Quorum sensing in the cyanobacterium Gloeothece PCC 6909.

Sharif, Dilara Islam

QUORUM SENSING IN THE CYANOBACTERIUM *GLOEOTHECE* PCC 6909

Dilara Islam Sharif
B.Sc (Hons) and M.Sc (Dhaka)

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This thesis is dedicated to my parents
To whom I shall always be deeply indebted

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SUMMARY

The thesis sought to study whether cyanobacteria utilise the process of quorum sensing during their growth, to identify any quorum sensing molecules produced by the cyanobacteria *Gloeothece* PCC 6909/1 and to study the effect of any such molecules in aexenic cultures of the organism. The study presents the first evidence of N-octanoyl homoserine lactone (C8-HSL) quorum sensing molecule from axenic cultures of the cyanobacterium PCC 6909 and its sheathless mutant PCC 6909/1 and that the production of this molecule followed a density dependent accumulation, a common feature of many quorum sensing systems. The response of the *Gloeothece* proteome to exogenous concentrations of C8-HSL was examined, indicating changes in 43 protein spots on a 2D-PAGE gel, thereby indicating a response through global changes in protein expression. Among the 15 proteins that showed more than 2 fold expression changes, RuBisCo, glutamate synthase, chorismate synthase, a LysR family of transcriptional regulator (all up regulated) enolase and aldolase (down regulated) could be identified. A number of phosphorylated proteins also showed increased accumulation suggesting changes in the phosphoproteome of *Gloeothece*. A response to C8-HSL was also detected in physiological changes of the organism through decreased accumulation of extracellular soluble carbohydrates and an increased acid phosphatase activity from cell extracts.

In conclusion, this study presents evidence that the cyanobacterium *Gloeothece* employs a C8-HSL based quorum sensing system through the accumulation and response to a C8-HSL signal. These findings can help increase our understanding of how colonial cyanobacteria encounter stress at high cell densities.

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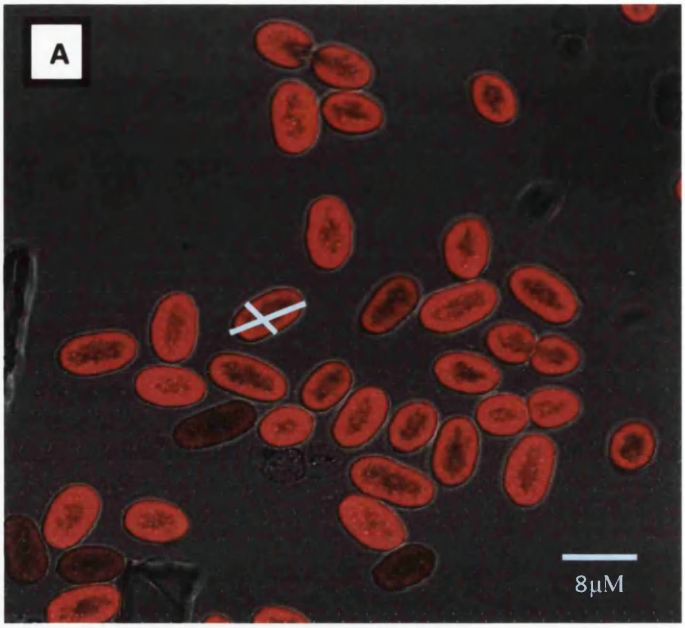
Abbreviations

ACP	Acyl carrier protein
AHL	Acylated homoserine lactone
AI-2	Autoinducer-2
BCA	Bicinchoninic acid
BG11	Cyanobacterial mineral medium with nitrate
$BG11^0$	Cyanobacterial mineral medium without nitrate
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
DHAP	Dihydroxyacetone phosphate
2D-PAGE	Two dimensional polyacylamide gel electrophoresis
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
F1, 6-BP	Fructose 1, 6-bisphosphate
G3P	Glyceraldehyde 3-phosphate
HPLC	High performance liquid chromatography
IgG	Immunoglobulin
LCMS	Laser scanning confocal microscopy
LC-MS	Liquid chromatography mass spectrometry
$[M+H]^+$	Protonated molecule
MS	Mass spectrometry
m/z	Mass to charge ratio
p	Level of significance
PBS	Phosphate buffer Saline

PEP	Phosphoenolpyruvate
2-PGA	2-phosphoglycerate
PMSF	Phenylmethylsulfonal fluoride
ρNPP	Para- nitrophenyl phosphate
ρNP	Para-nitrophenol
Psi	Pound-force per square inch
QS	Quorum sensing
RuBisCo	Ribulose bis phosphate carboxylase/oxygenase
SAM	S- adenosyl methionine
SB3-10	N-Decyl-N, N-dimethyl-3-ammonio-1-propanesulfonate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRM	Single reaction monitoring
TEMED	*N,N,N,N*-tetramethylethylenediamine
TCA	Trichloroacetic acid
TLC	Thin layer chromatogram
XIC	Extracted ion chromatogram
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside



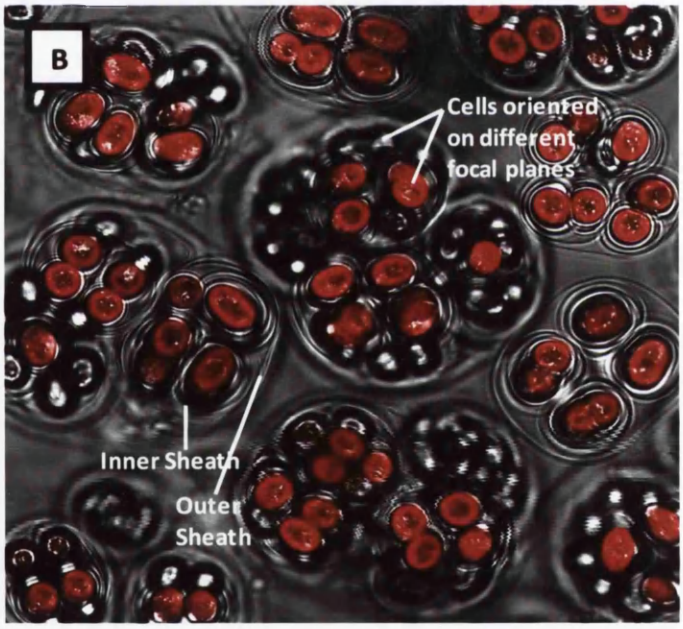


FIGURE: Confocal Laser Scanning Microscopic images of *Gloeothece* showing;

A. *Gloeothece* PCC 6909/1, sheathless mutant

B. Gloeothece PCC 6909 , wild type strain. Cells cluster as mirocolonies bound by inner and outer multilaminated sheaths.

CHAPTER 1

1. INTRODUCTION

1.1. What are Cyanobacteria?

Cyanobacteria are a group of gram negative photosynthetic bacteria capable of photosynthetic oxygen evolution akin to higher plants. They probably exhibit the widest range of diversity in respect to growth habitats of all photosynthetic organisms, as they are found in cold and hot, alkaline and acidic, marine, freshwater, saline, terrestrial, and symbiotic environments. They can synthesize chlorophyll A and have also been termed as blue green algae due to their ability to form phycobiliproteins which impart the blue-green coloration to cyanobacteria. Some cyanobacteria however, can also synthesize phycoerythrin, a red pigment that imparts red colouration to these cyanobacteria. The blue pigment Phycocyanin can constitute approx 60% of the soluble proteins in cyanobacteria under certain conditions. Although cyanobacteria are categorized as a single phylogenetic group they are diverse in their morphological structure, with cells ranging from coccoid to multicellular filamentous forms. Their diversity is also evident at the species level, with individual strains significantly differing in their genetic makeup.

To survive under diverse environmental conditions, cyanobacteria have developed unique adaptive capabilities that allow them to grow in environments that are considered extreme including conditions of high metal toxicity, low oxygen, free sulphide, UV radiation, desiccation and nutrient limitation (Schwarz and Forchhammer,

2005). Most of the studies on cyanobacteria have been carried out on *Synechocystis*, *Synechococcus*, *Anabaena* and more recently *Nostoc* sp. These cyanobacteria have a readily available genome sequence from which valuable information can be obtained. However, most of the other unsequenced cyanobacteria that are found to inhabit extreme conditions are poorly understood. One study revealed that most of the terrestrial coccoid cyanobacteria detected in biofilms present on buildings often failed to be detected in laboratory cultures (Crispim and Gaylarde, 2005). DNA amplification from these biofilm colonies revealed that they share a low homology to most aquatic species of cyanobacteria which comprise the majority of known sequences in present databases (Crispim and Gaylarde, 2005). Therefore, the study indicates the lack of sufficient information on terrestrial cyanobacterial populations that are found to inhabit extreme environments subjected to temperature and salinity extremes, repeated desiccation and high UV. Recently, these terrestrial cyanobacteria have attracted great interest in understanding evolution of life forms on earth. In this respect, the unicellular coccoid cyanobacteria can also be significantly important in understanding cooperative behaviour and the emergence of multi-cellularity. However, dense growth of these various unicellular coccoid cyanobacteria associated biofilms and cyanobacterial mats and their association with other bacteria make these cyanobacteria potentially valuable in the study of quorum sensing and inter-kindom signalling that may lead to an understanding of coordination and species survival within mixed microbial communities.

1.1.1. Growth of Cyanobacteria

Nutritional requirements for cyanobacterial growth are often reflected in their ability to colonize different habitats and, as a result, it is difficult to study specific requirements of individual cyanobacterium under laboratory conditions. Despite their diversity in nutritional requirements, most cyanobacteria that were initially isolated and subcultured in laboratory conditions are considered to be those that can provide adequate biomass rapidly, by their ability to grow exponentially and utilize nutrients at very high ambient concentrations. Several cyanobacterial growth media have been developed which provide higher concentrations of nutrients for growth. The most common growth media used for fresh water, soil and thermophilic cyanobacteria is BG11 (mineral medium with nitrate) and, in some cases, these are used with minor modifications to suit individual growth requirements of cultures (Stanier *et al.*, 1971). Although BG11 growth medium is generally used in the cultivation and growth of several cyanobacteria, the medium may not support optimal growth of some individual cyanobacteria that prefer their natural resources for growth and therefore, further investigation of their natural habitats may be required to obtain a balanced optimal growth for use in the laboratory. These cyanobacteria have been found to grow at a higher cell density when provided and maintained under their natural growth conditions. For example, the cyanobacterium *Gloeothece* when supplied with its natural growth substrate gypsum was found to double its biomass when compared with the normal substrate used under batch cultivation (Ortega-Calvo *et al.*, 1995). It was demonstrated that this cyanobacterium requires a high demand for sulphur for balanced growth due to the incorporation of large amounts of sulphur into its polysaccharide sheath (Ortega-Calvo *et al.*, 1995). Another cyanobacterium *Aphanothece sacrum,*

which is phylogenetically closely related to *Gloeothece*, could not be isolated using the conventional BG11 medium. However, designing a medium that closely resembled the nutrient concentrations of its natural growth habitat, it became possible to isolate this cyanobacterium in culture, retaining its natural form (Fujishiro *et al.*, 2004). In laboratory cultures these cyanobacteria may exhibit a linear growth rate compared to other cyanobacteria that are capable of growing exponentially, provided that adequate supply of nutrients is maintained. Linear growth in cyanobacteria can be common when grown under laboratory conditions and may result due to a limiting nutrient and cells may undergo an extended linear growth before entering into a stationary phase of growth (Foster *et al.*, 2007; Volk, 2007)

1.1.2. Gene expression in Cyanobacteria:

Gene expression in cyanobacteria has been studied extensively through artificially imposing various stressful conditions including high light stress (Hihara *et al.*, 2001; Mary *et al.*, 2004), high-temperature stress (Hirani *et al.*, 2001), hyperosmotic stress (Shapiguzov *et al.*, 2005), salt stress (Shoumskaya *et al.*, 2005), intense-light stress (Lazdunski *et al.*, 2004), heat stress (Rajaram and Apte, 2008). These studies have provided valuable information regarding gene expression in cyanobacteria that may occur when they encounter various levels of stress under natural conditions. To date most research in cyanobacterial gene expression has mainly focused on *Synechocystis* PCC 6803, due to the established gene sequence of this cyanobacterium (Ikeuchi, 1996). Experiments for example, the temporal program of gene expression during acclimation from low to high light intensity have been carried out on transcriptomic

analysis using microarrays to address diverse stress-induced modifications of gene expression within *Synechocystis sp* (Hihara *et al.*, 2001). Proteomics analyses have also been used in several studies and have provided a valuable tool to examine the functionality of the organisms (and their components), because the observed phenotype of an organism is a direct result of the action of the proteins rather than the genome sequence (Rosen *et al.*, 2004). With the use of proteomics it is possible to obtain valuable information and improved understanding of metabolic pathways, physiological responses and regulation in cyanobacteria (Kurian *et al.*, 2006a). Proteomic analysis can also provide a readily accessible, inexpensive tool for analyzing global protein expression patterns and post translational modifications with high resolution (Lowry *et al.*, 1951) (Rosen *et al.*, 2004) (Fulda *et al.*, 2006). However, the use of proteomics has been limited due to the cumbersome nature of current two-dimensional gel electrophoresis (2D-PAGE) techniques, low recoveries from 2D gels and non representative results (due to the exclusion of several protein types) (Santoni *et al.*, 1999). The whole proteome on a 2D gel has only been investigated for the cyanobacterium *Synechocystis* sp. (Fulda *et al.*, 2006), *Synechococcus* sp. strain PCC 7942 (Koksharova *et al.*, 2006) and *Anabaena variables* (Barrios-Llerena *et al.*, 2007). On the 2D proteome map carried out on *A. variables* authors claim to have identified 254 unique proteins, with significantly better coverage of basic and low abundance proteins than that reported in 2D analyses of, *Synechocystis sp.* Various other studies on 2D have mainly focused on subcellular fractionation methods, where different techniques are used to enrich proteins from separate fractions of cells such as the cytosol, membrane etc. Proteins resolved on 2D-PAGE from the cyanobacterium *Synechocystis* PCC 6803 allowed the identification of proteins from the periplasm (32 proteins) (Kurian *et al.*, 2006b), the plasma membrane (84 proteins) (Huang *et al.*,

2003), the thylakoid membrane (58 proteins) (Srivastava *et al.*, 2005) and the outer membrane (29 proteins) (Huang *et al.*, 2003). The 2D proteomic approach has also been used to provide information in relation to differential expression of proteins in response to various stress conditions, such as the response of *Synechocystis* in response to salt stress reviewed by Pandal, (2006) (Pandhal *et al.*, 2008). Proteins of the cyanobacterium *N. communi* have also been investigated through the 2D approach and involved the study of shock and acclimation proteins under UV-B stimuli and the study of proteins under photoautotrophy and diazotrophy growth conditions through partial fractionation of cytosolic, membrane-associated and membrane proteins (Ehling-Schulz *et al.*, 2002; Lowry *et al.*, 1951). A summary of all proteomics techniques employed on cyanobacteria before July 2003 has been summarized by Burja, (2003) (Burja *et al.*, 2003).

1.1.3. Intercellular signals studied in Cyanobacteria

Intercellular signals are released by bacteria in order to communicate with other bacteria of their own species or other host cells during a symbiotic association (Meeks *et al.*, 1978). Usually the signals are released extra-cellularly and a small concentration is required in order to produce a response. Various studies in cyanobacteria have focused on intracellular signalling systems but very little information is available on intercellular signals in cyanobacteria. The common areas where cyanobacterial intercellular signalling has been studied is in heterocyst formation (Yoon and Golden, 1998) and in symbiotic association during lichen formation, although the specific signals involved in the latter are not yet clear. In conditions of nitrogen limitation filamentous cyanobacteria are able to form specialized cells called heterocysts, which

are able to fix atmospheric nitrogen. Heterocyst formation has been studied at the molecular level, revealing that a diffusible peptide PatS, acts as a signal in heterocyst formation (Yoon and Golden, 1998). These findings indicate that intercellular signalling is involved in cooperative activities of cells to survive under nitrogen limitation in these heterocyst-forming cyanobacteria and open up the possibility of intercellular signalling to be present in other types of cyanobacteria in response to stress. Intercellular signalling can also take place during association formation. During symbiotic associations of cyanobacteria with plants, signals released by the plant partner can induce hormogonia (motile filaments) formation in the cyanobacteria (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994). For example, hormogonia formation in *Nostoc* is triggered by the release of an extracellular low molecular weight heat labile product by its host, hornwort (*Anthoceros punctatus*) during symbiosis (Campbell and Meeks, 1989). Others compounds, such as arabinogalactan-like proteins and phenolic compounds, have also been considered to be potential signals produced by plants that may potentially function in differentiation, attraction, defence and transcriptional activation in cyanobacteria (Bergman *et al.*, 1992). However, the role of cyanobacterial signals in such processes has only been postulated and may involve arabinogalactan proteins released by cyanobacteria to potentially induce changes in host plants (Bergman *et al.*, 1996).

1.1.4. Epilithic microbial communities

Detailed studies on epilithic microbial communities have only recently been undertaken. These complex communities have been found to cause damaging effects to buildings and monuments and have been placed as a recent concern in preservation of

important archaeological sites (de Los Rios *et al.*, 2004). The study of these communities has become important in understanding various biological processes such as weathering and biofilm formation. The microbiota on rock surfaces represents a complex ecosystem, which depends on various environmental factors as well as the the physiochemical properties of the rock materials. The complex ecosystem favours a microniche dominated by various microorganisms, including bacteria, cyanobacteria, algae and fungi and protozoa. Cyanobacteria being photolithotrophic have been considered as the first colonizers on rock surfaces. The use of in situ microscopy greatly facilitates the detection of epilithic microbial community on rock surfaces and shows that coccoid cyanobacteria comprise the major biomass within these habitats (de Los Rios *et al.*, 2004). Ottaoa-Morales et al. (2006), investigated the epilithic biofilms on rock surfaces associated with Mayan monuments at Uxmal (Yucatan, Mexico) by microscopy and cultivation methods and found that cyanobacterial populations of the genera *Xenococcus*, *Gloecapsa, Gloeothece, Synechocystis and Synechococcus* were the dominating organisms in these biofilms (Ortega-Morales, 2006). A separate study carried out by Garcia-Miguel et al. (1995) also reported that cyanobacteria were the most abundant organisms associated with the Pyramid of the Great Jaguar at Tikal, Guatemala. These authors identified *Phormidium, Plectonema, Scytonema, Chlorogloepsis* and *Gloecapsa* as the most representative cyanobacterial genera (Garcia de Miguel *et al.*, 1995). The difference in various predominant cyanobacteria in different locations may represent differences in ecosystems under which growth is favoured.

Biodeterioration of buildings and stone surfaces occurs as a result of complex microbial interactions (de Los Rios *et al.*, 2004) and understanding such interactions can be

crucial in designing effective treatment procedures aimed at inhibiting the growth of these organisms or their elimination. The chemical contributions of free-living phototropic cyanobacteria and heterotrophic bacteria in deterioration processes can be significant, since they produce and secrete a variety of potentially damaging metabolic products. The extracellular polymeric matrix (EPS) secreted by various microorganisms forms a structural part of biofilms. These substances can act as sorbents of metal ions and can initiate chemical effects, such as loosening of rocks through repeated hydration and dehydration (Mohamed, 2001). The hygroscopic properties of EPS and a consequent higher water content of the microenvironment around microbial communities may also enhance nutrient availability and interactions among microorganisms.

1.2. Quorum Sensing: diversity and variety

1.2.1. What is Quorum Sensing (QS)?

Intercellular communication and concerted multi-cellular activity in microorganisms is well documented and generally accepted to be common among bacteria. This type of communication allows bacteria to integrate intercellular signals with other information to respond via gene expression and cellular differentiation (Gray, 1997; Kaiser, 2001). Cell density has been found to be a major trigger used by many bacteria for concerted activities. This type of cell density dependent communication in which bacteria use chemical signals to assess the size of their population and to behave coordinately has been termed "Quorum sensing" (Fig. 1.3) (Fuqua and Winans, 1994).

In order to sense population density, bacteria release small signalling molecules. As the cell density of these bacteria increases, the concentration of signal also increases and begins to accumulate in the environment. The bacteria then use strategies in order to tune into a higher concentrations of these signals and convey the information into measurements of population density (Fuqua and Greenberg, 2002; Fuqua and Winans, 1994). Quorum sensing therefore, can be collectively described as a system whereby bacteria require a minimum population density or a quorum, in order to make decisions about gene expression. Most quorum sensing systems have been studied in proteobacteria and the most commonly encountered signal is the acylated homoserine lactones (AHLs).

1.2.2. History of Quorum Sensing

The identification of quorum sensing in microorganism can be dated back to 1968 when several reports on a pheromone-like system involved in fruiting body formation in *Myxococcus xanthus* (McVittie *et al.*, 1962), on streptomycin biosynthesis and aerial mycelium formation in *Streptomyces griseus* (Khokhlov *et al.*, 1967), on the induction of genetic competence in *Streptococcus pneumoniae* (Tomasz and Beiser, 1965) and on the control of bioluminescence in marine *vibrios* (Nealson and Markovitz, 1970) were reported. Studies on quorum sensing in the bioluminescent bacteria *Vibrio fischeri* initiated with an observation that *V. fischeri* when cultured in broth, exhibited a lag in luminescence gene (*lux*) expression in the early and mid-exponential phases of growth followed by a rapid increase in expression during the late exponential and early stationary growth phases. It was found later that an active component or "autoinducer" present in the cell free extract accumulated at a high enough concentration and was

responsible for the luminescence (Nealson *et al.*, 1970). This active component was later isolated and identified as *N*-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL) in 1981 by Eberhard et al (Eberhard *et al.*, 1981). The autoinducer was found to be released by the cells through simple diffusion and accumulated to a concentration that induced luminescence (Kaplan and Greenberg, 1985). It is worth mentioning that "autoinducer" was a term given to the diffusible molecules involved in autoinduction that is different from autoregulation. Autoinduction formerly was described as a communication mechanism whereby bacteria were able to sense their population density by monitoring the autoinducer concentration. This term was replaced by term "Quorum Sensing" (Fuqua and Winans, 1994) referring to an environmental sensing system that allows bacteria to monitor their own population density.

1.2.3. Identification of quorum sensing signals in different organisms

In general quorum sensing molecules can be classified into two categories, the oligopeptide signals directed by two component phosphorelay proteins in Gram positive bacteria (Dunny and Leonard, 1997) and the AHL's in Proteobacteria. However this classification is becoming dated with the diversity of other signalling systems that have now been documented. To date there have been numerous other quorum sensing molecules identified in various organisms. In Proteobacteria other signalling molecules such as 4-quinolones, fatty acids and fatty acid methyl esters have also been identified (Fig. 1.1). Gram positive bacteria are found to use other types of quorum sensing signals such as γ-butyrolactones, for example Khoklov's A-factor produced by *Streptomyces* (Fig. 1.1). Although no 'universal' bacterial quorum sensing system or

signal molecule family has yet been discovered, many Gram negative and Gram positive bacteria produce 'autoinducer-2' (AI-2) (Fig.1.1), a collective term for a family of interconvertible furanone compounds.

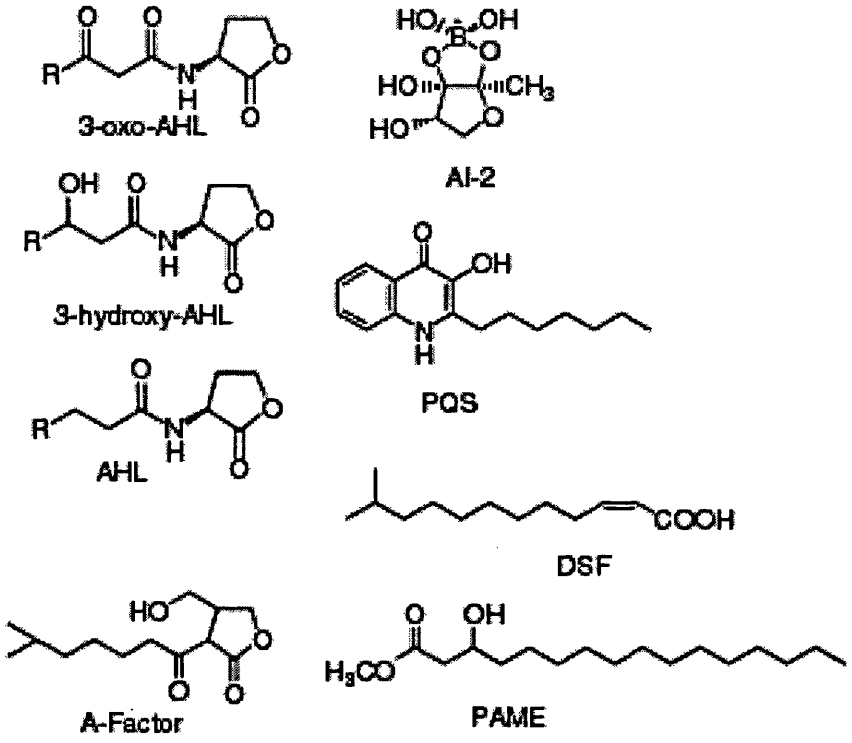


FIGURE 1. 1. STRUCTURES OF SOME REPRESENTATIVE QUORUM SENSING SIGNALLING MOLECULES.

3-oxo-AHL, N-(3-oxoacyl) homoserine lactone; 3-hydroxy-AHL, N-(3-hydoxyacyl) homoserine lactone and AHL, N-acylhomoserine lactone where R ranges from C1 to C15. The acyl side chains may also contain one or more double bonds: A-factor, 2-isocapryloyl-3 hydroxymethyl-g-butyrolactone; AI-2, autoinducer-2, furanosyl borate ester form; PQS, Pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; DSF, 'diffusible factor', methyl dodecenoic acid; PAME, hydroxyl-palmitic acid methyl ester. (Williams et al., 2007)

Recently, it has become apparent that diverse fungal species also use quorum regulation to affect population-level behaviours such as sporulation, biofilm formation and pathogenesis (Hogan, 2006). In *Candida albicans* two different autoinducers are involved in different functions. The autoinducer farnesol can effectively block *C. albicans* biofilm and hyphae development while in small numbers, whereas during the stationary phase of growth, the autoinducer, tyrosol, stimulates formation of hyphae (Hornby *et al.*, 2001). Quorum sensing has not been reported in any other higher organisms; however plants and mammals are reported to respond to AHL signalling molecules. It has been shown that plants also secrete substances that mimic several AHL compounds to which AHL dependent bacteria show a response (Teplitski *et al.*, 2000). These findings therefore demonstrate the diversity of chemical signals used in quorum sensing and inter-kingdom communication process.

1.3. Acylated homoserine lactones

Acylated homoserine lactones (AHL) have a common lactone ring with a fatty acyl chain attached to the amino nitrogen of a lactone ring through an amide bond. It has been found that the length of fatty acyl chain with various substitutions determines the specificity of the signal. Primarily substitutions have been detected at the carbon 3 positions which can be a hydroxyl, a carbonyl or fully reduced. (Fig 1.2). In most naturally occurring AHLs, the carbon chain ranges from 4 to 16 carbons in length, however a chain of 18 carbons has also been detected (Marketon *et al.*, 2002). The shortest naturally occurring AHLs so far identified are C4-HSL and 3-hydroxy-C4-HSL, which are produced by *Pseudomonas aeruginosa* (Winson *et al.*, 1995) and

Vibrio harveyi (Cao and Meighen, 1989). Several other bacteria such as *Rhizobium leguminosarum* (Gray *et al.*, 1996; Schripsema *et al.*, 1996) and *Rhodobacter sphaeroides* (Puskas *et al.*, 1997) are also capable of producing AHL containing double bonds such as 7,8-cis-*N*-(3-oxohexanoyl) homoserine lactone and 7,8-cis-*N*-(tetradecenoyl) homoserine lactones respectively. The first evidence that bacteria are also capable of synthesizing an odd chain length AHL was obtained from cultures of *Rhizobium leguminosarum*, which were found to produce C7-HSL (Lithgow *et al.*, 2000).

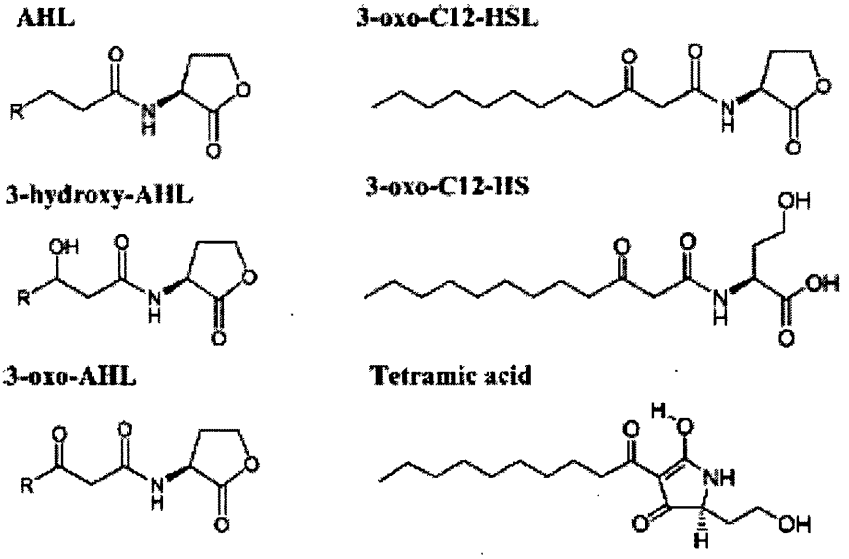


FIGURE 1. 2. THE AHLS AND THEIR HYDROLYSIS PRODUCTS.

AHL, N acylhomoserine lactone; 3-hydroxy-AHL, N-(3-hydroxyacyl) homoserine lactone; 3-oxo-AHL, N-(3-oxoacyl) homoserine lactone (ranges from C1 to C15). The acyl side chains may also contain one or more double bonds. 3-Oxo-C12-HSL, N-(3-oxododecanoyl)-L homoserine lactone; 3-oxo-C12-HS, N-(3-oxododecanoyl) L homoserine; tetramic acid, 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione. (Williams, 2007)

1.3.1. Functions of AHL quorum sensing system

Bacteria collectively produce a wide variety of quorum sensing signalling compounds. In some microorganisms these signals have a specific and narrow range of functions. For example, quorum sensing molecules of fungi and yeast are only involved in cell differentiation and antibiotic production. AHL's from gram negative bacteria are reported to be involved in a diverse range of functions (Table. 1.1). These functions include motility, growth inhibition, antibiotic production, conjugation, DNA transfer, virulence, biofilm formation, pigment production and luminescence (Table. 1.1).

Organism	AHLs	Phenotype
Aeromonas hydrophila	C4-HSL, C6-HSL	Biofilms, exoproteases, virulence
Aeromonas salmonicida	C4-HSL, C6-HSL	Exoproteases
Agrobacterium tumefaciens	3-Oxo-C8-HSL	Plasmid conjugation
Agrobacterium vitiae	C14:1-HSL, 3-oxo-C16:1-HSL	Virulence
Acidithiobacillus ferrooxidan	3-Hydroxy-C8-HSL, 3-hydroxy-C10-HSL, C12-HS 3-hydroxy-C12-HSL, C14-HSL, 3-oxo-C14-HSL, 3-hydroxy-C14-HSL, 3-hydroxy-C16-HSL	Not known
Burkholderia cenocepacia	C6-HSL, C8-HSL	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence
Burkholderia pseudomallei	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C14-HSL	Virulence, exoproteases
Burkholderia mallei	C8-HSL, C10-HSL	Virulence
Chromobacterium violaceum	C6-HSL	Exoenzymes, cyanide, pigment
Erwinia carotovora	3-Oxo-C6-HSL	Carbapenem, exoenzymes, virulence
Pantoea (Erwinia) stewartii	3-Oxo-C6-HSL	Exopolysaccharide
Pseudomonas aeruginosa	C4-HSL; C6-HSL, 3-oxo-C12-HSL	Exoenzymes, exotoxins, protein secretion, biofilms, swarming motility, secondary metabolites, 4-quinolone signalling, virulence
Pseudomonas aureofaciens	C6-HSL	Phenazines, protease, colony morphology, aggregation, root colonization
Pseudomonas chlororaphis	C6-HSL	Phenazine-1-carboxamide
Pseudomonas putida	3-Oxo-C10-HSL, 3-oxo-C12-HSL	Biofilm development
Pseudomonas fluorescens	3-Oxo-C10-HSL	Mupirocin
Pseudomonas syringae	3-Oxo-C6-HSL	Exopolysaccharide, swimming motility, virulence
Rhizobium leguminosarum bv. *viciae*	C14:1-HSL, C6-HSL, C7-HSL, C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL	Root nodulation/symbiosis, plasmid transfer, growth inhibition; stationary phase adaptation
Rhodobacter sphaeroides	7-*cis*-C14-HSL	Aggregation
Serratia sp. ATCC 39006	C4-HSL, C6-HSL	Antibiotic, pigment, exoenzymes
Serratia liquefaciens MG1	C4-HSL, C6-HSL	Swarming motility, exoprotease, biofilm development, biosurfactant
Serratia marcescens SS-1	C6-HSL, 3-oxo-C6-HSL, C7-HSL, C8-HSL	Sliding motility, biosurfactant, pigment, nuclease, transposition frequency
Serratia proteamaculans B5a	3-Oxo-C6-HSL	Exoenzymes
Sinorhizobium meliloti	C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16:1-HSL, C18-HSL	Nodulation efficiency, symbiosis, exopolysaccharide
Vibrio fischeri	3-Oxo-C6-HSL	Bioluminescence
Yersinia enterocolitica	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	Swimming and swarming motility
Yersinia pseudotuberculosis	C6-HSL, 3-oxo-C6-HSL, C8-HSL	Motility, aggregation

TABLE 1. 1. SOME EXAMPLES OF AHL-DEPENDENT QS SYSTEMS AND THE PHENOTYPES CONTROLLED
(Williams, 2007)

1.4. Homoserine lactone quorum sensing system

1.4.1. Basic model

Analysis of the bioluminescence genes involved in Quorum Sensing (QS) in *V. fischeri* has led to the basic model for QS, which is now a paradigm for many other similar quorum sensing systems. The paradigm states that transcription of QS target genes is activated at a certain population density refered to as the 'quorum size', which is proportional to the AHL concentration. There are several other recognized inter-bacterial systems that also mimic the basis of this system, for example, the QS system in *Pseudomonas* spp, *Rhizobium* spp. The basic model of how QS works in *V. fischeri* involves the presence of two proteins; a LuxI protein called AHL synthase which is responsible for the biosynthesis of 3-oxo-C6-HSL and the LuxR protein which is a signal receptor that when binds to 3-oxo-C6-HSL, leads to transcriptional activation of of the *luxICDABGEG* operon required for bioluminescence (Fuqua and Greenberg, 2002). The LuxI and the LuxR proteins are encoded by genes *luxI* and *luxR* and genes coding protein homologous are found in numerous other bacteria (Fig. 1.4). The LuxI and LuxR proteins as well as a LuxR binding site called a lux box (a 20 bp inverted repeat sequence situated within the *lux* regulon) are necessary for the activation of quorum sensing related gene expression (Stevens and Greenberg, 1997). At low cell densities, the *luxI* is constitutively expressed at a low basal level therefore the concentration of the 3-oxo-C6-HSL is low, and at high cell densities 3-oxo-C6 HSL accumulates to a concentration sufficient for binding to the LuxR protein which is known as the threshold concentration. Binding causes conformational changes of active LuxR-AHL complex that binds with other signal receptor complex to form dimers and

multimers which can bind to the specific *lux* box promoter sequences (Nasser and Reverchon, 2007) and activate the transcription of the bioluminescence genes (Fig. 1.3 & 1,4). Since *luxI* is also located in this operon, transcription initiates increased production of the LuxI protein and hence increased synthesis of 3-oxo-C6-HSL which initiates a positive feedback loop. (Fig. 1.4).

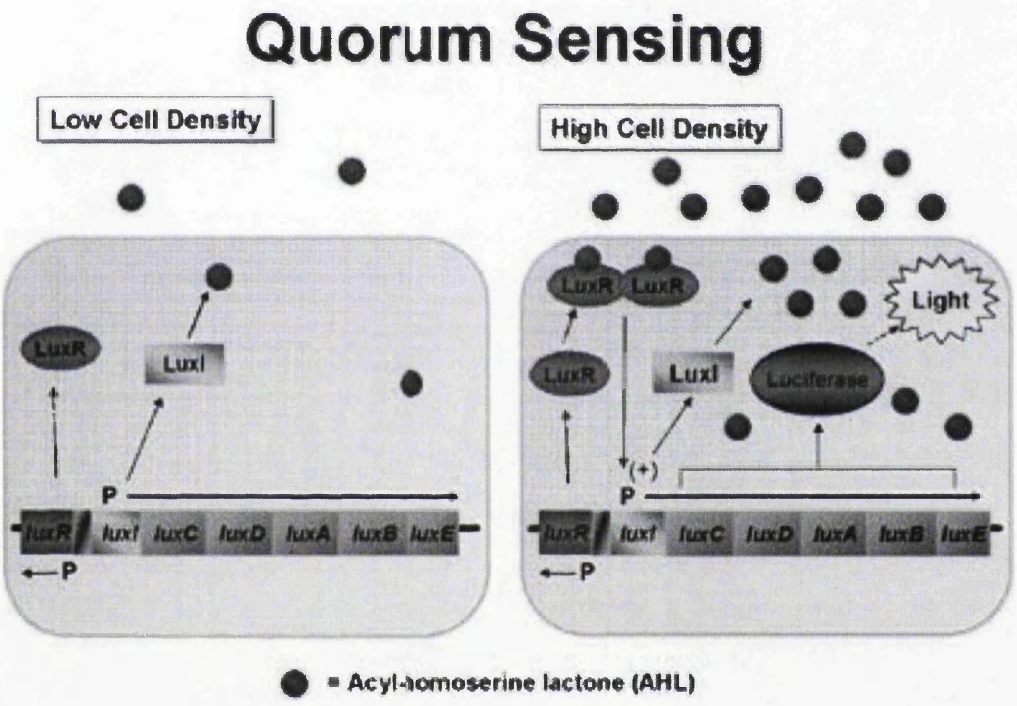


FIGURE 1. 3. QUORUM SENSING:DENSITY DEPENDENT ACCUMULATION OF SIGNALS RESULTING IN INDUCTION IN BIOLUMINESCENT GENE EXPRESSION IN *VIBRIO.FISCHERI.* *Source: http://www.che.caltech.edu*

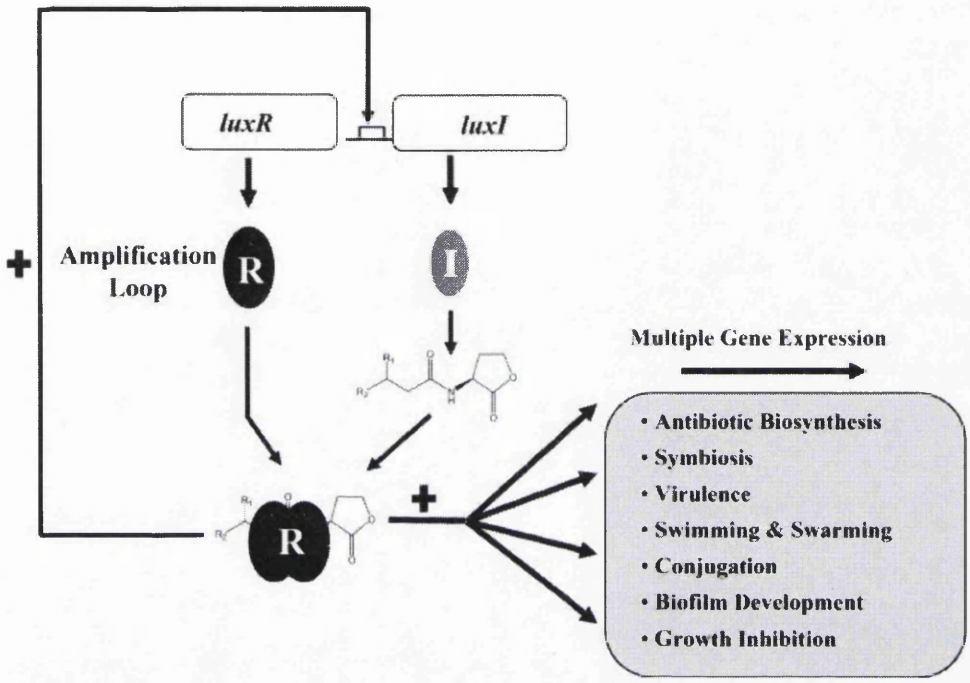


FIGURE 1. 4. MOLECULAR MECHANISM OF AHL-MEDIATED QS-DEPENDENT REGULATION OF MULTIPLE TARGET GENE EXPRESSION.

luxR and luxI represent orthologues of the corresponding V. fischeri genes and code for the AHL receptor and AHL synthase respectively. In many but not all systems the AHL is an autoinducer since it drives its own production via the amplification loop leading to the AHL synthase gene. (Williams, 2007)

1.4.2. Themes and variation in QS circuits

The previously described model represents a simplistic view of QS and great diversity exists in the molecular mechanism of *luxI/luxR* based QS system (Smith *et al.*, 2006) even within the same genus (Milton, 2006). The following complexities are summarized below:

1.4.2.1. Multiple QS molecules acting in a concerted manner:

In some systems several quorum sensing molecules can integrate information and act in a concerted manner to carry out quorum sensing gene expression (Fig. 1.5). For example, *Vibrio harveyi* uses three channel quorum sensing systems that act in a concerted manner to activate a master regulator $LuxR_{vh}$ that directly activates the lux operon (Fig. 1.5) (Defoirdt *et al.*, 2008; Swartzman *et al.*, 1992) (Defoirdt, Boon et al. 2008). Besides its cognate AHL which is 3-hydroxy-C4-HSL (Cao and Meighen, 1989), termed autoinducer 1 (HAI-1), *V. harveyi* integrates two other different quorum sensing molecules, one of which is AI-2 (Chen *et al.*, 2002) and the other one is named cholera autoinducer 1 (CAI-1, still unknown), in order to properly activate its QS regulated genes. The molecular mechanism works in a different manner to that of *Vibrio fischeri*; instead of quorum sensing molecules freely diffusing into the cells, they are detected at the cell surface by membrane bound, two component receptor proteins that feed a common phosphorylation / dephosphorylation signal transduction cascade (Taga and Bassler, 2003). The receptor of 3-hydroxy-C4-HSL is LuxN, which does not

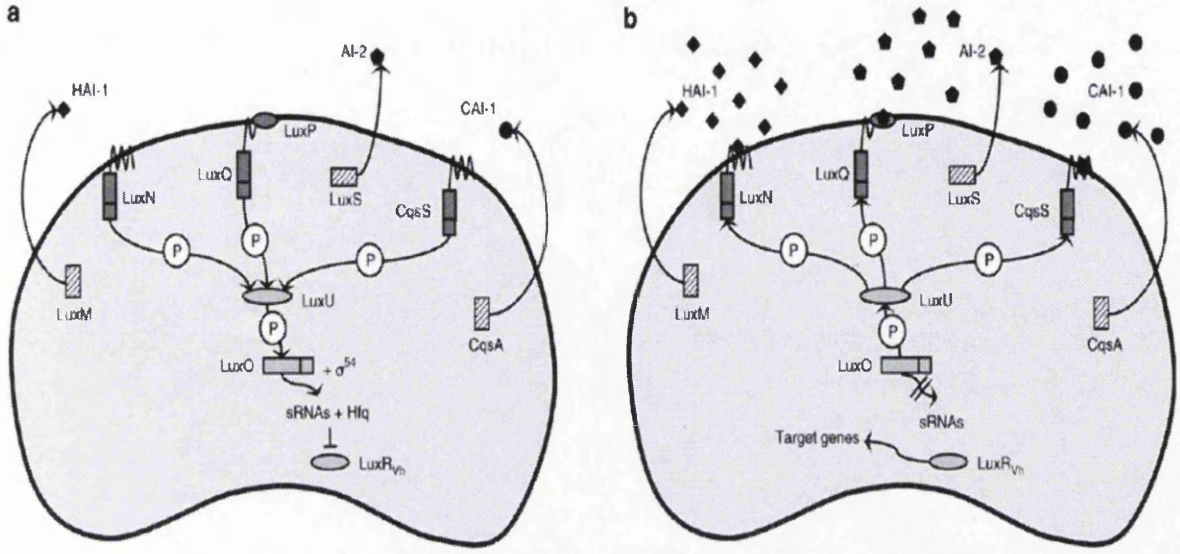


FIGURE 1. 5. QUORUM SENSING IN VIBRIO. HARVEYI; SIGNALS ACTING IN A CONCERTED MANNER

The LuxM, LuxS and CqsA enzymes synthesize the autoinducer 1(HAI-1), autoinducer 2 (AI-2) and cholera autoinducer 1 (CAI-1), respectively. These autoinducers are detected at the cell surface by the LuxN, LuxQ and CqsS two-component receptor proteins, respectively. (a) In the absence of autoinducers, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with s54 activates the production of five small regulatory RNAs (sRNAs). These sRNAs, together with the chaperone Hfq, destabilize the mRNA encoding the transcriptional regulator LuxRVh. Therefore, in the absence of autoinducers, the LuxRVh protein is not produced. (b) In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, which result in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed and the transcriptional regulator LuxRVh is produced. See text for more details. Circled "P" denotes phosphotransfer. (Defoirdt et al., 2008)

resemble any homology to LuxR family of transcriptional activators. All three channels enter information based on phosphorylation status which determines the transcription of target genes. This clearly represents the diversity of AHL systems in two different species and shows the involvement of phosphoryl cascades and cell surface receptors in AHL based quorum sensing. A detailed molecular mechanism of *V. harveyi* QS is discussed in the recent review by Defoirdt (Defoirdt *et al.*, 2008).

1.4.2.2. Multiple QS molecules acting in a hierarchical manner:

In addition to the concerted action of quorum sensing signals in *V. harveyi*, a complex hierarchical LuxI/LuxR circuit operates in *Pseuodomonas* sp (Fig. 1.6.). *Pseudomonas* an oppertunistic pathogen uses quorum sensing to control gene expression associated with the production of various virulence factors (elastase, exotoxin A, pyocyanin etc) and biofilm development that aids the organism in colonization and persistence within its host organism (Parsek and Greenberg, 2000; Throup *et al.*, 1995; Winson *et al.*, 1995; Winzer and Williams, 2001) There are two QS systems in *P. aeruginosa*, which have been extensively studied. The *las* system consists of LuxI/LuxR homologues, LasR Transcriptional regulator and the LasI synthase protein. LasI is essential for the production of the AHL signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL) (Pearson *et al.*, 1994). A second QS system in *P. aeruginosa* consists of the RhlI and RhlR proteins. The RhlI synthase produces the AHL *N*-butyryl-L-homoserine lactone (C_4-HSL), and RhlR is the transcriptional regulator (Pearson *et al.*, 1995; Winson *et al.*, 1995). Each transcriptional regulator binds to its cognate AHL in order to regulate gene expression (Fig. 1.6).

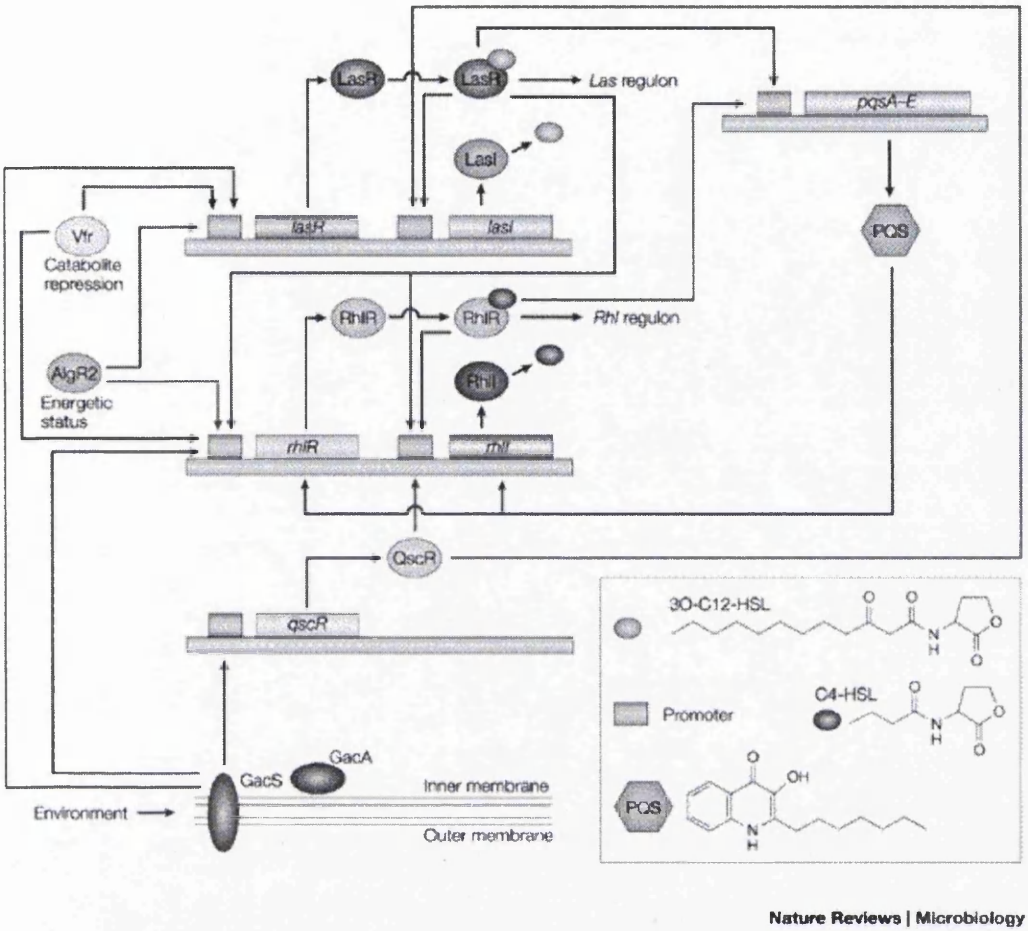


FIGURE 1. 6. A HIERARCHICAL MODEL OF QUORUM SENSING SYSTEM IN PSEUDOMONAS AERUGINOSA

For each circuit in the cell the interactions between the different QS systems are indicated by arrows. Black arrows indicate positive regulation and red arrows indicate negative regulation. Signals from the environment, the intracellular metabolic status of the cell and other regulators, such as RpoS, RsmA and MvaT, also interface with this cellular circuitr. (Lazdunski et al., 2004).

The LasI/LasR regulatory circuit exerts transcriptional control over the second quorum sensing circuit RhlI / RhlR and the two regulatory circuits act in a tandem to control the expression of various quorum regulated gene products (Latifi *et al.*, 1996). However, the *rhl* system can function independently of the *las* system. Recently, a novel additional quorum sensing signal 2-heptyl-3-hydroxy-4-quinolone (PQS) has also been

identified in *Pseudomonas* sp. PQS is also part of the quorum sensing system and its activity and synthesis is modulated through the *lasI* and *rhl* systems (Pesci *et al.*, 1999). LasR is responsible for activating the synthesis of PQS, thereby, creating a regulatory link between the two QS circuits (Diggle *et al.*, 2003; Gallagher *et al.*, 2002; McKnight *et al.*, 2000). A model of the *P. aeruginosa* quorum-sensing network has been defined by Fuqua *et al.* (Fuqua, 2006) Other hierarchical quorum-sensing cascades have also been identified in *Yersinia pseudotuberculosis* involved in the regulation of clumping and motility (Atkinson *et al.*, 1999), Ti plasmid conjugation in *Agrobacterium tumefaciens* (Welch *et al.*, 2000) and AHL autoinduction in *Ralstonia solanacearum* (Flavier *et al.*, 1997)

1.5. Integrating QS with other control circuits

1.5.1. Environmental factors

A number of bacterial systems are reported to integrate starvation and quorum sensing signals to regulate the transition into stationary phase. This may be advantageous to bacteria that have evolved to monitor crowding as a measure of competition for nutrients and induce responses appropriate for the anticipation of starvation. Such mechanisms can be utilized when bacteria are starved and crowded or when growing as a colony on a solid surface in nature. Moreover, the levels of expression of the AHL synthase gene and response regulators have been found to be affected by various environmental conditions, thus modulating the levels of AHL accumulation and gene expression irrespective of cell density. The quorum sensing system of *Pseudomonas* is

one of the best characterized LuxI/LuxR type QS system that have demonstrated that the quorum sensing regulon is novel and integrates and responds to nutritional factors and stress as well as high cell density (Soberon-Chavez *et al.*, 2005). In this system the stationary phase sigma factor RpoS, which is transcribed at high levels during stress conditions is linked through complex circuits to the QS system in *P. aeruginosa*. Both *lasR* and *rhlR* gene expression and autoinducer synthesis of the 3-oxo-C (12)-HSL could be prematurely activated during the stringent response. These findings suggest that the stringent response can activate quorum-sensing systems of *P. aeruginosa* independent of cell density (van Delden *et al.*, 2001). Moreover, a global regulator (GacA) of the two component system, that is activated in response to environmental stimuli, has also been found to positively regulate a third type of luxR homologous protein QscR, which can respond to AHL signals generated via a different R-I regulatory system as well as 3O-C12-HSL in *P. aeruginosa* (Lee *et al.*, 2006; Pessi and Haas, 2001). In a different study authors tested the expression levels of *lasI*, *lasR*, *rhlI*, and *rhlR* under 46 experimental conditions over a 30 h time course which indicated that the expression profiles and the timing of the expression peaks of both the synthase genes and the transcriptional regulator genes is dependent on growth conditions including nutritional conditions as well as addition of exogenous AHLs, mucin, DNA, subinhibitory concentrations of antibiotics, NaCl, and AI-2 (Duan and Surette, 2007). Previously, Kim et al. (2005) also reported that *lasR* expression in *Pseudomonas* is also affected by oxygen levels and iron concentration in the medium (Kim *et al.*, 2005). The authors claimed that no correlation could be established between cell densities and the activation of QS expression over this range of conditions, indicating the absence of critical cell density as a prerequisite for QS activation.

The acidophilic gram-negative bacterium *Acidithiobacillus ferrooxidans* oxidizes ferrous iron or reduced sulfur compounds to obtain energy for growth. AfeI, a LuxI homologue protein, present in this bacteria is responsible for the synthesis of various AHL molecules including 3-hydroxy-C_8-HSL, 3-hydroxy-C_{10}-HSL, C_{12}-HSL, 3-oxo-C_{12}-HSL, 3-hydroxy-C_{12}-HSL, C_{14}-HSL, 3-oxo-C_{14}-HSL, 3-hydroxy-C_{14}-HSL, and 3-hydroxy-C_{16}-HSL (Farah *et al.*, 2005). Reports have demonstrated that the transcription level of the *afeI* gene is modulated through sulphate and phosphate concentrations in the medium. Higher transcripts were obtained when cells were grown in sulfur and thiosulfate media than in iron-rich medium. Phosphate starvation was also found to induce an increase in the transcription levels of *afeI* which correlated with an increase in AHL levels and therefore, demonstrates how the levels of AHL can be modulated through nutrient concentrations (Farah *et al.*, 2005). The role of AHL in adaptation to stationary-phase survival in *Rhizobium leguminosarum* bv. *phaseoli* has also been documented. The authors show that survival under conditions of carbon or nitrogen limitation was cell-density dependent and these bacteria could survive starvation at low cell densities when treated with signal *N*-(3*R*-hydroxy-7-*cis*-tetradecanoyl)-L-homoserine lactone, indicating starvation and quorum sensing to be interconnected (Thorne and Williams, 1999). Examples of environmental conditions modulating QS regulators can also be evident in *A. tumefaciens*, where the *traR* gene is induced by nutrients called opines, released from plant tumors (Fuqua and Winans, 1994).

1.5.2. Growth phase regulation

Recent evidence has shown that each QS regulated gene in bacteria may have its own quorum size and so different genes can be activated at different population densities and

therefore represent early quorum activated genes and late quorum activated genes (Lupp and Ruby, 2005; Schuster *et al.*, 2003; Wagner *et al.*, 2003). However, in most cases QS dependent gene activation is found to occur as cultures approach the stationary phase of growth. This may be significant since, bacteria are often at high cell density during entry into stationary phase, and cells may monitor this crowding as a signal to prepare for entry into stationary phase. In stationary phase cells dramatically alter their patterns of gene expression to allow extended cell survival in response to starvation and as previously described quorum-sensing pathways converge with starvation-sensing pathways to regulate cell entry into stationary phase. Studies on various quorum regulated genes have been investigated through inducing an early activation of these genes by exogenously adding AHL to a low density culture (Schuster *et al.*, 2003). In *Pseudomonas aeruginosa* microarray analysis of genes regulated by QS was analysed by exogenously added and without added AHL (1 μM 3O-C12-HSL and 2 μM C4-HSL) in cultures of a mutant deficient in 3O-C12-HSL and C4-HSL production. Of the 616 genes whose expression was identified as being QS regulated, 394 were promoted and 222 were repressed (Schuster *et al.*, 2003). Expression of many genes involved in basic cellular processes, such as DNA replication, RNA transcription and translation, cell division, and amino acid biosynthesis, and genes involved in group behaviour, such as chemotaxis and biofilm formation, were found to be QS regulated. Various authors have reported a time dependent expression of QS genes where the expression of some genes were induced early in growth while most of the genes were induced in the late logarithmic to early stationary phase; and some genes were not induced until the stationary phase. The timing was found to be similar in the wild type and in the signal generation mutant grown in the presence of saturating levels of added signals indicating that the trigger for

quorum-controlled gene activation was not signal accumulation (Whiteley *et al.*, 1999). A possible explanation for such timing was given by Schuster *et al.* (2003) from an observation that lasR and rhlR, showed increased transcript abundance after the culture reached an OD_{600} of 0.8 (late logarithmic phase) and timings in many QS gene expression could be attributed to the levels of the receptor proteins that themselves may be regulated depending on energy status (Albus *et al.*, 1997), environmental conditions (Kim *et al.*, 2005) as well as the stringent response (van Delden *et al.*, 2001). The hypothesis was later tested and it was concluded that many quorum sensing target genes are directly responsive to receptor protein levels early in growth (Schuster and Greenberg, 2007). Moreover, the specificities of responses of many genes to AHL signals in *Pseudomonas* are in consert showing some genes to specifically respond to 3OC12-HSL; other genes respond to 3OC12-HSL, but their activation boosted by addition of C4-HSL; and other genes to C4-HSL, showing no response to 3OC12-HSL alone (Schuster *et al.*, 2003; Wagner *et al.*, 2003). These findings indicate the complexities of quorum sensing not just being dependent on a quorum which determines the population density for coordinate expression of genes, but a rather an elaborate pattern of expression of genes being triggered at different times during culture growth, and responding to different signals at various degrees. However, the authors Yarwood et al (2005) have claimed that the *Pseudomonas* quorum signaling appears similar to quorum signaling in other Proteobacteria and the timing and delay in expression of most QS genes in complex medium is attributed to the presence of an inhibitor regardless of growth phase (Yarwood *et al.*, 2005).

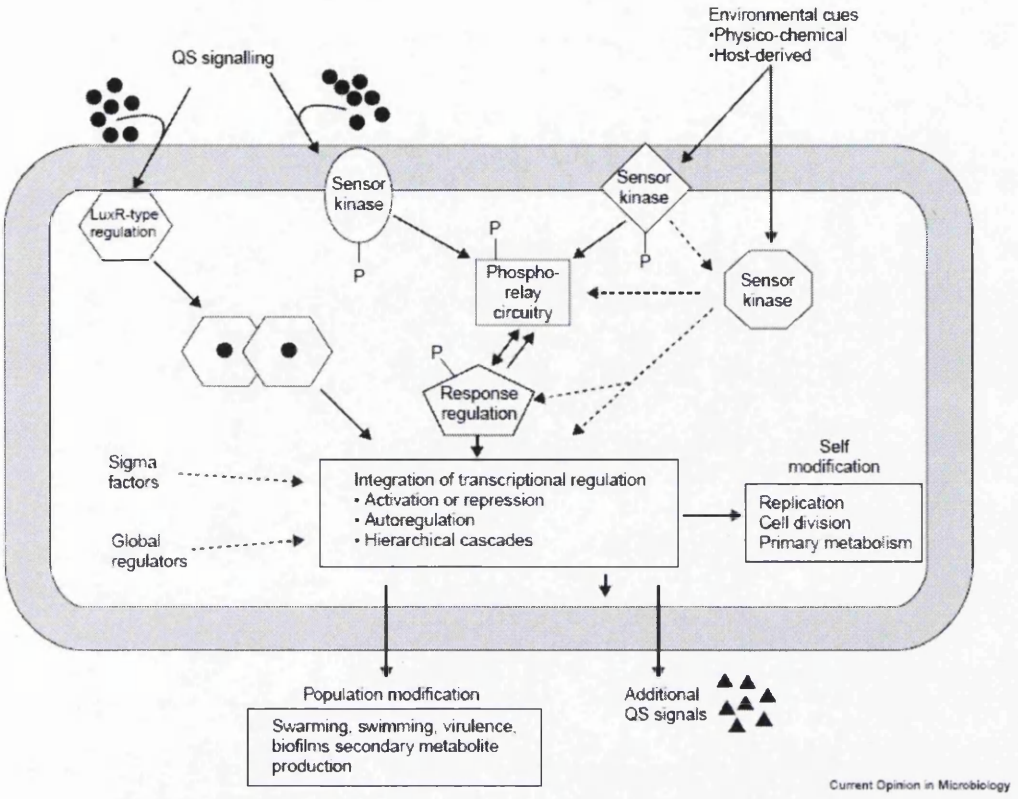


FIGURE 1. 7. A MODEL OF QUORUM SENSING INTEGRATING OTHER SIGNALLING SYSTEMS AND REGULATORY NETWORKS MAKING UP THE 'NEURAL' REGULATORY NETWORKS IN BACTERIA.

Integration of intra- and inter-QS signalling modules to facilitate adaptation to environmental stress. A single bacterial cell contains a diverse array of signalling modules that may act in parallel or may be organised into a hierarchical cascade to give rise to the concept of 'neural' regulatory networks in bacteria. QS, as the determinant of cell population density, is therefore just one parameter amongst many that the cell must integrate in order to determine subsequent behaviour. QS signal molecules, in common with other environmental signals, may be internalised or detected at the cell envelope such that signal transduction leads to self modulation or population modification. (Withers et al., 2001)

1.6. Accumulation of AHL

1.6.1. Accumulation in batch cultures

The accumulation of AHL has been primarily investigated in planktonic batch cultures maintained in closed systems. An increase in cell density is thought to be the primary cause of AHL accumulation in closed systems. A Fixed volume of culture in a flask allows AHL molecules to increase in concentration with time (unless they are degraded) and initiate a quorum sensing response at a specific point in the growth curve, coinciding with a threshold concentration of signal. The response thus may allow increased accumulation of signal at a rate greater than the exponential increase in cell numbers in several bacteria (Ravn *et al.*, 2001; Swift *et al.*, 1993). The level of accumulation of AHL in cultures has also been generally found to correlate with the expression level of the AHL synthase gene (LuxI homologues) (Duan and Surette, 2007).

