Quorum sensing for improved production of industrially useful products from filamentous fungi.

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QUORUM SENSING FOR IMPROVED PRODUCTION OF INDUSTRIALLY USEFUL PRODUCTS FROM FILAMENTOUS FUNGI

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ABSTRACT

Quorum sensing (QS) is a cell density dependant phenomena utilized as a communication process among microorganisms to regulate their physiological responses. QS depends on the production and release of low molecular weight, diffusible chemical signalling molecules, known as quorum sensing molecules (QSM) in the extracellular milieu. When the concentrations of the QSMs reach a critical threshold corresponding to a particular cell density, they bind to a receptor enabling them to function as transcriptional regulators. QS is widely studied in Gram-positive and Gram-negative bacteria in addition to unicellular fungi. Various recent studies report the presence of QS in filamentous fungi. Lipophilic molecules, including lactone-based molecules and the oxygenated poly-unsaturated fatty acids, oxylipins, are the major signalling molecules reported in filamentous fungi.

This study correlates the cell-densities of filamentous fungi to the regulation of their different physiological responses. Two fungal species have been investigated. The first is *Penicillium sclerotiorum*. It was found that addition of ethyl acetate extracts from high cell densities of *P. sclerotiorum* culture increases sporulation delays the onset of hyphal branching and enhances the production of the secondary metabolite, sclerotiorin in shaken flasks as well as stirred tank bioreactors (STR). GC-MS analysis of the high cell-density extracts revealed several molecules including the oxylipin ricinoleic acid that might be involved in the regulation of the *P. sclerotiorum* physiological responses via QS.

The second species studied was *Aspergillus terreus*. Here, the role of oxylipins as signalling molecules was investigated. It was found that the supplementation of linoleic acid, as an oxylipin precursor, enhances the production of the secondary metabolite lovastatin in shaken flasks and STRs. Studies using linoleic acid were extended, to investigate its effect on the cytosolic proteome profile of *A. terreus*. Results showed that several proteins were altered, mainly stress-related proteins and those involved in carbohydrate metabolism.

Furthermore, the effect of different oxylipins was investigated on intracellular cAMP levels. It was observed that addition of oxylipins induces a burst in cAMP levels; in particular 9-HpODE, the linoleic acid-derived oxylipin in *A. terreus,* induces cAMP levels in a dose dependant manner. *In silico* analysis of *A. terreus* genome revealed the presence of genes encoding the different components of G-protein/cAMP-mediated signalling. It is speculated that the addition of 9-HpODE activates the signalling mechanism in *A. terreus* by binding to G-protein coupled receptors. Upon activation, the secondary messenger cAMP is produced. cAMP then induces the expression of different genes, triggering different cellular responses such as sporulation, and secondary metabolism in *A. terreus*.

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Dedicated to the memory of my Grandmother Hassiba Chames

"Advance, and never halt, for advancing is perfection.
Advance and do not fear the thorns in the path,
for they draw only corrupt blood."

Gebran Khalil Gebran

(1883-1931)

AUTHOR'S DECLARATION

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

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

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

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

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LIST OF ABBREVIATIONS

°C.....	Degree centigrade
13- HpOTE.....	13S-hydroperoxy linolenic acid
13-HODE.....	13-hydroxy-octadecadienoic acid
13-HpODE.....	13-hydroperoxy octadecadienoic acid
1DE.....	One-dimensional electrophoresis
2DE.....	Bi-dimensional electrophoresis
3-oxo-C6 HSL.....	N-(3-oxo-hexanoyl)-L-homoserine lactone
8-HODE.....	8-hydroxy octadecadienoic acid
8-HODE.....	8-hydroxylinoleic acid
8-HOE.....	8-hydroxyoleic acid
8-HOTE.....	8-hydroxylinolenic acid
9-HODE.....	9-hydroxyoctadecadienoic acid
9-HpODE.....	9-hydroperoxy octadecadienoic acid
ABC.....	ATP-binding cassette
AHL.....	Acyl homoseriene lactone
AI-1.....	Autoinducer 1
AI-2	Autoinducer 2
AIP.....	Auto-inducing peptide
ANOVA.....	Analysis of variance
APS.....	Ammonium persulphate
BLAST.....	Basic Local Alignment Search Tool
BSA.....	Bovine serum albumin
BSTFA.....	N,O-bis (trimethylsilyl) trifluoroacetamide
cAMP.....	Cyclic adenosine monophosphate
CCD.....	Central composite design
CDW.....	Cell dry weight
CHAPS.....	(3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate)
CHO.....	Carbohydrate
CHP.....	Conserved Hypothetical protein
CSF.....	Competence stimulating factor
CSP.....	Competence Stimulating peptide
CV.....	Coefficient of Variation
DNA.....	Deoxyribonucleic acid
DOT.....	Dissolved oxygen tension
DTT.....	Dithiothreitol

EDTA.....	Ethylenediaminetetraacetic acid
EI.....	Electron ionisation
ELISA.....	Enzyme-Linked Immunosorbent Assay
EST.....	Expressed sequence tags
eV.....	Electron volt
GC-MS.....	Gas chromatography-mass spectrometry
GDP.....	Guanosine diphosphate
GMM.....	Glucose Minimal Medium
GPCR.....	G-protein coupled receptor
GST.....	Glutathione S-transferases
GTP.....	Guanosine triphosphate
HMG-coA.....	Hydroxy-3 methyl glutaryl coenzyme A
HP.....	Hypothetical Protein
HPLC.....	High-performance liquid chromatography
HSL.....	Homoseriene lactone
IEF.....	Isoelectric focusing
IPG.....	Immobilized pH gradient
LA.....	Linoleic acid
LC-MS.....	Liquid chromatography mass spectrometry
LOX.....	Lipoxygenase
m/z.....	Mass to charge ratio
MALDI-TOF.....	Matrix-assisted laser desorption/ionization-Time-of-flight
mAU.....	milli absorbance unit
min	Minutes
N_2.....	Nitrogen gas
NCBI.....	National Centre for Biotechnology Information
NIST.....	National Institute of Standards and Technology
ORF.....	Open reading frame
PAGE.....	Polyacrylamide gel electrophoresis
PDA.....	Potato dextrose agar
PDB.....	Potato dextrose broth
PKA.....	Protein kinase A
PKS	Polyketide synthases
Ppo.....	Psi producing oxygenases
Psi.....	Precocious sexual inducer
PUFA.....	Poly unsaturated fatty acids
QS.....	Quorum sensing
QSM.....	Quorum sensing molecule
RGS.....	Regulator of G-protein signalling
ROS.....	Reactive oxygen species
Rpm.....	Revolutions per minute

RSM.....	Response surface methodology
SAM.....	S-adenosyl-L-methionine
SDS.....	Sodium dodecyl sulphate
STR.....	Stirred tank reactor
TEMED.....	N,N,N’,N’-Tetramethylethylenediamine
TIGR.....	The Institute for Genomic Research
TLC.....	Thin layer chromatography
vol/vol.....	volume per volume
wt/vol.....	weight per volume

AIM AND OBJECTIVES

The overall aim of this work was to investigate quorum sensing for improved production of industrially useful products from filamentous fungi.

In this context, the following objectives have been addressed:

- Investigate the presence of putative quorum sensing molecules in the culture supernatant of the filamentous fungus *Penicillium sclerotiorum*, and study their effect on sporulation, lateral-hyphal branching and secondary metabolism of this fungus.

- Investigate the impact of concentration and addition time of 7-day old *P. sclerotiorum* culture extract on sclerotiorin production and cell dry weight in shaken flasks and bioreactor cultures of *P. sclerotiorum.*

- Identify some of the possible signalling molecules, lactone-based or oxylipins, in *P. sclerotiorum* cultures using GC-MS analysis.

- Investigate the role of oxylipins as potential quorum sensing molecules in *Aspergillus terreus*, by testing the effect of linoleic acid, an oxylipin(s) precursor, on the production of the secondary metabolite lovastatin in shaken flasks and 5L stirred tank bioreactor.

- Investigate the role of linoleic acid on the intracellular (cytosolic) protein profile of *A. terreus* by 2D gel electrophoresis followed by MALDI-TOF/TOF.

- Investigate the role of different oxylipins and their precursor (linoleic acid) on intracellular cAMP levels in *A. terreus.*

- Detect and identify different components (proteins) of G-protein/cAMP-mediated signalling pathways in *A. terreus*.

Chapter I

INTRODUCTION

1.1 Quorum Sensing

Microorganisms are no longer regarded as independent, solitary cells; beside their competition for survival and multiplication, they have developed a sophisticated communication process that allows them to adapt to local environmental changes. This includes their population cell density (Dong et al., 2001, Hardman et al., 1998), where at an appropriate local cell density the communicating microorganisms undertake a coordinated change in their gene-expression profiles to ensure an efficient biological effect (Elasri et al., 2001).

This regulatory process that correlates gene-expression to the cell density of the microbial population is well-known as quorum sensing (QS). QS depends on the production and release of small diffusible chemical signalling molecules in the extracellular milieu (Fuqua et al., 1994). The signalling molecules involved in cell-cell communication are considered as pheromones or autoinducers and defined as quorum sensing molecules (QSM). When the concentration of these molecule(s) reaches a critical threshold (corresponding to a particular cell density), they bind to receptor molecules which are either located on the surface or in the cytoplasm enabling them to function as transcriptional regulators (Miller and Bassler, 2001). Transcriptional regulators then induces or repress QS target genes as well as activate the genes encoding for quorum sensing signal synthesis; thus resulting in a positive feedback loop, autoinduction, to amplify the production of the corresponding signalling molecule (Williams, 2007). Quorum sensing thus provides bacteria and other microorganisms with the multicellular characteristics, a behaviour of higher organisms (Hooshangi and Bentley, 2008).

1.2 History of Quorum Sensing

1.2.1 Quorum Sensing in *V. fischeri*

Quorum sensing was initially discovered and characterized over 30 years ago in the luminous marine bacterium *Vibrio fischeri* (Nealson and Hastings, 1979). This bacterium lives in symbiotic association with various eukaryotic hosts including the bobtail sepiolid squid, *Euprymna scolopes* (Ruby, 1996).

V. fischeri colonizes the specialized light organs of its eukaryotic host; the host provides the bacterial species with the essential nutrients required for its survival and growth, in return *V. fischeri* culture supplies the host with light (Ruby and McFall-Ngai, 1992). Only at high cell densities of *V. fischeri*, the accumulated QSM elicit a signalling cascade to induce bioluminescence production via QS (Nealson and Hastings, 1979) .

Quorum sensing in *V. fischeri* is regulated by eight bioluminescence *lux* genes; arranged in two independent transcribed operon units (Figure 1.1). The luciferase structural operon (*LuxICDABE)* consists of *LuxI* followed by five genes *LuxCDABE* (Engebrecht et al., 1983). Alongside the *lux* operon, a single gene *LuxR* is transcribed in the opposite direction. *LuxI* encodes an autoinducer synthase, producing the autoinducer N-(3-oxohexanoyl)-homoseriene lactone (3-oxo-C6-HSL); whereas *LuxR* encodes for a transcriptional activator of bioluminescence (Eberhard et al., 1981). *LuxA* and *LuxB* genes encode the subunits of the heterodimeric luciferase enzymes which catalyses the oxidation of aldehyde and reduced flavin mononucleotide to produce a long chain fatty acid, water and flavin mononucleotide. Excess free energy is released as an oxidation-reaction by-product in the form of blue-green light (Whitehead et al., 2001). Finally, *LuxCDE* encodes for the multi-enzyme complex involved in the biosynthesis of luciferase substrates (Fuqua et al., 1994).

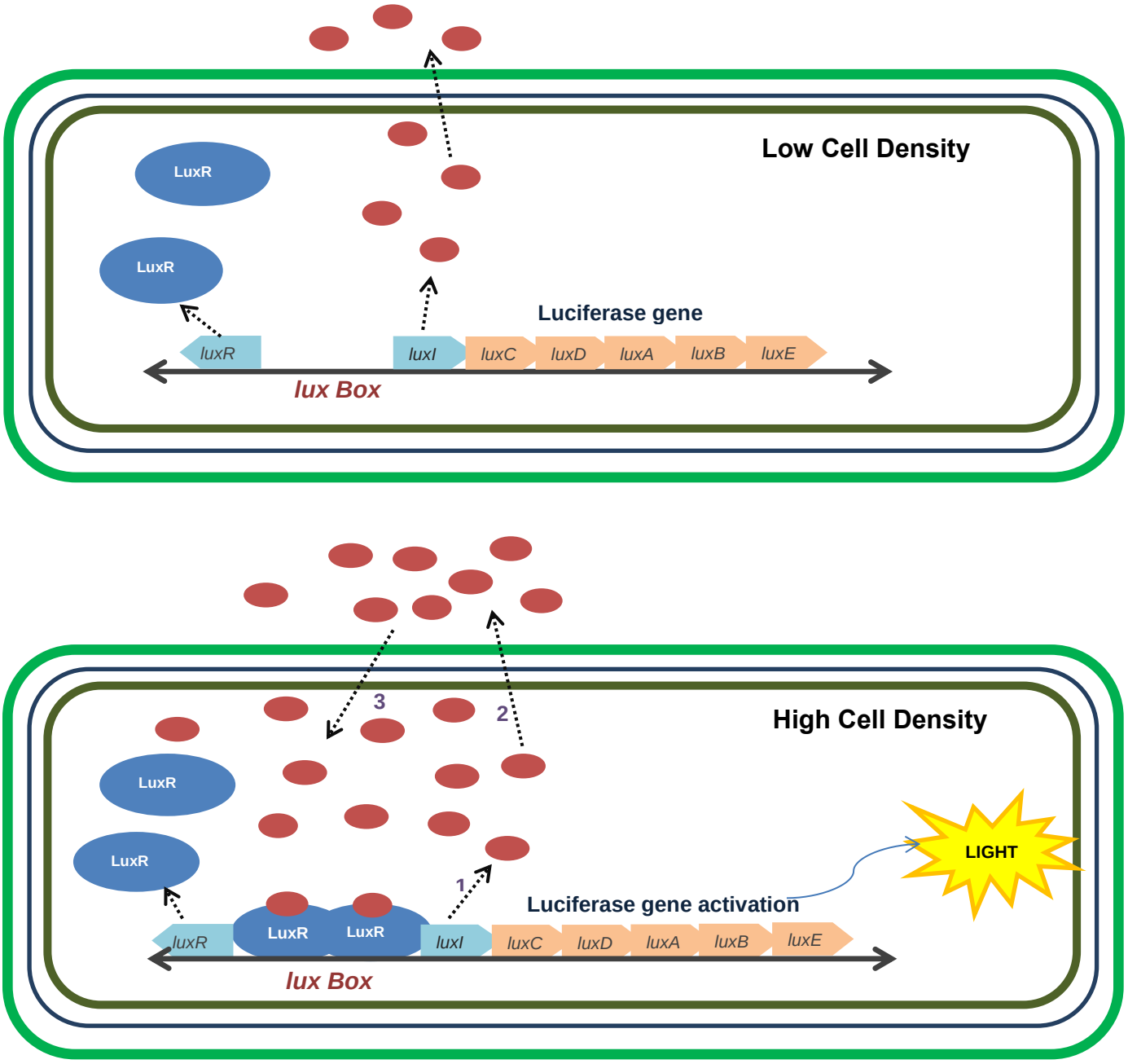


Figure 1.1: Quorum sensing in *Vibrio fischeri.* Bioluminescence production through the *lux* operon; at low cell densities the concentration of the QSM is low, and tends to freely diffuse across the cell membrane to the extracellular environment. However at high cell densities the QSM concentration increases, and accumulates beyond a threshold level (1-10μg/mL). The concentration is thus sufficient for the detection and binding of the QSM to the cytoplasmic response regulator. The response regulator-QSM binds to the Lux box and activates the transcription of *LuxICDABE* (Luciferase enzyme) resulting in light emission.

At low cell densities of *V. fischeri,* the QSM are produced by *LuxI* in low amounts (below critical threshold); thus they exhibit no evident physiological response (Engebrecht et al., 1983). However, as the local population of *V. fischeri* increases in the specialized light organ, the concentration of QSM proportionally increases beyond the threshold concentration (Eberhard et al., 1981). The QSM 3-oxo-C6-HSL then binds to the response regulator synthesised by *LuxR* (Hanzelka and Greenberg, 1995, Schaefer et al., 1996). Subsequently, the acyl-homoseriene lactone-response regulator dimer binds to the *Lux* box, activating *LuxICDABE* which in turn activates the production of additional QSM by autoinduction and light production/emission via quorum sensing (Engebrecht et al., 1983).

1.3 Quorum Sensing Systems

Quorum sensing mechanisms were later found to be prevalent in diverse bacterial species; utilizing three different quorum-sensing communication systems. The first system occurs exclusively in Gram-negative bacteria, the second exclusively in Gram-positive bacteria and the third is used by both Gram-negative and Gram-positive and is referred to as the universal QS system (Miller and Bassler, 2001). A range of physiological activities are regulated by these systems and include bioluminescence, toxin formation, biofilm formation, secretion of virulence factors, sporulation (conidiation), motility, conjugation, pigment production and secondary metabolites production (Miller and Bassler, 2001).

1.3.1 Quorum Sensing in Gram-negative Bacteria

Beside the well-studied QS system in the Gram-negative bacterium *V. fischeri*, it is currently well-established that the majority of Gram-negative bacteria share a similar mechanism. Quorum sensing in Gram-negative bacteria (Figure 1.2) is mediated by acylated homoseriene lactones (AHLs); that are catalysed by an enzyme belonging to the *LuxI* family. At high cell densities, the autoinducer AHLs bind to cytoplasmic DNA-binding response regulatory proteins belonging to the *LuxR* family (Engebrecht et al., 1983). The *LuxR* regulatory protein-AHL complex then recognizes and binds specifically to a quorum sensing regulated promoter; thus activating the transcription

of target genes (DNA-sequences) and induces a particular quorum sensing response (Fuqua et al., 2001). Examples of Gram-negative quorum sensing systems with their corresponding target functions are reported in Table 1.1.

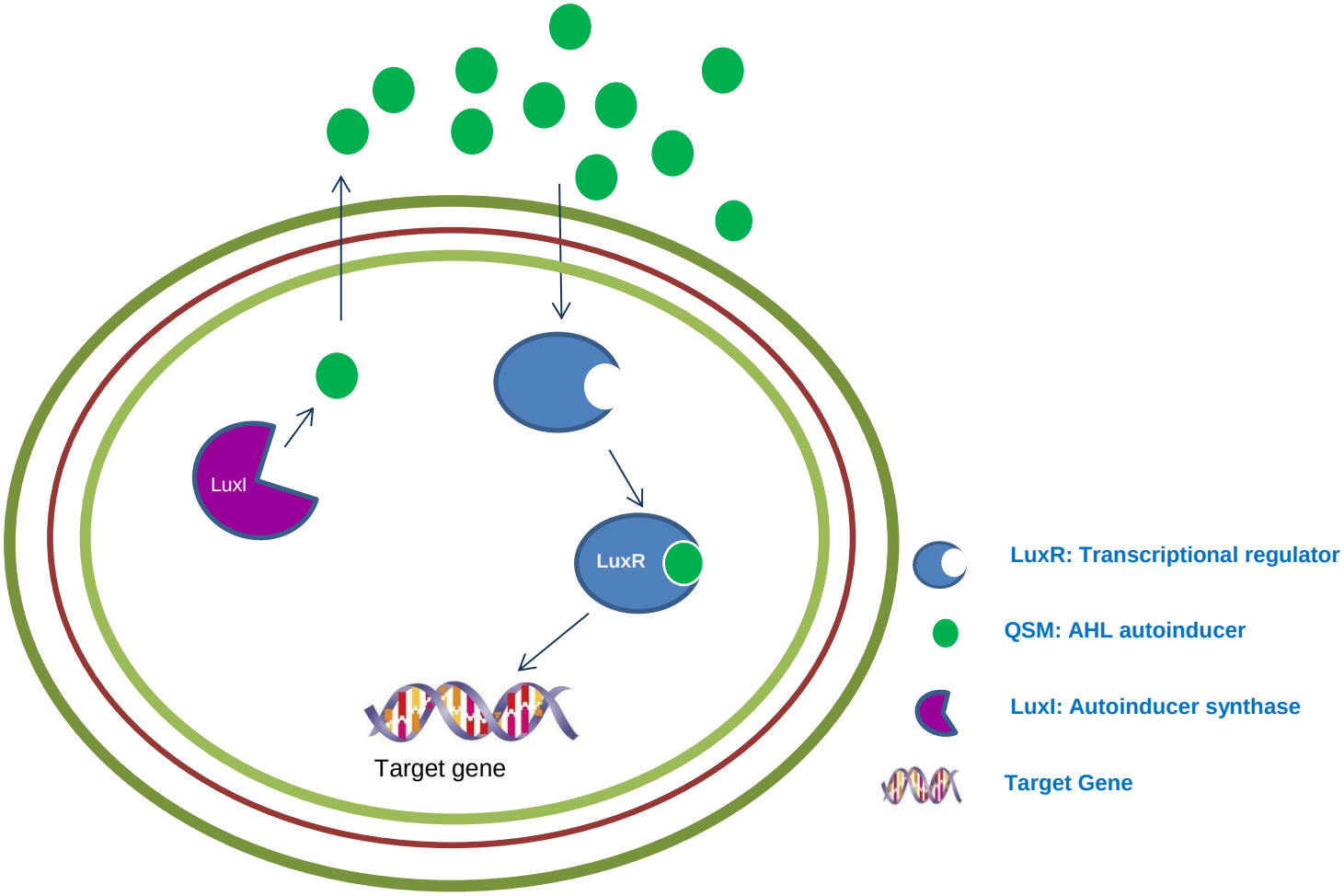


Figure 1.2: Quorum sensing in Gram-negative bacteria. The autoinducer synthase LuxI synthesizes the QSM (AHL); and releases it to extracellular environment. When the concentrations of AHL ≥ threshold; the AHL diffuses in and binds to LuxR. LuxR-AHL binds to QS regulated-genes to activate or repress them.

Table 1.1 Quorum sensing systems in Gram-negative bacteria. This table illustrates different Gram-negative bacteria with their corresponding AHL-based QS systems; in addition to the QS target gene(s) and/or function in the producing bacterial species.

Gram-negative species	QS System	AHL-based QSM	Target gene & function (Physiological Response)	Reference
Chromobacterium violaceum	CviI/CviR	N-hexanoyl-HSL	Violacein pigment, hydrogen cyanide, antibiotics, exoproteases and chitinolytic enzymes	(Chernin et al., 1998, McClean et al., 1997)
Pseudomonas aeruginosa	LasI/LasR	N-(3-oxododecanoyl)-HSL	*lasA, lasB, aprA, toxA* (Exoprotease, virulence factors, Biofilm formation)	(De Kievit and Iglewski, 2000, Davies et al., 1998, Pearson et al., 1994)
	RhlI/RhlR	N-butyryl-HSL	*lasB,rhlAB* (rhamnolipid), *rpoS* (stationary phase)	(Pearson et al., 1995, De Kievit and Iglewski, 2000, Latifi et al., 1996)
Agrobacterium tumefaciens	TraI/TraR	N-(3-oxooctanoyl)-HSL	*tra,trb* (Ti plasmid conjugal transfer)	(Zhang et al., 1993, Piper et al., 1993)
Aeromonas salmonicida	AsaI/AsaR	N-butanoyl-HSL	*AspA* (Exoprotease)	(Swift et al., 1999)

1.3.1.1 Acyl homoserine lactones

Acyl homoseriene lactones are produced by more than 50 different bacterial species. They differ from one another by the length and substituent of their acyl side chain (Figure 1. 3) (Fuqua and Eberhard, 1999). Each AHL corresponds to a particular LuxR-type protein; therefore allowing a high degree of selectivity and complexity. Some bacterial species have a single AHL synthase, producing one type of AHL, whereas others species such as *Pseudomonas aeruginosa* (as seen in Table 1.1) have multiple AHL synthases corresponding to more than one type of AHL (Pearson et al., 1995).

R_1	R_2
-H	$-C_nH_{2n}$
=O	$-C_nH_{2n+1}$
-OH	

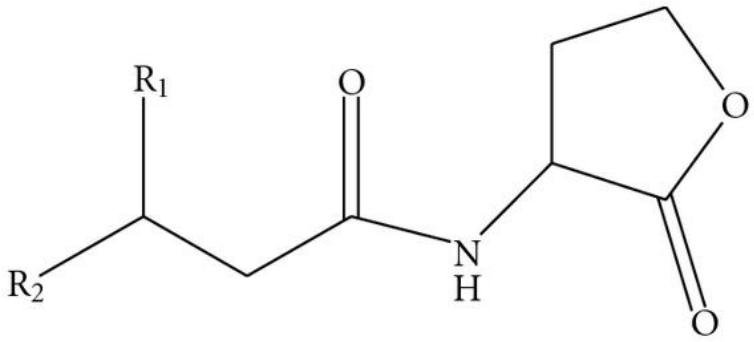


Figure 1.3: General Structure of the Acyl homoserine lactone (AHL). R_1 and R_2 refer to the different side chains that are connected to AHL. Image source (www.hindawi.com)

1.3.2 Quorum Sensing in Gram- positive bacteria

Gram-positive bacteria also utilize quorum sensing for gene regulation. QS in Gram-positive bacteria is mediated by processed oligopeptide autoinducers via a two-component signal transduction mechanism that consists of a histidine kinase and a response regulator protein that triggers signal transduction via phosphorylation and de-phosphorylation cascade (Figure 1.4). Translationally derived oligopeptides (auto-inducing peptides, AIPs) serve as the QSM.

AIP are small, precursor peptide synthesized in the cytoplasm by the ribosomes in the form of post-translationally modified (Class I) or un-modified (Class II) oligopeptides. Mature oligopeptides are released into the extracellular milieu via an ATP-binding cassette exporter protein (ABC transporters). AIPs are detected by the two-component signal transduction system. The sensor kinase auto-phosphorylates the histidine portion; the phosphate is then transferred to an aspartate residue located on the response regulatory protein which in turn activates the transcription genes that are under quorum-sensing control (Demain, 2010, Waters and Bassler, 2005). Gram-positive QS systems include virulence in *Staphylococcus aureus*, competence in *Streptococcus pneumoniae*, and competence and sporulation in *Bacillus subtilis.*

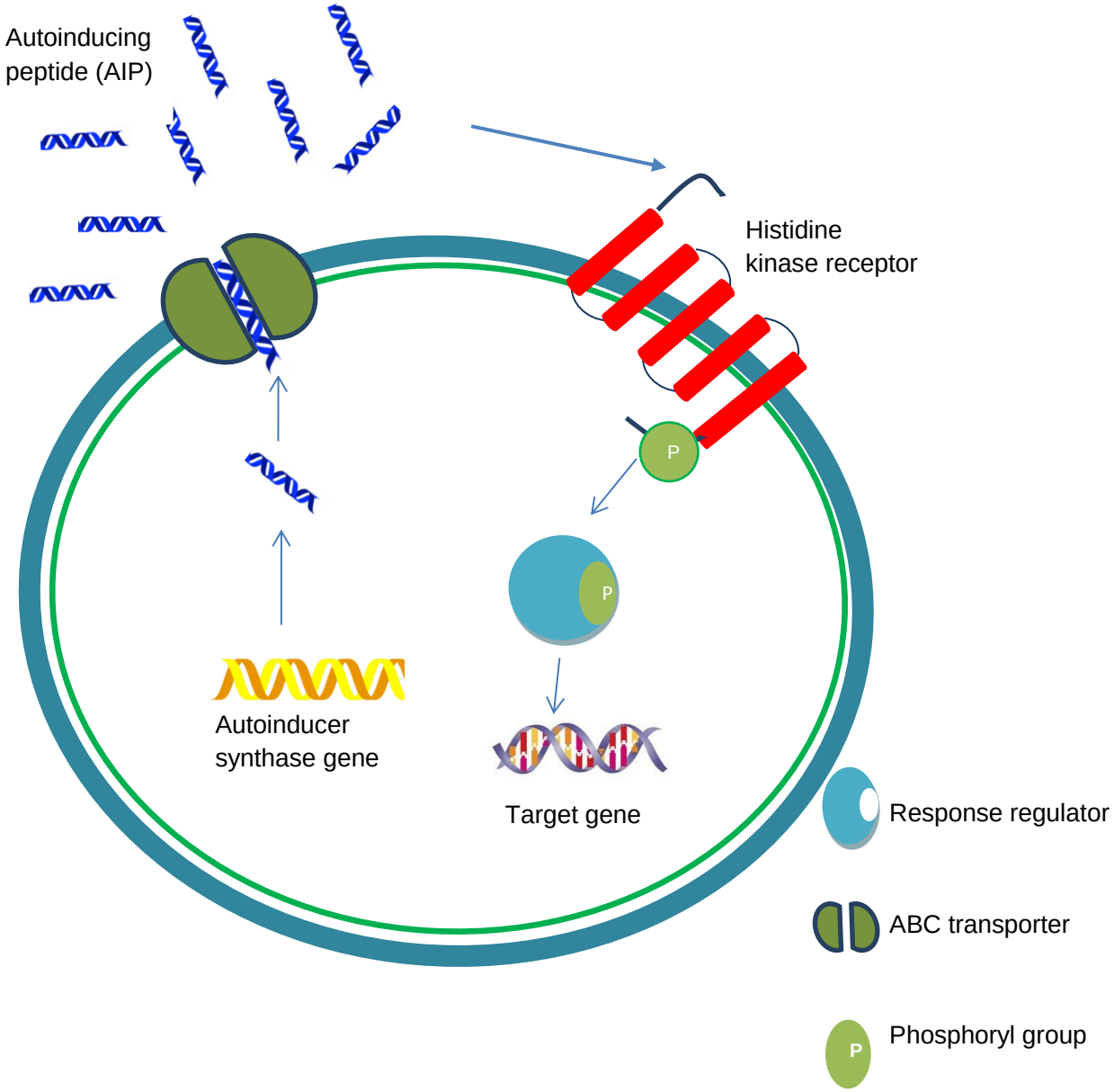


Figure 1.4: Quorum Sensing in Gram-positive bacteria. The autoinducer synthase gene produces autoinducing peptides (AIP) in cytoplasm and releases them to extracellular environment. AIPs are then recognised by histidine kinase receptor that will phosphorylates a response regulator and then activate/repress the target QS-regulated gene.

In the Gram-positive bacteria, *Streptococcus pneumonia,* the ComD/ComE competence system is utilized to regulate competence for the uptake of exogenous DNA molecules. This system utilizes a 17-amino acid peptide known competence stimulating peptide (CSP). CSP is produced from a 41-amino acid precursor peptide ComC. CSP is processed in the cytoplasm and secreted to the extracellular environment via a ComAB, an ABC transporter. At high cell densities the concentration of CSP increases and accumulates. The accumulated CSP is then detected by a two component sensor-kinase protein ComD, and induces the auto-phosphorylation of ComD. The phosphoryl group is then transferred to the response regulator ComE. The response regulator-phosphoryl group complex (Phospho-ComE) then activates the competence target genes ComX (Pestova et al., 1996).

Other examples of QS include the ones in filamentous Gram-positive bacteria, *Streptomyces* species. The QSMs produced by *Streptomyces* spp. are γ-butyrolactone auto-regulators. QS in *Streptomyces* spp. is used to regulate antibiotics production, sporulation and morphological differentiation (Horinouchi and Beppu, 1994).

1.3.3 Universal Quorum Sensing systems

The third quorum sensing system is universal; and is widely spread among both Gram-negative and Gram-positive bacteria (Surette et al., 1999). This system is referred to as LuxS or Autoinducer-2 (AI-2); LuxS is a highly conserved enzyme that catalyses the synthesis of AI-2 (Surette et al., 1999, Bassler et al., 1993). This system allows interspecies cell-cell communication; where bacteria can detect and respond to various autoinducers secreted by other species (Bassler, 1999). The universal QS system was originally discovered in the luminous marine bacterium *Vibrio harveyi* (Bassler et al., 1993).

V. harveyi have two quorum sensing systems: AI-1 and AI-2. AI-1(Autoinducer-1) is used for intraspecies cell-cell communication, and functions in a way similar to the *LuxIR* bioluminescence system in *V. fischeri* (Miller and Bassler, 2001), whereas the

second system AI-2, is utilized for interspecies communication. The presence of two quorum sensing systems provides *V. harveyi* with the advantage to monitor its own cell-population density in addition to the population density of other bacteria located in the surrounding local environment. In addition to *V. harveyi,* other bacterial species that utilize AI-2/LuxS system include *Streptococcus pyogenes, Klebsiella pneumonia, Enterococcus faecalis, Neisseria meningitides, Salmonella typhi, Escherichia coli,* and *Bacillus subtilis* (Surette et al., 1999, Bassler, 1999).

An example of interspecies quorum sensing system is the AI-2–Lsr system of *V. harveyi* and *E. coli*. AI-2 can be produced by both *V. harveyi* and *E. coli.* AI-2 production regulates light production and trigger *lsr* induction in *V. harveyi* and *E. coli* respectively. The induction of *lsr* genes in *E. coli* results in the consumption of AI-2 transporter which inhibits bioluminescence by *V. harveyi. E. coli* interferes with AI-2-mediated communication by blocking the QS-regulated extracellular toxin production in *V. harveyi* (Xavier and Bassler, 2005).

1.4 Quorum sensing in fungi

Quorum sensing was later found to be prevalent also in diverse fungal species. Fungi have two growth forms: unicellular (yeast) and filamentous. However some fungi exhibit a feature known as fungal dimorphism or polymorphism which is identified as an environmental inter-conversion between the yeast and mycelia morphologies (Romano, 1966).

For a compound to be classified as a signalling molecule, or QSM in bacteria as well as in fungi, it has to fulfil five criteria as summarized by Albuquerque and Casadevall (2012). The molecule should “ (1) accumulate in the extracellular environment during microbial growth; (2) accumulate in a concentration that is proportional to the population cell density with its effects restricted to a specific stage of growth; (3) induce a coordinated response in the entire population that is not simply an adaptation meant to metabolize or detoxify the molecule itself after a threshold

concentration is reached; (4) reproduce the QS phenotype when added to the culture exogenously; (5) not be solely a by-product of microbial catabolism."

1.4.1 Quorum sensing in unicellular and dimorphic fungi

Extensive studies of cell-density dependent regulation in the polymorphic fungi, originated in the opportunistic human pathogen *Candida albicans* (Hogan, 2006). *C. albicans* is part of the normal microbial flora of the mucosa and skin in humans; however it contributes to nosocomial infections such as bloodstream infections, urinary infections as well as mucosal infections of the oral cavity and vagina in immunocompromised patients (Greguš et al., 2010).

C. albicans undergo transition between a budding yeast, hyphae and pseudo-hyphae forms. The morphological switch between its various growth forms is the leading cause of its virulence and pathogenicity (Saville et al., 2003, Lo et al., 1997).

At low cell densities ($<10^6$ cells/mL), *C. albicans* develop into its filamentous form, that is required for tissue invasion and biofilm formation, whereas at high cell densities ($\geq 10^6$ cells/mL) it switches to budding yeasts (Figure 1.5). This alternating behaviour is under the direct control of two QS molecules: farnesol and tyrosol (Hornby et al., 2001).

Farnesol inhibits yeast-to-mycelium conversion through the inhibition of three germ-tube formation inducers: L-proline, N-acetylglucosamine and serum (Hornby et al., 2001). Even though farnesol blocks germ tube formation, yet it does not prevent the elongation of pre-existing Hyphae (Mosel et al., 2005). In addition to morphological regulation, farnesol induces the up-regulation of proteins required for the protection against oxidative stress (Shirtliff et al., 2009).

On the other hand, tyrosol, the second QS molecule in *C. albicans*, reduces the time required for the lag phase and induces filamentation and biofilm formation. Tyrosol is inactive in the presence of farnesol, thus indicating a superior role for farnesol (Chen et al., 2004, Alem et al., 2006).

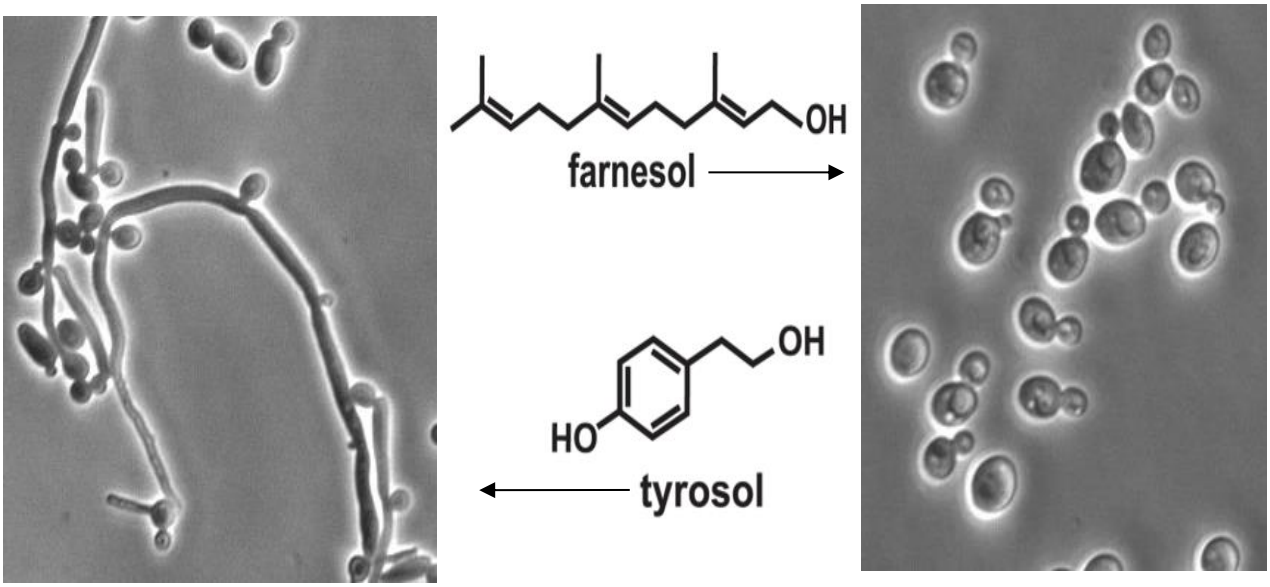


Figure 1.5: Quorum sensing in *Candida albicans*. Impact of the two QSM (farnesol and tyrosol) on the inter-conversion of *C. albicans* between its two growth forms (yeast and hyphae form) (Image source: www.vetnext.com).

Another example of fungal QS is in the budding yeast, *Sacchromyces cerevisiae*; where the two aromatic alcohols: phenylethanol and tryptophol act as QS molecules to stimulate morphogenesis and pseudohyphal growth formation The QSM(s) biosynthesis pathway is activated under nitrogen-poor conditions, whereas it is repressed under nitrogen rich conditions due to high levels of ammonia (Chen and Fink, 2006).

In addition to the quorum sensing mechanisms in *C. albicans* and *S. cerevisiae,* other putative QS mechanisms were previously reported in the yeast-to mycelial transition in the parasitic fungus *Histoplasma capsulatum* (Kügler et al., 2000, Klimpel and Goldman, 1988), *Ceratocystis ulmi* (Hornby et al., 2004),*Cryptococcus neoformas* (Lee et al., 2007) and the circadian rhythm clock of conidiation by *Neurospora crassa* (Roca et al., 2005).

1.4.2 Quorum sensing in filamentous fungi

1.4.2.1 Filamentous fungi

Filamentous fungi are eukaryotic microorganisms characterized by their ability to grow in mycelia form, producing hyphae (Prosser and Tough, 1991). Filamentous fungi can reproduce sexually and asexually. In some cases they are homothallic, i.e. reproduce both sexually and asexually like the model filamentous fungus *A. nidulans* (Pyrzak et al., 2008). Even though research on filamentous fungi is quite limited as compared to that of unicellular yeast; they are of extreme importance both industrially and medically. Filamentous fungi can be used to produce a range of medically important compounds e.g. human therapeutics (antifungal, antibacterial, statins) or may be industrially exploited for the production of specialty chemicals (organic acids, enzymes) (Demain et al., 2005, Goldman and Osmani, 2007, Sauer et al., 2008).

1.4.2.2 Quorum Sensing

Investigation of QS in filamentous fungi initiated and expanded over the past few years. Recently published studies have reported the presence of QS in various filamentous fungi including *A. nidulans, A. terreus,* and *P. chrysogenum, A. flavus* and *P. sclerotiorum* (Calvo et al., 2002, Schimmel et al., 1998, Sorrentino et al., 2010, Tsitsigiannis and Keller, 2007, Brown et al., 2008).

QS in filamentous fungi is rather challenging due to the nature of those microorganisms. However there are several putative quorum sensing molecules or signalling-like molecules that were reported to induce physiological changes in fungi including morphological changes (hyphal branching), sporulation, and secondary metabolite production (mycotoxins, antibiotics). Lipid-based molecules such as oxylipins and butyrolactones are the major reported signalling molecules in filamentous fungi (Tsitsigiannis and Keller, 2007, Schimmel et al., 1998).

1.5 Signalling molecules

1.5.1 Oxylipins

Oxylipins are a large family of structurally related and biologically active polyenoic fatty acids. They are generated by the oxidative metabolism of polyunsaturated fatty acids (PUFAs) such as linoleic acid, linolenic acid and oleic acid (Tsitsigiannis and Keller, 2007). Oxylipins are secondary metabolites that might act as signalling compounds leading to intra- and inter- cellular communication among the various living organisms including animals (mammals), plants, prokaryotes (bacteria) and fungi (Herman, 1998, Noverr et al., 2003)

Oxylipins are involved in the regulation of developmental processes as well as environmental responses. The occurrence, physiological role and the biosynthesis mechanisms of both mammalian and plant oxylipins is well characterized as opposed to that of fungal oxylipins (Brodhun and Feussner, 2011).

Oxylipins have various forms and functions; in mammals they are known as eicosanoids; and they include leukotrienes, prostaglandins, thromboxanes. Eicosanoids regulate inflammatory processes and other homeostatic responses (Funk, 2001). In plants, oxylipins are in the form of volatile aldehydes and jasmonates which regulate the plants defence mechanism against pathogen attacks, stress, physical damage by pests, plant cell death. Oxylipins also exhibit antimicrobial effects and contribute to the formation of phytohormones (Blée, 2002).

In prokaryotes, lipogenic diffusible molecules such as acyl homoserine lactones regulate a variety of the QS responses such as virulence, bioluminescence and biofilm formation (Bassler and Miller, 2013) whereas diols and lactones, in fungi, contribute to the major regulation and signalling mechanisms (Tsitsigiannis and Keller, 2007).

The first signalling mechanism involving fungal oxylipins was discovered in the model filamentous fungus *A. nidulans.* In *A. nidulans* the *Ppo* genes code for three dioxygenase enzymes: *PpoA, PpoB,* and *PpoC* that produces oxylipins (Figure 1.6) (Champe and El-Zayat, 1989, Tsitsigiannis et al., 2004a, Tsitsigiannis et al., 2004b).

Studies involving gene deletion showed that the deletion of one or more *Ppo* genes influence several physiological activities; including the production of the secondary metabolites: sterigmatocystin (a carcinogen mycotoxin) and penicillin (antibiotic), while the loss of all *Ppo* genes shifts the sporulation from asexual to sexual (Tsitsigiannis et al., 2004b, Tsitsigiannis et al., 2004a).

Another signalling mechanism was discovered in *A. terreus*. The addition of the linoleic acid as an oxylipin precursor to *A. terreus* cultures enhanced the production of the secondary metabolite lovastatin, decreased sporulation rate, and increased the transcriptional rate of lovastatin biosynthetic genes (*lovB* and l*ovF*). It also increased the transcriptional rate of putative oxylipin biosynthetic genes, dioxygenase enzymes (Sorrentino, 2009).

Oxylipins can also be involved in inter-organism signalling, such as in the plant-pathogen *Aspergillus ochraceus* where the deletion of the homolog of a *lox* allele demonstrated that lipoxygenase-derived oxylipins are involved in the formation of the mycotoxin ochratoxin as well as other developmental changes (Reverberi et al., 2010).

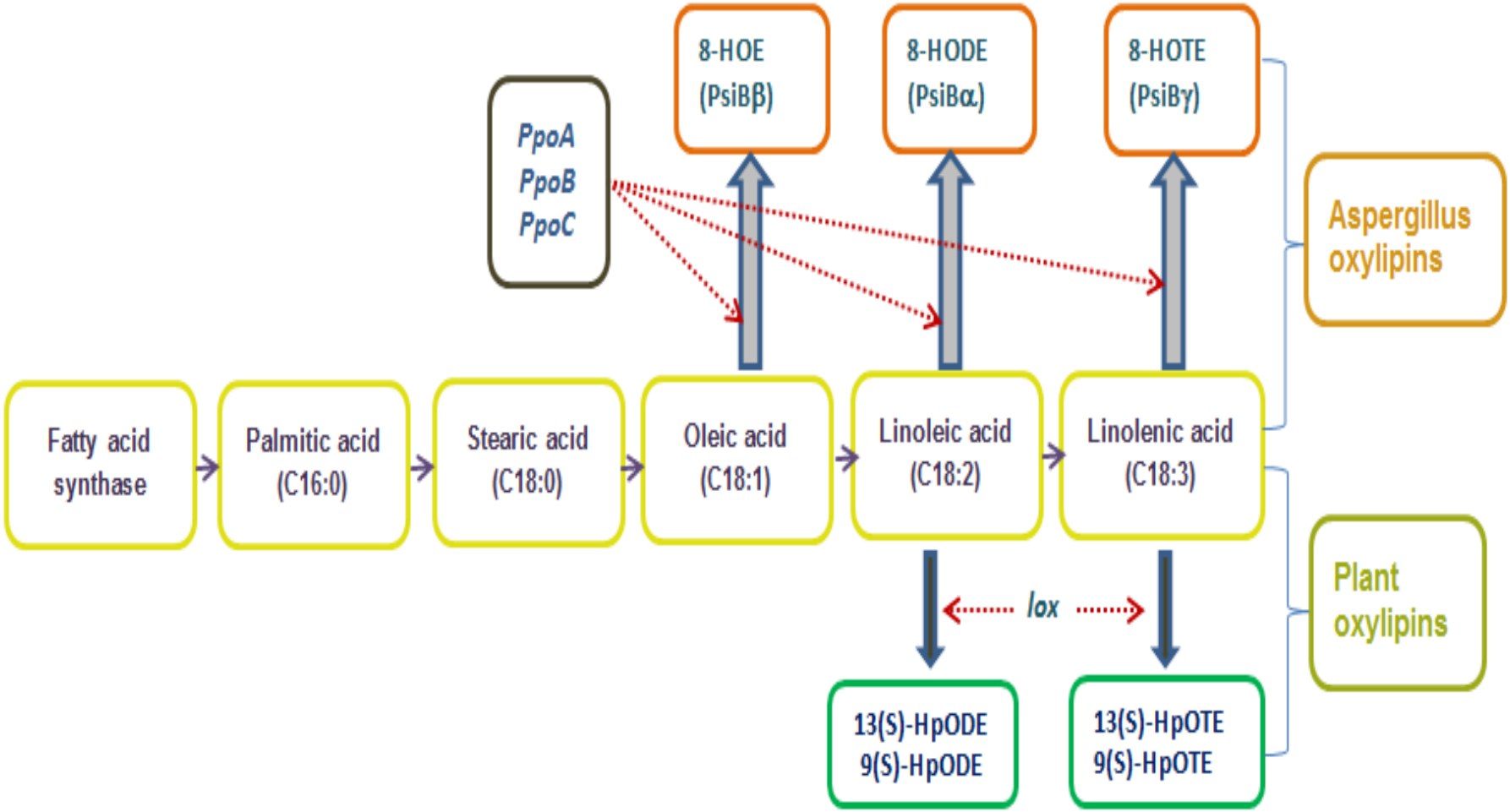


Figure 1.6: The basic pathway of fatty acid metabolism in *Aspergillus* spp. and plants. Fatty acids produce oxylipins through lox genes in plants and PpoA/B/C genes in *Aspergillus* spp. Abbreviations: Genes *lox* (Lipoxygenase), *PpoA, PpoB, PpoC:* psi producing oxygenases, *Aspergillus* oxylipins: 8-HODE (8-hydroxylinoleic acid); 8-HOE (8-hydroxyoleic acid), 8-HOTE (8-hydroxylinolenic acid); 9S-HpODE (9S-hydroperoxylinoleic acid) and 13S-HpODE (13S hydroperoxy linoleic acid), 9S-HpODE (9S-hydroperoxy linolenic acid), 13S- HpOTE (13S- hydroperoxy linolenic acid). Adapted from Tsitsigiannis and Keller (2007).

