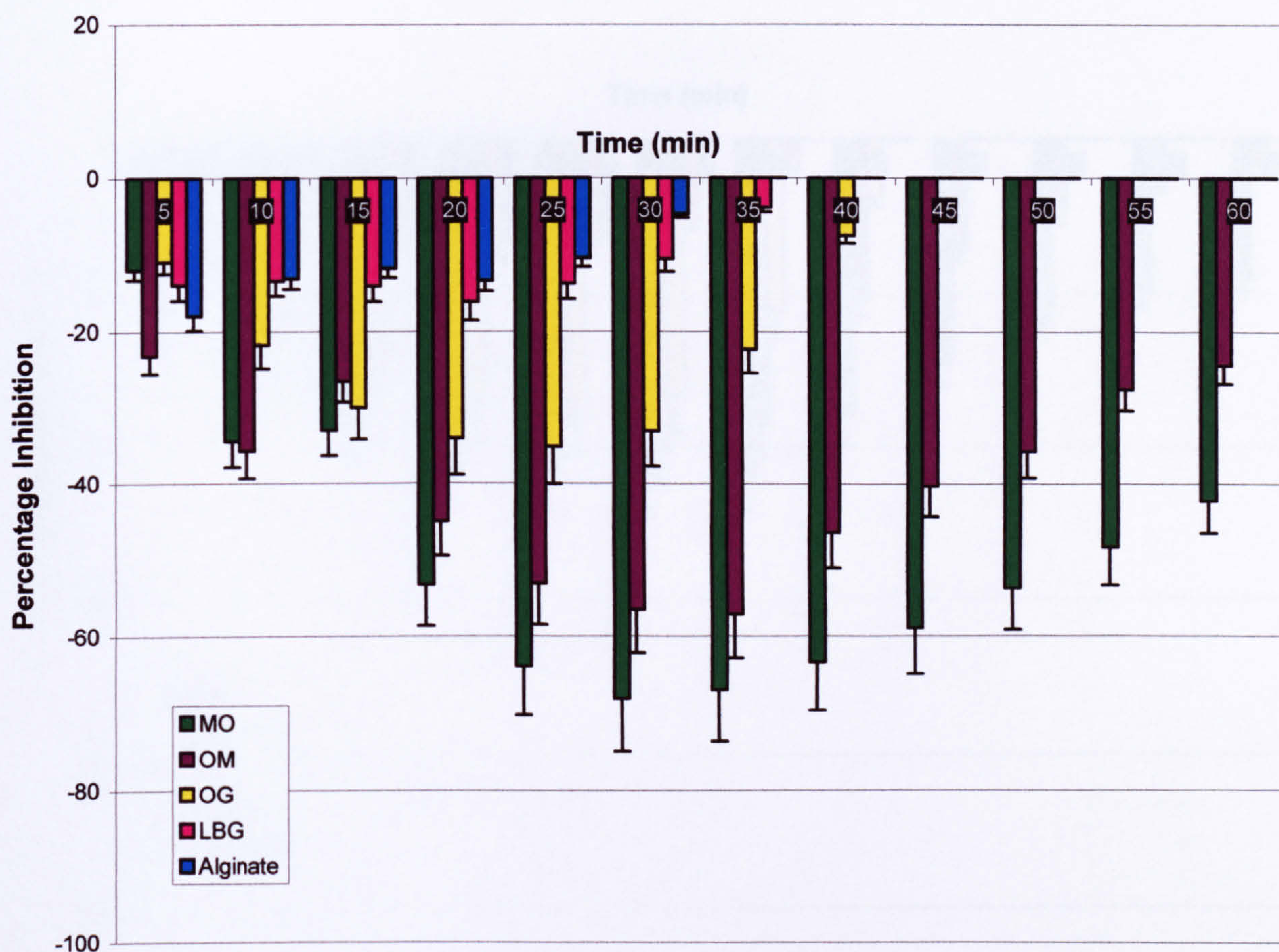


Fig 3.24 Percentage ROS produced after 72 hrs for *P. chrysogenum* P2 in presence of elicitors

Figure 3.24 shows the percentage inhibition of ROS compared to control when oligosaccharides and polysaccharides were added to *P. chrysogenum* P2 after 72 hrs. Inhibition of ROS is low with alginate and LBG showing least inhibition. MO an OM show greatest inhibition with 67% and 57% respectively, compared to control.

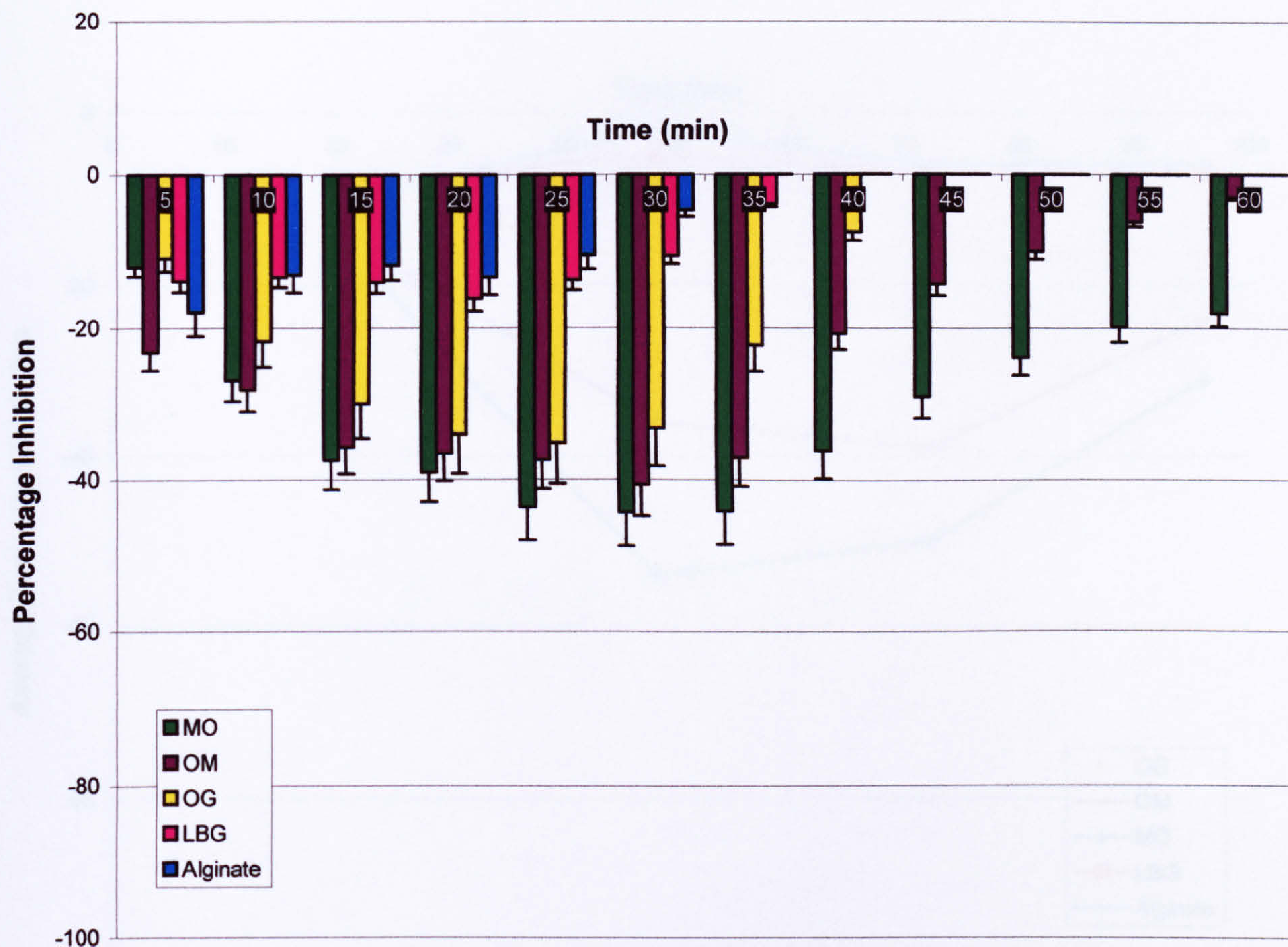


Fig 3.25 Percentage ROS produced after 96 hrs for *P. chrysogenum* P2 in presence of elicitors

Figure 3.25 shows the percentage inhibition of ROS compared to control when oligosaccharides and polysaccharides were added to *P. chrysogenum* P2 after 96 hrs. Inhibition of ROS was decreased for both oligosaccharides and polysaccharides. MO and OM, 44% and 41% showed the highest inhibition respectively, compared to control.