AHL molecules are generally released from the cells by simple diffusion and their amphipathic nature may allow these molecules to navigate through the phospholipid bilayer of cell membranes into aqueous intracellular and extracellular environments. The evidence that AHL freely diffuses out of the cells was obtained from studies carried out on *V. fischeri* and *Escherichia. coli* (Kaplan and Greenberg, 1985; Pearson *et al.*, 1997). In addition to its slow diffusion, C12-HSL in *Pseudomonas* is actively pumped from cells by the efflux pump MexAB-OprM, and perhaps other, efflux pumps (Evans *et al.*, 1998; Pearson *et al.*, 1999). Presumably, the difference in the length and /or the

degree of substitution of the acyl side chains determines whether an AHL signal is freely diffusible or is subject to active efflux.

1.6.2. Accumulation of AHL in natural environments:

The physiological concentrations of AHL required for induction of gene expression under natural environmental conditions is poorly defined. However, McLean et al. (1997) demonstrated that biofilms present on rocks submerged in flowing aquatic systems produce measurable amounts of AHLs (McLean *et al.*, 1997), supporting the idea that physiologically relevant concentrations of signal might be present in some environmental biofilm systems. Since, biofilm formation in certain organisms is dependent on an AHL QS system; it is likely that QS-relevant concentrations are found in biofilms. In nature microorganisms grow in close proximity to each other forming small microcolonies, which in turn can be arranged into biofilms, which are self encapsulated with extracellular polymeric matrix. The accumulation of signals sufficient to induce gene expression within biofilms can be dependent on a number of factors including the hydrodynamic environment, physical, chemical and biological variables that can impact on signal production, stability and distribution (Horswill *et al.*, 2007). The signal produced in the biofilm diffuses into the bulk fluid depending on mass transfer rate. Thus, not all of the signal will remain in the local environment. Mass transfer can produce nutrient gradients, and can influence QS (Bollinger *et al.*, 2001; Duan and Surette, 2007). For example, iron availability can modulate QS in *Pseudomonas aeruginosa* (Bollinger *et al.*, 2001; Duan and Surette, 2007), while other components of the nutritional environment can delay the onset of QS (Yarwood *et al.*, 2005). The hydrodynamic environment can also affect biofilm density and thickness

(Pereira *et al.*, 2002), which in turn can affect signal gradients. QS signal accumulation under natural conditions is thought to be a function of:

1. Signal-production rate;
2. The degradation rate or half-life of the signal;
3. The diffusion properties of the signal; and
4. The external hydrodynamic or mass-transfer conditions

AHL can accumulate under natural conditions in which diffusion of signal molecule is constrained. A much higher concentration (1mM) of AHL has been found to accumulate at the vicinity of a *P. aeruginosa* biofilm, which is 1000 times more than needed to induce QS in shaken cultures (Charlton *et al.*, 2000). A similar situation is found in *V. fischeri* which accumulates 3OC6-HSL to concentrations exceeding 100 nM in its host organism, which is greater than 40 times the concentration needed to induce QS induction and thus bioluminescence in liquid culture (Boettcher and Ruby, 1995). Autoinducer secretion and response by organisms may confer on the bacterium the ability to determine whether secreted molecules move away from the cell. This process, termed "diffusion sensing" could allow the cells to regulate the secretion of effectors, such as degradative enzymes, antibiotics, surfactants, and siderophores, to minimize losses to extracellular diffusion (Redfield, 2002). Clustering of bacterial cells as well as limitation in diffusion can account for a local increase in AHL concentration resulting in early activation of QS genes, which has been demonstrated through a model by Hense, *et al.* and hypothesized as "efficiency sensing" (Hense *et al.*, 2007). This model unifies the traditional population size concept of QS and that of diffusion sensing into a single process that measures cell density, mass-transfer properties, and spatial

distribution to determine the efficiency by which AHL producing cells express extracellular products and exhibit proper behavioural responses. The evidence that a small bacterial colony can undergo QS induction has been reported for *P. syringae* growing on dry leaves where signal diffusion is restricted (Dulla and Lindow, 2008). Authors reported that QS induction was common in *P. syringae* aggregates, as small as 33 cells on dry leaves and increases rapidly with increasing aggregate sizes >35 cells after 3 days (Dulla and Lindow, 2008). These, reports suggest that AHL can accumulate under natural conditions sufficient to induce QS induction within microcolonies.

1.7. Enzymatic synthesis of AHL

The structure of AHL suggests that this signal molecule is synthesised from fatty acid and amino acid precursors (Eberhard *et al.*, 1981). The involvement of SAM (S-adenosyl-L-methionine) as a substrate for AHL synthesis came from studies, that demonstrated when the intracellular pools of SAM in *E. coli* were depleted (by employing SAM hydrolase from bacteriophage T7), the synthesis of 3-oxo-C_8-HSL by the TraI (AHL synthase of *Agrobacterium tumefaciens*) protein was severely reduced in proportion to the observed reduction in SAM pools. This study provides indirect evidence for the role of SAM as a substrate in AHL synthesis (Val and Cronan, 1998). Other studies with an AHL synthase protein RhlI, purified successfully under denaturing conditions from *E. coli* analysed for enzymatic activity indicated that only ACP (acyl carrier protein) and SAM are required for synthesis of AHL. The biochemical mechanism for conversion of SAM and specific acyl-ACPs to AHL has been demonstrated for several LuxI-like proteins from different bacteria. These

enzymes include LuxI from *V. fischeri* (Hanzelka and Greenberg, 1996), TraI from *A. tumefaciens* (More *et al.*, 1996), and RhlI from *Pseudomonas aeruginosa* (Parsek *et al.*, 1999). The enzymatic reaction mechanism of C4-HSL synthesis was studied for RhlI from *P. aeruginosa* (Fig.1.8). The synthesis occurs in an ordered reaction with preliminary binding of SAM to RhlI. Butyryl-ACP is then bound to the enzymatic complex. Subsequent amide bond formation releases the the holo-ACP following which lactonization of homoserine ring occurs and the product, butyryl-HSL, is liberated. In the final step 5'-methylthioadenosine (5'-MTA) is released (Parsek *et al.*, 1999).

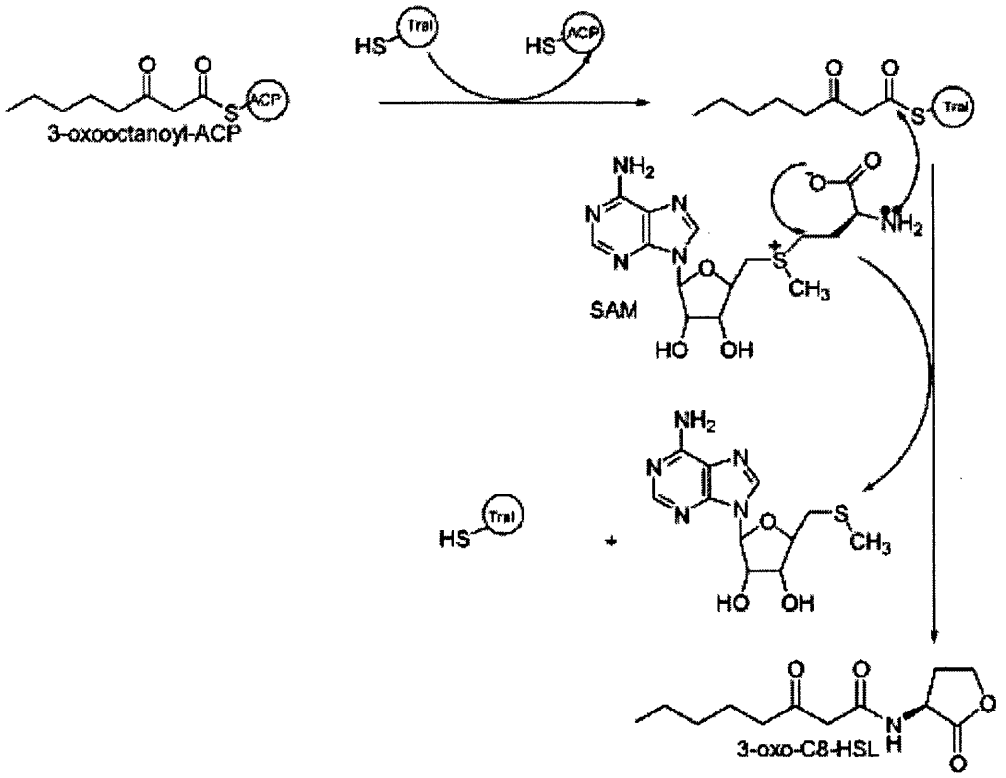


FIGURE 1. 8. THE BIOSYNTHESIS OF AHLS VIA LUXI FAMILY PROTEINS – A MODEL OF TRAI-MEDIATED BIOSYNTHESIS OF 3-OXO-C8-HSL.

TraI catalyses the formation of an amide bond between the amino group of S-adenosylmethionine (SAM) and the a-carbon of the fatty acid provided via the 3-oxooctanoyl-loaded acyl carrier protein (3-oxooctanoyl-ACP). Lactonization follows and the reaction products released are 3-oxo-C8-HSL and methylthioadenosine (MTA). For the LuxI orthologue, RhlI, the biosynthesis of C4-HSL is initiated by the binding of SAM and the appropriately charged ACP is subsequently bound to the complex (Parsek et al., 1999).

1.8. The AHL synthases:

The Lux I type proteins in various organisms are responsible for producing AHL, from precursors derived from fatty acid and amino acid metabolism (Jiang *et al.*, 1998; More *et al.*, 1996; Parsek *et al.*, 1999). The AHL synthases can synthesize more than one type of AHL depending on the fatty acid chain on the ACP. The AHL synthase SinI from *Sinorhizobium meliloti* Rm1021 is able to synthesize five different kinds of AHLs (Gonzalez and Marketon, 2003) and the *Yersinia pseudotuberculosis* AHL synthase YtbI, directs the synthesis of at least 24 different AHLs, among which four compounds predominate (3-oxo-C6-HSL, 3-oxo-C7-HSL, 3-oxo-C8-HSL and C8-HSL) (Ortori *et al.*, 2007). Other classes of AHL synthase proteins have also been identified and include the Lux M from *V. harveyi.* which also uses SAM and other acylated ACPs or acyl CoA as substrates (Bassler *et al.*, 1994). AinS from *Photobacterium (Vibrio) fischeri* and VanM from *Vibrio anguillarum* are homologous to the LuxM class of AHL synthases (Gilson *et al.*, 1995; Milton *et al.*, 2001) These AHL synthases however share no homology with LuxI type proteins. A third class of AHL synthase has been identified in in *P. fluorescens* F113, which is a novel class of acyl transferase enzyme called HdtS (Laue *et al.*, 2000).

1.9. The response regulators:

LuxR-type proteins share two regions of sequence conservation, an AHL binding domain and a DNA binding motif (Zhang *et al.*, 2002) and are reported to be membrane bound proteins (Kolibachuk and Greenberg, 1993; Qin *et al.*, 2000). Thus, LuxR proteins undergo conformational changes when bound to AHL and form dimers or multimers. The current model from TraR suggests that in the absence of a signal, TraR is in a monomer form and is localized to the inner membrane; the binding of AHL signal drives the dimerization of TraR and releases it from membranes into the cytoplasm (Qin *et al.*, 2000). The binding of AHL to these proteins stabilizes them, otherwise in the absence of signal they are targeted to degradation (Zhang *et al.*, 2002; Zhu and Winans, 1999, , 2001). The interaction between purified LuxR proteins with their cognate AHL is specific (Qin *et al.*, 2000; Welch *et al.*, 2000; Zhu and Winans, 1999).. However, other LuxR-like proteins such as the SdiA of *Salmonella enterica* serovar *typhimurium* recognize more than one AHL and may respond to signals from other bacteria where no AHL synthase is found. (Janssens *et al.*, 2007).

1.10. Detection of Homoserine lactones using bioreporters:

AHL signal molecules are produced by bacteria at very low concentrations generally detected within the pM –μM range. This precludes the use of most conventional methods using specific and non-specific chromogenic reagents for detection of these signals in culture supernatants (Brelles-Marino and Bedmar, 2001). Therefore, methods

for detection of AHL are based on detection through various sensitive bioreporter AHL sensors systems. These bioreporters have been developed to allow the fast screening of AHL compounds from various sources. They have also been suitable in partial characterization and quantification of AHLs using different methods (Fig1.9).

Bioreporters have been designed based on the fact that most gram negative bacteria undergo gene expression in response to levels of AHL molecules and most LuxR homologue proteins are activated in response to different AHL with the highest specificity towards its cognate AHL. Various analogues differing in side chain length and the nature of the substitution at carbon 3 can, in most cases, activate LuxR homologues but a much higher concentration than usually required (Eberhard *et al.*, 1986; Passador *et al.*, 1996; Schaefer *et al.*, 1996). Bioreporters are constructed in such a way that they are not capable of producing their own AHLs but can respond to the presence of exogenous AHLs. They are generally designed with a functional LuxR-family protein cloned together with a cognate target promoter usually that of a *luxI* synthase, which can positively regulate the transcription of reporter genes such as bioluminescence (Boettcher and Ruby, 1995; Swift *et al.*, 1993; Winson *et al.*, 1998) lacZ-ß-galactosidase (Cha *et al.*, 1998), green florescent protein (Andersen *et al.*, 2001) and violacein pigment production (Blosser and Gray, 2000; McClean *et al.*, 1997; Ravn *et al.*, 2001).

The differential specificity of LuxR homologous proteins towards AHL makes some bioreporters suitable for detecting long chain AHLs while others are suitable for detection of short chain AHLs or of oxo and hydroxyl containing groups (Cha *et al.*, 1998; McClean *et al.*, 1997; Winson *et al.*, 1998). The bioreporters can be classified

into two broad functional categories, depending on the range of AHL to be detected. These are: (1) Bioreporters detecting a narrow range of AHLs and (2) bioreporters detecting a broad range of AHLs. For the detection of AHL, bioreporters should be carefully selected based on the desired AHL detection and when screening isolates from various sources it has been found useful to use a combination of bioreporters or simultaneously use bioreporters (Ravn *et al.*, 2001) to identify bacterial populations for AHL production. Various bioreporters designed with different constructs with the range of AHL detected are listed in Table 1.2 (Steindler and Venturi, 2007).

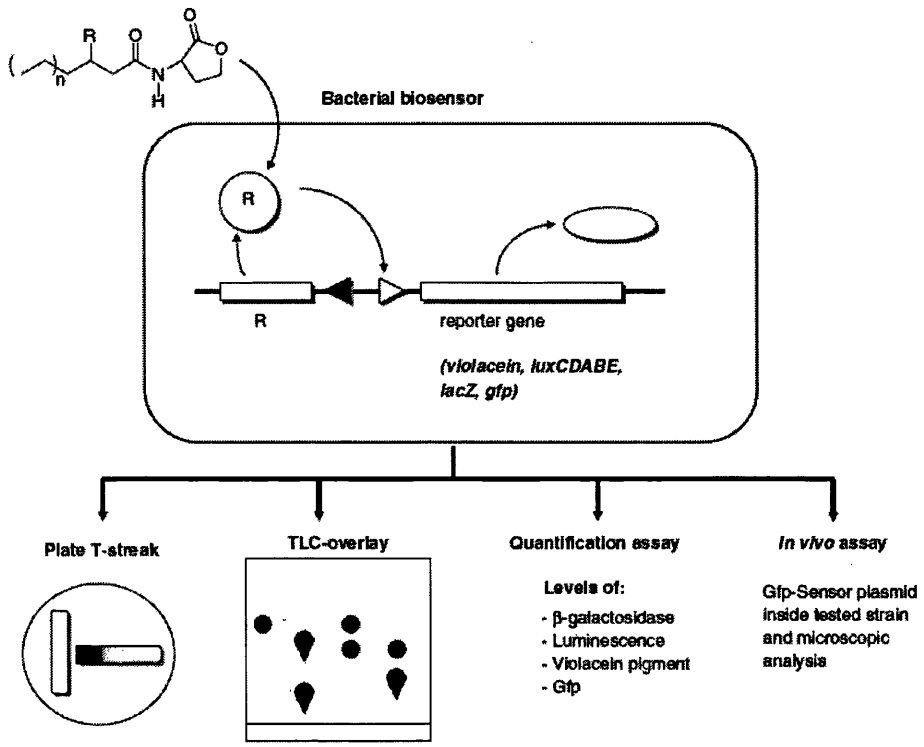


FIGURE 1. 9. CONSTRUCTION AND USE OF A BACTERIAL AHL BIOSENSOR.

At the top of the diagram, the structure of AHLs is shown. The letter 'R' refers to the moiety at position C3, which can be either unmodified or carries an oxo- or hydroxyl group. The letter 'n' refers to the length of the acyl chain, which is most commonly from 4 to 12 and in some cases bacteria can produce AHLs having chains of 14 to 18 carbons. The exogenous AHL interacts with a LuxR family protein inside the bacterial biosensor (non-AHL producer), which results in the transcription of a reporter gene(s) from a LuxR family-AHL regulated promoter as shown by the open triangle. The LuxR family gene is usually expressed from a constitutive promoter as shown with a filled triangle. The AHL biosensor can then be used in (i) a plate 'T' streak assay, resulting in the expression of the reporter phenotype in a gradient near the tested strain as indicated, (ii) a TLC overlay technique after separation of AHLs from a bacterial extract resulting in the detection of the reporter phenotype at the place where AHLs are found, (iii) a quantification assay by measuring levels of the reporter phenotype upon exposure to spent bacterial supernatants or bacterial organic solvent extracts and (iv) an in vivo assay using gfp-based biosensors. (Steindler and Venturi, 2007)

Strain/Plasmid sensor	Host	Based on QS system	Reporter system	Best responds to	Good detection
C. violaceum CV026	*C. violaceum*	CviI/R (*C. violaceum*)	Violacein pigment	C6-AHL	C6-3-oxo-AHL C8-AHL C8-3-oxo-AHL C4-AHL
pSB401	*E. coli*	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-AHL	C6-AHL C8-3-oxo-AHL C8-AHL
pHV200I[-]	*E. coli*	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-AHL	C6-AHL C8-3-oxo-AHL C8-AHL
pSB403	Broad host range	LuxI/R (*V. fisheri*)	*luxCDABE*	C6-3-oxo-AHL	C6-AHL C8-3-oxo-AHL C8-AHL
pSB536	*E. coli*	AhyI/R (*A. hydrophyla*)	*luxCDABE*	C4-AHL	
pAL101	*E. coli* (*sdiA* mutant)	RhlI/R (*P. aeruginosa*)	*luxCDABE*	C4-AHL	
pSB1075	*E. coli*	LasI/R (*P. aeruginosa*)	*luxCDABE*	C12-3-oxo-AHL	C10-3-oxo-AHL C12-AHL
pKDT17	*E. coli*	LasI/R (*P. aeruginosa*)	β-galactosidase	C12-3-oxo-AHL	C12-AHL C10-AHL C10-3-oxo-AHL
M71LZ	*P. aeruginosa lasI*[-]	LasI/R (*P. aeruginosa*)	β-galactosidase	C12-3-oxo-AHL	C10-3-oxo-AHL
pZLR4	*A. tumefaciens* NT1	TraI/R (*A. tumefaciens*)	β-galactosidase	C8-3-oxo-AHL	All 3-oxo-AHLs C6-AHL C8-AHL C10-AHL C12-AHL C14-AHL C6-3-hydroxy-AHL C8-3-hydroxy-AHL C10-3-hydroxy-AHL
pCF218 + pCF372	*A. tumefaciens* WCF47	TraI/R (*A. tumefaciens*)	β-galactosidase	As above with more sensitivity	As above with more sensitivity
pJZ384 + pJZ410 + pJZ372	*A. tumefaciens* KYC55	TraI/R (*A. tumefaciens*)	β-galactosidase	As above with more sensitivity	As above with more sensitivity
pSF105 + pSF107	*P. fluorescens* 1855	PhzI/R (*P. fluorescens* 2-79)	β-glucuronidase β-galactosidase	C6-3-hydroxy-AHL	C8-3-hydroxy-AHL
S. meliloti sinI::lacZ	*S. meliloti sinI::lacZ*	SinI/R (*S. meliloti*)	β-galactosidase	C14-3-oxo-AHL	C16:1-3-oxo-AHL C16-AHL C16:1-AHL C14-AHL
pJNSinR	*S. meliloti sinI::lacZ*	SinI/R (*S. meliloti*)	β-galactosidase	As above with more sensitivity	As above with more sensitivity
pAS-C8	Broad host range	CepI/R (*B. cepacia*)	gfp	C8-AHL	C10-AHL
pKR-C12	Broad host range	LasI/R (*P. aeruginosa*)	gfp	C12-3-oxo-AHL	C10-3-oxo-AHL
pJBA-132	Broad host range	LuxI/R (*V. fisheri*)	gfp	C6-3-oxo-AHL	C6-AHL C8-AHL C10-AHL

TABLE 1. 2. VARIOUS BIOREPORTER CONSTRUCTS USED IN DIFFERENT METHODS OF AHL DETECTION AND QUANTIFICATION
(Steindler and Venturi, 2007)

1.10.1. Biosensors for detection of a narrow range of AHL

E. coli cells have been transformed with various *lux*-based recombinant reporter plasmids. The plasmid pSB315 lacks a functional *luxI* homolog and is capable of responding to exogenous AHL when supplied with a long-chain fatty aldehyde such as dodecanal, which is essential for the light reaction (Swift *et al.*, 1993). A refined construct, with plasmid pSB401 has been constructed that responds to a wide range of AHLs but does not require the addition of exogenous aldehyde for the light reaction. The bioreporter has been constructed with the replacement of *luxR* and the *luxI* promoter regions with the *P. aeruginosa* luxR homolog, *rhlR,* and the *rhlR* promoters (Winson *et al.*, 1995). The lux-based reporters are most sensitive towards its cognate AHL but most are capable of detecting the 3-oxo and alkanoyl standards, however they do not detect N-butanoyl homoserine lactone or any of the 3-hydroxy forms (Cha *et al.*, 1998). Lux based biosensors have also been developed with different *luxR* homologues that could be used in combination to detect a broad range of AHL compounds (Swift *et al.*, 1993; Winson *et al.*, 1998)

Chromobacterium violaceum is a gram-negative bacterium commonly found in soil and water that produces an insoluble purple pigment called violacein through a quorum sensing system. McClean *et al* developed a bioreporter from this bacterium using mini Tn5 mutagenesis; hence the organism no longer was able to produce the pigment (McClean *et al.*, 1997). However when subjected to exogenous AHL, the bacterium is able to restore its pigment production. The bioreporter has prove to be extremely sensitive to short- and medium-chain length non substituted AHLs, but lacks sensitivity to AHL with longer chains and most of the 3-oxo-substituted AHLs (Cha *et al.*, 1998;

Latifi *et al.*, 1995; Milton *et al.*, 1997). Other bioreporters have been constructed based on AHL-induced bacterial swarming. The *Serratia. liquefaciens* MGI bioreporter cannot synthesise C4-HSL or C6-HSL but can restore serrawettin synthesis and swarming in the presence of exogenous supply of AHL (Lindum *et al.*, 1998).

1.10.2. AHL Biosensors for broad range and high sensitivity detection system

An *A. tumefaciens*-based AHL sensor has been developed based on the TraI/R quorum sensing system and has been particularly suitable for TLC analysis and AHL quantification using ß-galactosidase assays (Cha *et al.*, 1998; Zhu *et al.*, 1998). The traI/R quorum sensing system in *A. tumefaciens* is localized in a plasmid called the Ti plasmid. Activation of the traI/R system results in transcription of several genes involved in Ti plasmid conjugal transfer. *A. tumefaciens* NTL1 bioreporter involves a strain that lacks the Ti plasmid so the strain is not capable of producing AHL, a plasmid NTL4 is inserted that contains contains the *traR* gene and a *tra* operon involved in conjugal transfer, containing traG::lacZ reporter fusion, which is expressed in the presence of exogenous AHL (Cha *et al.*, 1998). A similar *A. tumefaciens* biosensor was constructed, called WCF47 (pCF218) (pCF372) that shows greater sensitivity to a range of AHL; the strain WCF47 contains a mutation in *traI* and consequently does not produce AHLs (Zhu *et al.*, 1998). Among the two plasmids, the plasmid pCF218 contains DNA that overexpresses TraR and the plasmid pCF372 contains *traI* promoter transcriptionally fused to *lacZ*. Recently a, highly sensitive bioreporter has been developed in *A. tumefaciens* KYC55 lacking the Ti plasmid which does not produce AHLs (Zhu *et al.*, 2003). It is a three plasmids system with (pJZ384) (pJZ410) (pJZ372)

inserted. Its higher sensitivity results from over-expression of the TraR protein through the incorporation of larger amounts of regulators making it suitable for detecting extremely small quantities of AHL. Detection of AHLs using *lacZ* expression involves the incorporation of 5-bromo-4-chloro-3-ß-D -galactopyranoside (X-Gal) in to the medium, which is hydrolysed to produce a blue coloured compound when lacZ is expressed. This can be visualized after an overnight incubation as blue induced spots on TLC plates or on agar petri dishes (Cha *et al.*, 1998). Due to the sensitivity of these reporters, only small volumes of AHL extracts from spent supernatants are required for their detection (Farrand *et al.*, 2002). Extracts of spent supernatants can also be assayed directly for a response and quantification of the amount of AHL can also be determined using a culture of the *A. tumefaciens* biosensor (Zhu *et al.*, 2003). When different constructs based on *tra* of *A. tumefaciens*, *lux* of *V. fischeri*, *las* of *P. aeruginosa* and pigment production of *C. violaceum*, were compared for their abilities to detect acyl-HSLs with chain lengths ranging from C_4 to C_{12}, the *traG*: *lacZ* fusion reporter from the *A. tumefaciens* Ti plasmid was found to be the most sensitive and versatile detector among the bioreporters tested (Cha *et al.*, 1998). The *A. tumefaciens* bioreporter was capable of detecting *3*-oxo substituted AHL derivatives with acyl chain lengths from 4-12 carbons as well as 3-unsubstituted AHL with low specificity and the exception of C4 AHL (Cha *et al.*, 1998).

Usually the bioreporter detection method for AHL involves the use of whole cell bioreporters, however recently, different assay systems have been developed using bioreporter systems. An assay based on cell free lysate of *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) has been reported, allowing the expression of ß-galactosidase upon addition of exogenous AHLs in cell-free solution (lysate from *Agrobacterium*

tumefaciens NTL4 (pCF218)(pCF372) culture) (Kawaguchi, Chen et al. 2008). This was found to significantly reduce assay times from greater than 24 h to less than 3 h, while maintaining a 10 fold increase in sensitivity (from 10nM to 30nM), especially when the assay was conducted with the luminescent substrate Beta-Glo(R) (Promega Coorporation). Another assay was designed with the bioluminescent bioreporter *Escherichia coli* ROlux2 (Perry *et al.*, 2005) that showed advantage in overcoming a concentration and extraction step in detection of AHL, with the use of small sample volumes from cultures of *V. fischeri*, that can also account for effects caused by the composition of the culture medium (Yan *et al.*, 2007).

1.11. Purification and characterization of AHL

1.11.1. Extraction of AHL

In order to identify AHL molecules, AHLs are extracted from the spent culture supernatant. Due to the characteristic of having lipid side chain AHL molecules can be easily extracted using organic solvents such as ethyl acetate and dichloromethane (Cha *et al.*, 1998; Holden *et al.*, 1999). Acidified ethyl acetate and dichloromethane have been a widely used solvent for extracting AHL molecules from different bacterial species. Extraction yields can depend greatly on the lipid side chain and yields can dramatically decrease with the polarity of the extracted AHL (Morin *et al.*, 2003). Therefore, identification of polar HSL can greatly depend on the extraction of AHL molecules to a sufficient amount for their detection. The extraction process is known to improve the sensitivity of detection process by removing the background effects caused by the complex sample matrix.

1.11.2. Detection and identification of AHL

The chemical properties of AHLs suggest that they should be stable enough to be isolated. A currently widely used technique to isolate AHL is as follows: AHLs are concentrated from spent culture filtrate by extracting into an organic solvent e.g. dichloromethane, ethyl acetate and purified to homogeneity by using semi-preparative high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) (Cha *et al.*, 1998; McClean *et al.*, 1997; Swift *et al.*, 1993). In some cases detection without extraction can involve growing the testing organism in 'T' streaks with a bioreporter, which results in expression of the reporter phenotype in a gradient in the presence of AHL. However, this method requires both the testing organism and the bioreporter to grow in the same growth medium and under the same growth conditions with possibly similar growth rates. Spent cell free culture supernatant, without solvent extraction can also be used for detection using bioreporters but consideration has to be given to negative results that may result from any inhibitory compounds present in the supernatant that may interfere with the detection process and the concentration of AHL present in the supernatant. However, similar interference may also arise from extracted samples, especially if the inhibitory compounds are co-extracted with AHL and cause interference with the bioreporter detection process. In several cases the use of chemically defined growth media has been preferable over the use of complex growth media that can reduce interference in AHL detection of the organism under study. Following extraction, the extract can either be used directly for detection of AHL using bioreporters or may involve a purification step involving reverse phase semipreparative columns. Fractions separated through columns are eluted with different gradients of solvent systems. Among the solvents used are methanol (Pearson *et al.*, 1994) and

acetonitrile (Lithgow *et al.*, 2000; Schripsema *et al.*, 1996) or an isocratic mobile phase of acetonitrile: water (McClean *et al.*, 1997). High pressure liquid chromatography or C18 reverse phase TLC plates can also be used to purify individual AHLs from extracts (Cha *et al.*, 1998; Makemson *et al.*, 2006). The identification of specific types of AHL using bacterial reporter strains is often cheap and easy to handle, when combined with TLC separation. A partial characterization of an individual AHL can be obtained by separation of samples containing supernatants or extracts and different AHL standards on C18 TLC plates following by soft overlay with bioreporter strain and then comparison of their relative mobilities to that of known standards on TLC plates. Although this approach can be used to give identification of the type of AHL, in some cases, bioreporters have been found to respond to other types of related compounds and give a positive bioreporter result for example, *C. violaceum* can respond to cyclic peptides besides its usual detection of AHL (Holden *et al.*, 1999). Therefore, a more confirmative approach involves identification through the fragmentation pattern of AHL using mass spectrometry. Although use of biosensors reveals local information about the occurrence of AHL in situ, they fail to provide information about a wide or complete spectrum of AHL compounds. Due to the limitation of bioreporters in the range of AHL detected with high sensitivity, analytical tools and separation techniques, including gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have been used for both quantitative and qualitative analysis of AHL in various studies (Fekete *et al.*, 2007). The structure of purified AHL can be identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Eberl *et al.*, 1996; Makemson *et al.*, 2006). A list of the methods developed for AHL analysis has been summarized by Fekete *et al*, 2007 (Fekete *et al.*,

2007). Some of these techniques have been used to verify the results of bioassay and identify with its structural information of the AHL species produced.

1.12. Measuring the concentration of AHL

Various studies measure AHL concentration indirectly by a semiquantitative method using bioreporter response. In such techniques the degree of response of bioreporter to standard concentrations of AHL is used as a standard to measure AHL concentrations in unknown samples (Quinones *et al.*, 2004). An agar well-diffusion assay based on *A. tumefaciens* PDZLR4 biosensor was used to quantify AHL from supernatants to study the kinetics of AHL production in different organisms (Ravn *et al.*, 2001). In such an assay the distance of diffusion of the autoinducer from the point of application is used indirectly to measure the concentration of the autoinducer to which the bioreporter responds. In other studies a lux plasmid-based bioluminescent sensor W3110 (pSB315) was developed capable of detecting 80pg ml^{-1} and was used to demonstrate density dependent production of AHL in several enteric bacteria (Swift *et al.*, 1993). Compared to the classical AHL analysis of bacterial cultures with biosensors, where selectivity and sensitivity is often limited, a rapid analytical technique using HPLC-MS can substantially improve qualitative and quantitative detection of AHL (Morin *et al.*, 2003) This technique, using a nano-liquid chromatography--electrospray ionization mass spectrometry setup, was found to be suitable for analysis of AHL from samples without prior sample preconcentration and with minimal cleanup (Frommberger *et al.*, 2004).

1.13. The cyanobacterium *Gloeothece* PCC 6909

Two strains of *Gloeothece* were used in this study, a wildtype PCC 6909 (Rippka *et al.*, 1971) previously known as *Gloeocapsa* UTEX 795 (Huang *et al.*, 2002) and sheathless mutant of *Gloeothece* PCC 6909/1 (Kallas *et al.*, 1983). The strains have been extensively studied by Gallon and collaborators mainly in the area of aerobic nitrogen fixation.

Gloeothece is a relatively slow growing organism. It cannot tolerate high light intensities at which most other cyanobacteria grow. Most favourable growth is reported to be within light intensities of 500-1000 Lux. Within this range the generation time of the wild type and mutant has been ca. to be 40 and 70 hours respectively (Kallas *et al.*, 1983). Growth can also differ depending on nitrogen sources; whether growing under nitrogen fixing conditions or in the presence of combined nitrogen. When grown under both low light intensities well below 500 Lux and micro aerobically, growth rates on nitrogen and combined nitrogen is similar. At light intensities above 500 Lux, under air, nitrogen fixing cultures grow much more slowly than combined nitrogen (Kallas *et al.*, 1983).

Different species of *Gloeothece* are reported to be detected from different habitats including hypersaline environments (Zhao *et al.*, 2005), hot deserts (Budel and Wessels, 1991), in epilithic surfaces on rocks and stones (Crispim and Gaylarde, 2005) and in lichen formation (Rai, 1990). This therefore may reflect the diversity within the species. However, most *Gloeothece* reported to date has been found to be associated with biofilms in archeologically important sites worldwide (Crispim and Gaylarde, 2005;

Ortega-Morales *et al.*, 2000). Various reports indicate that *Gloeothece*, as a cyanobacterium are able to grow on toxic metabolites such as black sulphated crusts and limestone buildings which may reflect the complexity in their metabolic processes (Saiz-Jimenez, 1997).

Since, quorum sensing functions in many bacteria are employed for survival (Quinones *et al.*, 2004) protecting the niche (Lynch *et al.*, 2002) competence (Dunny and Leonard, 1997) or escaping (Omata *et al.*, 1999), the possibility of an AHL quorum sensing system in cyanobacteria was sought for. The cyanobacterium *Gloeothece* was considered for the following reasons:

(1) *Gloeothece* can be obtained in pure culture form aiding investigation of the presence of an AHL quorum sensing system

(2) *Gloeothece* is a colonial cyanobacterium, with colonies surrounded by multilaminated sheaths. The colonies often associate with each other surrounded by slime creating macrocolonial aggregates such as in biofilms. The sheath and extracellular polysaccharide in *Gloeothece* may provide a diffusion barrier or allow accumulation through sequestering of signals (Charlton *et al.*, 2000). Coordinated behaviour through quorum sensing within colonies can be particularly suitable for the survival of the colony. From an evolutionary point of view signalling in a single colony can be seen as the most primitive method of species survival.

(3) *Gloeothece* is found in mixed biofilms on epilithic surfaces. Reports have indicated the presence of AHL in natural biofilms on rock surfaces (McLean *et al.*, 1997). The

habitat of *Gloeothece* can be represented by a habitat of low mass transfer and periods of desiccation that can allow *Gloeothece* cells to efficiently use AHL through its accumulation.

(4) *Gloeothece* shows the ability to form associations with other organisms. The close association of *Gloeothece* with other AHL producing bacteria may allow interbacterial cross talk and influence community structure (McLean *et al.*, 2005).

CHAPTER 2

The identification of acylated homoserine lactones (AHL) from cell free culture extracts of the cyanobacterium *Gloeothece*

2.1. Introduction

Quorum sensing is widely recognized among different phylogenetic groups including bacteria, fungi and yeast (Hogan, 2006) (Waters and Bassler, 2005). Even plants, where a quorum sensing system has not been recognized, are found to secrete AHL mimics and also show responses to several known bacterial AHL (Mathesius *et al.*, 2003). Cyanobacterial assemblies and phototrophic biofilms on stones have been reported as indicating the presence of AHL under natural conditions (Bachofen and Schenk, 1998; Braun and Bachofen, 2004). Although these reports provide evidence of physiological concentrations of AHL under natural conditions, they do not discriminate whether the AHL is produced by cyanobacteria or other associated gram negative bacteria. There are also no reports on the presence of AHL from axenic cultures to date.

Cyanobacteria belonging to the group of Chroococcales are unicellular or colonial cyanobacteria that have attracted great interest because of their great metabolic diversity and include members of *Synechocystis, Microcystis, Synechococcus, Prolchoron* and *Gloeothece*. Cyanobacteria such as *Microcystis* and *Gloeothece* represent colonial forms and have been found to be associated with blooms and biofilms where cell density is high. Several cooperative behaviours and metabolic activities have been noted in colonial cyanobacteria at high cell densities, including regulation of pigments in response to stress (Haibo and Baosheng, 2005; Paerl, 1984).

Moreover, the cyanobacterium *Microcystis aeruginosa* has been reported to have homologous proteins to bacteria that are directly under the control of an AHL quorum sensing system (Dittmann *et al.*, 2001). These findings suggest that it would be worth investigating for a quorum sensing system in colonial cyanobacteria.

Recently, great emphasis has been placed on the preservation of archaeologically important sites, which have been found to be attacked by micro-organisms growing in biofilms, causing their bio-deterioration and altering the aesthetic view. Among various cyanobacteria, *Gloeothece* has been found to comprise a major biomass in biofilms growing in important sites in urban places. These habitats can also be favourable for organisms that employ quorum sensing due to low mass transfer. Since quorum sensing is known to be involved in biofilm formation, a possible quorum sensing system in *Gloeothece* was investigated using axenic cultures of this cyanobacterium grown under laboratory batch cultivation.

Aims and Objectives:

This chapter was aimed towards testing the hypothesis that the cyanobacterium *Gloeothece* PCC 6909 produces AHL quorum sensing molecules with a view towards answering the following questions.

- Can AHL molecules be detected from axenically grown cultures of *Gloeothece* PCC 6909 and *Gloeothece* PCC 6909/1 (sheathless mutant)?
- Provided that AHL signals can be detected, what is the identity of the type(s) of AHL molecules?

The objectives are to:

- Detect the presence of AHL from extracted culture supernatants through the use of bioreporter NTL4 (pZLR4) that responds to a wide range of AHL compounds. The presence of AHL is detected by the production of ß-galactosidase that by the cleavage of X-Gal produces a blue colour complex, indicative of the presence of AHL.

- Following a positive indication of an AHL molecule, TLC and HPLC-MS and MS/MS is used to further confirm and gain information with regard to the identification and the specific type(s) of AHL molecules present in the culture supernatant.

2.2. Materials and Methods

2.2.1. Materials:

Cultures

Gloeothece strains PCC 6909 and a sheath less mutant of *Gloeothece* PCC 6909/1 were obtained from Pasteur culture collection, Institute Pasteur, Paris. CCAP 1303/1 was obtained from Culture Collection by Algae and Protozoa, Scottish Association of Marine Sciences.

AHL Bioreporters

Biosensor NTL4 (pZLR4): *Agrobacterium tumefaciens* bioreporter NTL4 (PZLR4) (Cha *et al.*, 1998) was a generous gift from Stephen K. Farrand.

Chemicals and equipments

All Homoserine lactone standards (standards of C4, C5, C6, C8, C10, C12, and C14) were purchased from Fluka and prepared as stock solutions in acetonitrile at a concentration of 1 nmol μl^{-1} (1mM). Appropriate working concentrations were prepared from the stock solutions and used for bioreporter (where stated) and TLC applications. Reagents for bioreporter detection such as X-gal, DAPI (4'-6-Diamidino-2-phenylindole), Gentamycin, were obtained from Melford, UK, Agro-medium was obtained from Q-Biogene, UK, Whatman filters, antibiotic assay discs, were from Anachem Ltd. All other chemicals were obtained from Sigma Chemical Company Ltd. or Fisons Scientific Equipment

Media

BG11^{o} cyanobacterial mineral media without nitrate (Appendix I)

BG11 cyanobacterial mineral media with nitrate

2.2.2. Methods:

2.2.2.1. Culture maintenance and growth conditions

Gloeothece strains PCC 6909 and PCC 6909/1, a sheathless mutant of PCC 6909, (Pasteur Culture Collection, Institut Pasteur, Paris) were grown under aerobic conditions in 250 ml conical flasks containing 100 ml of BG11^{0} medium (nitrate omitted), a nitrogen free medium used for diazotrophic growth (see appendix). Cultures were maintained at 26 °C under alternating 12 h light (30 μmol m^{-2} s^{-1}, over the waveband 400-700 nm, measured at the surface of the culture) and 12 h darkness on an orbital shaker (70 g).

2.2.2.2. Extraction of AHL from culture supernatants

Cultures were grown and maintained according to section 2.2.2.1. Supernatants from different age cultures were collected by harvesting the cultures by centrifugation and carefully decanting the supernatant. Culture supernatants were then extracted with organic solvent for subsequent detection of AHL using the bioreporter method.

- Cultures from 100 ml samples (unless otherwise stated) were centrifuged at 8000 g to remove cells.
- The resultant supernatant was adjusted to pH 2.0 and incubated overnight (ca. 16 h) to reverse lactonolysis (unless otherwise stated).
- The supernatant was then extracted (3 times) against dichloromethane (2:1 (v/v) culture medium: dichloromethane) with gentle shaking to mix the organic and aqueous layers. In cases where an emulsion was formed extraction was continued for 2-3 h. In cases where the emulsion was still present after 2 h the dichloromethane emulsion was taken and processed subsequently.
- The combined dichloromethane extracts were dried with anhydrous magnesium sulphate to eliminate any aqueous solution present in the extract and filtered through a Whatman no.1 to eliminate residual magnesium sulphate before subjecting to drying under rotary evaporation.
- The organic extract was dried under rotary vacuum evaporation.
- The resulting residue was re-dissolved in 1ml of acetonitrile (HPLC grade) and transferred to a glass vial.
- The solvent was removed under a stream of filtered nitrogen gas and the extract was stored at –20°C until required. Prior to use, for HPLC-MS, the dried residue was re-dissolved in 20-50 µl methanol/water (1:1 v/v) containing 0.1% (v/v) formic acid. For use in bioreporter assays the sample was re-dissolved in 20-50 µl acetonitrile.

2.2.2.3. Bioreporter assay

Extracted samples from *Gloeothece* cultures were used in bioreporter assay. The bioreporter assay plates were prepared according to the following method. Following incubation with samples a positive bioreporter test was indicative of the presence of AHL and was evaluated by visually detecting the presence of a blue colour zone surrounding the sample application site.

Bioreporter culture preparation

Stocks of the *Agrobacterium tumefaciens* NTL4 bioreporter were stored at -80 °C. When required, a sample of stock culture was transferred into 5 ml Agro-medium (Q-Biogene, UK) supplemented with gentamicin (5 μg ml^{-1}) and yeast extract (0.1% (w/v)) and was incubated overnight (ca.16 h) at 28 °C, on an orbital shaker (200 g). After this time, a 2 ml sample of the culture was used to inoculate 20 ml fresh medium, which was incubated (as previously) until late exponential phase (*ca.* five hours) and subsequently used for detection of AHL on bioassay dishes and prepared TLC plates.

Preparation of bioassay dishes

130 ml of sterile Agro-medium, supplemented with 1.2 % agar was melted and thermo stated at 45 °C. 5-bromo-4-chloro-3-indolyl-b-D-galactosidase, X-gal dissolved in dimethylformamide (DMFO) was added at a concentration of 40 μg ml^{-1} to this media. Bioassay dishes were then prepared by adding 10 ml of the bioreporter culture to the thermostated medium and transferring 30 ml of the resultant culture aseptically into

sterile Petri dishes. Appropriate negative controls were prepared by omitting the bioreporter culture from the bioassay dishes to test false positive due to the hydrolysis of X-Gal by components co-extracted with samples from *Gloeothece.*

Application of samples on bioassay dishes

Aliquots (5 µl and 10 µl) of extracted samples and standards in working concentrations of 1 pmol μl^{-1} prepared in acetonitrile were pipetted carefully onto individual antibiotic discs (Whatman Ref No.2017 006), which were air-dried or solvent evaporated on a heating block and subsequently placed on the agar surface of the bioassay dishes. The dishes were placed in an incubator for 16 h at 28 °C, at the end of which time plates were inspected visually for the indication of AHL by a resulting blue colour zone.

2.2.2.4. Detection and identification of AHL using Thin Layer Chromatography (TLC):

2.2.2.4.1. Preparation and development of chromatogram

Thin layer chromatography was carried out on C18 reverse phase plates (200 mm layer, 20- x 20 cm, Baker, UK). AHL standards (C-10, C-8, C-7, and C-6) were applied as a single spot on one end at the base of the TLC plate in the following concentrations:

N-Hexanoyl homoserine lactone (C6-HSL): 1 µl of this stock was applied on the plate to give an amount of 1pmol

N-Heptanoyl homoserine lactone (C7-HSL): Working concentration was prepared by diluting 1 µl of stock into 200 µl of acetonitrile. Two µl of this working concentration was applied on TLC plate to give an amount of 10 pmol.

N-Octanoyl homoserine lactone (C8-HSL): Working concentration was prepared for by dissolving 1 µl of standard in 100 µl of acetonitrile. One µl of this working concentration was applied to TLC plate to give an amount of 10 pmol.

N-Decanoyl homoserine lactone (C10-HSL): working concentration was prepared by adding 1 µl of standard to 50 µl of acetonitrile. 5µl of this was solution was applied on TLC plates to give an amount of 100 pmol. Sample extracts (10 µl-15 µl) to be analyzed were also placed as individual spots along the same line on the base of the plate. The plate was then placed in a methanol saturated chamber and the chromatogram was allowed to develop in a solvent system (methanol-water (60:40, vol/vol)) until the solvent front reached the top of the plate (ca, 5 – 6 h). After development the solvent was evaporated and the chromatogram was air-dried before overlaying with bioreporter culture.

2.2.2.4.2. Preparation of bioreporter overlay

The overlay was prepared by preparing 50 ml of bioreporter culture (section 2.2.2.3.1.) and transferring the culture into 100 ml of freshly melted Agro agar medium (0.7% agar) maintained at 45 °C containing 60 mg ml^{-1} of X-gal. The culture was mixed and poured immediately over the TLC plate framed with a zig. The resultant bioreporter chromatogram was allowed to cool and incubated at 28 °C for 12 - 18 h. Plates were

then visually inspected for the appearance of blue colour. The relative mobility (R_f) of the bioreporter response of the unknown sample was compared to the R_f of various AHL standards in order to obtain tentative information on the type of AHL present in *Gloeothece* samples.

2.2.2.5. Effect of pH on AHL

Gloeothece PCC 6909/1 was grown as described in section 2.2.2.1. in 130 ml BG11^o medium for 2 months after which the supernatant was collected by centrifugation at 8000 g . The supernatant was then divided in to two equal parts containing 65 ml each. One part of the sample was adjusted to pH 2.0 and incubated along with the other sample at 4 ^oC overnight. The following day both samples were extracted according to section 2.2.2.2. and analysed for AHL positive results by inoculating half (20 µl) of the extracted samples and 20 pmol of C8-HSL as a standard using the bioreporter technique described in section 2.2.2.3.

2.2.2.6. Mass spectrometric identification of AHL

2.2.2.6.1. Preparation of capillary column for HPLC

A column (15 cm x 300 µm i.d.) was prepared from dried stationary phase (Polaris C18, a gift from Varian UK). Slurry (1 mg stationary phase ml^{-1} isopropanol) was prepared and transferred to a high pressure column packing device attached to a cylinder of oxygen-free nitrogen (OFN). A frit was prepared at the end of a piece of 300 µm fused

silica (Composite Metal, UK) by dipping the fused silica into a reaction mixture of Kasil (PQ Corporation) / Formamide (Sigma Aldrich) (3:1 v/v) followed by heating to 100°C over a 15 min period. The silica was maintained at this temperature for 4 h and at the end of this period the unfretted end was passed into the bomb via an entrance sealed with a ferrule and into the stationary phase solution. Packing was achieved by application of pressure (40 bar) from the OFN cylinder.

2.2.2.6.2. Separation of commercial standard AHLs

Separation of AHLs with the standards stated (standards of C4, C5, C6, C8, C10, C12, and C14) was achieved using HPLC on a column of C18 stationary phase using a Dionex "Ultimate" pump and FAMOS autosampler (Camberley, UK). The column was connected to the mass spectrometer with a piece of fused silica (10 cm x 20 μm i.d.). Samples, in 20 - 50 μl methanol/water (1:1 v/v) containing 0.1% (v/v) formic acid were injected onto the column with the aid of a FAMOS autosampler system. 5 μl of sample was injected onto the column, which was eluted at a flow rate of 4 μl min^{-1} with a solvent consisting of 50:50 mixture of methanol, containing 0.1% (v/v) formic acid (A): water, containing 0.1% (v/v) formic acid (B), for 5 min, followed by a linear increase over 5 min to a final solvent mixture of A:B 95:5. This final solvent mixture was maintained for 30 min before a return to the initial eluent composition, which was maintained for 20 min, to re-equilibrate the column.

The eluent from the HPLC was linked directly to an LCQ Deca XP Ion Trap mass spectrometer (Thermo Electron, UK) fitted with a low flow adapter (allowing flow rates

below 10 µl min^{-1} without broadening of peaks). The mass spectrometer was operated in positive ion mode with a spray voltage of 3.5 kV, a capillary voltage of 23 V, a capillary temperature of 200 °C and a sheath gas flow of 30 (arbitrary units). For full scan analysis the mass spectrometer was set to scan from 50-500 Da with a maximum injection time of 50 msec. For tandem mass spectrometric analysis scans, an isolation window of ± 0.5 Da was used when selecting the desired parent ion. Collision energy of 35 arbitrary units and an activation time of 30 msec were applied.

2.2.2.6.3. Identification of AHL from *Gloeothece* samples

For identification of AHLs for *Gloeothece* extracts, 5 µl of sample was injected onto the HPLC column and separated and analyzed under conditions described in section 2.2.2.6.2. The mass spectrometer was operated once in full scan mode in order to determine if any signals indicative of the AHL standard compounds could be detected. If such signals were detected, MS/MS was employed to fragment the ions of interest. Only if the HPLC retention time, the full scan MS and subsequent fragmentation analysis were in agreement with that of the commercial standards were the AHLs reported as being present.

2.2.2.7. Detection for contamination

Cultures to be extracted for detecting AHLs were checked for contamination. Only axenic cultures were selected for subsequent extraction and detection of AHL. The

following procedures were tested in their suitability for detecting contamination in *Gloeothece* cultures.

2.2.2.7.1. Agarose plate technique

The agar plate assay is designed to support growth of any contaminating bacteria by providing favourable nutrients such as glucose and amino acids into the growth media of cyanobacteria to support bacterial growth. BG11+ agar plates were prepared by supplementing 0.5 % agar, $NaNO_3$ and casamino acids into the $BG11^0$ medium. After autoclaving and cooling at 55 °C the agar media was poured in to petri dishes and allowed to solidify. Samples were also tested on nutrient agar as a positive control, commonly used to support bacterial growth. A loop of sample from *Gloeothece* PCC 6909/1 cultures were streaked on the agar plates (BG11+ and nutrient agar) with a sterile loop on one half of the plate. On the other half of the plate a known non axenic culture of CCAP 1303/1 (culture collection by algae and protozoa, Scottish Association of Marine Sciences) was inoculated similarly. Plates were then incubated at 26 °C for 1-3 days to detect the presence of bacterial colonies.

2.2.2.7.2. Confocal microscopy

4', 6-diamidino-2 phenylindole (DAPI) stained mutant and wild-type cultures were examined for bacterial contamination using a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss, 07740 Jena, Germany). The 405 nm and 488 nm lasers were used to excite DAPI and chlorophyll a, respectively. Band-pass 420-480 nm

and long-pass 650 nm filters were used to observe blue and red fluorescence, respectively.

2.3. Results

2.3.1. Detection of AHL from culture supernatant of *Gloeothece*

Cultures of *Gloeothece* PCC 6909 were screened throughout growth for the detection of AHL. Several samples showed a positive bioreporter result within culture ages of 15 days to 80 days. A positive result was evident by the presence of a blue colour surrounding the sample application site (Fig. 2.1), suggesting that the bioreporter was capable of responding to a compound present in *Gloeothece* extracts. The area produced by the blue colour zone from the *Gloeothece* extract (Fig. 2.1) also increased with an increase in the quantity (µl) of culture extract, which was also evident with the application of different concentrations of standard AHL. These results indicated that an active component was present in *Gloeothece* extract which was capable of activating the bioreporter and could be potentially identified as AHL(s). These results were also confirmed against a negative control (without Bioreporter), used to test whether any factor in the *Gloeothece* extract could cause a false positive result by the cleavage of X-gal in the medium. A variation in concentration with different samples was also evident by comparing their zone sizes which represented different concentrations of AHLs extracted from different culture age and densities of samples studied in Chapter 3.

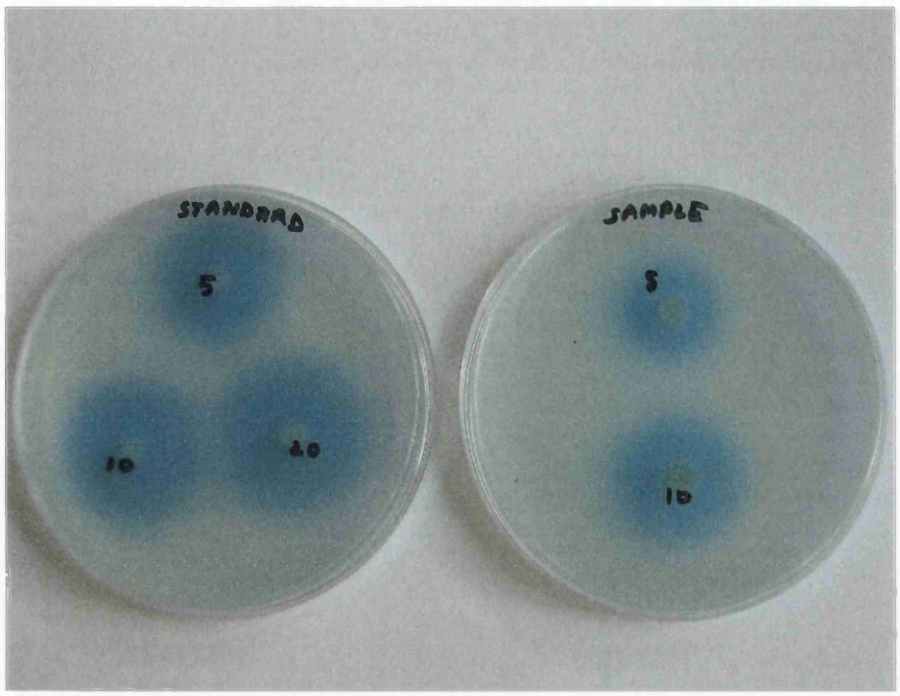


FIGURE 2. 1. AHL DETECTION FROM EXTRACTED CULTURES OF *GLOEOTHECE* PCC 6909 USING BIOREPORTER NTL4 (PZLR4)

Plates seeded with the bioreporter indicate the presence of AHL by a blue colour zone surrounding the sample application site. Samples of Gloeothece extract applied at different quantities (5µl and 10µl) (Right bioreporter plate) and standard AHL applied at different concentrations (1x, 2x, and 4x of an AHL working solution) (Left bioreporter plate) indicates response by AHL.

There were also some samples from cultures that did not show any positive bioreporter result which may indicate low concentrations of different AHL(s), not within the limit of the bioreporter detection or not being extracted under conditions used in this experiment or the absence of AHL. However, such results could also be due to a possible AHL degradation activity. For this, it became important to investigate if AHL detection is affected by other parameters for example, pH dependent variations,

extraction conditions, and signal degradation and low concentrations of AHL that may be produced during specific stages of growth (studied in chapter 3).

2.3.2. Effect of pH

Alkaline pH is known to cause degradation of AHL to its corresponding compound homoserine, which does not activate a bioreporter response. Therefore, to study whether pH of the growth medium has any effect on the degradation of AHL, *Gloeothece* growth medium was checked during various times of growth. It was found that the pH of *Gloeothece* culture media with an initial pH of 7.2 rises to pH 8.0 within 2-4 weeks of growth and reaches to about 8.4-8.5 during later stages (90 days) of growth. In order to reverse any effect that pH may have on the detected AHL concentration, cell free culture supernatant was routinely incubated at pH 2.0 overnight prior to extraction. Figure 2.2 shows the area of the bioreporter response to be similar from samples that were extracted from the same volume and same culture supernatant but under different pre-extraction conditions (pH adjusted to 2.0 and measured pH of the growth medium 8.1). These results indicate that equal amounts of AHL were extracted from each sample showing the reproducibility of the extraction method and at the same time indicating that AHL levels were not altered by different pre-extraction pH conditions. Results also indicate that alkaline pH of the culture medium during growth does not alter the concentration of the AHL detected; suggesting that high pH of *Gloeothece* cultures may not have any effect on the stability of the accumulated AHL. Pre-incubation at pH 2.0 was used as a method to standardise the extraction procedure, so that results could be compared across different growth samples of *Gloeothece*.

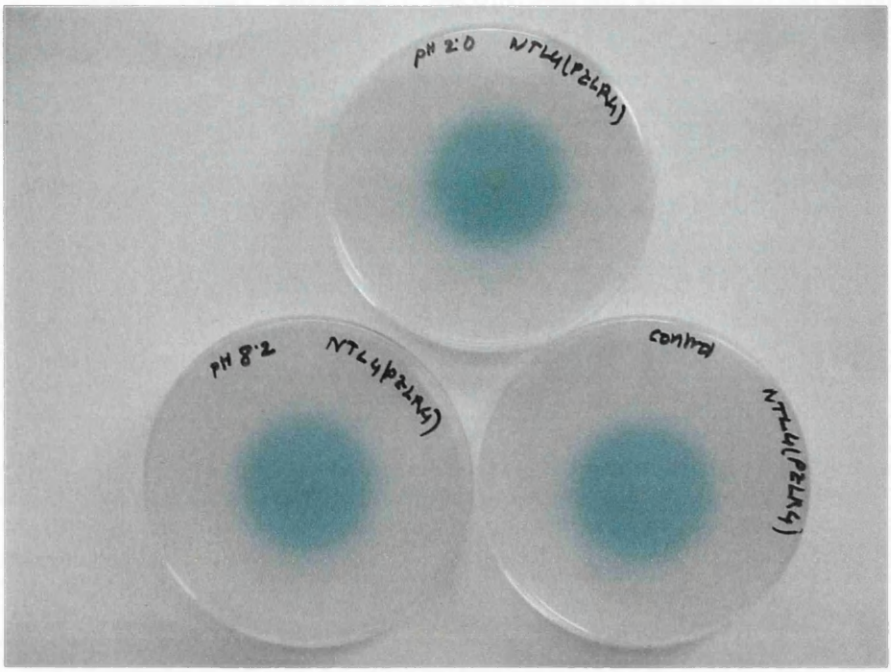


FIGURE 2. 2. THE EFFECT OF CULTURE PH ON AHL DEGRADATION.

AHL detected from different pre-extracted samples by the use of bioreporter NTL4 (pZLR4). Pre-extraction carried out at pH 2.0 (top plate), pH of the culture supernatant pH 8.2 (left plate) and a control of 20pmol of C8-HSL in acetonitrile (right plate)

2.3.3. Investigating potential presence of bacteria

Cultures of *Gloeothece* were frequently checked for contamination to confirm that the AHL detected was produced from *Gloeothece* and not by other contaminating bacteria present in the cultures. Figure 2.3 and 2.4 show the two separate techniques used to detect bacterial contamination in *Gloeothece* cultures. With the agar plate assay (Fig 2.3) bacterial growth was visualized in a non axenic culture of *Gloeothece* CCAP 1430/3 as colonies appear within 24 h of growth compared to the absence of any growth in an axenic culture of PCC 6909/1. The use of both nutrient agar plates and BG11 agar plates supplemented with casamino acids was found to support the growth of

contaminating bacteria. Microscopy was also used to detect bacterial contamination (Fig 2.4). Fig 2.4 shows bacterial contamination directly detected in cultures of PCC 6909/1 visualized by confocal laser scanning confocal microscopy useing of DAPI (4'-6-Diamidino-2-phenylindole). Careful inspection using LCMS showed the presence of bacteria visualized as blue fluorescent rods, mostly embedded within the slime matrix in proximity to *Gloeothece* cells (Fig 2.4).

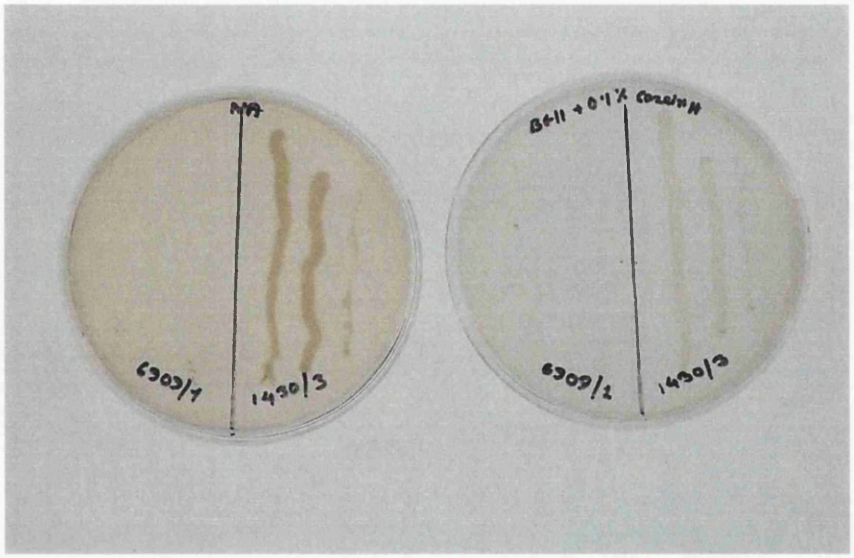


FIGURE 2. 3. BACTERIAL CONTAMINATION DETECTED USING AGAROSE PLATE TECHNIQUE.

