Optimization of Culture Conditions and Extraction Method for Phycocyanin Production from a Hypersaline Cyanobacterium

Submitted in fulfilment of the requirements of the degree of Master of Technology: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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DECLARATION

Optimization of Culturing Conditions and Extraction Method for Phycocyanin Production from a Hypersaline Cyanobacterium

Trisha Mogany

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

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Trisha Mogany **Date**

2014

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I, _____ (full name of student) and, _____ (full name of supervisor) do hereby declare that in respect of the following dissertation:

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- as far as we know and can ascertain:
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- All references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

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APPROVAL

I hereby approve the final submission of the following dissertation.

Prof. F. Bux
Supervisor
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This _____ day of February , 2014, at the Durban University of Technology.

*** SUBMISSION APPROVED FOR EXAMINATION**

SUPERVISOR	**DATE**

Prof. F. Bux

Doctoral Degree in Technology: Biotechnology

Durban University of Technology

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ABSTRACT

Cyanobacteria contain phycocyanin a light harvesting pigment found to have numerous biotechnological applications, such as: a natural colorant in food and cosmetics, fluorescent tags employed in clinical and immunological research and also in therapeutic processes. Successful phycocyanin production depends on growth characteristics, ability to accumulate high quantities of the pigment, and an effective downstream process. Therefore, the aim of this research was to optimize the extraction method and production by determining the optimal cultivation conditions for phycocyanin producing cyanobacterium. This cyanobacterium was isolated from a hypersaline water body in Kwa-Zulu Natal, and subsequently purified using traditional streak and spread plate techniques. Different cell disruption techniques and a range of buffers were evaluated for the extraction of phycocyanin. The buffer concentrations and pH was subsequently optimized. Results showed that maximum phycocyanin was extracted when cells were suspended in 50mM sodium phosphate buffer (pH-7.5) supplemented with 10 % lysozyme and then disrupted using the freeze–thaw method at -20 & 4°C. The UV-Vis absorption spectral scan of the crude extracted pigments showed a peak at 620 nm. This corresponds to phycocyanin production. Unwanted proteins were removed using a 25and 50% saturated ammonium sulphate precipitation, followed by dialysis. SDS-PAGE showed two subunits with molecular masses of 19 and 20 kDa. These masses corresponded to phycocyanin α and β subunits. Furthermore, a food grade purity ratio (A615/A280) of 1.20 was achieved. The effects of various abiotic factors (temperature, light and pH) on growth and phycocyanin production of the *Cyanothece* sp. was investigated. Temperature ranging from 20-45°C and pH (5-10) was evaluated for 2 weeks. Cultures were then subjected to four photoperiods (24:0, 18:06 12:12 and 8:16 h light: dark) three light intensities (25, 75 and 125 µmol photons per m^2 per $^{-s}$) at varying wavelengths i.e. blue, red and green and Grolux light. Ideal conditions were observed at 35°C, 125 µmol photons.m^2.s^{-1} of Grolux light for a 16:8 light and dark photoperiod. It was observed that the highest biomass and phycocyanin production was found to be at 35°C, temperatures below or above resulted in a decrease in both growth and pigment synthesis. Phycocyanin concentration changed in response to light quality and intensity. A significantly higher (p<0.05) phycocyanin yield was found when the culture was exposed to 125 µmol photons.m^2.s^{-1} of Grolux light compared with the other three light conditions. Using Design

of experiments, a series of fractional factorial experiments were carried out to optimize media components for pigment production. The final optimized growth medium was determined from a central composite design using response surface plots together with a mathematical point-prediction tool and consisted of 2g/L $NaNO_3$, 0.06g/L K_2HPO_4, 0.12 g/L $MgSO_4.7H_2O$, 0.033 g/L $CaCl_2.2H_2O$, 100g/L NaCl, 12mL minor nutrients and 0.5 trace metal. A 72 % increase in phycocyanin was observed. This research revealed that this particular *Cyanothece sp*. shows great potential as a reliable source of phycocyanin.

DEDICATION

This dissertation is dedicated to:

My husband, Kirshlin Mogany for his endless love and encouragement. The road we travel together was not easy but it was worth every step. Through good times and the worst times, you have been a there with me.

&

My son, Drelin Cahil Mogany, my true inspiration and hope. He has taught every challenge can be overcome with hardwork, patience and determination,

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LIST OF EQUATIONS

LIST OF ABBREVIATIONS

ANOVA- Analysis of variance

APC- Allophycocyanin

ASW- Artificial seawater

BG11- Blue-green medium

BLAST -Basic local alignment search tool

CO_2- Carbon di oxide

DCW- Dry cell weight

df - degrees of freedom

DNA- deoxy ribonucleic acid

DOE- Design of Experiments

EDTA- Ethelyene Diamine Tetracetic Acid

FFD- Fractional factorial design

F-value- Fisher's value

N- Nitrogen

NaCl- Sodium Chloride

NCBI- National Center for Biotechnology Information

NO_3- Nitrate

NSW-Natural seawater

OD- Optical density

OFAT- one factor at a time

OH- hydroxide

P- Phosphorous

PBPs -Phycobiliproteins

PBS - Phycobilisome

PC - Phycocyanin

PCR-Polymerase chain reaction

PE- Phcoerythrin

PO_4- Phosphate

PS-Photosystem

rRNA- Ribosomal ribonucleic acid

RSM - Response Surface Methodology

SDS-PAGE- Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis

UPGMA- Unweighted pair group method with arithmetic averages

UV – Ultraviolet

LIST OF MATHEMATICAL SYMBOLS

μ - Specific growth rate (hr^{-1})

λ- Wavelength (cm^{-1})

CHAPTER 1 : INTRODUCTION

1.1 General Introduction

The phycobiliproteins (PBPs) are a family of highly soluble light and stable pigments involved in light harvesting in cyanobacteria (blue-green algae, procaryotic), rhodophytes (red algae, eukaryotic) and cryptomonads. There are three basic types of biliproteins – phycoerythrin (PE, λ_{max} 560 nm), phycocyanin (PC, λ_{max} 615 nm, blue pigment) and allophycocyanin (APC, λ_{max} 652 nm, bluish green pigment). Cyanobacteria have all three types of phycobilins: APC and PC are always present and PE is found in some organisms and not in others, but forms the most spectroscopically variable class of PBPs. Light energy absorbed by PE migrates first to PC, then to APC and finally to chlorophyll *a* (Prasanna *et al.*, 2010).

In the last 10–15 years, there has been increasing interest in the potential uses of cyanobacterial PBPs in the commercial sector, as they have several applications. Thus, the first and most important application of PC is as a food pigment, replacing current synthetic pigments. Dainippon Ink & Chemicals (Sakura, Japan) has developed a product called "Lina" blue which is used in chewing gum, ice sherbets, popsicles, candies, soft drinks, dairy products (Spolaore *et al.*, 2006). Furthermore, a number of investigations have shown on PBP's health-promoting properties and a broad range of pharmaceutical applications. In addition, they are widely used in clinical and immunological research laboratories. Indeed, their properties make them powerful and highly sensitive fluorescent reagents, so they can serve as labels for antibodies, receptors and other biological molecules in a fluorescence-activated cell sorter (FACS), in immune-labelling experiments, fluorescence microscopy and diagnostics (Sekar & Chandramohan, 2008).

Phycocyanin has been reported to exhibit a variety of pharmacological properties. Niels (2008) reported the antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects of PC, when it was evaluated as an antioxidant in vitro, it was able to scavenge alkoxyl, hydroxyl and peroxy radicals and inhibited microsomal lipids peroxidation in vitro. It is used for the treatment of diseases such as Alzheimer's and Parkinson's including the prevention of oral and skin cancers (Cherng *et al.*, 2007). The extensive use of cyanobacterial

pigments has been in diagnostics as fluorescent tags or “phycoflour probes”. Phycoflours are conjugates of intensely fluorescent PBPs with molecules that confer biological specificity (Prasanna *et al.*, 2010).

Several multinational companies exploit these PBPs as a commercial commodity for the medical and biotechnology industry. Sekar & Chandramohan (2008), reported that their global market was estimated at more than US$ 50 million in 1997. Currently the prices of PBP products are 3 to 25/mg US $ for native pigments and can reach 1500/mg US $ for certain cross linked PBPs (with antibodies or other fluorescent molecules). Due to their limited pigment distribution, these pigments are rather expensive, thus obtaining them as pure compounds is a potentially attractive endeavour (Silva *et al.*, 2009). Since cyanobacteria are eco-friendly and renewable, there is an increasing tendency to use them as a source of natural colours. The success in the production of PBPs depends on the nature of organisms, its growth characteristics, availability of mass cultivation technology, the extent of accumulation of PBPs, as well as efficacy of downstream processing.

1.2 Aim and Objectives

The aim of this study was to increase phycocyanin production from the isolated cyanobacterium, by optimizing the culture conditions, and to determine a suitable extraction and purification method.

To achieve the aim the objectives were to:

i. Screen, isolate and purify suitable cyanobacteria.
ii. Identify the cyanbacteria based on morphology and phylogenetic analysis.
iii. Determine a suitable extraction method in order to recover majority of the phycocyanin.
iv. Determine the effects of abiotic factors on the growth and phycocyanin production.
v. Establish the essential nutrients for phycocyanin production, and to optimize their concentrations and cultivation conditions for increased production.

1.3 Thesis Outline

With the realization of the chemical potential of cyanobacteria in recent years, there has been an increase in their application, and products being commercialized. The data from this study can be used to evaluate the potential of cyanobacterium for high biomass concentration or high value PBPs. If feasible, it can be scaled-up for further study. This thesis describes the research on PC from *Cyanothece* sp. isolated from a hypersaline environment. This thesis comprises 7 chapters and is organized as follows:

In chapter 1 the introduction, research aim and objectives are presented.

Chapter 2 provides a comprehensive literature review, comprising of an introduction to cyanobacteria and giving detailed information of PBPs, specifically PC and their applications. This chapter also gives a background to the extraction and purification methods and the factors that affect biomass and PC production based on review of available reports, publications and communications.

The next 3 chapters (Chapter 3-5) comprises of an introduction, materials and methods, results and discussion sections.

Chapter 3 discusses the screening, isolation, purification and identification of the cyanobacterium. Both traditional and molecular methods such as DNA extraction, polymerase chain reaction, were used to identify the organism.

Chapter 4 presents the extraction and partial purification method used in this study. The first step was the evaluation of different cell disruption techniques, once this was determined various buffers are compared, followed by optimization of buffer concentrations and pH. Chapter 5 investigates the effects of abiotic factors such as light, temperature and pH on biomass generation and PC production.

Chapter 6 involves the optimization of the media composition used to cultivate the cyanobacterium, using statistical methods in order to increase the production of PC. A sequential approach comprising fractional factorial design and central composite design was used. These studies explored the importance of optimum levels of significant nutrients of the medium used for the production of PC.

Chapter 7 summarizes the significant findings of this research and gives recommendations for future research.

CHAPTER 2 : LITERATURE REVIEW

2.1 Introduction: Cyanobacteria

Cyanobacteria (blue-green algae) are Gram-negative oxygenic photosynthetic autotrophs, and are thought to be among the oldest life forms found to exist more than 3.5 billion years ago on Earth (Bandyopadhyay *et al.*, 2011). Globally, they play major roles in biogeochemical cycles of nitrogen, carbon and oxygen. Cyanobacterial photosynthesis is thought to have changed the atmosphere from CO_2-rich into an oxygen-rich environment, and providing the basis for today's varied life forms, as it facilitated the evolution of aerobic metabolism. Many species are able to fix nitrogen, and a large number of them do so under aerobic conditions. They are among a few groups that can perform oxygenic photosynthesis and respiration at the same time in the same compartment; therefore they can be a model to study important biological activities (Sharma *et al.*, 2010).

They are able to flourish in a wide range of environmental conditions such as fresh and marine water, even in some harsh conditions: hot springs, deserts, hypersaline waters, snow and ice, rocks, volcanic ash and mountain streams, as well as symbiotic relationships with multicellular eukaryotes (Darling & Mahon, 2011; Sharma *et al.*, 2010; Thajuddin & Subramanian, 2005; Vermaas, 2001). Cyanobacteria easily adapt and alter their morphology to fully utilize the available resources for maximal growth under different stress conditions. These adaptations include acclimation responses to environmental fluctuations including freezing, desiccation, high temperature, UV radiation, saline stresses, as well as low or high light intensities, and low nutrient conditions which allows them to thrive, as well as obtaining genetically heritable long-term responses, such as developmental changes or cellular differentiation, (Montoya, 2009; Singh & Montgomery, 2011; Tamary *et al.*, 2012).

Cyanobacteria were originally classified as "blue green algae" because of their microscopic morphology, presence of chlorophyll *a* and phycobilin including oxygen evolving photosynthesis. Chlorophyll *a* and several accessory pigments are embedded in the cyanobacterial internal membrane which has a highly organized structure and similar function to the eukaryotic thylakoid. Their cell walls contain peptidoglycan but lack a discrete nucleus, thus do not possess the histone proteins associated with eukaryotic chromosomes and gene structure (Silveira *et al.*, 2007). The genome size of cyanobacteria is

in the range, 1.6 ´ 109 to 8.6 ´ 109 Da, which is similar to that of other bacteria. Cyanobacteria have been studied for their interesting morphology, diversity and physiology but in recent years they have gained attention because of their potential applications in biotechnology. They are a source of pigments, vitamins, polysaccharides, proteins and other biologically active compounds with a high commercial value.

2.2 Marine and Hypersaline Cyanobacteria

Many cyanobacteria are widespread and abundant in most marine habitats (35-40% salinity), due to their ability to tolerate high salt concentrations and preference for alkaline conditions. Other cyanobacteria are found in hypersaline environments, which can grow in a salinity of 200% or higher. The mechanism of salt adaptation has been explained in terms of osmoprotective compounds and maintenance of low internal contents of inorganic ions (Nagasathya & Thajuddin, 2008). These substances even in high concentration are found to be compatible with cellular metabolism of the cyanobacteria. They are able to protect macromolecules against denaturation and thus improve the cellular function in a limited water environment. Some cyanobacteria are found to grow well in salt concentration two to three times high as those of seawater, these organisms are referred to as halotolerant. Halotolerant species such as *Calothrix scopularum* grow at a salt concentration of 175.5 g/L and below, whereas Halophilic species such as *Arthrospira subsala* can grow at salt concentrations above 175.5 g/L generally found in salt pans (Allen & Vermaas, 2010).

Chroococcus turgidus, is a cyanobacterium usually found under hypersaline conditions possess a thick mucilaginous capsule which is a protective adaptation. The Grande coastal lagoon is a brackish to hypersaline habitat with salinity gradients from 2-90 g/L sodium chloride (NaCl), pH values from 7.0 to 10.5 and temperatures from 18 to 31°C, and is found to harbour a range of algae and cyanobacteria (Montoya, 2009). The cyanobacterial species generally found to dominate a hypersaline environment include *Chroococcus turgidus, Gomphosphaeria aponina, Aphanothece stagnina, Spirulina subsalsa, Pleurocapsa entophysaloides, Nodularia spumigena, Microcoleus chthonoplastes, Oscillatoria limnetica, Orcuttia tenuis, Phormidium hypolimneticum, Lyngbya aestuarii, and Aphanothece spp*.

2.3 Morphological Classification of Cyanobacteria

Microorganisms are classified into taxonomic categories to assist in providing identification and a name. Microbiologists aim for a natural classification system which is based on ancestral relationships. The hierarchy of the taxa reveals the evolutionary or phylogenetic relationships between microorganisms. The two most commonly adopted classification systems include the Bacteriological approach as given in Bergey's Manual of Systematic Bacteriology and the traditional Botanical approach (Mur *et al.*, 1999). The bacteriological taxonomic system created by Takano *et al.* (1995) for cyanobacteria have been improvised by Komárek (2009). The traditional techniques for cyanobacteria identification and systematics is mainly based on morphological characters which includes cell size, shape and arrangement (filamentous, colonial or single cells), presence of flagella's, coloration and the presence of gas vacuoles and a sheath (Koijam *et al.*, 2009; Subashchandrabose *et al.*, 2011). Gross & Martin (1996) proposed that cyanobacterial taxonomy should follow the International Code of Nomenclature of Bacteria, and published a taxonomy of the cyanobacteria that was based on physiological, morphological and genetic criteria. Currently, there have been attempts to classify cyanobacteria using both the Botanical and Bacteriological Code of Nomenclature (Oren, 2004).

Cyanobacteria are monophyletic but morphologically diverse. In traditional classifications, morphological distinctions have been used to divide the group into five subsections (Table 2.1) (Tomitani *et al.*, 2006). Unicellular cyanobacteria may divide in one, two or three planes. The cells range in size from 0.5-1 μm to 40 μm in diameter (Koijam *et al.*, 2009). Filament branching may be true or false. Some cyanobacteria form a complex and multi-layered photosynthetic membrane system composed of mucilaginous envelopes or sheaths that bind filaments or groups of cells together (Smarda *et al.*, 2002). Some filamentous cyanobacteria form heterocysts, which are rounded, seemingly empty cells that are generally distributed along a filament or at one end of the filament. Some of the vital indicators used to identify cyanobacteria include the ability to fix nitrogen, ability to regulate buoyancy, their pigment content and mode of reproduction (Saker *et al.*, 2009).

Table 2.1: Morphological classification of cyanobacteria according to (Castenholz & Waterbury, 1989)

Sub section	Order	Description	Name
I.	Chroococcales	Unicellular rods and cocci , cell division by binary fission, non-filamentous	*Synechococcus*; *Cyanothece*; *Synechocystis*; *Chamaesiphon*; *Gloeobacter*; *Gloeothece*; *Halothece cluste*, *Microcystis*; *Aphanothece*
II.	Pleurocapsales	Unicellular ,reproduction by multiple fission without growth yielding beacoytes, cells single or aggregates	*Dermocarpa*; *Dermocarpella*; *Chroococcidiopsis*; *Myxosarcina*; *Xenococcus*; *Pleurocapsa*
III.	Oscillatoriales	Filamentous, binary fission in one plane trichomes , no true branching, no heterocysts	*Oscillatoria*; *Spirulina*; *Pseudoanabaena*; *Lyngbya*; *Phormidium*; *Plectonema*; *Trichodesmium*
IV.	Nostocaceales	Filamentous, cell division in one plane, heterocyst formed in low nitrogen environment	*Anabaena*; *Nostoc*
V.	Stigonematales	Filamentous, division occurring periodically, multiseriate trichomes that branch, heterocyst formed in low nitrogen, true branched trichromes	*Fischerella*; *Chlorogloea* or *Chlorogloeopsis*

Blue-green algae have an overall Gram negative cell wall; however, the peptidoglycan layer is comparatively thicker than Gram negative bacteria (Singh & Montgomery, 2011). Hoiczyk & Hansel (2000) reported that in unicellular cyanobacteria *Synechococcus* sp., the peptidoglycan layer thickness is about 10 nm, in filamentous species such as *Phormidium uncinatum,* it ranges from 15 to 35 nm and in large cyanobacteria such as *Oscillatoria princeps* it is more than 700 nm. Cyanobacteria such as *Phormidium* sp., *Synechococcus* sp., *Synechocystis* sp., *Microcystis* sp., *Gloecapsa alpicola, Cyanothece minerva,* and *Aphanothece halophytica* were found to possess an envelope that is covered by an exterior

layer such as carbohydrates pili or fimbrae, slime, capsule or sheath and S-layers (Hoiczyk & Hansel, 2000).