Average Percentage Inhibition Pattern of ROS for *P. chrysogenum* P2

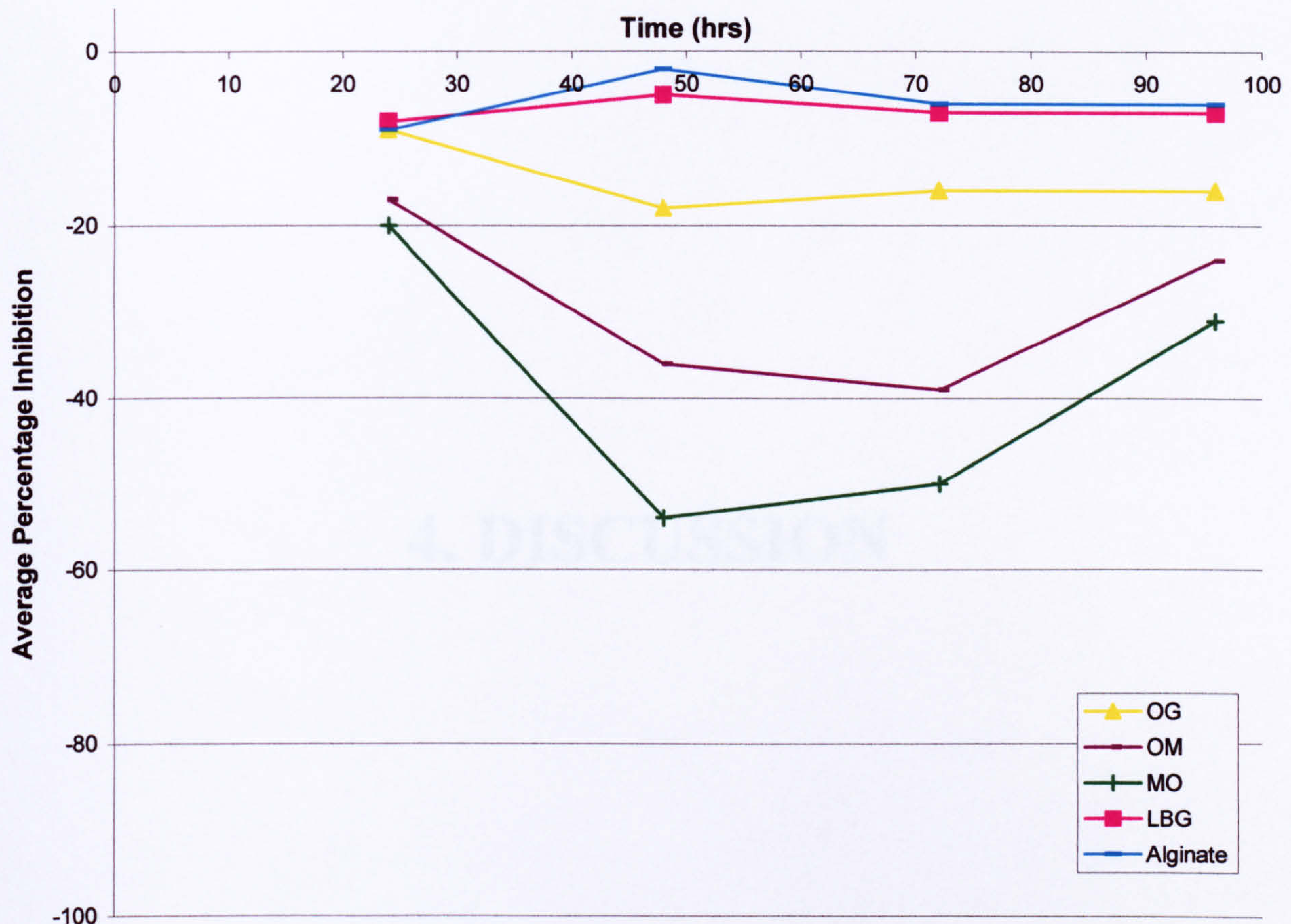


Fig 3.26 Average percentage inhibition of ROS produced for *P. chrysogenum* P2 in presence of elicitors

Figure 3.26 shows the average percentage inhibition of ROS for *P. chrysogenum* P2 over 96 hrs. OG and MO showed their highest average percentage inhibition at 48 hrs with 18% and 54%, respectively. OM showed highest average percentage inhibition at 72 hrs with 39%. LBG and Alginate both show highest average percentage inhibition at 24 hrs with 8% and 9%, respectively.

4. DISCUSSION

4.1 Effects of Oligosaccharides and Polysaccharides on *P. chrysogenum* P2 – Brief Review of Results

Oligosaccharides and polysaccharides had varying effects on the parameters investigated in this study. Penicillin G and 6-APA levels increased significantly in the presence of OG, OM and MO elicitors. MO followed by OM and OG showed the greatest effect. A similar pattern was observed when investigations were carried out on the effects of oligosaccharides on morphology changes in submerged cultures of *P. chrysogenum* P2. Clump area and hyphal tip numbers were shown to increase by the addition of oligosaccharide elicitors, with MO showing the highest effect followed by OM and OG.

Sporulation of *P. chrysogenum* P2 liquid cultures increased significantly in the presence of oligosaccharide elicitors. A similar pattern was observed to the above studies. MO showed highest induction of sporulation with OG showing the least effect when compared to control.

The pattern of germination in elicitor-supplemented cultures was less clear. Addition of OG was shown to inhibit germination of spores up to 48 hours whereas alginate stimulated germination. MO and OM appeared to have no effects on the germination of *P. chrysogenum* P2 spores, when compared to control. The effects of germination however, did not relate to the findings on the development of the clumps. OG, LBG and alginate retarded the development of clumps in the inoculum cultures. MO and OM showed no apparent effects.

Although elicitation in cultures of *P. chrysogenum* is now well established, the mechanisms by which this process is controlled or induced have not been investigated. The work presented in this thesis serves as a starting point for the elucidation of some possible elicitation mechanisms. Work carried out with ANTS-derived oligosaccharides showed that the ANTS-oligosaccharide unit does

pass through the cell wall. The intensities of fluorescence were visually different depending on the hyphal age. Production of penicillin G in shaken flasks supplemented with ANTS-oligosaccharide showed a similar pattern of overproduction compared to STR fermentations.

ROS production in *P. chrysogenum* P2 was also investigated. Both polysaccharides and oligosaccharides inhibited ROS production but the effect of LBG and alginate was less pronounced.

4.2 Structure and Size Relationship of Oligosaccharides to Overproduction

The three oligosaccharides used in this study had varying effects on the overproduction of penicillin G and 6-APA. The highest percentage increase in penicillin G and 6-APA was induced when MO was supplemented to *P. chrysogenum* P2 cultures, followed by OM and OG (Fig. 3.7, page 83). The reason for this is not fully understood, however, consideration must be given to two important points:

First, the DP of the different oligosaccharides: As shown in Table 1 (Table 2.4, page 60) OG and OM had a similar DP (size 7), however the MO preparation had a mixture of DPs (size 5-8). This could explain the variation between the maximum penicillin G and 6-APA production in these cultures as the previous work on plant cell cultures suggests the existence of a stringent response to the specific DP sizes. Work on plant cell cultures has shown that defined oligosaccharides of different DP have different effects on the signalling induced elicitation of metabolites (Côté and Hahn, 1994 and Promé, 1996). Baureithel *et al.*, (1994) and Ito *et al.*, (1997) investigated protein-binding factors with high affinity for chitin oligosaccharides in plant cell membranes. They found high affinity to oligosaccharides with a DP of between 5 to 8. Ariyo *et al.* (1997) have shown that oligosaccharides with DP of 7 to10 induced higher levels of penicillin G when compared with oligosaccharides of a lower DP.

The variation of DP in the case of MO may induce a number of mechanisms though simultaneous actions of these oligosaccharide fragments. Separation of pure DP oligosaccharides with sufficient quantities for elicitation studies could not be achieved using a 1 m Bio-Rad P2 gel column.

Second, there are structural differences to be taken into account: Most alginate consists of uronic acid units with the highest proportion being found in OM when compared to OG. These units are suggested to act on the cell surface membranes that could bring about the elicitation of penicillin G and 6-APA in *P. chrysogenum* P2. This stipulation is based on research on the membrane-associated effects and inducement of elicitation of physiological responses to oligo and poly-uronides reported in plants (Natsume *et al.,* 1994), animals (Bland *et al.*, 2001) and bacteria (Akiyama *et al.,* 1992). Taking into account these reports it can be suggested that analogous effects of high uronic acid prevalence can be implicated to the elicitation effects in *P. chrysogenum* P2. This could in part explain the differences in the overproduction of OM and OG in this system.

Comparing MO oligosaccharides to alginate-derived oligosaccharides the structures of the chains are of paramount importance to instigating regulatory signals in the production of secondary metabolites. It can be speculated that cell membrane receptors have recognition sites that may contain mannose receptors. The mannose receptors in the presence of mannan oligosaccharides of specific size and structure may regulate the production of penicillin G and thus 6-APA. Similar mechanisms of carbohydrate recognition have been reported in animal cells (Bland *et al.*, 2001) and it could be argued to be comparable to the systems present in fungi. In plant cell cultures of *Hypericum perforatum*, mannan has been shown to elicit a two-fold increase in hypericin levels and a four-fold increase in pseudohypericin (Kirakosyan *et al.*, 2000). This may offer different avenues for understanding the structural imperative that may govern the mechanism of elicitation.

4.3 Overproduction of Penicillin G and 6-APA in *P. chrysogenum* P2 STR Cultures

The use of OG, OM and MO as elicitors in *P. chrysogenum* P2 had a significant effect on the production of penicillin G, when compared to control. The percentage yields of penicillin increased considerably in the presence of these oligosaccharides as shown in the results section (Fig. 3.7, page 83). As mentioned before this increase was dependant on the oligosaccharide type. In the presence of elicitors the penicillin G production rate increased when compared to control cultures. The maximum increase was observed with MO elicited cultures (0.30 gh^{-1}) followed by OM (0.28 gh^{-1}) then by OG (0.24 gh^{-1}). The induction of higher production rates implies that the oligosaccharides had an effect on the penicillin G production pathway of the cell. This will be discussed further. The specific productivity increased, too, in the presence of these elicitors (Table 3.1, page 81).

A similar pattern was observed in the levels of extra-cellular 6-APA. Both the production rate and the specific activity increased when elicitors were added. 6-APA is condensed with phenylacetyl-CoA to synthesise penicillin G. The presence of extra-cellular 6-APA might indicate an overproduction of IPN synthase, thus an increase in the levels of isopenicillin N. There could also be effects on the levels of IPN amidolyase, which converts IPN to 6-APA. Tamerler *et al.* (2001) reported the production of extra-cellular IPN and ACV in OG, OM and MO supplemented *P. chrysogenum* P2 cultures. They observed that levels of ACV decreased over 120 hours, when compared to control, with MO showing the highest decrease followed by OM and OG. The IPN levels increased, showing a similar oligosaccharide effect profile. This work suggests that a mechanism of enhancement by which oligosaccharides act is through enzymatic influences on the

production of these two intermediates (i.e. ACV synthase and IPN Synthase). Gang *et al.* (2001) investigated the transcript levels of *pcb*AB, *pcb*C and *pen*DE in *P. chrysogenum* Wis 54-1255 cultures supplemented with alginate elicitors. The genes encode the enzymes ACV synthase, IPN synthase and 6-APA:CoA Acyltransferase, respectively. When the elicitors were added to the cultures there was an increase in the transcript levels of the three genes. The highest transcript levels were shown for the *pcb*C gene. The over-expression of this gene in elicited cultures and the observations by Tamerler *et al.* (2001) of an increase in IPN levels might suggest a reason for the increase in the extra-cellular 6-APA concentration. As the production rate of both IPN and 6-APA were increased it may be suggested that there is an increase in the levels of the IPN amidolyase enzyme, or possibly its activity. In this case the oligosaccharides, or breakdown products from them, interact with INP amidolyase with signals that control the activity of the enzyme or with systems that control the synthesis of the enzyme. This also highlights a possible mechanism for the enhancement of penicillin G concentration in *P. chrysogenum* P2 cultures supplemented with oligosaccharide elicitors.

There could be a number of speculative reasons why alginate and LBG-derived oligosaccharides instigate the overproduction of penicillin G in *P. chrysogenum* P2 cultures. The induced synthesis of penicillin G by elicitors suggests that these elicitors may function as activators of defence mechanisms in this system. In soil *Penicillium* sp. and bacteria such as *A. vinelandii* compete for the same carbohydrate for example sucrose exudates from roots of plants which could be a source of competition (Pindar and Bucke, 1975). *A. vinelandii* are bacteria capable of synthesising alginate as an extracellular polysaccharide. In harsh conditions they are known to breakdown this polysaccharide layer for use it a carbon source. These breakdown products might act as a trigger

for penicillin G production. With this in mind the physiological effects and the mode of action of oligosaccharides on fungi may be analogous to the physiological effects observed in plants and animals.