Axenic cultures of Gloeothece PCC 6909/1 at the left side of each plate. Bacterial contaminated cultures of 1430/3 at the right side of each plate.

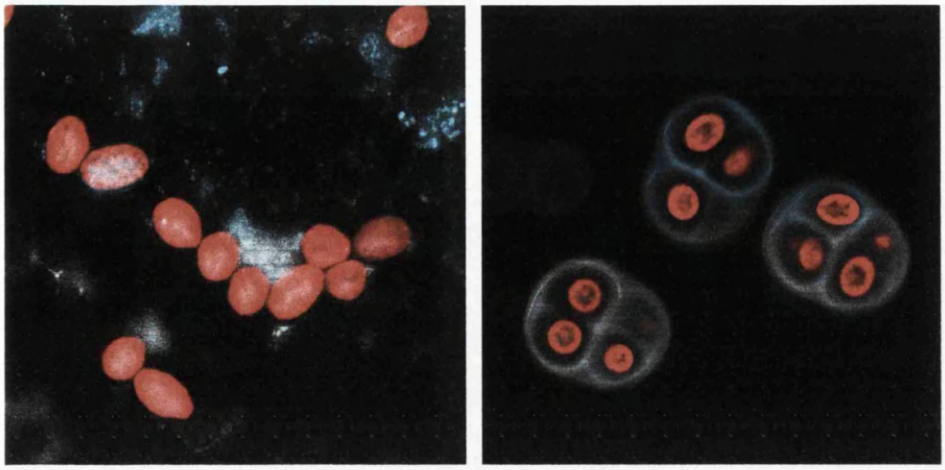


FIGURE 2. 4. BACTERIAL CONTAMINATION DETECTED USING CONFOCAL MICROSCOPE

Axenic cultures and contaminated cultures of Gloeothece; Cells stained with DAPI where bacteria are visualized as fluorescently stained small blue rod-like cells, mostly present in the slime (blue fluorescence) of autofluorescent (red) Gloeothece PCC 6909/1 (left) and the absence of bacteria in PCC 6909 (right).

2.3.4. Investigating the type(s) of AHL molecules extracted from *Gloeothece* cultures.

2.3.4.1. Analysis using Thin Layer Chromatogram (TLC)

Results obtained from TLC separation showing the type(s) of AHL present in extracts of both wild type and sheathless mutant cultures are presented in Fig. 2.5. The figure show a good TLC separation of various standards of AHL and *Gloeothece* extracts of both wild type and mutant. Standards of different AHLs, showed a separation with C12-HSL stationary at the origin and C6-HSL furthest from the base. Extracts of *Gloeothece* (wild type, lane 1 and mutant, lane 2) also showed migration of an active bioreporter responsive spot with an R_f value consistent with C8-HSL, indicating that both the wild

type and the mutant strain produces similar bioreporter active compound. The absence of a tailing reaction in both of the extracts (a characteristic of oxo-HSL) indicated the potential AHL lacks an oxo-group in its carbon atom and therefore, could potentially be identified as C8-HSL. The detection of only one spot from both cultures indicated the presence of only one type of AHL. From these results it is not possible to tell if C8-HSL is the sole AHL present in the sample since other AHLs present may not be within the detection limits of the bioreporter. However, due to other compounds that may have the same R_f value as C8-HSL, which could possibly activate a bioreporter response, further investigation into the identification of the AHL was necessary.

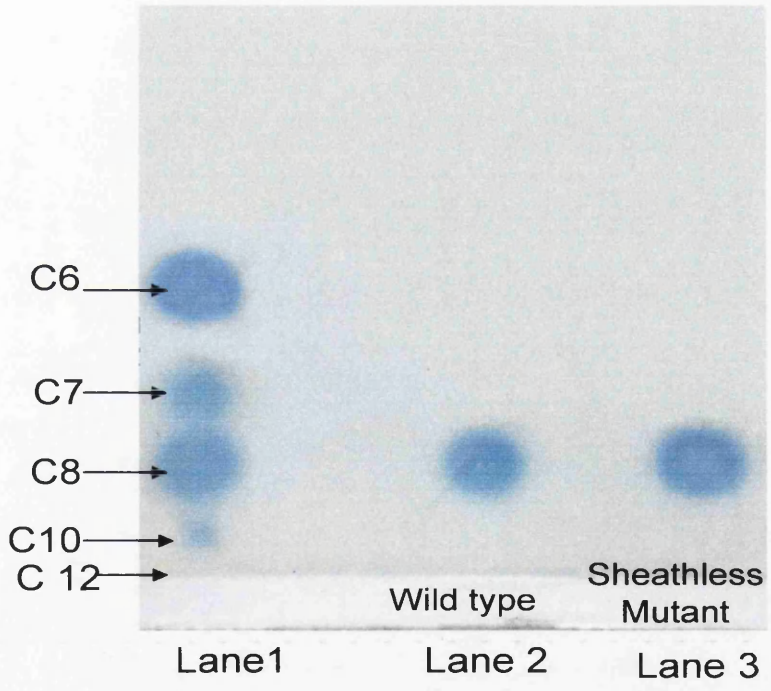


FIGURE 2. 5. TLC SEPARATION OF DIFFERENT AHL STANDARDS AND *GLOEOTHECE* EXTRACTS.

Reverse phase C18 TLC plate separation of different AHL standards (lane1) and extracts from wildtype PCC 6909 (lane 2) and sheathless mutant PCC 6909/1 (lane 3). Individual AHL detected using an overlay of bioreporter NTL4 (pZLR4).

2.3.4.2. Identification of AHL using HPLC-MS and MS/MS

In order to identify and further confirm the presence of C8-HSL in *Gloeothece* samples both samples extracted from cultures of PCC 6909 and PCC 6909/1 and standards were run on a HPLC column and analysed through MS. Fig. 2.6 shows the total ion chromatogram (XIC) of a test mix of seven AHLs, separated through HPLC with the position of C8-HSL indicated (upper trace) followed by the XIC of the *m/z* value for protonated C8-HSL (228) from the sample (middle trace) and the peak for the more sensitive single reaction monitoring study of C8-HSL in the sample (lower trace). The SRM shows a clear signal to be obtained by decreasing the background noise and thus increasing both specificity and sensitivity. On the basis of the retention time of the protonated molecular ion (*m/z* 228, Fig. 2.6), a full scan mass spectrum (Fig. 2.7) recorded at 18 min (the time at which authentic C8-AHL is eluted, Fig 3.6), and MS/MS analysis of the molecular ion (*m/z* 228, Fig. 2.8), of extracts from both strains of *Gloeothece* confirmed the presence of C8-AHL as the sole AHL in both the wild type and sheathless mutant. In each analysis, the data generated from the sample agrees well with the data obtained from a sample of authentic C8-HSL (Fig. 2.8). Furthermore, comparison of the product ions generated from HPLC-MS/MS analysis of the *Gloeothece* extract (Fig. 2.8) with the fragmentation pathway derived from examination of the MS/MS analysis of authentic C8-HSL (Fig. 2.9) indicated that no unexpected product ions were generated by the HPLC-MS/MS analysis of the extract.

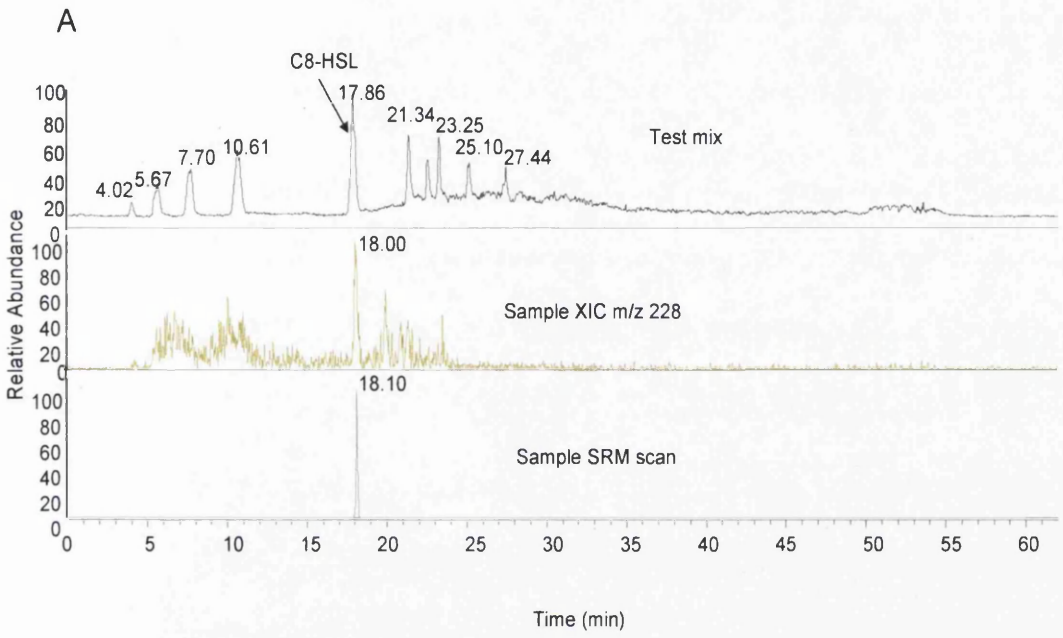


FIGURE 2. 6. HPLC-MS ANALYSIS OF AHL

Test mixture of HSLs (upper trace), extracted ion chromatogram for m/z 228 (C8-HSL) (middle trace) and more sensitive mass spectrometric identification of C8-HSL by single reaction monitoring (m/z 228 to m/z 102) (lower trace) (m/z=mass to charge ratio).

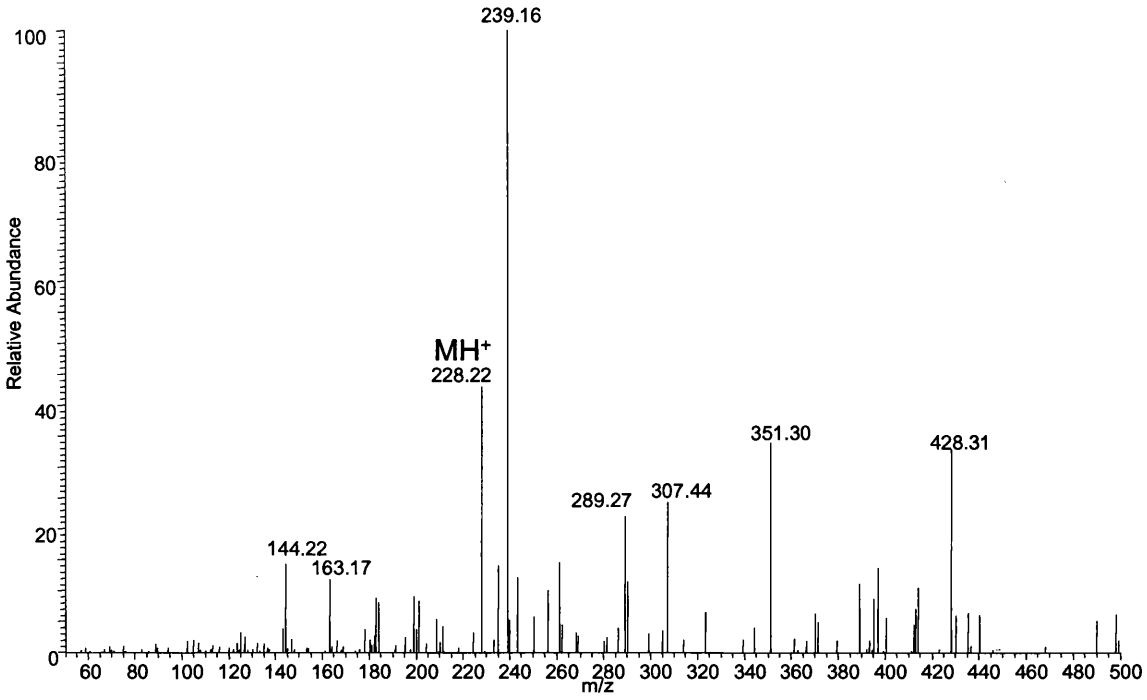


FIGURE 2. 7. FULL SCAN MASS SPECTRUM OF THE HPLC RUN AT 18 MINUTES

The spectrum shows the presence of the protonated C8-HSL (m/z 228) from extracted samples of Gloeothece (m/z=mass to charge ratio).

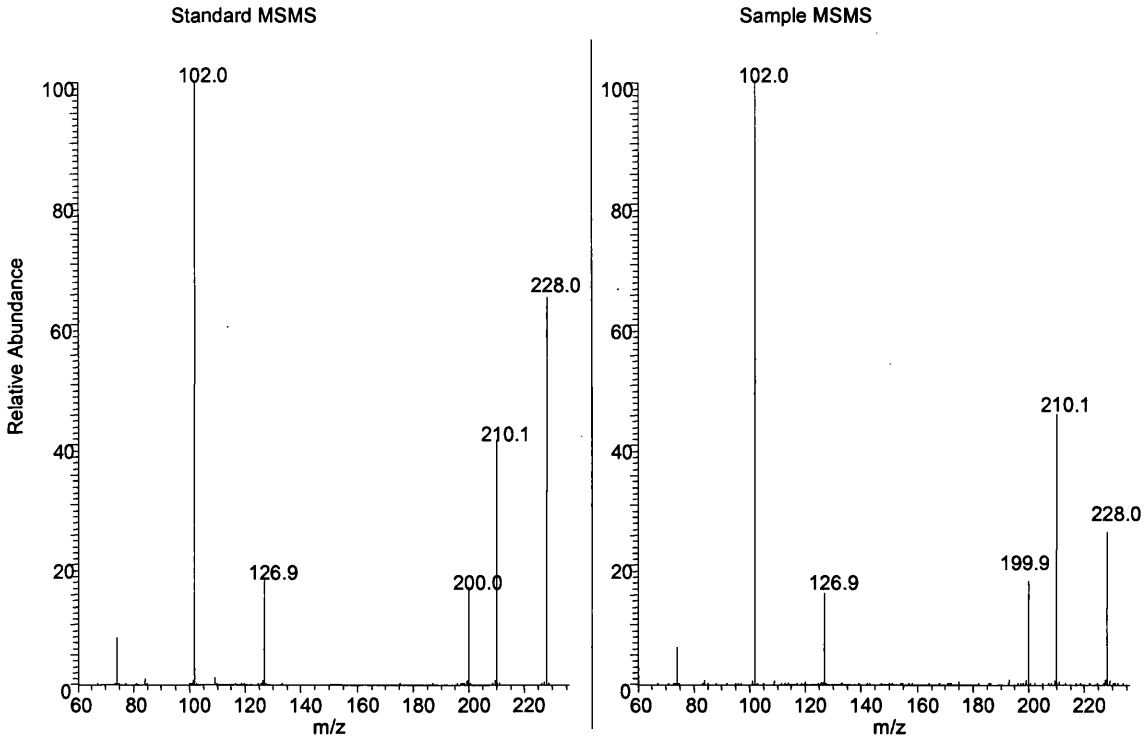


FIGURE 2. 8. COMPARATIVE MS/MS ANALYSIS OF THE COMMERCIAL STANDARD C8-HSL AND THE EXTRACTED PUTATIVE C8-HSL

(m/z=mass to charge ratio)

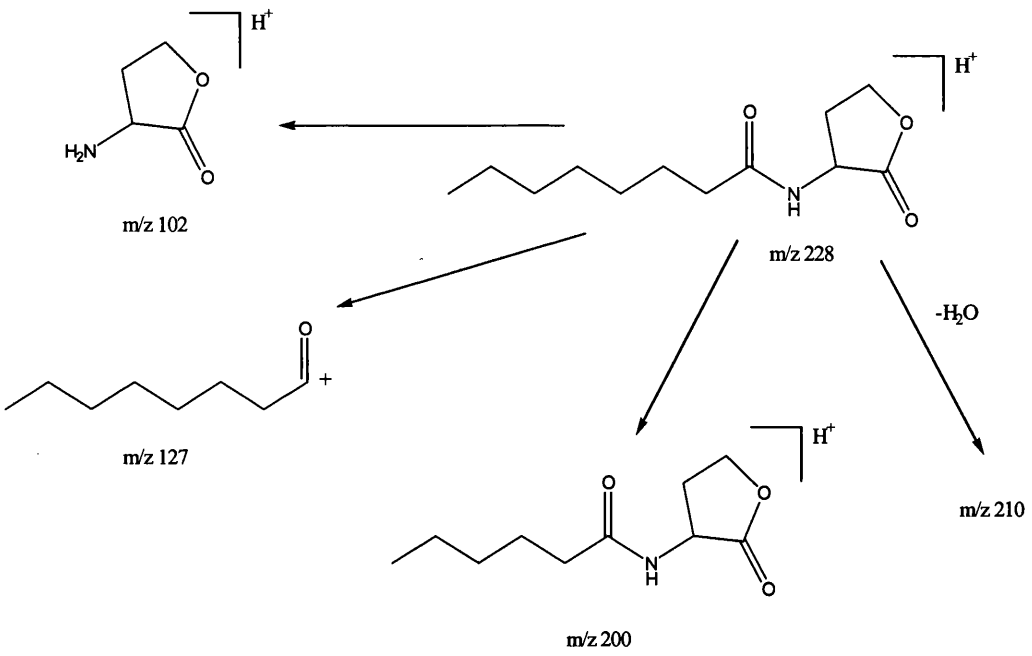


FIGURE 2. 9. FRAGMENTATION PATHWAY OF C8-HSL IDENTIFYING THE FRAGMENT IONS DETECTED BY HPLC-MS/MS ANALYSIS

(m/z=mass to charge ratio)

2.5. Discussion

Using bioreporter NTL4 (pZLR4), a bioreporter active compound was detected in culture extracts of *Gloeothece.* Further detection and identification of the active compound by TLC and HPLC-MS later confirmed the presence of C8-HSL in extracts of both PCC 6909 and PCC 6909/1 cultures of *Gloeothece.* These findings therefore, provide the first evidence of an AHL detected from axenic cultures of a cyanobacterium.

Cyanobacterial cultures can become contaminated during handling or during prolonged growth in culture vessels that may allow moisture to build up on the rim of the vessel which in contact with exposed to air can introduce contamination. Cultures were routinely checked for bacterial contamination. The agar plate technique can be used to test for bacterial contamination within a small volume of culture. It may also provide useful information on the nature of contamination and become useful in identifying different bacterial contamination in the cultures. However, an absence of growth in agar plates may not always be sufficient to confirm that the culture is axenic since, bacteria that are fastidious and require specific growth requirements (provided by its associated cyanobacteria), may not grow in these agar plates. In these situations laser scaning confocal microscopy (LCMS) can be useful in detecting low levels of contamination directly from cyanobacterial cultures. Only in a few occasions contamination was detected from *Gloeothece* cultures, especially when cultures were maintained in large flasks of 5L or more. When maintained in 100 ml flasks over several generations, only in some cases cultures became contaminated. With the use of agar plates, contamination

was detected within 24 h in both BG11 medium supplemented with casamino acids (0.2 %) and nutrient agar.

Since AHL are known to be excreted in to the extracellular environment and accumulate over time as cell density increases, *Gloeothece* spent culture supernatants were extracted with organic solvents in order to detect the presence of AHL molecules. Due to the characteristic of having a lipid side chain, AHL molecules could be easily extracted using organic solvents such as chloroform, ethyl acetate, dichloromethane. Acidified ethyl acetate and dichloromethane have been a widely used solvent for extracting AHL molecules from different bacterial species. In this study the C8-HSL was detected and found to be efficiently extracted using dichloromethane with good reproducibility. A good extraction yield for C8-HSL has also been supported by other studies (Morin *et al.*, 2003). The volumes of extraction (100 ml) were carefully chosen in order to extract AHL with ease and also that AHL extracted would fall within the limit of bioreporter detection (2 pmol-1 nmol for various AHLs). This could allow detection of different AHL in the range of 20 pM to 10 nM, concentrations that are reported to be detected within most bacterial AHL concentrations. Hence, even if AHL production by *Gloeothece* is lower than in other organisms, it was possible to detect AHL using the bioreporter. Results in the detection of AHL from *Gloeothece* extracts indicated a bioreporter active compound, which in the case of AHL could be present in a range of 2 pmol to 1 nmol of different AHL; a range by which the bioreporter responds to different AHL. It is generally known that bacteria can produce one or several types of AHL. However, since, the bioreporter can respond to many diferrent AHLs it does not allow a differentiation of whether there is one, or more than one, type of AHL present in the sample nor does it give any information on the type(s) of AHL

present. Further analysis of this bioreporter active compound indicative of AHL was undertaken in order to determine type(s) of AHL in *Gloeothece*. Identification of individual AHL was done by TLC combined with the bioreporter detection method. TLC allows the separation and detection of individual AHL(s), which, when compared to different standards (also run with the samples) can give a tentative identification of the type(s) of AHL present. On C18 reverse phase TLC plates, long chain AHL migrate less far up the TLC plate compared to short chain AHL due to increased hydrophobic interactions and hence, individual AHL's are separated depending on their hydrophobicity. Individual AHL separated on TLC plates can be detected after a response of the bioreporter overlayed on top of the TLC plate. A further confirmation of the identification of AHL(s) samples were subjected to HPLC-MS separation and tandem mass-spectrometric analysis. In preliminary detection of AHL, a negative control (without Bioreporter) was used to test whether any factor in *Gloeothece* extract could produce a false positive result by cleavage of X-gal. These results were found to be negative and were further supported from the identification of C8-HSL using HPLC-MS that showed that the positive bioreporter results were due to AHL present in the extract of *Gloeothece*. However, some samples collected from cultures within 7-14 days of growth did not show any bioreporter response. This could be a possibility that AHL are present and simply not detected due to lower concentrations or due to the sensitivity of the bioreporter and further investigation may be required using other techniques such as MS to detect their presence.

The pH of the growth medium of *Gloeothece* with an initial pH of 7.2 was found to rise to pH 8.0 within 2-4 weeks of growth and reach about pH 8.4-8.5 during later stages (90 days) of growth. Results indicated that the culture pH at later stages may not

significantly affect the concentration of AHL accumulated. If so, the precise conditions that make AHL more stable in *Gloeothece* cultures at these pH may need further investigation. Finally to summarise the questions placed at the beginning of this chapter,

- AHL is detected from axenic cultures of both *Gloeothece* PCC 6909 and PCC 6909/1 when grown under laboratory batch cultivation and conditions.
- AHL is identified as C8-HSL through both TLC separation and HPLC-MS detection.

The detection of AHL in *Gloeothece* is discussed within a wider context in chapter 6

CHAPTER 3

C8-HSL accumulation during growth in cultures of *Gloeothece* PCC6909/1

3.1 Introduction

Quorum sensing is known to be a density dependent mechanism where signals are released by the cells in proportion to cell density and hence used by the cells as a factor to determine population density (Waters and Bassler, 2005). The release of AHL by cells in a closed system, such as laboratory culture vessels may allow AHL to accumulate in the media. Experiments have been conducted using axenic cultures grown in such vessels under laboratory conditions to provide useful information on the amount of AHL that accumulates over time and also on AHL accumulation kinetics of the organism under study. A critical threshold concentration of AHL usually determines the quorum and initiates QS dependent gene expression that can also activate an auto amplification loop resulting in increased production of these molecules. The feedback type response during growth in batch cultures can be evident as increased production of autoinducer at a rate greater than the rate of growth (Ravn *et al.*, 2001). In most cases, quorum sensing activated of gene expression occurs as the cultures approach the stationary phase of growth, indicating that bacteria can use this mechanism to sense stress associated with high cell density (Swift *et al.*, 1993; van Delden *et al.*, 2001). A range of AHL concentrations has been reported to be produced by different bacterial cultures, under laboratory conditions ranging from nM to μM concentration (Burton *et al.*, 2005; Kaplan and Greenberg, 1985). Most bacteria are known to have more than

one type of AHL systems and individual types of AHL may accumulate over growth in different patterns from each other and also the concentration of individual signals may vary from one another and their production may occur at different stages of growth (Llamas *et al.*, 2005). Such an observation may represent early or late systems acting in a concerted or hierarchical fashion to carry out quorum sensing gene expression (Lupp and Ruby, 2005).

Aims and Objectives:

In the previous chapter C8-HSL was detected and identified from axenic cultures of *Gloeothece*. However, the concentration at which C8-HSL accumulates in cultures during growth of *Gloeothece* is not known. Also other types of AHL(s) may also be produced during growth that may not have been detected in previous results due to the sensitivity of the bioreporter. Therefore, this chapter is aimed to test the hypothesis that C8-HSL is a major quorum sensing molecule in *Gloeothece*, through answering the following questions.

- Does C8-HSL accumulate in cultures associated with particular stages of growth?
- Does C8-HSL accumulation show a pattern of autoinduction?
- Does C8-HSL represent the major type of AHL in *Gloeothece*?
- Does C8-HSL accumulate at a low concentration as reported by other bacterial AHL systems?

The main objectives are:

- To test a suitable and sensitive technique to measure AHL concentrations. Bioreporter and mass spectrometry (MS) analysis will be compared for quantification of C8-HSL concentrations accumulated over growth of *Gloeothece*. Suitability of both methods to C8-HSL will be determined through standard calibration curve preparation using different concentrations of C8-HSL and analysing for both sensitivity and selectivity.

- To detect the presence of other types AHL over growth of *Gloeothece* using MS.

3.2. Materials and Methods

3.2.1. Materials:

Solvents and chemicals were and purchased according to stated in section 2.2., Chapter 2.

3.2.2. Methods:

3.2.2.1. Bioreporter and High Performance Liquid Chromatography-Single Reaction Monitoring (HPLC-SRM) quantification method

In order to test the suitability and sensitivity of the bioreporter and MS for quantification of C8-HSL concentrations, a standard calibration curve was prepared using different concentrations of C8-HSL and compared for both sensitivity and selectivity.

3.2.2.1.1. Standard curve preparation using bioreporter NTL4 (pZLR4)

Bioreporter assay was performed according to section 2.2.2.3 (Chapter 2). C8-HSL concentrations between 2-50 pmol were prepared according to applied concentrations on bioassay plates. Applied concentrations were prepared from C8-HSL working concentrations. Two working concentrations were prepared. First working concentrations were prepared by adding 5 µl stock (1 nmol μl^{-1}) to 5 ml acetonitrile to give a final working concentration of 1 pmol μl^{-1}, which was applied for quantification in the range of 2-10 pmol. A second working concentration was prepared by adding 5

µl of stock (1 nmol μl^{-1}) solution to 1 ml of acetonitrile to give a final working concentration of 5 pmol μl^{-1}, which was used for quantification using the bioreporter assay in the range of 20-50 pmol. After application of samples the bioassay plates were placed in an incubator for 16 h at 28 °C, at the end of which time the response of the bioreporter to different C8-HSL concentrations was measured. Measurements were taken from the border of the disc to the border of the blue coloured zone and the values were used to calculate the area of bioreporter response for each concentration of C8-HSL. For standard curves the area of the bioreporter response was plotted against C8-HSL concentrations.

3.2.2.1.2. Standard curve preparation using HPLC-MS

SRM analysis was utilised for the analysis in which a time period was set for each AHL under investigation and during each such window the fragmentation transition from protonated molecule to the predominant fragment ion (*m/z* 102) was monitored in order to quantitate the AHLs. The HPLC separation and MS detection parameters were set as described in the previous chapter with the difference that a longer HPLC column was utilised (20 cm) resulting in increased retention times and improved resolution between the AHLs under investigation. For comparative analysis with the bioreporter a dilution series of a stock solution of C8-HSL (between 2.5 pmol and 50 fmol per injection) was applied to the detection methodology. The sensitivity of the method was determined by demonstrating the linearity of the mass spectrometric response versus analyte concentration over the defined range and the limits of detection of the HPLC-single reaction monitoring-MS/MS assay.

3.2.2.2 Determination of C8-HSL concentration during growth of *Gloeothece* PCC 6909/1

3.2.2.2.1. Build up of inoculums

To prepare cultures for experiments a dense culture was achieved through repeated subculturing into larger volumes of media and finally in 200-750 ml cultures in either 1 L or 2 L conical flasks (unless otherwise stated). The resultant cultures were grown and maintained in an orbital shaker for 1 month under conditions stated in section 2.2.2.1. Cultures were subcultured from a single flask, so that the final culture used in the experiments would represent a single homogenous culture of *Gloeothece*.

3.2.2.2.2. Culture preparation

For experiments that involved measurement of growth and C8-HSL concentration, a culture of PCC 6909/1 was grown according to section 3.2.2.2.1 in to 750 ml of BG11^{o} medium in a 2 L conical flask. The culture was grown for a further two months under appropriate conditions. Cells were then harvested under sterile conditions and the resultant pellet was resuspended, aseptically, in 200 ml sterile fresh BG11^{o} medium to use as an inoculum. Ten ml of this inoculum was then further used to inoculate 20 flasks (250ml), each containing 80 ml of fresh sterile BG11^{0} medium and grown maintained for a further 90 days. Two flasks were sacrificed and used for each growth point measurements and measurement of C8-HSL concentration.

3.2.2.2.3. Measurement of growth

Measurement through turbidity:

Growth was monitored by measuring changes in cyanobacterial concentrations through measurements of turbidity. Culture samples were pipetted into plastic cuvettes and gently agitated to keep cells in suspension. Turbidity was then measured at 436 nm against blank distilled water. 436 nm was found to be the maximum peak of absorbance of *Gloeothece* cultures monitored over the entire visible range. Measurements were plotted against sample collection days in order to obtain a growth curve of *Gloeothece* PCC 6909/1

Measurement through protein concentration:

The protein concentration during growth of *Gloeothece* PCC 6909/1 was measured through the Folin/phenol method as described by Lowry *et al.* (1951) (Lowry *et al.*, 1951). Before protein estimation cells from 100 ml of culture were pelleted by centrifugation at 10,000 g for 10 min at 4 °C and the supernatant discarded. The cell pellet was then dissolved in 3 ml of 1M NaOH and boiled for 30 min and then cooled. The protein extract was then re-centrifuged to eliminate light scattering material. 200 µl of protein extract was used for protein estimation according to the following procedure and the concentration of protein in the total extract was determined from a standard curve prepared against known concentrations of bovine serum albumin (BSA). The final concentration of protein in 3 ml of total extract was represented as the amount of protein in mg obtained from 100 ml of culture.

Folin/ Phenol method (Lowry method) of protein determination (Lowry *et al.*, 1951):

Solution A and solution B consisted respectively, of 2% (w/v) Na_2CO_3 in 0.1M NaOH and 0.5% (w/v) $CuSO_4$ with 1% (w/v) trisodium acetate ($Na_3C_6H_6O_7.2H_2O$). These were mixed in a 50:1 ratio to make solution C. The protein sample was made up to 1 ml through the addition of distilled H_2O and 5 ml of solution C was then added. After incubation at room temperature for 10 min, 250 µl of Folin-Ciocalteu's reagent was added. The solution was vortexed and left to stand at room temperature for 30 min. Absorbance was measured at 500 nm, using a Shimadzu UV-1601 Spectophotometer, against blank lacking protein. Standard curves were produced using stock (1 mg ml^{-1}) bovine serum albumin (Fig.I. shows a typical standard curve). The concentration of protein in the sample was determined upon analysis of the standard curves.

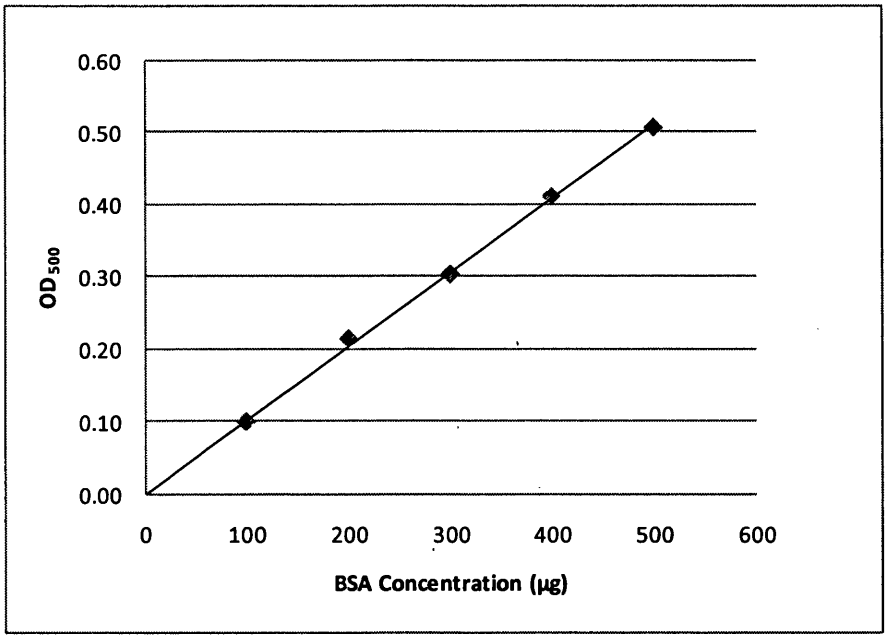


Fig. I. Typical calibration curve for the measurement of protein concentration by the Folin/phenol method (as described by Lowry *et al.* 1951) OD_{500}= Optical density at 500 nm

3.2.2.2.4. Determination of AHL concentration

For the determination of AHL concentration over growth of *Gloeothece* PCC 6909/1, cultures taken during individual growth points prepared in section 3.2.2.2.2. were harvested by centrifugation at 10,000 g and the cell free supernatants were collected and extracted according to the method described in Chapter 2 section 2.2.2.2. Extracted samples were stored at -20°C until all samples were collected during growth and ready to determine AHL concentration using HPLC-MS.

For quantitation of C8-HSL, an HPLC single reaction monitoring-MS/MS method was used. The transition from protonated C8-HSL (m/z 228) to the product ion at m/z 102 (±0.5 Da) was monitored. Quantitation of C8-HSLs took advantage of the proven linearity and reproducibility of the mass spectrometric response of C8-AHL described in section 3.2.2.1.2. A standard addition method was employed for C8-HSL quantification from samples by the following method. The experimental sample was divided into two equal fractions. To one of these, 5 ml of mobile phase was added, whereas 5 ml mobile phase containing 1 pmol of C8-HSL was added to the other. The amount of C8-HSL naturally occurring in the sample was calculated, as the increase in signal produced by the addition of C8-HSL to the experimental sample is equivalent to the response produced by 1 pmol of C8-HSL. The response of the sample to which no AHL had been added was then compared to this increased signal and expressed as a fraction. This allowed potential difficulties relating to altered sensitivity of the mass spectrometric system over time to be overcome.

3.2.2.3. Detection of other AHL compounds during growth

Samples prepared and extracted from *Gloeothece* PCC 6909/1 according to section 3.2.2.2. were used in detection of potential presence of other AHL using MS.

3.2.2.1.1. HPLC retention times for different standards

The same HPLC separation was used as in Chapter 2, but a longer HPLC column was used to gain better resolution. For each AHL the transition from protonated molecule to the fragment ion m/z 102 was monitored within a specific retention time window. The MS conditions were as Chapter 2.

3.2.2.1.2. Analysis of *Gloeothece* PCC 6909/1 samples

For analysis of the presence of other AHLs, samples collected during growth in section 3.2.2.2.4 were run on HPLC-MS and analysed as described in Chapter 2 Section 2.2.2.6.3.

3.3. Results

3.3.1. Comparison of the suitability of HPLC-Single Reaction Monitoring (HPLC-SRM-MS/MS) method and the bioreporter method in quantification of C8-HSL

3.3.1.1 Standard curve from (HPLC-SRM-MS/MS)

SRM analysis using HPLC-MS allowed quantification of C8-HSL through increasing the sensitivity of the method removing background noise, in this case the product ion at *m/z* 102 (± 0.5 Da) that resulted from the transition of protonated C8-HSL (*m/z* 228) was monitored. Results in Fig 3.1 show a standard curve prepared from the peak area generated by the product ion at *m/z* 102 (± 0.5 Da) at different C8-HSL concentrations (0.05-2.5 pmol). The sensitivity of the method was determined by analysing low concentrations of C8-HSL between 2.5 pmol and 50 fmol injection^{-1}. Results demonstrate that the mass spectrometric response is sensitive and is linear over the range of 50 fmol to 2.5 pmol with an R^2 value of 0.9913 and low standard error values (Fig. 3.1). Since, The SRM scan allows selective quantification of C8-HSL, it suggests that it is a suitable method to both detect and specifically quantitate C8-HSL concentrations from biological extracts that may have other types of AHL's.

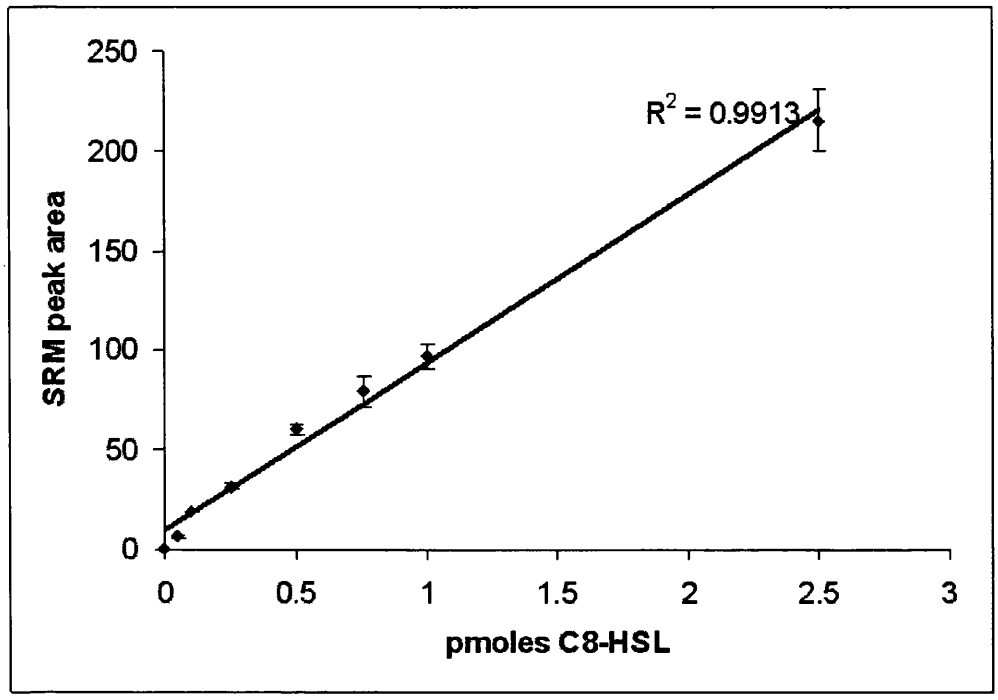


FIGURE **3. 1** STANDARD CURVE FOR MEASUREMENTS OF C8-HSL CONCENTRATION USING HPLC-MS-SRM

The standard curve prepared from the peaks are generated by the SRM scan of m/z 102 (± 0.5 Da), a product generated by the transition of protonated C8-HSL (m/z 228) at different concentrations (0.05-2.5 pmol) of C8-HSL.

3.3.1.2. Standard curve of AHL activity prepared using bioreporters

In the bioreporter method of quantification a standard curve was prepared using 2 -50 pmol of C8-HSL (Fig.3.2). The results indicated, that the linear response of the bioreporter NTL4 (pZLR4) is within the range of 2-30 pmol (Fig. 3.2). A similar response was obtained using another bioreporter *A. tumefaciens* NTL4 (pCF 218, pCF 317) (Fig. 3.5). The response of the bioreporter NTL4 (pZLR4) to different C8-HSL

concentrations (Fig. 3.3) as measured from the border of the disk to the border of the blue colour zone, showed a significant difference. However, the differences between triplicate samples for each of the concentrations showed negligible increase in diameter and therefore, an error value could not be calculated. Moreover, both bioreporters were not capable of detecting C8-HSL at levels less than 1 pmol. Results therefore suggest that the bioreporter is not as sensitive as SRM in quantification of C8-HSL and SRM is a good method when both selectivity and sensitivity is desired to identify and quantify C8-HSL cultures of *Gloeothece*.

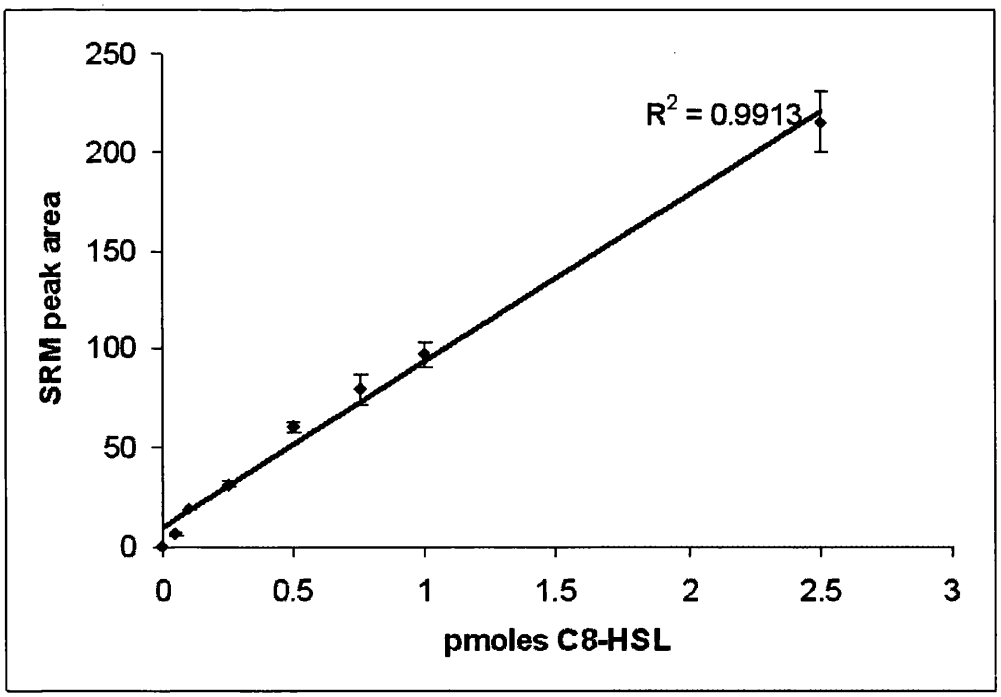


FIGURE **3. 2** . A STANDARD CURVE OF C8-HSL BIOREPORTER NTL4 (PZLR4)

The standard curve prepared from the response of the bioreporter to different concentrations of C8-HSL and plotted using the area produced by the bioreporter response against different concentrations of C8-HSL.

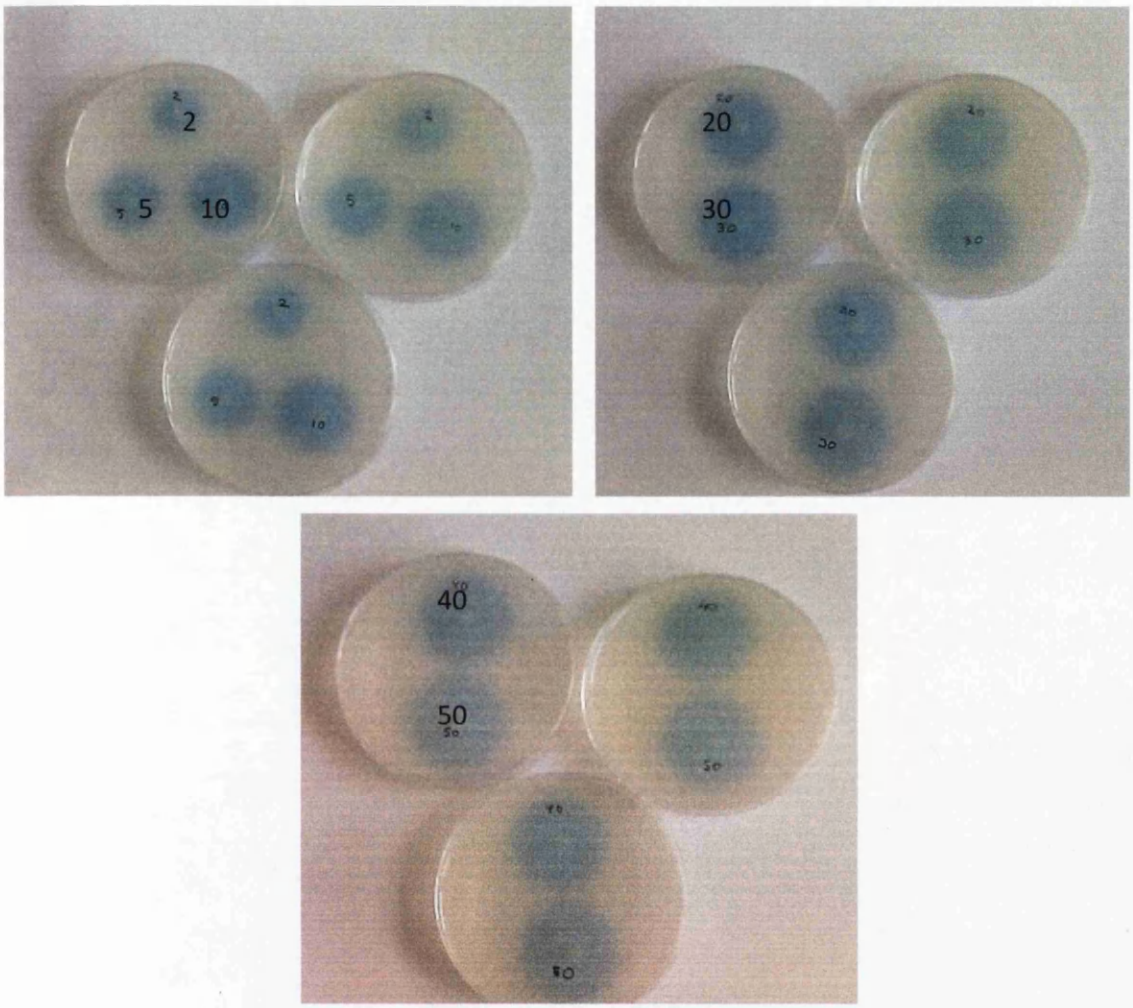


FIGURE **3. 3**. BIOREPORTER RESPONSE TO DIFFERENT CONCENTRATIONS OF C8-HSL

Bioreporter NTL4 (pZLR4) is used in measurements of standard area produced by bioreporter response at different concentrations of C8-HSL. Triplicate samples of each C8-HSL concentrations used, 2, 5, 10 pmol (top left), 30, 40 pmol (top right) and 40, 50 pmol (bottom), showing a bioreporter response (blue colour area) to different concentrations of C8-HSL.

Pmol of C8-HSL	Distance from the border of the disc to the border of the blue colour zone (mm)	Area of the blue colour Zone (mm^2)
2	6.5	132
8	9	254
10	9.5	283
20	12	452
30	14	615
40	15	706
50	16	803

TABLE 3. 1. AREA PRODUCED BY BIOREPORTER NTL4 (PZLR4) IN RESPONSE TO DIFFERENT CONCENTRATIONS OF C8-HSL.

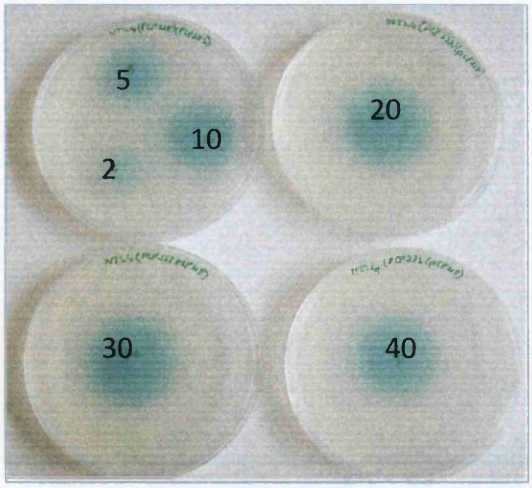


FIGURE **3. 4**. BIOREPORTER NTL4(PCF 218, PCF 317) RESPONSE TO DIFFERENT C8-HSL CONCENTRATIONS

Response of bioreporter, NTL4 (pCF218, pCF317) to different C8-HSL concentrations, 2, 5, 10 pmol (top left), 20 pmol (top right), 30 pmol (bottom left) and 40 pmol (bottom left) measured by the area of the blue colour zone.

3.3.2. C8-HSL accumulation during growth of *Gloeothece*

3.3.2.1. Measurement of cell biomass

Cell biomass of *Gloeothece* PCC 6909/1 was measured through increase in both turbidity and protein concentration. Figure 3.6 shows the results of turbidity measurements and protein concentrations plotted over 80 days of growth. Measurements of the cell density by both techniques show a good correlation (Fig. 3.6). Results show that cell density of *Gloeothece* increased in a linear fashion over 80 days. The trend in increase in both turbidity and protein concentration indicated that *Gloeothece* cultures at the end of 80 days may still show further growth.

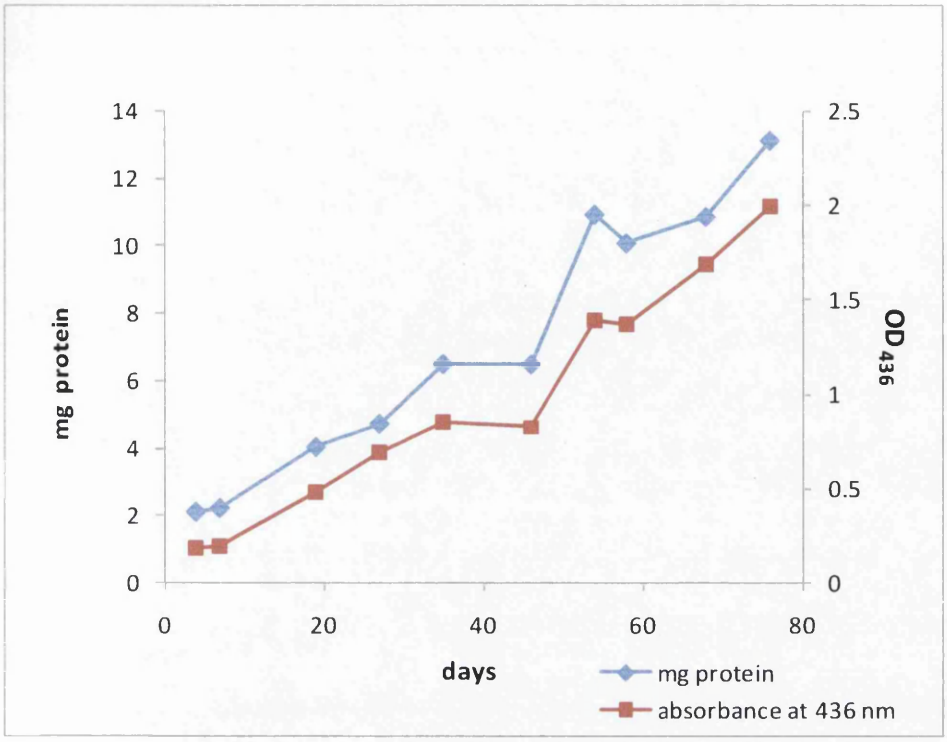


FIGURE **3. 5.**GROWTH PATTERN OF *GLOEOTHECE* PCC 6909/1

Growth of Gloeothece PCC 6909/1 plotted as increase in absorbance at 436 nm and an increase in protein concentration over 80 days. Protein concentration is expressed in mg of protein obtained from 100 ml of culture. OD_{436}*=Absorbance at 436 nm*

3.3.2.2. Quantification of C8-HSL over growth of *Gloeothece* PCC 6909/1

Samples of *Gloeothece* PCC 6909/1 were taken during individual growth points and C8-HSL was selectively quantified using HPLC SRM MS/MS. C8-HSL concentrations were plotted against turbidity (OD_{436nm}) measurements in order to determine the accumulation pattern of C8-HSL in relation to growth. Results show that C8-HSL accumulates in cultures during growth of *Gloeothece* PCC 6909/1 and increases as the density of the culture increases (Fig 3.6.a). A concentration of 3500 pM of C8-HSL was detected from the last sample collected during growth, representing the maximum

concentration of C8-HSL obtained during this study. Such concentration in *Gloeothece* PCC 6909/1 agrees well with AHL concentrations produced by other bacteria. Results for both growth and C8-HSL accumulation also indicate the possibility of further increases in C8-HSL concentration under conditions where the culture would be allowed grow for extended periods (over 80 days).

Results for C8-HSL accumulation in cultures also showed a rapid increase in concentration after 55 days which was found to occur just after a short (7 days) stationary phase of no increase in growth (Fig 3.6.a). The sudden increase in C8-HSL concentration coincides with the feedback type response resulting in increased production of AHL detected in many other bacterial systems. In order to investigate whether C8-HSL levels are induced at this point, the amount of C8-HSL was plotted against per mg of protein (Fig. 3.6.b). The data shows that from 55 days onward the accumulation of C8-HSL increases rapidly per mg protein, which is a characteristic of the AHL auto-induction phenomenon evident in many AHL quorum sensing systems. These results therefore suggest that C8-HSL in *Gloeothece*, may function as an autoinducer.

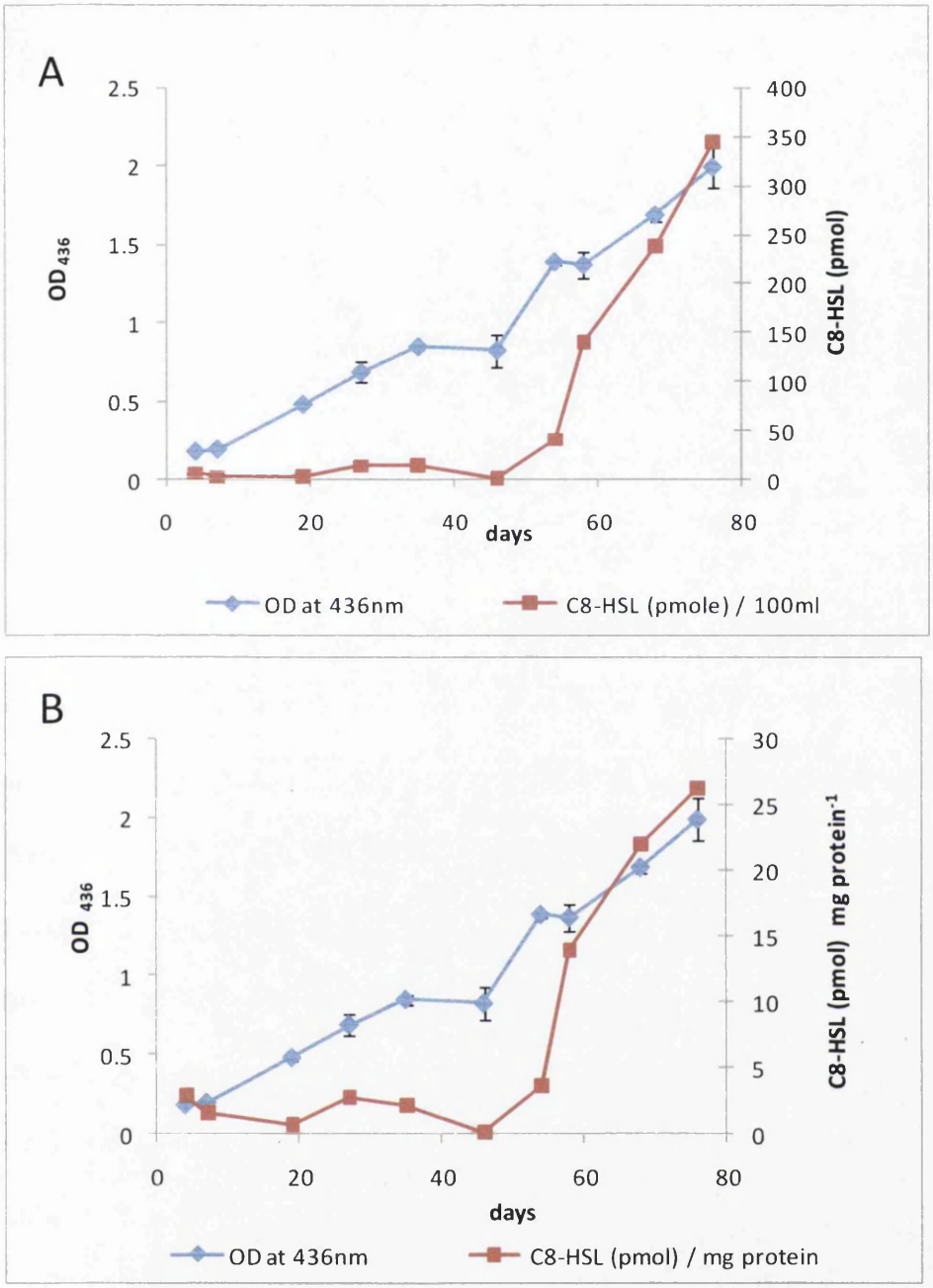


FIGURE **3. 6.** C8-HSL ACCUMULATION DURING GROWTH OF *GLOEOTHECE* PCC 6909/1

(A) *Total C8-HSL accumulation is expressed as pmol of C8-HSL obtained from 100ml of culture during growth of Gloeothece PCC 6909/1.* (B) *C8-HSL concentration expressed as pmol per mg of protein during growth of Gloeothece PCC 6909/1. Biomass is monitored as measurements in turbidity (OD $_{436}$). Bars represent standard deviation from the average of duplicate samples. OD_{436}=Absorbance at 436 nm*

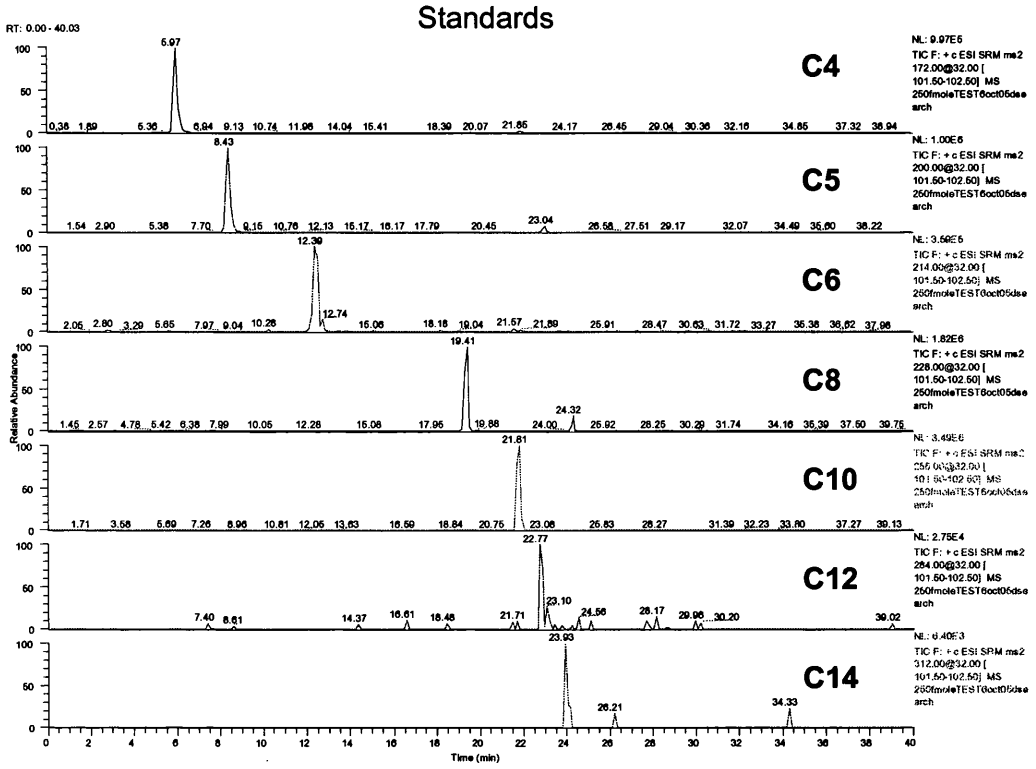
3.3.3. Determination of other AHL produced during growth of *Gloeothece* PCC 6909/1

The previous chapter confirmed the presence of C8-HSL in cultures of *Gloeothece* and C8-HSL was selectively quantified from samples during growth of PCC 6909/1. Samples taken during growth of PCC 6909/1 were also analyzed for the presence of other AHL species using mass-spectrometry. Standards representing authentic AHL's were subjected to HPLC-MS-SRM analysis and their retention times with characteristic fragmentation ions were recorded (Fig.3.7 a). Ten samples from each growth point were subjected to HPLC-MS-SRM analysis. Results in Fig 3.7 (b, c) show a representative SRM report for the first two samples analysed during growth (SRM of other samples are included in appendix III). In all samples C8-HSL was evident. However, some other AHL peaks were also evident, characteristic of C4-HSL and C6-HSL during growth (Table 3.2). Table 3.2 summarises all the different types of AHL's detected during growth from the SRM report. These results indicate that C8-HSL is the major AHL produced during growth of *Gloeothece* PCC 6909/1 and others such as C4-HSL and C6-HSL, which could be produced in comparably low concentrations, can be defined as minor AHL's.

