Investigation of a quorum sensing peptide in bacillus licheniformis and its novel antifungal property

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INVESTIGATION OF A QUORUM SENSING PEPTIDE IN *BACILLUS LICHENIFORMIS* AND ITS NOVEL ANTIFUNGAL PROPERTY

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Abstract

Quorum sensing molecules (QSMs) are involved in the regulation of complicated processes helping bacterial population benefit from their cell-density. This phenomenon has been recently studied in some fungal populations. Prokaryotes and Eukaryotes' co-evolution raises the prospect of the existence of inter-kingdom signalling pathways. The involvement of hormone-like molecules such as QSMs in microbial cells communication promise potential role of QS process in inter-kingdom cross-talk.

Bacterial antagonistic activity against fungi is considered as an important bio-control opportunity to control fungal invasion of plants. Several bacterial species such as *Bacillus* spp. have shown the ability to inhibit fungal growth. During the screening of antagonistic bacteria against *Aspergillus flavus* (*A. flavus*), *Bacillus subtilis* (*B. subtilis*) was identified as having high antifungal activity. The bacterium, *Bacillus licheniformis* (*B. licheniformis*) is related to *B. subtilis* genetically and is used at industrial-scale for production of the antimicrobial compound bacitracin. Although the *comQXPA* cluster involved in QS development has been identified in the genome sequence of *B. subtilis* and different *B. licheniformis* strains, the QS system in *B. licheniformis* was not previously investigated in detail, and its QSM (ComX pheromone) was not identified.

In this context, and given the importance of this antagonistic bacterium as an industrial workhorse, this study was aimed to use *B. licheniformis* NCIMB-8874 as a model antagonistic bacterium to investigate its effect, and the effect of its ComX pheromone on potential inhibition of fungal growth.

The results obtained from bioinformatics studies on *B. licheniformis* NCIMB 8874 genome sequence presented in this project confirmed the presence of essential quorum sensing-related genes, such as the *comQXPA* gene cluster. The cell-cell communication of *B. licheniformis* NCIMB-8874 was investigated through further elucidation of QS process in this bacterium. The detection of the QSM, ComX pheromone, was achieved through molecular biology and biochemical studies including over-production, purification and partial identification.

Subsequently, the potential influence of ComX pheromone and *Bacillus* cells on the growth of *A. flavus* was examined and concluded that the QSM could cause a significant reduction in the growth of *A. flavus* strains (NRRL 3357 and ESP 15).

This work reports for the first time the amino acid sequence of the purified ComX pheromone and its novel antifungal property. Pheromone as a QSM is a potential signal for communication of cells between kingdoms and could be applied for bio-control purposes. Identification of new antifungal peptides against *A. flavus* could lead to the development of biotechnological strategies which facilitate control of aflatoxin contamination.

توانا بود هر که دانا بود
ز دانش دل پیر برنا بود

"*The source of might is knowledge,*

Knowledge makes old hearts grow young again."

Abu 'l Qasim Ferdowsi (940-1020)

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LIST OF ABBREVIATIONS

°C	degree centigrades
3-oxo-C6 HSL	N-(3-oxo-hexanoyl)-L-homoserine lactone
A-Factor	2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone
ABC	ATP binding cassette
AIP	auto-inducing peptide
Agr	accessory gene regulator
AHL	N-acyl-homoserine lactone
AI-1	auto-inducer1
AI-2	auto-inducer2
ANOVA	analysis of variance
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pair
BLAST	Basic local alignment search tool
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CSF	competence and sporulation factor
Ct	threshold cycle
Da	daltons
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacitic acid
g	Gram
xg	times gravity
h	hour
HPLC	high performance liquid chromatography
HSL	homoserine lactone
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kb	kilobase
L	litre
LB	lysogeny Broth
m	Meter or milli (10^{-3})
µl	microlitre
µM	micromolar
M	molar
MALDI-MS	matrix assisted laser desorption ionization-mass spectra
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
mAU	milli absorbance units

min	minutes
mRNA	messenger RNA
MS/MS	tandem mass spectrometry
MU	Miller unit
m/z	mass to charge ratio
NCBI	National Center for Biotechnology Information
NCIMB	National Collection of Industrial and Marine Bacteria
NMR	Nuclear Magnetic Resonance
NRRL	Northern Regional Research Lab
OD	optical density
ONPG	Ortho-Nitrophenyl-β-galactoside
Opp	Oligopeptide permease
ORF	open reading frame
PCR	polymerase chain reaction
PDA	potato dextrose agar
PI-Factor	2,3-diamino-2,3-bis (hydroxymethyl)-1,4-butanediol
ppb	parts per billion
ppm	parts per million
qPCR	quantitative polymerase chain reaction
QS	quorum sensing
QSM	quorum sensing molecule
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RPMI 1640	Roswell Park Memorial Institute medium
RT-PCR	reverse transcriptase polymerase chain reaction
TBE	tris borate EDTA
TLC	thin layer chromatography
Tm	melting temperature
vol/vol	volume per volume
wt/vol	weight per volume

LIST OF PUBLICATIONS

1. **Esmaeilishirazifard, E.** and Keshavarz, T., (2014). Aflatoxin Occurrence. In: Faulkner. A.G. (editor) Aflatoxins: Food Sources, Occurrence and Toxicological Effects, 1st ed. Nova Science Publishers Inc., New York, USA, pp. 35-62.

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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.

This thesis is entirely my own work and that where any material could be construed as the work of others, it is fully cited and referenced, and/or with appropriate acknowledgement given.

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.

Signed: Elham Esmaeilishirazifard Date:

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Chapter I

INTRODUCTION AND LITERATURE RIVEW

1.1 Quorum sensing history and definition

Multicellularity is one of the crucial transitions to evolve a new and complex Earth ecology. Scientists have discovered biochemical pathways through which single cells might have developed group behaviour paving the way for evolution of multicellular complexity. These discoveries show how complex life might have evolved in the living world (Libby and Ratcliff, 2014). The development of animals and plants is governed by sophisticated systems which depend on the communication between cells. The initial steps in the formation of the organs from zygote in multicellular life rely on the transmission of molecular signals between cells. Although, bacteria are considered as non-differentiating uni-cellular microorganism, they employ mechanisms to facilitate responses, through altering their gene expression, to the changes in the growth environment. Their great similarity to signal transduction mechanisms in higher organisms shows that they are more capable of operating communicative behaviour than generally recognised (Williams, 1994). The evolution of complex life forms indicates that unicellular microbes were the important organisms to reshape the planet ecology. So Prokaryotes as the ancestor of the Eukaryotes have possessed a simpler means of communication with higher living organisms; their signalling pathways systems might have evolved to form complex ones in the Eukaryotes.

In macro-organisms, the signalling molecules are critical for conducting the activities of large groups of cells in organs. Studies have revealed that microorganisms communicate using chemical signal molecules such as small hormone-like molecules (Tomasz and Beiser, 1965; Nealson *et al*., 1970). In fact, bacteria have developed cell-density-dependant regulatory systems that modulate gene expression. This phenomenon that correlates bacterial cell-density with expression of specific genes encoding relevant biological phenotypes is named quorum sensing (QS) (Fuqua *et al*., 1994; Fuqua and

Greenberg, 1998). Thus, QS phenomenon enables bacteria to act as multicellular organisms (Waters and Bassler, 2005).

The first evidence of microbial cell-cell communication was introduced by Tomasz and Beiser (1965), when they suggested that a hormone-like extracellular product regulated competence in *Streptococcus pneumoniae* (*S. pneumoniae*). Later, researchers found that the molecule was a peptide acting as a common signal in cell-cell communication amongst Gram-positive bacteria (Dunny and Leonard, 1997). However, organized response in a microbial colony was officially reported on the luminous marine bacterium *Aliivibrio fischeri* (*A. fischeri*) in its symbiotic relationship with the Hawaiian squid, *Euprymna scolopes*. Bioluminescence was developed and controlled by one or more signalling molecules accumulating in the extracellular environment of *A. fischeri* as their cell-density increased and reached a critical number (quorum) (Nealson *et al*., 1970). This symbiotic story between the two organisms represents a cooperative development for both the fish and the bacterium: whilst the bacterium colonizes the light organs of its eukaryotic host to supply the squid with light to be used for defence or attraction, the bacterium exploits its host as a source of essential nutrients (Ruby and McFall-Ngai, 1992). *A. fischeri* at high cell-density (10^{11} cells.ml^{-1}) accumulates signal molecules (N-acyl homoserin lactone (AHL)) to an adequate concentration to induce the transcription of genes encoding the luminescence enzymes (Eberhard, 1972).

The mechanism of bioluminescence regulation was originally called auto-induction (Nealson, 1977). Later research suggested that auto-induction was only one of the common traits characterising the QS phenomenon. Pivotal to this population-dependant adaptive behaviour is an alteration in gene expression in response to the processing of diffusible molecules (often referred to as auto-inducers). Once the QS signal molecule(s)' concentration reaches a threshold or "quorum", a number of target genes get activated or repressed in processes that bring about a collective behavioural adaptation (Williams *et al*., 2007). Signal molecules implicated in cell-cell communication are known as auto-inducers and/or quorum sensing molecules (QSM) and their function is to regulate gene expression in other cells of the community to control bacterial responses (Nealson *et al*., 1970).

QSMs and signalling mechanisms based on cell-density are widespread among bacteria. This fact led scientist to hint that these molecules and systems were widespread in single-celled eukaryotes, such as fungi (e.g. yeasts). Evidence for density-dependant cell-cell communication in fungi has begun to emerge (Hogan, 2006). Among filamentous fungi, *Aspergillus* utilizes quorum regulation to affect morphogenesis and secondary metabolite production (Brown *et al*., 2009). Several studies report the effect of certain types of QSMs secreted from one group of fungi (e.g. farnesol from *Candida albicans* (*C. albicans*)) on the growth, morphology, sporulation (conidiation), apoptosis and metabolite production of other fungi (e.g. *Aspergillus nidulans* (*A. nidulans*) and *Saccharomyces cerevisiae* (*S. cerevisiae*)) (Brodhun and feussner, 2011). A QS-like behaviour in a filamentous fungus was first reported in *Aspergillus terreus* (*A. terreus*) (Schimmel *et al*., 1998). Almost after a decade, QS behaviour were reported in *A. flavus* (Brown *et al*., 2008 and 2009) and then in *A. nidulans* (Williams *et al*., 2012) (for futher details please refer to section 1.5).

1.2 QS in bacteria

Bacteria synthesise and secrete a variety of small molecules. The molecules have diverse specific roles in the modulation of metabolic activities representing natural microbial communities. They might be involved in cell-cell signalling and communication (Yim *et al*., 2006). The QSMs are small diffusible molecules in extracellular environment with the specific role in cell communication. Yim and colleagues (2006) suggested that many extracellular bacterial metabolites have the potential to function as signal molecules. However, it is crucial to differentiate between the real signal cell communication compound and other metabolites. Some important features to define a molecule as QS signal in bacteria and fungi, have been summarised by Albuquerque and Casadevall (2012). The QSM needs to fulfil all of the following criteria;

- The molecule is produced in a basal amount at low cell-density. But the QS response is only launched at a certain stage of microbial growth which is determined by its cell-density.

- The molecule is synthesised inside the cell, and secreted to the outside of the cell and will be recognised by a specific receptor.
- After reaching a critical threshold concentration, the molecules accumulated in the extracellular environment induce a coordinated response in the entire microbial population.
- The molecule elicits a similar response when added exogenously to the culture of the mutant which is unable to produce the signalling molecule.
- QSM is not a by-product of microbial metabolism or detoxification. The toxic metabolites or by-products which build up in the extracellular milieu might trigger stress response without affecting the expression of genes.

Some molecules secreted in the extracellular environment, such as antibiotics, are able to induce their own specific uptake machinery, upon influencing the expression of genes involved in other metabolic pathways. Therefore, it has been suggested that antibiotics might be considered as QSMs due to their capability to affect gene expression (Yim *et al*., 2006). Although some antibiotics like nisin and subtilisin produced by *Lactobacillus lactis* (Quadri, 2002) and *Bacillus subtilis* (Kleerebezem *et al*., 2004) respectively, are well established QSMs, there are a large number of antibiotics whose participation in cell to cell communication has not been established yet. Overall, QSMs are involved in the regulation of complicated processes to help bacterial population benefit from their cell-density (Winzer *et al*., 2002).

Since the discovery of cell to cell communication in numerous bacterial species, QS-regulated phenotypes include bioluminescence, antibiotic and exoenzyme production, exopolysaccharide production, virulence, conjugal plasmid transfer, biofilm formation, sporulation, biosurfactant production and growth inhibition (Lazdunski *et al*., 2004; Bassler and Miller, 2013).

A range of chemical compounds are detected as QS signal molecules, and in many bacterial species more than one class of compound engage in the network of regulatory systems that control the response of bacterial population to the environmental changes by regulating the diverse genes (Atkinson and Williams, 2009). QS mechanisms in diverse bacterial species could be classified in three different cell communication

systems. One system is mainly related to the regulation of cell signalling through QS in Gram-negative bacteria. Another system is widely found in the Gram-positive bacteria. In addition to these systems, there is a universal QS system which occurs in both groups of bacteria (Miller and Bassler, 2001).

1.2.1 QS in Gram-negative bacteria

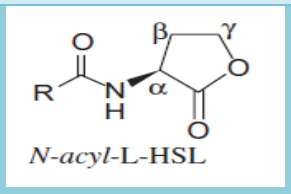
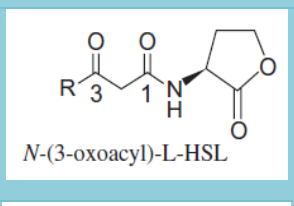
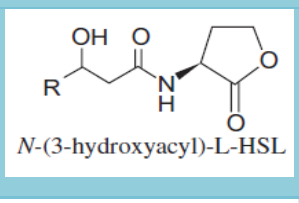
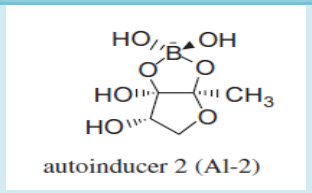
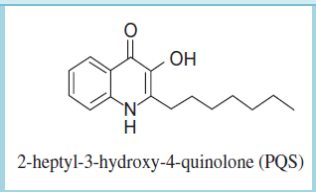
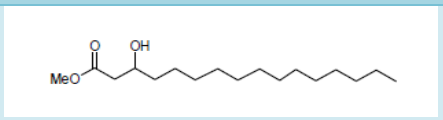
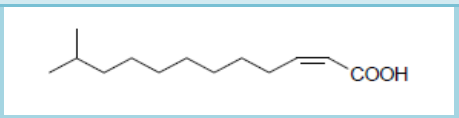
The identified QSMs in Gram-negative bacteria include the N-acylhomoserine lactones (AHLs), 2-alkyl-4-quinolones, γ-butyrolactones, furanones, long-chain fatty acid derivatives, fatty acid methyl esters, peptides, the 4,5-dihydroxy-2,3- pentandione (DPD) derivatives collectively referred to as auto-inducer2 (AI-2) and auto-inducer3 (AI-3, structure unknown). The structure of some of these molecules is presented in Table 1.1 (Winzer *et al*., 2002; Sperandio *et al*., 2003; Winzer and Williams, 2003; Vendeville *et al*., 2005; Williams, 2007).

Apart from the different QSMs with their own specific systems in Gram-negative family, the LuxIR (or a homologue) mechanism along with its QSM, AHL (or a homologue), is introduced as the model QS system in Gram-negative bacteria. This QS system is the first well-known QS mechanism which is established to regulate bioluminescence in *A. fischeri* (Eberhard *et al*., 1981). Most studies support the view that a universal gene regulation mechanism similar to the *A. fischeri* bioluminescence auto-induction system may exist in diverse bacterial species. Since the discovery of the QS phenomenon, the list of bacterial species known to produce AHL QS signals has expanded rapidly (Zhang, 2003; Dong *et al*., 2007; Williams *et al*., 2007; Atkinson and Williams, 2009).

AHL molecules are biosynthesised preliminarily by the *luxI* family of AHL synthases of which over 100 types are defined recently in bacterial genome databases (Atkinson and Williams, 2009). The LuxIR system uses the LuxI protein to synthesise the auto-inducer AHLs, and LuxR is the regulator that binds to the auto-inducer and affects gene expression. At high cell densities, the response to an AHL takes place in the cytoplasm through the interaction between QS signal and the transcription factor, a member of the LuxR family of transcriptional regulators. The combined LuxR regulatory protein-AHL

then recognizes and binds specifically to a QS-regulated promoter to activate the transcription of target genes (DNA-sequences). It induces a particular QS phenotype (Fuqua *et al*., 2001). This system exerts a high specificity, since the auto-inducer produced by one bacterial species can rarely interact with the LuxR-type regulator of another species. The chemical structure of AHL in different species is a common homoserine lactone moiety along with the variable acyl side-chains.

Table 1.1 Chemical structures of some QSMs present in Gram-negative bacteria.

Signal	Molecule structure	Organisms
Acyl-homoserin lactones (Atkinson and Williams, 2009)	 *N-acyl*-L-HSL	*Acinetobacter, Chromobacterium, Agrobacterium, Aeromonas, Burkholderia, Erwinia, Pseudomonas, Rhizobium, Serratia, Aliivibrio, Vibrio, Yersinia*
	 *N*-(3-oxoacyl)-L-HSL	
	 *N*-(3-hydroxyacyl)-L-HSL	
Auto-inducer2 (Atkinson and Williams, 2009)	 autoinducer 2 (AI-2)	*Vibrio harveyi*
Alkyl quinolones (Atkinson and Williams, 2009)	 2-heptyl-3-hydroxy-4-quinolone (PQS)	*Pseudomonas aeruginosa*
Fatty acid methyl esters (Flavier *et al.*, 1997)	 3-hydroxylpalmitic acid methyl ester	*Ralstonia solanacearum*
Long chain fatty acids (Huang and Lee Wong, 2007)	 cis-Δ2-11-methyl-dodecenoic acid	*Stenotrophomonas maltophilia*

Two other classes of AHL biosynthase have been identified. One is an enzyme consisting of amino acid composition distinct from LuxI homologues, but analogous in function. This type of QSM synthase is prevalent in *Vibrio* spp. and belongs to the LuxM protein family (Miltone *et al*., 2001). HdtS represents another class of AHL synthase which is distinct from LuxI and LuxM homologues. It is frequent in *Pseudomonas fluorescens* (Laue *et al*., 2000) and its homologue, Act, has been characterized in *Acidithiobacillus ferrooxidans* (Rivas *et al*., 2007). In many species, more than one AHL is synthesised by different LuxI homologues. Most of AHLs travel across the cell membrane by diffusion and bind LuxR-like response regulators. The LuxR protein (or homologue) functions as a sensor and transcription factor simultaneously. This group of signal transduction system is referred to as one-component signal transduction system as the fusion of the signal binding domain and transcription-regulating DNA-binding domain happens in this network. It is the most widspread signal transduction system in bacteria (Figure 1.1) (Ulrich *et al*., 2005).

Some examples of Gram-negative AHL-based QS systems and their corresponding functions are reported in Table 1.2.

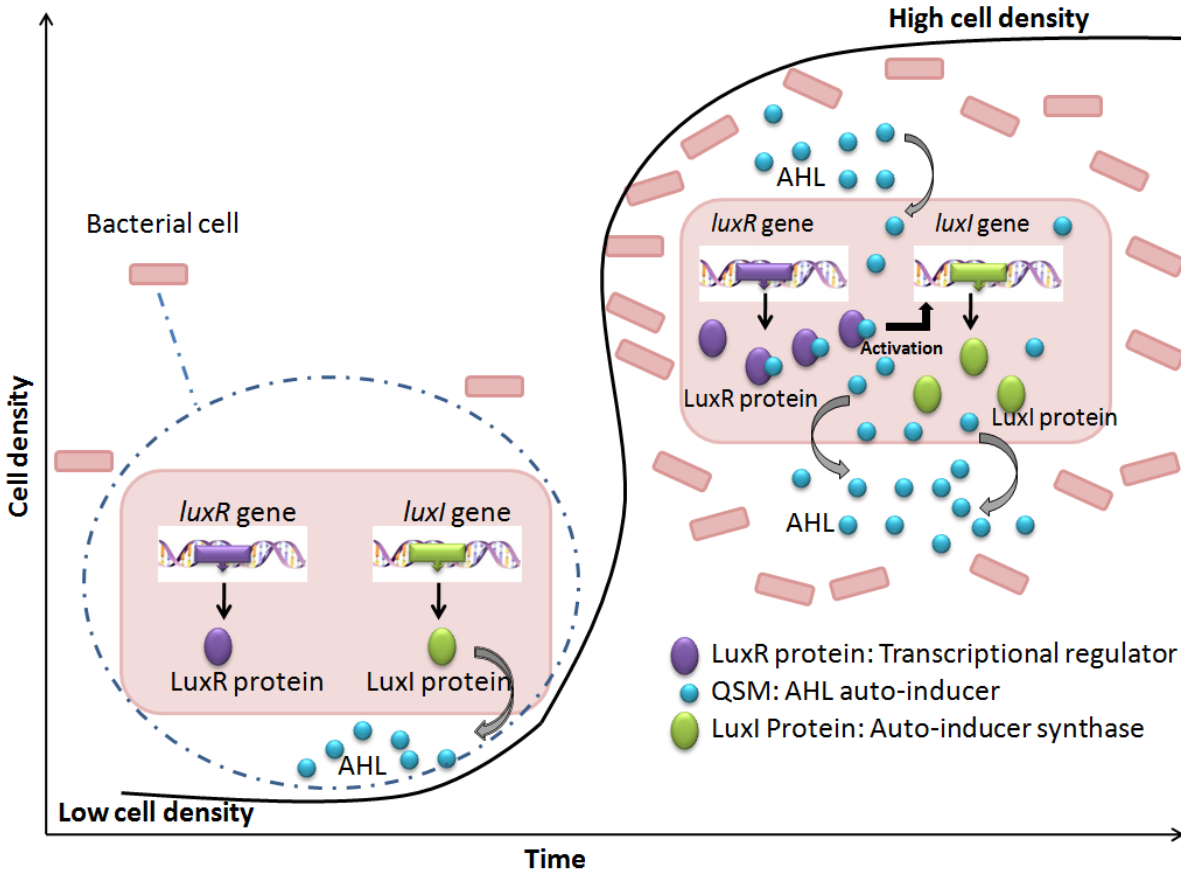


Figure 1.1 QS in Gram-negative bacteria. This schematic diagram shows the growth curve of bacteria and their QS process in low and high cell-density. The QSMs, Acyl homoserine lactones (AHLs), are synthesised by LuxI protein and diffused into the extracellular space. In higher concentrations, AHLs diffuse into the cytoplasm and bind to LuxR protein to activate or repress the transcription of QS-regulatory operon (Adapted from Lazdunski *et al*., 2004).

Table 1.2 Examples of AHL-dependent QS systems in various bacterial species

Bacterial species	AHL-based QSM	QS systems (LuxI-LuxR homologues)	Biological functions	Reference
Agrobacterium tumefaciens	3OC8HSL	TraI-TraR	Ti plasmid conjugation	Zhang *et al*., 1993 Piper *et al*., 1993
Erwinia stewartii	3OC6HSL	EsaI-EsaR	capsular polysaccharide biosynthesis, virulence	Beck Von Bodman and Farrand, 1995
Pseudomonas aeroginosa	3OC12HSL	LasI-LasR	virulence factors, biofilm formation	Pearson *et al*., 1995
	C4HSL	RhlI-RhlR	rhamnolipid production	Pierson *et al*., 1994
Rhizobium legominosarum	C6HSL C8HSL	RhiI-RhiR	rhizosphere genes expression and growth inhibition	Gray *et al*., 1996
	3H-7-cis-C14HL	CinI-CinR	plasmid pRL1JI transfer	Lithgow *et al*., 2000
Yersinia pseudotuberculosi	3OC6HSL C8HSL	YpsI-YpsR YtbI-YtbR	bacterial aggregation and motility	Atkinson *et al*., 2008

1.2.1.1 Acyl homoserine lactones

Acyl homoseriene lactones are produced as a QSM in more than 50 different bacterial species. AHLs are composed of a homoserine lactone ring attached to fatty acid chain. These QSMs vary in the length and substituent of their acyl side chain. The chain varies in length from 4 to 18 carbons, and may have a keto-group in position 3. For instance, in *A. fisheri*, the produced AHL is 3OC6 homoserine lactone molecule with a keto group on

the third of the 6 carbons of fatty acid chain, whilst in *Agrobacterium tumefaciens*, the LuxI homologue, TraI, produces the $3OC_8$ molecule with a keto group on the third of the 8 carbons of fatty acid chain (Table 1.1) (Atkinson and Williams, 2009).

Each AHL binds to a particular LuxR-type protein to exert a high degree of selectivity and complexity. The specificity of the LuxR-like protein for AHL is determined by the acyl binding site of the LuxR homologue as AHLs differ only in their acyl chains. AHL binding is high-affinity which represents the bacterial ability to sense relatively low concentrations of the small communicating signals. Some bacterial species have a single AHL synthase, producing one type of AHL, whereas other species such as *Pseudomonas aeruginosa* (as seen in Table 1.2) have multiple AHL synthases corresponding to more than one type of AHL (Pearson *et al*., 1995; Camilli and Bassler, 2006).

1.2.1.2 The first known QS system

The luminous bacterium (*A. fischeri*) generates light in the colonized light organs of its host by synthesizing the enzyme luciferase, an oxidase consisting of two different subunits (Ziegler and Baldwin, 1981). In the process of light generation, luciferase catalyses the oxidation of a reduced flavin as well as a long chain aldehyde which leads to biosynthesis of oxidized flavin, and the corresponding long chain fatty acid. The reducing power for the luminescence system is generated by an NAD(P)HFMN oxidoreductase (Jablonski and DeLuca, 1978). Other necessary components for the generation of light include enzymes that catalyse the synthesis of a specific fatty acid or aldehyde. Using Inhibitors in other studies it has been suggested that the auto-inducer (AHL) controls light production at gene transcription level (Nealson *et al*., 1970).

In light production, at least 10% of cellular energy is consumed through the biolumenesence procedure (Karl and Nealson, 1980). This energy commitment indicates that the system is well-regulated by the signalling molecules. After colonisation of the squid light organ by *A. fischeri*, the bacterium accumulates the signal molecules (AHL) to

a high concentration to induce the transcription of genes encoding the luminescence enzymes (Eberhard, 1972).

The QS system in *A. fischeri* is regulated by eight bioluminescence *lux* genes, which are located in two transcriptional units, operon I and operon R (Figure 1.2). *luxI* and five genes (*luxCDABE*) operate as the luciferase structural operon (LuxICDABE). Together with the Luxl operon, a single gene *luxR* is transcribed in the opposite direction which encodes for a transcriptional activator of bioluminescence. *LuxI* encodes an auto-inducer synthase to produce the auto-inducer N-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL). *luxA* and *luxB* genes encode the heterodimeric luciferase enzyme subunits. Luciferase enzyme catalyses the oxidation of two compounds, aldehyde and reduced flavin mononucleotide to produce a long chain fatty acid, flavin mononucleotide and water. Excess free energy is released during oxidation-reaction in the form of blue-green light. In addition, *luxCDE* involved in the biosynthesis of luciferase substrates by encoding for multi-enzyme complex (Eberhard *et al*., 1981; Engebrecht *et al*., 1983; Fuqua *et al*., 1994).

When the concentration of 3-oxo-C6-HSL produced by *luxI* is lower than the threshold due to low biomass concentration of *A. fischeri,* no physiological response is given. However, as the cell-density increases on the specialized light organ, the concentration of QSM increases beyond the threshold (Eberhard *et al*., 1981). Subsequently the complex QSM-LuxR activated transcription factor induces transcription of *luxCDABE* genes (Hanzelka and Greenberg, 1995). This causes the additional production of QSM through auto-induction that results in light generation via QS scenario (Engebrecht *et al*., 1983).

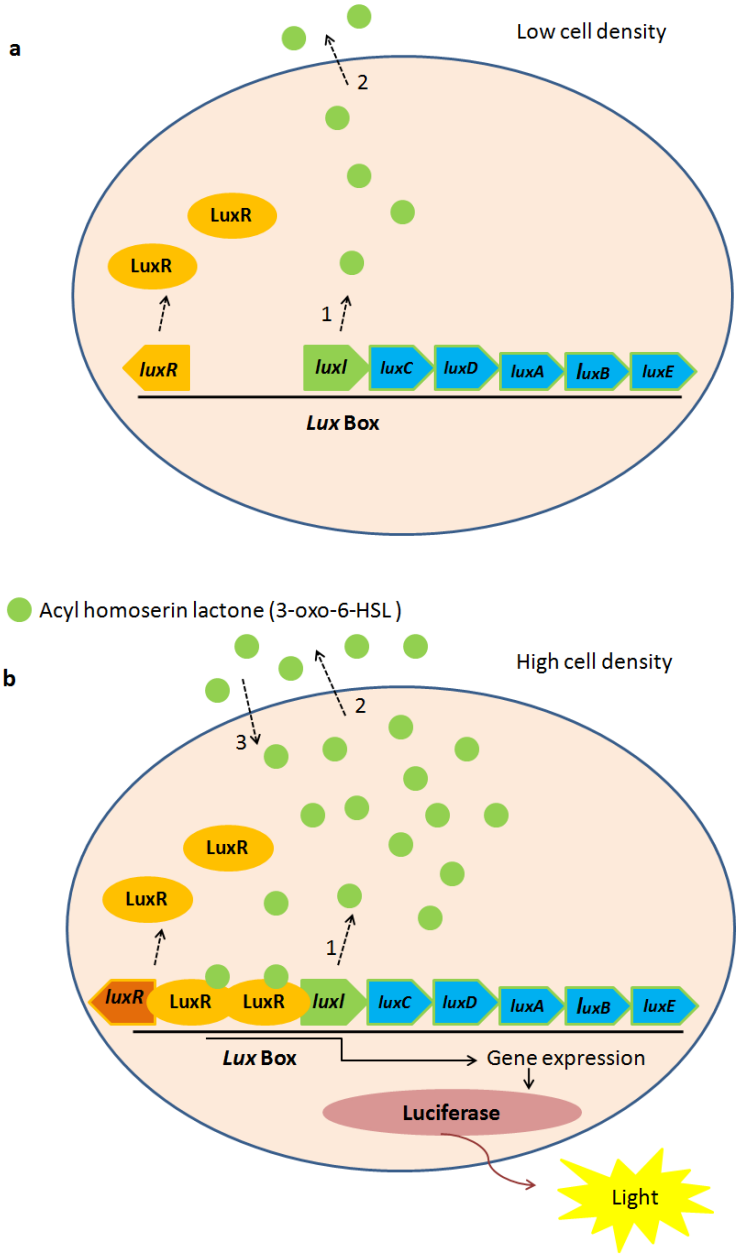


Figure 1.2 Bioluminescence in *A. fischeri* through the LuxIR operon. The *luxICDABE* genes required for light production. The *luxC*, *luxD* and *luxE* genes encode acid reductase which helps to produce the substrate for the light-producing enzyme luciferase which its subunits, α and β, are produced by *luxA* and *luxB* genes. The *luxI* gene encodes the enzyme (acylhomoserine lactone synthase) that produces the QS signal 3-oxo-C6-HSL. The single gene transcribed in the opposite direction, *luxR*, encodes the signal response regulator of *lux* box. (a) At low cell densities, the auto-inducer signals are produced in a smaller concentration and they diffuse through the cell membrane to the outside. (b) As the cell-density rises, QSM concentration increases and accumulates beyond a threshold level. At high cell-density, AHL molecule is bound to the cytoplasmic response regulator (LuxR). This combined AHL-LuxR could be attached to the *lux* box to activate the expression of *luxICDABE* genes, luciferase enzyme production, and then light emission.

1.2.2 QS in Gram-positive bacteria

The QS signalling communication in Gram-positive bacteria does not have any similarities with QS system in Gram-negative bacteria, as LuxIR and AHLs are not involved in this system. While Gram-negative bacteria use low molecular weight hydrophobic signal molecules, Gram-positive bacteria employ unmodified (e.g. the competence stimulating factors of *S. pneumoniae*) or post-translationally modified peptides such as the staphylococcal cyclic peptides (Williams *et al*., 2007).

Signalling peptides of Gram-positive bacteria are synthesized in cytoplasm by ribosomes, but the pathways employed for the biosynthesis of the extracellular final product are characterised by considerable diversity. Post-translationally modified peptides are identified in many Gram-positive bacteria as QS signal molecules. These peptides, referred to as auto-inducing peptides (AIPs), consist of 5 to 34 amino acids which contain unusual chemical architectures. Based on their unique structure, three different families of AIPs are known: (i) the oligopeptide lantibiotics, mainly the *lactococcal* nisins, (Quadri, 2002); (ii) the 16-membered thiolactone peptides, exemplified by the staphylococcal AIP-1 (Chan *et al*., 2004); and (iii) the isoprenylated tryptophan peptides, ComX and its variants, from *B. subtilis* and other *Bacillus* species. These are currently the only known members (Ansaldi *et al*., 2002; Okada *et al*., 2005).

Peptide signals are not diffusible across the membrane; hence signal delivery is mediated by dedicated oligopeptide exporters. These small signal molecules, either unmodified or post-translationally modified peptides, are secreted via an ATP-binding cassette (ABC) exporter protein. They are produced throughout the growth to reach a threshold concentration at a certain cell-density. Cell-cell communication in these bacteria is controlled by a two-component system, which can detect the accumulated peptides in the extracellular environment. This two-component system consists of a histidine kinase and a response-regulator. The external part of a membrane bound sensor kinase protein recognizes the auto-inducer and then triggers signal transduction by using phosphorylation, and activates a response regulator that binds to DNA and modulates transcription (Parkinson, 1995; Kleerebezem *et al*., 1997, Bassler, 1999 and 2002).

γ-butyrolactone acts as another type of auto-regulator in *Streptomyces* spp. and binds to cytoplasmic receptor proteins and therefore inhibits their binding to specific DNA targets. Most of these receptor proteins act as transcriptional repressors, so that binding of γ-butyrolactones to them inhibits their repression activity, and then it induces expression of target genes (Ohnishi *et al*., 2005).

Antibiotic production by *Streptomyces*, virulence gene expression in *Staphylococcus aureus* (*S. aureus*), and development of competence for DNA uptake by *B. subtilis* and *S. pneumoniae* are some of the processes regulated by QS in Gram-positive bacteria, as listed in Table 1.3. The general mechanism for peptide-mediated QS in Gram-positive bacteria is represented in Figure 1.3.

Table 1.3 QS signals in Gram-positive bacteria

QS molecule	Functions	Mechanisms	Organisms
Peptide (unmodified)	Competence	Two-component signal transduction	*Streptococcus pneumoniae* (Havarstein *et al.*, 1995)
Peptide (unmodified)	Bacteriocin production	Three-component system	*Lactobacillus spp.* (Brurberg *et al*., 1997)
Modified peptide	Competence	Two-component signal transduction	*Bacillus subtilis* (Okada *et al*., 2005)
Modified peptide	Nisin production	Two-component system-biosynthetic promoters	*Lactococcus lactis* (Kuipers *et al*., 1995b)
Modified peptide	Virulence	Two-component system-regulatory RNA promoter	*Staphylococcus aureus* (Balaban and Novick, 1995)
γ-butyrolactone	Secondary metabolism	Internalisation/Promoters for antibiotic biosynthetic genes	*Streptomyces griseus* (Miyake *et al*., 1990)

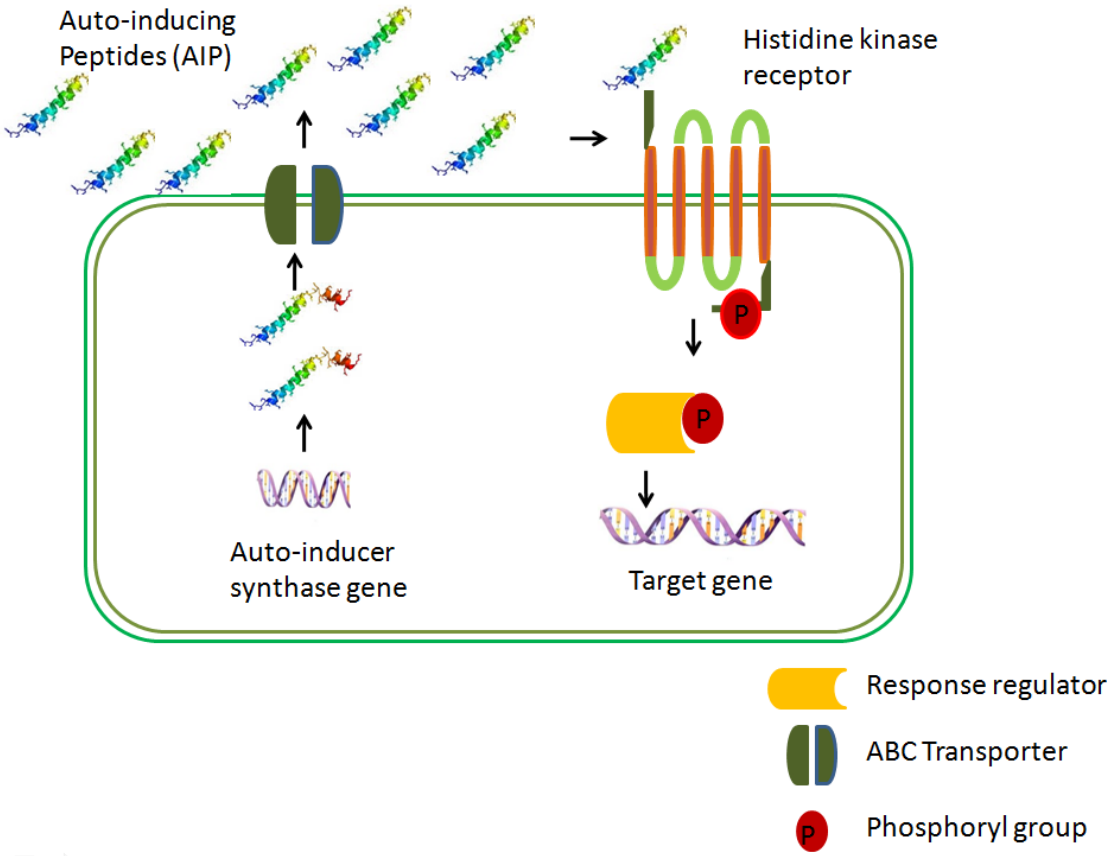


Figure 1.3 QS in Gram-positive bacteria. In most Gram-positive bacteria QS system, peptide auto-inducers (AIPs) after being synthesized (modified or unmodified) are exported via ABC transporters. Extracellular AIPs are recognised by two-component kinase proteins in the membrane. The receptors auto-phosphorylate, and subsequently transfer the phosphoryl group (P) to cognate response regulators. As a result, response regulator protein activate/repress transcription of QS-regulated target genes.

1.2.2.1 QS-mediated by unmodified peptides

Some of the auto-inducing peptides are linear, and unmodified and originally synthesized as precursor polypeptides characterised by a typical double-glycine-type leader peptide (containing N-terminal extensions). These leader peptides are cleaved during exporting across the cytoplasmic membrane by a dedicated ABC transporter. The ABC transporter is a maturation protease and leads to the biosynthesis of a mature leader protein. In this system of QS, the ABC transporter has a dual function; removal of the leader peptide from its substrate, and transportation of this peptide across the cytoplasmic membrane (Havarstein *et al.*, 1995).

These peptides act specifically as QSMs in some Gram-positive bacteria. They are reported in *S. pneumoniae* as the competence-inducing peptide ComC (Havarstein *et al.*, 1995). In *Lactobacillus sake* LTH673 (*L. sake*), the bacteriocin-inducing peptide IP-673 is employed as an unmodified peptide in QS system (Brurberg *et al.*, 1997). Plantaricin A in *Lactobacillus plantarum* C11 (*L. plantarum*) (Diep *et al.*, 1996) and CbnB2/CbnS in *Carnobacterium piscicola* LV17B (Kleerebezem *et al.*, 2001) are other examples of this type of QSM.

There is a QS circuit that helps to develop temporary expression of particular traits followed by changing to the original phenotypes. This system acts as an on-off switch control competence development in the Gram-positive bacterium *S. pneumoniae*. In this system, QSM is an oligopeptide auto-inducer named competence-stimulating peptide (CSP) to monitor cell-density. CSP precursor is encoded by *comC* and consists of 17 amino acids subjected to cleavage to generate the mature peptide (Havarstein *et al*., 1995). The *comA* gene encodes the transporter ComAB. This transporter exports and modifies CSP simultaneously (Hui *et al*., 1995). One operon comprised three different genes; *comC*, *comD* and *comE*. The two-component transduction system family includes homologues of the histidine kinase (encodes by *comD*) and response regulator proteins (encodes by *comE*). Extracellular CSP is detected by the membrane-bound sensor histidine kinase ComD, which transfers phosphate to the cytoplasmic response regulator ComE (Pestova *et al*., 1996). This circuit controls the transcription of different group of genes in a precise transient order. Following ComE phosphorylation, early genes (*comAB* and *comCDE*) are triscribed; this prompt producing of signals in higher level (Pestova *et al*., 1996). This cyclic system results in increasing competence when the bacterial cell-density reaches the threshold. On the other hand, ComE also activates transcription of *comX* and *comW* which are required for DNA uptake (Lee and Morrison, 1999; Luo *et al*., 2004).

QS system in *S. pneumoniae* is specialized to an efficient mechanism with which the process of competence development initiates and terminates rapidly (Tomasz and Hotchkiss, 1964). In comparison with non-competent cells, competent S*. pneumoniae* cells are unstable and subjected to autolysis (Dagkessamanskaia *et al*., 2004).

Interestingly, once the new genes are taken up by the competent cells, the advantage gained from acquiring DNA could be efficiently employed by the rapid QS circuit which helps to activate and terminate the process to minimize death through autolysis (Luo *et al*., 2004). The schematics of QS system has been shown in Figure 1.4.

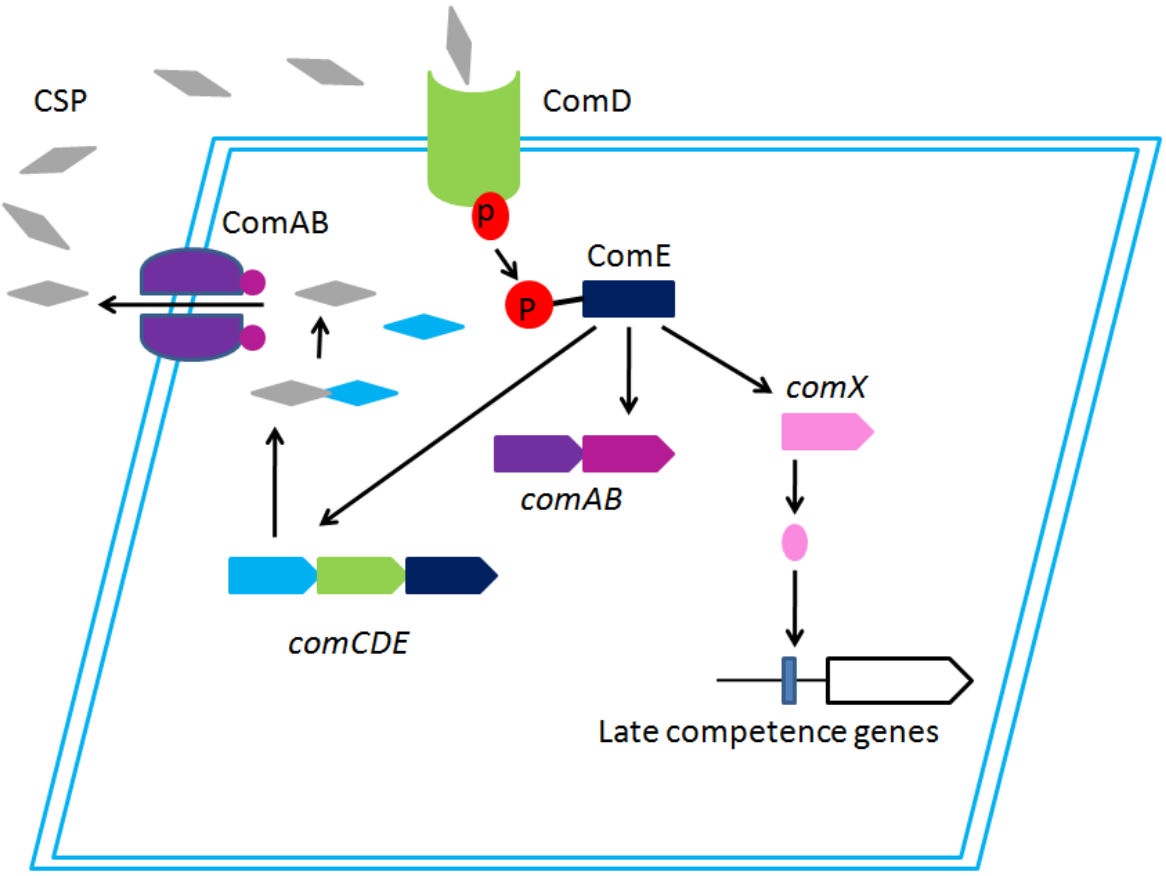


Figure 1.4 QS-regulated competence in *S. pneumoniae*. The induction of genetic competence is modulated by a CSP–mediated QS mechanism. Two genetic loci, *comAB* and *comCDE* are involved in QS system as the "early genes" to produce the associated products. *comAB*, encodes a cassette transporter-protein which is involved in the processing and export of the CSP. The loci, *comCDE*, respectively encode the precursor to the CSP, a histidine kinase as a CSP receptor, and a response regulator that activates both *comAB* and *comCDE* operons. QS signals initiate competence through activity of ComX which starts the transcription of competence-specific operons involved in DNA uptake (adopted from Cvitkovitch *et al*., 2003)

To indicate another scenario of QS mechanism in Gram-positive bacteria (unmodified peptide as QSM), Class II bacteriocin production regulation is interesting (Class I bacteriocins are post-translationally modified compounds named lantibiotics, refer to section 1.2.2.2). The QS signalling processes involved in the biosynthesis of Class II bacteriocin molecules and bacteriocin-like II peptides have been determined as a three-component regulation mechanism. In bacteriocin-producing bacteria such as *L. plantarum* and *L. sake*, just one operon is responsible for coding the induction factor, the response regulator, and the histidine kinase receptor. For instance, plantaricins (bacteriocin produced by *L. plantarum*) production is modulated by plantaricin A. This molecule acts as an induction factor which coded by gene *plnA*. This gene is on the same operon along with the genes encoding a two-component regulatory system (plnBCD) (Nes *et al*., 1996).

1.2.2.2 QS-mediated by modified peptides

Post-translationally modified peptides are identified in many Gram-positive bacteria referred to as auto-inducing peptides (AIPs), with three different families; the oligopeptide lantibiotics, the 16-membered thiolactone peptides, and the isoprenylated tryptophan peptides, ComX and its variants (Williams *et al*., 2007).

Lactic acid bacteria produce a wide spectrum of antimicrobial peptides, known as bacteriocins (classified as lantibiotics or Class I bacteriocins). These compounds are characterised by the sequence of the unusual amino acids lanthionine and 3-methyllanthionine residues. The structural diversity in lantibiotics is high which leads to sub-groups classification based on the structure of the mature peptide or the pre-peptide (Jung, 1991; de Vos *et al*., 1995; Jack and Jung, 2000). Lantibiotics as bacteriocin use a non-enzymatic system to disturb the cell membrane and/or inhibit the biosynthesis of cell wall. Most of these peptide antibiotics are synthesised as precursors in ribosome and following their post-translational modification to reach their biological activity, they are secreted extracellularly. These bacteriocins reveal remarkable antibacterial activities against a wide range of Gram-positive organisms. The lantibiotic-

mediated QS mechanism is unique since it specifically modulates a cell-density dependent system through application of potentially harmful signal molecule (Williams *et al*., 2007).

Nisin and lacticin 481 from *Lactococcus lactis* (*L. lactis*) (Siezen *et al*., 1996) are the best identified Class I bacteriocins amongst many lantibiotics. Nisin biosynthesis and regulation has been known as the best model for the study of post-translationally modified polypeptides. The dual function of nisin is efficient as it acts as antimicrobial agent against other bacteria and in parallel performs as a QS signal to regulate its production and control of immunity genes in *L. lactis* (Kuipers *et al*., 1995a and b, de Ruyter *et al*., 1996).

The biosynthesis of the auto-inducing nisin occurs through employing a cluster of eleven genes, *nisABCTIPRKFEG*. It is an auto-inducible system involving the two-component regulatory system NisK–NisR (Kuipers *et al*., 1995b; Dodd *et al*., 1996). The nisin pre-peptide is encoded by *nisA*. The post-translational modifications happen using the enzymes (encode by *nisB-C*, *nisP* and *nisT*) to modify and export the mature lantibiotic (de Vos *et al*., 1995). The two-component system responds to extracellular accumulation of nisin, comprising a histidine kinase and a response regulator. *nisK* and *nisR* genes respectively encode these two components (de Vos *et al*., 1995). As the concentration of secreted nisin increases, the QS signal is detected by the histidine kinase NisK, which starts the phosphorylation of the response transduction cascade, thereby leading to the activation of the *nisA* promoter and the *nisF* promoter to control respectively transcription of nisin biosynthetic machinery/*nisI* gene clusters and the genes involved in immunity (Kuipers *et al*., 1995a; de Ruyter *et al*., 1996; Kleerebezem *et al*., 1999).

QS moderated by employing thiolactone peptides is a well-studied system. This group of AIPs is structurally known by a 16-membered side chain-to-tail macrocyclic peptide with the extension of short peptide. The prototypical molecule in this family is the modified octapeptide AIP-1 employed by *S. aureus* (Ji *et al*., 1995 and 1997). A locus, *agrBDCA*, comprises genes required for AIP synthesis (*agrBD*) and AIP response (*agrCA*). The precursor protein (AgrD) has a thiolactone macrocyclic structure which is modified post-

translationally with AgrB (Zhang *et al*., 2002 and 2004). The AIPs are detected by the two component signal transduction system (TCSTS) including AgrC as a trans-membrane receptor kinase and AgrA as a response regulatory protein. While the AIP is sensed by its cognate AgrC, it leads to activating of the TCSTS which resulting in upregulation of the agr-mediated QS system.

Finally, the *S. aureus* QS system helps 517 nucleotide transcript, RNAIII, to initiate the transcription of genes that encode a variety of exo-proteins to repress the genes encoding cell surface proteins, e.g. spa (protein A) (Ji *et al*., 1995 and 1997; Dunman *et al*., 2001; McDowell *et al*., 2001). Consequently, the agr system effectively controls the virulence factor expression during the colonization and invasion phases of the staphylococcal infection. This family of thiolactone/lactone peptides with variable size depicts the principle of chemical architecture by Gram-positive bacteria employing as QSMs. The AIP structural variants confirm a high degree of selectivity recognised by different cognate sensors or receptors (Williams *et al*., 2007).

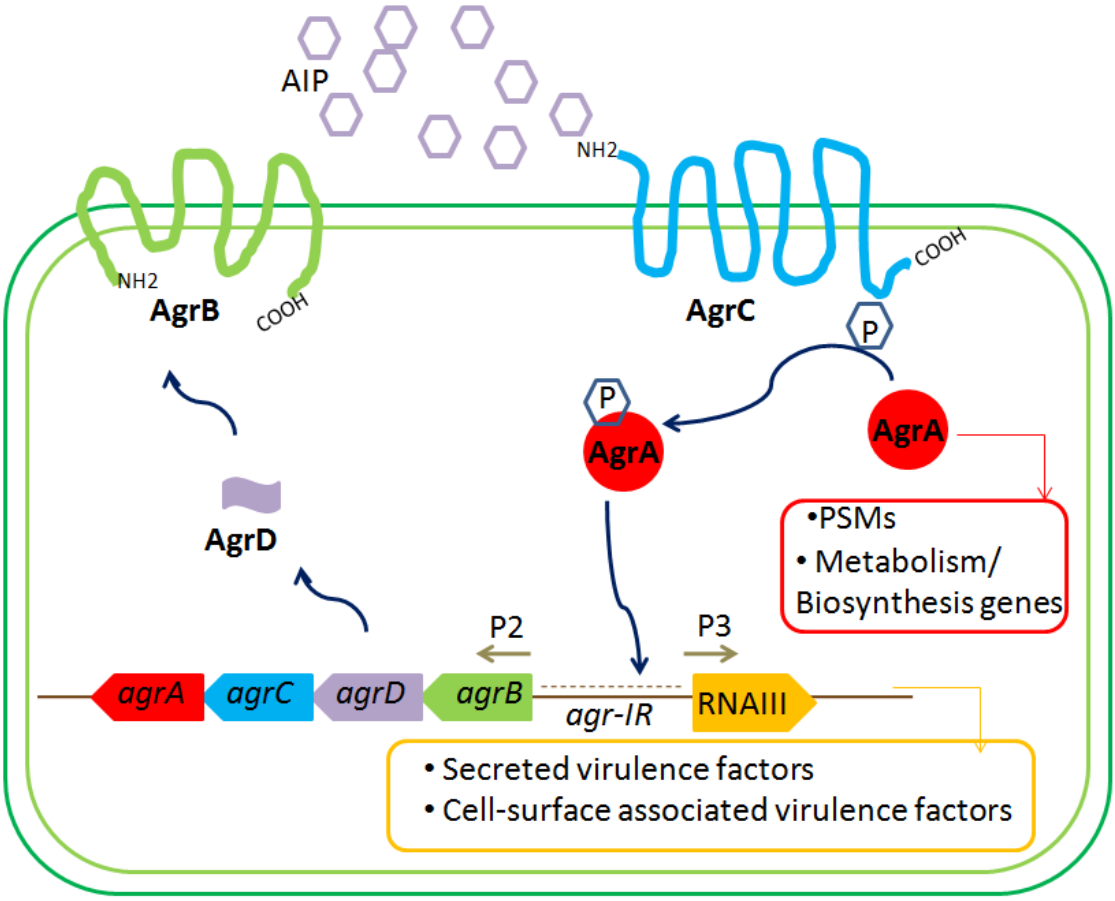


Figure 1.5 The "accessory gene regulator" (Agr) QS system in *S. aureus*. The agr gene cluster (*agrBDCA*) encodes an auto-activating QS system from the P2 promoter of RNAII and P3 promoter of RNAIII. RNAIII regulates the expression of numerous toxins. AgrB and AgrD are involved in the auto-inducer (API) production, while *agrC* and *agrA* encode the sensor kinase and the response regulator of the two-component transduction system, respectively. The interaction of API with AgrA (through AgrC) activates the signal transduction to transcribe both RNAII and RNAIII. In addition, AgrA modulates the expression of cytolytic phenol soluble modulins (PSMs) and several metabolism-related genes (Adapted from Painter *et al.*, 2014).