The specific effects on the possible genetic link (i.e. the increase in penicillin G regulatory genes transcription levels and thus an increase of their encoded enzymes) might suggest two activation mechanisms induced by oligosaccharides. Firstly, in order for the oligosaccharides to control the activity of enzymes (due to the increase of 6-APA) they must enter the cells. The same is true if the oligosaccharides work by interacting with DNA or RNA (due to the increased levels of penicillin G regulatory genes transcripts). Secondly, it could be said that there is a possibility that oligosaccharides act in a 'hormone-like' manner that trigger a metabolic cascade. In this case it can be suggested that oligosaccharides need not enter the cells but must be recognised by a binding site on the cell membrane. This has been reported as an elicitor induced mechanism in plants indicating the possibility of a similar mechanism in fungi (Véronési *et al.*, 1999).

For a direct enzymatic action high concentrations of oligosaccharides must be present. The work carried out used 150 mgL^{-1} of each elicitor. This indicates that at this low concentration the elicitors probably act on cells in a 'hormone-like manner'. Work carried out with ANTS-oligosaccharide derivatives shows conclusively that the oligosaccharides pass through the cell wall. This action was observed to be rapid. Whether the elicitors are then internalised or act on the cell membrane receptors is open to speculation. The fast action of ANTS-oligosaccharide elicitors has been noted in plant cell cultures. Oligosaccharides supplemented to cell cultures of *Taxus chinesis* were shown to elicit taxol (secondary metabolite) within 15 minutes of addition of the elicitor (Yuan *et al.*, 2001).

However, Holst *et al.*, (2001) present a compelling argument for a receptor based oligosaccharide induced mechanism for elicitation. The work presents a speculative model where oligosaccharides are perceived by membrane-bound receptors and they hypothesise that downstream signal transduction protein phosphorylation via a kinase that is either part of the cell membrane receptor itself or is in a complex with the membrane receptors.

As the concentration of the elicitors was very low, they could not act as a carbon source and their 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 23 - 26 gL^{-1}. The addition of 150 mgL^{-1} carbohydrate elicitor cannot have a notable impact on the *P. chrysogenum* P2 biomass in terms of carbon balance.

The *Penicillium* strain used in these studies is a high penicillin producer (P2). Previous work on elicitation in fungal cultures has used the wild-type strain (NRRL 1951) as well as P2. There were suggestions that high producers achieved their maximum production through strain selection methods, thus their overproduction mechanisms are probably stretched to the limit. However, this work showed, through reproducible results that this is not the case. *P. chrysogenum* P2 when grown in liquid cultures in the presence of the oligosaccharide elicitors showed enhanced production of penicillin G by up to 128% (Fig. 3.6, page 80). In light of these findings it is suggested that the maximum production capacity must be revisited.

4.4 Morphology Studies

All the elicitors used in these studies had an impact on the morphology of *P. chrysogenum* P2 culture.

Alginate was shown to increase the rate of germination by 9% compared to control. [This percentage may be considered to fall outside significant level, however, if consideration is given to the number of spores analysed and that the statistical variance of the triplicates was below 1 these results can offer scientific significance.] *A. vinelandii* produces alginate under favourable conditions in nature. If *Penicillium* coexists with bacteria such as *A. vinelandii* under favourable conditions, then it is possible that *Penicillium*'s regulatory mechanism would induce the germination of spores. OG showed an inhibition of germination. Larsen and Haug (1971) and Haug *et al.* (1974) showed that *A. vinelandii* has a higher proportion of guluronic acid than mannuronic acid residues. It can be suggested that in unfavourable environmental conditions alginate capsule breakdown of *A. vinelandii* will have a higher guluronic acid residues. This may act as an environmental stimulus to prevent the germination of *Penicillium* spores under limited nutritional conditions. Metabolites produced by *Fusarium acuminatum* have been shown to inhibit *P. digitatum* spore germination (Burmeister *et al.*, 1977).

LBG, MO and OM showed no effects on the germination of *P. chrysogenum* P2 spores.

Due to the early germination of spores in the presence of alginate, the germ-tube length is much longer at a later stage when compared to the control and other elicitor supplemented cultures. The addition of OG to the culture resulted in a decrease in germ-tube length, which could be due to its inhibitory characteristics. It is also important to consider that even after 48 hours incubation a high

percentage of spores (32%) did not germinate in the presence of OG. This in turn can pose a question: Does a difference in the state of the spores result in the germination of certain spores? Even though the germination was induced earlier in alginate-supplemented cultures the development of hyphal clumps was retarded. This could be explained by an evolutionary equilibrium developed to respond to external stimuli. This would ensure that in the presence of other micro-organisms (e.g. *A. vinelandii*, *P. aeruginosa* – in this case alginate producing micro-organisms) in nutrient poor environments there will be a developmental inhibition, possibly limiting fungal growth. This will ensure a steady rate of nutrient consumption.

Morphology changes in the STR cultures of *P. chrysogenum* P2 were more clearly defined compared to inoculum development. In all cases where oligosaccharide elicitors were supplemented to the cultures there was an increase in the clump area and number of hyphal tips. It is believed that production of secondary metabolites such as penicillin G occurs at the tips of hyphae (Prosser and Tough, 1991; Nielsen, 1993 and Peñalva *et al.*, 1998). Image analysis work showed that in the presence of elicitors the number of hyphal tips increased, compared to control. This increase together with the possible activation of penicillin G regulatory genes can explain the overproduction pattern observed in *P. chrysogenum* P2 STR cultures. This increase in hyphal tip numbers could relate to an increase in branching and clump size. Clumps sizes increased significantly in *P. chrysogenum* P2 cultures supplemented with oligosaccharides. The clumps were not only larger but also more filamentous. Even though it can be argued that smaller clumps will result in higher dissolved oxygen to the cell, the control clumps were observed to be more compact than the elicited cultures. This can cause autolysis at the centre of the pellet due to oxygen limitations and loose clumps are preferred as they benefit from better oxygen transfer.

The reasons for this morphological change in the presence of elicitors are to be speculated. If one looks at elicitation from a defensive mechanism way of action in nutrient rich media it may be assumed that the increase in clump size and thus hyphal growth will result in higher production rates of penicillin G. This will confer a competitive advantage on the fungus. If production is confined to the hyphal tip, then a cyclic pattern could operate where if elicitors are responsible for enhancing transcript levels of penicillin G genes they could also be responsible in activating genes responsible for the change in morphology of the clumps. There may be similar mechanisms to those reported when paramorphogens are used. Paramorphogens are compounds capable of inducing morphological changes in fungi. Work carried out on *Botrytis fabae*, for example, (Jejelowo and Trinci, 1988) showed an increase in hyphal branching and expansion of clumps.

In fungi there is evidence that calcium plays a fundamental role in hyphal extension and branching (Robson *et al.,* 1991; Jackson and Heath, 1993). Research by Levina *et al.* (1995) on *Neurospora crassa* showed the effects on hyphal tip growth by Ca^{2+}. It shows that tip growth is dependent on tip-gradient of Ca^{2+} concentration. This gradient dissipates in non-growing tips however it is present in all growing tips (Garrill *et al.*, 1993; Levina *et al.*, 1995 and Hyde and Heath, 1997). Although the role of Ca^{2+} as a secondary messenger in fungal growth and differentiation is not as well understood as that in mammalian or plant cell systems, it now seems clear that Ca^{2+} has a significant role in fungi (Jackson and Heath, 1993). Experimentally it has been found difficult to measure Ca^{2+} in fungi due to the problem of rendering Ca^{2+}-free media (Kovac, 1985 and Youatt, 1993). Calcium is believed to transduce stimuli at the cell surface, which may include chemical, electrical or physical signals, into specific intracellular effects. The regulation by calcium of diverse cellular processes underlines the complexity of the situation: several Ca^{2+}-mediated events may occur simultaneously. The action of Ca^{2+} in fungi is modulated through calcium-sensitive cell

process by selective binding to appropriate substrates (Carafoli, 1985 and Rasmussen and Rasmussen, 1990). There could be an activation of a pathway mechanism by elicitors that will result in an increase in intracellular calcium. This hypothesis is based on research in plant cell cultures that have shown that oligosaccharides induced elicitation of metabolites including defence metabolites results in elevated levels of cellular Ca^{2+} (Knight *et al.*, 1991 and Blume *et al.*, 2000). If in *P. chrysogenum* P2 cells Ca^{2+} concentrations rise as a direct effect of elicitors then there would be a case for the increase in clump size and hyphal tips in elicited cultures.

As there were no significant differences in the agitation rates of the control and elicited fermenters (to maintain the DOT above 30%) the possible effect of shear on the observed morphological differences can be disregarded.

4.5 Sporulation in *P. chrysogenum* P2

It was observed that in cultures *P. chrysogenum* P2 STR cultures supplemented with oligosaccharide elicitors sporulation was induced earlier (after 120 hours) than control cultures. Not only that but the concentration of spores increased too (Fig. 3.16, page 98).

The existence of polysaccharide molecules that play a role in differentiation and organogenesis of plant tissues are known. Polysaccharides and oligosaccharides have been shown to induce differentiation and organogenesis of strawberry callus and tabacco plants (Thanh *et al.*, 1985 and Bois, 1992). In fungi the first report of carbohydrates regulating conidiation was the observation by Morton (1961) that the presence of minor components of commercial glucose has a stimulating effect on the formation of conidial hyphae in *Penicillium*. Gelling agents such as isubol were reported to induce early sporulation of *A. flavus* and *P. chrysogenum* (Jain *et al.*, 1997).

In *P. chrysogenum* P2, MO was shown to induce the highest concentration of spores in submerged culture, followed by OM and OG. The reason for this enhancement effect and the differing extent of the effect on the concentration of spores by the oligosaccharide elicitors are yet to be understood. Again it might be inferred that oligosaccharides with different DPs or structures may have various regulatory functions. It can also be argued that time and level of sporulation in this case are related to the fungus' defence mechanism. If the response of the elicited cultures of *P. chrysogenum* P2 towards elicitor supplementation is perceived as a defence mechanism, then it stands to logic to assume that production of spores (which is the ultimate response to a hostile environment) is part of the defence mechanism. This can also be related to the nutrient depletion of the media. Elicited cultures of *P. chrysogenum* P2 were shown to have an increase in the rates

of penicillin G and 6-APA and possibly other metabolites. It may cause a faster rate of nutrient consumption that can in turn induce sporulation as a survival mechanism.