1.5.2 Butyrolactones

In addition to oxylipins, other lipid moieties such as butyrolactones are also involved in QS signalling mechanisms in filamentous fungi.

As previously reported, QS in Gram-negative bacteria is mediated by acylated homoserine lactones. Besides AHLs, other γ-butyrolactone-containing compounds act as signalling molecules in the filamentous bacteria *Streptomyces* spp. to regulate cellular differentiation and secondary metabolite production. They are also involved in the control of aerial mycelium, virulence factor production, biofilm and spore formation, bioluminescence, and conjugal transfer (Takano, 2006).

γ-butyrolactones in *Streptomyces* spp. are divided into three groups based on their fatty acid side chain substitution and their stereochemistry. Group I includes the virginae butanolide type; 6-α-hydroxy group from *Streptomyces virginiae*. Group II includes IM-2 type from *Streptomyces lavendulae* FRI-5; 6-β-hydroxy group, and Group III includes the A-factor type: 6-Keto-group from *Streptomyces griseus* (Takano et al., 2000, Kawabuchi et al., 1997, Kondo et al., 1989).

Because of their role as self-regulating factors in filamentous bacteria, extensive studies have been conducted to detect the presence of γ-butyrolactone-containing molecules in filamentous fungi, and to investigate their putative roles as QSM.

γ-butyrolactone -containing QS molecules (Figure 1.7) were identified in several filamentous fungi including butyrolactone I in *A. terreus*, multicolanic acid and its derivatives in *P. sclerotiorum*, and γ-heptalactone in *A. nidulans* (Raina et al., 2010, Schimmel et al., 1998, Williams et al., 2012).

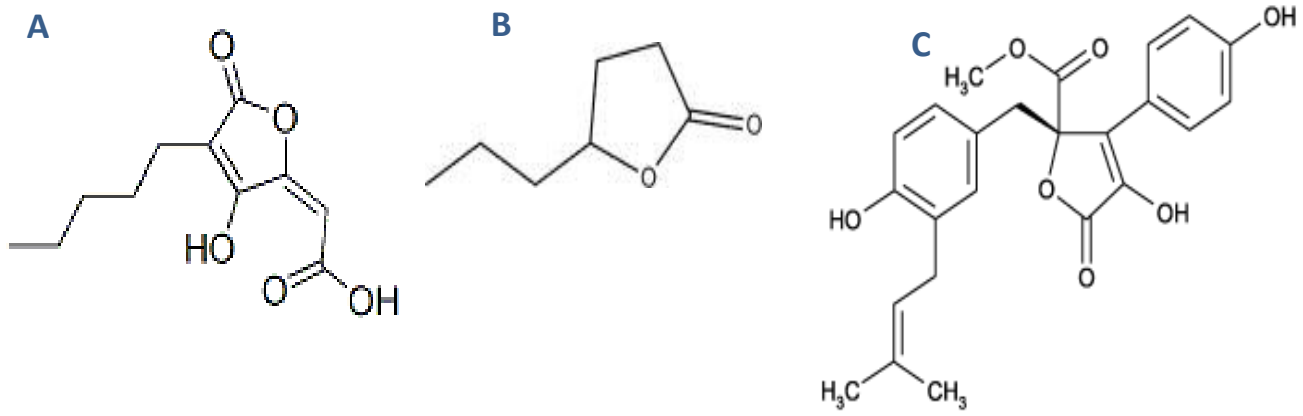


Figure 1.7: Structures of butyrolactone-containing signalling molecules. A) Multicolanic acid, B) γ-heptalactone, and C) butyrolactone I. (Image sources: www.guidechem.com, www.enzolifesciences.com, www.sciencedirect.com).

Butyrolactone I, a secondary metabolite produced by *A. terreus,* acts as a signalling molecule to enhance the production of the secondary metabolite lovastatin and to increase hyphal branching and submerged sporulation (Schimmel et al., 1998). Butyrolactone I was also found to have an auto-stimulatory function as well as induction of lovastatin biosynthetic genes (Raina et al., 2012).

Another γ-butyrolactone-containing molecule, γ-heptalactone, was recently purified and identified as a QSM in the filamentous fungus *A. nidulans.* γ-heptalactone alters the fungal growth profile by shortening the lag phase, and induces the production of the secondary metabolite penicillin (Williams et al., 2012).

1.5.3 Other quorum sensing signalling molecules in filamentous fungi

Besides oxylipins and butyrolactones other molecules purified from fungi demonstrated a QS effect in the producing filamentous fungi. Examples include the small peptide 1,3-diaminopropane produced by the fungus *P. chrysogenum* which significantly increases penicillin production as well as induces the expression of three genes involved in penicillin biosynthesis (Martín et al., 2011).

1.6 Fungal secondary metabolism, sporulation and hyphal branching

1.6.1 Fungal secondary metabolism

Filamentous fungi are considered to be exceptional microorganisms as they are a prolific source of secondary metabolites that result from unusual and unique biochemical pathways within the fungal kingdom. Secondary metabolites are bioactive, low molecular weight compounds produced during a restricted phase of the microbial life cycle; late exponential phase and stationary phase and they are accompanied with morphological differentiation of the growing fungi (Keller et al., 2005).

Studies on fungal secondary metabolites initiated in 1922 by Harold Raistrick, however it expanded later upon the discovery of the antibiotic penicillin from *P. notatum* by Alexander Fleming in 1928 (Fleming, 1944). Research on fungal secondary metabolism carried on over the years contributed to the remarkable discovery of thousands of molecules with diverse range of chemical structures and biological functions including bioactive compounds that inhibit the growth of bacteria, fungi, protozoa, parasites, insects, viruses and human tumour cells as well as those that are cytotoxic, mutagenic, carcinogenic, teratogenic, immunosuppressive, enzyme inhibitory, and allelopathic (Keller et al., 2005).

Fungal secondary metabolites are produced as families of related compounds and are classified into several classes including polyketides (e.g. aflatoxins, azaphilones), non-ribosomal peptides (e.g. sirodesmin, peramine and siderophores), terpenes (e.g. T-2 toxin, deoxynivalenol), and indole alkaloids(e.g. paxilline and lolitrems) (Keller et al., 2005, Yu and Keller, 2005).

1.6.2 Filamentous fungi sporulation

Filamentous fungi are characterised by both asexual and sexual reproduction, with asexual reproduction being the major reproduction form. Sporulation is the main type of asexual reproduction, and it is used by a wide range of medically, industrially and agriculturally important fungal species. Asexual sporulation is characterised by the

production and release of resistant structures known as spores. Sporulation occurs during the mitosporic phase (also known as anamorphic phase) of the fungal life cycle. The onset of asexual reproduction is controlled by various factors such as nutrient levels, CO_2 and light levels as well as the addition of external chemicals including quorum sensing molecules. Upon sporulation, spores will spread, colonise new surfaces and initiate germination. Spore germination leads to the formation of tubular hyphae, which would further undergo apical extension and branching (apical and lateral) to form a network of interconnected cells known as mycelium (Adams et al. 1998).

1.6.3 Hyphal Branching

The two main types of hyphal Branching are apical and lateral branching. Apical branching refers to the emergence of branches from the hyphal tips, however lateral branching is the emergence of branches from lateral sides of the hyphae, different than the hyphal tip. Hyphal branching is of major importance as it increases the surface area of the mycelia colony; as a result it improves the colonies access to nutrients. Other important role of hyphal branching is that they serve as a site of nutrients and signals exchange between the fungal hyphae within the same environment and the outside environment (Harris et al.2008).

1.7 The Genus *Penicillium*

The genus *Penicillium* comprises a large group of over 300 mould-forming ascomycetous fungal species, characterized worldwide. Scientific studies of the genus *Penicillium* were originally conducted in 1809 by the German naturalist and botanist Johann Heinrich Friedrich Link (1767-1850). Link reported in his scientific work *Observations in ordines plantarum naturals* the distinctive brush-like, asexual fruiting structure (conidiophore) of this fungus (Espinel-Ingroff, 2003, Pitt and Hocking, 1997, Pitt and Hocking, 1985).

The genus name was derived from the similarity between the microscopic features of the conidiophore (asexual fruiting structure) that resembles a broom and a

penicillium, which is the latin name for painters brush (Haubrich, 2003). Species belonging to the genus *Penicillium* of this species are of high industrial, nutritional, agricultural and medical importance. For instance *Penicillium* moulds such as *P. camemberti, P. roqueforti* are the moulds on the cheeses including camemberti cheese, roquefort and brie, while *P. naligovense* plays a key role in improving the taste of sausages and ham as well as prevention of bacterial and other moulds colonization. *P. digitatum is the major cause of green mould* post-harvest diseases in citrus fruits (Holmes and Eckert, 1995). *P. chrysogenum* widely produces, at industrial level, useful β-lactam antibiotics like penicillins and cephalosporins (Bruggink and Roy, 2001). At the same time some *Penicillium* spp. such as *P. citrinum* produces the mycotoxins: ochratoxin and citrinin (Calvo et al., 2002, Davis et al., 1975).

1.7.1 *Penicillium sclerotiorum*

P. sclerotiorum (syn*. P. multicolour*) is a saprophytic filamentous fungus; originally isolated in 1935 from the air in Java, Indonesia by Professor Boedijn and; later described in 1937 by Van Beyma (Curtin and Reilly, 1940). *P. sclerotiorum* has monoverticillate, vesiculate conidiophores and at optimum temperatures, it produces colonies of orange to red colours which obscure the blue-green conidia (Figure 1.8) (Rivera and Seifert, 2011, Curtin and Reilly, 1940).

P. sclerotiorum derives its name from the orange sclerotia produced by some strains. The saprophytic fungus *P. sclerotiorum* is mainly isolated from soil and rarely from textile, in-house dust (Vesper et al., 2005), endophyte of coffee Arabica berries (Vega et al., 2006) and diseased grape fruits and stems (De Lucca et al., 2008).

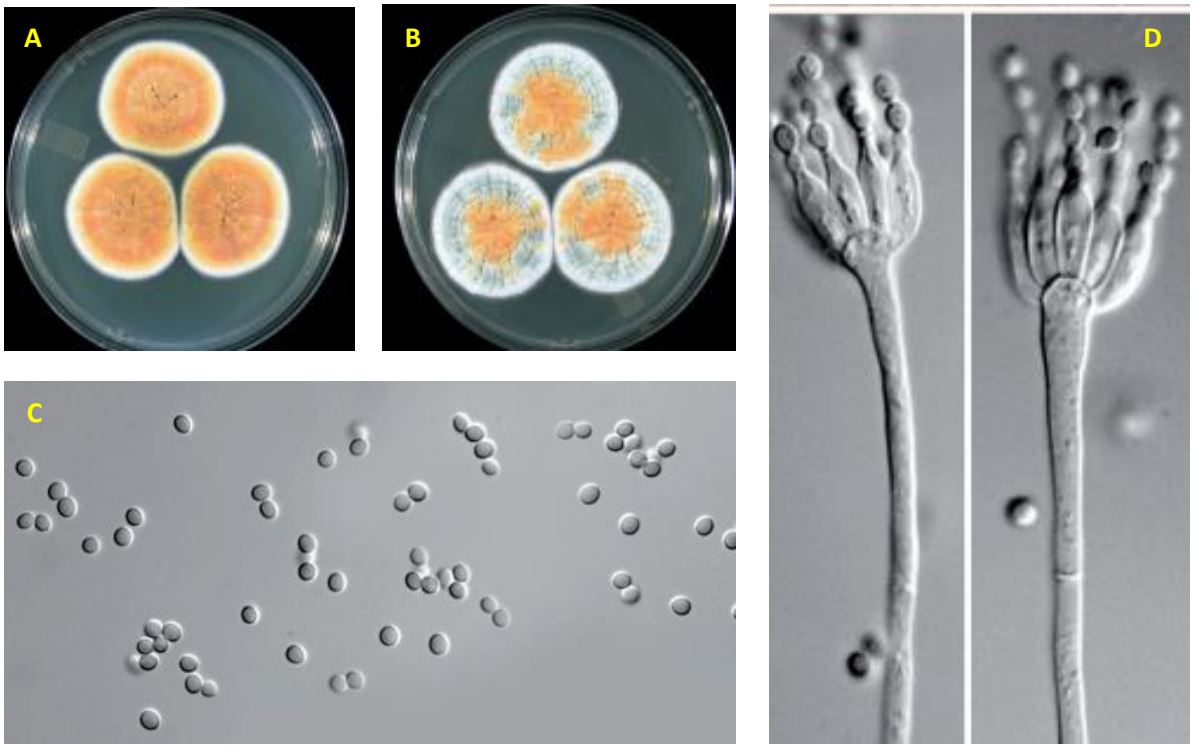


Figure 1.8: *P. sclerotiorum* culture. A& B) Colonies of *P. sclerotiorum* growing on different media, C) *P. sclerotiorum* spores D) *P. sclerotiorum* conidiophores (Rivera and Seifert, 2011).

P. sclerotiorum produces several commercially important secondary metabolites such as pencolide, rotiorin, azaphilones including sclerotiorin and isochromophilone IV (Lucas et al., 2007), extracellular xylanases (Knob and Carmona, 2010), and carotenes (Mase et al., 1957).

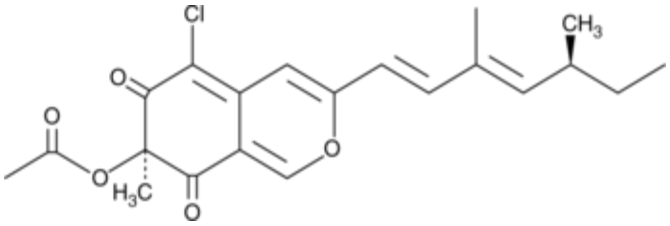
1.7.2 Sclerotiorin

Sclerotiorin is an azaphilone, chlorine-containing fungal secondary metabolite. Azaphilones are generally considered to be a relatively small subset of the polyketide class of compounds. They are structurally diverse pigments, mainly characterized by a highly oxygenated bicyclic core and a chiral quaternary centre which breaks the aromaticity of the ring structure (Turner, 1971, Gill M, 1987, Osmanova et al., 2010).

Over 170 different azaphilones have been identified so far, with the majority originally isolated as secondary metabolites of fungal species (Lin et al., 2012a).

Sclerotiorin is a yellow crystalline solid composed of nine-carbon aliphatic diene side chain, a bicyclic core and a chiral quaternary centre. Its structure and some of its reported characteristics are listed in Table 1.2.

Table 1.2: Sclerotiorin structure and some of its characteristics

	Appearance: yellow crystalline solid Molecular weight: 390 Melting point: 205-207°C Solubility: Highly soluble in chloroform, Ethyl acetate, diethyl ether, acetone, Ethanol and Methanol

(Image source: www.CaymanChemicals.com)

Sclerotiorin was originally isolated in 1940 by Curtin and Reilly from *P. sclerotiorum* as a halo-metabolite with the molecular formula of $C_{20}H_{20}O_5Cl$ (Curtin and Reilly, 1940). A new empirical formula was later assigned to sclerotiorin when it was re-isolated in 1952 from the filamentous fungus *P. multicolour* (Birkinshaw, 1952). The structure of sclerotiorin was first established in 1959, however its absolute configuration and stereochemistry were later determined in 1976 using x-ray crystallography (Dean et al., 1959, Whalley, 1976). In 1987 sclerotiorin was found to be a bioactive compound, as it was found to induce chlamydospore-like cells in the fungal plant pathogen *Chliobolus lunatus* (Natsume et al., 1988).

Similar to other polyketides, the biosynthetic production of sclerotiorin occurs via iterative type I polyketide synthase (PKSs). PKSs are multi-domain proteins with similar domain structures to the eukaryotic fatty acid synthases. These enzymes repetitively use one set of domains to assemble polyketides such as sclerotiorin

(Campbell and Vederas, 2010). PKSs operate via the acetate–malonate pathway, where it proceeds from the condensation of terminal two-carbon unit of acetate origin (acetyl-co-enzyme A) with chain-building blocks derived from malonate (malonyl-co-enzyme A) that are partially de-carboxylated to acetate upon their introduction to the terminal unit (Holker et al., 1964).

Sclerotiorin was originally synthesised, chemically, by Chong et al. (1969). Recently research started focusing on finding new methods and pathways for the chemical synthesis of sclerotiorin and its racemic azaphilones. This is mainly aimed at finding new analogues of sclerotiorin with improved biological activity (Lin et al., 2012b). Germain et al. (2011) reported its synthesis via enantio-selective syntheses methods using copper-mediated asymmetric de-aromatization employing bis-μ-oxo copper complexes prepared from readily available (þ)-sparteine surrogates. Lin et al. (2012a) also synthesised sclerotiorin, chemically, using two key steps: cycloisomerization and oxidation, followed by Sonogashira cross-coupling reaction to construct the required functionalized precursor.

Sclerotiorin has a wide range of biological activities; of pharmaceutical and industrial importance. In addition to its antibacterial, antifungal (Lucas et al., 2007, Chidananda et al., 2006), and anticancer activities (Giridharan et al., 2012), sclerotiorin also acts as an endothelin receptor binding molecule (Pairet, 1995), as well as it inhibits Grb-2-Sch interaction (Nam et al., 2000, Nam et al., 1998), gp120-CD4 binding (Matsuzaki et al., 1995), cholesteryl ester transfer protein (Tall, 1993, Barter and Rye, 1994, Tomoda et al., 1999) and the activity of lipase (Lucas et al., 2007), aldose reductase (Kador, 2006), soybean lipoxygenase (LOX-I) (Chidananda and Sattur, 2007, Chidananda, 2008), and monamine oxidase (Fujimoto et al., 1990).

1.8 The genus *Aspergillus*

The genus *Aspergillus* is one of the oldest fungal genera; with a worldwide distribution of over 250 species, characterized to date (Geiser et al., 2007). The name *Aspergillus* was originally given by the Italian priest and biologist, Pier Antonio Micheli in in 1729. The naming was a result of the high similarities between its spore-bearing structures, conidiophores, to the device aspergillum, a holy water sprinkler used by the Roman Catholic clergy during *asperges*, a part of the Christian liturgy (Ainsworth, 1976).

Different species of the genus *Aspergillus* were originally exploited for food production, as it was used for the production of traditional fermented foods such assake (rice wine), miso (soybean paste), and shoyu (soy sauce) for more than 1,000 years in Japan and other Far-East countries (Machida and Gomi, 2010, Bennett and Arnold, 2001).

Aspergillus species are characterised with an extreme ecological and metabolic diversity. The majority of *Aspergillus* species are saprophytic, living in soil, or present on stored food or in decaying vegetation. However members of this genus can also be pathogenic causing invasive diseases in plants, and animals (insects, poultry and mammals) as well as cause decay and deterioration when growing as food contaminates of cereals or oil seeds causing diseases in plants, and animals: insects, poultry and mammals (Bennett, 2010).

Aspergillus species secrete a variety of biologically active secondary metabolites including antibiotics, mycotoxins, immune-suppressants as well as cholesterol lowering agents. For example, *A. flavus* and *A. fumigatus* produce the mycotoxins aflatoxin and gliotoxin respectively. Aflatoxin is a carcinogen causing contamination in food crops, whereas gliotoxin can lead to Aspergillosis, an invasive pulmonary disease in immunocompromised patients. On the other hand, *A. niger* is used for the industrial production of citric acid and *A. oryzae* is exploited in the fermentation industry for the production of soy sauce, rice vinegars and other traditional Japanese foods and beverages, and *A. terreus* used for the production of the cholesterol

lowering agents, such as lovastatin (Bennett and Arnold, 2001, Alberts, 1988, Kamei and Watanabe, 2005).

1.8.1 *Aspergillus terreus*

A. terreus is an ascomycete filamentous fungus belonging to the genus *Aspergillus.* This species is ubiquitous in the environment, and it is mainly isolated from desert soils, grasslands, marine environments, composts as well as from contaminated corn, barley and peanuts crops (Kozakiewicz, 1989).

A. terreus cultures are characterised by their yellowish-brown colonies, long columnar conidia chains and a compact head (Figure 1.9). Unlike the model fungus *A. nidulans, A. terreus* is mainly known to reproduces asexually (Samson et al., 2011). However, very recently Arabatzis and Velegraki (2013) reported the presence of genes involved in sexual reproductions in *A. terreus.* In submerged cultures *A. terreus* produces aleuriospores instead of conidiophores. Aleuriospores are lateral cells exclusively produced by *A. terreus*; thus used as a screening method for this fungus in clinical samples (Baddley et al., 2003).

A. terreus produces a range of economically valuable secondary metabolites including statins, cholesterol lowering agents, such as lovastatin, simvastatin, pravastatin (Manzoni and Rollini, 2002, Alberts, 1988). Other secondary metabolites include sulochrin, antitumour metabolites terrain, butyrolactones, cyclosporineA and the organic acid, itaconic acid (Schimmel et al., 1998); however at the same time, *A. terreus* can also be pathogenic as it produces various mycotoxins such as gliotoxin, patulin, citrinin, citreoviridin leading to infectious diseases such as Aspergillosis in immunocompromised patients (Bräse et al., 2009).

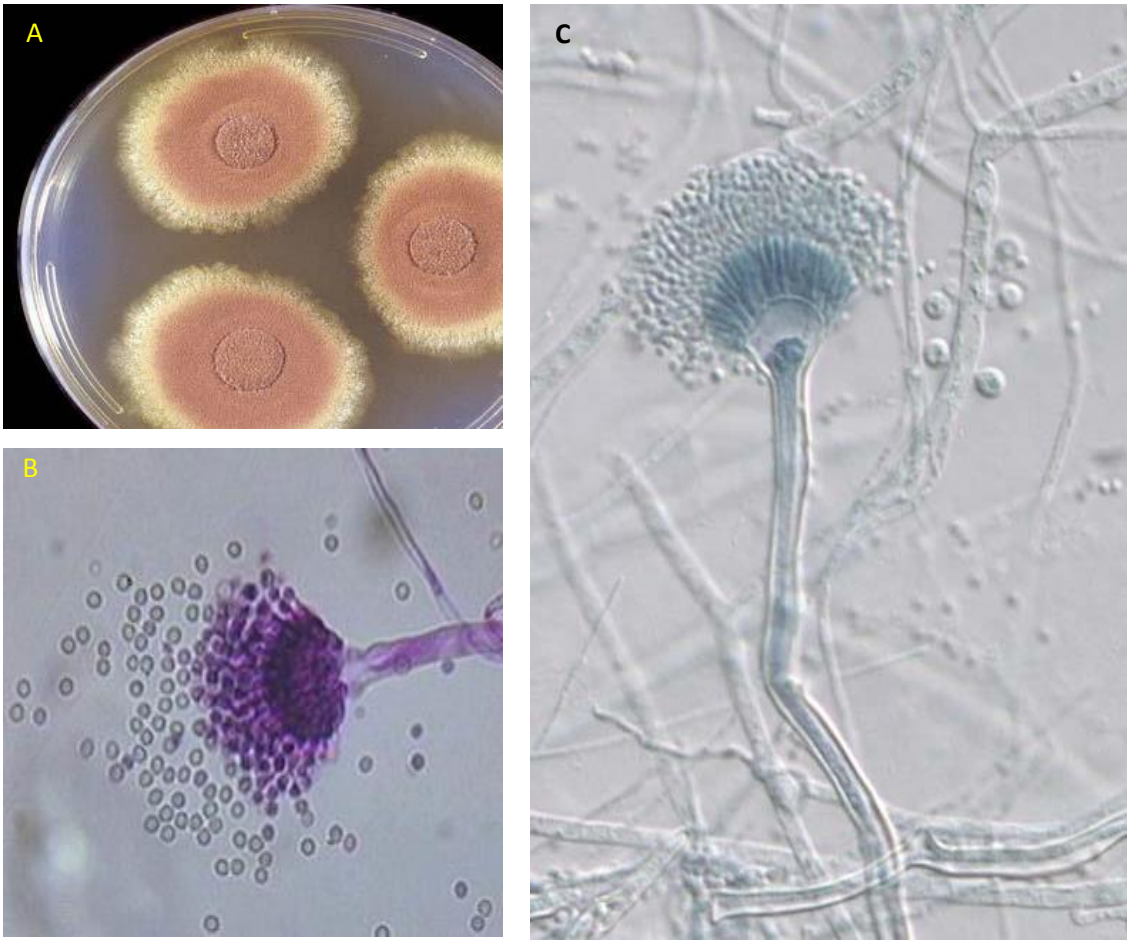


Figure 1.9: *A. terreus* morphology. *A. terreus* colonies on agar, B & C) Conidiophores with dispersed conidia. Image sources (www.doctorfungus.org, www.geniebio.ac-aix-marseille.fr)

1.8.1.1 *Aspergillus terreus* genome

The genome sequence project of the *A. terreus* clinical isolate strain NIH 2624 was funded by the National Institute of Allergy and Infectious Disease as part of a larger *Aspergillus* comparative genomics effort involving the Microbial Sequencing Centres at the Broad Institute and at The Institute for Genomic Research (TIGR). The Broad Institute used the genomic DNA provided by the University of Manchester to carry out the sequencing. The unannotated genome sequence was publicly released by the Broad Institute in 2005. The 11.0 x assembly consists of 267 contigs that cover 29.3 Mb; the contigs were linked to form 26 scaffolds. The assembly as well as the whole genome shotgun section of the NCBI are available on the Broad Institute

website. The haploid genome of *A. terreus* is approximately 35 MB spread over eight chromosomes (Birren B, 2004) (http://www.aspergillus.org.uk). A whole-genome shotgun sequencing in addition to the transcriptional, metabolite profile of another *A. terreus* isolate strain ATCC 20542 was conducted by the Microbia company (Askenazi et al., 2003).

1.8.2 Lovastatin

The major group of secondary metabolites produced by *A. terreus* are the statins (Manzoni et al., 1998). Extensive studies focused on statins because of their positive contribution to the medical industry; statins are the main leading drugs used to lower human serum cholesterol levels in patients suffering from hypercholesterolemia, thus reducing the risk of heart attacks (Manzoni and Rollini, 2002).

Lovastatin (also known as melvinolin, mevastatin, monacolin K) is one of the various statins produced by *A. terreus*. Lovastatin was originally isolated from *A. terreus* in 1978 and later from *Monascus ruber* in 1979 (Endo, 1979, Endo, 1980 , Alberts et al., 1980). However; it was not until 1987 that lovastatin was approved as a prescribed human cholesterol-lowering drug by the US Food and Drug Administration (1987).

Similar to other statins, lovastatin competitively inhibits (3S)-hydroxy-3 methyl glutaryl coenzyme A reductase (HMG-coA reducatse) activity by binding to its active site (Alberts, 1988, Manzoni and Rollini, 2002). HMG-coA reducatse catalyses the reduction of HMG-coA to melvanolate, a rate limiting step in the cholesterol biosynthesis pathway (Friesen and Rodwell, 2004, Burg and Espenshade, 2011).

1.8.2.1 Lovastatin Biosynthesis

The *A. terreus* lovastatin gene cluster is a 64 kb genomic region, and it contains 18 genes involved in lovastatin biosynthesis (Figure 1.10). Out of the 18 genes, only 5 were identified as essential enzymes required for lovastatin production (Hutchinson et al., 2000, Kennedy et al., 1999). These include the two genes *lovB* and *lovF* encoding for polyketide synthases: nonaketide synthase and diketide synthase respectively.

Other genes include *lovC* that encodes an enoyl reducatase, *lovD* that encodes a trans-esterase, and *lovA*, also known as cytochrome P450 genes encoding cytochrome P450 oxygenase. Additional genes involved in lovastatin biosynthesis include two regulatory genes, *lovE* and *lovH*, that are similar to transcription factors, as well as potential resistance genes and the three transporter genes and genes with unknown function referred to as *lovG* (Hutchinson et al., 2000).

Lovastatin is composed of two fungal type-I polyketide synthases (PKSs): diketide synthase (LovF) and nonaketide synthase (LovB). LovB and LovF work simultaneously, together with other enzymes to produce one product, lovastatin. LovB, the enoyl reductase (LovC) and the cytochrome P450 oxygenases (LovA) operate together to assemble nonaketide monacolin J, the immediate precursor of lovastatin. Simultaneously, LovF condenses 2 acetyl units, which are then reduced, and then undergo methylation by methyl transfer from S-adenosyl-L-methionine (SAM) via individual LovF catalytic domains to yield the five carbon unit, 2-methylbutyrate side chain of lovastatin. In order to connect the two polyketides, the transesterase (LovD), transacylates the acyl group from LovF to the C8 hydroxyl group of monacolin J to yield the final product, lovastatin (Xie et al., 2006).

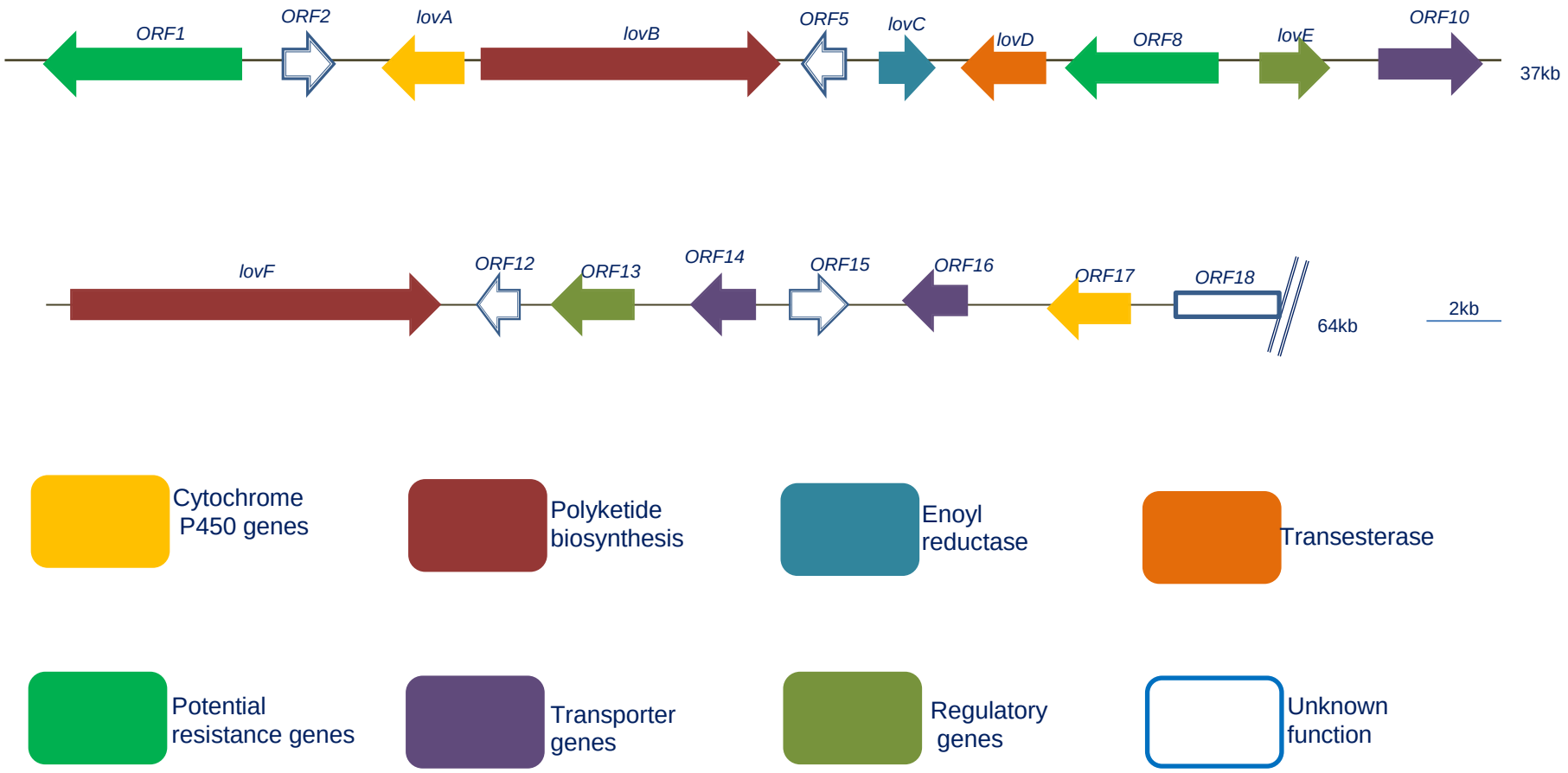


Figure 1.10: The lovastatin biosynthetic gene cluster in *A. terreus.* The lovastatin biosynthetic gene cluster consists of 18 putative open reading frames (ORFs). *lov* genes (*lovA, lovB, lovC, lovD, lovF)* encode essential enzymes required for lovastatin production. Adapted from Kennedy et al. (1999).

1.9 Proteomics

Proteomics is the large-scale study of the entire protein components present in a cell, tissue, or even the whole organism (Pandey and Mann, 2000). It is a powerful analysis tool used to obtain a systemic understanding of the molecular mechanisms at the protein level as it provides a global, integrated view of the proteins identity, localization, expression levels, interactions and post-translational modifications (Pandey and Mann, 2000, Blackstock and Weir, 1999, Barreiro et al., 2012).

Proteomics is complementary to genomics, as it provides both qualitative and quantitative information about gene expression (Banks et al., 2000). Fungal proteomics vastly progressed in the last decade; mainly because of the increasing availability of fungal genome sequences (Barreiro et al., 2012).

As in other organisms, fungal proteomics is performed at three levels: intracellular (cytosolic & inside organelles), extracellular (secretome) and membrane& organelle proteomes. Two main approaches are followed in proteomics: identification of individual proteins or generation of constituent protein data sets. Individual protein identification is performed using SDS-PAGE or 2D-PAGE fractionation followed by MALDI-TOF/TOF; whereas protein data sets are generated using shotgun proteomics, where the total fungal protein digests are analysed by tandem LC-MS. These proteomics techniques can be used to either prepare proteome reference maps that provide information on the whole set of proteins expressed at a particular time under certain experimental conditions, or can be used in comparative proteomics; where comparative analysis of fungal proteome is performed between different experimental conditions, strains, mutants (Barreiro et al., 2012).

1.9.1 Comparative proteomics

Comparative proteomics is more applied in research compared to proteome reference maps, as it provides a better understanding of the role of proteins in situ, by studying the impact of different environmental and external factors including the effect of different carbon sources, effect of added compounds, elicitors, antifungal drugs, impact of gene-deletion or strain improvement (Fernández-Acero et al., 2010, Cagas et al., 2011, Jami et al., 2010).

1.9.2 Proteomics in *Aspergillus* spp.

Proteomics research is spread over a wide range of filamentous fungi; some of the studies done in *Aspergillus* spp. are reported in Table 1.3. In this context and to date, there is only one study performed in *A. terreus by* Han et al. (2010). In this study the impact of different carbon sources including glucose, sucrose and starch on *A. terreus* extracellular proteome was investigated. A total of 82 protein spots were identified by 2-DE and nano LC-MS/MS; of the identified proteins the majority were hydrolytic enzymes in addition to various hypothetical and predicted proteins. As mentioned earlier, *A. terreus* genome was fully sequenced in 2005. However, only a very small percentage of the genome was annotated, therefore the majority of the proteins in *A. terreus* are regularly reported as hypothetical, conserved hypothetical or predicted.

Table 1.3: Applications of proteomic studies in different *Aspergillus* species. This table respresents the different applications of proteome-analysis prepared from *Aspergillus spp*.

Fungus	Proteome Type	Application	Reference
A. fumigatus	Intracellular	Human pathogenicity & Alcohol metabolism	(Kniemeyer et al., 2006)
		Oxidative stress response	(Lessing et al., 2007)
		Antibiotic resistance	(Gautam et al., 2008)
A. nidulans	Intracellular	Bacterial-fungal interaction	(Melin et al., 2002)
		Osmoadaptation	(Kim et al., 2007)
		Hypoxic responses	(Shimizu et al., 2009)
		Oxidative stress response	(Sato et al., 2009) (Thön et al., 2010) (Pusztahelyi et al., 2011)
		Conidia germination	(Oh et al., 2010)
A. niger	Intracellular	Biotechnological processes	(Sørensen et al., 2009)
A. fumigatus	Extracellular	Immunome	(Singh et al., 2010)
		Human pathogenicity	(Wartenberg et al., 2011)
A. oryzae	Extracellular	Food production	(Oda et al., 2006) (Machida et al., 2005)
A. fumigatus	Plasma-membrane Anchored proteins	Biomarker	(de Groot et al., 2009) (Ouyang et al., 2010)
	Conidial surface		(Asif et al., 2006)
	Cell wall and Plasma membrane	Antibiotic resistance	(Cagas et al., 2011)

1.10 G-protein Coupled receptors and cAMP signalling

A G-protein/cAMP-mediated signalling pathways involve four components: G-protein coupled receptors (GPCRs), heterotrimeric G proteins, the secondary messenger cAMP, and cAMP dependant protein kinases (Lafon et al., 2006).

Heterotrimeric G proteins, also known as G-protein complex, are composed of three subunits: Gα, Gβ and Gγ. In an inactive state the Gα subunits binds to GTP and GDP; and then hydrolyses the GTP to GDP, whereas the Gβ and Gγ form a dimer. The G protein complex is associated with the plasma membrane as well as a G-protein coupled receptor (Li et al., 2007).

GPCRs are a large family of integral plasma membrane localised proteins that respond to various extracellular stimuli, or ligands. Examples of ligands include peptides and non-peptides neurotransmitters, pheromones, nutrients (carbon and amino acids), growth factors, and lipids (e.g. oxylipins) (Marinissen and Gutkind, 2001).

Ligands bind to several GPCRs and activate them, thus inducing a conformational change, in which a GTP is exchanged to a GDP on the Gα subunit and the Gβγ dissociate leading to a signal transmission, via a secondary messenger such as cAMP, cGMP, diacylglycerol, inositol (1,4,5)-trisphosphate, phosphatidyl inositol (3,4,5)-trisphosphate (Marinissen and Gutkind, 2001, Birnbaumer, 2011). A receptor-mediated signalling pathway that involves cAMP as the secondary messenger is referred to as a cAMP signalling pathway. The activated effector molecules are then carried via secondary messengers towards the nucleus. In the nucleus the signalling molecules bind to the transcriptional factors leading to either activation or repression of target genes thus inducing a range of biological activities including secondary metabolism, development, and sporulation (Birnbaumer, 2011, Affeldt et al., 2012).

1.10.1 Signalling mechanisms in the genus *Aspergillus*

A typical quorum sensing mechanism requires three obligatory components: a signalling molecule, a target gene (response) as well as a developed signal

transduction pathway. It is now well established, in filamentous fungi, that oxylipins and other lipid moieties such as butyrolactones act as QSM to regulate various cellular responses such as the regulation of secondary metabolism (aflatoxin, sterigmatocystin), spore, and sclerotial production as well as *Aspergillus* development (Brown et al., 2008, Tsitsigiannis et al., 2004a, Tsitsigiannis and Keller, 2007, Raina et al., 2012).

However, there is limited knowledge on the signal transduction pathways utilised by the QSM to activate or repress target genes. Signalling mechanisms in mammals and plants uses G-protein signalling pathways to perceive signals (oxylipins) and regulate various cellular mechanisms. Very recently, a research project has started to investigate a hypothesis suggesting that fungal signalling occurs in a similar manner, where oxylipins are also perceived by GPCRs and follow G-protein/cAMP-mediated signalling pathways for signal transduction (Affeldt et al., 2012).

According to this hypothesis, oxylipins act as QSM. At low cell density cultures, there are low amounts of oxylipins that tend to shift away, thus leading to mycotoxin (aflatoxin) production as well as sclerotiorum production in *A. flavus*. However at high cell density culture, the amount of oxylipins increases proportionally. Upon exceeding threshold levels, the oxylipins binds to various GPCRs located on the cell surface, leading to receptor activation that is translated into various activities such as the regulation of the development and conidiation. Examples include spore germination in *A. flavus,* shift from asexual to sexual reproduction (sclerotial production) in *A. nidulans, A. flavus* and *A. paraticus* (Brown et al., 2008, Brown et al., 2009, Affeldt et al., 2012).

GPCRs as well as other components; G-protein and cAMP-mediated signalling were identified across the fungal kingdom, particularly in the model filamentous fungus *A. nidulans,* either through use of genetic screens, analyses of expressed sequence tags or partial examination of the genome (Lafon et al., 2006). By contrast there is limited information about the existence of putative GPCRs, and other components of G-protein/cAMP-mediated signalling pathways in *A. terreus.*

Chapter II

MATERIALS & METHODS

2.1 Chemicals and Reagents

All chemicals used in this study were obtained from Sigma-Aldrich Company Limited (Poole, Dorset, UK) unless stated otherwise. High performance Liquid Chromatography (HPLC) assays were performed using HPLC-grade solvents purchased from Fisher Scientific (Loughborough, UK).

2.2 *Penicillium sclerotiorum* Studies

2.2.1 Strain

Penicillium sclerotiorum IMI 104602 was obtained from CABI Biosciences (Surrey, UK).

2.2.2 Maintenance medium

P. sclerotiorum IMI 104602 stock culture was maintained on modified Czapek-Dox agar slants. Agar slants were inoculated using a prepared spore suspension (section 2.2.3). The slants were regularly sub-cultured at 28^0C for 6-8 days until sporulation, and subsequently stored at 4^0C for later use. The modified Czapek-Dox medium (Table 2.1) was prepared in 1 litre of distilled water; the medium was initially stirred and boiled for 1 minute to ensure homogeneity, then poured into universal bottles and sterilized in an autoclave at 121°C for 15 minutes.

Table 2.1 Modified Czapek-Dox agar medium

Ingredients	Concentration (g/L)
Sodium nitrate	2.00
Potassium chloride	0.50
Magnesium glycerol-phosphate	0.50
Potassium sulphate	0.35
Ferrous sulphate	0.01
Sucrose	30.00
Agar	12.00

2.2.3 Spore suspension

For the preparation of spore suspension, spores were harvested from *P. sclerotiorum* culture grown on modified Czapek-Dox agar 20mL universal bottle slants using 5mL of sterile 0.01% tween 80 solution (vol/vol) containing approximately 20 glass beads. The spores were then counted using a haemocytometer under the microscope.

2.2.4 Media

2.2.4.1 Raulin-Thom medium

P. sclerotiorum was grown in 500mL shaken flasks using 100mL of Raulin-Thom medium for the production of putative quorum sensing molecule(s). The liquid medium was inoculated using 1mL of 10^7 spore suspension and grown at 27°C at 180rpm. Raulin-Thom media was prepared as indicated in Table2.2. The pH of the medium was adjusted to 3.5 using 1M HCL prior to autoclaving. All medium constituents were autoclaved at 110°C for 10 minutes except for ferrous sulphate; which was filter-sterilized using 0.22µm cellulose acetate membrane filter (VWR, UK).

Table 2.2 Raulin-Thom medium

Ingredients	Concentration (g/L)
Ammonium tartrate	2.60
Ammonium di-hydrogen phosphate	0.40
Ammonium sulphate	0.16
Tartaric acid	2.60
Magnesium carbonate basic	0.28
Potassium carbonate	0.40
Zinc sulphate heptahydrate	0.07
Copper (II) sulphate pentahydrate	0.005
Ferrous sulphate heptahydrate	0.06
Sucrose	30.00

2.2.4.2 Growth medium for sclerotiorin production

P. sclerotiorum was grown in 500mL shaken flasks using 100mL of potato dextrose broth (PDB) for sclerotiorin production. All medium constituents were autoclaved at 121°C for 15 minutes. The liquid medium was inoculated using 1mL of 1 x 10^7 spore suspension and grown at 27°C at 220 rpm for 8 days.

2.2.5 Assays

The cell-dry weight (biomass), total carbohydrate assay (carbohydrate consumption), pH, and sclerotiorin production levels in shaken flasks were assayed throughout the course of the fermentation.

2.2.5.1 Cell Dry weight

Fungal growth was estimated by measuring the cell dry weight (CDW) at the end of the fermentation. *P. sclerotiorum* cultures growing in shaken flasks were filtered on a pre-weighed Whatmann no.1 filter paper (VWR, UK). The biomass samples were then washed thoroughly with 100mL of distilled water and oven-dried at 60°C

for 48 hours. The filter papers with fungal biomass were weighed again and the CDW was calculated by subtracting the total weight to that of the filter paper alone. The CDW tests were done in triplicate.

2.2.5.2 Total Carbohydrate assay

A total carbohydrate assay was performed using phenol-sulphuric acid assay as described by Chaplin and Kennedy (1994). A 1mL sample was taken from the shaken flasks or bioreactor, and centrifuged at 12,000 rpm for 10 minutes. The pellet was discarded and the supernatant was 100 x diluted for the assay. Phenol solution (0.2mL of 5% w/v) was added to 0.2mL of the diluted sample, standard, and blank in a clean, dry glass test tube. Concentrated sulphuric acid (1mL) was then rapidly added to the surface of liquid. The glass test tubes were vortexed, followed by 30 minutes incubation at room temperature. The absorbance was read at 490nm using a UV-spectrophotometer (Jenway, Fisher Scientific, UK). A standard curve was prepared using glucose concentrations in the range of 0-100 µg/mL. Carbohydrate consumption assay was performed in the fume cupboard for safety. All tests were performed in triplicates.