Although the morphological classification for cyanobacteria has been used for many years, the information can be unreliable, because in certain taxa, some morphological characteristics change with changing environmental conditions, and the phenotypic plasticity is high (Jodłowska & Latała, 2013). In addition, new cyanobacterial species are usually identified based on the difference of a single character, and it is estimated that 50% of cyanobacterial strains in culture collections have been incorrectly identified or have been assigned to the wrong taxonomic groups (Komárek, 2006). Some cyanobacteria appear to be polyphyletic and cannot be distinguished by morphological characteristics, therefore, are especially difficult to identify and classify (Lau *et al.*, 2005). In recent years the application of molecular based identification for marine cyanobacteria in order to characterize phylogenetic relationships has increased considerably and helped to solve some of the problems.

2.4 Molecular Methods for Cyanobacteria Identification

2.4.1 DNA Extraction

The efficiency of DNA isolation determines the overall yield of DNA and thus the sensitivity of the procedure. Isolation of good quality of DNA in sufficient quantity from cyanobacteria proves a challenge as some of the cells are often resistant to traditional DNA extraction procedures, therefore making it difficult to break cell walls. Different lysis methods, such as enzymatic, mechanical, freeze/thaw or using glass beads in the presence of a detergent, such as SDS to disintergrate the cell walls are the commonly used, in particular for complex cell walls of some bacteria, fungi and plants (Billi *et al.*, 1998). It is known that DNA extraction from marine algae is encumbered by the large quantity of polysaccharides and polyphenolics present in these algae (Murray & Thompson, 1980).

2.4.2 Polymerase Chain Reaction (PCR) and Gene Sequencing

The evolutionary relationships within cyanobacteria can be determined by using molecular techniques, including DNA base composition, DNA and RNA hybridizations, gene sequences, and PCR (Jodłowska & Latała, 2013). Polymerase Chain Reaction is a powerful and sensitive technique which amplifies specific DNA sequences exponentially through a three-step process done in multiple cycles. The main optimization for PCR variables includes magnesium (Mg^{2+}) concentrations, buffer pH, and cycling conditions. Within cycling conditions, the annealing temperature is of utmost importance.

The assessment of the phylogeny of organisms through gene sequence analysis has increased dramatically since the advent of PCR and automated sequencing. A number of genes have been used as evolutionary markers for inferring phylogenetic relations and delineation of cyanobacterial taxonomy due to their conserved function and universal presence. At the end of 1998, 171 16S rRNA genes were sequenced from cyanobacteria (Schluchter & Bryant, 2002). These 171 cyanobacterial rDNA sequences (81 complete sequences comprising nearly 1500 nucleotides and 90 partial sequences) provided the basis for modern cyanobacterial taxonomy (Paerl *et al.*, 1994). DNA base composition has been determined for several cyanobacterial strains in the Pasteur Culture Collection. Generally, large differences in DNA base composition reflect the fact that strains are not closely related (Vaidya *et al.*, 2003).

The 16S rRNA gene has commonly been used a marker because it is universal, and is structurally and functionally conserved. Furthermore, a large number of 16S rDNA sequences available and a wide variety of quick search tools are freely provided on the internet. These include tools such as the basic local alignment search tool (BLAST) provided by the National Center for Biotechnology Information (NCBI), (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi), and the taxonomic classifier and sequence match tools provided by the Ribosomal Database Project II. The phylogenetic relationships between species are often visualized by using phylogenetic trees generated by methods such as neighbour-joining, unweighted pair group method with arithmetic averages (UPGMA), maximum parsimony or maximum likelihood (Tamura *et al.*, 2004). The maximum likelihood methods are based on the actual sequence data whereas the neighbour-joining method and UPGMA are based on distance matrixes calculated from sequence distances using models such as the Kimura or the F84 model (Vandamme, 2009).

It is likely that neither morphological nor molecular methods alone can fully resolve cyanobacterial classification and further work to resolve this complex issue is required. The correct classification is impossible without the careful combination of genetic data with morphological diversity and variation, ecological and ecophysiological characteristics, ultra structural studies. Additionally without the correct application of convenient formal prescriptions for designation of taxa and strains classification cannot be achieved.

2.5 Phycobilisomes and Phycobiliproteins

The light reactions in all photosynthetic organisms like cyanobacteria, and red-algae begin with the absorption of photons by antenna complexes called phycobilisomes (PBS). These are multimeric highly organized complexes that are composed of two types of proteins (i) brilliantly coloured proteins i.e. PBPs, which absorb and transmit light energy to the photosynthetic reaction centers and (ii) non-pigmented linker polypeptides, which are responsible for the assembly of the PBS (Sun *et al.*, 2009).Phycobiliproteins harvest the portion of light (480 to 650 nm) which is not absorbed by chlorophyll *a*, this make the process of photosynthesis more efficient.

Phycobiliproteins can make up 40 to 60% of the total proteins in cyanobacteria (Benedetti *et al.*, 2006; Glazer, 1989; Soni *et al.*, 2008). Phycobilisomes anchor on the stroma side surface of the thylakoid membrane and commonly on top of photosystem II (PSII). They transfer energy to the membrane PSII complexes, however under certain conditions the energy can also be supplied to photosystem I (PSI) by means of PBSs moving on thylakoid membranes and attaching to PSI (Ayyaraju *et al.*, 2012). The PBP are highly water-soluble and are not extractable by organic solvents because they are covalently attached to polypeptides. Phycobiliproteins can be classified into three main groups: phycoerythrin (PE, λ_{max}~545–565 nm), phycocyanin (PC, λ_{max}≈620 nm), and allophycocyanin (APC, λ_{max}≈650 nm) according to the number and type of a chromophore group (Tamary *et al.*, 2012).

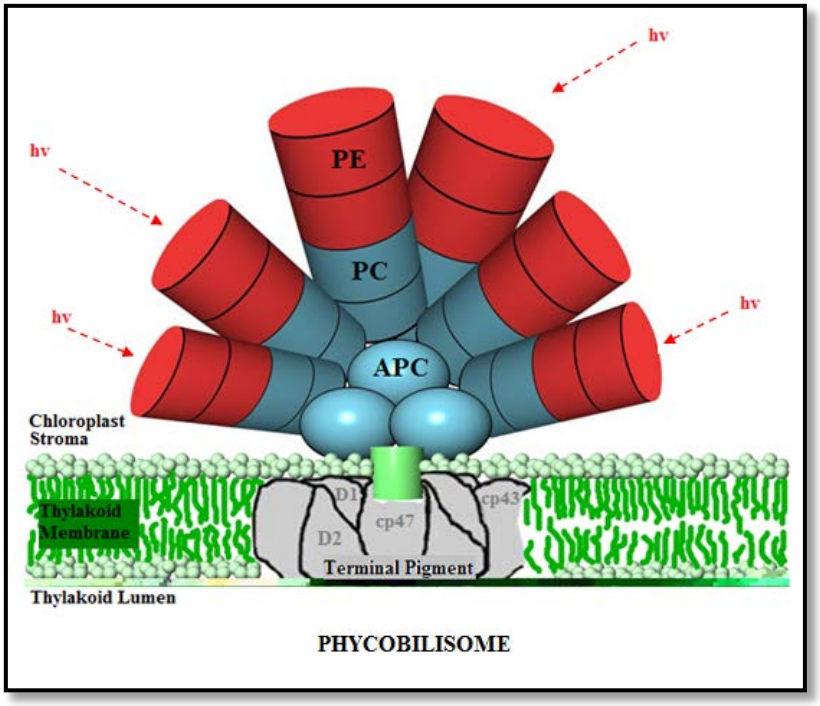

Figure 2.1: Structure of cyanobacteria phycobilisome consisting of a central core with rods that radiate outwards. Six rod substructures are attached to the core, three on each membrane-distal side. This figure is a modified version as drawn by (deMarsac, 2003).

The outer membrane of PSII contain a donut shaped PBS. The PBS consists of a core composed of stacked PBP trimeric discs and radiating rods composed of stacked PBP hexameric discs. The core is associated a membrane which is the closest to chlorophyll in the energy transfer pathway. It is composed of three AP structures that run parallel to one another, and there are six or more rods of PBPs that branch out from the core. (Figure 2.1) Each rod contains the PC and PE biliproteins. The light energy shown in the red arrows are first absorbed by PE, transferred to PC then AP, finally reaching the chloroplast of PSII.

The collection of polypeptides known as "linker" polypeptides mediate the assembly of the PBS and regulate the spectroscopic properties of the individual PBPs within the PBS by interacting with the bilins near the central cavity of the trimmers (Montgomery, 2005). Each PBP is mainly composed of two different equimolar polypeptides known as α and β subunits. The α subunit has a molecular weight between 10 to19 kDa and β subunit has between 14 to 21 kDa (Schluchter & Bryant, 2002). The (αβ) monomer is composed of either trimer (αβ) 3 or hexamer (αβ) 6 which are common aggregation states. Each α and β subunit has one to three covalently attached bilin chromophores, which contributes to each PBP's unique spectroscopic properties The linker polypeptides are colourless with the exception of the γ – subunit linker (Piven *et al*., 2005). The morphology of PBS varies with the organisms. These particles may be ellipsoidal, hemidiscoidal, or bundles of rod shaped elements. The differences in gross morphology do not reflect fundamental differences in the placement of the major phycobiliproteins or in the fundamental properties of the particle (Glazer 1989).

2.6 Biosynthesis, Structure and Function of PBPs

Phycobilins are chromophores, open-chain tetrapyrrole systems which are covalently bound to proteins. They are biosynthetically derived from 5-aminolae-vulinic acid. The most commonly occurring phycobilins are phycocyanobilin (PCB) and phycoerythrobilin (PEB) (Morisset & Kremer, 1984). These phycobilins are isomeric and have linear tetra pyrrolerings which are attached to the cysteine amino acid of the apoprotein by thioether linkages (Figure 2.2). Typical of free phycobilins is an ethylidene group in a ring form, which is not present in vivo. Except for these phycobilins, two others phycourobilin (PUB) and PXB (phycobiliviolin-like chromophore) have been identified. There are three types of PBPs which differ in their phycobilin composition. Allophycocyanin (APC) is only present in the

PBS core and binds the blue PCB. The basal part of the rods is composed of phycocyanin (PC). This PBP binds only PCB or a combination of PCB and the red PEB (Six *et al.*, 2007).

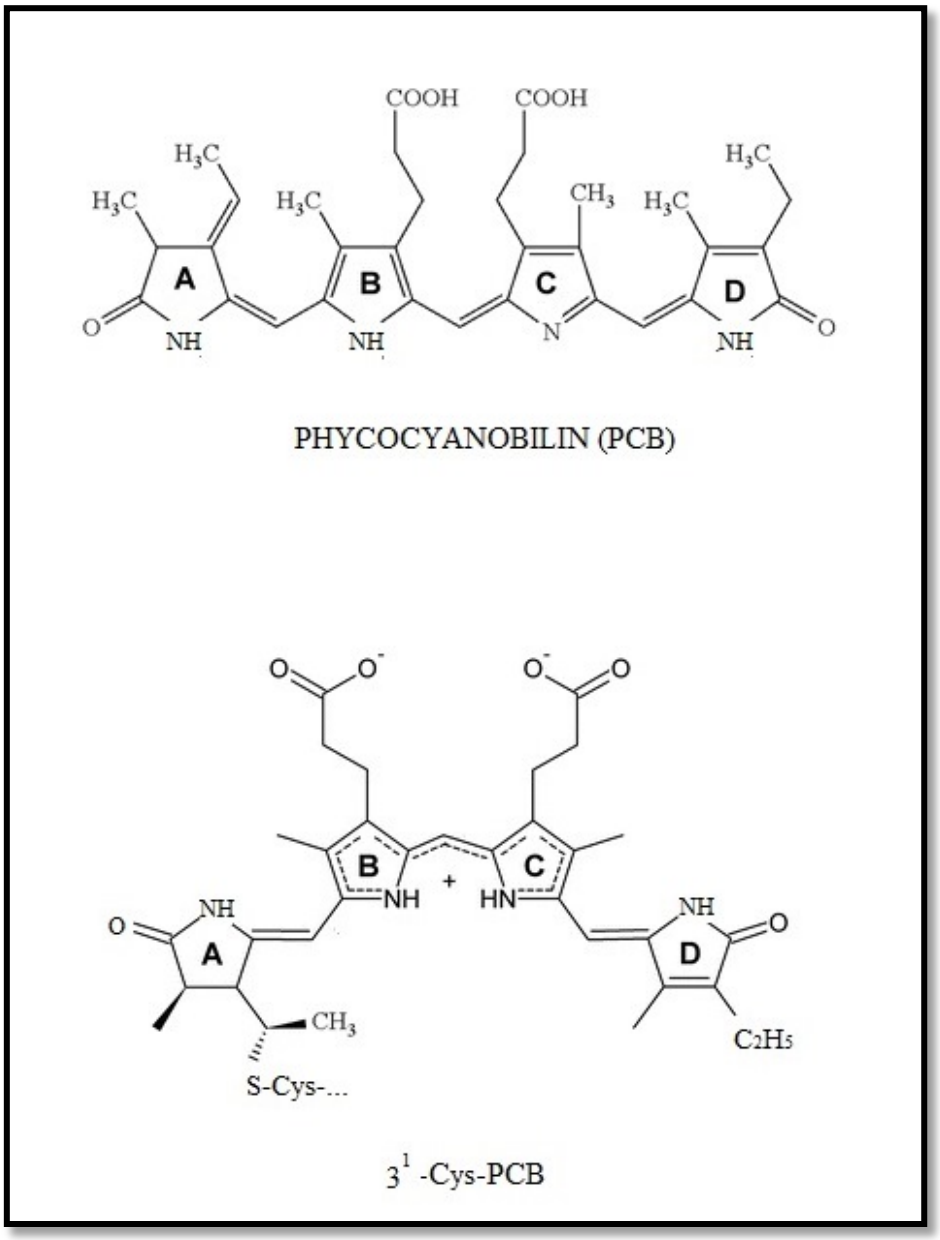

Figure 2.2: Structure of linear tetrapyrrole chromophore, PCB in their free forms (above) and PCB which is covalently attached at C-31 to the apoprotein via cysteine thioether bond (below). This figure is a modified version as drawn by (Bohm *et al.*, 2007).

2.7 Characteristics and Applications of Phycobiliproteins

Phycobiliproteins are stable and highly soluble proteins that can absorb light in the visible range of 450 to 665 nm, which covers the absorption of light wavelengths between the two maxima, covered by chlorophyll *a* i.e 420 and 680 nm (Sekar & Chandramohan, 2008). Because of their role in light collection, PBPs have evolved to maximize both absorption and fluorescence and to minimize the quenching caused either by an internal energy transfer or by external factors such as changes in pH or ionic composition. Consequently, their absorption and fluorescence characteristics are exceptional; with quantum yields up to 0.98 and molar extinction coefficients of up to $2.4 \times 10^6 m.cm^{-1}$.

Furthermore, they have the following characteristics which make them ideal for fluorescent tags: (i) red shifted excitation and emission resulting in less interference from biological matrices, (ii) a large stokes shift, therefore, interference from Rayleigh and Raman scatter and other fluorescing particles is less significant or absent, (iii) resistance to quenching by naturally occurring biological substances because the fluorophores are protected by covalent binding to the protein backbone, (iv) high solubility in an aqueous environment (Montgomery, 2005; Sekar & Chandramohan, 2008). Phycobiliproteins can be covalently conjugated to proteins such as antibodies and other molecules to make probes with greatly enhanced detectability without modification of their spectral characteristics. They can be excited by common lasers such as helium-neon, krypton or argon lasers.

2.7.1 Phycobiliproteins as Dye and Food Additives

Due to the toxic effect of several synthetic dyes, there is an increasing demand for natural colourants in food, pharmaceuticals, cosmetics, textile and printing industries. Phycobiliproteins, specifically PC and PE are used as a natural protein dye in the food and cosmetic industries (Pulz & Gross, 2004; Sharma *et al.*, 2010). The common method for human intake of PC is in a non-purified form via *Arthrospira platensis* health food products (Niels, 2008). The Japanese Company Dai Nippon Ink and Chemical Company extracts the blue PC from *Arthrospira platensis* and sells it as a natural blue pigment for use in health foods and cosmetic products (Prasanna *et al.*, 2010). Furthermore, PC is also used in the colouring of many food products such as fermented milk products, ice creams, soft drinks, desserts, sweet cake decoration, milk shakes, chewing gum and jellies (Santiago-Santos *et al.*, 2004).

Sekar & Chandramohan, (2008) reported that although PC is less stable in heat and light, it is considered more versatile than gardenia and indigo, showing a bright blue colour in jelly, gum and coated soft candies. It was found that the blue pigment produced from *Phorphyridium aerugineum* does not change with pH and was stable under light, however, it was found to be sensitive to heat. Within a pH range of 4 to 5, the blue colour produced is stable at 60°C for 40 minutes. This property was important for food uses, since many food items are acidic, particularly drinks and confections. The blue colour was added to beverages without heat application (Pepsi® and Bacardi Brezzer®) which did not lose their colour for at least 1 month at room temperature. The colour was stable in dry preparations such as sugar flowers for cake decorations which maintained their colour for several years of storage.

2.7.2 Phycobiliproteins as Fluorescent Probes

Phycobiliproteins act as valuable probes in fluorescent based detection system and possess certain characteristic's which make them ideal fluorescent tags. These include (i) a broad and high absorption of light from a variety of light sources, (ii) an intense emission of light: 10 to 20 times brighter than small organic fluorophores, (iii) low background which allows multicolor detections, (iv) excitation and the emission of spectra does not overlap compared to conventional organic dyes, (v) fluorescence retention period is longer, (vi) their simultaneous use by Fluorescence resonance energy transfer (FRET) with conventional chromophores with the same light source.

Phycobiliproteins are bound to immunoglobulins, protein A and avidin to develop fluorescent probes. This also shows that phycobiliproteins conjugates are excellent reagents for the two colour fluorescence analysis of single cells using FACS. These conjugates have also been widely used in histochemistry, flow cytometry, fluorescence microscopy and immunoassays. Fluorescent labelling reagents are an essential component of in a huge industry built on sensitive fluorescence detection. This powerful multi-chromophore protein-labelling reagent has changed the flow cytometry industry and permitted sensitive two-colour lymphocyte subset analysis using a single argon ion laser. Another important use of PBP is in fluorescence immunoassay technique (FIT), a process used for the identification of various proteins or enzymes in diseased cells. Table 2.2 outlines the applications of the commercially available PBPs.

Table 2.2: Commercially used products from cyanobacterial PBPs and uses reported by (Sekar & Chandramohan, 2008)

Name of company	Type of PBP	Product	Uses
Cyanotech Corporation	R-PE APC, and Cross linked APC, and C-PC	Fluorescent tags, Markers	Flow cytometry, food and cosmetic colouring
PROzyme Inc.	R-PC, C-PC, APC, GT5 APC and Crosslinked APC, RPE, B-PE and YPE	Fluorescence tags, markers	Multicolour fluorescence applications, fluorescence resonance energy transfer (FRET)
Dojindo Molecular Technologies	R-PE, B-PE and APC	Fluorescent labelling kits	Immunoblotting and immunostaining
Martek Bioscience Corporation	R-PE, B-PE, APC and R-PC	PBXL-1, PBXL-3 and P3L SensiLight™dyes.	Microarray imaging, immunohistochemistry and immunodiagnostic (e.g., sandwich, competitive displacement assays, microtiter, flow cytometry, microscopy)
Invitrogen-Molecular probes	APC, B-PE, R-PE, Crosslinked (APC-XL), conjugated PE biotin, and R-PE pyridyldisulphide	Fluorescent labels, tags and markers	Flow cytometry and fluorescent microscopy

In addition, PBP's are widely used in laboratory tests and immunological assays, due to their properties such as high fluorescence, good storage stability at temperatures between 4 and 10 °C, isoelectric point (IP) close to 4.65, making them easily linkable to antibodies and other proteins by conventional techniques without changing its spectral characteristics, have high molar absorbance coefficient and emission, oligomeric stability and high photo-stability (Oi *et al.*, 1982).