As alginate elicitors have been shown to increase the transcript levels of penicillin G genes there could be a similar mechanism at play with regards to sporulation. Such regulatory genes might exist in *P. chrysogenum*. In *A. nidulans* wetA gene is required for synthesis of cell wall layers that make conidia impermeable and it is suggested to be spore-specific gene expression (Marshall and Timberlake, 1991). A further study by Prade and Timberlake (1994) demonstrated that there is a similar gene in *P. chrysogenum* through homologous gene studies. The paper concluded that the mechanisms controlling sporulation in *A. nidulans* and *P. chrysogenum* are evolutionary conserved and thus similar in function. In the case of *A. nidulans* it was shown that the initiation of conidiophore formation could either occur as a response to intrinsic signals or to environmental stresses such as nutrient deprivation (Adams *et al.*, 1998). I

It should be noted that both the control and elicited STR cultures stopped producing penicillin G and 6-APA after approximately 120 hours. This indicates that there was a limiting factor in the *P. chrysogenum* P2 STR culture. This could explain the induction of sporulation, however, the overproduction of spores in elicited cultures compared to control is significantly different.

If elicitors induce higher calcium levels in the cells this can also lead to the induction of sporulation in *P. chrysogenum* P2 oligosaccharide supplemented cultures. In other systems it has also been shown that elevated levels of calcium could influence conidiation in ascomycetes (Hadley and Harrold, 1958; Righelato *et al.*, 1968; Ugalde and Pitt, 1983; Adams *et al.*, 1998).

4.6 ROS Production in *P. chrysogenum* P2

This study has attempted for the first time to show the effects of elicitors on ROS in *P. chrysogenum* P2 cultures. The results showed a pattern of inhibition of ROS among the elicitors-added cultures regardless of the time of addition of these elicitors. At 24 hours there was some inhibition of ROS, however, overall there were lower levels of ROS including control compared to 48, 72 and 96 hours. The reasons for this are not clear however it can be suggested that a 'younger' culture has a more efficient means to deal with oxidative stress. After 48 hours a distinct pattern can be observed between the effects of polysaccharides and oligosaccharides. Polysaccharides have less inhibitory action on ROS than oligosaccharides and in the case of alginate slightly stimulatory at 48 hours (Fig. 3.23, page 109). The order in which elicitors inhibited ROS was: MO-OM-OG-LBG-alginate, with MO showing the highest inhibition and alginate the lowest. The highest inhibition of ROS was shown at 48 hours. This coincides with the optimum time for the addition of elicitors to *P. chrysogenum* cultures. At 48 hours the penicillin G precursor, PAA, is added too. The inhibition of ROS decreases with the age of the cultures for all elicitors used, after 48 hours.

The reasons for these changes and observations in *P. chrysogenum* P2 are not clear. ROS including the superoxide anion, hydrogen peroxide and hydroxyl radicals are continuously generated in aerobic micro-organisms. The decomposition of hydrogen peroxide is of primary importance to avoid oxidative injury. GSH has been shown to take part in the protection against oxidative stress caused by hydrogen peroxide in *P. chrysogenum* (Pócsi *et al.*, 1997). GSH may also contribute to the penicillin G biosynthesis as it may serve as an important S-source in the biosynthetic process (Stevens *et al.*, 1953), it may take part in the activation of the penicillin side-

chain precursor PAA (Ferrero *et al.*, 1990) and it may maintain a suitable reduced environment for the penicillin enzymes such as IPN synthase (Perry *et al.*, 1988 and Bainbridge *et al.*, 1992). The latter being because in penicillin G production, IPN synthase requires satisfactory oxygen supplementation (Henriksen *et al.*, 1997). These complex interactions might serve to explain in part the effects of elicitors on ROS. GSH appears to have a number of beneficial functions in the production of penicillin G. This might imply that there is a need of some sort for regulatory pathways to control the levels of GSH in the cell. A number of studies have shown that when levels of GSH decline, cells normally increase the production of GSH and the reduction of glutathione disulphade (Verduyn *at al.*, 1988; Shi *et al.*, 1994 and Sweiry *et al.*, 1995). If the presence of GSH in *P. chrysogenum* P2 cells increases due to any number of regulatory systems then this may explain the reduction of ROS. With MO showing the greatest effect on the overproduction of penicillin G it can be suggested, based on the discussion above, that it will have the greatest effect on the inhibition of ROS, followed by OM and then OG.

P. chrysogenum has also been shown to have a very good mechanism for protecting cells from ROS damage through its ability to produce high levels of catalase (Pócsi *et al.*, 1997). This work showed that very high levels of extracellular hydrogen peroxide did little to inhibit the function of *P. chrysogenum* cells. Not only did the cells function but high catalase and glutathione peroxidase activity was observed. These high activities explained the remarkable resistance of *P. chrysogenum* to oxidative stress caused by high concentrations of peroxides. The occurrence of ROS has been demonstrated in plants to be regulated by changes in the intracellular Ca^{2+} concentrations through the addition of oligosaccharides (Angelova *et al.*, 2000). This ROS response is part of a number of tools that plants employ in their defence mechanism. In micro-organisms such as *Escherichia coli* and *Salmonella typhimurium* it was demonstrated that apart

from cellular generation of ROS, when these bacteria are exposed to stress conditions the formation of ROS greatly increases (Farr and Kogoma *et al.*, 1991). A similar action may be found in fungi when they are exposed to stress conditions. In the case of elicitation in *P. chrysogenum* cultures it can be suggested that elicitors as inducers of defence mechanism (i.e. penicillin G overproduction) might trigger the overproduction of enzymes such as catalase to protect the cell from a perceived external attack. In this case the higher levels of catalase, for example, can reduce significantly the levels of ROS. However, there may be a multi-cascade mechanism that is inclusive of a number of pathways.

The reasons for the action of the polysaccharides LBG and alginate on ROS are less clear. In the case of alginate it can be speculated that the production of ROS was similar to that of control because there was no apparent effect of the polysaccharides on the overproduction of penicillin G. Angelova *et al.* (2000) showed that addition of alginate to cultures of *A. niger* did not cause an increase in SOD levels. SOD plays an important part in the cell's defence abilities against ROS (Wang and Schellhorn, 1995).

5. FUTURE WORK

Cytoplasmic calcium ion gradient has been implicated in regulation of several signal transduction processes in living organisms. Calcium ion gradient activation in the cytoplasm is one of the early events associated with signal transduction following binding of elicitors to the cell membrane in plant cells (Messiaen *et al.*, 1993 and Yuan *et al.*, 2001). It will be useful to elucidate the role of Ca^{2+} in the enhancement of secondary metabolite production by oligosaccharides in fungal cultures as such information is clearly lacking in the literature. Calcium gradients will also exist between organelles. Through localisation experiments using dyes such as Fura-2, Calcium Green 1, Indo-1 together with real time microscopy further work in this area could enhance the understanding of Ca^{2+} involvement in the overproduction of secondary metabolites. Other methods that could be explored in ascertaining the levels of cellular calcium make use of microelectrodes and microinjections with dyes.

The effects of oligosaccharides on possible morphological changes in other fungal fermentations should be investigated further. It would be useful to investigate whether this effect is generic or species dependent. This could also further enhance the knowledge on the physiological requirements for production of secondary metabolites.

The effects of elicitors should be investigated further to fully understand the maximum possible threshold effects on overproduction. In this study, it was shown that when a mixture of oligosaccharide DPs, i.e. mannan oligosaccharides, was supplemented to *P. chrysogenum* P2 submerged cultures it had a superior effect, compared to alginate oligosaccharides, on the overproduction of secondary metabolites. Studies on multiple DP additions together with additions of a combination of oligosaccharide elicitors to fungal cultures will give an insight into the

boundaries of overproduction in this system. Fed-batch cultures should also give an insight into this. Mannan oligosaccharides showed the greatest effects on overproduction of secondary metabolites but the reasons for this are not understood. Whether structural differences are at play or the mixture of DPs remains to be elucidated. Bland *et al.* (2001) have shown that DP 7 oligosaccharides were more effective at eliciting a response in white blood cell systems. Methods must be developed and employed to ensure the separation of these DPs in quantities large enough for fermenter studies to further understand this mechanism.

This study, as well as previous studies, has indirectly implicated a number of penicillin G enzymes in the overproduction of the antibiotic. Further understanding of the mechanism of elicitation should focus on the regulatory activities of elicitors on such enzymes. Looking at levels of these enzymes and where possible their regulatory genes will shed light on a possible mechanism of elicitation. This interaction with enzymes or genes needs then to be related to a direct action of elicitors on such systems. Work on plant cell cultures has shown that oligosaccharide elicitors activate genes and thus enzymes through cell membrane receptors. Such studies must be undertaken in fungal systems to determine if receptors are involved and to identify their exact characteristics. These studies will help to understand why different sizes as well as structural characteristics have distinct effects on the overproduction.

Genetic analysis of genes in *P. chrysogenum* P2 will not only help identify the exact genes involved in the enhancement but also clarify whether the effects are solely at transcription level or at translation level as well.

This study has shown that oligosaccharides have a significant effect on sporulation in *P. chrysogenum* P2. A new method for production of large number of spores will have applications both in industry and agriculture. It would be useful to investigate whether this effect is strain or species dependent. It is interesting to note that in animals, exposure of immune cells to antigens (considered as elicitors in plant and microbial cultures) leaves a memory of the antigens on the cells. The antigen induces an immunogenic reaction in the cell on a repeated encounter. In this context it will be illuminating to know if the spores produced by cultures exposed to oligosaccharide elicitors have different metabolic profiles compared to the original spores unexposed to the same oligosaccharide elicitors.

Also comparable to animal and plant cells, this study has investigated for the first time the production of ROS in *P. chrysogenum* P2 exposed to oligosaccharides and polysaccharides. Further studies in this area are needed to formulate a possible mode of action and a possible pattern. Whether this observed effect is strain dependent or species dependent remains to be studied. It would be of great interest to see whether a method could be developed based on the inhibition or possible stimulation of ROS as a screening protocol for potential elicitors in fungal systems. As previous studies have shown the concentration, time of addition and DP play a crucial role in the overproduction of secondary metabolites in *P. chrysogenum*. This work is both labour intensive and time consuming and because of this it limits the number of elicitors that can be investigated. If a method based on ROS could be developed it could revolutionize the area of elicitation and bring about a rethink of the modes employed in industry on strain selection for high producing strains.

As this study has shown that elicitors inhibit the production of ROS it is important to investigate the roles of such enzymes as catalase and superoxide dismutase as part of the cellular response to ROS. It would also be interesting to examine the production of specific ROS in response to elicitors through the use of specific fluorescent dyes.