The third group to be categorized in the modified QSMs is the auto-inducer ComX in *B. subtilis*. The two component system, ComP-ComA, senses ComX, which results in upregulating the expression of genes required for competence development (Tortosa and Dubnau, 1999). The chemical structure of the hexapeptide ComX from *B. subtilis* RO-E-2 was defined with the conserved tryptophan residue which was modified by a geranyl group at the C3 of the indole side-chain, as well as stereospecific intramolecular (indole C2/Na) cyclization to build a rigid tricyclic structure. These post-translational

modifications are crucial for biological activity. On the other hand, the modification has a key role in the interaction of ComX with its cognate receptor, ComP (Okada *et al*. 2005) (for further information please refer to section 1.3).

1.2.2.3 γ-butyrolactones as signalling molecules

The diffusible molecules which named auto-regulators modulate the secondary metabolite production in many actinomycetes (Horinouchi and Beppu, 1992). The activity of these auto-regulators is under QS control. *Streptomyces griseus* (*S. griseus*) and *Streptomyces natalensis* (*S. natalensis*) QSMs are respectively 2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone (A-Factor) and 2, 3-diamino-2, 3-bis (hydroxymethyl)-1, 4-butanediol (PI signalling factor) which are employed in the QS system to produce the antibiotics streptomycin and pimaricin (Miyake *et al*., 1990; Recio *et al*., 2004).

The A-Factor as a QSM is required for production of secondary metabolites such as streptomycin and grixazone and also is the essential factor for formation of aerial mycelia and sporulation (Bibb, 2005). Although this butyrolactone molecule bears resemblance to the homoserine lactone (HSL) signalling molecules in QS system of Gram-negative bacteria but its mechanism does not employ LuxI/LuxR-like systems. When A-Factor concentration rises to threshold, it binds to ArpA (its cognate membrane receptor protein), which employs as a repressor by binding to a specific DNA sequence in the promoter region of the operons required for antibiotic production and morphological differentiation. Following binding A-Factor/ArpA, the promoter releases and allows the transcriptional activation of these operons. *adpA* is one of ArpA target promoters which regulates the transcription of StrR and GriR, responsible for streptomycin and grixazone production, respectively. The expression of other genes of the *adpA* regulon which are responsible for morphological differentiation is also modulated by AdpA (Figure 1.6) (Natsume *et al*., 2004).

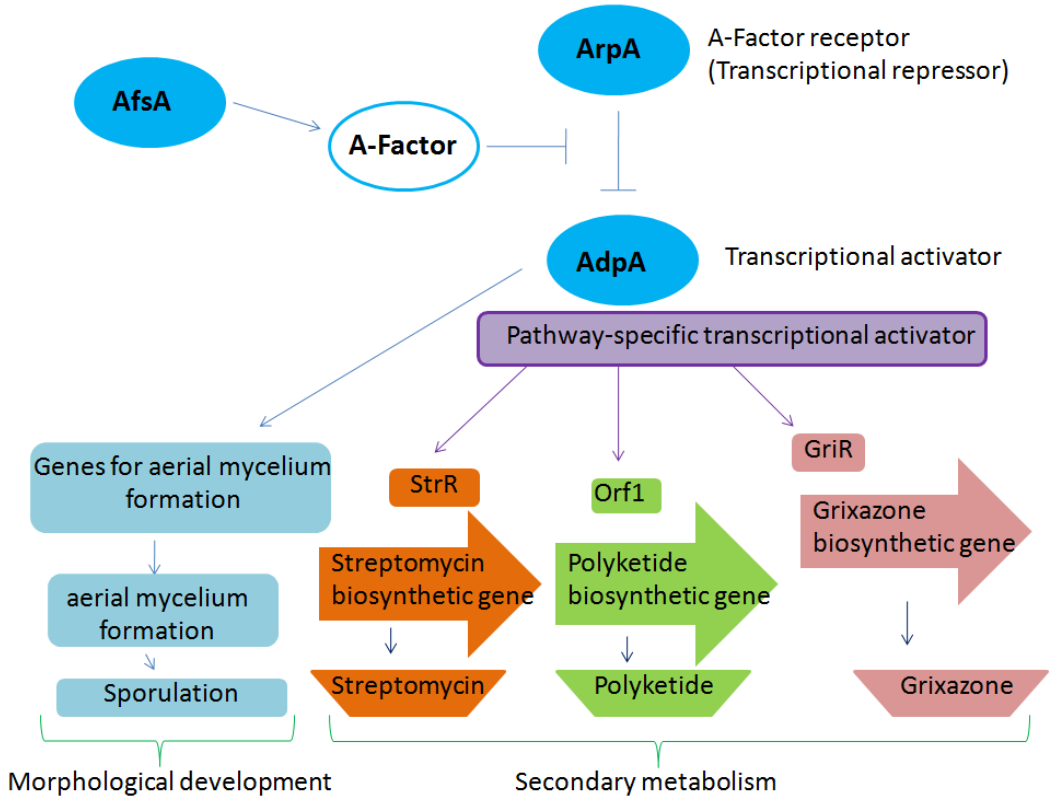


Figure 1.6 A-Factor-mediated regulatory systems for secondary metabolite production and morphological development in *S. griseus* (Adapted from Ohnishi *et al.,* 2005).

An auto-inducer named PI-Factor acts as an essential factor for antifungal pimaricin production at high cell densities of *S. natalensis* used in food industry for mould prevention. The mechanism for the production of pimaricin is similar to the latter QS process. Since pimaricin biosynthesis was induced by both factors (A-Factor or PI-Factor) by addition of them into the deficient mutant strain culture, it has been suggested that QS signals might be exchanged between members of different *Streptomyces* strains (Anton *et al*., 2004; Recio *et al*., 2004).

1.2.3 Universal QS systems

The third QS system is spread among both Gram-negative and Gram-positive bacteria. LuxS is a highly conserved enzyme that catalyses the synthesis of Auto-inducer-2 (AI-2) (Bassler *et al*., 1993; Surette *et al*., 1999). Although LuxS and AI-2 were described

originally in *Vibrio harveyi* (*V. harveyi*), AI-2 is reported in more than 70 bacterial species, and homologous of the enzyme LuxS have been found in several bacteria whose genomes have been sequenced. This system allows interspecies cell-cell communication; where bacteria can recognise and respond to various auto-inducers secreted by other species. On the other hand, AI-2 molecules produced by heterologues organisms are able to activate the luminescence system in a *V. harveyi* reporter strain. All the evidence suggest the hypothesis that the AI-2 may be a universal signal molecule which appears to be employed by different organisms for different purposes (Bassler *et al.*, 1993, 1997; Bassler, 1999; Schauder *et al.*, 2001).

Two QS systems in *V. harveyi* monitor the cell-population density of its own cells and other bacteria located in its surrounding environment. These two QS systems comprise the QSMs such as AI-1 and AI-2. The first one is used for its intraspecies cell-cell communication in a similar system as the LuxIR bioluminescence system in *A. fischeri* (Miller and Bassler, 2001), whereas the second system AI-2, is applied to interspecies communication. Other bacterial species that utilize AI-2/LuxS system include *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Neisseria meningitides*, *Salmonella typhi*, *Escherichia coli* (*E. coli*), and *B. subtilis* (Surette *et al*., 1999, Bassler, 1999).

For instance, the interspecies cell communication through QS system is reported as AI-2-Lsr system in *V. harveyi* and *E. coli*. The auto-inducer (AI-2) can be produced by both species and modulates light production and triggers lsr induction in *V. harveyi* and *E. coli*, respectively. The induced lsr gene in *E. coli* leads to the inhibition of bioluminescence in *V. harveyi* due to the consumption of AI-2 transporter. As a result, *E. coli* blocks the QS-regulated extracellular toxin production in *V. harveyi* (Xavier and Bassler, 2005).

1.3 QS in *B. subtilis*

The QS system in Gram-positive bacteria employs peptides as auto-inducer signalling molecules. In the cytoplasm, the auto-inducing peptides are produced as precursors of

the QSM and then cleaved, modified and exported. Once in the extracellular environment, the peptides are detected via a two-component system (Kleerebezem *et al*., 1997). In *Bacillus* species, QS is moderated by small peptides that control competence, sporulation and the production of certain secondary metabolites in a cell-density dependent manner.

1.3.1 QS-mediated regulation of competence

The development of competence requires several regulatory genes to control the natural ability of a microorganism to take up exogenous DNA. Naturally, at the onset of the stationary phase of microbial growth, a group of cells in the culture differentiates to become competent cells. This process leads to the production of specialized proteins concerned with the uptake of DNA in a way that is independent from its nucleotide sequence (Spizizen, 1958).

Genes regulating competence in *Bacillus* can be classified into two groups; early and late. For example, *comA*, *comP*, *comQ* and *comX* have been named as early genes because of their expression throughout growth whereas the expression of late genes (e.g. *comK*, *comF* and *comG*) increases sharply during the transition to the stationary phase. The early genes are incorporated in the QS government of competence development. The products of these genes do not develop proteins or enzymes which have a direct contribution to the DNA-binding system, however they contribute to the detection of cell-density and the transfer of information to the late genes. The late genes are directly responsible for the generation of proteins which make the actual DNA-binding and competence apparatus. (Van Sinderen and Venema, 1994; Hahn *et al*., 1996).

Regulation of early competence development in *B. subtilis* involves two peptides both accumulating in the extracellular environment at high cell-density. One peptide, known as the ComX pheromone, involves strictly in the regulation of competence development, while the second signalling molecule, CSF (Competence and Sporulation

Factor) controls competence as well as sporulation in *B. subtilis* cultures (Magnuson *et al*., 1994, Solomon *et al*., 1995).

Two concurrent pathways modulate the cell responses to the above mentioned two signalling factors. The ComX activation requires the histidine kinase (encoded by *comP*, a member of the two-component transduction system), while the oligopeptide permease (encoded by *spoOK*) is engaged in the response to CSF (Solomon *et al*., 1995; Perego *et al*., 1996). Both CSF and the ComX pheromone operate together to activate the response regulator/transcription factor ComA (Roggiani and Dubnau, 1993). A general scheme of the QS-regulated development of competence in *B. subtilis* cells is illustrated in Figure 1.7.

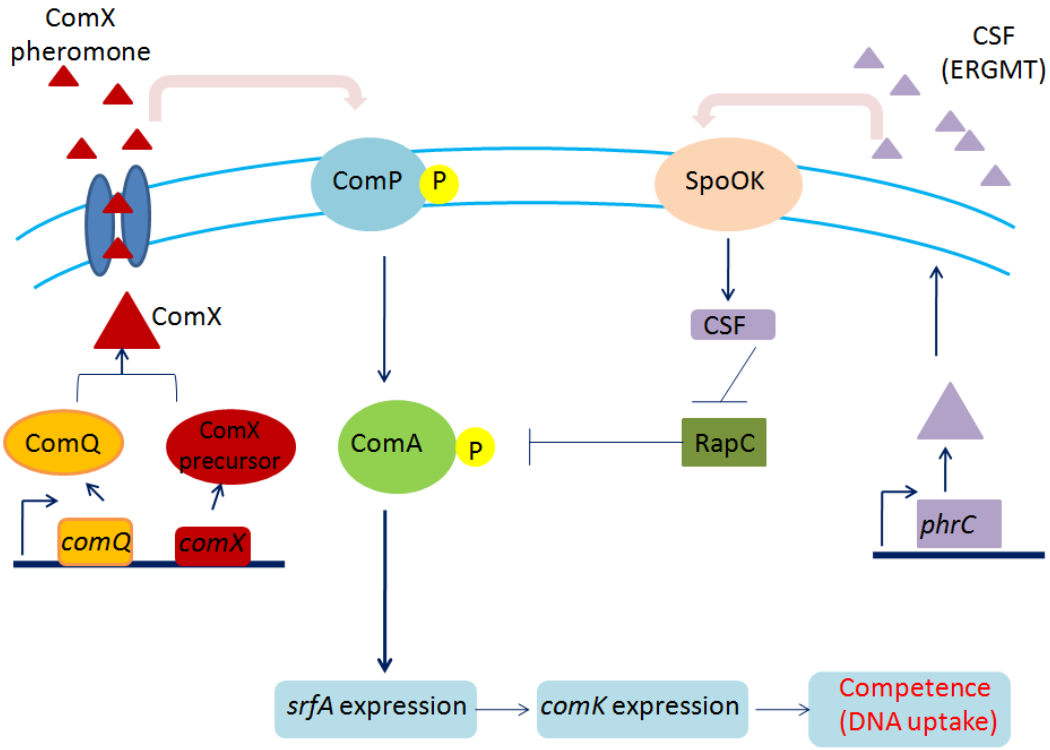


Figure 1.7 A schematic model for regulation of competence through two extracellular signalling peptide-mediated QS in *B. subtilis*. Both ComX and CSF activate the transcription of *SrfA* operon by stimulating the transcription factor ComA through phosphorylation via two separate pathways. One of them needs ComX to be processed and secreted by ComQ. The histidine kinase sensor protein (ComP) interacts with ComX pheromone leading to phosphorylation of ComA response regulator. CSF encoded by phrC is re-internalised via two intracellular targets generated through processing by the *spoOK* gene. At low concentrations, CSF inhibits RapC phosphatase leading to increased accumulation of ComA-p which induces competence (Adapted from Guan *et al*., 2015).

1.3.1.1 The ComX Pheromone

Magnuson and co-workers identified the ComX pheromone (1994) which is encoded by *comX* gene on the *comQXPA* cluster. ComX is a 55-residue precursor which is cleaved at the C-terminus to a 9 or 10-amino-acid peptide, modified by the addition of an isoprene group on a tryptophan and then secreted to the extracellular environment. A partial overlapping has been observed between the 5' end of *comX* and the 3' end of *comQ*, which codes for a protein concerned with the maturation and modification of the pheromone (Magnuson *et al.*, 1994). Since the discovery of ComX in *B. subtilis* 168, this process has been thoroughly investigated which led to the characterisation of several aspects of this system.

Contrary to the majority of the AHL signals generated by Gram-negative bacteria, accumulation of the pheromone in the cell culture supernatant was found to be directly related to cell growth. So the production of this signalling molecule is not auto-induced. In modified strains over-expressing *comX*, a 10-fold increase in the production of ComX has been detected when compared to the wild-type strain. These findings suggest that, in the wild-type cells, *comX* expression limits ComX production, as the concentration of pheromone in the extracellular milieu executes a major role in controlling of the expression of quorum-responsive genes, such as *srfA* (Schneider *et al.*, 2002).
The *comQXPA* locus has been recognised in other members of the *Bacillus* family closely related to *B. subtilis*, but it is highly variable. The polymorphism is associated with the specificity of QS response and therefore it is a feature of the proteins such as ComQ, the ComX precursor polypeptide and the sensor domain of ComP, but not ComA (Tran *et al.*, 2000, Tortosa *et al.*, 2001).

Genetic and biochemical analysis carried out on natural isolates of *B. subtilis* allowed to classify the pheromones of *Bacillus* spp. into 4 pherotypes (variable types of the ComX pheromone which are slightly different in amino acid sequences and post-traslational modifications). However, the *comQXP* locus appears to be highly polymorphic (Tortosa *et al*., 2001). This classification was performed according to the capability of each *Bacillus* strain to activate a QS response in other strains. Both the sequence of the

mature peptide and its modification residue can vary in different pherotypes of pheromone (Ansaldi *et al.*, 2002).

According to Tortosa and colleagues' studies (2001) on the pheromone pehrotypes, the existence of at least four pherotypes in the studied *Bacillus* strains was demonstrated. One group consisted of *B. subtilis* 168 and *B. mojavensis* RO-C-2 (Table 1.4). A second group consisted of *B. subtilis* RS-B-1 and the *B. mojavensis* isolates RO-H-1 and RO-B-2 (Table 1.4). It was predicted that the pheromone of RO-B-2 might share 5 out of 10 identical amino acid residues and 1 similar residue with the two other members of this group. The third group contained a single member, *B. subtilis* RO-FF-1, that showed little similarity to the others in its mature pheromone sequence. The fourth group consisted only of *B. natto* NAF4 which has unique predicted mature pheromone sequence. The ComX precursor protein has a long C-terminal extension in *B. natto* NAF4.

Table 1.4 Characteristics of the ComX variants (adopted from Ansaldi *et al.*, 2002).

Strain	*Bacillus* group	Actual mass [a]	Calculated mass [b]	Modification mass [c]	Cleavage site sequence [d]	Phenotype
168	*B. subtillis* 168	1363.1	1157.2	205.9	ADPITRQ**W**GD	1
RO-C-2	B. mojavensis	967.6	761.8	205.8	TRE**W**DG	1
RO-H-1	B. mojavensis	974.4	854.0	120.4	MLD**W**KY	2
RO-B-2	B. mojavensis	1172.0	1036.1	135.9	YTNGN**W**VPS	2

a. Actual masses were measured by mass spectrometry analysis of the fractions
b. Calculated masses were derived from the theoretical mass obtained from the sequences.
c. Modification masses were obtained by subtracting the calculated mass from the actual mass.
d. The cleavage sites are presented by various sequences.
The alignment of pre-ComX amino acid sequences containing cleavage sites, bold conserved W (Tryptophan) residue and predicted ComX sequnces have been presented bellow (adopted from Tortosa *et al.*, 2001).

168 ---MQDLINYFLNYPEALKKLKNKEACLIGFDVQETETIIKAYNDYYL-ADPITRQ**W**GD----------
RO-C-2 --- MQDLINYFLSYPEVLKKLKNREACLIGFS SN ETETIIKAYNDYHL-SSPTTRE**W**DG----------
RO-H-1 --MQEMVGYLIKYPNVLREVMEGNACLLGVDKDGSECIINGFK---GLEIYSMLD**W**KY----------
RO-B-2 --MQEIVGYLVKNPEVLDEVMKGRASLLNIDKDQLKSIVDAFG-----GLQIYTNG**W**VPS----------

Characterization of the diverse ComX molecules is based on two main criteria: the N-terminal cleavage site and the molecular weight of the post-translationally modified peptide. Different cleavage sites have been identified in various ComX precursors (Table 1.4). These sites generate diverse mature peptides whose length can range from 5 to 10 amino acids, though each of them possesses a conserved tryptophan residue (Ansaldi *et al.*, 2002). Since it is featured in the ComX pheromone developed in *B. subtilis* 168, the

post-translational modification of the peptide occurs on this tryptophan residue (Magnuson *et al.*, 1994). All the investigated ComX variants so far have been demonstrated to be modified by isoprenylation, although different pherotypes were varied in the mass of the isoprene group, thus suggesting that the modification may depict a major element of specificity. The mass of the isoprene group for each pheromone can be obtained by subtracting the calculated mass of the mature peptide from the actual mass measured by mass spectrometry (Ansaldi *et al.*, 2002). Based on this assumption, the ComX purified by *B. subtilis* 168 has been subjected to a farnesylation addition because the mass (206 Da) matches that of a farnesyl group, whilst the 136 Da modifications associated with ComX from *B. subtilis* isolates RO-B-2 and RO-E-2 correspond to geranyl groups (Ansaldi *et al.*, 2002). Other pheromones' mass of 120 Da does not correspond to any simple isoprenoid, thus displaying that the mode of modification of the tryptophan residue might not be a simple isoprenylation (Ansaldi *et al.*, 2002). Further studies on the structure of ComX RO-E-2 confirmed a substitution of a tryptophanyl proton with a geranyl group at position 3 of its indole ring, which leads to the formation of a tricyclic structure (Okada *et al.*, 2005, Okada *et al.*, 2007b). The structure of ComX RO-E-2 which has been investigated through mass spectrometry and NMR is illustrated in Figure 1.8.

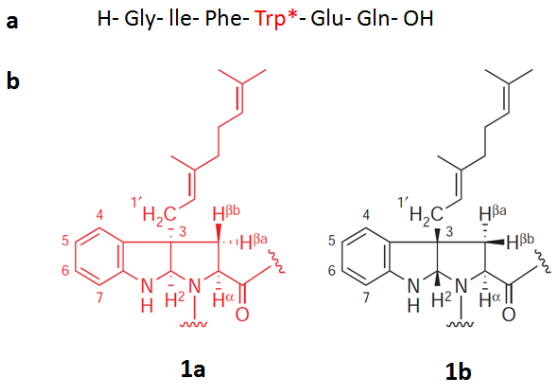


Figure 1.8 Structure of the ComX pheromone (a) Amino acid sequence of the ComX pheromone from *B. subtilis* strain RO-E-2. The Trp* residue is modified by a geranyl group. (b) The possible structures of the modified tryptophan residue are shown in 1a and 1b. 1a is proposed as the most likley structure of modified tryptophan residue which could have a dihedral angle near 90° between H-α and H-βb in the stable conformation (for further details please refer to Okada *et al.*, 2005).

The ComX pheromone has been compared to the A-Factor produced by the yeast *S. cerevisiae*, which acts as an inducer of the mating process, due to the detected analogies in their functions (they are both concerned with genetic exchange), and their biosynthesis (Chen *et al.*, 1997). Both signals are synthesized as precursors, and then they are cleaved and modified by isoprenylations prior to secretion into the extracellular medium. However, whilst the modification reaction leading to the formation of the active A-Factor involves isoprenylation of a cysteine residue (Chen *et al.*, 1997) a unique post-translational modification on the ComX pheromone occurs through a cyclization reaction prior to the attachment of the isoprene moiety to the conserved tryptophan residue (Okada *et al.*, 2005, Okada *et al.*, 2007a). Nonetheless, both the farnesylation of A-Factor and the geranylation/farnesylation of the ComX pheromone appear to be absolutely essential for their biological activity (Gibbs, 2005).

ComQ performs a role as the enzyme for the maturation of ComX precursor which is encoded by *comQ* gene located directly upstream of *comX* in the chromosome as part of a peptide signalling cassette. The first evidence that ComQ partakes in the final production of the ComX pheromone was given by a *comQ* knock-out which resulted in a reduced *srfA* (surfactin biosynthetic operon) expression compared to the wild-type (Magnuson *et al.*, 1994). Co-expression of *comQ* and *comX* in *E. coli* leads to the production and secretion of active pheromone in the medium, showing that *comQ* is the only dedicated protein essential for the processing and the release of active competence pheromone (Tortosa *et al*., 2001). Moreover, induction of QS related genes (*srfA*) at low cell-density could be mediated via the over-expression of only *comQ* and *comX*. Following the identification of isoprenoid binding site in ComQ, the discovery has revealed that mutations in this region could prevent ComX maturation. This confirmed the role of ComQ in the processing of the ComX pheromone (Schneider *et al.*, 2002).

1.3.1.2 The ComP-ComA two component transduction system

The two-component ComP-ComA system exhibits the core of prokaryotic signalling touching phosphorylation transduction cascades (Alex and Simon, 1994; West and

Stock, 2001). The two-component system includes several distinguishing domains usually structured on two conserved proteins: a histidine kinase/sensor and a response regulator that are phosphorylated on histidine and aspartate residues, respectively.

External signal detected by the sensor domain of the histidine kinase triggers the enzyme which catalyzes an ATP-dependent trans-autophosphorylation reaction. A specific histidine residue is phosphorylated which results in generating a phosphoimidazole. The phosphoryl group is subsequently transferred by the response regulator to an aspartate residue located on its own regulatory domain. Phosphorylation leads to the activation of the effector domain of the response regulator which then triggers the specific output response (Alex and Simon, 1994; West and Stock, 2001).

The histidine kinase ComP, concerned with the regulation of competence in *B. subtilis*, is the 769-amino-acid long polypeptide. This molecule is encoded by *comP*, located on the *comQXPA* gene cluster (Weinrauch *et al.*, 1989 and 1990). The protein comprises a membrane-localized sensor domain at the N-terminus domain and a conserved C-terminal cytoplasmic transmitter domain (Weinrauch *et al.*, 1990).

ComA is the only product of the *comQXPA* cluster showing a conserved sequence which displays its role as a response/transcription regulator not involved in the determination of specificity of the QS system (Tortosa *et al*., 2001). ComA comprises two domains: the response regulator containing an invariable aspartate residue at position 55 which is the site of phosphorylation by ComP at the N-terminus (Guillen *et al*., 1989, Weinrauch *et al*., 1989), and the C-terminal effector region which demonstrates high homology with the DNA-binding domain of several transcription factors (Roggiani and Dubnau, 1993). The effector domain sequence alignment and the secondary structure prediction have underlined that ComA is a member of the NarL family of response regulator proteins.

Investigation of ComA interaction with the *srfA* operon led to the identification of two DNA regions essential for the positive control of *srfA* transcription (Roggiani and Dubnau, 1993). Although the *srfA* operon is the best characterised binding target of ComA (Hahn and Dubnau, 1991; Nakano and Zuber, 1993; Roggiani and Dubnau, 1993),

this regulatory protein has been revealed to contribute to the direct control of the several other operons. Microarray analysis has distinguished almost 89 genes, in 35 operons, not involved in competence development, to be affected by ComA (Comella and Grossman, 2005). Examples of these genes are: *degQ*, encoding a regulator protein involved in degradative enzyme production (Msadek *et al.*, 1991); *rapA* (Mueller *et al.*, 1992), *rapC* (Solomon *et al.*, 1996), *rapE* (Jiang *et al.*, 2000), and *rapF* (Jarmer *et al.*, 2001) each encoding a regulatory protein of the Rap family. Expression of numerous genes which is controlled by ComA, indicates the affinity of this regulator protein for its DNA binding site. This might represent the most important feature for the coordination of transcription regulated by population density (Griffith and Grossman, 2008). At low cell-density, ComA is mainly at an inactive non-phosphorylated state, and a small concentration of ComA is activated by phosphorylation. In this scenario, the active ComA is only able to bind to high affinity sites, such as the regulatory region of *rapA*, thus certainly moderating the transcription of target genes at low population. On the other hand, at high cell densities, phosphorylated ComA accumulates in the cytoplasm and its concentration allows binding to low affinity degenerate sites, such as those present in *srfA* (Griffith and Grossman, 2008).

1.3.1.3 The competence and sporulation factor (CFS)

The competence and sporulation factor (CSF) is the second signalling peptide concerned with the cell-density dependent control of competence development in *B. subtilis*. It was originally characterised as a peptide (609 Da) following the discovery of ComX (Solomon *et al.*, 1995). CSF is a 5-amino-acid peptide (ERGMT) encoded by the last 5 codons of a 40 codons open reading frame, named phrC (Carter *et al.*, 1990). The product of *phrC* from *B. subtilis* has been characterised as a 40-amino-acid polypeptide (Lazazzera, 2001).

Whilst the ComX pheromone requires the histidine-kinase ComP for its recognition and signalling, CSF sensing needs the Opp encoded by *spoOK*. SpoOK is a member of the ATP-binding cassette (ABC) carrier family that utilize ATP hydrolysis for the import and

export of several compounds, including oligopeptide transport through *B. subtilis* cell membrane (Rudner *et al.*, 1991, Perego *et al.*, 1991, Higgins, 1992).

CSF was recognised as an auxiliary competence pheromone to be a modulator of the timing and levels of competence (Solomon *et al.*, 1996). To investigate whether various CSF molecules might be produced by diverse *Bacillus* strains, *rapC-phrC* operons from six strains (which have different *comQXP* sequences) have been analysed (Tortosa *et al.*, 2001, Pottathil *et al.*, 2008). All the strains were identified with a conserved sequence of a mature CSF peptide. Additionally, their corresponding PhrC polypeptide showed a similar functional signal and peptidase cleavage sites (Becker *et al.*, 2004), although amino acid sequences in RapC and PhrC from different strains varied. But as most of the amino acid substitutions are conserved, they cannot alter the function of these proteins (Pottathil *et al.*, 2008). These findings suggest that *Bacillus* strains which produce the different ComX pherotypes are characterised by identical CSF peptides, indicative of CSF ability to mediate communication between strains that cannot communicate via ComX. It has been shown that the competence and sporulation factor is neither strain specific nor species specific, as CSF homologues have been identified in *Bacillus mojavensis* (*B. mojavensis*), a species closely related to *B. subtilis* (Pottathil *et al.*, 2008).

At least three roles have been attributed to the pentapeptide CSF: stimulation of expression of genes activated by the phosphorylated ComA (at low concentrations), inhibition of those same genes after reaching a threshold concentration, and activation of sporulation under specific conditions (Solomon *et al.*, 1996; Lazazzera *et al.*, 1997). The contribution of CSF in the activation of sporulation, requires the inhibition of a phosphatase, RapB, a negative regulator of the phosphorelay required for the initiation of sporulation (Perego, 1997).

Since the two extracellular competence signals in *B. subtilis* are involved in divergent processes, with the ComX pheromone stimulating a kinase and CSF inhibiting a phosphatase, it is not unexpected that CSF is considered an auxiliary competence pheromone, whereas the ComX pheromone is known as the major competence pheromone (Lazazzera *et al.*, 1999a).

1.4 QS in *Bacillus licheniformis*

B. licheniformis strains belong to the genus *Bacillus*, as rod-shaped Gram-positive bacteria including both pathogenic and non-pathogenic strains. Under conditions of stress, the cells form endospores to survive long periods of time in a dormant state. *Bacillus* species are typically motile because of their flagella. *B. licheniformis* is predominantly found in soil as endospores, and is a facultative anaerobe (Claus and Berkeley, 1986). Since 1972, different *B. licheniformis* strains have been used in the fermentation industry for production of proteases, amylases and specialty chemicals (Schallmey *et al.*, 2004). The industrial relevance of *B. licheniformis* is also related to the production of antimicrobial compounds such as bacitracin (Johnson *et al.*, 1945), and the bio-surfactant, the surfactin-resembling lichenysin (Yakimov *et al.*, 1995). *B. licheniformis* is genetically related to *B. subtilis,* whose modulation of competence and sporulation is under the control of a QS mechanism.

1.4.1. Identification of competence-mediated genes in *B. licheniformis*

Competence and QS cognate genes in *B. licheniformis* was characterised by preliminaryly establishing genetically co-linear regions with *B. subtilis* genome using physical mapping. Co-linear scaffolding technique allowed Lapidus and co-workers (Lapidus *et al.*, 2002) to compare co-linear regions between *B. licheniformis* ATCC 14580 genome, whose sequence had not been published yet at the time, and the entirely sequenced genome of *B. subtilis* 168. Since these two genomes are 70% identical at the nucleotide level this investigation helped to characterization of more than 60% of all the genes of *B. licheniformis*, comprising relevant genes which show significant homology with competence-modulating genes of *B. subtilis*, such as the *comQXPA* cluster, *clpC*, *comK*, *comC*, and the entire *comE*, *comF* and *comG* operons.

The gene encoding ComS, the small protein responsible for ComK activation, was not identified in *B. licheniformis*. In the *B. subtilis* genome, *comS* is located within the *srfA* operon, encoding surfactin biosynthetic machinery. This operon consists of three long ORFs, which were not detected in any co-linear region (Lapidus *et al.*, 2002).

Recently, the identification of a putative ComS protein in *B. licheniformis* was identified by investigating of conserved ComS peptides in different *Bacillus* species (Hoffmann *et al.*, 2010). The putative ComS of *B. licheniformis* varies from its counterparts of *B. subtilis* and *B. amyloliquefaciens,* by an N-terminal extension of 16 amino acids, a four amino acids insertion at position 27, and a different core sequence for MecA binding (Hoffmann *et al.*, 2010).

1.4.2 The *comQXPA* cluster of *B. licheniformis*

The *comQXPA* gene cluster function as an essential genomic region in the regulation of competence development in *B. subtilis*. This key gene cluster also identified in *B. licheniformis* genome which is only present in *Bacillus* strictly related to *B. subtilis* (Nakano and Zuber, 1991, Magnuson *et al.*, 1994). According to the present genomic data, in the phylogenetic trees, *B. licheniformis* is located far from *B. subtilis* 168, hence it is not surprising that the ComQ and ComX proteins of *B. licheniformis* ATCC 14580 represent higher similarity to their counterparts in *B. mojavensis*, a closer related strain, than to those in *B. subtilis* 168 (Lapidus *et al.*, 2002). The response regulator ComA, which regulates the expression of the surfactin biosynthetic gene in *B. subtilis*, was shown to play a similar role in *B. licheniformis* by inducing transcription of the lichenysin synthase genes. This regulatory function was proved by the identification of a putative ComA Box in the promoter region of the lichenysin operon (Yakimov and Golyshin, 1997, Yakimov *et al.*, 1998). Figure 1.9 presents the schematic model to show a comparison between *B. subtilis* and *B. licheniformis* QS systems.

The gene encoding the histidine kinase ComP was characterised to have a 1288-bp insertion sequence in *B. licheniformis* genome. This region dedicated to the recognition of the ComX pheromone and the phosphorylation of ComA in *B. subtilis*. The insertion sequence, named as IS3Bli1, encodes a 278-amino-acid protein belonging to the IS3 family of transposases of IS elements (Nagai *et al.*, 2000).

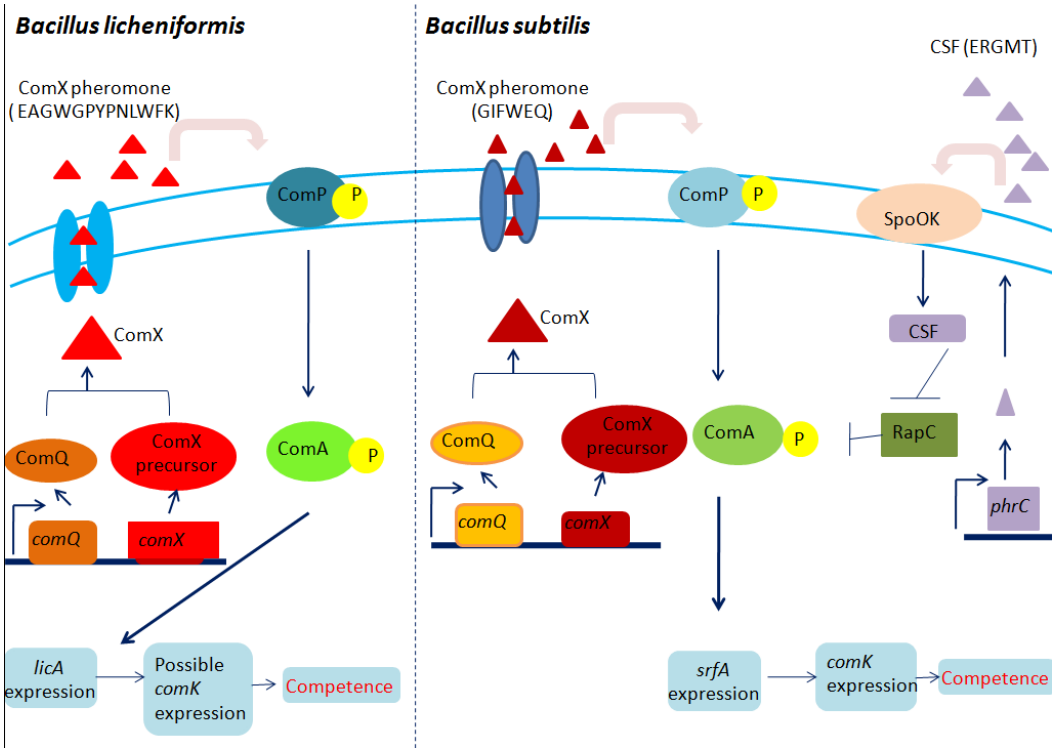


Figure 1.9 A schematic model to compare *B. subtilis* and *B. licheniformis* regulation of competence through QS signalling peptides. In *B. subtilis*, two molecules, ComX and CSF, could activate the transcription of *srfA* operon by stimulating the transcription factor ComA through phosphorylation via two separate pathways. Whearse *B. licheniformis* developes possible QS regulatory system by producing one molecule, the ComX pheromone, which has different sequence from *B. subtilis*'s pheromone (please refer to the section 3.3.2.2). In both bacteria, the ComX is processed and secreted by ComQ. The histidine kinase sensor protein (ComP) interacts with ComX pheromone leading to phosphorylation of ComA response regulator. CSF encoded by *phrC*, in *B. subtilis*, is re-internalised via two intracellular targets generated through processing by *spoOK* gene. At low concentrations, CSF inhibits RapC phosphatase leading to increased accumulation of ComA-p which induces competence. *licA* gene encodes lichenysin in *B. licheniformis* comparing to *srfA* gene in *B. subtilis* which encodes surfactin (*B. subtilis* related information is adapted from Guan *et al*., 2015).

Apart from competence development the *comQXPA* system of *B. subtilis* is contributed to the regulation of several cell density-dependent phenomena, such as surfactin, poly-glutamic acid and degradative enzymes production and surface attachment (Weinrauch *et al.*, 1990; Nakano and Zuber, 1991; Magnuson *et al.*, 1994). The disruption of the sensor kinase of the system by the insertion of IS elements leads to impairment of all the processes by interrupting communication between cells (Nagai *et al.*, 2000). These findings suggest a strong competition among *Bacillus* for the benefits arising from

communication between closely related neighbours. Recently, the investigation on *B. licheniformis* strains DSM13 (Veith *et al.*, 2004) and the isogenic ATCC 14580 (Rey *et al.*, 2004) compared two genomes showing that they only differ in the orientation of the IS element, possibly suggesting a flexible position within the genome (Hoffmann *et al.*, 2010).

Although QS is well established in *B. subtilis* and in spite of the above studies, no precise report has been published on cell-cell communication signalling pathways in *B. licheniformis* and the production of signalling molecules in this organism has not been verified so far. However, according to De Vizio (2011), *B. licheniformis* NCIMB 8874 cell-cell communication through *comQXPA* cluster operates in analogy with the well established *comQXPA*-controlled pathway of *B. subtilis*.

1.5 QS in fungi

The ubiquity of QS networks in bacteria has led scientists to suggest that these molecules and systems were also widespread in other single-celled eukaryotes, such as fungi (e.g. yeasts) (Hogan, 2006). Evidences of density-dependent regulation systems for cell-cell communication in fungi have begun to emerge since the last decade. However, research has been carried out particularly in pathogenic fungi, as QS pathways are considered a potential therapeutic target.

1.5.1 QS in dimorphic fungi

Dimorphic fungi (e.g. *C. albicans and Histoplasma capsulatum*), like bacteria, possess certain characteristics such as biofilm formation and pathogenesis which are modulated in a density-dependant process. The QSMs in fungi are small alcohols with different structures from those in bacteria such as AHL and modified peptides. These small alcohols regulate dimorphism and biofilm formation in response to the external environment. The first fungal QSMs were identified in *C. albicans* (Hornby *et al*., 2001; Chen *et al*., 2004). *C. albicans* is a dimorphic fungal human pathogen and its ability to

switch between the yeast and the filamentous form depends on cell-density, in a phenomenon known as inoculum size effect.

C. albicans produces the sesquiterpene farnesol (Hornby *et al*., 2001) and the aromatic alcohol tyrosol (Chen *et al*., 2004) while another yeast, *S. cerevisiae*, produces the aromatic alcohols phenylethanol and tryptophol (Chen and Fink, 2006). The latter three molecules are derived from the aromatic amino acids; tyrosine, phenylalanine and tryptophan, respectively.

Upon reaching a critical threshold concentration of QSMs a change in the cell physiology is observed. Dimorphic fungal secretion and recognition system of QSMs as well as their structures are similar to that used by Gram-positive bacteria. In both microorganisms, the QSMs are secreted to the extracellular milieu and then recognised by receptors which transport the signal across the cell membrane to initiate the target gene transcription (Waters and Bassler, 2005). QSMs produced by dimorphic fungi, as mentioned above, are farnesol, tyrosol, phenylethanol and tryptophol.

The sesquiterpene farnesol was identified from a filtrated supernatant of the stationary phase culture of *C. albicans*. It prevented the change from the yeast form to the mycelial form at high cell densities by inhibiting the *Candida* cAMP/PKA pathway (Hornby *et al*., 2001; Hall *et al*., 2011; Lu *et al*., 2014). Besides, further research revealed that farnesol was capable of inhibiting biofilm formation by *C. albicans* through sterol biosynthetic pathway (Ramage *et al*., 2002; Yu *et al*., 2012) and that it was involved in oxidative stress resistance with similar mechanism as used for inhibition of yeast-hyphal transition (Westwater *et al*., 2005; Deveau *et al*., 2010) (Figure 1.10).

Farnesol does not affect the growth-rate of *C. albicans*. However, it does exert a potent growth inhibitory influence on several other fungi, including *A. nidulans* (Semighini *et al*., 2006) and *S. cerevisiae* (Machida *et al*., 1999). Likewise, farnesol has antibacterial activity against two bacterial species, *Propionibacterium acnes* (Kubo *et al*., 1994) and *Haloferax volcanii* (Tachibana *et al*., 1996). Taking into account the above findings, it has been shown that farnesol, besides its QS functions, is also employed by *C. albicans* to reduce competition from other microbes.

A second QSM produced by *C. albicans* is tyrosol. Tyrosol increased the effects of farnesol at high cell-density which inhibits filamentation in fungal cultures and biofilm formation. The evidence that tyrosol, even at 16-fold molar excess, would not alter the activity of farnesol indicates the dominant effect of farnesol in *C. albicans* (Alem *et al*., 2006).

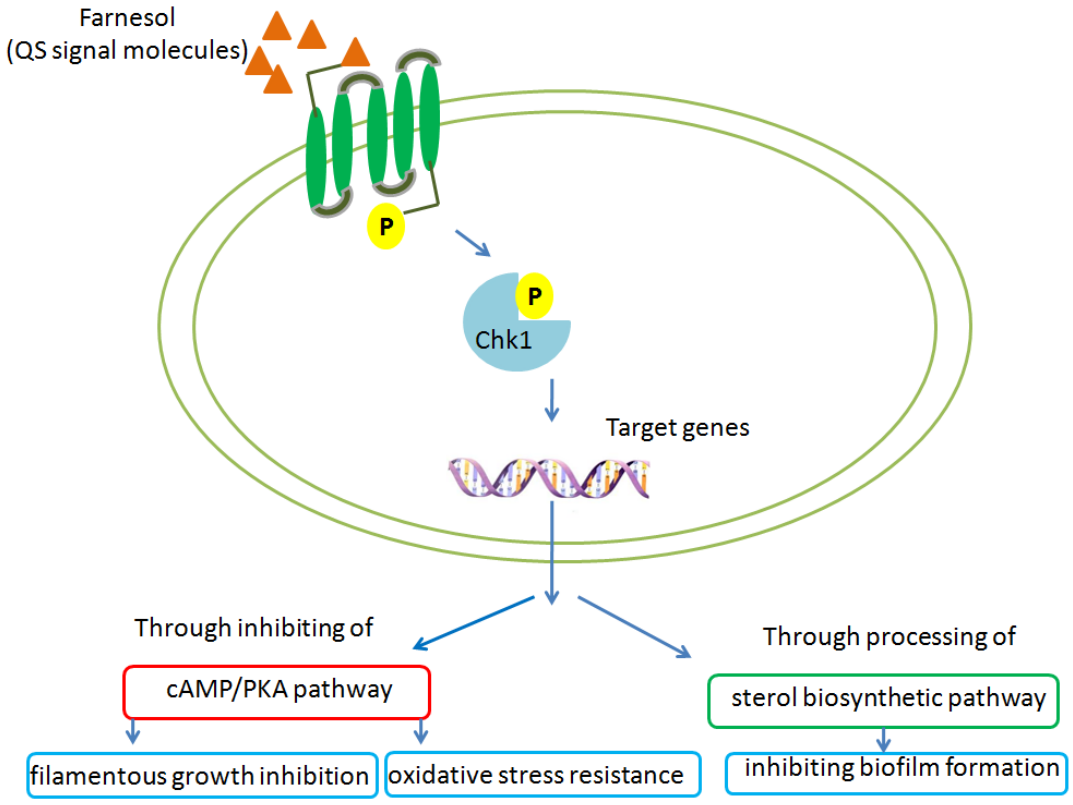


Figure 1.10 QS in *C. albicans*. Farnesol as a fungal QSM with regulatory properties is excreted continuously by *C. albicans.* When it accumulates beyond a threshold level, it is recognised by a two-component system which includes the histidine kinase Chk1. The signal affects the target genes, thus blocking the conversion from yeast to mycelium morphology, formation of biofilms, and enhancing the resistance to oxidative stress.

In the budding yeast *S. cerevisiae*, two QSMs, phenylethanol and tryptophol, have been identified. Chen and Fink (2006) showed that these QSMs were capable of pseudohyphal growth stimulation in *S. cerevisiae* cultures. Pseudohyphal growth is the elongation of the cells thereby they switch to a unipolar budding pattern and remain physically attached to each other (Chen and Fink, 2006).

1.5.2 QS in filamentous fungi

The best known cell-cell communication in filamentous fungi is the secretion and recognition of mating pheromones. In the presence of mating pheromones, two haploid cells fuse to become diploid (Bolker and Kahmann, 1993). This mechanism prevents the formation of higher ploidy cells which would disrupt the normal life cycle. Mating pheromones of several fungal families have been characterised such as ascomycetes, basidiomycetes and zygomycetes. For instance, in the basidiomycete fungus *Ustilago maydis* (*U. maydis*), the mating pheromones regulate filamentous growth and pathogenesis (Spellig *et al*., 1994). It is interesting to note that the secretion and recognition of the fungal peptide pheromones, as well as their structure, are reminiscent of the QS systems used by Gram-positive bacteria. In fact, in both systems, the signals are secreted to the extracellular environment, and are subsequently recognised by dedicated receptors that transfer the signal to the cell. The receptors are G protein-coupled molecules in *U. maydis* while Gram-positive bacteria employ a two-component sensor kinase (Bakkeren *et al*., 2008).

Moreover, few more examples of QS-like molecules have been reported which highlight the fact that QS is ubiquitous among fungi as it is among bacteria. However, in all cases, very little is known about the pathways, receptors or signal transduction mechanisms.

A QS-like behaviour in filamentous fungi was first reported in *A. terreus* (Schimmel *et al*., 1998). A wide range of secondary metabolites, including the γ-butyrolactone containing compound butyrolactone I have been produced by this species (Rao *et al*., 2000). Butyrolactone I was capable of inducing morphological changes when exogenously added to *A. terreus* cultures. These morphological alterations include increasing in hyphal branching and submerged spore formation as well as production of other secondary metabolites such as lovastatin (cholesterol-lowering drug) and suchlorin (Schimmel *et al*., 1998).

A novel role for butyrolactone I as a QSM in *A. terreus* has been proposed based on its ability to affect lovastatin production. The exogenous addition of butyrolactone I to the cultures of *A. terreus* resulted in 2.5-fold increase in lovastatin production as compared

to the control cultures (Raina *et al*., 2012). Moreover, presence of γ-heptalactone as a QSM has been reported in *A. nidulans*. The addition of the stationary phase extract of *A. nidulans* culture supernatant to a low-density cell culture of *A. nidulans* resulted in a 37.80 % increase in penicillin production (Williams *et al*., 2012).

Filamentous fungi such as *A. nidulans*, *A. flavus* and *Aspergillus niger* secrete lipid molecules named oxylipins, with putative signalling properties (Tsitsigiannis *et al*., 2005; Brown *et al*., 2008; Wadman *et al*., 2009). Oxylipins comprise a large group of oxygenated C18, C20 and C22 lipids derived from polyunsaturated fatty acids (Noverr *et al*., 2002). These molecules are employed as signals of intra- and inter-species communication in both prokaryotic and eukaryotic microbes (Mosblech *et al*., 2009). Oxylipins are heterogeneous in type and function in different organisms, and their biosynthesis is normally a consequence of abiotic and biotic stresses. Oxylipins have a common role in signalling processes in both prokaryotic and eukaryotic cells, being mainly involved in cellular development and stress response (Andreou *et al*., 2009). For instance, the regulation of sexual (cleistothecium) and asexual (conidium) development in *A. nidulans* is dependent on the ratio of different oxylipins (Tsitsigiannis *et al*., 2005). Similarly, the ratio of oxylipins exert the influence on the alteration of sclerotial and conidial production in *A. flavus* (Calvo *et al*., 1999). In addition, the impact of linoleic acid as an oxylipin on lovastatin production has revealed the involvement of oxylipins as QSMs in *A. terreus*, and their potential use as enhancers of fungal secondary metabolite production (Sorrentino *et al*., 2010).

Since microbial QS is responsible for the cell density-dependent regulation of genes through cell-cell interactions and formation of multicellular structures then these reports suggest the potential role of oxylipins as QSMs in filamentous fungi (Erb-Downward and Huffnagle, 2006; Tsitsigiannis and Keller, 2007).

Overall, it has been shown that QS in fungi is a prevalent and unique network system which is employed to modulate response to their cell-density and their environment (Chen *et al*., 2004; Chen and Fink, 2006; Sorrentino *et al*., 2010; Raina *et al*., 2012; Williams *et al*., 2012). However, this area of research is still in its infancy, with a great

deal of investigation to elucidate the signalling pathways as well as the chemical identification of putative QSMs in fungal cells.

1.6 The genus *Aspergillus*

Aspergillus species are Ascomycetous fungi belonging to the subkingdom of Dikarya which contains the phyla Ascomycota and Basidiomycota (Spatafora and Robbertse, 2010). Ascomycota are reported as the largest phylum of fungi with more than 63,000 species (Kirk *et al*., 2008). Among three subphyla, Taphrinomycotina, Saccharomycotina, and Pezizomycotina, the latter is the one named as filamentous fungi in fungal biology which includes more than 60,000 species. This group contains some well-studied organisms such as *Neurospora*, *Penicilium* and *Aspergillus* (Spatafora and Robbertse, 2010). *Aspergillus* is a filamentous fungus with tubular and elongated structures called hyphae. Most cellular activities are through hyphal growth. Hyphal cytoplasm contains most of the organelles found in other heterotrophic eukaryotic organisms, such as nuclei, mitochondria and vacuoles (Bennett *et al*., 2010; Roberson *et al*., 2010).

Aspergillus is a genus of high industrial, agricultural and medicinal importance. This genus represents a large family of fungi, with over 185 recognised species (Samson, 1991). Most species are saprophytes that grow on a large number of substrates from plants to animal waste and thus they are very important in nutrient cycling and detoxification (Geiser, 2009). The Aspergilli can also cause decay and deterioration of stored products as well as causing disease in plants (e.g. ear rot in maize by *A. flavus*), insects (e.g. infection in living *Galleria mellonella* by *A. flavus*), poultry, humans and other mammals (e.g. Aspergillosis by *Aspergillus fumigatus*) (Kamei and Watanabe, 2005).

1.6.1 *Aspergillus* secondary metabolites and QS

Pathogenic and saprophytic fungi could lead to significant damage to agricultural crops. Among these microorganisms, *Aspergillus* species such as *A. flavus* occur as a

saprophyte in the soil worldwide. This species causes pre-harvest and post-harvest diseases on several agricultural seed crops. The fungus also causes animal and human diseases either through consumption of contaminated feed (causing aflatoxicosis and/or liver cancer) or through invasive growth (causing aspergillosis). Secondary metabolites from fungi consist of small bioactive molecules harmful (e.g., aflatoxins) or beneficial (e.g., lovastatin) to human. This fungal pathogen can produce the polyketide-derived carcinogenic and mutagenic secondary metabolite, aflatoxin as well as other secondary metabolites including cyclopiazonic acid (CPA), aflatrem and penicillin. Yield loss in agricultural crops due to aflatoxin contamination has been reported in million-dollars in the United States (Georgianna *et al*., 2010; Amaike and Keller, 2011).

Recently, evidences for density dependant cell-cell communication of fungi have begun to emerge (Hogan, 2006). Among filamentous fungi, *Aspergillus* utilizes quorum regulation to affect morphogenesis and secondary metabolite production (Brown *et al*., 2009). Several studies report the effect of certain types of QSMs secreted from one group of fungi on the growth, morphology, sporulation (conidiation), apoptosis and metabolite production of other fungi (Brodhun and feussner, 2011). A QS-like behaviour in a filamentous fungus was first reported in *A. terreus* (Schimmel *et al*., 1998). This fungus produces a wide range of secondary metabolites, including the γ-butyrolactone. By adding γ-butyrolactone to *A. terreus* cultures, morphological changes such as increasing in hyphal branching, spore formation and production of lovastatin as a secondary metabolite were induced (Schimmel *et al*., 1998; Rao *et al*., 2000). Linoleic acid-derived oxylipins (large family of oxidized fatty acids) have been reported to be engaged in the QS responses in *Aspergillus* cultures. The recent investigation on the impact of linoleic acid on lovastatin production in *A. terreus* revealed that production of lovastatin was enhanced up to 1.8-fold (Sorrentino *et al*., 2010). Furthermore, in *A. nidulans*, oxylipins are involved in the production of asexual and sexual spores, as well as penicillin (Tsitsigiannis *et al*., 2005). The QSM, γ-heptalactone, in *A. nidulans* resulted in 37.80 % increase in penicillin production (Williams *et al*., 2012).

Moreover, in *A. flavus* the switch from conidium to sclerotium and the production of aflatoxin are cell density-dependent and related to oxylipins (Brown *et al*., 2008 and

2009). From recent researches, it seems that QS is an established mechanism employed by fungi to modulate response to each other and their environment by using unique systems compared to the bacterial QS (Sorrentino, 2009). Curiously, instances of small molecule exchange between bacteria and eukaryotes have also been reported (Mullard, 2009). These molecular exchanges between two kingdoms hint that the inter-kingdom cross-talk through QS phenomenon might be developed (refer to section 1.7). Having considered the above, the microbial communication is a vital process which could be involved in the bio-control solution (refer to the section 1.8).

1.6.2 Aflatoxin and its gene cluster

Aspergillus species produce aflatoxins, citrinin and patulin. *A. flavus* and *A. parasiticus* are the predominant species responsible for aflatoxin contamination of crops prior to harvest or during storage. The word "aflatoxin" comes from "*Aspergillus flavus* toxin" (Miller, 1995; Yu *et al*., 2004; Gokmen *et al*., 2005). Aflatoxins are considered as the most important mycotoxins because of their occurrence, toxicological effects and impact on human well-being and crop trade (Gnonlonfin *et al*., 2013).