2.7.3 Phycobiliproteins as a Pharmaceutical Agent

In the last decade, the screening of cyanobacteria for antibiotics and pharmaceutically active compounds has received increasing interest. The nutraceutical and pharmaceutical potential shown by PC includes antioxidant, anti-inflammatory, neuroprotective and hepatoprotective activity. Results and evidence suggest PBPs act as antioxidants, inhibiting neuronal death by a mechanism that involves the scavenging of free radicals (Niels, 2008). When PC was evaluated as an antioxidant using a chemiluminescence (CL) assay, it was able to scavenge alkoxyl and hydroxyl radicals. Phycocyanin also reduces the levels of tumor necrosis factors (TNF-α) in the blood serum of mice-treated with endotoxin and showed neuroprotective effects in the rat cerebellar granule cell cultures (Romay *et al.*, 2003). Benedetti *et al.*, (2006) reported *Aphanizomenon flos-aquae* as a source of PC, indicating that it is a strong antioxidant, protecting human erythrocytes and plasma samples against oxidative damage in vitro. The virtual lack of toxicity of PC suggests that it can be used in the treatment of some neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases (Ramos *et al.*, 2010). Phycocyanin derived from Spirulina platensis powerfully influenced serum cholesterol concentrations and imparted a stronger hypocholesterolemic activity (Nagaoka *et al*. 2005).

An inhibitory effect of PC on allergic inflammatory reactions has also been reported by Kova´rova´-Kovar *et al.* (2000), as it is able to protect red blood cells against lysis induced by peroxyl radicals. They also reduced edema, histamine (Hi) release, myeloperoxide (MPO) activity and the levels of prostaglandin (PGE2) and leukotriene (LTB4) in the inflamed tissues. In plasma samples, they inhibited the extent of lipid oxidation induced by a pro-oxidant agent, cupric chloride. The anti-allergic effect of PC was demonstrated in experimental models of ovalbumin-induced ear swelling in sensitized mice, skin reactions to Hi and compound 48/80 in rats, and Hi release by compound 48/80 from isolated rat

peritoneal mast cells. These findings suggest PC could be considered for their potential benefits in the prevention of many pathological disorders associated with oxidative stress and inflammation (Rosgaarda *et al.*, 2012).

2.8 Isolation and Purification of Phycobiliproteins

The extraction of PBPs is one of the essential steps in the accurate identification and quantification of these proteins. Extraction of PBPs from cyanobacteria is notoriously difficult due to the extremely resistant cell wall and the small size of the cells (Moraes *et al.*, 2011; Stewart & Farmer, 1984). Various methods can be employed for extraction and purification of phycobiliproteins, but no standard technique to quantitatively extract pigments from micro algal suspensions exists (Viskari & Colyer, 2003). Furthermore, the purity and recovery of PBPs is relatively low because, it is highly sensitive to light, oxygen and moisture, hence, it is needed to process it along with efficient preservatives. Purity of the pigments plays a major role for commercial application particularly as fluorescence tags. A method that works well for one organism may not be a suitable method for another organism.

2.8.1 Extraction Methods Used for Isolation of Phycobiliproteins

Several different physical and chemical extraction methods, which combine disruption of cell walls and extraction of the water-soluble PBPs into aqueous media exist (Niels, 2008). One of the common methods used for extraction is to homogenise the cell suspension in dilute phosphate buffer, which results in osmotic shock that results in the breakage of cell walls (Sun *et al.*, 2009). Adams *et al.*, (2002) extracted PC from dried *Arthrospira. platensis* biomass, by re-suspending the biomass in 0.1 M phosphate buffer, at pH 7. Phycocyanin has been successfully extracted from wet biomass, when exposed to repeated freezing and thawing cycle. Patel *et al.*, (2005) reported this to be an effective method in cell disruption in *Spirulina* sp., *Lyngbya* sp., and *Phormidium* sp. Abalde *et al.*, (1998) found that the highest PC concentration was obtained when the *Synechococcus* sp. biomass was subjected to cycles of freezing at −21°C and thawing at ±4°C. Often it is beneficial to first freeze the sample in liquid nitrogen thereafter, grind the frozen sample. Some protocols require other mechanical disruption by grinding, sonication, or by use of a French Press (Furuki *et al.*, 2003; Lawrenz *et al.*, 2010). Sonication is a commonly used method for the extraction from *Synechococcus* sp. promoting cell breakage, and the use of silica beads further aids the disruption process

(Abalde *et al.*, 1998). Cell disruption by a French press depends on the blunt force used to treat the samples as they are squeezed through a small orifice by the press, which breaks the cells (Stewart & Farmer, 1984). Nitrogen cavitation is a more gentle method of cell disruption that has not been used as much as the other techniques for the extraction of PBPs (Viskari & Colyer, 2003). A combination of EDTA and lysozyme was used for PBPs extraction by many researchers (Hemlata *et al.*, 2011). Phycobiliproteins from *Arthrospira platensis* have previously been extracted using lysozyme treatment which was more effective than mechanical methods, since a higher yield of PBP was obtained. Furthermore, this method was easier to handle (Santiago-Santos *et al.*, 2004).

2.8.2 Purification of Phycobiliproteins

Purification of PC is a multistep procedure involving a combination of various methods. The first step after extraction of the PBP is precipitation using ammonium sulphate, centrifugation and dialysis to obtain a crude extract (Ramos *et al.*, 2010). Ammonium sulphate is used as a purifying agent since it readily precipitates PBPs whilst reducing the quantity of sample to be handled. Proteins precipitation occurs due to the salting out effect, as a result of the competition between protein and saline ions for water molecules, leading to the removal of water from protein. A greater protein-protein interaction occurs, which becomes stronger than protein-water interaction, resulting in the aggregation of protein molecule followed by their precipitation (Silva *et al.*, 2009). Patel *et al.* (2005) reported that the crude extract of PC from *Arthrospira* sp., *Phormidium* sp. and *Lyngbya* sp. was fractionated by precipitation with solid ammonium sulphate [$(NH_4)_2SO_4$] first at 25% w/v and then at 50% w/v saturation. Similarly Kamble *et al.*, (2012) and Minkova *et al.*, (2007) precipitated crude PC from *Arthrospira sp.* and *Arthronema africanum* with $(NH_4)_2SO4$ till 50% saturation.

Following precipitation of the PBP, chromatographic methods which include ion exchange chromatography and gel filtration, chromatography on hydroxyapatite and expanded bed adsorption chromatography can be performed (Bermejo *et al.*, 2003; Galland-Irmouli *et al.*, 2000; Soni *et al.*, 2006). Besides this common process, attempt were made to optimize PC purification by including the use of rivanol (Minkova *et al.*, 2007), chitosan and charcoal (Patil & Raghavarao, 2007), and hydrophobic interaction chromatography (Soni *et al.*, 2008).

These techniques have been employed to obtain PC of food, reactive and analytical grades. The purity of PC extracts is evaluated based on the ratio between absorbencies from PUB at 620 nm, and aromatic amino acids in all proteins at 280 nm (Niels, 2008). Phycocyanin with A620/A280 greater than 0.7 was considered food grade, while A620/A280 of 3.9 was considered reactive grade and A620/A280 greater than 4.0 analytical grade (Kamble *et al.*, 2012; Muthulakshmi *et al.*, 2012). Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS- PAGE) are also employed to complement or enhance the purity evaluation. Electrophoresis is also used as a simple, less-expensive and reliable method for separation and identification of the PBP. Under the given electrophoretic conditions, the separation of a complex PBP depends partially on the different iso-electric points (IEP) of the PBPs (Morisset & Kremer, 1984). The relative density of the PBP bands reflects the approximate proportions

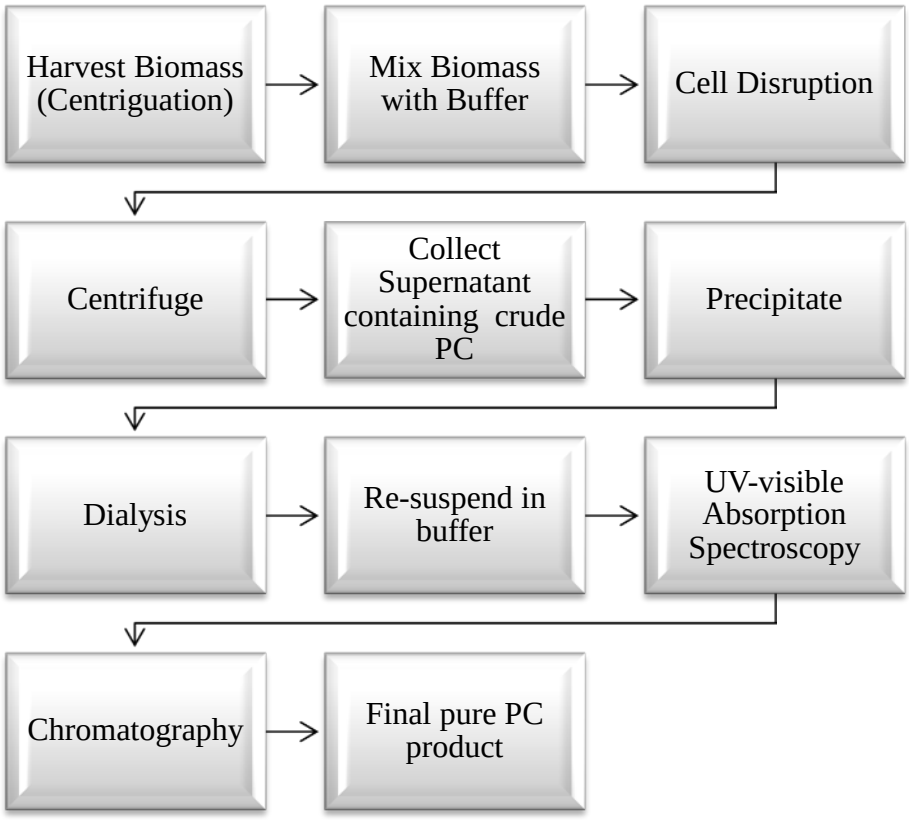

Figure 2.1: Schematic diagram showing the overview of PC extraction and purification process.

2.9 Factors Affecting Growth of Cyanobacteria and Phycobiliprotein Production

The conditions that affect growth include both chemical substrates (e.g. macro- and micro-nutrients) and physical "substrates" such as temperature and irradiance. Light and temperature have been widely accepted as the driving factors that influence the overall biochemical composition in cultures (Carvalho *et al.*, 2009). Although, conditions for culturing blue-green algae are carried out in according to previous literature, it is important to determine the conditions for optimal growth as it has been reported that growth rate for the same species of algae culture can differ at different locations (Harrison & Berges, 2005). The following section discusses the different factors, the inter-relation of these factors, and their effects on cyanobacteria and PBPs production.

2.9.1 Light

Light intensity, duration and quality directly affect the photosynthetic complex thus the growth and pigment production in cyanobacteria. Therefore, light has been exploited for enhancing pigment production. Cyanobacteria grow in a range of differing light environments, which has led them to evolve mechanisms to detect the incident illumination and modify their photosynthetic system. The light is captured by the PSII light-harvesting antenna which assures photo protection. Changes in the pigment composition are usually due to differences in the relative rates of synthesis and total amount of PBP (Islam *et al.*, 2003). Light a major parameter in cell photo-acclimatization, in a source of which cyanobacteria changes its physiological properties, thereby optimizing light harvesting and utilization (Carvalho *et al.*, 2009).

In order to accommodate fluctuations in light intensity and quality, the photosynthetic apparatus can be adapted. This can occur through either short-term modification, which does not require protein synthesis. However, cyanobacteria are also capable of altering i.e. (i) the total number of photosystems, (ii) the ratio of PSI to PSII and (iii) PBS structuring response to prolonged changes. These long-term acclimation responses require the induction of genes and de novo synthesis of proteins (Prasanna *et al.*, 2004). The regulation of light harvesting process is useful for the balance between the absorption and the usage of the energy of light to minimise the photo-oxidative damages (Bercea *et al.*, 2012).

2.9.1.1 Light Intensity and Photoperiod

Cyanobacteria favour low light because of their low specific maintenance energy rate and their pigment composition (Kumar *et al.*, 2011). In light limited conditions, the cells maximize use of the available energy via increasing pigmentation (Hemlata & Fatma, 2009).reported that low irradiances actually broadens the overall light absorption band in such a way that the balance of light energy distribution between the two photo systems is maintained that optimizes the rate of light energy conversion.

The decrease in contents of chlorophyll *a* and other light harvesting pigments with increasing irradiance has been extensively described by Prasanna *et al.*, (2010). High light intensity can also lead to photo inhibition, which is caused by photo oxidation reaction occurring within the cyanobacterium cells (Dautania & Singh, 2012). Since excess light cannot be absorbed by the photosynthetic apparatus it causes damages to the PS II reaction centers resulting in a decrease in the quantum yield of CO_2 evolution (Bercea *et al.*, 2012; Wahidin *et al.*, 2013). An increase in irradiance results in an increase in the PSI/PSII ratio and in the number and/or size of PBPs (Poza-Carrión *et al.*, 2001). Large antenna is necessary for an efficient capture of the light in light-limited conditions, but it becomes unstable when the light is abundant or excessive (Rap *et al.*, 1985; Walter *et al.*, 2011). Excessive light may also result in formation of reactive oxygen species (ROS) such as hydroxide radical (•OH), singlet oxygen ($^1O_2$), superoxide radical (SOR; $O_2•^-$) and hydrogen peroxide (H_2O_2) which are harmful (Kumar *et al.*, 2010).

Poza-Carrión *et al.*, (2001) and Singh *et al.*, (2010) reported that high irradiance significantly decreased the total PBP content cultures of *Nostoc* sp. strain UAM206. The synthesis of various cellular components is affected by the presence or absence of light-irradiance. In continuous cultures it was found that the total of DNA, RNA and proteins increased at an apparent constant rate during a light-dark cycle. Changes in light regimes have been shown to bring about differences in pigment and biochemical composition of microalgae (Dautania & Singh, 2012). The importance of the photoperiod has been demonstrated by photosynthesis i.e. a process which delivers energy for carbon assimilation in the light and nitrogen assimilation in the light and dark period. The light/dark photoperiod is beneficial, as cell numbers are sustained in the exponential phase for longer.

2.9.1.2 Light Quality

Phycobilisomes adapt to the quality of light, through a complex process, which enables them to change the PC/PE composition to efficiently absorb prevalent wavelengths of light (Simeunović *et al.*, 2012). Based on the responses to light quality, cyanobacteria have been divided into three groups: Group 1- cyanobacteria can alter their PBS number and size, but do not change the absorbance characteristics of the PBPs. Group two- can alter their levels of PE, and Group 3- cyanobacteria have the ability to change the ratio of PC to PE and vice versa (Adams *et al.*, 2002). This control of pigmentation by group three cyanobacteria is known as complementary chromatic adaptation (CCA). This process is characterized by a preferential synthesis of PBPs with absorption spectra complementary to the incident light wavelengths (Sinha *et al.*, 2002). For example, light shift from white light to red light increases the PC and chlorophyll synthesis of *Anacystis nidulans*. Vijaya & Anand, (2009) found that red or blue light was essential for PC production in *Synechococcus* sp. NKBG 042902. (Kilani & Lebeault (2007) demonstrated that under red light *Fremyella diplosipho* accumulates high levels of PC and very little PE. Furthermore, the ratio of phycobilins to one another and/or the ratio of PBS to Chlorophyll vary depending upon the colour of light in which they are growing in. The relative levels of RNAs encoding PC and APC vary with light intensity (Venugopal *et al.*, 2006).

2.9.2 Temperature

Temperature affects the structure of cell components especially proteins and lipids, as well as the reaction rates and biosynthetic pathways. Increasing temperature will increase the substrate- saturated reaction rate of RUBISCO enzyme which directly affects the efficiency of photosynthesis, thus the potential growth rates of cyanobacteria. There are also secondary effects on metabolic regulatory mechanisms, cell permeability and cell composition (Boussiba & Richmond, 1980; Carvalho *et al.*, 2009). Chaneva *et al.* (2007) reported that low and high temperatures caused changes which decreased the *Arthronema africanum* biomass and its PBPs content. Gigova *et al.*, (2012) reported *Synechocystis* sp. regulated also its PBP content in response to temperature. High temperature causes pigment degradation, whereas low temperature reduced metabolic activity of the cell. Kumar *et al.* (2011) found that the optimal temperature under controlled conditions for *Arthrospira* sp. was 32°C to 37°C Jodłowska & Latała (2013) reported increase in the temperature up to 30°C which resulted in

an increase in *Geitlerinema. amphibium* biomass production as well as the cell size and pigment levels which lead to changes in absorption efficiency. Islam *et al.* (2003) found that extended growth temperature stimulated carbohydrate biosynthesis with the decreasing of protein. Murthy *et al.* (2004) demonstrated that the increase in temperature up to 60 °C caused a decrease in PC.

2.9.3 pH

The pH gradually increases as bicarbonate is added to the culture medium. This is a result of the dissolving of the bicarbonate to produce CO2, which releases OH- during cultivation. Since the increased pH acts as an auto inhibitor of cell growth, it has been suggested that controlling the pH of the culture medium is necessary (Pandey *et al.*, 2010). Cyanobacteria are alkalophiles that have the ability to maintain an internal constant pH between 7.1 to 7.5 when exposed to an external pH from 5 to 10. The pH has a direct effect on the physiological properties of cyanobacteria, as well as the solubility and availability of carbon source and other nutrients (Usharani *et al.*, 2012). Moreover, the uptake of phosphate is influenced by pH; uptake rates decrease in acidic and relatively alkaline environments. Furthermore changes in pH affect the activity of intra- and extracellular enzymes, the osmotic potential of the cytoplasm, as well as the transport of substances across the cytoplasmic membranes, and photosynthetic electron transport (Poza-Carrión *et al.*, 2001). The maintenance of an optimal pH in the culture is of great importance for gas exchange between the atmosphere and the culture medium, since it is dependent on the partial pressure gradients of the gases across the gas-liquid boundary layer (Castenholz & Waterbury, 1989).

The pH of the medium also influences the production of PBPs. Studies by Deshmukh & Puranik (2012) showed that the pH of the medium (between 6.5 and 9.0) did not influence the growth rate of cyanobacterium, *Gloeotrichia natans,* but it did affect PBP content, as shown by a colour change. It was observed that at pH 7.0, the culture was green-brown with PBPs constituting up to 10% of the total protein, whereas an increase to pH 9.0, resulted in brownish-black culture with 28% PBPs. Hong and Lee (2008) reported that pH of 7.3 and 7.5 were optimal for *Synechocystis* sp. PCC 6701 growth and PBP production, respectively.