6. CONCLUSION

This work has brought together some important findings in relation to physiology and morphology changes in submerged cultures of *P. chrysogenum* P2 when supplemented with oligosaccharide elicitors. Whether these changes are strain- or *Penicllium* sp.-specific remains to be seen but clearly the changes suggest an added route, elicitation, for better understanding of secondary metabolite production. Understanding the mechanism of elicitation will open the door to a range of possibilities in different bio-industries.

The overproduction of spores is of great importance to industry especially in the bio-control sector. Some elicitors have been implicated in the stimulation of spore germination and development. In this case strains that are shown to grow faster will give shorter periods for production of the desired product. This has economic benefits for the biotechnology and pharmaceutical industries. With regards to the effects of elicitors on the morphology of *P. chrysogenum* P2, there could be a potential for improvement of oxygen transfer in aerobic fungal systems where improved oxygen transfer is beneficial. This could be useful in the biosynthesis of target products.

This study combined with other reports has suggested that elicitor-induced overproduction is linked to enzymatic function. This is of great importance as it has wider implications in the search for more efficient enzymatic systems for both academic research and industry.

Carbohydrate elicitors are "natural" compounds and they are effective at very low concentrations. Their industrial use should not pose any serious and expensive validation problems. These elicitors could be considered as a cheap and environmentally-friendly means for the enhancement of desired products.

7. REFERENCES

Abraham , E. P., Huddleston, J. A., Jayatilake, G. S., O'Sullivvan, J., and White, R. L. (1981). Conversion of δ-(L-α–aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N incell-free extract of Cephalosporin. In *Recent Advances in the Chemistry of β-lactam antibiotic* (G. I. Gregory, ed.), Royal Society of Chemistry, London. pp: 125-134.

Adams, T. H., Wiser, J. K., and Yu, J. H. (1998). Asexual reproduction in *Aspergillus nidulans*. *Microbiol. Mol. Biol. Rev.* **62**: 33-57.

Adlard, M. W., Godon, B. M., Keshavarz, T., Bucke, C., Lilly, M. D., Bull, A. T., and Holt, G. (1991). An HPLC procedure for monitoring penicillin-G production in cultures of *Penicillium chrysogenum*. *Biotechnol. Tech.* **5**: 121-126.

Akiyama, H., Endo, T., Nakakita, R., Murata, K., Yenemoto, Y., and Okayama, K. (1992). Effect of depolymerised alginates on the growth of *Bifidobacteria*. *Biosci. Biotech. Biochem.* **56**: 355-356.

Albersheim, P., and Valent, B. S. (1978). Host-pathogen interactions in plants: Plants when exposed to oligosaccharides of fungal origin defend themselves by accumulating antibiotics. *J. Cell Biol.* **78**: 627-643.

Alexopoulos, C. J., and Mims, C. W. (1979). Introductory mycology. Wiley & Sons, New York.

Amanullah, A., Blair, R., Nienow, A. W., and Thomas, C. R. (1999). Effects of agitation on mycelial morphology and protein production in chemostat cultures of recombinat *Aspergillus oryaze*. *Biotechnol. Bioeng.* **62**: 434-446.

Angelova, B. M., Pashova, S. B., and Slokoska, L. S. (2000). Comparison of antioxidant enzyme biosynthesis by free and immobilized *Aspergillus niger* cells. *Enzyme Microb. Technol.* **26**: 544-549.

Ariyo, T. A., Bucke, C., and Keshavarz, T. (1997). Alginate oligosaccharides as enhancers of penicillin production in cultures of *Penicillium chrysogenum*. *Biotech. Bioeng.* **53**: 17-20.

Ariyo, T. A., Bucke, C., and Keshavarz, T. (1998). Enhanced penicillin production by oligosaccharides from batch cultures of *Penicillium chrysogenum*. *FEMS Microbiol. Lett.* **166**: 165-170.

Asilonu, E., Bucke, C., and Keshavarz, T., (2000). Enhancement of chrysogenin production in cultures of *Penicillium chrysogenum* by uronic acid oligosaccharides. *Biotechnol. Lett.* **22**: 931-936.

Aust, S. D., Morehouse, L. A., and Thomas, C. E. (1985). Role of metals in oxygen radical reactions. *Free Radica. Biol. Med.* **1**: 3-25.

Axelrod, D. E., Gealt, M., and Pastushok, M. (1973). Gene control of developmental competence in *Aspergillus nidulans*. *Dev. Bio.* **34**: 9-15.

Baldwin, R. R., Campbell, H. A., Thiessen, R., and Loment. G. J. (1953). The use of glucose oxidase in processing of food with special emphasis on desugaring egg white. *Food Technol.* **7**: 275-282.

Bainbridge, Z. A., Scott, R. I., and Perry, D (1992). Oxygen utilisation by isopenicillin N synthase from *Penicillium chrysogenum*. *J. Chem. Tech. Biotechnol.* **55**: 233-238.

Barnett, H. L., and Lilly, V. G. (1955). The effect of humidity, temperature and carbon dioxide on sporulation of *Choanephora cucurbitarum*. *Mycologica* **47**: 26-29.

Baureithel, K., Felix, G., and Boller, T. (1994). Specific, high affinity binding of chitin fragments to tomato cells and membranes. Competitive inhibition of binding by derivatives of chitooligosaccharides and a Nod factor of *Rhizobium*. *J. Biol. Chem.* **269**: 17931-17938.

Behrens, O. K. (1949) Biosynthesis of penicillins. In: *The chemistry of penicillin*, (H. T. Clarke, J. R. Johnson and R. Robinson, eds.). Princeton University Press, New Jersey. pp: 657-679.

Belozerskaya, T. A., and Potapova, T. V. (1993). Intrahyphal communication in segmented mycelium. *Exp. Mycol.* **17**: 157-169.

Bland, E. J., Keshavarz T., and Bucke C. (2001). Using 2',7'-dichlorodihydrofluorescein-diacetate to assess polysaccharides as immunomodulating agents. *Mol. Biotechnol.* **19**: 125-131.

Blume, B., Nürnberger, T., Nass, N., and Scheel, D. (2000). Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell* **120**: 1425-1440.

Brakhage, A. A. (1998). Molecular regulation of β-lactam biosynthesis in filamentous fungi. *Microbiol. Mol. Biol. Rev.* **62**: 547-585.

Bois, F. (1992). The influence of some natural cell wall derived precursors on organogenesis and differentiation of wild strawberry (*Fragaria vesca* L) callus-cultures. *Plant Cell Tissue* **28**: 91-96.

Burmeister, H. R., Vesonder, R. F., and Hesseltine, C. W. (1977). Swelling of *Penicillium digitatum* conidia by a *Fusarium acuminatum* NRRL 6227 metabolite. *Mycopathologia* **62**: 53-56.

Bruner, R., and Rohr, M. (1975). Phenylacetyl:coenzymeA ligase. *Methods Enzymol.* **43**: 476-481.

Bucke, C. (1987). Cell immobilization in Ca-alginate. In *Methods in enzymology, immobilized enzymes and cells,* Part B (K. Mosbach, ed.) Academic Press . pp 175-189.

Calam, C. T. (1987) Process development in antibiotic fermentations. Cambridge studies in biotechnology 4, Cambridge University Press, Cambridge.

Carafoli, E. (1987). Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56**: 395-433.

Carlile, M. J. (1970). The photoresponses of fungi. In: *Photobiology of Microorganisms* (P. Halldal, ed.). Wiley & Sons, Chichester. pp: 309-344.

Carlile, M. J., and Matthews, S. L. (1988). Chemotropism of germ-tubes of *Chaetomium globosum*. *Trans. Br. Mycol. Soc.* **90**: 643-644.

Carlile, M. J., and Tew, P. M. (1988). Chemotropism of germ-tubes of *Phytophthtora citricola*. *Trans. Br. Mycol. Soc.* **90**: 644-646.

Chang, Y. C., and Timberlake, W. E. (1992). Identification of *Aspergillus* brlA response elements (BREs) by genetic selection in yeast.*Genetics* **133**: 29-38.

Clipson, N. J. W., Jennings, D. H., and Smith, J. L. (1989). The response to salinity at the microscopic level of the marine fungus *Dendryphiella salina* Nicot and Pugh as investigated stereologically. *New Phytol.* **113**: 21-30.

Christiansen, T., Spohr, A. B., and Nielsen, J. (1999). On-line study of growth kinetics of single hyphae of *Aspergillus oryzae* in a flow-through cell. *Biotechnol. Bioeng.* **63**: 147-153

Cole, G. (1986). Models of cell differentiation in conidial fungi. *Microbiol. Rev.* **50**: 95-132.

Cole, M. (1966). Microbial synthesis of penicillins II and I. *Process Biochem.* **1**: 334- 338, 373-377.

Collins, Y. E., and Stotzky, G. (1989). Factors affecting the toxicity of heavy metals to microbes. In: *Metal ions and bacteria* (T. J. Beveridge, and R. J. Doyle, eds.). Wiley & Sons, Canada.

Côté, F., and Hahn, M. G. (1994). Oligosaccharins: structures and signal transduction. *Plant Mol. Biol.* **26**: 1379-1411.

Cross, C. E., van der Vliet, A., Louie, S., Thiele, J. J., and Halliwell, B. (1998). Oxidative stress and antioxidants at biosurfaces: plants, skin, and respiratory tract surfaces. *Environ. Health Perspect.* **106**: 1241-1251.

Cunliffe, B., Trinci, A. P. J, Robson, G. D., Wiebe, M. G., and Naylor, T. W. (1990). Microbial growth dynamics (R. K. Poole, M. J. Bazin, and C. W. Keevil, eds.). IRL Press, Oxford.

D'Souza, C., Sharma, C. B., and Elbein, A. D. (1992). Biosynthesis of lipid-linked oligosaccharides. *Anal. Biochem.* **203**: 211-217.

Dea, I. C. M., and Morrison, A. (1975). Chemistry and interactions of seed galactomannans. *Adv. Carbohydr. Chem. Biochem.* **31**: 241-312.

Dekker, R. F. H., and Richards, G. N. (1976). Hemicellulases: Their occurance, purification, properties and mode of action. *Adv. Carbohydr. Chem. Biochem.* **32**: 277-352.

Demain, A. L. and Masurekar, P. S. (1974). Lysine inhibition of in vivo homocitrate synthesis in *Penicillium chrysogenum. J. Gen. Microbiol.* **82**: 143-151.

Demain, A. L. (1983). Biosynthesis of β-lactam antibiotic. In *Antibiotics containing the β-lactam structure* (A. L. Demain and N. A. Solomon, eds.). Springer-Verlag, Berlin. pp: 189-228.