Aflatoxins found in food are classified as B1, B2, G1, and G2. 'B' and 'G' refer to the blue and green fluorescent colours produced by these toxins under UV light during the thin layer chromatography plate visualization; the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Because of their high toxicity, the USA Food and Drug administration (FDA) regulates the amount of allowable aflatoxin contamination as 20 ppb in crops or 0.5 ppb in milk for humans. More than 100 countries have similar aflatoxin regulatory laws, but allowable threshold levels vary (Pitt, 2000; Lanyasunya *et al*., 2005; Amaike and keller, 2011).

Since the discovery of aflatoxins, *A. flavus* has become the most widely reported food-borne microorganism. This reflects its importance in health care and economy (Pitt, 2000). *A. flavus* is an ubiquitous and morphologically complex species, and is geographically distributed worldwide (Tran-Dinh *et al*., 1999).

Aflatoxins are polyketide synthases (PKSs)-derived mycotoxins, synthesized from a large cluster near one telomere of chromosome 3 of *A. flavus* (Figure 1.11) (Amaike and keller, 2009). Mapping of *A. parasiticus* and *A. flavus* genomic DNA has established that the genes in the aflatoxin biosynthetic pathway are clustered. In general, the aflatoxin gene cluster in *A. parasiticus* and *A. flavus* consists of 25 genes, approximately 70 kbp. This cluster is of critical importance since its genetics has been a model for better understanding of fungal secondary metabolism. So it has been subject to many reviews (Georgianna and Payne, 2009). The aflatoxin cluster is conserved to varying degrees in several fungi including *Aspergillus* species such as *A. parasiticus*, *A. ochraceoroseus*, *A. flavus* var. *parvisclerotigenus*, *A. toxicarius*, *A. nomius*, *A. pseudotamarii*, *A. zhaoqingensis*, *A. bombycis*, *A. rambellii*, *A. nidulans*, *A. oryzae*, *A. sojae* and related fungi such as *Emericella venezuelensis*, *Dothistroma* sp. and *Emericella astellata*. However, only some *Aspergillus* species produce aflatoxin (Amaike and Keller, 2011).

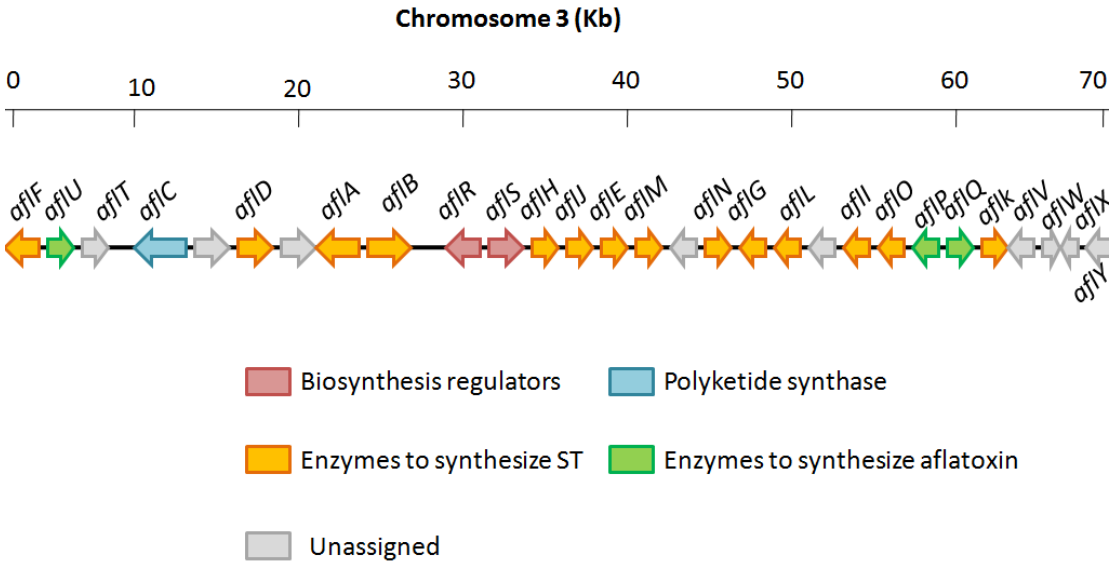


Figure 1.11 Aflatoxin cluster in *A. flavus*. The aflatoxin cluster is located near the telomere of chromosome 3 and composed of approximately 30 different genes. *aflA*, *aflB* (fatty acid synthase), and *aflC* (polyketide synthase) are required to synthesize the first stable aflatoxin precursor, norsolorinic acid (NOR). NOR is synthesized to sterigmatocystin (yellow genes) and then to aflatoxin (green genes). Gray genes have not been assigned a function (adopted from Amaike and keller, 2011).

The specific pathway regulatory gene, *aflR*, is located in the cluster. A Zn(II)2Cys6 type transcriptional factor encoded by *aflR* regulates expression of the aflatoxin/sterigmatocystin biosynthetic genes. Over-expressed aflR results in increasing aflatoxin production and up-regulates other biosynthetic genes in the aflatoxin biosynthesis pathway. While, in *aflR* deletants, aflatoxin/sterigmatocystin biosynthetic genes, and their products, are not expressed. AflR is able to bind the consensus motif 5′-TCGN5CGR-3′ found in the promoter regions of many aflatoxin and sterigmatocystin genes (Fernandes *et al*., 1998). A second binding site 5′-TTAGGCCTAA is reported as important in autoregulation of *aflR* transcript in *A. flavus* and *A. parasiticus* (Chang *et al*., 1995). Expressed divergently from *aflR* is *aflS* (formerly termed *aflJ*), whose product also regulates aflatoxin production through binding and activating AflR in *A. parasiticus* and *A. flavus* and presumably other Aspergilli (Du *et al*., 2007). Progress in understanding of the biology of *Aspergillus* has greatly improved with the new techniques in genome sequencing and the developed molecular tools that enable rapid genetic analysis of individual genes within the genome. Particularly, the genetics of aflatoxin synthesis is regarded as a model for better understanding of fungal secondary metabolism through its role in the identification of the secondary metabolite clusters in chromatin regulation of such clusters through histone modifications (Amaike and Keller, 2009).

1.7 Inter-kingdom communication between bacteria and fungi through QS

The co-evolution of prokaryotes and eukaryotes raises the prospect of the existence of inter-kingdom signalling pathways; promoting parasitic and symbiotic relationships. While the members of each biological kingdom possess hormone-like molecules responsible for cell-cell communication, the members of any given kingdom also respond to the signals produced by the members of another kingdom (Fox, 2004; Rumbaugh, 2007). The signal molecules which are derived from bacteria may not be limited to signalling between bacterial species alone but also modify the behaviour of eukaryotic organisms. This is not a one-way interaction since eukaryotes may also control bacterial signalling networks and therefore moderate bacterial behaviour.

Bacterial signalling may also be interrupted following enzymatic degradation of the signal molecules (for example, plant defence signal such as salicylic acid up-regulates the expression of an AHL-degrading enzyme on *A. tumefaciens*) or by the effect of agonist/antagonist molecules (such as AHL-antagonists produced by plants and fungi) that block QS (Bauer and Mathesius, 2004; Dudler and Eberl, 2006; Williams, 2007).

QS is a process for intra- and/or interspecies communication of microbes based on the cell-density (Lu *et al*., 2014). In terms of inter-kingdom cross-talk, QS plays a major role. For instance, one of the bacterial QSMs, AHL, not only directs bacterial compound synthesis, but it is also recognized by eukaryotic cells such as animal cells, plants, seaweed and fungi (Dudler and Eberl, 2006). On the other hand, several reports indicate that fungi interfere with bacterial QS by producing AHL antagonists (Rasmussen *et al*., 2005).

Besides, fungi affect bacterial QSMs through their QS systems. As an example, the addition of farnesol as a fungal QSM from *C. albicans* to the cultures of *P. aeruginosa*, led to decrease the production of *Pseudomonas* QSM (*Pseudomonas* quinolone signal (PQS)). This reduction in the QSM production occurred through farnesol inhibition of PQS-stimulated transcription. It shows that farnesol and the related compounds can participate in interspecies interactions (Cugini *et al*., 2007). Moreover, Lee and colleagues (Lee *et al*., 2015) have recently reported that farnesol, as a fungal QSM, is a novel blocker of signalling pathway (e.g. STAT3) in mice and exerts both anti-proliferation and apoptotic activities to inhibit tumour growth.

When the QS compounds of the filamentous fungi (e.g. *A. terreus*, *A. flavus* and *A. nidulans*) were exogenously added to their own fungal cultures, they effected morphological changes in the microbes as well as their secondary metabolite production such as lovastatin, penicillin and aflatoxin (Schimmel *et al*., 1998, Brown *et al*., 2008 and 2009; Williams *et al*., 2012). From the recent researches, it seems that QS is an established process used by fungi to modulate mutual responses to each other and their environment (Sorrentino, 2009).

Overall, fungi coexist with bacteria in the environment, and bio-chemical exchanges between them are the method of communication. Instances of small molecule exchange between bacteria and eukaryotes and the interspecies microbial communication through QS are the strong evidences of inter-kingdom communication particularly via QS systems (Mullard, 2009; Lu *et al*., 2014).

1.8 *Aspergillus* bio-control and future directions

Aflatoxin contamination of crops remains a critical problem worldwide with additional health threats in increasing numbers of *A. flavus*–induced aspergillosis. Pre-harvest control of *A. flavus* has traditionally depended on selecting resistant crop-lines to help protection of crops under unfavourable environmental conditions (e.g. drought) which favour aflatoxin contamination (Campbell and White, 1995). In addition, irrigation is a key to avoid drought stress (Payne, 1998). Efforts have also focused on identifying plant proteins that are important for defence against *A. flavus* invasion, including resistance-associated proteins (e.g. pathogenesis-related protein 10 (PR 10)) (Chen *et al*., 2010). Likewise, effort has been focused on specific molecules such as oxylipins which are involved in signalling pathways across different kingdoms such as fungi, plants and animals. These molecules could regulate sclerotia and conidia production and secondary metabolism such as aflatoxin in *A. flavus*. They may play important role in *Aspergillus* – host interaction which could apply to control aflatoxin production as a chemical agent or its relevant gene to make a resistant transplant. Further identification of the QSMs and their relevant genes that are responsible for interactions between bacterial cells, unicellular and mycelial fungal cells or inter-kingdom communications (bacteria and fungi /plant and fungi) would be of critical importance in future control strategies. Moreover, the understanding of the biology of this fungus has progressed with the advent of the genome sequence and improved molecular tools allowing rapid genetic analysis of individual genes within the genome as well as specific regulators. Other available control methods, such as optimal cultural practices (e.g. date of planting and harvesting, choosing the cultivar and a suitable region for planting) have diminished, but not eliminated, pre-harvest aflatoxin contamination in crops. Furthermore, in recent

years public concern over pesticide residues in the environment, food and feed, has led to a limitation and reduction of some chemical fungicides commonly used to control plant pathogens and post-harvest plant diseases. Consequently, alternative methods for controlling these pathogens and diseases are needed. Biological control or use of microbial fungicides is an alternative strategy to the application of chemical fungicides. Identification of new antifungal peptide molecules against *A. flavus* may lead to the development of novel applied biotechnological strategies that could facilitate control of aflatoxin contamination. In addition, genetic engineering of plant resistance to fungal invasion through the use of genes related to the bacterial antifungal peptide molecules will prove useful. During the screening of bacteria for antagonistic activity against *A. flavus in vitro*, a *B. subtilis* isolate, AU195, was identified as having the highest antifungal activity. The potential of *Bacillus* spp. to synthesize a wide variety of metabolites with antibacterial and antifungal properties has been intensively exploited in medicine and industry (Leifert *et al*., 1995; Munimbazi and Bullerman, 1998; Moyne *et al*., 2001). In most cases, although these strains were highly effective against the fungal growth under laboratory conditions, they do not show high efficacy in the fields as it is not feasible to bring the bacterial cells to the *Aspergillus* infection sites under field conditions (Dorner, 2004).

In the current study, identification of new antifungal, QS peptide molecules from antagonistic bacteria like *Bacillus* (*B. licheniformis* NCIMB 8874), against *A. flavus* was investigated. This ongoing research could lead to the development of new biotechnological strategies. These strategies would facilitate aflatoxin contamination control as well as genetic engineering of plant resistance to fungal invasion through the use of genes related to the bacterial antifungal peptide molecules. The novel knowledge will contribute to the development of inhibitors of aflatoxin, design of the bio-competitive *Aspergillus* strains, application of bio-control bacterial strains and improvement in host- resistance against fungal invasion or toxin production.

1.9 Aim and objectives

While QS has been explored broadly to investigate a range of activities in both Gram-positive and Gram-negative bacteria, little has been reported on QS in the industrially important bacterium *B. licheniformis*. Besides, no research has been carried out so far to explore the role of QS in inter-kingdom communication. Since antagonistic bacteria have the ability to inhibit fungal growth and production of secondary metabolites, it is of interest to investigate the potential inhibitory activity of *B. licheniformis* QSM.

In this context, the overall aim of this study is to identify *B. licheniformis* QSM and investigate the effect of this antagonistic bacterium and its QSM on the growth of a filamentous fungi such as *A. flavus*.

In order to achieve this aim, the following objectives are explored:

- To investigate the putative QS genes (*comQX*) of *B. licheniformis* NCIMB 8874 and over-express *comQX* genes using gene cloning techniques.
- To purify the ComX pheromone of *B. licheniformis* NCIMB 8874 using biochemical techniques on a recombinant *E. coli* culture, constructed for over-production of the pheromone in the supernatant.
- To investigate the effect of *B. licheniformis* cells, and their QSM on growth of *A. flavus.*
- To implement the whole genome sequencing of *B. licheniformis* NCIMB 8874 using next generation sequencing platform to determine genomic variance to reference genomes in the *comQXPA* region.

CHAPTER II

MATERIALS AND METHODS

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2.1 Chemicals and reagents

Most of the chemicals used in this study were obtained from Sigma Aldrich Company (Dorset, UK) and Fisher Scientific (Loughborough, UK). High Pressure Liquid Chromatography (HPLC) grade reagents and water were used to analyse samples for HPLC. All other quantitative and qualitative assays were carried out using analytical grade reagents. General purpose reagents were used for media preparation. Materials for molecular biology studies were obtained from Qiagen Ltd. (Crawley, UK), Promega (Southampton, UK) and Fisher Scientific (Loughborough, UK). PCR Master Mix (Promega) was used for conventional polymer chain reaction. Primers for conventional PCR and quantitative PCR (qPCR) were purchased from Thermo Fisher Scientific (Invitrogen).

2.2 Microorganisms and plasmid

A. flavus, NRRL 3357 (aflatoxigenic strain) and ESP 15 (non-aflatoxigenic strain) were kindly obtained from Prof. Naresh Magan, Department of Environmental Science and Technology, Cranfield University.

B. licheniformis NCIMB 8874 was available in the Culture Collection of the University of Westminster, London, UK. It had been purchased from National Collection of Industrial and Marine Bacteria, USA. *B. subtilis* JRL293 [amyE:(*srfA-lacZ*, cat),trp, phe] and *E. coli* strain BL21 (DE3) were provided from the Culture Collection of the University of Westminster, London, UK.

The bacterial plasmid pET-22b(+) used for gene cloning in this study was obtained from Thermo Fisher Scientific (Novagen). This is an expression vector with *T7* promoter and

terminator. It has N-terminal *pelB* signal sequence for sub-cellular targeting, ampicillin resistance and restriction enzyme cloning sites (Figure 2.1).

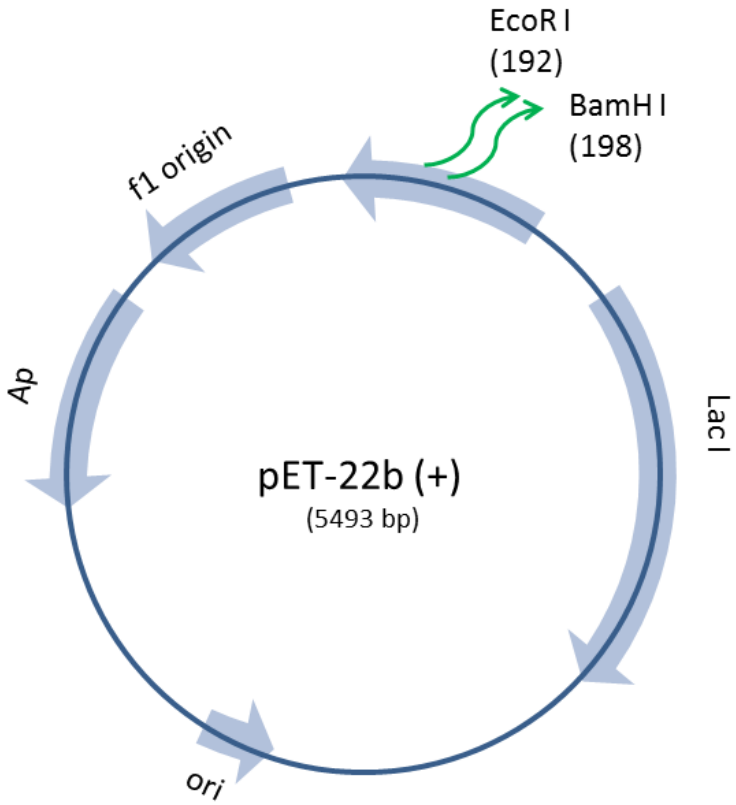


Figure 2.1 Plasmid used for *comQX* genes cloning, Plasmid pET-22b(+)

2.3 Media and growth conditions

All media used in this study were sterilized at 121 °C for 15 min. The media and all the additional supplements were obtained from Sigma Aldrich Company (Dorset, UK).

2.3.1 Media for growth and maintenance of *B. licheniformis* NCIMB 8874 and *B. subtilis* JRL293

Lysogeny broth (LB) and LB agar (LBA) (Table 2.1) were used for the maintenance of *B. licheniformis* NCIMB 8874. Medium was prepared by dissolving 20 gL^{-1} LB powder in distilled water and autoclaved at 121 °C for 15 min. Agar was added to the broth to

make up LB agar at a concentration of 12 gL^{-1}. Maintenance medium for *B. subtilis* JRL293 was supplemented with chloramphenicol (Sigma) (5 $\mu g.ml^{-1}$). Chloramphenicol stock solution (5 $mg.ml^{-1}$) was prepared in ethanol and then filter sterilised before adding to the medium.

Table 2.1 Lysogeny Broth composition

Component	**Concentration (gL^{-1})**
Tryptone	10
Yeast extract	5
Sodium Chloride (NaCl)	10

All the bacterial strains were stored as glycerol stocks at -80 °C. The stocks were prepared by growing cells from a fresh colony in LB to optical density (OD) 600 nm ~ 1 and adding 15% (v/v) sterilised glycerol before freezing. Fresh colonies were obtained by streaking a loopful of the frozen bacteria from stocks onto LB agar plates. The plates were then incubated at 37 °C for 18-24 h and stored at 4 °C.

2.3.2 Medium for growth and maintenance of *A. flavus* strains

Potato dextrose agar (PDA) was used for propagation and sporulation of *A. flavus* strains. The medium was prepared by dissolving 39 gL^{-1} of PDA in distilled water. The solution was autoclaved and then divided onto Petri dish plates and Universal bottles under aseptic conditions to form the slant media. The PDA medium was then inoculated using 200 µl of the spore suspension from the working glycerol stock solution which was stored at -20 °C. These inoculated plates/slants where incubated at 26 °C for approximately seven days. The fully sporulated slants were stored in sterile airtight containers at 4 °C for future use.

Glycerol stocks were used for maintenance of fungal isolates at -20 °C. The stocks were prepared by storing, in 15% v/v glycerol solution, spores from a fresh sporulating colony taken from PDA medium. The stocks were stored at -20 °C.

For spore inoculating the PDA plates (e.g. experiment of testing the effect of different treatments on fungal growth), spores were extracted from the fully sporulated slants/plates using 5 ml of sterile 0.01% Tween 80 (v/v) in distilled water supplemented with 2 mm glass beads (VWR). Spores were counted using a haemocytometer and adjusted to the desired inoculums concentration before inoculating the PDA plates.

2.3.3 Growth medium for *E. coli* BL21 (DE3) harbouring recombinant plasmid

Cultivation of *E. coli* expression strain (BL21 (DE3)), which was transformed by recombinant plasmid, was conducted in M9 minimal salts solution. The 5x concentrated stock solution was made by stirring to suspend 56.4 g powder (Table 2.2) in 1 L distilled water and then autoclaved for 15 min at 121 °C. The 5x M9 concentrated stock was diluted to a 1x working solution by adding 200 ml M9 stock to 800 ml sterile water, supplemented with a mix of filter-sterilised amino acids (leucine, phenylalanine, histidine, serine, 40 $\mu g.ml^{-1}$ each; glutamine, 400 $\mu g.ml^{-1}$), and ampicillin (100 $\mu g.ml^{-1}$). According to the manufacturer instruction, additional supplementation of filter-sterilised 20% (w/v) glucose, 1 M magnesium sulfate and 1 M calcium chloride was required in order to complete M9 minimal medium preparation. Filter sterilization was carried out through a 0.22 µm filter (Millipore).

Table 2.2 M9 minimal salts solution composition before adding the supplements

Component	**Concentration (gL^{-1})**
Sodium phosphate (Na_2HPO_4)	33.9
Potassium Phosphate (KH_2PO_4)	15
Ammonium Chloride (NH_4Cl)	5
Sodium Chloride (NaCl)	2.5

2.3.4 Medium for growing of the reporter strain used in β-galactosidase activity assay

LB was the liquid medium to grow the reporter strain, *B. subtilis* JRL293, in the bioassay experiment and it was prepared as described in section 2.3.1. The medium was supplemented with chloramphenicol (Sigma) (5 $\mu g.ml^{-1}$).

2.4 Assay procedures

2.4.1 Optical density measurement

The optical density (OD) of bacterial cultures was read at 600 nm wavelength using JENWAY 6300 spectrophotometer (Fisher Scientific), where the uncultured medium (LB or M9 minimal medium) was used as a blank. Absorbance readings above 0.5 were diluted using fresh medium.

For the β-galactosidase activity assay, the optical density measurement was carried out at 420 and 550 nm wavelength following Miller's protocol to calculate the Miller Unit (section 2.4.4).

2.4.2 Pheromone purification

The whole procedure of pheromone purification is presented in Figure 2.2.

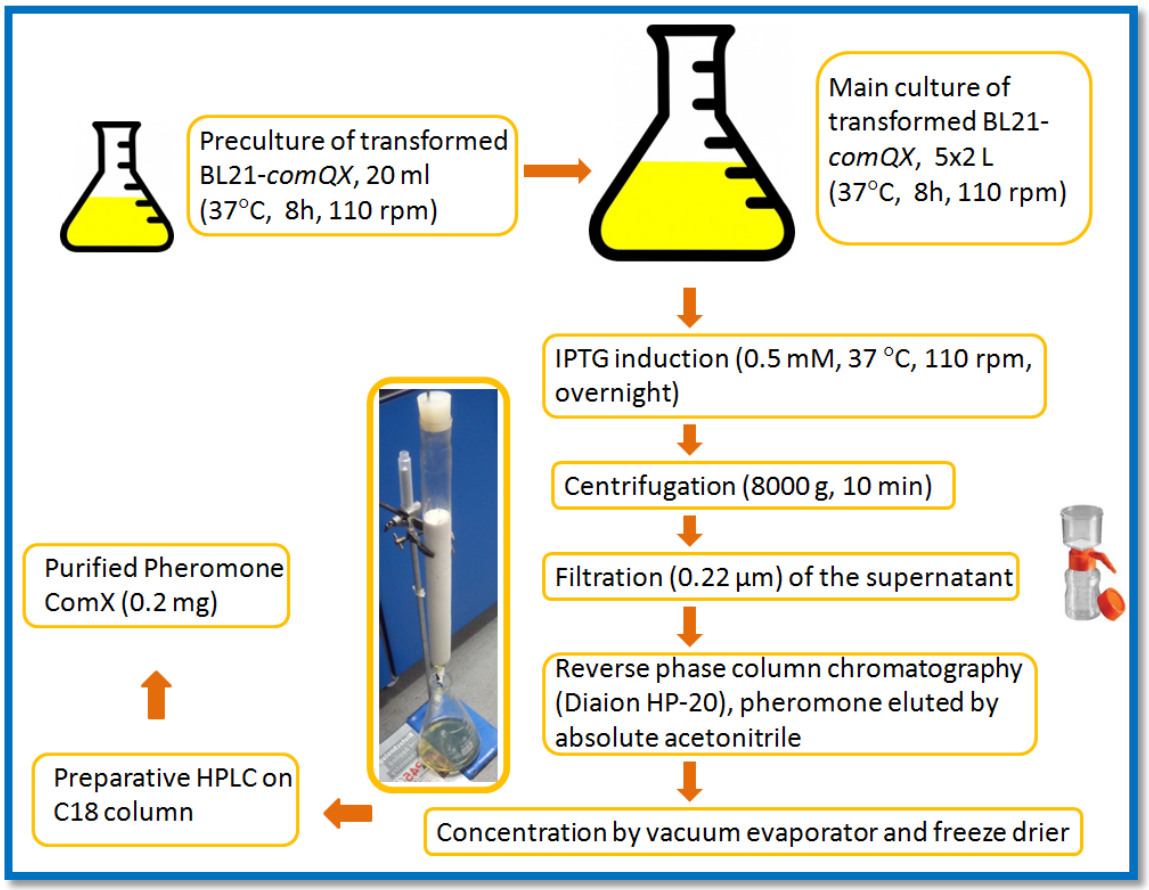


Figure 2.2 Purification scheme of the ComX pheromone

2.4.2.1 Pheromone over-production

E. coli expression strain (BL21 (DE3)) transformed by recombinant plasmid (carrying *comQX* genes, described in section 2.5.8.4) was grown overnight in the completed M9 minimal salts medium described in section 2.3.3. At stationary phase, this pre-culture (20 ml) was added to 1980 ml of the supplemented M9 medium to make 2 L bacterial culture (5 flasks in total to prepare 10 litters culture) and then incubated at 37 °C and 110 rpm for 8 h. *comQX* gene expression was induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C and 110 rpm overnight. The culture broth (10 L) divided into the smaller centrifuge buckets and was centrifuged for

10 min at 8,000 g using Sorvall RC 3BP+ centrifuge (Thermo Fisher Scientific). The supernatant was filtered through a 0.22 µm vacuum filtration unit Corning (Sigma) and kept at 4 °C for future use. The same procedure was performed for culturing the non-recombinant *E. coli* BL21 (DE3) in 2 L medium as a control.

2.4.2.2 Pheromone extraction using reverse-phase chromatography

Reverse-phase chromatography method was used for the initial purification and concentration of the filtered supernatant (as described in section 2.4.2.1). Diaion HP-20 is a non-polar copolymer styrene-divynilbenzene adsorbent resin with spherical particles of 0.5 mm diameter. The column which was used to be packed with resin had a bed volume of 290 ml (Bed volume= 3.14x [1/2 inside diameter]^2 x length of the bed). To start resin wetting procedure, the dry resin was covered with methanol up to 5 cm. After stirring the resin, the mixture was allowed to stand for 15 min. Methanol was decanted and replaced with distilled water. To prepare the column, 3 cm deionised water was added to the empty column and then the resin was poured slowly into the column. When the column was half filled, the excess water was drained through the bottom of the column. Care was taken to avoid the liquid level fall below the top of the resin bed. To backwash the column, 30 min introducing a slow upward flow of deionised water was sufficient to prepare the bed. In addition, the bed was backwashed after the adsorbate was adsorbed to resin and prior to elution of the adsorbate. The backwash was used to clean the packed resin from any suspended solids, accumulated dirt (from the sample loading step) and broken resin particles. This also allowed the resin beads to be classified by size. Proper backwash rate is of great importance and must be followed as suggested in the protocol.

The resin loaded with the supernatant of the bacterial cultures (10 L of *E.coli* BL21 carried recombinant plasmid-*comQX* and 2 L of intact *E. coli* BL21 separately) with the rate of 8 bed volumes (2300 ml) per hour. After the completion of resin loading, the column was washed with 2 bed volumes (580 ml) of deionised water. The elution solution was collected using 3 bed volumes (870 ml) of absolute acetonitrile and kept at

4 °C for future use. Finally, the column bed was equilibrated with 2 bed volumes loading of deionised water.

2.4.2.3 Drying the eluted solution from reverse chromatography

In order to concentrate the eluted solution from the reverse-phase chromatography, acetonitrile was evaporated to 5 ml in a vacuum rotary evaporator. Then the small amount of solution was frozen at -80 °C to transfer into the vacuum freeze dryer "Savant Modulyo D" (Thermo Fisher Scientific) for 20 h to ensure complete drying. The dried samples of the eluted solution from the supernatant of *E.coli* BL21 carrying the recombinant plasmid was kept at -20 °C for further work; purification through HPLC, β-galactosidase bioassay and fungal growth experiments.

2.4.2.4 Pheromone detection and collection using preparative High Performance Liquid Chromatography (HPLC)

The dried extract from reverse-phase chromatography was analysed through HPLC for the presence of the ComX pheromone. The column was C18 (Thermo Fisher Scientific, 5 µm x 4.6 x 150 mm) with Acclaim 120, C18 5 µm Guard Cartridges (4.6 x 10 mm) on a Dionex ICS-5000 HPLC instrument (Thermo Fisher Scientific). The dried extract was re-dissolved in 200 µl acetic acid, 600 µl acetonitrile and 1200 µl deionised water (1:3:6) to prepare a solution of 25 $mg.ml^{-1}$ which was passed through 0.22 µm filter (Millipore) to run in HPLC. Two different sequences of amino acids were synthesised as standard which obtained from Pepceuticals Ltd. (Leicestershire, UK). These standard samples were used for pheromone quantification and also to confirm the retention time. The mobile phase of 20% acetonitrile in 0.1% aqueous ammonium acetate (w/v) at the flow rate of 1.0 $ml.min^{-1}$ washed the system for 5 min equilibration and continued to another 5 min after injection the sample into the column. The run was continued with a linear gradient of 20-55% acetonitrile in 0.1% aqueous ammonium acetate for 20 min. According to the retention time of the two standard samples, the most active fractions

(standard 1 retention time: 14.5-15.5 min, and standard 2 retention time: 12.5-13.5 min) were collected and purified using Automated Fraction Collector Dionex UltiMate 3000 (Thermo Fisher Scientific). The pheromone molecule was detected at 210 nm.

2.4.2.5 Pheromone identification using MS/MS and MALDI-MS

MS/MS and MALDI-MS used to determine the mass spectrometry and the sequences of amino acids in collected samples from HPLC. This work was performed by Proteomics Services at department of Biology, York University. The dried fractions were dissolved in 500 µl of 50% acetonitrile and 0.1% formic acid and then analysed on Bruker ultraflex III MALDI-TOF/TOF instrument (Bruker, MA, USA). There were two dominant ions in the MS spectrum at 1209.74 and 1564.78 mz^{-1}. The peptide sequences of these ions were identified confidently.

2.4.2.6 Pheromone identification using Nuclear Magnetic Resonance (NMR)

NMR was used to determine the structural characterisation of the pheromone molecule. Purified fraction which was positive for the presence of pheromone was analysed on Bruker Avance III 700 MHz-^{1}H at NMR centre, Department of Structural and Molecular Biology, UCL, London. This instrument runs under TOPSPIN 2.4 for automation. Data were acquired for 1D analysis. Chemical shifts were referenced to the solvent peak deuterated acetonitrile.

2.4.3 Pheromone assay (70 min assay)

Pheromone activity was assayed based on its ability to induce expression of *srfA-lacZ* in cells at low cell-density. *B. subtilis* JRL293 carrying the *srfA-lacZ* fusion strain was used as the reporter strain. Also, *E. coli* (wild type) used as a control positive to present the basal level of enzyme activity. In the standard assay, the cell-density of the overnight cultures (LB) of the reporter strain was recorded by measuring the absorbance at

600 nm using JENWAY 6300 spectrophotometer. The cell cultures were diluted in fresh medium (LB) and grown to OD 600 of 0.80. Then 0.5 ml of the diluted culture was added to 0.5 ml of the supernatants. The supernatants were obtained from the culture of *E. coli* BL21 containing recombinant plasmid-*comQX* before and after the addition of IPTG and from *B. licheniformis* NCIMB 8874 culture in the late exponential phase. In addition, β-galactosidase activity of a more concentrated supernatant from the induced recombinant *E. coli* BL21 was tested by applying 0.5 ml filtered extract of IPTG induced supernatant (1 $mg.ml^{-1}$). BSA (50 $\mu g.ml^{-1}$) was added to the sample potentially containing the ComX pheromone to prevent non-specific loss of activity. The mixture (reporter strain and supernatant) was incubated at 37 °C for 70 min in a rotary shaker at 200 rpm and then assayed for β-galactosidase specific activity (Magnuson *et al*., 1994). The procedure is presented in the Figure 2.3.

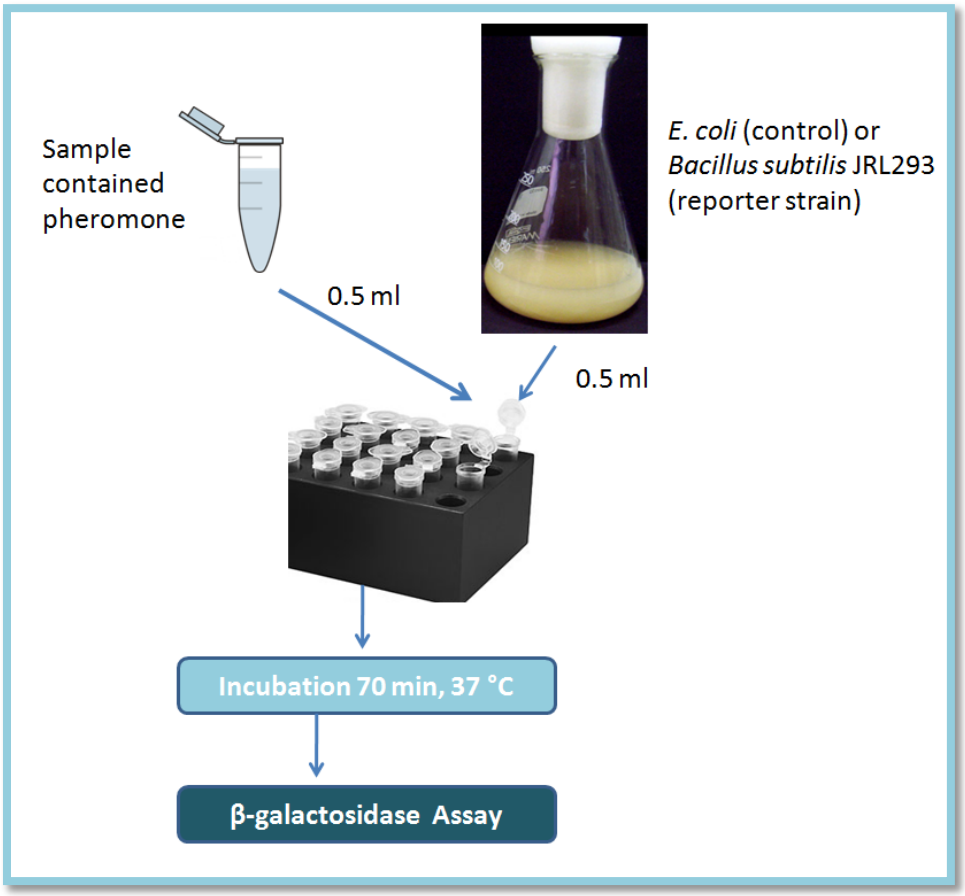


Figure 2.3 Diagram of the pheromone assay

2.4.4 β-galactosidase assay

β-galactosidase activity was estimated by colorimetric assay. A suitable antibiotic (chloramphenicol, final concentration of 100 $\mu g.ml^{-1}$) was added to the samples which were obtained from the final 70 min incubation (see section 2.4.3), and kept for 15 min on ice. Before adding the warmed permeabilisation solution (Table 2.3) to the samples; concentration of the samples was measured at OD 600. The assay was carried out in test tubes by adding 0.5 ml permeabilisation solution to 0.5 ml sample. Then 100 µl chloroform and 50 µl 0.1% SDS (sodium dodecyl sulphate) were added to the mixture in order to complete permiabilisation of the diluted cells. The tubes were incubated at 28 °C for 5 min. The reaction was started by adding 0.2 ml of the substrate buffer (Table 2.4) to each tube and mixing thoroughly. The mixture was kept in the incubator at 28 °C for 100 min. The reaction was then stopped by addition of 0.5 ml of a 1 M sodium carbonate (Na_2CO_3) solution following the development of yellow colour. One ml of the samples was transferred to a 1.5 ml tube (Fisher Scientific) and spun for 5 min at maximum speed (14000 rpm) using Centrifuge 5418 R (Eppendorf, Germany) to remove debris and chloroform. Then the optical density of the clear reaction solution was recorded at 420 nm and 550 nm wavelengths using a JENWAY 6300 spectrophotometer. β-galactosidase specific activity was calculated according to Miller (Δ A 420/min.$ml^{-1}$of culture OD 600) x 1000 (Miller unit, MU) (Miller, 1972).

Table 2.3 Concentration of the components in permeabilisation buffer

Components	**Concentration (gL^{-1})**
Sodium Monohydrogen Phosphate Heptahydrate (Na2HPO4.7H2O)	16.08
Sodium Dihydrogen Phosphate Monohydrate (NaH2PO4.H2O)	6.24
Potassium Chloride (KCl)	0.75
Magnesium Sulfate (MgSO4)	0.12
β-mercaptoethanol	0.05M (3.52 $ml.L^{-1}$)

Table 2.4 Concentration of the components in substrate buffer

Components	**Concentration (gL^{-1})**
Sodium Monohydrogen Phosphate Heptahydrate(Na2HPO4.7H2O)	16
Sodium Dihydrogen Phosphate Monohydrate(NaH2PO4.H2O)	6.2
o-nitrophenyl-β-galactoside	3

2.4.5 Antifungal susceptibility assays for pheromone

This test was performed with two different methods which were described in sections 2.4.5.1 and 2.4.5.2.

2.4.5.1 Micro-broth dilution antifungal assay

The activities of the purified pheromone determined against *A. flavus* (aflatoxigenic strain) using micro-broth dilution assay (du Toit and Rautenbach, 2000). All procedures were performed under aseptic conditions. The broth dilution assays were performed in sterile 96-well microtiter plates. Fungal spore suspension was prepared using the extracted spores from the fully sporulated plates in 5 ml of sterile 0.01 % Tween 80 (v/v) supplemented with 2 mm glass beads (VWR). Spores were counted using a haemocytometer and adjusted to the desired concentration ($2x10^6$ $spore.ml^{-1}$) to inoculate 5 ml the half strength potato dextrose broth (PDB) medium. The prepared broth spore suspension (90 µl) was added to the wells (Troskie *et al*., 2012). Each well contained a total of $1.8x10^4$ spores. Dried pheromone mixture was dissolved in 1% Dimethyl sulfoxide (DMSO) to a concentration of 13 $\mu g.ml^{-1}$. Serial dilutions (6.5, 3.25, 1.62, 0.8, 0.4, 0.2, 0.1, 0.05 $\mu g.ml^{-1}$) were made using 1% DMSO and then 10 µl of these diluted pheromone mixture were added to the wells containing 90 µl broth suspension. Control culture (positive) received 10 µl of 1% DMSO instead of pheromone. Control negative was a combination of half strength PDB (90 µl) and 1% DMSO (10 µl) to confirm the sterile PDB and DMSO used in the wells. All wells contained a final volume

of 100 µl. Subsequent to the pheromone addition, the microtiter plate was covered tightly with tinfoil, sealed with parafilm and incubated at 27 °C for 24 h and 48 h. The absorbance of the cultures in the wells was spectrophotometrically determined at 595 nm using a VersaMax Tunable microplate reader (VWR) after 24 h and 48 h incubation.

2.4.5.2 Reference method for broth dilution antifungal susceptibility testing of filamentous fungi (CLSI M38-A)

According to the M38-A standard protocol for moulds, the medium RPMI-1640 (Sigma) containing L-glutamine and phenol red (as a pH indicator) (Sigma) was used for growth of *A. flavus* in antifungal susceptibility study (CLSI, 2008). This medium did not contain bicarbonate as this component has the antifungal activity. The medium was supplemented with filter-sterilised 0.2% glucose and buffered to a pH of 7.0 with 0.165 $mol.L^{-1}$ 3-(N-Morpholino) propanesulfonic acid (MOPS) (Sigma) (CLSI, 2008). The spore suspension was prepared according the protocol described in section 2.4.5.1 using supplemented RPMI-1640 medium. The inoculated RPMI-1640 (90 µl) was added to each well. The microtiter plate was incubated at 27 °C for 48 h after the addition of 10 µl pheromone solution (13, 6.5, 3.25, 1.62, 0.8, 0.4, 0.2, 0.1, 0.05 $\mu g.ml^{-1}$) to the associated wells. After the incubation the absorbance of the culture in each well was measured using a VersaMax Tunable microplate reader (VWR) at 530 nm.

2.4.6 Aflatoxin extraction

Aflatoxin extraction was carried out according to the method of Nesci and co-workers (2005). The agar medium and fungal biomass (1 cm x 1 cm) was taken from the cultures of *A. flavus* (NRRL 3357 [aflatoxigenic strain] and ESP 15 [non-aflatoxigenic strain]) and incubated at 26 °C for 12 days. The sample was transferred to an Eppendorf tube and then 500 µl chloroform was added. The mixture was shaken at 850 g for 20 min. The chloroform extract was dried under nitrogen gas. The residue was re-dissolved in 10 µl

chloroform for screening by Thin Layer Chromatography (TLC) (described in the section 2.4.7).

2.4.7 Aflatoxin detection by Thin Layer Chromatography (TLC)

After extraction, aflatoxin was detected by loading approximately 15 µl of each sample onto silica TLC plates (10 cm X 20 cm) containing a fluorescent indicator (254 nm) (Sigma) along with aflatoxin standard (aflatoxin B1) (Sigma). TLC plates were then developed in chloroform/acetone (9:1, V/V) (Munimbazi and Bullerman, 1998). Photographs of TLC plates were taken following exposure to UV radiation using a digital camera. The extracted Aflatoxin was detected by measurement of fluorescence of spots using Rf values and colours. This result was compared with the obtained outcome from aflatoxin standard sample (Aflatoxin B1 obtained from Sigma).

2.4.8 Fungal growth-rate experiment

In order to study the effect of *B. licheniformis* NCIMB 8874 and its over-expressed pheromone on the growth of *A. flavus* strains, NRRL3357 and ESP15, the following experiment was carried out where several treatments were applied to the fungal culture. The treatments included washed and unwashed *B. licheniformis* cells, *B. licheniformis* culture supernatant, and the over-produced QSM (all the treatments and associated controls are listed and labelled in Table 2.5). The unwashed cell treatments comprised 1 ml of *B. licheniformis* NCIMB 8874 cell suspension in LB at concentrations of 10^4 cfu.ml^{-1} and 10^9 cfu.ml^{-1} (labelled a and b, respectively). Besides, the washed cells of *B. licheniformis* NCIMB 8874 (1 ml) were also used at the concentrations of 10^4 and 10^9 cfu.ml^{-1} (labelled j and k, respectively). The cells were washed three times with sterile water by spinning at 7000 rpm for 10 min (BIOFUGE PRIMO R centrifuge, Thermo Fisher Scentific).

In order to test the potential inhibitory activity of *Bacillus* QSM (over-produced QS molecule from recombinant *E.coli* and also QS molecule from exponential phase of

B. licheniformis) on *Aspergillus* growth, different treatments were used as following: 1) the filtrate supernatant of IPTG-induced transformed *E. coli* BL21 strain carrying recombinant plasmid (*comQX*) containing over-expressed pheromone (1 ml) (from now on referred to as "the IPTG-induced *comX*-supernatant", labelled c), 2) the solution obtained from the reverse-phase chromatography filtration of 10 litters of IPTG-induced transformed *E. coli* BL21 strain with recombinant plasmid (*comQX*) at two different concentrations of 5 and 30 $mg.ml^{-1}$ (500 µl) (from now on referred to as filtered extract of transformed *E. coli*, labelled f and g), 3) *B. licheniformis* NCIMB 8874 supernatant which were obtained from the late exponential growth phase (1 ml) (labelled i).

The negative controls were: 1) the filtrate supernatant of transformed *E. coli* BL21 strain (*comQX*) before adding IPTG (1 ml) (from now on referred to as "the non-induced *comX*-supernatant", labelled d), 2) the filtrate supernatant of *E. coli* BL21 strain (1 ml) (from now on referred to as host strain supernatant, labelled e) and, 3) 1% DMSO which was used as a solvent for the filtered extract (500 µl) (labelled h). The main positive control was *Aspergillus* strains without any additional components to the PDA plates.

The specified controls for each treatment were applied to allow the proper comparison with treated plates. These specific controls were LB (1 ml), M9 minimal medium (1 ml), 1% DMSO (500 µl) and sterile water (1 ml).

All the treatments were added to the allocated plates before adding the melted PDA medium. The plates containing the treatments were pour-plated with 20 ml of melted PDA, and after solidifying, 10^8 $spore.ml^{-1}$ of *Aspergillus* strains were spot inoculated in the centre of the plates. *Aspergillus* spore suspension was prepared in semi-solid agar (0.2% agar and 0.05% Tween 80). Each treatment was applied in three replicates. The inoculated plates were incubated at 27 °C for 7 days. The fungal colony area measurement was performed taking daily plate images using UVITEC Imaging System (Cambridge, UK). Following the collection of the images over 7 days, the images were analysed with new algorithm (please see section 2.4.8.1) for the accurate measurement of fungal colony area. After the incubation period, controls and treated plates were frozen at -20 °C for future use. The diagram in Figure 2.4 shows the stages that were followed to perform this experiment.

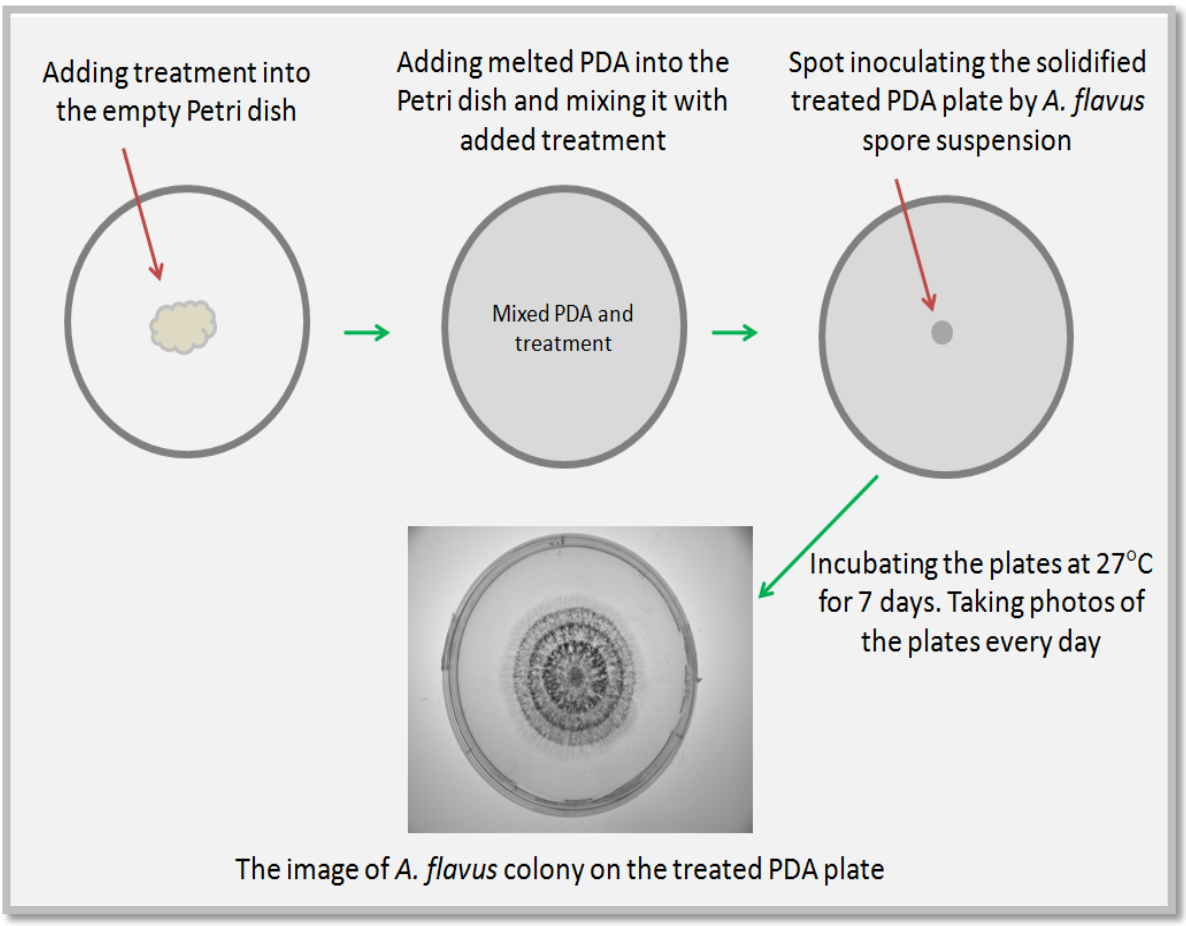


Figure 2.4 Diagram of the fungal growth-rate experiment

Table 2.5 List of the treatments used in fungal growth-rate and fungal dry weight experiment

order	Treatments	Referred to as	Associated control
a	*B. licheniformis* NCIMB 8874 suspension with 10^4 cfu.ml^{-1} concentration	*Bacillus* suspension of 10^4 cfu.ml^{-1}	LB
b	*B. licheniformis* NCIMB 8874 suspension with 10^9 cfu.ml^{-1} concentration	*Bacillus* suspension of 10^9 cfu.ml^{-1}	LB
c	Filtrate supernatant of IPTG-induced transformed *E. coli* BL21 with recombinant plasmid (*comQX*)	IPTG-induced *comX*-supernatant	M9 minimal medium
d	Filtrate supernatant of transformed *E. coli* BL21 strain with recombinant plasmid (*comQX*) before adding IPTG	Non-induced *comX*-supernatant	M9 minimal medium
e	Filtrate supernatant of *E. coli* BL21 strain	Host strain supernatant	M9 minimal medium
f	Solution of the dried sample obtained from the reverse-phase chromatography filtration of 10 litters IPTG-induced transformed *E. coli* BL21 strain with recombinant plasmid (*comQX*) in the concentration of 5 mg.ml^{-1}	Filtered extract of transformed *E. coli* in 5 mg.ml^{-1}	1%DMSO
g	Solution of the dried sample obtained from the reverse-phase chromatography filtration of 10 litters IPTG-induced transformed *E. coli* BL21 strain with recombinant plasmid (*comQX*) in the concentration of 30 mg.ml^{-1}	Filtered extract of transformed *E. coli* in 30 mg.ml^{-1}	1%DMSO
h	DMSO (1%)was used as a solvent for the filtered extract	DMSO	Sterile water
i	*B. licheniformis* NCIMB 8874 supernatant obtained from the late exponential phase of its growing	*Bacillus* supernatant in exponential phase	LB
j	*B. licheniformis* NCIMB 8874 LB suspension in the concentration of 10^4 cfu.ml^{-1} was washed three times	Washed *Bacillus* suspension in 10^4 cfu.ml^{-1}	Sterile water
k	*B. licheniformis* NCIMB 8874 LB suspension in the concentration of 10^9 cfu.ml^{-1} was washed three times	Washed *Bacillus* suspension in 10^9 cfu.ml^{-1}	Sterile water

2.4.8.1 Image analysis

In addition to the classic methods based on the manual measurement of fungal colony radii or diameters used for a selected group of the cultured plates, a computer image processing software (ColonyAreaAnalyzer) was used in order to measure precisely the fungal growth area based on their digital images. The algorithm has been developed at Institute of Astronomy, Cambrideg University.

The ColonyAreaAnalyzer software is developed in Python based on Open Source Computer Vision Library (OpenCV), a library of C/C++ programming designed for high-throughput and real-time image processing applications. The library has also a Python interface and supports all types of operating systems. Images were captured using the UVP BioImaging Systems. Dimensions of captured images were set to 1.3 mega pixels and were taken with an exposure time of 15.0 ms. On average, it took ~0.4 sec for each image to be processed on a single-core desktop computer.

The software analyses Petri dish images in two stages. In the first stage, an image was taken and read into the memory. Then the computer algorithm segmented the image in order to create a cut-out image of the object. This cut-out image was used as an input for the second stage. In stage two, a mask was created using the cut-out image in order to characterize the shape (centroid and area) and intensity of the growth. The schematic summary of fungal colony image processing is presented in Figure 2.5.

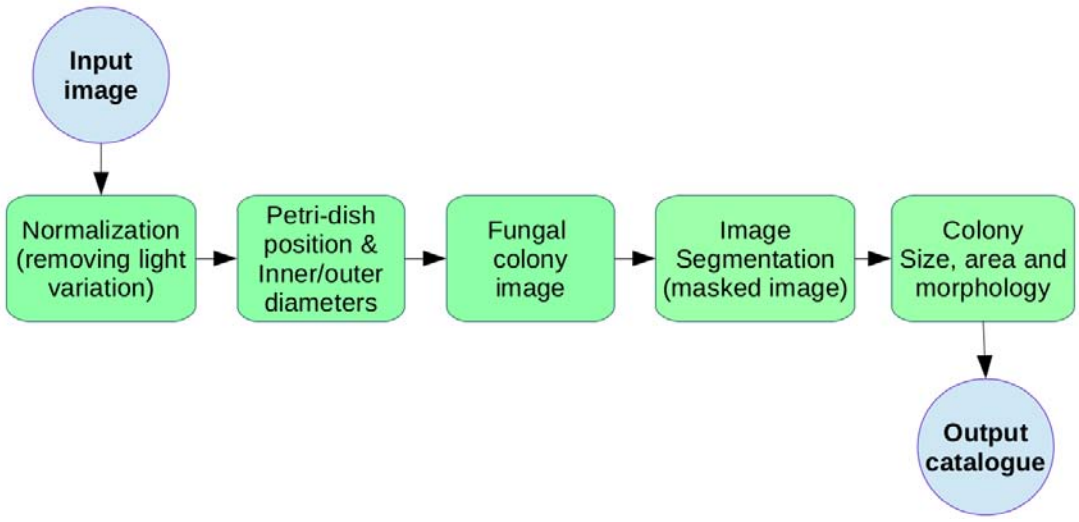


Figure 2.5 A schematic summary of major steps involved during image analysis

Following processing all input images, the software produces draft images associated to individual original/input images together with a final catalogue of extracted parameters describing various aspects of detected colonies (Figure 3.18). This, for instance, includes parameters like (i) plate and colony central positions and the offset between the plate and colony centres, (ii) area of the colony and the fraction of the plate's area covered by the colony, (iii) colony morphological parameters such as eccentricity and solidity, and (iv) colony light intensity calculated based on integrated intensities of all pixels within the colony divided by their mean value, normalized by 1.0 (Table 3.5).