2.9.4 Nutrients

For successful cultivation of cyanobacteria, the media composition requires modification (Bano & Siddiqui, 2004). Marine cyanobacteria have additional requirement for Na^+, Cl^-, Mg^+ and Ca^+ ions for their optimal growth therefore, several liquid media have been modified for the culture of marine and freshwater species (Takano *et al.*, 1995). Sometimes certain marine cyanobacteria require complex salts, vitamins, organic carbon, nitrogen and trace elements, which may result in the difficulty to isolate and culture. Nutrients are inorganic or organic compounds other than carbon dioxide or water are used for growth. Their presence in the cell is necessary for cellular function. The availability and quantity of nutrient components, such as sulphur (S), nitrogen (N), phosphorus (P), carbon (C), and iron (Fe) influences the production of pigments by cyanobacteria (Tarko *et al.*, 2012). These elements are needed in the ratio in which they occur in living cells (in weight units: 42 C, 8.5 H, 57 O, 7 N, 1 P and 0.7 S). Cyanobacteria also exhibit a drastic response in limited supply of these nutrients which can lead to a decrease in pigments in the cell. The composition or abundance of PBS can be caused by accessibility of these nutrients (Collier & Grossman, 1994).

When cyanobacteria are maintained under conditions of starvation for an essential nutrient, they turn yellow due to the degradation of PBPS. This process is termed chlorosis or bleaching (Schwarz & Forchhammer, 2005). The pattern of PBS degradation consists of two basic phases: (i) the PBS loses the PC hexamers and linkers located most distal to the core during a 'trimming' process which reduces 50% of the PBS size and (ii) under continued nutrient starvation core degradation occurs, where the remaining PBS structure is completely destroyed (Peter *et al.*, 2010).

2.9.4.1 Carbon

Carbon is an essential nutrient for cyanobacteria cultivation and can be taken up from inorganic and organic forms. Inorganic carbon is utilized through the CO_2 concentrating mechanism, an active function that enables the cyanobacteria to acquire and concentrate inorganic carbon from the extracellular environment. Cyanobacteria have the ability to utilize both CO_2 and HCO_3 as an inorganic carbon source (Giorgos & Dimitris, 2011). Carbon has been investigated as a potentially limiting factor. Poza-Carrión *et al.*, (2001) reported that the concentration of dissolved inorganic carbon (DIC) during cultivation has an effect on the

relative pigment content of cyanobacteria. Under low DIC concentrations, there is a decrease in the PC content and an increase in the PSI/PSII ratio including the excitation of PSI units.

2.9.4.2 Nitrogen

Nitrogen (N) content in cyanobacteria may amount to more than 10% of the biomass and is the third most important nutrient (Xiao *et al.*, 2007). A deficiency in nitrogen will affect the quantity of PBP and therefore photosynthesis (Collier & Grossman, 1994). The main sources of N are ammonia (NH_4), which can be taken up by its passive diffusion or its protonated form $NH4^+$. Some cyanobacteria can compensate nitrogen deficiency by through fixation of atmospheric nitrogen. Cyanobacteria, such as *Oscilatoria*, *Anabaena* and *Spirulina*, are diazotrophic, which means that they are capable of utilizing nitrogen as their sole nitrogen source by the reduction of $NH_4^+ > NO_3^- > N_2$. NH_4 is the preferred nitrogen source for cyanobacteria, and they do not utilize other N sources until NH_4 is used up (Giorgos & Dimitris, 2011).

Generally, nitrogen stress results in a decreasing the rate of PBP production in cyanobacteria. Lewitus & Caron (1990) reported that during the initial stages of nitrogen depletion constant growth rates and chlorophyll *a* production were maintained while PBP content in *Anacystis nidulans*, *Cyanophora paradoxa*, *Arthrospira Platensis*, and *Agmenellum quadruplicatum* decreased. When cyanobacterial cells are exposed to N limitation, the PBPs are selectively degraded by chlorosis. This is a specific acclimation process which allows cells to survive persistent periods of nitrogen (Simeunović *et al.*, 2012). Chlorosis involves degradation of PC and APC inducing a loss of chlorophyll *a*, which affects the absorption thus the energy transfer for photosynthesis (Bohm *et al.*, 2007). A small polypeptide (NblA) was identified that triggers the proteolytic degradation of PBPs; however, its actual function is still unknown.

Increasing the nitrogen supply has been shown to increase protein content and the inverse relationship has been described for carbohydrates. Also increase nitrogen enhances the growth rate of cyanobacteria. (Martins et al., 2011) reported that the growth rates, PC and PE content and photosynthesis of a light green *H. musciformis* strain correlated positively with the increase of nitrate availability. When cyanobacteria are grown under all unbalanced nutrient conditions i.e. nitrogen saturation, the excess nitrogen is incorporated into

cyanophycin (Kolodny et al., 2006). Cyanophycin is a non-ribosomally synthesized peptide, composed of arginine and aspartic acid, which accumulates in at different rates and to different extents, depending on the source of nitrogen (ammonium or nitrate). Cyanobacteria that synthesize cyanophycin tend to use it as a nitrogen source during nitrogen starvation before using the sources of cellular nitrogen such as proteins.

2.9.4.3 Phosphorous and Iron

Phosphorous is another essential macro-nutrient for growth and accounts for 0.6% of the dry biomass. Although, cyanobacteria do not need large amounts of phosphorous, it is an important growth limiting factor. It is also a key element in many physiological and biochemical processes, (Wu *et al.*, 2013). Most often, a low phosphorous concentration is related to low cell densities and limit the amount of biomass produced. Dejsungkranonta *et al.* (2012) reported the shortage of phosphorous (10 mg/L K_2HPO_4) caused a markedly decreased of biomass *Arthrospira platensis* arginyl-poly-L-aspartic acid) under phosphorous starvation conditions. Cyanobacteria have high ability for absorption of phosphate even under limited conditions. When *Synechococcus* cells were starved for phosphorous, they significantly induced phosphate uptake, where a 50-fold increase in V_{max} for phosphate transport was observed (Schwarz & Forchhammer, 2005). Additionally, the lack of sodium and magnesium and potassium ions decreases the uptake rate of phosphate (Giorgos & Dimitris, 2011). The utilization of phosphate is energy dependent and its uptake rate is slower in dark than in light environments.

Iron has also been identified as important limiting nutrient cyanobacterial productivity. It is a major component of ferredoxin, one of the primary constituents of PSI (Paerl *et al.*, 1994). The nitrogenase enzyme, required for nitrogen fixation, contains iron in both of its subunits Ethylenediaminetetra-acetic acid (EDTA) is a low molecular weight synthetic compound that can chelate either ambient iron making it bioavailable, or other trace metals, mitigating their toxicity (Bhaya *et al.*, 2002). *Lyngbya majuscula* grown under treatment of iron chelated EDTA acid (FeEDTA), resulted in an elevated photosynthetic rate which highlights the importance of bioavailable iron species. The stimulation of photosynthesis through FeEDTA addition has been previously recorded in *Lyngbya majuscula* (Gross & Martin, 1996).

Pandey & Pandey (2008) observed maximum biomass accumulation of *Nostochopsis lobatus* occurred when cultures were supplemented with phosphorous and iron. Furthermore addition of phosphorous and iron in the medium substantially raised the antioxidant capacity of this cyanobacterium. Thus iron is a critical nutrient for the improvement of antioxidant activity. A study conducted by Swingley *et al.* (2005) described iron stress had a large effect on cell physiology of *Acaryochloris marina* cultures, as well as the down-regulation of PBPs, specifically PC. Converse the use of a high iron concentration results in fast growing cultures with a high density.

2.9.4.4 Sulphur

Generally, in an aqueous environment sulphur as well as hydrogen and oxygen are available in excess concentrations. The findings of a study by Wakte *et al.* (2011) confirmed that metal ions, specifically $MgSO_4$, have a significant effect on cell mass production and PC production in *Arthrospira platensis*. It has been identified that if there is a lack of nitrogen compounds, sulphur compounds, or phosphorus compounds, *Synechococcus* PCC 7942 produces a protein factor (NblC) that initiates the degradation of PBS (Tarko *et al.*, 2012). However, different cyanobacteria strains react differently to sulphur starvation; for example, Collier & Grossman, (1994) reported a limited supply of sulphur causes rapid chlorosis in *Synechococcus* sp.; while *Synechocystis* sp. does not degrade its PBS in response to sulphur deprivation (Peter *et al.*, 2010). The enzyme thioredoxin peroxidase which is essential for growth under high photon flux is induced during sulphur starvation due to oxidative stress (Schwarz & Forchhammer, 2005).

2.9.4.5 Growth of Cyanobacteria

The lifetime of cyanobacteria has 4 main phases. These phases' include the lag phase, exponential phase, stationary phase and death phase. In lag phase slight increase in cell biomass occurs. Following this phase in the exponential phase the cell biomass starts increasing as a function of time according to a logarithmic function. Then in stationary phase the limiting factors and the growth rate are balanced so no increase or decrease is observed. In death phase cell density decreases rapidly and the culture eventually collapses.

2.10 Statistical Optimization Methods Using Design of Experiments

Very few strains of cyanobacteria have the ability to produce a high quantity of PC, therefore, there is a need to look for high yielding strains and to improve production by manipulation of culture conditions. The conventional and classical method for optimizing medium components is by 'one factor at a time' (OFAT) approach. This is done by varying a single factor at a time, while keeping the other factors at constant levels. The OFAT method often is expensive, requires a considerable amount of experimental work, and is time-consuming especially for numerous factors (Singh *et al.*, 2009). Moreover, since it does not consider potential interactions among factor, this leads to misinterpretation of results (Cheng *et al.*, 2012). The major benefit of applying a factorial design is the reduced number of experiments that need to be carried out using a choice of the best experimental points to get maximum information (Fannin *et al.*, 1981). This results in an efficient experimental design. Furthermore, to avoid misleading conclusions, a factorial design is often necessary when interactions may be present. Finally, the factorial design allows the effect of a given factor to be determined at several levels of the other factors, so the conclusions are valid over a larger range of experimental conditions.

According to Anderson & Whitcomb (2009) Design of Experiments (DOE) is a planned approach for determining the causes and effect relationships including interactions of different parameters. It can be applied to any process with measurable inputs and outputs. It is an analysis tool that is used by researchers, field engineers and scientists to find the optimal point for their processes. Therefore, using statistical experimental design such as fractional factorial design (FFD) and response surface methodology (RSM) has been applied to overcome the drawback of the OFAT method (Hong & Lee, 2008). Since the 1990s the statistical analysis of DOE has been simplified using computer software that was specially developed to assist the researcher in optimizing the well-defined process. Software like Design Expert® has reduced the difficulty of statistically data analysing for moderately skill researcher, this with known inputs and outputs widening the usage of this method. The potential of DOE is such that it could be manipulated to benefits the manufacturer from various aspects in processing and manufacturing of a product.

The common DOE procedure allows a preliminary screening of between five and ten experiments factors in a limited number of experiments, a reduced factorial design experimental protocol on two levels of concentrations (Montgomery, 2005). In this process,

several medium factors are simultaneously compared and their effects are observed and ranked based on analysed properties or parameters, normally defined as response variables. Once the response variables have been determined, statistical performance parameters are generated from subsequent computations based on the DOE procedure, and then used to assess the relevance of the observed effects.

2.10.1 Fractional Factorial Design

Fractional factorial design is a fraction of full factorial design. One is capable of determining the main effects and the lower-order interactions by performing a fraction of the complete factorial design without loss of any information. The most common are 2k−p designs in which the fraction is 1/2p. Often 2k and 2k−p are used to identify or screen for important factors (Gohel *et al.*, 2006). The factorial design offers four runs at the high level for A and the same for the low level. Similarly, factors B and C also benefit from having four runs at both high and low levels. In order to provide similar power of replication for the OFAT experiment, the DOE must provide four runs each at the high levels of each factor versus four at the base line. This necessitates a total of 16 runs for OFAT versus only 8 for the two-level factorial (Figure 2.4).

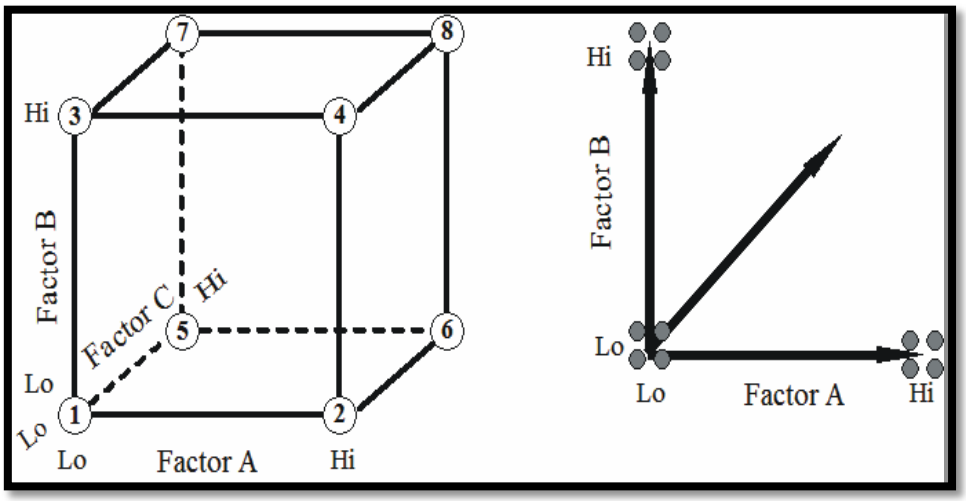

Figure 2.2 : Three-factor (2^3) design and an OFAT equivalent replication (Anderson & Whitcomb, 2009).

2.10.2 Response Surface Methodology

Response surface methodology is a statistical tool that leads to the optimization of parameters, where maps are generated based upon mathematical models. RSM is a

combination of mathematical and statistical techniques and it is useful for the modelling and analysis of this kind of problems (Niladevi *et al.*, 2009). RSM attempts to analyse the effect between several explanatory variables and one or more response variables. The main idea of RSM is to use a set of designed experiments to obtain an optimal response. Furthermore, RSM also generates a mathematical model. The graphical representation of the model has led to the coined term RSM (Montgomery, 2005). The independent variables are presumed to be measurable and continuous, and can be controlled with negligible error, whereas the response is postulated to be a random variable. The practical application of RSM requires developing an approximating model for the true response surface.

2.10.3 Data Analysis

2.10.3.1 Analyses of Variance (ANOVA)

Analysis of variance can be used to analyse variations in a response; it is used to determine exactly which of the factors significantly affected the dependent variable. It compares the hypothesis of difference means of two or more factors, provided the means are normally distributed. A $p<0.05$ and below indicate that there is a significant difference in the means of the selected factors with a confidence of 95% (Boddy & Smith, 2009). This analysis method can be used to determine if the model used to predict the PC concentration is significant or not, thus determining if all the data points fit the model. Analysis of variance was used in this study to determine if the dependant and the independent variables had a linear relationship.

The F-test compares the variance among the factors means versus the variance of individuals within the specific treatments. The F distribution is a probability used to compare variances by examining their ratio. If they are equal, or have no effect, the F-value is 1. The larger the ratio, the large the F-value and the more likely that the variance contributed by the model is significantly larger than the error. High values of F indicate that one or more of the factors differ from another.

2.10.3.2 Box-Cox Plot

The Box Cox plot is a tool to help determine the most appropriate power transformation to apply to response data. A transformation is needed if the residuals are a function of the magnitude of the predicted values. The blue line indicates the current transformation, if any,

the green line represents the best model and the red lines are the confidence interval surrounding it. A lambda value of 1 indicates that no transformation is required, this means that if the blue line falls within the red lines, then no transformation is required. The power transformation allows transformation to any power in the range –3 to +3, provided the data are positive (Sakia, 1992).

2.10.3.3 Pareto Charts

Pareto chart allows one to look at magnitude and importance of an effect. It ranks the effects of the media components according to the most significant. The chart shows two different *t*-values, one being the Bonferroni limit and the other a standard *t*-value. The effects that fall above the Bonferroni limits are significant and those that are above the *t*-value are possibly significant and therefore, if not selected should be selected (Anderson & Whitcomb, 2005; Barrentine, 1999).

2.10.3.4 Cube Plots

These indicate three factor effects at a time. This plot shows predicted values from the model for combinations of both upper and lower levels of any three effects (factors) selected. These plots are used to ascertain the steepest ascent or descent of an optimization experiment. The cube plot was used in this research to further investigate the effects that were determined to be important to increase PC production.

2.10.3.5 Contour Plot

These show the relationship between three factors represented as two dimensional, two factors for the X and Y axes, while factor Z is the contour lines, which are plotted as curves and the areas between them are coloured coded to represent interpolated values i.e. using know values to determine the unknown (Anderson & Whitcomb, 2009). These contour plots give a two dimensional view of selected factors. They help predict the areas of highest PC production in screening and optimization experiments.

CHAPTER 3 : PURIFICATION, CULTIVATION AND IDENTIFICATION OF CYANOBACTERIUM

3.1 Introduction

Cyanobacteria also known as blue-green algae are oxygenic photoautotrophic prokaryotes, comprising more than 150 genera and 2000 species. The characteristic bluish-green colour pigment found in the cells is due to the presence of PC; this gives them the common name "blue-green algae" (Mohite & Wakte, 2011). Cyanobacteria are known to inhabit various aquatic and terrestrial environments which cannot sustain the growth of other microalgae (Lopes *et al.*, 2012). This includes fresh and marine water, alkaline and acidic environments (Badger *et al.*, 2006) as well as volcanic ash, desert sand, rocks cold and hot springs (Mur *et al.*, 1999). These microorganisms inhabiting coastal environments have the ability to tolerant salt rather than require it for their growth. It is reported that cyanobacteria found in hypersaline environments, such as salt works and salt marshes, have the ability to grow at combined salt concentrations as high as 170 to 230 g/L (Murthy *et al.*, 2004). These organisms are characterized by a wide variability of metabolic mechanisms and strategies, which allows them to withstand stress conditions such as low and high light intensity, fluctuating temperature and low nutrient availability (Lopez-Rodas *et al.*, 2006; Schwarz & Forchhammer, 2005).

The conventional method for identification of cyanobacteria is based on these morphological characteristics by means of microscopy. The features that are commonly used to characterize cyanobacterial species includes; cell size, cell division planes and shape, cell dimension and cell numbers in a colony, presence of filaments, the filament structure, vegetative cells, trichome width, heterocysts and akinete (Kamble *et al.*, 2012). The cell wall structure is similar to that of a Gram negative bacterium; however the peptidoglycan layer is comparatively thicker than Gram negative bacteria (Hoiczyk & Hansel, 2000). According to the botanical system of classification for cyanobacteria, i.e. five orders Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales, which also reflects the subsections used in the bacteriological classification (Choi *et al.*, 2008; Smarda *et al.*, 2002). Among this, subsection I, i.e. Chlorococcus contains 14 genera of unicellular cyanobacteria

with cylindrical, ellipsoidal or rod- shaped cells that reproduce by equal binary transverse fission or budding, which include: *Gloeobacter', 'Chamaesiphon', 'Cyanobacterium', 'Cyanobium', 'Cyanothece','Synechococcus', 'Dactylococcopsis', 'Gloeothece', 'Microcystis', 'Synechocystis', 'Chroococcus' and 'Gloeocapsa'*.