Demain, A. L., and Elander, R. P. (1999). The β-lactam antibiotics: past, present, and future. *Antonie van Leeuwenhoek* **75**: 5-18.

Demple, B. (1996). Redox signaling and gene control in the *Escherichia coli* soxRS oxidative stress regulon. *Gene* **179**: 53-57.

Dey, P. M. (1978). Biochemistry of plant galactomannans. *Adv. Carbohydr. Chem. Biochem.* **35**: 341-376.

Dickson, B. (1995). Nuclear factors in sevenless signalling. *Trends Genet.* **11**: 106-111.

Dorge, T., Carstensen, J.M., and Frisvad, J.C. (2000). Direct identification of pure *Penicillium* species using image analysis. *J. Microbiol. Methods* **41**: 121-133

Duffy, J. B., and Perrimon, N. (1994). The pathway in drosophila: lessons on receptor tyrosine kinase signaling and pattern formation. *Dev. Biol.* **166**: 380-395.

Edwards, S. (1994). Biochemistry and physiology of the neutrophil. Cambridge University Press, Cambridge.

Elander, B. P. (1980). New genetic approaches to industrially important fungi. *Biotech. Bioeng.* **22**: 49-61.

Emri, T., Pócsi, I., and Szentirmai, A. (1999). Analysis of the oxidative stress response of *Penicillium chrysogenum* to menadione. *Free Radical Res.* **30**: 125-132.

Eriksen, S. H., Soderblom, T. B., Jensen, B., and Olsen, J. (1998). Uptake of phenylacetic acid by two strains of *Penicillium chrysogenum*. *Biotechnol. Bioeng.* **60**: 310-316.

Farr, S. B., and Kogoma, T. (1991). Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol.Rev.* **55**: 561-585.

Fenice, M., Federici, F., Selbmann, L., and Petruccioli, M. (2000). Repeated-batch production of pigments by immobilised *Monascus purpureus*. *J. Biotechnol.* **80**: 271-276.

Fernández-Canón, J. M., Reglero, A., Martínez-Blanco, H., and Luengo, J.M. (1989). Uptake of phenylacetic acid by *Penicillium chrysogenum* wis 54-1255: a critical regulatory point in benzylpenicillin biosynthesis. *J. Antibiot.* **42**: 1398-1409.

Ferrero, M. A., Reglero, A., Martín-Villacorta, J., Fernández-Cañón, J. M., and Luengo, J. M. (1990) Biosynthesis of benzylpenicillin (G), phenoxymethylpenicillin (V) and octanoylpenicillin (K) from glutathione S-derivatives. *J. Antibiot.* **43**: 684-691.

Filipiak, M., Rudra, G., and Gosciminska, E. (1996). Enzymatic membranes for determination of some disaccharides by means of an oxygen electrode. *Biosens. Bioelectron.* **11**: 355-364.

Fishman, P. H. (1976). Biosynthesis and function of gangliosides. *Science* **194**: 906-915.

Flattery-O'Brien, J., Collinson, L. P., and Dawes, I. W. (1993). *Saccharomyces cerevisiae* has an inducible response to menadione which differs from that to hydrogen peroxide. *J. Gen. Microbiol.* **139**: 501-507.

Fleming, A. (1929). On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in their use in the isolation of *B. influenzae*. *Brit. J. Pathol.* **10**: 226-236.

Fry, S. C. (1989). The structure and function of xyloglucan. *J. Exp. Bot.* **40**: 1-11.

Fujiki, K., Matsuyama, H., and Yano, T. (1994). Protective effect of sodium alginates against bacterial infection in common carp, *Cyprinus carpio*. *J. Fish Dis.* **4**: 349-355.

Gacesa, P. (1988). Alginates. *Carbohydr. Polym.* **8**: 161-182.

Gang, L., Casqueiro, J., Gutierrez, S., Kasalkova, K., Castillo, N., and Martin, J. F. (2001). Elicitation of penicillin biosynthesis by alginate in *Penicillium chrysogenum* exerted on *pcb*AB, *pcb*C and *pen*DE genes at transcriptional level. *J. Microbiol. Biotechnol.* **11**: 1-8.

Gadd, G. M. (1995). Signal transduction in fungi. In: *The growing fungus* (N. H. R. Gow, and G. M.Gadd, eds.). Chapman & Hall, London. pp. 183-210.

Garrill, A., Jackson, S. L., Lew, R. R., and Heath, I. B. (1993). Ion channel activity and tip growth: tip-localized stretch-activated channels generate an essential Ca^{2+} gradient in the oomycete *Saprolegnia ferax*. *Eur. J. Cell Biol.* **60**: 358-365.

Gooday, G. W., and Trinci, A. P. J. (1980). Wall structure and biosynthesis in fungi. In: *The eukaryotic microbial cells series* - 30. (G. W. Gooday, D. Lloyd, and A. P. J. Trinci, eds.). Cambridge University Press, Cambridge.

Gomez, R., Schnabel, I., and Garrido, J. (1988). Pellet growth and citric acid yield of *Aspergillus niger*. *Enzyme Microb. Technol.* **10**: 188-191.

Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* **10**: 751-757.

Grignon, C. (1991). pH and ionic conditions in the apoplast. *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* **42**: 103-110.

Hadley, G., and Harrold, C. E. (1958). The sporulation of *Penicillium notatum* in submerged liquid culture. *J. Exp. Bot.* **9**: 408-425.

Halliwell, B. (1998). Biomarkers of oxidative damage associated with human disease. *Pathophysiology* **5**: 267.

Halliwell, B. (1999). Antioxidant defence mechanisms: from the beginning to the end (of the beginning). *Free Radical Res.* **31**: 261-272.

Haug, A., Larsen, B., and Smidsred, O. (1974). Uronic acid sequence in alginate from different sources. *Carbohydr. Res.* **32**: 217-225.

Hayes, B. K., Esquivel, F., Bennink, J. R., Yewdell, J. W., and Varki, A. (1995). Structure of the N-linked oligosaccharides of MHC class I molecules from cells deficient in the antigenic peptide transporter. *J. Immunol.* **155**: 3780-3787.

Herbash, G. J. M., van der Beck. C. P., and Vanijk, P. W. M. (1984). The penicillins: properties, biosynthesis and fermentation. In: *Biotechnology Industrial Antibiotics* (E. Vandamme, ed.). Marcel Dekker, New York. pp. 44-140.

Henriksen, C. M., Nielsen, J., and Villasden, J. (1997). Influence of dissolved oxygen concentration on the penicillin biosynthetic pathway in a steady-state cultures of *Penicillium chrysogenum*. *Biotechnol. Prog.* **13**: 776-782.

van der Holst, P. P. G., Schlaman H. R. M., and Spaink H. P. (2001). Proteins involved in the production and perception of oligosaccharides in relation to plant and animal development. *Curr. Opin. Struct. Biol.* **11**: 608-616.

Hönlinger, C., and Kubicek, C. P. (1989). Regulation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N biosynthesis in Penicillium chrysogenum by the alpha-aminoadipate pool size. *FEMS Microbiol. Lett.* **53**: 71-75.

Hughes, R. C. (1975). The complex carbohydrates of mammalian cell surfaces and their biological roles. *Essays in Biochemistry* **11**: 1-36.

Hunt, J. A., Gray, C. H., and Thorogood, D.E. (1956). Enzyme tests for the detection of glucose. *Br. Med. J.* **4**: 586-588.

Hyde, J. G., and Heath, I. B. (1997). Ca^{2+} gradients in hyphae and branches of *Saprolegnia ferax*. *Fung. Genet. Biol.* **21**: 238-247.

Inanova, A. E., and Marfenina, O. E. (2001). Effect of ecological factors on spore germination and the viability of the mycelial fragments of microscopic fungi. *Mikrobiologiia* **70**: 235-240.

Ito, Y., Kaku, H., and Shibuya, N. (1997). Identification of a high-affinity binding protein for N-acetylchitooligosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labelling. *Plant J.* **12**: 347-356.

Jackson, S. L., and Heath, I. B. (1993). Roles of calcium ions in hyphal tip growth. *Microbiol. Rev.* **57**: 367-382.

Jain, N., Gupta, S., and Babbar, S. B. (1997). Isubgol as an alternative gelling agent for microbial culture media. *J. Plant Biochem. Biotechnol.* **6**: 129-131.

Jamieson, D. J., Stephen, D. W. and Terrière, E. C. (1996). Analysis of the adaptive oxidative stress response of *Candida albicans*. *FEMS Microbiol. Lett.* **138**: 83-88.

Jarvis, F. G., and Johnson, M. J. (1947). The role of constituents of synthetic media for penicillin production. *J. Am. Chem. Soc.* **69**: 3010-3017.

Jazlova, P., Martinkord, L. and Kren, V. (1996). Secondary metabolites of the fungus *Monascus*: a review. *J. Ind. Microbiol.* **16**: 163-170.

Jejelowo, O. A., and Trinci, A. P. J. (1988). Effect of the paramorphogens, 3-O-methyl-D-glucose, glucosamine and L-sorbose, on the growth and morphology of *Botrytis fabae*. *Trans. Br. Mycol. Soc.* **91**: 653-660.

Jennings, D. H. (1979). Membrane transport and hyphal growth. In: *Fungal walls and hyphal growth* (J. H. Burnett, and A. P. J. Trinci, eds.). British Mycological Society, University Press, Cambridge.

Jennings, D. H. (1990). Nutrient acquisition by fungi – the relation between physiological understanding and ecological reality. *Plant Sci.* **100**: 153-162.

Jennings, D. H. (1995). The physiology of fungal nutrition. University Press, Cambridge.

Jennings, J. C., Apel-Birkhold, P. C., Mock, N. M., Baker, C. J., Anderson, D. J., and Bailey, B. A. (2001). Induction of defence responses in tobacco by the protein Nep1 from *Fusarium oxysporum*. *Plant Sci.* **161**: 891-899.

Katz, D., Goldstein, D., and Rosenberger R. F. (1972). Model for branch initiation in *Aspergillus nidulans* based on measurements of growth parameters. *J. Bacteriol.* **109**: 1097-1100.

Katz, V., Fuchs, A., and Uwe C. (2002). Pre-treatment with salicylic acid primes parsley cells for enhanced ion transport following elicitation. *FEBS Lett.* **520**: 53-57.

Kawasaki, L., Wysong, D., Diamond, R., and Aguirre, J. (1997). Two divergent catalase genes are differentially regulated during *Aspergillus nidulans* development and oxidative stress. *J. Bacteriol.* **179**: 3284-3292.