2.4.8.2 Statistical analysis

All statistical analyses for significant differences between the means of 11 experimental treatments and their control in fungal area over 7 days were determined by one-way analysis of variance (ANOVA). Data analysis was performed using Microsoft Excel 2007 software (Microsoft, USA) to test the normal distribution of the data. Data sets of test/control in individual days were compared using unpaired T-test and significant differences were given as $P \leq 0.05$ where a P value equal or below 0.05 was regarded as statistically significant. All results are presented as mean ± standard error of two independent experiments where all tests and controls were carried out in triplicate. The charts were drawn using Anaconda-2.2.0 software (Continuum Analytics, Inc.). A linear regression model was fitted to each set of test/control data points. Estimated slopes of the fitted linear model to test and control data were compared against each other in order to monitor the rates of fungal growth, and the way they are affected, in the presence of treatments.

2.4.9 Fungal dry weight measurement

Fungal strains (*A. flavus* NRRL 3357 and ESP 15) grown in the shaken tubes contained 10 ml PDB with all the applied treatments as described for the fungal growth-rate experiment (section 2.4.8, Table 2.5). Each tube was inoculated with the prepared spore suspension with the final concentration of 10 spores.ml^{-1}. The cultures were incubated for 7 days in the incubator shaker "New Brunswick Scientific Excella E25" (Fisher Scientific) at 180 rpm, 27 °C. Then they were harvested and filtered using grade No. 54

filter paper (Whatman international Ltd., UK). These filter papers were kept at 70 °C for 24 h, cooled down to room temperature in desiccators and pre-weighted. Filter papers containing fungal mass were dried at 70 °C in the oven for 48 h, cooled down to room temperature in a desiccators before weighing. The difference between the weight of the filter paper and the combined weight of the filter paper together with fungal dried mycelia was considered as fungal dry weight.

2.5 Molecular biology studies

2.5.1 Measurement of nucleic acid concentration

Nucleic acid purity and concentration ($ng\mu l^{-1}$) were determined by measuring the Optical Density (OD) at 260 and 280 nm using Nanodrop 1000 spectrophotometer (software v3.7.1) (Thermo Scientific, UK). The A260/A280 absorbance ratio of 1.8 to 2 indicates that isolated nucleic acid (DNA or RNA) is pure and not degraded indicating it has a good quality without protein interference.

2.5.2 Genomic DNA isolation

Genomic DNA of *B. licheniformis* NCIMB 8874 was isolated using DNeasy Blood and Tissue kit (Qiagen). *B. licheniformis* NCIMB 8874 single colonies were inoculated in 5 ml LB and incubated overnight on an incubator shaker "New Brunswick Scientific Excella E25" (Fisher Scientific) at 200 rpm, 37 °C for 15 h. Cultures (1.25 ml) at OD 600 of 2 (corresponding to 2 x 10^9 cells) were transferred in a micro-centrifuge tube and cells harvested by centrifugation at 7500 rpm for 10 min. Genomic DNA extraction was carried out following the instructions provided by the manufacturer. The bacterial pellets were resuspended in 180 µl enzymatic lysis buffer and incubated for 30 min at 37 °C. Proteinase K (25 µl) and Lysis Buffer (AL) (200 µl) provided with kit was added to the lysate and the mixture was incubated at 56 °C for 30 min. Samples were then treated with 200 µl absolute ethanol and thoroughly mixed by vortexing. The obtained mixture was then transferred into a DNeasy Mini spin column and centrifuged

at 8000 rpm for 1 min. The DNA was first washed with 500 µl Washing Buffer 1 (AW1), then with 500 µl Washing Buffer 2 (AW2), both buffers contained ethanol. After the ethanol was removed from the DNeasy column membrane by centrifugation at 8000 rpm for 3 min, the DNA was eluted in a clean micro-centrifuge tube. DNA elution was carried out by adding 200 µl Elution Buffer (AE) and spinning at 8000 rpm for 1min. Genomic DNA concentration was evaluated using Nanodrop 1000 spectrophotometer (Thermo Scientific, UK). DNA samples were stored at -20 °C for future use.

2.5.3 Primers designed for *B. licheniformis* NCIMB 8874 QSM-encoding genes

Primers for performing Polymerase Chain Reaction (PCR) on *B. licheniformis* NCIMB 8874 were designed using Primer 3 software. PCR as the initial step for cloning procedure was applied to investigate *B. licheniformis* NCIMB 8874 QSM. Primers were engineered to amplify the genes encoding the ComX pheromone, using the genome sequence from *B. licheniformis* NCIMB 8874 (De Vizio, 2011). Forward primer hybridized to a sequence upstream of *comQ* contained a NdeI site while downstream *comX* was amplified by reverse primer containing a BamHI site. The annealing temperature was around 53 °C and the expected PCR product fragment was approximately 1070 bp. Primers were custom prepared by Invitrogen (Thermo Fisher Scientific).

comQ forward primer: ACGTCATATGAATCATTTTATAGACGTTGAGATTCC

comX reverse primer: ACGTGGATCCTTATTTGAACCATAAATTAGGGTAAG

2.5.4 Conventional PCR

The PCR was performed using genomic DNA isolated from *B. licheniformis* cells and primers designed for amplification of *comQ-comX* (from now on referred to as *comQX*) genes. The amplification reaction was carried out by utilizing PCR master mix (Promega,

Southampton, UK). The PCR reaction was prepared according to the manufacturer instructions (Table 2.6). In the MJ Mini Thermal Cycler (Biorad, UK), the amplification program was started with initial denaturation at 95 °C for 2 min and followed with 30 cycles of denaturation at 94 °C, annealing at 53 °C and extension at 72 °C all for 1 min. Final extension was for 5 min at 72 °C. PCR products (~1000 bp) were run on a 0.8% agarose gel using 1 Kb DNA ladder (New England Biolabs, Hitchin, UK) as a DNA size marker. To confirm that the product corresponded to the target sequence, the purified PCR was sent for sequencing (the UCL DNA Sequencing Service). The purified PCR product was used for the cloning experiment.

Table 2.6 PCR composition for 25 µl reaction

Component	**Volume (µl)**	**Final concentration**
PCR Master Mix (2X)	12.5	1X
Forward Primer (10 µM)	2.5	1.0 µM
Reverse Primer (10 µM)	2.5	1.0 µM
DNA Template	1-5	<250 ng
Nuclease-Free Water	1-5	NA

2.5.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used for analysis of PCR products obtained from amplification process and also for digested plasmid/insert fragments. The same method was also used to purify DNA fragments generated in PCR. Different agarose concentrations were used for gel preparation depending upon the expected fragments or product size of the target DNA. DNA Fragments between 100 and 500 bp were run on a 3% agarose gel using Hyper ladder V Bioline (London, UK) as a DNA size marker. Products with sizes >500 bp were visualised on a 0.8-1% agarose gel where 1 Kb DNA ladder (New England Biolabs) or 1 kb plus DNA ladder (invitrogen) were selected as a size marker. During the course of this project 0.8% agarose gels were prepared in

1X Tris Acetate EDTA (TAE) buffer (pH 8.5). The composition of 50X TAE buffer is given in Table 2.7. PCR products or DNA fragments to be analysed through electrophoresis (10 µL) were mixed with 2 µL of 6X Blue/Orange Loading Dye (Promega) before loading onto the gel wells. The gel was run at 100 V for 60 min (Mini-Sub Cell GT System, Biorad, UK) to allow the DNA fragments to separate. After electrophoresis the gels were stained with ethidium bromide (10 $mg.ml^{-1}$) and placed on a UV transilluminator (UVITEC, UK) to visualise the DNA fragments.

Table 2.7 Composition of 50X TAE buffer

Components	**Weight or Volume**
Tris Base	252 g
Glacial Acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml

2.5.6 DNA extraction from an agarose gel

DNA fragments were run on a suitable agarose gel (with bigger wells) as described in section 2.5.5. After visualising the bands on the gel (using UV transilluminator), the target fragment was cut from the agarose gel using a sterile scalpel. The DNA was extracted and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

2.5.7 DNA sequencing of the PCR product

The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen). The purification was carried out according to the manufacturer instructions. In the last step of the purification procedure, while the PCR product was eluted from the column, the nuclease free water was applied as an elution buffer. The purified PCR product was sent

for sequencing (the UCL DNA Sequencing Service). The obtained nucleotide sequences were analysed using Basic Local Alignment Search Tool (BLAST).

2.5.8 *comQX* genes cloning of *B. licheniformis* NCIMB 8874

To investigate *B. licheniformis*' QSM, ComX pheromone, *comQX* genes from the extracted DNA of *B. licheniformis* NCIMB 8874 were cloned in one vector. The modified vector plasmids were derived from the pET-22b(+) allowed the over-production of the ComX pheromone in *E. coli* (Ansaldi *et al*., 2002).

2.5.8.1 Preparation of *E. coli* chemically competent cells

To prepare chemically competent cells, fresh LB broth was inoculated with an *E. coli* culture (TOP10 or BL21 (DE3), Table 2.8) and incubated at 250 rpm, 37 °C for 18-20 h. Cells were grown in this condition until the optical density at 600 nm reached 0.4. The culture was then transferred to ice bath to chill for 10 min. Cells were centrifuged at 3000 g and gently re-suspended in the cold TSS buffer (pH 6.5, filter sterilized) (Table 2.9). The re-suspended cells in TSS were further incubated in ice for at least 20 min prior to dividing in 1.5 ml tube of 100 µl aliquot and storage at -80 °C.

Table 2.8 *E. coli* strains for cloning and *comX* pheromone expression

Strains	Genotype	Source
TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*	Life Technologies (Thermo Fisher Scientific)
BL21 (DE3)	*F- ompT gal dcm lon hsdSB(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Stratagene (La Jolla, California)

Table 2.9 Composition of TSS buffer (50 ml)

Components	Weight or Volume
Polyethylene Glycol (PEG 8000)	5 g
Magnesium Chloride (MgCl2) (1 M)	1.5 ml
Dimethyl sulfoxide (DMSO)	2.5 ml
LB broth	to 50 ml

2.5.8.1.1 Transformation of chemically competent cells [*E. coli* (TOP10 or BL21 (DE3))]

Following the addition of the recombinant plasmid DNA (10 ng) into the aliquot (100 µl) of thawed competent cells, the cells were Incubated on ice for at least 5 min. Cells were heat-shocked at 42 °C for 45 sec and subsequently incubated on ice for another 5 min. LB broth (500 µl) was added to the cells and incubated at 200 rpm, 37 °C for an hour. Transformed cells were either grown directly in LB broth or agar plates supplemented with ampicillin (50 $\mu g.ml^{-1}$).

2.5.8.2 Restriction double digest

To create the sticky ends of the target sequence, hereafter will be referred to as insert, purified PCR products were cut using restriction enzymes, BamHI and NdeI, (Invitrogen) following the manufacturer's protocol. Restriction double digests were typically set in a total volume of 20 µl. The appropriate buffer (10X buffer K) and 1 µl of each restriction enzyme (BamHI and NdeI) were added to 1 µg DNA. The reaction mixtures were incubated at 37 °C overnight. Plasmid vectors were digested using the same procedure. The digested products were cleaned as described in section 2.5.6 using the QIAquick gel extraction kit (Qiagen).

2.5.8.3 Ligation reaction and PCR colony

Ligation was performed (10 µl) by incubating the mixture of purified linearised plasmid vector (10 ng.µl^{-1}) and digested insert DNA (30 ng.µl^{-1} or more) with T4 DNA ligase (0.5 µl) and buffer (1 µl) (Invitrogen) for 2 h at 26 °C. Ligation products were transformed into *E. coli* TOP10 (as a non-expression host) as described in section 2.5.8.1.1. To determine a positive colony i.e. colony that contains a plasmid vector with the DNA insert, PCR (called PCR colony) was performed using half of each colony as template. PCR mixture containing sequencing primers (*T7* promoter and terminator, invitrogen) were inoculated with the bacterial colonies obtained from transformation and then PCR was carried out following the standard protocol. Intact vector was used as a negative control. PCR products were analysed for the presence of insert using agarose gel electrophoresis. Positive clones were propagated using a plasmid miniprep kit (Qiagen) and the accuracy of the sequence was determined using the UCL DNA sequencing service.

2.5.8.4 Cloning of plasmid construct into expression host cells (*E. coli* BL21 (DE3))

The bacterial strain, *E. coli* BL21 (DE3), applied as a host to express the ComX pheromone. Its competent cells were transformed (as described in the section 2.5.8.1.1) with the propagated plasmid construct which contained the desirable insert (*comQX*). The grown transformed bacterial colonies on the LB plates (contained ampicillin, 0.05 mg.ml^{-1}) were used to purify the plasmid through plasmid miniprep kit (Qiagen) and sent to the UCL DNA sequencing service to determine the accuracy of the sequence. The transformed *E. coli* BL21 (DE3) kept in glycerol stock at -20 °C for further use to over produce the ComX pheromone. The schematic diagram of gene cloning procedure is presented in Figure 2.6.

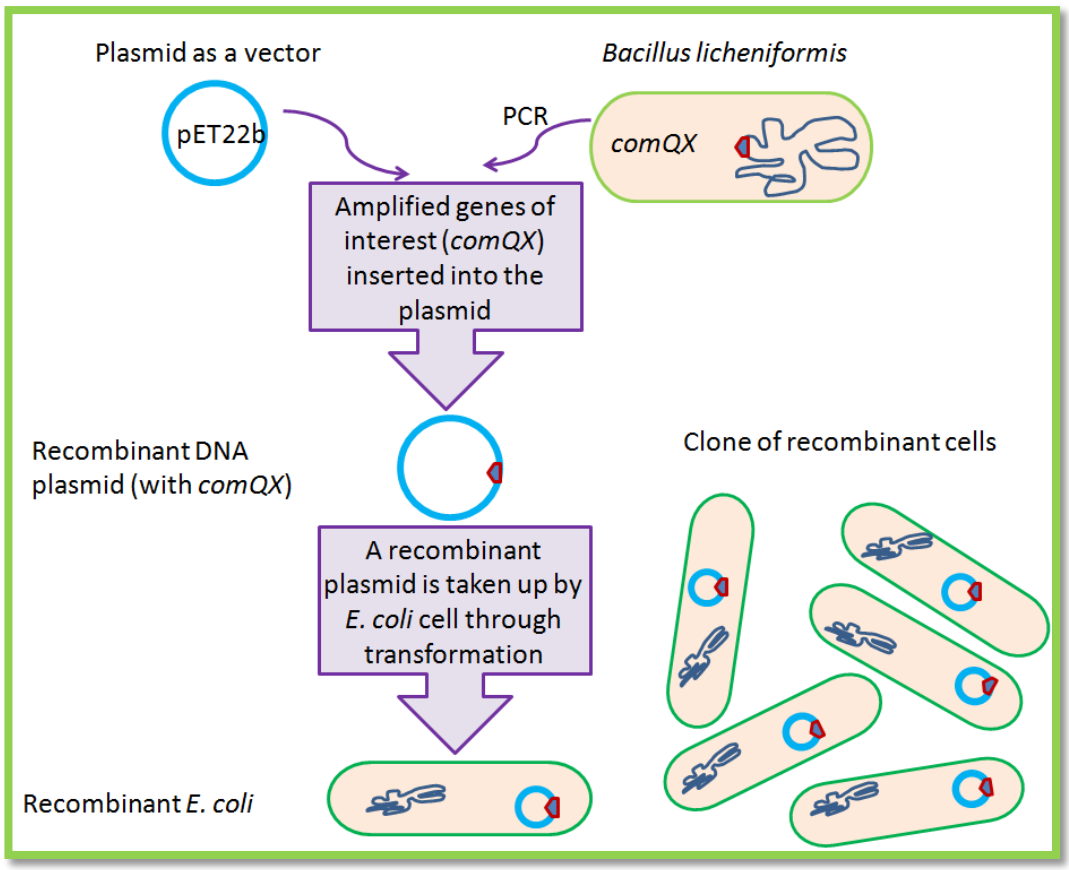


Figure 2.6 Diagram of *comQX* gene cloning procedure

2.6 Transcriptomic studies

2.6.1 Total RNA extraction from *A. flavus* treated/untreated cultures

A. flavus which was grown on PDA medium with different treatments (section 2.4.8) kept at -20 °C for this experiment. The cultures were harvested using the scalpel to collect 100 mg fungal biomass. RLC buffer (450 µl) (Qiagen, UK) was added to 100 mg of the fungal mass in Lysing Matrix Y tubes (Q-BIO Gene) and the cells were disrupted in a bench-top homogeniser (FastPrep® 24, MP Biomedicals, UK). Following the cell disruption step, total RNA was extracted from the lysing matrix using RNeasy Mini Plant kit (Qiagen) according to the manufacturer's instructions. The lysate transferred into the QIA shredder spin column and centrifuged for 2 min at full speed (14000 rpm) and then the supernatant was carefully collected. Prior to transfer to RNeasy spin column, the collected supernatant was mixed with 0.5 ml absolute ethanol. The 15 sec spinning

at 10000 rpm was applied on the column before and after the addition of 700 µl buffer RW1. To wash the spin column membrane, 500 µl buffer RPE was used twice with spinning at 10000 rpm for 15 sec and 2 min. The extracted RNA was eluted from spin column membrane into 30 µl nuclease-free water. To avoid RNase contamination, sterile disposable plastic-ware and RNase-free water (Sigma-Aldrich, UK) were used. RNA purity and concentration ($ng.\mu l^{-1}$) was determined by measuring the absorbance at 260 and 280 nm using Thermo Scientific NanoDrop 1000 (FisherScientific, UK). The absorbance ratio A260/A280, between 1.8 and 2, indicates that isolated RNA is pure, not degraded and of good quality without protein interference. RNA integrity was verified by gel electrophoresis on a 1% (w/v) agarose gel in 1xTBE buffer and visualised after Ethidium bromide ($10\ mg.ml^{-1}$) staining using a UV trans-illuminator (UViTec, UK). RNA samples were then stored in the -80 °C freezer.

2.6.2 DNase treatment of extracted RNA and RNA cleanup

All RNA samples were treated by using QIAGEN RNase-Free DNase Set (Qiagen, UK) to digest contaminating genomic DNA in RNA solutions prior to RNA cleanup and concentration. According to the manufacturer's protocol, RNA samples were diluted to 87.5 µl using RNase-free water. RDD buffer (10 µl) and 2.5 µl DNase I (from 1500 Kunitz units in 550 µl RNase-free water stock solution) were added to the diluted RNA and mixed gently. The mixture incubated at 25 °C for 10 min. To deactivate the digestion reaction, immediately preceded to RNA cleanup step as explained in the section 2.6.3.

2.6.3 RNA cleanup and concentration

Following DNase treatment which was performed on the extracted RNA samples, the enzymatic reaction was used to desalt and concentrate the RNA samples. RNeasy MinElute Cleanup kit (Qiagen, UK) was applied to carry out several steps of washing and centrifugation with using buffers and RNeasy MiniElute spin columns. The cleaned RNA was eluted from the membrane of the column with 14 µl RNase-free water. RNA purity

and concentration ($ng.\mu l^{-1}$) was determined by measuring the absorbance ratio, A260/A280, using Thermo Scientific NanoDrop 1000 (Fisher Scientific, UK). Cleaned RNA samples were stored at -80 °C.

2.6.4 cDNA synthesis

Complementary DNA (cDNA) synthesis from *A. flavus* mRNA was carried out using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, UK) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA in an overall reaction volume of 20 µl. In the initial incubation step, the cleaned RNA (500 ng to 1 µg), 1 µl oligo $(dT)_{18}$ primer and 1 µl Random Hexamer Primer were added to the appropriate amount of nuclease-free water to make 12 µl primary reaction volume and then incubated at 65 °C for 5 min using Prime Thermal Cycler (Techne, Staffordshire, UK). After denaturing the secondary structure of RNA and annealing of primers to the template RNA in the first incubation period, the following reagents were added to complete the reaction mixture: 5X Reaction Buffer (4 µl), RiboLock RNase Inhibitor ($20\ U\mu l^{-1}$) (1 µl), 10 mM dNTP Mix (2 µl) and Revert Aid M-MulV RT ($200\ U\mu l^{-1}$) (1 µl). Subsequent to mixing the reagents, they were incubated for 5 min at 25 °C followed by 60 min at 42 °C using Prime Thermal Cycler (Techne, Staffordshire, UK). The reverse transcription reaction products can be directly used for quantitative PCR. The resulting cDNA products were stored at -80 °C to be used later.

2.6.5 Three-step real time quantitative PCR (qPCR)

A three step qPCR experiment was conducted to determine any changes in the expression level of aflatoxin biosynthetic gene, *aflP* or formerly *omt-1* (encoding the enzyme to synthesize aflatoxin), in the treated *A. flavus* cultures. The synthesised cDNA from mRNA templates (section 2.6.4) was used as a template for real time qPCR experiment. In this study, the housekeeping gene was *ITS* (Table 2.11).

In order to generate the standard curve, the 1254 bp fragment of *omt-1* gene was amplified with the primers F-OMT and R-OMT and used as standard stock solution (OMT). The concentration of OMT PCR product was determined and the numbers of copy were calculated (DNA Copy Number calculator, Thermo Fisher Scientific). The stock solution was serially diluted by a factor of 10 and used as a copy number standard during each setup of the qPCR reaction to confirm the efficiency of the primers. In addition, to confirm the accuracy of the PCR product, it was sent to GATC, Biotech (Germany) to be sequenced.

The Real time PCR was carried out using Fast SYBR Green Master Mix Kit in a total reaction volume of 20 µl (Applied Biosystems, Thermo Fisher Scientific). Two sets of qPCR experiments were performed using two different sets of primers (F-*omt*/R-*omt* and F-*alfp*/R-*alfp*, Table 2.11). The following cycling conditions used to perform real time quantitative PCR experiments: Initial activation step to activate AmpliTaq Fast DNA Polymerase at 95 °C for 5 min; this was followed by a three-step cycling consisting of denaturation of cDNA template at 95 °C for 5 min and a primer annealing step at 50 °C for 15 sec and extension step at 72 °C for 20 sec. All real-time PCR cycling experiments were carried out using a Rotor-Gene Q 2plex HRM system PCR machine (Qiagen Ltd., Crawley, UK). A melt curve analysis of PCR amplicons was also performed to determine the specificity of primers. The PCR composition is shown in the Table 2.10. Details of all primers used in the real time qPCR experiments and their conditions are given in Table 2.11. All the primers were custom prepared by Invitrogen (Thermo Fisher Scientific). To confirm the accuracy of the qPCR products, they were sequenced at GATC, Biotech (Germany).

Table 2.10 qPCR composition for 20 µl reaction

Component	**Volume (µl)**	**Final concentration**
Fast SYBR Green Master Mix (2x)	10	1X
Forward Primer (2 µM)	2	0.2 µM
Reverse Primer (2 µM)	2	0.2 µM
DNA Template	Variable in different samples	20 ng
Nuclease-Free Water	Variable	NA

Table 2.11 Sequences of the primer sets used in PCR and three-step qPCR experiments on *A. flavus* NRRL 3357

Gene	**Primer name**	**Primer sequences**	**Expected product size (bp)**	**Annealing temperature (° C)**	**Reference**
aflP (*Omt-1*)	F-OMT	GGCCCGGTTCCTTGGCTCCTAAGC	1254	54	Rodriguez *et al*., 2012
	R-OMT	CGCCCCAGTGAGACCCTTCCTCG			
aflP (*Omt-1*)	F-*omt*	GGCCGCCGCTTTGATCTAGG	123	60	Rodriguez *et al*., 2012
	R-*omt*	ACCACGACCGCCGCC			
aflP (*Omt-1*)	F-*aflP*	CCAGCGCTTCATTTTCGAGG	122	60	Designed, using Primer3
	R-*aflP*	ACCGCATCACTTCGTCACAT			
ITS	ITS1	TCCGTAGGTGAACCTGCGG	600	60	White *et al*., 1990
	ITS4	TCCTCCGCTTATTGATATGC			

2.6.6 Reverse transcriptase polymerase chain reaction (RT-PCR) and analysis of the products on the gel electrophoresis

The synthesised cDNA from all RNA samples were amplified using the conventional PCR. The amplification reaction was carried out by utilizing PCR master mix (Promega, Southampton, UK). The PCR reaction was prepared according to the manufacture instructions. The RT-PCR composition is described in Table 2.12. The PCR conditions were optimised for applying combined primes; *aflP* primers for aflatoxin gene (F-*aflP*/R-*aflP*) and *ITS* primers for housekeeping genes (ITS1/ITS4) (details are given in Table 2.11). Using TC-PLUS Thermal Cycler (Techne, Staffordshire, UK), the amplification program was started with initial denaturation at 94 °C for 2 min and followed with 35 cycles of denaturation at 94 °C, annealing at 55 °C and extension at 72 °C all for 1 min. Final extension was for 5 min at 72 °C. On completion of the PCR reaction, 8 µl of each PCR product was subjected to electrophoresis in a 2% agarose gel containing ethidium bromide. Following electrophoresis, bands corresponding to transcripts of the study gene, *aflP*, and the reference gene, *ITS*, were noted. The gel was photographed using UVITEC Imaging System (Cambridge, UK).

Table 2.12 RT-PCR composition for 50 µl reaction

Component	Volume (µl)	Final concentration
PCR Master Mix (2X)	25	1X
F-*aflP* Primer (10 µM)	2.5	0.5 µM
R-*aflP* Primer (10 µM)	2.5	0.5 µM
ITS1 Primer (10 µM)	2.5	0.5 µM
ITS4 Primer (10 µM)	2.5	0.5 µM
DNA Template	4	<250 ng
Nuclease-Free Water	11	NA

2.7 Whole genome sequencing of *B. licheniformis* NCIMB 8874 using Ion Personal Genome Machine (PGM) System

The genome sequences of *B. licheniformis* NCIMB 8874 were determined on the PGM (Life Technologies, Thermo Fisher Scientific, UK). Following the first stage of sequencing procedure which was library construction, the template was prepared and run on the PGM to accomplish the sequencing process. The sequencing work flow is presented in Figure 2.7.

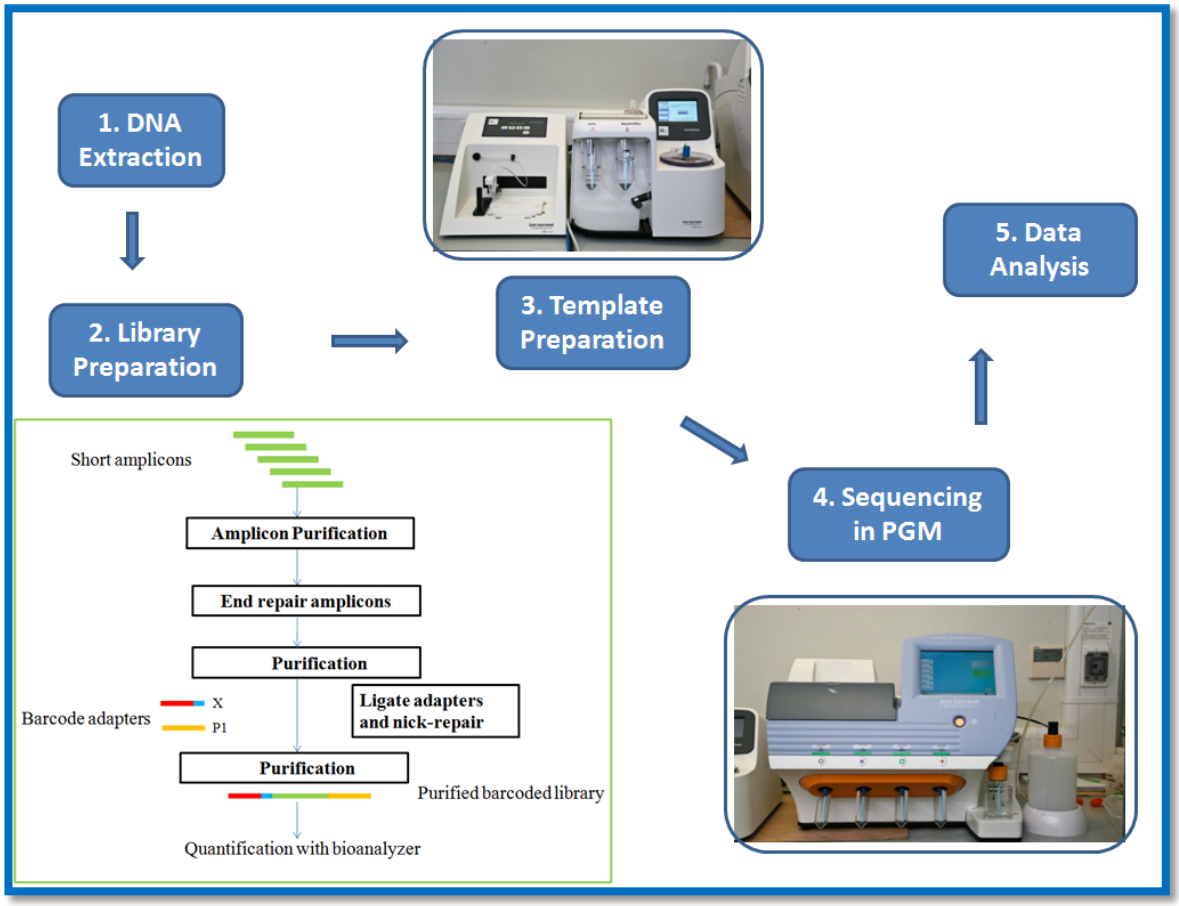


Figure 2.7 Bacterial whole genome sequencing work-flow using the next generation sequencing system (PGM)

2.7.1 Preparing short amplicon (<350 bp) libraries using the Ion Plus Fragment Library Kit

After extracting DNA from *B. licheniformis* NCIMB 8874 culture according to the instruction described in the section 2.5.2, the genome was sheared using S220 Focused-ultrasonicator (Covaris Inc., MA, USA).

There were several steps to prepare short amplicon libraries from the sheared genome. The process was performed according to the manufacturer instruction (Publication Part Number MAN0006846, Revision 3) as following:

2.7.1.1 Amplicons purification (Agencourt AMPure XP Reagent)

After resuspension of Agencourt AMPure XP Reagent at room temperature for 30 min, 36 µl (1.8 × sample volume e.g. 20 µl) of the resuspended Agencourt reagent was added to the sheared genome. Following mixing the bead suspension with the DNA and incubation at room temperature for 5 min, the tube was placed on a magnet (DynaMag2, Magnet, Thermo Fisher Scientific) until the solution became clear. The supernatant was removed without disturbing the pellet. Two washing steps with ethanol were done with using 12 µL of freshly prepared 70% ethanol when the tube was still on the magnet. The clear supernatant was removed and the samples were kept on the magnet for 3 min to air-dry the beads. To elute the purified DNA, 8 µl Nuclease-free Water was added directly to each bead pellet to disperse the beads without using the magnet. After pipetting the mixture, the tube was returned to the magnet. Supernatant (6 µl) containing the purified amplicons was transferred into the new centrifuge tube. The purified PCR products were stored at -20 ˚C.

2.7.1.2 Quantification of short amplicons

The concentrations of purified amplicons were quantified through this assay accurately. It was performed using Qubit assay based on the manufacturer instruction. The Qubit

dsDNA HS Assay Kit (Invitrogen, Thermo Fisher scientific) was designed specifically for appyling in Qubit 2.0 Fluorometer instrument (Thermo Fisher scientific).

2.7.1.3 End-repair

End repair was needed to ensure that each amplicon was free of overhangs, and contained 5’ phosphate and 3’ hydroxyl groups. End-repair reagents were provided in the Ion Plus Fragment Library Kit (Cat, 4471252). According to the related protocol, 10-100 ng of the short amplicon in a total volume of 79 µl Nuclease-free Water for 100 µl reaction volume should be prepared (Table 2.13). The end-repair reaction was incubated for 20 min at room temperature. Then the sample proceeded with another purification step.

Table 2.13 End-repair composition

Component	**Volume**
Short amplicons (10-100 ng)	79 µl
End Repair Buffer (5X)	20 µl
End Repair Enzyme	1 µl
Total Master Mix	21 µl
Total	100 µl

2.7.1.4 Another purification step for amplicons

This purification was slightly different from the previous one. In both washing steps, 100 µl of freshly prepared 70% ethanol was used. Another difference was in the elution step. To elute the purified DNA, 7 µl Low TE was added to each bead pellet and then 5 µl supernatant containing the purified amplicons was transferred to new tube. The purified sample was stored at -20 ˚C.

2.7.1.5 Ligate adapters and nick-repair

The reagents were combined as indicated in Table 2.14 for barcoded libraries, and then were mixed well. Then the prepared mixture was incubated in TC-PLUS Thermal Cycler (Techne, Staffordshire, UK) for 15 min at 25 ˚C and followed by another incubation for 5 min at 72 ˚C to carry out the ligation of the adapters to blunt-ended library fragments. The nick-repair was performed to yield library fragments flanked by adapters.

Table 2.14 Barcoded libraries

Component	Volume
DNA	25 µl
Ligage Buffer (10X)	10 µl
Ion P1 Adapter	2 µl
Ion Xpress Barcode*	2 µl
dNTP Mix	2 µl
Nuclease-free Water	49 µl
DNA Ligase	2 µl
Nick Repair Polymerase	8 µl
Total	100 µl

*Barcode chosen

2.7.1.6 Purify the adapter-ligated and nick-translated DNA

Agencourt AMPure XP Reagent (28 µl = 1.4 × sample volume e.g. 20 µl) was added to the adapter-ligated and nick-translated DNA. The rest of purification procedure was perfomed as described in section 2.7.1.1. The supernatant (4 µl) containing the eluted DNA was transferred to the new tube. The purified DNA was kept at -20 ˚C for future use.

2.7.1.7 Quantifying the prepared library using Bioanalyzer

Agilent 2100 Bioanalyzer instrument (Agilent Technologies Inc., USA) and Agilent High Sensitivity DNA Kit were used to assess the library size distribution. The molar concentration of library (pmol.L^{-1}) was determined by using the Bioanalyzer software (Agilent 2100 Expert). This assay was repeated for diluted libraries (1/10, 1/100 and 1/1000) due to the high concentration of library which was quantified in the first run. The DNA library was prepared at the highest possible concentration (27 pM). The dilution factor that resulted in a concentration of ~20 pM was calculated with using the following formula. This concentration is suitable for downstream template preparation:

Dilution factor = (Library concentration in pM)/20 pM

The diluted library was stored at 4 °C no longer than 48 h. This library dilution was applied in template preparation using an Ion template preparation kit.

2.7.2 Template preparation

Library was used as a template for clonal amplification on Ion Sphere Particles during the emulsion PCR according to the Ion OneTouch™ 200 Template Kit v2 manual (Publication Number 4478372-revision B) in the Ion OneTouch™ Instrument. The Ion OneTouch System (Life Technologies, Thermo Fisher scientific) includes the Ion OneTouch Instrument and the Ion OneTouch ES Instrument. The template-positive ISPs was enriched with the Ion OneTouch™ ES.

2.7.2.1 Ion Sphere Quality Control

The Ion Sphere™ Quality Control program on the Qubit 2.0 Fluorometer (Thermo Fisher scientific) labelled the Ion Sphere Particles (ISPs) with two different fluorophores, Alexa Fluor 488 and Alexa Fluor 647. The ratio of the Alexa Fluor 488 fluorescence (all ISPs present) to the Alexa Fluor 647 fluorescence (templated ISPs) yields the percentage of templated ISPs. The percentage between 10 and 30 considered as the optimal amount

of library. This assay was performed according to the manufacturer instruction in Ion Sphere Quality Control guide (Life Technologies, Thermo Fisher scientific).

2.7.3 Run the sample on the PGM

The template ISPs in an optimum concentration was loaded onto an Ion 316 chip, and subsequently sequenced on PGM using 105 sequencing cycles according to the Ion Sequencing 200 kit user-guide. One hundred and five sequencing cycles resulted in an average reading length of 200 nucleotides approximately.

2.7.4 Sequencing data analysis

The sequencing data as well as the summary of the details of the run were available through the Ion Reporter 5.0 software provided on the Ion Reporter server system. Assembled DNA sequences data in FASTA format from the Ion Reporter 5.0 software was submitted online to xBASE Annotation Service (http://static.xbase.ac.uk) which provided the comprehensive coverage of all bacterial genomes. The phylogenetic analysis was carried out by aligning amino acid sequences of *comQXPA* cluster from *B. licheniformis* NCIMB 8874 strain with homologues proteins from other *Bacillus* which were obtained from NCBI nucleotide/protein database using “Clustal Omega” as a multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

CHAPTER III

RESULTS

Chapter III

RESULTS

3.1 Introduction

The focus of this chapter is on the detection, purification, and partial identification of a putative QSM (ComX pheromone) of *B. licheniformis*, an industrial antagonistic bacterium, and also investigation of the effects of the bacterial strain cells and its QSM on the growth of the plant pathogenic fungus, *A. flavus* (both aflatoxigenic and non-aflatoxigenic strains). This research led to the detection and purification of a pheromone, a small QSM oligopeptide, in *B. licheniformis* for the first time. Following this, the research was directed at the investigation of the inhibitory activity of *Bacillus* QSM as a natural antifungal agent. The ComX pheromone has the potential to be used in developing a bio-control strategy in agriculture. In this context, the chapter begins to present the results of all experiments including molecular biology and chemical compound purification studies on *Bacillus*. This chapter also covers the morphological studies on *Aspergillus* colonies under different treatments to investigate the potential inhibitory effects of *B. licheniformis* and its culture supernatant.

The result chapter is divided into ten main sections. Molecular biology experiments on *B. licheniformis* including QS gene cloning and over-expression are studied in section 3.2. Pheromone extraction, purification and detection are presented in section 3.3. Next section (3.4) describes aflatoxin extraction and verification from *Aspergillus* aflatoxigenic and non-aflatoxigenic strains which were used in the experiments explained in the next two sections. Sections 3.5 and 3.6 show the results of the investigation on *Bacillus* and its QSM effects on *Aspergillus* growth-rate and biomass, respectively. Sections 3.7 and 3.8 are devoted to the results of two assays, β-galactosidase assay and antifungal susceptibility assay, on the purified pheromone respectively. Transcriptomic studies on the effect of *B. licheniformis* and its QSM on the expression of aflatoxin gene are presented in section 3.9. Finally, the genomic studies of *B. licheniformis* QS genes are presented in section 3.10.

3.2 Molecular biology studies on *B. licheniformis* to clone and over-express the QS genes

According to the previous studies on *B. subtilis*, ComX pheromone is a 10-amino-acid peptide (Okada *et al*., 2005). Two genes, *comQ* and *comX*, are required for ComX pheromone biosynthesis. Based on the information on *B. licheniformis* sequencing of QS genes (De Vizio, 2011), this study was extended to investigate *B. licheniformis* QSM (ComX pheromone) following a gene cloning procedure and pheromone purification methods which have been adopted during the last decade on *B. subtilis* (Okada *et al*., 2005).

Primers were designed for the genes encoding the ComX pheromone (Figure 3.1), using the genome sequence from *B. licheniformis* NCIMB 8874 (De Vizio, 2011). Introducing two restriction sites, BamHI and NedI, in primers' sequence led to the expectation of, approximately, 1070 bp fragments as the PCR product (Figure 3.2). The recombinant plasmid was used to transform the *E. coli* strains (TOP10 and BL21 (DE3)) in order to clone and over-express *comQX* genes. The double digestion on plasmid and *comQX* PCR product (using BamHI and NedI) and ligation reaction to ligate the digested plasmid and insert were performed before carrying out *E. coli* TOP10 transformation. To verify that proper digestion was done on the PCR fragments and the plasmids, these digested products were run on 1% agarose gel electrophoresis (Figure 3.3).

Subsequently gel extraction method was performed to purify the digested plasmid and the insert. Following the ligation reaction, transformation of TOP10 was carried out using the whole ligated products. In order to verify the positive Top10 colonies (harbouring the ligated plasmid-insert), each colony was examined by *T7* colony PCR (Figure 3.4). The confirmed positive colonies were used to purify the recombinant plasmids for sequencing, as well as transforming competent *E. coli* BL21 (DE3) cells to over-express the *comQX* genes. DNA sequencing was used to confirm the correct sequence of the insert in the plasmid. The sequenced *comQX* genes were aligned with reference genomes of *B. licheniformis* using the BLAST algorithm. This alignment represented 99% identities and 86% query coverage between the sequenced genes and

comQX genes in *B. licheniformis* 9945A (Figure 3.5). All molecular biology experiments were described in details in section 2.5.

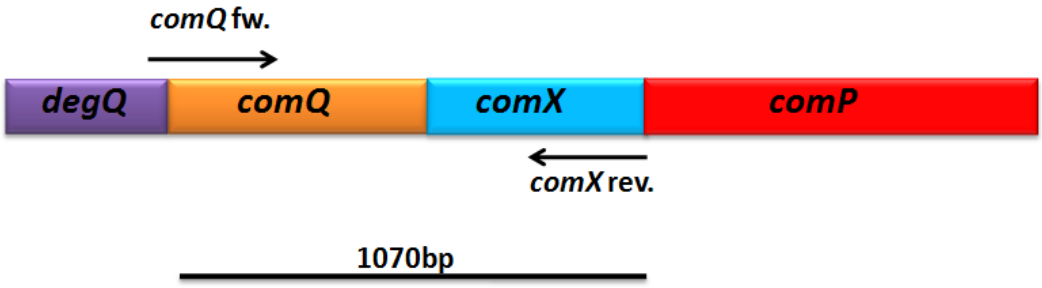


Figure 3.1 Schematic representation of primer design for *comQX* amplification and expected PCR product size. The sequences of the designed primers which used for amplifying the interested genes are presented below:
comQ forward primer: ACGTCATATGAATCATTTTATAGACGTTGAGATTCC
comX reverse primer: ACGTGGATCCTTATTTGAACCATAAATTAGGGTAAG

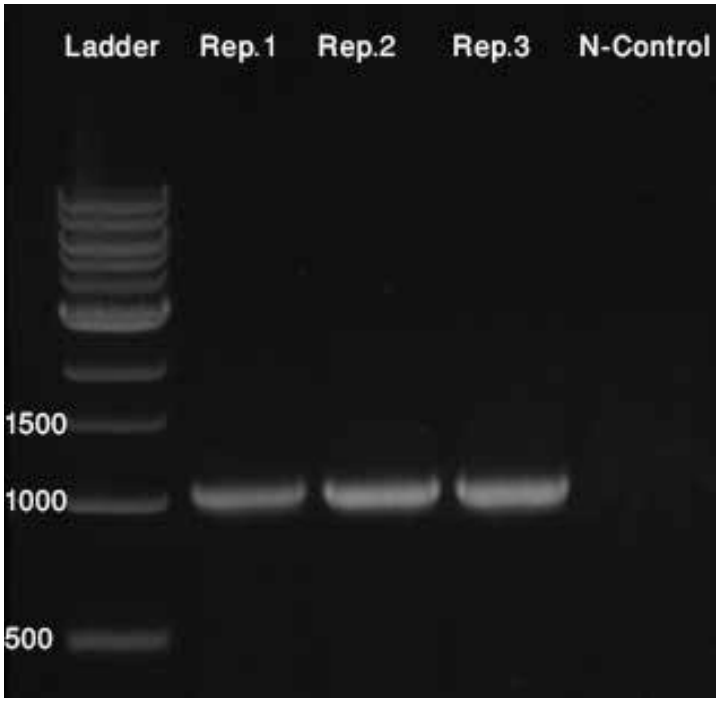


Figure 3.2 Verification of *comQX* PCR products (1070 bp) from *B. licheniformis* NCIMB 8874. DNA ladder (1 Kb) was used (Biolabs). The numbers indicate the size (bp) of the marker. Rep1, Rep2 and Rep3 are three products of PCR reaction replicates (1070 bp). The last lane is indicated as the negative control (N-Control) of PCR reaction without DNA template.

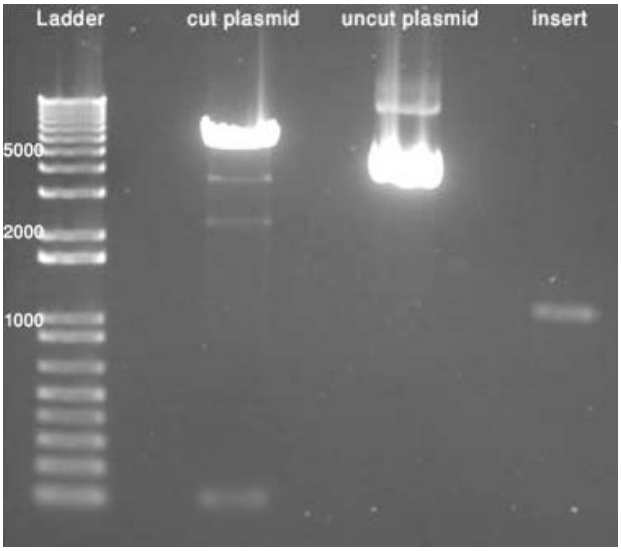


Figure 3.3 The products obtained from the restriction digestion (using restriction enzymes, BamHI and NedI) of plasmid and PCR product (insert) on 1% agarose gel electrophoresis. 1 Kb Plus DNA ladder (Invitrogen) was used as a marker. The numbers indicate the size (bp) of the marker.

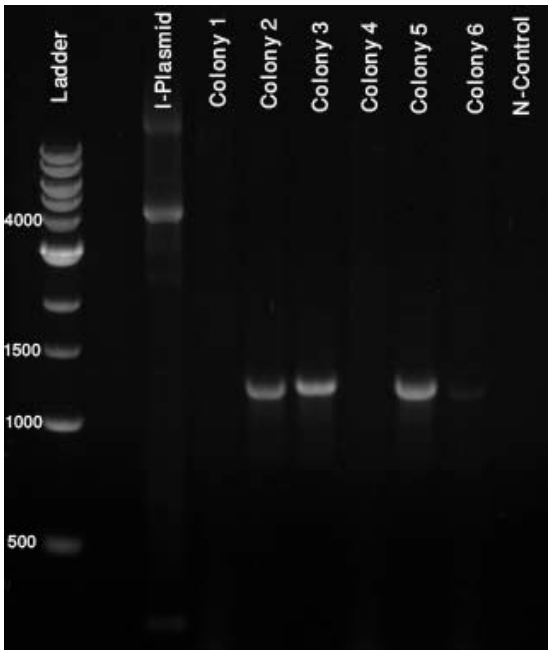


Figure 3.4 Colony PCR products on 1% agarose gel electrophoresis. Ladder was 1 Kb DNA (Biolabs). The numbers indicate the size (bp) of the marker. Intact plasmid and negative control is labelled as I-Plasmid and N-Control. Colonies 2, 3, 5 and 6 show the fragments in the predicted size (~1400 bp which comprises inserts (1070 bp) and *T7* covering area in the plasmid (334 bp)) to confirm successful ligation of the insert with pET22 (b)

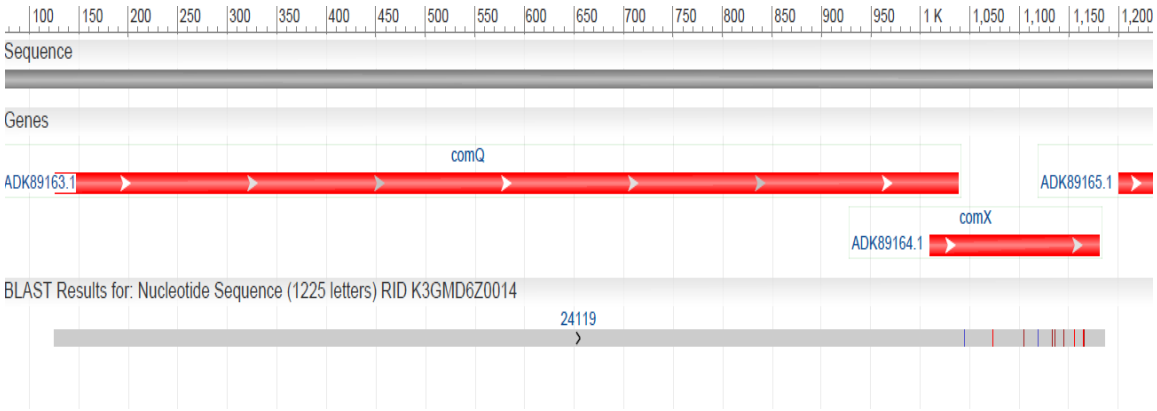


Figure 3.5 Graphics of the BLAST alignment of the sequenced *comQX* genes with *B. licheniformis* 9945A

3.3 Pheromone over-production, extraction, purification and detection

3.3.1 Pheromone over-production (using the transformed *E. coli* BL21 (DE3)) and extraction

The *E. coli* expression strain (BL21 (DE3)) transformed by recombinant plasmid (carrying *comQX* genes) was grown in 5 batches of 2 L shaken flasks. Incubation was continued after the addition of the inducer, IPTG, overnight to allow *comQX* genes expression. Before and after the addition of IPTG, 50 ml samples from each of the 2 L cultures were collected, and the filtered supernatants were stored at 4 °C to use as supplements to the fungal cultures (to study fungal growth-rate). The culture (pooled to a total of 10 L) was centrifuged and filtered to be used for pheromone extraction. The same procedure was performed for culturing the non-recombinant *E. coli* BL21 (DE3) as a control.

Reverse-phase chromatography method was used for the initial separation and concentration of the filtered supernatant from *E. coli* cultures using Diaion HP-20 as a polymeric adsorbent resin. After the loading of the supernatant of the *comQX* over-expressed *E. coli* culture, the elution solution was collected using absolute acetonitrile and then dried for future use (details explained in section 2.4.2) (Figure 3.6).

Figure 3.6 Resin column reverse-phase chromatography used for the initial extraction of pheromone

3.3.2 Pheromone purification, detection and partial identification

3.3.2.1 Pheromone detection and collection using preparative High Performance Liquid Chromatography (HPLC)

The dried extract from reverse-phase chromatography of the transformed *E. coli* BL21 culture supernatant was analysed using a gradient HPLC programme (section 2.4.2.4). The samples of ComX pheromone were collected and detected. To find the standard peptide sequences for the HPLC run, the whole genome sequence of *B. licheniformis* NCIMB 8874 and the sequence of the recombinant plasmid (carrying *comQX*) were studied. The potential pheromone sequence as standard 1 was obtained from the whole genome sequence of *B. licheniformis* NCIMB 8874 and the corresponding amino acid sequences were identified through xBase (Chaudhuri and Pallen, 2006). This sequence was compared also through BLAST to *B. licheniformis* 9945A ComX sequence with 100% identity. The amino acid sequence of standard 2 was based on the sequence of the recombinant plasmid. These sequences (Table 3.1) were

used to synthesize the standard pheromone samples which were applied to confirm the appropriate retention time of pheromone in HPLC run. Based on the retention time of the two standard samples, the most active fractions (retention time for standard 1 was 14.5-15.5 min and for standard 2 was 12.5-13.5 min) were collected and purified using the HPLC's Automated Fraction Collector Dionex UltiMate 3000 (Thermo Fisher Scientific). The pheromone molecule was detected at 210 nm. All the chromatograms of the standards and the extracted samples are presented in the Figures 3.7, 3.8 and 3.9.

Table 3.1 Oligopeptide molecules used as standard samples in HPLC. The sequences of these molecules were identified as potential sequences of ComX pheromone based on genome sequence of *B. licheniformis* NCIMB 8874 and recombinant plasmid.To confirm the appropriate retention time of pheromone in HPLC run, the standard samples are essential.

Standards	Amino acid sequences
Standard 1	**WGPYPNLWFK** (Trp-Gly-Pro-Tyr-Pro-Asn-Leu-Trp-Phe-Lys)
Standard 2	**KSWGGGGFWI** (Lys-Ser-Trp-Gly-Gly-Gly-Gly-Phe-Trp-Ile)

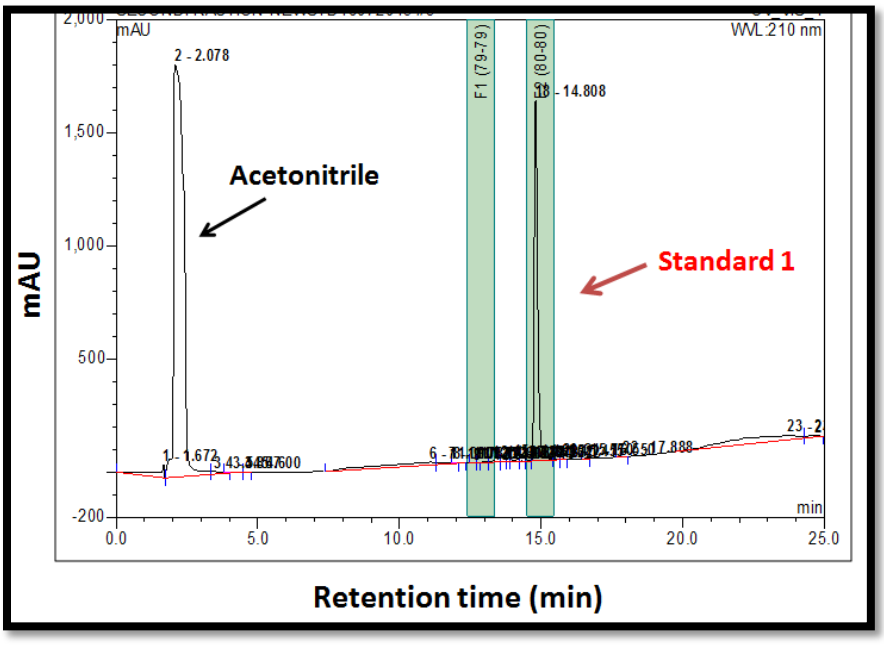


Figure 3.7 Chromatogram of standard 1 molecule (400 µg.ml^{-1}) during gradient HPLC. Analysis was carried out using a C18 silica HPLC column and the molecule was eluted after 14.8 min (retention time). The labelled peak shows standard 1 molecule in the chromatogram.