Cyanobacteria however, belong to a complex group of microorganisms which are usually difficult to recognize based on few morphological characters alone. The major concerns are the absence of distinctive characters easily observed and some features can and be lost or changed during cultivation (Neilan, 2002; Nowruzi *et al.*, 2012). Cyanobacteria identification at genus level is often difficult when the morphological characteristics are not significantly different from other genera (Nübel *et al.*, 1997). For some taxa, a limited number of sequences are currently present in worldwide databases under the presumption that they were correctly identified (Gupta & Mathews, 2010). Due to the numerous challenges associated with morphological identification of cyanobacteria, molecular approaches entailing the evaluation of the phylogeny based on the sequence analysis of the 16S rDNA region have gained considerable interest in recent years (Conradie *et al.*, 2008). The 16S rRNA gene is the best-studied sequence and therefore the most commonly used evolutionary marker (Case *et al.*, 2007). Furthermore the sequences are independent from culture or growth conditions and can be recovered by PCR from minute amounts of DNA extracted from either natural environments or laboratory cultures (Nowruzi *et al.*, 2012). Evolutionary relationships are generally revealed using molecular structures and sequences rather than the use of conventional phenotypes (Conradie *et al.*, 2008).

Classification of cyanobacteria should be a combination of both molecular and morphological methods in order to obtain accurate and precise identification (Pandhal *et al.*, 2008). Recently increasing attention has been paid to the biotechnological application of cyanobacteria (Dufosse *et al.*, 2005; Subashchandrabose *et al.*, 2011; Abed *et al.*, 2008). This has necessitated the discovery, identification, isolation and culture of marine blue green algae (Nagle *et al.*, 2010).

The focus of this chapter was to isolate and identify high phycocyanin producing cyanobacteria from marine environments. Purification of cyanobacteria was done using traditional streak and spread plating techniques. Thereafter the pure isolate was classified based on the morphology of the cyanobacterium; and the identification confirmed using molecular methods. Finally different several different media was assessed for the growth and optimum biomass production of the cyanobacterium.

3.2 Materials and Methods

3.2.1 Screening and Isolation

Water samples were collected from various marine environments in Kwa-Zulu Natal with the aim of isolating and identifying cyanobacteria for the production of phycocyanin. Samples were collected using the grab sample technique. Glass bottles were immersed into the water-body and samples withdrawn. Scrapings of submerged rocks and loose sediments were also sampled using metal spatulas. The salinity, temperature and pH of the water was measured using a multiparameter YSI 556 MPS system (Yellow Spring Systems, USA). The samples were initially filtered with a 100 µm filter (Whatman filters, Kent, United Kingdom) to remove debris and unwanted microorganisms.

Media for the isolation was prepared by mixing filtered (55 mm, Whatman filters, Kent, United Kingdom) and sterilized natural brackish or seawater (same source from where samples were taken) with either ASW or BG11 medium which favours the growth of blue green algae (Appendix 1).One ml of the water sample was inoculated on agar plate using the spread and streak plating technique. For plating, a low agar concentration (10g/L) was used (Xiao *et al.*, 2007), due to the impurities in the agar which inhibit the growth of cyanobacteria (Ferris & Hirsch, 1991). Inoculated plates were incubated (orbital shaker incubator LM575D, LASEC, S.A) under continuous irradiance of 100 µmol photons $m^{-2}s^{-1}$ measured using a lux light meter (Major Tech, South Africa) at 27°C and slow swirling at 90 rpm. After 3 to 4 weeks, colonies were selected based on colour and size differences and transferred to fresh agar plates. Single colonies were viewed at 1000X magnification with oil immersion (Nikon eclipse 80*i* phase contrast Microscope, USA).

3.2.2 Cultivation and Maintenance of the Cyanobacterium Culture

Out of the isolates, only one cyanobacterium was successfully isolated from Listers Point, False bay, St Lucia, KwaZulu Natal (27 ˚58'9.5" S and 32˚23'0"E). Salinity of the water sample (120 g/L) was checked using a multiparameter YSI 556 MPS system (Yellow Spring Systems, USA). The salinity of all media was kept at ~120 g/L by the addition of SAXA natural sea salt. A pure stock culture was maintained using ASW and BG11 medium in 250mL Erlenmeyer flasks, each containing 200mL medium and exposed to previously described irradiance conditions. Routine sub-culturing for the stock was performed using aseptic microbiological techniques. Wet mounts were prepared and viewed under a light microscope (Olympus microscope) prior to sub-culturing to check for contamination. Sub-culturing involved the transfer of inoculum from a late exponential/stationary growth phase culture into fresh, autoclaved medium. The interval of transfer was approximately 14 to16 days. This was determined from the growth curve of the cyanobacterium. The sub-cultured cells were used as inoculum in all the other experiments.

3.2.3 Analytical Determination of Growth

3.2.3.1 Light Scattering (Turbidity)

The cyanobacterial density was determined by Optical Density (OD) using a Spectrophotometer (SpectroquantR Pharo 300, Merck Germany). A wavelength spectrum (400 to 700 nm) was performed to determine the absorbance maxima (λ_{max}) of the cyanobacterium culture. Optical density of the cyanobacetrium suspension was measured at an absorbance of 630 nm, using culture medium (BG11) as a blank. Spectrophotometric determinations of biomass were periodically verified by DCW measurements. Correlation between the dry weights of microalgae was taken to measure the growth rate of the cyanobacterial sample.

3.2.3.2 Biomass (Dry Cell Weight)

The biomass concentration was measured as dry weight as it is one of the most direct and cost effective means to estimate biomass production. Dry weight can be measured through sampling, separation, drying and weighing. The rate of increase in biomass concentration is expressed by the growth rate (μ). A 2 mL microtube was dried in the oven for 3 hr at 90°C, placed in a desiccator for 24 hr and weighed. Thereafter, a sample was added to the

microtube and centrifuged (Heraeus Biofuge *fresco*, Germany) at 855xg for 15 min at 4^{o}C. The supernatant was discarded and the pellet was washed with 0.05 M HCl in order to remove insoluble salts and non-biological adhering materials such as mineral precipitates. The microtube with the sample was dried in the oven at 60^{o}C for 4 h or constant weight. After cooling the microtube in desiccators, it was weighed.

The calculations were carried out using the following formula:

(weight of microtube + 2 mL sample) - (weight of microtube only) x 5OO = dry cell weight (g/L)

(Eq 3.1)

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3.2.3.3 Growth rate

The growth rate of the cyanobacteria was determined using the biomass results. At the exponential growth phase, the increase in biomass (d.X) is proportional to the amount of biomass (X) generated and to the elapsed time interval (dt).Thus specific growth rate represents the rate of growth per unit amount of biomass and has the dimension of reciprocal time

The specific growth rate was measured by using the formula:

$\mu = (ln\ X_2 - ln\ X_1)/ (t_2 - t_1)$ **(Eq 3.2)**

Where μ = specific growth rate constant (day^{-1})

X_1 and X_2 are biomass concentration at time interval t_1 and t_2

3.2.4 Tentative Identification of Cyanobacteria

Tentative identification of cyanobacteria was made with reference to standard taxonomic keys in Bergey's manual of systematic bacteriology (Volume 3). The morphology of the colonies was studied after 3 to 4 days of growth at 27°C on BG-11 plates. The cellular properties of the cyanobacterial cells were determined using a light microscope (Nikon

eclipse 80*i* Phase Contrast, Japan). The identification was thereafter confirmed using molecular analysis (Lopez-Cortes *et al.*, 2001).

3.2.5 Molecular Confirmation using 16 S rDNA Phylogeny

3.2.5.1 DNA Extraction and Purification

Genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (modified and optimized for this organism) to reduce contaminations of polysaccharides as follows: Approximately 1.5mL of sample was added to a 2mL microtube (Eppendorf) with 3-4 glass beads and homogenized for a minute to break cell walls. Cells were re-suspended in 1 mL lysis buffer containing (2% CTAB, 180 µg proteinase K, 100 mM Tris (pH 8.0), 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.2% β-mercaptoethanol, 1% SDS) and then incubated at 65 °C in a water bath for 90 minutes. DNA was purified successively by one chloroform:isoamyl alcohol (24:1), one phenol:chloroform:isoamyl alcohol (12:12:1) and one chloroform:isoamyl alcohol (24:1) extractions. The microtube was inverted several times and then centrifuged at 16060xg for 30 minutes to remove the cell debris. Supernatant was transferred into a new microtube, and repeated until no further debris was visible at the interface. The upper transparent layer was transferred to a new tube, and 0.6 mL cold isopropanol was added and kept at 4/-20 °c overnight to precipitate the DNA. Following the precipitation, the DNA pellet was washed with 70% ethanol. The supernatant was removed after centrifugation at 855xg at 4℃ for 20 minutes, and then the sample was left to dry under sterile conditions. DNA was re-suspended in TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA; pH 8.0) and stored at -20℃ for later use.

The DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA. The purified DNA was also evaluated through PCR analysis.

3.2.5.2 Polymerase Chain Reaction

I. First Round PCR Amplification

The first round of PCR amplification was performed using universal bacterial primer pairs 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 1492R (5'GGTTACCTTGTTACGACTT3'). PCR amplification was carried out using a verity 096 well thermal cycler (Applied Biosystems,S.A). The reaction mixture (25 µl) contained approximately 100 ng genomic DNA as a template, 5 pmol of each forward 27F (Wilmotte *et al.*, 1993) and reverse (1492R) primer 200µM deoxyribonucleotide triphosphate (dNTP) mix (Invitrogen, San Diego, California), 10X buffer, 0.5 U of Taq DNA polymerase. The optimized PCR condition included: initial denaturation at 95°C for 5 min, followed by 39 cycles of denaturation at 95°C for 30 s, primer annealing at 52°C for 45 s, and extension at 72°C for 2 min, and a final 8-min extension at 72°C.

II. Nested PCR using Cyanobacterium Specific Primer Pair

After the first round of PCR using universal primer pairs, a nested PCR using cyanobacterium 16S rDNA specific primers was performed. The reaction mixture (25 µl total volume) contained 1 x Gold Taq PCR buffer, 1 U of GoldTaq DNA polymerase (Applied Biosystems), dNTP at a concentration of 0.2 mM , 5 pmol of each cyanobacteria specific forward and reverse primer (Table 3.1) and 1 µl product from first round of amplification. The PCR conditions optimized include, initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 59°C for 1.5 min , and extension at 72°C for 2 min, followed by a final extension at 72°C for 5 min. All the PCR amplification reactions were carried out using Veriti 96 well thermal cycler (Applied Biosystems).

Table 3.1 : Cyanobacterial specific -primer sequence and target sites (Nübel *et al.*, 1997). The primers were synthesized commercially by Inqaba Biotech

Name of primer	Sequence (5'-3')	Target site
CYA106F	CGG ACG GGT GAG TAA CGC GTG A	106–127
CYA359F	GGG GAA TYT TCC GCA ATG GGd	359–378
CYA781R(a)*	GAC TAC TGG GGT ATC TAA TCC CAT T	781–805
CYA781R(b)*	GAC TAC AGG GGT ATC TAA TCC CTT T	781–805

*Reverse primer CYA781R was an equimolar mixture of CYA781R(a) and CYA781R(b).

3.2.6 Gel Electrophoresis

The PCR products were confirmed using 1 % agarose gels on Tris-borate-EDTA (TBE) buffer. This was done to visualize the amplified fragments to ensure that the fragments are of similar length, that only one fragment is obtained and that clear bands are visible. Ethidium bromide was added to the gel mix to a final concentration of 0.2 µg/mL. The PCR products were mixed with 6X loading dye (0.25 % w/v bromophenol blue and 40 % w/v sucrose) (Thermo Scientific* Fermentas) and run at 80 volts for 60 min (Bio-Rad Power Pac 300, South Africa). The gel was viewed using a gel documentation system (Vacutec G: Box, South Africa) under UV light.

3.2.7 Sequence Analysis

Sequencing of PCR amplified 16S ribosomal DNA was done at a commercial lab (Inqaba Biotechnical Industries, Pty Ltd, South Africa). The obtained sequences were aligned using FinchTV (Geospiza) and were then compared to the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) The sequence showing >97% similarities were selected and edited using BioEdit and was aligned using multiple alignments programme (CLUSTAL_X). A phylogenetic tree was constructed using neighbour joining method (MEGA, 5.2 beta 2).

3.2.8 Media Selection for Cyanobacterium

The cyanobacterial strain was sub-cultured in six different media: Bold's Basal Medium (BBM), Blue Green-Medium 11 (BG11), Artificial Seawater Medium (ASW), ASN-III, F/2-Si Medium, and Allen and Arnon Medium (ATCC 1142). Media compositions are listed in Appendix 1. The growth medium was prepared, transferred into 250 mL Erlermyner flasks and sterilized at 121°C for 15 min. Thereafter, a 10% v/v pure culture of the cyanobacterium in the exponential growth phase was inoculated into 150 mL of each medium and incubated in an orbital shaker at 90 rpm.The flasks were exposed to a light intensity of 100 µmol.m-^{2}.s-^{1} with a light/dark cycle of 16/8 h at 27 °C. All tests were carried out in triplicate.

3.3 Results

3.3.1 Preliminary Screening for Cyanobacteria

Multiple sampling regimes were undertaken in various aquatic environments in KZN; specifically targeting cyanobacteria, of special interest was False Bay, a hypersaline water body. Samples 001 and 003 were taken from estuaries, near the mouth of the river thus coming under influence from ocean currents. Green microalgae and diatoms were isolated from these samples. Although sample 002 was turbid no microorganisms were successfully isolated on BG11 or ASW media. Filamentous algae were isolated from samples 004 and 006. Cyanobacteria were only present in the False Bay sample.

Table 3.2: Sampling details of the seven samples collected in KZN

Sample no	Location	Co-ordinates	Type of water body	Salinity (g/L)	Sample description	Microorganism Present
KZN 001	Umtentweni	30 ˚ 43'S 30 ˚ 28'E	Estuary	44	Murky	Green microalgae Diatoms
KZN 002	Hibberdene	30 ˚ 57'S 30 ˚ 56'E	River	33	Turbid	No growth
KZN 003	Margate	30 ˚ 79'S 30 ˚ 40'E	Estuary	29	Green	Green microalgae Diatoms
KZN 004	Amazimtoti	30 ˚ 53'S 30 ˚ 2.9'E	Beach	34	Turbid/green	Filamentous green Algae Diatoms
KZN 005	St Lucia	27˚58'9.5" S 32˚ 23'0"E	Eustary Lake	120	Red bloom	Cyanobacteria Diatoms
KZN 006	Umbilo	30 ˚ 59'S 29 ˚ 254'E	River	45	Dark green murky	Filamentous green algae

3.3.2 Isolation and Purification of Cyanobacteria

The cyanobacterium strain used for this study was isolated from False bay, St Lucia, KwaZulu Natal. A wet mount was prepared and examined under the light microscope. As seen in Figure 3.1 a-c, three types of microorganisms were initially detected from these samples initially, which included diatoms, ciliates and cyanobacteria. The samples were then filtered using a 30 micron filter (Lasec, South Africa) to eliminate the ciliates (Buskey & Hyatt, 1995). The contaminants were identified using molecular techniques. The salinity of the media was maintained at 120 g/L by the addition of natural SAXA sea salt. Germanium IV (GeO2), 5 mg/L was added to the culture medium to remove the diatoms, *Navicula sp*. Further enrichment and isolation of the cyanobacterium was carried out using ASW and BG11 medium (Appendix1). After repeated streak plating, single blue-green colonies were obtained.

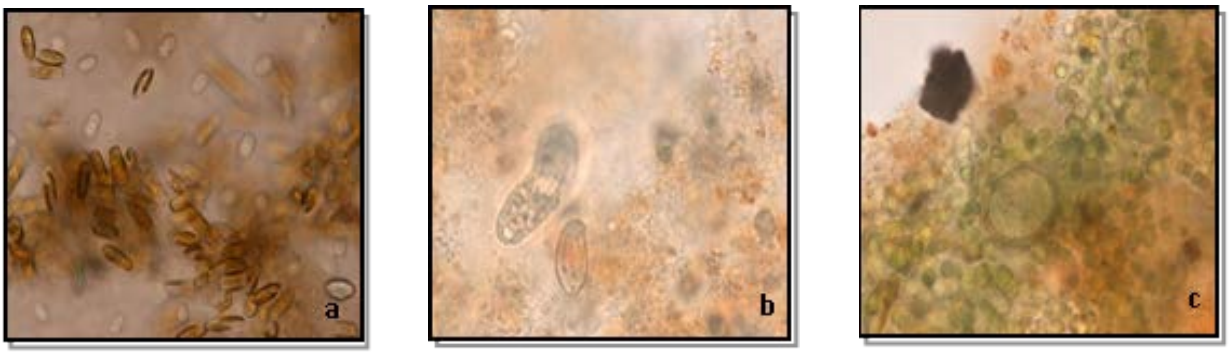

Figure 3.1: Micrograph showing (a) diatom; *Navicula sp*., (b) ciliate and (c) blue/green algae or cyanobacteria at 1000X magnification.

3.3.3 Morphology

3.3.3.1 Cellular Morphology

Table 3.3 summarises the cell morphology of the isolated cyanobacterium. The organism was able to grow in extremely high salt content (120 g/L of NaCl) and maintained buoyancy. The unicellular cyanobacteria seen in Figure 3.2a, showed the following characteristics: division in only one plane; cells widely oval shaped; and approximately 3 to 5 µm in diameter. The sheath layers were absent; however, it had a loosely adherent capsule. Figure 3.2b shows the gram stained positive cells even after repeated trails, which is unusual for cyanobacteria cells.

Cyanobacteria contain the following storage products: cyanophycean starch which does not react with iodine, cyanophycin (CP) particles and, volatin (polyphosphate particles). Figure 3.2c shows the CP particles in the cyanobacterium (dark blue to violet) and centroplasm light blue.

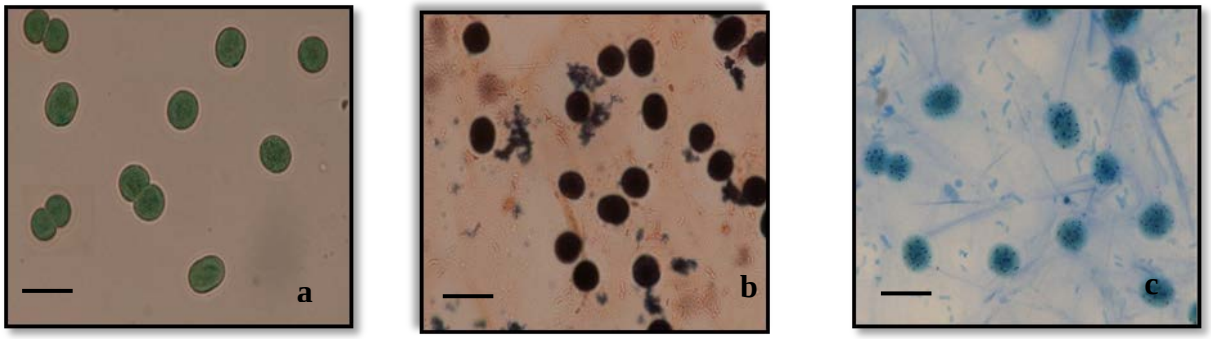

Figure 3.2 : Cyanobacterium viewed under the light microscope at 1000 x magnification, a) wet mount of purified cells in singles and undergoing binary fission (b) Gram stained positive cells (c) methylene blue stained cells showing storage products. Scale bars = 100 µm.

Table 3.3 : Summary of cellular morphology of isolated cyanobacterium

Features	Description
Cell colour	Blue-green
Cell shape	Unicellular round, widely oval to almost cylindrical with widely rounded ends
Cell size	3-5 um
Gram Stain	Positive
Cell arrangement	Unicellular
Motility	Non-motile
Colony	Sphericial/pearls
Pigment	PC and APC

3.3.3.2 Colonial Morphology

The colonies appear to be spherical (or pearls) on the agar. Besides the progression of growth on plates, there was a colour change from light green to dark/deep blue green. A distinct life cycle can be observed in the form of the various colony appearances on the culture media. At the onset of incubation, round rigid cells tended to prevail. Colonies on both agar plates and liquid medium is pigmented, with coloration ranging from green to blue green. The cells also produce abundant quantity of mucilaginous slime.