Keshavarz, T., Walker, E., Eglin, R., Lilley, G.; Holt, G. and Lilly, M.D. (1989). Immobilization of *Penicillium chrysogenum*: spore growth on celite. *Appl. Microbiol. Biotechnol.* **30**: 487-491.

Kimura, S., Amachi, S., Ohno, N., Takahashi, H., Shinoyama, H., and Fujii, T. (2002). Relationship between conidial enzymes and germination of the apple blue mold, *Penicillium expansum*. *Biosci., Biotechnol., Biochem.* **66**: 1126-1129.

Kirakosyan, A., Hayashi, H., Inoue, K., Charchoglyan, A., and Vardapetyan, H. (2000). Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures. *Phytochemistry* **53**: 345-348.

Kisser, M., Kubicek, C. P., and Roehr, M. (1980). Influence of manganese on morphology of cell wall composition of *Aspergillus niger* during citric acid production. *Arch. Microbiol.* **128**: 26-33.

Klionsky, D. J., Herman, P. K., and Emr, S. D. (1990). The fungal vacuole: composition, function, and biogenesis. *Microbiol. Rev.* **54**: 266-292.

Knight, M. R., Campbell, A. K., Smith, S. M., and Trewavas, A. J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**: 524-526.

Konings, W. N., Poolman, B., and Driessen, A. J. M., (1992). Can the excretion of metabolites by bacteria be manipulated? *FEMS Microbiol. Rev.* **88**: 93-108.

Kovac, L. (1985). Calcium and *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **840**: 317-323.

Kuby, J. (1997). Immunology, 3rd ed. W. H. Freeman & Co., New York.

Kumar, H. P. M., Hague, C., Haley, T., Starr, C. M., Besman, M. J., Lundblad, R. L., and Baker, D. (1996). Elucidation of N-linked oligosaccharide structures of recombinant human factor VIII using fluorophore assisted carbohydrate electrophoresis. *Biotechnol. Appl. Biochem.* **24**: 207-216.

Kurylowicz, W., Kurzatkowski W., and Kurzatkowski, J. (1987). Biosynthesis of benzylpenicillin by *Penicillium chrysogenum* and its golgi apparatus. *Archivum Immunologiae et Therapiae Experimentalis* **35**: 699-724.

Lara, F., Mateos R. C., Vazquez, G., and Sanchez, S. (1982). Induction of penicillin biosynthesis by L-glutamate in *Penicillium chrysogenum*. *Biochem. Biophys. Res. Commun.* **105**: 172-178.

Larsen, B., and Haug, A. (1971). Biosynthesis of alginate. 3. Tritium incorporation with polymannuronic acid 5-epimerase from *Azotobacter vinelandii*. *Carbohydr. Res.* **20**: 225-232.

Lee, J., Dawes, I. W., and Roe, J. H. (1995). Adaptive response of *Schizosaccharomyces pombe* to hydrogen peroxide and menadione. *Microbiol.* **141**: 3127-3132.

Lemmon, M. A. (1994). Regulation of signal transduction and signal diversity. *Trends Biochem. Sci.* **19**: 459-463.

Levina, N. N., Lew, R, R., Hyde, G. J., and Heath, I. B. (1995). The roles of Ca^{2+} and plasma membrane ion channels in hyphal tip growth of *Neurospora crassa*. *J. Cell Sci.* **108**: 3405-3417.

Losel, D. M. (1990). Lipids in the structure and function of fungal membranes. In: *Biochemistry of cell calls and membranes in fungi* (P. J. Kuhn, A. P. J Trinci, M. J. Jung, M. W. Goosey and L. G. Copping, eds.) Springer-Verlag, Berlin.

Mallakin, A., McConkey, B. J., Miao, G., McKibben, B., Snieckus, V, Dixon, D. G., and Greenberg, B. M. (1999). Impacts of structural photomodification on the toxicity of environmental contaminants: photooxidation products. *Ecotoxicol. Environ. Saf.* **43**: 204-212.

Manachere, G. (1980). Conditions essential for controlled fruiting of macromycetes. *Trans. Br. Mycol. Soc.* **75**: 255-270.

Manavathu, E. K., and Thomas, D. S. (1985). Chemotropism of *Achlya ambisexulalis* to methionine and methionyl compounds. *J. Gen. Microbiol.* **101**: 65-70.

Marero, L. M., Je, H. J., Joong, H. S., Ho, J. L., In, C. S. and Lee, H. J. (1997). Effect of fungal elicitation on indirubin production from a suspension culture of *Polygonum tinctorium*. *Enzyme Microb. Technol.* **21**: 97-101.

Marshall, M. A., and Timberlake, W. E. (1991). *Aspergillus nidulans* wetA activates spore-specific gene expression. *Mol. Cell. Biol.* **11**: 55-62.

Mendez, C., and Salas, J. A. (1998). ABC transporters in antibiotic producing actinomycetes. *FEMS Microbiol. Lett.* **158**: 1-8.

Messiaen, J., Read, N. D., Vancutsem, P., and Trewavas, A. J. (1993). Cell-wall oligogalacturonides increase in cytosolic free calcium in carrot protoplasts. *J. Cell Sci.* **104**: 365-371.

Mooney, J. L., and Yager, L. N. (1990). Light is required for conidiation in *Aspergillus nidulans*. *Genes Dev.* **4**: 1473-1482 .

Morton, A. G. (1961). Induction of sporulation in mould fungi. *Proc. Royal Soc.* **B153**: 548-569.

Müller, C., Spohr A. B., and Nielsen, J. (2000). Role of substrate concentration in mitosis and hyphal extension of *Aspergillus*. *Biotechnol. Bioeng.* **67**: 390-397.

Munkres, K. D. (1992). Selection and analysis of superoxide dismutase mutants of *Neurospora*. *Free Rad. Biol. Med.* **13**: 305-318.

Natsume, M., Kamo, Y., Hirayama, M., and Adachi, T. (1994). Isolation and characterization of alginate-derived oligosaccharides with root growth-promoting activities. *Carbohydr. Res.* **258**: 187-197.

Nayler, J. H. (1991). Early discoveries in the penicillin series. *Trends Biochem. Sci.* **16**: 195-197.

Nelson, W. L., and Cretcher, L. H. (1929). The alginic acid from *Macrocystit pyrifera*. *J. Am. Chem. Soc.* **51**: 1914-1920.

Nielsen, J. (1993). A simple morphologically structured model describing the growth of filamentous microorganisms. *Biotechnol. Bioeng.* **41**: 715-727.

Packer, H. L., and Thomas, C. R. (1989). Morphological measurements on filamentous microorganisms by fully automatic image analysis. *Biotechnol. Bioeng.* **35**: 870 –881.

Papagianni, M. (1999). Fungal morphology. In: *Citric acid biotechnology* (B. Kristiansen, M. Mattey and J. Linden, eds.). Taylor and Francis, London. pp: 69-84.

Papagianni, M., and Moo-Young, M. (2002). Protease secretion in glucoamylase producer *Aspergillus niger* cultures: fungal morphology and inoculum effects. *Process Biochem.* **37**: 1271-1278.

Pastushok, M., and Axelrod, D. E. (1976). Effect of glucose, ammonium and media maintenance on the time of conidiophore initiation by surface colonies of *Aspergillus nidulans*. *J. Gen. Microbiol.* **94**: 221-224.

Paul, G. C., Kent, C. A., and Thomas, C. R. (1993). Viability testing and characterisation of germination of fungal spores by automatic image analysis. *Biotechnol. Bioeng.* **42**: 11-23.

Paul, G. C., Priede, M. A., and Thomas, C. R. (1999). Relationship between morphology and citric acid production in submerged *Aspergillus niger* fermentations. *Biochem. Eng. J.* **3**: 121-129.

Paul, G. C., and Thomas, C. R. (1998). Characterisation of mycelial morphology using image analysis. In: *Relation between morphology and process performance* (K. Schugerl ed.). Springer, Berlin. **60**: 1-59.

Peberdy, J. F. (1985). Biology of *Penicillium*. In: *Biology of Industrial Microorganisms* (A. L. Demain and N. A. Solomon, eds.), Biotechnology Series, Benjamin Cumminns Co. Inc., London, pp: 407-431.

Peñalva, M. A, Rowlands R. T., and Turner, G. (1998). The optimization of penicillin biosynthesis in fungi. *Trends Biotechnol.* **16**: 483-489.

Penninckx, M. J., and Elskens, M. T. (1993). Metabolism and functions of glutathione in micro-organisms. *Adv. Microbial Physiol.* **34**: 239-301.

Perry, D., Abraham, E. P., and Baldwin, J. E. (1988). Factors affecting the isopenicillin N synthetase reaction. *Biochem. J.* **255**: 345-351.

Petruccioli, M., Federici, F., Bucke, C., and Keshavarz, T. (1999). Enhancement of glucose oxidase production by *Penicillium variabile* P16. *Enzyme Microbiol. Technol.* **24**: 397-401.

Pindar, D. F., and Bucke, C. (1975). The biosynthesis of alginic acid by *Azotobacter vinelandii*. *Biochem. J.* **152**: 617-622.

Pócsi, I., Emri, T., and Szentirmai, A. (1997). Glutathione metabolism and protection against oxidative stress caused by peroxides in *Penicillium chrysogenum*. *Free Radical Biol. Med.* **23**: 809-814.

Prade, R. A., and Timberlake, W. E. (1994). The *Penicillium chrysogenum* and *Aspergillus nidulans* wetA developmental regulatory genes are functionally equivalent. *Mol. Gen. Genet.* **244**: 539-547.

Promé, J. C. (1996). Signalling events elicited in plants by defined oligosaccharide structures. *Curr. Opin. Struct. Biol.* **6**: 671-678.

Prosser, J. I., and Tough, A. J. (1991). Growth mechanisms and growth kinetics of filamentous microorganisms. *Crit. Rev. Biotechnol.* **10**: 253-274.

Ramos, F. R., López-Nieto, M. J., and Martín, J. F. (1985). Isopenicillin N synthetase of *Penicillium chrysogenum*, an enzyme that converts δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. *Antimicrob. Agents and Chemother.* **27**: 380-387.

Ramussen, H., and Ramussen, J. E. (1990). Calcium as intracellular messenger: from simplicity to complexity. *Curr. Top. Cell. Reg.* **31**: 1-109.

Rattray, L., Prosser J., Silcock D., Glover, A., and Killham, K. (1994). Spatial characterisation of luminescence marked bacterial cells by CCD image-enhanced microscopy. *Binary* **6**: 49-54.

Rees, T. (1977). Conservation of carbohydrate by the non-photosynthetic cells of higher plants. *Symp. Soc. Exp. Biol.* **31**: 7-32.