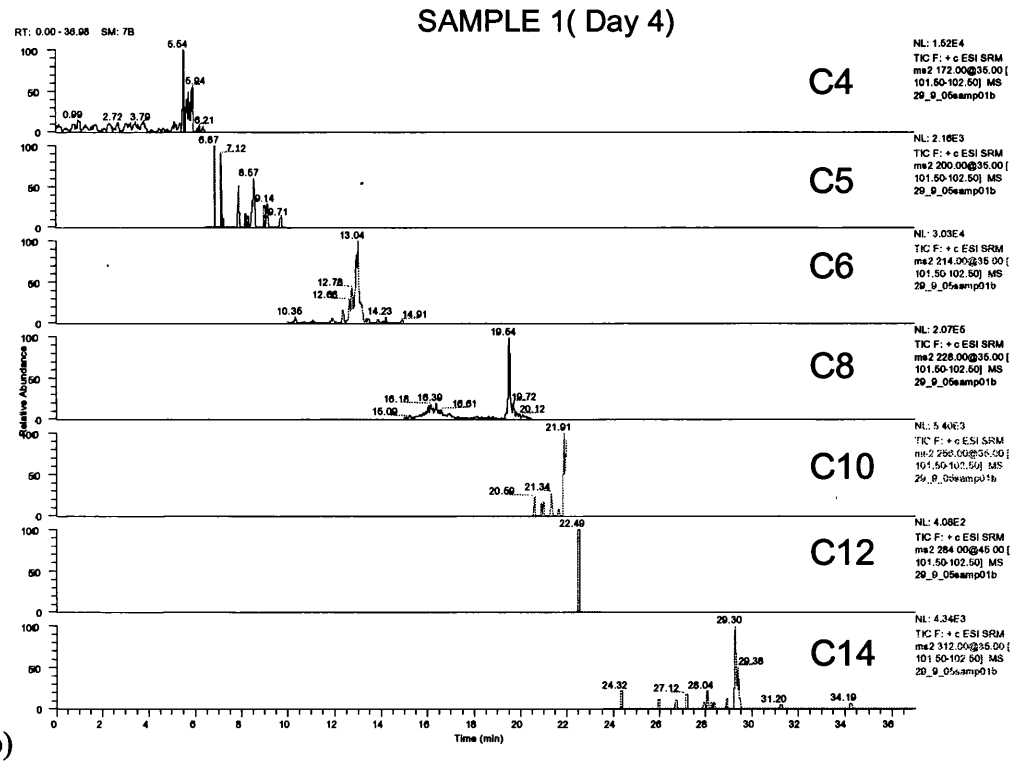
HSL peak	SAMPLE									
	1	2	3	4	5	6	7	8	9	10
C4	X	X	O	x	x	X	X	x	x	O
C5	X	x	x	x	x	x	x	x	x	x
C6	O	O	O	O	O	O	O	O	O	O
C8	O	O	O	O	O	O	O	O	O	O
C10	X	x	x	x	x	x	O	x	x	x
C12	X	x	x	x	x	x	x	x	x	x
C14	X	x	x	x	x	x	x	x	x	x

TABLE 3. 2. SUMMARY OF HPLC-MS-SRM RESULTS ON ANALYSIS OF DIFFERENT TYPES OF AHL DURING GROWTH OF *GLOEOTHECE* PCC 6909/1.

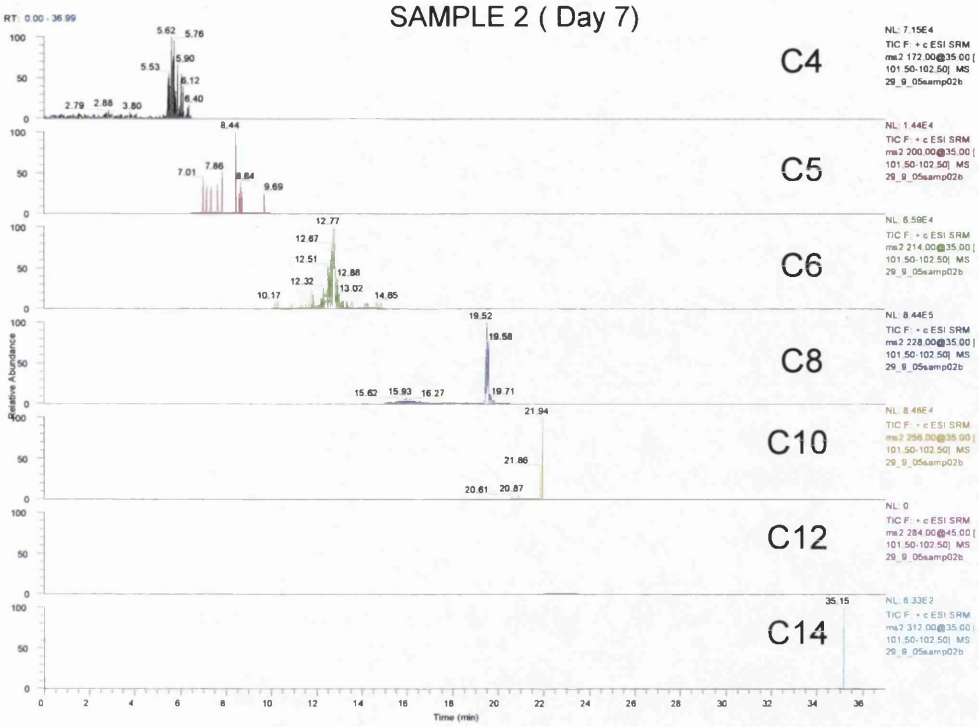
Circles represent the presence and cross represents the absence of different HSL monitored by SRM peak generated by the characteristic HSL, from 10 different samples obtained throughout growth of Gloeothece PCC 6909/1.


Standards
C4
C5
C6
C8
C10
C12
C14
Relative Abundance
Time (min)

(a)


SAMPLE 1(Day 4)
C4
C5
C6
C8
C10
C12
C14
Relative Abundance
Time (min)

(b)



(C)

FIGURE **3. 7**. ANALYSIS OF OTHER TYPES OF AHL PRODUCED DURING GROWTH OF GLOEOTHECE PCC6909/1 USING HPLC-MS-SRM ANALYSIS.

Different AHL produced during growth of Gloeothece PCC 6909/1 analysed through HPLC-MS-SRM. (a) Different AHL standards, C4-HSL, C5-HSL , C6-HSL , C8-HSL , C10-HSL, C12-HSL analysed; (b) Representative samples of Gloeothece PCC 6909/1 analysed for C4-HSL, C5-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL from sample collected at day 4 and (c) and at day 7 respectively.

3.4. Discussion

In order to measure C8-HSL concentrations associated with cell density, representative measurements of *Gloeothece* biomass were required. Since a well established growth curve of PCC6909/1 has not yet been determined, especially over such a long period of 180 days, both culture conditions and biomass measurement techniques were optimized in order to determine growth of PCC 6909/1. When both cultures of *Gloeothece* were studied under conventional batch cultivation in large flasks, it was observed that aerating the flasks in some cases allowed significant evaporation of the culture media, resulting in an increase in culture density. When duplicate cultures were used to measure cell density, results showed variation in between samples. Evaporation could partially be reduced when cultures were bubbled through water saturated chambers. However, the chambers needed to be filled with sterile water often and thus the culture was found to suffer from risk of becoming contaminated through repeated handling. This process was therefore omitted, as it imposed difficulty in handling without the risk of contamination. In order to reduce evaporation and contamination at the same time, cultures were prepared in 250 ml conical flasks with 100 ml of sterile BG11^{o} medium. Cultures maintained under such conditions during longer periods showed little evaporation of growth medium and axenic cultures were also maintained over long periods. For this reason, in the experiment where growth was studied, cultures were prepared in 250 ml conical flasks. However, due to the volume of samples required for C8-HSL determination a series of cultures were prepared that represented cultures to be in the same growth stage and at each sampling points individual cultures were sacrificed, whilst the others were allowed to grow under the same conditions. All flasks needed to be prepared from the same starting inoculum with the same concentration of

cells. Therefore, in each growth experiment an inoculum was prepared from a single population of 50-70 days dense culture (OD_{436}>1.0) which was split in equal volumes to be used as a starter inoculum. This technique was found give better representation of the growth, allowed ease in handling, prevented contamination and also showed less variation between samples.

Gloeothece is a nitrogen-fixing cyanobacteria surrounded by a polysaccharide sheath. Several methods to determine biomass were tested as part of an undergraduate project including, turbidity, protein concentration, chlorophyll-a concentration and phycobiliprotein concentration in their ability to be used as reliable indicators of cell biomass in *Gloeothece* (Emily, 2008), (see Appendix II). Several of these methods have also been also been previously used to quantify cell abundance in phytoplankton. For example, in studies of *Anabaena,* methods such as turbidity (Babu *et al.*, 1998), measurement of dry mass (Moreno *et al.*, 2003) and biovolume (Cromar and Fallowfield, 2003) have been used which have provided reliable methods of biomass for these organisms. For *Gloeothece,* cell biovolume (as determined by microscopic length and width measurements) was calculated and was found to give useful results to compare the other parameters in order to test their reliability as indicators of biomass (Emily, 2008), (see Appendix II). Biovolume is a measurement of the cell abundance and mean cell volume and was performed using laser scanning confocal microscope and image software. When analysing for significant correlation of other parameters with biovolume, the turbidity results for both the wild type and the mutant strains were shown to be highly significant (p<0.001) and therefore, could be used as a highly reliable indicator of biomass for both the wildtype and sheathless mutant (Emily, 2008) (,see Appendix II).

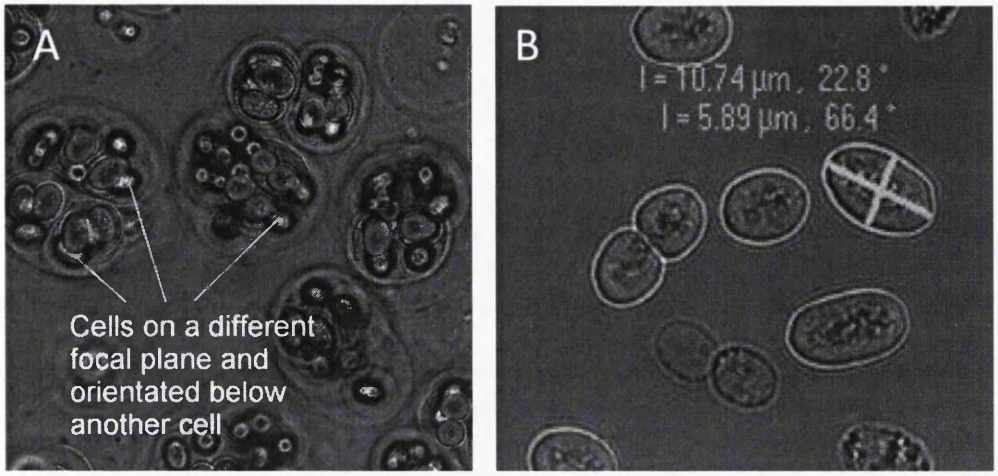


Figure. II. (A) Stacked wild type cells on different focal planes; (B), Image taken using the confocal microscope and LSM Image Examiner showing the length and diameter of the cell, which was then used to calculate cell volume.

However, due to the possibility that a significant amount of C8-HSL could be bound or retained within the sheath of the wildtype and interfere with the concentration and accumulation pattern over growth, the mutant strain was subsequently used in the experiment in determination and C8-HSL accumulation over growth. During the experiment, parameters such as protein concentration and turbidity measurements were used to measure cell biomass in order to provide a higher level of confidence in the growth pattern of *Gloeothece* PCC 6909/1. Growth of *Gloeothece* PCC 6909/1 when carried out over 80 days in shaken flasks under diazotrophic growth conditions showed a linear trend in growth. Linear growth has also been reported by cyanobacteria grown in batch cultures, and is thought to be associated with limitation of a specific nutrient (Foster *et al.*, 2007). Growth was also followed by a short phase where no further

increase in turbidity was observed and occured from day 35 lasting for about 7 days. It could be possible that culture at this stage enter a short stationary phase. Various other reports on growth of *Gloeothece* measured through protein concentration in ASM (ASM-1) media indicate cells enter a stationary phase in 20-30 days (Gallon *et al.*, 1988) , which agrees well with these findings. However, there are no reports on growth carried out for further extended periods (80 days).

C8-HSL concentrations over growth of *Gloeothece* PCC 6909/1 could be measured by both bioreporter and MS. In this study comparison made between the bioreporter and MS techniques showed MS quantification method to be both reliable and sensitive to C8-HSL. The bioreporter quantification method was simpler to perform but proved to be less suitable especially when dealing with unknown concentration of AHL in samples. Several other AHLs detected during growth in *Gloeothece* (Fig 3.8), may require complicated separation process before quantification can be performed using bioreporter. The bioreporter also showd to be less sensitive to C8-HSL concentrations compared to HPLC-SRM. The SRM technique proved to be more sensitive and selective and therefore, was used in quantification of C8-HSL during growth of *Gloeothece* PCC 6909/1 as also proved by other authors (Morin *et al.*, 2003).

The accumulation pattern of C8-HSL over growth suggested that the initial levels of C8-HSL in cultures may be low as 10 pmol but increases as the cell density increases. The highest concentration detected in the nM range in *Gloeothece* PCC 6909/1 was found to fall within the lower range of concentrations produced by other bacterial species (Burton *et al.*, 2005). C8-HSL accumulation in cultures could also be calculated reflecting cell numbers (cell abundance) using equations that relate turbidity to cell

abundance (see Appendix II) and can be useful for comparative studies of C8-HSL production by the cyanobacterium *Gloeothece* to that of other AHL producing bacteria. A C8-HSL autoinduction type phenomenon observed in this study was found to be associated with a positive feedback type response allowing increased accumulation of C8-HSL (Fig 3.6). The positive feedback type response was detected through measurements of C8-HSL concentrations per milligram of protein which was found to increase rapidly after the end of a short stationary phase. This may indicate that cells could undergo gene expression associated with increased levels of C8-HSL production at this stage. The autoinduction type trend in accumulation of C8-HSL observed in *Gloeothece* has been found to show similarity with several other bacterial systems (Ravn *et al.*, 2001; Swift *et al.*, 1993). Autoinduction type mechanisms are known to occur associated with quorum sensing gene expression and therefore, C8-HSL autoinduction type phenomena observed could be used to further support the proposal that C8-HSL is used as a quorum sensing molecule in *Gloeothece*. However, in order to provide evidence that *Gloeothece* PCC 6909/1 activates quorum responsive genes, further investigation may be necessary to test if *Gloeothece* is able to respond to C8-HSL. It is known that quorum sensing can allow stationary phase survival and allow a certain clone of cells to grow further, which may explain the observations detected in *Gloeothece* where further growth was observed after a short stationary phase. This could lead to the possibility that C8-HSL production in *Gloeothece* allows stationary phase survival.

Several bacteria are known to produce more than one type of AHL (Krick *et al.*, 2007). However, in the previous chapter only C8-HSL was detected from samples using the TLC separation. In order to investigate whether other kinds of AHLs are produced

during growth, detection for other AHL were carried out on samples collected at individual growth points. Samples were analysed by HPLC-MS due to the sensitivity in detection of AHL's (Morin *et al.*, 2003). In some samples C4-HSL was detected and C6-HSL was detected in all samples during growth. It could be possible that these AHLs were not detected in previous results (Chapter 2) using TLC separation because of the low sensitivity of the bioreporter to C6-HSL (detection limit >300 pmol) (Cha *et al.*, 1998). Moreover, the bioreporter used is not capable of detecting C4-HSL (Cha *et al.*, 1998). Therefore, it could be possible that C6-HSL is another AHL that is synthesized during growth may suggest it to be another component of a quorum sensing system in *Gloeothece*. In review of the questions asked at the beginning of this chapter the answers can be given as follows:

- C8-HSL accumulates in cultures in relation to growth showing a density dependent accumulation.
- C8-HSL accumulation shows a pattern of a positive feedback type response allowing increased accumulation of C8-HSL after a particular point in relation to growth.
- Other AHLs are detected in cultures of *Gloeothece* during growth.
- C8-HSL accumulates up to concentrations in the nM range and agrees with concentrations reported for other bacterial AHL systems.

CHAPTER 4

Determination of de novo protein synthesis in response to C8-HSL in cultures of *Gloeothece* by the use of a reverse immunoprecipitation technique.

4.1. Introduction

Immunoprecipitation is a technique generally employed to precipitate a soluble protein (antigen) out of solution using a specific antibody to that protein. The protein bound antibody complexes thus formed can be removed from the bulk solution by capture with an antibody-binding protein. The most common antibody binding protein used is protein-A. Protein-A is a 42,000-Dalton polypeptide originally isolated from the cell walls of *Staphylococcus aureus*. In a number of species, the affinity protein-A relies on the specific interaction of protein-A with the Fc region (heavy chain) of antibodies, binding at least 2 molecules of IgG per molecule of Protein-A (Richman *et al.*, 1982). It has a high binding affinity for rabbit IgG. The molecule contains four binding sites, but only two Fc domains can be bound at any one time. Since the antibody combining site is left free, protein-A, when covalently coupled to stationary supports like agarose or sepharose beads, provides an excellent reagent for isolating immune complexes or immunoglobulins from a crude solution through immunoprecipitation. However, a slightly different technique that involves precipitation of all the proteins (antigens) in protein lysate, while leaving in solution proteins of interest for which no antibodies are present, has not been found to be used previously. Ideally this type of reverse

immunoprecipitation technique would require preparation of a mixture of antibodies raised against the whole protein lysate.

In the previous chapters, C8-HSL was detected from culture supernatants of *Gloeothece* and was found to accumulate over growth showing a feedback type response associated with increased production of C8-HSL. Such a feedback type response in many organisms is involved with quorum sensing gene expression and altered protein expression levels. In some bacteria exogenous addition of AHL in cultures lead to expression of de novo synthesis of new proteins not previously expressed. These findings, if detected, may suggest that *Gloeothece* cells respond to a threshold concentration of C8-HSL through gene expression leading to altered protein expression.

Aims and Objectives

The aim was

- To test the hypothesis that immunoprecipitation technique can be used as a suitable technique to pre-fractionate samples.
- To visualise any proteins that are newly produced in response to C8-HSL in *Gloeothece*.

The main objectives are

- To optimize an immunoprecipitation condition using different concentrations of *Gloeothece* protein extract, whole cell antibodies and protein-A concentrations for efficient immunoprecipitation of total *Gloeothece* proteins in the extract.

- To immunoprecipitate whole cell proteins from C8-HSL treated *Gloeothece* cells with whole cell antibodies raised against untreated C8-HSL *Gloeothece* whole cell protein, assuming that no antibodies are present to the those proteins that are newly synthesized in response to C8-HSL. The immunoprecipitation will allow precipitation of all of the proteins while leaving only those proteins that are newly synthesized in response to C8-HSL in the supernatant.

- To detect retained proteins in solution of a C8-HSL treated sample after immunoprecipitation by SDS-PAGE following its identification through mass-spectrometry.

4.2. Materials and Methods

4.2.1. Materials:

Protein-A sepharose 4B, rabbit IgG, protease inhibitors, Dimethylpimelimidate, BCA protein estimation kit were commercially available from Sigma Aldrich. Microcon YM-3 filters were purchased from Amicon Bioseparations. Gel electrophoresis equipment and associated chemicals were purchased from Bio-Rad Laboratories and Sigma Aldrich. All other chemicals were from Sigma or Fluka Biochemika.

Rabbit serum containing antibodies against nitrogen fixing *Gloeothece* cells were provided as a generous gift from Prof. John Gallon, Swansea University. Serum samples were kept frozen at -20 °C. Prior to use, serum samples were defrosted and centrifuged at 10,000 g for 10 min for the purification of immunoglobulin fractions.

4.2.2. Methods:

4.2.2.1. Purification of whole cell protein antibodies of *Gloeothece* PCC 6909

The main advantages of protein-A affinity purification of antibodies are that it is relatively simple and gives high yield of pure antibodies in a single step. The principle involves purification of antibodies through affinity binding of antibodies to protein-A beads under physiological conditions. Once antibodies are bound, the contaminants are washed off. The antibodies are then eluted at a low pH that alters the binding properties of antibodies to protein-A and allows the antibodies to be collected in a purified form.

The technique is suitable when the affinity of the antibody for protein-A is sufficient to allow high-capacity binding.

4.2.2.1.1. Preparation of Protein A-sepharose bead columns

Protein-A sepharose beads were weighed equivalent to 1ml of swollen beads and allowed to swell in 10 mM Tris (pH 8.0) overnight. The beads were then washed in buffer (10 mM Tris, pH 8.0) through centrifugation at 4000 g for 5 min and resuspended again in the same buffer. Protein-A columns were then prepared by settling 1ml of the beads into the column by gravity. The bead column prepared was capable of binding 20 mg of rabbit IgG according to the binding capacity of Protein-A.

4.2.2.1.2. Antibody Purification on protein-A columns by "Low salt" technique

Antibodies from serum samples were purified according to the following technique:

- pH of the crude antibody preparation was adjusted to pH 8.0 by adding 0.1 volume of 1.0M Tris (pH 8.0)

- 1ml of antibody solution was passed through protein-A sepharose bead column, which is known to bind 20 mg of antibody/ml of wet beads (serum contains approximately 10mg ml^{-1} of total IgG)

- Column was washed with 10 ml (10 column volumes) of 100 mM Tris (pH 8.0)

- Again columns were washed with 10 mM Tris (pH 8.0)

- Antibodies were eluted stepwise with 500 µl of 100 mM glycine (pH 3.0)

- Eluted fractions were collected in 1.5 ml conical tubes containing 50µl of 1 M Tris (pH 8.0) and the solution was then gently mixed.

The columns were washed with elution buffer before subjecting the column to the next batch of purification.

4.2.2.1.3. Antibody purification by protein-A antibody purification kit (Sigma Stock No. PURE-1A)

Total of 6 ml of rabbit serum containing antibodies against N_2 fixing *Gloeothece* proteome was subjected to purification according to the users' manual. During purification 2 ml of serum was used in each batch of purification and the process was repeated 3 times. Columns were washed after each batch and the final purified antibody from three runs were pooled and combined.

4.2.2.1.4. Detection of Immunoglobulin containing fractions

Bradford spot test

10 µl of sample from each fraction in duplicate were applied to a parafilm. Next 2 µl of Bradford dye concentrate (Appendix I) was applied to the samples and mixed well by pipetting slowly. Colour development was monitored within 2 min. Suitable controls

were prepared by applying 10µl of buffer instead of samples. The drops that turned blue were selected as the samples containing immunoglobulin fractions.

All the purified immunoglobulin fractions were combined and the amount of antibody in the total fraction was measured through turbidity and Bradford protein estimation (see following sections) and used to calculate the average amount of antibody purified per ml of serum sample.

4.2.2.2. Quantification of protein and antibody concentrations.

Antibody was quantified by the Bradford assay from a standard curve prepared from commercially available purified rabbit IgG antibodies at concentrations from 0 to 100 µg (Fig. III). Extracted *Gloeothece* protein samples, both soluble and detergent treated and immunoprecipitated samples (where stated) were measured for protein concentration using the Bicinchoninic Acid Kit (BCA protein estimation kit, Sigma Aldrich) due to interference of detergent with Bradford assay and was carried out according to instructions provided by the manufacturer. Protein concentrations of *Gloeothece* extract were estimated from a standard curve prepared using 0 to 100 µg of bovine serum albumin (BSA) (Fig. IV).

4.2.2.2.1. Bradford assay (Bradford, 1976)

Preparation of Coomassie blue reagent: Coomassie blue reagent was prepared from Bradford dye concentrate (Appendix I) by dissolving the dye in a ratio of 1:4 (v/v) in distilled H_2O.

Sample (100 µl containing 10-100 µg protein) was mixed with 5 ml of Coomassie blue reagent and the absorbance (595 nm) was recorded after 10 min. Calibration curves were prepared by using BSA and rabbit IgG as standard protein. Typical curves for both proteins obtained are presented in Fig. III and IV respectively.

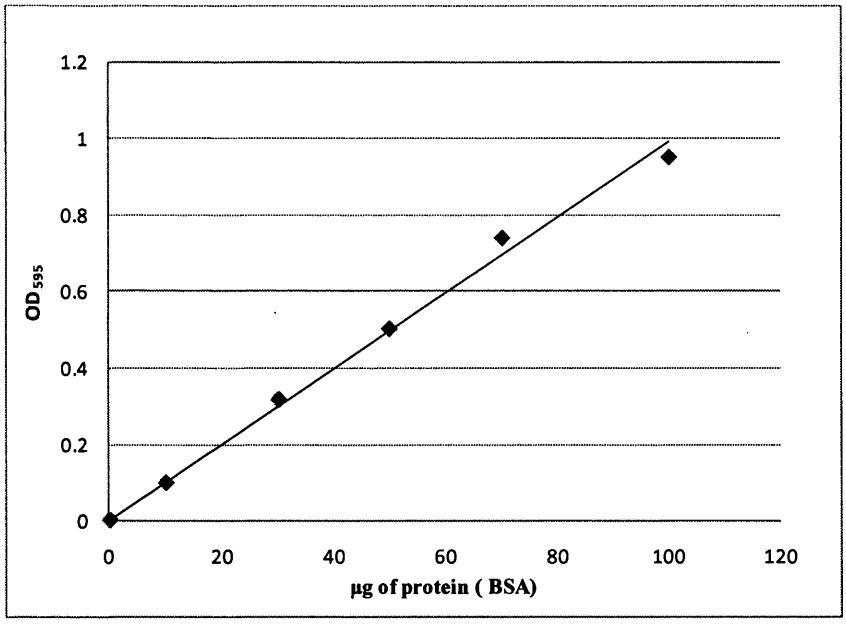


Fig. III. A typical calibration curve of Bradford protein estimation method using BSA.

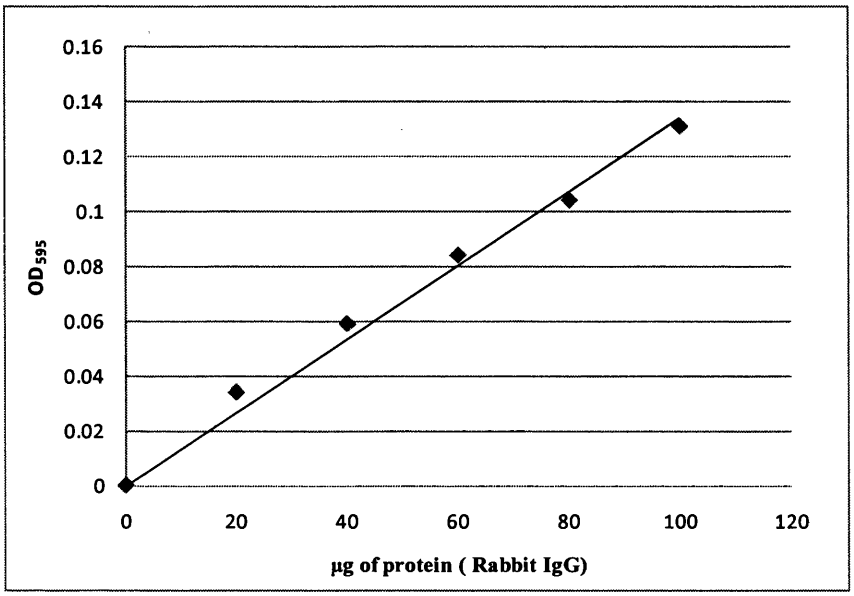


Fig. IV. A typical calibration curve of Bradford protein estimation method using commercial rabbit IgG.

4.2.2.2.2. Absorbance at 280nm

Antibody concentration was also measured through OD measurements at 280 nm, using the equation 1 OD_{595nm} = 0.8 mg of IgG.

4.2.2.3. Assessing the purity of the antibody

The purified antibody from serum was assessed by running samples on SDS-PAGE. 24 µg of purified antibody was compared against 30 µg of serum sample, 72 µg of wash solution and 10 µg of commercial rabbit IgG on a 10% SDS-PAGE (Laemmli, 1970) (section 4.2.2.4). Protein estimations were done by the Bradford assay (see section 4.2.2.2.1). Apart from purified antibodies, protein estimation from serum samples and

wash solutions were determined from BSA standard curve (section 4.2.2.2.1). Protein concentration of the purified antibody was estimated from commercial rabbit IgG standard curve (section 4.2.2.2.1).

4.2.2.4. Separation of proteins by SDS-PAGE

4.2.2.4.1. Sample preparation

Samples to be analysed by SDS-PAGE were estimated for protein concentration. The amount of protein was kept within a volume of 30 µl. A volume of 5X concentrated sample buffer (Appendix I) at a ratio of 5:1 was added to the samples. The samples were then boiled for 5 min with 2-mercaptoethanol, at a final concentration of 5% (w/v). Samples were then centrifuged at 3000 g (MSE microcentaur centrifuge) and applied to the polyacrylamide gel.

4.2.2.4.2. Electrophoresis of samples

Gels were prepared and run using the Bio-Rad Mini Protean II System. The protocol was an adapted from that described by Laemmli (1970). Separation of proteins was performed on a 10% resolving gel and a 4% stacking gel (Appendix I). The components of the gels are listed in Appendix I. All parts of the gel equipment were washed with detergent solution followed by cleaning with ethanol in order to remove dirt and grease. The equipment was then assembled and the resolving gel prepared as outlined in Appendix I., with addition of TEMED last. The solution was then poured between plates leaving sufficient space for the stacking gel. A solution of water saturated butanol

was then gently introduced on the top of the resolving gel to create a level of interface and also to exclude O_2. The gel was then left at room temperature to polymerize. The butanol layer was next removed and the gel rinsed with resolving gel buffer (Appendix I). The stacking gel was then prepared with contents listed in Appendix I. The solution was introduced above the polymerised resolving gel and a 10-well comb inserted and allowed to polymerize. The combs were then removed and the wells were washed with staking gel buffer.

The polymerized gels were removed from the sitting stand and assembled in the running tank. Sufficient reservoir buffer consisting of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS, was poured in to the inner reservoir to cover the top of the gel. Molecular weight markers were loaded first in a well from on one side followed by the addition of protein samples (prepared as described bellow) to the other wells. Reservoir buffer was then added to the outer reservoir and the electrophoresis apparatus was connected to a power supply (Bio-Rad Model 200/2.0). Proteins were concentrated on top of the stacking gel by application of 50 V, after which the voltage was increased to 200 V until the bromophenol dye in the samples reached the bottom of the gel. Gels were then removed from the outer casting and stained for visualization of protein bands.

4.2.2.4.3. Detection of proteins

Coomassie blue staining:

Proteins were located by the use of different Coomassie blue staining solutions according to the desired sensitivity. The compositions of all the staining solutions are

specified in Appendix I. When staining with "Blue silver" (Coomassie Blue G-250 colloidal stain); a very sensitive stain that can detect 1 ng protein/ band (Candiano *et al.*, 2004), gels were kept in the staining solution overnight for the protein bands to develop. The gels were then washed repetitively with distilled water to remove background stain. With the Coomassie blue-R-250 stain, gels were stained for 2-3 h after which gels were destained in a solution of 45% (v/v) methanol; 10% (v/v) glacial acetic acid, with several changes and then kept immersed in distilled water. All staining procedures are reported to be compatible with detection of peptides by mass spectrometry.

Silver staining:

For a highly sensitive detection of proteins, a silver staining protocol was used, which depends on the reduction of metallic silver onto protein surfaces. The silver staining protocol is listed in section 5.2.2.5.3. All solutions were applied sequentially with the timing specified therein. After colour development the gels were repeatedly washed with several washes of distilled water. Gels were then kept immersed in distilled water.

4.2.2.5. Total protein (antigen) extraction from *Gloeothece*

4.2.2.5.1. Preparation of control and C8-HSL treated cultures

The culture from which the inoculums for the experimental cultures were prepared was washed under sterile conditions by centrifugation at 7000 g for 10 min and the supernatant decanted in order to eliminate any endogenous AHLs. The pellet was then

resuspended aseptically into fresh sterile $BG11^{0}$ medium. The culture was shaken for a few minutes for complete resuspension of the cell pellet. The resultant inoculum was then aseptically transferred in equal volumes using a sterile pipette to inoculate 100 ml of fresh $BG11^{0}$ medium in 250 ml conical flasks, hence the resulting experimental cultures contain the same amount of cells representing clonal cultures. The cultures were then allowed to grow for a further 7 days after which the cultures were treated accordingly.

For C8-HSL treated and control cultures, commercially available synthetic C8-HSL was solubilised in ethanol and then added to one or a set of experimental cultures aseptically in equal volumes (50-100 µl) that would result in the final desired concentration of 1 µM C8-HSL in the cultures. Control samples, were treated with the same volume of ethanol instead of C8-HSL. After treatment the cultures were allowed to grow for a further 7-10 days before the cultures were harvested.

4.2.2.5.2. Extraction of proteins for immunoprecipitation and SDS-PAGE

C8-HSL treated and control cultures of *Gloeothece* PCC 6909 and/or PCC 6909/1 prepared according to section 5.3.4.1. were harvested by centrifuged at 7000 g (Beckman, J2-21/E) for 5 min and washed in wash buffer containing 50 mM Tris-HCl pH 7.5 and recentrifuged. The pellet was resuspended in 1ml ice cold chilled extraction buffer (20 mM Tris-HCL pH 7.5, 1mM Na-EDTA, 100 mM NaCl, 2 mM $MgCl_2$, and protease inhibitors; 0.05 mM Leupeptin, 0.001mM Pepstatin, 0.5 mM PMSF) and broken through a passage by the French Press (SLM-AMINCO) at 10,000 psi. The broken cell suspension was centrifuged at 10,000 g to remove cell debris and the

supernatant was collected and referred to as soluble proteins. For detergent extraction, samples were extracted in extraction buffer containing 2% IPGCAL under the same conditions and referred to as detergent extracted proteins. The protein content of extracts was estimated according to section. 4.2.2.2. and subsequently used for immunoprecipitation and SDS-PAGE.

4.2.2.6. Immunoprecipitation with soluble antigen

Immunoprecipitation with *Gloeothece* whole cell antibody was carried out by adding antibody to *Gloeothece* soluble protein extract at a ratio of 1:2 (protein to antibody) unless otherwise specified. The mixture was incubated in an end over turner at 37 °C for 1 h for antibody antigen interaction to occur. Afterwards, swollen protein-A sepharose beads were added at twice the ratio of 1:0.1 (Antibody: protein-A) than the normal (1:0.05) capacity to bind antibodies, unless otherwise stated. The mixture was incubated for a further 1 h, for subsequent binding of antibody antigen complexes after which the complexes were precipitated by a brief centrifugation at 5000 g for 1 min. The supernatant was then carefully aspirated and estimated for protein concentration and or analyzed by running the samples on a 10% SDS-PAGE.

4.2.2.7. Optimization of immunoprecipitation conditions

In order to optimize antigen-antibody to protein-A binding capacity to remove immunocomplexes formed, samples were used in the following concentrations; 20 µg of *Gloeothece* extract in a volume of 50 µl. Antibody was used (purified from Pure-1A

antibody purification kit) at a concentration of 2 μg μl^{-1} and protein-A sepharose beads swollen overnight was used in a final concentration of 0.5 μl of protein-A beads μl^{-1}.

4.2.2.7.1. Optimizing antibody ratio

Antibody was added to the antigen (protein 20 μg), at an antigen: antibody of 1:2, 1:3, 1:4 (antibody of 40 μg, 60 μg and 100 μg) and immunoprecipitated according to section 4.2.2.6. protein-A beads were added at an amount of 36 μg of wet beads in a volume of 60 μl that resulted in a ratio of 1: 0.09, 1:0.06 and 1:0.3 (w/v) (antibody: protein-A) respectively. The protein-A concentrations added were expected to bind antibodies within the range of 40-100 μg (36 μl of protein-A is known to bind 1.2 mg of IgG) for efficient precipitation of antigen antibody complexes and free antibodies. After immunoprecipitation the supernatant was collected and 40 μl of samples were applied and separated on SDS-PAGE according to section 4.2.2.4.

4.2.2.7.2. Optimizing protein-A concentration

For determination of an optimum concentration of protein-A sepharose beads, an antigen: antibody ratio of 1:3 was selected and the ratio of beads were varied to the antibody at 1:0.3, 1: 06 and 1:1 (w/v). Immunoprecipitation of all the samples was carried out according to section 4.2.2.6. After immunprecipitation, the supernatant was collected and loaded on to an SDS-PAGE in a total volume of 40 μl, along with molecular weight standards, Protein A beads and an IgG standard (rabbit IgG, Sigma)

4.2.2.7.3. Immunoprecipitation using a higher concentration of protein extract and concentrating samples after precipitation

Controls and C8-HSL treated samples were prepared as before section 4.2.2.5. The protein concentration in the extract was determined using the Bradford assay. The concentration of protein in both samples was adjusted to $2.5\mu g^{-1}$ and a total of 50 µg was used for immunoprecipitation. Antibody ratios to protein were used at 2:1 and 4:1 and protein-A beads were used at a ratio to antibody at 1:5. The total volume of sample before immunoprecipitation was adjusted to 250 µl for the 2:1 ratio and 530 µl for the 4:1 ratio of samples with antibody. Volumes were adjusted to maintain a good mixing condition and a high local antibody concentration. Following immunoprecipitation, samples were concentrated using microcon filters (MW cut off 10 kDa) and 20 µl of each sample was loaded and run on a 10% SDS-PAGE. Protein bands were visualized using silver stain.

4.2.2.8. Immunoprecipitation with immobilized antibody sepharose beads:

4.2.2.8.1. Immobilization of antibodies on protein-A sepharose beads

Coupling of antibody to protein-A beads was used, where the interaction of antibody to protein-A is stabilized through cross-linking with a bifunctional coupling reagent. This technique provides a good antibody orientation but is expensive. The coupling was carried out according to the following method: 200 mg of Protein A sepharose beads

were allowed to swell at room temperature with gentle rocking for 1.5 h and then incubated overnight at 4 °C. To the 800 µl of swollen protein-A beads (500 mg Protein-A swells to 2 ml of wet beads) 16 mg of antibody was added (generally 20 mg of antibody binds to 1 ml of wet beads) mixed and incubated at room temperature for 1 h with gentle rocking. Beads were then washed with 8 ml (x10 volumes) of 0.2 M sodium borate (pH 9.0) by centrifugation at 3000 g for 5 min. The beads were again resuspended in 8 ml of 0.2 M sodium borate (pH 9.0) and an equivalent of 10 µl of beads (100 µl) was removed and kept for analysis. 41.44 mg of dimethylpimelimidate (solid) was added to the resuspended bead slurry to give a final concentration of 20 mM. The solution was then allowed to mix for 30 min at room temperature on a rocker. Again equivalent of 10 µl of coupled beads (100 µl) was removed and the reaction was stopped by washing the beads in 0.2 M ethanolamine (pH 8.0) and incubating for 2 h at room temperature in 0.2 M ethanolamine with gentle shaking. Beads were then washed and resuspended in 8 ml of PBS (consisting of 0.19 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 7.4 mM Na_2HPO_4, pH 7.5) and used as a stock solution. Considering 16 mg of IgG to be coupled to the beads with no loss of IgG, the bead stock solution would have 2 µg of IgG present per µl of bead solution.

4.2.2.8.2. Checking efficiency of coupling

The efficiency of coupling was checked by boiling samples of beads taken before and after coupling in Laemmli sample buffer (Appendix I). 100 µl of samples before and after coupling were mixed with 5x Laemmli sample buffer and loaded on a 10% SDS PAGE in volumes of 10 µl and 20 µl along with antibody standard (20 µg) and molecular weight markers. After electrophoresis the gels were stained with commassie

blue stain (4.2.2.4.3). A good coupling was checked by heavy chain bands (55,000 Mw) in the "before" and not in the "after" lanes. In cases where small amounts of heavy chain is still present a prewash step of the coupled beads with 100 mM glycine (pH 3.0) was done to remove any non-covalently bound antibodies to the protein A molecules.

4.2.2.8.3. Immunoprecipitation using immobilized antibody sepharose beads

Immunoprecipitation was carried out by mixing antibody (coupled beads) with 100 µg of *Gloeothece* protein (control and or C8-HSL treated) at different ratio as 1:2, 1:4, 1:6 and 1:8 in a total volume of 400 µl adjusted with PBS buffer (consisting of 0.19 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 7.4 mM Na_2HPO_4, pH 7.5) and constant rocking for 2 h at room temperature. The bound proteins were removed by settling the beads at a low pulse in centrifuge for 10 s. The unbound antibody was recovered gently by aspiration and not upsetting the layer of settled beads. 100 µl of samples were taken and analysed using the BCA protein determination kit.

4.2.2.9. Comparison of coupled and uncoupled immunoprecipitation:

50 µg of soluble protein and detergent soluble protein (section 4.2.2.5.2) was used in immunoprecipitation. For uncoupled immunoprecipitation 200 µg of IgG was used and for coupled immunoprecipitation 100 µl of the stock bead solution which contains 200 µg of IgG was used allowing an antigen to antibody ratio of 1:4. Volumes of samples (soluble and detergent soluble) was adjusted to 300 µl with PBS buffer and incubated in

an end over turner at 37 °C in a waterbath for 2 h. After which, for uncoupled immunoprecipitation, 150 µl of protein-A sepharose with a binding capacity of 300 µg of antibody was added to the solution and incubated for another hour. After which samples were collected after a brief pulse in centrifuge. 100 µl were from each sample was used for protein estimation using the BCA protein estimation kit. The total amount of protein retained in total of 450 µl solution was calculated from which the percentage of protein retained in each sample was estimated. An amount of 10 µg of immunoprecipitated samples were then taken and run along with 10 µg of extracts on a 10% SDS-PAGE to visualize specific proteins that are retained after immunoprecipitation.

4.2.2.10. Immunoprecipitation with C8-HSL treated *Gloeothece* extracts.

Immunoprecipitation was carried out with both control and C8-HSL treated extracts according to section 4.2.2.9. The amount of protein used in immunoprecipitation was 100 µg and 200 µg respectively. The coupled antibody beads were used as described in section 4.2.2.9. but at different ratios of 1:5 and 1:10 respectively. The amount of protein retained in each solution was calculated as described in section 4.2.2.9.

4.2.2.11. Testing the binding specificity of *Gloeothece* whole cell antibodies to *Gloeothece* extracts by Dot blot analysis.

The binding of whole cell antibodies to *Gloeothece* extract was determined by Dot blot analysis. The protocol was modified from Stal & Bergman (1990) (Stal and Bergman

1990). Dot blot is a technique can be used as a simplified version of western blot using antibodies which bind to specific antigen in a mixture of proteins applied as a dot on nitrocellulose membranes. Before primary antibody is added the uncoupled sites on the membrane are blocked by the addition of protein solution, otherwise non-specific reactions occur. Recognition of the primary antibody is accomplished by the addition of secondary antibody. The secondary antibody is usually conjugated with a marker protein, for example, horseradish peroxidase. Upon addition of a suitable substrate, such as 4-chloro-1-naphthol, a coloured precipitate is produced, thus allowing visualization of specificity of antibody

4.2.2.11.1. Determination of antibody titre for use in dot blot:

An antibody titre was determined through many fold dilution of antibody so that the detection of antigen remains possible while reducing the non-specific background. Nitrocellulose membranes were cut into small strips. *Gloeothece* protein extract of 2µl and 5µl was applied on one end of each nitrocellulose membrane strip and allowed to air dry. Strips were then soaked in TBS-milk solution, containing 10mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl and 3% milk powder overnight with gentle rocking (Stovall Belly Dancer orbital shaker) at room temperature for blocking non specific binding of antibodies. The membranes were then washed for 1 h with TBS-Tween buffer (10 mM Tris-HCl, pH 7.5, containing 0.9% (w/v) NaCl and 0.1% (v/v) Tween 80) changing the buffer every 10 min. Each membrane was then incubated for 1 h with antibody diluted at concentrations of 0.002 (v/v), 0.001 (v/v), 0.0005 (v/v) and 0.0002 (v/v) with TBS-Tween buffer. Following incubation with antibody membranes were again washed with TBS milk solution changing every 10 min. Membranes were then incubated for 1 h with

the secondary antibody, goat anti-rabbit, conjugated to horseradish peroxidise (HRB), which was in a dilution of 0.001 (20 µl in 20 ml) in TBS-Tween buffer. Following, strips were washed with several washes of TBS milk and just before developing they were washed only with TBS buffer to remove the milk. The antigen- antibody complex coupled with HRB was then visualized using the developing solution containing 2.8 mM 4-chloro-1-naphthol dissolved in 16.5% (v/v) methanol, to which 0.04% (v/v) H_2O_2 was added immediately before use. After development the colour reaction was stopped with several washes of distilled water. An antigen antibody binding was indicated by visualization of staining at the sample application site. The membranes were placed in the dark until photographed.

4.2.2.11.2. Determination of the specificity of whole cell antibodies against *Gloeothece* whole cell protein extract.

Dot blot analysis was carried out according to method in section 4.2.2.10.1. *Gloeothece* soluble protein extracts were applied to nitrocellulose membranes (10 µl) Dilutions of 0.002 (v/v) of purified whole cell antibodies, serum samples and null antibody (antibody raised without *Gloeothece* antigens) was used to test the binding efficiency to *Gloeothece* proteins. A negative control (without any primary antibody) using only secondary antibody (HRB) was also carried out to test non specific binding of the secondary antibody to *Gloeothece* protein antigens.

4.3. Results

4.3.1. Antibody Purification

The average amount of the purified antibody obtained from 1ml of serum sample through the "Low salt" method was estimated to be 2.5 mg (1.7-3.3 mg), through measurements in optical density. Antibodies alternatively purified using an antibody purification kit yielded 4.0 mg of purified antibody from 1 ml of serum samples, through similar OD measurements. Although, protein estimation using OD measurements showed higher amounts of antibody to be purified using the antibody purification kit, the amounts obtained by both techniques were found to be considerably low compared to known amounts of antibody concentrations present in rabbit serum, which is approx. 10 mg ml^{-1}.

Since OD measurements in some cases may not accurately reflect the actual concentration; an alternative "Bradford" protein quantitation method was used to measure the amount of antibody purified using both techniques. The amount of purified antibody from 1ml of serum samples, obtained through the "Low salt" technique and the antibody purification kit was estimated to be 18.36 mg and 20.8 mg respectively. These amounts were found consistent with the binding capacity of the column (10-20 mg of IgG) but relatively higher than the level of immunoglobulin present in serum (1 ml of Rabbit serum contains 10 mg IgG). A higher protein concentration may reflect contamination of the purified immunoglobulin fractions with serum proteins.

4.3.2. Checking the purity of the antibody on SDS-PAGE

To test the purity of anybodies, antibodies obtained by Low salt technique were run on a SDS-PAGE along with molecular weight markers, rabbit IgG (control), serum samples and wash solution (solution after column wash Fig. 4.1.). Result in Fig. 4.1 show two characteristic bands in the purified antibody sample that can be comparable to the heavy and the light chain of antibodies of the commercial purified rabbit IgG. The molecular weight of these bands estimated through comparison with the molecular weight markers, was found to represent the molecular weights of the heavy chain (55 KDa) and light chain (25 KDa) bands of antibodies and thus further confirming that these bands were derived from antibody. However, when comparing the purified antibody sample with serum sample, two bands in the molecular weight of 66 KDa and 14 KDa were found to be similar to bands present in the serum with the former being more prominent than the later. This suggested some contamination of the purified antibody with proteins from the serum sample and may explain the reason for a high antibody concentration estimated by the Bradford method.

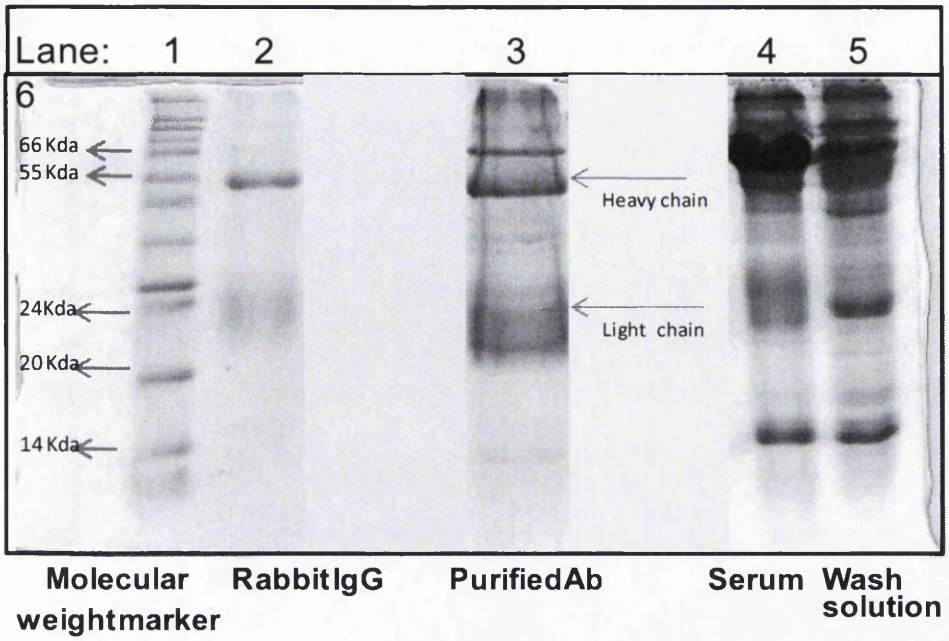


FIGURE **4. 1.** SDS-PAGE ON ANTIBODY SAMPLES PURIFIED THROUGH THE "LOW SALT" TECHNIQUE

Samples of purified whole cell antibodies (lane 2) by Low salt technique were run on 10% SDS-PAGE, along with commercial rabbit IgG, (lane 2) and serum sample (lane 4), wash solution (lane 5) and molecular weight markers (lane 1) in order check the purity of antibodies. After electrophoresis gels were stained with coommassie blue silver stain in order to visualize protein bands.

4.3.2. Checking the affinity of whole cell antibody to *Gloeothece* soluble antigens

4.3.2.1. Determination of antibody titre

Before using the purified antibody for immunoprecipitation, the specificity of the purified whole cell antibody to *Gloeothece* antigens was checked using the dot blot technique. A preliminary test was undertaken to determine an antibody concentration at

which antigen antibody binding was observable with minimum background. Different dilutions of purified antibody concentrations were used to determine a concentration at which an antigen-antibody complex can be detected. Results in Fig 4.2 A, show complex formation visualized by staining, at antibody dilutions of above 0.001% (v/v).

4.3.2.2. Antigen antibody binding test

Antibody dilutions of 0.002 (v/v) were subsequently used to test the presence of specific antibodies to *Gloeothece* proteins. Non-specific binding of the purified antibody was tested against serum sample, blank sample and null antibodies. Fig 4.2 B, shows the blotting results of each of these samples with *Gloeothece* proteins. Staining was observed with the purified antibody and serum sample, with the former being darker than the serum sample, suggesting both serum and purified antibody samples contains antibodies specific to *Gloeothece* proteins and also that the purified antibody contains a higher concentration of such antibodies. However, results obtained from the blank control (only using 2^{nd} Antibody) and null antibodies (antibodies not raised against *Gloeothece* antigens), showed no staining (Fig 4.2 B). Hence indicating that the staining observed with *Gloeothece* antibodies and serum samples were not caused due to nonspecific binding of the secondary antibody or cross reactivity with any other antibodies (null antibody) present in serum. Results therefore, suggest that the purified antibody contains antibodies specific to whole cell *Gloeothece* proteins and therefore, could be potentially used to precipitate *Gloeothece* proteins from solution.

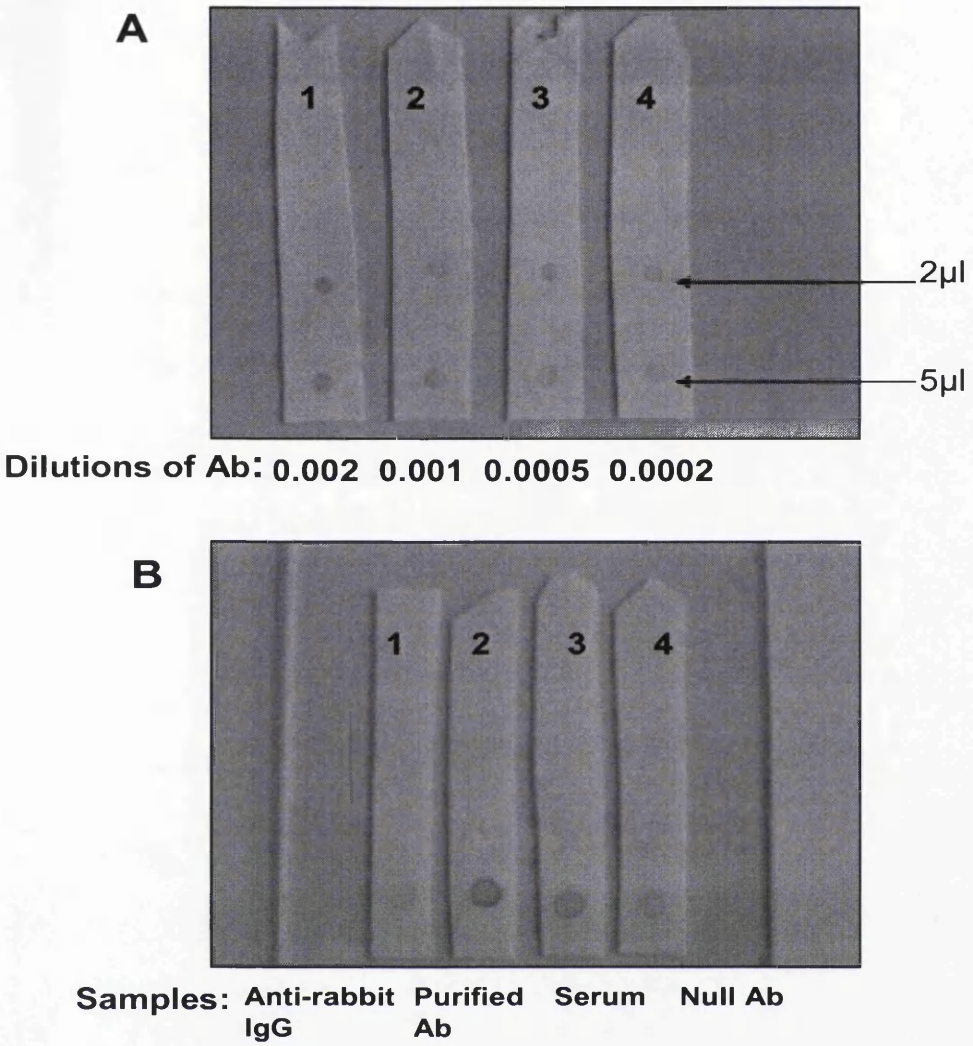


FIGURE **4. 2.** DOT BLOT OF WHOLE CELL ANTIBODIES ON *GLOEOTHECE* PROTEIN EXTRACTS.

A. *Antibody stock solution in dilutions of, 0.002% (v/v) (sample1), 0.001% (sample 2), 0.0005% (v/v) (sample 3), 0.0002% (v/v) (sample 4) were used to blot 5 µl and 10 µl of Gloeothece extract.*

B. *Specificity of the purified antibody to Gloeothece proteins (sample 2) were compared against Anti-rabbit IgG (sample 1), serum (sample 3) and null antibody (sample 4) through dot blot. All samples were visualized after specific staining.*

4.3.3. Optimization of antigen, antibody and protein-A ratio for immunoprecipitation.

4.3.3.1. Optimization of antibody to antigen ratio:

Gloeothece PCC 6909 proteins were used at different antibody ratios to determine an optimum ratio at which all of the proteins could be precipitated. Previously, an antibody to antigen ratio of 1:1 was suggested to be optimum in different experiments, therefore, an antibody to antigen ratio of 1:2, 1:3 and 1:4 were initially tested. Results in Fig 4.3 show that no other visible bands were observed at a ratio of 1:2, except for two bands, one band of high molecular weight that was previously found to be a contaminant derived from serum sample (Fig 4.1) and one low molecular band, that was comparable to a band derived from *Gloeothece* extract, thus indicating an efficient immunoprecipitation at this antigen antibody ratio. A sample of *Gloeothece* extract also run in parallel, comparable to an amount (5 μg), of a 75 % precipitation of proteins, indicated that a low molecular band for *Gloeothece* extract would be observed when 75 % of all the proteins are precipitated. Tthis was found to be consistent with the observed results in all immunoprecipitated samples, suggesting the low molecular weight bands in the immunoprecipitated samples are derived from *Gloeothece* extract. However, even with a high antibody ratio the bands did not show any changes which may suggest that these proteins are not precipitated with increasing concentrations of antibodies

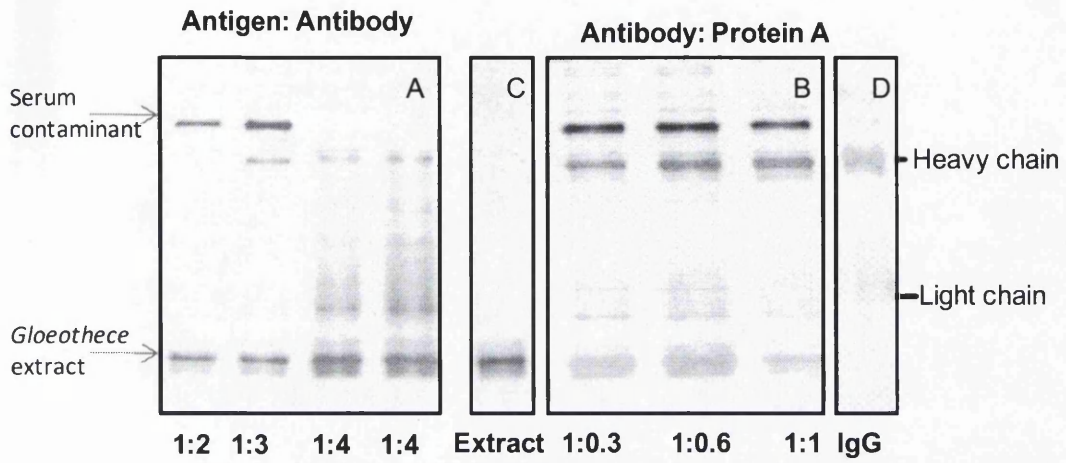


FIGURE **4. 3.** IMMUNOPRECIPITATION ON *GLOEOTHECE* PROTEIN EXTRACT AT VARYING ANTIBODY TO ANTIGEN AND ANTIBODY TO PROTEIN-A RATIOS.

A. Extracted proteins from Gloeothece PCC 6909 immunoprecipitated with whole cell antibodies and at different antigen to antibody ratio of 1:1, 1:2, and 1:4; B. Proteins precipitated using antibody with varying ratio of protein-A to the antibody concentrations; C. Gloeothece protein loaded at 0.25 % of the concentration of used in immunoprecipitation and D. purified rabbit IgG. All samples were separated on 10% SDS-PAGE and visualized through silver staining.

With a further increase in antibody concentration at ratios of 1:3 and 1:4, more bands were observed in the immunoprecipitated samples (Fig. 4.3.). A comparison of such bands with rabbit IgG, showed that these bands were similar to the heavy chain and light chain bands of antibodies, indicating that an increase in antibody ratio during immunoprecipitation may result in free antibodies being left over in solution.

4.3.3.2. Optimization of antibody to protein-A ratio

Different protein-A ratios to antibody were tested in order to investigate whether increasing protein-A sepharose beads resulted in removal of contaminating antibodies. Generally a ratio of 1: 0.05 antibody to protein-A sepharose beads is used to bind all antibodies and higher ratios of 1: 0.3, 1: 0.6 and 1:1 ratio of Protein A-sepharose beads were tested. Results, show that there was no change in the appearance of bands with an increase in protein-A sepharose beads (Fig. 4.3), suggesting that a higher concentration of protein-A sepharose beads was incapable of removing contaminating antibodies after the immunoprecipitation reaction.

4.3.5. Immunoprecipitation using coupled antibody to protein-A sepharose beads

4.3.5.1. Coupling antibody to protein-A sepharose beads:

Previous results showed that immunoprecipitation using soluble antibodies resulted in substantial amounts of antibody and *Gloeothece* proteins to be left in solution. Therefore, immobilization of antibody to protein-A sepharose beads was undertaken. Efficient coupling of antibody to the beads was checked by running samples taken before and after coupling on a SDS-PAGE (Fig. 4.4). Results show a good coupling, indicated by the appearance of heavy chain bands from samples taken before and not after the coupling method. Since heavy chains are immobilized to protein-A, samples after coupling lose their heavy chains along with sepharose beads when subjected to

SDS-PAGE, allowing the visualization of only light chain bands. However since a small amount of heavy chain bands was still seen from samples after coupling (Fig. 4.4), coupled beads were washed again to insure complete removal of non specifically bound antibodies. Each wash solutions during coupling were also collected, concentrated and run on the same SDS-PAGE (Fig. 4.5). Results show sequential removal of almost all antibodies by the 3rd washing step (Fig. 4.5).

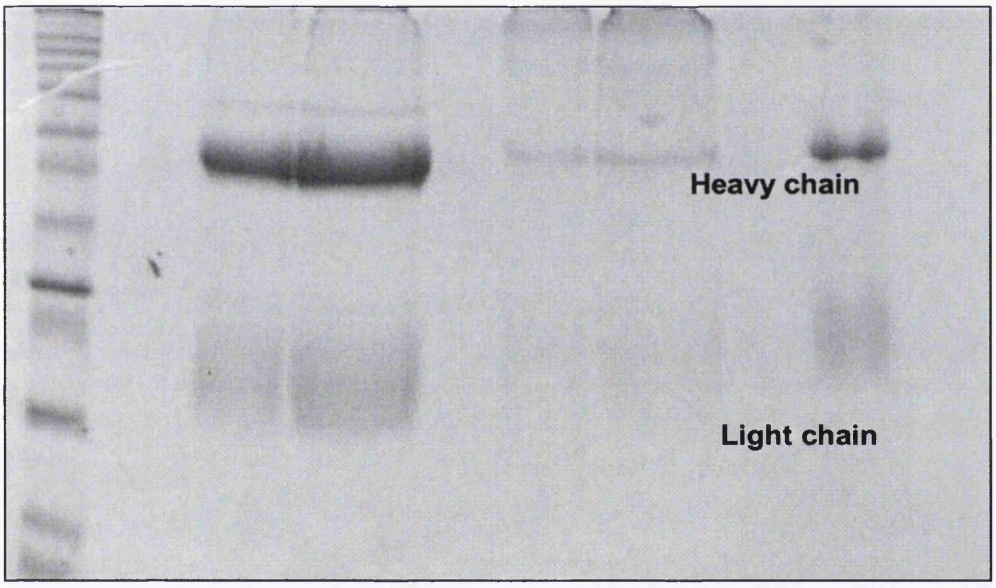


FIGURE **4. 4.** CHECKING THE EFFICIENCY OF ANTIBODY COUPLED TO PROTEIN A SEPHAROSE BEADS USING SDS-PAGE.

Samples taken before and after the coupling method were separated through SDS-PAGE. Protein bands were separated on a 10% SDS-gel and observed after staining the gel with Coommassie blue R-250. A sample of purified rabbit IgG and molecular weight markers were also run along with the samples on the same gel.