2.2.5.3 Sclerotiorin Detection and Quantification

2.2.5.3.1 Sample preparation

Quantification of sclerotiorin was performed according to the method by Weng et al. (2004) with minor modifications in regards to the amount of mycelia, and volume of methanol used for extraction. *P. sclerotiorum* mycelia were collected from shaken flasks and/or bioreactor and frozen overnight at -80^{0}C. The frozen mycelium was then freeze-dried under vacuum pressure at -50^{0}C in a vacuum freeze dryer (Savant Modulyo D, Thermo Fisher Scientific, Loughborough, UK). Freeze-dried mycelia (0.4g) were extracted 60-fold (v/w), twice, using HPLC-grade methanol and placed in a 50mL volumetric flask. Methanol was then added to make a final volume of 50mL. The methanol extract was diluted twice with methanol and filtered using 0.45µm cellulose acetate membrane filter (VWR) and assayed using HPLC system (Dionex).

2.2.5.3.2 HPLC method

Quantitative analysis of sclerotiorin standards and samples were carried out using HPLC system (Dionex) equipped with a Phenomenex (C18, 5µM x4.6mm x150mm) reverse phase column, and a UV-VIS detector. The mobile phase was acetonitrile: water (65:35 v/v) with a flow-rate of 1 mL/min, injection volume of 20µL and peak detection was at 370nm.

2.2.5.3.3 Sclerotiorin standard curve

A standard curve was prepared using a sclerotiorin standard over a concentration of 0-100 µg/mL (Cayman Chemicals, UK). Sclerotiorin eluted after 2.7 minutes under these conditions. All tests were performed in triplicate.

2.2.6 Solvent extraction

As previously mentioned, *P. sclerotiorum* culture broth was grown in Raulin-Thom medium for the production of putative quorum sensing molecule(s). Four different time-points were selected from the growth profile (Figure 2.1). The days at which *P. sclerotiorum* culture broth was selected for extraction were days 2, 4, 6 and 8 post-inoculation. The culture broth(s) obtained from these days were filtered using Whatmann no.1 filter paper (VWR) via a Buchner filter pump. The broth was then extracted three times with an equal volume of ethyl acetate using a separatory funnel until the organic phase was colourless. The aqueous solution was discarded; whereas the organic phase containing the putative quorum sensing molecule(s) was concentrated in a Rotavapor RE-120 (Büchi). The resulting pellet was re-suspended in 1/50th of the initial volume of ethyl acetate (VWR) to obtain 50 fold concentrated culture extracts. The ethyl acetate extracts from 2, 4, 6 and 8 day-old cultures referred to as day2- extract, day4- extract, day6- extract, and day8- extract respectively, were stored at 4°C for future use. A schematic diagram (Figure 2.1) illustrates the solvent extraction process.

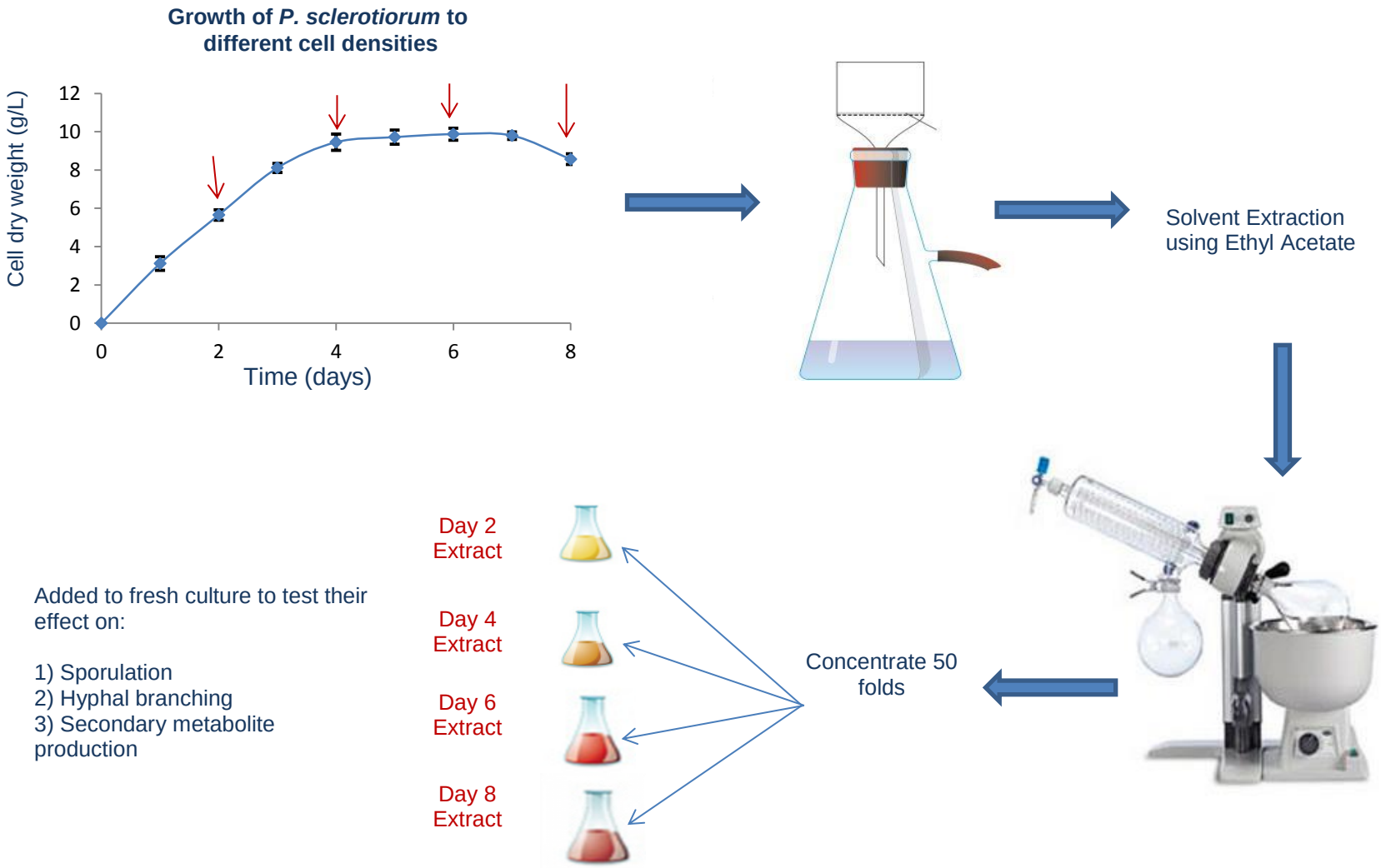


Figure 2.1: Schematic diagram of solvent extraction process. The selected time points from the growth profile, and the subsequent procedures followed for ethyl acetate extracts preparation

2.2.7 Impact of ethyl-acetate culture extracts

The effect of day2- extract, day4- extract, day6- extract and day8- extract was investigated on hyphal branching, sporulation and secondary metabolite production.

2.2.7.1 Hyphal Branching

Ethyl acetate extracts (100µL) were added to 18 hours old *P. sclerotiorum* liquid culture. The effect of extracts on hyphal branching was studied after 24 and 36 hours. Two control samples (negative and positive control) were maintained. Negative control represents a *P. sclerotiorum* culture with nothing exogenously added; whereas the positive control was treated with 100µL of filter sterilized ethyl acetate (solvent control). An average of 100 hyphae, from each aliquot representing each condition, were viewed under the Leica microscope (Leica, UK) and included for analysis. The percentage of hyphae with lateral branches was calculated by dividing the number of branched-hyphae to the total number of hyphae (branched or unbranched). The mean of three replicates corresponding to each condition (test and controls) was then tabulated as seen in Figure 3.4.

2.2.7.2 Sporulation assay

Sporulation assay was performed on agar surface culture to study the effect of the ethyl acetate extracts on *P. sclerotiorum* sporulation. The plates were prepared by pouring 5mL of soft Czapek-Dox modified agar layer inoculated with 1 x 10^7 *P. sclerotiorum* spores over 20mL of modified Czapek-Dox agar in 90mm petri dishes. A volume of 20µL of ethyl acetate extracts was placed on 6mm Whatmann filter discs. The discs containing the ethyl acetate extracts were air dried for approximately 1 hour and placed on top of the agar surface layer. Pure ethyl acetate was used as control. A schematic diagram of the sporulation assay setup is illustrated in Figure 2.2.

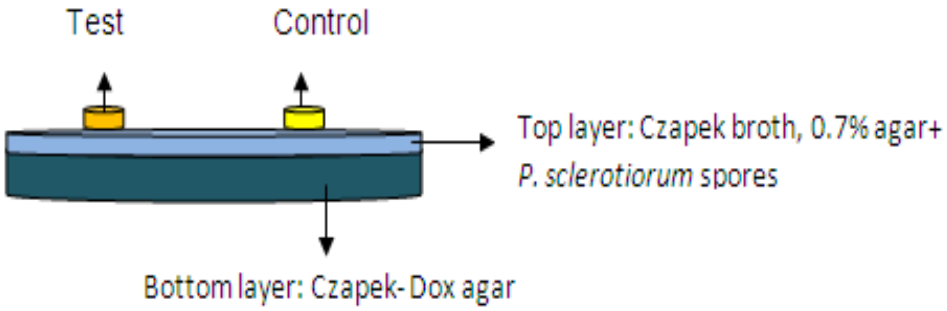


Figure 2.2: Sporulation-assay setup . The sporulation assay was prepared in 90mm petri dishes using two layers of Czapek-Dox agar. The bottom layer was prepared using Czapek-Dox agar, and the top layer with Czapek-Dox broth, 0.7% agar and *P.sclerotiorum* spore suspension. Two 6mm filter discs (one test and one control) were placed on the surface of petri-dish. The test contained 20µL of ethyl acetate extracts, whereas the control contained only ethyl acetate.

Agar plates were incubated at 28^0C for 7 days and checked visually every day. Cores of 12mm were removed on the 5^{th} and 7^{th} day from the areas surrounding the discs and shaken vigorously for 4 minutes in universal bottles containing 3mL sterile water, 0.01% tween 80, and glass beads. Spores were counted using a haemocytometer under the microscope.

2.2.7.3 Sporulation on plates

A visual assay was performed to investigate how the concentration of culture extracts affects sporulation. Two volumes of day 6-extract, 30µL and 50µL, were dissolved in methanol to a final volume of 100µL to make a concentration of 30% and 50%. The same volume of methanol (100µL) was used as control. The two extracts and the solvent control were then spread over the surface of modified Czapek-Dox agar (25mL) in 90mm petri dishes. The plates were then spot-inoculated in the centre with 10µL of 10^7 spores/mL *P. sclerotiorum* spore suspension. Macroscopic images of the plates were taken after 10 days of incubation at 28^0C

2.2.7.4 Secondary metabolite production (plates)

The effect of the ethyl acetate extracts on secondary metabolite production (sclerotiorin) on agar surface culture was investigated; sclerotiorin production was observed as exudates (pigment production) which create a change in colouration

on the surface of plates. The plates were prepared by pouring 25mL of modified Czapek-Dox agar onto 90mm Petri dishes. Ethyl acetate extracts (30µL) were dissolved in 1mL of methanol and spread over the agar surface. The plates were air dried for approximately 2 hours in the laminar flow cabinet. When dried, the plates were inoculated using 13mm *P. sclerotiorum* agar plugs at the centre of the petri dishes. The plates were incubated at 28^0C for 10 days. Macroscopic images of the plates were taken on the 10^{th} day; illustrating the difference in sclerotiorin production, as indicated by the orange colour formation.

2.2.8 Secondary metabolite production studies

Response surface methodology (RSM) was performed using Design Expert software (Trial version, Stat-Ease, Minneapolis, USA) to examine the addition effect of *P. sclerotiorum* 7-day old culture extract on the production of the secondary metabolite sclerotiorin and the cell dry weight in *P. sclerotiorum* liquid culture. The two studied factors were the concentration (% vol/vol) of the added culture extract and its time of addition.

Flasks (500mL) containing 100mL of potato dextrose broth (PDB) were inoculated with 1mL of *P. sclerotiorum* spore suspension (1 x 10^7spores /mL). *P. sclerotiorum* 7-day old culture extract (prepared in Raulin-Thom medium, as seen in Figure 2.1) was dissolved in ethyl acetate to a final volume of 200µL. The culture extract was exogenously added at the concentration(s) and time(s) determined by the RSM design experiment (Appendix section 8.1). Flasks were incubated at 27°C and shaken at 220 rpm. Each flask was harvested after 8 days of the fermentation and assayed for the cell dry weight, and sclerotiorin production yield (method under section 2.2.5).

2.2.8.1 Response Surface methodology- Experimental design

Optimization of sclerotiorin production upon the addition of culture extract was investigated using RSM. A two-factor central composite design (CCD) was constructed consisting of 28 runs, including 4 centre points, 4 replicates of axial star points, and 2 replicates of factorial points. The design variables were the percentage (%vol/vol) of culture extract added (X1, %) and the addition time (X2,

Days), whereas the two monitored responses were the cell-dry weight (R1, g/L) and sclerotiorin yield (R2, mg/g).

Table 2.3 Code and level of variables chosen for the RSM experiment. The impact of two different factors (Percentage of added extract % (vol/vol) & its time of addition) on sclerotiorin production and cell dry weight were investigated using Design Expert software.

Factors	Code	Levels			α = 0.5	
		-1	0	1	-α	+α
Concentration/ Percentage of added extract %(vol/vol)	A	0.0375	0.075	0.1125	0	0.15
Time of addition (days)	B	1.5	3	4.5	0	6

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

(Equation 1)

$$y = \beta_0 + \beta_{ii}X_i + \beta_{ii}X_i^2 + \beta_{ij}X_iX_j + e$$

Where y is the response, β_0 the constant coefficient, X_i (i= 1–2) are non-coded variables, β_is are the linear, and β_{ii}s are the quadratic, and β_{ij}s are the second-order interaction coefficients. Data were processed for Eq. (1) using Design-Expert 7.0.0 program (Trial version, stat-ease, Minneapolis, USA) including ANOVA to obtain the interaction between the process variables and the response. The quality of the fit of polynomial model was expressed by the coefficient of determination R^2.

2.2.9 Thin Layer chromatography

Further separation and purification of 7-day old extract was performed using thin layer chromatography (TLC). Analytical and preparative TLC were performed using 0.2mm silica gel plates (60 Å) and fluorescent indicator (200 x 200 mm, Merck Kieselgel).

2.2.9.1 Analytical TLC

For analytical TLC, samples of the ethyl acetate extract were spotted on the TLC plates; then placed in the development chamber containing the mobile phase. Solvents with different polarity including hexane:ethyl acetate (3:7) and hexane:ethyl acetate (6:4) were tested as the mobile phases for optimal separation of the molecules within the extract. Upon development; the separated compounds on the silica gel plates were visualized as dark spots under ultraviolet light (254 nm) in a fluorescent green background.

2.2.9.2 Preparative TLC

The compounds separated by TLC, were scraped off their corresponding silica and transferred to clean glass beakers. The compounds were extracted from the silica gel with 5mL of the developing solvent (hexane:ethyl acetate 6:4). The silica was removed by filtration using glass wool, and the collected solvent was evaporated under nitrogen gas (N_2). The pellets were stored at 4^0C for later analysis using Gas Chromatography-Mass Spectrometry (GC-MS).

2.2.10 Gas Chromatography-Mass spectrometry

2.2.10.1 Derivatization for GC-MS analysis

The dry extracts were dissolved in 0.5mL of HPLC-grade methanol, vortexed and kept in the freezer at -20^0C; 200µL of each extract in addition to 20µL of 0.5 mg/mL of Margaric acid (heptadecanoic acid) were transferred to a 2mL screw top vial. The samples were evaporated to dryness under nitrogen gas (N_2), then treated with 50µL of acetonitrile and 50µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethyl chlorosilane (vol/vol), and then heated in an oven at 80°C for 30 minutes.

2.2.10.2 GC-MS analysis method

GC-MS was performed with an HP-5973B MSD bench-top quadrupole (Hewlett Packard) connected to an HP-6890 series GC system (Hewlett Packard). Sample injection was accomplished by means of an HP-7683 series auto-sampler (Hewlett Packard). Helium was used as a carrier gas at the flow rate of 1mL/min. The injection port was operated either in the split mode (10%) or in the splitless mode, and injection volume was 1µL. The column was a DB-5MS+DG 30m x 0.25mm I.D. x 0.25µm (J&W Scientific) with 10m Duro-Guard directly inserted into the ion source. The oven temperature was programmed from 50°C to 290°C at the rate of 10°C per minute, and the final temperature was held for 6 minutes. The mass spectrometer was operated in the electron ionization (EI) mode at ionization energy of 70eV, scanning over the mass range m/z 10 to 600. Data were collected with a G1701BA Enhanced ChemStation software version B.01.00 (Hewlett Packard).

2.2.11 Bioreactor Studies (2.5L Stirred Tank Reactor)

Two 2.5L Stirred Tank Reactors (STR- Electrolab Ltd, UK) were used for bioreactor studies. The 2 STRs (one control and one test) containing 1.5L PDB media were inoculated with 1% spore suspension $1x10^7$ *P. sclerotiorum* spores/ mL.

The bioreactors had an internal diameter of 10cm; shaft length of 20 cm and two Rushton turbine impellers placed at a distance of h/2, h represents the height of the liquid. The bioreactors together with the medium were sterilized at 121ºC for 15 minutes. The dissolved oxygen tension (% DOT), pH and temperature were monitored throughout the course of fermentation. The air flow rate and temperature were set to 1.0 vvm and 27ºC respectively. The stirrer speed was set at 220 rpm.

Ethyl actetate extract was exogenously added at the optimized conditions (0.15%, after 2.06 days) determined by RSM in shaken flask studies. Samples were assayed for sclerotiorin production on days 5-8 of the fermentation. Cell dry-weight was assayed at the end of fermentation.

2.3 *Aspergillus terreus* Studies

2.3.1 Strain

Lovastatin producer strain of *A. terreus* (MUCL 38669) was purchased from the Mycothèque de l'Université Catholique de Louvain, Belgium.

2.3.2 Maintenance Medium

A. terreus (MUCL 38669) stock cultures were maintained on potato dextrose agar (PDA) slants. The slants were grown at 28°C for 7 days and subsequently stored at 4°C. Spores were harvested by using 5mL sterile solution of 0.01% Tween 80 (vol/vol) solution in distilled H_2O supplemented with glass beads (VWR) and then counted using a haemocytometer under the microscope.

2.3.3 Growth media

Lovastatin production in *A. terreus* was studied via a two-stage process: *A. terreus* growth stage and lovastatin production stage.

2.3.3.1 *Aspergillus terreus* growth stage

A. terreus was grown in 500mL Erlenmeyer flasks, containing 100mL of inoculum medium (Table 2.4 and Table 2.5). The pH of the inoculum medium was adjusted to 6.8 using 0.1 M NaOH prior to autoclaving. A spore suspension of 10^6 spores/ mL was used to inoculate *A. terreus* growth medium. The inoculated flasks were incubated at 27°C at 220 rpm for 24 hours.

Table 2.4: *A. terreus* growth medium

Ingredients	Concentration (g/L)
Corn steep liquor	5
Tomato paste (Tesco stores)	40
Oat flour (Tesco stores)	10
Glucose	10
Trace element solution	10 mL

Table 2.5: The trace element solution ingredients

Ingredients	Concentration (g/L)
$FeSO_4 \cdot 7H_2O$	1
$MnSO_4 \cdot 4H_2O$	1
$ZnSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.1
H_3BO_3	0.056
$CuCl_2 \cdot 2H_2O$	0.025
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.019

2.3.3.2 Lovastatin Production step

For the production of lovastatin 10mL of *A. terreus* mycelia grown in the growth medium was used to inoculate 500mL Erlenmeyer flasks containing 90mL of the production medium (Table 2.6). The pH of the solution was adjusted to 7.4 by using 0.1 M NaOH before autoclaving. The inoculated flasks were incubated at 27°C at 220 rpm for 10 days.

Table 2.6: Lovastatin production medium

Ingredients	Concentration (g/L)
Lactose	50
Glucose	25
Peptonised milk (oxoid)	24
Yeast extract	2.5

2.3.4 Shaken Flask Studies

The impact of linoleic acid on lovastatin production was initially validated in 500mL shaken flasks. The shaken flasks containing 90mL of lovastatin production media were inoculated with 10mL (10%) of 1-day old vegetative inoculum that was grown in *A. terreus* growth media in shaken flasks for 1 day at 27°C and 220 rpm. Linoleic

acid (LA) was added on the day of inoculation (fermentation day 0) at a final concentration of 0.1%, whereas ethanol was used as solvent control. Lovastatin production, total carbohydrate consumption, pH were assayed throughout the course of fermentation; whereas the cell dry weight was assayed on the final day of fermentation. Total carbohydrate and cell dry weight were assayed as in section 2.2.5.

2.3.5 Bioreactor studies (5L Stirred Tank Reactor)

2.3.5.1 Lovastatin production

Two 5L Stirred Tank Reactors (Electrolab Ltd, UK) were used to for bioreactor studies. The two bioreactors had an internal diameter of 16 cm, shaft length of 31.5cm and two impellers; a Rushton turbine and a Variable Pitch impeller.

The 2 STRs (one control and one test) were operated with a total working volume of 4L. The reactors containing 3.7L of lovastatin production media were inoculated with 7.5% of a 1-day old vegetative inoculum that was grown in *A. terreus* growth media in shaken flasks for 1 day at 27° C and 220 rpm.

Linoleic acid was added on the day of inoculation (day 0 of fermentation) at a final concentration of 0.1%, whereas the same volume of ethanol was used as solvent control. The dissolved oxygen tension (% DOT), pH and temperature were monitored throughout the course of fermentation. The air flow rate and temperature were set to 0.5vvm and 27ºC respectively. The fermentation was carried out for 6 days. Samples were taken and analysed for lovastatin production.

2.3.6 Lovastatin Detection and Quantification

2.3.6.1 Sample preparation

Samples were collected from fermentation carried out in shaken flasks and bioreactors. Lovastatin was extracted from the collected samples by adding an equal amount of HPLC-grade methanol to the culture. Samples were then properly shaken, filtered using 0.45µm cellulose acetate membrane filter (VWR) and analysed via HPLC.

2.3.6.2 Preparation of lovastatin standards

The major form of lovastatin in fermentation broth is the open hydroxy acid form, also called mevinolinic acid. Lovastatin was freshly prepared from the lactone form since its open hydroxy acid form is unstable. Conversion of lovastatin from the lactone form to the β-hydroxy acid form was performed according to the method by Yang and Hwang (2006); 2 mg of lovastatin was dissolved in 10mL of 0.1 N NaOH prepared in 50% aqueous acetonitrile solution. The sample was kept at 45°C for 1 hour followed by neutralisation with 0.1 N HCl prepared in acetonitrile. For calibration purposes, a standard solution of lovastatin was prepared at concentrations ranging from 0.025 to 0.1 mg/mL.

2.3.6.3 HPLC method

Quantitative analysis of lovastatin standards and samples were carried out using HPLC system (Dionex) with UV-VIS detector using a Phenomenex (C18, 5µM x4.6 mm x150 mm) reverse phase column equilibrated at 25°C. Samples were analysed using an isocratic method. The mobile phase was acetonitrile (HPLC-grade) and aqueous 0.1 % H_3PO_4 at a ratio of 55:45 (vol/vol). The flow rate was 1 mL/min, the injection volume 25µL and peak detection was at 238 nm.

2.3.7 Microscopic images

The impact of linoleic acid on *A. terreus* mycelia and hyphal branching was studied after 24 hours. Samples from the fermenter were placed on microscopic slides and stained with lactophenol blue. All plates were observed using a Leica microscope (Leica, UK) at 20x magnification.A total of six images representing each condition (Test, Control).

2.3.8 Proteomics

The impact of linoleic acid on the expression of *A. terreus* intracellular (cytosolic) proteins was examined. *A. terreus* mycelia were harvested from shaken flasks on the fourth day. *A. terreus* culture was filtered using Whatmann filter paper (VWR) via a Buchner filter pump and the collected mycelia were properly washed using sterile distilled water and frozen overnight at -80˚C and subsequently freeze-dried in a vacuum freeze dryer (Savant modulyo D, Thermo Fisher Scientific, Loughborough, UK).

2.3.8.1 Cell lysis and total protein extraction

Freeze-dried mycelium (50mg) was added to Lysing matrix C tubes (Fisher Scientific, UK) which contained glass beads. Lysing buffer (1mL) (Table 2.7) was added to the Lysing matrix C tubes. All tubes were homogenised using a homogeniser (FastPrep® 24, MP Biomedicals, UK) at a speed of 6.5 for 10 minutes in 30 seconds of alternating homogenizing and cooling on ice to avoid sample overheating.

Table 2.7: Lysing Buffer components

Components	Concentration
Tris buffer pH 7.5	20 mM
EDTA	1mM
DTT	1mM
Fungal protease inhibitor cocktail	40 µL/mL

2.3.8.2 Cell Fractionation

Cell fractionation was carried out according to the method described Cagas et al. (2011). Samples obtained from protein extraction were centrifuged at 5000 x g for 10min at 4°C. The resulting pellet which contains the cell wall and plasma membrane fraction was discarded, whereas the supernatant was further centrifuged in an ultracentrifuge (Sorvall® Discovery™ 100SE, Kendro®) at a speed of 30,000 rpm for the period of 1h at 4°C in order to isolate the cytoplasmic proteins. The obtained cytoplasmic proteins were subsequently stored at -20°C and used for the 2-dimensional gel electrophoresis proteomic studies (2D- gels).

2.3.8.3 Protein precipitation

Protein precipitation was performed by adding 150µL of cytosolic protein sample (~150-300 µg of protein) to 600µL of methanol and mixing by vortexing. Upon mixing, 150µL of chloroform and 450µL of ultrapure water were added and thoroughly mixed. The mixture was then centrifuged at 12,000 rpm for 5 min at 4°C

to obtain two layers separated by a white precipitation disc. The upper layer was discarded, whereas the precipitation disc was kept. Then 450µL of methanol was added, mixed and again centrifuged at 12,000 rpm for 5 min at 4°C, the obtained supernatant was discarded and the pellet was air dried. Precipitated proteins were then re-suspended in 200µL of rehydration buffer containing (in 10mL) 6M urea, 2M thiourea, 0.5% (v/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate),and 0.5% (v/v) pharmalyte pH 3-10 (Amersham, UK). After precipitation the protein content was quantified using Bradford assay (Sigma, UK). A protein standard curve was prepared using bovine serum albumin (BSA) at a concentration 0-2.0 mg/L. Absorbance of standard and samples was measured using the spectrophotometer at 595 nm.

2.3.8.4 1-D Gel electrophoresis: Isoelectric focusing

For one dimensional gel electrophoresis; purified cytoplasmic protein fraction of *A. terreus* were iso-electrically focused on immobilised pH gradient gel strips (IPG) strips (7cm length-NL) covering a pH range from 3 to 10 (GE Healthcare, UK). A total of 100µg of proteins in a total of 130µL of rehydration buffer (6M Urea, 2M thiourea, 0.5% (v/v) CHAPS, 0.5% (v/v) pharmalyte pH 3-10 (Amersham, UK), 0.4% (w/v) DTT, and Bromophenol Blue) was applied to the 7cm-NL Immobiline IPG strips pH 3-10, overlaid with mineral oil and left for overnight rehydration in a reswelling tray (Amersham, UK) in order to allow the proteins to adsorb onto the 7cm IPG. All experiments were carried out in replicates of 6, using protein samples from different fermentation batches.

One dimensional gel electrophoresis was carried out on a Multiphore II (Pharmacia Biotech) electrophoresis system. Temperature during isoelectric focusing was maintained at 17^{o}C using Amersham Multiptemp III thermostatic circulator cooling unit. Strips were placed gel-side-up onto the dry strip aligner tray and electrode strips were soaked with distilled water and placed at the anode and cathode ends of the IPG strips. Mineral oil was then poured onto the cooling ceramic block to cover the IPG strips. The aligner tray was then placed over the block. Electrodes where then connected to the isoelectric focusing (IEF) electrophoresis unit and the IPG strips were focused using an Amersham EPS 3501 power pack for a total focusing time of 4h and 5min, with the following voltage profile: A linear increase

from 0 to 300 V for 30 min, followed by a linear increase from 300 to 600 V for 30 min and then a linear increase from 600 to 3500 V for 3h and 5 min.

The IPG strips were equilibrated following the end of first-dimension electrophoresis. Equilibration was performed in two steps to allow protein unfolding and better transfer from the strips to the SDS-PAGE. The first equilibration buffer containing 5mM Tris-Hcl, pH 8.4, 6M Urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS), 0.02% Bromophennol Blue, 50mM DTT for 15 min on a rocking platform at a speed of 40. The strips were then further equilibrated in the second equilibration buffer containing 5mM Tris-Hcl, pH 8.4, 6M Urea, 30% glycerol, 2% SDS, 0.02% Bromophennol Blue, and 100mM iodoacetamide while also rocking at a speed of 40 for 15 min. After the second equilibration, the strips were loaded on a 12.0% SDS-PAGE gel for the second dimension gel electrophoresis.

2.3.8.5 Two-dimensional (2-D) gel electrophoresis and protein identification

For two dimension gel electrophoresis; the equilibrated strips were loaded onto a 1mm thick 8 x 7 cm, 12.0% (w/v) SDS-PAGE resolving gel. Molecular weight marker was loaded onto a small piece of filter paper and placed on the low pH end of the strip on the gel. IPG strips were sealed onto the SDS-PAGE resolving gel (Table 2.8) by adding the sealing buffer gel (Table 2.9) over the strips. SDS-PAGE electrophoresis was carried out using a Bio-Rad protein electrophoresis unit at 150 V for about 60-70min until the dye front reached the bottom of the gel. After Two dimensional electrophoresis, gels were stained with Coomassie brilliant blue G-250 dye in 10% (v/v) acetic acid by microwave-heating the gels followed by de-staining with 10% (v/v) acetic acid by, again microwave-heating the gels to remove the excess dye until the protein spots became visible. Gels were then stored in 2% acetic acid at 4^{o}C.

Table 2.8: Components of resolving gel

Ingredients	Volume
1.5MTris-Hcl pH 8.8	2.5mL
30% Bio-Rad Acrylamide Mix	4.0mL
10% SDS stock solution	100µL
10% of ammonium persulfate (APS)	100µL
N,N,N',N'-tetramethylethylenediamine (TEMED).	10µL
Distilled water	3.3mL

Table 2.9: Components of sealing gel

Ingredients	Amount
Agarose	0.25 g
Laemmli electrolyte buffer (running buffer)	50mL
Bromophenol blue	0.002 % (200µL)

Table 2.10: Components of Laemmli electrolyte buffer (Running Buffer)

1% SDS (w/v)
0.25M Tris base
1.92 M Glycine

2.3.8.6 Image analysis

Stained 2-D gels were scanned using GS800 densitometer (Biorad, UK) and the resulting images were taken using Quantity II software. All images were converted to a TIFF file and analysed using Progenesis PG240 Samespot software (Nonlinear Dynamics, UK).

A total of six gel images representing each condition (test, control) were uploaded onto the progenesis software for analysis. Gels representing test cultures were compared to those of the control. The protein concentrations were determined by the spot intensities. Fold changes in protein spots between the two conditions (test,

control) were calculated based on the *p*-value from one way ANOVA and only spots with *p*-value ≤0.05 and maximum fold change ≥1.3 were considered in the final analysis.

2.3.8.7 Protein identification

Spots that differed significantly in the intensity between test and control cultures were cut out from the 2-D gels and sent for analysis. Analysis of the protein spots was carried by the University of York Mass Spectrometry unit (York, UK) where spot samples were identified by MALDI-TOF/TOF which allows identifications by including tandem spectral data in Mascot search. The resulting data was further identified by the input of the resulting sequences into protein Basic Local Alignment Search Tool (BLAST).

2.3.9 cAMP Quantification

The impact of three pure oxylipins: 9(S)-HODE, 9(S)-HpODE, 13(S)-HODE (Cayman Chemicals, UK) and linoleic acid (as an oxylipins precursor) on cAMP levels in *A. terreus* was examined according to the method developed by Affeldt et al. (2012).

2.3.9.1 Culture conditions

A. terreus (10^6 spores /mL) was grown in 1L Erlenmeyer flasks, containing 400mL of glucose minimal media (Table 2.11) at 27° C at 220 rpm. The mycelia was harvested after 24 hours by vacuum filtration, and washed with sterile GMM. *A. terreus* mycelia were divided into 200mg fractions that were used to inoculate 20mL sterile GMM media in 50mL conical tubes. The *A. terreus* subcultures were subsequently equilibrated at 27°C at 220 rpm for 1hour.

Table 2.11: Components of Glucose Minimal Medium (GMM)

Ingredients	Concentration (g/L)
$NaNO_3$	6.0
KCl	0.52
$MgSO_4 \cdot 7H_2O$	0.52
KH_2PO_4	1.52
Glucose	10
Trace elements stock solution	1mL

Table 2.12: Trace elements solution for GMM

Ingredients	**Concentration** (g /100 mL H_2O)
$ZnSO_4 \cdot 7H_2O$	2.2
H_3BO_3	1.1
$MnCl_2 \cdot 4H_2O$	0.5
$FeSO_4 \cdot 7H_2O$	0.5
$CoCl_2 \cdot 5H_2O$	0.16
$CuSO_4 \cdot 5H_2O$	0.16
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.11
Na_4EDTA	5.0

2.3.9.2 Treatment

After equilibration, *A. terreus* cultures were treated with pure oxylipins in ethanol, linoleic acid or with pure ethanol as a solvent control. Three oxylipins; 9(S)-HODE, 9(S)-HpODE, 13(S)-HODE were added at a final concentration of 10μM in a final volume of 12.5μL ethanol. Linoleic acid and the solvent control (ethanol) were added at the same volume of 12.5μL. When other concentrations of oxylipins

(9(S)-HpODE) were needed, a stock solution was either diluted or concentrated using ethanol so that the final added volume was kept constant at 12.5µL.

After the addition of oxylipins, linoleic acid and ethanol to the equilibrated *A. terreus* cultures, the samples were shaken by hand for twenty seconds precisely. The fungal mycelia were then separated using Whatman Filter papers no. 1 (11µm). The mycelia was then scraped off and, placed into cryogenic vials and flash frozen in liquid nitrogen. Samples were stored at -80°C for 15 hours until extraction, and later cAMP and protein quantification.

2.3.9.3 Extraction

Frozen mycelia were ground to a fine powder using a clean, pre-chilled mortar and pestle in liquid nitrogen and weighed on a microbalance. Grounded mycelia was re-suspended and homogenised in 50 mM Tris.HCl/4 mM EDTA (pH 7.5) using a homogeniser (FastPrep® 24, MP Biomedicals, UK) at a speed of 6.5 for 30 seconds. Samples were then boiled for five minutes and then acidified via the addition of concentrated HCl to a final concentration of 0.1M. Samples were then incubated for ten minutes on ice and centrifuged at 13,000 rpm at 4°C for five minutes.

The obtained supernatant was transferred to a clean cryogenic vial and flash-frozen in liquid nitrogen. Acidified supernatants were split into two fractions; one used for quantification of cAMP levels, whereas the second was used for protein quantification.

2.3.9.4 Quantification of cAMP

Cyclic AMP (cAMP) levels were measured using the direct cyclic AMP Enzyme-Linked Immunosorbent Assay (ELISA) kit (Enzo Life Sciences, Exeter, UK) according to the manufacturer's directions. The cAMP ELISA is a competitive immunoassay for the quantitative determination of cyclic AMP levels in samples treated with 0.1 M HCl. Standards and the fungal samples (prepared as above) were added to 96 well plates that are coated with a secondary antibody, GxR IgG antibody. A blue solution of cAMP, conjugated to alkaline phosphatase, was then added, followed by a yellow solution of rabbit polyclonal antibody to cAMP. During

a simultaneous incubation at room temperature the antibody binds to the cAMP in the sample or the conjugate in a competitive manner. The plate was then washed, leaving only bound cAMP. Afterwards a pNpp substrate solution was added, with subsequent incubation. During incubation, the substrate was catalysed by the alkaline phosphatase on the cAMP conjugate, thus generating a yellow colour. The enzyme reaction was then stopped upon the addition of a Stop solution.

The optical density of the generated yellow colour was measured at 405nm using a Versa-Max Microplate reader (Molecular Devices, UK). The intensity of the yellow colour is inversely proportional to the concentration of cAMP in either the standards or the samples. cAMP concentrations of tested samples from each plate were calculated from the individual plate's standard curve. The R^2 for the standard curves was> 0.98 for both assays. Two technical replications were performed on each sample. The standard curve represented in Figure 3.28 illustrates on the x-axis the log of given standard cAMP concentrations , whereas the primary y-axis shows the ratio of %B/Bo, representing the ratio of the absorbance of the given standards to that of the maximum binding (Bo), as supplied by the kit.

2.3.9.5 Protein Quantification

The protein content in the samples was quantified using Bradford assay (Sigma, UK). In order to neutralize the 0.1 M HCl in the samples, an equal volume of 0.1 N NaOH was added to each subsample. A protein standard curve was prepared using bovine serum albumin (BSA) standards at a concentration 0-2.0 mg/L. Absorbance of standard and samples was measured using a spectrophotometer at 595 nm.

2.3.10 Identification of G-protein/cAMP-mediated signalling pathway components in *A. terreus*

G-protein and cAMP-mediated signalling pathways involve G-protein coupled receptors (GPCRs), heterotrimeric G-protein constituents, the secondary messenger cAMP, cAMP-dependant protein kinase (PKA). The majority of the components of these signalling pathways have been previously identified in *A. nidulans, A. fumigatus, A.oryzae* either through the use of genetic screens, analyses of expressed sequence tags (ESTs) or partial genome examination.

2.3.10.1 Homology search using BLASTp

An in silico genomic exploration of *A. terreus* genome was performed to identify components of G-protein/cAMP-mediated signalling in this species as well as two other *Aspergillus* species: *A. clavatus*, and *A. flavus*. components of G-protein/ cAMP-mediated signalling were previously identified in *A. nidulans, A. fumigatus* and *A. oryzae* (Han et al., 2004, Lafon et al., 2006).

The search included: I) putative GPCRs that are distributed over nine classes GPCRs (gprA-Q) and nopA II) Heterotrimeric G-protein constituents III) proteins with a regulator of G-protein signalling (RGS) domain IV) genes that encode adenylase cyclise, two catalytic subunits and regulatory subunit of cAMP-dependant protein kinase (PKA).

The 18 GPCRs are distributed among nine classes and theyinclude *gprA(PreB), gprB(PreA), gprC-E, gprF, gprG, gpH, gprI, gprJ, gprL, gprK, gprM, gprN, gprO, gprP, gprQ,* and *nopA*. Heterotrimeric G-protein constituents include three Gα subunits (FadA, GanA, GanB), one Gβ subunit (SfaD), one Gγ subunit (GpgA). Proteins with a regulator of G-protein signalling (RGS) domain are: FlbA, RgsA-C.

The Basic Local Alignment Search Tool (BLASTp) available from the National Centre for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/BLAST/ , NCBI) was used to explore the genome of *A. terreus*, *A. clavatus,* and *A. flavus.* Similar proteins identified in *A. terreus*, *A. clavatus,* and *A. flavus* with 100 % coverage and more than 30% identity to the model filamentous fungi *A. nidulans* was reported.

2.3.10.2 Protein alignments and phylogenetic analyses

The identified components of G-protein/cAMP-mediated signalling in *A. terreus*, *A.clavatus,* and *A. flavus, A. nidulans, A, fumigatus,* and *A.oryzae*were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic trees with a neighbour joining approach were then generated using these alignments without the omission of positions containing high number of gaps.

Chapter III

RESULTS

3.1 *Penicillium sclerotiorum* studies

3.1.1 Overview of the results

The first part of this chapter (Section 3.1) describes the work done using the filamentous fungus *P. sclerotiorum.* This work addresses the detection and initial identification of putative quorum sensing molecule(s) in the ethyl acetate culture extracts of the fungus.

Quorum sensing molecules are produced and released to the extracellular environment by the communicating microorganism(s). Their concentration(s) increases with the microbial cell density and a physiological response is made only when their concentration reaches a critical threshold.

The first steps in this study were performed to 1) investigate the presence of putative QSM(s) in the culture, 2) to determine whether their concentration is correlated with the fungal cell density, and 3) to indicate the time-point at which the threshold concentration is attained.

Extract(s) were prepared from the supernatant(s) of *P. sclerotiorum* cultures. Different time points of the fungal growth curve, corresponding to different fungal cell-densities, were selected for solvent extraction. The effect of the prepared culture extracts were tested on three physiological responses. The three physiological traits investigated in this study were: sporulation (spore production), hyphal morphology (i.e. branching) and secondary metabolism (sclerotiorin).

The possibility of using culture extract(s) to improve the productivity of useful secondary metabolites produced during fungal fermentations was then explored. A statistical approach using response-surface methodology (RSM) was designed to test optimal concentrations of day 7- culture extract and its time of addition that could lead to enhanced yields of sclerotiorin, an industrially useful secondary metabolite produced by *P. sclerotiorum.* The optimal conditions proposed by this model were then validated in shaken flasks. A scale-up to a 2L bioreactor was also performed to investigate whether the optimal conditions for sclerotiorin production are maintained at a larger scale (compared to shaken flasks).

Initial investigation to identify the putative quorum sensing molecules was then carried out, through separation of the culture extract using TLC (thin layer chromatography) followed by analysis using GC-MS (Gas chromatography-Mass spectrometry).

3.1.2 Preparation of *P. sclerotiorum* culture extracts

Initial studies conducted within our research group by Raina et al. (2010) suggested a putative quorum sensing behaviour in *P. sclerotiorum.* However, no studies were previously conducted to correlate the increasing fungal cell density to the accumulation of quorum sensing molecules in the supernatant of the culture, and their impact on different physiological traits regulated by quorum sensing.

P. sclerotiorum was grown in Raulin-Thom medium to different cell densities. Fermentation samples were collected at four different time points of growth as indicated by the arrows on Figure 3.1. The selected time-points were days 2, 4, 6 and 8 of the fermentation. Day 2-extract indicates mid-exponential phase; day 4-extract, end of exponential phase or early stationary phase; day 6-extract, mid-stationary phase, and day 8-extract, late stationary phase or early-death phase.

After the removal of the fungal cells from the culture broth via vacuum filtration, the culture supernatants collected at the different cell densities were extracted with ethyl acetate and concentrated 50 folds. The concentrated extracts were tested for their effect on three QS-regulated physiological traits: sporulation, hyphal branching and secondary metabolite production (sclerotiorin).

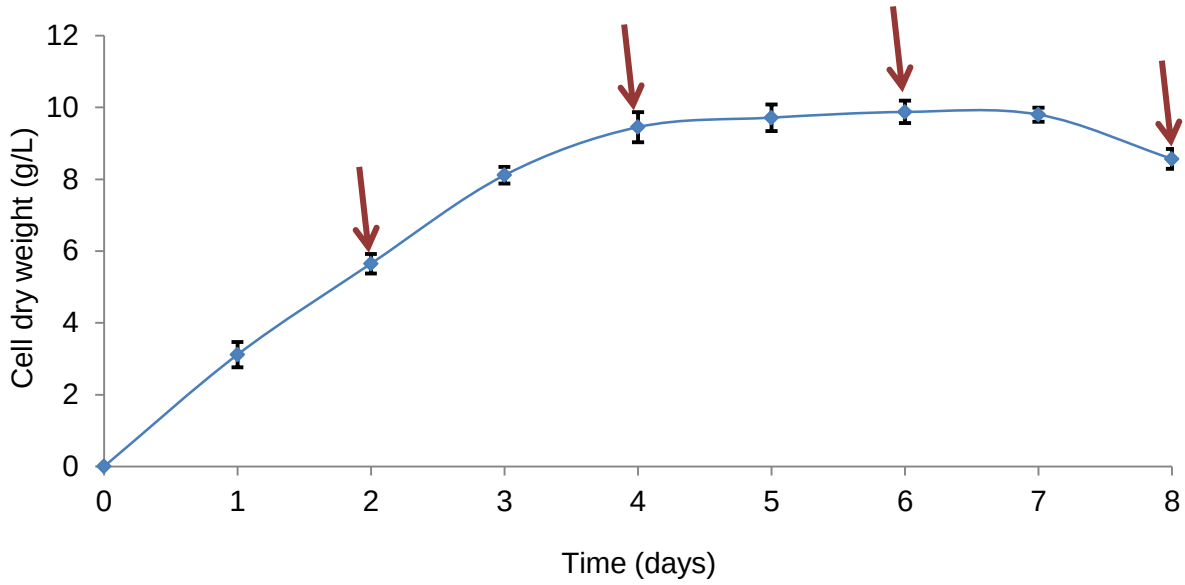


Figure 3.1: Growth profile of *P. sclerotiorum* in Raulin-Thom medium. *P. sclerotiorum* spores were inoculated in Raulin-Thom medium (10^7 spores/mL) and incubated at 27℃ at 180 rpm for 8 days for the production of putative quorum sensing molecule(s). Cell dry weight was measured throughout the fermentation (Days 0 to 8). The reported values are means of three replicates. Error-bars represent standard error of means. The arrows represent the days when culture broth was collected for solvent extraction.

3.1.3 Sporulation

3.1.3.1 Sporulation assay

For the sporulation assay, 30μL of the culture extracts were added to the 6mm filter discs on Czapek-Dox agar plates. Same volume of pure ethyl acetate was used as solvent control. Czapek-Dox agar plates were inoculated with *P. sclerotiorum* spore suspension. The spore production of *P. sclerotiorum* was counted after 5 and 7 days of incubation. Addition of low cell density culture extracts; day 2-extract did not have any significant effect on spore production as compared to the control ($p>0.05$).This indicates that the quorum sensing molecule(s), had a concentration below the threshold; thus exerted no significant changes on the spore production or the QSM was synthesised by *P. sclerotiorum* at the later stages of growth.