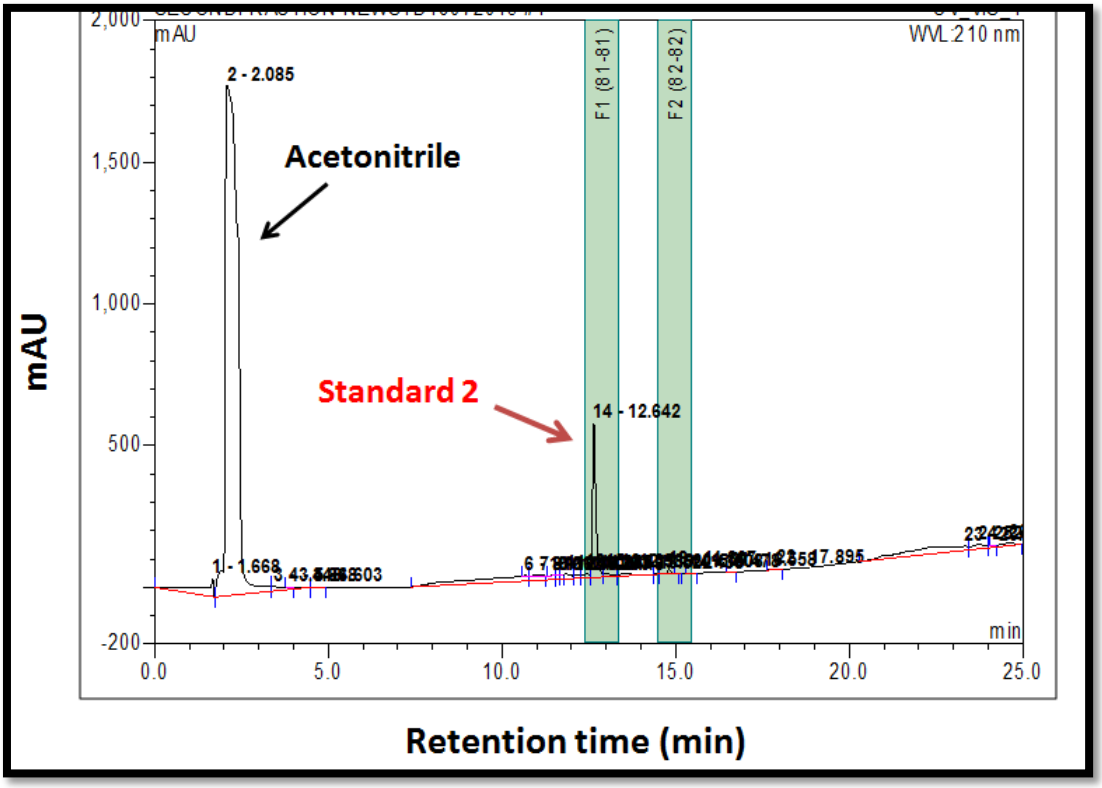


Figure 3.8 Chromatogram of standard 2 molecule (200 µg.ml^{-1}) during gradient HPLC. Analysis was carried out using a C18 silica HPLC column and the molecule was eluted after 12.6 min (retention time). The labelled peak shows standard 2 molecule in the chromatogram.

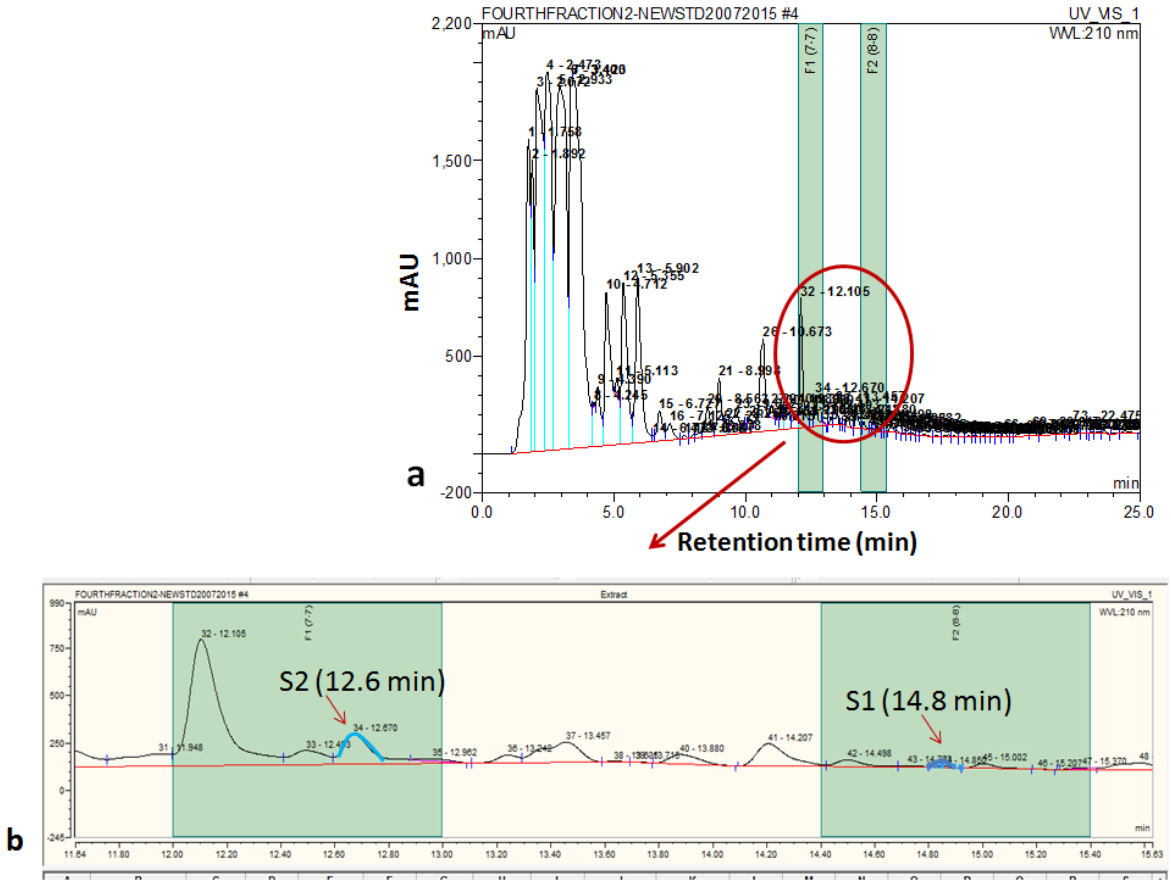


Figure 3.9 Chromatogram of the filtered extract supernatant of transformed *E. coli* BL21 (carrying *comQX*) culture during gradient HPLC. (a) the chromatogram shows 25 min HPLC run (b) the zoomed fraction areas of one run. Collection of the samples was performed from two fractions (presented in green time windows). First fraction was collected between 11.90 min and 12.90 min to cover the specific retention time of standard 2 (12.6 min). Second fraction was collected between 14.40 min and 15.40 min according to the retention time of standard 1 (14.8 min). The red arrows presented in image b (S1 and S2) point at the interested peaks in the fractioning time which are representing the retention time of standard 1 and standard 2.

3.3.2.2 Pheromone identification using MS/MS and MALDI-MS

MS/MS and MALDI-MS were used to determine the mass spectrometry of the collected samples from the preparative HPLC in the two defined retention times. Based on the results, the amino acid sequences were identified of the two molecules which were presented in the collected samples from the specific retention time. There were two dominant ions in the MS spectrum at 1209.74 mz^{-1} (Mass 2) and 1564.78 mz^{-1} (Mass 1) (Figure 3.10). The peptide sequences of these ions were identified confidently (Figures

3.11 and 3.12). The amino acid sequence of Mass 1 (EAGWGPYPNLWFK) is strongly matched with the sequence of standard 1 (WGPYPNLWFK). Although the peptide sequence of Mass 2 (FSLIEGFKRI) was not comparable with the standards' sequence, it was identical with the sequence of the ComX precursor in *B. licheniformis* 9945A, available on the UniProt (D9YRL0) (MQEIVSFLVEHPEVLEQVIAGKASLIGVDKDQVFSLIEGFKRIEAGWGPYPNLWFK).

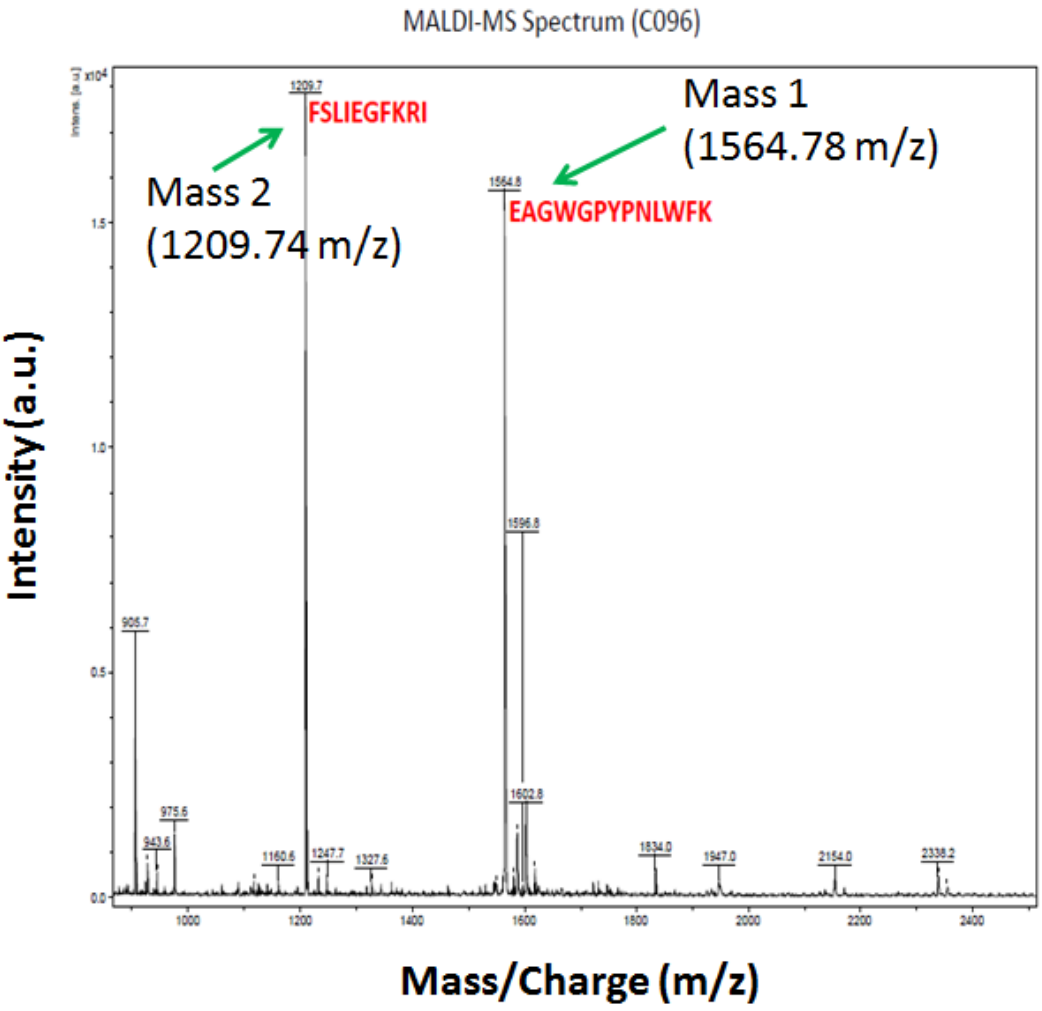


Figure 3.10 MALDI-MS Spectrum of the collected sample from HPLC. Mass 1 and Mass 2 are labelled to indicate two dominant ions in the MS spectrum

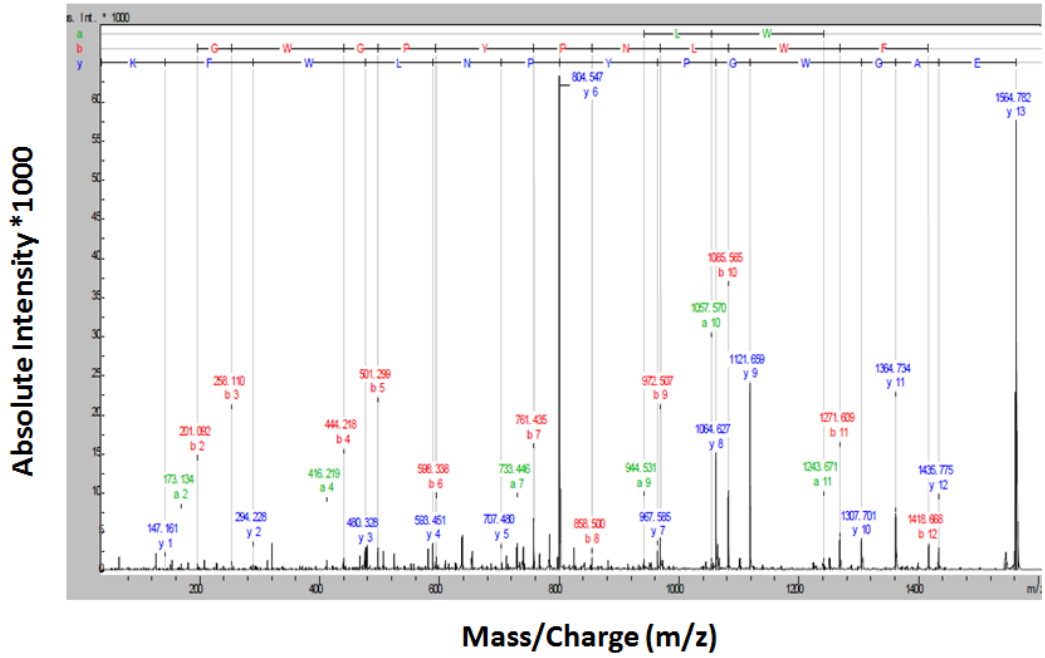


Figure 3.11 MS/MS spectrum of Mass 1 which is shown as one of the dominant ion peak in the MALDI-MS spectrum in Figure 3.10. This spectrum presents the amino acid sequences of the molecule as following **EAGWGPYPNLWFK**.

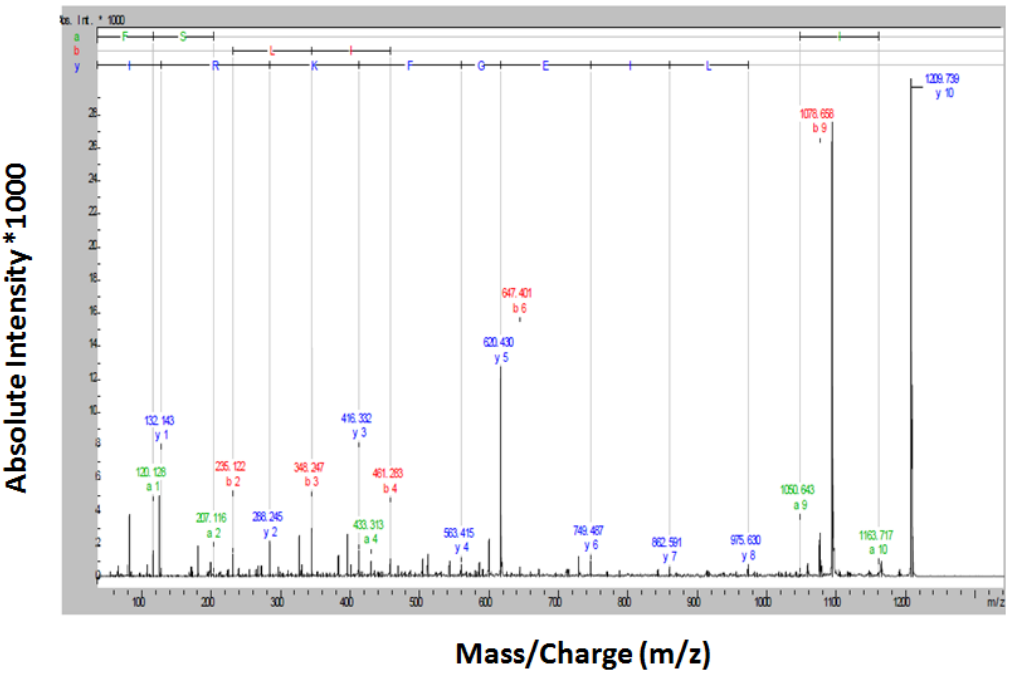


Figure 3.12 MS/MS spectrum of Mass 2 which is shown as one of the dominant ion peak in the MALDI-MS spectrum in Figure 3.10. This spectrum presents the amino acid sequences of the molecule as following **FSLIEGFKRI**.

3.3.2.3 Pheromone identification using Nuclear Magnetic Resonance (NMR)

NMR was used to determine the structure of the pheromone molecule. Purified fractions, which were positive for the presence of pheromone, were analysed using NMR for 1D analysis (700 MHz ^{1}H). As a result of the low concentration of the sample (0.26 $mg.ml^{-1}$, the sufficient concentration of sample for NMR analysis might be $> 2\ mg.ml^{-1}$) and the possible degradation of amino acids, the data was not sufficiently clear and distinctive to identify the structures of the amino acids presented in the fraction (Figure 3.13).

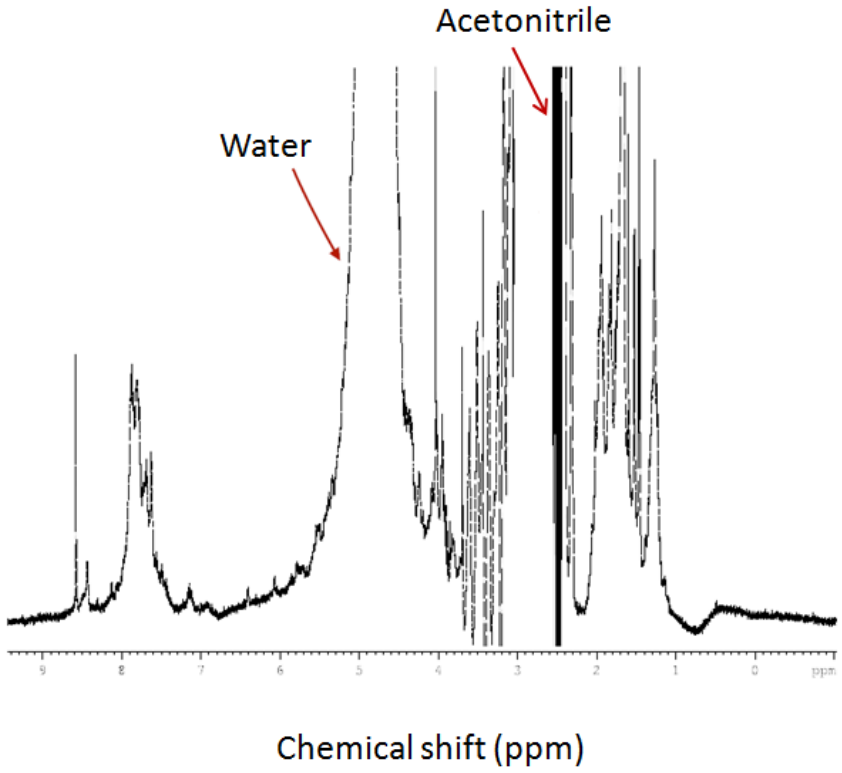


Figure 3.13 NMR spectrum of purified pheromone collected from preparative HPLC. There are no peaks relevant to the amino acids.

3.4 Aflatoxin extraction and detection from *A. flavus* strains

In order to determine the presence of aflatoxin in the aflatoxigenic strain of *A. flavus*, the putative aflatoxin was extracted from *A. flavus* NRRL 3357 (aflatoxigenic strain) and ESP 15 (non-aflatoxigenic strain) cultures (section 2.4.6) and detected using Thin Layer Chromatography (TLC) method following the protocol described in section 2.4.7. The

obtained result from the chloroform/acetone (9:1) TLC plate is shown in Figure 3.14. After developing the aflatoxin spots, the retention factor was calculated. The retention factor (Rf) was 0.44 for both the pure aflatoxin extract from aflatoxigenic strain of *A. flavus* and the standard aflatoxin B (labeled as Control in Figure 3.14).

It is a traditional practice to use TLC to identify/detect aflatoxin on the basis of Rf value and fluorescence. In the present study, the initial detection and confirmation of aflatoxin presented in *A. flavus* NRRL 3357 isolates (AF-Pos1, AF-Pos2, AF-Pos3) is based on the similarities of their Rf values (0.44) with that of standard aflatoxin (Control). As a result of confirming the presence of aflatoxin component in aflatoxigenic strain through TLC, *A. flavus* NRRL 3357 isolate was used confidently for the fungal growth rate experiment.

Figure 3.14 TLC analysis of the extracted aflatoxin from *A. flavus* strains. The aflatoxin was extracted from 12-day fungal cultures using chloroform (see section 2.4.6). Each extract (15 µl) was analyzed by TLC using chloroform/acetone (9:1) as solvent system. When the plate was visualized under the UV light, aflatoxin compound appeared as fluorescent spot. The Control sample was the aflatoxin B standard. AF-Pos 1,2,3 present three extracts of aflatoxigenic strain and AF-Neg 1,2,3 indicate the extracts of non-aflatoxigenic strain.

3.5 Effect of *B. licheniformis* NCIMB 8874 and its QSM on the growth-rate of *A. flavus* strains

Several treatments were applied to study the effect of *B. licheniformis* NCIMB 8874 and its over-expressed pheromone on the growth of *A. flavus* strains. All the treatments with their associated concentrations (Table 2.5 and Table 3.4) were added to the allocated plates before pouring the melted PDA medium (section 2.4.8). The spot inoculated plates with fungal spore suspention were incubated at 27 °C for 7 days. The images of the plates were taken every 24 h during the 7-day study. Image analysis of the photos of plates is described in section 3.5.1. All results are presented as mean ± standard error of two independent experiments performed in triplicates.

Four models (linear, power law, exponential and linear with y-fixed intercept) were fitted to the data points of tests and controls to find the best fit. To check the goodness of each model, every observed distribution was compared to its fitted model using the calculated P-value. All the comparative data (min, max and mean of P-values) are presented in Table 3.3. According to the overall statistics associated to the distribution of P-values in all plots, the best fit is linear without y-fixed intercept ($y = a*x + b$) with the mean of P-values of 0.999. Although the linear regression without y-fixed intercept is the best fit, any of these models arrive at the same conclusion.

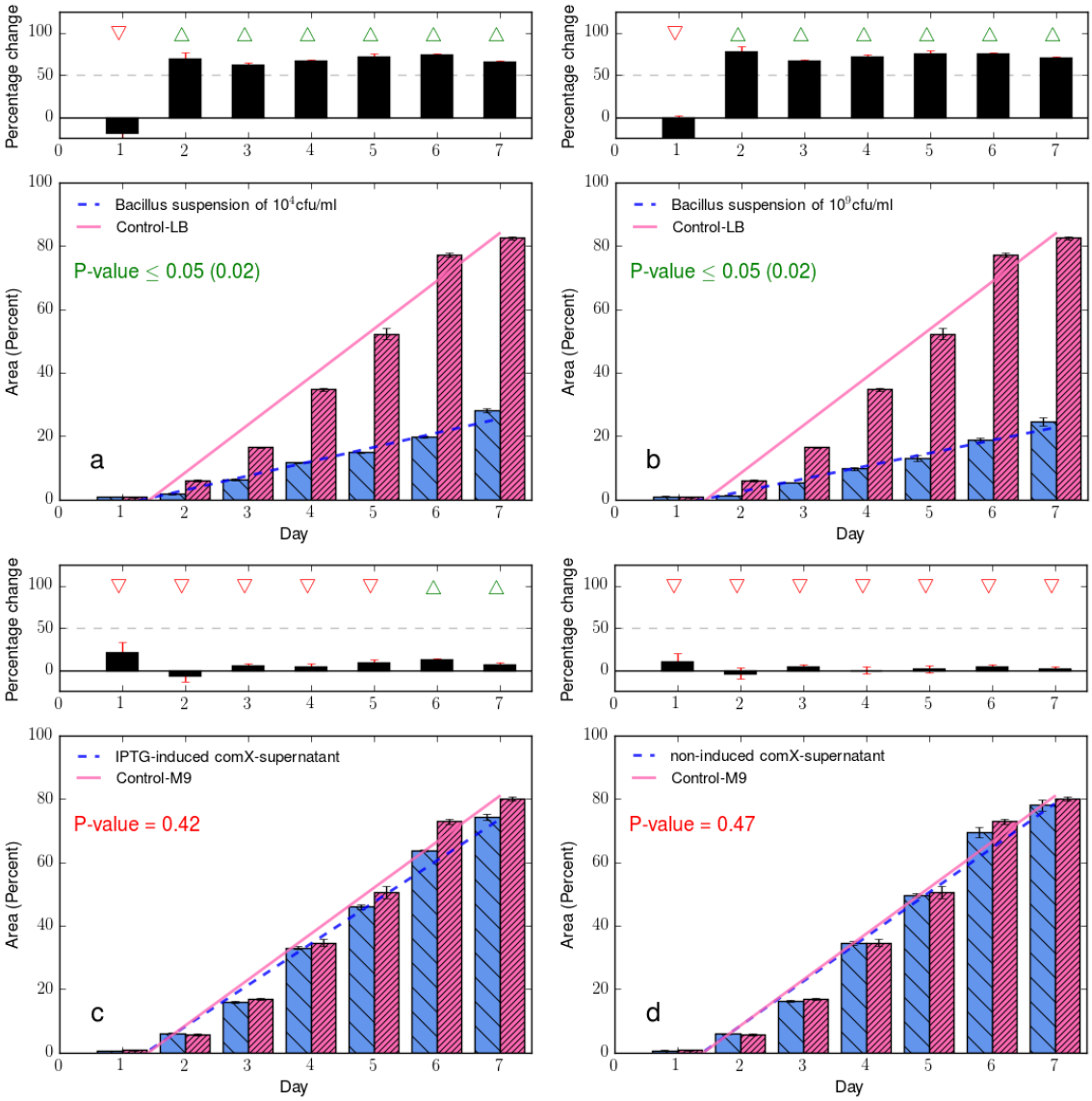
All the fungal growth profiles and the percentage changes in the fungal colony area of the tests compared to their associated controls during the 7 days are presented in Figure 3.15. The line of best fit for each group of data (test and control separately) is presented in the same growth chart in Figure 3.15. The slops of these lines are shown in Table 3.2. The profiles and slops are named according to the used treatments from (a) to (K) (refer to the Table 2.5 for the complete description of the treatments).

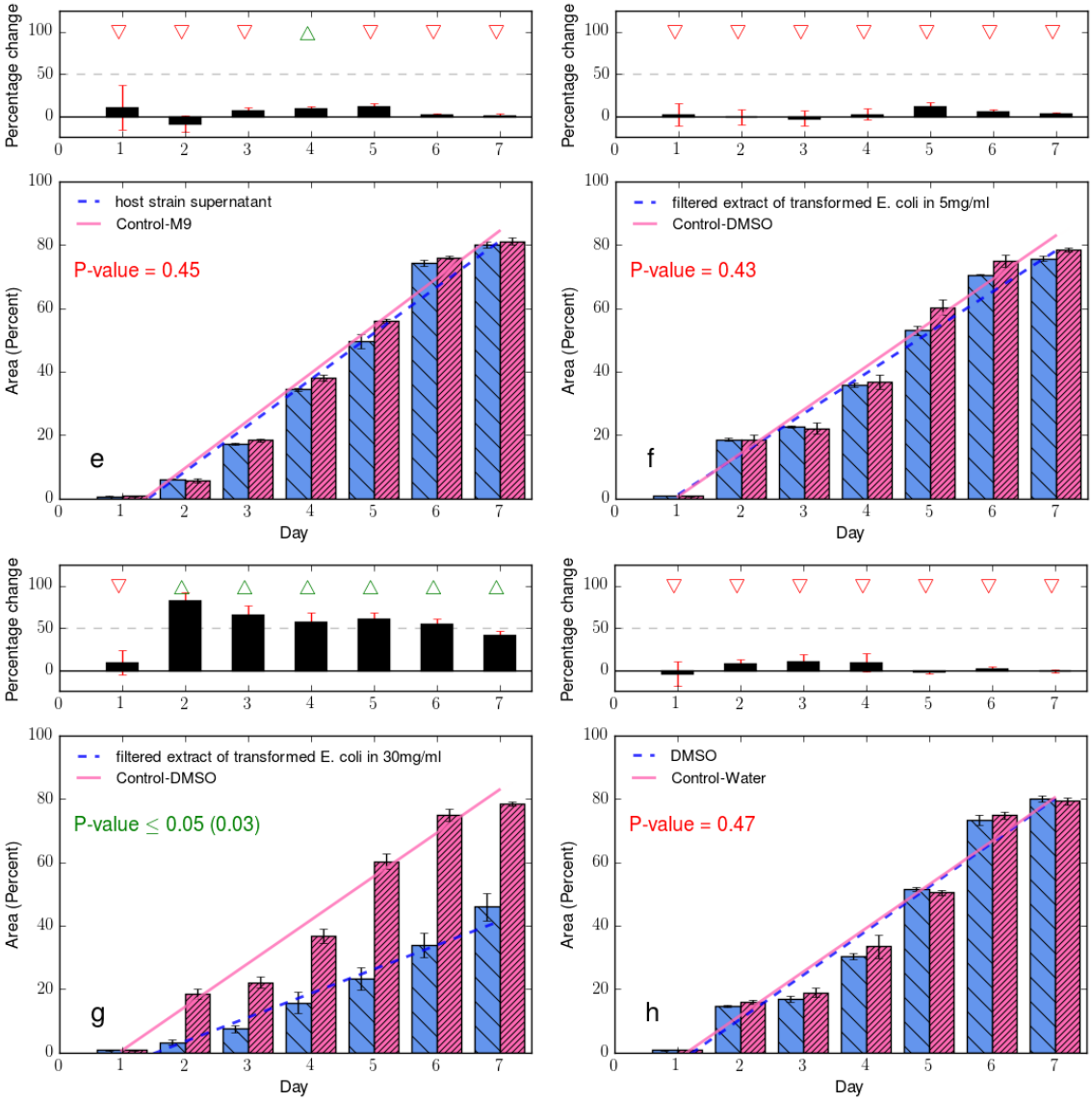
The results reveal that out of the 11 treatments, only four of them cause a notable reduction in the fungal growth-rate. The results rank in descending order as following; *Bacillus* suspension of 10^9 cfu.ml^{-1} (a), *Bacillus* suspension of 10^4 cfu.ml^{-1} (b), filtered extract of transformed *E. coli* in 30 mg.ml^{-1} (g) and washed *Bacillus* suspension in 10^9 cfu.ml^{-1} (k). These four treatments cause the high daily reduction (40-70%) in the fungal

colony area of the tests in comparison to their controls. However, only the first three tests (a,b,g) show statistically significant changes overall one week of fungal growth compared to the controls ($P \leq 0.05$). The slopes of the lines of best fit for these four treatments are notably steeper compared to the slopes of the associated controls. This shows the evident change in the fungal growth-rate as a result of the presence of these treatments on the fungal plates. The test slopes range from 4.06 to 7.87, whilst the control's slopes range from 13.73 to 15.12. There were smaller changes in the test growth-rate compared to the controls for the IPTG-induced *comX*-supernatant (c) and *Bacillus* supernatant at the exponential phase (i). Their slopes are 13.09 (control is 14.5) and 13.81 (control is 15.12), respectively. The rest of the treatments show a negligible inhibitory effect on the fungal growth. Similar results were obtained from non-aflatoxigenic strain of *Aspergillus* (Please see the Chapter Appendices).

Besides, the average increase of fungal colony area per day measured during 5 days of monitoring test plates between day 2 and 7 which presents in Figure 3.16. The results of average increase in fungal growth over 5 days are comparebale with the results of fungal colony area changes (Figure 3.15). The presence of *Bacillus* suspension of 10^9 $cfu.ml^{-1}$ (a) and *Bacillus* suspension of 10^4 $cfu.ml^{-1}$ (b) in the plates causes the increase of ~1200 $mm^2.day^{-1}$ in fungal area, whearse filtered extract of transformed *E. coli* at the concentration of 30 $mg.ml^{-1}$ (g) and washed *Bacillus* suspension in 10^9 $cfu.ml^{-1}$ (k) show the increase of ~2100 $mm^2.day^{-1}$. However, the fungal growth rate in presence of other treatments is ~ 3000 $mm^2.day^{-1}$ and above. Similar results were obtained from non-aflatoxigenic strain of *A. flavus* (Please see the Chapter Appendices).

Overally, the results obtained from this experiment indicate that *B. licheniformis* cell suspentions, both wased and unwashed, and its concentrated over-produced ComX pheromone reduce fungal growth rate in the solid medium. Different statistical analysis methods applied in this study (Figure 3.15 and 3.16) show identical outcomes of fungal growth area reduction as a result of the presence of different treatments in the plates.


Percentage change
Area (Percent)
Day
Bacillus suspension of 10^4cfu/ml
Control-LB
P-value ≤ 0.05 (0.02)
a
Bacillus suspension of 10^9cfu/ml
Control-LB
P-value ≤ 0.05 (0.02)
b
IPTG-induced comX-supernatant
Control-M9
P-value = 0.42
c
non-induced comX-supernatant
Control-M9
P-value = 0.47
d


Percentage change
Area (Percent)
Day
host strain supernatant
Control-M9
P-value = 0.45
e
filtered extract of transformed E. coli in 5mg/ml
Control-DMSO
P-value = 0.43
f
filtered extract of transformed E. coli in 30mg/ml
Control-DMSO
P-value ≤ 0.05 (0.03)
g
DMSO
Control-Water
P-value = 0.47
h

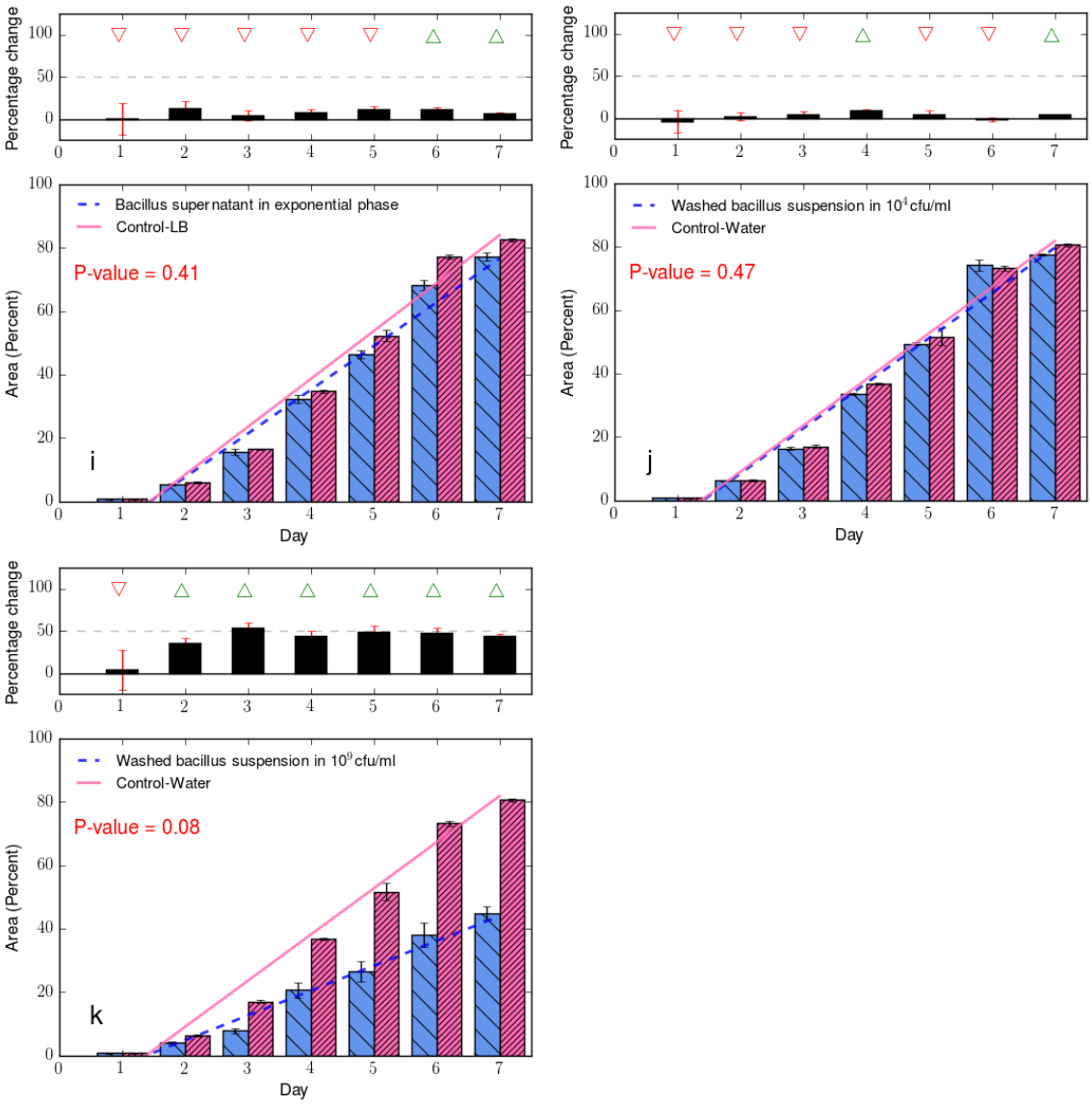


Figure 3.15 Fungal colony area changes during 7 days of monitoring test plates and associated control plates. Tests labelled from (a) to (k) (Table 3.4). The line of the best fit is drawn for both sets of data (test/control) in each profile. Red dotted line is associated to the control and blue dashed line is associated to the test. The written P-value for the growth profile shows the statistical significance of the overall change of the test compared to its control measured over the 7 days. The upper plot shows colony area percentage changes between test and its control in each individual day of monitoring period. The statistical significance of the percentage change is shown in green and red triangles. Green upward triangle represents the significant difference between test and control per individual day ($P \leq 0.05$) otherwise it is red downward triangle.

Table 3.2 The slope values of the best fit lines of the fungal growth data obtained from the tests and their associated controls. In the observations column, the notable differences between the test and the control slopes (the ratio of over 1.8) are shown as * and the negligible differences are presented as N.

Test labels	Slope1 (Test)	Slope2 (Control)	Ratio of the slopes (Control/Test)	Observations
a	4.52	15.12	3.35	*
b	4.06	15.12	3.73	*
c	13.09	14.5	1.11	N
d	14.04	14.5	1.03	N
e	14.55	14.98	1.03	N
f	12.83	13.73	1.07	N
g	7.6	13.73	1.81	*
h	13.91	13.76	0.99	N
i	13.81	15.12	1.1	N
j	14.27	14.58	1.02	N
k	7.84	14.58	1.86	*

Table 3.3 The list of P-values of each observed distribution compared to its fitted model (four possible models)

Model	Min P-value	Max P-value	Mean P-value	Standard Deviation
linear fit (y = a*x + b)	0.999	0.999	0.999	0.000
power-law ($y = a*x^b$)	0.946	0.998	0.975	0.00014
exponential ($y = a*exp^{b*x}$)	0.922	0.965	0.941	0.0001
linear fit with fixed y-intercept (y = a*x)	0.763	0.867	0.793	0.0007

Table 3.4 List of the treatments and associated controls applied to the cultures of *A. flavus* strains

Test labels	Treatments	Associated controls
a	*Bacillus* suspension of 10^4 cfu.ml^{-1}	Control-LB
b	*Bacillus* suspension of 10^9 cfu.ml^{-1}	Control-LB
c	IPTG-induced *comX*-supernatant	Control-M9A
d	Non-induced *comX*-supernatant	Control-M9A
e	Host strain supernatant	Control-M9
f	Filtered extract of transformed *E. coli* in 5 mg.ml^{-1}	Control-DMSO
g	Filtered extract of transformed *E. coli* in 30 mg.ml^{-1}	Control-DMSO
h	DMSO	Control-Water
i	*Bacillus* supernatant in exponential phase	Control-M9
j	Washed *Bacillus* suspension in 10^4 cfu.ml^{-1}	Control-Water
k	Washed *Bacillus* suspension in 10^9 cfu.ml^{-1}	Control-Water

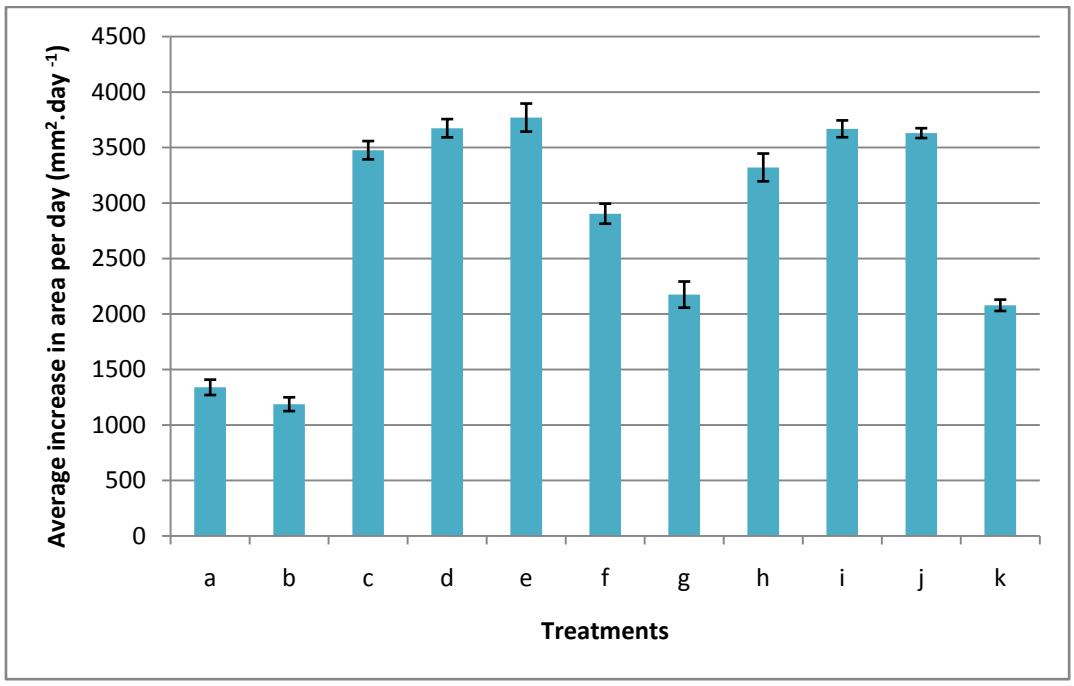


Figure 3.16 The average increase of fungal colony area per day measured during 5 days of monitoring test plates between day 2 and 7. Treatments labelled from (a) to (k) (Table 3.4).

3.5.1 Image analysis of the fungal colonies to study the effect of *B. licheniformis* NCIMB 8874 and its QSM on growth-rate of *A. flavus* strains

3.5.1.1 Image processing method

In order to investigate the inhibitory effect of the different treatments on the growth-rate of *A. flavus* strains, the fugal growth on PDA plates in presence and absence of the treatments was studied. Fungal growth was monitored over 7 days by taking the images of the plates on daily basis. A computer image processing software (ColonyAreaAnalyzer) was used in order to measure precisely the fungal colonies growth area based on their digital images. The software which has been developed especially for the current project, analysed Petri dish images. The ColonyAreaAnalyzer software was developed in Python, based on Open Source Computer Vision Library (OpenCV), a library of C/C++ programming designed for high-throughput and real-time image processing applications.

Images were captured using the UVP BioImaging System which comprised CCD (charge-coupled device) camera. Dimensions of the captured images were set to 1.3 mega pixels and were taken with an exposure time of 15 msec. On average, it took ~0.4 sec for each image to be processed on a single-core desktop computer.

Digital images of agar plates were processed to detect and characterise the morphology of the filamentous fungus *A. flavus* NRLL 3357 and ESP 15. The processing happened on the images through software initialized by image calibration. To do so, a flat field image i.e. a blank CCD image was obtained with no agar plate, but with the same exposure time as the one with an agar plate (Figure 3.17a). Image analysis and segmentation (the process of partitioning a digital image into multiple segments) were then performed in several steps (Figure 3.17).

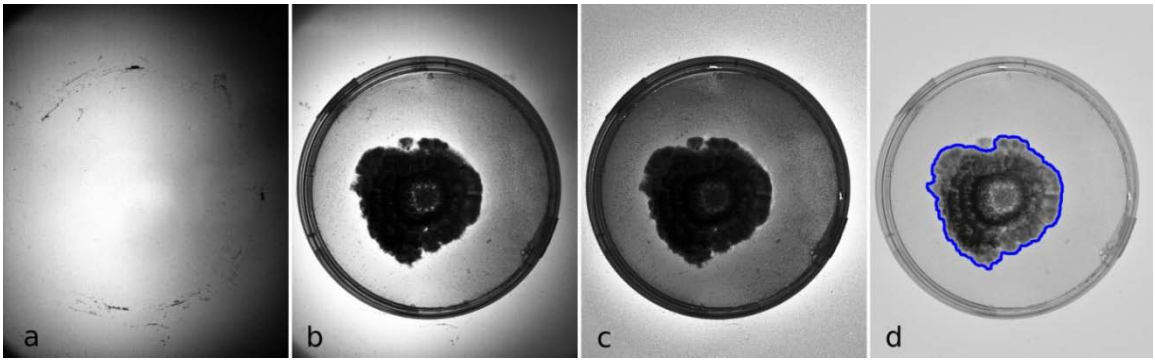


Figure 3.17 Flat field correction: (a) A blank/single-channel (flat) image of the darkroom screen. (b) Image of a Petri dish. (c) The same image as in 'b' but after applying the flat field correction. To enhance the effect of flat field correction, image contrasts have been increased to the same level. (d) A sample of the processed image where the contour represents the computed area.

Following processing of all input images, e.g. images within a given directory, the software produces draft images associated to the individual original/input images as shown in Figure 3.18 together with a final catalogue of extracted parameters (one row per each plate) describing various aspects of the detected colonies. This includes parameters like (i) plate and colony central positions, (ii) offset between the plate and colony centres, (iii) area of the colony, (iv) fraction of the plate's total area covered by the colony, (v) colony morphological parameters such as eccentricity and solidity, and (vi) colony light intensity, i.e. integrated intensities of all pixels within the colony divided by their mean value, normalized by 1.0 (see Table 3.5). In the present study, among extracted parameters, the fungal colony area was used as a percentage of the plate's area covered by *Aspergillus* colony. In future, other parameters such as light intensity could be analysed for investigation of fungal sporulation.

The image processing method developed for the present study was applied to measure fungal colony area on the solid medium accurately and easily. The calculated parameter was analyzed statistically and the outcomes are presented in section 3.5. This automated technique could lead to robust measurement of the observable parameter less laboriously and provides the possibility of keeping the record of the images and re-analysis of data.

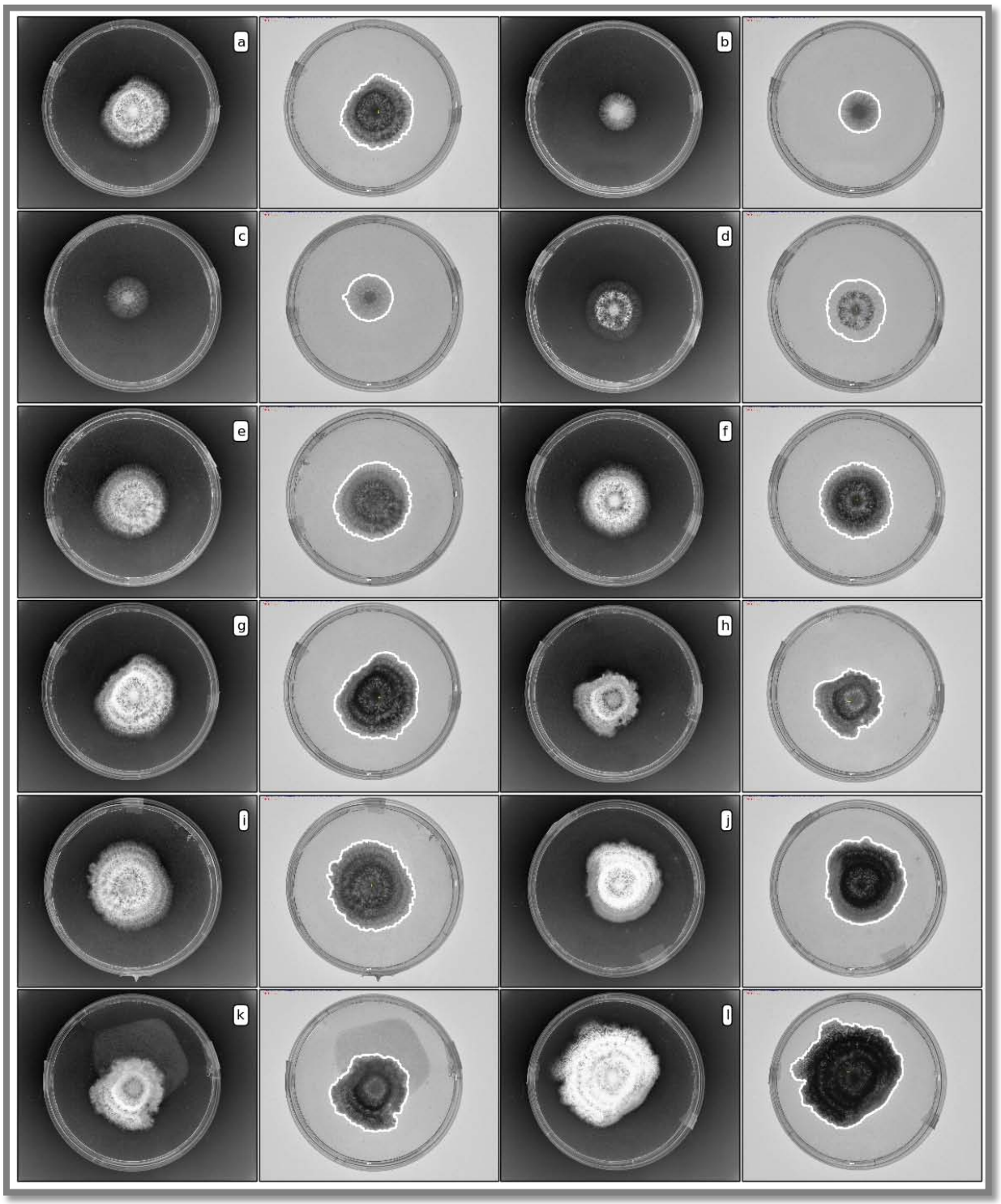


Figure 3.18 Sample of 12 input and output images from the test samples. Original labelled images are inverted in order to visually enhance the identification of the fungus colonies. Next to the original images, are flat-field corrected images where the identified colonies are marked by the white contour. Output parameters associated to each image are given in Table 3.5.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Image	DCentreX	DCentreY	FCentreX	FCentreY	Offset(pix)	Offset(mm)	Area(pix)	RelativeArea	Area(mm^2)	Eccentricity	Solidity	FFlux	FFluxStd
a	604	491	633	518	40	8	109776	0.197	3976	0.429	0.957	0.57	0.167
b	605	497	627	511	26	5	37449	0.067	1352	0.191	0.981	0.47	0.123
c	615	497	587	468	40	8	46844	0.084	1696	0.235	0.96	0.42	0.082
d	608	501	607	533	32	6	80268	0.144	2907	0.317	0.973	0.4	0.108
e	608	493	610	509	16	3	120365	0.216	4360	0.259	0.964	0.58	0.179
f	615	491	609	515	25	5	127547	0.229	4622	0.371	0.968	0.53	0.142
g	601	489	638	528	54	10	149910	0.269	5430	0.514	0.963	0.65	0.19
h	604	503	568	550	59	11	95226	0.171	3452	0.332	0.937	0.56	0.149
i	616	494	597	490	19	4	165530	0.296	5975	0.445	0.949	0.59	0.141
j	615	499	649	462	50	10	153318	0.277	5591	0.319	0.97	0.7	0.199
k	611	500	599	552	53	10	126902	0.228	4602	0.327	0.95	0.63	0.155
l	613	495	564	448	68	13	234427	0.428	8639	0.393	0.941	0.77	0.182

Table 3.5 Sample of output parameters, associated to Petri dish images shown in Figure 3.18. For each input image (column1), output parameters include: plate central position (columns 2 and 3), colony central position (columns 4 and 5), offset between the plate and colony centres in pixel/physical units (columns 6 and 7), colony area in pixel/physical units (columns 8 and 10), fraction of plate total area covered by the colony (column 9), colony morphological parameters (columns 11 and 12) and colony light intensity, i.e. integrated intensities of all pixels within the colony divided by their mean value, and its standard deviation (columns 13 and 14).

3.5.1.2 A Comparison of the fungal growth measurement techniques

Over the 7-day incubation period of the fungal plates, fungal growth was monitored every day. Two techniques for colony area measurement were applied. Besides the classic measurement method based on the manual measurement of fungal colony radii or diameters using a ruler, a computer image processing software (ColonyAreaAnalyzer) was used in order to measure precisely the fungal growth area based on their digital images.

The growth of fungal colony, on a given plate, can be quantified either manually or computationally. In the manual method, circular growth is assumed for the colony and the diameter of the colony is measured by a ruler. However due to the non-circular, asymmetric, or irregular shape of the colonies, which is frequent in real cultures, the measured colony area by manual assessment, tends to be different from the actual colony area. This is better shown in Figure 3.19, where a colony, as detected by the software (blue contour), is compared with the one observed manually (red circle).

In Figure 3.20 panels, a comparison is made between colony areas, as calculated manually and those measured computationally. Figure 3.20a shows a strong correlation between both measurements of automated versus manual data, with a Pearson correlation coefficient $r^2 \sim 0.98$, as measured from the linear regression:

Area (automatic) = 0.84*Area (manual) + 0.05.

Therefore, area measured manually is likely to be larger than the actual fungal colony area since the slope in the above equation is less than unity. This is further represented in Figure 3.20b, showing the distribution (histogram) of ratio of the manual area over automated area measurements, for each data point in the Figure 3.20a. Based on the log-normal fit to the data points in Figure 3.20b, the outcome of the fit suggests a mean of 1.2 with a standard deviation of 0.07. It is clear that in majority of cases, the manual measurement peaks around 1.2 and therefore over-predicts the actual colony area by ~20 percent. This is quantified by fitting a log-normal function to the histogram of the data points. This is a significant drawback of the manual method that could result in erroneous conclusions.