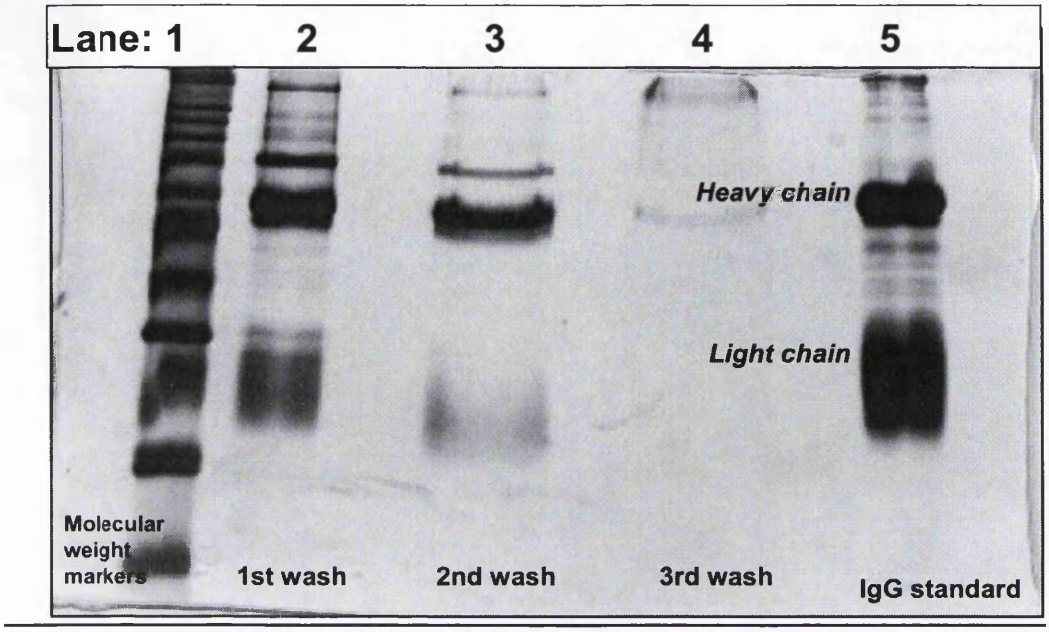


FIGURE **4. 5.** CHECKING THE WASHING STEP FOR REMOVAL OF FREE ANTIBODIES DURING COUPLING OF ANTIBODIES TO PROTEIN A-SEPHAROSE BEADS

Washed solution obtained form 1st, 2nd and 3rd washing step during coupling of antibody to protein-A sepharose beads, purified IgG from rabbit serum and molecular weight markers were run on a 10% SDS-PAGE and visualized through silver staining.

4.3.5.2. Immunoprecipitation using coupled and soluble antibodies:

Immunoprecipitation using coupled antibodies were compared to that of soluble antibodies. Higher concentrations of proteins were used in order to carry out quantitative measurement on proteins retained after each immunoprecipitation step and calculate the percent of non-precipitated proteins in solution. Table 4.1. gives the percentage of non-precipitated proteins obtained using different protein extracts and antibodies in immunoprecipitation . Results show that a comparatively low percent of

non-precipitated proteins was left in solution using soluble *Gloeothece* proteins immunoprecipitated with coupled antibody beads. However, when comparing the efficiency of coupled antibody with soluble antibody in immunoprecipitation, a higher percentage of non-precipitated proteins were retained in solution using different *Gloeothece* extracts (both soluble and detergent extracted) immunoprecipitated with soluble antibodies. In order to visualize the proteins that are precipitated and that are retained in solution, immunoprecipitated samples obtained from both techniques were run on SDS-PAGE along with *Gloeothece* extract. Fig. 4.6 show the appearance of protein bands in samples after immunoprecipitation.

Type of Ab used in immunoprecipitation	Immunoprecipitated sample (% of proteins retained in solution)	
	Detergent extracted protein	soluble protein
Coupled Ab	49.5(Lane 2)	36.6(Lane 5)
Soluble Ab	77.1(Lane 3)	50.4(Lane 6)

TABLE 4. 1. COMPARION BETWEEN TWO DIFFERENT PRECIPITATION METHODS.

Table shows the comparison of the percentage of proteins retained in solution after immunoprecipitation by two different methods using coupled antibodies and soluble antibodies. The table also shows the difference in precipitation using soluble proteins and detergent extracted proteins by the two different methods.

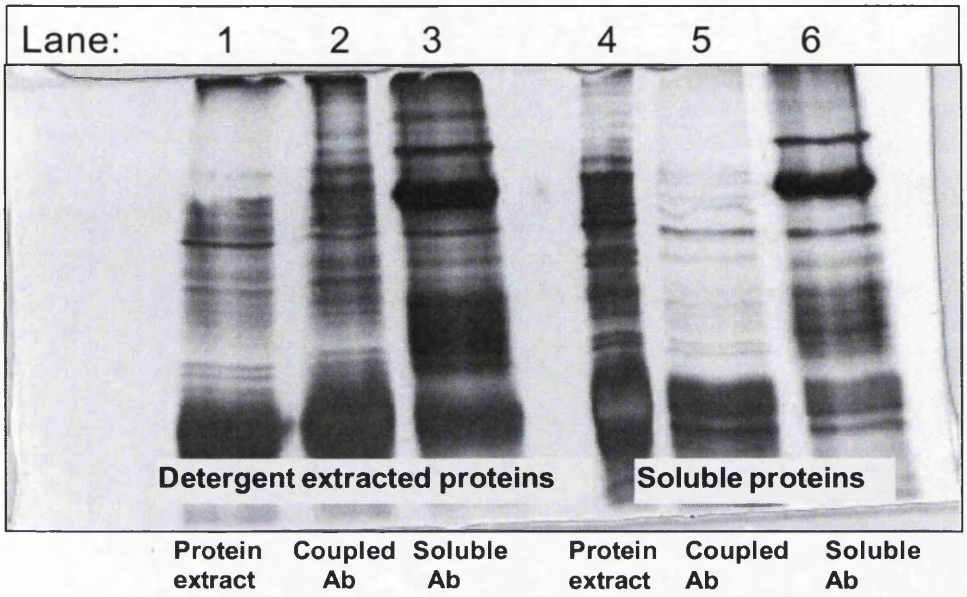


FIGURE **4. 6**. COMPARISION OF IMMUNOPRECIPITATION OF GLOEOTHECE PROTEINS USING COUPLED AND UNCOUPLED ANTIBODIES.

Immunoprecipitated samples of Gloeothece soluble (lane 5, 6) and detergent extracted proteins (lane 1, 2) precipitated using coupled antibody (lane 2, 5) and soluble antibody (lane 3, 6) separated on a 10% SDS-PAGE. Protein extracts of both soluble (lane 4) and detergent extracted proteins (lane 1) of Gloeothece PCC 6909 were also separated along with the immunoprecipitated samples for comparison. Gels were stained with silver stain for visualization of protein bands.

A greater number of proteins bands were detected in the detergent extracted samples (Fig 4.6, lane 2, 3) compared to the soluble protein samples (Fig 4.6. lane 5, 6), indicating that the whole cell antibodies used in this experiment are more specific for the soluble proteins in *Gloeothece*. A comparatively higher amount of non-precipitated proteins was observed from samples immunoprecipitated with soluble antibodies (Fig.

4.6. lane 3, 6) The samples also showed an increased abundance of antibody bands of the heavy chain and light chain, which were absent from samples precipitated with coupled antibodies (lane 2, 5). Results therefore, indicate that the use of coupled antibody in immunoprecipitation can eliminate interfering antibodies and allow visualization of other proteins of interest. Most of the other protein bands that were detected in all immunoprecipitated samples were found to be derived from *Gloeothece* extracts (lane 1 and 4).

4.3.6. Immunoprecipitation with C8-HSL treated samples

Control samples, of soluble and detergent extracted proteins and C8-HSL treated soluble proteins of *Gloeothece* were immunoprecipitated using coupled antibodies. Different concentrations of samples were used in order to test the reproducibility and the extent of protein precipitation. Protein concentrations in the supernatant of immunoprecipitated samples were determined. Results in Table 4.2 show the percent of proteins retained in each sample after immunoprecipitation. Results indicate that although some precipitation of proteins can be achieved, significant amount of proteins are retained in solution. Variations in the amount of antibody-sepharose beads to that of protein concentration in ratio of 1:5 and 1:10 did not show any significant changes, in terms of decreasing the percentage of un-precipitated proteins in solution (Table 4.2). An increase in the amount of protein used in immunoprecipitation in all cases, was found to reduce the percentage of proteins being precipitated (Table 4.2.). In control and C8-HSL treated samples, a large variation in the amount of protein precipitation was observed (Table 4.2), with less amounts of proteins being precipitated in the C8-HSL treated sample. Such difference along with observed differences in the control

samples therefore, may not indicate changes upon treatment of C8-HSL but instead may reflect the possibility that, the precipitation technique lacks the reproducibility to observe any differences that may arise solely due to C8-HSL treatment.

Samples		Total Protein (µg)	Protein:Protein A-Antibody (v/v)	protein retained (µg)	Protein retained (%)
Control	Detergent extracted	100	01:05	54	54
		200	01:05	129	64.5
	Soluble protein	100	01:05	39	39
		100	01:10	138	56
		200	01:05	56	69
HSL treated	Soluble protein	100	01:05	62	62
		200	01:05	219	100

TABLE 4. 2. IMMUNOPRECIPITATION OF CONTROL AND C8-HSL TREATED SAMPLE

4.4. Discussion

The hypothesis, that whole cell antibodies can be used to precipitate all proteins in *Gloeothece*, was tested in order to determine if the method could be employed to detect any changes in proteins that may result due to treatment of cultures with C8-HSL. In order to use whole cell antibodies raised against *Gloeothece* proteins, an antibody purification step was carried out from serum samples in which the antibodies were raised. Antibodies were primarily purified by using the "Low salt" technique. This technique employs affinity purification of antibodies using protein-A bead columns. The "Low salt" technique can be a good method to purify antibodies that have high binding affinity for protein-A, such as rabbit polyclonal antibodies and therefore, were considered to be a suitable method to purify *Gloeothece* polyclonal antibodies (serum sample) raised in rabbit. The purified antibodies were found to be relatively pure, with some contamination derived from serum. Results by dot blot analysis also showed that these antibodies have specificity towards *Gloeothece* proteins. The dot blot technique was used as an efficient technique to test whether an antibody concerned has specificity towards its antigen. An antigen-antibody complex formed as a result can be visualized by chemiluminescence techniques through binding of 2^{nd} antibodies. However, no information could be obtained on the extent of individual antigen – antibody complex formation and also on how these antigen-antibody complexes behave in solution. When whole cell soluble antibodies were used to immunoprecipitate *Gloeothece* proteins, variations were observed in the amount of precipitated proteins, which largely depended on the amount of protein extract used in immunoprecipitation. This result may arise due to various factors, for example, protein modification, low affinity antibodies, low concentrations of a particular antibody, high concentrations of a particular protein.

Immunoprecipitated samples were also found to retain some antibodies in solution that could not be eliminated through increasing protein-A concentrations which suggests that these antibodies may have lost or have low specificity towards protein-A. These antibodies could represent any of the other antibody isotypes (classes) named IgA, IgG, IgD, IgE, and IgM in serum which may have low specificity towards protein-A and co-purified during protein-A affinity purification. Another possibility could be that some antibodies may have lost their binding capacity to protein-A after the purification step. However, such observations of antibodies retained in solution may not be due to loss or changes in the binding properties of protein-A, since most *Gloeothece* proteins were found to be absent in the immunoprecipitated samples when low concentrations of protein was used, which supports the idea that protein-A-sepharose beads are efficient in removing the antigen antibody complexes. The problem of antibody carryover was obviated by using coupled antibodies to protein-A sepharose beads during immunoprecipitation. Coupling of antibodies was found to be a good technique in removing antibodies that may have lost specificity for protein-A thus preventing the appearance of antibody bands and associated *Gloeothece* proteins on SDS gels. However, *Gloeothece* proteins were still found to appear on SDS-PAGE gels of the immunoprecipitated samples using coupled antibodies and could not be eliminated by increasing the ratio of coupled antibodies. These findings suggest other alternative possibilities for *Gloeothece* proteins being retained other than low concentrations of antibodies. *Gloeothece* proteins in the low molecular region and other proteins that appeared throughout the SDS gels of the immunoprecipitated samples may represent proteins that could not be precipitated due to modifications or due to their high abundance. High abundant proteins such as phycobiliproteins may not all become precipitated due to lack of proportionally high concentrations of antibodies. Some

proteins may also undergo modification or may be synthesized during particular growth stages that may not represent the actual state and age of extract used to produce whole cell antibodies. Under these circumstances the whole cell antibody may not contain antibodies to these proteins. However, in most cases a low amount of protein extract was found to give a greater percent of precipitation than the use of high amounts of protein extract.

In this experiment a complex mixture of *Gloeothece* whole cell antibodies were used to precipitate proteins extracted from *Gloeothece* as a pre-fractionation method to isolate proteins that were newly synthesized upon treatment of *Gloeothece* with C8-HSL. The results obtained supports the fact that this could be difficult to achieve, because antigen antibody complex formation depends on a number of factors, which includes the affinity of antibody to its antigen, the avidity between the antibody antigen complexes and the antigen antibody ratio. The following terms can be discussed separately on basis of this experimental condition.

Affinity of Antibody: The affinity of an antibody increases with repeated exposure to an antigen. Regardless of this knowledge, whole cell antibodies raised against *Gloeothece* extracts were found to be specific towards *Gloeothece* proteins. However, it is not possible to test the affinity of an antibody towards its specific protein antigen in a complex mixture. In such a context the extent of antigen antibody complex formation and the amount of proteins precipitated may be influenced by the affinity of antibodies towards each protein.

Avidity between the antibody antigen complexes: Avidity is the functional affinity of multivalent antigen binding to multivalent antibody molecules. When using whole cell antibodies the type of antibodies (divalent, multivalent) to its specific protein antigens is not known. With *Gloeothece* proteins nothing is known about individual proteins and their interaction with antibodies, how they behave in solution once complex is formed and the strength at which the complexes are held together. In a complex mixture the avidity may be influenced by the concentration of each *Gloeothece* protein to its antibody, which in turn may influence the amount of proteins precipitated during immunoprecitation.

Antigen antibody ratio: Since complex formation also depends on antigen antibody ratio, individual concentration of each antibody to their specific *Gloeothece* proteins may not be optimum in a mixture of antibodies to *Gloeothece* proteins in order to precipitate all protein. It would be impossible to empirically determine a single mixture of antibody concentration at which all proteins will form a complex with their specific antbodies. In certain cases, for example, for the high abundant phycobiliproteins, there may not be an optimum concentration of specific antibodies present in a mixture to precipitate all phycobiliproteins.

The results therefore, show that a complete precipitation of all proteins is not possible, However, considering the fact that some proteins are retained after immunoprecipitation, it may be used as a pre-fractionation method, but may suffer due to its lack of reproducibility in any comparative analysis.

CHAPTER 5

Analysis of protein expression changes in cultures of *Gloeothece* in response to C8-HSL

5.1. Introduction

In most cases quorum sensing (QS) dependent gene expression leads to direct transcriptional activation of quorum regulated genes however, in some cases direct repression of target genes has been observed in some bacteria such as *Yersinia enterocolitica* (Throup *et al.*, 1995), *Serratia liquefaciens* (Christensen *et al.*, 2003; Lindum *et al.*, 1998) through the decreased accumulation of polypeptides on 2D gel. 2D-PAGE is a widely used technique to analyse global changes in protein expression patterns. The technique separates proteins in the 1st dimension based on isoelectric pH and in the 2nd dimension on molecular weight (Mw). Therefore individual proteins are separated and highly resolved on polyacrylamide gels.

In most cases QS dependent gene expression occurs during the late logarithmic or stationary phase of growth when cell density is high and AHL accumulates to a critical threshold concentration. In some cases it is possible to early activate QS genes by addition of exogenous AHL to low density cultures during their early stages of growth, for example bioluminescence genes in *V. fischeri*, can be induced early by the addition of exogenous 3-oxo-C6-HSL to the cultures. Addition of exogenous AHL has also been used to stimulate various physiological responses characteristic of a stationary phase

culture and has been used to understand AHL regulated phenotypes in various bacteria through the use of 2D proteomics (Sauer and Camper, 2001). Most studies have been carried out with the addition of exogenous AHL to mutants defective in AHL production or response to determine genes that are directly under quorum sensing control. These studies revealed that AHL can function through global changes in gene expression affecting a large number of genes, involved in various processes (Schuster *et al.*, 2003). However, studies on wildtype strains (lacking mutations in AHL synthesis/ response) have also been carried out through proteomic approach in cultures of *Sinorhizobium meliloti* to which the addition of AHL affected over 100 proteins resolved by 2D-PAGE, which were found to be involved in diverse functions of carbon and nitrogen metabolism, energy cycles, metabolite transport, DNA synthesis and protein turnover (Chen *et al.*, 2002). Proteomic approaches have also been employed in *Pseudomonas aeruginosa*, in the identification of multiple quorum-sensing regulated proteins (Riedel *et al.*, 2003), in *Vibrio fischeri* in identification of new proteins regulated by quorum sensing (Qin *et al.*, 2007), in *P. putida* in surface associated growth (Sauer and Camper, 2001) and in analysis of quorum sensing response in *Rhizobium leguminosarum biovar viciae* (Cantero *et al.*, 2006).

In previous chapters C8-HSL was identified from *Gloeothece* cultures which accumulated in cultures during growth. Its accumulation indicated a feedback response through increased production of C8-HSL during a short stationary phase; such responses may have occurred through activation of gene expression as evident with many other AHL based quorum sensing systems in various bacteria. Further to strengthen the hypothesis that C8-HSL is used as a quorum sensing signal in

Gloeothece the organism should show a response to its own produced C8-HSL at the level of gene (and therefore protein) expression.

Aims and Objectives

The aim of this chapter was to test the hypothesis; *Gloeothece* responds to C8-HSL through global changes in gene expression, with a view towards answering the following questions.

- Does *Gloeothece* responds to its own signal through changes in gene expression?
- How does *Gloeothece* respond? (Through upregulation, downregulation,or through global changes in protein expression patterns. Is there changes in the phosphopoteome).

Since the genome of *Gloeothece* is not yet sequenced the objective will be to investigate a response to C8-HSL through a proteomic approach. The approach involves: treating early growth stage cultures at low cell densities with exogenous C8-HSL and then analysing changes in the proteome to such treatment. In studying a quorum sensing response with a wild type strain capable of producing its own AHL, it is required that the level of AHL be lowered or removed from cultures so that the effects of AHL could be reliably detected. Therefore, the approach will be to wash off any endogenous AHL from *Gloeothece* cultures and then expose the cultures to a concentration of synthetic C8-HSL. SDS-PAGE will be used initially to study any changes that may arise from C8-HSL treatment followed by further analysis and identification of proteins by

optimizing the 2D-PAGE technique for *Gloeothece* proteins. Specific enrichment of phosphoproteins from samples will also be undertaken in order to enable the specific analysis and identification of phosphoproteins that may undergo expression changes by the use of SDS-PAGE.

5.2. Materials and Methods

5.2.1. Materials:

All 2D-PAGE equipments and reagents (IPG strips, IpG buffer, covering oil, precast gels and gel buffers, 2D quant kit) were from GE Healthcare, UK. All other chemicals were purchased from that described in previous chapters and Sigma Aldrich and Fisher Scientific, UK.

5.2.2. Methods:

5.2.2.1. Analysis of protein expression changes in response to C8-HSL using SDS-PAGE

5.2.2.1.1. Culture preparation for SDS-PAGE:

C8-HSL treated and control cultures for the experiment were prepared as described in chapter 4, section 4.2.2.5.1. For C8-HSL treated cultures, commercially available synthetic C8-HSL was added to one of experimental cultures aseptically in equal volumes (50-100 µl) that would result in the final desired concentration of 5 µM of C8-HSL in the cultures. Control samples, were treated with the same volume of ethanol instead of C8-HSL. After treating the cultures (C8-HSL, ethanol), the cultures were allowed to grow for a further day (unless otherwise stated) before the cultures were harvested.

5.2.2.1.2. Extraction and separation of proteins on SDS-PAGE

C8-HSL treated and control cultures of *Gloeothece* PCC 6909 and/or PCC 6909/1 prepared according to section 5.2.2.1.1. were harvested by centrifugation at 7000 g (Beckman, J2-21/E) for 5 min and washed in wash buffer containing 50 mM Tris-HCL pH 8.0 and recentrifuged. The pellet was resuspended in 1ml ice cold chilled extraction buffer (20mM Tris-HCL pH 8.0, 1mM Na-EDTA, 100mM NaCl, 5mM DTT, and protease inhibitors; 0.05mM Leupeptin, 0.001mM Pepstatin, 0.5mM PMSF) and broken through a passage by the French Press (SLM-AMINCO) at 10,000 psi. The broken cell suspension was centrifuged at 10,000 g and the supernatant was collected. Prior to SDS-PAGE samples were dissolved in Laemmli sample buffer (5x) Samples of *Gloeothece* PCC 6909/1 were further processed by precipitation with 10% TCA (10% TCA-acetone, 5mM DTT) for 2 h at -20°C with occasional vortexing. Precipitated proteins with other cell fractions were centrifuged at 12,000 g for 10 min. The pellets were further washed with several washes of ice cold acetone and redissolved in Laemmli sample buffer (x1) without the addition of bromophenol blue dye and mercaptoethanol. The protein content of the samples was determined using BCA protein estimation kit as described in chapter 4 section 4.2.2.2. An amount of 40µg of protein was boiled with ß-mercaptoethanol and subsequently run on 10% and or 7% SDS-PAGE prepared according to chapter 4, section 4.2.2.4. SDS-PAGE and visualization of protein bands was carried out with colloidal coomassie "blue silver" stain (Candiano *et al.*, 2004) according to chapter 4 section 4.2.2.4. and 4.2.2.4.3 respectively.

5.2.2.1.3. Analysis of expression changes in response to C8-HSL

Changes in protein expression were analysed visually by observing changes in the intensity of staining of corresponding bands in the control and C8-HSL treated sample. An upregulation in protein expression was considered if the C8-HSL treated sample showed protein bands of a greater intensity compared to its corresponding band/spot in the control sample. Similarly downregulation in protein expression was considered when the intensity of the C8-HSL treated sample showed a lower intensity than its corresponding band/ spot in the control sample. Protein bands/spots that showed differential expression were excised and trypsin digested according to section. 5.2.2.7.2. for identification through MS.

5.2.2.1.4. Protein identification through MS

Protein bands of interest were sliced with a sharp blade and placed in an eppendorf tube. Destaining of gels prior to trypsin digestion and trypsin digestion were carried out according to section 5.2.2.7. Digested proteins were further analysed and identified though MS according to section 5.2.2.8.

5.2.2.2. Optimization of protein extraction conditions from *Gloeothece* PCC 6909 prior to 2DE-PAGE

5.2.2.2.1. Removal of sheath material from *Gloeothece* PCC 6909

In order to remove sheath material from cultures of *Gloeothece* PCC 6909, cultures were centrifuged to pellet cells. Cells were then resuspended in fresh sterile BG11^{o} media and passed twice through a Yeda press at a nitrogen gas pressure of 1,000 psi. Cells were intermittently visually inspected to observe if colonies were ruptured. The cell suspension was then collected and recentrifuged at 7000 g for 5 min to pellet cells. The pelleted cells were again resuspended in a small volume of BG11^{o} medium and layered on top of a 10 % Ficoll solution in a centrifuge tube. Ficoll is a neutral, highly branched, high-mass, hydrophilic polysaccharide which dissolves readily in aqueous buffer. Ficoll is a registered trademark owned by GE Healthcare companies and commonly used to separate blood components. The tube containing *Gloeothece* cell suspension with Ficoll was then centrifuged at 10,000 g for 20-30 min until the cells pelleted at the bottom of the tube leaving the fractionated sheath and slime on the top layer of the Ficoll solution. The cell pellet was collected, washed several times with extraction buffer and then finally redissoved in extraction buffer for cell breakage.

For removal of sheath material by the use of glass beads, pelleted cells were dissolved in 10 ml of Tris-HCl pH 8.0 and homogenized by shaking with glass beads (0.18 mm in diameter) of cell to glass bead ratio 1:2 at room temperature in a vibrogen shaker for 10 min allowing sheath and slime material to be separated from the cells by friction with the glass beads. Cells were then centrifuged and purified through Ficoll gradients described as before.

5.2.2.2.2. Use of different rehydration buffers

Cells obtained after removal of sheath material described in section 5.2.2.2.1. were dissolved in 1 ml of extraction buffer containing 20 mM Tris-HCl pH 8.0, 1 mM Na-EDTA, 100 mM NaCl, 5 mM DTT, and protease inhibitors 0.05 mM Leupeptin, 0.001 mM Pepstatin, 0.5 mM PMSF and broken through a passage by the French Press (SLM-AMINCO) at 10,000 psi. The broken cell suspension was centrifuged at 12,000 g to remove cell debris and the supernatant was collected. The protein content of the supernatant was determined by the BCA protein estimation kit. A volume of supernatant containing approximately 80 µg of protein was taken and the supernatant was split in two equal volumes representing 40 µg of protein. Each extract was precipitated with 10 % TCA (10% TCA-acetone, 5 mM DTT) for 2 h at -20 °C with occasional vortexing. Proteins were precipitated by centrifugation at 12,000 g for 10 min. The pellets were further washed with several washes of ice cold acetone and allowed to air dry. Samples were then prepared by dissolving one pellet in rehydration buffer containing 8 M urea, 2% CHAPS, IPG buffer 0.5% (v/v), and the other pellet in 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10 and IPG buffers at pH 4-7 0.5% (v/v), pH 3-10 0.25% (v/v) (Mechin *et al.*, 2003). To aid in the solubilisation of the pellet samples they were sonicated in a sonicating bath at low temperature. Following solubilisation, samples were recentrifuged to remove insoluble particles. Protein estimation was carried out using the "2D quant kit" (GE Healthcare) according to user's instructions. Reducing agents such as DTT and TCEP were added from appropriate stocks to obtain a final concentration of 20 mM DTT and 5 mM TCEP respectively. 60 µg of protein in a volume adjusted to 450 µl was rehydrated with 24 cm 3-10NL IEF strips overnight.. 2D-PAGE was carried out according to the procedure described in

section 5.2.2.5. Following 2D-PAGE protein spots were visualized through silver staining as described in section 5.2.2.5.3. A 2D work flow in Fig. V is used to show these steps briefly.

5.2.2.2.3. Use of fractionation and precipitation:

Samples were processed according to the 2D workflow (Fig. V) to obtain soluble and membrane soluble protein fractions. In order to obtain high protein content sheathless cells were prepared from 500 ml of a 2 month old culture. The cell pellet was dissolved in 3 ml of extraction buffer containing 20 mM Tris-HCL pH 8.0, 1 mM Na-EDTA, 100 mM NaCl, 5 mM DTT, and protease inhibitors 0.05 mM Leupeptin, 0.001 Pepstatin mM, 0.5 mM PMSF and broken through a passage by the French Press at 10,000 psi. In order to prepare soluble and membrane soluble fractions, the extract was briefly centrifuged at 10,000 g for 5min. The supernatant was collected and subjected to ultracentrifugation (Beckman Coulter) at 80,000 g for 1 h at 10 °C. The clear blue supernatant was collected and considered as the soluble protein fraction. The pellet was redissolved in rehydration buffer (Mechin *et al.*, 2003) containing 5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10 and IPG buffer at pH 4-7 0.5% (v/v) pH 3-10 0.25% (v/v) with the aid of a sonicating bath. After solubilisation, the sample was centrifuged again at 10,000 g to remove insoluble particles and the supernatant was collected for use as membrane protein fraction. The protein content of both samples was estimated using the "2D quant kit". In order to prepare precipitated samples of both soluble and membrane fractions 100 µg of proteins from each sample was subjected to 10 % TCA (10 % TCA-acetone, 5 mM DTT) for 1 h at -20 °C. Both precipitated samples were

resolubilised in the above rehydration buffer. Samples were estimated for protein content as before. The volume of all samples was adjusted to 450 µl with rehydration buffer and a final concentration of 20 mM DTT and 5 mM TCEP. Rehydration was carried out with a 24 cm 3-10NL IEF strips overnight after which 2D-PAGE was carried out according to section 5.2.2.5. Following 2D-PAGE protein spots were visualized through silver staining as described in section 5.2.2.5.3.

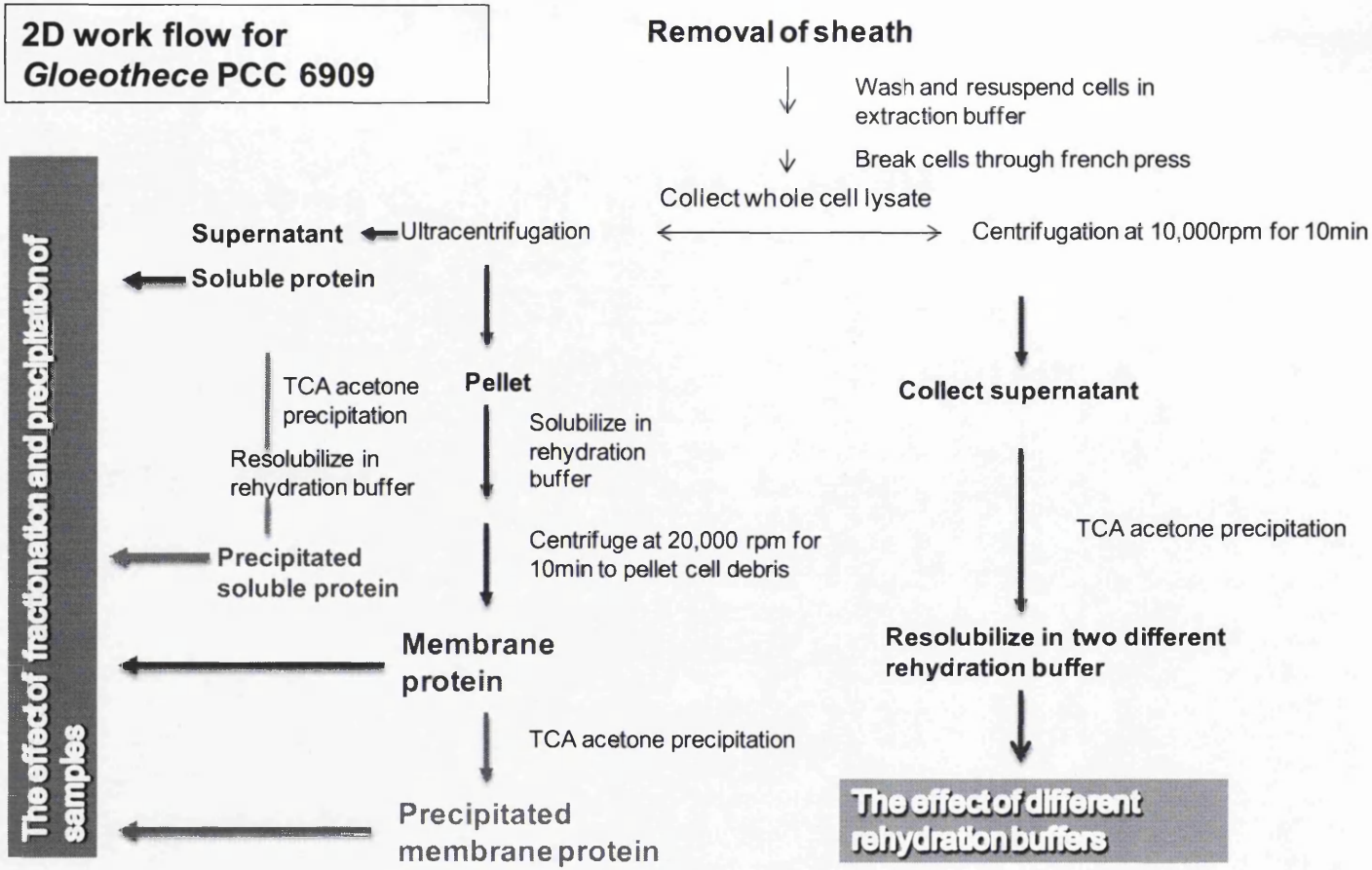


FIGURE V. 2D WORK FLOW SHOWING SAMPLE PREPARATION STEPS IN THE ANALYSIS OF THE EFFECT OF FRACTIONATION METHOD AND IN THE USE OF DIFFERENT REHYDRATION BUFFERS.

5.2.2.3. An optimized technique for protein extraction from PCC 6909/1 prior to 2D-PAGE

A large volume of cells of PCC 6909/1 were harvested and centrifuged at 7000 g (Beckman, J2-10/E) for 5 min and washed in wash buffer containing 50 mM Tris-HCl pH 8.0 and recentrifuged. The pellet was resuspended in 3 ml ice cold extraction buffer (20 mM Tris-HCL pH 8.0, 1 mM Na-EDTA, 100 mM NaCl, 2 mM MgCl, 5 mM DTT, and protease inhibitors 0.05 mM Leupeptin, 0.001 Pepstatin mM, 0.5 mM PMSF) and broken through a passage by the French Press (SLM-AMINCO) at 10,000 psi. The broken cell suspension was directly precipitated with chilled TCA-acetone (10% TCA-acetone, 5 mM DTT) in a ratio of 7:1 TCA-acetone to sample and kept for 2 h at -20 °C. Precipitated proteins with other cell fractions were centrifuged at 8000 g for 10 min. The pellets were further washed with several washes of ice cold acetone to remove pigments until the wash acetone became light yellow and clear in appearance. The pellets were then dried under air or through a stream of nitrogen until the pellet appeared to be light blue and powdery and was kept frozen at -80 °C until further use.

The pellets was dissolved in 3ml of rehydration buffer (Mechin *et al.*, 2003) consisting of 5 M urea, 2 M Thiourea, 2% CHAPS, 2% SB3-10, 20 mM DTT, 5 mM TCEP, carrier ampholytes; 4-7, 0.5%; 3-10, 0.25% but omitting the reducing agents and ampholytes at this stage. Sonication was performed under ice in a sonicator in order to completely dissolve the pellets. To remove cell debris and obtain a clear supernatant, centrifugation was performed at 10,000 g for 5 min and the resultant supernatant was ultracentrifuged at 80,000g for 1 h at 10 °C. The clear blue supernatant was recovered and used for protein estimation using a 2D quant kit (GE Healthcare). Proteins were kept in aliquots at -80 °C until use. 40 µg and 80 µg of proteins were used for

rehydration of 24 cm 3-10 NL and 4-7 NL IEF strips. Sample volumes were adjusted to 450 µl using rehydration buffer and adding required amounts of DTT and TCEP from stock solutions and IPG buffers. Rehydration and 2D-PAGE was carried out under conditions described in section 5.2.2.5. using labcast 2D gels for the optimization process and precast 2D gels (GE Healthcare, UK) for expression analysis. Following 2D-PAGE protein spots were visualized through silver staining as described in section 5.2.2.5.3. A 2D work flow of this process is diagrammatically presented in Fig. V1

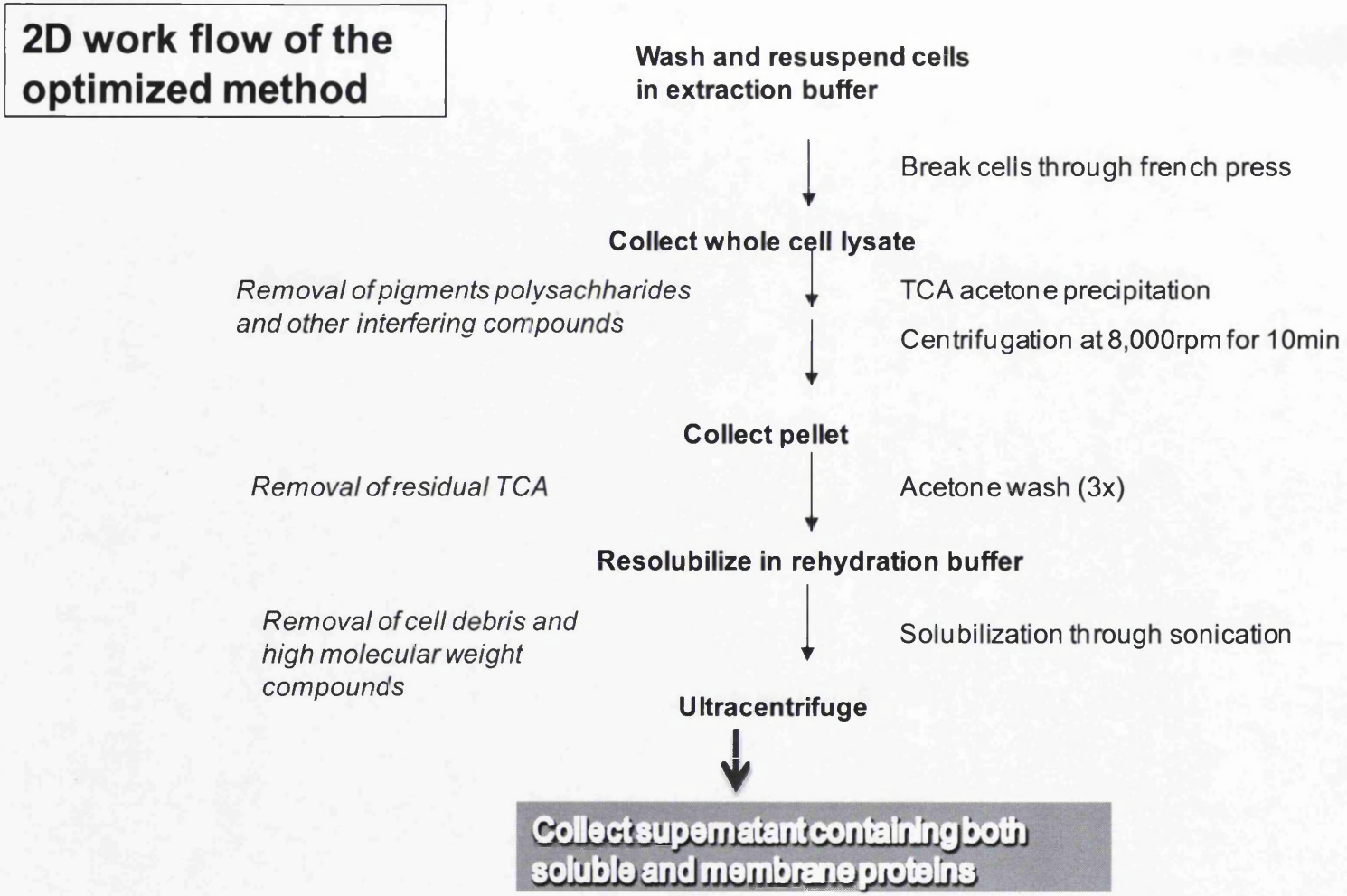


FIGURE VI. 2D WORK FLOW SHOWING AN OPTIMIZED PROCESS IN SAMPLE PREPARATION FOR 2D-PAGE FROM PROTEIN EXTRACTS OF *GLOEOTHECE* PCC 6909/1.

5.2.2.4. Experimental manipulations of the cultures for 2D-PAGE analysis

5.2.2.4.1. Build up of inoculums

To prepare cultures for 2D-PAGE experiments, a dense culture was prepared according to section 3.2.2.2.1.

5.2.2.4.2. Elimination of endogenous AHL and addition of C8-HSL

To eliminate any effects caused by endogenous AHLs, cells were washed and resuspended in fresh $BG11^0$ medium, as follows: one liter of *Gloeothece* PCC6909/1 (60 days old) was harvested under sterile conditions by centrifugation at 7000 g for 10 min at 20 °C. The resulting pellet was resuspended in 100 ml of sterile $BG11^0$ medium and 50 ml was used to inoculate each of two flasks containing 750 ml sterile $BG11^0$ medium. The resulting two separate cultures were grown for a further 10 days under the conditions described previously, after which 1 flask was treated with C8-HSL (final concentration, 10 μM) and the other flask was used as a control (ethanol). After a further 24 h the flasks were re-treated with C8-HSL or ethanol as appropriate, grown for another 24 h and then proteins were extracted for analysis.

5.2.2.4.3. Extraction of protein by the optimized method:

Cells of each culture extracted separately according to the optimized method described in section 5.2.2.3.

5.2.2.5. 2D-PAGE

5.2.2.5.1. 1st dimension IEF

Proteins were extracted from the C8-HSL-treated and control cultures. Three samples from each of the extracts were subjected to 2D-PAGE. 70 µg of total protein was used to rehydrate IEF strips (24 cm, GE healthcare) 3-10 and 100 µg was used rehydrate IEF strips 4-7 in an IPGphor strip holder. First dimension IEF was run with a horizontal electrophoresis apparatus (Ettan IPGphor, Amersham Pharmacia Biotech) at 20 °C. Strips were rehydrated, at low voltage at 15 V for 14 h following which IEF was carried out initially at a low voltage at 200 V for 1 h in order to remove any interference with contaminating salts. IEF was carried out at 500 V for 1 h, 1000 V for 1 h and then 8000 V for 8 h 20 min. After the end of IEF, strips were gently washed and stored at -80 °C for subsequent 2nd dimension SDS-PAGE.

5.2.2.5.2. 2nd Dimension SDS-PAGE

Before 2nd dimension SDS-PAGE, strips were taken out of -80 °C freezer and kept at room temperature for few minutes. Following this, equilibration was carried out with equilibration solution containing 2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.002% bromophenol blue according to the following procedure: Strips were placed in individual tubes. In the first equilibration, 10 ml of equilibration solution containing 100 mg of DTT was added to each tube and placed on its side on a rocker for 10 min. The second equilibration was performed similarly with 250 mg of iodoacetamide solution instead of DTT. The 2nd equilibration helps to prevent point

streaking and other artifacts. After equilibration the strips were gently placed on top of SDS gels that were prepared from the components of resolving gel buffer as outlined in Appendix I. 2nd dimension SDS-PAGE was then carried out in Ettan Dalt*six* electrophoresis system at 100 mA (Amersham Pharmacia Biotech) until the bromophenol dye reached the bottom of the gel.

The 2nd dimension gel electrophoresis for expression analysis of control and C8-HSL treated samples were carried out on 12.5% precast gels using Ettan Dalt*six* electrophoresis system (Amersham Pharmacia Biotech). Gels were run at 25°C; using Ettan Dalt*six* electrophoresis system at a maximum of 100 mA for approx 5 h until the bromophenol blue dye font reached the bottom of the gel. Co-migrating broad range standards (BioRad) were used for estimation of molecular weight.

5.2.2.5.3. Detection of proteins by silver staining:

Protein spots were located by staining gels with silver stain, using a method modified from Shevchenko et al. (1996) (Khokhlov *et al.*, 1967), which is compatible with MS identification of proteins. Gels were stained according to each step in Table II with strict maintenance in timing. Following visualization of protein spots gels were washed with several washes of distilled water and stored in distilled water at 4 °C.

Step	Reagents	Time
Fixation	Methanol: water: acetic acid (50: 38: 12)	Overnight
Remove fixing solution	Ethanol (50%)(x2)	20 min
Sensitization	0. 02% $Na_2S_2O_3$. $5H_2O$	1 min
Wash	H_2O (x3)	20s
Silver	Silver nitrate 0.2%(w/v) 0.026%(v/v) formaldehyde	20 min
Wash	H_2O (x2)	20 s
Development	6% Na_2CO_3, 0.0004% (v/v) $Na_2S_2O_3$. $5H_2O$, 0.017%(v/v) formaldehyde	Until colour development

Table. II. Silver staining method

5.2.2.6. 2D-PAGE protein expression analysis

Silver stained gels of control and C8-HSL treated were scanned using a scanner (Amersham Pharmacia Biotech). Gel alignment, matching and statistical analysis was performed using the 2-DE software "Progenesis Same Spot" (Non Linear Dynamics Ltd). Basically gels were first grouped into two individual groups in three replicates and assigned as control and treated. A reference gel was selected among each group based on a gel that showed a good representation of all the spots in the triplicate gels. The reference gel was the gel to which all other gels of that group were aligned. Spots were selected in each group based on expression changes as shown by fold change. Only spots that showed no or marginal change were omitted. Background subtraction and

normalization was performed on each gels by selecting "lowest to boundary method", which divides each spot volume by the total volume of spots present in each gel. Usually this eliminates errors associated with pipetting and staining. Spots statistically different, were reviewed and selected by the t-test ($p<0.05$). These spots were then analyzed for expression changes and spots that showed an approximate two fold or greater expressions were visually identified on the gel and trypsin digested for tentative identification by mass-spectrometry.

5.2.2.7. Protein digestion and identification

5.2.2.7.1. Destaining gel slices for protein identification:

Gels slices of proteins stained by Coomassie colloidal blue G-250 were destained with a solution consisting of 40% acetonitrile and 250 mM ammonium bicarbonate which was used according to the protocol described by Castellanos-Sera *et al.* (2005) (Castellanos-Serra *et al.*, 2005). Gel slices containing the proteins of interest were cut into 1 mm squares and placed in to an eppendorf tube. 50 µl of destaining solution was applied to each tube until full decoloration. In some cases the destaining solution was changed with a fresh solution to aid destaining of gel slices. Gel slices were than washed with 50 µl of water and then dehydrated two times 10 min with 100 % acetonitrile. After this stage samples were kept frozen at -80 ^{o}C until trypsin digestion.

Silver stained 2D Gel spots of interest were excised and destained according to the following method. Each gel slice containing the proteins of interest were cut into 1mm squares and placed in to an eppendorf tube as above. Each gel slice was then

sequentially destained and dehydrated using a volume of 50 µl according to the protocol listed in Table III, following which gels were dried in an Eppendorf Speed Vac (Eppendorf, UK).

Step	repeats	Time	solution
1	2X	5 min	water
2	2X	5 min	25 mM NH_4HCO_3
3	1X	Until complete	1% H_2O_2 in 25 mM NH_4HCO_3
4	2X	5 min	water
5	1X	5 min	1% Formic acid
6	1X	5 min	Water: acetonitrile: formic acid (50:50:1)
7	1X	10 min	Acetonitrile

TABLE III. Silver removal using hydrogen peroxide and ammonium bicarbonate (Sumner *et al.*, 2002)

5.2.2.7.2. Trypsin digestion:

The dehydrated gel pieces were re-swollen in a solution of trypsin (Promega sequencing grade) (50 ng μl^{-1} in 50 mM ammonium bicarbonate) for 45 min on ice. After this time excess trypsin was removed and the gel piece covered in ammonium bicarbonate and incubated overnight at 37 °C. Next the peptides were extracted from the gel piece. Firstly the present solution was removed and kept, next 50 µl of 5% formic acid in 70 % acetonitrile was added to the gel piece which was then sonicated for 5 min before removal of the liquid and its addition to the previously removed liquid. This process

was repeated three times and then the total peptide mixture was reduced to dryness in the Speed Vac. Prior to analysis, the peptide mixture was desalted using ziptips (Millipore, UK) following manufacturer's instructions and reducing the eluent to dryness and resolubilising in 0.1% formic acid in water for analysis.

5.2.2.8. Protein identification using liquid chromatography-electrospray ionization- tandem mass spectrometry (LC-ESI-MS/MS)

Extracted peptides were analyzed by nano reverse phase liquid chromatography (Ultimate Pump, LC-Packing, Dionex, Netherlands) using an electrospray ion trap MS (LCQ Deca XP, Thermo Electron, Hemel Hempstead, UK). LC-ESI-MS/MS separations were performed using a 10 cm x 75 mm I.D. pulled-tip capillary column, that was slurry packed in-house with 3 μm, 100 Å pore size C18 silica bonded stationary phase (PepMap, Dionex, Camberley, UK). The autosampler was fitted with a 5 μl injection loop and was refrigerated at 4°C during analysis. After injecting 7.5 μl of the peptides, the sample was eluted onto the analytical column, over which a 5 min wash with 98% buffer A (0.1% formic acid in water v/v) was applied and peptides were then eluted using a stepwise gradient of 0% solvent B (0.1% formic acid in acetonitrile v/v) to 65% solvent B in 100 min and then to 100% B in 10min with a constant flow rate of 0.2 μl min^{-1}. The electrospray MS was operated in a data-dependent mode in which each full MS scan (*m/z* 475-2000) was followed by three MS/MS scans, in which the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. The temperature of the heated capillary and electrospray voltage was 160°C and 2.0 kV,

respectively. The data were searched against the NR database using Sequest in Bioworks 3.2 (ThermoElectron, Hemel Hempstead, UK) and results were filtered within Bioworks for high stringency cross correlation (X_{corr} 1+, 2+, 3+ = 1.8, 2.2, 2.5).

5.2.2.9. Analysis of the Phosphoproteome of *Gloeothece* in response to C8-HSL

5.2.2.9.1. Culture preparation and Phosphoprotein Extraction:

C8-HSL and control treated cultures were prepared as described in section 5.2.2.1. using the mutant PCC 6909/1. For this experiment cell cultures were allowed to grow for 7 days after which 25 μM of C8-HSL was inoculated in the treatment flask. Then the following day again 25 μM of C8-HSL was inoculated and cultures were harvested after 1 h of incubation. In each case control cultures were prepared by inoculating with the same volume of ethanol. Different set of controls were processed similarly without the addition of C8-HSL.

For Phosphoprotein purification a phosphoprotein purification kit (Qiagen) was used according to the user's manual. All AHL treated and control samples were processed individually. Cells were first washed with 10 mM Tris-HCl, pH 8.0 and the cell pellets were resuspended in the cold "extraction buffer" provided with the kit basically containing the detergent Chaps and protease inhibitors. The cell suspensions were then passed through the French Press at 10,000 psi and the extracts were centrifuged at 9,000 g for 30 min at 4°C. The clear supernatant was collected and the protein concentration of the extract was determined using the 2D-Quant Kit (GE Healthcare UK). The protein

content of the control and AHL treated cultures were adjusted to the same concentration using the phosphoprotein extraction buffer with a total of 1.5 mg of protein. Phosphoproteins from the extract were then purified using the affinity columns according to the user's instruction provided with kit. The phosphoprotein enriched fractions collected from the column were detected using the "Bradford spot test" (Chapter 4, section 4.2.2.1.4.). Phosphoprotein containing fractions were then combined and concentrated to approx 150-200 µl using the nano-sep ultrafiltration columns. 10 µl of sample was used for protein estimation using the 2D quant kit.

5.2.2.9.2. Phosphoprotein detection on SDS-PAGE:

SDS gels were prepared according to chapter 4 section 4.2.2.4. 30 µg of each AHL treated and control samples were dissolved in 5x sample buffer, boiled for 5 min and loaded in equal protein concentrations of 6 µg on a 7% SDS-PAGE (prepared with reagents in Appendix I). Gels were then stained with colloidal commassie blue G-250 (appendix I)) and analysed for expression changes according to section 5.3.4.3.

5.2.2.9.3. Gel digestion for MS:

Phosphoprotein bands showing differential expression were cut and put in to eppendorf tubes. The gel slices were then destained as described in section 5.2.2.7. with 50 µl of colloidal commassie blue destain for 30 min (section 5.2.2.7.1) and then repeated until full decolouration. Gel slices were washed with 50 µl of water and then dehydrated twice for 10 min with 100% acetonitrile. After this stage samples were kept frozen at -80 °C until trypsin digestion.

5.2.2.9.4. Trypsin digestion:

Trypsin digestion of gel slices were carried out according to section 5.2.2.7.2. using trypsin (Promega sequencing grade). Extractions of peptides and Zip tip of the samples were carried out as described in section 5.2.2.7.2.

5.3. Results

5.3.1. Analysis of protein expression patterns on SDS-PAGE

Protein samples extracted from *Gloeothece* PCC 6909/1 when separated on a 10% gel, (Fig 5.1) indicated the separation of proteins within the range of 200 KDa-10 KDa, with the appearance of most proteins present above 15 Kda; however, some faint bands were also detected in the lower range below 10 Kda. Most proteins within the region could be further separated and visualized by running samples on a 7.5% polyacrylamide gel (Fig. 5.1,B). Figure 5.1 show the separation of proteins on a 7.5% gel and also indicate that most protein bands on a 10% gel can be further separated and detected on the 7.5% gel.

In order to determine protein expression changes in response to C8-HSL, gels were analysed visually. Comparison of proteins bands in the control and C8-HSL treated samples separated on 10 % SDS gel, showed detectable changes in the intensity of some protein bands indicating possible protein expression changes in response to C8-HSL. Some bands from the C8-HSL treated sample were comparatively darker compared to their corresponding control bands indicating up-regulated proteins (Fig 5.1, red arrows) and only one band was comparatively lighter indicating down-regulated proteins (Fig 5.1 black arrows). Differentially expressed protein bands when further analysed on a 7.5% gel, showed no detectable changes in intensity to their corresponding control bands (Fig. 5.1, B). Such results may be due to the separation of these proteins, which were stacked together on the 10% gel and hence appeared as a single band and visualized by its overall increase in intensity.

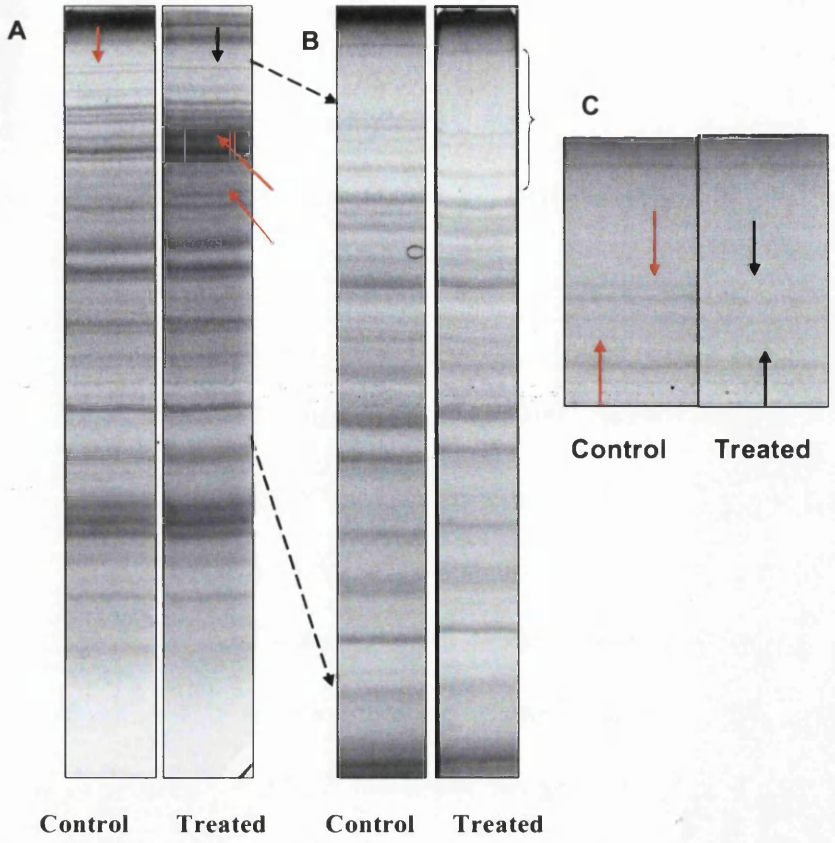


FIGURE **5. 1**. SAMPLES FROM PCC 6909/1, UNTREATED (CONTROL) AND 5 μM C8-HSL TREATED (TREATED) SEPARATED THROUGH SDS-PAGE

Protein bands were visualized by staining gels with Coomassie blue silver (G-250). Left hand pair (A) shows control and treated samples run on a 12% SDS-PAGE and right hand pair (B) shows control and treated samples run on a 7% gel. An area separated on 7.5% gel (brackets) is further magnified for visualization of protein bands (C). Broken arrows indicate the region on a 10% gel that is separated and represented on the 7.5% gel. Red arrows indicate bands containing upregulated proteins and black arrows indicate bands containing down regulated proteins.

However, it was possible to detect two down-regulated protein bands on a 7.5% gel, a magnified view of the region which has been presented in Fig 5.1(c). These observations indicate that C8-HSL treatment in *Gloeothece* in early growth stage causes changes in protein expression.

In order to evaluate the results and check if C8-HSL treatment in the wild type PCC 6909 caused similar changes in protein expression, samples from both control and C8-HSL treated cultures of PCC 6909 were run on a 7.5% SDS-PAGE. Separation of proteins from both the control and C8-HSL treated samples allowed identification of some protein bands that were differentially expressed (Fig 5.2, protein 1-8). In C8-HSL treated sample, one band of a high molecular weight was detected as down-regulated when compared to its corresponding band in the control sample (Fig 5.2, protein 3). This band was also identified in the mutant strain and showed similar down-regulation pattern. Therefore, results indicate that treatment of *Gloeothece* cells with C8-HSL causes changes in protein expression in both the mutant and the wildtype background. However, in order to demonstrate that expression changes in individual strains are solely due to C8-HSL treatments, experiments were carried out using clonal cultures therefore, any differences observed in protein expression would be expected to result from the effect of C8-HSL addition to *Gloeothece* cultures.

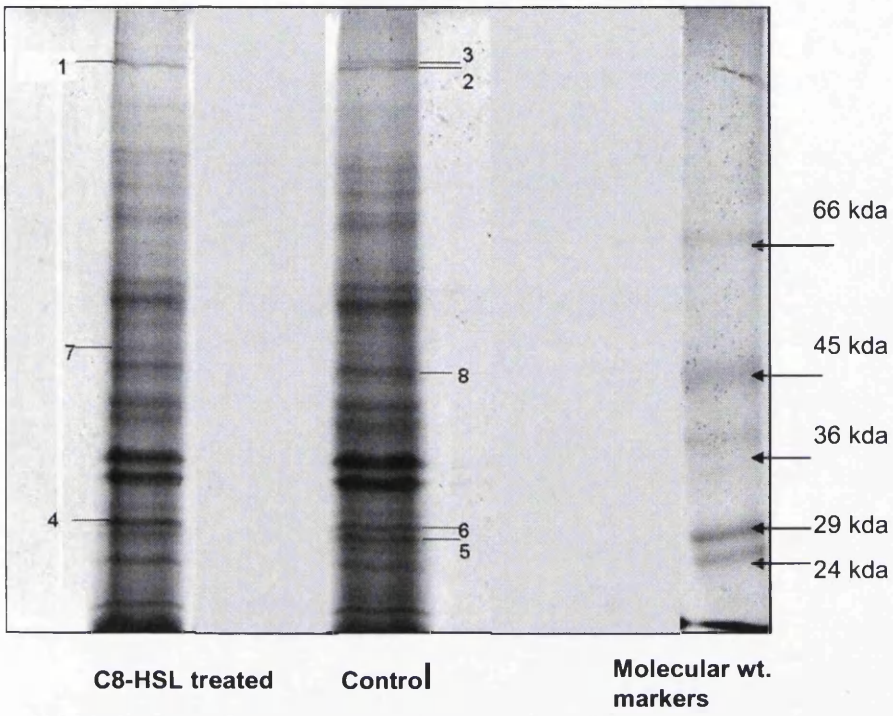


FIGURE **5. 2.** SAMPLES OF PCC 6909 CONTROL AND HSL TREATED SAMPLES SEPARATED THROUGH SDS-PAGE

Control and C8-HSL treated samples were run along molecular weight markers and separated on a 7% gel. Protein bands were visualized through Coomassie blue (G-250) stain. Protein bands numbered (1-7) were further analysed for expression changes.

5.3.1. Identification of proteins from bands differentially expressed through SDS-PAGE

In order to identify proteins that may be differentially expressed, corresponding protein bands on gels from both control and C8-HSL treated samples of PCC 6909 (Fig 5.2, band 4&6) that showed differential expression were subjected to mass-spectrometric identification. Since, *Gloeothece* does not have its genome sequenced; the peptide fragments generated by mass spectrometry were searched against a database of all organisms. Table 5.1 gives a list of proteins identified from the control and C8-HSL treated protein bands. All the identified proteins passed a good acceptance criteria set for mass spectrometric identification of proteins. Several proteins identified from the control sample were also identified from the C8-HSL treated sample (Table 5.1), indicating that the protein bands correspond to each other. Due to the detection of several proteins in one single band, samples separated on SDS-PAGE were shown not to be sufficient to identify which proteins are differentially expressed in response to C8-HSL treatment. Therefore, for further analysis of the expression changes, an alternative technique 2D-PAGE was undertaken.

	Protein identification	peptide	z	X Correc	MW	Ions
	Regulators of stationary/sporulation gene expression [Croco.]	R.VNLTDFYDAVLAAK.G	2	3.83	18453.9	20/26
	Transcriptional regulator [Pseudomonas aeruginosa UCBPP-PA1]	K.SKEQFGQALLEDYFR.V	2	2.64	21756.7	15/28
Control	Peroxiredoxin [Haemophilus somnus 2336]	K.MFIEPQEPGDPFK.V	2	3.50	26484.1	19/24
band 6	Response regulators consisting of a CheY-like receiver doma	K.HSEILNYGPLTLVPER.F	2	4.40	27949.8	19/30
	Amidases related to nicotinamidase [Nostoc punctiforme PCC	R.TLGVSPNAWTVNDAIADITRPQK.P	3	2.84	28293.1	26/88
	adenosine 5'-phosphosulfate reductase alpha subunit [Syntrophobacter]	R.ENKFYAFK.A	1	2.11	33740.3	7/14
	oligopeptide ABC transporter (permease) [Bacillus licheniformis ATCC 145]	R.YLNPEIRLK.E	1	1.89	35327.7	10/16
	Dipeptide transport ATP-binding protein dppD [Leptospira interrogans Ser]	K.AEFLLTSVGITDIQQR.L	2	2.74	36700.3	15/30
	phosphoribulokinase [Synechocystis sp. PCC 6803]	K.VVVIEGLHPLYDER.V	2	2.69	37914.1	16/26
	Regulators of stationary/sporulation gene expression [Croco.]	R.VNLTDFYDAVLAAK.G	2	3.07	18453.9	19/26
	Peroxiredoxin [Haemophilus somnus 2336]	K.MFIEPQEPGDPFK.V	2	3.20	26484.1	17/24
AHL treated	uridylate kinase [Zymomonas mobilis subsp. mobilis ZM4]	R.EAGYQLCIVVGGGNIFRGLAATAR.G	3	2.92	26515.4	21/92
band 4	Response regulators consisting of a CheY-like receiver doma	K.HSEILNYGPLTLVPER.F	2	4.02	27949.8	22/30
	dynein-associated protein, putative [Plasmodium falciparum 3D7]	K.YENPIIIEK.E	1	1.99	30841.7	12/16
	adenosine 5'-phosphosulfate reductase alpha subunit [Syntrophobacter]	R.ENKFYAFK.A	1	2.23	33740.3	10/14
	Dipeptide transport ATP-binding protein dppD [Leptospira interrogans Ser]	K.AEFLLTSVGITDIQQR.L	2	3.04	36700.3	17/30
	capsid portal protein [Enterobacteria phage 186]	R.FREVNDWLGMEVIR.F	2	2.88	38585.7	13/26

TABLE. 5.1. PROTEINS IDENTIFIED THROUGH MS ANALYSIS (FROM C8-HSL TREATED AND CONTROL) SEPARATED ON SDS-PAGE IN FIG 5.2.