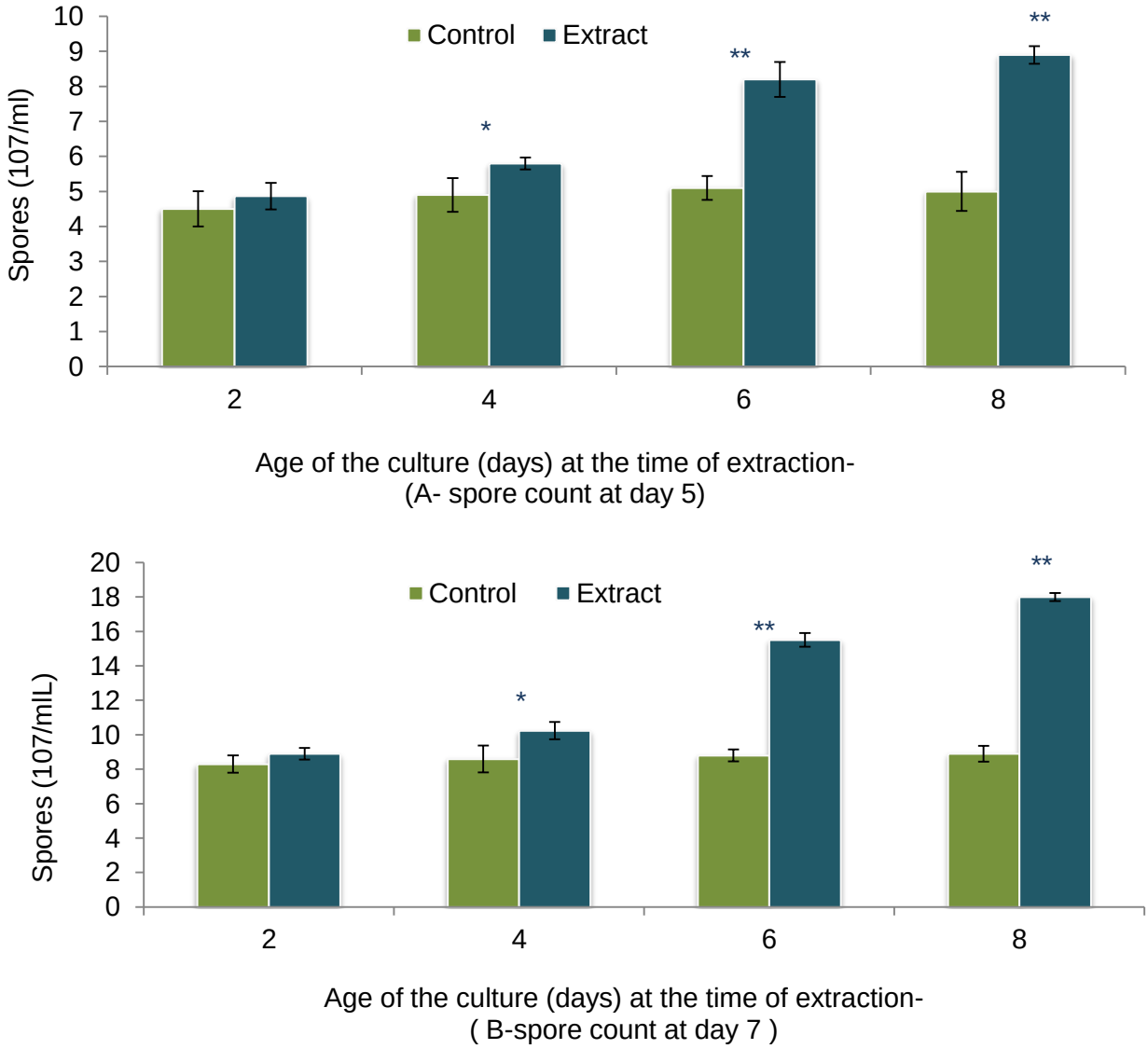


Figure 3.2: Effect of culture extract(s) prepared from different cell densities of *P. sclerotiorum* culture on the spore production. Spore count was done on the 5thday (Fig 3.2a) and 7th day (Fig 3.2b) of the fermentation. Values are means of three replicates. Error-bars represent standard error of means. * indicates $p<0.05$ ** $p<0.005$ (by Unpaired T-test assuming equal variances)

By contrast, agar plugs removed from areas surrounding filter discs treated with extracts from high cell densities; day 6-extract and day 8-extract, had a significant increase in the spore count as compared to the control. For instance the spore count from culture areas surrounding filter discs treated with day 6-extract and day 8-extract, increased by 1.8 and 2 folds after 7 days respectively ($p\leq0.005$) (Figure 3.2b).

3.1.3.2 Sporulation on agar plates

To determine how the volume of the culture extract effects sporulation; two volumes of day 6-extract (30μL and 50μL dissolved in a total of 100μL of methanol) were added to the surface of a Czapek-Dox modified agar plate, which was then spot-inoculated in the centre with 10μL of *P. sclerotiorum* spore suspension.

The effect of the two different high cell density extract volumes was visually checked after 10 days, and compared to the control where no extract was added. Macroscopic images of the plates showed that the addition of day-6 extract as well as its volume induces sporulation of the fungus. Control plates (solvent only, no extract) had limited or no sporulation as compared to the plates in which 30μL and 50μL of extract was added respectively (Figure3.3). Sporulation is indicated by the green colour of *P. sclerotiorum* spores.

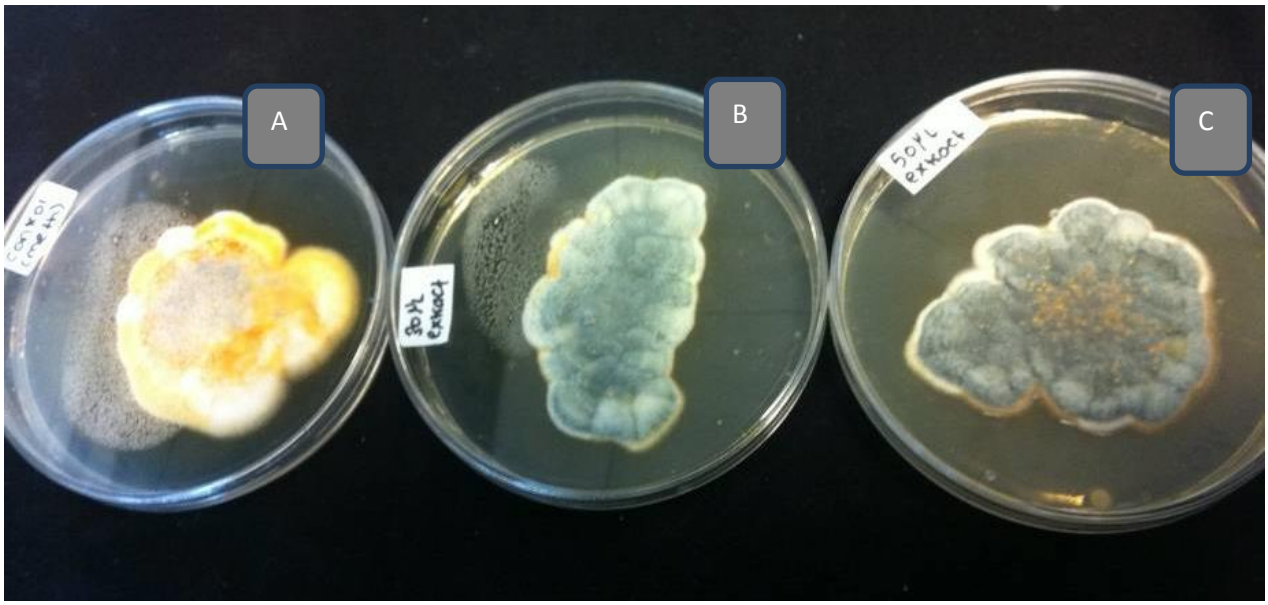


Figure 3.3: Effect of different amounts of the high cell density culture extract (day-6 extract) on *P. sclerotiorum* sporulation. A high cell density culture extracts (day 6-culture extract), dissolved in methanol as a solvent, and was added to the surface of the agar plate. The plates were then spot inoculated at the centre by *P. sclerotiorum* spore suspension (1x 10^7spores/mL). Plate A) Control: methanol only, plate B) 30 μL of extract and C) 50 μL extract.

3.1.4 Hyphal Branching

The second physiological trait investigated in this section was hyphal branching. The effect of the culture extracts from different *P. sclerotiorum* cell densities was tested on *P. sclerotiorum* hyphal branching in liquid culture. The different extracts were added at 18 hours post inoculation. Microscopic images were taken at 24 hours and 36 hours. There was no significant effect on hyphal branching in all conditions after 24 hours (results not shown).

Addition of low cell density culture extracts (day 2-extract and day 4-extract) did not have significant effects on the number of branched hyphae as compared to the solvent control. However when *P. sclerotiorum* liquid cultures were treated with extracts from higher cell density (day 6-extract and day 8-extract); there was a delay in the onset of branching of the hyphae of the fungus *P. sclerotiorum*; this was indicated by the decrease in total number of hyphae containing branches at the same time-point (at 36 hours) (Figure3.4).

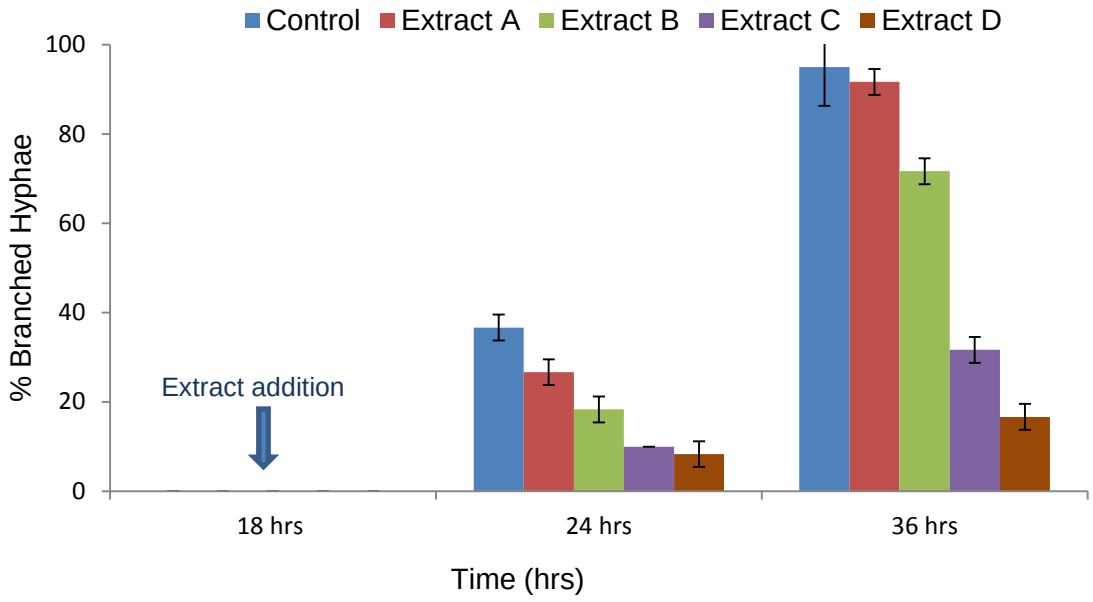


Figure 3.4: The effect of *P. sclerotiorum* culture extracts on the percentage of branched hyphae in *P. sclerotiorum* submerged culture. The ethyl acetate extracts were added at 18 hours post inoculation. Percentage of hyphae with branches was counted after 24 hours and 36 hours. Each bar represents the mean obtained from three replicates corresponding to each test and control. **Control:** 100µL filter sterilized ethyl acetate only; Extract A (day2- Extract)-extract from 2-day old culture; Extract B (day4-extract)-extract from 4-day old culture; Extract C (day6- Extract):extract from 6-day old culture and Extract D (day8- Extract): extract from 8-day old culture.

Results showed a correlation between the age of the culture extract, cell density, and overall branching of the fungus. The addition of culture extracts delayed the onset of lateral branching by this fungus as illustrated by the microscopic observations (Figure3.5) of samples taken at different time-points.

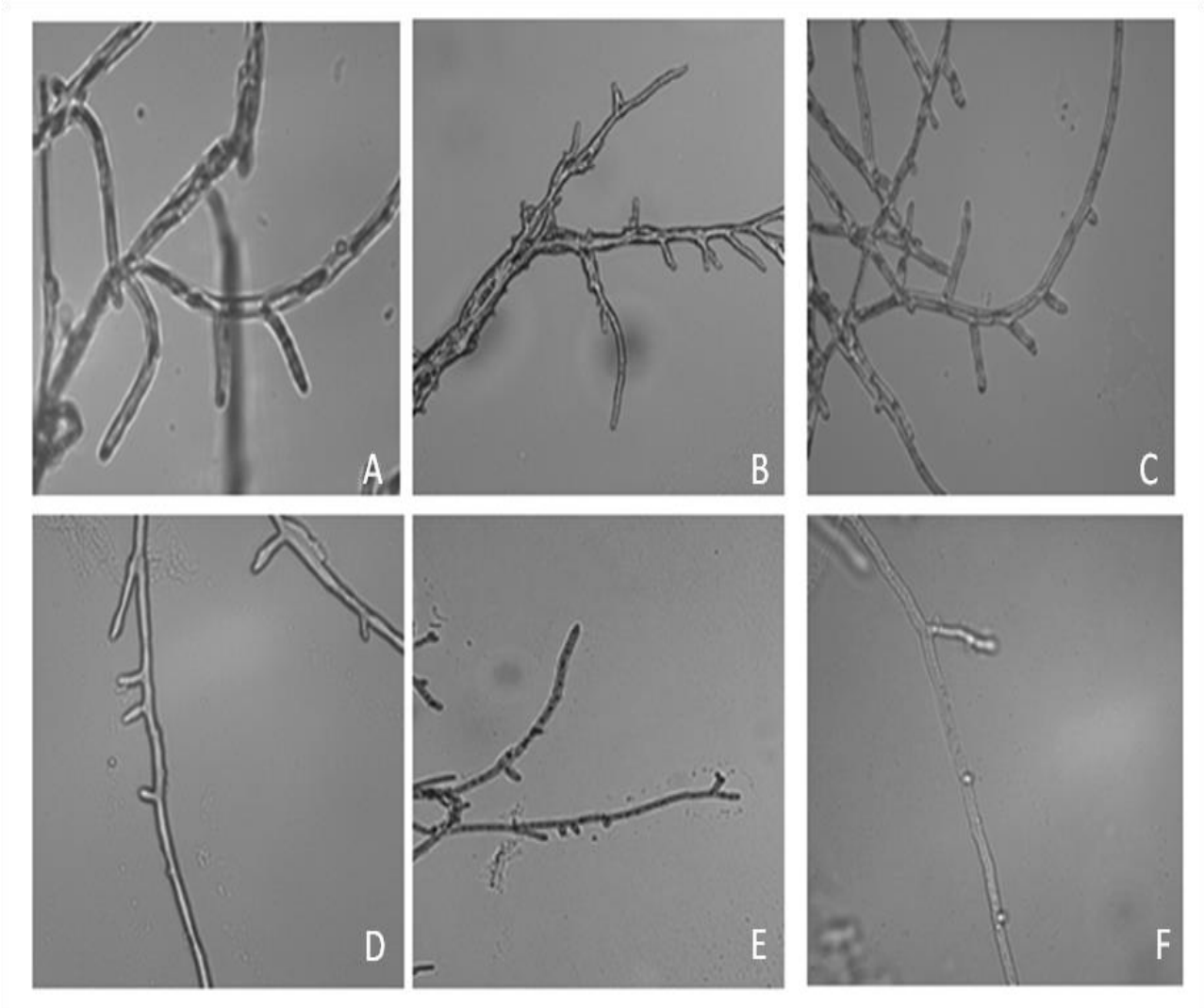


Figure 3.5: Effect of different cell density culture extracts on the hyphal branching in *P. sclerotiorum* submerged culture. Representative images of each condition were taken using a Leica Microscope (Leica, UK) at 4,000 x magnification. Ethyl acetate extracts (100µL) from different days of *P. sclerotiorum* culture were added to the culture at 18hrs post-inoculation. The images were taken at 36hours post inoculation A) Negative control (Nothing was added to the *P. sclerotiorum* culture) B) Positive control, addition of 100µL filter sterilized ethyl acetate C)day 2-extract was added D) day 4-extractwas added E) day 6-extract was added F) day 8-extract was added.

3.1.5 Secondary metabolite production

Two experiments were conducted to investigate whether the addition of the culture extracts has any impact on the production of sclerotiorin, a secondary metabolite produced by the fermentation of *P. sclerotiorum.* In the first experiment extracts from different cell densities were tested on sclerotiorin production by *P. sclerotiorum* on solid surface (agar plates), whereas the second experiment tested sclerotiorin production in submerged cultures of *P. sclerotiorum* using RSM.

3.1.5.1 Secondary metabolite production on agar plates

The effect of the extracts on secondary metabolite production was macroscopically visualised on plates (Figure 3.6). Agar plates that were inoculated at the centre using *P. sclerotiorum* agar plugs were treated with ethyl acetate extracts from different cell-densities. The production of industrially useful secondary metabolite, sclerotiorin was identified by the strong orange colour on plates and in submerged culture (Lucas et al. 2010). It was shown that extracts from low cell-density cultures had no detectable effects on the secondary metabolite production as compared to the control; this was indicated by the absence of orange colour indicative of sclerotiorin presence (Figure 3.6).

Plates treated with day 4-extract, day 6-extract and day 8-extract corresponding to plates C, D, E respectively in Figure 3.6 showed that extracts from higher cell densities; i.e. later periods of the fungal growth profile increased secondary metabolite production compared to the control plate and plates treated with extracts from low cell density, day 2-extact, corresponding to plates A, and B respectively; the result was evidenced by the intense orange colour of sclerotiorin.

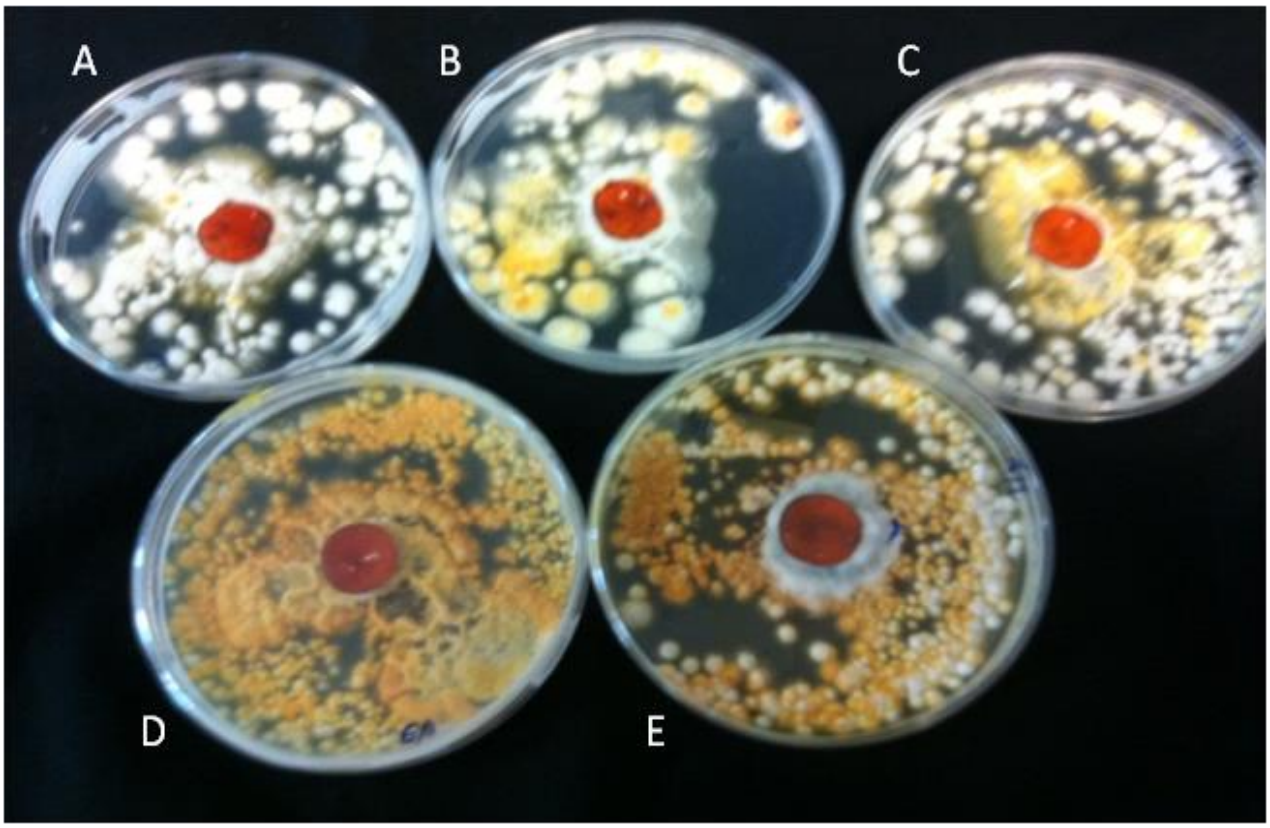


Figure 3.6: Effect of culture extracts from different cell densities of *P. sclerotirum* on the production of the secondary metabolite, sclerotiorin, by *P. sclerotiorum*. The extracts were added to the agar surface then inoculated by *P. sclerotiorum* agar plugs. Plates A) Control: 3mL filter sterilized ethyl acetate only B) day2- Extract C) day4- Extract D) day6- Extract E) day8- Extract.

3.1.5.2 Secondary metabolite production in liquid culture

Based on the above experiments, it was found out that higher cell density extracts day 6-extract and day 8-extract had major effect on the spore production, hyphal branching and secondary metabolite production (agar surface) of the fungus. This finding suggested that the extract contains a high concentration of putative quorum sensing molecules. Therefore it was decided to test how that high cell density extracts contribute to secondary metabolite production in liquid culture (shaken flasks).

The effect of high cell density extract, prepared from day 7 of the growth profile (average day between day 6 and day 8); its % concentration (vol/vol) and the time of addition to the culture were investigated on the production of sclerotiorin. This

approach was carried out using response surface methodology (RSM) in order to find the optimal conditions for the enhanced production of sclerotiorin in shaken flasks.

A two-factor central composite design (CCD) was constructed consisting of 28 runs, including 4 centre points, 4 replicates of axial star points, and 2 replicates of factorial points. The design variables were the percentage of culture extract concentration added (X1, %) and the addition time (X2, days), whereas the two monitored responses were the cell dry weight (R1, g/L) and sclerotiorin yield (R2, mg/g). The sclerotiorin yield, quantified via HPLC, and the cell dry weight were measured on the 8th day of the course of fermentation. Sclerotiorin standards in the range of 0-100 µg/mLwere run on HPLC (Figure3.7) to obtain a standard curve (Figure3.8).

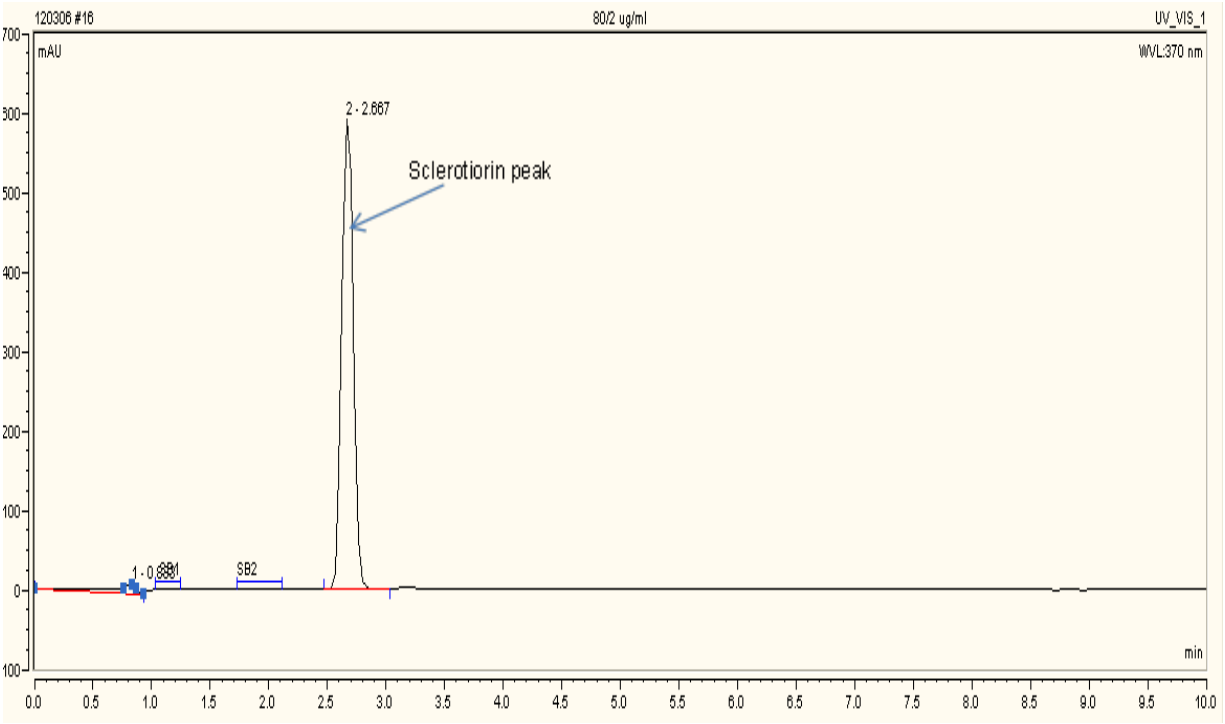


Figure 3.7: HPLC chromatogram of a sclerotiorin standard. Sclerotiorin eluted after 2.7min.

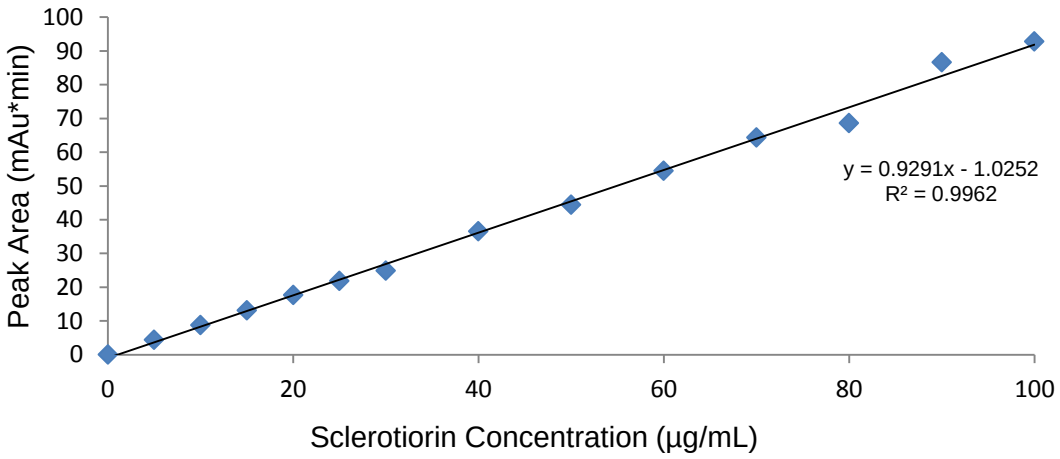


Figure 3.8: Sclerotiorin standard curve. The standard curve was prepared using a sclerotiorin standard over a concentration of 0-100 µg/mL using HPLC system.

The 3D response surface and the 2D contour plots for the two responses (sclerotiorin production and the cell dry weight) are the graphical representations of the regression equation. Both plots are represented in Figure 3.9. The main goal of response surface is to detect the optimum values of the two tested variables such that the response is maximized. Each plot depicts the interaction of the two variables and represents an infinitive number of their combinations. Based on the obtained results, it was found that the addition of high cell density extracts has a limited or no impact on the cell dry weight (Figure 3.9a and Table 3.1a). However, sclerotiorin production increases with the increasing concentrations of the added culture extract (Figure 3.9b). Point prediction of the design expert software was used to determine the optimum values of the factors for maximum sclerotiorin production as it is difficult to analyse the response surface plots simultaneously. The optimum values (with a desirability of 1.00) for maximum production was a concentration of 0.15% (0.1472%)(vol/vol) crude extract; to be added after 2.06 days. The predicted optimum values for the extract concentration and its addition time were validated in shaken flask studies and taken to scale-up in a 2L stirred tank reactor.

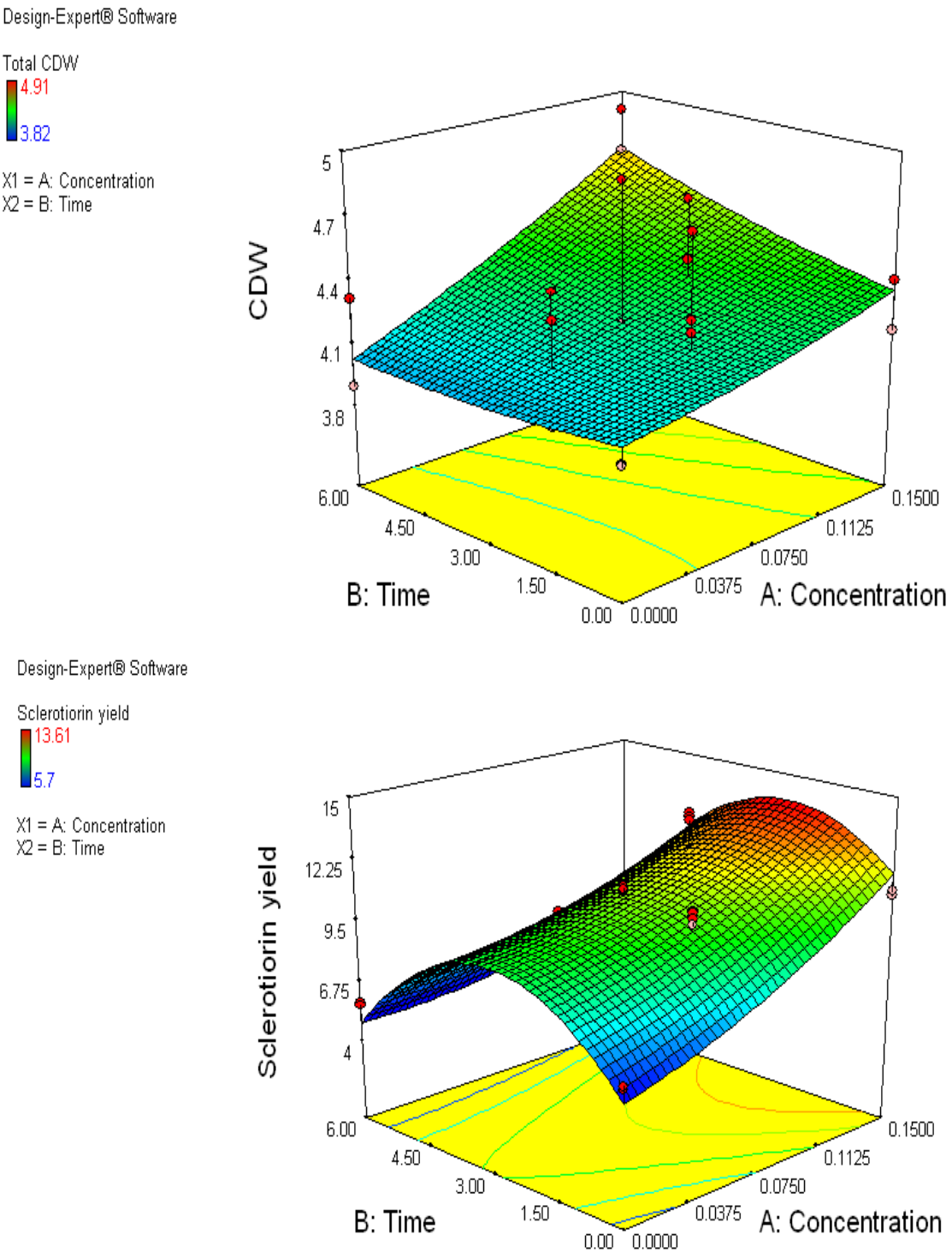


Figure 3.9: Response surface plot (3D) showing the effect of the high cell density culture extract of *P. sclerotiorum* on sclerotiorin production and cell dry weight. The effect of two factors concentration of culture extract and its time of addition were investigated on A) cell dry weight (CDW g/L) and B) Sclerotiorin yield (mg/g of CDW) in shaken flasks.

3.1.5.3 Statistical Analysis

Table 3.1: ANOVA for response surface quadratic model. a) ANOVA table response for the cell-dry weight, and b) ANOVA table for sclerotiorin prodcution

a) Cell dry weight

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	0.73	5	0.15	2.01	0.1176 (not significant)
Residual (error)	1.6	22	0.073		
Lack of fit	0.27	3	0.09	1.28	0.3095 (not significant)
Pure error	1.33	19	0.07		
Total	2.34	27			

R^2 = 0.3131; CV = 6.33 %; SS, sum of squares; DF, degrees of freedom; MS, mean square; Adj R^2 = 0.1570

The ANOVA of quadratic regression model demonstrates that the model is not significant, also evident from the Fisher's *F*-test with a probability value [($P_{model} > F$) = 0.1176)]. On the other hand the low value of the coefficient of variation (CV %= 6.33) indicates precision and reliability of the experiment.

b) Sclerotiorin Production

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	126.22	5	25.24	23.72	<0.0001 (significant)
Residual (error)	23.41	22	1.06		
Lack of fit	22.88	3	7.63	271.00	<0.0001 (significant)
Pure error	0.53	19	0.028		
Total	149.63	27			

R^2 = 0.844; CV = 10.77 %; SS, sum of squares; DF, degrees of freedom; MS, mean square; Adj R^2 = 0.808

The ANOVA of quadratic regression model demonstrates that the model is highly significant, also evident from the Fisher's *F*-test with a very low probability value [($P_{model} > F$) = 0.0001)]. On the other hand the low value of the coefficient of variation (CV %= 10.77) indicates precision and reliability of the experiment.

3.1.5.4 Results Validation

The optimum conditions were validated in shaken flasks. High cell density extracts were added after 2.06 days at a concentration of 0.15 % (vol/vol) as determined by the Design Expert software. Sclerotiorin yield, pH and total carbohydrate consumption were determined throughout the course of the fermentation. A control culture was run in parallel, to be used for comparison. It was found that the addition of extract at the optimum conditions (concentration and addition time) had no impact on the total carbohydrate consumption (Figure 3.11) and the pH (Figure 3.12). However, the increase of sclerotiorin production yield (Figure 3.13) was validated, and the maximum reported sclerotiorin yield was13.89 mg/g, around 2.1 folds increase compared to the control.

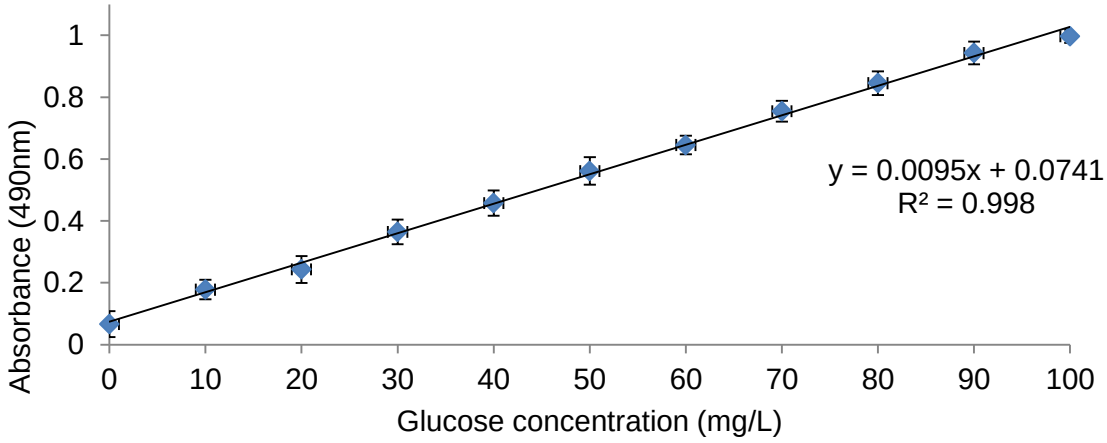


Figure 3.10: Glucose standard curve. The standard curve was prepared using glucose over a concentration of 0-100 µg/mL using phenol sulphuric acid assay. Values are means of three replicates. Error-bars represent standard error of means.

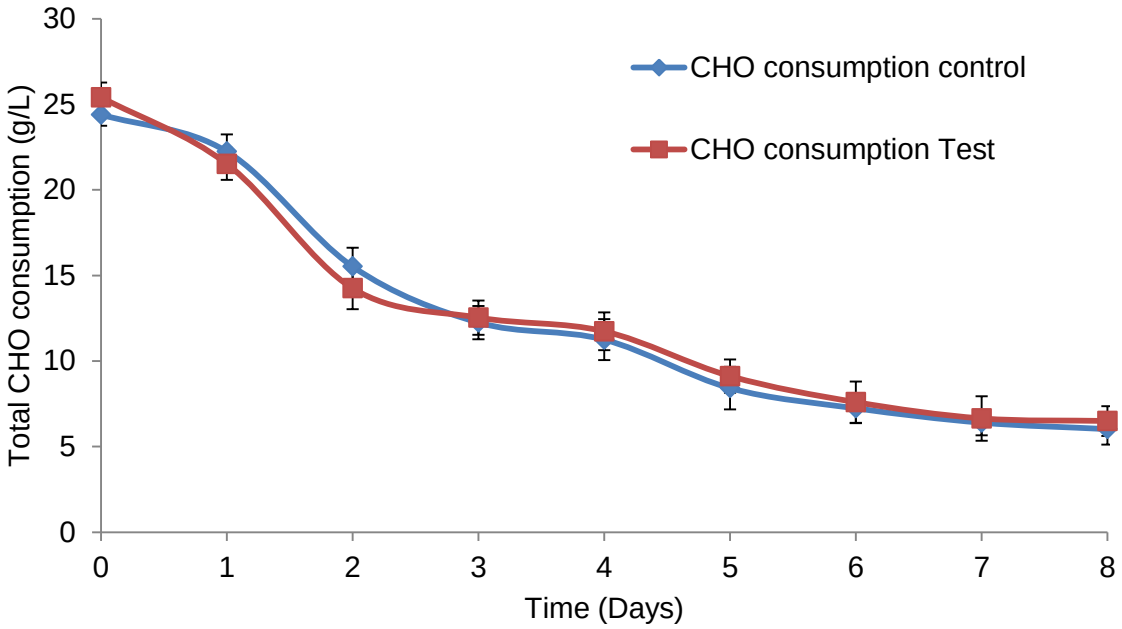


Figure 3.11: Effect of high cell density culture extract on the carbohydrate consumption by *P. sclerotiorum*. Test culture refers to the addition of culture extract at a concentration of 0.15 % after 2.06 days. Values are means of three replicates. Error-bars represent standard error of means.

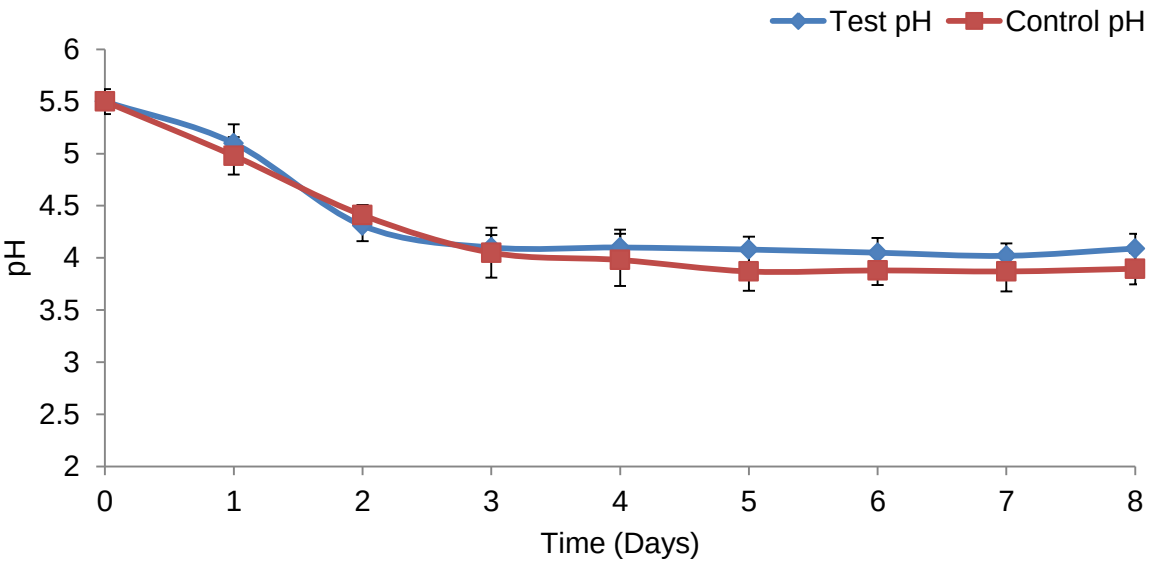


Figure 3.12: Effect of optimized conditions on the pH by *P. sclerotiorum.* Test culture refers to the addition of culture extract at a concentration of 0.15 % after 2.06 days. Values are means of three replicates. Error-bars represent standard error of means.

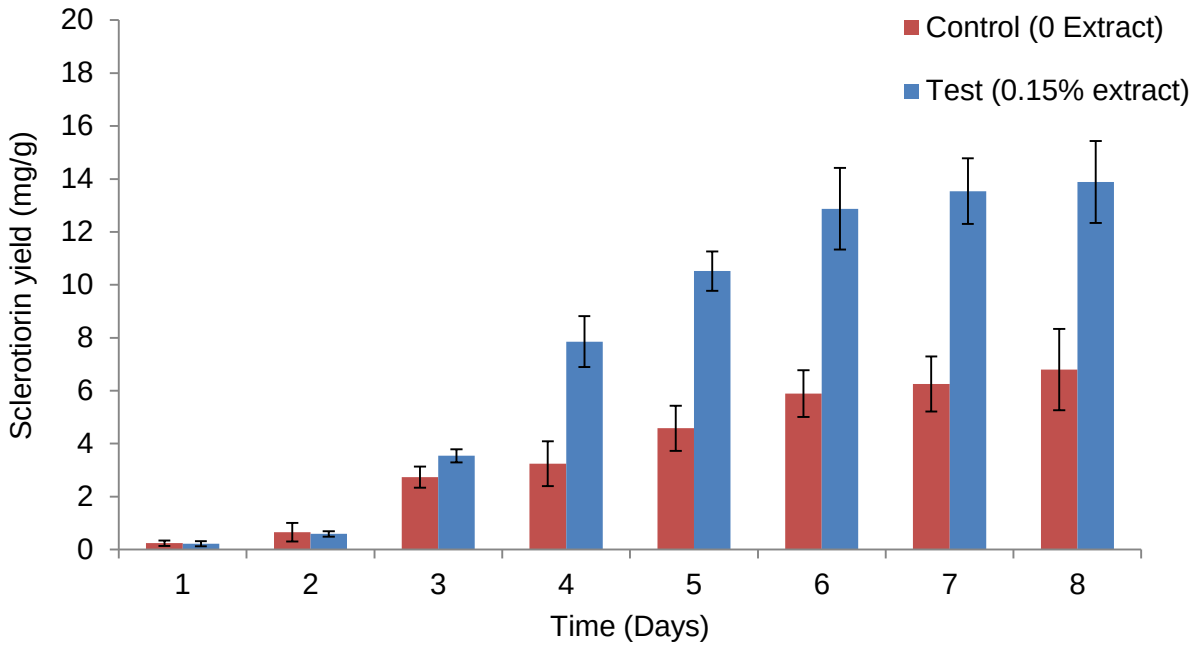


Figure 3.13: Effect of optimized condition on sclerotiorin production by *P. sclerotiorum.* Test culture refers to the addition of culture extract at a concentration of 0.15 % after 2.06 days. Sclerotiorin production yield is mg of sclerotiorin/g of *P. sclerotiorum* CDW. Values are means of three replicates. Error-bars represent standard error of means.

3.1.6 Bioreactor Studies

The obtained results were also used for scale-up from shaken flasks to a 2L stirred tank bioreactor (Figure3.14). The scale-up was performed to investigate whether sclerotiorin production is maintained and/or enhanced at a larger working volume, and to validate that results obtained in 500mL shaken flasks can be scaled-up.

Figure 3.14: *P. sclerotiorum* fermentation in 2L STR. The orange colour represents *P. sclerotiorum* mycelial pellets.

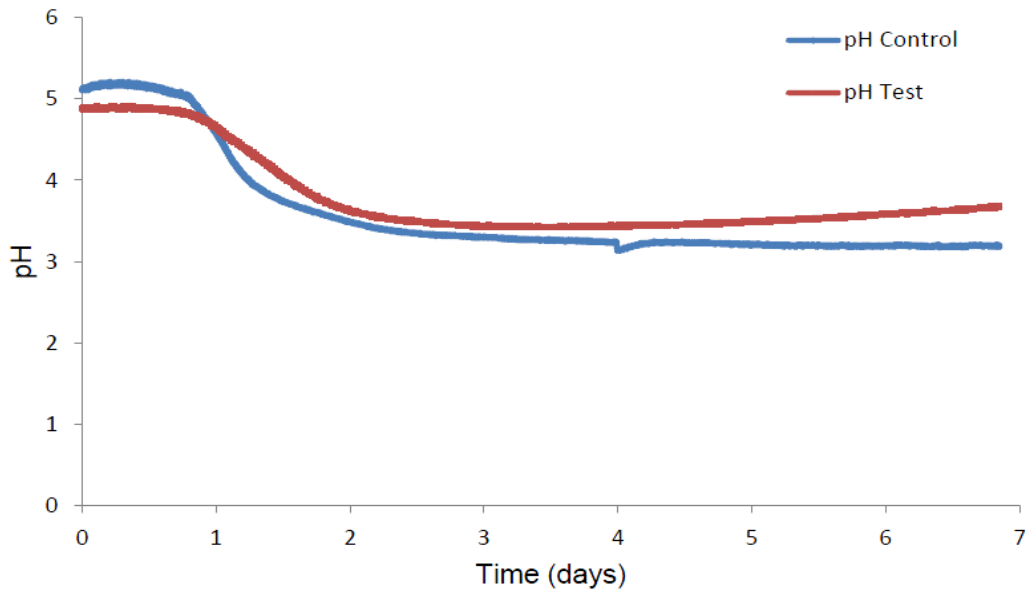


Figure 3.15: Effect of optimized conditions on the pH profile of *P. sclerotiorum* In culture in a 2L stirred tank reactor. Test culture refers to the addition of culture extract at a percentage of 0.15% (vol/vol) after 2.06 days.

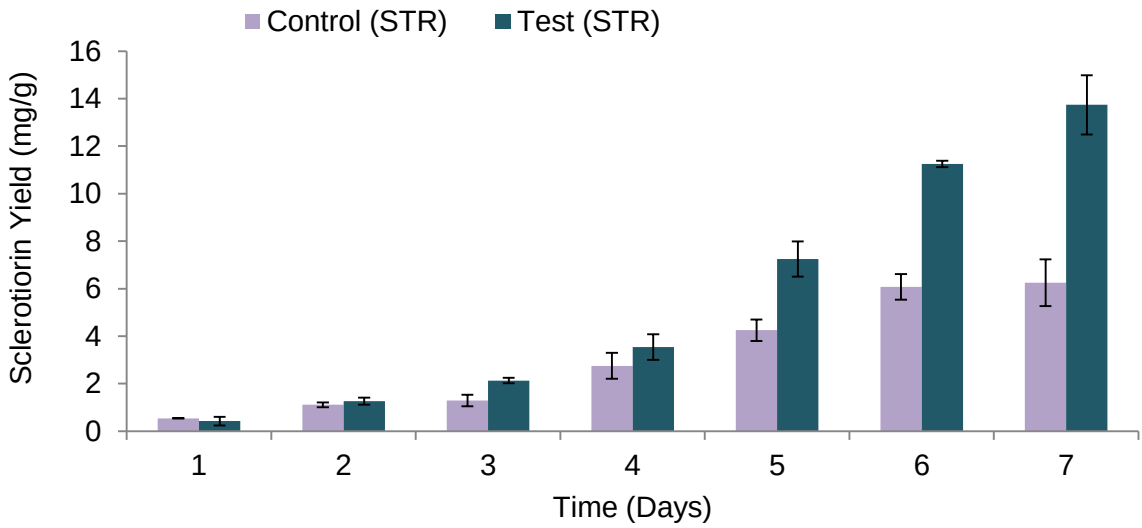


Figure 3.16: Effect of optimized conditions on sclerotiorin production by *P. sclerotiorum* in 2L Stirred tank bioreactor. Test culture refers to the addition of culture extract at a percentage of 0.15% after 2.06 days. Sclerotiorin yield was measured via HPLC. Values are means of three replicates. Error-bars represent standard error of means.