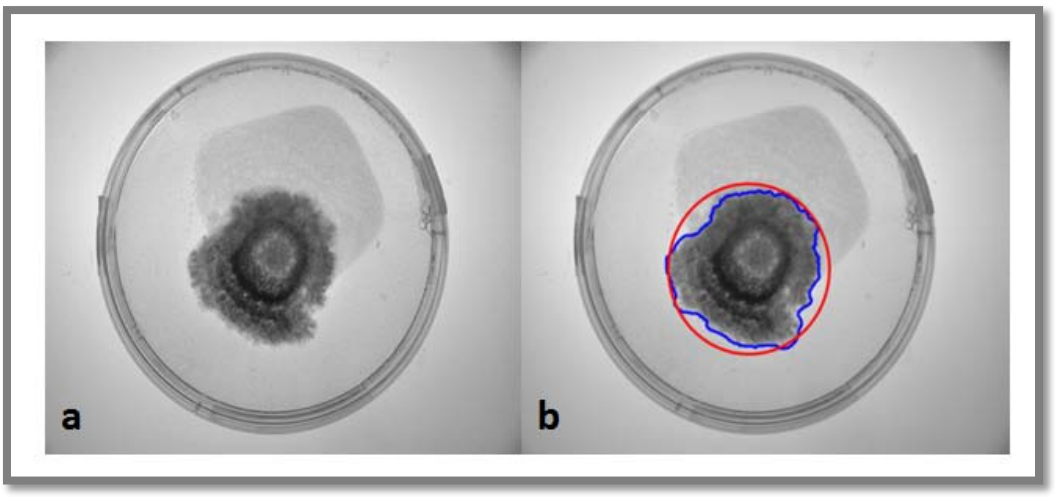


Figure 3.19 Comparison between manual and automated measuring of the fungal colony area (treated *A. flavus* NRRL3357 by IPTG-induced supernatant of transformed *E. coli*, day6). a) The original image b) The processed image; red circle indicates the manual measured area and blue contour shows the computed area.

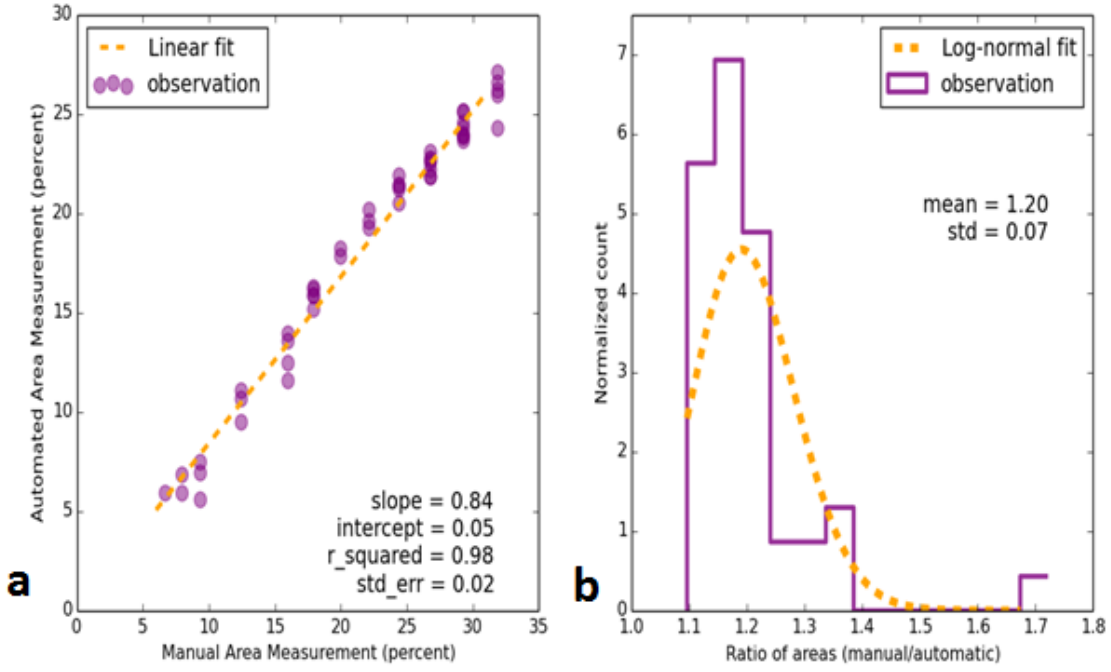


Figure 3.20 The plot of manual measurements versus the associated automatic measurements for *A. flavus* NRRL3357 growth area at day 4. a) Manual versus automatic measuring data points and its linear fit. b) The distribution (Histogram) of the ratio of the manually measured area over the associated automated one.

3.6 Effect of *B. licheniformis* NCIMB 8874 and its QSM on *A. flavus* NRLL 3357 and ESP 15 dry weight

Shaken fungal cultures were treated as described before (Table 2.5) to investigate the effects of different treatments on *Aspergillus* dry weight (section 2.4.9). The calculated fungal dry weight (*A. flavus* NRLL 3357) as a plot is reported in Figure 3.21. The list of the treatments and controls is shown in Table 3.4.

The results show significant difference ($P \leq 0.05$) between the test and the control dry weight for the following treatments (Table 2.5): high and low concentrations of the unwashed *B. licheniformis* NCIMB 8874 cells (a, b), washed high concentration of the *Bacillus* cells (K), IPTG-induced supernatant of the transformed *E. coli* cells (c) and high concentrated filtered extracts of the transformed *E. coli* BL21 (g). Other treatments did not seem to affect the biomass production significantly. The dry weight experiment on *A. flavus* ESP 15 shows the similar results to NRLL 3357 strain and is not presented here.

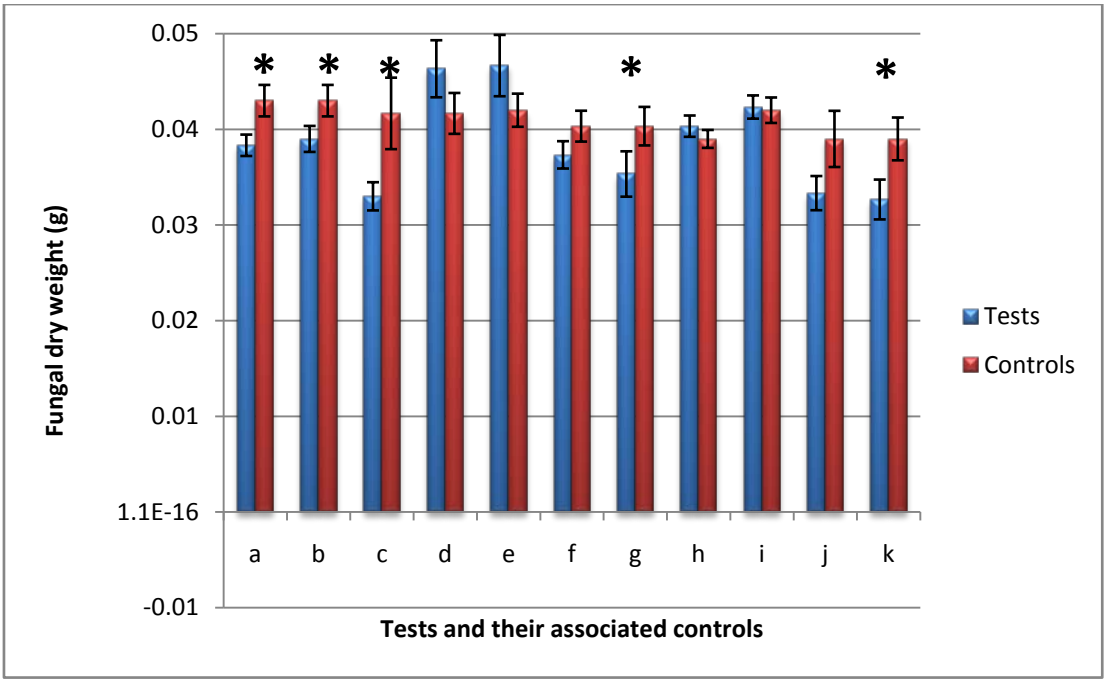


Figure 3.21 Fungal dry weight data of *A. flavus* NRLL 3357 (Calculated weight (g) of fungal dry mass which was grown in 10 ml PDB in the presence of different treatments). (*) indicates significant difference between the test and the control (P≤0.05). All the treatments and associated controls (a-k) are listed below:

a *Bacillus* suspension of 10^4 cfu.ml^{-1}- Control LB
b *Bacillus* suspension of 10^9 cfu.ml^{-1}- Control LB
c IPTG-induced *comX*-supernatant- Control M9A
d Non-induced *comX*-supernatant- Control M9A
e Host strain supernatant- Control M9
f Filtered extract of transformed *E. coli* in 5 mg.ml^{-1}- Control DMSO
g Filtered extract of transformed *E. coli* in 30 mg.ml^{-1}- Control DMSO
h DMSO- Control Water
i *Bacillus* supernatant in exponential phase- Control M9
j Washed *Bacillus* suspension in 10^4 cfu.ml^{-1}- Control Water
k Washed *Bacillus* suspension in 10^9 cfu.ml^{-1}- Control Water

3.7 β-galactosidase assay to test the bioactivity of the pheromone

In this study, the reporter strain, *B. subtilis* JRL293, carried the *srfA-lacZ* fusion. This strain was constructed in order to monitor the expression of *srfA* operon through *lacZ* expression. As *srfA* is required for competence development, it is induced by accumulated molecules in the medium. The addition of these molecules induces an increase in *srfA* expression, which can be detected by measuring the resulting

β-galactosidase activity (Figure 3.22). Therefore, the level of β-galactosidase activity is related to the increase in *srfA* expression induced by pheromone accumulated in the extracellular medium of the cultures to be tested.

The bioactivity of pheromone presented in several samples was analysed through β-galactosidase assay. These samples are including; the supernatants which were obtained from the transformed *E. coli* BL21 cultures before and after the addition of IPTG as well as the supernatant from *B. licheniformis* NCIMB 8874 culture in the late exponential phase. Besides, the filtered extract from concentrated supernatant of induced recombinant *E. coli* BL21 was also tested (sections 2.4.3 and 2.4.4).

β-galactosidase activity of the above samples is demonstrated in Figure 3.23. Test samples reveal values from 49 to 61 Miller Unit (MU). Among all the tests, the supernatant of *B. licheniformis* NCIMB 8874 in the late exponential growth-phase (61 MU) and the transformed *E. coli* BL21 supernatant after the addition of inducer IPTG (59 MU) present high enzyme activity which could indicate the high pheromone bioactivity and high concentration of accumulated pheromone in the extracellular medium of tests. The next lower level of enzyme activity is presented in the filtered extract supernatant of the induced *E. coli* BL21 (49 MU). Other samples including non-induced transformed *E. coli* and finally control positive, *E. coli* (wild type), display 20 MU and 14 MU enzyme activities, respectively. All data was collected from three independent experiments which were performed in triplicate.

This bioassay could be easily used to measure the level of β-galactosidase activity in *bacillus* reporter strain carrying the *srfA-LacZ* cassette in presence of different test samples. This bioactivity determined the related increase in *srfA* gene expression induced by the ComX pheromone accumulated in the extracellular medium of the cultures to be tested. The produced pheromone in *B. licheniformis* supernatant at the exponensial phase and in the induced *E. coli* supernatant (carrying *comQX*), either non-concentrated or concentrated solutions, induced *srfA* expression which trigged high β-galactosidase activity.

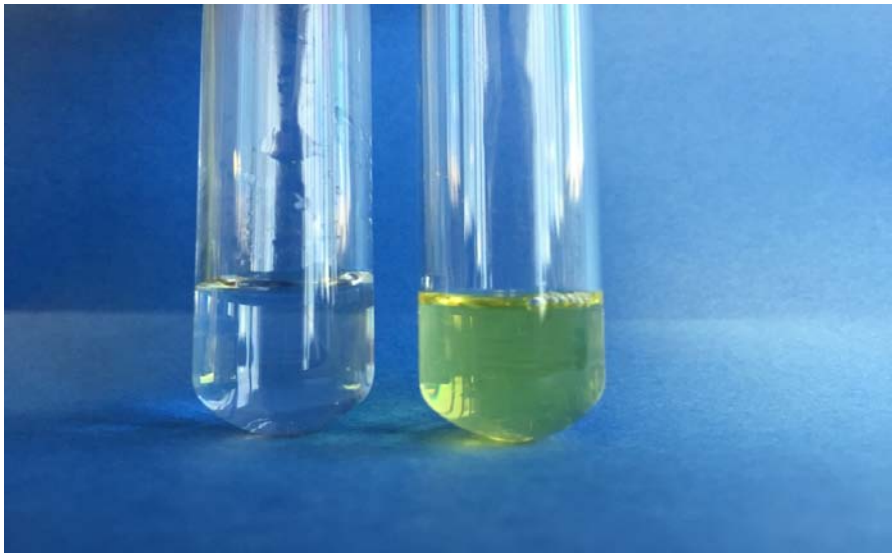


Figure 3.22 The colorimetric β-galactosidase assay. The clear reaction shows the negative control (water) in comparison to the developed yellow colour in the test reaction.

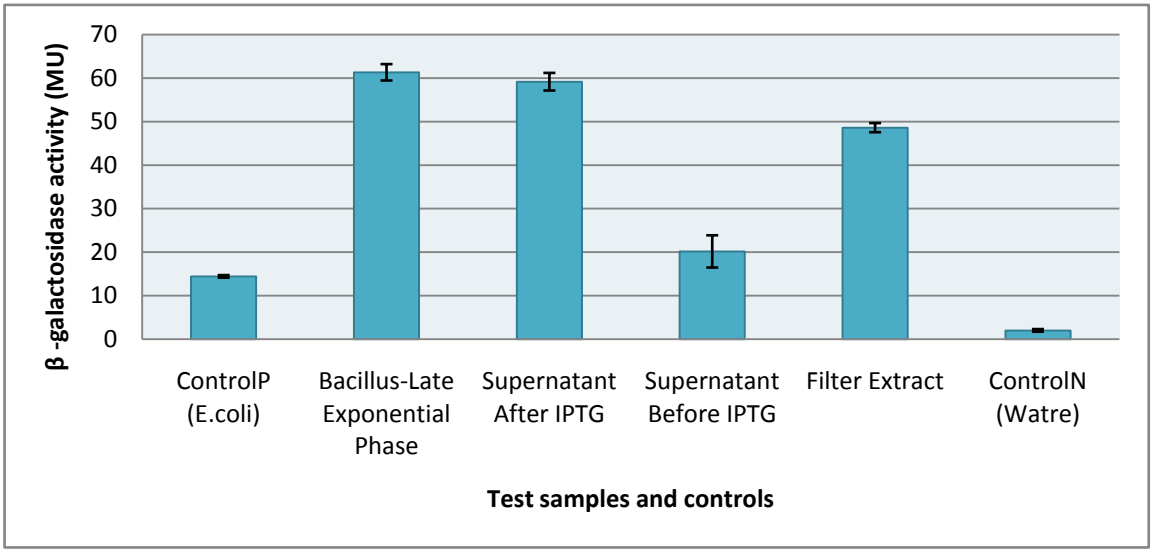


Figure 3.23 β-galactosidase activities of different samples. *E. coli* (wild type) is as a control positive (Control P). The samples are tested in *B. subtilis* JRL293 reporter strain, including supernatant of *B. licheniformis* in the late exponential phase, supernatant of the transformed *E. coli* BL21 after and before the addition of IPTG and the filtered extract solution from the transformed *E. coli* BL21 supernatant. Negative control (Control N) is made by the addition of water as a negative sample to the reporter strain. Bars=Standard Deviation, Repeats=3.

3.8 Antifungal susceptibility assays to test pheromone

Two different antifungal susceptibility assays were carried out to verify the threshold concentration of the purified pheromone with notable antifungal activity. The results of the two methods are presented in the following sections.

3.8.1 Micro-broth dilution antifungal assay

The antifungal activity of the purified pheromone against *A. flavus* (aflatoxigenic strain) was tested using micro-broth dilution assay. This assay was performed in 96-well microtiter plates using serial dilutions (6.5, 3.25, 1.62, 0.8, 0.4, 0.2, 0.1, 0.05 $\mu g.ml^{-1}$) of the pheromone (13 $\mu g.ml^{-1}$) (2.4.5.1). After the incubation period (24 h and 48 h), the absorbance of the cultures in the wells was spectrophotometrically determined at 595 nm based on the protocol (Troskie *et al*., 2012). Figure 3.24 shows the images of fungal mycelia from the control well (containing no pheromone) and the test well (containing 13 $\mu g.ml^{-1}$ purified pheromone).

The results show that the mycelium growth in presence of 0.05 to 6.5 $\mu g.ml^{-1}$ purified pheromone was over 90% of fungal growth in the control well. Whereas 13 $\mu g.ml^{-1}$ purified pheromone resulted in lower fungal growth by 78-80% of control level, regardless of incubation time (24 h or 48 h). However, the significant outcome ($P \leq 0.05$) was at 13 $\mu g.ml^{-1}$ purified pheromone after 24 h incubation. Figure 3.25 present that the outcome from 24 h incubation is statistically significant at 13 $\mu g.ml^{-1}$ pheromone while 48 h incubation does not display any reliable statistical results (Figure 3.26).

According to the scoring system of the standard protocol in antifungal susceptibility tests (CLSI, 2008); when the growth of the test sample is 25% of control, it is classified as “slight growth, score 1”; 50% of control “prominent reduction in growth, score 2”; 75% of control “slight reduction in growth, score 3”; and “no reduction in growth, score 4”. Hence, the present significant outcome (obtained from the highest pheromone concentration after 24 h at 595 nm) falls under the “no reduction in growth, score 4” classification since the lowest percentage is 78%. The test was performed in two independent experiments and four replicates.

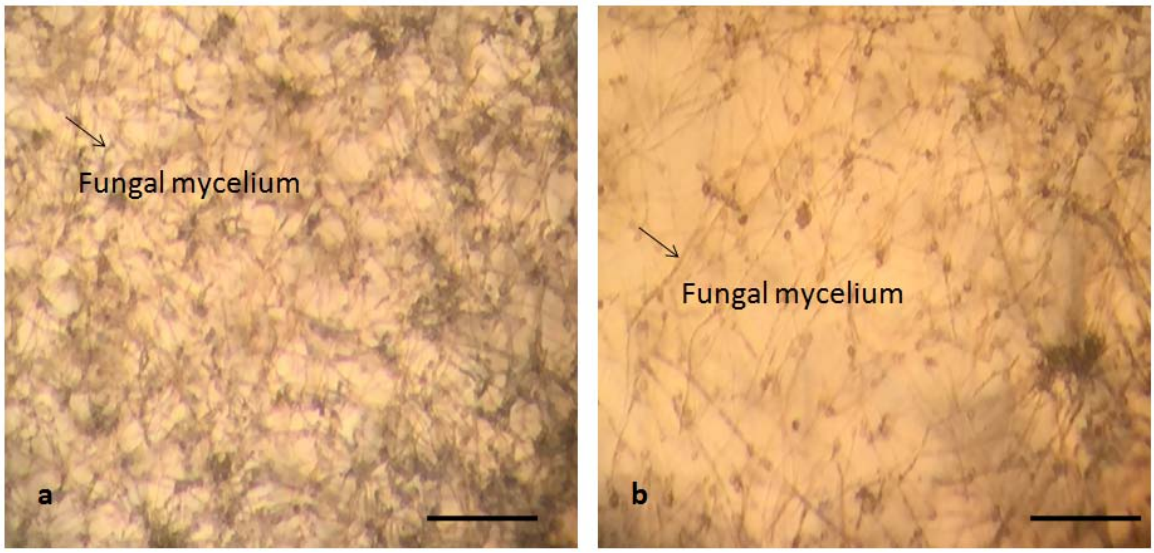


Figure 3.24 Microscopic visualisation of the mycelial growth of *A. flavus* NRRL 3357 in two wells of the plate of micro-broth dilution antifungal assay after 48 h incubation at 27° C. a) the image was taken from control well contained PDB+DMSO which shows the normal fungal mycelia gwoth with no addition of pheromone b) this image shows the fungal mycelia growth in the test well contained PDB+13 µg.ml^{-1} purified pheromone which represents the inhibitory effect of pheromone on fungal vegetative growth. Images were captured using a Leica light microscope, x100 magnification and scale bar 100 µm.

3.8.2 CLSI M38-A antifungal assay

The experiment was performed based on the micro-broth dilution assay, but using different medium. The medium RPMI-1640 was applied as suggested in M38-A reference protocol (section 2.4.5.2). In this assay the absorbance was measured at 530 nm only after 48 h incubation (Figure 3.27). The fungal growth after 24 h was insufficient to be recognised under the dark background caused by RPMI-1640 medium (Figure 3.28).

The results show that the growth level of the fungus, under the exposure of pheromone at the concentrations of 0.05 to 6.5 µg.ml^{-1}, is similar to the control. The significant reduction in the growth of the fungus was only under addition of 13 µg.ml^{-1} purified pheromone ($P \leq 0.05$) where the growth was 23% of the control growth. This is classified as "slight growth, score 1" according to the standard protocol (CLSI, 2008). The results are presented in the Figures 3.27 and 3.28. The experiment was repeated twice, each in four replicates.

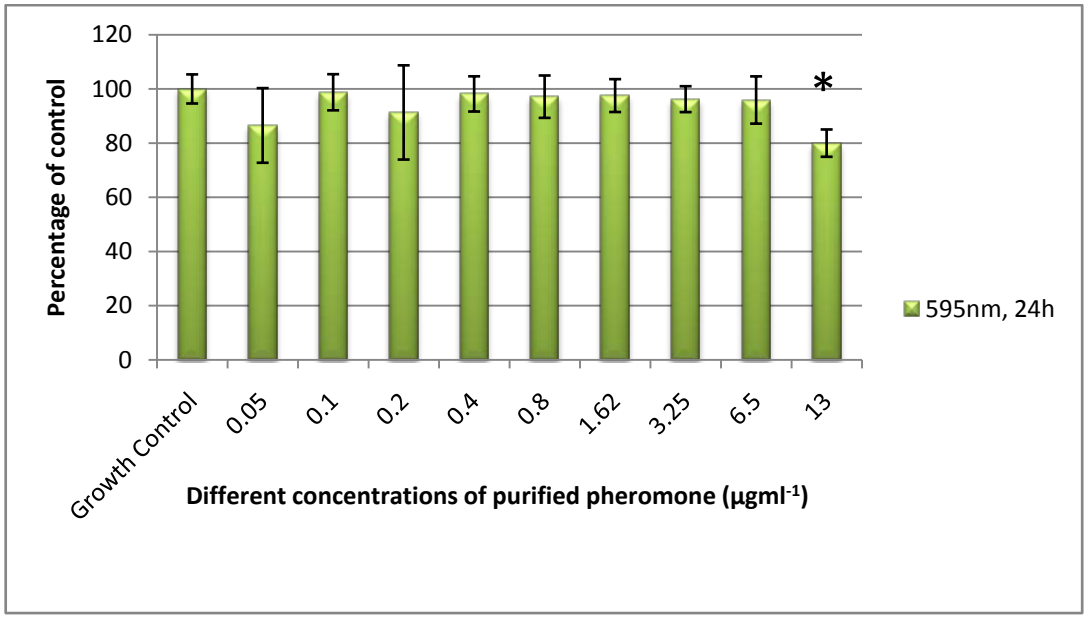


Figure 3.25 Micro-broth dilution antifungal assay at the absorbance of 595 nm after 24 h. Antifungal susceptibility of *A. flavus* was tested by using serial dilution of pheromone in the microtiter plates of PD medium (X axis). The calculated values show the growth percentage of the tests compared to the control.

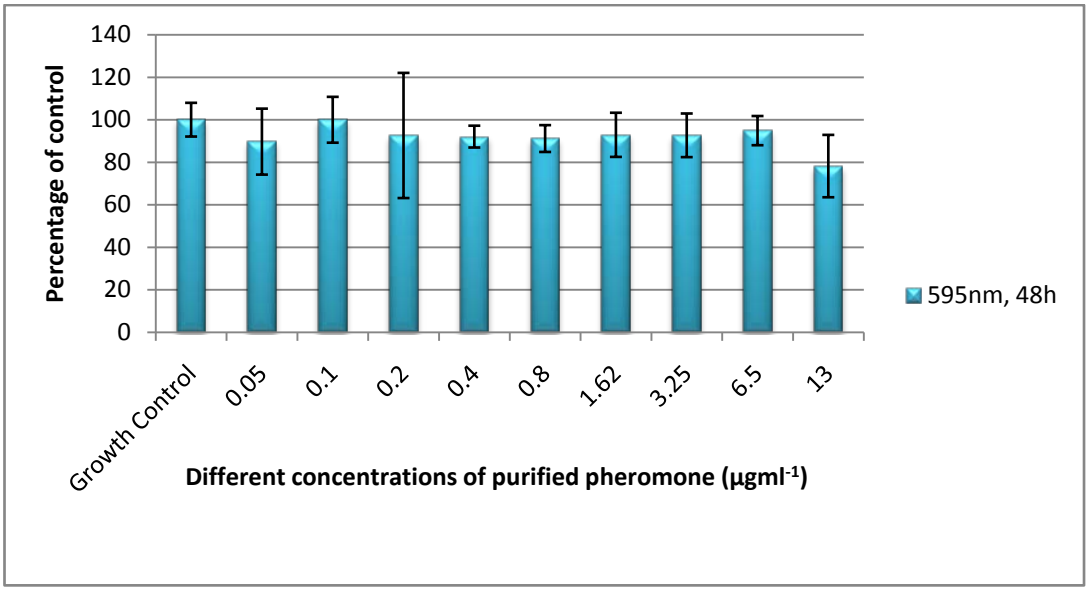


Figure 3.26 Micro-broth dilution antifungal assay at the absorbance of 595 nm after 48 h. Antifungal susceptibility of *A. flavus* was tested by using serial dilution of pheromone in the microtiter plates of PD medium (X axis). The calculated values show the growth percentage of the tests compared to the control.

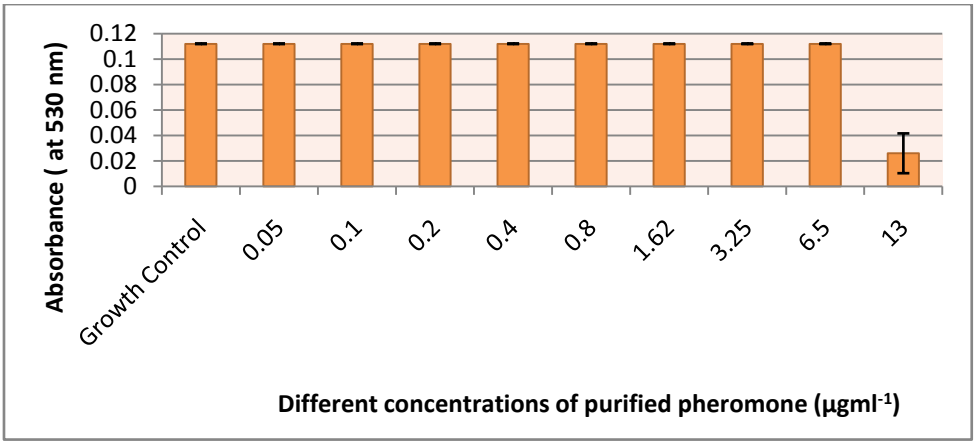


Figure 3.27 CLSI M38-A antifungal assay. Antifungal susceptibility of *A. flavus* was tested by using double dilution series of pheromone (X axis) in the microtiter plates of RPMI medium. Y axis represents the absorbance at 530 nm after 48 h.

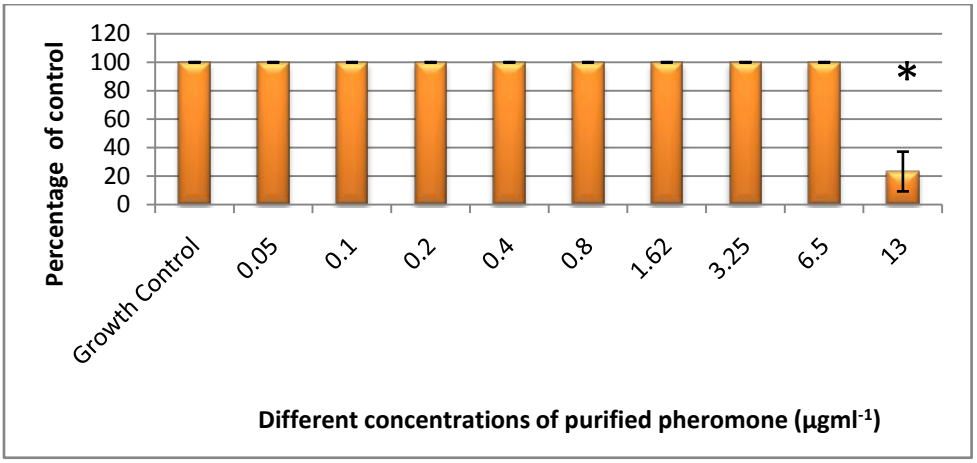


Figure 3.28 The plot for the percentage of growth control in CLSI M38-A antifungal assay. The absorbance was measured at 530 nm after 48 h. Antifungal susceptibility of *A. flavus* was tested by using double dilution series of pheromone in the microtiter plates of RPMI medium (X axis). The calculated values show the growth percentage of the tests compared to the control.

3.9 Transcriptomic studies

The transcriptomics can be considered as a precursor for the proteomics which studies the entire set of proteins expressed by a genome. In transcrptomic studies, the analysis of relative mRNA expression level is applied to predict the total amount of the corresponding protein present in the cell. In this study, the expression level of aflatoxin synthesis gene (*aflP*) was investigated to determine the possible effect of the

treatments on aflatoxin production in *Aspergillus* colonies. The outcomes obtained from Q-PCR and RT-PCR methods were presented in the following sections.

3.9.1 Total RNA extraction/clean up of treated and untreated *A. flavus* cultures

The total RNA was extracted from the fungus grown on the plates obtained from the fungal growth-rate experiment (different treatments) using RNeasy Mini Plant kit (Qiagen) (section 2.6.1). Following RNA extraction, samples were treated to digest contaminating genomic DNA by using QIAGEN RNase-Free DNase Set prior to RNA cleanup and concentration (section 2.6.2 and 2.6.3). RNA purity and concentration were determined by measuring the absorbance ratio, A260/A280. The integrity of all the extracted RNA samples was verified through running agarose gel electrophoresis (1%).

3.9.2 cDNA synthesis

The purified RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (section 2.6.4). The reverse transcription reaction products were used directly for quantitative PCR and RT-PCR. The cDNA products were stored at -80 °C to be used later.

3.9.3 Making OMT fragments as standard in qPCR

Prior to preparation of qPCR reaction, one PCR was performed using *A. flavus* extracted DNA to amplify the large fragment of *aflP* gene (aflatoxin biosynthetic gene, formerly named *omt-1*). The obtained PCR product was applied as a standard in qPCR to make standard curve. The 1254 bp fragment was amplified using the primers F-OMT and R-OMT (detailed in Table 2.11) as standard stock solution (OMT). The concentration of OMT PCR product was determined and the number of copies was calculated. The stock solution was serially diluted by a factor of 10 and used as a copy number standard during each setup of the qPCR reaction to confirm the efficiency of the primers. The accuracy of the PCR product was confirmed after sequencing and verifying the sequencing data in BLAST. The gel electrophoresis image of OMT fragments is presented in Figure 3.29.

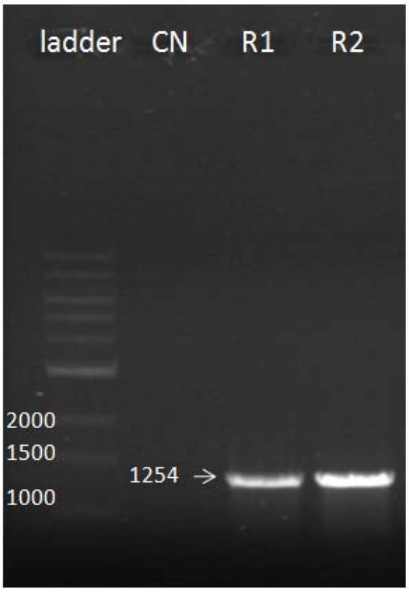


Figure 3.29 Verification of OMT products (1254 bp). DNA ladder (1 Kb, Biolabs) was used as a marker. The numbers indicate the size (bp) of the marker. Rep1, Rep2 are two products of the PCR reaction replicates. The first lane after the ladder was indicated as the negative control of PCR reaction without DNA template.

3.9.4 Three-step real time quantitative PCR

The treated *A. flavus* cultures were used for transcriptomic studies. In order to determine any changes in the expression level of the fungal mRNA of *aflP* gene, which encodes the enzyme to synthesize aflatoxin, a three-step qPCR experiment was conducted. The synthesised cDNA from mRNA templates (section 2.6.4) were used as a template for real time qPCR experiment. In this study, the housekeeping gene was *ITS* (Table 2.11).

Two different primer sets (F-*omt*/R-*omt* and F-*aflP*/R-*aflP*; Table 2.11) were chosen and tested by conventional PCR. Although the first primer set F-*omt*/R-*omt* has recently been reported and tested for *aflP* gene studies (Rodriguez *et al*., 2012), it has not amplified the interested region of cDNA samples. Subsequently, the second set F-*aflP*/R-*aflP* was designed and applied. The primer set of F-*aflP*/R-*aflP* was designed according to the genomic and mRNA sequencing data in BLAST as a suitable primer for qPCR which would span exon-exon junction to differentiate the amplified genomic DNA as a potential contamination from cDNA's amplified products. As a result, the qPCR

products obtained from cDNA fragments were 121 bp and from genomic DNA contamination were 181 bp (Figure 3.30). It shows that the intron size is 60 bp. Following testing different PCR conditions through gradient PCR experiment, the optimum annealing temperature for qPCR was verified. After many trial qPCR runs under different qPCR conditions, the generated melting curve of the chosen set of primer was not as expected as the standard melting curve which would appear in the normal real time PCR. However, the sequenced qPCR products showed that the gene was amplified, and the qPCR products appeared as sharp bands on the gel electrophoresis (Figure 3.30). This could be due to the primer dimer formation (a by-product in PCR which consists of primer molecules that have attached to each other) through the real time PCR.

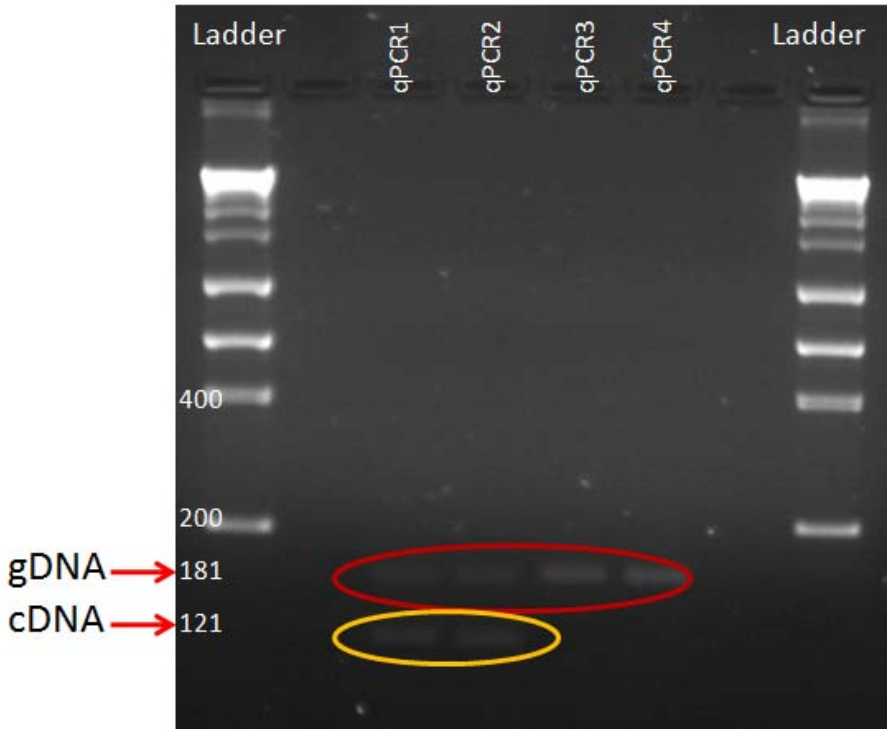


Figure 3.30 Verification of qPCR products of cDNA and extracted DNA of *A. flavus* NRRL 3357 (121 and 181 bp). HyperLadder 1 Kb (Bioline) was used as a marker. The numbers indicate the size (bp) of the marker. qPCR1 and qPCR2 represent the products of 121 bp (obtained from amplifying the related region on cDNA) and 181 bp (obtained from amplifying the related region on genomic DNA contamination) from contaminated cDNA template. qPCR3 and qPCR4 are the products obtained from the extracted DNA with the product size of 181 bp.

3.9.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and analysis of the products on the gel electrophoresis

To verify possible changes in the expression level of *aflP* gene, the reverse transcriptase polymerase chain reaction was carried out. This experiment was employed as an alternative method for testing the gene expression level since the qPCR experiment could not be optimised in this study to give the comparable analysis of *aflP* expression level. The synthesised cDNA from all RNA samples were amplified using the conventional PCR. The PCR conditions were optimised for the application of combined primers; *aflP* primers for aflatoxin gene and *ITS* primers for housekeeping genes (details are available in Table 2.11). Following the completion of the PCR reaction, 8 µl of each PCR product was run through a 2% agarose gel containing ethidium bromide. Following electrophoresis, bands corresponding to the transcripts of the study gene, *aflP*, and the reference gene, *ITS*, were noted. The image of the gel is shown in the Figure 3.31 and the list of samples is presented in Table 3.6. The results confirm the presence of *aflP*-amplified fragments (121 bp) from cDNA in the samples obtained from Aflatoxigenic strain of *A. flavus* (NRRL 3357) (control) (labelled 6 and 14) and the washed *Bacillus* suspension of 10^4 cfu.ml^{-1} (labelled 12). *ITS* gene was amplified in almost all the samples (600 bp) (except 10 and 12). The fragments of 181 bp were amplified by *aflP* primers from the genomic DNA templates.

Overally, in this sudy, using RT-PCR technique demonstrates the expression levels of *aflP* and *ITS* genes in tested samples through visually comparison analysis. This method helps to start the initial investigation about the effect of different treatments on the expression levels of aflatoxin synthesis and houskeeping genes.

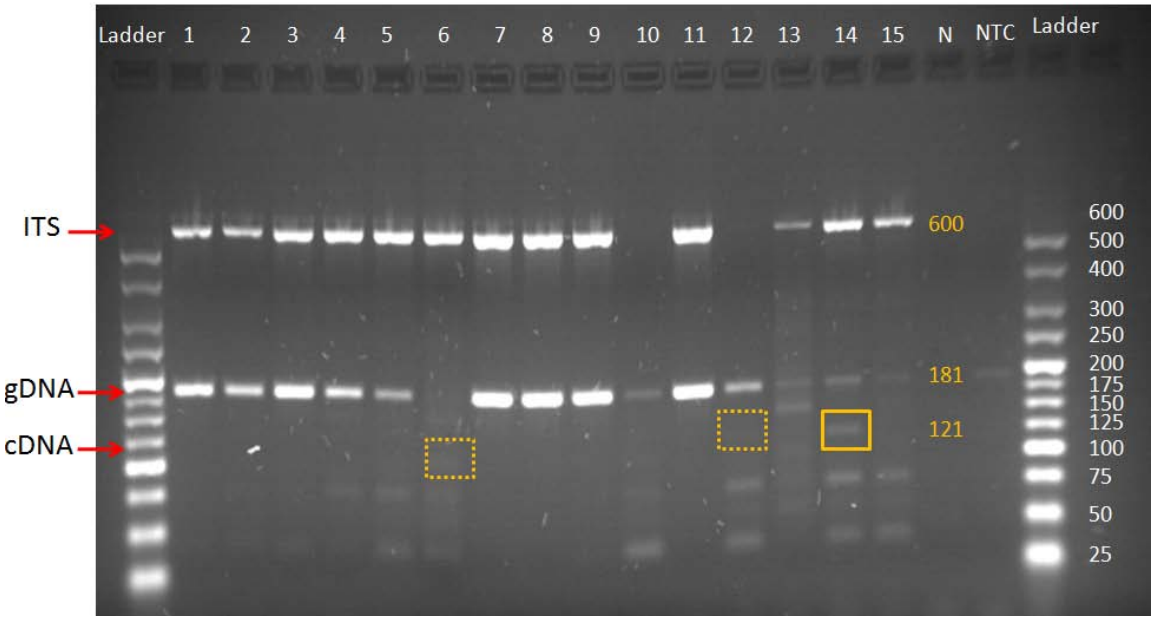


Figure 3.31 Verification of RT-PCR products of cDNA of *A. flavus* NRRL 3357 (which was under different treatments) using *aflP* primers (121 and 181 bp) and *ITS* primers (600 bp). HyperLadder 25 bp (Bioline) was used as a marker. The numbers indicate the size (bp) of the marker. The list of the RT-PCR products is presented in Table 3.6. The label "N" indicates the negative control for RT-PCR and "NTC" is a negative control for reverse transcriptase reaction which does not have the reverse transcriptase enzyme in the reaction.

Table 3.6 List of cDNA samples obtained from *A. flavus* NRRL 3357 under different treatments used in RT-PCR experiment (Figure 3.31)

Lane labels in the gel	cDNA Samples
1	Host strain supernatant
2	IPTG-induced *comX*-supernatant
3	Filtered extract of transformed *E. coli* in 5 mg.ml^{-1}
4	Filtered extract of transformed *E. coli* in 5 mg.ml^{-1} (repeat)
5	Filtered extract of transformed *E. coli* in 30 mg.ml^{-1}
6	Aflatoxigenic strain of *A. flavus* (NRRL 3357)
7	DMSO
8	*Bacillus* suspension of 10^9 cfu.ml^{-1}
9	*Bacillus* suspension of 10^4 cfu.ml^{-1}
10	*Bacillus* supernatant in exponential phase
11	Washed *Bacillus* suspension in 10^9 cfu.ml^{-1}
12	Washed *Bacillus* suspension in 10^4 cfu.ml^{-1}
13	Non-aflatoxigenic strain of *A. flavus* (ESP 15)
14	Aflatoxigenic strain of *A. flavus* (NRRL 3357) (repeat)
15	Aflatoxigenic strain of *A. flavus* (NRRL 3357) (repeat)

3.10 Whole genome sequencing of *B. licheniformis* NCIMB 8874 using Next Generation Sequencing System (Ion Torrent Personal Genome Machine)

The whole genome of *B. licheniformis* NCIMB 8874 was sequenced for the first time using the high throughput Next Generation Sequencing System of Personal Genome Machine (PGM). Following the first stage of sequencing procedure, library construction, the template was prepared through emulsion PCR automated system and then run on the PGM to accomplish the sequencing process (section 2.7.1-2.7.3). The report of the sequencing run is presented in Figure 3.32. The report shows that the Ion Sphere Particles (ISP) which carried the genome fragments as templates were loaded into the chip with ~50% efficiency. The usable reads were 70% and the read-length average was 194 bp (very close to the expected amplicon size, 200 bp).

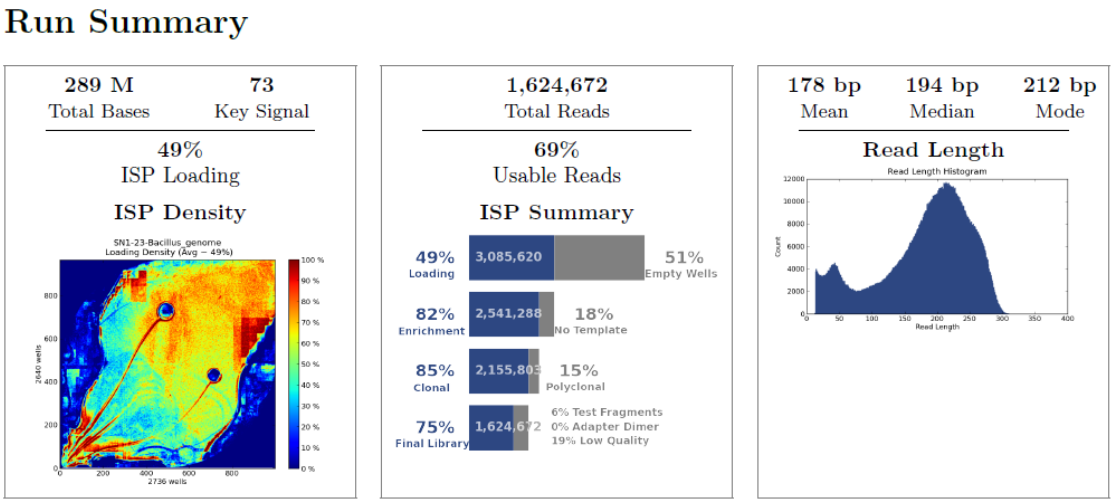


Figure 3.32 Run report of whole genome sequencing of *B. licheniformis* NCIMB 8874 on PGM. Ion Sphere Particle (ISP) refers to the particles which carried the templates of 200 bp to be sequenced.

3.10.1 Data analysis of the sequenced genome of *B. licheniformis* NCIMB 8874

Assembled DNA sequences data in FASTA format was obtained from the Ion Reporter 5.0 software. The assembly resulted in a single nucleotide sequence consisting of ~226 contigs and the coverage was 60x. These sequence data have been submitted to the

GenBank data bases under the accession number MBGK01000000. Details of data submission can be found at GenBank: http://www.ncbi.nlm.nih.gov. The assembled sequence was annotated through xBASE Annotation Service (http://static.xbase.ac.uk). This service provides the protein sequences of the whole genome. The phylogenetic analysis was carried out by aligning amino acid sequences of *comQXPA* cluster from the strain with homologous proteins from other *Bacillus* which obtained from NCBI nucleotide/protein database using "Clustal Omega" as a multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

3.10.2 Bioinformatics analysis of QS-related genes on *B. licheniformis* NCIMB 8874 genome

Following the sequencing and data analysis of the sequenced bacterium (3.9.6 and 3.9.6.1), bioinformatics studies led to the confirmation of the presence of the genes involved in cell-cell communication in *B. licheniformis* NCIMB 8874. QS-related genes previously identified and annotated in other *Bacillus* were compared to the *B. licheniformis* NCIMB 8874 genome as homologues. The genome sequence of *B. licheniformis* strain NCIMB 8874 used for this study was obtained from the alignment of output sequences from PGM.

3.10.2.1 The *comQXPA* gene cluster

Bioinformatics studies were performed for the identification of the competence regulating locus, *comQXPA*, in *B. licheniformis* NCIMB 8874 genome. The *comQXPA* gene cluster comprises the genes encoded the isoprenyl transferase ComQ, responsible for the maturation of the pheromone; the precursor protein of the ComX pheromone; and the two-component signal transduction system ComPA. The organisation and localisation of the *comQXPA* locus on *B. licheniformis* NCIMB 8874 are presented in Figure 3.33.

The investigation of the *comQXPA* locus of *B. licheniformis* NCIMB 8874 was performed at the protein level with comparison analysis of other *Bacillus* in order to determine the conservation of *comQXPA* genetic organisation.

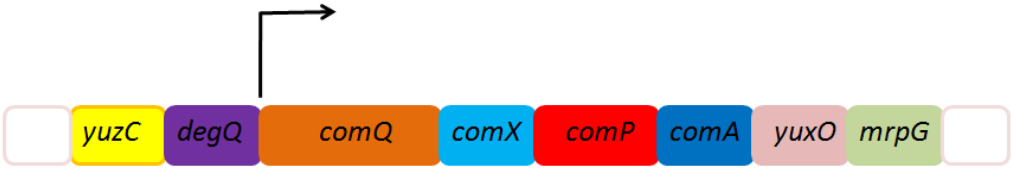


Figure 3.33 Genetic organisation and localisation of the *comQXPA* cluster in *B. licheniformis* NCIMB 8874 (adopted from De Vizio, 2011).

3.10.2.2 Conservation of proteins encoded by the *comQXPA* locus

In order to investigate the proteins encoded by the QS gene cluster in *B. licheniformis* NCIMB 8874, their evolutionary comparison with other *Bacillus* was needed. Alignment of the amino acid sequences was carried out using homologues from other *Bacillus* (listed in Table 3.7) and *B. licheniformis* NCIMB 8874 protein sequences, with Clustal Omega. The colour code used to indicate amino acids according to their physicochemical properties is as following: Red (small and hydrophobic); Blue (acidic); Magenta (basic); Green (hydroxyl and amine), and Gray (others).

The selection of *Bacillus* species for the amino acid alignment studies was mainly based on their available annotated *comQXPA* cluster in the gene bank. As the ComX pheromone of *B. mojavensis* R-O-B2 was isolated by Ansaldi and co-workers (2002), this bacterium was selected despite the incomplete annotation of its competence cluster.

Table 3.7 List of *Bacillus* species used for comparative analysis of QS-related genes in *B. licheniformis* NCIMB 8874.

NCBI Accession number	Organism	Competence	Genome annotation
NC_000964	*B. subtilis subsp.* 168	competent	Annotated (Kobayashi *et al.*, 2003)
NC_006270	*B. licheniformis* ATCC 14580	non competent	Annotated (Rey *et al.*, 2004)
GQ505081.1	*B. licheniformis* 9945A	competent	*comQXPA*, *comS* and *mecA* annotated (Hoffmann *et al.*, 2010)
GQ505080.1	*B. licheniformis* F11	non competent	*comQXPA*, *comS* and *mecA* annotated (Hoffmann *et al.*, 2010)
NC_009725	*B. amyloliquefaciens* FZB42	competent	Annotated (Chen *et al.*, 2007)
AF456135.1	*B. mojavensis* R-O-B2	not identified	*comQXP* annotated (Ansaldi *et al.*, 2002)
NZ_ACWC00000000	*Bacillus sp*. BT1B_CT2	not identified	Annotated (unpublished)
NC_014479	*B. subtilis subsp. spizizenii* W23	not identified	Annotated (unpublished)

3.10.2.2.1 The isoprene synthases ComQ

In *B. licheniformis* NCIMB 8874, ComQ was identified as a 303-amino-acid protein. Its comparison with homologues from genetically related *Bacillus* (Uniprot database) confirmed ComQ protein as a member of the FPP/GGPP synthase family engaged in isoprene biosynthesis. The percentage identities between ComQ of *B. licheniformis* NCIMB 8874 and other *Bacillus* species are listed in Table 3.8. The multi-alignment of the ComQ amino acid sequences is illustrated in Figure 3.34. To determine the protein conservation amongst selected *Bacillus* species, Figure 3.35 provides a phylogenetic tree.

Table 3.8 Percentage identities of ComQ protein of *B. licheniformis* NCIMB 8874 compared to homologues from other *Bacillus*. This was obtained from the alignment of the sequences using Clustal Omega.

Accession	Organisms	Length	%Identity
ADK89163.1	*B. licheniformis* 9945A	303	100
Q65FH4	*B. licheniformis* 14580	289	97
ADK89154.1	*B. licheniformis*_F11	289	97
EFV71237.1	*B. sp.* BT1B CT2	293	93
ADM39121.1	*B. subtilis subsp. Spizizenii* W23	286	52
AAL67730.1	*B. mojavensis* R-O-B2	286	51
CAB07902.1	*B. Subtilis* 168	299	43
ABS75210.1	*B. amyloliquefaciens* FZB42	286	40

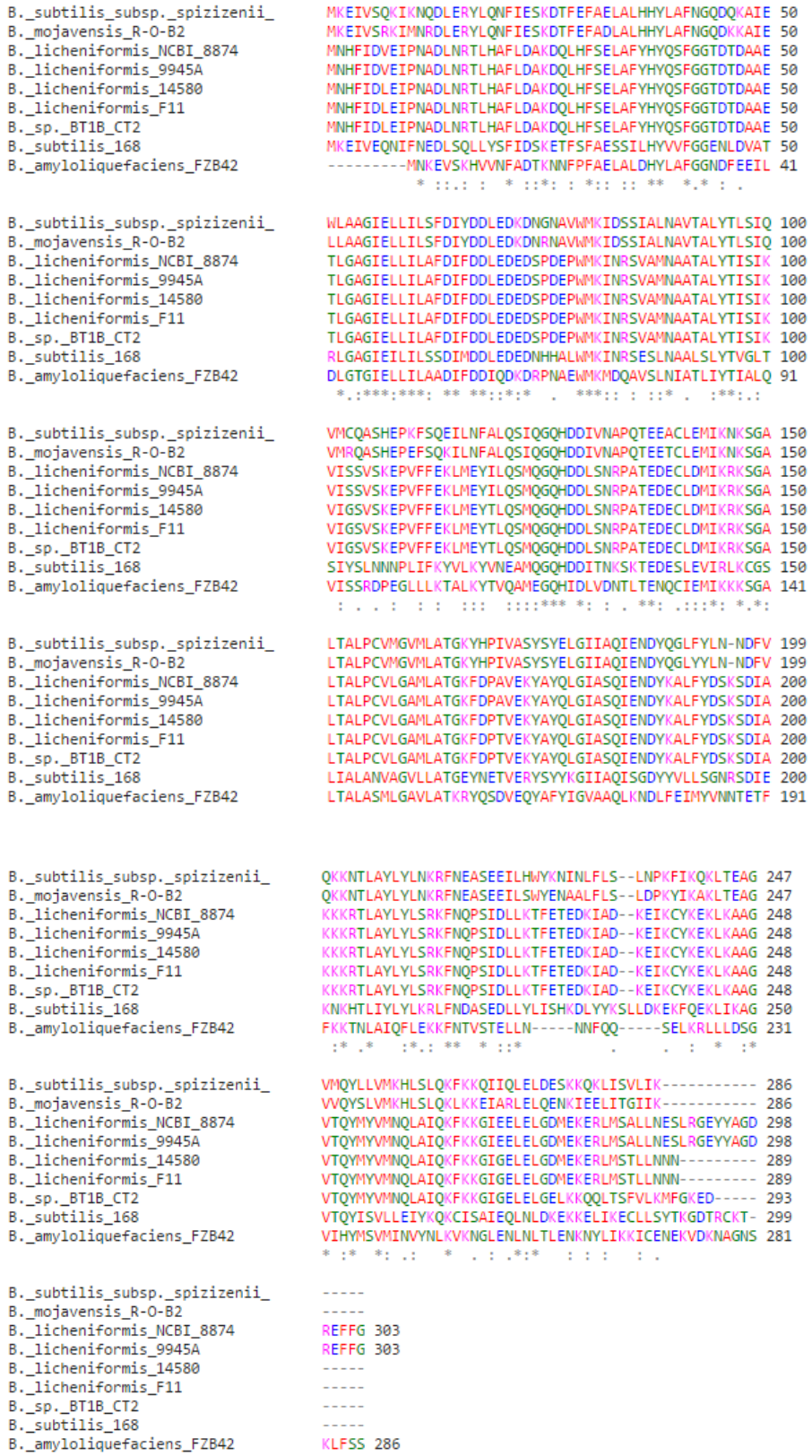


Figure 3.34 Clustal Omega multiple sequence alignment of ComQ. Conserved amino acids are indicated with the same colours in all rows.