5.3.4. Analysis of protein expression patterns on 2D-PAGE:

5.3.4.1. Optimization of sample preparation from PCC 6909

5.3.4.1.1. Removal of Sheath Material

Various techniques were carried out in order to attempt to remove sheath from the cells, such as the use of Yeda press and also through vigorous shaking of cell suspensions with 0.18 mm glass beads. The amount of unsheathed cells obtained through both techniques was found to constitute only a small fraction of the initial sample, indicating a low recovery of unsheathed samples for subsequent processing. IEF followed by 2D-PAGE separation of proteins from these samples showed no resolved protein spots on 2D gels. A large smear of proteins was observed in the low molecular weight region. In order to investigate if results were due to inefficient extraction and sample preparation, samples were processed according to the 2D workflow (Fig. V) and tested for their compatibility to visualize proteins on 2D-PAGE.

5.3.4.1.2. Effect of different rehydration buffers:

In order, to test the compatibility of two commonly used rehydration buffers in the solubilisation of proteins, *Gloeothece* extracts were prepared according to the 2D work flow (Fig. V) and separated on 2D-PAGE. After staining of gels, results showed few protein spots, with majority of the proteins appearing as a smear at the bottom of the gel, indicating loss and lack of separation of proteins on 2D gels.

5.3.4.1.3. Fractionation and precipitation of samples

A fractionation and precipitation step was undertaken as part of sample preparation (2D work flow, Fig. V). Fractions containing soluble proteins and membrane soluble proteins were compared against each other and also against their precipitated counter parts. (Fig. 5.3 & 5.4). A comparison of protein spots on 2D gels showed similar numbers of spots and patterns in distribution, indicating, precipitation did not result in a substantial loss of soluble proteins. However, some proteins in the precipitated soluble fraction showed distorted protein spots (Fig. 5.3) that may have arisen due to an inefficient equilibration carried out before the 2^{nd} dimension SDS-PAGE. In comparison, membrane proteins showed an increased amount of protein spots on 2D gels, of the non-precipitated membrane fraction (Fig. 5.4), indicating that a large number of membrane protein spots could be resolved by 2D-PAGE under these conditions. The membrane precipitated fraction, however failed to show any separation of proteins that was observed by the presence of a large smear of proteins in the low molecular weight region of the gel (Fig. 5.4). This result was found to be similar to the previously mentioned 2D-PAGE results (section 5.3.4.1.2.), where a precipitation step was carried out during sample preparation of whole cell *Gloeothece* proteins, indicating that precipitation of these proteins may result in poor solubilisation and subsequent exclusion of proteins on 2D gels.

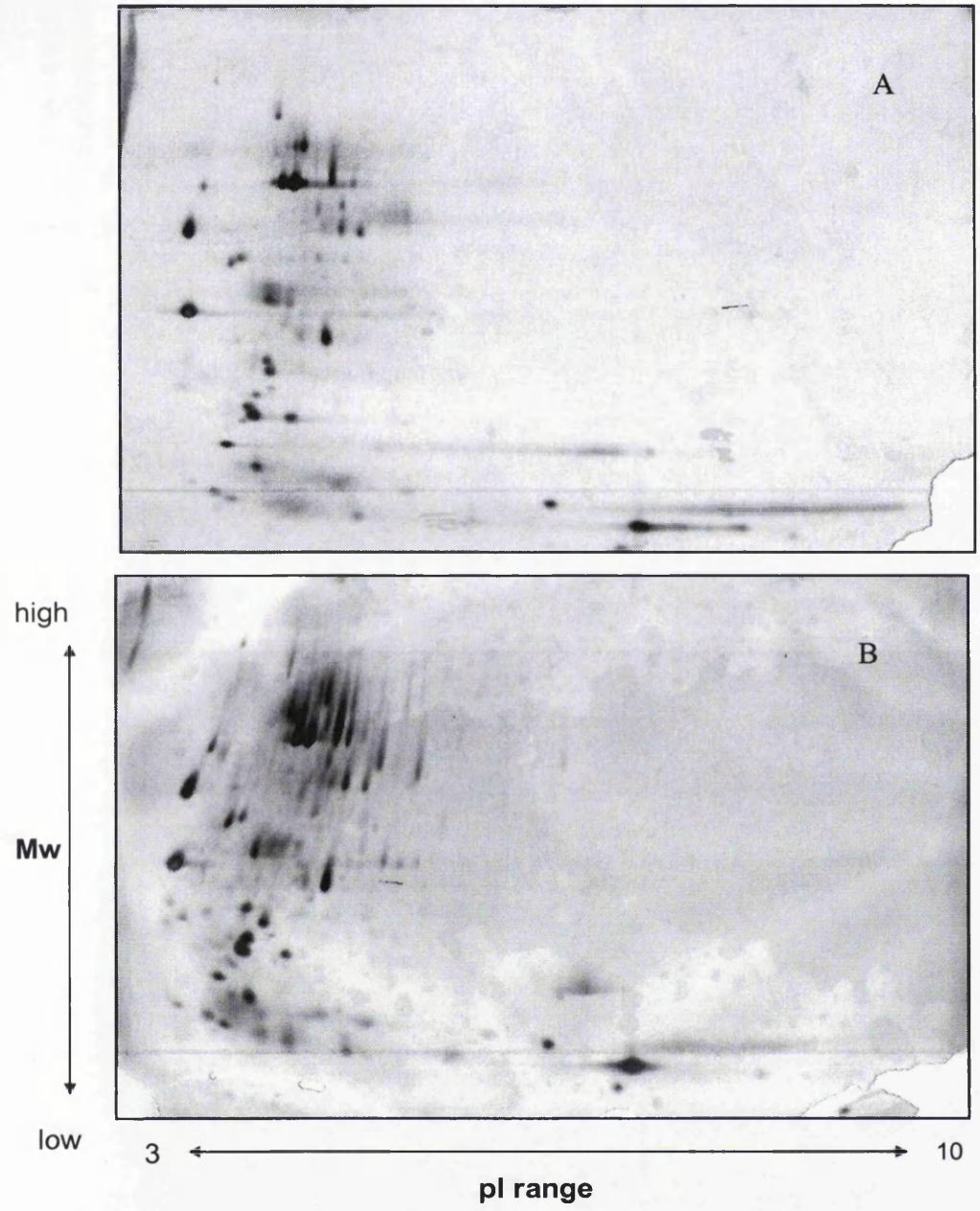


FIGURE **5. 3**. SOLUBLE PROTEINS FROM *GLOEOTHECE* PCC 6909 SEPARATED THROUGH 2D-PAGE.

Samples were prepared by fractionation and separated in the 1st dimension on a 3-10 pI range and on the 2nd dimension on a 10% gel (laemmli). (A), represents a sample without TCA acetone precipitation and (B) represents a TCA acetone precipitated sample.

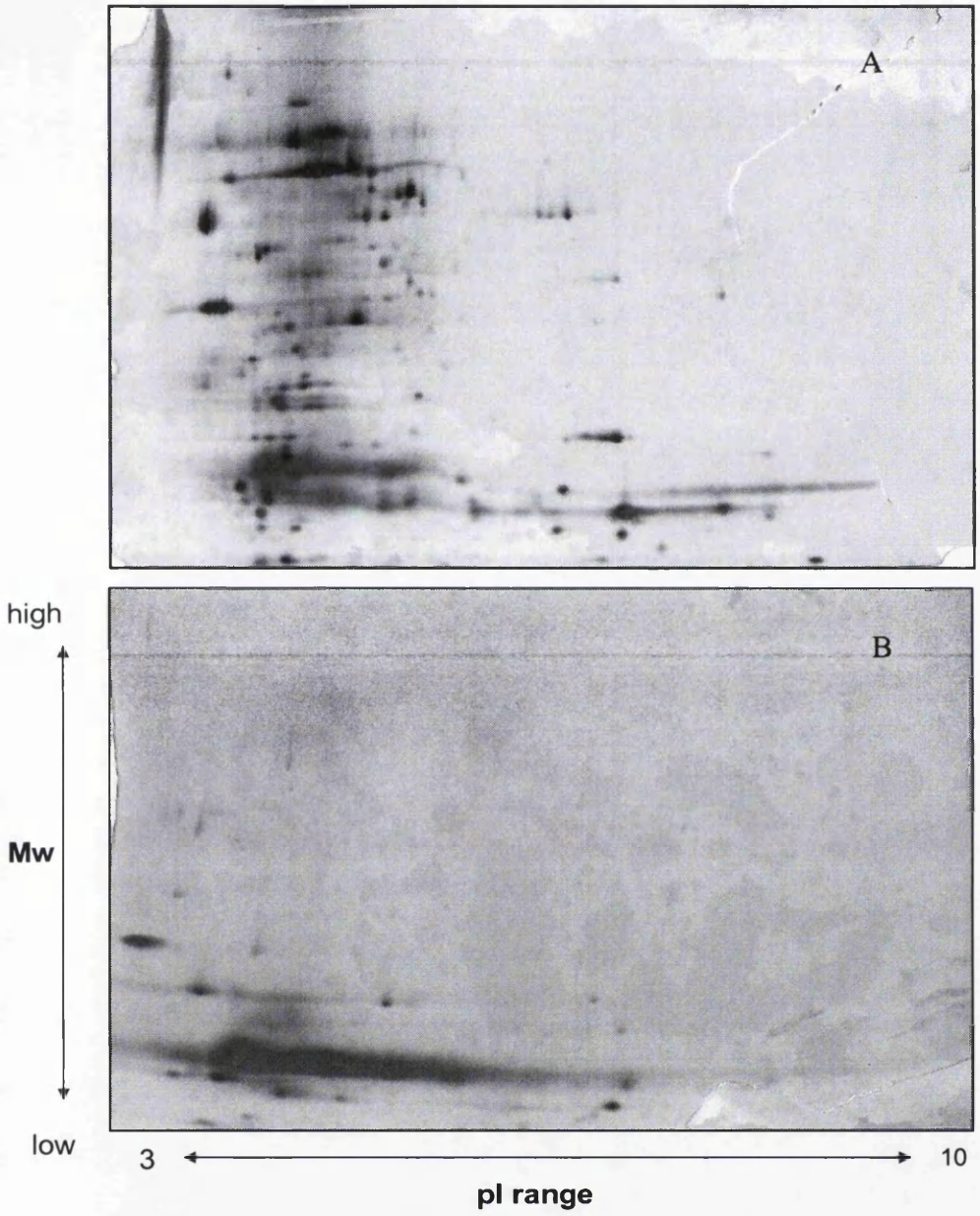


FIGURE **5. 4**. DETERGENT SOLUBLE MEMBRANE PROTEINS FROM *GLOEOTHECE* PCC 6909 SEPARATED THROUGH 2D-PAGE

Samples were prepared by fractionation and separated in the 1st dimension on a 3-10 pI range and on the 2nd dimension on a 10% gel (laemmli). (A), represents a sample without TCA acetone precipitation and (B) represents a TCA acetone precipitated sample

5.3.5. Optimization of sample preparation from PCC 6909/1

Due to the possibility of sheath material interfering with complete separation of proteins on 2D gels, samples were subsequently processed and used in optimization of sample preparation from the sheathless mutant PCC 6909/1. Samples were prepared according to the 2D work flow (Fig. VI) designed to optimize sample preparation for 2D-PAGE experiments. TCA acetone precipitation of whole cell proteins was performed just after cell breakage. This process was found to remove interfering substances with greater efficiency through increasing solvent accessibility to most parts while precipitating both membrane and soluble proteins. Precipitation of whole cell extracts was found to result in efficient removal of solvent extractable pigments such as chlorophyll. Samples prepared under such conditions, when run on 2D-PAGE showed an overall increase in the number of spots detected with a good representation of the whole proteome of *Gloeothece* over a wide pI range and a negligible background smearing (Fig. 5.5). Fig. 5.5 shows 2D separation of protein on a 3-10 pI and a 4-7 pI range IEF system. Protein spots separated on a 3-10 pI range gel shows most proteins to be present within the pI range of 4-7. Further separation of protein on a 4-7 pI range shows an increased abundance of protein spots detected within this range. Most proteins were highly resolved, indicating a good separation of proteins with no proteolysis. However, a large smear of proteins in the low molecular weight region was subsequently identified as high abundance phycobiliproteins. In some cases interferences at the acidic end on the gel (Fig. 5.5, top gel) were detected and found to be the cause of an incomplete wash with acetone after TCA acid precipitation, causing interference with separation of proteins during the 1st dimension IEF.

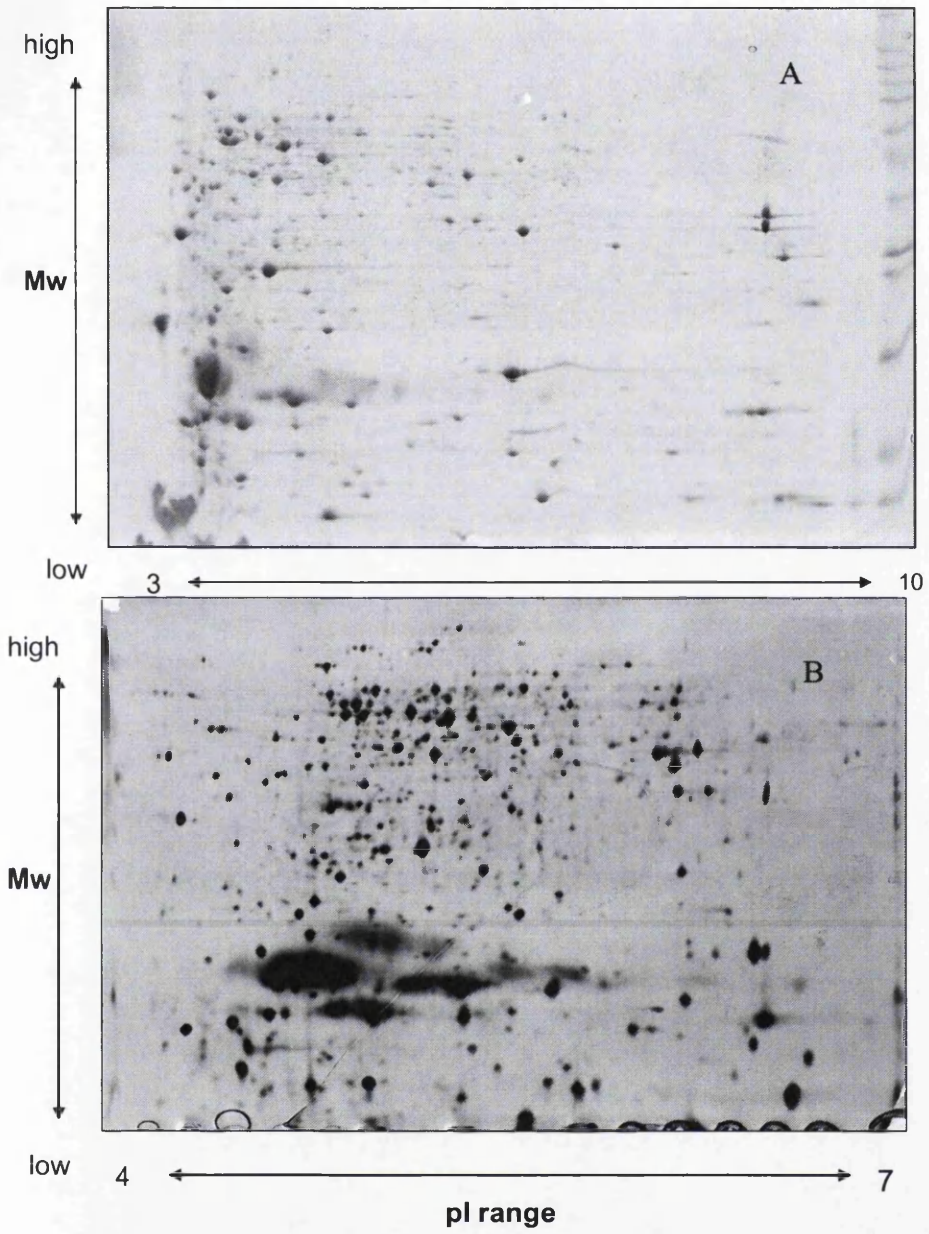


FIGURE **5. 5**. *GLOEOTHECE* PROTEINS EXTRACTED WITH THE OPTIMIZED METHOD SEPARATED ON A 2D-PAGE

Proteins from PCC 6909/1 were extracted using the optimized method and separated on 2D-PAGE.The top gel represents proteins separated on a 3-10 pI range and bottom gel represents proteins separated in the 4-7 pI range. Proteins were separated in the 2nd dimension on a 10% gel (laemmli) and protein spots were visualized after silver staining.

However, centrifugation of the precipitated sample at a relatively low speed (8,000 g) for a longer time (10 min), and allowing subsequent washes with acetone resulted in removal of these interferences (Fig. 5.5 bottom gel). Sample preparation using this optimized technique therefore allowed efficient recovery of whole cell proteins (membrane and soluble proteins) in one step. The extracted samples were used in subsequent experiments in analysis of protein expression changes in response to C8-HSL.

5.3.6. Changes in protein expression in *Gloeothece* PCC6909/1 in response to C8-HSL

5.3.6.1. Analysis of proteins separated on 2D gels within the 3-10 pI range

A silver-stained 2D gel showing the separation of proteins achieved in the 3-10 pI range is presented in Fig. 5.6. This gel was selected as a reference gel to which all other protein spots in both AHL treated and control gels were matched in order to identify differentially expressed proteins (showing differential expression > 2 fold). The differentially expressed proteins are indicated with arrows on the gel. Two hundred and thirty four protein spots of interest were primarily selected using the 2D software based on their differential expression levels. Among them several proteins were found to be differentially expressed with a significant difference measured through the t-test ($p<0.05$). Within these, 27 proteins showed more than 1.5 fold expression changes and are summarized in Table 5.2. The table gives the list of protein spots with their assigned spot number (as referred by the 2D software) and their t-value with corresponding

expression changes. From the table it is evident that, for certain proteins, the expression changes are highly significant with a significance level $p<0.05$ measured through the students t-test (Table 5.2). Such a significance level can strongly support the hypothesis that C8-HSL induces changes in protein expression. However, significant changes in less than 1.5 fold were not considered. 14 protein spots showed greater than 2-fold expression changes. Differentially expressed proteins were mainly found to be within the 4-7 pI regions, with only one spot detected at the basic end of the gel (spot 240). Proteins that showed differential expression ($p< 0.05$) with more than 1.5 fold changes were subjected to trypsin digestion and the peptides were identified through mass spectrometry.

FIGURE **5. 6**. A REFERENCE GEL IN THE 3-10 PI RANGE SELECTED TO ANALYZE DIFFERENTIALLY EXPRESSED PROTEINS.

Red arrows indicating upregulated proteins and black arrows indicate downregulated proteins in response to C8-HSL treatment in Gloeothece PCC 6909/1. Numbers correspond to proteins presented in table 6.2.

spot number	Pvalue	Expression change
124	0.027	-2.5
125	0.012	-2.5
136	0.024	2.4
170	0.001	-2.2
202	0.019	-1.9
207	0.023	-1.9
213	0.034	-1.9
237	0.01	-1.8
240	0.043	1.7
242	0.001	-1.7
248	0.04	-1.7
251	0.013	-1.7
257	0.038	-1.6
264	0.034	-1.6
265	0.002	1.6
271	0.011	-1.5
274	0.045	-1.5
1102	0.05	-2.5
1158	0.05	2.5
2184	0.05	-2
7746	0.005	3.2
7747	0.028	2.4
7775	0.044	3
7791	0.05	-2.4
7801	0.004	-2.4
7819	0.048	-2.1
7821	0.043	-2.4

TABLE 5. 2. SUMMARY OF DIFFERENTIALLY EXPRESSED PROTEINS ON A 3-10 PI RANGE 2D GEL.

Protein spots assigned by the 2D software with their p-value and expression changes for Gloeothece PCC 6909/1 proteins run on a 3-10 pI range 2D gels in Fig. 5.6.

5.3.6.2. Analysis of proteins on 4-7pI range

Triplicate gels of each control and C8-HSL treated proteins separated on a 4-7 pI range on 2D gels were analysed. The reference gel against which all other gels were aligned is presented in Fig 5.7. The expression level, of 43 proteins showed differential expression ($p<0.05$, students t-test). However proteins that showed more than 1.5 fold expression changes were selected for further analysis. Table 5.3. gives a list of proteins that exhibit a statistically significant difference (t-test $p<0.05$) with an expression change within 1.5 fold. Several proteins were found to show increased expression and also decreased expression in the response to C8-HSL with a high abundance showing increased expression levels indicating upregulated proteins. Among 31 upregulated proteins 15 showed more than two fold expression changes and only 4 proteins among 6 downregulated proteins showed more than 2 fold expression changes. These findings suggest that cells mainly respond through the upregulation of various proteins. Some of these proteins also exhibited a highly significant difference ($p<0.01$, Table 5.3). Many of the proteins differentially expressed represent low abundant proteins observed from their size and intensity on the 2D gels. A representative example of one of the protein images derived from the 2D software (protein 3 in Fig.5.7), showing differential expression of that protein, (based on comparison of the triplicate analyses for C8-HSL-treated and control culture) is presented in Fig. 5.8.

FIGURE **5. 7**. A REFERENCE GEL IN THE 4-7 PI RANGE SELECTED TO ANALYZE DIFFERENTIALLY EXPRESSED PROTEINS.

Red arrows indicating upregulated proteins and black arrows indicate downregulated proteins in response to C8-HSL treatment in Gloeothece PCC 6909/1. Numbers correspond to proteins presented in table 6.3.

spot number	p value	Expression change
1	0.002	4.2
3	0.002	-4
4	0	3.6
5	0.025	3.5
6	0.043	-3.1
8	0	3.1
9	0.011	3
10	0.008	-3
15	0.028	2.5
16	0.013	2.5
17	0.001	2.5
19	0.029	2.4
22	0.004	2.4
29	0.045	-2.1
30	0.042	2.1
31	0.004	2.1
41	0.019	1.9
46	0.036	-1.8
55	0.045	1.7
56	0.036	1.7
57	0.027	1.7
60	0.002	1.7
61	0	1.7
62	0.043	1.7
64	0.042	1.7
75	0.032	1.6
76	0.006	-1.6
78	0.032	1.6
82	0	1.5
84	0.002	1.5
689	0.058	2
4994	0.034	1.6
5014	0.018	2.6
5015	0	1.6
5050	0.012	1.6
5065	0.007	1.5

TABLE 5. 3. SUMMARY OF DIFFERENTIALLY EXPRESSED PROTEINS ON A 4-7 PI RANGE 2D GEL.

Protein spots assigned by the 2D software with their p-value and expression changes for Gloeothece PCC 6909/1 proteins run on a 4-7 pI range 2D gels in Fig. 5.7.

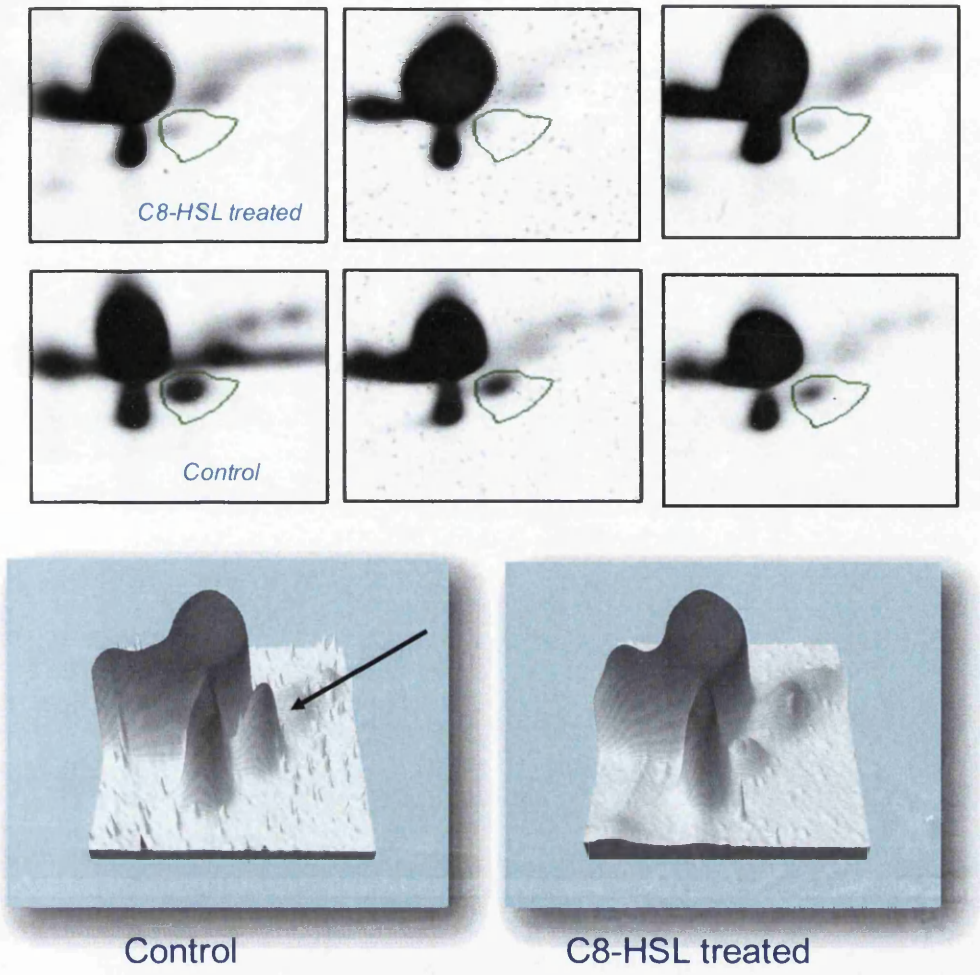


FIGURE **5. 8**. A PROTEIN SHOWING DIFFERENTIAL EXPRESSION ON A 4-7PI RANGE 2D GEL IN RESPONSE TO C8-HSL TREATMENT.

Expression changes of a protein detected on 2D gels. A protein detected in each control & C8-HSL treated sample showing decreased expression in response to C8-HSL (top figure) and a 3D view of that protein detected through 2D software which was subsequently identified as an aldolase (bottom figure).

5.3.6.3. Tryptic digestion and protein identification

Several proteins differentially expressed in both the 3-10 and 4-7 pI range 2D gels were subjected to tryptic digestion and the sequences of the resultant peptides were identified

by HPLC-MS/MS combined with database searches for protein identification. Identification of proteins from *Gloeothece,* for which the genome has not been sequenced, relies on homology between the same proteins from different species and the occurrence of conserved sequences in those proteins. Among proteins that showed a significant change in expression in response to treatment with C8-HSL, 7 were identified in the database search, based on matching the identity of more than one peptide (Table 5.4). All the peptides reported in Table 5.4 passed the acceptance criteria set, judging the XCorr of individual peptides, for determination of peptide sequences from MS/MS data (Personal communication with software manufacturer). However, identification of 3 proteins (proteins 240, 124, and 10, Table 5.4) is more tentative, depending on a single peptide match. Consequently, in the case of these proteins, the results of the database search were combined with matching other experimentally-determined characteristics. For example, protein 240 produced only 1 peptide which led to a tentative identification of the protein as membrane bound O-acyl transferase. The theoretical pI value of this protein is very basic, (approximately 10) and such a value is consistent with the position of the *Gloeothece* protein (at the basic end of the 2D gel - Fig. 5. 6). Encouragingly, although searches were carried out using a proteomic database based on all the available proteomes, many of the protein identifications shown in Table 5.4 are from bacterial species. This suggests that, in these cases, reliance on conservation of amino acid sequences for identification of proteins from the *Gloeothece* was sufficient. Further discussion on the significance of identified proteins is discussed in the final discussion section.

No.	old chang	P value	Protein	Species	peptides	z	Xcorr	Ions matched
1158	2.474	0.058	Glutamate synthase	*Plectonema boryanum*	R.ADRPGGLLMYGIPNMK.L	3	3.06	23/60
(Fig. 5.6)					R.GQSLVVWAFNEGR.G	2	3.74	19/24
				Bacillus halodurans	K.TNNFPEFTGR.V	2	2.47	15/18
					K.AGHWVTVFER.E	2	2.36	14/18
				Synechocystis sp. PCC 6803	R.AANNPWPEWPK.V	2	2.29	12/20
7775	3.001	0.044	ribulose-1,5-bisphosphate	*Synechoco*	K.TPIIMHDYLTGGFTANTTLAK.F	3	4.52	30/80
(Fig. 5.6)			carboxylase/oxygenase	*Prochloron sp.*	R.FLFVQEAIEK.A	2	3.25	16/18
					K.TFQGPPHGIQAER.D	2	2.80	15/24
				Euonymus	K.TFQGPPHGIQVEK.N	2	2.47	14/24
					R.NKGLLLHIHR.A	2	2.45	13/18
7747	2.439	0.028	Chorismate synthase	*Nostoc punctiforme PCC 73102*	K.GLGEPVFDK.L	2	2.27	13/16
(Fig. 5.6)					K.YRPSHADATYDAK.Y	3	2.94	23/48
				Thermosynechococcus elongatus	R.SGGVQGGISNGENIILR.V	2	3.24	19/32
						3	3.28	25/64
				Brucella suis 1330	R.CGAVIDTASLIR.K	2	2.57	16/22
240 (Fig. 5.6)	1.73	0.043	membrane bound O-acyl transferase		R.RANEASLFIDFVK.R	2	2.27	13/24
3	-4.029	0.0026	aldolase		K.GILAADQSTGSIAK.R	2	2.96	20/26
(Fig. 5.7)					K.LQSIGZTBTZZBR.R	3	2.99	16/24
8	2.059	0.043	ABC transporter	*Nostoc sp.*	K.QMPAGYETQIGEGGGMLSGGQR.Q	3	3.10	26/84
(Fig. 5.7)			ABC-type amino acid transporter		K.SYSAILGPGLSSQASAISPIAEK.G	3	2.66	28/88
30	-2.452	0.027	ATP synthase beta subunit /	*Penthorum sedoides*	R.FLSQPFFVAZVFTGSPGK.Y	2	4.02	21/34
(Fig. 5.7)			ATPase beta subunit	*Lactobacillus gasseri*	R.GMEVEDTGAPISVPVGK.D	2	3.52	18/32
				Synechococcus sp. PCC 7002	R.IFNVLGEPVDEK.G	2	2.95	17/22
				Amphidinium operculatum	R.VANAALTMAEYFR.D	2	2.93	16/24
				1711264A CF1	R.FVQAGSEVSALLGR.M	2	2.90	21/26
124 (Fig. 5.6)	2.402	0.0245	transcriptional regulator LYSR-type	*Escherichia coli O157:H7*	R.VDATFSSGCQLMINAIR.K	3	2.85	23/64
136	-1.949	0.0187	enolase	*Paramecium multimicronucleatum*	K.VQIVGDDLLVTNPTR.V	2	4.09	19/28
(Fig. 5.6)				*Azotobacter vinelandii*	R.SGETEDTTIADLAVATR.A	2	3.32	22/32
						3	3.95	30/64
				Clostridium acetobutylicum	K.GIQLGVANSILIK.L	2	3.00	18/24
10 (Fig. 5.7)	3.02	0.0084	PS II Mn-stabilizing polypep. precur.	*Cyanothece*	K.RQEAEYVPGK.L	2	2.41	14/18

TABLE. 5.4. IDENTIFICATION OF PROTEINS DIFFERENTIALLY EXPRESSED (ON 3-10 & 4-7 PI RANGE 2D GELS) IN RESPONSE TO C8-HSL TREATMENT IN CULTURES OF *GLOEOTHECE* PCC 6909/1

5.3.7. Phosphoprotein analysis

In order to test the reproducibility of the phosphoprotein purification system, duplicate samples extracted from a control culture of PCC 6909/1 were separately purified and run on a 7% SDS-PAGE (Fig. 5.9). Fig 5.9 show that proteins separated on a SDS-PAGE from a separately purified control culture to be similar, indicating the reproducibility of the purification system. In order to test if C8-HSL addition to *Gloeothece* cultures resulted in changes in the phophoproteome, proteins extracted from C8-HSL treated and control cultures were purified and run along the other two control samples (Fig. 5.9). Results in Fig 5.9 show that some bands in the C8-HSL treated sample are more heavily stained than their corresponding control bands indicating upregulated expression or differential phosphorylation status of these proteins. Protein bands that showed changes were further subjected to trypsin digestion and the proteins were identified by MS. Results in Table 5.5 show the identification of these proteins. All proteins identified passed good acceptance criteria judged through the mass-spectrometry. Several of these proteins were also characterized as phosphorylated proteins, thus further confirming changes in the phosphoproteome of *Gloeothece* in response to C8-HSL treatment.

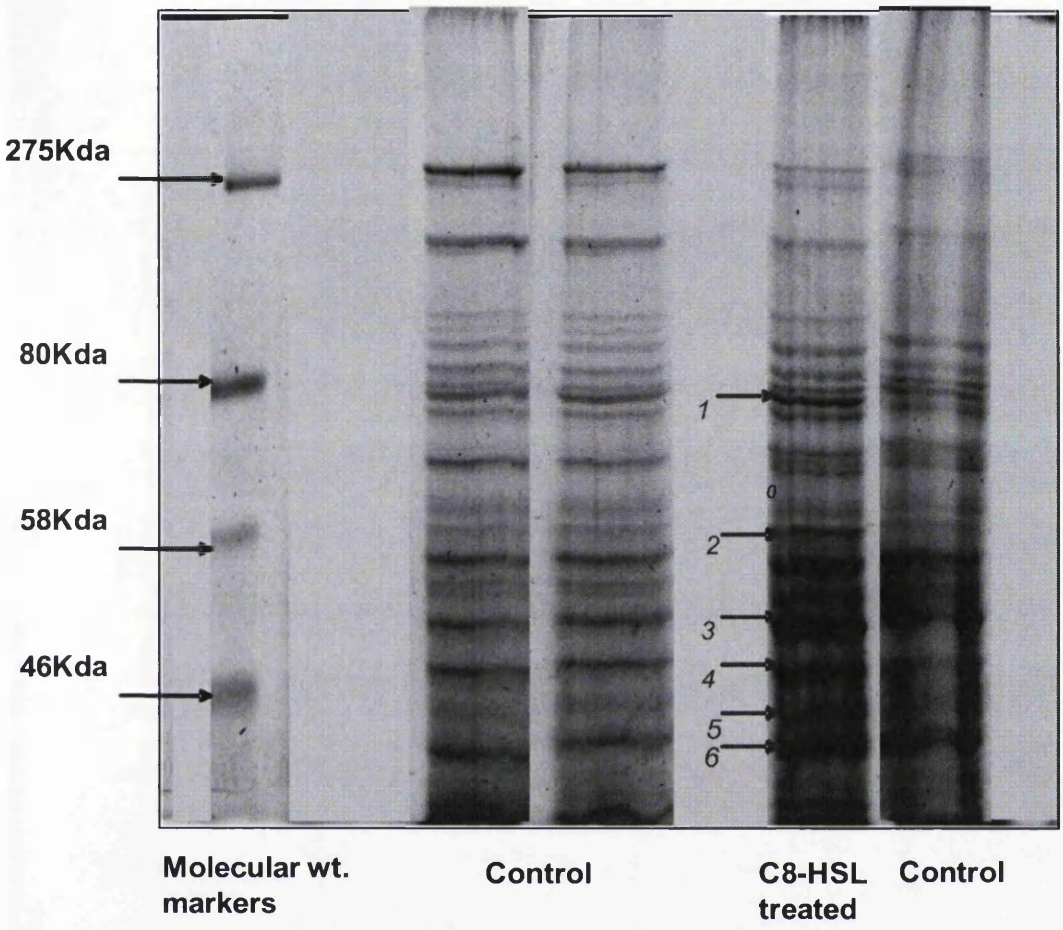


FIGURE **5. 9**. PURIFIED PHOSPHOPROTEINS SEPARATED ON SDS-PAGE

Phosphoproteins purified from Gloeothece PCC 6909/1 run on a 7% SDS-PAGE; Two samples separately purified from a control culture and samples purified from a C8-HSL and control culture were run along molecular weight markers and visualized through Coomassie blue G-250 (blue silver) stain.

Sample	Peptide identity	Peptide	Organism	Z score	X corr.	ion matched
1	ABC transporter oligopeptide binding protein	R.EPTCLDPHVQGDMPQVFVAR.Q	*Zanthomonas axonopodis*	3	2.96	29/76
	ABC-type dipeptide/oligopeptide/nickel transport systems	R.PSIWILIAILGLLSWESTAR.V		3	2.96	25/76
	ABC transporter ATP-binding protein alr1927	K.QMPAGYETQIGEGGGMLSGGQR.Q	*Nostoc sp.*	3	2.74	32/84
2	60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1)	K.EALATLVVNR.L		2	2.53	14/18
	60kD chaperonin 2	R.LEDALNATK.A	*Thermosynechococcus elongatus* BP-1	2	2.29	14/16
	60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1)	R.IEDALNATK.A		1	1.81	10/16
	60 kDa chaperonin 2 (Protein Cpn60 2) (groEL protein 2)	K.IALVQDLVPVLEQVAR.A		2	3.34	19/30
	60 kDa chaperonin 1 (Protein Cpn60 1) (groEL protein 1)	R.IAENAGQNGAVIAER.V		2	4.00	20/28
	Hsp60	K.VGAATETELK.E	*Bacillus thuringiensis*	2	2.72	16/18
3	6-phosphogluconate dehydrogenase	R.LPQNLTQAQR.D	*Nostoc sp* .PCC 7120	2	2.75	16/18
4	ribulosebisphosphate carboxylase	K.TFQGPPHGIQAER.D		2	2.73	16/24
5	phycobiliprotein ApcE	R.AYSQSISYLESQVK.S	*Synechococcus Sp* . PCC 7002	2	2.44	17/26
6	P-type ATPase, putative	K.YIPYFQKYVEK.I	*Plasmodium berghei*	2	2.24	15/20
		R.FPLLWKNGNMVLLR.K		2	2.26	14/26

TABLE 5. 5 MS IDENTIFICATION OF PURIFIED PHOSPHOPROTEINS DIFFERENTIALLY EXPRESSED ON SDS-PAGE

5.3. Discussion

Results in this chapter indicate that C8-HSL treatment of *Gloeothece* cells results in changes in protein expression. Preliminary investigation carried out on samples separated on SDS-PAGE indicated some proteins to be differentially expressed. SDS-PAGE was found to be a very good technique since it required less sample preparation and most proteins were found to be readily solubilised in sample buffer containing SDS. Moreover, the technique suffers from less interference caused by the presence of salts in the extraction buffer than 2D-PAGE. However, due to the presence of sheaths in the wildtype PCC 6909, protein bands separated on SDS-PAGE were found to be less sharper compared to the mutant strain Fig. 5.1 & 5.2). However, this could also arise due to removal of contaminants by a precipitation step carried out on the mutant but omitted in the wildtype. The percentage of protein loss during precipitation was investigated through measuring protein concentration before and after precipitation. It was found that precipitation could result in 22% loss in proteins. However, most of the loss during the investigation could arise from inefficient resolubilization of proteins after precipitation. The protein estimation carried out before and after precipitation may also suffer inappropriate comparison using detergent solubilised samples after but not before precipitation. Although SDS-PAGE showed indication of protein expression changes in response to treatments, the technique suffered from the inability to separate proteins of similar molecular weight masses and hence, when combined with MS for identification, it was not found to be a suitable technique to identify proteins differentially expressed. 2D-PAGE, which is a combination of isoelectric focusing and SDS-PAGE, was found to greatly resolve this problem through separating individual proteins based on their isoelectric point and molecular weight masses. However, first dimension isoelectric

focusing (IEF) was found to be subjected to interference caused by *Gloeothece* extracts, possibly due to the presence of ionic substances, such as lipids and nucleic acids in the extract, and therefore a sample preparation and optimization step was required to remove such contaminants.

In testing the techniques involved in removing the sheath of *Gloeothece*, for subsequent sample preparation for 2D-PAGE, it was apparent that only a small fraction of sheath free cells could be obtained and often large volumes of samples were necessary for processing to obtain enough proteins for the experiments. The technique was also found to be time consuming and also costly since huge amounts of cultures were needed to be built up over time and the amount and cost of Ficoll used was not feasible. Several initial attempts using whole cell proteins extracted from these samples to separate proteins on 2D gels failed. A fractionation technique to fractionate samples into a soluble fraction (soluble protein) and a detergent soluble membrane fraction (membrane protein) was also undertaken to identify the fraction causing interference. Results showed that using both fractions proteins could be detected and resolved on 2D gels. One technique that was found to be useful in making this difference with previously undetected spots was ultracentrifugation. This technique is useful for removing high molecular weight contaminants like polysaccharides and nucleic acids, allowing the separation of proteins. However, with the preparation of membrane soluble fraction which was basically extracted from the pellet obtained from ultracentrifugation, the possibility of the membrane soluble fraction contaminated with other high molecular weight contaminants cannot be overruled. A background smear on 2D gels of the membrane protein fractions (Fig 5.4) could be an indicative of the presence of these contaminants such as nucleic acids. A second ultracentrifugation step of membrane

proteins could however be useful in eliminating background smear but could not be undertaken due to the low recovery of membrane proteins. Another failure in 2D-PAGE was detected with samples prepared from the precipitation of the membrane soluble fraction. Membrane proteins are generally known to be difficult to resolubilize once precipitated and the presence of interfering substances, for example, viscous sticky substances like slime and sheath material in the membrane samples may interference with resolubilization and subsequent processing for 2D-PAGE. It is also not known if the detergent present in the membrane solubilised fraction interferes with the precipitation process. Since precipitation is a good method to remove contaminants, mostly carbohydrates, pigments and polyphenolic compounds, it was still considered to be useful towards successful 2D-PAGE and subsequently employed during an optimized method for protein extraction from *Gloeothece* PCC 6909/1. The activities of many proteases are also known to be inactivated during the precipitation step and therefore beneficial in early sample preparation. An optimized technique involving precipitation of proteins was considered while proteins are in detergent free extraction buffer. Therefore, the aim was to optimize a protein extraction condition from *Gloeothece* PCC 6909/1 that would allow one step extraction of both membrane and soluble protein fractions and then applying the methods of sonication and ultracentrifugation on the total extracted proteins for efficient processing for 2D-PAGE. Using the optimized method of extraction, more than 400 protein spots were detected on 2D-PAGE gels, which are considered to be significantly higher than the number of spots detected from other cyanobacteria, including *Synechocystis spp* (Fulda *et al.*, 2006), *Synechococcus sp.* strain PCC 7942 (Koksharova *et al.*, 2006) and *Anabaena sp* (Barrios-Llerena *et al.*, 2007).

The amount of protein loaded for 2D gels in the 4-7 pI range was optimized and adjusted to 100 μg for detection through silver stain. This concentration allowed visualization of low abundance proteins on the gel. Various studies on gene expression analysis mainly focused on these low abundance proteins that may undergo expression changes as a response to a stimulus while keeping most of the high abundance housekeeping proteins normally functional. However, since quorum sensing in many cases is associated with stress, it may also cause changes in the expression of important metabolic pathways and hence the overall expression pattern of the whole proteome in *Gloeothece* was investigated in both the 3-10 and 4-7 pI range. The 3-10 pI range was initially used to find the region on 2D gels in which most proteins showed expression changes. Although a generalized view of expression changes based on acidic protein or basic proteins was not evident, most diferentially expressed proteins were found to be distributed between 4-7 pI ranges.

5.3.1. Rationale for the AHL concentration used in the protein expression experiment

To see if *Gloeothece* responses to the C8-HSL produced and what kind of proteins might be expressed, protein expression was studied by treating the cultures with synthetic C8-HSL followed by 2D gel electrophoresis. For the analysis of protein expression, C8-HSL was added to a concentration of 10 μM. This concentration is high compared with the highest steady-state concentration observed in cultures of *Gloeothece* (Chapter 3 Fig. 3.7A). However, there are reports of pH-dependent degradation of AHLs in culture medium (Yates *et al.*, 2002). For example, at pH 7.2 (the pH of the medium in which the *Gloeothece* cultures were maintained) 70 % of C4-

HSL (the chain length closest to C8-HSL and for which data are available) is converted to the inactive form. Using this as a model, it is expected that the concentration of the active form of C8-HSL that would remain in the experimental cultures from the addition of C8-HSL to an apparent concentration of 10 μM, would be 3.0 μM.

5.3.2. Expression changes of proteins on a 2D gel

In this experiment the two treatment samples were derived from a single parent and therefore, the two individual treatment groups were clonal in nature. This is thought to eliminate any variations among the two groups (despite any mutations that could take place) and in use of any biological replicates, that are generally used to encounter variations among natural populations. To evaluate results in expression changes, technical replicates were used and expression changes were analysed by applying standard statistical methods. The number of differentially expressed proteins by C8-HSL treatment could be represented as 11 % of total protein detected. However, the number of proteins can be affected by a number of factors. Proteins expressed differently can be obscured by phycobiliproteins, these are the light harvesting pigments in cyanobacteria which constitute about 60 % of the total soluble protein with a molecular mass of 15 Kda that have been found to appear as a smear on the gel (Fig. 5.6 & 5.7, lower right hand corner) and were easily identified from this region by LC/MS/MS. The expression of many genes involved in quorum sensing may depend on other factors and may not be induced to externally added C8-HSL at early stages of growth and many late genes may not respond to exogenously added AHL until the culture reaches the stationary phase of growth (Schuster *et al.*, 2003; Whiteley *et al.*,

1999). Also many proteins, especially hydrophobic membrane proteins are difficult to resolve by 2D proteomics and progress is still being made towards optimising conditions for their efficient extraction.

The difficulty in protein identification and assigning proteins to a particular metabolic pathway in *Gloeothece* is mainly due to the unknown genome sequence and lack of reports on different metabolic pathway in this organism. Therefore, the LC/MS/MS data files were searched against databases of all organisms. Encouragingly, many of the proteins shown in Table 5.4 are from bacterial species, which suggested, that the reliance on sequence homology and conservation for the identification of proteins from *Gloeothece* to be sufficient in this case. The main reason of having less peptide matches may be due to the evolutionary heterogeneity of cyanobacterial species which show more divergent protein sequences between individual strains than for the same proteins in higher organisms, therefore only those proteins that showed the greatest level of confidence in the results were presented.

5.3.3. Phosphoproteins

Phosphorylated proteins in the cyanobacterium *Gloeothece* have not been investigated before. Since phosphorylated proteins are known to comprise a small fraction (2-10%) of the total proteins, a specific enrichment technique that allows purification of phosphorylated proteins from non phosphorylated ones has great advantage. In this study a specific phosphoprotein purification kit was used to purify phosphorylated proteins. The kit uses specific affinity columns to which the phosphoproteins bind. The phosphoproteins are then eluted from the column with the buffer provided. An equal

concentration of proteins were loaded in to the column that resulted in approximately same concentrations of phosphoproteins to be purified from both samples, suggesting that the recovery of phosphoproteins could be reproducible. Protein estimation before and after purification showed, that the phosphoproteome of *Gloeothece* PCC 6909/1 comprises of only 2.3% of the total proteome (1.5 mg initial protein resulted in 35 μg of total phosphoprotein).

Many phosphoproteins are found to accumulate under various stress conditions. In order to investigate if C8-HSL addition to the medium resulted in any changes in the phosphoproteome of *Gloeothece* cells, PCC 6909/1 the sheathless mutant was treated with C8-HSL in early stages of growth. To ensure that sufficient time is given with the treatment to allow detection of any possible differences that may be caused by the treatment, cells were treated first with 25 μM C8-HSL for a longer time (48 h) and then again with 25 μM C8-HSL for a shorter time (1 h) just before extraction of proteins. Also to ensure that any differences were not caused by any external stimulus samples were processed rapidly and simultaneously as possible. Samples were initially separated on a 10% gel that allowed visualization of any changes in protein expression and also allowed to detect the molecular weight region where most proteins were separated. Proteins bands were observed in the high molecular weight region above 45 Kda and therefore, samples were further analysed to a 7% gel (Fig. 5.9). The proteins identified through MS were interestingly found to be phosphorylated from various reports judging them as phosphoproteins. However, it is not possible to tell if these proteins become phosphorylated and de-phosphorylated in response to C8-HSL and what the phosphorylation status of each protein is. Much more work may be needed to identify if any of these proteins undergo phosphorylation in response to C8-HSL treatment. The

current knowledge from these results holds the possibility that there may be some changes associated with C8-HSL treatments.

A broad discussion on proteins identified is discussed in the final discussion section, and finally to summarize the questions asked at the beginning of this chapter:

- *Gloeothece* responds to C8-HSL through changes in gene expression as observed through expression changes of various proteins.
- *Gloeothece* responds through both upregulation and downregulation of proteins, of the protein which showed a change although most proteins responded through an upregulation.
- Proteins identified to be differentially expressed were involved in various cellular processes suggesting *Gloeothece* responds to C8-HSL through global changes in protein expression.
- Significant changes in the phosphoproteome of *Gloeothece* were observed identifying various proteins that are regulated through a stress response in other organisms.

CHAPTER 6

C8-HSL mediated changes in physiological conditions

6.1. Introduction

Quorum sensing (QS) is generally associated with gene expression that results in phenotypic changes in microorganisms. These changes, associated with AHL signals, in gram negative bacteria are diverse and include the secretion of various enzymes, exopolysaccharide production, swarming, biofilm formation, stationary phase survival (see Table 1.2). It is often very difficult to tell by the nature of AHL molecules what phenotypic changes are associated with the organism as the AHL signals are highly specific to individual bacteria in their effect on gene expression. For example, the CepI/R QS system of *Burkholderia cepacia* is responsible for the production of C8-HSL that results in positive regulation of extracellular virulence factors, swarming motility and biofilm formation (Conway *et al.*, 2002; Degrassi *et al.*, 2002; Huber *et al.*, 2001; Lewenza *et al.*, 1999) whereas, in some *Rhizobium* sp, the C8-HSL regulated QS system is involved in nodulation, plasmid transfer and other unknown functions (Gonzalez and Marketon, 2003). Most cases studies examining phenotypic changes associated with particular types of AHL have been carried out with mutants defective in the production of their cognate AHL. The mutant physiology is then compared with the wild-type physiology.

Since quorum sensing in several bacteria is associated with stress at high cell density physiological responses that occur in response to such stress are found to be associated

with a quorum sensing response. In most cases, physiological changes associated with stress in cyanobacteria may involve entry into a dormant stage, such as heterocysts, due to nutrient limitation. Others may involve, exopolysaccharide production, accumulation of intercellular reserve materials (Schwarz and Forchhammer, 2005). In previous Chapters, C8-HSL was identified as a quorum sensing molecule showing a density dependent accumulation and also was capable of inducing changes in protein expression in low density cultures suggesting a quorum sensing system to be operative in the cyanobacterium *Gloeothece*. Therefore, investigation of any physiological responses that may be associated with a quorum sensing system in *Gloeothece* was investigated.

Aims and Objectives

The aim was to detect for C8-HSL mediated physiological changes in *Gloeothece* associated with growth, chlorophyll concentration, the release of extracellular carbohydrate and acid phosphatase activity, which could be useful in answering the following important questions:

- Does C8-HSL produce any effect on growth?
- Does C8-HSL produce any effect on chlorophyll concentration?
- Does C8-HSL produce any effect on extracellular carbohydrate?
- Does C8-HSL produce any effect on non-specific acid phosphatase activity?

The objective will be to wash any AHL produced by *Gloeothece* cultures and then treat early growth stage cultures with C8-HSL. Changes in any of the physiological response

of the C8-HSL treated culture is then compared to a control culture in which C8-HSL has not been added.

6.2 Materials and Method

6.2.1. Materials:

Para-nitrophenyl phosphate, wheat germ acid phosphatase were purchased from Sigma Aldrich, UK. All other chemicals were purchased according to companies stated in other chapters.

6.2.2. Method:

6.2.2.1. Determination of the effect of C8-HSL on changes in cell biomass and chlorophyll concentration

6.2.2.1.1. Sample preparation

To investigate changes in biomass and chlorophyll concentration *Gloeothece* PCC 6909 was grown in 3 L of sterile BG 11° medium in large 5 L pot for 1 month at 26 °C under alternating 12 h light (30 μmol m^{-2} s^{-1}), over the waveband 400-700 nm (measured at the surface of the culture) and 12 h darkness in order to obtain sufficient cell material. The cultures were maintained through continuous bubbling with sterile air that was passed through double sterile filters in order to supply oxygen and maintain the cells in suspension. In order to prepare an inoculum for the two experimental cultures, samples were washed out of any endogenously produced AHL according to the following procedure. The cells were first allowed to settle at the bottom of the flask by stopping the air supply. The media was then carefully and aseptically removed from the flask. Five hundred ml of fresh sterile BG11° media was then added to the culture from which 400 ml of culture was collected and washed in preparation for an inoculum for each experimental culture. Equal amounts of inoculums were added to two experimental

flasks containing 3 L of fresh sterile BG11^{o} medium and cultures were maintained with a stream of water-saturated, sterile filtered air to prevent evaporation under appropriate growth conditions. Cultures were allowed to grow for a further 7 days, after which C8-HSL was added in to a final concentration of 100 μM in one flask and the same volume of ethanol was added to the other flask, used as control. From each experimental flask 25 ml of sample were collected at different growth points for up to 30 days. Samples were analysed on each day of collection for growth and chlorophyll measurements.

6.2.2.1.2. Measurement of biomass and chlorophyll concentration

For determination of cell biomass, turbidity of the culture was measured through absorbance at 436 nm according to Chapter 3 section 3.2.2.2.3.1. For the analysis of total chlorophyll concentration, 3 ml of culture was used and chlorophyll concentration was measured using phytoplankton pulse amplitude modulator (phyto-PAM; waltz, Germany) against calibrated chlorophyll measurements for *Gloeothece* cells, according to the users guide. Chlorophyll concentration was measured as mg ml^{-1} and expressed as the percent increase over the growth of *Gloeothece.*

6.2.2.2. Determination of the effect of C8-HSL on extracellular carbohydrate release

6.2.2.2.1. Culture preparation for carbohydrate estimation

The effects of C8-HSL on extracellular total carbohydrate concentration were investigated on cultures of *Gloeothece* PCC 6909/1. Ten flasks containing 100 ml of

sterile $BG11^0$ medium were inoculated with the same amount of inoculum in each flask prepared according to section 5.2.2.1.1. so that each flask in the experiment represented cultures at the same stage during growth. The resultant cultures were grown and maintained for 7 days, after which concentrations of C8-HSL or ethanol were added to the cultures. Duplicate flasks were set aside for each C8-HSL treated and control samples (ethanol treated). C8-HSL was then added to each set of experimental cultures in the following final concentration of 5 μM, 500 nM and 50 nM. All cultures were grown and maintained for a further 7 days after which the cultures were harvested by centrifugation at 10,000 g and the supernatant was collected and analysed for carbohydrate content. A set of control samples were used (Control-7d) as a reference sample to calculate the increase in carbohydrate content during the 7 days incubation and was analysed for carbohydrate content just before the addition of C8-HSL to the cultures. In order to determine total carbohydrate supernatants were directly used in analysis. For determination of soluble and particulate carbohydrate, 2 ml of each sample was placed in dialysis tubing (Mw cut-off 7,000 Da) and dialysed against distilled water overnight at 4°C. Particulate carbohydrate was calculated from the samples after dialysis and the soluble carbohydrate was calculated from the difference in total and particulate carbohydrate.

6.2.2.2.2. Phenol-sulphuric acid method of carbohydrate estimation

A colorimetric method of carbohydrate quantitation, the phenol-sulphuric acid method (Dubois *et al.*, 1951), was used to determine the total carbohydrate concentrations in the samples. The method is sensitive in the nmol range and suitable in detection of a wide

range of carbohydrates, including sugars, methylated sugars, neutral sugars and acidic polysaccharides, which comprise most of the components of microbial EPS (Underwood and Peterson, 1995). A clear aqueous solution of the carbohydrates to be analyzed was placed in a test-tube and then phenol and concentrated sulphuric acid were added. The sulphuric acid causes all non-reducing sugars to be converted to reducing sugars. The yellow-orange colour solution produced as a result of the interaction between the carbohydrates and the phenol is read at absorbance 490 nm which is proportional to the total sugars present in the sample.

A calibration curve was initially prepared using standard known concentrations of glucose, using the following method. To 1 ml of sample or standard, 1 ml of 5 % phenol solution was added after which 5 ml of concentrated sulphuric acid was added directly and rapidly on top of the samples. The mixture was incubated at 25-30 ^{o}C for 20 min after which the reading of the sample was taken by absorbance at OD 490 nm using a UV spectrophotometer. The readings were plotted against glucose concentrations (μg) from which carbohydrate concentration of the unknown sample were calculated using equations obtained from the standard curve (Fig.VII). The concentrations of the unknown samples were expressed as μg of glucose equivalent ml^{-1}.

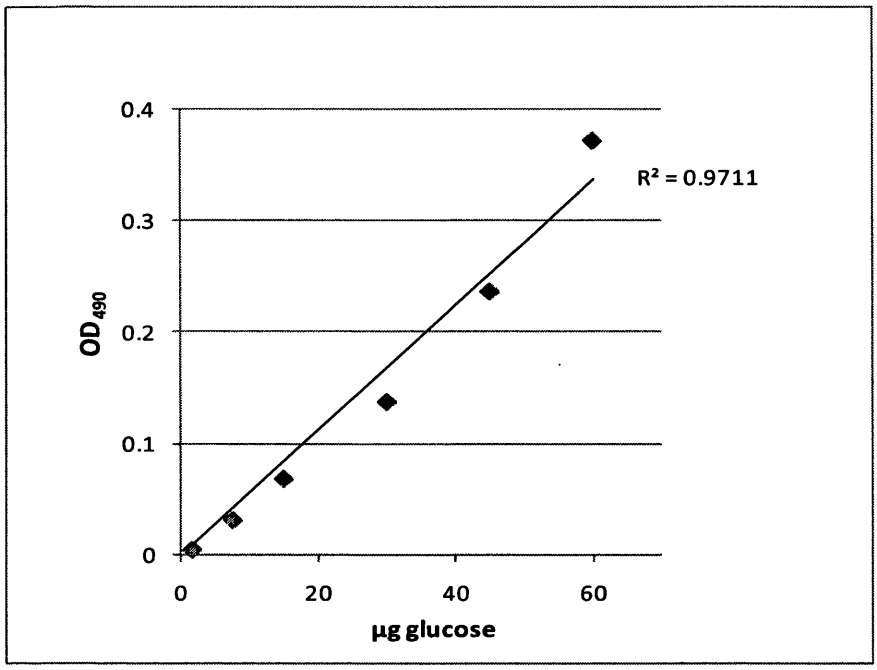


Figure. VII. Standard calibration curve of phenol sulphuric acid method of total carbohydrate estimation. (Dubois, M. et al, 1956)

6.2.2.3. Carbohydrate estimation over growth of *Gloeothece* PCC 6909/1

Total extracellular carbohydrate released during growth of PCC 6909/1 was estimated over a period of 180 days from samples collected at various growth points in section 3.2.2.2. Total carbohydrate was estimated by absorbance at 490 nm according to section 6.2.2.2.2. The values were plotted against individual sample collection points to obtain a pattern of release. The increase in carbohydrate content during growth was calculated from day 7 and was plotted as percent increase in carbohydrate content during growth.

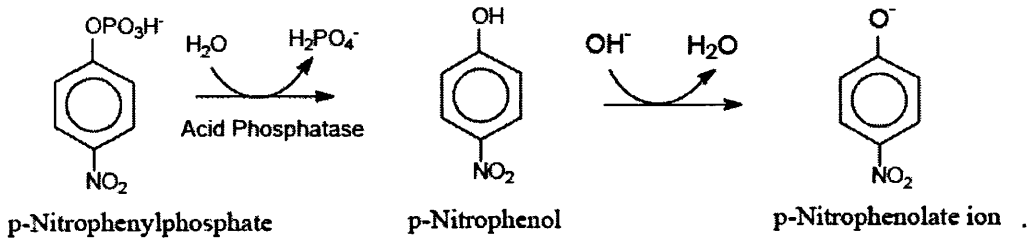
6.2.2.4. Extraction of *Gloeothece* acid phosphatase

Cultures of *Gloeothece* were extracted with different extraction buffers for detection of acid phosphatase activity. Cultures were first centrifuged at 10,000 g for 10 min to pellet cells. The pellet was then re-dissolved in a small volume of cold extraction buffer (1 ml). For most assays samples were extracted with an extraction buffer containing 100 mM sodium citrate buffer (pH 5.0), 1mM EDTA, 50 mM NaCl and protease inhibitors; 0.5 mM PMSF, 1μM pepstatin, 50 μM Leupeptin. For samples where comparisons were made between acid phosphatase activity of membrane and soluble protein and between control and C8-HSL treated cultures, samples were extracted with extraction buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM DTT, 1 mM EDTA and protease inhibitors 0.5 mM PMSF, 1 μM pepstatin, 50 μM Leupeptin. Cells were then broken by passing them through a chilled French press at 1000 psi. The collected extract was centrifuged at 10,000 g to remove cell debris and the cleared supernatant was assayed for acid phosphatase activity. In cases where, acid phosphatase activity was determined later, the samples were kept in aliquots and frozen at -20 °C.

6.2.2.5. Standard assay of acid phosphatase

Principle:

Non-specific acid phosphatases catalyze the hydrolysis of the substrate ρ- nitrophenyl phosphate (ρNPP) to ρ-nitrophenol (ρNP) and inorganic phosphate. The amount of liberated ρNP is determined by photometric measurement. The addition of excess alkali quantitatively converts the ρNP to its anion which is bright yellow and has a λmax at 405 nm of the visible spectrum.



p-Nitrophenylphosphate p-Nitrophenol p-Nitrophenolate ion .

6.2.2.5.1. Standard wheat germ acid phosphatase assay:

The acid phosphatase assay was carried out in 96 well microtiter plates. In order to establish a valid acid phosphatase assay in a 96 well microtiter plate format, enzymatic reactions were carried out under the following conditions. To initiate the reaction 0.0028 units of commercially available wheat germ acid phosphatase (Sigma Aldrich, UK) in 10 µl of citrate buffer (pH 5.5) was added to each well containing the substrate ρNPP (2 mM) in 50 mM sodium citrate buffer (pH 5.5). The total reaction volume was 130 µl. Samples were incubated under a series of incubation periods and the reaction was terminated at various time intervals between 5-30 min by the addition of 20 µl of

1M sodium hydroxide. Suitable controls were prepared using an enzyme assay mixture together with NaOH to terminate any reaction and to eliminate measurements due to any pre-formed ρNP present in the reaction mixture. After the termination of all reactions colour developed was recorded by absorption at 405 nm using a Beckman UV microtitre plate reader.