3.1.7 Thin Layer chromatography

The high cell density extracts obtained from the culture supernatant of *P. sclerotiorum* is a complex mixture of several compounds. The putative quorum sensing molecules contributing to the changes are constituents of this mixture. Thin layer chromatography (TLC) was performed to partially resolve and separate the constituent molecules.

Analytical TLC was performed using hexane:ethyl acetate at two ratios (6:4) and (3:7). The solvent mixture of hexane:ethyl acetate of (6:4) as a mobile phase resulted in a better separation of molecules. The RF values obtained in solvent mixture of hexane:ethyl acetate of (6:4) as a mobile phase were between (0.08) and (0.83); thus providing a good resolution (Figure 3.17a).

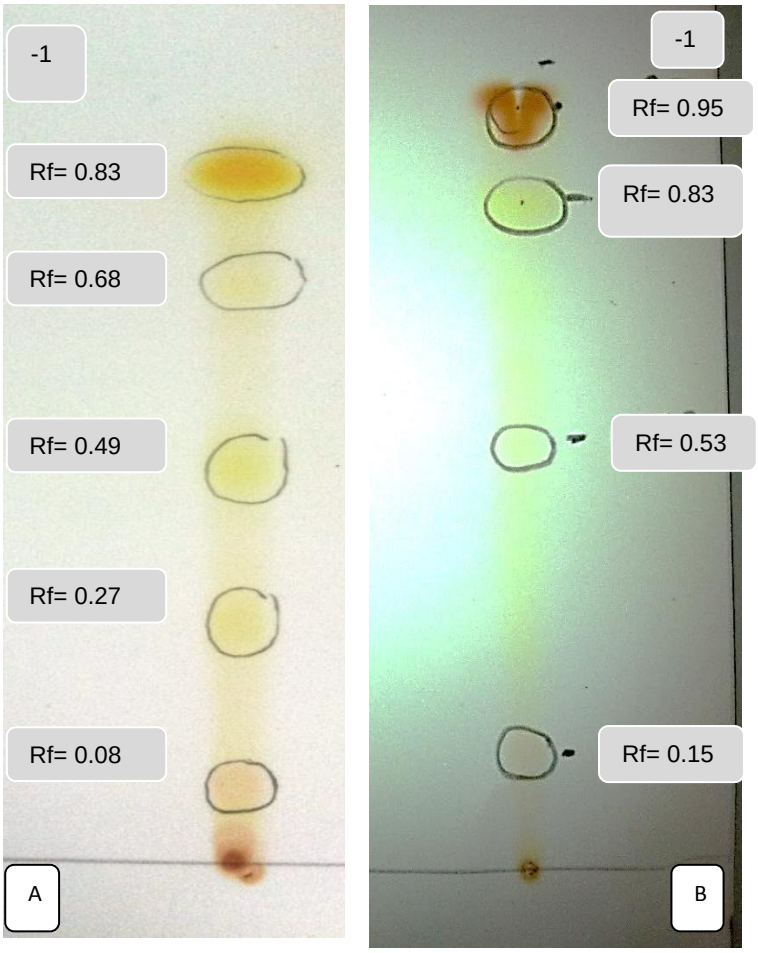


Figure 3.17: Analysis by TLC of different quantities of extracts of *P. sclerotiorum* at high cell densities. Supernatant of P. sclerotiorum, grown for 7 days, was extracted with ethyl acetate. The extract were analysed by TLC using both hexane:ethyl acetate (6:4) (panel A) and hexane:ethyl acetate (3:7) (panel B) as solvent systems for plate development.

Extracts from different cell concentrations (Days 4,5,6,7, and 8) were then separated via TLC, using the mobile phase of hexane: ethyl acetate (6:4). The TLC results in Figure 3.18 showed that the separation of the different components of the culture extracts as indicated with the several RF spots on the TLC plate. The extract was then analysed via GC-MS. The GC-MS profile is represented in Figure 3.19. Some of the identified compounds are represented in Table 3.2. The structure of ricinoleic acid and its ion chromatogram are represented in Figure 3.20 and Figure 3.21 respectively.

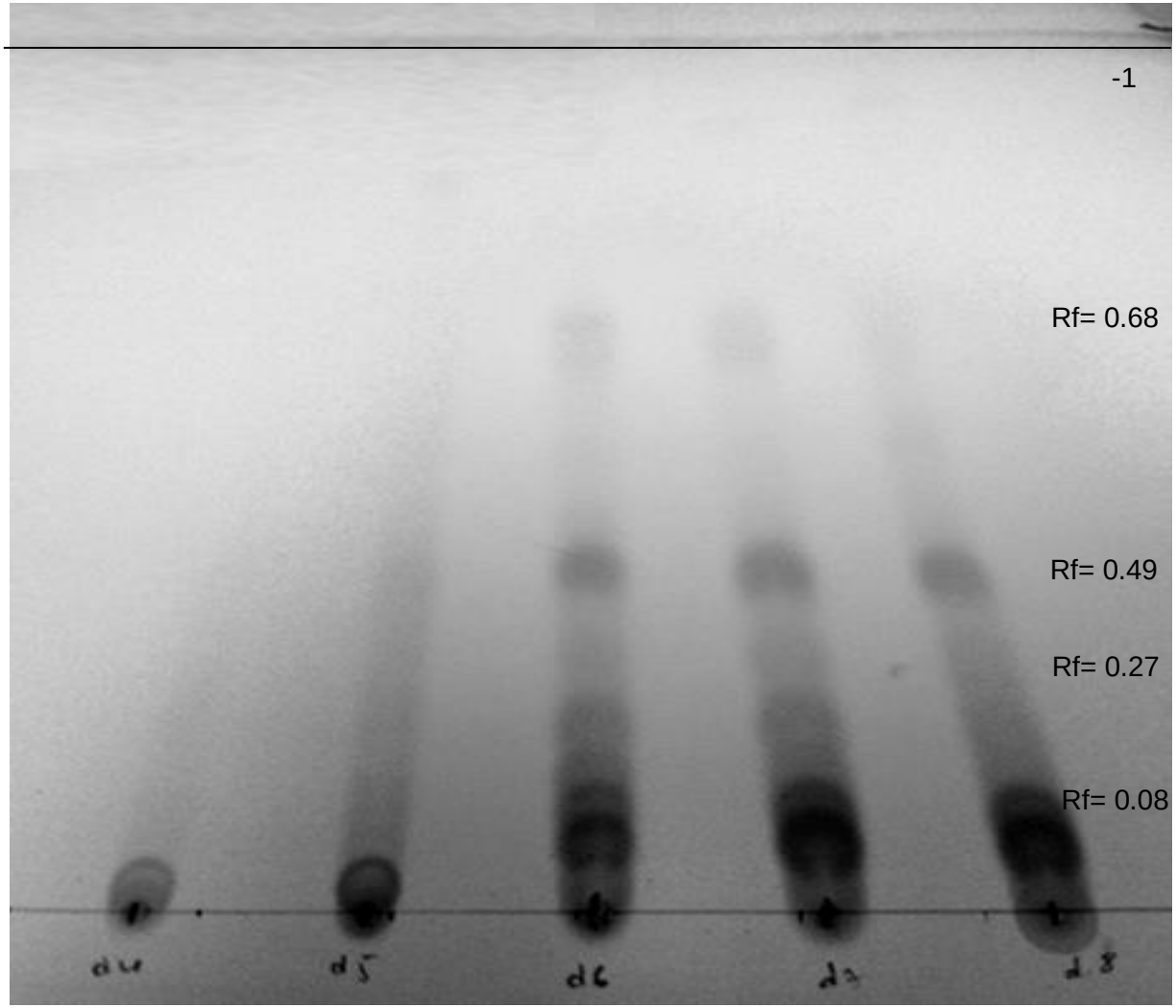


Figure 3.18: Analysis by TLC of extracts of *P. sclerotiorum* cultures at different cell concentrations. Supernatants of *P. sclerotiorum* cultures grown for 4, 5, 6, 7, and 8 days were extracted with ethyl acetate. A volume of 1 mL of each extract was analysed by TLC using hexane:ethyl acetate (6:4) as solvent system for the plate development. UV-visible compounds presented in the different extracts appear as dark spots when the plate was visualised at 254 nm.

3.1.8 Gas Chromatography-Mass spectrometry

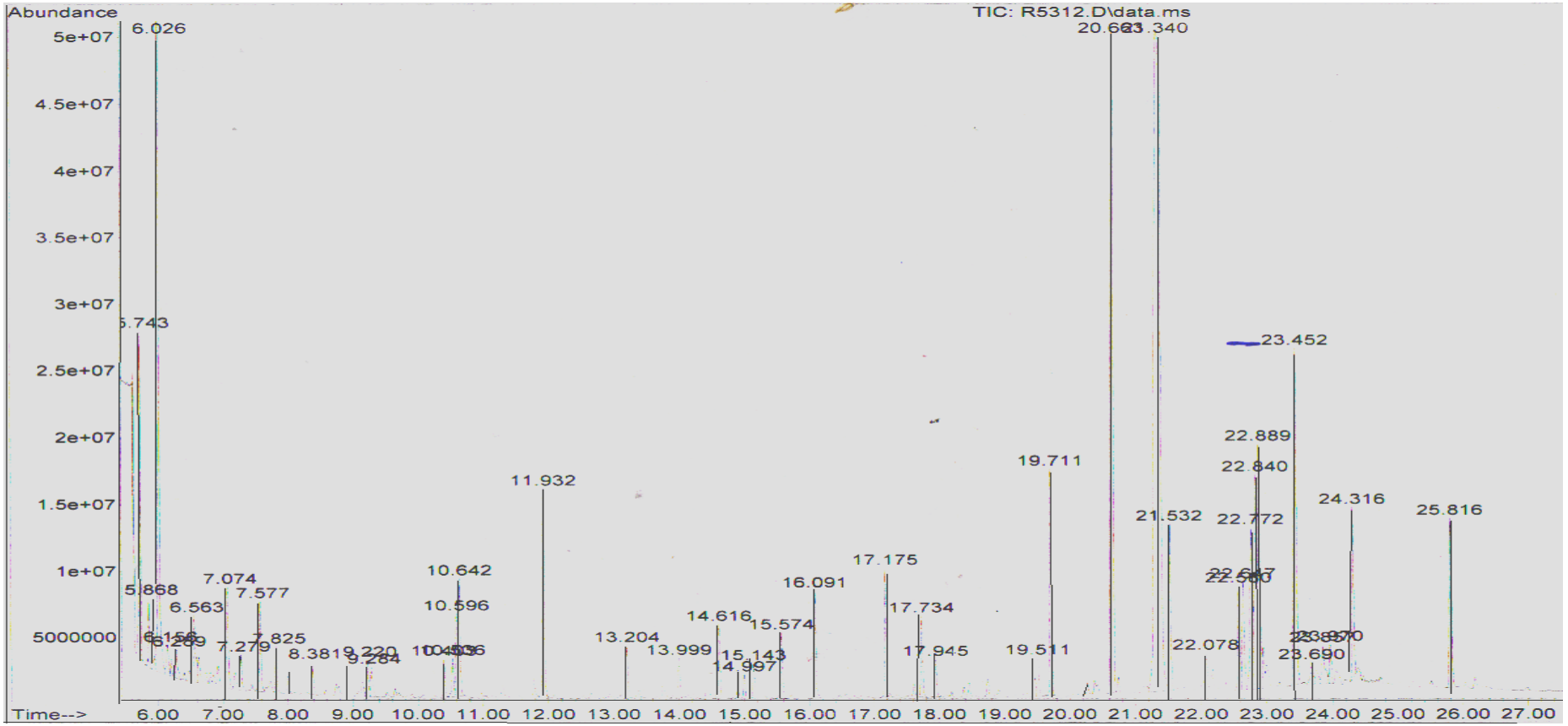


Figure 3.19: GC analysis of ethyl acetate extract of *P. sclerotiorum* culture supernatants grown in Raulin Thom medium for 6 days (derivatised with TMS). Analysis was carried out on a fused silica column (30 m x 0.25 mm I.D. X 0.25µm) coated with phenyl arylene polymer (J&W Scientific). Helium was used as the carrier gas at a flow-rate of 1 mL/ min. The temperature of the oven of the HP-6890 gas chromatograph, fitted with splitless mode injection. The oven temperature was programmed from 50° C to 290° C at the rate of 10° C per minute, and the final temperature was held for 6 minutes. Some of the peaks are be identified by reference to the retention times in Table 3.2.

Table 3.2: Compounds identified by GC-MS in *P. sclerotiorum* supernatant extracts. The analysis was carried out on an extract of a 7-day old culture supernatant of *P.sclerotiorum grown* in Raulin-Thom medium. The identification of the compounds present in the extract was performed by comparison of retention times and mass spectra with reference compounds contained in the NIST Mass Spectral library.

Retention Time (min)	Compound	Retention Time (min)	Compound
5.743	Benzene	25.816	Monostearin
5.868	3-Amino-N-butyric acid	15.574	Dodecanoic acid (Lauric Acid)
6.026	Trifluoromethyl ketone	17.175	Azelaic acid
7.279	2-Pyrrolidinone	19.711	Hexadecanoic acid (Palmitic acid)
7.577	Propanoic acid	20.663	Internal Standard-Heptadecanoic acid
7.825	Hexanoic acid	21.340	11-cis-octadecanoic acid
9.284	Butanoic acid	21.532	Octadecanoic acid (*Stearic acid)*
10.40	Benzoic acid	22.647	Myristic acid
10.642	Glycerol	22.840	9,12-octadecadienoic acid (Linoleic acid)
11.932	Nonanoic acid	22.89	Ricinoleic acid
14.616	Octanoic acid	24.316	9,12,15-octadecatrienoic acid (α-linolenic acid)

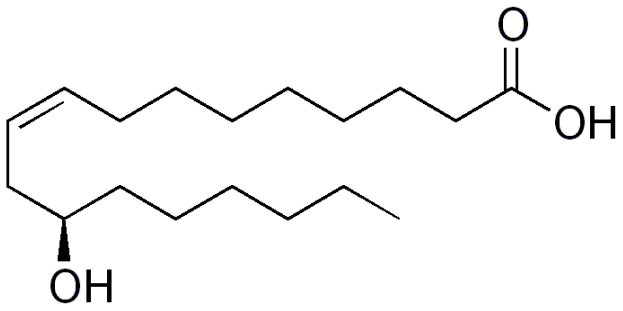


Figure 3.20: Structure of 12-hydroxy-9-cis-octadecenoic. The molecular formula for this compound is $C_{18}H_{34}O_3$and the molecular weight is 298.461 g/mol.

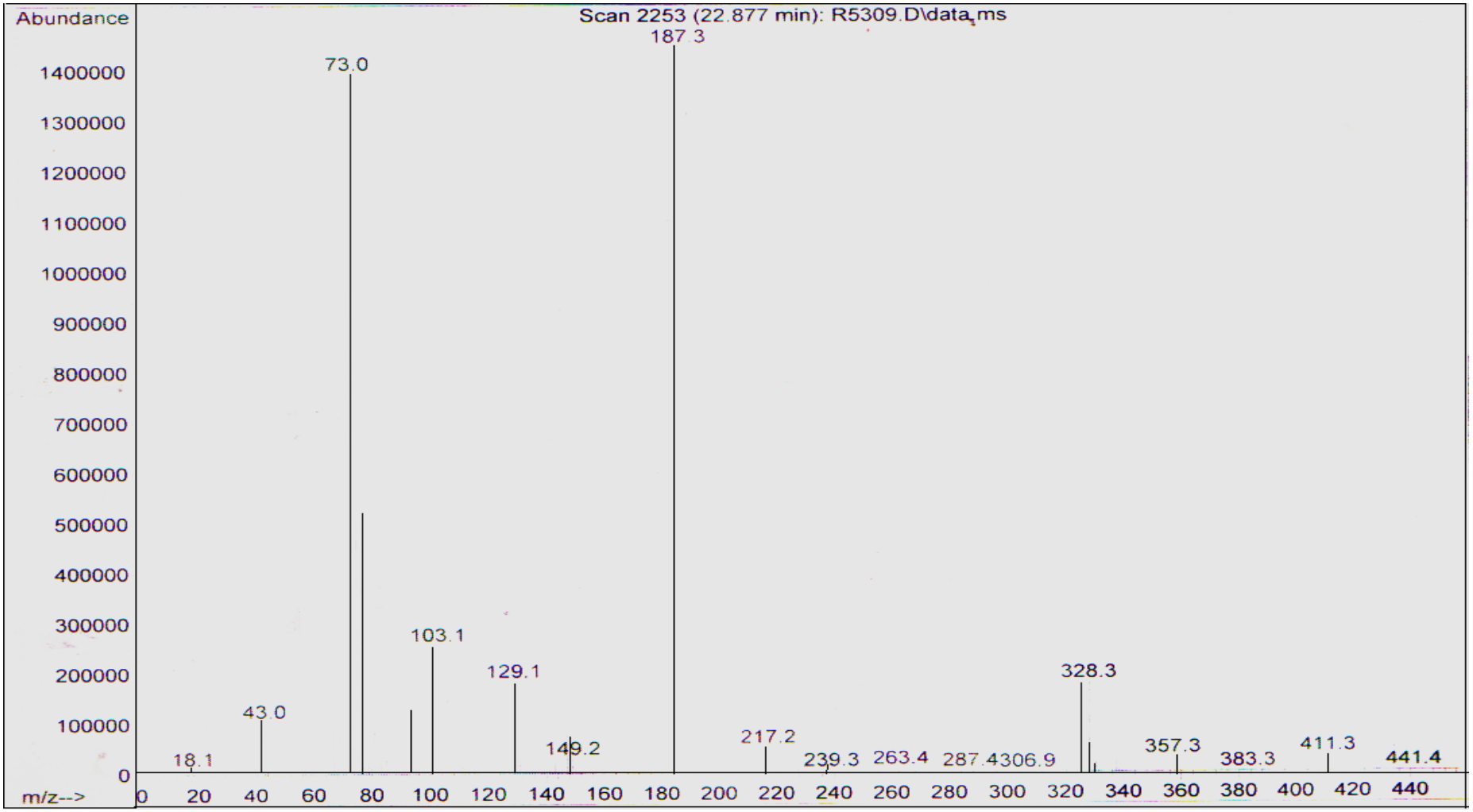


Figure 3.21 : Ion chromatogram of the compound with retention time of 22.89 min in the ethyl acetate extract of *P. sclerotiorum* culture supernatants grown for 7 days (derivatised with TMS) -Analysis was carried out on a fused silica column (30 m x 0.25 mm i.d.) coated with phenilarylene polymer (J&W Scientific). Helium was the carrier gas at a flow-rate of 1 mL/min. The temperature of the oven of the HP-6890 gas chromatograph, fitted with split injection (ratio 100:1), was programmed from 50° C to 290° C at the rate of 10° C per minute, and the final temperature was held for 6 minutes.

3.2 *Aspergillus terreus* studies

The second part of this chapter (section 3.2) describes the work done on the filamentous fungus *A. terreus.* This work addresses the role of oxylipins as signalling molecules, as well as the potential presence of G-protein/cAMP mediated signalling pathways in *A. terreus*. A previous study by Sorrentino et al. (2010) reports that the addition of linoleic acid at a concentration of 0.1% on the day of inoculation enhances the production of the useful secondary metabolite, lovastatin by *A. terreus,* as well as increases the transcription of lovastatin biosynthetic genes: *lovB* and *lovF*.

Linoleic acid is a polyunsaturated fatty acid (PUFA) that is converted to various oxylipins upon oxygenation. Oxylipins are regarded as signalling molecules, and they contribute to various developmental and morphological changes in fungal cultures including *A. nidulans, A. flavus* (Brown et al., 2008, Brown et al., 2009, Tsitsigiannis and Keller, 2007, Calvo et al., 2002). They are also involved in fungal interactions with plant and animal cells (Tsitsigiannis and Keller, 2007, Brodhun and Feussner, 2011).

This study investigates the impact of linoleic acid as an oxylipins precursor on lovastatin production and hyphal morphology of *A. terreus* in stirred-tank bioreactor (5L). In addition to bioreactor studies, the effect of linoleic acid on the expression of cytosolic proteins (intracellular proteome) was investigated via comparative proteomics. The expression of cytosolic proteins was studied using 2D- gel electrophoresis. Obtained 2D gels were analysed using Progenesis Spot analysis software and later identified using MALDI-TOF/TOF.

The impact of commercial oxylipins (9-HODE, 9-HpODE, 13-HODE) and linoleic acid on the intracellular cAMP levels in *A. terreus* was investigated to determine a putative link between linoleic acid (oxylipins precursor), and oxylipins to GPCRs (G-protein coupled receptors) signalling via the alteration of the cAMP levels.

A search of the different components of the G-protein and cAMP mediated signalling pathways was conducted by homology search of the *A. terreus* genome sequence using BLASTp as well as protein alignments and phylogenetic analyses to different *Aspergillus* spp.

3.2.1 Shaken Flask studies

In order to validate the results obtained in the previous study conducted within our research group by Sorrentino et al. (2010), linoleic acid was added at 0.1% concentration at day 0 of the fermentation to 100mL of *A. terreus* cultures in 500mL shaken flasks. The impact of linoleic acid on pH, total CHO consumption and lovastatin production was measured throughout the course of fermentation (10 days). Results shown in figure (3.20) confirm previous results suggesting that the addition of linoleic acid at a concentration of 0.1% enhances lovastatin production by 1.7 folds with no significant effect on pH and total carbohydrate consumption.

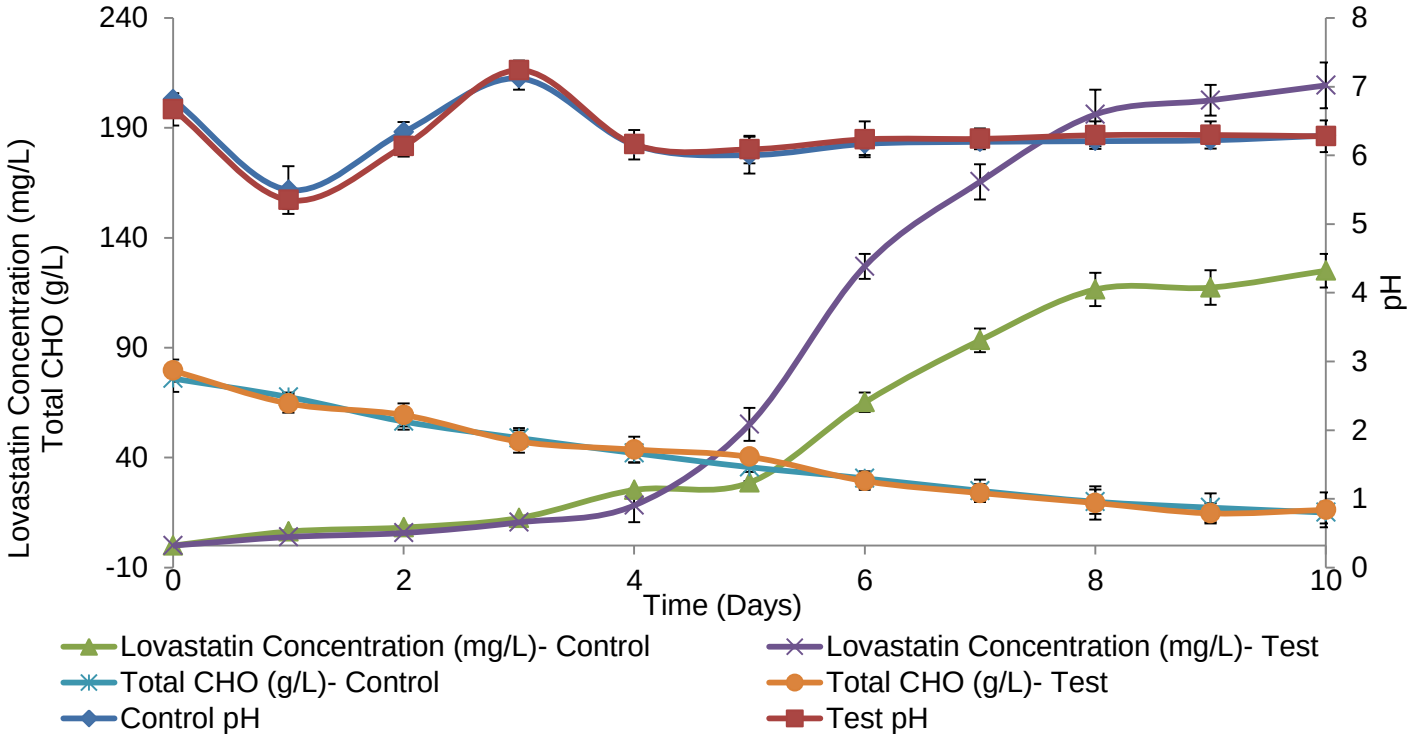


Figure 3.22: Effect of linoleic acid addition on lovastatin production, pH and total carbohydrate consumption in *A. terreus* cultures. Linoleic acid was exogenously added at a concentration of 0.1% on the day of inoculation (Fermentation day 0). *A. terreus*cultures were grown in lovastatin production medium and incubated for 10 days at 28˚C at 220 rpm. Lovastatin production, pH and total carbohydrate consumption was monitored throughout the fermentation. Values are the mean of three replicates, and error bars represent standard error of means.

3.2.2 Bioreactor Studies

In order to test whether the results obtained in shaken flasks were maintained upon scale up, a 5L stirred tank reactor (STR) was used. The original configuration of the bioreactors was prepared using two Rushton turbine impellers placed with a spacing of height/2 (height of liquid in the bioreactor). *A. terreus* fermentation in the 5L STRs under these parameters was repeated twice leading in both times in to a fungal coagulation on the upper sides of the fermenter (Fig 3.23a).

Figure 3.23: Fermentation of *A. terreus* in 5L Stirred tank reactor. a) Overgrowth of *A. terreus* in the headspace and the surface walls of the 5L bioreactor, b) *A. terreus* growth after bioreactor adjustments.

Therefore, the impeller types and positioning in the fermenter were changed. The new setup consisted of one Rushton turbine impeller, and one variable pitch impeller inserted at an angle of 45 degrees, placed at a spacing of height/3 (height of the liquid in bioreactor). The stirrer speed was set to 300 rpm. Inoculation was done in a similar manner to the shaken flasks, as one-day old *A. terreus* culture, grown in *A. terreus* growth medium, was used for inoculation; linoleic acid was added at 0.1% concentration to the test bioreactor, and the same volume of ethanol was added in the control bioreactor.

3.2.2.1 Lovastatin production

Samples from the reactors were taken throughout the course of fermentation (6 days) and assayed for lovastatin production (Figure 3.24a). pH and DOT (% air saturation) measurements were automatically logged on (Figure 3.24b and c).

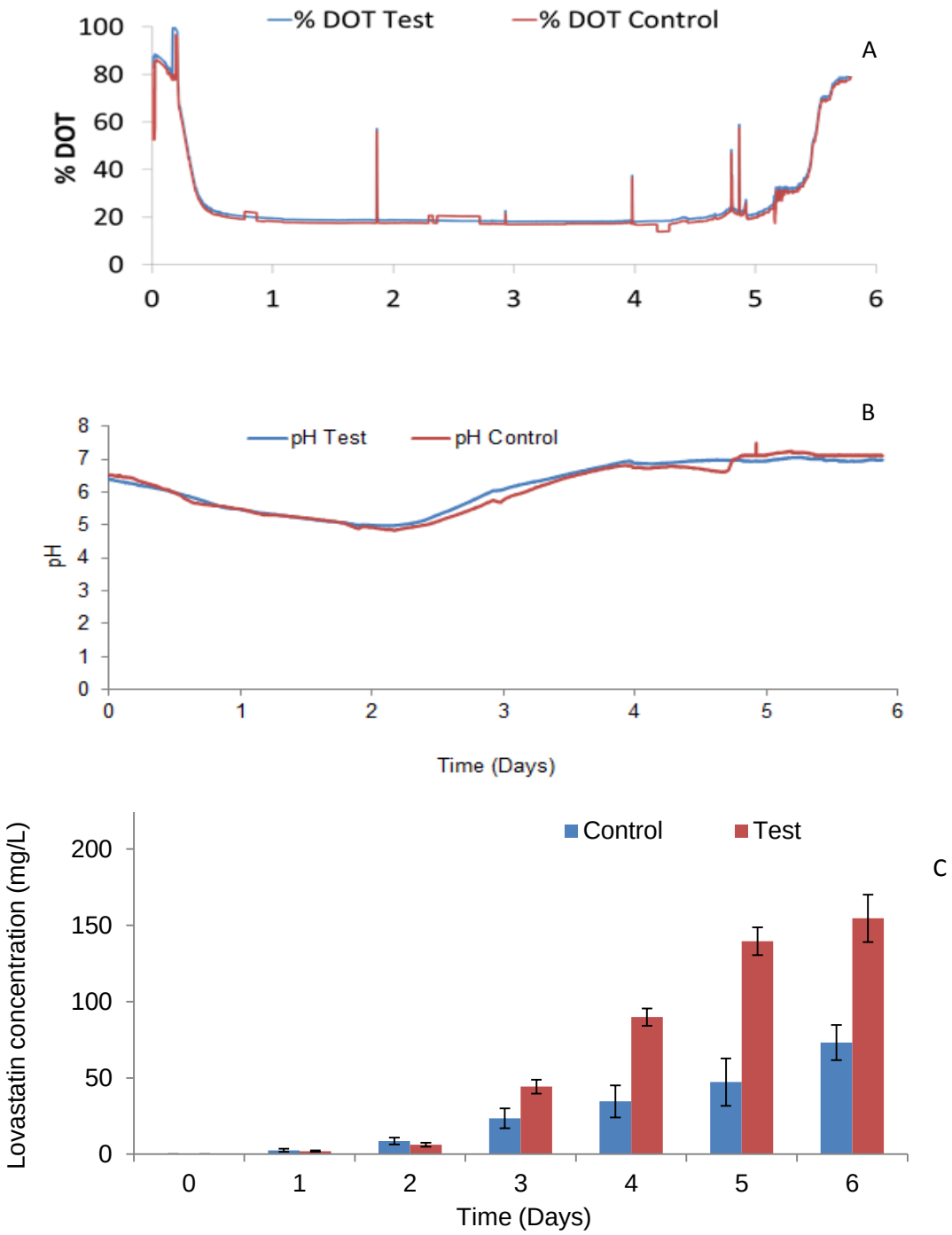


Figure 3.24: Effect of Linoleic acid addition on *A. terreus* culture in 5L stirred tank reactor. A) % Dissolved Oxygen Tension (DOT), B) pH and C) Lovastatin concentration

3.2.2.2 Microscopic Images

Microscopic slides were prepared to check whether the exogenous addition of linoleic acid have any impact on the hyphal morphology of *A. terreus* in the 5L STR. Based on the images, it was observed that the fungal samples taken from the fermenter treated with linoleic acid had branched hyphae as compared to control samples with relatively no branching (Figure 3.25).

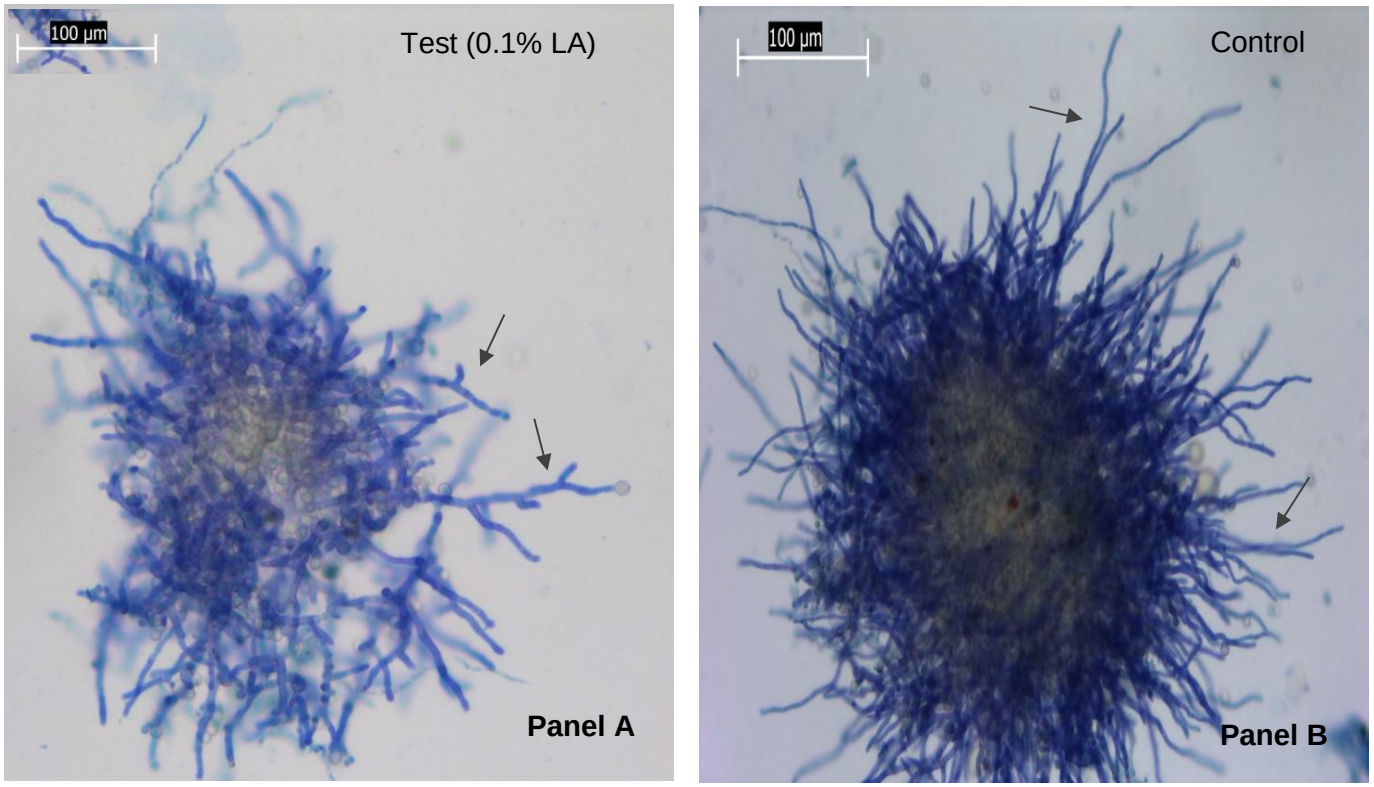


Figure 3.25: Microscopic images of *A. terreus* mycelia. *A. terreus* hyphae from 5L STR were stained with lactophenol blue and viewed under the microscope at 20 x magnification. Panel A represent samples from the test fermenter (treated with 0.1 % LA) and Panel B represent samples from the control fermenter.

3.2.3 Proteomics studies

In order to have an improved understanding of events occurring at the molecular level, a classic intracellular proteome study was carried out on the filamentous fungus *A. terreus.* The proteomic response of *A. terreus* to 0.1% linoleic acid supplementation (on the day of inoculation, i.e. day 0) was evaluated by 2D gel electrophoresis (PAGE). 2D- PAGE allows protein separation based on their size and charge on a gel matrix. 2D gels provide a comprehensive overview of the proteins expressed or induced as a result of changes in the environment such as linoleic acid supplementation.

3.2.3.1 Comparative 2-D gel electrophoresis

There are three proteomics levels to be analysed including intracellular (cytosolic & inside organelles), extracellular (secretome) and membrane & organelle proteomes. The whole proteome of *A. terreus* was not used for this study, as the protein spots were too numerous and clustered (data not shown) resulting in insufficient resolution and inadequate identification of the individual spots. Therefore to overcome this problem, cell fractionation using ultracentrifugation was carried out to separate various cellular compartments.

Of the various fractions, only the cytosolic fractions were further analysed. The cytosolic fraction was used based on the possibility of identifying proteins involved in signalling mechanism, or lovastatin biosynthetic pathways. The study of intracellular proteome was also used as an approach to understand the alterations of *A. terreus* metabolic process in response to linoleic acid supplementation. The obtained gels contained about 400-500 resolvable proteins, mainly soluble cytosolic proteins.

2D reference gels were obtained after 6 replicates representing the two conditions, the test (*A. terreus* culture treated with 0.1% linoleic acid) and the control culture (without linoleic acid supplementation, using ethanol as the solvent). The expressed cytosolic proteins obtained under two conditions are illustrated in Figure 3.26.

The 2D gels were aligned automatically for comparison using the Progenesis Same Spot software. The gels were then excised, and a total of 24 proteins within the cytosolic fraction were identified by MALDI-TOF/TOF mass spectrometry.

3.2.3.2 Protein Identification

The choice of protein spots to be identified was based on the statistical tables obtained after gels analysis using “Progenesis Same Spot” software; protein spots of 1.3 folds abundance or more were considered significant for further analysis. The reference image (Figure 3.27) illustrates the protein spots selected for identification using MALDI-TOF/TOF.

Based on the identified protein spots it was observed that more than half of the spots were classified as hypothetical or predicted proteins. This outcome was mainly due to the limited annotation of the fully sequenced *A. terreus* genome; with only 7.5% of the open reading frame have previously assigned functions. Putative functions of the hypothetical proteins, where applicable, was predicted and assigned by searching the spectral data against NCBI databases restricted to fungi via BLASTp search. Some spots appeared in the gel more than once with the same molecular weight; however with different abundance and PI values.This is possibly due to posttranslational modifications. On the other hand, some protein spots were identified and assigned to different types of protein. The identified protein spots are represented in Table 3.3.

The identified proteins (Table 3.3) that showed alteration in their expression levels in response to linoleic acid supplementation were classified into three major categories; detoxification and stress-related proteins (including oxidative stress), primary metabolite biosynthesis and energy metabolism (e.g. carbohydrate metabolism processes), and the third category include additional proteins putatively involved in secondary metabolite production and signalling mechanisms as well as those predicted, hypothetical proteins with either unknown or suggested functions.

The first category includes proteins that are involved in detoxification pathways such as glutathione S-transferase (GST), superoxide dismutase, spermidine synthase and HSP70.

The second category of proteins include: Fructose bis-phosphaealdolase, glyceraldehyde-3-phosphate dehydrogenase, a protein similar to alcohol dehydrogenase, aldose-6-phosphate reductase, and mannose-6-phosphate isomerase and other organelles biogenesis such as nucleoside disphosphate sugar epimerase.

The third category includes hypothetical and predicted proteins as well as other proteins such as ATP Citrate synthase subunit-1 (ATP- citrate lyase), peroxisomal membrane protein, non-heme chloropeoxidases, and transaldolase-like proteins.

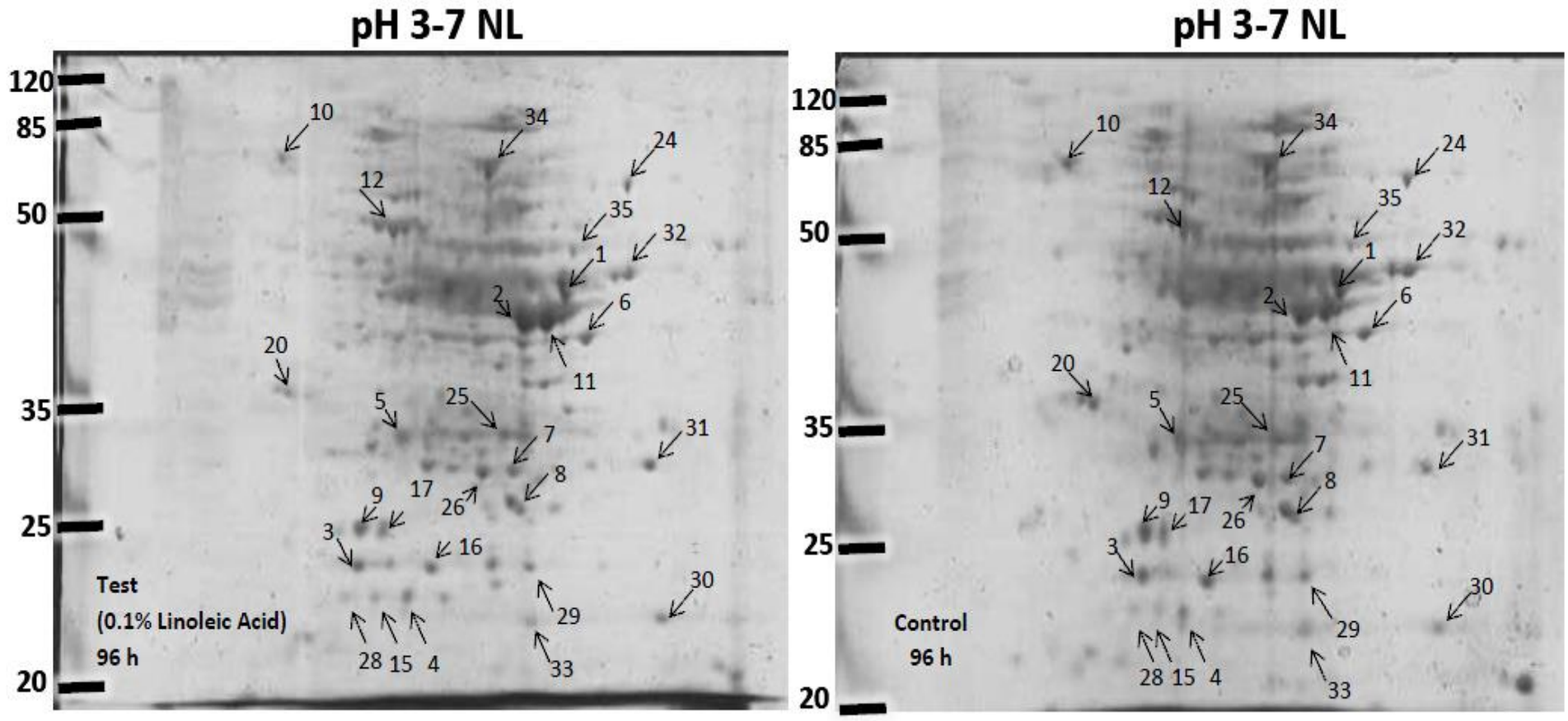


Figure 3.26: Comparative 2-D gel electrophoresis of cytosolic protein profile of *A. terreus* strain (MUCL 38669) grown with and without 0.1% linoleic acid in the medium. Purified cytosolic proteins were focused on a pH 3-7 non-linear (NL) IPG strips and further separated by 2-D gel electrophoresis. For simplicity of data analysis, the protein spots were given a different numbering to the reference image. A key is included in the appendix, correlating the spot numbers between the reference image and identified proteins.

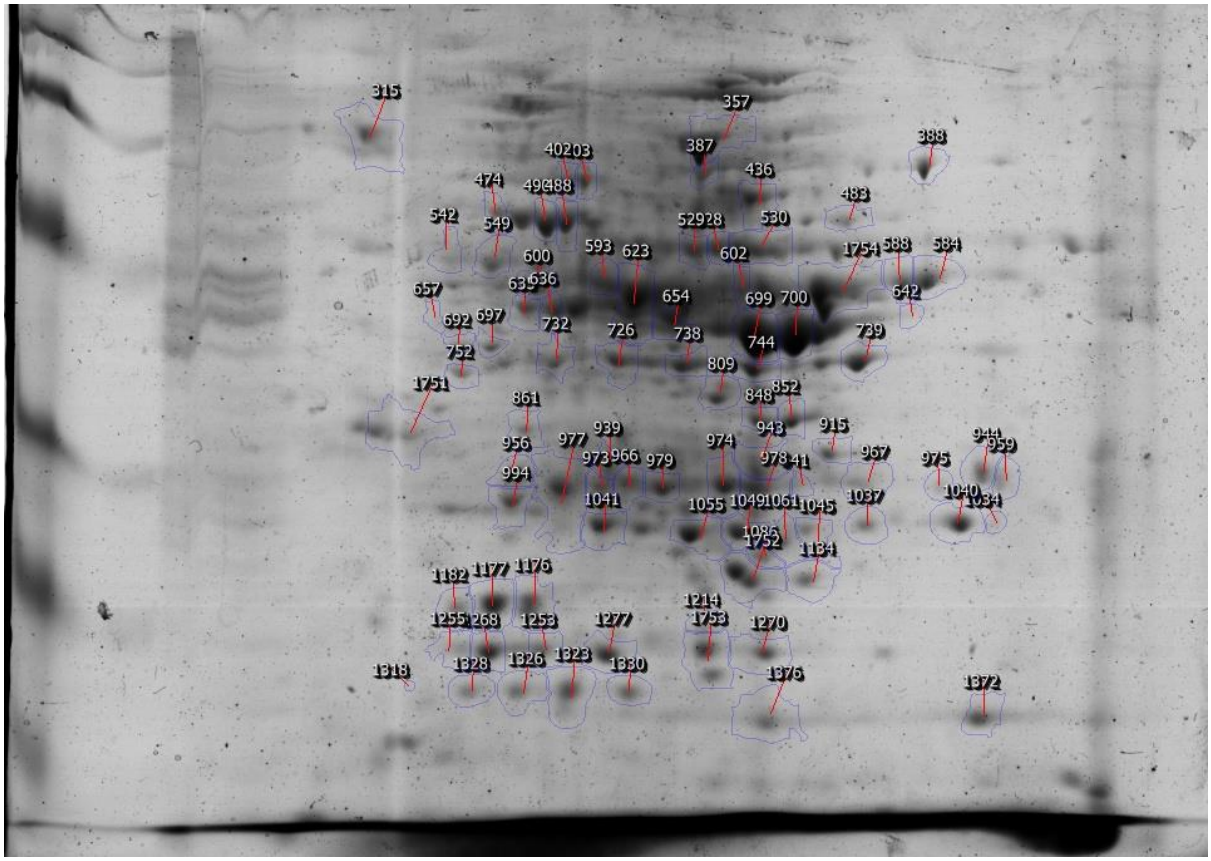


Figure 3.27: Reference 2D gel electrophoresis of cytosolic protein extracts of *A. terreus* culture. The reference gel was generated by Progenesis same Spot software after aligning the test and control gels. Proteins were stained with Commassie blue. The numbers refer to proteins whose level changed upon the addition of 0.1 % linoleic acid to *A. terreus* cultures.