3.3.4 Phylogenetic Analysis

3.3.4.1 DNA Extraction and PCR

DNA was extracted in triplicate from a 14-day old culture using the modified CTAB extraction method. DNA yield was from 100 to 600 ng/µl. DNA was quantified using the NanoDrop (ND 1000 Spectrophotometer). The ratio of A260/A280 was between 1.7 and 1.9. DNA with a purity ratio of >1.8 was diluted to 100 ng/µl and used as template DNA for PCR analysis. PCR with universal bacterial primers resulted in amplification of 1,7 kb (data not shown). Further amplification was performed using the initial PCR product, after nested PCR using cyanobactreial primer pair, a shorter PCR product of approximately 300 bp size was obtained (Figure 3.3). The nested PCR products were thereafter extracted from the gel using the Zymoclean™ Gel DNA Recovery Kit (USA) and sent for sequencing at a commercial lab (Inqaba Biotech, S.A). DNA was successfully amplified, using both sets and primers, i.e. CYA359F & CYA781R and CYA106F & CYA781R.

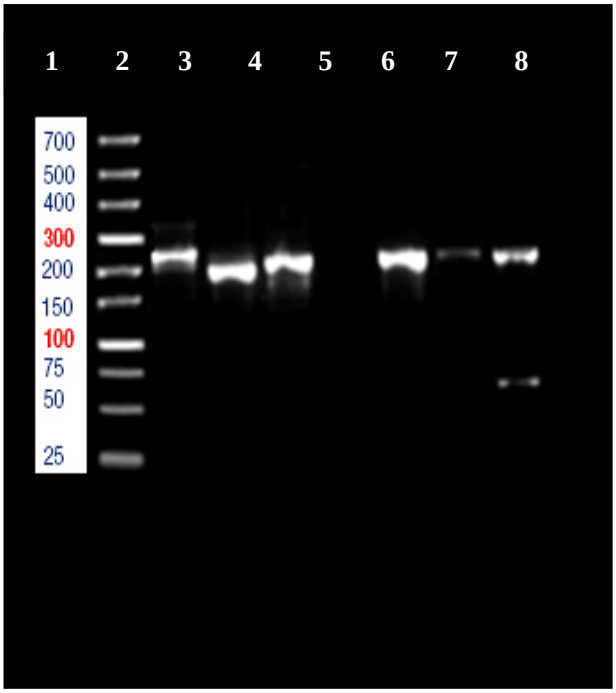

Figure 3.3: Electrophoresis of nested PCR in 1.0% (w/v) agarose gel. Lane 1: 100 bp DNA step ladder as a marker (M), Lane 2-4: DNA amplification with 100 ng of DNA Primer CYA359F and CYA781R , Lane 6-8 Primers CYA106F and CYA781R.

3.3.4.2 Phylogenetic Analysis of the PCR Product

The BLAST analysis of the obtained sequence (Appendix 2.1) with the NCBI database showed 97-99% similarity to Halothece and Cyanothece species reported in the Genbank. Furthermore, a phylogenetic tree was constructed on selected cynaobacterial species which showed >97% similarity to the obtained sequence.The tree was constructed using the Neighbour joining method (Figure 3.4). The evolutionary distances were computed using the Maximum Composite Likelihood method (Walter *et al*., 2011). The results obtained showed that the organism is within the Cyanobacteria genera, being most closely related with identified *Cyanothece* sequences. It formed a distant relatedness to the genera Halothece and Euhalothece. The isolated *Cyanothece* sp KF017202 was deposited in the Culture Collection of Algae and Protozoa (CCAP).

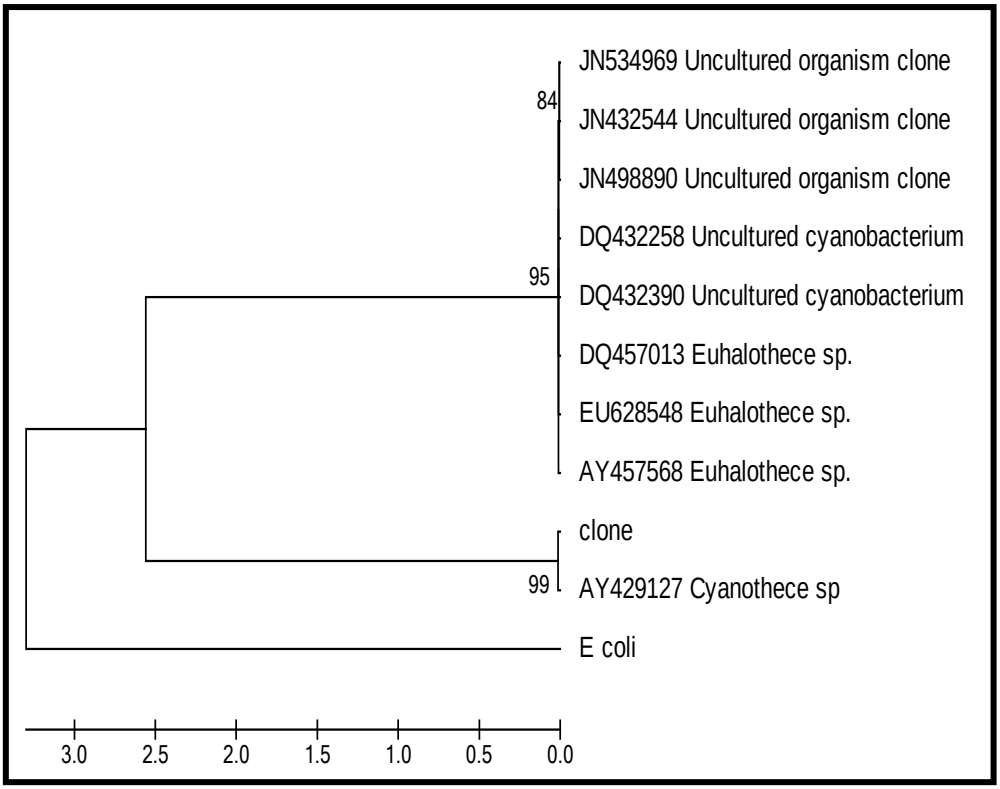

Figure 3.4 : Phylogenetic tree was constructed using the Neighbor-Joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Gregory, 2008). *E. coli* was used as an outgroup.

3.3.5 Media Assessment for Growth of Cyanobacteria

Growth kinetics of the *Cyanothece* sp. grown in the six different media was evaluated over duration of 19 days till it reached stationary phase. All the media investigated supported the growth of the *Cyanothece* sp., however, variable biomass results were noted (Figure 3.5). Biomass concentration cultured in BG 11 and ASW was significantly different ($p< 0.05$) from NSW (control). The maximum biomass was achieved using BG-11 medium followed by the ASW medium producing 1.98 g/L and 1.83 g/L of biomass respectively. Although a high amount of biomass was obtained in ASW medium, the culture appeared to be a light-green instead of blue-green (Figure 3.6b). By the end of cultivation (day 17), the *Cyanothece* culture grown in NSW medium turned brown (Figure 3.6c). Similar results were found in ASN medium, cells appeared to have lost most of their colour within a week. Although, a

lower biomas yield (0.76 g/L) compared to the other media was produced on day 17, a relatively high growth rate (0.0091.day^{-1}) was observed for ASN-III- medium (Table 3.4).

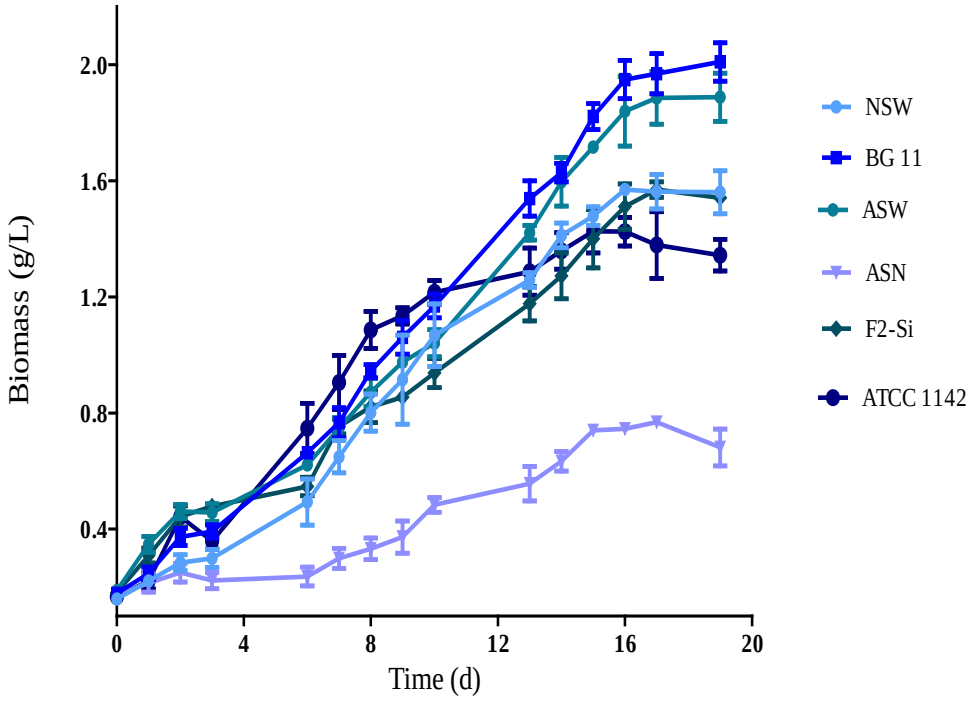

Figure 3.5: Biomass produced by *Cyanothece* sp. cultivated in six media over 19 days. Points are means of triplicate samples with standard error of means.

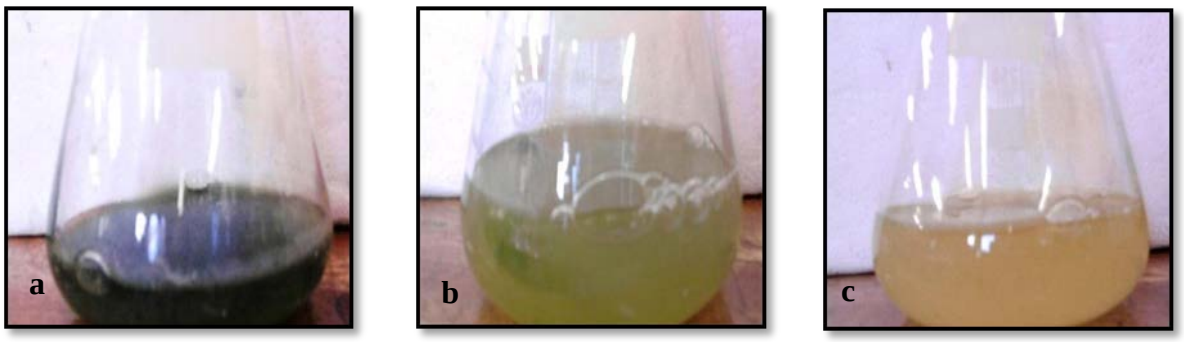

Figure 3.6 : *Cyanothece* sp. in broth cultures after 19 days of growth in (a) BG11 media showing blue-green biomass, (b) ASW media, showing olive-green biomass, and (c) NSW brown biomass.

The results shown in Table 3.4 were achieved by exponential regression of growth curves to the stationary phase. High values of the correlation coefficient (0.965-0.989) obtained for the regression, indicated that the *Cyanothece* sp., displayed exponential growth and fast adaptation to the media cultivation conditions. Highest specific growth rate (μ) 0.107 day^{-1} was achieved using the BG11 medium. *Cyanothece* sp. grown in ASW and NSW displayed a growth rate of 0.101 and 0.098 day^{-1} respectively. Results showed that ATTC 1142 medium had a significantly lower specific growth rate than all other media

Table 3.4: Specific growth rate (day^{-1}) and correlation coefficient (r^2) of *Cyanothece* sp. grown in the six different media

Medium	Specific growth rate (day^{-1})	correlation coefficient (r^2)
NSW	0.098	0.965
BG 11	0.107	0.980
ASW	0.101	0.972
ASN III	0.091	0.989
F_2-Si	0.077	0.974
ATTC 1142	0.037	0.989

Microscopic observations of *Cyanothece* sp. grown in each medium, revealed that the cells cultivated in ASW were brown and fragmented. The normal morphology of the cells (intact, round to oval, healthy and bright blue-green) was only observed in BG11 medium, (Figure 3.7a). *Cyanothece* sp. cultured in NSW medium was blue-green and healthy for a week, thereafter the cells began to change colour. By the end of the cultivation period they appeared unhealthy, with cultures turning yellow. At this stage the cell walls shrunk and contracted (Figure 3.7b).This indicates that the medium affected the pigment production. Similar results were found in ASN and F2-Si media.

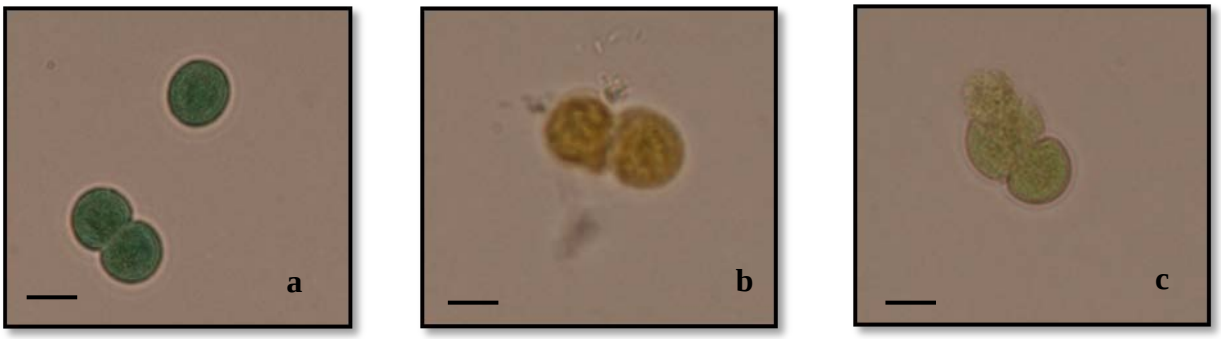

Figure 3.7: *Cyanothece* sp. cultivated in (a) BG11 media showing actively dividing, round-oval, blue-green cells, (b) NSW media, where the cells become brown and shrunk, (c) ASW media, the cells were light green and burst, observed under 1000x magnification. Bars are 10 µm.

3.4 Discussion

3.4.1 Preliminary Screening

Cyanobacteria are a highly diverse and an unexploited group of microorganisms; which presents an opportunity for discovery of novel compounds and their potential applications. All samples were streaked onto BG11 and ASW media. Green algae and diatoms grew rapidly on the agar plates (sample KZN 001 and 003). Samples 004 and 006 were found to only have slow growing filamentous algae. Of the seven samples, only one strain of cyanobacteria was isolated from KZN 005.

3.4.2 Isolation and Purification

Lake St. Lucia, which is a World Heritage Site in Africa is the largest estuarine that forms part of the iSimangaliso Wetland Park (Cyrus *et al.*, 2010). However, in recent years drastic changes to the natural conditions of the lake have been observed. Low freshwater inflow and high evaporation rates have resulted in a reversed salinity gradient, with hypersaline conditions in the upper regions, i.e. False Bay and North/South lakes (Muir & Perissinotto, 2011). Only a few organisms possess the ability to flourish in high salinity conditions. In this study the salinity of the water sample obtained from False Bay was 120 g/L (Table 3.2). Carrasco, (2011) previously reported the salinity of False Bay to exceed 200 g/L. The salt tolerant microorganisms that were found to dominate in these high salt conditions belonged to the following taxa; Ciliated Protozoa, Bacillariophyceae and Cyanophyceae.

The ciliate was adapted to the high salinities and found to feed on the cyanobacteria. Carrasco & Perissinotto, (2012) reported similar findings, where the heterotrichous ciliates were a dominant species in hypersaline environments, grazing on the cyanobacteria. Therefore the sample was filtered using are 30 micron filter to remove the ciliates. GeO2 was used remove the diatoms, *Navicula sp.,* since GeO2 is readily used by diatoms which lead to silicon being substituted by germanium. This results in a significant reduction of the diatoms' growth rate without affecting the other algae species (Azam *et al.*, 1973). These findings are in agreement with earlier reports from the same habitat in St Lucia. Muir & Perissinotto (2011) and Philippis *et al.*, (1998) reported 15 cyanobacterial strains isolated from hypersaline and saline habitats in Italy, Getzira, Republic of Somalia and Lake Abijata, (Ethiopia). These organisms were found to belong to the *Cyanothece* group. Lopez-Cortes *et al*.(2001) also found

Cyanothece and related genera in Marine hypersaline shrimp ponds in Baja California (Mexico).

3.4.3 Morphology

The isolated cyanobacterial cells were unicellular, round, widely oval to almost cylindrical with widely rounded ends under the microscope (Figure 3.2a). The diameters of the cells were ranged from 3 to 5 µm with a fine margin around the cells (Komarek, 2005). Montoya, (2009) reported that marine cyanobacteria are capable of coping with osmotic and ionic stress by producing osmoprotective compounds. Furthermore they are able to accumulate osmolytes. For long-term osmotic stabilization, organic solutes such as disaccharides sucrose and trehalose, glucosylglycerol, and glycine betaine accumulate in the cells (Nagasathya & Thajuddin, 2008). Thus far, external surface layers (S-layers) have been observed in 60 strains of 23 species, belonging to 12 genera of unicellular Chroococcales. *Cyanothece minerva* and *Cyanothece aeruginosa* was found to be covered by with these S-layers (Hoiczyk & Hansel, 2000; Smarda *et al.*, 2002). Although not presented in the preliminary electron microscopy investigations, the presence of S layers was observed. They function as protective coats or molecular sieves or are involved in cell adhesion and signalling. The mode of division of the isolate examined showed that they are reproduced by binary fission, which are a characteristic feature of subsection 1 Chroococcales (Malakhov *et al.*, 1995). Thus based on the morphological characteristics, the isolated strain of cyanobacterium was identified as *Cyanothtece sp*. belonging to Chroococales.

Cyanobacteria are generally described as Gram negative prokaryotes. They have an outer membrane and lipopolysaccharides (LPS), similar to Gram-negative bacteria, but they possess a significantly thicker, highly cross-linked peptidoglycan layer, characteristic of gram-positive bacteria (Hoiczyk & Hansel, 2000). The thickness of the murein layer of cyanobacterial cell walls and the degree of cross-linkage in the peptidoglycan layer is similar to layers found in Gram positive bacteria. Therefore cell walls react positively in the Gram reaction (Malakhov *et al.*, 1995 and Stewart *et al.*, 2006). However, in response to the high salinity, the *Cyanothece* sp. were found to adapt through production of general and specific stress proteins, changing the protein composition of extracellular layers (Pandhal *et al.*, 2008). A similar observation was also evident in this study. In Figure 6b, the cells stained

purple, indicating a Gram positive reaction which showed the cyanobacterium had to possess a higher degree of cross-linking between the peptidoglycan chains to stain Gram positive. The presence of an outer peptidoglycan structure largely determines the different properties of the two cell wall types in terms of mechanical stability, permeability, and resistance toward chemical substances including environmental stress.