6.2.2.5.2. Acid phosphatase assay using *Gloeothece* extract

The enzymatic activity in *Gloeothece* extracts was assayed according to the following procedure in a 96 well microtitre plate. The reaction mixture consisted of 1mM ρNPP, 50mM sodium citrate buffer (pH 5.0) and 10 µl of *Gloeothece* extract (unless otherwise stated) in a total volume of 150 µl. The reaction mixture was incubated at room temperature at various time intervals between 0-2.5 h. The reaction was allowed to stop by the addition of 20 µl of 1 M NaOH. Suitable controls were prepared by adding NaOH initially to the assay mixture in order to terminate any reaction. Colour developed was recorded by absorption at 405 nm using a microtitre plate reader. Enzymatic activity of samples (where stated) was calculated by the ρNP molar absorption coefficient of 17,000 M^{-1} cm^{-1} (Paliwal *et al.*, 2007) One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 µM of ρNP min^{-1} under these conditions.

6.2.2.6. Determination of a pH optimum of acid phosphatase activity

Acid phosphatase assays were carried out with *Gloeothece* PCC 6909/1 extract according to section 6.2.2.5.2 with minor modifications. The pH of optimum acid phosphatase activity in extracts was determined by incubating extracts under different pH assay conditions. The reaction mixture, consisted of the substrate ρNPP (2 mM) adjusted to different pH (3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 8.0) using buffers, sodium citrate (100 mM) and Tris-HCl (100 mM). Twenty µl of *Gloeothece* extract was added to each reaction mixture to initiate the reaction resulting in a total volume of 150 µl. A set of control samples was also used at individual pH. Samples were incubated for 2 h after which 20 µl of 1M NaOH was added to terminate the reaction. Optimum pH activity was then determined from taking absorbance at 405nm.

6.2.2.7. Effect of various substances and metal ions

Acid phosphatase assays were carried out according to section 6.2.2.5.2 with minor modifications as follows. 15 mM stock solutions of different metal ions Mg ($MgCl_2$), Ca ($CaCl_2$), Mn ($MnCl_2$), Cu ($CuCl_2$) were prepared. 10 µl of the stock solution was added to the reaction mixture resulting in a final concentration of 5 mM. Reaction mixtures containing final concentrations of 10 mm DTT, 20 mM tartrate, 13 mM EDTA, 0.4% (v/v) ß-mercaptoethanol and 0.3% (v/v) triton X-100 were also prepared. Enzymatic reactions were carried out with 20 µl of *Gloeothece* extract in a final volume of 150 µl with the pH adjusted with citrate buffer (pH 4.0). The reaction was terminated after 2 h of incubation with 20 µl of 1 M NaOH and the absorbance read at 405 nm.

6.2.2.8. Effect of culture age

The effect of culture age on acid phosphatase activity was tested from extracted samples of a 4 week and 8 week old culture. Cultures were extracted from 100 ml cultures, prepared according to section 3.2.2. Following growth, one culture was extracted at 4 weeks and the other was allowed to grow and then extracted at 8 weeks according to section 6.2.2.4. Extracted samples were then subjected to protein estimation using a 2D quant kit (GE Healthcare, UK) according to user's instructions. Acid phosphatase activity was determined according to section 6.2.2.5.2; using 2.5 mg of extract in a total reaction mixture (150 µl) containing 1 mM ρNPP in citrate buffer (pH 4.0). The reaction was incubated for 1 h and terminated by the addition of 20 µl of 1M NaOH after which the absorbance was read at 405 nm. The amount of activity was calculated using the equation in section 6.2.2.5.2.

6.2.2.9. Membrane and soluble protein

Gloeothece PCC 6909 cultures were extracted to obtainin soluble and membrane protein as follows. Briefly cells were centrifuged and resuspended in extraction buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2mM DTT, 1 mM EDTA and protease inhibitors 0.5 mM PMSF, 1 µM pepstatin, 50 µM Leupeptin and then broken through a French press at 10,000 psi. After centrifugation at 20,000 g for 30 min the supernatant was collected as soluble protein and the pellet was redissolved in the same extraction buffer containing 2% Igepal CA-630 (non ionic detergent) and treated as the membrane soluble protein. The protein content of both fractions was determined using

2D quant kit. Acid phosphatase activity was then determined according to section 6.2.2.5.2 using 2 mg of protein in 100 mM citrate buffer (pH 4.0) with incubation for 1h, after which the reaction was terminated with NaOH and the absorbance read at 405 nm.

6.2.2.10. Effect of acid phosphatase activity in response to C8-HSL in *Gloeothece*

6.2.2.10.1. Preparation of Control and C8-HSL treated cultures

Cultures of *Gloeothece* PCC 6909/1 were prepared for use as an inoculum in experimental cultures by washing the cultures to eliminate endogenous AHL and preparing cultures according to Chapter 4 section 4.2.2.5. After growth of experimental cultures for 7 days one culture was treated with C8-HSL at 10 μM concentration and the other was treated with ethanol (control culture). Cultures were then extracted the following day according to section 6.2.2.4. for determination of acid phosphatase activity. Protein concentration in each extract was determined using the 2D quant kit.

6.2.2.10.2. Acid phosphatase activity of C8-HSL treated and control cultures

Acid phosphatase assay was carried out according to section 6.2.2.5.2 using 2 mM substrate ρNPP in citrate buffer (pH 4.0) and by the addition of 100 μg of *Gloeothece*

protein extract in a total reaction volume of 150 µl. Equal volumes of extract of both control and C8-HSL treated samples were used during the assay. The reaction mixture was incubated at room temperature at various time intervals from 0-2.5 h after which the reaction was terminated by the addition of 40 µl of 1M NaOH. The acid phosphatase activity in the control and C8-HSL treated sample was calculated according to section 6.2.2.5.2; and expressed in units (U) of enzymatic activity.

6.3. Results

6.3.1. The effect of C8-HSL on cell biomass

The effect of C8-HSL on cell biomass of *Gloeothece* was assessed at various time points after the addition of C8-HSL (day 0), through turbidity measurements. The measurements obtained for both C8-HSL treated and control cultures were plotted during growth and represented in Fig 6.1. The characteristic pattern in increase in biomass during growth in both cultures was found to follow a similar trend. No apparent differences were observed in C8-HSL treated culture when compared to the control culture indicating that C8-HSL treatment did not alter changes in cell biomass of *Gloeothece* PCC 6909 under the conditions investigated.

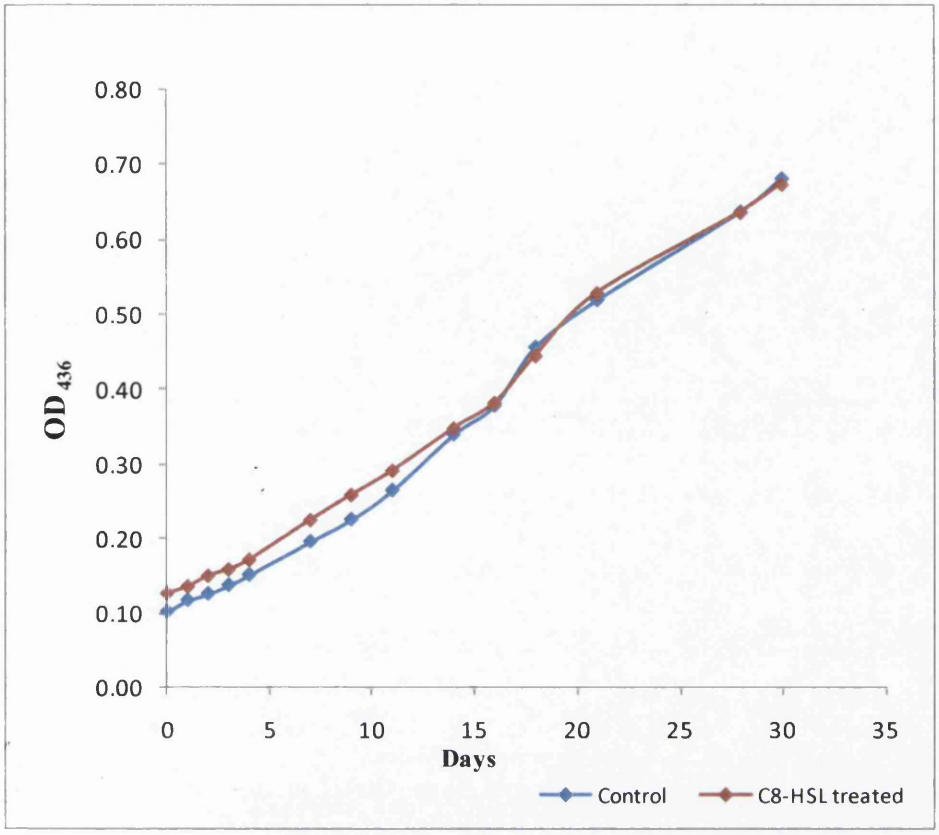


FIGURE. 6.1. THE EFFECT ON CELL BIOMASS IN CULTURES OF *GLOEOTHECE* PCC 6909 UPON TREATMENT WITH C8-HSL.

Cultures of PCC 6909, treated (100μM of C8-HSL at day "0") and control cultures (ethanol) monitored over 30 days for changes in cell biomass. Cell biomass was measured at different time points during growth as measurements in turbidity. Each point in turbidity represents a mean of triplicate measurements. OD_{436}=Optical density at 436nm.

6.3.2. The effect of C8-HSL on total chlorophyll concentration

The effect of C8-HSL on total chlorophyll concentration was monitored on cultures of *Gloeothece* PCC 6909 at various time points during growth after the addition of C8-HSL. The concentration of chlorophyll was measured and represented as a percent increase in chlorophyll concentration (Fig 6.2). Results in Fig 6.2 show that chlorophyll concentration increases in both control and C8-HSL treated cultures over 15 days and then gradually declines. However, no apparent changes in chlorophyll concentrations could be observed between the two cultures (treatment culture and the control culture), indicating C8-HSL addition to the cultures under conditions described does not cause any effect on total chlorophyll concentrations in *Gloeothece*.

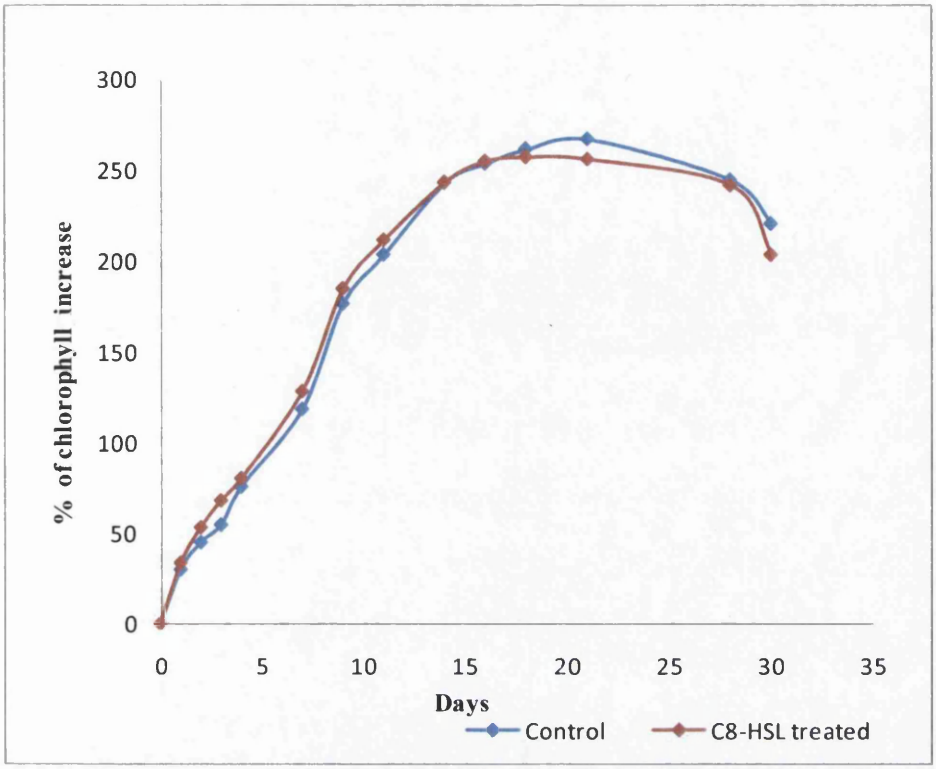


FIGURE 6.2. CHANGES IN CHLOROPHYLL CONCENTRATION IN CULTURES OF GLOEOTHECE PCC 6909 UPON TREATMENT WITH C8-HSL.

Cultures of PCC 6909 treated (100 μM of C8-HSL at day "0") and control cultures (ethanol) monitored over 30 days for changes in Chlorophyll concentration. Chlorophyll concentrations measured at individual points during growth is plotted as percentage increase in chlorophyll concentration from day 0. Each point in growth represents a mean of triplicate measurements.

6.3.3. Changes in carbohydrate release upon treatment of cultures of *Gloeothece* PCC 6909/1 upon treatment with C8-HSL

6.3.3.1. Changes in total carbohydrate

The effect of different concentrations of C8-HSL on the concentration of total extracellular carbohydrate in cultures of *Gloeothece* PCC 6909/1 was assessed. Fig. 6.3 shows total carbohydrate concentration (mg ml^{-1}) in various samples treated with both C8-HSL and control cultures. The total carbohydrate concentration in the control sample was found to be among the highest (representing approx. 55 μg ml^{-1} of glucose equivalent) and showed an increase of approximately 315 % during the 7 day period monitored. However, the total carbohydrate released across different C8-HSL treatment groups was found to be significantly lower than control samples. All samples in the treatment group accumulated similar concentrations of total carbohydrate (representing approx. 20 μg ml^{-1} of glucose equivalent) a 53 % increase within 7 days after the addition of C8-HSL. Low concentrations of total carbohydrate detected from various C8-HSL treated cultures compared to control cultures indicate that cells may respond to C8-HSL through decreasing the release of total extracellular carbohydrate under the experimental conditions.

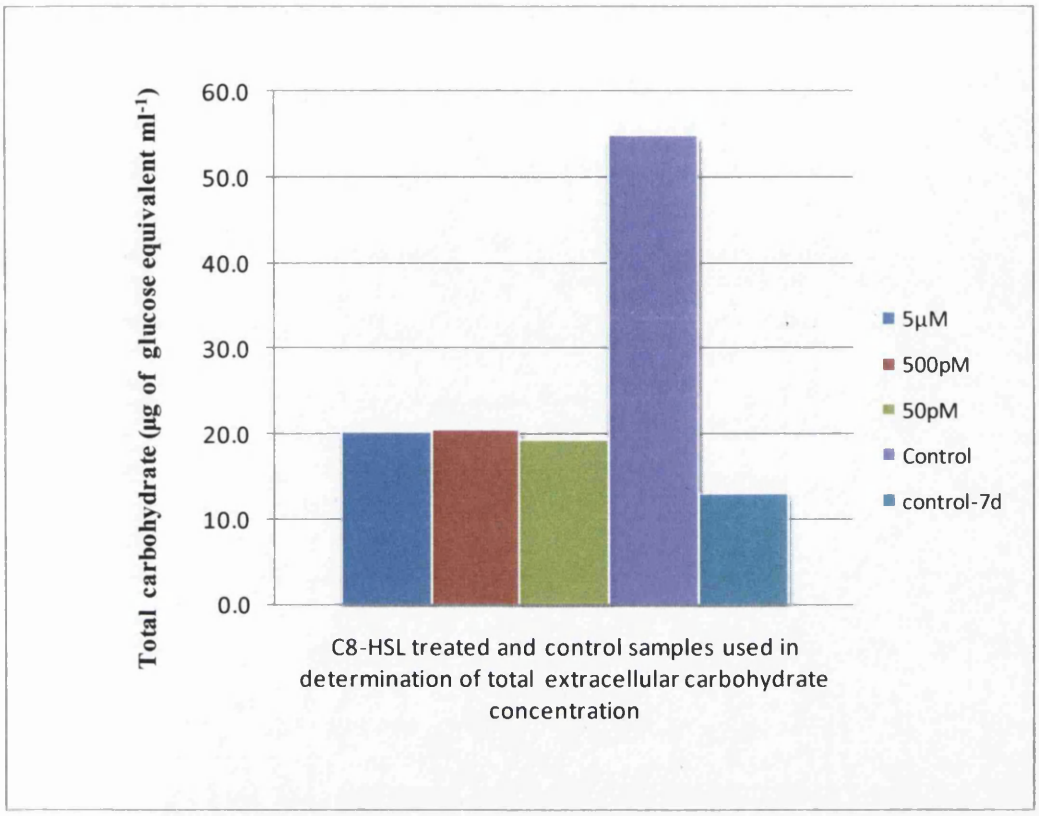


FIGURE 6.3. CHANGES IN TOTAL CARBOHYDRATE CONCENTRATION IN RESPONSE TO DIFFERENT CONCENTRATIONS OF C8-HSL.

Cultures of PCC 6909/1 treated with different concentrations of C8-HSL (5 µM, 500 nM and 50 nM) and control samples (ethanol) were estimated for total extracellular carbohydrate concentration 7 days after the addition of C8-HSL to the cultures. Cultures were also estimated for total carbohydrate on the day (control-d7) of addition of C8-HSL. The total amount of carbohydrate is estimated as µg of glucose equivalent ml^{-1} *and is calculated from the mean of two duplicate samples.*

6.3.3.2. Changes in soluble and particulate carbohydrate

To analyse the changes for reduced accumulation of total carbohydrate in C8-HSL treatment cultures, total carbohydrate was separated into soluble carbohydrate and particulate carbohydrate and their concentrations determined. Fig 6.4 shows the concentrations of soluble and particulate carbohydrate obtained from each sample. The accumulations of particulate carbohydrate over 7 days in all cultures were almost similar, with an average concentration of 19 μg ml^{-1} of glucose equivalent. Results therefore indicate C8-HSL does not cause any changes in extracellular particulate carbohydrate concentrations. Analysis of the soluble carbohydrate concentration across different samples showed higher concentrations of soluble carbohydrate present in control samples compared to all C8-HSL treated samples. Results show that the average concentration of soluble carbohydrate in all C8-HSL treated samples to be 1.5 μg of glucose eq. ml^{-1} which is significantly lower than the average concentration in the control samples which is 33 μg of glucose eq. ml^{-1}. The concentrations in the treated samples were also proportionally lower than the initial concentration detected 7 days earlier (3.6 μg of glucose eq ml^{-1}). These results therefore, indicate that C8-HSL addition to *Gloeothece* cultures in the range of 50 nM- 5μM results in no further increase in soluble carbohydrate during growth under the experimental conditions. In fact a lower concentration detected after 7 days of growth may indicate reutilization rather than release of the soluble carbohydrates by cultures of *Gloeothece.*

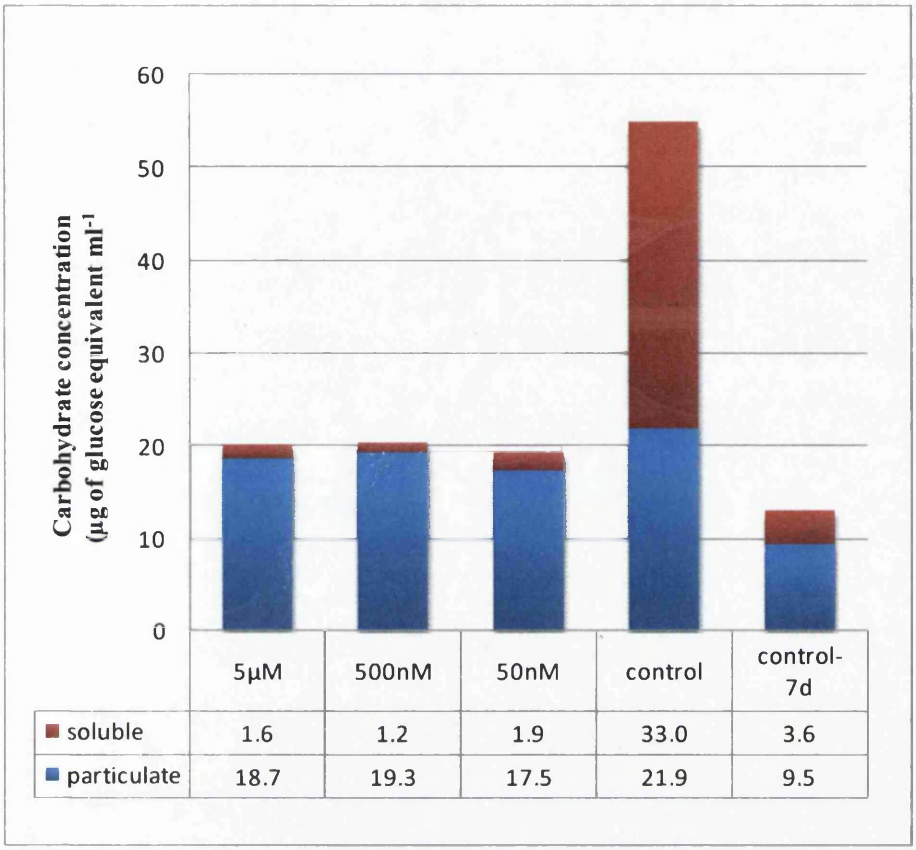


FIGURE 6. 4. CHANGES IN SOLUBLE AND PARTICULATE CARBOHYDRATE CONCENTRATIONS IN RESPONSE TO DIFFERENT CONCENTRATIONS OF C8-HSL.

Cultures of PCC 6909/1 treated with different concentrations (5µM, 500nM and 50nM) of C8-HSL and control samples (ethanol) estimated for soluble and particulate carbohydrate release 7 days before (control-d7) and 7 days after (control) the addition of C8-HSL to the cultures. The amounts represent soluble and particulate carbohydrate from the mean of two duplicate cultures. Carbohydrate concentration is expressed as µg of glucose equivalent ml^{-1}. Different colours in the bars represent the amount and proportions of soluble (red) and particulate (blue) carbohydrate in each sample.

6.3.3.3. Release of total carbohydrate during growth of *Gloeothece* PCC 6909/1

Total extracellular carbohydrate concentration at various points during growth of PCC 6909/1 was measured (Table 6.1) and represented as percentage of carbohydrate increase in relation to growth (Fig 6.5). Results indicate that increase in total extracellular carbohydrate concentration parallels increase in turbidity, reaching a maximum concentration of 549µg of glucose eq. ml^{-1} .

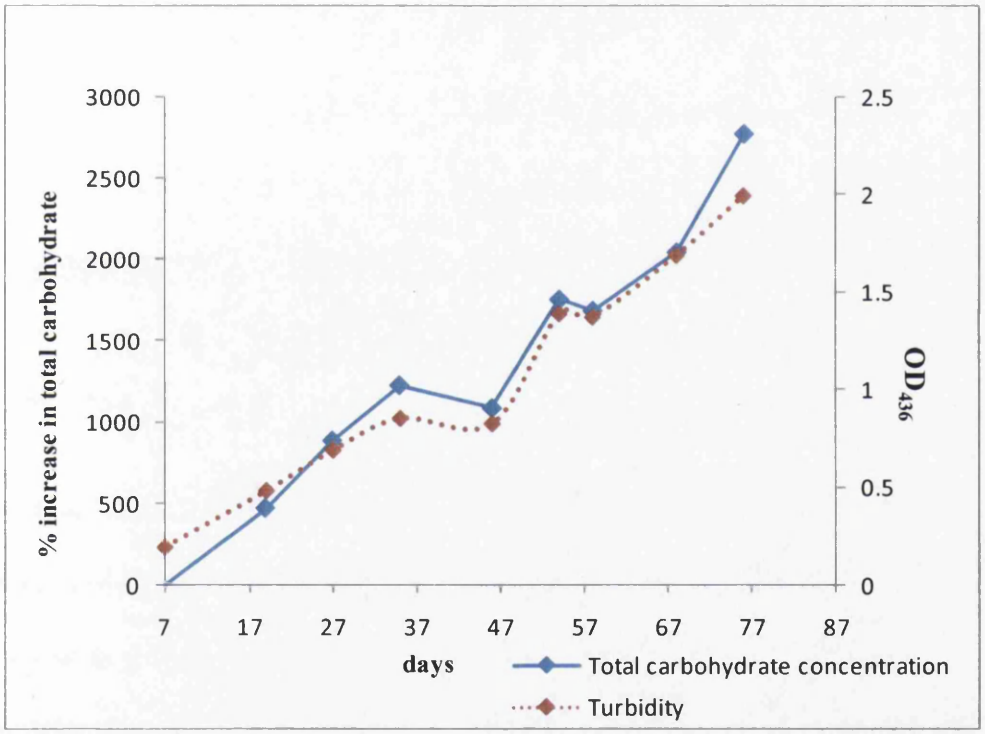


FIGURE 6.5.TOTAL CARBOHYDRATE RELEASE DURING GROWTH OF *GLOEOTHECE* PCC 6909/1

Total carbohydrate (solid line) in culture supernatant of PCC 6909/1 estimated over a period of 80 days. The concentration of total carbohydrate at each point is calculated from the average values obtained from two duplicate cultures and represented as a percent increase from day 7. Increase in cell biomass (dashed line) is measured through an increase in turbidity. OD_{436}=Optical density at 436nm.

days	OD 490nm		average	µg of glucose eq./ml	%increase
	S1	S2	S1+S2		in total carbohydrate
7	0.00	0.02	0.01	19	0
19	0.16	0.15	0.15	109	471
27	0.27	0.29	0.28	188	885
35	0.39	0.38	0.39	253	1225
46	0.32	0.36	0.34	226	1085
54	0.55	0.54	0.55	354	1754
58	0.55	0.51	0.53	341	1685
68	0.62	0.66	0.64	410	2045
76	0.87	0.84	0.86	549	2775

TABLE 6. 1. TOTAL EXTRACELLULAR CARBOHYDRATE RELEASE DURING GROWTH OF *GLOEOTHECE* PCC 6909/1.

Total carbohydrate measured through absorbance at 490nm; S1 & S2 represent sample collected from duplicate cultures 1 & 2 respectively.

6.3.4. Optimization of acid phosphatase assay conditions

6.3.4.1. Validation of acid phosphatase assay procedure:

In order to establish the validity of the assay procedure incubation was carried out with commercially available wheat germ acid phosphatase over various time intervals. Fig 6.6. show that product formation was linear with time and over the incubation period employed, thus providing the validity of the assay procedure.

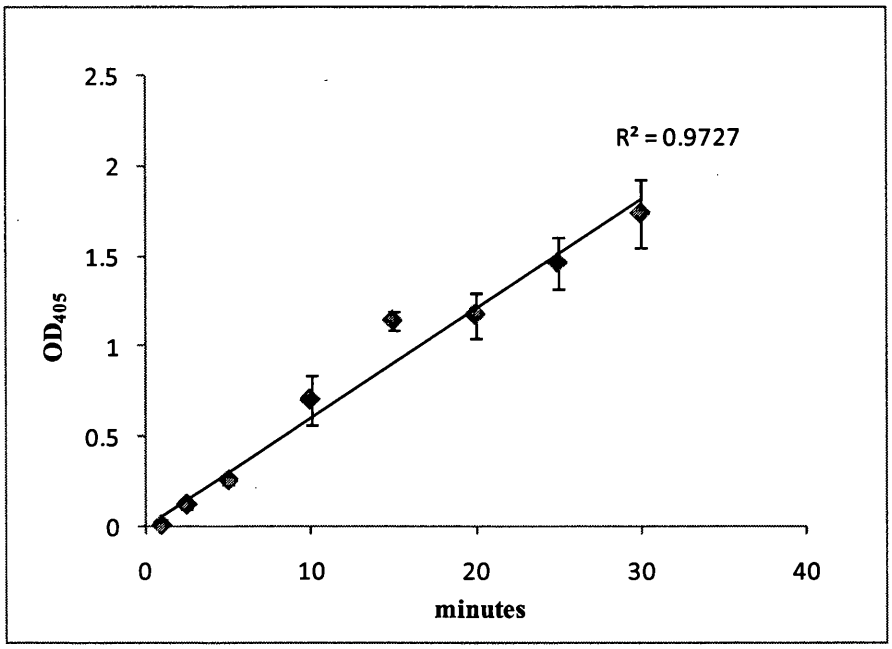


FIGURE 6.6. TIME COURSE FOR WHEAT GERM ACID PHOSPHATASE ACTIVITY

0.0028 units of wheat germ acid phosphatase incubated for over 30 minutues with substrate ρNPP at pH 5.0. Product formation measured by absorbance at 405nm. Bars represent standard deviation from mean reading of three replicate samples.

6.3.4.2. Time course of acid phosphatase activity in *Gloeothece* extracts

A time course experiment was carried out with *Gloeothece* PCC 6909 extracts to find whether acid phosphatase activity was detectable. Since nothing is known about the activity of acid phosphatases in extracts, a fixed volume of extract was incubated with the substrate ρNPP and its activity determined over 120 min (Fig 6.7). Results show that the acid phosphatase activity was detected in *Gloeothece* extracts. The activity was found to increase over time showing a linear response, thus validating the assay method for measuring acid phosphatase activity in *Gloeothece* extracts.

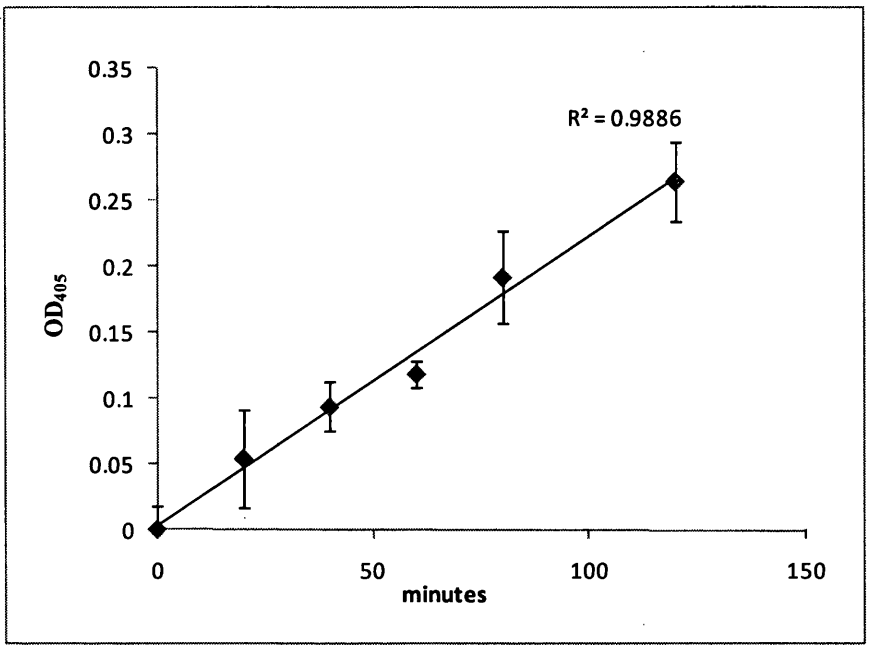


FIGURE 6.7. TIME COURSE FOR *GLOEOTHECE* ACID PHOSPHATASE ACTIVITY

Acid phosphatase activity in Gloeothece extracts incubated with substrate pNPP over 30 minutes at pH 5.0. Product formation measured by absorbance at 405nm. Bars represent standard deviation from mean reading of three replicate samples.

6.3.4.3. Determination of pH optimum

In order to investigate the optimim pH of acid phosphatase acitivity in *Gloeothece* extracts, extracts were incubated in a range of different pH conditions. Fig 6.8 shows acid phosphatase activity in *Gloeothece* extracts over the pH range of 3.5-8. Results show that the acid phosphatase activity was pH dependent and that the highest activity was obtained from extracts incubated at pH 4.0. No activity at pH 8.0 was observed, indicating the absence of a alkaline phosphatase in the extract. The pH dependent

sigmoidal curve obtained suggests that *Gloeothece* extracts contain acid phosphatase activity which has a pH optimum at pH 4.0.

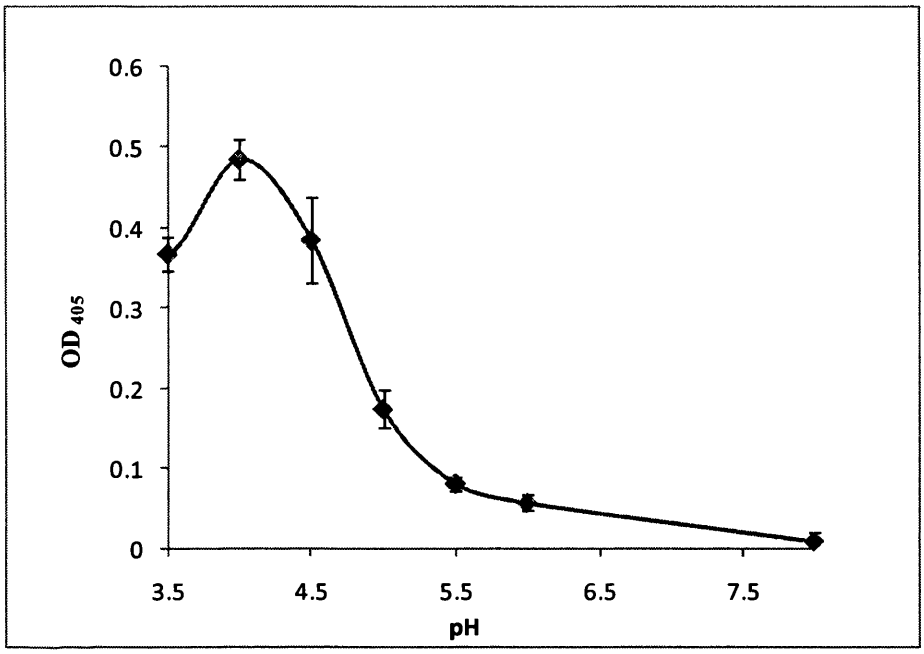


FIGURE 6.8. pH DEPENDENCY OF ACID PHOSPHATASE ACTIVITY IN *GLOEOTHCE*

Acid phosphatase activity in Gloeothece extracts (20 µl) of incubated with substrate pNPP over 2h at different pH (pH 3.5, 4.0, 4.5, 5.0, 5.5, and 8.0). Product formation is measured by absorbance at 405nm. Bars represent standard deviation from mean reading of three replicate samples.

6.3.5. General characterization of acid phosphatase activity in *Gloeothece*

6.3.5.1. Acid phosphatase activity in membrane protein and soluble protein fractions

Gloeothece extract was fractionated into soluble and membrane protein fractions in order to determine a fraction in which most of the acid phosphatase activity could be detected. Acid phosphatase activity in both fractions was determined at pH 4.0 using the same concentration of protein. A higher acid phosphatase activity was detected in the soluble fraction with no activity detected in the membrane soluble fraction (Fig 6.9). Results indicate that acid phosphatase activity was mainly confined in the soluble fraction of *Gloeothece* extracts, which could lead to subsequent characterization as a periplasmic or cytoplasmic acid phosphatase.

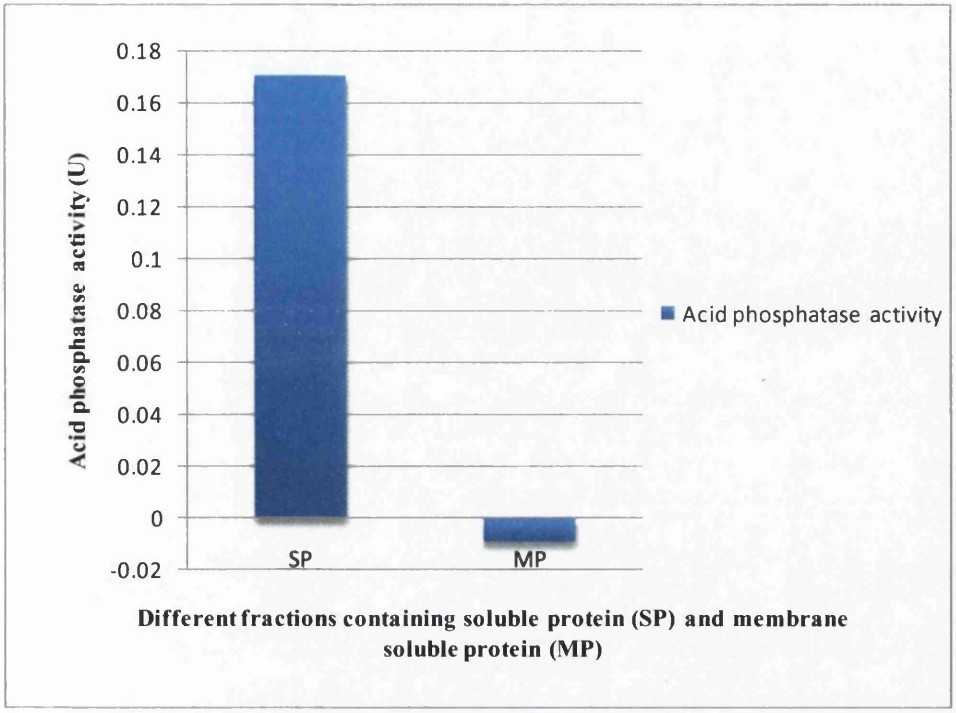


FIGURE 6. 9. ACID PHOSPHATASE ACTIVITY IN DIFFERENT FRACTIONS OF *GLOEOTHECE* EXTRACTS

Acid phosphatase activity detected in 2 mg of Gloeothece soluble protein (SP) and membrane soluble protein (MP) incubated with substrate ρNPP over 2 h at pH 4.0. Product formation is measured by absorbance at 405 nm and expressed as units of enzymatic activity (U). One U = the amount of enzyme required to produce 1 μM of ρNP min $^{-1}$ under the conditions. Measurements represent a mean of two duplicate sample readings.

6.3.5.2. Activity of acid phosphatase with varying culture age

Acid phosphatase activity was measured from cultures grown over four weeks and six weeks. Protein concentrations of the extracts were measured and the same concentration of protein was used in order to compare enzymatic activities. Fig 6.10 show the results

obtained from different culture age samples. Acid phosphatase activity was found to double (2.5x) in activity in the six week old culture when compared to the four week old culture (Fig. 6.10). Results therefore indicate that increasing concentrations of acid phosphatase activities could be detected as *Gloeothece* culture ages.

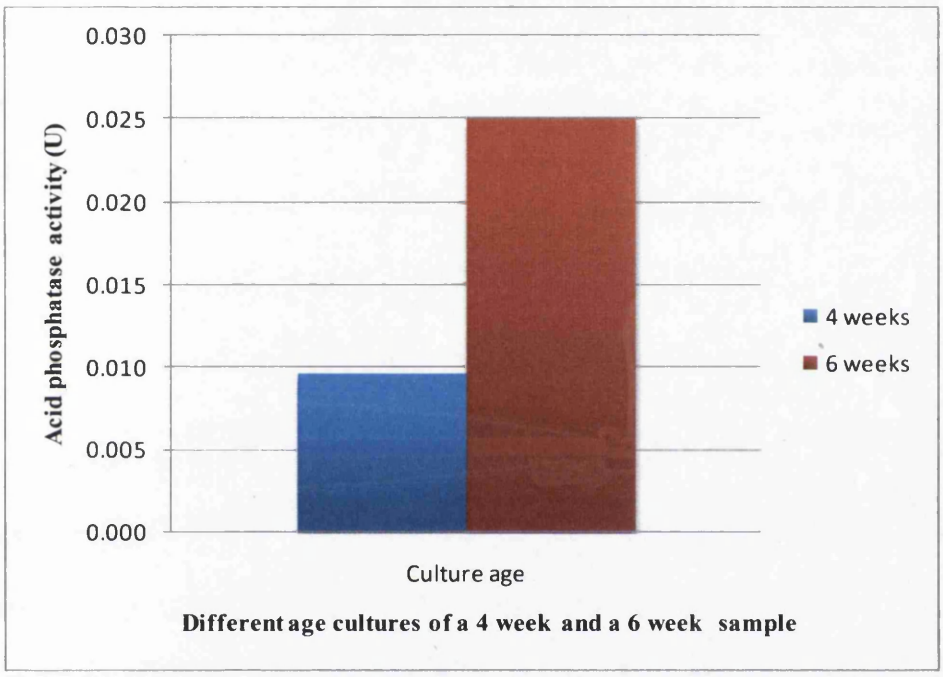


FIGURE 6.10. ACID PHOSPHATASE ACTIVITY IN DIFFERENT AGE CULTURES OF *GLOEOTHECE*

Acid phosphatase activity detected from 2.5 mg of extract incubated with substrate pNPP over 1 h at pH 4.0. Product formation is measured by absorbance at 405 nm and calculated and expressed as units of enzymatic activity (U). One U = the amount of enzyme required to produce 1 μM of pNP min $^{-1}$ under the conditions Measurements represent a mean of two duplicate sample readings.

6.3.5.4. Effect of various substances on acid phosphatase activity

The effect of different compounds on acid phosphatase activity was tested. The acid phosphatase activity obtained from incubation with different compounds was expressed as a percent change in activity relative to that of a control (100 %) (Fig. 6.11). All the metal ions at 5 mM concentrations showed almost no effect on acid phosphatase activity except for Mg which showed a higher activity than other metal ions. The use of reducing agents such as mercaptoethanol 0.4 % (v/v) and DTT (10 mM) and a chelating compound EDTA (13 mM) showed no marked change in acid phosphatase activity relative to the control. In the presence of tartrate (20 mM; inhibitor of some acid phosphatases), acid phosphatase activity decreased to 25 % of that of the control but showed no inhibition in activity in *Gloeothece* extracts. The effect of Triton X-100, 0.3 % (v/v) was tested, which resulted in reduced enzymatic activity upon incubation with this detergent. Results therefore indicate that acid phosphatase activity in *Gloeothece* extracts was sufficiently stable to reducing agents, chelating compounds and various metal ions in the concentrations employed.

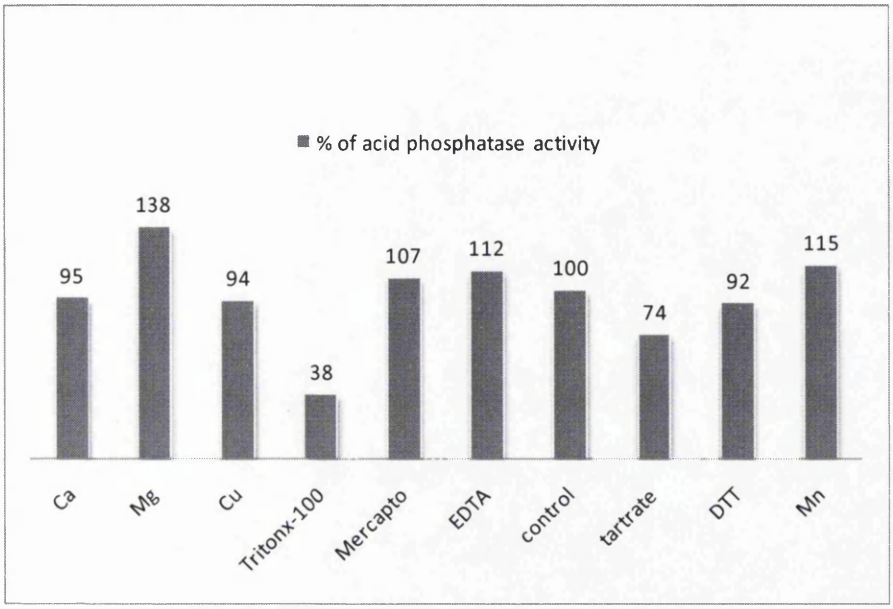


FIGURE 6.11. ACID PHOSPHATASE ACTIVITY IN *GLOEOTHECE* EXTRACTS IN THE PRESENCE OF VARIOUS SUBSTANCES

Relative acid phosphatase activity in Gloeothece extract incubated with substrate ρNPP and various metal ions (Ca, Mg, Mn, Cu), reducing agents (DTT, mercaptoethanol), detergent (Triton X-100), chelating compound (EDTA) and acid phosphatase inhibitor (tartrate) over 1h at pH 4.0. Relative activity is expressed as % of activity to that of the control (100 %). Measurements represent a mean of two duplicate sample readings.

6.3.6. Changes in acid phosphatase activity in cultures treated with C8-HSL:

Changes in acid phosphatase activity in *Gloeothece* cultures treated with C8-HSL were compared to that of control. A time course experiment was carried out on both extracts over of 2.5 h. Fig. 6.12 show a linear response in enzymatic activity by the release of the product ion ρNP monitored by absorbance at 405 nm (Fig. 6.12). Product formation in the C8-HSL treated sample was detected to be higher over time compared to the control sample, indicating a greater increase in enzymatic activity in the C8-HSL treated sample over time. Calculation of the enzymatic activity at 2 h of incubation (Fig. 6.13) in both samples shows a higher enzymatic activity in the C8-HSL treated sample compared to the control. The increase in activity represented 11 % that of the control sample (Fig 6.13).

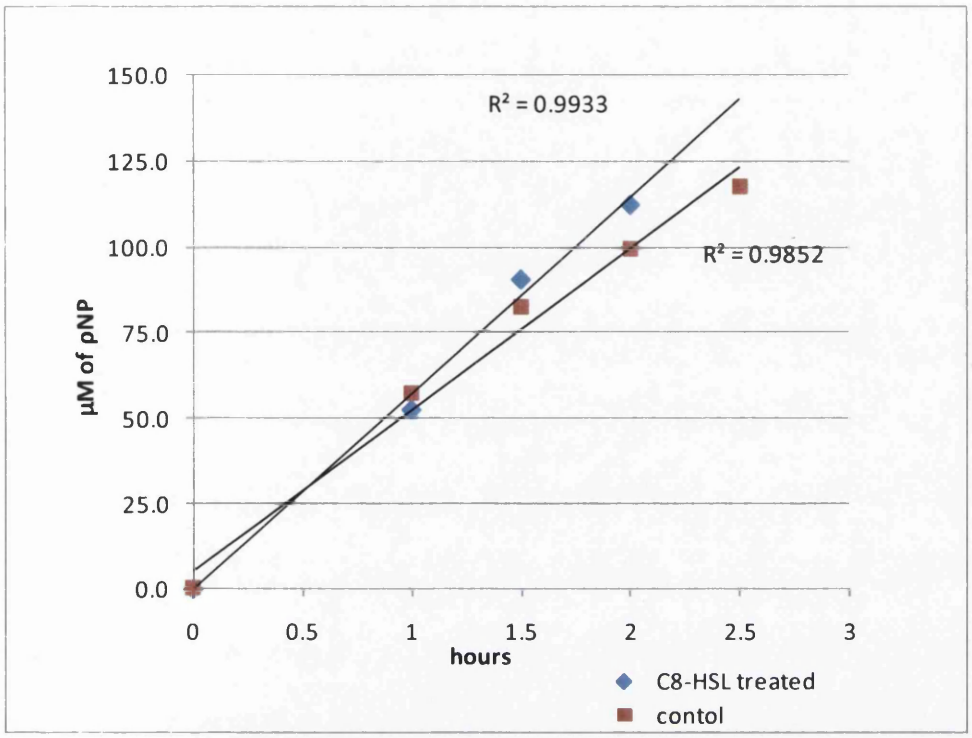


FIGURE 6.12. TIME COURSE OF ACID PHOSPHATASE ACTIVITY OF CONTROL AND C8-HSL TREATED EXTRACTS

Acid phosphatase activity time course using 100 µg of Gloeothece extract incubated with substrate pNPP over 2.5 h at pH 4.0. Product formation measured by absorbance at 405 nm and expressed as µM of pNP release. Measurements represent a mean of two duplicate sample readings.

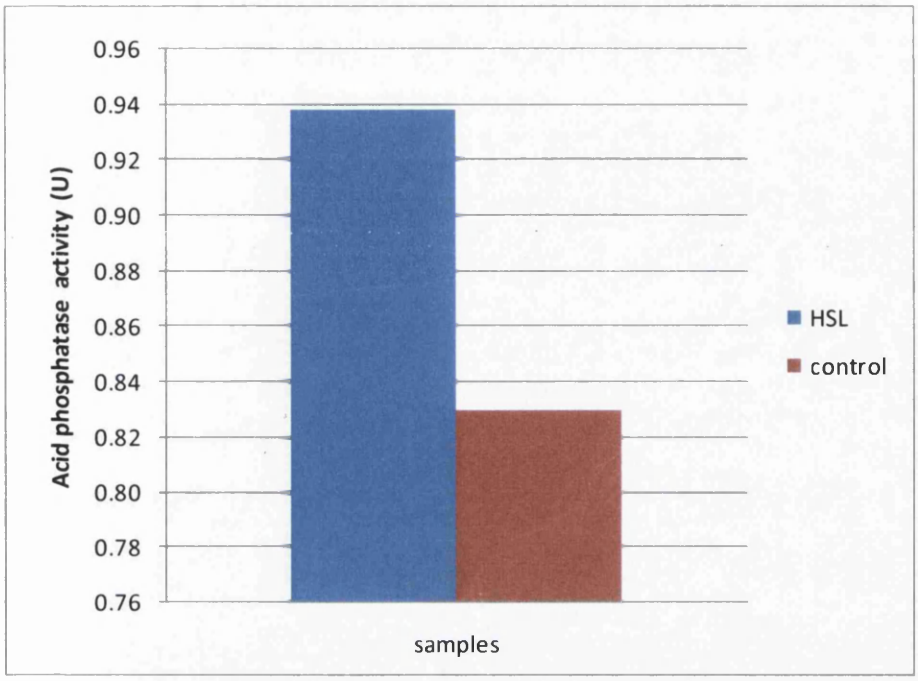


FIGURE 6.13. ACID PHOSPHATASE ACTIVITY IN CONTROL AND C8-HSL TREATED SAMPLES OF *GLOEOTHECE* EXTRACTS

Acid phosphatase activity (U) in Gloeothece extracts of control and C8-HSL treated samples incubated with substrate ρNPP over 2 h. One U = the amount of enzyme required to produce 1 μM of ρNP min $^{-1}$ under the conditions. Measurements represent an average of two duplicate sample readings.

6.3. Discussion

This Chapter shows that the addition of C8-HSL to cultures of *Gloeothece* induces changes in some physiological conditions. Preliminary studies on turbidity and chlorophyll concentrations were investigated in order to detect any changes in biomass or chlorophyll concentrations in response to C8-HSL. The effect of C8-HSL in terms of changes in biomass and chlorophyll concentrations was considered due to the fact that some colonial cyanobacteria can coordinate their pigment concentrations under increased self shading of the cells (Agusti and Philips, 1992). This situation may be particularly important when cells reach high cell density. Growth may also be changed under conditions that allow cells to enter a dormant stage particularly under nutrient limitation, which could also arise when cells encounter high cell densities. Although not much information is available in this regard for *Gloeothece*, the possibility of such changes in response to C8-HSL was considered. Growth inhibition has been reported for the bacterium *R leguminosarum* in response to its signal *N*-(3-hydroxy-7-*cis* tetradecenoyl)-L-homoserine lactone (3OH-$C_{14:1}$-HSL) (Lithgow *et al.*, 2000). However, under the experimental conditions used in this study no apparent changes in cell biomass or chlorophyll concentrations were observed in cultures of *Gloeothece*. One possibility could be that the physiological effects do not take place until additional mechanisms are first activated. However, a range of other possibilities may exist which can depend on one or several factors (Chapter 1) including:

- The presence of other signals that need to act along side C8-HSL in *Gloeothece.*

- The rate of synthesis and turnover of the receptor or transcriptional regulators required to carry out expression changes.
- The growth stage dependency of activation by C8-HSL.
- The integration of other regulatory circuits to bring about changes for example, environmental cues.
- Rapid mass transfer of C8-HSL signals in a highly aerated culture, allowing little binding or rapid signal dissociation.
- The sheath of PCC 6909 acting as a diffusion barrier allowing low concentration of signals to penetrate in order to activate a response.

All experiments cultures were prepared by washing away endogenous produced AHL by *Gloeothece* and then resuspending the same culture into two equal parts in fresh $BG11^0$ medium. As mentioned in the previous chapter, this is thought to eliminate differences caused by culture variations of growth stage and allows the use of a single homogenous culture, where any changes may be attributed to the treatment of C8-HSL to the cultures. However, in some cases changes in a physiological response may not be evident when cultures are already under the effect of the signal. This may occur if AHL molecules have already activated a response and if the response persists even after the culture has been washed out of AHL signals. For example, it has been observed in vitro that the transcriptional regulator TraR of *Agrobacterium tumefaciens* strongly binds to its cognate signal 3-oxo-C8-HSL and that the TraR remains active for as long as 8 h even after removal of the exogenous signal (Qin *et al.*, 2000).

During growth in batch cultures, extracellular polysaccharides are released by cultures of *Gloeothece* and are excreted into the extracellular environment. Both PCC 6909 and PCC 6909/1 are known to produce extracellular polysaccharide although the latter lacks its cell bound polysaccharide sheath. Various authors have characterized the sheath composition of *Gloeothece* and a soluble polysaccharide with a chemical composition similar to that of inner and outer sheath fractions was also found to be released into the extracellular medium (Tease and Walker, 1987). Investigation of a physiological response in total carbohydrate accumulation in *Gloeothece* PCC 6909/1 showed that C8-HSL addition within the concentration range of 50 nM to 5 μM resulted in decreased accumulation of extracellular carbohydrate, that were mostly characterized as soluble carbohydrates. The percent increase in total carbohydrate during growth of *Gloeothece* PCC 6909/1 from 7 days was monitored (Fig 6.5) which showed that total carbohydrate can increase to approximately 300 % during the first 7 days of measurement. This increase was found to be consistent with an increase detected in the control sample where a 360 % increase was detected after the addition of C8-HSL (Fig. 6.3). In contrast, the total carbohydrate increase in all C8-HSL treated samples was only 5.3 % indicating that C8-HSL resulted in decreasing the accumulation of total carbohydrate (Fig.6.3). However, separation of this total carbohydrate through dialysis tubing with a molecular weight cut off 7 KDa indicated that the changes are mainly in the extracellular soluble carbohydrates that are less than 7 KDa. These carbohydrates may represent various forms of monosaccharides and disaccharides. The ratio of soluble carbohydrate was also found to increase during growth in the control cultures, and accounted nearly 50 % of the total carbohydrate (Fig 6.4). Evidence of soluble polysaccharides occupying 40-70 % of total extracellular polysaccharides has been reported for various *Cyanothece* sp, some of which showed a dramatic decrease in the

soluble-to-total-carbohydrate ratio under conditions of nitrogen limitation (De Philippis *et al.*, 1998). The behaviour, which has been linked to the accumulation of intracellular carbohydrate reserves, has also been observed in the EPS-producing cyanobacterium *Cyanospira capsulata* under conditions of nitrogen limitation (De Philippis *et al.*, 1996). Therefore, decrease in the soluble-to-total-carbohydrate ratio in *Gloeothece* upon C8-HSL treatment could also be indicative of a stress response, allowing the cyanobacterium to build up intracellular carbohydrate reserves, which can be reutilized to provide an energy supply for growth. The evidence of QS involved in intracellular accumulation of polyhydroxybutyrate (PHB) storage granules has been evident in *V. harveyi* (Miyamoto *et al.*, 1998).

A study in non-specific phosphatase was carried out in response to C8-HSL treatment. Previously the presence of non-specific acid phophatase in *Gloeothece* has not been investigated and there is very little information concerning acid phosphatase activity in other cyanobacteria. Acid phosphatases usually play a key role in phosphate acquisition; however, except for a few enzymes performing specific metabolic functions it is difficult to ascribe a precise role to the majority of them. Induction of phosphatase activity is a common phenomenon among organisms acquiring phosphorous from the environment underlying an adaptive responses to phosphate starvation in various organisms and plants. Other functions may include metabolism of reactive oxygen species by purple acid phosphatases/ tartrate resistant acid phosphatases to prevent the formation of reactive oxygen radicals (Rossolini *et al.*, 1998). In *E.coli* and in *Thermotoga maritima* the surE gene product, an acid phosphatase, is important in stationary phase survival (Huang *et al.*, 2003; Schenk *et al.*, 2000) Although the physiological role and biochemical function of the surE gene

product is unknown, homologues of this gene are also present in numerous other organisms including cyanobacteria. In this study detection and characterization of the acid phosphatase was undertaken. Although detailed investigation is required for a total characterization of the specific enzyme (s), results can be used to give a preliminary idea of the acid phosphatase activity. Results in this study indicate that acid phosphatase activity is sufficiently stable to tartrate, EDTA, and reducing agents, which may allow characterization as a class I non specific acid phosphatase (Rossolini *et al.*, 1998). The activity also showed no specific metal requirements, although a slightly higher activity was detected in the presence of Mn^{2+} and Mg^{2+}. The acid phosphatase activity obtained was higher in *Gloeothece* extracts, extracted using Tris-HCl (pH 7.5) as an extraction buffer over citrate buffer (pH 5.0), which could be attributed to a higher extraction efficiency using Tris and the higher pH. However, under both extraction conditions the activity was not found to be sufficiently stable for a long time, preventing useful comparisons. During acid phosphatase activity a higher background absorbance was found to be derived from *Gloeothece* extract which may be the result of other phenolic compounds with similar absorbencies. Also a small increase in absorbance was detected with the substrate ρNPP which may be due to the presence of ρNP as a contaminant. The absorbance however was not found to increase with time and the use of appropriate controls ρNPP, *Gloeothece* extracts and NaOH used simultaneously eliminated most of these problems and allowed estimation of acid phosphatase activity from extracts. Studies carried out to identify a fraction of soluble and membrane soluble fraction showed that most activity was detected in the soluble fraction. However, a loss in activity due to the presence of detergents in the membrane fraction was tested by incubating soluble fractions with detergent, which showed no loss in activity, indicating the phosphatases activity to be in a soluble periplasmic or cytoplasmic compartment.

An increase in acid phosphatase activity in *Gloeothece* cultures was observed with culture age and also an increase was observed with the addition of C8-HSL. This could be of considerable significance with the findings that show C8-HSL concentrations to accumulate during the later stages of growth in *Gloeothece* (Chapter 3) and may indicate a possibility that *Gloeothece* induces acid phosphatase activity at high cell density through C8-HSL. Although the activity in the C8-HSL treated sample increased by 11 % in 2 h compared to the control, most often it can be difficult to obtain sufficient information using whole cell extracts due to the presence of various inhibitors and compounds that may alter the acid phosphatase activity. In such cases it could be possible that an inhibitory effect was eliminated in response to C8-HSL treatment and the increase reflected such a condition. However, the possibility of such explanation may need further justification by determining the inhibitors and purification of the enzymes. Future experiments with the purification of the enzyme may lead to a better understanding of its characteristics and the effect by C8-HSL.

Finally, a summary of the main findings of this chapter are as follows:

- C8-HSL does not produce any effect on cell biomass.
- C8-HSL does not produce any effect on chlorophyll concentration.
- C8-HSL decreases extracellular accumulation of soluble carbohydrate
- C8-HSL affects changes in non-specific acid phosphatase activity.

CHAPTER 7

Final Discussion

7.1. Overview

As some activities of gram negative bacteria are co-ordinated in a density-dependent manner via quorum-sensing (QS), we examined the cyanobacterium *Gloeothece* for evidence of such a system. Evidence that AHL(s) accumulates in the culture medium of both the wild type (PCC6909) and the 'sheathless' mutant (PCC6909/1) was derived initially from bioassays using an *A. tumefaciens* reporter system (Fig. 2.1). Further mass spectrometric analysis identified C8-HSL as the major AHL accumulated by both strains (Figs.2.7-2.9). Prior to this study there have been only two reports of AHL production in cyanobacteria (Bachofen and Schenk, 1998)(Braun and Bachofen, 2004). However, the cultures involved were taken from open lake water and were likely to contain numerous other micro-organisms, including AHL producing gram negative species. In such conditions, the origin of the signalling molecule is unclear. The *Gloeothece* cultures used in this study were grown axenically and were periodically tested for the presence of other micro-organisms by a combination of laser scaning confocal microscopy and application of the cultures to a suitable bacterial growth medium on agar plates. Only axenic cultures were used during this study. This, then, is the first report of AHLs produced by an axenic strain of a cyanobacterium.

By itself, accumulation of AHL in a culture is not sufficient to indicate that an AHL-based signal system operates in that organism. It is possible that the C8-HSL identified in cultures of *Gloeothece* has no physiological/biochemical role. However, further evidence presented in this report suggests that this is not so. Quorum-sensing is a form of intercellular

signalling that is dependent on the density of the population, and so changes in gene expression, that arise via such signalling, co-ordinate with the growth stage of a culture (Miller and Bassler, 2001). The pattern of accumulation of C8-HSL in *Gloeothece* cultures appeared to be a density dependent one (Fig. 3.6 A, B). A significant increase in C8-HSL concentrations was apparent when cell numbers were expressed per mg of protein; this predicts that after 55 days, C8-HSL accumulates at a greater rate per cell signifying QS induction. Such a pattern of induction also correlated with the well known *V. fischeri* bioluminescence pattern and the AHL production kinetics of various other bacteria (Ravn *et al.*, 2001)(Swift *et al.*, 1993). This signifies that in *Gloeothece* C8-HSL accumulation is density dependent and a critical threshold concentration of C8-HSL is required for QS induction process after which C8-HSL accumulates at a greater rate. The concentration of C8-HSL detected in the medium of PCC6909 /1 reached the low nM range (Fig. 3.A). Such low concentrations have previously been reported to be within the range over which quorum-sensing systems appear to operate in other organisms (Burton *et al.*, 2005; Kaplan and Greenberg, 1985). In response to treatment with synthetic C8-HSL, changes in expression of various proteins were observed in cultures of *Gloeothece* (Figs. 5.6 & 5.7, Table 5.2-5.4), the response predicted a global change in protein expression similar to that of a response by a QS molecule. The level of change (43 proteins in total, ca. 11% of the total proteins detected on 2D gels) is consistent with reports in changes in protein expression observed in other organisms during quorum-sensing (Christensen *et al.*, 2003) (Riedel *et al.*, 2003). From these results it is evident that the C8-HSL released by *Gloeothece* acts as a QS signalling molecule in this organism.