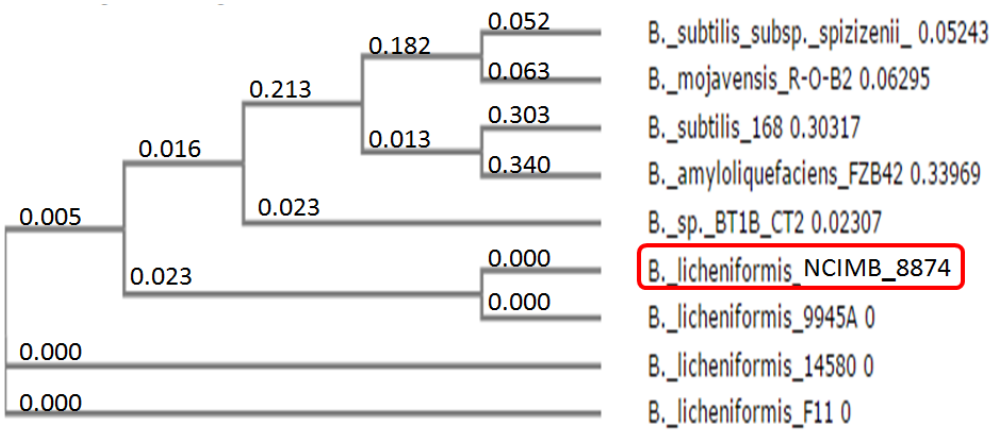


Figure 3.35 ComQ phylogenetic tree based on protein sequences aligned using Clustal Omega. The red box denotes *B. licheniformis* NCIMB 8874. The distance values show the number of substitutions as a proportion of the length of the alignments.

The alignment of *B. licheniformis* NCIMB 8874 ComQ with other homologues showed that the highest degree of identity appeared in other *B. licheniformis* strains such as 9945A, F11 and ATCC 14580. In particular, the ComQ sequences of two strains, NCIMB 8874 and 9945A, are 100% identical, whist 97% identity was detected with counterparts, F11 and ATCC 14580. ComQ from *B. amyloliquefaciens* FZB42 appears to be the most divergent, with only 40% identity.

Although the alignment of ComQ sequences (Figure 3.34) highlights the polymorphism of the protein at the amino acid level, the derived phylogenetic tree (Figure 3.35) shows three distinct groups of *Bacillus* species based on ComQ relative conservation.

3.10.2.2.2 The ComX pheromone precursor

The precursor of ComX pheromone is a 56-amino-acid protein encoded from *comX* locus in *B. licheniformis* NCIMB 8874. Table 3.9 presents the resulted identities from the alignment of the pre-ComX of *B. licheniformis* NCIMB 8874 with selected homologues. The Clustal Omega alignment of the amino acid sequences of ComX precursor is depicted in Figure 3.36. The phylogenetic tree presents the conservation of the pre-ComX protein amongst different *Bacillus* species in Figure 3.37.

Table 3.9 Percentage identities of pre-ComX protein of *B. licheniformis* NCIMB 8874 compared to homologues from other *Bacillus*. This was obtained from the alignment of the sequences using Clustal Omega.

Accession	Organisms	Length	%Identity
ADK89164.1	*B. licheniformis* 9945A	56	100
EFV71236.1	*B. sp.* BT1B CT2	57	57
AAL67716.1	*B. Subtilis* R-o-FF1	57	50
AAL67716.1	*B. Subtilis* 168	57	50
AAF82177.1	*B. mojavensis* R-O-H1	53	49
AAL67731.1	*B. mojavensis* R-O-B2	54	48
ABS75209.1	*B. amyloliquefaciens* FZB42	57	40
Q65FH5	*B. licheniformis* 14580	54	38
ADK89155.1	*B. licheniformis* F11	47	31
AAL67740.1	*B. subtilis subsp. Spizizenii* W23	58	30
AAL67740.1	*B. subtilis* R-O-E2	58	30
AAL67728.1	*B. mojavensis* R-O-C2	56	26
AAL67737.1	*B. Subtilis* R-o-F3	73	22

Figure 3.36 Clustal Omega multiple sequence alignment of pre-ComX. Conserved amino acids are indicated with the same colours in all rows.

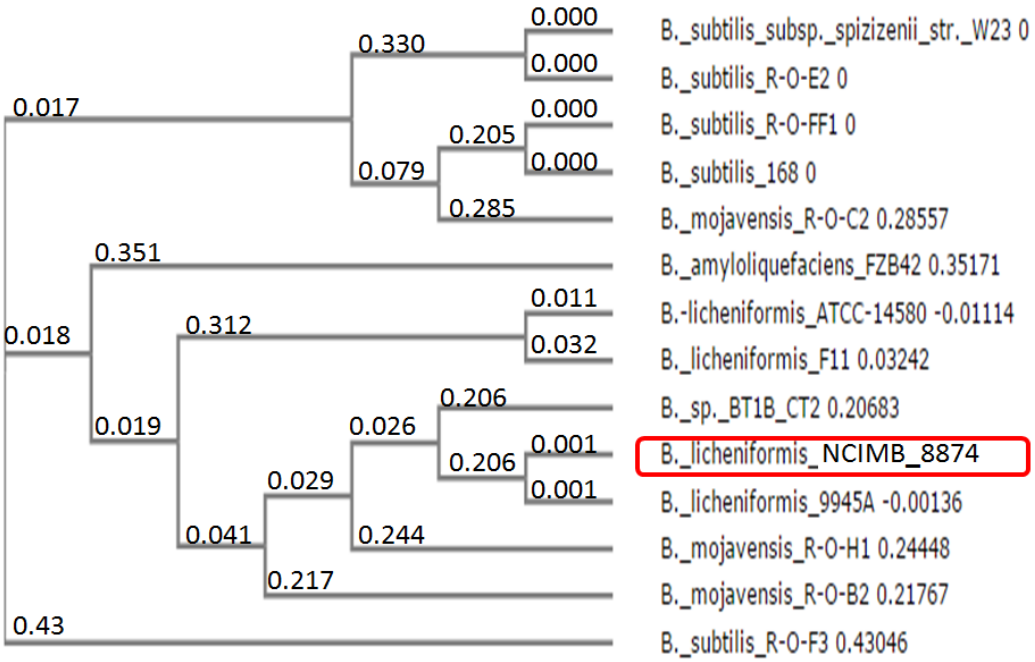


Figure 3.37 Pre-ComX phylogenetic tree based on protein sequences aligned using Clustal Omega. The red box denotes *B. licheniformis* NCIMB 8874. The distance values show the number of substitutions as a proportion of the length of the alignments.

According to the results, the precursor of the ComX pheromone is highly polymorphic. Among *Bacillus* species, *B. licheniformis* 9945A and *B. sp.* BT1B CT2 share 100% and 57% identity respectively. The other percentage identities range from 50 to 22 (Table 3.9 and Figure 3.36). However, similarly to what is shown for ComQ, the pre-ComX sequences of the *Bacillus* species could be classified into three main phylogenetic groups (Figure 3.37).

3.10.2.2.3 The sensor kinase ComP

The *comP* nucleotide sequence of *B. licheniformis* NCIMB 8874 translated to a 771-amino-acid protein which performed as the sensor histidine kinase of the ComPA two-component system. *B. licheniformis* NCIMB 8874 ComP was aligned with its counterpart of selected *Bacillus*. Results of this alignment are listed in Table 3.10 and illustrated in Figure 3.38. The phylogenetic tree presents in Figure 3.39.

Table 3.10 Percentage identities of ComP protein of *B. licheniformis* NCIMB 8874 compared to homologues from other *Bacillus*. This was obtained from the alignment of the sequences using Clustal Omega.

Accession	Organisms	Length	%Identity
ADK89165.1	*B. licheniformis* 9945A	771	100
EFV71235.1	*B. sp.* BT1B CT2	766	93
Q65FH9	*B. licheniformis* 14580	408	90
ADK89156.1	*B. licheniformis* F11	773	90
Q99027.3	*B. Subtilis* 168	769	67
ADM39119.1	*B. subtilis subsp. Spizizenii* W23	774	66
A7Z882	*B. amyloliquefaciens* FZB42	766	65
ABB16431.1	*B. mojavensis*	559	62


B._licheniformis_NCBI_8874
B._licheniformis_9945A
B._sp._BT1B_CT2
B._licheniformis_F11
B._licheniformis_14580
B._amyloliquefaciens_FZB42
B._subtilis_subsp._spizizenii_
B._subtilis_subsp.
B._mojavensis

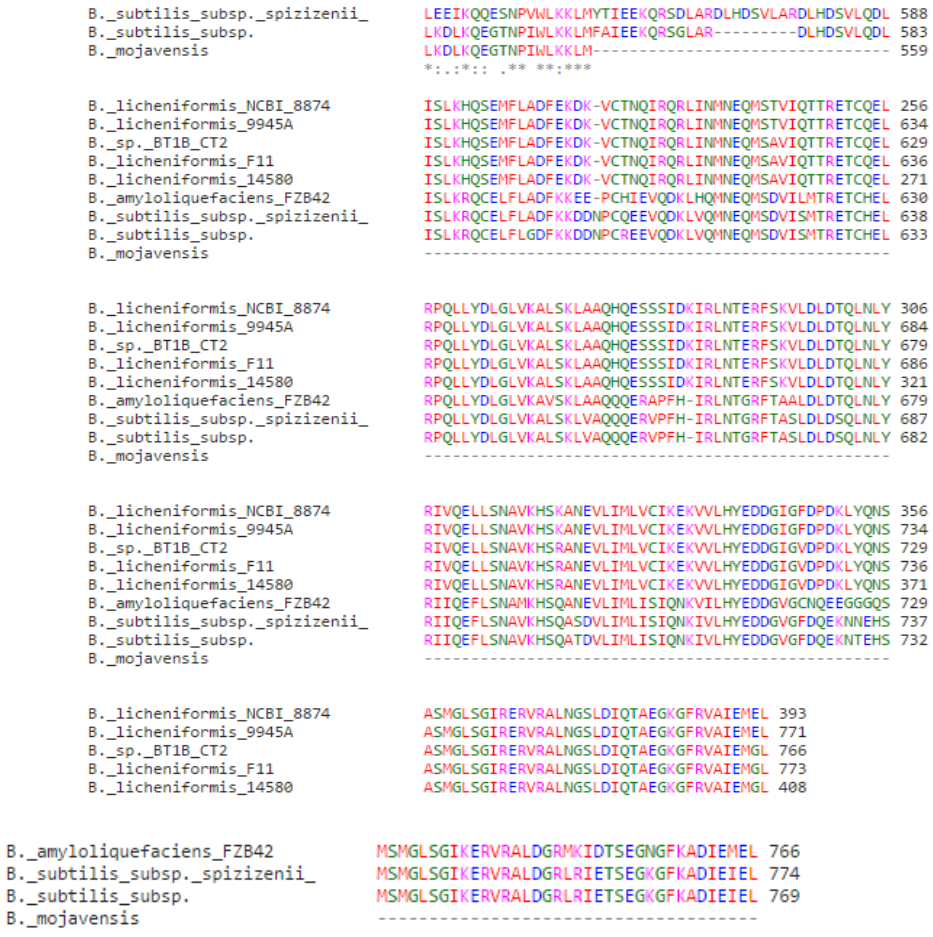


Figure 3.38 Clustal Omega multiple sequence alignment of ComP. Conserved amino acids are indicated with the same colours in all rows.

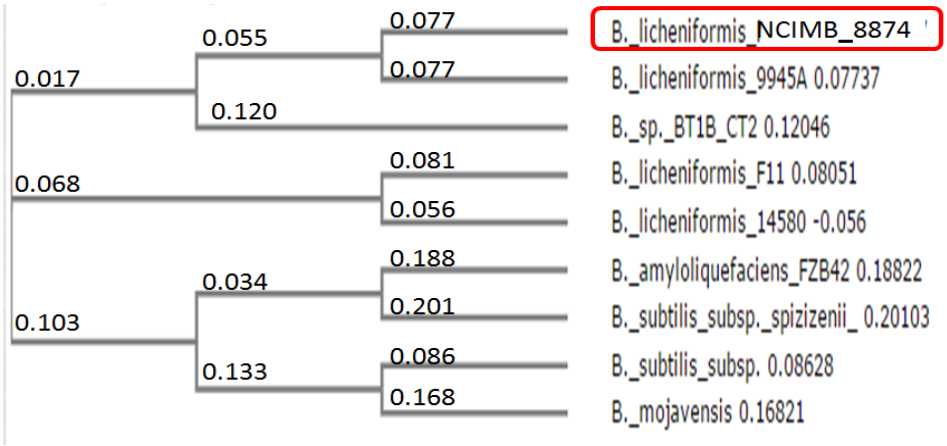


Figure 3.39 ComP phylogenetic tree based on protein sequences aligned using Clustal Omega. The red box denotes *B. licheniformis* NCIMB 8874. The distance values show the number of substitutions as a proportion of the length of the alignments.

The results obtained from the amino acid sequence alignment between ComP of *B. licheniformis* NCMB 8874 and selected homologues from other species reveal a variable distribution of identities ranged from 100% (*B. licheniformis* 9945A) to 62% (*B.* mojavensis). This range represents the ComP polymorphism, which is illustrated in Figure 3.38. The polymorphism is found only in the N-terminal portion of the protein, whereas the C-terminus appears to be conserved in these species. The phylogenetic congruence of ComP in selected *Bacillus* is shown in Figure 3.39. On the tree, ComP homologues are grouped in three distinct clusters.

3.10.2.2.4 The response regulator ComA

The 212-amino-acid protein (ComA) in *B. licheniformis* NCIMB 8874 was compared to its counterparts to study its conservation in *Bacillus*. The results of this comparative analysis of amino acid alignment are presented in Table 3.11. This multiple sequence alignment of ComA amino acid sequences is illustrated in Figure 3.40. The phylogenetic distribution of the response regulator in different *Bacillus* species is shown in Figure 3.41.

Table 3.11 Percentage identities of ComA protein of *B. licheniformis* NCIMB 8874 compared to homologues from other *Bacillus*. This was obtained from the alignment of the sequences using Clustal Omega.

Accession	Organisms	Length	%Identity
Q65FI0	*B. licheniformis* 14580	212	99
EFV71234.1	*B. sp.* BT1B CT2	212	99
ADK89157.1	*B. licheniformis* F11	212	99
ADK89166.1	*B. licheniformis* 9945A	212	99
P14204.1	*B. Subtilis* 168	214	79
ADM39118.1	*B. subtilis subsp. Spizizenii* W23	214	79
ABS75207.1	*B. amyloliquefaciens* FZB42	214	77

The alignment results shown in Table 3.11 and Figure 3.40 confirm ComA as the most conserved protein of the QS-regulating cluster. The results show 99% identity between ComA amino acid sequence of *B. licheniformis* NCIMB 8874 and homologues from other *B. licheniformis* strains and *Bacillus sp.* BT1B_CT2. The lowest identity is 77% with ComA

of *B. amyloliquefaciens* FZB42 (Table 3.11). The phylogenetic tree drawn from the alignment (Figure 3.41) demonstrates three groups of comA homologues.

```
B._licheniformis_ATCC_14580        MRRLLVIDDHPAVMEGTKAILETNQDFVVDCLSPEASEQFLKKHDFSAYD 50
Bacillus_sp._BT1B_CT2              MRRLLVIDDHPAVMEGTKAILETNQDFVVDCLSPEASEQFLKKHDFSAYD 50
B._licheniformis_F11               MRRLLVIDDHPAVMEGTKAILETNQDFVVDCLSPEASEQFLKKHDFSAYD 50
B._licheniformis_9945A             MRRLLVIDDHPAVMEGTKAILETNQDFVVDCLSPEASEQFLKKHDFSAYD 50
B._licheniformis_NCBI_8874         MKKILVIDDHPAVMEGTKAILETNQDFVVDCLSPEASEQFLKKHDFSAYD 50
B._subtilis_subsp.subtilis_168     MKKILVIDDHPAVMEGTKTILETDSNLSVDCLSPEPSEQFIKQHDFSSYD 50
B._subtilis_subsp._Spizizenii_     MKKILVIDDHPAVMEGTKTILETDSNLSVDCLSPEPSEQFIKQHDFSSYD 50
B._amyloliquefaciens_FZB42         MKKILVIDDHPAVMEGTKTILETDTNLSVDCLSPDASEQFVLRHDFSAYD 50
                                   *:::***************:****: :: ******:.****: :****:**

B._licheniformis_ATCC_14580        LILMDLNLG-EINGMDLSKKILETCSSCKIIVYTGYEVEDYFEEAIRSGM 99
Bacillus_sp._BT1B_CT2              LILMDLNLG-EINGMDLSKKILETCSSCKIIVYTGYEVEDYFEEAIRSGM 99
B._licheniformis_F11               LILMDLNLG-EINGMDLSKKILETCSSCKIIVYTGYEVEDYFEEAIRSGM 99
B._licheniformis_9945A             LILMDLNLG-EINGMDLSKKILETCSSCKIIVYTGYEVEDYFEEAIRSGM 99
B._licheniformis_NCBI_8874         LILMDLNLG-EINGMDLSKKILETCSSCKIIVYTGYEVEDYFEEAIRSGM 99
B._subtilis_subsp.subtilis_168     LILMDLNLGGEVNGMELSKQILQENPHCKIIVYTGYEVEDYFEEAIRAGL 100
B._subtilis_subsp._Spizizenii_     LILMDLNLGGEVNGMELSKQILQENPHCKIIVYTGYEVEDYFEEAIRAGL 100
B._amyloliquefaciens_FZB42         LILMDLNLGDDISGIELSKKILKENPLCKIIVYTGYEVEDYFEESIRAGL 100
                                   ********* ::.*::***:**:  . ****************:**:*:

B._licheniformis_ATCC_14580        HGAISKTESKEKIIEYIYQVLDNKIVVDYDYFKQLITQQKSK-SNSQQTQ 148
Bacillus_sp._BT1B_CT2              HGAISKTESKEKIIEYIYQVLDNKIVVDYDYFKQLITQQKSK-SNSQQTQ 148
B._licheniformis_F11               HGAISKTESKEKIIEYIYQVLDNKIVVDYDYFKQLITQQKSK-SNSQQTQ 148
B._licheniformis_9945A             HGAISKTESKEKIIEYIYQVLDNKIVVDYDYFKQLITQQKSK-SNSQQTQ 148
B._licheniformis_NCBI_8874         HGAISKTESKEKIIEYIYQVLDNKIVVDYDYFKQLITQQKSK-SNSQQTQ 148
B._subtilis_subsp.subtilis_168     HGAISKTESKEKITQYIYHVLNGEILVDFAYFKQLMTQQKTKPAPSSQKE 150
B._subtilis_subsp._Spizizenii_     HGAISKTESKEKITQYIYHVLNGEILVDFAYFKQLMTQQKTKPAPSSQKE 150
B._amyloliquefaciens_FZB42         HGAISKTESKEKIMQYIYHVLNGQILVDFSYFKQLMTQQKTKTSSSPQSE 150
                                   ************ :***:**:.:*:**: *****:****:* : * *.:

B._licheniformis_ATCC_14580        HDLLTERECLILKEVEKGYTNQEIADALHLSKRSIEYSLTSVFSKLNVGS 198
Bacillus_sp._BT1B_CT2              HDLLTERECLILKEVEKGYTNQEIADALHLSKRSIEYSLTSVFSKLNVGS 198
B._licheniformis_F11               HDLLTERECLILKEVEKGYTNQEIADALHLSKRSIEYSLTSVFSKLNVGS 198
B._licheniformis_9945A             HDLLTERECLILKEVEKGYTNQEIADALHLSKRSIEYSLTSVFSKLNVGS 198
B._licheniformis_NCBI_8874         HDLLTERECLILKEVEKGYTNQEIADALHLSKRSIEYSLTSVFSKLNVGS 198
B._subtilis_subsp.subtilis_168     QDVLTPRECLILQEVEKGFTNQEIADALHLSKRSIEYSLTSIFNKLNVGS 200
B._subtilis_subsp._Spizizenii_     QDVLTPRECLILQEVEKGFTNQEIADALHLSKRSIEYSLTSIFNKLNVGS 200
B._amyloliquefaciens_FZB42         QDRLTPRERHILQEVEKGLTNQEIADALHLSKRSIEYSLTSIFNKLNVGS 200
                                   :* ** **  **:***** ***********************:*.******

B._licheniformis_ATCC_14580        RTEAVLIAKSEGVL 212
Bacillus_sp._BT1B_CT2              RTEAVLIAKSEGVL 212
B._licheniformis_F11               RTEAVLIAKSEGVL 212
B._licheniformis_9945A             RTEAVLIAKSEGVL 212
B._licheniformis_NCBI_8874         RTEAVLIAKSEGVL 212
B._subtilis_subsp.subtilis_168     RTEAVLIAKSDGVL 214
B._subtilis_subsp._Spizizenii_     RTEAVLIAKSDGVL 214
B._amyloliquefaciens_FZB42         RTEAVLIAKSDNVL 214
                                   **********:.**
```

Figure 3.40 Clustal Omega multiple sequence alignment of ComA. Conserved amino acids are indicated with the same colours in all rows.

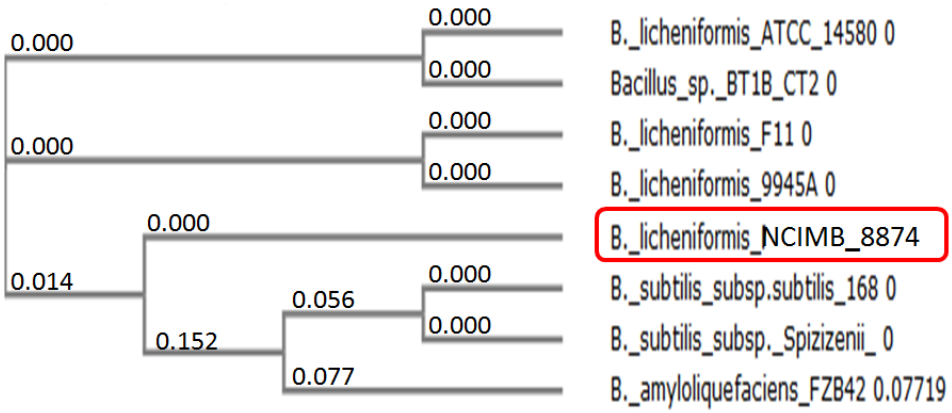


Figure 3.41 ComA phylogenetic tree based on protein sequences aligned using Clustal Omega. The red box denotes *B. licheniformis* NCIMB 8874. The distance values show the number of substitutions as a proportion of the length of the alignments.

3.10.2.3 The 16S rRNA gene sequences to study of *Bacillus* phylogeny

In order to investigate the phylogenetic relatedness of different *bacillus* species, 16S rRNA gene sequences were studied. The 16S rRNA is used for phylogenetic studies as it is highly conserved between different species of bacteria. Available 16S rRNA gene sequences in *B. licheniformis*, *B. subtilis* and *B. movajensis* (details available in Table 3.12) were aligned using Clustal Omega. The phylogenetic tree is shown in Figure 3.42. The percent identity matrix is presented in Figure 3.43.

Table 3.12 List of *Bacillus* species, their accession numbers for available 16S rRNA gene sequences and associated gene size.

Accession	Organisms	Length of the gene (bp)
D31739.1	*B. licheniformis* B-6-4J	1467
LN681568.1	*B. Subtilis* DSM 10T	1502
AB021191.1	*B. mojavensis* IFO15718	1526

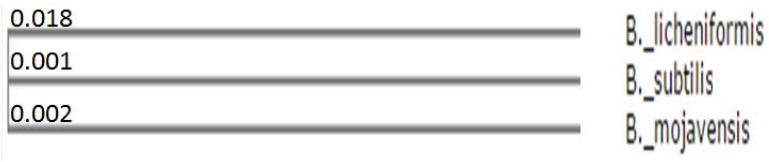


Figure 3.42 The 16S rRNA phylogenetic tree based on aligned gene sequences using Clustal Omega. The distance values show the number of substitutions as a proportion of the length of the alignments.

B. licheniformis	100.00	97.95	97.82
B. subtilis	97.95	100.00	99.67
B. mojavensis	97.82	99.67	100.00

Figure 3.43 The percent identity matrix, created by Clustal Omega through 16S rRNA gene alignments of three *Bacillus* species. This matrix shows the pairwise identity score which could indicate how much two species are close.

CHAPTER IV

DISCUSSION

Chapter IV

DISCUSSION

4.1 Introduction

Quorum sensing molecules (QSMs) are involved in the regulation of complicated processes in microbes helping population benefit from their cell-density. The cell to cell communication between microorganisms, such as bacterial-fungal species, through inter-kingdom cross-talk has been reported in many studies recently (Dudler and Eberl, 2006; Williams, 2007); so this communication could be via QS signals.

Bacterial antagonistic activity against fungi is considered as an inter-kingdom communication, and is an important bio-control strategy to control fungal invasion of plants. Several bacteria like *Bacillus* have shown the ability to inhibit *A. flavus* growth (Palumbo *et al*., 2006; Reddy *et al*., 2010). The bacterium *B. licheniformis* is related to *B. subtilis* genetically and has potential industrial application in producing antimicrobial compounds. The study of QS system in *B. licheniformis* is in its infancy (De Vizio, 2011) and its QSM (ComX pheromone) is neither identified nor reported yet.

Taking into account the above background, this project was aimed to conduct novel research on QS potential of *B. licheniformis*. The strain of *B. licheniformis* NCIMB-8874 was chosen as a model for the investigation of its QSM and its effect on *A. flavus* growth as an antagonist.

In this research project, cell-cell communication of *B. licheniformis* NCIMB-8874 was investigated through further elucidation of its QSM (ComX pheromone). The QSM was purified and detected by applying variety of genomic studies, molecular biology and biochemical investigations. Additionally, the influence of QSM (as an ingredient in the supernatant and in the form of purified compound) and the effect of bacterial cells on *A. flavus* colony area was examined. In this chapter a further detailed discussion of the

outcomes of the experiments is presented, and the results are discussed in the context of published literatures.

4.2 Molecular biology studies on *B. licheniformis* NCIMB-8874 QSM

QS system in Gram-positive bacteria employs peptides as signalling molecules. In the cytoplasm, these peptides are produced as precursors of the QSM and then cleaved, modified and exported. Once in the extracellular environment, the peptides are detected via two-component systems (Kleerebezemet *et al*., 1997). The studies have shown that QS in *Bacillus* species is moderated by small peptides that control competence, sporulation and the production of certain secondary metabolites in a cell-density dependent fashion.

Molecular biology studies by Ansaldi *et al.* (2002) confirmed that the *comQXPA* gene cluster plays an essential role in the regulation of competence development in *B. subtilis* QS mechanism. Since this gene cluster is present in *Bacillus* species closely related to *B. subtilis*, it is also recognised in *B. licheniformis* genome (Magnuson *et al*., 1994) as *B. licheniformis* is related to *B. subtilis* genetically. De Vizio identified that a *B. licheniformis* NCIMB 8874 cell-cell communication operates in analogy with the *comQXPA*-controlled pathway of *B. subtilis*. The genome sequences of several *B. licheniformis* strains, made available recently, as well as further molecular investigations, have provided information on cell-cell communication in *B. licheniformis* (De Vizio, 2011). Although QS is well established in *B. subtilis*, further investigation of cell-cell communication and signalling molecule in *B. licheniformis* was required as its QSM's sequence was not reported in previous studies. Moreover, the over-production and extraction of QSM in this strain could lead to further novel findings in bio-inhibitory activity of the strain and its metabolites through the present research.

At industrial scale, different *B. licheniformis* strains have been safely used in the fermentation industry for production of proteases, amylases and specialty chemicals (Schallmey *et al*., 2004). The industrial application of *B. licheniformis* also induces the

production of several antimicrobial compounds, such as bacitracin (Johnson *et al*., 1945) and the surfactin-resembling lichenysin (Yakimov *et al*., 1995).

According to the previous molecular and biochemical studies of QSMs, in *B. subtilis*, ComX pheromone is a 10-amino-acid peptide with a modification on the tryptophan residue (Okada *et al*., 2005). Two genes, *comQ* and *comX*, are required for ComX pheromone production. *comX* encodes the 55-amino-acid precursor of ComX pheromone. *comQ* is located immediately upstream of *comX* on the chromosome, and its null mutants are unable to produce ComX pheromone (Magnuson *et al*., 1994). Tortosa *et al.* (2001) reported that co-expression of *comQ* and *comX* in *E. coli* leads to the production of active pheromone in the medium, demonstrating that ComQ is the only dedicated protein required for processing, modification, and release of active ComX pheromone. In the current study, the finding about the importance of the co-expression of *comQ* and *comx* for pheromone production in *B. licheniformis* NCIMB 8874, helped to design a suitable primer set (Figure 3.1 and 3.2) and subsequently to conduct successful *comQX* gene cloning to produce ComX pheromone of *B. licheniformis* NCIMB 8874.

Some investigations in *B. subtilis* have focused on the production of the pheromone as a post-translationally modified peptide which requires processing of the precursor to 10 amino acids, modification of the tryptophan residue and export from the cell by ComQ (Lazazzera *et al*., 1999b). It has also been confirmed that the pheromone was formed by isoprenylation of an inactive precursor peptide (Schneider *et al*., 2002). Okada and colleagues identified *B. subtilis* pheromone structure for the first time and reported the sequence and the structure of 6-amino-acid peptide as QSM (Okada *et al*., 2005).

Some information was already available on *B. licheniformis* sequencing and QS genes (De Vizio, 2011), and here we extended the QS study (ComX pheromone) by partially employing the gene cloning procedure and the pheromone production / purification methods (sections 2.4.2 and 2.5) which have been adopted during the last decade on *B. subtilis* with some modifications.

The results of current study show that the adopted primer design, the restriction sites/the vector selection and the gene cloning procedure were well optimised, operated and accomplished successfully (Figures 3.1 to 3.5). Using the plasmid pET-22b as the vector, and the restriction enzymes (BamHI and NdeI) introduced in *B. subtilis* QS studies (see the methods in Schneider *et al*., 2002; Ansaldi *et al.,* 2002), led to successful cloning of *comQX* genes of *B. licheniformis* NCIMB-8874. The cloned *comQX* genes was also sequenced and its identity verified (Figure 3.5).

The present molecular investigation on *B. licheniformis* NCIMB-8874 QSM led to the genomic, molecular and chemical identification of pheromone in this *Bacillus* strain. The study continued with the new finding about the antifungal property of this QS peptide. The present study has revealed the antifungal characteristic of *B. licheniformis* ComX pheromone for the first time. The ComX pheromone as a natural antifungal compound can be applied in a sustainable bio-control strategy.

This finding as well as examples of growth inhibitory activity of other QSMs such as farnesol against *Pseudomonas* and mice tumour cells (Cugini *et al*., 2007; Lee *et al*., 2015), encourage further investigations on potential antimicrobial activity of QSMs in other microorganisms (Please note the Future Work Chapter, section 6.5).

4.3 Pheromone over-production, purification and detection from *B. licheniformis* NCIMB-8874

This project aimed to purify, and identify the QSM, ComX pheromone, of *B. licheniformis*. For this purpose, the QSM was over-produced using transformed *E. coli* BL21 (carrying *comQX*). The filtered extract supernatant from this transformed *E. coli* culture was run in HPLC in order to purify the pheromone present in extract. In addition to the traditional purification techniques such as crystallization, extraction and distillation, the first instruments for preparative column chromatography were developed in the 1960's. The advantages were that the compounds could be applied in high concentration and that the purification step was a simple filtering procedure washing away every compound that was not attached to the resin. The only method

that fulfils the requirements for automated and easy-to-use purification of large numbers of compounds is preparative HPLC (Unger, 1994; Huber, 2004). In this study, the obtained product containing the QS peptide was collected from preparative HPLC (Figure 3.9) and subsequently was analysed through MS-MS and MALDI-MS.

Tandem Mass spectrometry (MS-MS) involves multiple steps of mass spectrometry and is applied to pure samples as well as complex mixtures. A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio (Boggess, 2001). MALDI-MS is a soft ionization technique used in mass spectrometry, allowing the analysis of bio-molecules such as DNA, proteins, peptides and sugars which tend to be fragile and fragment when ionized by more conventional ionization methods. In proteomics, MALDI-MS is used for the rapid identification of proteins isolated by using SDS-PAGE or chromatography (such as preparative HPLC). The most popular analytical application of MALDI-MS is to reveal amino acid sequence of peptides (Karas and Kruger, 2003). In the present study, these techniques were used to identify the amino acid sequence of the ComX pheromone confidently (Figures 3.10-3.12).

In the present study, two sequences were identified from two main masses obtained from MS-MS analysis (Figure 3.10). The first sequence (Mass1, 13-amino-acid peptide, Figure 3.11) was perfectly matched with pre-ComX sequence of *B. licheniformis* NCIMB-8874 which is located towards the end of C-terminus of precursor polypeptide. This sequence is the most likely sequence of ComX pheromone as its region is comparable with the region which corresponds to ComX in the previous studies on *B. subtilis* (Schneider *et al*., 2002).

The results confirmed the presence of the QS peptide (Mass1) which possessed identical amino acid sequence with the sequence of HPLC standard 1 (C-terminus region of precursor polypeptide). The identification of amino acid sequences of ComX pheromone in *B. licheniformis* is a novel finding. This is the first report of the amino acid sequence of ComX pheromone from *B. licheniformis* NCIMB-8874 specifying the 13-amino-acid residue, Glu-Ala-Gly-Trp-Gly-Pro-Tyr-Pro-Asn-Leu-Trp-Phe-Lys. This sequence is totally different from the QS peptide sequence of *B. subtilis* Ro-E-2 with 6-amino-acid residue, Gly-lle-Phe-Trp-Glu-Gln (Okada *et al*., 2005). As the percentage

identity of *comX* gene in the nucleotide level between *B. licheniformis* NCIMB-8874 and *B. subtilis* Ro-E-2 is only 31%, it confirms that the ComX pheromone amino acid residue in these two strains vary both in size and sequence. In addition, the variety of the amino acid sequence of ComX peptide was confirmed based on the evident polymorphism, through the phylogenetic studies on the conservation of ComX protein among different *Bacillus* species and *B. licheniformis* strains (details available in the section 4.10). This result also agrees with De Vizio's (2011) bioinformatics studies on *B. licheniformis* NCIMB-8874.

Moreover, the characterised 13-amino-acid residue of pheromone in *B. licheniformis* NCIMB-8874 shows that the N-terminal cleavage site in this *Bacillus* strain is different from the sites characterised by Ansaldi *et al.* (2002) who reported diverse mature peptides with the length of 5 to 10 amino acids.

Interestingly, the second sequence (Mass 2, 10-amino-acid peptide, Figure 3.12) is comparable with the sequence of the adjacent region to the pheromone sequence in the pre-ComX molecule in *B. licheniformis*. This is a first report which reveals, in addition to ComX pheromone, the presence of another molecule with identical sequence to different region of pre-ComX. The role of this molecule in QS system has not yet been known.

Okada and colleagues (2005) reported the structure of *B. subtilis* QS peptide, ComX pheromone. They worked on *E. coli* ED413 as the host expression strain carrying *comQ* and *comX* constructs. They employed C18 Sep-Pak Cartridge (Waters) using Acetonitrile/Trifluoroacetic acid as mobile phase to filter the culture supernatant. This method of filtration was tested in the current research as a trial method and was found unsuitable for the large volume (10 L) of the culture supernatant since the large volume could not be filtered through the small C18 Sep-Pak Cartridge which was available. So the reverse-phase chromatography resin column was used as an alternative method for filtering of *E. coli* BL21 (DE3) culture supernatant. Besides, the next stage of peptide purification was designed and optimised according to the available C18 HPLC column (refer to the section 2.4.2.4).

NMR and X-ray crystallography are two methods that can be applied to the study of 3-dimentional molecular structures of peptides at atomic resolution. NMR is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation. The most common studied nuclei are ^{1}H and ^{13}C. NMR spectroscopy allows determining the molecular structure in solution phase (Filler, 2009). Therefore, in the current study, NMR ^{1}H was used to determine the chemical structure of purified pheromone solution collected from preparative HPLC. The report from Okada and colleagues verified the structure of the pheromone using NMR ^{1}H. In this study, although the amino acid sequence of ComX pheromone from *B. licheniformis* NCIMB-8874 was identified confidently through MALDI-MS and MS-MS, the purity of the peptide molecule was insufficient to reveal the structure using NMR. Due to the low concentration of the sample (0.26 $mg.ml^{-1}$) and the possible degradation of the amino acids through processing, the NMR study was inconclusive.

4.4 Investigation on the effect of *B. licheniformis* NCIMB-8874 and its QSM on *Aspergillus* growth-rate

Several bacterial species, such as *B. subtilis*, *Lactobacillus* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp. have the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus* spp. under laboratory conditions. Palumbo and colleagues (2006) reported that a number of *Bacillus* species (*B. subtilis* and *B. licheniformis*), *Pseudomonas*, *Ralstonia* and *Burkholderia* strains isolated from California almond nut and flower samples could completely inhibit *A. flavus* growth and aflatoxin production. Several strains of *B. subtilis* and *R. solanacearum* isolated from non-rhizosphere maize soil are also capable of inhibiting aflatoxin accumulation (Nesci *et al*., 2005). The inhibitory effect of antagonistic microorganisms on aflatoxin production and accumulation indicates that the repressed aflatoxin does not have enough potency to exert any influence on the growth of the antagonist, in reverse. This premise is supported by the present results. Indeed, this work shows that the aflatoxigenic strain, under the co-culture condition with *Bacillus* antagonist, is unable to produce the same level of aflatoxin secreted naturally in the pure culture of *Aspergillus*.

This deficiency in aflatoxin production under the co-culture condition appears to be a result of the repression by the antagonistic activity of *B. licheniformis,* present in the co-culture. The strong evidence provided by the results to verify this assumption is that the *Bacillus* cells have grown naturally on the co-culture plates (Figure 3.15a and b).

In a recent study, the bio-control agents *B. subtilis*, *P. fluorescens* and *Trichoderma viride* have shown >65% inhibition in *A. flavus* growth (Reddy *et al*., 2010).

Unravelling the molecules of antagonists which have a potent inhibitory effect on another microorganism will provide a better insight into ways to overcome the infections in plants or mammalians caused by harmful microorganisms. Recently, an example of the inhibitory activity of *B. licheniformis* strain associated with a marine organism was reported. It appears that the strain has anti-biofilm activity on *E. coli* and *P. fluorescens*. It is found to secrete a complex exo-polysaccharide compound (consisting of monomeric units of α-D-galactopyranosyl-(1→2)-glycerol-phosphate) having the potential for biofilm inhibition in different strains (Sayem *et al*., 2011). Moyne and colleagues (2001) reported that *B. subtilis* AU195 with high antagonistic activity against *A. flavus* possessed two lipopeptides with antifungal activity named Bacillomycin D. The above reports are the examples of antimicrobial compounds secreted by antagonists.

In another line of research, *A. fumigatus* biofilm formation was inhibited by co-culturing with *P. aeruginosa* (Ramage *et al*., 2011). Secreted heat-stable factors (decanol and decanoic acid) were also shown to exhibit biofilm inhibition. Subsequently, co-culture of *P. aeruginosa* QS mutants did not significantly inhibit *A. fumigatus* biofilms and vegetative growth to the same extent as the wild type *Pseudomonas*. Based on these findings, it was hypothesized that QSMs affected the fungus growth. Also it was demonstrated that biofilm could be inhibited in a concentration-dependent manner by short carbon chain molecules (decanol and decanoic acid) analogous to *Pseudomonas* QSMs (Ramage *et al*., 2011). Overall, it has been suggested that small diffusible and heat-stable molecules may be responsible for the competitive inhibition of filamentous fungal growth in environments with potential for polymicrobial colonisation such as the

lung, and this could be exploited as a potential therapeutic strategy (Mowat *et al*., 2008; Seidler *et al*., 2008).

Some clues exist that many QSMs function as more than just signals. Non-signalling roles for QSMs have been reported in such important processes as nutrient scavenging, ultra-structure modification and competition. It is now known that some bacterial communication molecules do not just interact with regulatory proteins to initiate changes in gene expression. They also possess the capability to sequester limited nutrition resources, intercalating into membranes or killing other organisms (as antimicrobial compound) (Schertzer *et al*., 2009). There are some other instances of the QSMs which exhibit antimicrobial activity. Gram-negative QSM (AHL) and its derived tetramic acid were shown to be bactericidal against Gram-positive bacteria (Kaufmann *et al*., 2005). Tetramic acids have been known to display mycotoxic, antibacterial and antiviral activities (Wang *et al*., 2003; Evans *et al*., 2006; Yu *et al*., 2007).

Moreover, it has been reported that the principal QS signal in Gram-negative symbiotic bacteria (AHL), does not only direct bacterial compound synthesis, but it is also recognized by eukaryotic cells such as animal cells, plants, seaweed and fungi. For instance, some AHLs secreted from symbiotic bacteria have immunomodulatory capabilities and can modulate gene expression in animal cells. This evidence shows the possible evolutionary approach of eukaryotes by the association of free-living bacteria with a host prokaryote (Dudler and Eberl, 2006).

Furthermore, some studies are focused on the fungal QSM, farnesol, which is secreted from the dimorphic fungus, *C. albicans*. Semighini and colleagues (2006) reported that externally added farnesol to *A. nidulans* culture triggers morphological features characteristic of apoptosis. It has been proved that growth and development of *A. nidulans* are reduced when it is co-cultured with *C. albicans*. Therefore, farnesol has been suggested as a compound which mediates an unexpected interaction between these two species. This indicates the mechanism that may be employed by fungi to antagonize the growth of competitors. Other reports reveal the inhibitory effect of farnesol on the growth of different fungal species such as, *A. niger*, *Fusarium graminearum*, *Candida dubliniensis* and *Penicillium expansum*. The growth

inhibition has been found by exogenous addition of farnesol in these fungal cultures. It induces the apoptosis-like phenotype in all these species (Henriques *et al*., 2007; Lorek *et al*., 2008; Semighini *et al*., 2008; Liu, *et al*., 2009 and 2010).

The current study examines the antagonistic effect of *B. licheniformis* NCIMB-8874 on *Aspergillus* growth under co-culture conditions. The results show that unwashed (at low and high concentrations; the ratios of slopes of the fitted lines to the data points are 3.35 and 3.73 respectively, Table 3.2, Figures 3.15a and b) and washed (only at high concentration; the ratio of slopes is 1.86, Table 3.2, Figure 3.15k) *B. licheniformis* cells cause significant reduction in *A. flavus* growth. The influence of *Bacillus*-cell treatments could be attributed to the antagonistic activity of *B. licheniformis* against *Aspergillus*. These results are comparable with the previous studies on antagonising microorganisms which have been explained earlier (Moyne *et al*., 2001; Nesci *et al*., 2005; Reddy *et al*., 2010).

However, since cell-free supernatant of *B. licheniformis* from the exponential phase (the ratio of slopes is 1.1, Table 3.2, Figure 3.15i) showed negligible decrease in the fungal growth-rate, it may be argued that the presence of bacterial cells in the fungal culture (either unwashed or washed cells) is the effective factor on fungal growth reduction rather than the cell-free supernatant. In this scenario, the bacterial cells would compete against fungus and/or secrete/employ their antimicrobial molecules under the co-culture condition. Likewise, the cell-free supernatant contains the compounds released into the medium during the growth of pure bacterial culture. However, molecules in the cell-free supernatant were produced under the non-competing condition of the pure bacterial culture which in turn may exhibit a different matrix of molecules from the one presented in the co-culture of *Bacillus* and *Aspergillus*. Collectively, under the multi-microbial environment, the behaviour of the cells and consequently the composition and concentration of the secreted compounds might be different when compared to a pure microbial culture. In agreement with this observation, Losada *et al*. (2009) performed co-cultivation competition assays among different species of *Aspergillus* and reported that the extracts from *Aspergillus* species had greater antifungal activity when

they were grown in the presence of a competitor. Using gas chromatography, they determined that the composition of extracts changed due to competition.

Based on the results which show the significant influence of *Bacillus* cells as well as negligible effect of cell-free supernatant on fungal growth reduction, It could be concluded that the presence of the cells might enhance the effect of the secreted antimicrobial compounds on fungal development as the cells would produce more (or different) antimicrobial molecules in their competition with the fungus. Besides, the extent of the contribution of the cells alone, without considering the effect of their secreted molecules, on reducing the fungal growth is not known. In order to implement this research, a mutant strain of *B. licheniformis*, impaired in producing all the potential antimicrobial compounds as well as QSM, is needed. The mutant could be tested under the co-culture condition with the fungus in order to demonstrate its effect on reducing the rate of fungal growth in comparison to the wild type *B. licheniformis* (Please note the Future Work Chapter, section 6.5).

The results showed that both washed and unwashed *Bacillus* cells exerted inhibitory effects on fungal growth, but to different degrees. The reason for the lower antifungal effect of the high concentration of the washed cells compared to the unwashed *B. licheniformis* cells appears to be due to the loss of putative antimicrobial molecules/QSM through the washing process. However, as the washed cells grow on the plates impregnated with *Aspergillus*, they may produce fresh antimicrobial molecules/QSM that affect the fungus albeit at lower levels.

The unwashed cells (at low and high concentrations) had (Figures 3.15a and b), notable inhibitory effect on fungal growth. This could be attributed to the presence of sufficient amounts of antimicrobial molecules in cell suspensions at both concentrations. However, only high concentrated washed cells could exert to some extent similar reduction as that of unwashed cells on the growth of fungus.

The effect of high concentrated washed cells on the fungal growth might be related to the inhibitory role of the secreted QSM, as well as the presence of bacterial cells as a

competitor in the co-culture plates. The QS peptide could be produced and accumulated in higher concentration when the cell-density is high.

Accordingly, it seems that the washed cells act efficiently on the fungal growth reduction if they are at higher density. In this scenario, the presence of the secreted antimicrobial/QS molecules is important and they are provided by cells at higher concentration. Indeed, this evidence is more prominent since the results show that the low concentrated washed cells (e.g. those having a ratio of slopes of 1.02, Table 3.2, Figure 3.15j) were ineffective on lowering the rate of fungal growth. This draws the conclusion that the concentration of the secreted molecules is as important as the presence of the washed bacterial cells in the co-culture to reduce the fungal growth.

The supernatant of IPTG-induced recombinant *E. coli* (carrying the *comQX* genes), showed little effect on decreasing the fungal growth-rate (the ratio of the slopes is 1.11, Table 3.2, Figure 3.15c) whereas the supernatants of non-induced recombinant *E. coli* and non-recombinant *E. coli* (the ratio of the slopes in both treatments is 1.03, Table 3.2, Figures 3.15d and e) did not affect fungal development as they did not contain the ComX pheromone molecule. Although the effect of IPTG-induced recombinant *E. coli* supernatant on decreasing of fungal growth-rate was negligible, the filtered extract of this supernatant (prepared from 10 L of IPTG-induced transformed *E. coli*) appeared to have potent inhibitory effect on *Aspergillus* colony development. The filtered extract's influence on fungal colony was notable at concentration of 30 $mg.ml^{-1}$. This finding draws attention to the impact of over-produced pheromone on the lowering of the fungal growth (the ratio of the slopes is 1.81, Table 3.2, Figure 3.15g). It appears to be related to the larger amount of accumulated ComX pheromone in the concentrated filtered extract compared with the induced supernatant which was collected from the smaller volume of transformed *E. coli* BL21.

In a complementary analysis, the average increase of fungal colony area per day, measured during 5 days of monitoring test plates, (Figure 3.16) showed a comparebale outcome with the results of fungal colony area changes (Figure 3.15).

4.4.1 Bacterial inter-kingdom communication with fungi and QS

The co-evolution of prokaryotes and eukaryotes raises the prospect of the existence of inter-kingdom signalling pathways; promoting parasitic and symbiotic relationships (Fox, 2004; Rumbaugh, 2007). Bacterial QS signals (e.g. AHL) may be interrupted by AHL-antagonist molecules produced by plants and fungi (Bauer and Mathesius, 2004; Dudler and Eberl, 2006; Williams, 2007).

In terms of inter-kingdom cross-talk, QS plays a major role. For instance, AHL as a bacterial QSM can be recognized by eukaryotic cells such as animal cells, plants, seaweed and fungi (Dudler and Eberl, 2006). Besides, farnesol as a fungal QSM from *C. albicans* led to decrease in the production of *Pseudomonas* QSM. It is suggested that farnesol and the related compounds may participate in interspecies interactions (Cugini *et al*., 2007).

Fungi coexist with bacteria in the environment, so bio-chemical exchange between them is a method of communication. The QS-like behaviour in filamentous fungi was reported in *A. terreus* (Schimmel *et al*., 1998) for the first time. Almost after a decade, QS behaviour was reported in *A. flavus* (Brown *et al*., 2008 and 2009) and then in *A. nidulans* (Williams *et al*., 2012). When their QS compounds were exogenously added to the fungal cultures, they effected morphological changes through hyphal branching and spore formation as well as secondary metabolite production like lovastatin, penicillin and aflatoxin. From the recent researches, it seems that QS is an established mechanism used by fungi to modulate mutual responses to each other and their environment (Sorrentino, 2009). Also, instances of small molecule exchange between bacteria and eukaryotes have been reported (Mullard, 2009). Given the above knowledge, it can be suggested that the communication between *A. flavus* as a filamentous fungus, which has its own QS network, and *B. licheniformis* antagonist might occur through their QS systems. This needs to be further investigated (Please note Future Work chapter, section 6.8). As this study reveals the significant influence of *B. licheniformis* NCIMB-8874 QSM (high concentrated filtered extract, Figure 3.15g) on *Aspergillus* growth, this could be a strong evidence to suggest the possible inter-kingdom cross-talk through their QS systems.

In this study, the results obtained from the treatments on the fungus plates, confirmed the antagonistic activity of *B. licheniformis* NCIMB-8874 against *A. flavus*, and the effect of the natural antifungal peptide, crucial for bacterial competence. The results (Figures 3.15a, b, g and k) promise a possible application of this bacterial strain through production of a natural antifungal agent to promote a sustainable bio-control strategy for agricultural crops. This innovative approach exploits the inter-kingdom cross-talk. Furthermore, this study provides a strong body of evidence for future investigation on possible application of genetic engineering of plant resistance to fungal invasion through the use of genes related to the bacterial antifungal peptide molecules.

In another line of research, Lee *et al*. (2015) have recently suggested that farnesol as a fungal QSM is a novel blocker of signalling pathway (e.g. STAT3) and exerts both anti-proliferation and apoptotic activities in multiple myeloma to inhibit tumour growth *in vitro* and *in vivo*. This new finding hints the prospect of the application of QSMs as anti-tumour agents. As such, reports about antifungal, anti-tumour and apoptotic properties of QSMs suggest potential application of the signal molecules to control fungal plant diseases as well as therapeutic relevance of these compounds in human tumour control.

4.5 Image analysis of the fungal colonies

A novel digital image processing method was applied for measuring fungal colony area on agar plates. This method helped to maximize the accuracy of the fungal area measurement. The advantages of this computational technique adopted to this work are summarized below.

There are many advantages in adopting an automated technique over the traditional manual method in characterizing the growth of fungal colonies on agar plates. Images can be saved as digital files which help researchers to access and re-analyse them without adverse effects of time on agar plates.

The results of automated and manual methods, used in this project, agreed with each other as far as the trend is concerned (Figure 3.19). Based on Figure 3.20b, the manual

estimation often is larger than the automated one by a factor of ~ 1.2 which over-predicts the actual colony area by ~ 20 percent. The difference in the results is expected as the fungal colonies are not always circular in their shape and in most cases are irregular (Figure 3.19). Manual measurements are often based on assumption of a perfect circle for each colony. This is a significant drawback that could result in erroneous conclusions. The digital image processing records accurately all areas of the circle circumference. This leads to robust measurement of the observable parameter.

Furthermore, the automated method based on the processing of digital images, provide the possibility of keeping an accurate record of the images of the colonies that could be referred to the digital archive without any change over time. Besides, this method enables researchers to measure parameter such as light intensity (relative brightness) of image pixels to study the fungal sporulation. The light intensity might be difficult or even impossible to measure using manual method.

This method is less laborious, and the digital images are processed very fast. This helps to re-process the overall data within seconds in order to extract new information or apply corrections to the current measurements. For example, the total processing time for a dataset, consisting of ~ 300 images (each 1280*1024 pixels), is around 100 seconds (~ 330 milliseconds per image). Processing the same size dataset, obtained from the traditional manual measurement of fungal colony diameters, to perform statistical analysis, takes at least a few hours.

Finally, adopting the automated method reduces the risk of spreading spores while plates are accidentally flipped over during manual assessment.

4.6 Studies on the effect of *B. licheniformis* NCIMB-8874 and QSM on fungal dry weight

According to the results of fungal dry weight (Figure 3.21), the treatments such as low and high concentrated unwashed *Bacillus* cells, high concentrated washed *Bacillus* cells as well as the high concentration of filtered extract of the transformed *E. coli* appear to

have significant effect on the reduction of fungal dry weight. These results are comparable with the effects of the same treatments on fungal growth-rate. However, the IPTG-induced supernatant of the transformed *E. coli* cells shows a significant reduction on fungal dry weight while this IPTG-induced supernatant shows a negligible reduction in the fungal growth-rate. This may be attributed to the minor growth reduction in every single day of monitoring of the fungal colonies on the plates which is not sensed significantly while the overall growth reduction, by the dry weight assay, collected over 7 days show significant change in the whole fungal mass.