In cyanobacteria, the PS II contains only small fraction of chlorophyll a, instead they possess unique light harvesting pigment proteins. The blue green colour is indicative of the predominant accessory pigments, PC and APC produced by the isolate. Some cyanobacteria have two nitrogen storage compounds with high nitrogen content, which are PC and cyanophycin. Phycocyanin is primarily an accessory pigment, whereas cyanophycin is considered to function primarily as a nitrogen reserve (Kolodny *et al.*, 2006). Blue green algae store nitrogen as CP, a polymer generally composed of equal amounts of arginine and aspartate. CP synthesis is typically induced when growth is restricted due to phosphorus or light limitation. In Figure 3.2c intracellular dark granules identified as CP were clearly seen as cyanophycin production is common among nitrogen-fixing cyanobacteria. Elvitigala *et al.*, (2009) and Bandyopadhyay *et al.* (2011) reported *Cyanothece sp.* has the ability to fix atmospheric nitrogen, however, this hypothesis was not established in this current study.

3.4.4 Phylogentic Analysis

Although the morphological evaluation of cyanobacteria is necessary for understanding their diversity, often this method can lead to misidentification. Besides morphological examinations, it is currently accepted that characterization and taxonomy of cyanobacteria necessitates a multidisciplinary approaches (Komarek, 2005). Among the molecular methods, the analysis of the 16S rRNA gene sequences has proved to be a useful tool for exploring phylogenetic relationships among cyanobacteria. Therefore a combination of morphological and molecular techniques can provide an insight into existing genotypes and their approximate distribution in various ecosystems. PCR amplification and sequencing was done using cyanobacterial specific primers CYA359F and CYA781 R (Nübel *et al.*, 1997). CYA781R(a) and CYA781R(b) differ by two polymorphic bases situated at positions 7 and 23 (5V to 3V). These primers have the advantage of producing an amplicon which corresponds to variable regions V3 and V4, and contains important information for

phylogenetic assignment (Boutte *et al.*, 2006).

The obtained sequences showed more than 97% sequence similarity to the reported *Cyanothece* sp. in the GenBank. The unicellular cyanobacterium phylotype recovered from this study shared high affinity to the *Cyanothece* clade and a sister group in the clade composed of *Euhalothece* sp. in the phylogenetic tree (Figure 3.4). Although, the cell morphology, i.e cell shape and division are similar, the cluster of *Cyanothece* sp. to which the isolate belongs seems to be different from *Euhalothece*. The 16S rRNA partial sequencing of the isolate, shared only one close phylogenetic association with *Cyanothece* sp. strain in the GenBank, which could possibly be due to the novelty of the isolated strain and also could be as a result of insufficient corresponding sequences available in the database.

3.4.5 Assessment of Media for Growth of Cyanobacteria

Marine cyanobacteria have additional requirement for Na^+, Cl^-, Mg^+ and Ca^+ ions for their optimal growth, therefore, several liquid media have been modified for the culture of marine and freshwater species, e.g., ASN-III, MN, and BG11, ASP-2 and SAG1 (Bano & Siddiqui, 2004). In order to select for the appropriate culture medium for growth of *Cyanothece* sp., the culture was incubated into six different media of varying nutrient composition. The best medium for promoting the robust growth of *Cyanothece* sp, and supporting the highest growth rate of $0.117.day^{-1}$ was BG11 medium, (BG11 >NSW >ASW >F_2-Si> ASN III >ATTC 1142) as seen in Table 3.4. The growth of the culture in NSW was hampered by the lack of key micronutrients. BG11 medium contains sufficient concentrations of nutrients required for this cyanobacterium to grow. Similar results were observed by Swingley *et al.*, (2005) who found that *Acaryochloris marina* grew to a low cell density on ASW medium, and growth seemed to be improved on an artificial marine version of the traditional BG-11medium.

The alteration of the photosynthetic apparatus due to nutrient-limiting conditions causes apparent changes in the cyanobacteria. During nutrient starvation they turn yellow and this process is known as chlorosis or bleaching, which involves either active degradation of the PBPs (Schwarz & Forchhammer, 2005), or the cessation of pigment synthesis and continuation of cell division which results in a dilution of pigments (Adams *et al.*, 2002). Degradation of PC was observed in cyanobacteria cultures grown in NSW (Figure 3.6b and

3.7b). Chlorosis occurred in culture grown in ASW; however, pigment degradation did not occur which can be confirmed by an intense increase in growth rate (0.101 day^{-1}) of *Cyanothece* sp. Furthermore, ASW as well as lower quantity of nutrients compared to BG11 medium, which might have led to the utilisation of the nutritive components. A decrease in PBS is measured by the loss of PC.

In summary, a marine unicellular cyanobacterium belonging to the genus *Cyanothece* was isolated from False bay, St Lucia. Cyanothece sp. was able to flourish in high salt concentration (120g/L), and produced blue light harvesting pigment, PC. The cyanoabcterium produced a high biomass yield and maintained the blue green colour when it was cultivated in BG11 medium compared to NSW and artificial media.

CHAPTER 4 : EXTRACTION AND PARTIAL PURIFICATION OF PHYCOCYANIN

4.1 Introduction

Phycocyanin is water-soluble antennae-protein pigment involved in light harvesting in cyanobacteria, red algae and cryptophytes (Patel *et al.*, 2005; Patil & Raghavarao, 2007; Sharma *et al.*, 2010). The brilliant colour of the pigment originates from covalently attached linear tetrapyrrole prosthetic groups (Benedetti *et al.*, 2006). PC is a dark blue in solution with an absorbance wavelength between 615 and 620 nm, and has a fluorescence emission at 640 nm (Chakdar & Pabbi, 2012; Lawrenz *et al.*, 2010; Niu *et al.*, 2007). It has a molecular mass of 140–210 kDa with two subunits, α and β (Santiago-Santos *et al.*, 2004). There are equal numbers of α and β subunits. However, the numbers of α and β pairs may vary among the different species.

Extraction of PBP's from cyanobacteria can be extremely difficult due to the small size (0.2-2 μm) of some cells (Mur *et al.*, 1999), and they possess exceptionally resistant multi-layered cell walls (Viskari & Colyer, 2003). Since the proteins are regularly arranged in parallel rows on the thylakoid membrane, the efficiency of extraction depends on the rupture of the cell-wall. Extraction of the crude protein involves a multi-step treatment procedure. This is done by (i) enzymatic digestion of the cell walls, and (ii) repeated freezing and thawing cycles (Guangce, 2002), or (iii) mechanical disruption by sonication, cell milling with glass beads and hand-grinding (Furuki *et al.*, 2003). Since PBP's are water soluble, they are commonly extracted by suspending in a phosphate buffer (Gupta & Sainis, 2009; Lawrenz *et al.*, 2010), thereafter centrifuged to remove cell debris and contaminants (Guangce, 2002).

The purification of the crude protein occurs with a variety of procedures including centrifugation, fractional precipitation, dialysis, and column chromatography. Ammonium sulphate precipitation (NH_4SO_4) is a commonly used protein purification technique, as it is relatively inexpensive, requires simple equipment, and it can be applied on large scale (Kamble *et al.*, 2012; Tripathi *et al.*, 2007). The purity of the protein is an important factor that determines the cost and its application. Harsh cell disruption methods release various other proteins, which in turn results in a lower purity ratio in crude extracts. The lower purity results in an increase in the amount of purification steps coupled with a decrease in yield. The

purity of PC is generally evaluated based on the absorbance ratio of A620/A280. A purity of 0.7 considered as food grade, 3.9 as reactive grade and greater than 4.0 as analytical grade (Silva *et al.*, 2009).

PC is used as natural dyes for food (candy, ice creams, dairy products and soft drinks), and cosmetics replacing synthetic colourings (Deshmukh & Puranik, 2012; Patel *et al.*, 2005). Furthermore it has potential health benefits as an antioxidant and possesses significant immune enhancing and antiviral properties (Hemlata *et al.*, 2011). They are also widely used in biomedical research and pharmaceutical industries (Tripathi *et al.*, 2007). Owing to their highly fluorescent properties, they can serve as labels for antibodies, receptors and other biological molecules in a fluorescence-activated cell sorter, flow cytometry, fluorescence immunoassay, fluorescence microscopy and diagnostics (Viskari & Colyer, 2003).

Although, various methods with numerous steps have been developed for the extraction and purification of PC from cyanobacteria, the purity and recovery is relatively low because, it is highly sensitive to light, oxygen and moisture. Furthermore, the scale-up of these procedures is expensive and difficult. Extraction and purification processes account for 50 to 90% of the production cost of PBP's (Gupta & Sainis, 2009; Patil *et al.*, 2008). Hence there is a need for efficient and economical extraction methods that will yield a high purity product. Complete extraction of PBPs is one of the vital steps in the accurate identification and quantification of these proteins from cyanobacteria. Therefore, the objective was to find a suitable extraction method to recover majority of the PC, this was achieved by comparing different cell disruption methods and extraction buffers. Thereafter, the purity and concentration of PC was determined by performing ammonium sulphate to precipitation and dialysis.

4.2 Materials and Methods

4.2.1 Chemicals

All chemicals were purchased from Sigma-Alrich® (St. Louis, MO, USA) or Merck (Germany), unless otherwise indicated. All buffers and reagents used in this study were prepared in type 1 ultrapure (18.2MΩcm) water and supplemented with 0.01% sodium azide.

4.2.2 Extraction Procedure

Extraction protocol (Appendix 3.1) was optimized by firstly determining the ideal buffer for PC extraction. The buffers include: acetate (pH-6.0), double distilled water, potassium phosphate (pH-7.5), sodium phosphate (pH-7.5), Tris chloride (pH-7.4) and Tris Base/EDTA (pH 7.4). Thereafter the concentration of selected buffer was varied from 10 mM – 1M. Following the buffer selection, enzymatic disintegration of the cell wall and mechanical disruption methods were compared. For enzymatic cell lysis lysozyme (100 µg/mL) was also added to the sample in the selected buffer, and incubated in a shaking bath at 65 °C for 2 h (Muthulakshmi *et al.,* 2012). Mechanical cell disruption was performed using the following two techniques: Sonication on ice for 30 s using 8W pulses (XL -2000 series Minsonix Ultarsonic liquid processor , Trilab support, S.A) (Lawrenz *et al.*, 2010), and bead beating for 1 min pulses, three times (Mini bead beater, Biospec Products,USA).

4.2.3 Partial Purification using Ammonium Sulphate Precipitation and Dialysis

The supernatant was precipitated with 30% w/v and 50% w/v NH_4SO_4 at 4°C. Finely powdered NH_4SO_4 was gradually added in the crude extract to achieve 30% saturation with continuous stirring for 1 h. The precipitate from 30% w/v solution was discarded (Silva *et al.*, 2009). The supernatant was further brought to 50% w/v saturation and allowed to stand at 4°C overnight. The precipitated proteins was recovered by centrifugation (Heraeus Biofuge *fresco,* Germany) at 9500x g for 30min.The precipitate is re-dissolved in 0.005M phosphate buffer pH 7.0 and dialyzed overnight using a dialysis tube (Muthulakshmi *et al.,* 2012). Prior to dialysis the membrane had to be treated using a series of solutions and boiling steps (Appendix 3.2).

4.2.4 Estimation of PC

Absorption spectra was determined by scanning the protein sample in a range of 250 to 750 nm wavelengths using a Merck spectroquant Pharo 300 (Merck,Germany). The concentration of the total crude pigment PC was determined at 620 and 650 nm as reported by Chaiklahana *et al.* (2012) and Moraes *et al.*(2011). Phycocyanin concentration and extraction purity (EP) were calculated by the following equation:

$$(PC)\ \mu g/mL = OD620 - 0.7\ (OD650)/7.38 * 1000 \quad \textbf{(Eq 4.1)}$$

$$(EP) = OD620/OD280 \quad \textbf{(Eq 4.2)}$$

4.2.5 Gel Electrophoresis

Electrophoresis specifically SDS-PAGE was performed using Biorads Any kDa MiniProtean TGX precast gel in a MiniProtean II apparatus (Bio-Rad®). The optimal separation range is between 10 to 200 kDa. Unstained protein standards, broad range 10 – 250 kDa (Bio-Rad®) were used as molecular weight markers and commercial PC and APC was purchased from Sigma-Alrich®. Samples were pre-incubated with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol at 90–95°C for 5 min. Gels were run at room temperature at 200 V for 30 min. Thereafter the gel was stained with Coomassie Brillant Blue (Bio-Rad®) for 4 hours, and destained overnight using a solution containing acetic acid and ethanol (1:4). Destained gels were rinsed thoroughly using distilled water, thereafter viewed over a white light box and image taken using white-light transillumination. The gel was covered with cling wrap and stored at 4°C with distilled water.

4.3 Results

4.3.1 Optimization of Extraction Procedure

Pigment recovery can be greatly affected by the extraction buffers and procedure used, thus optimization of the extraction process for maximum protein recovery is crucial. The factors optimized were mechanical cell disruption such as sonication and bead beating; varying the chemical composition of extraction buffer i.e. varying the ionic strength and pH

For the comparative study of different extraction methods of PC from *Cyanothece* sp., four lysis methods, i.e. sonication, bead beating and freeze/thaw and heat shock was carried out using for wet biomass. The freezing and thawing method resulted in the highest PC yield of 718.73 µg/mL, followed by sonication and bead beating with a PC concentration of 650.98 and 629.48 µg/mL, respectively. There was a significant difference (p<0.05) between the freeze/thaw and other methods (Figure 4.1).

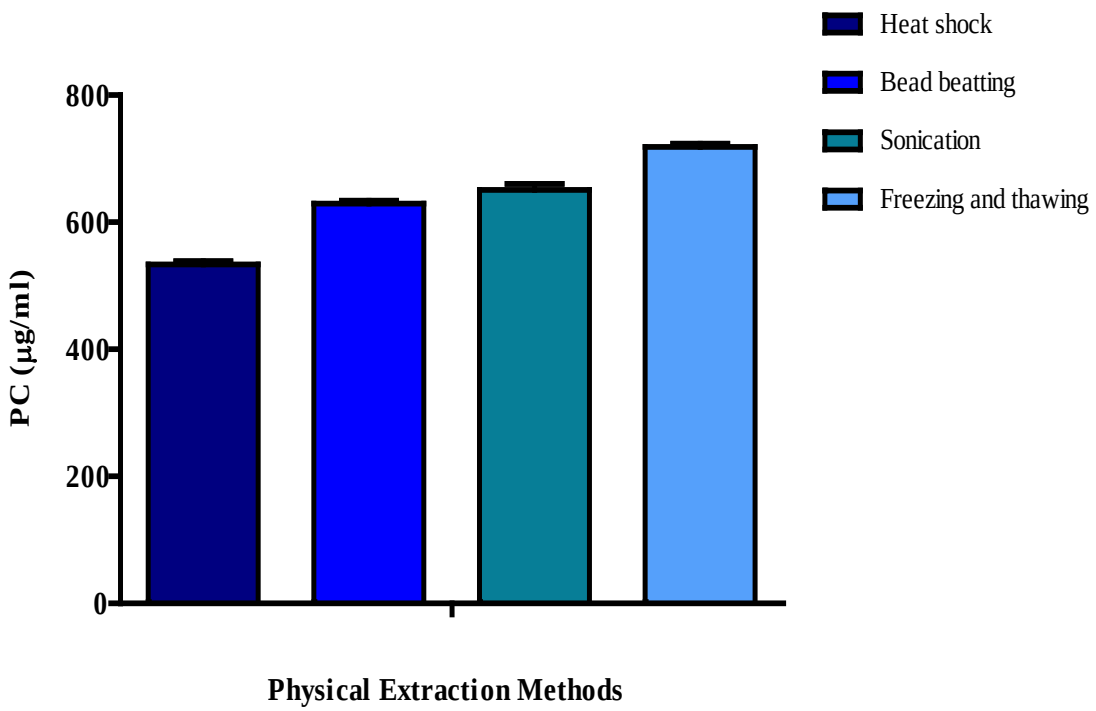

Figure 4.1 : Yield of PC extracted from *Cyanothece sp*. using different cell disruption methods. The cells were centrifuged and re-suspended in ultrapure water then subjected to the different lysis techniques. Data were expressed as the means and standard deviations of 3 replicates.

The quantities of the crude extracts using different buffers are given in Table 4.1. During comparison of buffers, sodium phosphate buffer (pH 7.0) resulted in the highest yield (810.71µg/mL) of PC. As the PBP's are water-soluble, ultrapure water (18.2MΩ) was used as extraction solvent. However, the concentration of PC extracted using water was significantly lower (p<0.05) compared to sodium phosphate buffer. Potassium phosphate buffer and ultrapure water (18.2MΩ) yielded similar PC concentration. The least PC concentration of 191.65 µg/mL was extracted using Tris Chloride buffer.

Table 4.1 : Effect of different buffers on the extraction of total phycocyanin in *Cyanothece* sp. using freeze thaw method. Data were expressed as the means and standard deviations of 3 replicates.

Buffer	PC (ug/mL)
Acetate buffer (pH-6.8)	339.482±1.73
ultrapure water (18.2MΩ)	704.631±0.62
Potassium phosphate buffer (10mM ,pH-7.0)	698.358±0.36
Sodium phosphate buffer (10mM, pH-7.0)	**810.710±0.72**
Tris chloride buffer (pH-7.4)	191.653±1.48
Tris Base 1M pH 7.4 + EDTA	573.176±1.79

It can be seen from Table 4.2, the ionic strength of the sodium phosphate buffer, influenced the extraction of the PBPs. With decreasing ionic strength, the PC concentration increased, except at the lowest ionic strength (10 mM), where the least amount (755.588 of ug/mL) of PC was recovered. The highest extraction was obtained by osmotic shock using 50 mM sodium phosphate buffer, yielding 1039.463 ug/mL of PC.

Table 4.2: Phycocyanin extraction from *Cyanothece* biomass with different concentrations of sodium phosphate buffer. Data were expressed as the means and standard deviations of 3 replicates.

Sodium phosphate buffer (mM)	PC (ug/mL)
10	800.588±0.57
50	**1039.463±0.36**
100	978.764±0.87
500	982.957±1.41
1000	869.272±0.93

The pH of the sodium phosphate buffer was varied in order to evaluate the effect of pH on the phycobiliprotein extraction. The maximum extraction was achieved at pH-7 yielding 1081.35 µg/mL PC (Table 4.3). At a pH above 7.5 or below pH-7 there was a significant decreases in PC concentrationwas found (p<0.05).

Table 4.3: Total PC extracted from *Cyanothece* sp. biomass using 50 mM sodium phosphate buffer at varying pH. Data were expressed as the means and standard deviations of 3 replicates.

pH of Sodium phosphate buffer	PC (ug/mL)
6	987.465±0.71
6.5	998.764±0.67
7	1010.305±0.34
7.5	**1081.451±0.23**
8	991.034±0.43

Improvement of the cell lysis technique after buffer optimization was carried out to determine if a significant increase in PC yield may be achieved. Freeze thaw was used in combination with sonication, bead beating and lysozyme (Figure 4.2). The combinations with bead beating and sonication resulted in a 15% and 18 % increase compared to freeze/thaw alone. However no significant difference was achieved between combinations of bead beating and sonication. The combination of freeze/thaw and heat with lysozyme resulted in a 36% increase in PC extracted and making it the most efficient cell disruption method.