Table 3.3: Summary of all the identified protein spots involved in various cellular activities in the cytosolic fraction of *A. terreus*

Spot No.	Name	MW	Pi	Match to	Fold increase	Score	Pathways involved in/ Proposed function based on region
28	Hypothetical protein similar to alcohol dehydrogenase	59793	5.79	gi|114190454	1.5	232	catalyses the oxidation of ethanol to acetate via acetaldehyde
24	glyceraldehyde-3-phosphate dehydrogenase	40092	6.01	gi|115443264	1.4	416	Glycolysis/ glucogenesis, dihydroxyacetone cycle
6	Single peptide match to non-hemechloroperoxidase	29692	6.36	gi|115491601	1.3	122	Hydrolase activity, catalytic activity, aromatic compound metabolism, cellular metabolism
1i	Glyceraldehyde 3-phosphate dehydrogenase	40092	6.01	gi|115443264	1.4	283	Glycolysis/ glucogenesis, dihydroxyacetone cycle
1ii	Hypothetical protein similar to aldehyde reductase;Aldose-6-phosphate reductase (NADPH)	34593	6.81	gi|115391717	1.4	219	Aldo-keto reductases are a superfamily of soluble NADPH oxidoreductases. They reduce aldehydes and ketones to primary and secondary alcohols.
17	hypothetical protein similar to mannose 6-phosphate isomerise	17794	5.17	gi|115492805	1.3	503	D-mannose degradation, GDP-mannose biosynthesis
29	Conserved hypothetical protein	34122	6.18	gi|115390615	1.3	175	Region of Nucleoside-diphosphate-sugar epimerases
8	Superoxide dismutase(mitochondrial precursor)	24959	8.64	gi|115391187	1.3	208	superoxide radicals degradation
27	Single peptide match to protoplast secreted protein 2 precursor	21845	5.85	gi|115387237	1.3	84	---
26	superoxide dismutase, mitochondrial precursor	23305	6.43	gi|115492231	1.4	153	superoxide radicals degradation
25	Conserved hypothetical protein similar to Glutathione S-transferase	26358	6.25	gi|115401134	1.3	136	glutathione-mediated detoxification
3	hypothetical protein similar to peroxisomal membrane protein	18554	5.35	gi|115386734	1.4	358	Peroxiredoxin family; present as peroxisomes in cytosol
16	Predicted protein	19582	6.04	gi|115491593	1.3	141	-
5	Spermidine synthase	33185	5.35	gi|115492019	1.3	192	spermidine biosynthesis (polyamine biosynthesis)

7	superoxide dismutase, mitochondrial precursor	23305	6.43	gi|115492231	1.4	203	superoxide radicals degradation
12 i	Fructose-bisphosphate aldolase	39564	5.58	gi|115396484	1.5	232	formaldehyde assimilation III (dihydroxyacetone cycle) , gluconeogenesis I , glycolysis I
12 ii	a single peptide match to hypothetical protein; transaldolase like protein	35651	5.57	gi|67515917	1.5	49	Pentose phosphate pathway, Biosynthesis of secondary metabolites, formaldehyde assimilation III (dihydroxyacetone cycle)
20 i	Single peptide matches to nitroreductase family protein *A. fumigatus*	24356	5.63	gi|70997834	1.4	160	Nitroreductase family protein
20 ii	Single peptide matches to nitroreductase family protein *A. niger*	24356	5.63	gi|145249980	1.4	51	Nitroreductase family protein
11	Conserved hypothetical protein	34122	6.18	gi|115390615	1.5	333	Region of Nucleoside-diphosphate-sugar epimerases
35	ATP-citrate synthase subunit 1	51370	5.82	gi|115388095	1.3	172	Catalyzes the formation of cytosolic acetyl-CoA. Biosynthesis secondary metabolites
31	hypothetical protein similar to nit protein 2 (nitrilase family protein)	31902	6.29	gi|115390066	1.5	241	-
9	Hypothetical protein similar to mannose 6-phosphate isomerise	17794	5.17	gi|115492805	1.5	280	D-mannose degradation, GDP-mannose biosynthesis
4	Single peptide match to conserved hypothetical protein	34122	6.18	gi|115390615	1.3	137	Region of Nucleoside-diphosphate-sugar epimerases
34	Hsp 70	68354	5.05	gi|115385867	1.3	241	Chaperon, protein export
2	Conserved hypothetical protein	34122	6.18	gi|114193736	1.6	343	Region of Nucleoside-diphosphate-sugar epimerases

3.2.4 Oxylipins link to GPCR signalling

Previous observations by Sorrentino et al. (2010) indicated that linoleic acid supplementation alters sporulation levels, increases the transcriptional levels of lovastatin biosynthetic genes *lovB* and *lovF*, as well as induction of cytosolic proteins. Despite extensive knowledge of how oxylipins are synthesised in filamentous fungi (mainly *Aspergillus* genus) and the impact of linoleic acid on *A. terreus,* there is limited knowledge on how oxylipins and their precursor linoleic acid are perceived as signals; little is known on their signal transduction pathways.

G-protein coupled receptors were found to act as oxylipin receptors in mammals and plants, thus involved in oxylipins signalling mechanisms. Several GPCRs have been identified across the *Aspergillus* genus (Seo et al., 2004,Grice et al.,2013, Gehrke et al., 2010).

Alterations in cAMP levels are a direct consequence of G-protein signalling, as cAMPs are known to act as secondary messengers in the G-protein signalling pathway (Yu and Keller, 2005). Previous studies reported that cAMP is involved in the signalling mechanism of the model filamentous fungus *A. nidulans* as well as alteration of cAMP when exposed to pure oxylipins (Fillinger et al., 2002, Shimizu and Keller, 2001, Shimizu et al., 2003, Roze et al., 2004, Lafon et al., 2006).

A hypothesis by Affeldt et al. (2012) states that linoleic acid and its derived oxylipins are perceived by fungal GPCR(s) that in turn stimulate a burst in cAMP levels. One approach to investigate this hypothesis in *A. terreus* was to check whether the exogenous addition of linoleic acid and pure oxylipins alters cAMP levels in *A. terreus* in a similar manner to *A. nidulans* (Affeldt et al., 2012).

3.2.4.1 Oxylipin link to cAMP levels in *A. terreus*

The levels of cAMP were measured in *A. terreus* exposed to pure oxylipins as well as linoleic acid using the direct cAMP colorimetric assay (ENZO Life Sciences, UK). The reactions were performed in 96 well-plate reader and measured at an optical density of 405mm. Bradford assay was performed to quantify the protein levels in the samples since results are presented as yields of cAMP/ mg of protein.

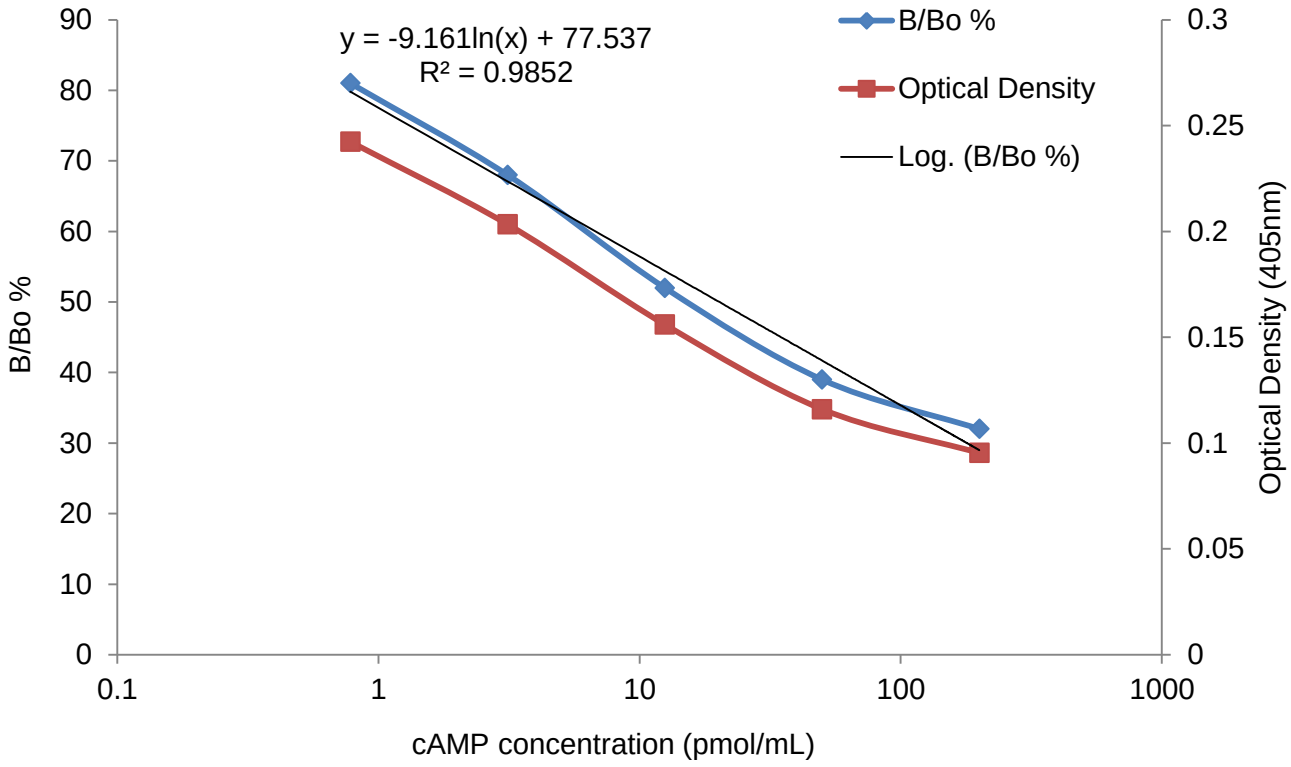


Figure 3.28: Standard curve of cAMP concentrations. The standard curve was prepared using direct cAMP colorimetric assay kit. %B/Bo represents the ratio of the absorbance of a particular sample or standard in a particular well to that of the maximum binding (Bo).

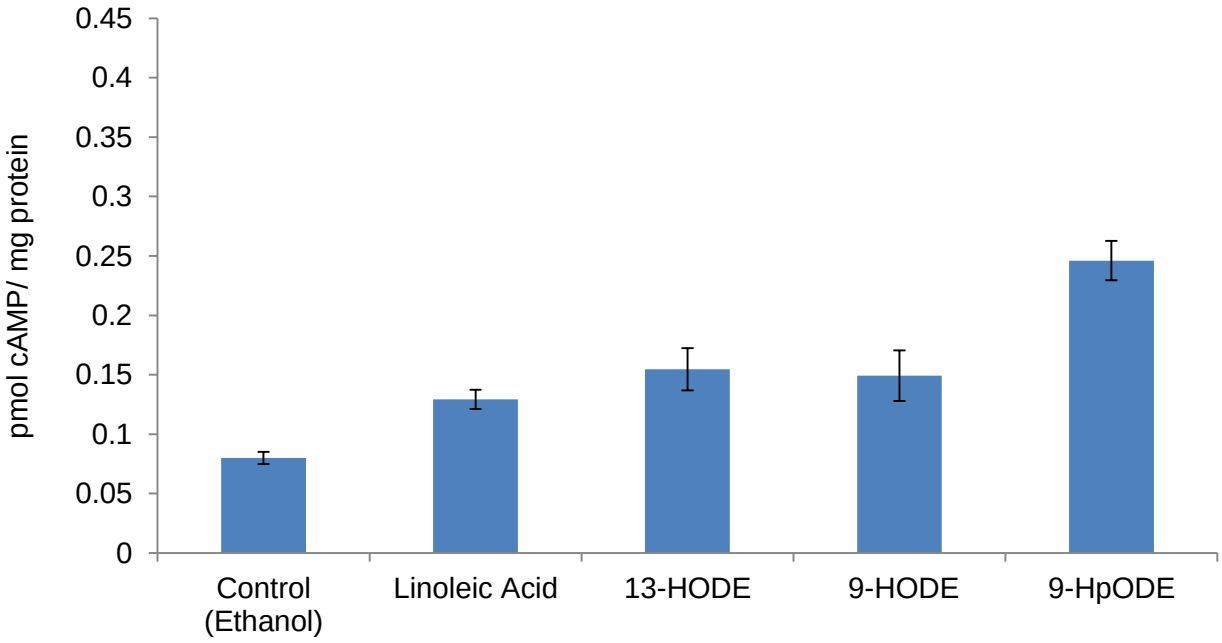


Figure 3.29: Effect of three pure oxylipins; 9-HODE, 9-HpODE, 13-HODE and linoleic acid on cAMP levels in *A. terreus*. *A. terreus* samples were treated with ethanol (solvent control) or an equivalent volume of pure oxylipins, or linoleic acid, dissolved in ethanol to achieve the final concentrations of 10 µM. cAMP levels were calculated as yield cAMP:pmol or cAMP/ mg of Protein. cAMP quantification was determined from the optical densities, and calculated from the standard curve. Values are means of three replicates. Error-bars represent standard deviation.

The response of *A. terreus* to three pure oxylipins; 9(S)-HODE, 9(S)-HpODE, 13(S)-HODE and the oxylipin precursor, linoleic acid was investigated (Figure 3.29). The three oxylipins were metabolites generated by plant and mammalian lipoxygenases (Tsitsigiannis and Keller, 2007). The three oxylipins and linoleic acid were added at a final concentration of 10µM. Ethanol was used as a solvent control. It was found that *A. terreus* produced more cAMP after a twenty seconds exposure to oxylipins or linoleic acid as compared to the control using ethanol. 9(S)-HpODE caused the highest response, as compared to the other two oxylipins, linoleic acid as well as the control (ethanol) (Figure 3.29). Statistical analysis of the obtained values using ANOVA showed a significant change ($P<0.005$). Unpaired T-test between the control and the different treatments showed a significant changes $P<0.005$.

3.2.4.2 Impact of increasing concentrations of 9-HpODE on cAMP levels.

The obtained results illustrated that the plant oxylipin, 9(S)-HpODE increases cAMP levels when exogenously added to *A. terreus*, therefore *A. terreus* samples were then treated with increasing concentrations of 9(S)-HpODE in ethanol, at concentrations of 1, 10 and 100 µM. It was found that 9(S)-HpODE exhibit a burst in cAMP levels in a dose dependant manner (Figure3.30). Statistical analysis carried out using unpaired T-test illustrated that the addition of 1µM 9-HpODE had no significant effect on cAMP levels as compared to the control (treated with ethanol). The addition of 10 and 100 µM 9-HpODE had a significant impact on cAMP levels (P<0.005). However the increase in cAMP levels was not significant when the concentration of 9(S)-HpODE was increased from 10 to 100 µM (P>0.05). This might indicate a saturation of oxylipins binding to putative receptors in *A. terreus.*

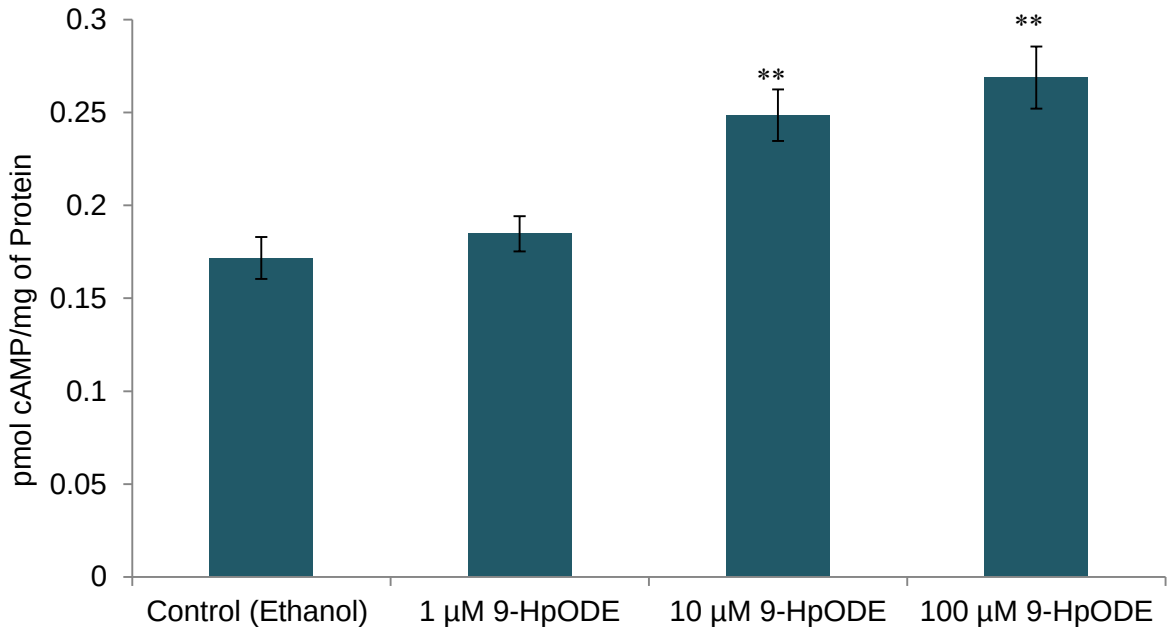


Figure 3.30: Effect of increasing concentrations of 9(S)-HpODE on cAMP levels in *A. terreus*. *A. terreus* samples were treated with ethanol (solvent control) or an equivalent volume of the plant oxylipins 9(S)-HpODE dissolved in ethanol to achieve the final concentrations of1,10 and 100 µM; Statistical Analysis was carried out on each condition with respect to the control culture: * indicates P<0.05, ** P<0.005.

3.2.5 Identification of G-protein/cAMP mediated signalling pathway components in *A. terreus*

Several components of the G-protein and cAMP–mediated signalling pathways have been identified in the model filamentous fungus *A. nidulans,* and *A. fumigatus* and *A. oryzae* (Lafon et al., 2006). In order to identify the similar components in *A. terreus*, an in silico genomic exploration of *A. terreus* genome was performed to identify putative components of G-protein/cAMP-mediated signalling in this species.

The search included: I) putative GPCRs that are distributed over nine classes GPCRs (gprA-Q) and nopA II) Heterotrimeric G-protein constituents III) proteins with a regulator of G-protein signalling (RGS) domain IV) genes that encode adenylase cyclase, two catalytic subunits and regulatory subunit of cAMP-dependant protein kinase (PKA). The 18 GPCRs are distributed among nine classes and they include *gprA(PreB), gprB(PreA), gprC-E, gprF, gprG, gpH, gprI, gprJ, gprL, gprK, gprM, gprN, gprO, gprP, gprQ,* and *nopA* . Heterotrimeric G-protein constituents previously identified in *A. nidulans* include three Gα subunits (FadA, GanA, GanB), one Gβ subunit (SfaD), one Gγ subunit (GpgA). Proteins with a regulator of G-protein signalling (RGS) domain are: FlbA, RgsA-C.

The Basic Local Alignment Search Tool (BLASTp) available from the National Centre for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/BLAST/ , NCBI) was used to explore the genome of *A. terreus*, *A.clavatus, A. fumigatus, A. oryzae* and *A. flavus.* Similar proteins identified with more than 98 % coverage and more than 40% identity to the model filamentous fungi *A.nidulans* were reported. Putative GPCRs are reported in (Table 3.4); whereas other components are reported in Table3.4 .Genes encoding putative GPCRs in *A. terreus* were aligned together with those of *A. nidulans,* and *A. flavus*. A phylogenetic tree was built (Figure 3.31) and further alignments are present in the appendix.

Table 3.4 Classes of GPCRs in different *Aspergillus* spp. Genes corresponding to GPCRs were identified in *Aspergillus* spp., grouped within nine classes according to the phylogenetic analysis in Fig 3.31; and represented by the gene entry, number of amino acids, and the relative domains

Class	Gene	*A.nidulans*	*A. terreus*	*A.oryzae*	*A.fumigatus*	*A. flavus*	*A. clavatus*
I	gprA (preB)	AN2520.0(377)-Ste2 GPCR	ATEG_03500 (373) HP- Ste2 GPCR	AO070286000075(369)- Ste2 GPCR	Afu3g14330(369) -Ste2 GPCR	AFLA_060740(374)-Ste2 GPCR	ACLA_041790(370)-STE2 GPCR
II	gprB (preA)	AN7743.2(426)-Ste3 GPCR-Pheromone A receptor	ATEG_08338(442) – Predicted protein- HP STE3 Pheromone A receptor	AO070325000167(465)- Ste3 GPCR	Afu5g07880(460) -Ste3 GPCR	AFLA_061620(465)-PreA-Ste3 GPCR-Pheromone A receptor	ACLA_012620(442)-Ste3 GPCR
III	gprC	AN3765.2(439)-HP-Rhodopsin-like GPCR-G protein-coupled glucose receptor regulating Gpa2	ATEG_04369 (446) - CHP- G protein-coupled glucose receptor regulating Gpa2	AO07030900034 (444)- Family A GPCR-like	Afu7g04800(480) - Rhodopsin-like GPCR; Family A GPCR-like	AFLA_074150A (444)- CHP-G protein-coupled glucose receptor regulating Gpa2	ACLA_006460 (445) – CHP -G protein-coupled glucose 9receptor regulating Gpa2
	gprD	AN3387.2(427) Family A-GPCR like-G protein-coupled glucose receptor regulating Gpa2	ATEG_01243 (416) - HP transmembrane receptor Secretin family	AO0703040000(431) Family A-GPCR like	Afu2g12640 (418) Family A-GPCR like	AFLA_135680 (415) G protein-coupled receptor GprD	ACLA_006460 (445) – CHP. Transmembrane receptor Secretin family
	gprE	AN9199.2(493)- HP	-	-	-	-	-
IV	gprF	AN 12206(370)-Ortholog have cationic amino acid transmembrane transporter activity	ATEG_08003 (374) – CHP- PQ loop repeat	-	Afu4g04100 (391) putative GPCR and PQ loop repeat protein	AFl2G_09986 (389)-HP similar to PQ loop repeat protein	ACLA_001070(383) PQ loop repeat protein
	gprG	AN10166 (424) Ortholog to PQ loop repeat protein	-	AO070285000037(426)- PQ loop repeat protein	Afu1g11900(431) -PQ loop repeat protein	AFLA_067770 (426).-PQ loop repeat protein	ACLA_023700 (433) - PQ loop repeat protein
	gprJ	AN5720.2(318) - PQ loop repeat protein and putative GPCR	ATEG_02934(321)-CHP- PQ loop repeat	Ao070324000130 (313)- PQ loop repeat	Afu1g06840(326) - PQ loop repeat protein	AFLA_127870 (322)-PQ loop repeat protein	ACLA_027730(326)-PQ loop repeat protein

V	gprH	AN8262(404)-Secretin-like GPCR	ATEG_08000 (424) – HP- Transmembrane receptor (Secretin family)	AO07031000038 (422)- Secretin-like GPCR-Family A GPCR-like	Afu5g04140(413) -Secretin-like GPCR	AFLA_006920 (428)-cAMP receptor-like protein, putative	ACLA_001040 (413)-CHP
	gprI	-	-	-	Afu3g00780 (355)	-	-
	gprL	-		-	Afu3g01750(384) - Secretin-like GPCR	-	-
VI	gprK	AN7795(563)-RGS	ATEG_08180 (562)-CHP- RGS domain	AO070333000017 (560)- RGS domain	Afu4g01350 (559) RGS domain	AFLA_009790(560)-CHP- RGS domain	ACLA_067510(562)-RGS domain
VII	gprM	AN6680.2 (489)	ATEG_06459 (508)-HP	AO070289000022(490)	Afu7g05300(499)	AFLA_075000 (490)-CHP	ACLA_007000 (503) -CHP
	gprN	AN5508.2(535)	-	-	-	-	-
VIII	gprO	AN4932(319) – Putative GPCR and Haemolysin III-related proteins	ATEG_04586 (322) CHP	AO070338000011(318)-Haemolysin III-related proteins	Afu3g10570(321) Haemolysin III-related proteins	AFLA_032130(282) - Haemolysin III-related proteins IZH family channel Protein (Izh3)	ACLA_038460(322) Haemolysin III- related proteins IZH family channel Protein (Izh3)
	gprP	AN5151.2(498)-Haemolysin III-related proteins	ATEG_03715(498)-HP- Haemolysin III-related proteins	AO0702370000 (502) Haemolysin III- related proteins	Afu6g07160 (419) Haemolysin III-related proteins	AFLA_088190(502) Haemolysin III-related proteins IZH family channel Protein (Izh3)	ACLA_088960 (497) Haemolysin III- related proteins. IZH family channel protein (Izh3)
	gprQ	-	-	AO070321000028 (314) Putative GPCR; haemolysin III-related protein	-	-	-
IX	nopA	AN3361.2(320) Bacterio-rhodopsin like protein	ATEG_08691 (250) CHP- Bacterio-rhodopsin-like protein	AO070281000037(312) Bacterio-rhodopsin- Family A GPCR-like	Afu7g01430 (304) Bacterio-rhodopsin Family A GPCR-like	AFLA_117970 (312)Bacterio-rhodopsin-like protein (opsin)	ACLA_066140 (299)-Bacterio-rhodopsin-like protein (opsin)

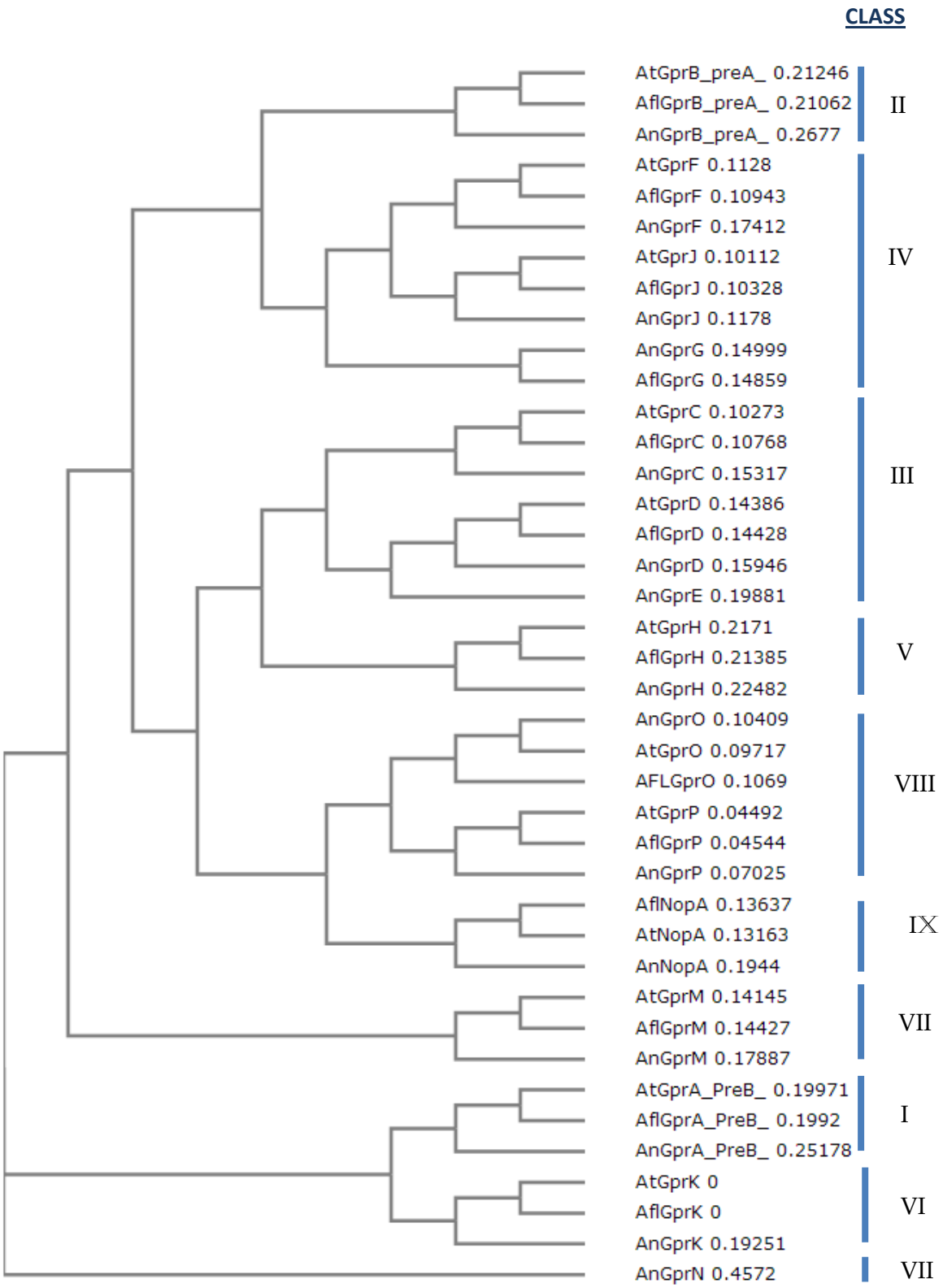


Figure 3.31: Phylogeny of putative GPCRs identified in the *A. nidulans, A. terreus* and *A. flavus* proteomes. The sequences of putative GPCRs were aligned and an unrooted tree was generated using CLUSTALW2.The GPCRs are grouped into nine classes. Abbreviations: Genes corresponding to *A. terreus* are represented by At; *A.nidulans*by An and *A. flavus* by Afl.

Table 3.5: Heterotrimeric G-protein, RGS-proteins, and cAMP-signalling components in *A. terreus, A. nidulans*, and *A. fumigatus.*

Protein	Function	Entry/locus (Size in aa number)		
		A. nidulans	*A. fumigatus*	*A. terreus*
FadA (GpaA)	α- subunit	AN0651.2(353)	Afu1g13140(353)	ATEG_00509 (353) G-alpha subunit
GanA	α- subunit	AN3090.2(361)	Afu3g12400(359)	ATEG_04149 (359) G-alpha-2 subunit
GanB (GpaB)	α- subunit	AN1016.2(356)	Afu1g12930(356)	ATEG_00488 (363) G-alpha-3 subunit
GaoC	α- subunit	Not Present	Not Present	Not Present
SfaD	β-subunit	AN0081.2 (352)	Afu5g12210(387)	ATEG_02052(320) G-beta subunit
GpgA	γ-subunit	AN2742.2(95)	Afu1g05210(114)	ATEG_02589 (90) hypothetical protein
RgsA	GanB regulator	AN5755.2(362)	Afu6g06860(363)	ATEG_09016 Predicted protein
RgsB	ScRax1p orthologue	AN3622.2(371)	Afu4g12640(356)	ATEG_03148(369) hypothetical protein
RgsC	ScMdm1p orthologue	AN1377.2(1255)	Afu1g09040(1274)	ATEG_08529(1221) hypothetical protein
FlbA	FadA regulator	AN5893.2(719)	Afu2g11180(718)	ATEG_01459 (714) developmental regulator flbA
RgsD	RgsAparalogue	Not Present	Afu5g00900(288)	Not Present
CyaA	Adenylate cyclase	AN3913.2(2132)	Afu6g08520(2057)	ATEG_05799(2152) hypothetical protein
CapA	Adenylate cyclase- associated protein	AN0999.2(529)	Afu1g12760(526)	ATEG_00465(531) hypothetical protein
PdeA	Low-Affinity cAMPphosphodiesterase	AN0829.2(561)	Afu1g14890(513)	Not Present
PdeB	High-Affinity cAMPphosphodiesterase	AN2740.2(952)	Afu1g05230(997)	ATEG_02567(952) hypothetical protein
PkaR	PKA regulatory subunit	AN4987.2(412)	Afu3g10000(413)	ATEG_04524(378) cAMP-dependent protein kinase regulatory Subunit
PkaA	PKA catalytic subunit	AN6305.2(472)	Afu2g12200(510)	ATEG_01157(478) cAMP-dependent protein kinase type 2
PkaB	PKA catalytic subunit	AN47172.2(396)	Afu5g08570(396)	ATEG_05778(316) similar to protein kinase A catalytic subunit 2
SchA	Ser/Thr protein kinase related to the PKA catalytic subunit	AN4238.2(919)	Afu1g06400(875)	ATEG_02982(879) serine/threonine-protein kinase sck1

Chapter IV

DISCUSSION

4.1 Quorum Sensing in *Penicillium sclerotiorum* culture

For a molecule to be classified as a QSM in microbial cultures, it should fulfil several criteria. 1) The molecule should accumulate in the extracellular environment during the microbial growth. 2) The quorum sensing molecules are produced throughout the microbial growth, and their production and accumulation is proportional to the microbial cell density. 3) However the quorum sensing response is only exerted at particular stages of the growth. 4) The molecule should be capable of regulating its own production such as acyl-homoserine lactones in Gram-negative bacteria (Fuqua and Eberhard, 1999) and A-factor (γ-butyrolactone molecules) in *Streptomyces* species (Horinouchi and Beppu, 1994). 5) The molecule must exert a coordinated response in the host organism which does not involve metabolising or detoxifying the molecule itself, examples include the regulation of physiological activities such as sporulation and secondary metabolism. 6) The exogenous addition of the quorum sensing molecule to a mutant strains, deficient in the production of QSM, must restore the quorum sensing response (Albuquerque and Casadevall, 2012).

Studies correlating cell-densities to quorum sensing behaviour are mainly reported in bacterial and unicellular fungal cultures (Surette et al.,1999, Singh et al.,2000, Hornby et al., 2004). A study by Brown et al. (2008) investigated the impact of cell density on the morphological transition in *A. flavus*, where extracts from spent medium of cultures with low cell densities stimulated sclerotial formation and extracts prepared from spent medium of cultures with high cell densities stimulated conidiation.

The objective of the current study was to correlate and investigate the impact of the fungal cell-density to the regulation of different physiological responses, frequently regulated by quorum sensing. *P. sclerotiorum* was chosen for these studies due to the putative quorum sensing behaviour previously detected in this fungus by Raina et al. (2010) and its capacity to produce the secondary metabolite, sclerotiorin. Therefore, to address this objective, the supernatants of *P. sclerotiorum* were collected at different cell densities, extracted using ethyl acetate and exogenously added to fresh *P. sclerotiorum* cultures. The effect of *P. sclerotiorum* culture extracts was investigated on spore production, hyphal morphology and secondary metabolism in *P. sclerotiorum* cultures.

The extract prepared from the culture supernatant of *P. sclerotiorum* contained solvent-extractable compound(s) capable of exerting an effect on the producing fungus just when their endogenous concentration had reached a threshold concentration. The solvent- extracted compounds could be considered as putative QSM(s) because lipophilic molecules as lactones and oxylipins (major fungal QSM) are efficiently extracted using ethyl acetate (Pearson et al., 1994, Shaw et al., 1997, Champe et al., 1987).

The active compounds fulfil several criteria required for QS; a) the putative QSM were produced, and released into the extracellular environment at an increasing concentration concomitant with the culture cell density, b) at low cell densities, the levels of putative quorum sensing molecules, were either below the threshold levels or too low to exert a significant physiological change. However at higher cell densities the concentration of those molecules increased proportionally and accumulated beyond a threshold concentration, contributing to several physiological changes in this fungus.

4.2 Sporogenic effect

Sporulation is one of the various physiological traits regulated by quorum sensing in bacteria and fungi. For instance, the competence-sporulation system, ComP/ComA in the Gram positive bacterium *Bacillus subtilis* have two main quorum sensing molecules 1) ComX and 2) the competence sporulation factor

(CSF). At low internal concentrations of CSF, competence is induced; however at high concentrations of CSF, competence is inhibited and sporulation is induced (Miller and Bassler, 2001).

To investigate the sporogenic effect of the putative presence of quorum sensing molecule(s) in the *P. sclerotiorum* culture extract(s), the extracts from different cell densities of *P. sclerotiorum* cultures were exogenously added to the cultures of this fungus. It was found that the addition of extracts from high cell-density induced sporulation, with a 2 folds increase in the sporulation rate as compared to the control (Figure 3.2). Furthermore, a correlation was observed between the age of the culture from which the extract was prepared and the extent of sporulation. It was also found that the concentration of the added extract had an impact on the sporulation as macroscopically visualized on plates (Figure 3.3).

The data obtained from GC-MS analysis (Table 3.2) revealed that the extract contained a range of compounds such as linoleic acid as well as, the oxylipin ricinoleic acid (Figure 3.19). The presence of those molecules in the culture extract indicates their putative involvement in the sporogeneic effects of this fungus, either individually or in a concerted manner.

Ricinoleic acid, 12-hydroxy-9-cis-octadecenoic acid, is a monounsaturated fatty acid with a hydroxyl group on the 12^{th} carbon (Figure 3.20). It was previously reported to induce the sporulation in *A.* flavus. The side group on the fatty acid backbone of ricinoleic acid is thought to be the main contributor to the sporogenic effect as compared to other fatty acids (Calvo et al., 1999). Additionally, the presence of linoleic acid in the extract suggests the possibility of its involvement in the sporogenic effect of *P. sclerotiorum*.

Fungal oxylipins, mainly linoleic acid and its derivatives, were found to function as hormone-like signals and had an impact on the sporulation of several *Aspergillus* strains including *A. nidulans, A. flavus*, *A. parasiticus* and *A. fumigatus* (Calvo et al., 1999) as well as in other filamentous fungi. For instance the endogenous oxylipins derived from linoleic acid, the Psi factors, function as hormone-like signals, to coordinate the sexual (ascospores) and asexual spore (conidia)

development in the model filamentous fungus *A. nidulans* (Tsitsigiannis et al., 2004a).

In addition to its impact on *Aspergillus* spp., linoleic and linolenic acids were found to induce sporulation in the anamorphic fungus *Alternaria tomato,* whose asexual conidia were produced only under illumination of light (Hyeon, 1976). Another identified sporogenic substance, the linoleic acid related compound glycerol mono linoleate, was isolated from the acetone extracts of the brown rot fungus *Sclerotinia fructicola* (Katayama and Muramo, 1978). Linoleic acid was also identified as the major component of the active fraction of the acetone extract from mycelia of the filamentous fungus *Neurospora crassa*, which enhanced the sexual spore production upon its exogenous addition to agar medium of this fungus (Nukina et al., 1981).

The sporogenic effect of linoleic acid and the oxylipin ricinloeic acid on *P. sclerotiorum* culture is only an assumption based on the fact that those molecules were previously reported to increase sporulation levels in other filamentous fungi. Therefore to determine the exact correlation between those molecules and sporulation, it is suggested to study the effect of their exogeneous addition on to *P. sclerotiorum* cultures.

Sporogenic effects of different lipophilic molecules were also previously reported in fungi. The γ-butyrolactone containing molecule, butyrolactone I isolated from the filamentous fungus *A. terreus* caused an increase in the number of visible and viable spore formations with approximately 3 folds or more in submerged fermentation (Schimmel et al., 1998).

Other examples where lipophilic molecules regulate sporulation are in the filamentous bacteria *Streptomyces* spp*.* For instance, the bacterium, *Streptomyces griseus* produces small signalling molecules, γ- butyrolactones, that are also known as A-factors. A-factors induce sporulation as well as the secondary metabolite production in this bacterium (Horinouchi and Beppu, 1994).

Other molecules reported to induce sporulation include the diterpene, conidiogenone isolated from *Penicillium cyclopium*. Conidiogenone is produced during the growth phase under submerged conditions, and it triggers signal

transduction pathways when it accumulates beyond threshold levels of 350pM (Roncal et al., 2002).

4.3 Hyphal branching

Hyphal morphology is also one of the various physiological traits regulated by quorum sensing (Schimmel et al., 1998). Hyphal elongation and hyphal branching, which emerge from the lateral walls of the main hyphae, are two fundamental processes required for efficient colonization and substrate utilization during fungal growth (Yarden, 2004).

Extracts from different cell densities of *P. sclerotiorum* cultures were exogenously added to the *P. sclerotiorum* submerged cultures of this fungus. *P. sclerotiorum* submerged cultures includes hundreds of fungal hyphae. Even though about 100 hyphae were included in analysis for each condition, yet this number was just considered a small representation; therefore this was a preliminary study conducted to investigate the impact of the extracts on lateral branching. Representative microscopic images showed that *P. sclerotiorum* cultures treated with extracts from low cell densities (days 2, 4) had little or no effect on the lateral branching of *P. sclerotiorum* hyphae as compared to the control, which is characterised by extensive branches (Figure 3.5). On the other hand, *P. sclerotiorum* samples treated with extracts prepared from higher cell density cultures (day 6 and 8) had limited, or almost no branching (Figure 3.5). So, it was concluded that the decrease in percentage of lateral branching was due to either a delay in the onset of branching or hyphal inhibition (Figure 3.4). These indicated that the putative presence of a quorum sensing molecule(s) in the extract that delayed the onset of hyphal branching in a proportional way to the cell density.

It is known for a long time that nutrient concentration has no significant influence on lateral branching in fungi (Trinci, 1969). The effect of exogenous addition of endogenous signalling molecules on hyphal branching has been studied with different outcomes; for instance, γ-butyrolactone, a secondary metabolite by *A. terreus*, was shown to induce hyphal branching upon its exogenous addition (Schimmel et al., 1998); whereas the addition of benzoic acid, an endogenous

molecule produced by *Pleurotus ostreatus*, was shown to inhibit hyphal branching in that filamentous fungus (Lettera, 2010).

Another study conducted by Spohr et al. (1998) showed that the substitution of the *A. oryzae* fresh medium with spent medium resulted in an increase in apical hyphae extension as well as increase in branching. The presence of an endogenous compound in *A. oryzae* was suggested to contribute to the induction of hyphal growth and branching.

The data obtained from GC-MS analysis (Table 3.2) reported the presence of benzoic acid in the culture extract; however it is not established yet whether benzoic acid is involved in the delay of hyphal branching in *P. sclerotiorum* in a similar manner as to *Pleurotus ostreatus.*

4.4 Secondary metabolism

4.4.1 Secondary metabolism on agar plates

The objective of this part of the study was to investigate how *P. sclerotiorum* culture extracts prepared from different cell densities impact secondary metabolite production. The industrially useful secondary metabolite, sclerotiorin was identified by the strong orange colour on plates (Lucas et al., 2010). The effect of the different cell density extracts on the onset of secondary metabolite production was macroscopically visualised on agar plates. The obtained results supported the putative presence of a regulating molecule in the extract, in which its concentration was proportional to the population cell density since only plates treated with extracts from higher cell densities (Figure 3.6, Plates D, and E); i.e. later stages of the stationary phase showed an increased in the orange colour formation compared to those treated with extracts from low cell density (Plates B and C) and control (Plate A). Therefore it is again suggested, that extracts from high cell densities, contain putative molecules that induce the production of secondary metabolites.

4.4.2 Secondary metabolism studies using RSM

Response surface methodology (RSM) is defined by Myers and Montgomery (2002) as a collection of mathematical and statistical methods that are used to

develop, improve, or optimize a product or a process. RSM has been widely used in fermentation experiments to determine the interaction between two or more variables. For instance Tanyildizi et al. (2005) investigated the effects of different macronutrients on α-amylase production by *Bacillus subtilis.* Another study by Ambati and Ayyanna (2001) investigated the quantitative effects of substrate concentration, pH, temperature, ammonuium nitrate, potassium ferrocyanide and time of fermentation on citric acid production by *A. niger.*

Response surface methodology was used in this study to investigate the role of two variables: the addition time and % concentration (vol/vol) of the culture extract prepared from high cell density *P. sclerotiorum* cultures on sclerotiorin production. It was observed that both variables played a key role in the regulation of sclerotiorin production as well as its final yield, but they had no significant impact on the cell dry weight as determined by ANOVA (Table 3.1).

Sclerotiorin production increased proportionally with the concentration of the added extract, however the optimum addition time, as determined by the Stat-Ease design software, is around day 2 of the fermentation (Figure 3.9). In fact sclerotiorin production increased by 2.1 folds when the extract was added at 0.113% (vol/vol) on day 3 as compared to the control culture; whereas it had no impact on sclerotiorin production when added at a later stage of the fermentation (day 6). The results can be observed from the 3D plot in Figure 3.9b. therefore, it is speculated that the addition of quorum sensing molecules, at early stage of fermentation, trigger changes the secondary metabolite production pathways, including the induction of sclerotiorin production.

The obtained results are also comparable to those examples where established quorum sensing molecules were exogenously added to filamentous bacteria, *Streptomyces* spp. For instance, the exogenous addition of the autoinducer γ-butyrolactone A-factor to *Streptomyces griseus* cultures caused earlier production of streptomycin (Beppu, 1992). Similarly the addition of the autoregulator virginiae butanolide C to *Streptomyces virginiae* resulted in 2.5 fold increases in the production levels of the secondary metabolites viginamycin M and S compared to the control that had no virginiae butanolide C added (Yang et al. 1996). Moreover it was also found that the secondary metabolite pristinamycin is regulated by the

A-factor in the filamentous bacteria *Streptomyces pristinaespiralis* (Paquet et al. 1992).

In fungi, secondary metabolism was also found to be regulated by quorum sensing. For instance the exogenous addition of butyrolactone I enhanced the production of the secondary metabolite lovastatin by three folds and that of sulochrin by two folds (Schimmel et al., 1998).

In a very recent work, Raina et al. (2012) suggested a new role for butyrolactone I as a quorum sensing molecule in the filamentous fungus *A. terreus.* The exogenous addition of butyrolactone I at nan-molar concentrations resulted in an increase of 2.5 folds in the production of secondary metabolite lovastatin and also led to an increase in the levels of endogenous butyrolactone I, through a novel auto-stimulatory effect (Raina et al., 2012).

The optimum concentration and the addition time of the culture extract that were determined by RSM were then validated in shaken flasks and then used for scale-up to a 2L bioreactor. In the 2L bioreactor, high cell density culture extract was added at a final concentration of 0.15 % after 2.06 days. Sclerotiorin production in the 2L bioreactor was enhanced by 2.2 folds (Figure3.13). The similar increase in sclerotiorin production in the 2L bioreactor confirms that the results obtained in shaken flasks are scalable; it thus opens up an opportunity for utilization of culture extract as method for enhanced industrial production of this metabolite. Similar results were reported by (Williams, 2009) where partially purified supernatant extracts from *A. nidulans* enhanced the production of the secondary metabolite penicillin in 2L and 20 L bioreactors in a similar manner to 500mL shaken flasks (Williams, 2009).

4.5 TLC and GC-MS analysis

As previously discussed, the active compounds within the ethyl acetate extract could be considered as QSM. Those QSM contributed to the sporogenic effect, onset of lateral branching, and secondary metabolism in *P. sclerotiorum*. The active compounds were separated using analytical and preparative TLC. The whole extract in addition to the separated fractions were subsequently identified using GC-MS analysis.

Results obtained from TLC (Figure 3.18) highlighted that components with an Rf of 0.49 were only available at a high cell density (corresponding to days 5, 6, 7 and 8 of *P. sclerotiorum* culture). Analysis of whole extracts, as well as compounds of an Rf of 0.49 indicated the presence of several compounds including the oxylipin ricinoleic acid in the culture supernatant of *P. sclerotiorum* (Table 3.2).