Righelato, R. C., Trinci, A. P. J., Prit, S. J., and Peat, A. (1968). The influence of maintenance energy and growth rate on the metabolic activity, morphology and conidiation of *Penicillium chrysogenum*. *J. Gen. Microbiol.* **50**: 399-412.

Robinson, P. M. (1973). Oxygen-positive chemotropic factor for fungi. *New Phytol.* **72**: 1349-1356.

Robinson, P. M. (1980). Autrotropism in germinating arthrospores of *Geotrichum candidum. Mycol. Soc.* **75**: 151-153.

Robson, G. D., Wiebe, M. G., and Trinci, A. P. (1991). Exogenous cAMP and cGMP modulate branching in *Fusarium graminearum. J. Gen. Microbiol.* **137**: 963-969.

Rolinson, G. N. (1979). 6-APA and the development of the β-lactam antibiotics. *J. Antimicrob. Chemother.* **5**: 7-14.

Sanchez, S., Flores, M. E., and Demain, A. L. J. (1988). Nitrogen regulation of penicillin and cephalosporin fermentations. In: *Nitrogen source control of microbial processes*. CRC Press, Boca Raton.

Sautour, M., Rouget, A., Dantigny, P., Divies, C., and Bensoussan, M. (2001). Prediction of conidial germination of *Penicillium chrysogenum* as influenced by temperature, water activity and pH. *Lett. Appl. Microbiol.* **32**: 131-134.

Schügerl, K., Gerlach S. R., and Siedenberg, D. (1997). Influence of the process parameters on the morphology and enzyme production of *Aspergilli. Adv. Biochem. Eng. Biotechnol.* **60**: 195-267.

Sekiguchi, J., Gaucher, G. M., and Costerton, J. W. (1975). Microcycle conidiation in *Penicillium urticae:* an ultrastructural investigation of conidial germination and outgrowth, *Can. J. Microbiol.* **21**: 2059-2068.

Shanks, J. V., and Bhadra, R. (1998). Characteristics of selected hairy root lines of *Catharanthus roseus*. In: *Hairy roots* (P. Doran, ed.). Harwood Academic, Amsterdam. pp: 51- 63.

Shi, M. M., Iwamoto, T., and Forman, H. J. (1994). γ-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. *Am. J. of Physiol.* **267:** L414-L421.

Sigler, K., Chaloupka, J., Brozmanová, J., Stadler, N., and Höfer, M. (1999). Oxidative stress in microorganisms. Microbial vs. higher cells - damage and defenses in relation to cell aging and death. *Folia Microbiol.* **44**: 587-624.

Skjak-Braek, G., and Espevik, T. (1996). Application of alginate gels in biotechnology and biomedicine. *Carbohy. Eur.* **14**: 19-25.

Skromne, I., Sánchez, O., and Aguirre, J. (1995). Starvation stress modulates the expression of the *Aspergillus nidulans* brlA regulatory gene. *Microbiology* **141**: 21-28.

Slavik, J., and Kotyk, A. (1984). Intracellular pH distribution and transmembrane pH profile of yeast cells. *Biochim. Biophys. Acta.* **766**: 679-684.

Slayman, C. L. (1992). Some remarks on the integrated actions of pumps, cotransporters and channels. *J. Exp. Biol.* **172**: 267-270.

Smith, J. E. (1978). Asexual sporulation in filamentous fungi. In: *The filamentous fungi* (J. E. Smith and D. R. Berry, eds.). Wiley, New York. pp. 214-239.

Solaiman, D. K., Ashby, R. D., and Foglia, T. A. (2001). Production of polyhydroxyalkanoates from intact triacylglycerols by genetically engineered *Pseudomonas*. *Appl. Microbiol. Biotechnol.* **56**: 664-669.

Somerson, N. L., Demain, A. L., and Nuheimer, T. D. (1961). Reversal of lysine inhibition of penicillin production by α-aminodipic acid. *Arch. Biochem. Biophys.* **93**: 238-252.

Spencer, B., and Maung, C. (1970). Multiple activities of penicillin Acyltransferase of *Penicillium chrysogenum*. *Biochem. J.* **118**: 29-30.

Spencer, B. (1975). Phenylacetyl coenzyme A hydrolase. *Methods Enzymol.* **43**: 482-487.

Srinivasan. V., Ciddi. V., Bringi, V., Shuler, M. L. (1996). Metabolic inhibitors-elicitors and precursors as tools for probing yield limitation in taxane production by *Taxus chinensis* cell cultures. *Biotechnol. Prog.* **12**: 457-465.

Stanford, E. C. C. (1886). On alginic acid and its compounds. *J. Soc. Chem. Ind.* **5**: 218-130.

Stevens, C. M., Vohra, P., Inamine, E., and Roholt, O. H. (1953). Utilisation of sulphur compounds for *Penicillium chrysogenum*. *J. Biol. Chem.* **205**: 1001-1006.

van Suijdam, J. C., Kossen, N. W. F., and Paul, P. G. (1980). An inoculum technique for the production of fungal pellets. *Eur. J. Appl. Microbiol. Biotechnol.* **10**: 211-221.

Sweiry, J. H., Sastre, J., Viña, J., Elsässer, H. P., and Mann, G. E. (1995) A role for γ-glutamyl transpeptidase and the amino acid transport system xc- in cystine transport by a human pancreatic duct cell line. *J. Physiol.* **485**: 167-177.

Taber, W. A. (1966). Mophogenesis in basidiomycetes. In: *The fungi* (G. C. Ainsworth and A. S. Sussman, eds.). Academic Press, New York. **2**: 387-412.

Tamerler, C., Ariyo, B., Bucke, C., and Keshavarz, T. (2001). Effect of mannan and alginate oligosaccharides on production in bioreactors of penicillin G and its biosynthetic intermediates. *Ann. Microbiol.* **51**: 53-60.

Takeuchi, Y., Schmid, J., Caldwee, J. H., and Harold, F. M. (1988). Transcellular ion currents and extension of *Neurospora crassa* hyphae. *J. Membrane Biol.* **101**: 33-41.

van Thanh, T. K., Toubart, P., Cousson, A., Darvill, A. G., Gollin, D. J., Chelf, P., and Albersheim, P. (1985). Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharides. *Nature* **314**: 615-617.

Timberlake, W. E., and Marshall, M. A. (1988). Genetic regulation of development in *Aspergillus nidulans*. *Trends Genet.* **4**: 162-169.

Treskatis, S. K. (1996). Morphology and antibiotic production in *Streptomycetes*. University of Tubingen, Germany.

Tucker, K. G., Kelly, T., Delgrazia, P., and Thomas, C. R. (1992). Fully automatic measurement of mycelial morphology by image analysis. *Biotechnol. Prog.* **4:** 353-359.

Tucker, K. G., and Thomas, C. R. (1992). Mycelial morphology: the effect of spore inoculum level. *Biotech. Lett.* **14**: 1071-1074.

Ugalde, U. O., and Pitt, D. (1983). Morphology and calcium-induced conidiation of *Penicillium cyclopium*. *Trans. Brit. Mycol. Soc.* **80**: 319-325.

Vanek, Z., and Hostalek, Z. (1986). Overproduction of microbial metabolites. (M.A. Stoneham, ed.). Butterworth Publishers, London. pp: 308.

Vecht-Lifshitz, S. E., Magdassi, S., and Braun, S. (1990). Pellet formation and cellular aggregation in *Streptomyces tendae*. *Biotechnol. Bioeng.* **35**: 890-896.

Verduyn, C., Guiseppin, M. L. F., Schemers, W. A., and van Dijken, J. P. (1988). Hydrogen-peroxide metabolism in yeasts. *App. Env. Microbiol.* **54**: 2086-2090.

Véronési, C., Pouénat, M., Rickauer M., and Esquerré-Tugayé, M. (1999). Regulation of tobacco lipoxygenase by methyl jasmonate and fatty acids. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie* **322**: 491-497.

Vetvicka, V., Thornton, B. P., and Ross, G. D. (1996). Soluble β-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. *J. Clin. Invest.* **98**: 50-61.

Wainwright, N.I. (1988). Metabolic diversity of fungi in relation to growth and mineral cycling in soil. *Trans. Br. Mycol. Soc.* **90**: 159-164.

Wang, P., and Schellhorn, H. E. (1995). Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans*. *Can. J. Microbiol.* **41**: 170-176.

Weete, J. D. (1980). Lipid biochemistry of fungi and other organisms. Plenum Press, New York.

White, R. L., John, E. M. M., Baldwin, J. E, and Abraham, E. P. (1982). Stiochiometry of oxygen consumption in the biosynthesis of isopenicillin from a tripeptide. *Biochem. J.* **203**: 791-793.

White, S., Berry B. R., and McNeil, B. (1999). Effect of phenylacetic acid feeding on the process of cellular autolysis in submerged batch cultures of *Penicillium chrysogenum*. *J. Biotechnol.* **75**: 173-185.

Whitehead, I. M., and Threlfall, D. R. (1992). Production of phytoalexins by plant tissue cultures. *J. Biotechnol.* **26**: 63-81.

Wiebe, M. G., and Trinci, A. P. J. (1991). Dilution rate as a determinant of mycelial morphology in continuous culture. *Biotechnol. Bioeng.* **38**: 75-81.

Wirtanen, G., Ahola, H., and Mattila-Sandholm, T. (1995). Evaluation of cleaning procedures in elinmination of biofilm from stainless steel surfaces in open process equipment. *Trans. Inst. Chem. Eng.* **73**: 9-16.

Yang, K., Wang, J., Li, X., Feng, X., and Duan, S. (2001). Strain selection of *Metarrhizium anisopliae* by image analysis of colony morphology for consistency of steroid biotrasformation. *Biotechnol. Bioeng.* **75**: 53-62.

Yamada, H. (1994). Pectic polysaccharides from Chinese herbs: structure and biological activity. *Carbohy. Polymers* **25**: 269-276.

York, W. S. (1984). Inhibition of 2,4-dichlorophenoxyacetic acid stimulated elongation. *Plant Physiol.* **75**: 295-297.

Youatt, J. (1993). Calcium and microorganisms. *Crit. Rev. Microbiol.* **19**: 83-97.

Yuan, Y., Li, C., Hu, Z., and Wu, J. (2001). Signal transduction pathway for oxidative burst and taxol production in suspension cultures of *Taxus chinensis* var. mairei induced by oligosaccharide from *Fusarium oxysporum*. *Enzyme Microb. Technol.* **29**: 372-379.

Zhang, K., and Petty, H. R. (1994). Influence of polysaccharides on neutrophil function: specific antagonists suggest a model for cooperative saccharide-associated inhibition of immune complex-triggered superoxide production. *J. Cell. Biochem.* **56**: 225-235.