7.2. The significance of quorum sensing in *Gloeothece* under natural conditions

The environments that *Gloeothece* inhabit are diverse, ranging from hypersaline salt lakes to deserts and in association with lichens, which may reflect species diversity of these cyanobacteria. In this study only one strain of *Gloeothece* has been investigated for its potential to use a QS mechanism. The strain under investigation is an isolate from biofilms on rock surfaces and obtained in pure culture from the Pasteur Culture Collection (PCC). Various studies examining the microbial community on stone surfaces show that coccoid cyanobacteria can play a significant role in biodeterioration of stone monuments (Crispim and Gaylarde, 2005). Various pictures taken using microscopic techniques show the distribution of cyanobacteria within or on the stone surfaces to be arranged in small microcolonies surrounded by extracellular slime (de Los Rios *et al.*, 2004), which may reflect how *Gloeothece* grows on these rock surfaces. The habitat of *Gloeothece* on epilithic surfaces together with its ability to form colonial aggregates may give these organisms a selective advantage to use C8-HSL based QS system. In biofilms, conditions can be suitable for autoinducer accumulation and concentration of autoinducers can build up significantly higher than that of laboratory liquid cultures. Concentrations of up to 600 μM have been measured in the vicinity of a *P. aeruginosa* laboratory biofilms (Charlton *et al.*, 2000), so under biofilm conditions *Gloeothece* may produce more AHL than observed in laboratory cultures. However, due to the heterogeneity of biofilms it has always been convenient to study individual species in a pure culture in planktonic form. Other factors, like desiccation have been found to increase the local concentrations of AHL under natural environments due to limitation in flow rates. Reports showed that when *Pseudomonas syringae* was grown

on dry leaves, 69 % of the population underwent QS induction compared to 0.04 % when grown on wet leaves. The level of induction on dry leaves increased with both time and the size of the aggregate indicating that desiccation can contribute to significant accumulation of AHL (Dulla and Lindow, 2008). In this regard epilithic surface may be a good environment for significant accumulation of AHL in *Gloeothece* biofilms, owing to the fact that these habitats can remain desiccated for long periods. AHL released by *Gloeothece* can accumulate under such conditions allowing rapid induction of QS gene expression. In this case QS in *Gloeothece* can be used as means of "Diffusion sensing" proposed by Redfield (Redfield, 2002). The diffusion sensing hypothesis states that micoorganisms release chemical signals in order to monitor diffusion. In low diffusional conditions signals can accumulate and microorganisms sense these signals in order to release various extracellular enzymes that would otherwise be a wasteful secretion in environments subjected to a high rate of diffusion. The hypothesis can prove to be useful in testing whether *Gloeothece* senses desiccation stress through the release of C8-HSL molecules. Another interesting observation is that under desiccated conditions, QS induction can occur in a relatively small quorum size (as small as 16 cells) (Dulla and Lindow, 2008), which suggests that *Gloeothece*, growing under natural biofilms conditions may also be capable of QS induction within individual colonies supported by the fact that even a single cell could produce enough AHL to be perceived by adjacent cells within a few micrometers (Steidle *et al.*, 2001). The sheath layers and extracellular slime released by *Gloeothece* cells can also provide a diffusion barrier, resulting in accumulation of high local concentrations of AHL within each colony sufficient for QS induction. In this regard C8-HSL identified in *Gloeothece* can be used as means of intra-colonial communication or "efficiency sensing" as proposed by Hense *et al.* (Hense *et al.*, 2007). The hypothesis states that not

only cell density and mass transfer affect the induction process by signal accumulation but also clustering of cells or colonial aggregates can also affect QS induction, regardless of population density.

7.3. Accumulation of AHL in *Gloeothece*

In natural environments bacteria seldom encounter conditions that permit periods of exponential growth. Rather, bacterial growth is likely to be characterized by long periods of nutritional deprivation punctuated by short periods of fast growth, a feature that is commonly referred to as the feast-or-famine lifestyle.(Kolter *et al.*, 1993). The stationary phase of growth may be regarded as a stage where no further increase in cell division is observed. In laboratory cultures this stationary phase represents the cessation of cell division due to nutrient deprivation, although the cells may still remain metabolically active (Kolter *et al.*, 1993). From growth studies on *Gloeothece*, an increase in C8-HSL concentration was evident just after a short stationary phase, indicating QS type induction at this stage resulting in increased production of C8-HSL. The abundance of cells calculated at this stage by an equation that converts turbidity measurements in to cell abundance (Emily, 2008) (Appendix II) shows that the cells can reach $1.7x\ 10^6$ cells ml^{-1}, which is similar to other cyanobacterial populations at their stationary phase of growth. The significance behind such an observation may lead to speculation that QS induction may allow cells to overcome stress associated with the stationary phase of growth and thus allow further growth. The involvement of QS in stationary phase survival has been reported in various bacteria, where QS induction activates the stationary phase sigma factor, resulting in transcription of several genes that allow the organism to survive the stationary phase (Bertani and Venturi, 2004). The

QS signal 3-OH-C14:1-HSL in *R. leguminosarum bv. phaseoli* has been found to play a role in adaptation to stationary phase, from an observation reported that when cells become starved by limited carbon or nitrogen source, cultures that enter the stationary phase at high cell densities shows no loss of viability over long periods (20–60 days), while cultures that enter stationary phase at low cell densities rapidly lose their viability (Thorne and Williams, 1999). A similar situation was evident while growing *Gloeothece* cultures in this laboratory. Dense cultures were able to survive for a longer periods than a low density culture. Owing to the fact that a highly dense culture should undergo nutrient limitation rapidly and enter stationary phase earlier than low density cultures, the observation seems to be one of a QS where dense cultures were able to survive longer, which may indicate the involvement of C8-HSL based QS identified in this study. However, it would be interesting to observe if addition of extracellular C8-HSL to low density starved cultures promoted similar starvation survival as observed in cultures of *R. leguminosarum bv. phaseoli* entering stationary phase at low cell density. The accumulation pattern of C8-HSL during growth of *Gloeothece* in this study may represent a pattern when using $BG11^0$ as growth medium. Since the AHL release and response to AHL in certain cases is dependent on growth phase and media composition (Brelles-Marino and Bedmar, 2001; Pongsilp *et al.*, 2005), the accumulation of C8-HSL in *Gloeothece* may therefore, vary with the use of different growth media or conditions and may not show a response similar to that observed in this study.

During growth of *Gloeothece* the pH of the culture medium becomes alkaline within weeks, although the exact reason for this is not known. The degradation kinetics of C8-HSL suggests that at pH 8.0 the half life of C8-HSL may be in days. However from an experiment carried in comparing extraction efficiecies of C8-HSL at different pH

showed that the same amounts of AHL could be extracted from culture supernatants of *Gloeothece* indicating no pH dependent lactonolysis (Fig. 2.2). The reason for such an self evident observation is not clear but it may be possible that signals are protected from lactonolysis through unknown mechanisms. The experiment was conducted on only one sample during growth and therefore the hypothesis that *Gloeothece* cultures provide protection of C8-HSL against pH dependent lactonolysis was not thoroughly tested. However, a protective mechanism involving QS molecules has been reported. The *P. aeruginosa* quinolone signal PQS which resembles AHL molecules can be localised in membrane vesicles that serve to protect and traffic these molecules within a population (Mashburn and Whiteley, 2005). Another function of AHL at alkaline pH have been reported to be involved in rearrangement of AHL molecules into tetramic acid that can act as iron chelating compounds (Kaufmann *et al.*, 2005) (Fig. 1.2). AHL has also been identified from halophilic microorganisms (Llamas *et al.*, 2005) although how they protect AHL or use AHL as a signalling molecule is still unknown.

7.4. Physiological responses in *Gloeothece*

The habitat of *Gloeothece* represents environments where nutrients can often be limiting and cells may encounter various levels of stress, such as desiccation, UV damage, and nutrient limitation and light limitation. Growth in such habitats may represent the ability of *Gloeothece* cells to overcome stressful conditions through the use of QS. Quorum Sensing is generally known to be associated with a coordinated physiological response. Therefore, various physiological changes generally associated with stress were investigated. Commonly stress may be associated with changes in

growth rate, accumulation of intercellular reserve materials. In *R. leguminosarum*, QS was found to be involved in growth inhibition by the signalling molecule 3-OH-C14:1-HSL(Gray *et al.*, 1996; Wilkinson *et al.*, 2002). Growth inhibition is maximal when C8-HSL or 3-oxo-C8-HSL is added together with 3-OH-C14:1-HSL (Wilkinson *et al.*, 2002). Chlorophyll concentrations may also represent the viability of cultures of cyanobacteria. Studies have shown that cell biomass and chlorophyll concentrations of *Gloeothece* are not significantly altered by the addition of C8-HSL to early stage cultures, indicating there may be no correlation with C8-HSL response and growth. These observations may also indicate that C8-HSL may not be used as energy sources as used by some other organisms (Huang *et al.*, 2003). However, further investigation is necessary to prove such a hypothesis. Since, C8-HSL accumulates in cultures during later stages of growth it could be speculated that C8-HSL has some functions in regulating stress response that may be observed as a decrease in soluble carbohydrate release and increase in intracellular reserves. Upon addition of C8-HSL, soluble carbohydrate accumulation was found to be reduced. This could be correlated with the fact that cyanobacteria release carbohydrates during growth under nutrient replete conditions, however, certain cyanobacteria when encountering nutrient limitation reduce the levels of soluble extracellular carbohydrates and store them as reserve carbohydrate granules in the cells (De Philippis *et al.*, 1996). For example, with enhanced carbon flux the cyanobacterium *Cyanospira capsulata* channels its excess carbon preferentially into the synthesis of an overflow product like EPS, whereas, with the diversion of carbon flux from the process of nitrogen assimilation, the synthesis of carbon reserves is mostly favoured (De Philippis *et al.*, 1998). It would be interesting to investigate whether a decrease in soluble carbohydrate release correlates with intercellular accumulation of carbohydrate reserve granules in *Gloeothece*. The study of

another physiological response such as acid phosphatase activity was investigated. Acid phosphatases are non specific enzymes which promote the degradation of complex phosphorus compounds into orthophosphate and an organic moiety, and are thus believed to have an essential function in various cellular and metabolic processes (Rossolini *et al.*, 1998). Acid phosphatases are grouped into various categories and are reported to be present in forms of life. Some non-specific acid phosphatases have been reported to be regulated in response to phosphate limitation in cyanobacteria while other functions may involve roles in oxidative damage, intracellular signalling and metabolism in other bacteria (Rossolini *et al.*, 1998; Schenk *et al.*, 2000). No particular role of acid phosphatases has been found to be associated with QS, however, various non specific acid phosphatases such as purple acid phosphatase and the stationary phase survival protein SurE (acid phosphatase) are interesting enzymes that are reported to be induced under bacterial stress conditions (Huang *et al.*, 2003; Schenk *et al.*, 2000). A cyanobacteria database search was carried out on SurE protein and purple acid phosphatase, revealing that most cyanobacteria contain enzymes homologous to these proteins for which their physiological role has not yet been investigated. The presence and activity of acid phosphatase was investigated in *Gloeothece*. An increase in enzymatic activity was detected in the C8-HSL treated samples compared to controls, indicating that acid phophatase activity in *Gloeothece* may be regulated through C8-HSL. However, experiments were conducted on crude extracts and enzymatic activity was found to be stable over a short period of time. Therefore, results may need further confirmation by purifying the acid phosphatase containing fraction and testing for activity.

7.5. Altered protein expression in respose to AHL

In order to investigate whether *Gloeothece* responds to its own C8-HSL, changes in protein expression were analysed by SDS-PAGE & 2D-PAGE. Analysis was undertaken by treating early stage cultures with exogenous C8-HSL. 2D-PAGE has been used as a method to study QS gene expression in a number of bacteria (Cantero *et al.*, 2006; Chen *et al.*, 2002; Riedel *et al.*, 2003). The addition of synthetic AHL has been found to induce early activation of various quorum responsive gene expression in both *Vibrio fischeri* and *Pseudomonas aeruginosa* (Cao and Meighen, 1989; Whiteley *et al.*, 1999). In this experiment the nature of the two treated samples designated as C8-HSL treated and control, were derived from a single parent culture of *Gloeothece* cells and therefore, the two treatments were clonal in nature. Biological replicates are generally used to encounter variations among natural populations and using clonal cultures was thought to eliminate any such variations among the two groups (despite any mutations that could take place). However, to evaluate results obtained in expression changes in response to C8-HSL, technical replicates were used and expression changes were analysed by applying standard statistical methods. The number of differently expressed proteins by C8-HSL treatment constituted about 11 % of total protein detected. However, this value may be arbitrary depending on other factors, such as the expression of proteins that are obscured by Phycobiliproteins (the light harvesting pigments in cyanobacteria), which constitute about 60 % of the total soluble protein with a molecular mass of 15 KDa and appear as a smear on the 2D gel (see Figure 5.6 & 5.7, lower right hand corner, easily identified from this region by LC/MS/MS). A potential limitation of this experiment is the possibility that the

expression of many genes involved in QS may also be time dependent. Therefore, many late genes may not respond to exogenously added AHL until the culture reaches the stationary phase of growth (Schuster *et al.*, 2003; Whiteley *et al.*, 1999). Furthermore, the extraction of many hydrophobic membrane proteins may not be achieved by 2D proteomics and may require optimization for their efficient extraction. Despite these limitations, the 2D experiment allowed the observation of proteins whose expression was altered in response to the C8-HSL.

The difficulty in protein identification and assigning proteins to a particular metabolic pathway in *Gloeothece* is mainly due to the unknown genome sequence and lack of reports on different metabolic pathways in this organism. Therefore, the LC/MS/MS data files were searched against databases of all organisms. Encouragingly, many of the proteins homologues shown in Table 5.4 & 5.5 are from bacterial species, which suggests, that the reliance on sequence homology and conservation for the identification of proteins from this cyanobacteria was sufficient in this case. The main reason for having less peptide matches may be due to the evolutionary heterogeneity of cyanobacterial species which show more divergent protein sequences for the same proteins in higher organisms. Only those proteins that showed the greatest level of confidence were presented. However, careful consideration should be made in assigning proteins to a particular role related with QS, because there may be other subtleties such as cofactor generation and other side products that might be met through regulating a specific protein at high cell density. Moreover, the function of several QS regulated proteins is still unresolved and it is thought that through regulating various cellular processes, bacteria are able to change their physiology in response to high cell density.

7.6. Functions of proteins identified differentially expressed on 2D gels

The largest increase in expression in response to C8-HSL was observed for RuBisCo which catalyses carbon dioxide fixation in *Gloeothece*. Generally, AHLs activate expression of genes that are advantageous to a species when they are at a population density perceived as a quorum. An increase in the expression of RuBisCo in response to a QS signal may offer such an advantage. The efficiency of RuBisCo is relatively low and its capacity to combine CO_2 with its co-substrate, Ribulose-1, 5-bisphosphate (RuBP), can be a major limitation on the rate of net CO_2 assimilation. A rise in cell density would eventually lead to limitations in availability of various nutrients (including CO_2), eg from limited diffusion in biofilms. In such circumstances, an increase in the amount of RuBisCo may be expected to lead to restoration in the efficiency of CO_2 fixation. This can be supported by the fact that RuBisCo transcripts in many cases are found to increase in response to inorganic carbon limitation (Shively *et al.*, 1998). An AHL-based quorum-sensing system may, therefore, modify the population and allow it to cope with lower CO_2 concentrations. Furthermore, RuBisCo is increased in expression following treatment of *Medicago truncatula* with nM concentrations of AHL (Mathesius *et al.*, 2003). A LysR type transcriptional regulator found to be upregulated (2.4-fold) in *Gloeothece* is known to be the transcriptional regulator of RuBisCo in most microorganisms (Shively *et al.*, 1998). In contrast to RuBisCo, expression of enolase (2-phosphoglyceratelyase) and aldolase, both of which are associated with carbohydrate metabolism, are down regulated by treatment with C8-HSL (Table 5.4). A decrease in the activity of enolase, which catalyses interconversion of 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP) may be expected to

reduce flux through the glycolytic/gluconeogenic sequence. Similarly, a decrease in the activity of aldolase, which catalyses the interconversion of fructose 1, 6-bisphosphate (F1, 6-BP) with glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), may also reduce flux through the same pathway. Such a reduction, coupled with increased RuBisCo could lead to increases in intermediates in this pathway, such as G3P, DHAP and intermediates such as 2- and 3-phosphoglycerate (2PGA & 3PGA respectively). Increases in the latter group would maintain the concentration of RuBP.

3PGA is also a precursor in the synthesis of the a-keto acids corresponding to the amino acids, serine, glycine and cysteine and so may lead to increase in the synthesis of these amino acids. In this regard, the increase in glutamate synthase (GS) in response to C8-HSL treatment (Table 5.4) would be significant. Glutamate, together with glutamine, plays a central role in the assimilation of NH_4^+ into amino acids and then into other nitrogen-containing biomolecules. Glutamate is the source of amino groups for most other amino acids and an increase in its availability may lead to a general increase in amino acid synthesis. There is some evidence that this may be so. Chorismic acid is a key intermediate in the synthesis of the aromatic amino acids and Chorismate synthase exhibits a 2.5-fold increase in response to C8-HSL treatment. Chorismate is synthesised from PEP and erythrose 4-phosphate (E4P), the latter arising from the activities of the oxidative- and reductive pentose phosphate pathways. The combined changes in aldolase, enolase and RuBisCo activities, discussed above, would be expected to lead to an increase in E4P that would then be available for chorismate synthesis. Chorismate is also the biosynthetic precursor of 2-heptyl-3-hydroxy-4-quinolone, a cell–cell signal that is unique to *P. aeruginosa* (PQS) and has been found to be an important metabolite in colonization during cystic fibrosis lung infection (Brown *et al.*, 2008; Gallagher *et*

al., 2002). Therefore, it is possible that through the regulation of various enzymes involved in carbon, nitrogen, amino acid biosynthetic pathways, *Gloeothece* regulates activities to increasing cell density.

The increased expression of an ABC-type transporter observed (Table 5.4 and Table. 5.5) would be expected to increase the capacity for trans-membrane transport of metabolites (Higgins, 1992). There is evidence that cells of *Gloeothece* excrete amino acids during times when nitrogen-fixation exceeds the capacity for growth and that these are re-imported at times of limited nitrogen fixation (Flynn and Gallon, 1990). The increase in the transporter protein may reflect this situation. An acyl transferase is also up regulated in response to AHL and potentially may be involved in the demonstrated autoinduction of C8-HSL biosynthesis. Although no LuxI and LuxR homologues have been detected in cyanobacteria, alternative pathways for AHL biosynthesis are known, eg, the HtdS utlising pathway in *Pseudomonas fluorescens*. The HtdS enzyme involved belongs to the lysophosphatidic acid acyltransferase (LPAAT) family and has been reported to direct the synthesis of some AHLs (Laue *et al.*, 2000). It is possible then, that C8-HSL production in *Gloeothece* occurs through such a route, though the genome of *Gloeothece* has not yet been characterised. The up regulation of an acyl transferase may reflect the requirement of a C8 carbon chain being available for AHL biosynthesis and may arise as an identified protein through homology to other similar enzymes such as the HtdS acyltransferase enzyme. On the other hand various proteins identified by MS that showed differential accumulation on SDS gels through phosphoprotein analysis indicate changes in the phosphoproteome of *Gloeothece* in response to C8-HSL. Several of these identified proteins are reported to be phosphoproteins that accumulate under stress conditions, such as heat shock or

starvation (Hengge-Aronis, 1999; Rosen *et al.*, 2004). Identification of phosphoproteins revealed several of these proteins to accumulate, such as ABC transporters, which can be upregulated through a bicarbonate stress. Also chaperons such as GroEL are also upregulated in response to stress (Omata *et al.*, 1999; Yamazawa *et al.*, 1999). The function of GroEL in proper folding of LuxR type regulators in QS has also been reported (Manukhov *et al.*, 2006).

7.7. Biofilm growth and AHL based quorum sensing in *Gloeothece*

While the current studies have been carried out with cultures of dispersed, 'planktonic' cells, in its natural environment *Gloeothece* frequently occurs as a component of biofilms and there is evidence, at least for some species, that QS influences biofilm development (Lynch *et al.*, 2002). However, assessing the significance of AHL accumulation within a biofilm is difficult, in part because a biofilm presents a complex environment for intercellular signalling to operate within. Much higher local densities of cells are likely to be encountered in such communities and so the distribution and accumulation/uptake of metabolites, including signal molecules is likely to be affected by such community structure (Horswill *et al.*, 2007). Furthermore, biofilms are normally a mixture of many microbial species and, even for a single species, strain diversification may result from the occurrence of microniches within the biofilm (Davey and O'Toole G, 2000). It is certain then, that signal production and perception, and the response to the signal is much more complex in biofilms than in cell cultures. Consequently, it is difficult to determine what the consequences may be for *Gloeothece* and its associated organisms of C8-HSL secretion into a biofilm. For example, it is

recognized that signal distribution is not likely to be uniform throughout the biofilm, being affected by factors such as mass transfer, the spatial distribution of cells, in particular formation of clusters and microniches, signal degradation by other species and differential rates of signal synthesis by individual cells in the film (Dunny and Leonard, 1997; Hense *et al.*, 2007; Horswill *et al.*, 2007). Such factors mean that it is most unlikely that all *Gloeothece* cells in a biofilm will be exposed to the same concentration of C8-HSL at a given time. It is possible, therefore, that within a biofilm there are clusters of *Gloeothece* cells, some of which have reached the concentration of signal molecule required to trigger a response, whilst others have not. Cells within densely clustered colonies, or cells deep within the biofilm, are likely to encounter nutrient or light limitation and may tend towards decreased metabolic activity. At the same time, restricted signal diffusion may lead to accumulation of C8-HSL to a sufficient concentration to trigger a response. It is possible that these stressed cells can undergo coordinated activities through quorum sensing, for example, through changes in carbohydrate and amino acid metabolism discussed. It should not be overlooked that biofilms frequently represent mixed communities of organisms. The involment of AHL in community structure has been previously carried out by McLean *et al.* (2005) who showed that C8-HSL can influence the recruitment of bacteria to biofilms (McLean *et al.*, 2005), Allison *et al.* (1998) showed that supplementation with C6-HSL and *Pseudomonas fluorescens* spent culture medium could enhance biofilm formation by this same organism (Allison *et al.*, 1998). Valle *et al.* (2004) showed that AHLs can influence sludge community composition (Morin *et al.*, 2003). AHLs have occasionally been used as medium supplements for the culturing of some fastidious organisms, eg heterotrophic bacteria from the Central Baltic Sea (Bruns *et al.*, 2002). In these regards the role of C8-HSL in *Gloeothece* may be of considerable importance in the recovery of

starved biofilms (Batchelor *et al.*, 1997) and in association with other bacteria in biofilms. It may be possible that other bacteria could also respond to C8-HSL produced by *Gloeothece* as means of inter-kingdom cross-talk. It is also recognized that some species can degrade AHLs and can use them as a carbon or nitrogen source (Flagan *et al.*, 2003; Leadbetter and Greenberg, 2000). Even if the available AHLs do not supply sufficient carbon for growth, their degradation by some species would likely influence signal gradients to the extent that some *Gloeothece* cells in the biofilm would not experience sufficient HSL concentration to trigger a response.

The current results indicate that elements of a QS system operate in *Gloeothece* but its function, especially in the context of the biofilm, is unclear. It remains to be established what factors influence the accumulation of AHLs and what are the consequences, in terms of cell and community function. Its relation to other species that are found in association with *Gloeothece* has still to be investigated.

7.8. Future direction

With the identification of AHL molecules from *Gloeothece* cultures, evidence of a QS system has been established in the cyanobacterium *Gloeothece*. This opens up a wide spectrum of potential future investigations which may involve identification of other AHL signalling systems in different *Gloeothece* sp. and also in different species of cyanobacteria, which may broaden our understanding of these molecules as universal signals in interspecies communication, such as the AI-2 signals from various bacteria (Chen *et al.*, 2002). Since, this study is centred on C8-HSL QS in *Gloeothece*, future

investigation can be carried out on identification of other QS molecules, eg AI-2 or other peptide signals of gram positive bacteria. These signals have not been reported to be produced by cyanobacteria and hold for potential future investigation on the detection of other types of QS systems in cyanobacteria. In this study the presence C6-HSL was detected throughout growth by MS, and further investigation on its accumulation pattern in cultures of *Gloeothece* can potentially give overall information on how these AHL signals operate in *Gloeothece*.

Regarding the detection of C8-HSL in *Gloeothece* cultures, identification of an AHL synthase gene and a receptor for C8-HSL could support the findings of a QS system in *Gloeothece*. In an attempt to identify an AHL synthase gene, progress has been made towards purifying the genomic DNA from this organism in creating a DNA library in *E.coli* harbouring a *luxR* activated reporter expression. So far successful isolation of DNA from PCC 6909/1 has been achieved with high purity. With this successful accomplishment, sequencing of the genome of *Gloeothece* has been proposed and has been sent of for sequencing accepted by the Craig Venter Institute, Rockville, USA. Therefore, a future investigation on the AHL synthase gene and receptors with underlying molecular mechanism involved in QS in *Gloeothece* holds promise of a potential future direction.

The accumulation pattern of C8-HSL has been investigated in $BG11^0$ medium, which is a nitrate free medium. Since various nutritional conditions and growth media can alter the accumulation pattern of AHL, it would be interesting to further investigate how C8-HSL accumulates in BG11 medium in the presence of nitrate or under different cultural conditions such as low aeration or light conditions. This could give an indication of

how C8-HSL in *Gloeothece* accumulates under stress conditions. In most experiments the sheathless mutant was used for studies. For the wildtype strain, investigation can be carried out to find weather significant amount of AHL is accumulates within the sheath, layers of *Gloeothece*. The properties of AHL suggest that there may be possibility of AHLs to partition within the hydrophobic sheath. , This could show whether the wildtype strain accumulates AHL more efficiently in order to rapidly undergo QS autoinduction.

Various proteins differentially expressed in response to C8-HSL await future investigation. For example, RubisCo, which is an important enzyme in CO_2 fixation can be analysed for activity in *Gloeothece* extracts to increasing concentrations of C8-HSL. Moreover, several phosphoproteins identified can be further analysed for their phosphorylation dephosphorylation status to C8-HSL treatments through MS. Further studies into a physiological response may involve investigation on the accumulation of carbon reserve material such as glycogen granules that may be synthesized in response to C8-HSL as through decreased accumulation of soluble carbohydrate observed in this study. Moreover, a general characterization of the acid phosphatase detected in *Gloeothece* may also provide useful information on the particular involvement of this enzyme associated with a QS response. Since AHL has also been found to be involved in species composition and community structure, further investigation could be carried out on the bacterial species that associate with *Gloeothece.* During this study it was observed that contaminated cultures were healthier than axenic cultures, in this case investigation if C8-HSL produced by *Gloeothece* has any role in synergistic association with associated bacteria remains promising.

7.9. Conclusion

In conclusion, C8-HSL based QS have been established in the cyanobacterium *Gloeothece* under laboratory experimental conditions. By itself, this is the first evidence of AHL signals detected outside the group of proteobacteria and the first identification of a QS system in cyanobacteria. With increasing evidence that micro-organisms apply QS and the diversity in the chemistry of such molecules, it may be possible that other novel molecules may work in QS in cyanobacteria. Studies on *Gloeothece* QS was important, in this case because it provides a good model to study the physiological process underlying unicellular colonial forms that show great adaptability to various environmental conditions. Further work into AHL based QS system in cyanobacteria may give us a deeper understanding into the mechanism of these highly stringent regulatory systems.

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APPENDIX I

(Buffers, stains and media)

5x Sample Buffer

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenol blue

1x Running Buffer:

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

1x Resolving Gel Solution

	7%	10%	12%
H_2O	15.3 ml	12.3 ml	10.2 ml
1.5 M Tris-HCl, pH 8.8	7.5 ml	7.5 ml	7.5 ml
20% (w/v) SDS	0.15 ml	0.15 ml	0.15 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	6.9 ml	9.9 ml	12.0 ml
10% (w/v) ammonium persulfate (APS)	0.15 ml	0.15 ml	0.15 ml
TEMED	0.02 ml	0.02 ml	0.02 ml

Stacking Gel Solution (4% Acrylamide):

H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (APS)	0.025 ml
TEMED	0.005 ml

Bradford dye concentrate :

100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95% ethanol.After 100 ml of concentrated phosphoric acid was added. The volume of the solution was then adjusted to 200 ml with distilled H_2O.

Blue Silver (Coomassie G-250 colloidal stain) (Candiano, 2004)

0.12% Coomassie blue G-250

10% ammonium sulphate

10% phosphoric acid

20% methanol

Commassie blue R-250 (Weber & Osborn)

0.1% (v/v) Coomassie Blue R-250

40% Methanol

10% (v/v) glacial acetic acid

BG11 mineral medium for cyanobacteria

Ingredient	Amount (g L^{-1})
$NaNO_3$	1.5
$K_2HPO_4.3H_2O$	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.001
EDTA (disodium magnesium salt)	0.001
Na_2CO_3	0.02
Trace metal mix A5+Co*	1 ml L^{-1}
Deionized water	1000 ml
pH after autoclaving and cooling	7.4

*Trace metal mix A5+Co contains (g L^{-1}): H_3BO_3, 2.86; $MnCl_2.H_2O$, 1.31; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.390; $CuSO_4.5H_2O$, 0.07; $Co(NO_3)_2.6H_2O$, 0.0494

APPENDIX II

(Correlations between the parameters chlorophyll a, protein, turbidity with biovolume in *Gloeothece*)

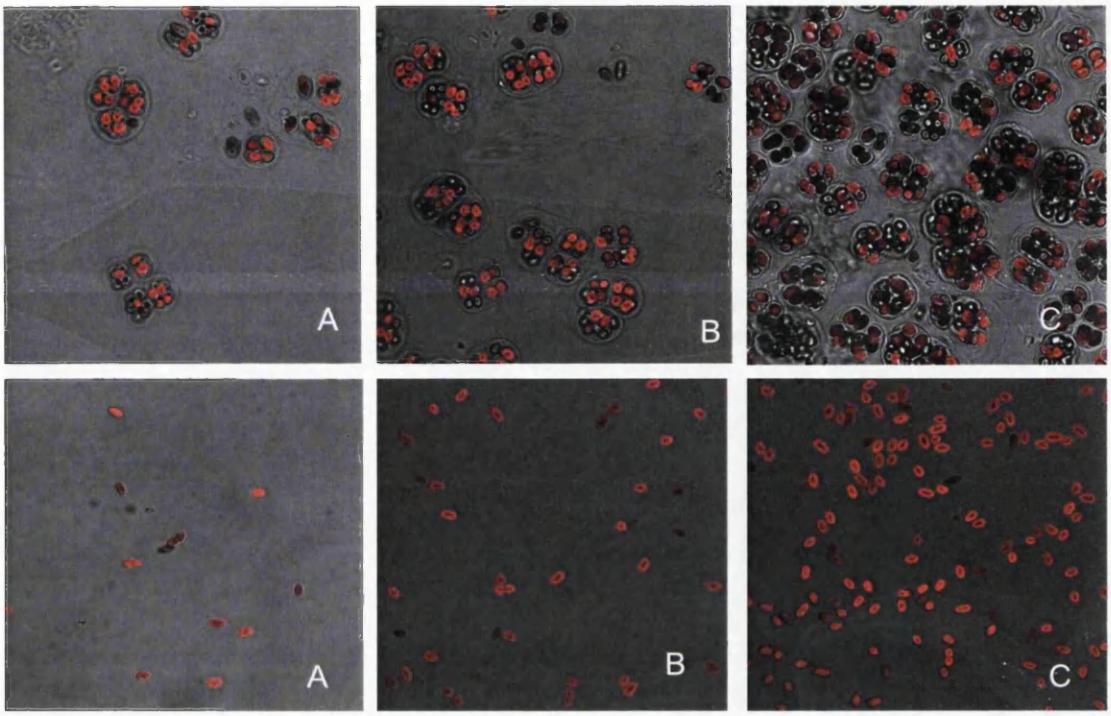


Figure: *Gloethece* images for the wild type and mutant strain at a) 2 weeks; b) 4 weeks and c) 6 weeks. The number of cells increases over time (a-c) for both the wild type and mutant strains.

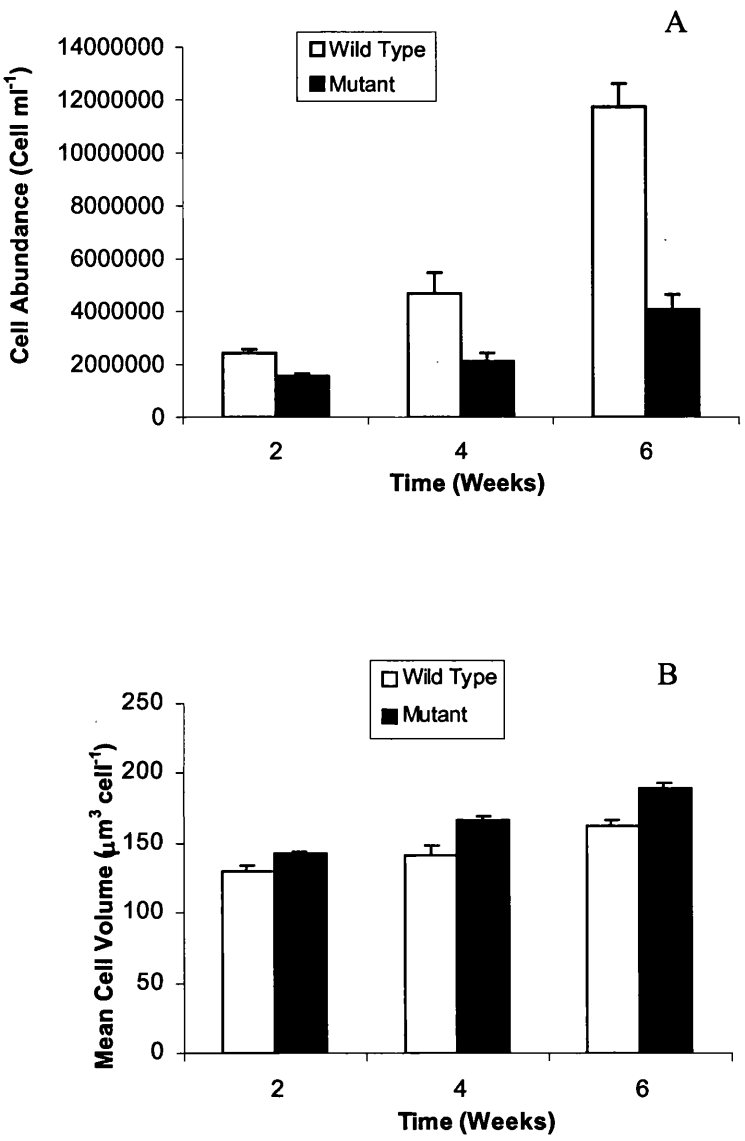


Figure: a) Change in cell abundance for wild type and mutant species of *Gloeothece* over a 6-week study period (± SE). b) Change in mean cell volume (MCV) for 2 species of *Gloeothece* over a 6-week study period (± SE).

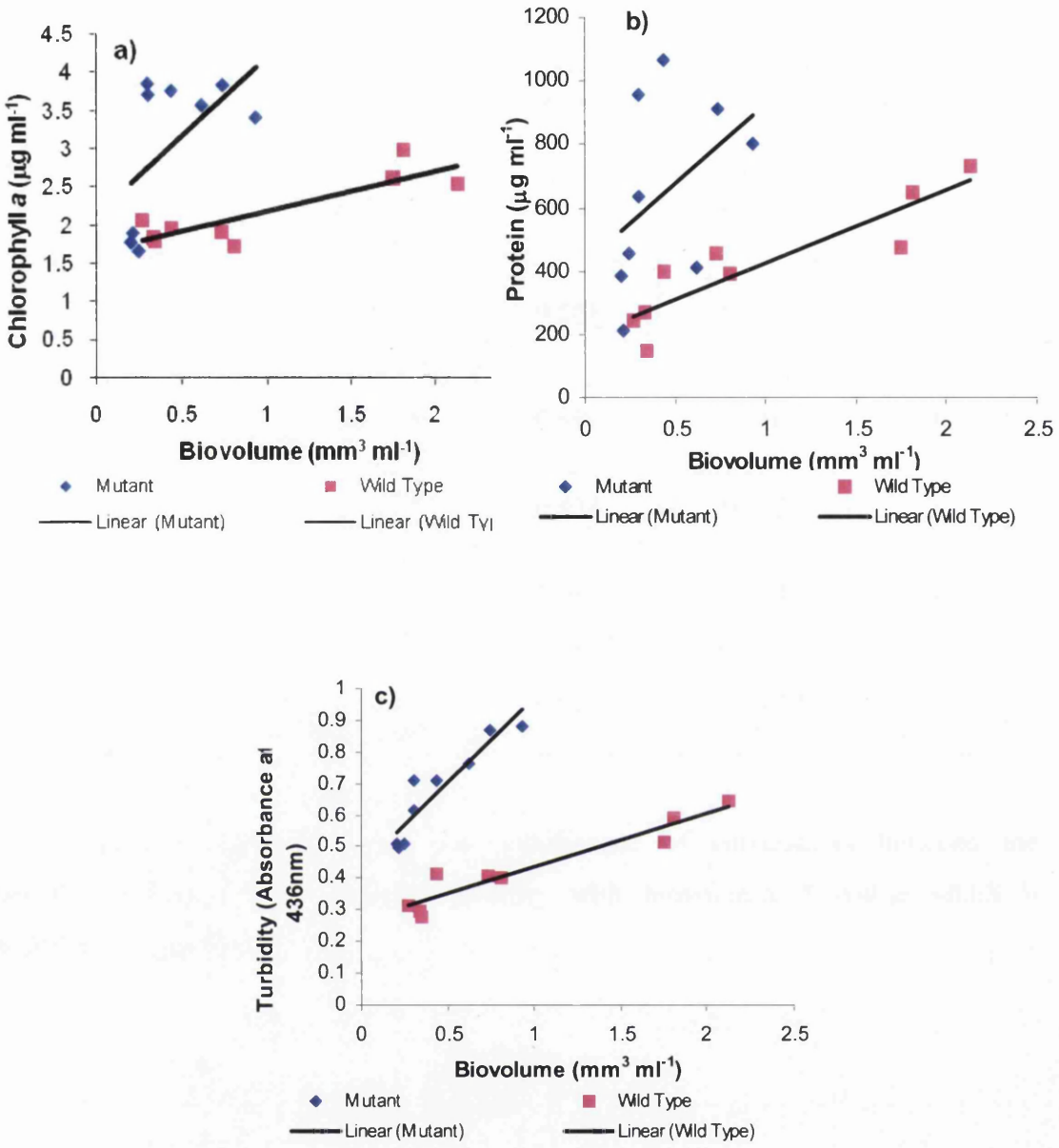


Figure: Correlation of wildtype and mutant strains of *Gloeothece* between the biovolume and a) Chlorophyll *a*; b) Protein; c) Turbidity; a-e shows the correlation between turbidity, chlorophyll *a*, protein, and phycobiliprotein concentration and the biovolume of the cells. The mutant strain chlorophyll *a* content correlates poorly with biovolume, whereas the wild type strain shows a significant correlation (a) (Table)

Parameter	Strain	Equation of the Line	Pearson's Correlation Co-efficient (*r*)	Significance (*p*)	Number of Variables (*n*)
Chlorophyll *a*	Wild Type	y = 0.5178x + 1.6642	0.855	0.03*	9
	Mutant	y = 2.0727x + 2.132	0.561	0.116	9
Protein	Wild Type	y = 229.51x + 197.31	0.899	0.001*	9
	Mutant	y = 493.8x + 427.56	0.434	0.243	9
Turbidity	Wild Type	y = 0.1706x + 0.2661	0.954	<0.001*	9
	Mutant	y = 0.5318x + 0.437	0.925	<0.001*	9

Table: Statistical analysis testing the significance of correlations between the parameters chlorophyll *a*, protein, turbidity with biovolume. *p-value <0.05 = correlation is significant.

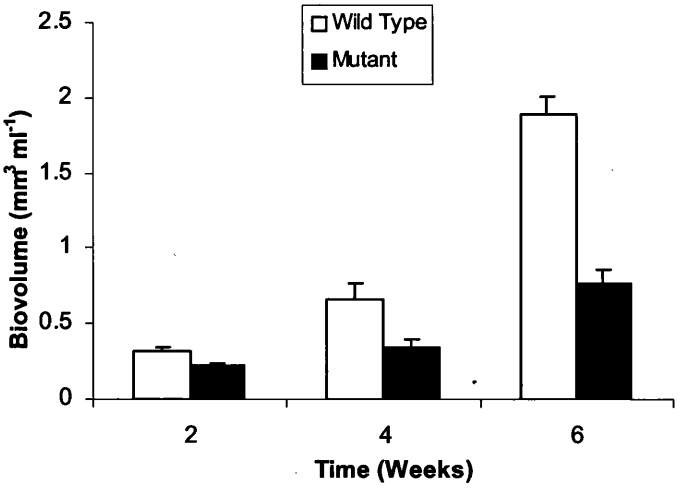
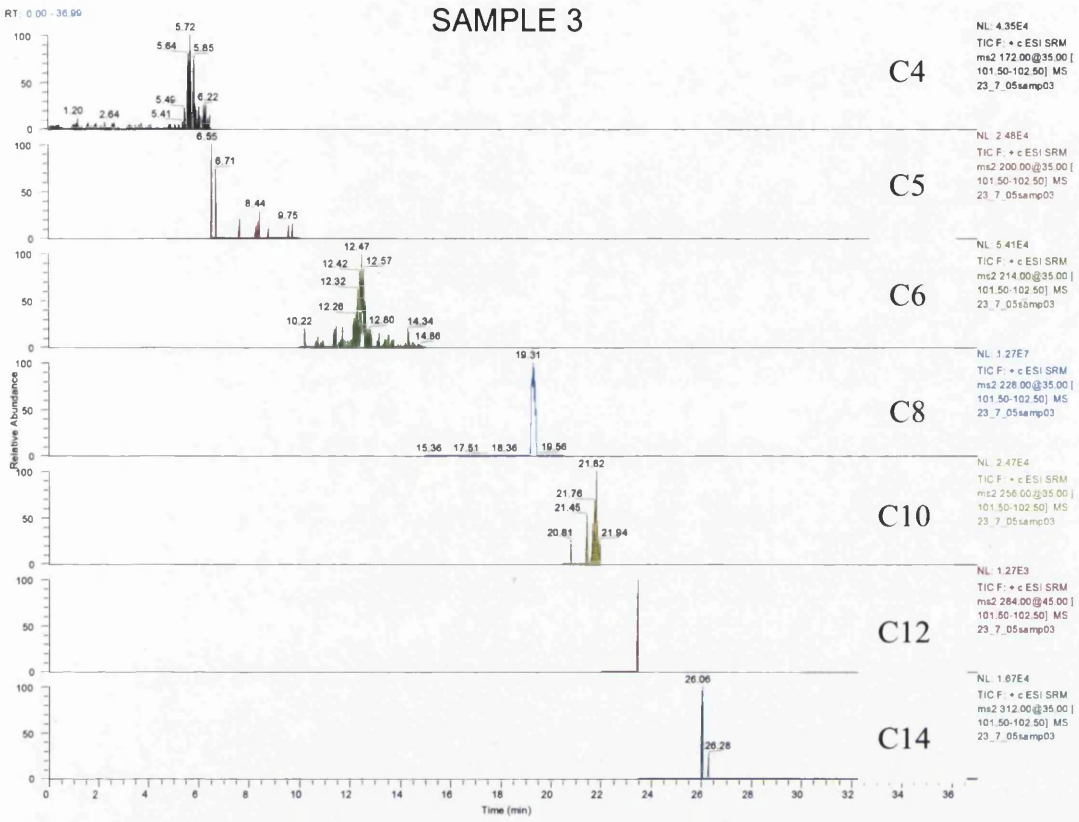


Figure: Change in biovolume in 2 strains of *Gloeothece* over a 6-week study period (± SE).

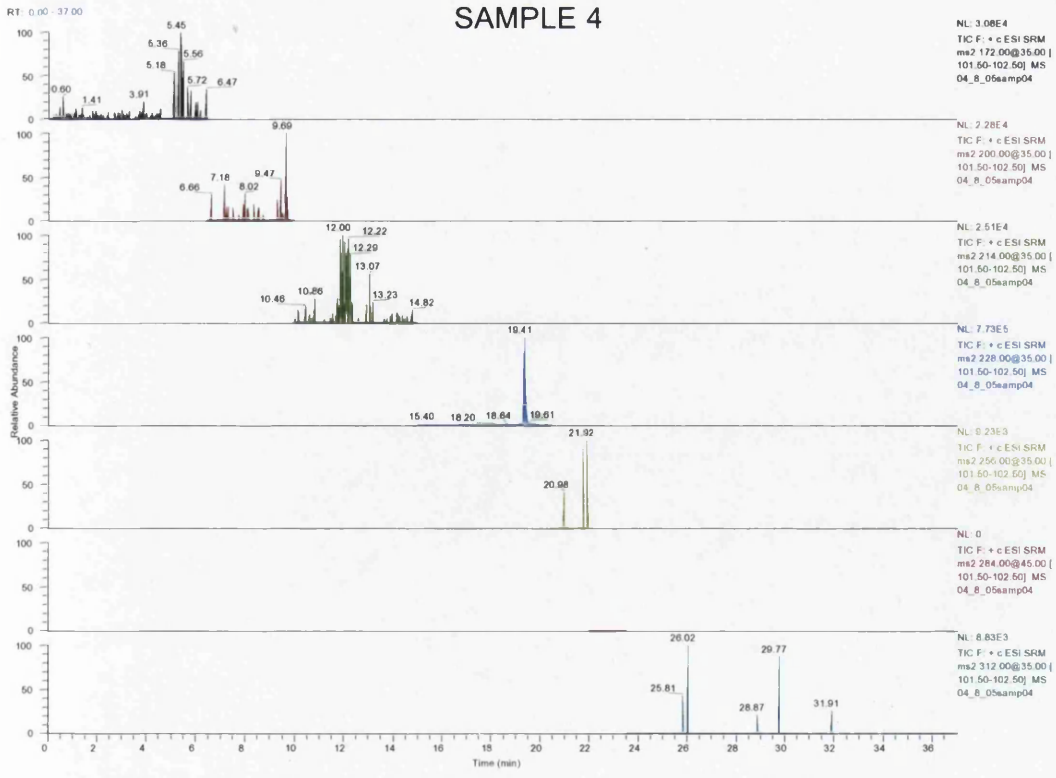
APPENDIX III

(HPLC-MS-SRM Report on samples collected during growth of *Gloeothece*)

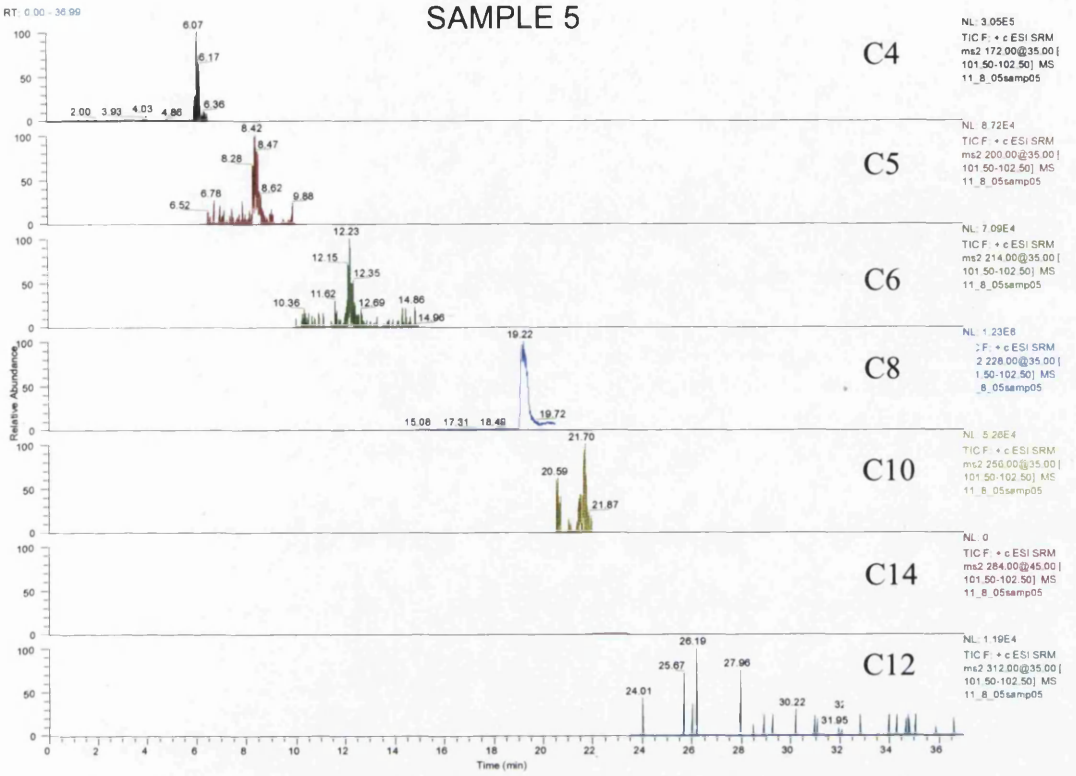
Detection for the presence of different AHL during growth in *Gloeothece* PCC 6909/1



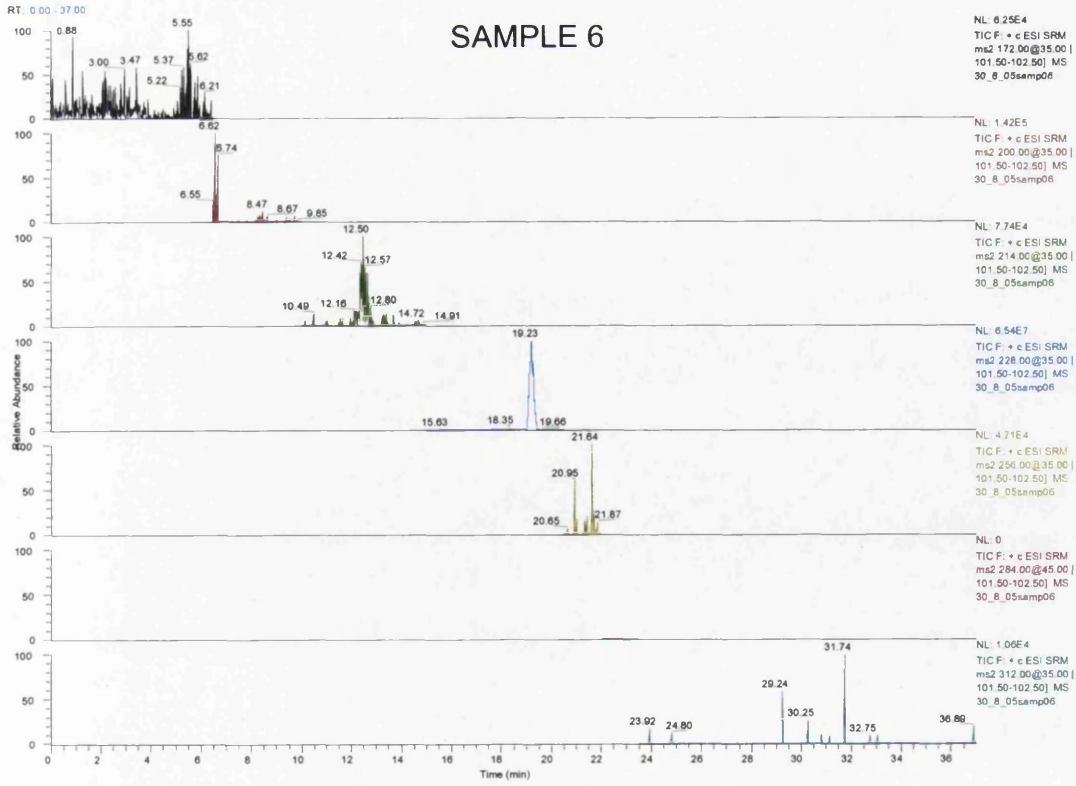
HPLC-MS-SRM of Day 19



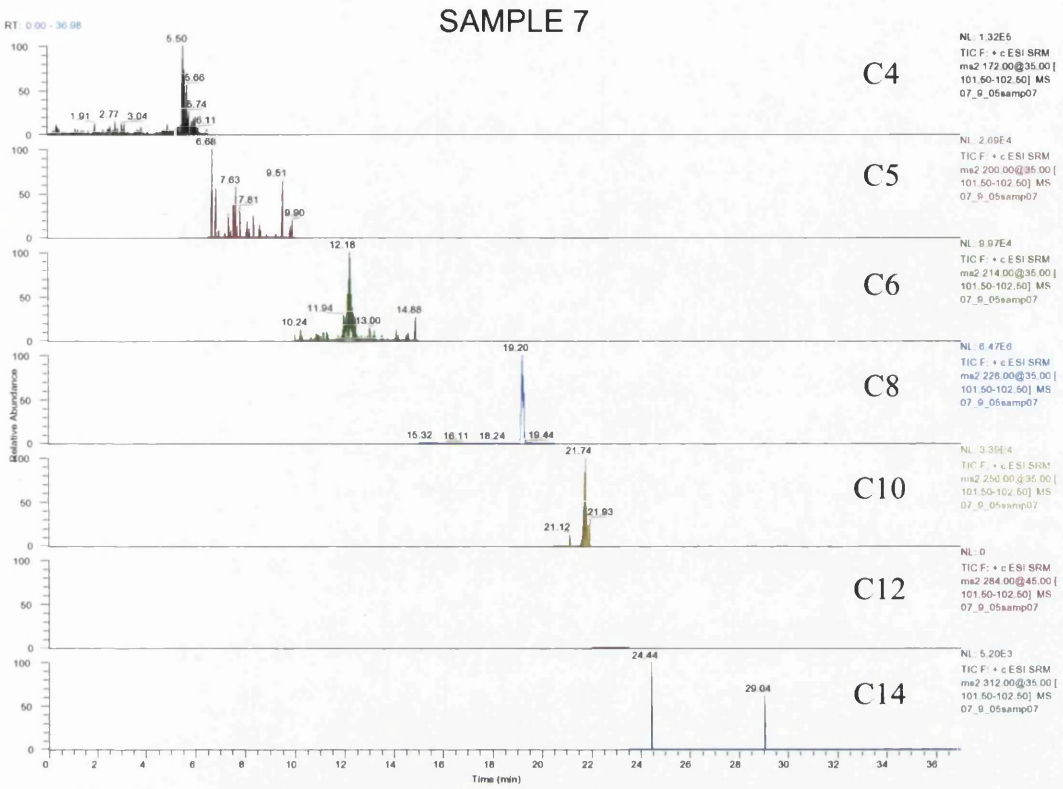
HPLC-MS-SRM of Day 27



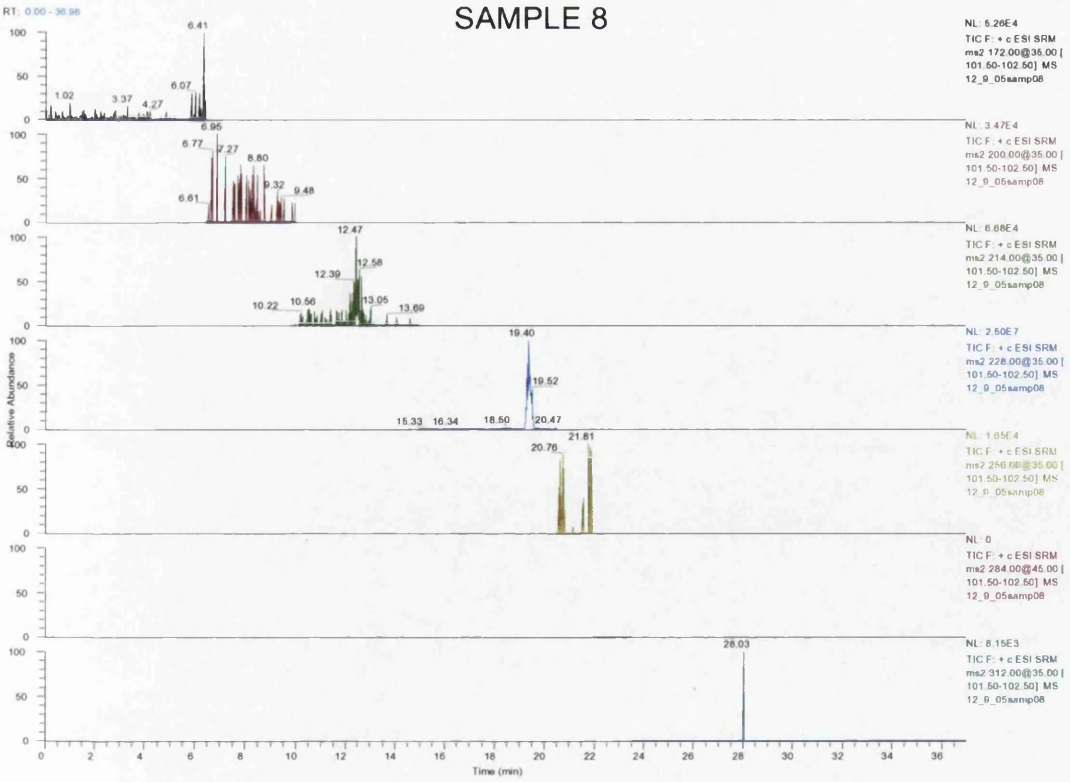
HPLC-MS-SRM of Day 35



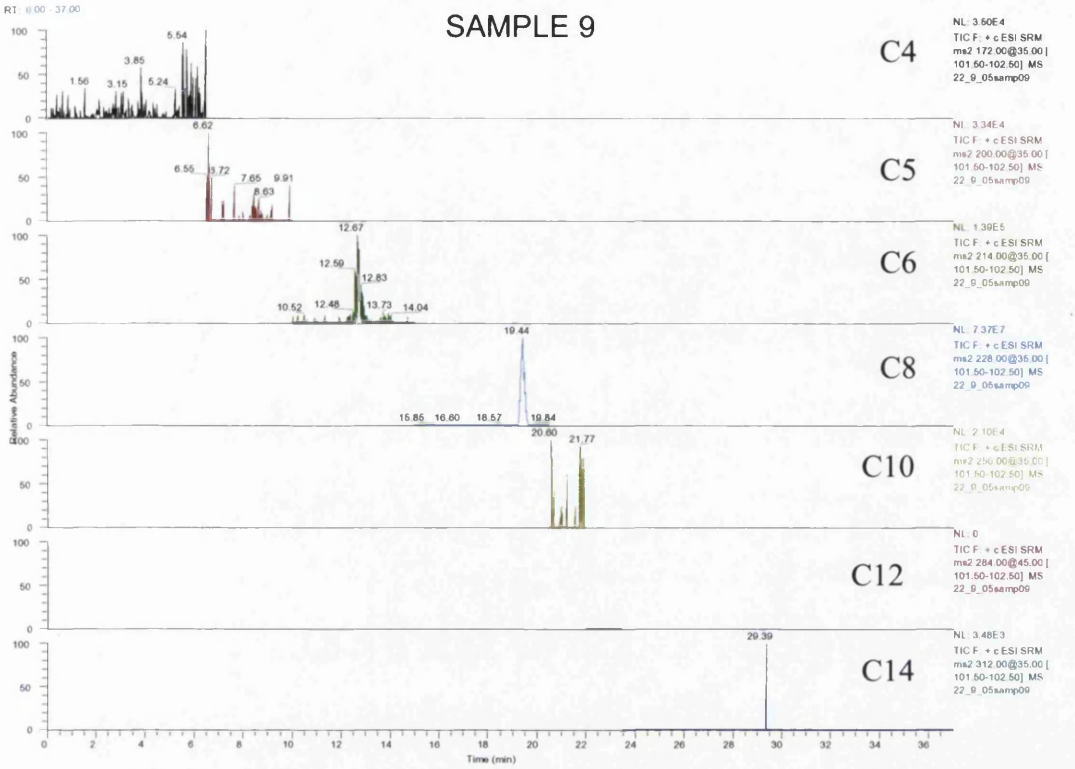
HPLC-MS-SRM of Day 46



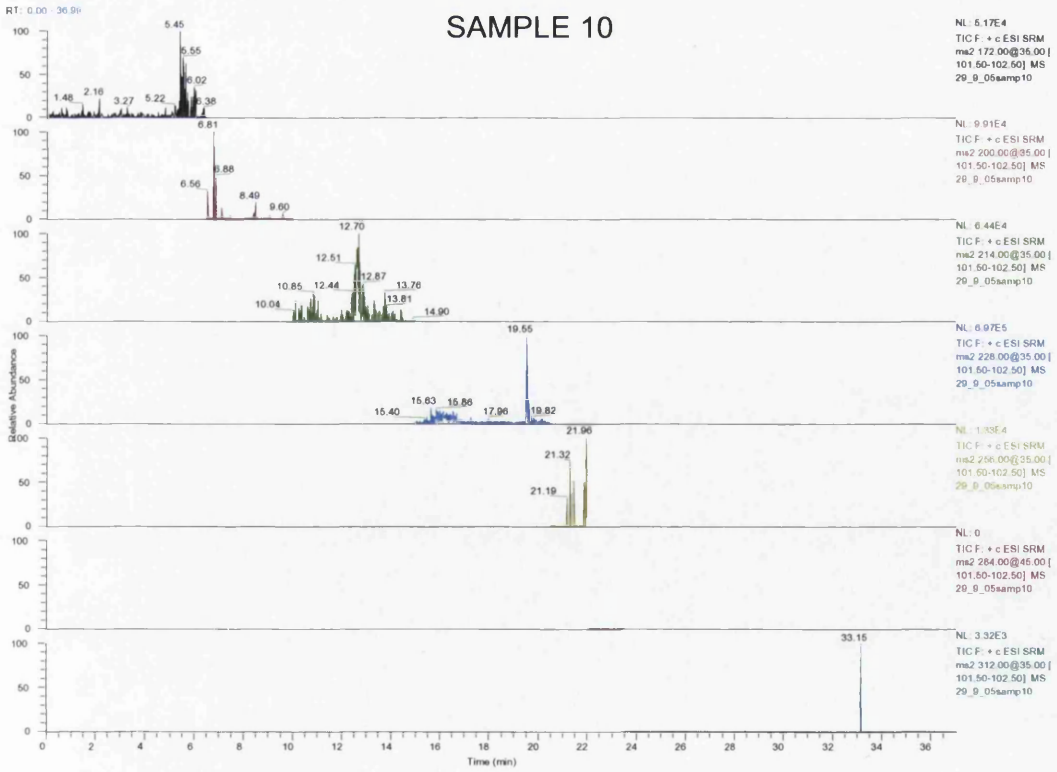
HPLC-MS-SRM of Day 54



HPLC-MS-SRM of Day 58



HPLC-MS-SRM of Day 68



HPLC-MS-SRM of Day 76

APPENDIX VI

(Publications and abstracts)

Publication generated out of this study:
(Sharif *et al.*, 2008)

Sharif, D. I., Gallon, J., Smith, C. J. & Dudley, E. 2008 Quorum sensing in Cyanobacteria: N-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium Gloeothece PCC6909. *Isme J.*

Peer reviewed abstract:

- Gordon Conference -"Microbial Stress Response", 6-11 July 2008
- 162nd SGM meeting, Edinburgh- "Cyanobacteria what they are and what they do", 2-3 April 2008