Ricinoleic acid is produced by the oxylipins producing enzymes: fungal dioxygenase and linoleate- diol synthase (Su and Oliw, 1996, Tsitsigiannis et al., 2005). Oxylipins were previously reported to act as quorum sensing molecules in fungi (Tsitsigiannis and Keller, 2007). For instance the deletion off the *Ppo* genes, oxylipins producing dioxygenase genes, affected the production of two secondary metabolites; the mycotoxin sterigmatocystin and the antibiotic penicillin as well as shifted the reproduction from asexual to sexual in *A. nidulans* (Tsitsigiannis et al., 2005). It is therefore speculated that oxylipins play a similar role in *P. sclerotiorum* regulating sporulation, lateral branching and secondary metabolism.

4.6 Summary of *Penicillium sclerotiorum* studies

This study confirms the presence of a regulatory mechanism used by *P. sclerotiorum* to regulate three different physiological traits. This could be considered as quorum sensing process as it fulfils a couple of the essential criteria previously mentioned. 1) The culture extract containing active compounds was prepared from *P. sclerotiorum* culture supernatant; thus it confirms the first criteria that the molecules are excreted to the extracellular environment during the microbial growth. 2) The active compounds accumulated in a concentration that was proportional to the population cell density since extracts from higher cell-densities exhibited significant effects as compared to those prepared from lower cell densities cultures. 3) Three physiological traits were altered upon the exogenous addition of the high-cell density extract: sporulation, hyphal branching and sclerotiorin production. Further studies are required to purify and characterise the main molecule(s) contributing to the quorum sensing mechanism. Furthermore, the impact of the exogenous addition of the purified molecule on *P. sclerotiorum* culture should be investigated.

Aspergillus terreus Studies

4.7 Lovastatin production and morphology in *A. terreus*

Oxylipins are ubiquitous molecules, derived from polyunsaturated fatty acids (PUFAs) by the addition of oxygen into its fatty acid backbone using the oxygenase class of enzymes such as lipoxygenase and dioxygenases. Linoleic, linolenic and oleic acids are the main fatty acids previously reported to generate fungal oxylipins (Tsitsigiannis et al., 2005).

Oxylipins have endogenous functions, and are involved in inter-organismal signalling. Endogenous functions of oxylipins include the regulation of fungal development, sporulation, morphology and secondary metabolite production (Tsitsigiannis and Keller, 2007).

Previous studies reported the putative presence of linoleate dioxygenase enzymes in *A. terreus*, whose activity is believed to be involved in the oxygenation of linoleic acid into its corresponding oxylipins, 9-HpODE (Sorrentino, 2009, Jernerén et al., 2010). The impact of linoleic acid, as an oxylipin precursor, on lovastatin production was investigated in shaken flasks and in 5L bioreactor. Its impact on *A. terreus* morphology was also observed in a bioreactor.

4.7.1 Shaken Flask Studies

Regulation of secondary metabolite production using linoleic acid addition was previously reported by a study conducted within our research group by Sorrentino et al. (2010), where they demonstrated that the supplementation of 0.1% linoleic acid on the day of inoculation (fermentation day 0) to *A. terreus* cultures in 500mL shaken flasks enhances the production of lovastatin. The results obtained were confirmed in this study, with a similar increase of 1.7 folds in lovastatin production by *A. terreus*.

The role of PUFAs as signalling molecules as well as their impact on fungal mycelia and secondary metabolism has been extensively studied. For instance, a research conducted by Yang et al. (2000) investigated the impact of three PUFAs, oleic acid, palmitic acid and linoleic acid, on mycelial growth and polysaccharide formation by the fungus *Ganoderma lucidum*. Their research showed that

stimulation or inhibition of mycelial growth is effected by both the length of the carbon chain and the saturation level of the fatty acid. The production of polysaccharide increased, proportionally, upon the supplementation of increasing concentrations of palmitic acid and oleic acid. Furthermore, the polysaccharide production was suppressed after the addition of linoleic acid and oleic acid. The effect of linoleic acid on lovastatin production in *A. terreus* could be a direct consequence of linoleic acid as a carbon source and/or due to oxylipins derived from the oxygenation of linoleic acid.

4.7.2 Bioreactor studies

Bioreactor studies using a 5L stirred tank reactor (4.5L working volume) were carried out to investigate whether linoleic acid supplementation enhances lovastatin production in bioreactor in a manner similar to the shaken flasks. The objective behind bioreactor studies was to compare lovastatin production and pellet morphology in the bioreactor supplied with linoleic acid to that of the control bioreactor (with no added linoleic acid).

The original configuration of the 5L bioreactor included two Rushton turbine impellers placed at a spacing of h/2, (h represents the height of the liquid in the reactor). Visual examination of the culture operated under these conditions showed a built up in the mycelial cake on the walls of the reactor and surface of the culture. This growth resulted in a lack of culture homogeneity thus fermentation inefficiency. This was also confirmed by the oxygen transfer limitation that was indicated by the drop in the dissolved oxygen tension below 20%.

In order to overcome these problems, mainly the formation of surface cakes (Figure 3.23a), the impeller types and position were adjusted. One of the two Rushton-turbine impellers was replaced by a variable-pitch impeller (with blades set at a 45° angle), which was placed near the top whereas the second impeller, Rushton-turbine impeller, was placed near the bottom of the reactor. This was arranged such that the spacing between the impellers was adjusted to h/3. The bioreactor adjustments were reflected in improved culture homogeneity (Figure 3.23b) compared to previous runs. Very little wall growth of *A. terreus* was observed in the fermentation, mainly towards the end of the fermentation, starting on the 5^{th} day.

Similar adjustment of the impeller types and their position in the reactor was previously carried out by Patrick et al. (1995) in order to improve culture homogeneity and enhance the production of the metabolite swainsonine by the filamentous fungus *Metarhizium anisopliae*. In particular, a surface mycelial cake, cultural non-homogeneity, and reduced productivity were initially observed when three Rushton-turbine impellers were used. To overcome the observed problems the upper Rushton-turbine was replaced by a Variable-pitch impeller, with blades set at an angle of 45°.

There are a range of factors that impact lovastatin production, biomass concentration as well as pellet morphology in mechanically agitated submerged batch fermentation of *A. terreus.* Some of these factors include shear stress (agitation speed), mode of aeration (O_2 or air-sparged), and number and type of impellers (Casas López et al., 2005).

Results obtained from the batch fermentation of *A. terreus* in 5L STR showed an increase in lovastatin production by 2.1 folds as compared to the results obtained under control conditions, with no significant impact on the pH and %DOT between the two bioreactors (Figure3.24, a&b). Lovastatin production was enhanced in 5L STR in a similar manner to those in shaken flasks upon linoleic acid supplementation (Figure3.24 c). However, on the other hand, the overall lovastatin concentration in STR was lower as compared to those obtained in shaken flasks.

Several studies previously reported lovastatin production via batch-fermentation in stirred tank bioreactors. For instance, Casas López et al. (2005) found that the agitation speed , and the mode of aeration (O_2 or air enriched) does not impact the biomass concentration, however it affects the pellet morphology, broth rheology and lovastatin titres by *A. terreus* in submerged batch fermentation in STRs.

A recent study by Pawlak et al. (2012) showed that lovastatin titres decrease by 87% upon fermentation scale-up from shaken flasks of 150mL to 5.3L working volume stirred tank reactor. This decrease was also evident from the scale-up studies from shaken flasks to 5L STR in the current study, with a 41% drop in the overall lovastatin concentration (control, no added linoleic acid) and a 26% drop in lovastatin concentration for linoleic acid-treated cultures. The percentage decrease in lovastatin production in this study was much less as compared to that obtained

by Pawlak et al. (2010) the observed results are most probably due to the applied adjustments to the bioreactor.

Aspergillus terreus can grow in a variety of morphological forms, ranging from the dispersed mycelia to tightly packed pelleted form (Rodriguez-Porcel et al., 2005). The morphology form is affected by the culture conditions, agitation speed, and sample time. Microscopic images of *A. terreus* pellets revealed morphological changes. Samples from the linoleic acid treated bioreactors illustrated that the *A. terreus* hyphae had lateral branches, unlike those from the control bioreactor with relatively longer non branched hyphae. As all parameters were maintained between the bioreactors, this illustrates that morphological changes were a result of linoleic acid supplementation, where linoleic acid and its derived oxylipins act as signalling molecules to induce morphological changes in this fungus. These results were similar to those by (Schimmel et al., 1998) where the addition of the butyrolactone I similarly resulted in short, branched hyphae as compared to the control cultures with no butyrolactone I added. Other studies by Radman et al. (1994) investigated the relationship between the production of the secondary metabolite penicillin G and the morphological effects of *P. chrysogenum.* It was demonstrated that the addition of oligosaccharide elicitors resulted in an increase of 120% of penicillin G levels together with an increase of 47% in hyphal tip numbers (Radman et al., 2004).

It is believed that lovastatin production occurs at the tips of hyphae. The increase in transcriptional activation of lovastatin biosynthetic genes (*lovB* and *lovF*) (Sorrentino et al., 2010) in addition to the morphological changes could explain the overproduction of lovastatin production in *A. terreus* cultures.

4.8 Proteomics of *Aspergillus terreus*

Proteomic studies were carried out in *A. terreus* in an attempt to obtain a systemic understanding of the molecular mechanism of the quorum sensing signalling process in this fungus at protein levels. Only limited work had been conducted on the proteome of *A. terreus,* with an exclusive study carried out by Han et al., (2010) on the extracellular proteome of *A. terreus* grown on different carbon sources: glucose, sucrose and starch. The majority of the identified proteins were hydrolytic enzymes, such as hydrolases, glycosylases and proteases.

In the present study the cytosolic proteome profile of *A. terreus* was investigated in response to linoleic acid supplementation. The cytosolic fraction was chosen in order to investigate changes in proteins involved in biochemical pathways in this fungus. The cytosolic fraction is comprised of dissolved ionic solutes, small molecule metabolites and macromolecules such as nucleic acids and proteins (Ito et al., 2014) . The most common biochemical reactions occurring in cytoplasm of living organisms are glycolysis, the oxidative branch of the pentose phosphate pathway, protein biosynthesis and degradation, signal transduction, primary and secondary metabolite biosynthesis and transportation, stress response signalling, and the accumulation of enzymes required for defence and detoxification pathways. In addition to the synthesis of nuclear-encoded organellar proteins (Ito et al., 2014)

A total of 24 proteins within the cytosolic fraction were identified by MALDI-TOF/TOF mass spectrometry. The discussion of the identified proteins illustrated their up-regulation in response to the treatment of *A. terreus* cultures with 0.1% linoleic acid. The identified proteins (Table 3.3) were classified into three major categories; detoxification and stress-related proteins (including oxidative stress), primary metabolite biosynthesis and energy metabolism (e.g. carbohydrate metabolism processes), and the third category includes additional proteins putatively involved in secondary metabolite production and signalling mechanisms as well as those predicted, hypothetical proteins with either unknown or suggested functions.

4.8.1 Detoxification and other stress-related proteins

Aerobic organisms are continuously exposed to reactive oxygen species (ROS) that are subsequently produced in living cells as a result of different metabolic processes as well as environmental stresses: biotic and abiotic (Barzilai and Yamamoto, 2004, Hayes et al., 2005). ROS such as superoxides, hydroxyl radicals, as well as hydrogen peroxide and glutathione have a powerful antioxidant activity that cause oxidative damage to the nucleotides (DNA), proteins and lipids if they were not efficiently detoxified and removed from the cells (Cabiscol et al., 2010).

Glutathione S-transferase (GST), superoxide dismutase, spermidine synthase and HSP70 were identified among the up-regulated proteins in *A. terreus* cytosolic proteome in response to linoleic acid treatment. Detoxification pathways in *A. terreus* were probably induced due to either an increase in the metabolic activities such as glycolysis pathways, or due to an induction of lipid oxidation or oxylipin production pathways.

Glutathione S-transferases (GST) are a small group of ubiquitous cytosolic proteins, containing a redox-active sulfhydryl group. GSTs are involved in the cellular defence mechanisms against endogenous xenobiotics and ROS. GST combines glutathione with the xenobiotics and ROS, which are then excreted to vacuoles and detoxified. In addition to their detoxification pathways; GSTs were previously reported to function in the synthesis of the mammalian oxylipin prostaglandin (Oakley, 2005). The up-regulation of this enzyme in the cytosol of *A. terreus* suggests its involvement, as a stress-related response protein, in the defence mechanisms triggered by the stress induced upon the supplementation of linoleic acid (Tsitsigiannis and Keller, 2007). Superoxide dismutase is another up-regulated protein. Superoxide dismutase is a detoxifying enzyme, mainly involved in the dismutase of superoxides O_2^- into oxygen and hydrogen peroxide, since the oxygen radicals produced in the cells are toxic to the biological systems (Alvarez et al., 1987, Scandalios, 1993).

Spermidine synthase was among the upregulated proteins upon linoleic acid supplementation in this study. Spermidine synthase is a key enzyme for the biosynthesis of polyamines (Kasukabe et al., 2004). Polyamines are ubiquitous,

small, aliphatic, nitrogen compounds that have a vital role in the growth and the development of various organisms (Sacramento et al., 2004). Spermidine, spermine and putrescine are the three most, common, naturally occurring polyamines (Tabor and Tabor, 1985). They were previously reported to have key functions in the cell differentiation processes including sporulation, spores germination and morphological switch from budding yeasts to mycelial growth in dimorphic fungi (Herrero et al., 1999, Marshall et al., 1979). For instance mycelial growth and sclerotiorum germination in *Sclerotiorum rolfsii* were directly correlated to increased levels of the polyamine peutrescine, where the high levels of spermine was correlated with sclerotiorum formation (Gárriz et al., 2008). In addition, deletion of spermidine synthase in *A. nidulans* has been associated with defects in transitions from germ tube to hyphae, hyphal growth, and secondary metabolite production (Jin et al., 2002). Therefore the up-regulation of spermidine synthase is correlated with an increase in polyamines, which might be an indicator of the morphological changes of *A. terreus* resulting in response to linoleic acid addition. The up-regulation of the heat-shock protein Hsp70 is suggested to be a consequence of the oxidative stress induction (Hahn and Thiele, 2004, Plesofsky-Vig and Brambl, 1993).

In this context, it is reported that the oxidation of linoleic acid by *A. terreus* leads to the production of hydroxyperoxides and other oxygen radicals (Jernerén et al., 2010). The presence of hydroperoxides triggers oxidative stress, and thus induces a stress response. Therefore it is speculated that the up-regulation of stress-related proteins is a consequence of linoleic acid oxygenation into its corresponding hydroperoxides. This is supported by a previous study that showed reactive oxygen species and detoxification mechanisms increased in *S. cerevisiae* upon its treatment with linoleic acid hydroperoxides (Evans et al., 1998).

Besides oxygenases-derived pathways, oxylipins can be derived from PUFAs through an additional reaction sequence known as free-radical-catalysed non-enzymatic lipid peroxidation. This pathway is catalysed by reactive oxygen species (Durand et al., 2009). Therefore another suggestion is that stress related response proteins were up-regulated in response to an increase in ROS required for oxylipin biosynthesis.

4.8.2 Carbohydrate transport and metabolism

Several proteins involved in the metabolic pathways were identified in the cytosolic protein profiles of *A. terreus* as up-regulated proteins upon linoleic acid supplementation. The upregulated proteins include: fructose bis-phosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, a protein similar to alcohol dehydrogenase, aldose-6-phosphate reductase, and mannose-6-phosphate isomerase.

Fructose bisphosphate aldolase is a heat-stable enzyme involved in both glycolysis and gluconeogenesis pathways. This enzyme catalyses the reversible reaction by converting fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Say and Fuchs, 2010). The second up-regulated protein in this category is glyceraldehyde-3-phosphate dehydrogenase; a protein family present in different subcellular locations and has a main role in glycolysis pathways. Other previously reported function of glyceraldehyde-3-phosphate-dehydrogenase is in the pathogenic fungus *Paracoccidioides brasiliensis* where it contributes to the fungal adhesion thus causing pulmonary mycosis (Barbosa et al., 2006).

A protein similar to alcohol dehydrogenase was also among the up-regulated proteins in this study. Alcohol dehydrogenases include a group of enzymes that catalyses the oxidation of ethanol to acetate via acetaldehyde. Acetate is a precursor molecule for the formation of acetyl-coA, one of the building blocks of polyketide biosynthesis, such as lovastatin (Remize et al., 2000). Aldose-6-phosphate reductase was another up-regulated protein. It belongs to the well conserved enzyme family of aldo-keto reductases. This enzyme is NADPH dependant and it catalyses the reduction of aldehyde form of glucose to its corresponding sugar alcohol, sorbitol (Karuna Sree et al., 2000). Mannose-6-phosphate isomerase is another up-regulated protein. This enzymes catalyses the inter conversion of mannose-6-phosphate and fructose-6-phosphate and it is classified as enzymes of isomerases and epimerase activities (Kalckar and Ullrey, 1986).

4.8.3 Other proteins

Other up-regulated proteins include ATP Citrate synthase subunit-1 (ATP- citrate lyase), peroxisomal membrane protein, non-heme chloropeoxidases, and transaldolase-like proteins.

ATP Citrate synthase subunit-1 is a cytosolic enzyme required for the production of acetyl-coA. The metabolite acetyl-coA is one of the main molecules required in the biosynthesis of cellular molecules as well as the production of fatty acids and sterols. It's also required for protein acetylation, including histone acetylation in the nucleus. ATP-citrate lyase was also found to be involved in developmental effects of *A. nidulans*, where its loss resulted in the reduction of asexual spore production and a complete absence of sexual development. On the other hand the absence of ATP-citrate lyase effected the maturation of the fruiting body in *Sordaria macrospora*. The effects are not repaired via acetate addition, thus it indicated the importance of this enzyme in fungal developmental processes (Hynes and Murray, 2010, Nowrousian et al., 1999). ATP citrate lyase was up-regulated upon linoleic acid addition. It is suggested that the up-regulation of ATP citrate lyase contributed to an increase in the production of acetyl coA, the building block of the polyketides such as lovastatin. A peroxisomal membrane protein was also up-regulated in this study; peroxisomes are ubiquitous organelles that are characterised with a rich protein matrix surrounded by a single membrane. In filamentous fungi, peroxisomes play a major role in primary metabolism of several carbon sources required for growth, including fatty acids. Those proteins assist the transport of acetyl-coA and NADP (H) through the membrane. There is also increasing evidence that they are involved in the secondary metabolism, as well as differentiation and development (Bartoszewska et al., 2011).

Several other identified proteins were either predictable or reported as conserved hypothetical with no role suggested by Mascot search. The presence of hypothetical and predictable proteins is due to incomplete annotation of the *A. terreus* genome of *A. terreus*. Only approximately 7.5% of the open reading frames encoded for proteins have been assigned functions. Figure 4.1 shows how different identified proteins are involved in different metabolic pathways in *A. terreus.*

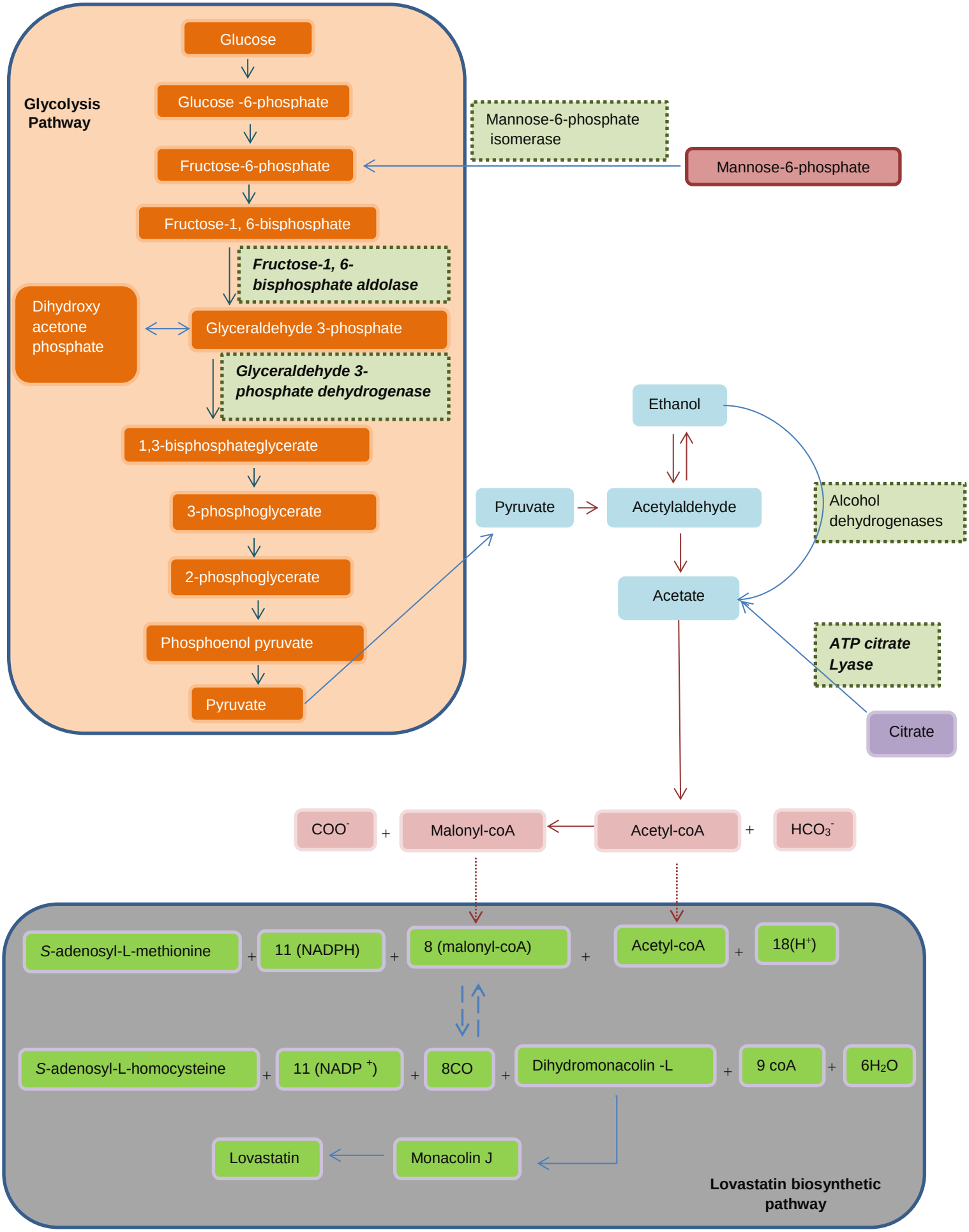


Figure 4.1: Role of identified proteins in different metabolic pathways

4.9 Oxylipin link to cAMP levels

The exogenous additions of three pure oxylipins as well as linoleic acid triggered a burst of the cAMP levels in *A. terreus*. Alteration of cAMP levels is considered as an indication of G protein signalling (Affeldt et al., 2012). For instance, the binding of the oxylipin 9(S)-HODE to a mammalian GPCR inhibit cAMP accumulation (Obinata et al., 2005). Therefore, the results suggest that the added oxylipins are putatively perceived as external signals, and triggered an internal response in the fungi.

The oxylipin 9(S)-HpODE had the major impact on the alterations of cAMP levels in *A. terreus* (Figure 3.29). The addition of increasing concentrations of 9(S)-HpODE triggered a burst of cAMP levels in a dose-dependent manner (figure 3.30). These results are supported by a previous study by Jernerén et al. (2010) who studied the oxylipins profiling of *A. terreus.* They showed via LC-MS/MS analysis that the oxygenation of the fatty acid linoleic acid leads to the production of the oxylipins 9(R)-HpODE, a stereoisomer of 9(S)-HpODE, in addition to the allene oxide 9(R)-EODE. This is subsequently hydrolysed into α- ketols (9-hydroxy-10-oxo-12(*Z*)-otadecenoic acid) and γ-ketols (13-hydroxy-10-oxo-11(*E*)-otadecenoic acid).

The oxygenation of linoleic acid into its oxylipins is achieved by dioxygenase enzymes, unlike in other *Aspergillus* spp. that uses the lipoxygenase enzymes. *A. terreus* lacks lipoxygenase enzymes (Jernerén et al., 2010).

The oxylipin 9-HpODE was previously found to decrease sporulation rate when added exogenously to *A. terreus* (Sorrentino, 2009). Another study by Calvo et al. (1999) showed that the addition of 9-HpODE to *A. nidulans* cultures induced cleistothecial formation when added at low cell levels, but results in conidial formation when added at high levels.

A previous study by Affeldt et al. (2012) showed that the model filamentous fungus *A. nidulans* respond to several pure plant oxylipins by accumulating cAMP in a dose dependant manner. Studies on *A. flavus* also revealed that oxylipins regulate secondary metabolism and spore development via a density-dependant mechanism similar to quorum sensing (Brown et al., 2008, Brown et al., 2009).

It is suggested that the addition of oxylipins, such as 9-HpODE activate the signalling mechanism by binding to a seven transmembrane-spanning G-protein coupled receptor (GPCRs). Oxylipins-bound GPCRs catalyse the exchange of GDP to GTP of the Gα subunit, which causes subsequent dissociation of Gα-GTP and Gβγ, and thus activation of various signalling mechanisms (Han et al., 2004). Upon activation, the secondary messenger cAMP is produced. cAMP then induces the expression of different genes, triggering different cellular responses such as sporulation, and secondary metabolism in *A. terreus* (Han et al., 2004, Lengeler et al., 2000, Neves et al., 2002). A schematic diagram illustrating the hypothetical signalling mechanism in *A. terreus* is present in Figure 4.2.

4.10 Putative G-protein/cAMP mediated signalling components in *A. terreus*

In-depth studies on G-protein signalling; its key components, functions and downstream signalling mechanisms, in *Aspergillus* spp. mainly focused on the model filamentous fungus *A. nidulans* and the pathogenic fungus *A. fumigatus* (Shimizu and Keller, 2001, Han et al., 2004, Yu, 2006).

In an attempt to investigate the presence of putative G-protein-cAMP signalling mechanism(s) in *A. terreus*, a homology search of *A. terreus* proteome was conducted via BLASTp. Different components of G-protein/cAMP mediated signalling that were previously identified in *A. nidulans*, and other deduced *Aspergillus* spp. proteomes were used as queries in similar analyses in *A. terreus*.

4.10.1 G-protein Coupled Receptors

Identified genes encoding putative GPCRs in the genomes of *A. terreus, are* reported in table 3.3. Phylogenetic tree analysis representing the different classes of the GPCRs in *A. nidulans, A. terreus* and *A. flavus* is represented in Figure 3.31

The identification of genes encoding putative G-protein-coupled receptors in *A. terreus* suggests that the G-protein coupled receptors could be used as the quorum sensing receptors to sense oxylipins in *A. terreus.*

Affeldt et al. (2012) investigated the possibility that the two GPCRs: GprC and GprD could be involved in oxylipin perception in *A. flavus* and *A. nidulans*. It was found that GprC and GprD are required in *A. flavus* for the regulation of spore,

sclerotioral and aflatoxin production, as well as the transition of *A. flavus* to a high-density developmental state. In *A. nidulans,* it was found that oxylipins stimulate a burst in cAMP levels and that the GPCR, *gprD* is required for cAMP accumulation in *A. nidulans.*

GprC and GprD belong to class III of GPCRs. Class III GPCRs share a high degree of similarity to Gpr1p of *S. cerevisiae* and Git3p glucose sensors of *Schizosachromyces pombe* (Kraakman et al., 1999, Welton and Hoffman, 2000, Xue et al., 1998). It was previously suggested that Class III GPCRs function as putative glucose (carbon) sensors *as they share a common ancestor with* Gpr1p and Git3p (Lafon et al., 2006).

Other studies on GprD in *A. nidulans* suggested that GprD is involved in the down-regulation of sexual development in *this fungus, as the absence of the receptor GprD is mainly associated* with germination and hyphal growth defects (Han et al., 2004).

4.10.2 Phylogenetic analysis

A phylogenetic tree representing the nine classes of GPCRs in *A. terreus, A. flavus* and *A. nidulans* (Figure 3.31) suggests a high similarity between the different *Aspergillus* spp., mainly *A. nidulans* where it is currently established that oxylipins act as signalling molecules to induce developmental and morphological changes by using G-protein/cAMP mediated signalling pathways (Affeldt et al., 2012).

4.10.3 Other components of G-protein/ cAMP mediated signalling

Other components of the G-protein/cAMP- mediated signalling pathways, including the heterotrimeric G proteins (α, β, and γ subunits), regulators of G-protein signalling proteins, in addition to components of cAMP and PKA signalling components were also identified in *A. terreus (*Table 3.5). The presence of *A. terreus* genes corresponding to G-protein/cAMP signalling pathways supports the putative presence of a signalling mechanism in *A. terreus* similar to the ones identified in *A. nidulans* and *A. fumigatus* (Affeldt et al., 2012, Lafon et al., 2006, Brodhagen and Keller, 2006).

4.11 Summary of *Aspergillus terreus* studies

The addition of linoleic acid at 0.1% was found to enhance the production of lovastatin in shaken flasks and in bioreactor (stirred tank reactor). Addition of linoleic acid was also found to alter the expression of different cytosolic proteins involved in primary and secondary metabolism in addition to proteins that exhibit stress related responses. Addition of pure oxylipins and linoleic acid induces a burst in intracellular cAMP levels in *A. terreus* tissues. The oxylipin 9(S)-HpODE resulted in the major increase of cAMP levels. Increasing concentrations of this oxylipin induced a burst in cAMP levels. Several components of the G-protein-cAMP signalling pathways were identified in *A. terreus*. G-protein-cAMP signalling pathways include GPCRs, heterotrimeric G proteins (α, β, and γ subunits), regulators of G-protein signalling proteins, in addition to components of cAMP and Pka signalling components. The identification of the putative components of the G-protein and cAMP–signalling pathways in *A. terreus* indicates that this fungus potentially uses these pathways for quorum sensing mechanisms.

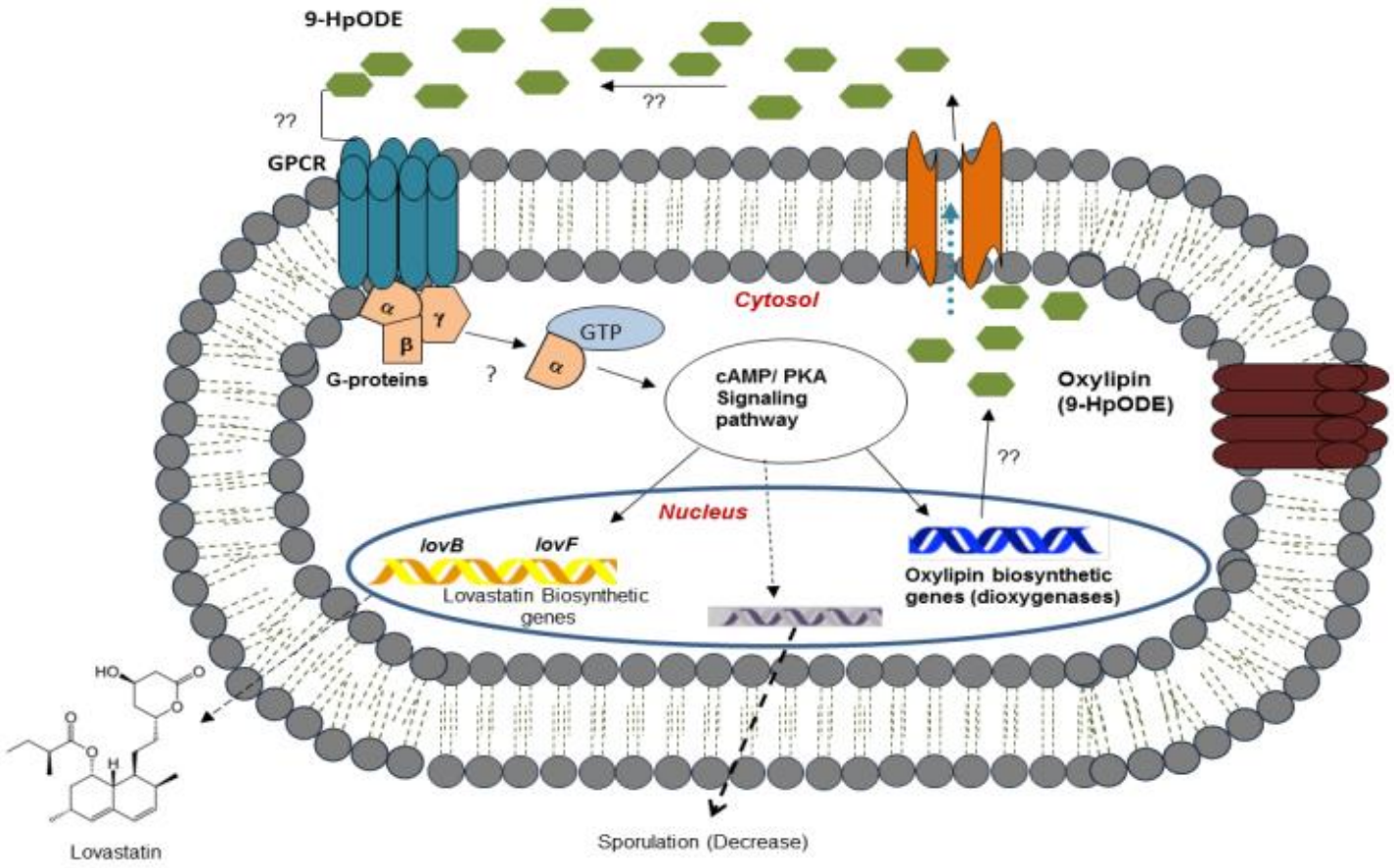


Figure 4.2: A schematic diagram of *A. terreus* hypothetical QS signalling mechanism. Oxylipins, such as 9-HpODE activate the signalling mechanism by binding to a seven transmembrane-spanning G-protein coupled receptor (GPCRs). Oxylipins-bound GPCRs catalyse the exchange of GDP to GTP of the Gα subunit, which causes subsequent dissociation of Gα-GTP and Gβγ, and thus activate the production of the secondary messenger cAMP. cAMP/ PKA signalling pathways then trigger the expression of target genes; including lovastatin biosynthetic genes, and oxylipins biosynthetic genes (autoinduction). Gene activation induces several physiological responses such as sporulation, and lovastatin production.

Chapter V

CONCLUSION

The overall aim of this project was to investigate the quorum sensing mechanisms for the improved production of industrially useful products from filamentous fungi. Investigation of QS mechanisms was carried out in two filamentous fungi: *Penicillium sclerotiorum* and *Aspergillus terreus.*

5.1 *Penicillium sclerotiorum* studies

The first chosen study was carried out to investigate the correlations between *P. sclerotiorum* cell densities, to the quorum sensing mechanism in this fungus. It was observed that the addition of *P. sclerotiorum* culture extracts from higher cell density culture supernatant to fresh *P. sclerotiorum* cultures increases sporulation rates; delays the onset of hyphal branching, and enhances the production of the secondary metabolite sclerotiorin. The impact of high cell densities culture extract on different QS regulated traits is an important feature for the presence of potential QS molecules.

Through the aid of response surface methodology, it was found that both the concentration of the added culture extract as well as its time of addition has an impact on sclerotiorin production, with no effect on the cell dry weight. The optimum conditions for the addition of high-cell density *P. sclerotiorum* culture extract (prepared from day 7 of culture) were determined by RSM. The optimum concentration was 0.15%, to be added after 2.06 days.

The optimum concentrations determined by RSM were used for scale up studies to 2L STR. A 2.2 fold increase in sclerotiorin production was observed in 2L STR treated with high-cell density *P. sclerotiorum* culture extract (prepared from day 7 of culture), as compared to control STR. Therefore this demonstrates that the results obtained in shaken flasks are conserved upon scale-up. These results

confirms that exogenous addition of culture extract, or established QS molecules could be exploited for improved production of fungal secondary metabolites.

Analysis of culture extract via analytical TLC revealed that compounds with an Rf of 0.49 are mainly present in culture extract of higher cell densities. GC-MS of the whole extract, as well as different fractions separated using preparative TLC revealed several compounds which might have a putative role in quorum sensing.

The presence of ricinoleic acid, 12-hydroxy-9-cis-octadecenoic acid, was detected in the fraction of Rf: 0.49 as well as in the whole extract via GC-MS. This compound is an oxylipin, similar to the oxylipins previously identified to regulate growth and development of the model filamentous fungus *A. nidulans.* These results represented a further evidence for the QS-like behaviour in *P. sclerotiorum.* Oxylipins such as ricinoleic acid could be one of the putative QSM in *P. sclerotiorum*. Further studies are need to be carried out to isolate, purify and identify the signalling molecule(s) contributing to the quorum sensing mechanism in *P. sclerotiorum.*

5.2 *Aspergillus terreus* studies

The second part was carried out to investigate the impact of the oxylipin precursor, linoleic acid on lovastatin production and the cytosolic profile of *A. terreus.* Additionally, the impact of different oxylipins and linoleic acid on cAMP levels was investigated. A homology search in *A. terreus* genome was carried out to identify different components of G-protein/cAMP mediated signalling pathways.

The impact of linoleic acid addition on lovastatin production in shaken flasks, and 5L STR demonstrated an increase in the production of the secondary metabolite, lovastatin. In the 5L STR, lovastatin production was enhanced by 2.1 folds thus suggesting that the experimental conditions are scalable. However the overall obtained lovastatin concentration was lower in bioreactor as compared to those of shaken flasks. This could offer the opportunity to utilize QS as a method to enhance the large scale production of lovastatin in bioreactors. The addition of linoleic acid to *A. terreus* cultures was found to alter the expression of different cytosolic proteins, mainly proteins that are stress-related or involved in the carbohydrate transport and metabolism. The results suggested that linoleic acid

supplementation induces metabolic pathways that could be involved in enhanced production of lovastatin.

Three pure plant oxylipins (9-HpODE, 13-HODE, 9-HODE), and linoleic acid were found to induce a burst in cAMP levels, a down-stream event in GPCR activation, in *A. terreus* with 9-HpODE showing the highest impact. The addition of increasing concentrations of 9-HpODE showed an increase in cAMP levels in a dose dependant manner. Therefore it is speculated that 9-HpODE could be considered as the signalling molecule in *A. terreus*. 9-HpODE is sensed by receptors, and translated with an increase in cAMP levels. These results represent additional evidence that oxylipins could be perceived by putative fungal GPCR(s) in *A. terreus*.

It is well established that cAMP pathway is a downstream event of GPCR activation, therefore it is suggested that G-protein/cAMP-mediated signalling could be used as the quorum sensing process in *A. terreus.* A homology search of *A. terreus* proteome was conducted via BLASTp to identify several components of the G-protein-cAMP signalling pathways involved in the putative signalling mechanism. G-protein-cAMP signalling pathways include GPCRs, heterotrimeric G proteins (α, β, and γ subunits), regulators of G-protein signalling proteins, in addition to components of cAMP and PKA signalling components. Proteins previously identified in *A. nidulans*, and other deduced *Aspergillus* spp. proteomes were used as queries in similar analyses in *A. terreus,* and two additional *Aspergillus* spp.: *A. flavus and A. clavatus.* The identification of the putative components of the G-protein and cAMP–signalling pathways in *A. terreus* reinforces the hypothesis that filamentous fungi potentially use these pathways for quorum sensing mechanisms.

This thesis describes a potential role of oxylipins as QS molecules in filamentous fungi. In addition to G-protein/cAMP mediated signalling to be considered as a QS pathway. This work also paves the way to exploitation of these molecules in bioprocesses for improved production of fungal secondary metabolites.

Chapter VI

FUTURE WORK

Following the investigation carried out in this thesis, several points need to be addressed to further understand the role, and mechanism of oxylipins signalling in quorum sensing pathways in filamentous fungi.

Penicillium sclerotiorum studies

6.1 Identification of the Quorum Sensing Molecule

GC-MS of the ethyl acetate extract of high cell density *P. sclerotiorum* culture supernatant revealed the presence of more than 100 compounds. Lipophilic molecules such as the oxylipin, ricinoleic acid, and the γ-butyrolactone containing molecules multicolic acid and its derivatives were suggested as putative signalling molecules in this study and the previous study by Raina et al. (2010).

Further steps are required to purify and identify the molecule(s) contributing to the quorum sensing responses in *P. sclerotiorum*. Quorum sensing molecules are produced at very low concentrations, that they cannot usually be detected by conventional methods. Prior to purification and identification, large scale production of the culture ethyl acetate extract should be carried out. Furthermore, the ethyl acetate extract of *P. sclerotiorum* culture supernatant should then be fractionated by HPLC. Different fractions shall be tested for the presence of the bioactive compound.

The development of a bioassay is required as a rapid screening method for testing the presence of the active metabolite(s) within the ethyl acetate fraction of high cell density *P. sclerotiorum* culture extract. One suggestion for a qualitative bioassay

could be done according to the method developed by Martín et al. (2011). The presence of a bioactive compound in the culture extract (or any obtained fraction) could be tested by sclerotiorin induction. Sclerotiorin is an antibiotic, and has antimicrobial properties against different Gram-positive and Gram-negative bacteria as well as some fungal species (Lucas et al., 2007). The antimicrobial properties of sclerotiorin inhibits the growth of bacteria, this could be detected by the presence of inhibition halo zones. The inducing capability of the fractions is correlated to the diameter of inhibition halo zones. QSM will induce sclerotiorin production; as an antibiotic sclerotiorin would then contribute to an increase in the diameter of inhibition halo zones. Another screening method to detect the presence of QSM in the ethyl acetate fractions is to construct a reporter gene, which could be used as a marker for sclerotiorin production. However the lack of the genome sequence is considered a limitation for the suggested detection method.

The active fraction could then be purified and concentrated by a series of steps, such as solid phase extraction, HPLC-MS, adsorption column chromatography. When the compound(s) is purified and isolated, its structural elucidation could be carried out via NMR spectrometry. The identified molecule could then be confirmed as a quorum sensing molecule by studying the direct impact of its exogenous addition to *P. sclerotiorum* cultures.

6.2 Genome sequencing of *P. sclerotiorum*

Research on quorum sensing in bacteria as well as in fungi mainly focus on identifying the molecular events, gene(s) transcription and signalling pathways involved. Therefore the presence of an annotated genome sequence could facilitate the understanding of the quorum sensing mechanism at the molecular levels.

The availability of the genome sequence of *P. sclerotiorum* would allow the design and manufacture of genomic microarrays which could then be applied to determine the transcriptional response of *P. sclerotiorum* to the presence of the quorum sensing molecules. The use of whole-genome microarrays would also

offer the possibility to gain a picture of the global pattern of *P. sclerotiorum* gene expression that is regulated by the identified quorum sensing molecules.

Aspergillus terreus studies

6.3 Construction of GPCR mutants

Linoleic-acid derived oxylipins regulate the development, secondary metabolism (mycotoxins, antibiotics), sporulation and morphology of different *Aspergillus* species (Tsitsigiannis and Keller, 2007). Oxylipins were also reported to be involved in quorum sensing pathways of *A. flavus* (Brown et al., 2008).

In mammals, oxylipins such as prostaglandins and leukotrienes are perceived by G- protein coupled receptors. Oxylipins such as 13-HpODE were previously found induce a cAMP burst in *A. nidulans,* and the loss of the *A. nidulans* GPCR, *gprD*, prevents the accumulation of cAMP. It was also found that *A. flavus* requires two putative GPCRs (GprC and GprD) to regulate spore, sclerotial and aflatoxin production (Affeldt et al., 2012).

Several putative GPCR were identified in *A. terreus* in this study, similar to those identified in the model filamentous fungus *A. nidulans.* The exogenous addition of oxylipins, in particular 9-HpODE, was found to induce a burst of cAMP levels in *A. terreus.*

Construction of *A. terreus* mutants for the different identified GPCRs, mainly those encoding GprD and GprC, would be useful to investigate the connection between the *A. terreus* putative GPCR genes and oxylipin signalling mechanism via cAMP mediated pathways. This could clarify if quorum sensing in *A. terreus* involves G-protein/cAMP mediated signalling.

6.4 Construction of dioxygenase mutants

Previous BLAST search into *A. terreus* genome revealed the presence of five putative oxylipin biosynthetic genes that are homologues of *A. nidulans Ppo* genes. Supplementation of linoleic acid to *A. terreus* cultures also caused an increase in the expression levels of three out of the five putative oxylipin

biosynthetic genes ATEG_02036, ATEG_03992 and ATEG_04755 (Sorrentino, 2009).

Construction of *A. terreus* mutants for each one of the three putative oxylipin biosynthetic genes could be useful in investigation of the connection between the *A. terreus* putative dioxygenase genes and oxylipin formation, as well as study of the functional roles of those genes on *A. terreus* morphology and development.

The proteome profile of *A. terreus* wild type strain and the dioxygenase deficient strains could be studied upon the supplementation of linoleic acid or the oxylipin 9-HpODE. The impact of linoleic acid on sporulation and lovastatin production could also be performed in both *A. terreus* wild type strain and the dioxygenase mutant strains. Different responses would be expected from the two strains due to alteration of the oxylipin profile in the mutant strain(s). The involvement of those genes in oxylipin formation could therefore be confirmed.

6.5 Bioreactor studies

Linoleic acid supplementation was found to enhance the overall production of lovastatin in shaken flasks as well as in the 5L conventional Stirred tank reactor. The overall lovastatin concentration decreased in the stirred tank bioreactor as compared to the shaken flasks. To further enhance large scale production of lovastatin, alternative bioreactors could be investigated as compared to the conventional stirred tank bioreactor. Two suggested bioreactors to be investigated are bubble column or air lift bioreactors as they previously showed to improve lovastatin production titres compared to STR (Rodriguez-Porcel et al., 2006, Casas-López et al., 2005).