4.7 Confirmation of pheromone's biological activity using β-galactosidase assay

Biological sensors (biosensors) are the bioassays which use biological molecules or constructs to detect the presence of a specific chemical compound. The use of reporter strains for the identification of QSMs is a well established bioassay in both Gram-negative and Gram-positive bacteria. This practice has been originally developed for the detection of AHLs in Gram-negative bacteria. *E. coli* cells carrying the *luxCDABE* operon are an example of *lux*-based biosensor used to detect AHLs through development of bioluminescence (Winson *et al*., 1998a and 1998b). Another example of AHLs bioassay is through using the production of purple pigment in *Chromobacterium violaceum* (McClean *et al*., 1997; Blosser and Gray, 2000).

The first biosensor for Gram-positive bacteria was developed by Magnuson and co-workers (1994) using *B. subtilis srfA-lacZ* fusion-carrying reporter strain for the identification of the ComX pheromone. Different reporter strains have been created and used for the isolation of ComX variants in different *Bacillus* strains or for detection of the pheromone in the course of purification (Ansaldi *et al*., 2002, Okada *et al*., 2004, Okada *et al*., 2005). The confirmation of using *B. subtilis* reporter strain in the investigation of the competence pheromone in supernatants of *B. licheniformis* was reported by De Vizio (2011). A similar approach has been reported for the investigation of AI-2 signal in *B. subtilis* where supernatant from the Gram-positive *B. subtilis* at high

cell-density was tested on low cell-density culture of *V. harveyi*, a Gram-negative bacterium (Lombardia *et al*., 2006).

In the current study the reporter strain *B. subtilis* JRL293 was used in order to monitor the expression of *srfA* operon (required for competence development) through *lacZ* expression. The *srfA* is induced by ComX molecules accumulating in the extracellular medium. *B. subtilis srfA-lacZ* cultures at low cell densities show *srfA* expression at basal level. The addition of an inducer molecule to activate *srfA* increases the *srfA* expression, which can be detected measuring the resulting β-galactosidase activity. Accordingly, the level of β-galactosidase activity will indicate *srfA* expression level induced by signalling molecules (pheromone) accumulated in the extracellular medium of the tested cultures (Magnuson *et al*., 1994).

The bioassay shows that among the samples used in this research (Figure 3.23), the supernatant of *B. licheniformis* NCIMB-8874 in the late exponential growth-phase, as well as IPTG-induced supernatant of transformed *E. coli* cells result in the highest expression of the *srfA-lacZ* reporter construct, hence it indicates an enhancement in the specific activity of β-galactosidase as a result of the high pheromone bioactivity in these two samples. The filtered extract of the transformed *E. coli* supernatant shows the second highest specific activity of β-galactosidase since the bioactivity of the pheromone presented in the filtered extract is less than the IPTG-induced supernatant. It may be attributed to the partial inactivation of the pheromone bioactivity during the purification process. The non-induced supernatant of the transformed *E. coli* cells shows lower enzyme activity by 1/3 fold compared to the induced supernatant as a result of *srfA* expression at the basal level. Low concentrated *E. coli* shows the enzyme activity of 14 MU as a control positive to present the basal level of enzyme activity. Collectively, the lesser pheromone bioactivity may be attributed to the lower concentration of the produced bioactive QSM in the tested samples.

4.8 Antifungal susceptibility assays to test the ComX pheromone

The rise of antimicrobial resistance in recent years as well as the harmful impact of conventional/chemical antibiotics on the environment demand the search for novel antimicrobials (Steinstraesser *et al*., 2009). However, determination of potential antifungal activity necessitates a standardised accurate quantitative test. Several antifungal growth assays have been established with their own advantages and disadvantages (Broekaert *et al*., 1990). The disc-plate diffusion assay has been performed using substance saturated paper discs placed at the edge of an emerging fungal lawn which is a commonly utilised method (Franrich *et al*., 1983; Mauch *et al*., 1988; Broekaert *et al*., 1990). However, the disc-plate diffusion assay and radial assays are only semi-quantitative tests since the extent of fungal growth inhibition and the *in situ* concentrations could not be evaluated and determined accurately (Broekaert *et al*., 1990).

Other methods are developed for more accurate quantification such as measurement of the length of fungal hyphae (Broekaert *et al*., 1988) and the dry mycelial weight (Odebode *et al*., 2006). These methods are still not ideal as they are arduous and hyphal length measurement can only be applied to fungal germlings (Broekaert *et al*., 1990). Broekaert and colleagues (1990) developed a quantitative broth dilution assay based on the absorbance at 530 nm in order to eliminate the complications in fungal growth inhibition tests. This antifungal susceptibility test was set as the standard antifungal activity reference test (M38-A2) by The Clinical and Laboratory Standard Institute (CLSI, 2008). Moreover, although the CLSI recommends adjustment of the turbidity of the inoculum by spectrophotometer at 530 nm, the current study has employed 530 nm for the test which followed CLSI (RPMI-1640 medium, Figures 3.27 and 3.28) and also 595 nm for the PDB broth experiment (Figures 3.25 and 3.26) as suggested by Troskie and co-workers (2012).

As the broth assays have been usually done in 96-well microplates, this assay could be used for high throughput analyses. However the broth assay is highly dependent on spectrophotometric analysis. Since fungi can be found in diverse niches and have different growth phases, it is therefore advisable to apply more than one growth

medium and growth phase to test a wide range of possibilities which supports reliable and comparable results. Broth dilution assay only assess antibiotics against fungi in a liquid environment (Troskie *et al*., 2012). To test the inhibitory effects of fungicide on different growth stages of *Aspergillus*, samples were taken after 24 h and 48 h. The results vary as the different stages of the fungal growth (24 or 48 h growth) show variable responses to the antimicrobial compounds. In the PDB medium the significant results were obtained after 24 h incubation. Longer incubation time resulted in no inhibition. This could be attributed to the morphogenic effect of the antifungal due to hyperbranching through the increasing of incubation time. Since one of the effects of morphogenic antifungals is hyperbranching of filamentous fungi due to multiple germination tubes, high density growth occurs in one small area (Troskie *et al*., 2012). However, the significant results in RPMI-1640 medium were achieved after 48 h incubation. These differences in the results obtained from PDB and RPMI-1640 tests may be attributed to the growth medium with low nutritional value (RPMI-1640) which allows the slow growth. Therefore it results in reliable absorbance measurement after 48 h despite the possible morphogenic effect of antifungal compound which causes fungal hyperbranching.

According to the results (Figures 3.25 and 3.28), the variation in fungal growth on different media (PDB and RPMI-1640) suggests that the fungi will be more susceptible to fungicidal action in certain media. Thus the growth medium could have an influence on the activity of the antifungal compounds tested. The antifungal activity of pheromone was confirmed with higher level of *Aspergillus* growth inhibition using RPMI-1640 medium than PDB. However, the minimum inhibitory concentration (MIC) of the pheromone as the potential fungicide compound is constant in both tests (13 $\mu g.ml^{-1}$). The variation in the inhibitory activity of the antifungal compound as a result of using different media in this study is comparable with the recent study by Troskie and colleagues (2012). They reported differences in *Botrytis cinerea* and *Fusarium solani* growth using different media with the two known fungicides, bifonazole and gramicidin S.

As reported in the literature (Troskie *et al*., 2012), it appears that broth dilution inhibition assays, based on spectrophotometric measurements, are suited to the more homogenous bacterial growth in suspension than heterogeneous fungal growth. The bacterial growth increases the optical density of the liquid medium uniformly giving a generally consistent increase in spectrophotometric measurements. Nevertheless, fungicides could have morphogenic and non-morphogenic activity on the growth of filamentous fungi. Morphogenic antifungals will alter the morphology of fungi while non-morphogenic antifungals, although inhibiting fungal growth, would not cause any alterations in morphology. One of the effects of morphogenic antifungals is hyperbranching of filamentous fungi which occurred due to multiple germination tubes, leading to high density growth in one small area (Troskie *et al*., 2012). The variable influence of fungicides on fungal growth will consequently affect on optical density; hence assays based on spectrophotometric measurements could lead to variance in determination of growth inhibition of filamentous fungi. The different results reported in this study from two different antifungal susceptibility tests agreed with this fact.

Based on the CLSI interpretations, the significant reduction in *Aspergillus* growth in the present study will be interpreted in two ways, firstly as “no reduction in growth” (fungus grows up to 78% of growth control) using PDB and secondly as “slight growth” (fungus grows up to 23% of growth control) using RPMI-1640.

4.9 Transcriptomic studies

Studies have shown that several strains of *B. subtilis* and *R. solanacearum* are capable of inhibiting aflatoxin accumulation (Nesci *et al*., 2005). Although the current research shows the inhibitory activity of *B. licheniformis* against *A. flavus* which causes the reduction in fungal growth, the direct effect of *B. licheniformis* on aflatoxin production in this study through the traditional purification of the produced aflatoxin from *Aspergillus* cultures was not investigated. However, transcriptomic study on the expression level of aflatoxin synthesis gene was performed and the results are discussed below.

Aflatoxins are secondary metabolites produced by a complex biosynthetic pathway. They are known as the most important mycotoxins produced by several Aspergilli and species assigned to other genera. Among these, the most economically important producer of aflatoxins is *A. flavus* and its relatives. Aflatoxin contamination occurs frequently in various food products. Sterigmatocystin is an intermediate compound in the procedure of aflatoxin biosynthesis. Molecular and genomic studies have led to the confirmation of aflatoxin and sterigmatocystin biosynthetic pathway which provided insight into the metabolism and effect of aflatoxins (Baranyi *et al*., 2013).

Early attempts tried to confirm aflatoxin production in fungi using 3 genes. Those attempts did not lead to reliable results (Shapira *et al*., 1996). Three different PCR systems have been used for the detection of aflatoxin producing isolates targeting the genes involved in the biosynthesis of aflatoxins: 1. a multiplex PCR assay targeting the *aflD* (*nor-1*), *aflR*, *aflP* (*omt-1*) genes, (Shapira *et al*. 1996), 2. PCR assays targeting the *aflP* (*omt-1*), *aflD* (*nor-1*), *aflM* (*ver-1*) genes individually (Farber *et al*., 1997) and 3. PCR assays amplifying individual sequences of the *aflRS*, *aflJ* and *aflO* (*omtB*) genes (Rahimi *et al*., 2008). Moreover, two regulatory (*aflR* and *aflS*) and three structural genes (*aflD*, *aflO*, *aflQ*) from the aflatoxin gene cluster of *A. flavus*, were targeted with specific primers for PCR to highlight their expression in mycelia cultivated under specific conditions (Levin, 2012). In the current study the targeted gene was *aflP* as an encoding gene of O-methyltransferase A or II which converts sterigmatocystin to O-methylsterigmatocystin as well as demethylsterigmatocystin to dihydro-O-methylsterigmatocystin (Abdel-Hadi *et al*., 2011).

Besides these PCR studies, some recent researches introduced real-time quantitative PCR (qPCR) which provides a tool for accurate and sensitive quantification of target DNA, that could be applied to detect and quantify aflatoxins producing moulds in foods (Mule *et al*., 2006; Gonzalez-Salgado *et al*., 2009; Rodriguez *et al*., 2011 and 2012). Some studies evaluated fungal contamination and aflatoxin production in crops applying qPCR techniques (Richard *et al*., 2009; Mideros *et al*., 2009).

In addition, qPCR has simplified the conventional fungal culturing techniques in the medium, with the continuous monitoring of sample amplification. This technique allows

easy identification of aflatoxigenic fungal samples using either non-specific fluorescent dyes, such as SYBR Green, which can also give a signal for primer-dimers and non-specific amplified products (Kubista *et al*., 2006), or a sequence-specific hydrolysis probe (TaqMan) (Rodriguez *et al*., 2012). In the current study, SYBR Green qPCR system has been applied to determine the expression level of *aflP* gene in *A. flavus* cultures which had been under the exposure of different treatments. The purpose of this experiment was to determine the impact of the applied treatments on aflatoxin production of the cultures through examining the change in the gene (encoded aflatoxin biosynthesase) expression level. The first attempt to optimise qPCR using the established primers (*omt* primers) (Rodriguez *et al*., 2012) did not work as the published sequence of primers was impaired and not compatible with *Aspergillus* sequence. So the second set of primers was designed (*aflP* primers). In the course of the second qPCR experiment, it became evident that production of the primer-dimers, and the potential non-specific amplification of *aflP* gene were inevitable with the new designed *aflP* primers. Therefore, SYBR Green method could not result in the conclusive qPCR analysis in this research.

Recently, an accurate qPCR (TaqMan) method was applied to investigate the potential impact of the interactions between water stress, temperature and CO_2 exposure on the gene expression of aflatoxin biosynthetic genes (*aflD*, *aflR*) in *A. flavus* (Medina *et al*., 2014).

To discriminate aflatoxigenic strains from non-aflatoxigenic strains of *A. flavus*, a reverse transcription-polymerase chain reaction (RT-PCR) protocol was elaborated by Degola and colleagues (2007). In another study by Leema and colleagues (2011), RT-PCR was used to investigate the expression level of *aflR*, *aflJ*, *aflC* (*pksA*) and *aflD* (*nor-1*) genes in *A. flavus* isolates from human keratitis.

In the current research, to establish whether the studied treatments exert any influence on the expression level of *aflP* gene in *A. flavus*, RT-PCR method was applied as an alternative method (Figure 3.31). The amplified bands associated to *aflP* gene (121 bp) from cDNA samples appeared in two samples; the control positive samples (aflatoxigenic strain of *A. flavus* without the addition of treatments) and the treated

samples with low concentrated washed *Bacillus* cells. It could be stated that low concentrated washed *Bacillus* cells did not affect *aflP* expression level, but other treatments exerted severe effect on the expression of *aflP* which causes the reduction/ elimination of the expression of the gene. However, the sharpness of the 121 bp bands (Figure 3.31, lanes 6, 12 and 14) even in the positive control is not high which could indicate the low expression level of *aflP* gene under the experimental condition. In contrast to another study, a sharp and intensive band was presented as the amplified fragment from *aflP* gene using different primer set in RT-PCR (Scherm *et al*, 2005). The current result is not comparable to the Scherm and colleagues report as the primers for *aflP* gene are not the same. In addition, several non-specific amplified bands on the electrophoresis gel are the evidence for the weak amplification of *aflP* gene using this primer set. However, this may be attributed to the inefficiency of the selected primers for this region of cDNA rather than the low level of *aflP* gene expression.

The most recent finding on tracking the best reference genes on RT-qPCR in filamentous fungi revealed a promising group of 6 genes. These genes presented weak expression changes and no tendency to up- or down-regulation over the whole set of conditions which were identified for *Talaromyces versatilis*. This group included *ubcB*, *sac7*, *fis1* and *sarA* genes, as well as *TFC1* and *UBC6* that were previously validated for their use in *S. cerevisiae* (Llanos *et al*., 2015). Unlike these reported reliable reference genes, in the current study, the weak expression of *ITS* (as a reference gene) was obtained from two tests, *Bacillus* supernatant in exponential growth-phase and low concentrated washed *Bacillus* cells (Figure 3.31, lanes 10 and 12). The results display the possible impact of these two treatments on the expression level of the conserved gene such as *ITS*. From these results, it could be concluded that *ITS* gene might not be a suitable reference gene as it would not belong to a reliable conserved region for RT-PCR and qPCR studies since its expression changed over the set of conditions due to using different treatments.

4.10 Bioinformatics studies on QS-related genes of *B. licheniformis* NCIMB 8874 genome

4.10.1 Conservation of proteins encoded by the *comQXPA* locus

The genetic characterization of competent pheromone was discovered for the first time in *B. subtilis* subsp. *subtilis* 168 (Magnuson *et al*., 1994). In this bacterium, cell-cell communication controls through the *comQXPA* locus. The products of this system are the ComX pheromone and the two-component transduction system ComP and ComA. They regulate the occurrence of natural competence in this bacterium (Weinrauch *et al.*, 1990, Dubnau *et al.*, 1994). The regulatory machinery leads to the development of competence, and related secondary metabolite production. The system is activated by accumulation of the pheromone in the extracellular milieu (Magnuson *et al.*, 1994).

Following the discovery of *comQXPA* cluster, each protein encoded by this locus has been investigated and compared with homologues in related species. These studies have shown that the competence regulating locus is highly polymorphic, with the polymorphism stretching through *comQ*, *comX* and the region of *comP* encoding the N-terminal part of the protein, whilst the C-terminus of *comP* and *comA* were proved to be highly conserved (Tran *et al.*, 2000, Tortosa *et al.*, 2001). Bioinformatics analysis performed on the recently acquired genome sequence of *B. licheniformis* NCIMB 8874 confirmed this hypothesis since *comQ* and *comX* coding regions of strains WX-02 and NCIMB 8874 were found to share only 85% identity at the nucleotide level. However these regions in ATCC 14580 strain show 93% identity with NCIMB 8874 strain of *B. licheniformis* as ATCC 14580 strain was known as *B. licheniformis* representative strain (normally used as a reference strain) whose genome was used to compare with *B. subtilis* 168 genome in the co-linear scaffolding technique by Lapidus and co-workers (Lapidus *et a*l., 2002).

Previous research has revealed that the *Bacillus* pheromones can be classified in four pherotype groups depending on their amino acid sequences and the nature of the post-translational modifications (operated by ComQ) on their tryptophan residues. Accordingly, the pheromones belonging to the same group are able to generate a cross-

induction phenomenon (Ansaldi *et al.*, 2002). In this context, the confirmed polymorphism in the *comQXP* locus suggests a striking pattern of specificity in pheromone interactions with receptor protein ComP. These findings also suggest that ComQ is the only dedicated protein required for the processing of active pheromone (Tran *et al.*, 2000, Tortosa *et al.*, 2001).

The present study demonstrated that the ComX pheromone generated by *B. licheniformis* NCIMB 8874 was able to activate a QS response in *B. subtilis* reporter strain through the β-galactosidase activation pathway. Therefore, comparative analysis was performed between the *comQXPA* loci of NCIMB 8874 strain and other selected *Bacillus*. This evaluation leads to investigate the relationship between the polymorphism of the competence regulating gene cluster and the specificity exerted in the QS system.

To this end, the products of the *comQXP* locus were individually aligned with homologues from selected *Bacillus*. To show the relative conservation of each protein, a phylogenetic tree was drawn, as illustrated in Figure 3.35 for ComQ, Figure 3.37 for ComX, and Figure 3.39 for ComP. Overall, the phylogenetic relationships between these selected strains based on four studied proteins showed that each protein could be classified in three different main phylogenetic groups. However, De Vizio (2011) reported four main phylogenetic groups related to each protein. This could be attributed to the different sequencing platforms used in these studies. 454 GS Junior and Illumina GAII sequencing platforms were utilised in De Vizio study while Ion Torrent next generation sequencing platform was used in the present study.

B. licheniformis strains were usually found in the same cluster, as well as a not identified species, namely *Bacillus sp*. BT1B_CT2. Interestingly, three polymorph proteins, including ComQ, ComX precursor and ComP, in two strains of *B. licheniformis*, ATCC 14580 and F11, were classified under a distinct group. Previously, it has been reported that these two strains of *B. licheniformis* harboured non-functional QS systems (Hoffmann *et al*., 2010). ComA congruence, shown in Figure 3.40, indicated that, whilst this protein is conserved in the same bacterial species, the conservation does not extend to the genus. This comparative analysis of *B. licheniformis* NCIMB 8874 may

present the potential conservation occurrence in ComA between the strains as well as across the species.

Although *B. subtilis subsp. subtilis* and *B. licheniformis* NCIMB 8874 were found to be classified under different phylogenetic groups for ComX and ComP, their positions on pre-ComX evolutionary tree are not too distant, thus confirming the possibility of cross induction between the two species. The amino acid sequence alignments between the two ComX precursor proteins, however, showed that their conservation is only restricted to the N-terminal ends. Moreover, high diversity in the amino acid sequence in C-terminus marked the pheromone-forming region, where the tryptophan residue is located.

Classification of this pheromone under a particular pherotype based on its amino acid sequence is not possible since little has been known about the mechanism of the modification by ComQ. As experimental evidence suggests that the ComX pheromone of *B. licheniformis* NCIMB 8874 is able to induce a QS response in *B. subtilis* reporter strain derived from *B. subtilis subsp. subtilis*, it may be concluded that the two molecules (ComX of both strains) possess similar modification although the amino acid sequences are certainly different.

4.11 The 16S rRNA study on *Bacillus*

The 16S rRNA gene sequence has been the most common genetic marker to study bacterial phylogeny and taxonomy. There are some reasons which make this marker well known in phylogenetic studies, including (i) its presence in almost all bacteria, (ii) the function of this gene over time has been conserved which helps to do evolutionary studies, (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes. Although it has been demonstrated in many reports that 16S rRNA gene sequence comparison between two close neighbour with a percent identity of <97% represents a new species, the meaning of similarity scores of >97% is not as clear. The latter value can represent a new species or indicate clustering within the previously defined taxon. However, 16S rRNA gene sequence data can be used for multiple purposes, there are no

defined “threshold values” (e.g., 98.5% similarity) to make a universal agreement which directs us towards the identification of the rank of species (Janda *et al*., 2007).

In the present study, the phylogenetic relatedness of different *Bacillus* species was investigated using 16S rRNA gene sequences. Available 16S rRNA gene sequences in *B. licheniformis*, *B. subtilis* and *B. movajensis* were aligned. The phylogenetic tree (Figure 3.42) shows three main groups, each belongs to one *bacillus* species. The percent identity matrix (Figure 3.43) presents that *B. licheniformis* and *B. subtillis* exhibit the similarity score of 97.95%. Also, it shows the similarity score of 97.82% between *B. licheniformis* and *B. mojavensis*. Wherase, *B. subtilis* and *B. movavensis* show a higher identity, 99.67%. These phlogenetic results indicate that even these species have the percent identity of >97% but they are clustering in three groups. This observation agrees with Janda and collegues studies (2007) about undefined universal threshold values to identify the rank of species.

Moreover, comparing the phylogenetic results obtained from the conserved gene such as 16S rRNA with ComQXPA in *Bacillus* species, it has also been demonstrated that *B. licheniformis* is phylotenetically closer to *B. subtilis* than *B. mojavensis* within ComQ, ComA and ComP (Figures 3.35, 3.39 and 3.41).

CHAPTER V

CONCLUSION

Chapter V

Conclusion

The aim of this study was to identify *B. licheniformis* NCIMB 8874 QSM through genomic and biochemical studies and then investigate the effect of this antagonistic bacterium and its QSM on the growth of *A. flavus*. The chosen *Bacillus* strain in this research, *B. licheniformis* NCIMB 8874, is an industrial antagonistic bacterium and *A. flavus* is a plant colonising fungus with the capability of producing the important toxin, aflatoxin.

After the successful gene cloning procedure which was applied to over-express the QS genes (*comQ* and *comX* are responsible for production of the ComX pheromone) of *B. licheniformis*, the study provided evidence for the presence of the QSM in the filtered extract of the supernatant of transformed *E. coli* BL21 carrying *comQX* genes. The sequence of this 13-amino-acid peptide as a novel QSM has been identified and its congruence with its nucleotide sequence has been confirmed. The over-production and purification of this QS compound through reverse-phase chromatography led to the identification of ComX pheromone for the first time.

This project has provided evidence that applying QS-related gene cloning technique could be a suitable biotechnology method for biosynthesis of the QS peptide, ComX pheromone, in larger amounts. However, this peptide signal needs further elucidation of its chemical structure.

Furthermore, this study reports a 10-amino-acid peptide with a sequence comparable to the sequence of the adjacent region to the pheromone sequence in the pre-ComX molecule. This is also the first report on the existence of a different molecule from QSM in *Bacillus* QS system which is secreted into the extracellular environment. This small peptide needs further investigation on its possible role in QS system.

Moreover, the over-produced pheromone in the transformed *E. coli* supernatants (in both low concentrated supernatant and high concentrated induced filtered extract

supernatant) has shown an almost identical QS response in *B. subtilis* reporter strain as the response by the pheromone produced in the late exponential-phase of *B. licheniformis*. Nevertheless, in the future studies, *B. licheniformis* reporter strains need to be established in order to detect QSM bio-activity more accurately. Also these reporter strains appear to be necessary to verify the effect of QSM on quorum sensing-regulated secondary metabolites production (Please note the Future Work Chapter, section 6.3).

On the genomic study, the sequenced recombinant plasmid (carrying *comQX*) obtained from the cloned recombinant *E. coli* BL21 confirmed the existence of *comQX* genes identical to its counterpart from *B. licheniformis* 9945A.

In the bioinformatics analysis of *B. licheniformis* NCIMB 8874 genome sequence, the comparative analysis at protein level between *B. licheniformis* NCIMB 8874 and other *Bacillus* determined the different level of the conservation in *comQXPA* genetic organisation. Although polymorphisms were identified in three genes of this cluster, ComQ, precursor-ComX and ComP, the ComA was confirmed as the most conserved protein in this cluster.

The present study has also established, for the first time, the confirmed antifungal influence of ComX pheromone on *A. flavus* which causes the fungal growth reduction as a natural antimicrobial compound.

This project has revealed that the ComX pheromone of *B. licheniformis* NCIMB 8874, as a bacterial QSM, is a potential signal not only in the bacterial system but also in communication between microbial kingdoms and has the potential to be applied for bio-control purposes. Identification of new natural antifungal signalling peptides against *A. flavus* could lead to the development of biotechnological strategies which facilitate control of aflatoxin contamination through the sustainable agricultural approach. Moreover, transcription studies are needed to further explore the results of fungal growth reduction at the gene expression level (e.g. aflatoxin biosynthesis genes).

QS is a mechanism for intra- and/or interspecies microbial communication. This research on a bacterial QSM and its inhibitory effect on fungal growth hints at the

existence of inter-kingdom cross-talk. This communication might occur through the available QS systems in both communicating sides. Therefore, the investigation of putative fungal QS which might be involved in this cross-talk is desirable. Indeed, unravelling the mechanism of the interaction between the two kingdoms (e.g. *Bacillus* and *Aspergillus*) might help to achieve a better understanding of other examples of pathogen-host or antagonist- agonist interactions through QS.

Furthermore elucidation of the mechanisms of QS in fungal pathogens will lead to a better understanding of fungal pathogenesis facilitating the development of novel antifungal and antibacterial approaches to combat human diseases.

Following on from the novel antifungal characteristic of the ComX pheromone in *B. licheniformis*, the investigation could be continued to find the potential antifungal and antimicrobial activity of other QSMs generated from different industrial bacterial strains to be applied to the therapeutic purposes. Moreover, it is desirable to develop a method for large-scale production of these compounds. Indeed, it would have economic benefits for the pharmaceutical and biotechnology industries as the optimised large-scale method would enhance the production of a natural, commercially useful antimicrobial molecules.

CHAPTER VI

FUTURE WORK

Chapter VI

FUTURE WORK

Taking into account the results of the present research and the derived conclusions, the following suggestions for future work may be considered. There are a number of ideas to further clarify the QS system in *Bacillus licheniformis* and *Aspergillus flavus* through which there is a possible avenue for their cross-talk.

6.1 Investigation of QS system in *B. licheniformis* NCIMB 8874 through microarray analysis on QS genes expression

Whereas cell-cell communication in *B. subtilis* has been investigated extensively, very little about this process is known in *B. licheniformis*. Given the fact that the research so far has shown that QS is slightly different in *B. licheniformis* compared to *B. subtilis*, the competence and sporulation factor as the second signalling molecule of *B. subtilis* has not yet been reported in *B. licheniformis.* Therefore, further investigation will be needed to elucidate the regulation of cell-density dependent phenomena in *B. licheniformis*.

Moreover, in the present study, a 10-amino-acid peptide has been identified in the pheromone purification procedure. It has a compatible sequence with the sequence of one region in pre-ComX. This might be determined as a novel molecule in the QS regulatory mechanism whose role in this system needs to be investigated.

There are some reports of applying microarray analysis to investigate QS pathway in *B. subtilis* strains. For instance, Berka and colleagues (2002) compared the expression profiles of wild-type and *comK* mutant, *mecA* mutant and a *comK*/*mecA* double mutant, using DNA microarrays. Moreover, using the whole genome DNA microarray technique was applied to identify genes whose expression is affected by *comA*. This technique was also used to identify genes affected by the ComX pheromone and the histidine kinase ComP (Comella and Grossman, 2005). DNA microarrays containing PCR products of the

annotated open reading frames covering >99% of the genome were used in these studies. In the experimental procedure, labelled cDNAs were subjected to several incubations, washing and treating steps to be prepared for hybridization.

In addition, RNA-sequencing is a new technique using Next Generation Sequencing (NGS) facilities to reveal the presence and quantity of RNA in a biological sample. Since this method has been recently established, there are lower numbers of reports on bacterial/fungal gene expression investigations than human studies (Maher *et al.*, 2009). However, the microarray analysis is more popular in microbial genetic studies and the established methods for particular research subjects such as bacterial QS pathway investigations are available which would help to expand this research in the future.

The recently acquired genomic sequence of *B. licheniformis* NCIMB 8874 could be used to design probes for gene expression profiling analysis by using microarray technology. This would allow comparison of the transcription levels in *B. licheniformis* cultures grown in the absence and presence of the ComX pheromone. This would generate a comprehensive profile of genome wide changes in gene expression in response to the addition of the signalling molecule and other treatments.

This method also has the great potential to be applied to investigate the effect of *A. flavus* on *comQX* gene expression and the production of ComX pheromone by *B. licheniformis* under the co-culture condition. Therefore, it is useful to investigate the competition between *Bacillus* and target fungus in relation to bacterial QSM gene expression and peptide production. Likewise, the comparative analysis of the washed and unwashed *Bacillus* cells could be applied to co-culture with *Aspergillus*. Here, again, the microarray analysis would help to clarify the effect of washing process on the production of QSM from the bacterial cells under the co-culture condition.

6.2 Scale-up of pheromone production in recombinant *E. coli* fermentation

One of the major concerns for the biotechnology industry is to maintain the productivity of products at large scale as they are successfully produced in small scale. Based on the present study, the recombinant *E. coli* Bl21 (DE3) carrying *comQX* genes will be used for the over-production of ComX pheromone.

E. coli is the best-established production organism in industrial biotechnology. However, when aerobic fermentations are performed at high growth-rates, considerable amounts of acetate are accumulated as by-product. This by-product has negative effects on growth and protein production. To overcome this problem, some strategies for reducing or eliminating unwanted acetate production have been adopted. The procedures include approaches that optimize the protein production process (Eiteman and Altman, 2006).

Therefore, scale-up and optimisation of the enhanced pheromone production in large scale would offer the possibility to extend the exploitation of recombinant *E. coli* for therapeutic purposes.

Moreover, it would be of interest to analyse by NMR the structure of highly concentrated and purified pheromone obtained from the scale-up experiments.

6.3 Construction of *B. licheniformis* reporter strains

The application of bioassays in the investigation of microbial cell-cell communication is a well-established practise. Bioassays have been developed to identify/detect the auto-inducer mediated QSMs by exploiting an easily observed phenotype characterised in bacteria, such as pigmentation or bioluminescence. In bacterial species which do not possess an easy-to-detect phenotype, this is created by introducing a reporter gene.

Reporter genes, such as the β-galactosidase encoding gene *lacZ*, could be used to investigate the biosynthetic gene expression of any secondary metabolite (e.g. the lichenysin biosynthetic operon) which is regulated by QS in *B. licheniformis* NCIMB 8874.

This experiment could be performed by constructing a reporter strain carrying a fusion of this operon promoter and the *lacZ* gene (*licA-lacZ* fusion) through gene cloning technique. This construct would lead to the study of the production of lichenysin, or other secondary metabolites modulated by QS in a cell-density dependant manner, by means of β-galactosidase assay. The level of β-galactosidase activity will indicate the secondary metabolite gene expression level induced by signalling molecules accumulated in the extracellular medium of the tested cultures.

Furthermore, the reporter strains could be exploited to examine the effect of exogenous addition of ComX on the production of secondary metabolites. By adding different concentrations of pheromone at various times to the cultures of *B. licheniformis* reporter strains, the best conditions for optimisation of the production of quorum sensing-regulated secondary metabolites would be established.

6.4 Construction of *B. licheniformis* NCIMB 8874 *comX* mutant

Construction of *B. licheniformis* mutants for *comX* gene will be useful to study the effect of *B. licheniformis* mutant cells under washed or unwashed condition (as treatments in co-culture with fungus) on *A. flavus* growth-rate.

This study would lead to perform the comparative analysis of the effect of the QS deficient strain and wild type strain on fungal growth-rate. The investigation will allow further elucidation of the role of bacterial cells alone without the contribution of their QSM on reducing *Aspergillus* growth in the competitive condition of co-culture by fungus. The extent of the involvement of the secreted QSM on fungal growth inhibition through antagonistic activity of *Bacillus* could therefore be confirmed.

A null allele is a mutant copy of a gene at a locus that completely lacks the normal function of that gene. This can be the result of the complete absence of the gene product such as RNA or protein at the molecular level, or the expression of a non-functional gene product. To make a null allele using microsatellites is very common. Microsatellites are the repetitive sequence of DNA and applied as molecular markers

amplifying them through PCR. Unlike the point mutations, which affect only a single nucleotide, microsatellite mutations lead to the gain or loss of an entire repeat unit (Tautz and Schlotterer, 1994). Therefore, using microsatellite marker would be a suitable method to make *B. licheniformis* mutant of *comX* gene.

6.5 Investigation antimicrobial property of QSM in other bacteria (e.g. *B. subtilis*)

Recently, the antifungal effect of a QSM from *P. aeruginosa* was reported. This bacterium inhibited *A. fumigatus* biofilm formation under co-culturing condition. Subsequently, co-culture of *P. aeruginosa* QS mutants did not significantly inhibit *A. fumigatus* biofilms and filamentation compared to the wild type *Pseudomonas* (Ramage *et al*., 2011). Accordingly, the present study on *B. licheniformis* reports another example of QSM antifungal effect on *Aspergillus*. In this line of research, the investigation could be continued to confirm the potential antifungal and antimicrobial activity of other QSMs generated from different Gram-positive and Gram-negative bacterial species such as *B. subtilis* and *Pseudomonas* spp. following the techniques used in this study such as QS gene cloning, QSM over-production, QSM purification and co-culturing test with particular microorganisms to study the antimicrobial effect of studied QSM.

6.6 Further investigation of aflatoxin biosynthesis and the expression of related genes by microarray analysis

The qPCR and RT-PCR methods used in this work were not sufficient to show changes in the expression level of *aflP* gene in *A. flavus*. So, to complete the findings, microarray analysis needs to be carried out on the genes (structural and regulatory) related to aflatoxin biosynthesis.

A whole-genome microarray of *A. flavus* has been used to study the regulation of aflatoxin biosynthesis genes. Price *et al*. (2005) used a whole-genome microarray approach to analyse the influence of substrate composition and pH on the activation of

aflatoxin biosynthesis genes. O'Brian and colleagues used this technique and found that temperature affected functioning of the genes, within transcript levels of *aflR* and *aflS* proteins in *A. flavus* (O'Brian *et al*, 2007). The recent study by Abdel-Hadi *et al.* (2012), applied a microarray analysis to examine the effect of combinations of water activity and temperature on the activation of aflatoxin biosynthetic genes (30 genes) in *A. flavus.* The relative expression of 10 key genes (*aflF*, *aflD*, *aflE*, *aflM*, *aflO*, *aflP*, *aflQ*, *aflX*, *aflR* and *aflS*) in the biosynthetic pathway was examined in relation to different environmental factors and phenotypic aflatoxin B1 production. In these experiments, cDNA synthesis and labelling were performed prior to putting the samples in an automatic hybridization station. After hybridization, the array was scanned with a laser system.

In particular, the availability of the genome sequence of *A. flavus* NRRL 3357 allows the design of partial genomic microarrays which could then be applied to determine the transcriptional response of *A. flavus* once it is under the exposure of bacterial cells and their secreted pheromone. Comparative analysis could be undertaken with other tests of various supplementations to *A. flavus* cultures. This will offer the possibility to gain a picture of the global pattern of aflatoxin associated gene cluster expression affected by *B. licheniformis* QSM and cells.

6.7 Investigation of QSM in *A. flavus* NRRL 3357

The instances of small molecule exchange between bacteria and eukaryotes suggest that the inter-kingdom cross-talk through QS phenomenon might exist/ be developed.

In this context, it can be suggested that *A. flavus*, through the QS process, could communicate with the antagonistic bacteria through their QS system. As this study reveals the significant influence of bacterial QSM on *Aspergillus* growth, an understanding of the possible QS signalling pathways in filamentous fungi, such as *Aspergillus,* would be an interesting line of research. Considering the reported QS behaviour in the filamentous fungi (e.g. *A.terreus*, *A. nidulans* and *A. flavus*), investigating the involvement of *A. flavus* QSM or QS system in its communication with

antagonistic bacteria via their QS mechanism can be suggested. This line of research will offer a better understanding of cell-cell communication networks which are involved in the inter-kingdom cross-talk. Further elucidation of the mechanisms of QS in fungal pathogens will lead to a better understanding of fungal pathogenesis promoting novel antifungal approaches to combat human diseases.

This would lead eventually to possibilities to interfere with the fungal signalling systems, blocking their successful colonization, and also would develop transgenic plants to be used in sustainable agriculture.

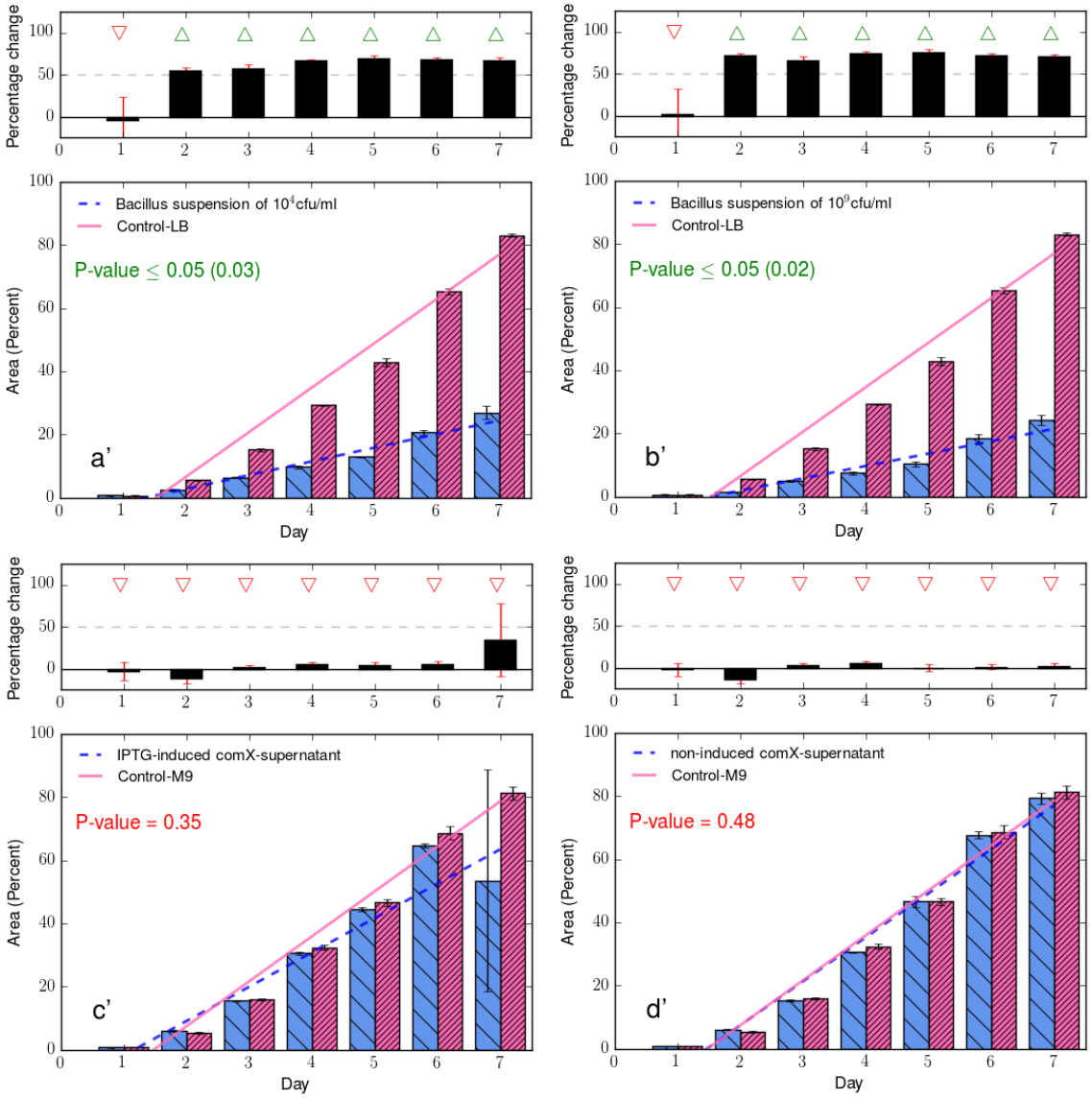
CHAPTER VII

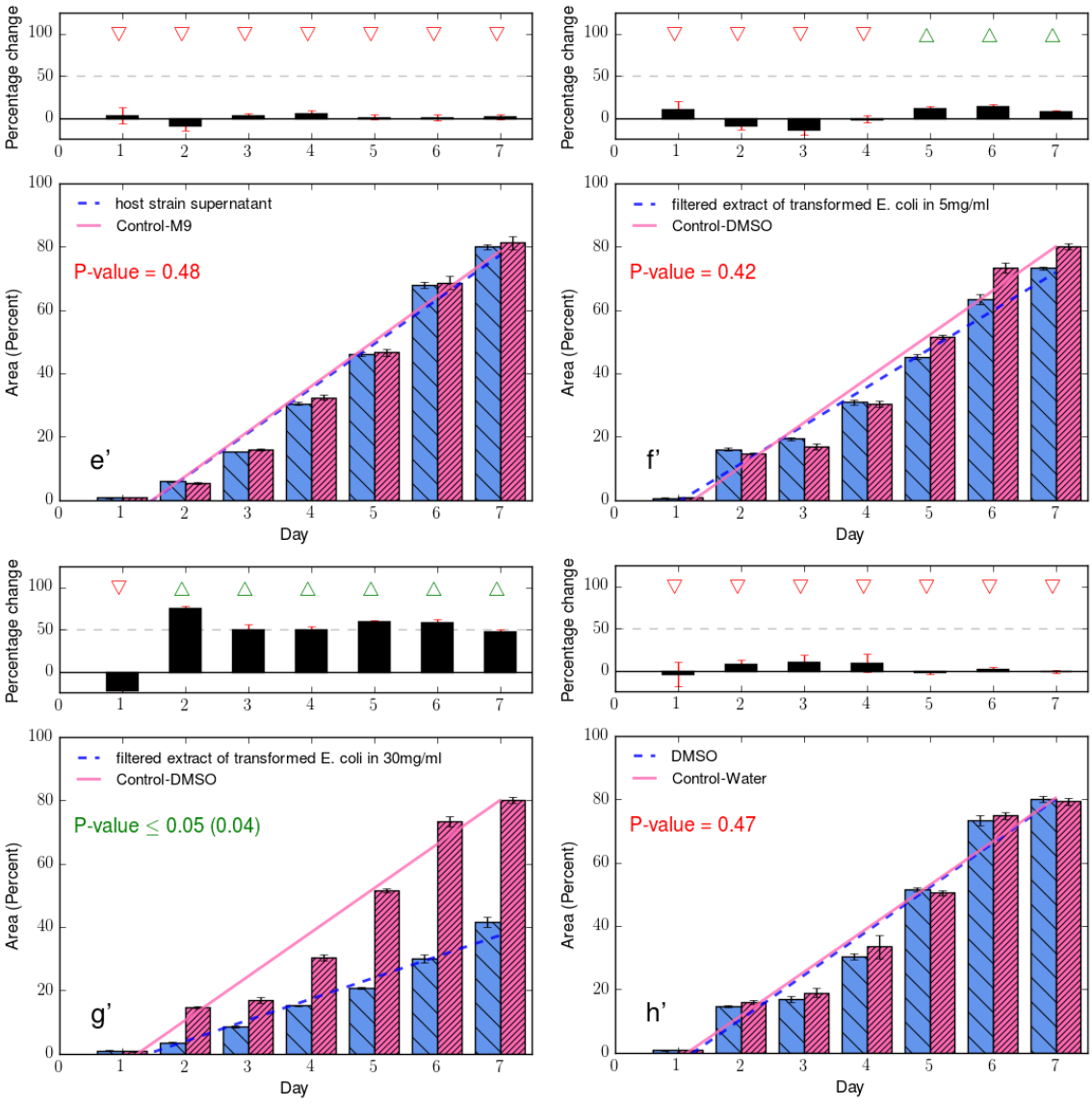
APPENDICES

CHAPTER VII

APPENDICES

7.1 Effect of *B. licheniformis* NCIMB 8874 and its QSM on the growth-rate of *A. flavus* ESP 15 (non-aflatoxigenic strain)




Percentage change
Area (Percent)
Day
host strain supernatant
Control-M9
P-value = 0.48
e'
filtered extract of transformed E. coli in 5mg/ml
Control-DMSO
P-value = 0.42
f'
filtered extract of transformed E. coli in 30mg/ml
Control-DMSO
P-value ≤ 0.05 (0.04)
g'
DMSO
Control-Water
P-value = 0.47
h'

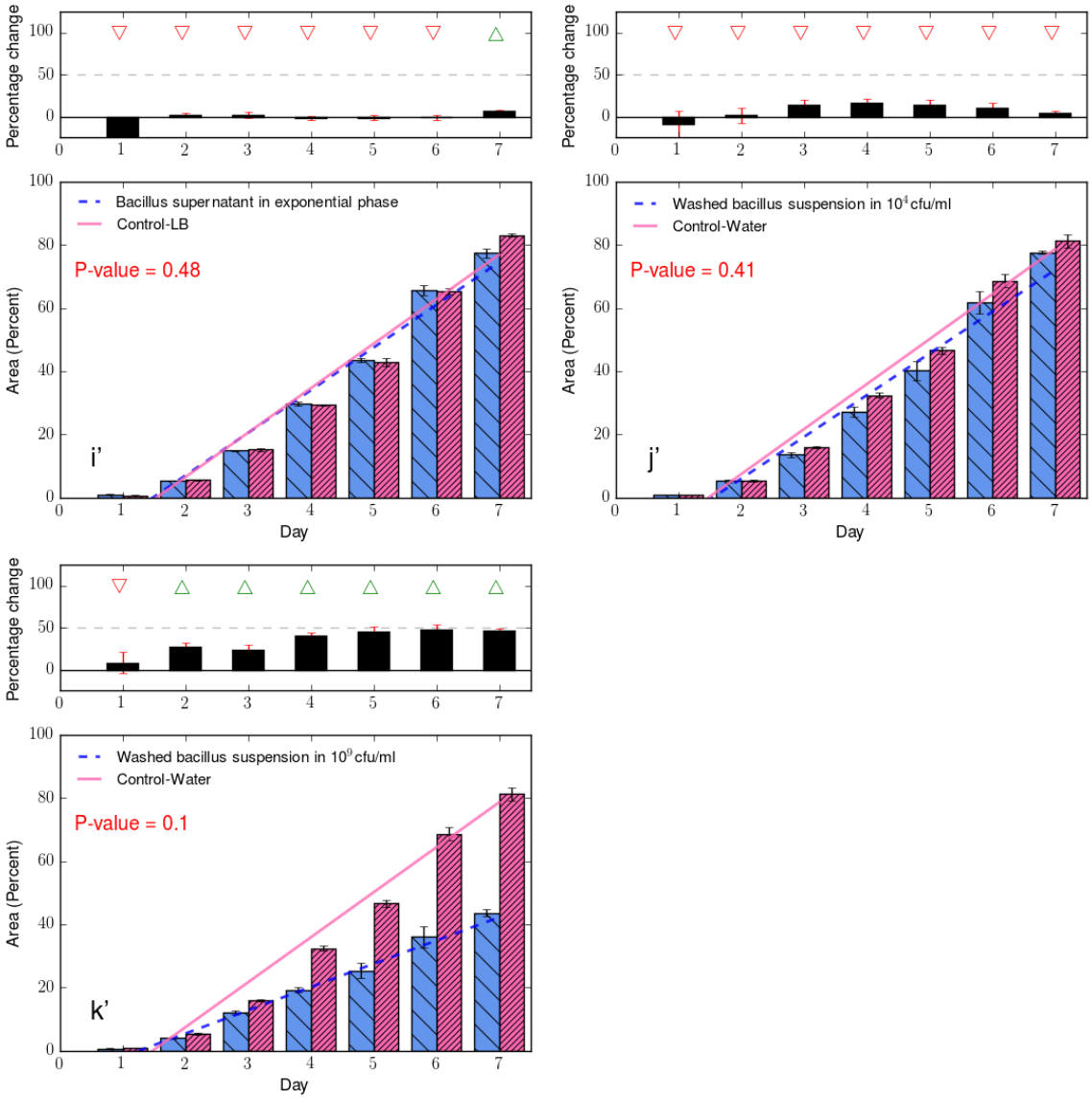


Figure 7.1 Fungal colony area changes during 7 days of monitoring test plates and associated control plates. Tests labelled from (a) to (k) (Table 7.3). The line of best fit is drawn for both sets of data (test/control) in each profile. Red dotted line is associated to the control and blue dashed line is associated to the test. The written P-value for the growth profile shows the statistical significance of the overall change of the test compared to its control measured over the 7 days. The upper plot shows colony area percentage changes between test and its control in each individual day of monitoring period. The statistical significance of the percentage change is shown in green and red triangles. Green upward triangle represents the significant difference between test and control per individual day ($P \leq 0.05$) otherwise it is red downward triangle.

Table 7.1 The slope values of the best fit lines of the fungal growth data obtained from the tests and their associated controls. In the observations column, the notable differences between the test and the control slopes (the ratio of over 1.8) are shown as * and negligible differences are presented as N.

Test labels	Slope1 (Test)	Slope2 (Control)	Ratio of the slopes (Control/Test)	Observations
a'	4.34	14.1	3.25	*
b'	3.92	14.1	3.6	*
c'	10.88	14.25	1.31	N
d'	13.94	14.25	1.02	N
e'	14.02	14.25	1.02	N
f'	12.09	13.91	1.15	N
g'	6.7	13.91	2.08	*
h'	13.91	13.76	0.99	N
i'	13.51	14.1	1.04	N
j'	13.22	14.25	1.08	N
k'	7.37	14.25	1.94	*

Table 7.2 The list of P-values of each observed distribution compared to its fitted model (four possible models)

Model	Min P-value	Max P-value	Mean P-value	Standard Deviation
linear fit (y = a*x + b)	0.999	0.999	0.999	0.000
power-law ($y = a*x^b$)	0.946	0.998	0.975	0.00014
exponential ($y = a*exp^{b*x}$)	0.922	0.965	0.941	0.0001
linear fit with fixed y-intercept (y = a*x)	0.763	0.867	0.793	0.0007

Table 7.3 List of the treatments and associated controls applied to the cultures of *A. flavus* ESP 15

Test labels	Treatments	Associated controls
a'	*Bacillus* suspension of 10^4 cfu.ml^{-1}	Control-LB
b'	*Bacillus* suspension of 10^9 cfu.ml^{-1}	Control-LB
c'	IPTG-induced *comX*-supernatant	Control-M9A
d'	Non-induced *comX*-supernatant	Control-M9A
e'	Host strain supernatant	Control-M9
f'	Filtered extract of transformed *E. coli* in 5mg.ml^{-1}	Control-DMSO
g'	Filtered extract of transformed *E. coli* in 30mg.ml^{-1}	Control-DMSO
h'	DMSO	Control-Water
i'	*Bacillus* supernatant in exponential phase	Control-M9
j'	Washed *bacillus* suspension in 10^4cfu.ml^{-1}	Control-Water
k'	Washed *bacillus* suspension in 10^9cfu.ml^{-1}	Control-Water

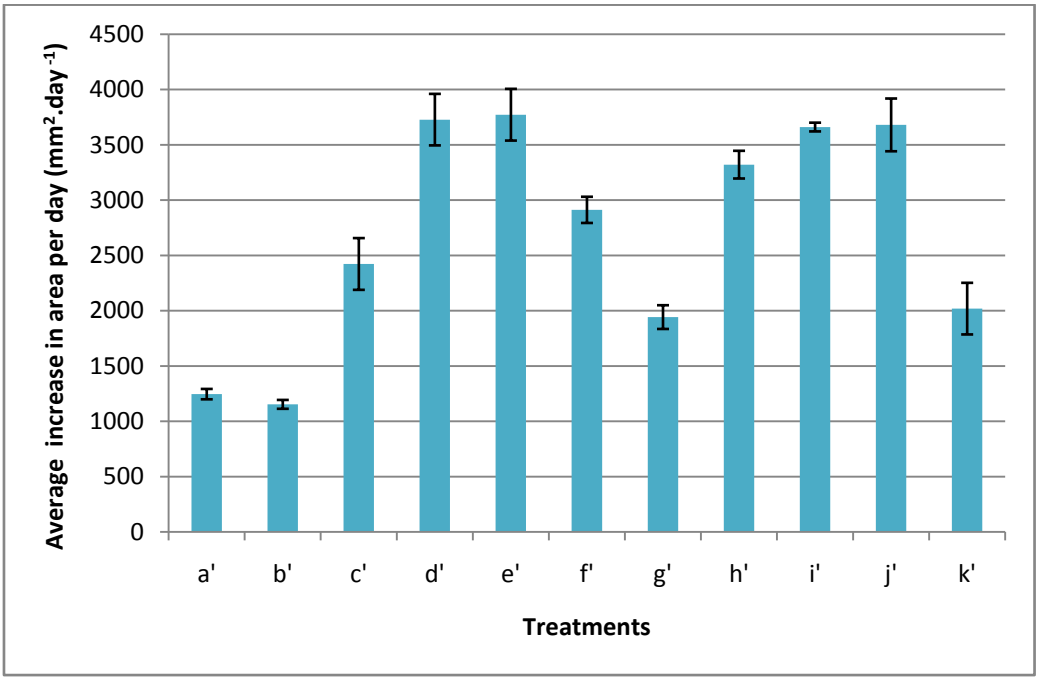


Figure 7.2 The average increase of fungal colony area per day measured during 5 days of monitoring test plates between day 2 and 7. Treatments labelled from (a') to (k') (Table 7.3).

CHAPTER VIII

REFERENCES

Chapter VIII

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