Chapter VII
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Chapter VIII

APPENDIX

8.1 Responses: Sclerotiorin yield (mg/g) and Total CDW (g/L)

Run	Extract addition %	Addition Time (days)	Total CDW (g/l)	Sclerotion Yield (mg/g)
1	0.0000	6.00	4.32	5.7
2	0.0750	4.50	4.23	8.79
3	0.0750	1.50	4.12	10.76
4	0.0750	1.50	4.32	10.51
5	0.0375	3.00	4.45	8.45
6	0.0750	4.50	4.11	8.76
7	0.0750	3.00	4.2	10.87
8	0.0750	4.50	4.06	9.12
9	0.0000	0.00	4.02	6.28
10	0.1500	6.00	4.91	6.78
11	0.0375	3.00	3.82	8.37
12	0.0750	1.50	4.26	10.23
13	0.1500	0.00	4.17	10.7
14	0.0750	1.50	4.72	10.87
15	0.0000	6.00	3.9	5.8
16	0.0375	3.00	3.91	8.24
17	0.1125	3.00	4.05	13.61
18	0.0000	0.00	4.01	6.44
19	0.0750	4.50	4.05	8.65
20	0.0375	3.00	4.32	8.41
21	0.0750	3.00	3.98	10.99
22	0.1125	3.00	4.41	13.45
23	0.1125	3.00	4.7	13.26
24	0.0750	3.00	4.87	11.12
25	0.1500	6.00	4.71	6.67
26	0.1500	0.00	4.41	10.91
27	0.0750	3.00	4.15	10.91
28	0.1125	3.00	4.21	13.45

8.2 Multiple Sequence Alignments of GPCRs identified in *A. terreus, A. nidulans and A. flavus.*

Class I: PreB

```
AtPreB          -----MGSTFDPFSQSITLRGPAENEITIPIETVDFFYQYCIRICINYGAQLGASIVLFI 55
AflPreB         MTMAIMDSKFDPYSQNLTFHAADGTPFQVPVMTLNDFYQYCIQICINYGAQFGASVIIFI 60
AnPreB          -----MASNFDPWNQTITFRLEDGTPFDISVDYLDGILQYSIRACVNYAAQLGASVILFV 55
                     * *.***:.*.:*::    . : :.:  :: : **.*: *:**.**:***:::*:

AtPreB          ILLLLTRPEKRRSSVFVLNITALLLNVSRLICQLIYFTSEFVRLYAYFGNDFSRVPPSAY 115
AflPreB         ILLLLTRPDKRASSVFFLNGGALLLNMGRLLCHMIYFTTDFVKAYQYFSSDYSRAPTSAY 120
AnPreB          ILVLLTRAEKRASCLFWLNSLALLLNFARLLCDVLFFTGNFVRIYTLISADESRVTASDL 115
                **:****.:** *.:* **  *****..**:*.:::** :**: *  :. * **...*

AtPreB          ADSILGVVLVTLLIVCIEASLVLQAQVVCANLRRGYRRILLAVSIVMALVPIIFRFVYMV 175
AflPreB         ANSILGVVLTTLLLVCIETSLVLQVQVVCANLRRRYRTVLLCVSILVALIPVGLRLGYMV 180
AnPreB          ATSIVGAIMTALLLTTIEISLVLQVQVVCSNLRRIYRRALLCVSAVVATATIAIRYSLLA 175
                * **:*.::.:**:. ** *****.****:**** **  **.** ::*  .: :*   :.

AtPreB          QNCWHIMHAAETIRLNWLESATNVVITISICFFSAVFITKLGFAMHLRKRLGMTDFGPMK 235
AflPreB         ENCKTIVQTDTPLSLVWLESATNIVITISICFFCSIFIIKLGFAIHQRRRLGVRDFGPMK 240
AnPreB          VNIRAILEFSDPTTYNWLESLATVALTISICYFCVIFVTKLGFAIRLRRKLGLSELGPMK 235
                 *   *:.   .    **** :.:.:*****:*. :*: *****:: *::**: ::****

AtPreB          VIFVMGCQTMAIPAIFSILQYIVSVPELASNIFSLVTISLPLSSIWAGFTIDQRARDSGM 295
AflPreB         VIFVMGCQTLTVPALLSILQYAVSVPELNSNIMTLVTISLPLSSIWAGVSLTRSS-STEN 299
AnPreB          VVFIMGCQTLVIPVIVSITHYVSDVPELQTNVLTIVALSLPLSSIWAGTTIDKPVTHSNV 295
                *:*:*****:.:*.:.** :*  .**** :*::::*::********** :: :    :

AtPreB          SSSRNNLFQALSFSKEAKTRQSS---TVTYASQPSSKSHTLCYAELSPAKQQ-------- 344
AflPreB         SPSRGALWNRLTDSTGTRSNQTSSTDTAVAMTYPSNKSSTVCYADQSSVKRQ-------- 351
AnPreB          R----NLWQILSFS-GYRPKQSTYIATTTTAT-TNAKQCTHCYSESRLLTEKESGRNNDT 349
                     *:: *: *   :..*::   *..  : .. *. * **::    ..:

AtPreB          -SDAEAAYGIAVERDISIHSCRKDGKAPNV 373
AflPreB         -YDPEQGHGISVEHDVSVHSCQRL------ 374
AnPreB          SSKSSSQYGIAVEHDISVRSARRESFDV-- 377
                  ...  :**:**:*:*::*.::
```

Class II: PreA

```
AtPreA        -----MALYAQTLLVPILSVVSMVLSITPLILHWQNRNWPATCLICWCLGLNLFNVVNAL 55
AflPreA       MSAPSTTPYAQAVIIPSFSILCMLLTIPPLILHWKNRNLPVVSLICWLLCLNLFNIINAF 60
AnPreA        MDAPKTSPSAEAVVMQALSAIAVIITSSTLALHWKNRNFPAAIMMGWLILLNLFNVINAF 60
                    :  *:::::  :* :.:::: ..* ***:*** *.. :: * : *****::**:

AtPreA        IWPNDDIDSWWSGVGLCDLEVKIMIASYVAVPGNLLCIFRNLASVLDTRRATLVPSKQQR 115
AflPreA       IWPSDDMDNWWNGAGLCDVEVKVMIASYVAVPGNLLCIFRTLASMIDTRRAMLVPSKQQR 120
AnPreA        IWPTDDVDNWWDGAGLCDIEVKMLISSYVAIPGSLVCIFRSLACVLDTSRATLVQTKRQR 120
              ***.**:*.**.*.****:***::*:****:**.*:****.**.::** ** ** :*:**
```

```
AtPreA        WRNHFIDILFCIVVPVMAMITHIIYQKSRYVLWGISGCVNSFDESWVSFVLSFIWPPIIC 175
AflPreA       WWNIGIDMLFCVIVPVVAIATHIVYQASRYILVGISGCVNSFDESWVSLILAWIWPLVIC 180
AnPreA        WITRSVEVLFCVLVPLLAAATHLIYQGNRYFIFAVSGCVSGLDRSWVSFALGYLWP---- 176
              * .  :::***::**::*  **::** .**.: .:****..:*.****: *.::**

AtPreA        FIAGYYCGLVLYRLHKYRSQFGSILESANSNLSKSRFLRLFFLSFVMLLAIIPTQIFVIY 235
AflPreA       LIAGYYCSLVVYRLHRYRNQFGDILQASNSNLNKSRFMRLFLLSFIVLLAILPVQTYVVY 240
AnPreA        --------LVAY----------------------------------------------MVY 183
                      ** *                                               ::*

AtPreA        KNVQYSYPWHRYSWSTAHSP--DWGTIPKWRSDGEVFFDRWVPITSGFLFFLFFGCGHDA 293
AflPreA       KNIELSLPWHAYSWRVAHGP--HWNKIQKIPSGGDAFFDRWFPIASGFMLFILFGCGRDA 298
AnPreA        VQVKLSMPWHPFSWSLLHGPGSSWRVIERIPTGGAVYFDRWIPVASAYTAFAFFGTGRDA 243
               ::: * *** :**   *.*   *  * :  :.* .:****.*::*.:  * :** *:**

AtPreA        ARIYRTVLRFFGFT--CVRGPPLASTVSSG--TGSMNSRAKILGRKKRSSD-----SRTY 344
AflPreA       SRMYGSYLRLLRLDRCFVRTQDSSSDASRSNTSGFSNSRFGLLFHKAWTST-----AETC 353
AnPreA        SRMYRSFLRPFGLD-CCFSPMENKTSGSFPHTSGSAGSRAHLIPGRGKAQSDLYHYARKN 302
              :*:* : ** : :    .      :  *    :*  .**  ::  :  :.      :..

AtPreA        VNNITSTRVAGTIYEDLEMGASVP--RGPKRS---AWLR-----LFK------LSSRVRK 388
AflPreA       AENPATANSIDRSSDDIEKGRASS--PKPQRGQNMSWLKHPWSFLHRPLHRSSTSSGERI 411
AnPreA        SIDYTSSSRASSKDEDLEKGIPVSSVTENQVVSKKGWLKTGFAWLGNQFPFSLSPSACRE 362
                : :::   .   :*:* * . .     :     .**:     * .       .*  *

AtPreA        ----PPTPDLSTPL--NLICTNAWAGTSMSRGSSDFTETP----TRRDFIRVKQVISQES 438
AflPreA       ----LSRPNLSTPS--NTVSANAWAGSSQSRGSSDLTYMP----GREAFIRVKRDISQES 461
AnPreA        KIRSRSTPHLVVPASSNTVCTNAWAGSRQSRGSIDFDDSSESLSSKMDFIRVKQVIRQER 422
                   . *.* .*   * :.:*****:  **** *:   .     :  *****: * **

AtPreA        EVQL 442
AflPreA       ELRK 465
AnPreA        ETHV 426  * :
```

Class III: GprC

```
AtGprC          MSLVQVLSALTARHDEAHTLSPRLAYTE--NGPTWDINPLPSDQRKGLIAVSVMAILSFI 58
AflGprC         MSLAQVLSVLASQDDEGNSLARRLAYTD--AGPTLDIDPLPSAQRKGLIAVTVMAFLSFI 58
AnGprC          MSLFKAFLSLTSSNDGSHPLFPRVAYVEGDNGSGLDIDPLPEEHRRGLVAVFVMAILSTV 60
                *** :.:  *:: .* .:.*  *:**.:   *.  **:***. :*:**:** ***:** :

AtGprC          ATLSLICFVTYRLVFWRGNYQRYIGYNQYVILIYNLVLADLQQSLAFLICIKWIVEDKIT 118
AflGprC         ATLVLLLFITYRLVFWRSNYARYIGYNQYIVLIYNLVLADLQQSLAFLICLKWITENKIE 118
AnGprC          ATFMLISFITYRLIFWRGSYQRYIGYNQYVVLIYNLVIADIQQSLAFLICAKWYADNKIS 120
                **: *: *:****:***..* ********::******:**:********* ** .::**

AtGprC          ASSSACFLQGFWLQVGDPSSGLFVLAIAFHTFLLVTMGHKLSHRVFVAFVVGLWGFVAVL 178
AflGprC         ASSAACFLQGFWLQIGDPGSGLFVLAIAVHTFILVALGHKLSHRVFVCGVVGVWLFVAIL 178
AnGprC          ANTAACFLQGFWLQVGDPASGIFVLAIAFHTFYLVALGKKLSYRVFVSIVVGLWVFVAIL 180
                *.::**********:***.**:******.*** **::*:***:****. ***:* ***:*

AtGprC          VIIPIASHGRYVFIPSGAWCWINEEYEPIRLWTHYIWIFLAEFGTVCLYAIMWFQLRRRI 238
AflGprC         VIIPLAAHGRFVFIPSGAWCWISEEYEPIRLWTHYIWIFLAEFGTVCLYAIMWFQLRRRI 238
AnGprC          VIIPIAAHGRQVFTPSGAWCWIDESYEPIRLGTHYFWIFFAEFGTVSLYAILWFQLRRRI 240
                ****:*:*** ** ********.*.****** ***:***:******.****:********

AtGprC          AQSAILGASHIESLKRLRRVIGYMVIYPVAYIVLSLPLAAGRMATARGETPSVVYFCVAG 298
AflGprC         KQSAILGNSQTESLKRLRRVIGYMIIYPVAYIVLSLPLAAGRMATAQGQTPSIAFFCVAG 298
AnGprC          KQSAILGSSHIESLRRLRRVVGYMVIYPIAYIILSLPLAAGRMATARGDTPSVVYFCVSG 300
                 ****** *: ***:*****:***:***:***:*************:*:***:.:***:*
```

```
AtGprC          ALITSSGLVDVLLYTLTRRNLILESEPSADRSYNRFASSSKNPRTTDHLTTITADP-KLT 357
AflGprC         AVITSSGLVDVLLYTLTRRNLILESEPSRDRSYNRFASSVN--RKTDHLTTITAAEGKHT 356
AnGprC          ALITSSGLVDVLLYTLTRKNLIIESEPSLDKSYQKFASGKN------RLN--TTTDLKNM 352
                *:*****************:***:***** *:**::***. :      :*.  *:   *

AtGprC          RADISALRTRRGRDDDVDDG-TIRDGSTDNIVQNSG--VELAPIGKVYQHTTIEITHEPA 414
AflGprC         RTDISVLRTHRHREDDDEFGHTVREGSTDNIVQPSG--MELAPLGKVYQHTTIEITHEPA 414
AnGprC          RTDISALHTYKGNDEEEQTR----DGSTDDIVKNTGGGVELAPIGKVYQHTTIEITSEPA 408
                *:***.*:* : .::: :      :****:**: :*  :****:************ ***


AtGprC          YPSEG-DSQRSSKDSLRHAHAKVNESSSRMWGK 446
AflGprC         YPEAE-SSDRSSKGSIGDG--KGPAQSARMWGR 444
AnGprC          YPSNGDASGRSSADSLQPT--GKMSPTARRWGR 439
                **.    * *** .*:          ::* **:
```

Class III: GprD

```
AtGprD          MSTLIRALHAL---HRQDAL--VSRDVQPKTEPLHGADHAGFIAIGIIGLTSFVATFGLL 55
AflGprD         MIALIRMLHLL---NGEDAQTHFTKRQNATQQPLTGGDRAGFIAMGVVALCSFIASFCLL 57
AnGprD          MAALYRAILLLRSDHGLDGEHHLVPRQSHTVETLDGSTKAGFIAMGICGLVSFIATLSLL 60
                * :* * :  *   :  *.   .    . . :.* *. :*****:*: .* **:*:: **

AtGprD          AFLTFRFIFWRRYYKHPLAANQYVVLIYNLLLVDIQQSIAFMLCLYWASQGRVTMPSAAC 115
AflGprD         SFLTYRFIFWKRYYKRPLAANQYVVLIYNLLLIDIQQATAFVLCLYWVSRGHVDYPSAAC 117
AnGprD          LFLTYRFIFWKRYYKRPLAHNQYVVLIYQLLLVDLQQATAFLLCLHWVTKGAVYYPSAAC 120
                 ***:*****:****:*** ********:***:*:**: **:***:*.::* *  *****

AtGprD          YLQGWWIQTGDPGSGLFVLAIALHTGAVVLRGRQLPFPIFIACVTGLWLFILILGFIPVG 175
AflGprD         VLQGWWIQIGDPGSGLFIMAIAMHTGAVVLRGRQLPHRTFVCCVIGLWAFIIVLGLIPVG 177
AnGprD          ILQGWWIQTADPGSGLFVIAIAMHTGAVVLRGRQLSFRAFVACVIGLWAFILVLGFITVG 180
                 ******* .*******::***:************..  *:.** *** **::**:*.**

AtGprD          LFGDEVFVISEAGWCWLSPDHETERLWVHYLWIFLAEFGTVILYGVMFFYLRRRMKEAAL 235
AflGprD         LFGSKTFVISEAGWCWLGPEHETERLWVHYLWIFLAEFGTVVFYGMLFFHLRRRMKQAAM 237
AnGprD          LYGSKTFVISEAAWCWLSPEHENERLWGHYLWIFLAEFGTVVLYGIMFFYLRRRMVHAAK 240
                *:*.:.******.****.*:**.**** ************::**::**:***** .**

AtGprD          LRHGPQENLKRLNRVVVYMVIYPLVYLLLSLPLAAGRMSTARHVAPSRTYFAVAGCLMGL 295
AflGprD         LRQGHQESLKRLNRVVIYMVIYPIVYLVLSLPLAAGRMSTARHIVQSRGYFAVAGSLMAL 297
AnGprD          LRPNHQDSLKRLNRVVIYMVIYPFAYILLSLPLAAGRMSSARHVIPSRQYFAAAGSLMAL 300
                ** . *:.********:******:.*::***********:***:  ** ***.**.**.*

AtGprD          SGLVDVLVYTLTRRHLLTDTEISASD---KLYQISGSQAYQTHISTTGRDSTKKSKSRGR 352
AflGprD         SGLVDVVVYTLTRRHLLLDTEISTSD---KMYAYSNSNAYQTHITTTTRE-NKKPRMGSR 353
AnGprD          SGLADAAVYTLTRRQLLLDTDLSQSDGPYNRYAYSGSHTYHTQVTSTTGG---RERKRGR 357
                ***.*. *******:** **::* **   : *  *.*::*:*::::*      : :  .*

AtGprD          FRRGLQSINETVDDDRADSTEDIVR-----KGDWEMHDMR-NAVYQETTIEISHEPADPD 406
AflGprD         LRRGLQTINDTIND--GDSTEDLRK-----DGDMEMADLG-HGVYQETTIEISHEPADPD 405
AnGprD          FRKGMQTLNETIQDDRDDSTEEIVKGGRNDSGDVEMVNYTGHGVYQETTIEITHEVADPR 417
                :*:*:*::*:*:::*   ****:: :     .** ** :   :.**********:** ***

AtGprD          EFHRRGRDSP 416
AflGprD         EFHGNKRNSG 415
AnGprD          EFPQRERHSG 427
                **  . *.*
```

Class IV: GprF

```
AtGprF          MFPSPMGNLDLDALSGICGSISIACWVVVFSPQIIENFRRSSADGLSLLFLVVWLAGDVF 60
AflGprF         MFHPPVGNIDLEALSGICGSISIACWVVVFSPQIIENFRRGSADGLSLLFLVVWLAGDVF 60
AnGprF          MFPPQTPNIDLEALSGICGSISIACWVVVFSPQIIENFSRGSADGLSLLFLVVWLAGDVF 60
                ** .   *:**:************************** *.******************

AtGprF          NILGAVLQGVLPTMIILAVYYTLADIVLLAQCFYYRGFTLRDEPSPPESRAPEEAEDNLE 120
AflGprF         NILGAVLQGVLPTMIILAVYYTLADIVLLGQCFYYRGFTLRDEPSPPSSRAQESDAEDQ- 119
AnGprF          NILGSVLQGVLPTMIILAVYYTLADVVLLAQCLYYRGFTFKDDASTAFTPEEPDEIETP- 119
                ****:********************:***.**:******::*:.*.. :    .  :

AtGprF          GGSTAATRKPTERSYLLPSETNGQSYQTNGHDHH-----------QHPIPHHQ-QRRHSA 168
AflGprF         ---PSFPGKVSERTALLSAKANGHSYQAHSQEHTGNGYPTPGPSGQQSFPYHQNHRRHSA 176
AnGprF          --SPVIARKPTERTSLLPTLET-QNYDAGTSAAP-----------SHQDDNAASAPLLAP 165
                   .  . * :**: **.:  . :.*::                .:          :.

AtGprF          TSFRDILHTHIDGTHLSPATPFIEPPSPKETRAARSISALQSLLFNACAVALVCAAGVLG 228
AflGprF         TSFRDILHPNVDGTHLSPVTPFVEP-SAKETRPVRRLSALQNALFNLSAVVLVCAAGVLG 235
AnGprF          SHARHRRHS-IDGTHLSPATPFVEP-SASGKR--KTTSTLQTVLFNLCAVALVCVAGILG 221
                :  *.  *. :*******.***:** *.. .*  :  *:**. *** .**.***.**:**

AtGprF          WYFTPGS--------SKNKEPTDDT--LAFDTLGQVFGYLCAVLYLGSRLPQILLNYRRK 278
AflGprF         WYVSPGS--------SKAEDKQDDSGSLSFDTLGQVFGYLCAALYLGSRLPQILLNYRRK 287
AnGprF          WYVSLSTPATPGKHTTHRHRKPADANPISFDTLGQVFGYLCAVLYLGSRLPQIMLNYKRK 281
                **.: .:        :: .    *:  ::*************.**********:***:**

AtGprF          STEGVSLLFFLFACIGNLTYVLSILAFSPVCRG----DRAGHCRPGEAAAIYGRYILVNL 334
AflGprF         STDGVSLLFFLFACIGNLTYVLSILAYSPVCKGVYPQGRVSQCRPGEAAALYGRYILVNL 347
AnGprF          STDGVSLLFFLFACIGNLTYVLSILAYSPICER------PRHCEPGEVGKLYGRYILVNL 335
                **:************************:**:*.         :*.***.. :*********

AtGprF          SWLIGSFGTLFLDMCIFVQFFMYQDNIYMDEEG-SQETVRG 374
AflGprF         SWLIGSLGTLFLDMVIFVQFFLYQDNGYGETEGTSEETLRG 388
AnGprF          SWLVGSFGTLFLDMCIFIQFFMYKDN-----NARSTMVVS- 370
                ***:**:******* **:***:*:**     :. *  .:
```

Class IV: GprJ

```
AtGprJ          MSLLRSLGTATAGESVPLTPREAASGLLGSISMTCWIFLLVPQLIENYRNGNAEAISLLF 60
AflGprJ         MPSLWSQVSMASAEPVPLTTREAASGLLGSISLTCWIFVLVPQLIENYRNGNAEAISLVF 60
AnGprJ          ----------MAGEFVPLTPREAASGLLGSISLTCWIFLLVPQLIENYRNGNAEAISLLF 50
                          :.* ****.************:*****:*******************:*

AtGprJ          LFVWFVGDITNLVGGLWAGLVPVIVAIAVYFCIADGVLIGQCLYYKARNARLEALRRRRR 120
AflGprJ         LAVWFVGDVTNLIGGLWAGLVPVIVAIAVYFCIADGVLIGQCLYYKMRNSRLQAFHRRR- 119
AnGprJ          LFVWFVGDITNLAGSAWAGLVPVVVAIAVYFCIADGVLIGQCLYYRARNSRGAAAHLRE- 109
                * ******:*** *. *******:*********************: **:*  * : *.

AtGprJ          SSTETPDPSTPLLGRRFSDTLPEAV-ARRRASVSSQRRGVDGGARQ--ADDTLAKIVEEN 177
AflGprJ         SSVETPDPTTPLLGRRFSDSLREGP-ASRRRSCSSQHR--DGHANG--PDDTLAKIVEEN 174
AnGprJ          SSSATPEPTTPLLGRRYSDALREGDGSRRRRDSRPSYSAVNGSQQAQLPDDTLAKLVEEN 169
                **  **:*:*******:**:* *.  : ** .  ..    :*  .   .******:****
```

```
AtGprJ          EAGQQAWVKNLSSVLAICVIGMAGWTIAWQSGVWKPAPRE-VNGGVDMAAGAQVVGYFSA 236
AflGprJ         EVGRSAWVKNFSSVLAICVIGMGGWTIAWQSGVWQPAPQE-DAGGAEIAVGAQVVGYFSA 233
AnGprJ          RIGQGAFVKNSSSVLAICVIGMVGWTVAWQTGVWRPAPLGGTSGGVDMAPGAQVLGYISA 229
                . *: *:*** ********** ***:***:***:***     **.::* ****:**:**

AtGprJ          LCYLGARLPQIYKNYCDKSCEGLSLLFFILSLMGNLTYGAGILCHSTEKNYIVTNTPWLI 296
AflGprJ         ICYLGARLPQIYKNWKDKSCEGLSLLFFILSLLGNLTYGAGILCHSTEKNYIVTNIPWLL 293
AnGprJ          VCYLGARLPQIYKNYCDKSCEGLSLLFFILSLMGNLTYGAGILCHSTERNYFLTNLPWLI 289
                :*************: ****************:***************:**::** ***:

AtGprJ          GSLGTMVEDVIIFVQFRIR-MTDMIA--- 321
AflGprJ         GSLGTMVEDITIFIQFRLYAVADSTAAGP 322
AnGprJ          GSLGTMVEDAIIFAQFRLYAVQDSAAEFA 318
                *********  ** ***:  : *  *
```

Class V: GprH

```
AnGprH          MSLSAGQLFAISATERTCSTVSLFATSIIIVSFVSSQSFRKPINRLVFYASFGNIMANVA 60
AflGprH         MSLSEQQLYAISATERVCSAISLTGTAIIVISFLGSSAFRKPINRLVFYASWGNLMTNIA 60
AtGprH          MPPTPDQLYAISVTERVCSVVSLLGTGIVVVSFLYSSAFRKPINRLVFYACWGNVMANIA 60
                *. :  **:***.***.**.:** .*.*:::**: *.:************.:**:*:*:*

AnGprH          TLISQSGIWAGTDSNLCQIQAFLIQWFMPADALWTFAMAFNVYLTFFRKYNSDQLRRLEW 120
AflGprH         TVISQSGIHMGLNSPLCQFQAFLIQWFMPADALWTFAMACNVYLTFFYKYNSEQLRRLEW 120
AtGprH          TVVSQSGIRAGLSSPLCQFQGFLIQWFMPADALWTLAMACNVYLTFFRKYNSDQLRKQEW 120
                *::*****  * .* ***:*.***************:*** ******* ****:***: **

AnGprH          KYVMLCYGLPFIPAFTYFFIRTESRGKVYGSAILWCWVAPSWDFLRIAVFYGPVWFVIFV 180
AflGprH         KYVLFCYGLPFIPSLAYFFIKTKARGKVYGYAVLWCWVSQTWDWLRIAVFYGPVWFFIAL 180
AtGprH          KYLLFCYGLPFIPAFVYFFVRSRSRGRVYGSAVLWCWISPSWDYLRLAVFYGPVWFVIFL 180
                **:::*********::.***::::.**:*** *:****:: :**:**:*********.* :

AnGprH          TLAIYARIGELVWRRRRQLKEVGGLDTT--------------------LDVSIPDDPPFS 220
AflGprH         TLAIYIRTGSVIWERRRQLRQLGNLDSADSYPRYEGEPTSEHYEGFEMRDTQRPLEPTHT 240
AtGprH          TLGIYARTGRVIYRKRRELEQLGTIESN----------IDIENISIENVDLETPLAQTVS 230
                **.** * * :::.:**:*.::* :::                      * . *   . :

AnGprH          K-VTEIRIEREDAASYRPEAGPSTEQDPPFSCA--QSIPRPYSVNVQAGSSQLELGAIRS 277
AflGprH         RPTTEIRVTSEITRSQQGSCPTAEELCNPVS-----PFYNPYSVTIEGGFMSRQSSGLP- 294
AtGprH          K-VTRVHITSELSNDCEASGSNRDSSLPTTTPTNIRPPYDPYSITIEGGARIERNGSVGY 289
                : .*.:::  * : . . .     .   . :     .   ***:.::.*    . ..:

AnGprH          EEPFDGPPTREYRSSSTALEVNTAAWAYTKYAMLFFVALLVTWVPSTINRVYAFIRPDAS 337
AflGprH         LKAIKHSHSESWSRRRAMSDTSTAAWAYTKYAMLFFVALIVTWVPSTVNRAYSLAYPNSH 354
AtGprH          ETARYDEQQWLAQRRALGPDASQAAWVYTKYAMLFFIALIITWVPSTINRVYALAFPDAF 349
                  .               :.. ***.*********:**::******:**.*::  *::

AnGprH          NFGLNYASSFVLPLQGFWNSLIYISISWRCFKPALSDLHN----RLLWYTHLG--AGHRR 391
AflGprH         NFALNYVSSFVLPLQGFWNSIIYMSISWPAFKTVYRDFRRGSLVRDPGYRNREEYEGHVE 414
AtGprH          NFPLNYIASFVLPLQGFWNSIIYVSISWPAFKATFEEWKMG---RRQCHAHSWYNAGNPV 406
                ** *** :************:**:**** .**..  : :     *   : :     *:

AnGprH          PS---SGQDSTRRLTN-- 404
AflGprH         QTY--SSNDSVRRLTR-- 428
AtGprH          VSKRPCYASGARQQFRSF 424
                 :   .  ...*:  .
```

Class VI: GprK

```
AtGprK          MGSEMGVTPDSKPQAVYSPVSIFWACWCAVWTTAVLCGMAFLIIRRDSPVIRVRGLWLSL 60
AflGprK         MGSEMGVTPDSKPQAVYSPVSIFWACWCAVWTTAVLCGMAFLIIRRDSPVIRVRGLWLSL 60
AnGprK          MGSELGITADQKPQAVYSPVAIWWVVWGCFWTAVVASGMAFLIIRRDSPIVRVRSLTLSL 60
                ****:*:*.*.*********:*:*. * ..**:.* .***********::***.* ***

AtGprK          AAVLFLHAYWAPVQFGVMIGPIMPGDAQYWIMGTYLPIGIALFHASNARFLHVAKLQKKY 120
AflGprK         AAVLFLHAYWAPVQFGVMIGPIMPGDAQYWIMGTYLPIGIALFHASNARFLHVAKLQKKY 120
AnGprK          GGVAFLHIYYWSVQFGVMIGPLMPGDSQYWIMGTWLPCGLALFHGANSYFLHIAKMQKKY 120
                ..* *** *: .*********:****:*******:** *:****.:*: ***:**:****

AtGprK          IRPGSSYDDMPAHMQRRPGLIVRFKRLDYTNKTLVVVAIAIVIQLFLTILMWLISRKWHP 180
AflGprK         IRPGSSYDDMPAHMQRRPGLIVRFKRLDYTNKTLVVVAIAIVIQLFLTILMWLISRKWHP 180
AnGprK          VKYSFLTDSTPDAKRQPSGLLSRFRRLDYSARVVILVAIAMFVQVFLTILMWVISRKFHS 180
                :: .   *. *   :: .**: **:****: :.:::****:.:*:*******:****:*.

AtGprK          SWGIPGTEVHGTKMQQLSAMGAGWEWWATIVGQFIWAWIVAPIVLWKARHIQDTQGWRVQ 240
AflGprK         SWGIPGTEVHGTKMQQLSAMGAGWEWWATIVGQFIWAWIVAPIVLWKARHIQDTQGWRVQ 240
AnGprK          SWGAPGTEVHGPPMQQLVEQGRGWEWWPGVFWQFFWSWVVAPIVLWRSRHIHDTQGWRTQ 240
                *** *******. ****   * *****. :. **:*:*:*******::***:******.*

AtGprK          TMACVIANLPATPMWLIAVYVPGFEKVNAVWLPPQWICLSIIVMEIFTIFLPCWEVIRHQ 300
AflGprK         TMACVIANLPATPMWLIAVYVPGFEKVNAVWLPPQWICLSIIVMEIFTIFLPCWEVIRHQ 300
AnGprK          TIGCALSGLHATPMWLIALYVPAMEKVNAYWIPPQWICVSIMFLEIFTVLLPCWEVMRHQ 300
                *:.*.::.* ********:***.:***** *:******:**:.:****::******:***

AtGprK          SLRKETLETIAEWEAKTKGKGSEAKSVSDATTMIDSMISGFKSTNASIDS-KSSRASILT 359
AflGprK         SLRKETLETIAEWEAKTKGKGSEAKSVSDATTMIDSMISGFKSTNASIDS-KSSRASILT 359
AnGprK          SLHKDTLEAIKQWEIRTKGANSDSKSLGSTATMVDSMMSGWKSNNESVISDSSARESILN 360
                **:*:***:* :** :*** .*::**:..::**:***:**:**.* *: * .*:* ***.

AtGprK          MGALEHVLERNPTPLLEFSALHDFSGENIAFLTSVAEWKSSLPKPIRDGTAAPDDPNTRE 419
AflGprK         MGALEHVLERNPTPLLEFSALHDFSGENIAFLTSVAEWKSSLPKPIRDGTAAPDDPNTRE 419
AnGprK          MSALEYVLERNPTPLQRFSALNDFSGENVAFLTSIAEWKTLLPKALCEGDEAADD-MTKE 419
                *.***:********* .****:******:*****:****: ***.: :*  *.**  *:*

AtGprK          LIRERFNRALQIYTEYISVRHAEFPVNISSQDLRRLEGIFDAAAQTCYGD-QVEADPATP 478
AflGprK         LIRERFNRALQIYTEYISVRHAEFPVNISSQDLRRLEGIFDAAAQTCYGD-QVEADPATP 478
AnGprK          MVREAFNRALYIYAEFISTRHAEFPVNISSQDLKKLDNIFETPARILYGDEKAEVDPATP 479
                ::** ***** **:*:**.***************::*:.**::.*:  *** :.*.*****

AtGprK          FDKFTFDPPSPRHSESSEQPIKQPASAPKYWGEVPESFNATVFDAAEESIKYLVLTNTWP 538
AflGprK         FDKFTFDPPSPRHSESSEQPIKQPASAPKYWGEVPESFNATVFDAAEESIKYLVLTNTWP 538
AnGprK          FNNP--GMPSPTNSNFSESSVAAIKNRAQYMGEIPKGFTAGVFDDAENSIKYLVLTNTWP 537
                *::   . *** :*: **..:    . .:* **:*:.*.* *** **:************

AtGprK          KFIK-NRRISADSANTLKP-EEYEMA 562
AflGprK         KFIK-NRRISADSANTLKP-EEYEMA 562
AnGprK          KFVKRQRRISTESTETLNAGNTAEFV 563
                **:* :****::*::**:. :  *:.
```

Class VII: GprM

```
AtGprM          MANSTLHGLCPEPFYQESLFPSTGGFIGGRYCQTVPLGNERLSCCLPCPLTEWTYDDQLL 60
AflGprM         MTNQTLHGLCPEPFYQESFFPSTGGFTRGRYCAQLPLADGNVSCCIPCPLSDWMYGEDIT 60
AnGprM          MSGNNLHGECPVPYYQESFFDTDGGFLQGRLCQAL----GNVTCCLPCPQASWMWGEDIQ 56
                *:...*** ** *:****:* : ***  ** *  :     .::**:*** :.* :.:::

AtGprM          SQTKVASWLGVAVLPLCIFLLVSYAVLPVKYTHRHYLSICFTLGICFMEIAFIIPLGVKP 120
AflGprM         TKAQAASWLSVGVLPLCIFLLVSYAVLPAKWTHRHYLSICFTMGICFMELAFIIPLGAKP 120
AnGprM          EQTKIASWVSVAVLPLCIFLLVSYAVLPAKRTHRHYLSICFTLGICCMQIAFIIPLGTKP 116
                 ::: ***:.*.****************.* ***********:*** *::*******.**

AtGprM          EQCHNAITPNDMYTDVSCAFTGALLLFGGWIVVVWSFIRTVAFHLQVCWEVIIGKKFMWG 180
AflGprM         DQCYNQITPNDMHSNLSCAWSGTLLLFGGWAVVTWSFIRTLAFHLQVCWEVILGNKFMWG 180
AnGprM          DQCYNEITPRDMYSSLSCAFTGAMLLFGGWVVVVFSFIRTVAFHLQVCWEVILGPRFMWG 176
                :**:* ***.**::.:***::*::****** **.:*****:**********:* :****

AtGprM          SFICGWGIPALCLTLMLIYTGVSYRFGNVCHINIKNSTSDYWAPVMAFSAASLLLHVATL 240
AflGprM         AFICGWGIPGIGITVMLILTGVSFRFGDMCHINIQYGTKDYWAPVTAFAGASLVLQLATM 240
AnGprM          AFICGLGVPIIGTSVMLVLTGVSFRFGDVCHINIDKSLQDYWIPVIAFSGAALIMQFTTM 236
                :**** *:* :  ::**: ****:***::*****. . .*** ** **:.*:*:::.:*:

AtGprM          GYCIHVYIRSLFDSSDSTTNSSGLPSYTGSVRTMTARQAYRRIRRVLQLQWRGISLVLII 300
AflGprM         AYCIHVYVKSIFDTADSSTNSSHLPSYTSSVRTVTARQAYRRIRRVLKLQWRGVALVLII 300
AnGprM          GYCVHIYIKSLYDTA-STTNSSNVHSYTASAVTLSARQAYRRVRKILKLQWRSNCLVLVI 295
                .**:*:*:::*::*:: *:**** : ***.*. *::*******:*::*:****. .***:*

AtGprM          IGNVIFFAVVFIDMNNELALTPENMEKAIPWLACLMETKGEKNRCLDQADAVRPNKATLL 360
AflGprM         IGNVIFFAVVFIKVNNKTNLTPENLQRAQPWLLCLVSTKGDVKKCQNLAHAIGPNEDTIL 360
AnGprM          LANVLIFAVIFIRTNNQTEMTAENLEKALPWLLCLAASEGDAKKCVDKAEEFGPKKATLL 355
                :.**::***:**  **:  :*.**:::* *** **  ::*: ::* : *. . *:: *:*

AtGprM          AVLILLSLVGFWNFILFARPSMFVGWVDLFKNGFGGGGPRRHEFVSVDARNKRLPDTRTY 420
AflGprM         ALLILLSLVGFWNFILFARPGMFLGWAEFFKIKFG----QNHEFVSADARAK-TPDTRTY 415
AnGprM          ALLILLTLVGFWNFILFARPSMFVGWWDLLFRSKDN---AQFEFVSADARKK-RSDASNY 411
                *:****:**************.**:** :::    .     ..****.*** *  .*: .*

AtGprM          EMLT-----SSGISKTPEPLVRSPSPARTAGTLSPVGGHNHYGREARYVRPSMSFSGPRP 475
AflGprM         EMLE-----SSGL-KSPEPIVRAPSAAR---TMSPEG--EHYGREARYVRPSMSFSSPRP 464
AnGprM          EMLSPYKNPEPYVEPSAEPQIRSPSPAYTSRTLSPDLN-NHYGRDARYVRPSMSFSVPRP 470
                ***      .. :  :.** :*:**.*    *:**    :****:*********** ***

AtGprM          PGSSQGQGRDWDPQATFARGQSPEYHHQQNLSR 508
AflGprM         PSASQ--GRDWDPQSTFAQSS-----HQHTLSK 490
AnGprM          PTSPG---RDWDPATTFAQGYR---------- 489
                * :.    ***** :***:.
```

Class VII: GprO

```
AtGprO          MPTRQRRPTENRPKGSGSATSEAVSVAETVLEQPPRVIKKLLHWDELPHWQRDNHHIHSG 60
AnGprO          MPPRQRRPTEIHKKDPG--GCQTASVATAVLEDPPRAIRSLVHWDELPHWQRDNQHIHTG 58
AFLGprO         MPSRQRRPTEKAQESKA---STPAAMAATVMKQPPQVLKKLLHWDDLPHWQRDNHHIHT- 56
                **.*******   :. .   . ..::* :*:::**:.::.*:***:********:***:

AtGprO          YRPASYSFLGSLHSLTYVHNETVNIYTHLLPSLLSVPAAVVLYQALAPRYATATHGDIGA 120
AnGprO          YRPASYSFRGSFQSLTYIHNETVNIYTHLLPSVLAIPAAIQLYRALAPRYETATDGDIRA 118
AFLGprO         ----------------------------------IPAAVWLHRALAPRYETATRADIIS 81
                                                  :***: *::****** *** .** :
```

```
AtGprO          FSCFFAGAAFCLGMSATYHTISNHSPVVARIGNALDYIGIVGLIVGSFVPSVYYGFYCMP 180
AnGprO          FGCFFVGAAFCLGMSAFYHTISNHSPTVARIGNTFDYIGIVGLIVGSFVPSVYYGFYCVP 178
AFLGprO         FACFFVGAAVCLGMSATYHTISNHSPTVARIGNALDYAGIVALIVGSFVPSVFYGFYCEP 141
                *.***.***.****** *********.******::** ***.**********:***** *

AtGprO          NLQRLYWTMICGIGLGCVFVSVLPQFRTPGWRPFRAAMFVGMGLSAVFPVLHGLRLYGRK 240
AnGprO          ELQRLYWSMICTIGLGCIVVSIFPKFRTPRWRPFRAAMFVGMGLSAVFPVVHGLQLYGRD 238
AFLGprO         GLQRLYWTMICTIGIGCVFVSIMPQFRTPRWRPFRAAMFVGMGLSAVFPVIHGLRMYGLE 201
                 ******:*** **:**:.**::*:**** ********************:***::** .

AtGprO          LMMRQIGLGWLLLQGLLYILGAAIYAARVPERLRPGRFDLLGSSHQIFHVLVVCAAVAHL 300
AnGprO          QMMRQIGLGWLLLQGFLYILGAGIYAARVPERLRPGGFDLWGSSHQIFHVLVVCAAVAHL 298
AFLGprO         QMTRQIGLGWLLLQGFLYILGAGIYAARVPERLRPGQFDLWGSSHQIFHVLVVCAAVAHL 261
                 * ************:******.************* *** ********************

AtGprO          TGLLSAFDYRHSGIAETCPRI 321
AnGprO          TGLLRAFDYRHNGTAGSCQV- 318
AFLGprO         TGLLRAFDYRHSGIAESCSWN 282
                **** ******.* * :*
```

Class VII: GprP

```
AtGprP          MACSAQSMTIESYPDEALSTSYAPTPSEE-TALVRDRRRRHSFHTARKLSCDYDADAVFL 59
AflGprP         MACSAQSVTIESYPEEAAVSSAYATPSEETTALLRDRRRRHSFHAARKLSCDYDADAVFL 60
AnGprP          MACSTGSVTIESYLDTAVSS---ATPSEE-TALLRDRKRRHSFHTARKLSCDYDADAIFL 56
                ****: *:***** : *  :   .***** ***:***:******:************:**

AtGprP          RVELFLAEMERRMHWIEEYRQAHMVQIDSSLRRGYATLEAVRDSCSFASGELMGGGKKRA 119
AflGprP         RVELFLAELERRLHWLENYRRSHMVQIDASLRRGYATLEAVRDSCSYASGELMGGGKKRA 120
AnGprP          RVELFLAELERRLHWIEQYRKSHMVQIDTSLRRAYATLEAVRDSCSHASGELMGSGKKRA 116
                ********:***:**:*:**::******:****.************.********.*****

AtGprP          KILVETLEDRYKDALATKETLEQKAQVGIRLMESFLTELESRAHAVRDRGIYGTLDDGWK 179
AflGprP         KILVETLEDGYKDALATKETLEQKAQAGVRLMESFLSELEARAHAVRDRGFYGTLDDGWK 180
AnGprP          KILVETLESRYNDALATKETLEQKAQAGVRLMESFLTELESRADAVRDRGVYGALDDGWK 176
                ********. *:***************.*:*******:***:**.******.**:******

AtGprP          AVDSKLVQAREAVDEGMERA---KEALRESIDRAIVLAQEKRLITYADLPHPWRINPHIL 236
AflGprP         AVDSKLVHAREVVDEGMERARKAKDALRENIDQAIALAQEKRLIAYADLPHPWRVNPHIL 240
AnGprP          AVDSTLVQAREVMDEGIERARQVKDALRENIDHAIMLAKEKRLISYSDLPAPWRINPHIL 236
                ****.**:***.:***:***   *:****.**:** **:*****:*:*** ***:*****

AtGprP          QGYRFTHSKVECFTSMFTFSNELVNIWSHLIGLFIVLSVAFYFYPLNPNFHLSTKTDVFV 296
AflGprP         EGYRFTHSKVECFTSMFTFSNELVNIWSHLIGLFIVLSVAFYFYPLNPNFHLSTKTDVLI 300
AnGprP          SGYRFHSSKVECLTSVFTFSNELVNIWSHLIGLIIVLSIAFYFYPLNPNFHLSTNSDTLV 296
                .****  *****:**:*****************:****:***************::*.::

AtGprP          AAVFFFAACKCLVCSTLWHTMNSIANQGLMERFACVDYTGISMLVAASIVTTEYTAFYCE 356
AflGprP         AAVFFFAACKCLVCSTLWHTMNSIANQGLMERFACVDYTGISMLVAASIVTTEYTAFYCE 360
AnGprP          AAVFFFAACKCLVCSTLWHTMNSIADQPLMERFACVDYTGISLLVAASIVTTEYTAFYCE 356
                *************************:* **************:*****************

AtGprP          PVSRWTYILATMSLGIGGVVLPWHPTFNRADFAWVRVAFYVTLALTGFAPLAQLTYTRGF 416
AflGprP         PVSRWTYILLTMSLGIGGVILPWHPTFNRADFAWARVAFYVTLALTGFAPLAQLTYTRGF 420
AnGprP          PTSRWVYILLTMSLGIGGVILPWHPTFNRADWAWVRVAFYVTLALTGFAPLAQLTYARGF 416
                *.***.*** *********:***********:**.*********************:***
```

```
AtGprP          EWCLYFYAPVVKSILVYFAGACIYASQVPERWRPGLFDYVGGSHNIWHFAVLGGILFHYC 476
AflGprP         AWCLYFYAPVVKSILVYFVGACIYASQVPERWRPGLFDYVGGSHNIWHFAVLGGILFHYC 480
AnGprP          SWCLYFYAPVMKSILVYFVGACVYASQIPERWKPGLFDYIGGSHNIWHLAVLGGILFHYL 476
                 *********:*******.***:****:****:******:********:**********

AtGprP          AMQDLFAVAFQRAQGECPNLSA 498
AflGprP         AMQDLFAGAFQRAKGECPHLTS 502
AnGprP          AMQDLFANAFQRAKGECPNLTS 498
                ******* *****:****:*::
```

Class IX: NopA

```
AtNopA          ------------------------------------------------------------
AflNopA         -MIDPPAEMIDFFDTL-----KTKPLPVPT---TTPTSVAPVPTVVPGHDLIFQELSKTS 51
AnNopA          MIEDALKKTVTVTQTLTETVTKAVPSHDPTSSWTTTTSVAPIPTVIPDHP-TFQAVDTAA 59


AtNopA          ----------MALSSLVFYILAARAPLSKRVFHSLVSIATTVSFIVYLALATGQGITWKH 50
AflNopA         QRTLWVVVVLMAISAIVFYILASRAPLTKRVIHNLIAISTTISFITYLALATGEGITYKH 111
AnNopA          KRTLWVVTVLMALSSLVFYILSNRVQLPKRVIHYLVATATTVSFIIYLALATGQGMDWKY 119
                          **:*::*****: *. *.***:* *:: :**:*** *******:*: :*:

AtNopA          DLIREHHKHVPNVTDEYYRQVFWLRYLNWFLTEPLILANLALLSGLPGAHLLSAVVADYV 110
AflNopA         DILTIHNKHVPNTHRDIYRQVLWLRYLNWFLTNPLALINLALLSGLPGAHLLVAIVADWI 171
AnNopA          DTYNHKHKHVPDTEYGIVRQVLWLRYVNWFLTGPLILASLTLLSGLPGASLFAAIVADWV 179
                *    ::****:.     ***:****:***** ** * .*:******** *: *:***::

AtNopA          MLGSGLLGTFAGHTARRWAWFAISAIGYLTTVYHIGINGSRAAVNKDVQIKRFFGTISAV 170
AflNopA         MLGTGILGTYAGHTPRRWVWFTISAIGYLTTVYHIGVNGGRSAVNKDAQTKRFFGTVSGV 231
AnNopA          MLGTGLFGTYAPNTSRKWIWFALSAIAFITLIYHIGIKGTRAANNRDSHTRRLFSAIASV 239
                ***:*::**:* :*.*:* **::***.::* :****::* *:* *:* : :*:*.::::.*

AtNopA          TLFVKALYPVAIAAGPLALKMNVNAETVIFAIYDIFTQGIIGYWLLIAHDSSPGLTLSVD 230
AflNopA         SLLIKALFPVAIAAGALALKIGIDAETIIFAIHDIFLQGIIGYWLIFAHDAAPGITLLVD 291
AnNopA          ALLAKALYPITLAAGPLSLKLGLTGETILFAIHDIVIQGILGYWLVIANDAATGTNLYVD 299
                :*: ***:*:::***.*:**:.: .**::***:**. ***:****::*:*::.* .* **

AtNopA          GFWSSGIGNEGSIRIEEE-GA 250
AflNopA         GFWSHGHGNEGAIRITEEEGA 312
AnNopA          GFWSSGLGNEGAIRINEEEGA 320
                **** * ****:*** ** **
```