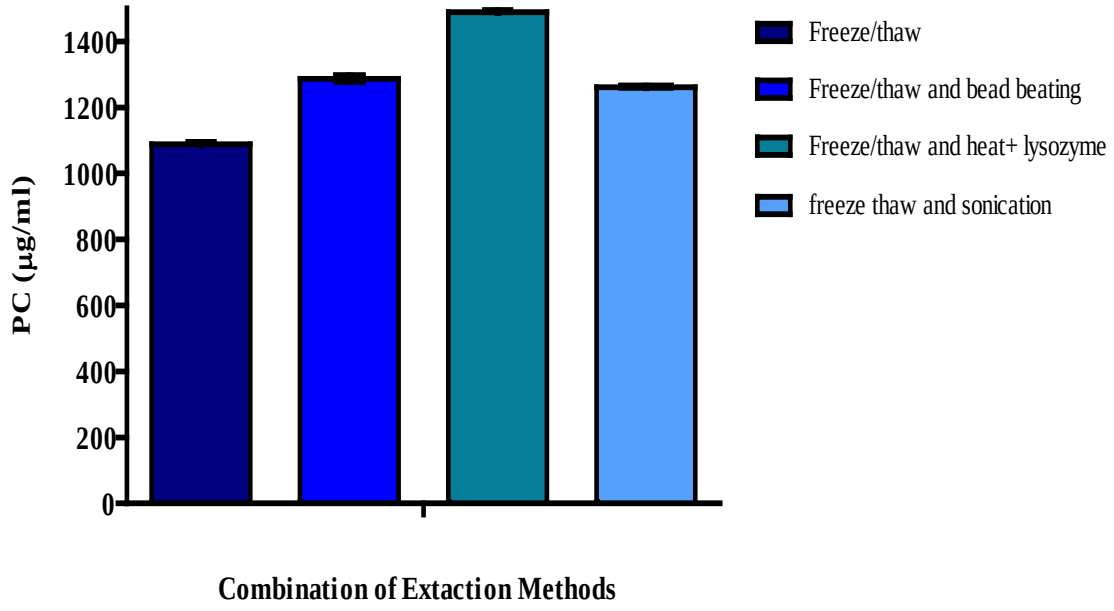

Figure 4.2: Extraction of PC using 50 mM sodium phosphate buffer (pH7) with a combination of physical disruption methods. Data were expressed as the means and standard deviations of 3 replicates.

4.3.2 Electrophoresis

Electrophoresis samples showed only two bands corresponding to α and β subunits of the cyanobacteria PCs. The molecular weights of subunits were determined by running the low molecular weight markers (Sigma-Alrich® SDS) along with the samples. The molecular weight of the extracted PC was determined by SDS-PAGE. The proteins were visualised with Coomassie brilliant blue (R250) staining. In Figure 4.3, the intense bands located near the 15, 20 and 25 kDa in lanes 5 and 6 indicated the presence of PBP, and other faded bands correspond to contaminant proteins. Phycocyanin showed two bands of ~17 and ~20 kDa molecular mass correspond to the characteristic α and β subunits, respectively. The single band of ~15 kDa represents APC.

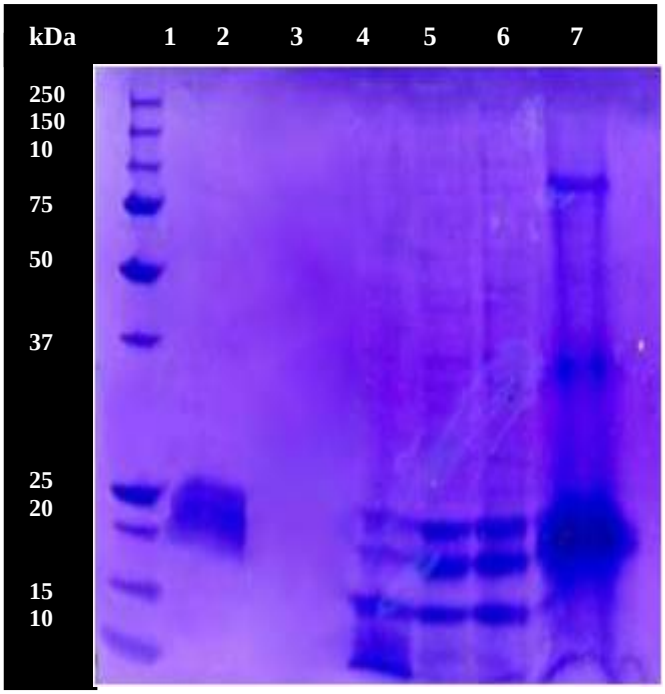

Figure 4.3 : SDS-PAGE of the PC from *Cyanothece* sp. stained with Comassie brilliant blue and viewed under white light. Lane 1, marker; lane 2, commercial standard APC, lane 3, blank, lane 4 crude PC and lanes 5 & 6 precipitated PC and APC and lane 7 commercial standard PC.

4.3.3 Purification

The crude PC extract was partially purified using NH_4SO_4 precipitation followed by dialysis. The first step in the fractional precipitation started with 30% w/v NH_4SO_4 saturation, which mainly salted out proteins with little improvement in the purity ratio. The PC was then salted out with a 50% w/v NH_4SO_4 and dissolved in phosphate buffer. The results of the fractionation process were improved as it eliminates other proteins, thereby increasing the purity ratio of PC (Muthulakshmi *et al.*, 2012). At each PC purification step the purity increases; however a decrease in PC was found (Table 4.4). After dialysis a food grade PC was isolated.

Table 4.4: Spectrophotometric purity and concentration of phycocyanin from *Cyanothece* sp. after different steps of purification. Results reported are the average of 6 independent experiments.

Purification step	Extraction purity (615/280 nm)	PC (µg/mL)
Crude Protein	0.250	6212.012±1.21
Ammonium Sulphate 30% w/v	0.289	4323.921±0.98
Ammonium Sulphate 60% w/v	0.976	4897.056±0.54
Dialysis	1.205	3354.898±0.79

The absorption spectrum of the *Cyanothece* sp. crude protein extract (Figure 4.5- dark blue line) shows 2 distinct peaks. The major peaks at 620 nm correspond to the absorbance of PC. The other peaks at between 300-350 nm is indicative of other unwanted proteins and nucleic acids extracted. After precipitation at 30% saturation using NH_4SO_4, the unwanted proteins (white pellet) were discarded (Figure 4.4b). The blue partial purified PC pellet can be seen in Figure 4.4c. The absorption spectrum (Figure 4.5- light blue line) confirms this, since only one peak at 620nm was present.

Figure 4.4: showing (a) crude extract obtained from *Cyanothece* sp. to be fractionated by precipitation with NH_4SO_4 at 30-50% saturation at 4°C, (b) the 30% fractionated white pellet containing unwanted proteins and (c) the 50% pellet fractionated by precipitation containing PC.

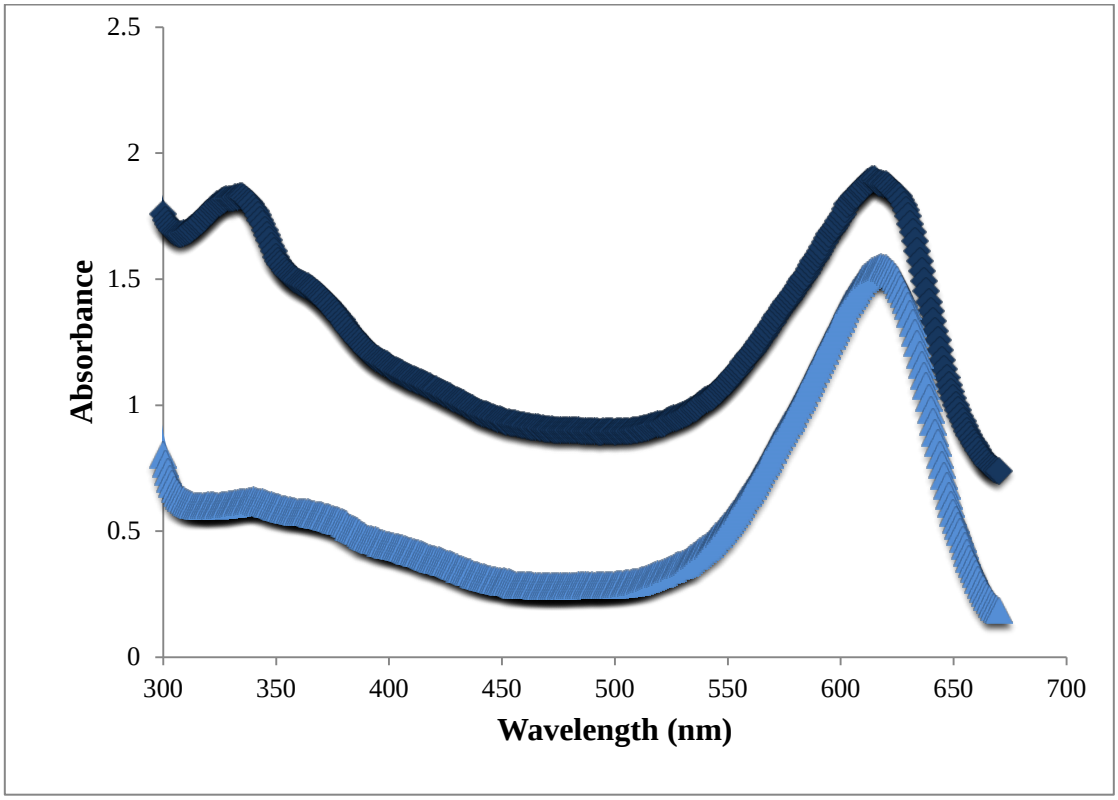

Figure 4.5: Absorption spectra of crude extract (dark blue line ▬) showing a high absorbance at 620 nm, and precipitated protein (light blue line ▬) showing a decrease in the peak at 320 nm.

4.4 Discussion

Extraction and purification methods are amongst the most crucial aspects in obtaining maximum protein yields. Cyanobacteria generally have thick cell walls, which are exceptionally tough to break and allow the release of PBPs into the extracting medium (Hemlata *et al.*, 2011). A method that is effective for extraction and purification of PBPs in one organism may not necessarily be the most suitable procedure for another organism (Kamble *et al.*, 2012). Therefore the extraction and purification techniques for PC from *Cyanothece sp*. were optimized in the current study and standardized for further work.

4.4.1 Comparison of Cell Disruption

Cyanothece sp. cells were successfully ruptured by sonication and bead beating, resulting in similar PC yields (Figure 4.1). Sonication is commonly used to promote cell disruption by supplying extra energy to the sample solution (Viskari & Colyer, 2003). This is achieved by cavitation whereby the rapid formation, growth and bursting of small gas bubbles creates high pressures and temperature (Vandeventer *et al.*, 2011). Bead beating achieves cellular disruption by rapid mixing of cells with beads by high frequency oscillation in a closed tube. The most effective cell disruption technique yielding 720.73 µg/mL was obtained when *Cyanothece* sp. cells were broken by freezing at – 20°C and thawing at 4°C. The freeze-thaw method has been previously found to be effective for various cyanobacteria including *Synechococcus* sp. IO9201 (Abalde *et al.*, 1998), *Synechococcus bacillaris* (Lawrenz *et al.*, 2010) and *Arthrospira* sp., (Patel *et al.*, 2005). The advantage of freeze-thawing method is that it is a mild and non-denaturing technique. Sharp ice crystals form during the freezing process and then contract during thawing thus breaking the cell wall and releasing the PBP into the extracting medium (Soni *et al.*, 2006).

4.4.2 Selection of Buffer

A suitable buffer must be selected for the extraction of maximum PBPs and to ensure that proteins are not denatured due to a shift in pH. Sodium phosphate buffer yielded the highest PC (810.71 µg/mL), whereas the Tris chloride buffer resulted in the lowest yield of PC at 191.65 µg/mL (Table 4.1). The results are comparable to those achieved by other researchers that found sodium phosphate to be a good extraction buffer (Kamble *et al.*, 2012; Ramos *et al.*, 2010; Reisfl *et al.*, 1998). Sodium phosphate buffer is also being reported to have

inhibitory effect on enzymes providing protein stability for several weeks at 4°C and provides a stabale neutral pH (Hemlata *et al.*, 2011). It is important that the protein is initially extracted in a neutral solution, so as not to alter its structure.

Regarding the ionic strength of the sodium phosphate buffer, results showed that it influenced the recovery of PC. The ionic strength of 50 mmol/L extracted the highest amount of PC for this cyanobacterium (Table 4.2). A further increase in the buffer's ionic strength resulted in a significant decrease in the PC yield. Similarly Ramos *et al.* (2010) reported that by increasing the ionic strength reduces the PBP extraction except at the lowest ionic strength tested i.e 50 mmol/L.

The pH of the buffer during extraction and purification must be maintained to ensure the stability of the PC molecules. The highest PC was obtained when extracted using buffer with pH 7.5 (Table 4.3). This is in agreement with work done by Doke (2005) who showed that the highest concentration of PC was obtained with the phosphate buffer at pH 7.0 as compared to pH 6.0 and 8.0. Similarly Ramos *et al.* (2010) achieved a 59.1% initial recovery of PBPs. Chaiklahana *et al.*, (2012) reported that the PC is highly soluble at pH 7.0, but insoluble in acidic solutions. Similar results were observed, based on the colour and clarity of the solutions at pH 7. A lower concentration of the PC and turbidity was observed in acidic solutions. Silva *et al.* (2009) and Patil & Raghavarao (2007) also showed PC was unstable at extreme pH values. Furthermore decolouration occurs below 5.0 and above 8. (Hemlata & Fatma, (2009) reported that extreme buffer pH causes internal electrostatic attraction by changing the charge on proteins giving net positive charge and at this stage protein unfolds and the bound solvent is lost, resulting denaturation of the protein.

4.4.3 Optimization of Extraction Method

The combination of the cell disruption methods using sodium phosphate buffer at optimized buffer conditions (50mM, pH 7) are shown in Figure 4.2. The combinations of freezing /thawing with bead beating and freezing/thawing with sonication both resulted in a 1.4 fold increase compared to the freeze/thawing method alone. The disruption of cells by freeze–thawing cycle (-20 & 4°C) supplemented with 10 % lysozyme was significantly higher (p<0.05) than in other combination disruption techniques. The combination of the freeze/thawing cycle and heating at 37°C with lysozyme resulted in a 1.6 fold increase in PC

extracted compared to freeze thaw method. Santiago-Santos *et al* (2004) had also successfully used lysozyme for the extraction of PC from *Calothrix* sp. According to Boussiba & Richmond (1980) the extraction of PC from *Arthrospira. platensis* treated with lysozyme was better than mechanical methods since it was easier to handle and a higher yield was obtained.

4.4.4 Purification

The absorption spectrum of the PC monomer and all aggregates exhibit a strong excited state band at ~615 nm (Niels, 2008; Romay *et al.*, 2003). Blue coloured pigment with single absorption maximum at 620 nm confirmed that the extract contained PC. Success of purification protocol was also revealed by UV-vis spectra (Figure 4.5). A single peak at 620 nm suggested absorbance maxima of PC while the decrease in absorbance between 300-350 nm suggested the removal of other contaminants. Patel *et al.*, (2005) reported that A_{260nm} and A_{280nm} indicate the contamination of extracted nucleic acids and aromatic amino acid rich proteins respectively.

Ammonium sulphate fractionation at 25% w/v and 50% w/v was used to purify the crude protein extract. This two-step precipitation salts out undesirable proteins and whilst concentrating the PC (Kamble *et al.*, 2012). Addition of 25 % and 50% ammonium sulphate gave a 0.289 and 0.976 purity ratio and yields of 4324 and 4897 µg/mL respectively (Table 4.4). Crude extracted precipitation with 30% w/v NH_4SO_4 removed majority proteins except PC; thereafter the resulting extract was precipitated with 50% NH_4SO_4 to concentrate the PC (Figure 4.4). Dialysis of the crude PC from isolated *Cyanothece* sp. was performed to improve the purity. The purity achieved after precipitation and dialysis was 1.2, which met the PBP purity standard for foods and feeds (Table 4.4). At a low temperature (25°C), NH_4SO_4 is highly water soluble and has ability to maintain the integrity of protein. Furthermore it has a bacteriostatic effect which is useful in purification of proteins (Soni *et al.*, 2006). Various purity values of PC using ammonium sulphate saturation, depending on strain of cyanobacterium were found. Patel *et al.*, (2005) reported the purity values of 2.66, 1.62 and 1.46 for *Spirulina* sp., *Phormidium* sp. and *Lyngbya* sp., respectively. A purity value of 2.12 for PC was found for *Anabaena* sp. (Hemlata *et al.*, 2011). Lower PC purity values of 0.37 and 0.26 were reported for *Nostoc muscorum* and *Aphanozomenon-flos-aquae*,

respectively. Phycocyanin extracted from *Oscillatoria* sp. had a purity value of 1.26 after NH_4SO_4 precipatation (Soni *et al.*, 2006).

4.4.5 Electrophoresis

The purity of the PC obtained from precipitation was confirmed using the SDS-PAGE as shown in Figure 4.3. From the SDS–PAGE, it could be observed that, the some of the contaminant proteins present in the crude extract were partitioned to the bottom phase during NH_4SO_4 precipataion hence, an increase in the purity of PC was observed. Phycocyanin generally exists in hexameric state i.e. (αβ). SDS-PAGE analysis of PC revealed that the apparent molecular mass of subunit was (~17 kDa) and (~19 kDa) for the α and β. This is in agreement with the earlier reports. (Niu *et al.*, 2007) reported α and β subunits of 18 kDa and 21 kDa from *Arthrospira Platensis*. The α and β subunits from *Phormidium fragile* were found to be 19 kDa and 20 kDa respectively, (Soni *et al.*, 2006); *Arthrospira* sp and *Arthronema africanum* α and β subunits were 17 kDa and 19 kDa (Kamble *et al.*, 2012 and Minkova *et al.*, 2007). (Pankaj *et al.*, 2010) reported α and β subunits of 17 kDa and 20 kDa from *Nostoc muscorum*. Some bands were still visible apart from the PC indicating the presence of other proteins. Further purification of the PC is needed in order to remove the other contaminant proteins, which could be achieved by using chromatography technique such as ion-exchange chromatography.

sodium phosphate buffer (10 mM; pH-7.5), and subjecting the biomass suspension to a freezing and thawing cycle. A the purification of the crude PC using ammonium sulphate precipitation

CHAPTER 5 : EFFECT OF ABIOTIC FACTORS ON GROWTH OF *CYANOTHECE* SP. AND PHYCOCYANIN PRODUCTION

5.1 Introduction

Environmental factors greatly influence the growth of cyanobacteria as well as photosynthetic pigments such as Chl, PC, APC and PE within the cell. Therefore, successful cultivation of cyanobacteria and PC production requires modification of the media composition as well as optimisation of other physico-chemical parameters (Vijaya & Anand, 2009). Walter *et al.* (2011) and Hemlata & Fatma (2009) reported abiotic factors such as light intensity, light wavelength and temperature have a strong influence the rate of photosynthesis and biomass productivity as well as the PBP content and composition. The size, structure and number of PBS in cyanobacteria are sensitive to environmental growth conditions. Cyanobacteria display considerable differences in their sensitivity, physiological and biochemical responses, as well as adaptive strategies to these factors (Gigova *et al.*, 2012).

Many researchers have shown that light sources of different wavelength affect metabolism and growth of cyanobacteria, as well as the pigment composition and structure, (Babu *et al.*, 1991). A study by Godínez-Ortega *et al.* (2007) showed that irradiance at different quantities and intensities, as well as spectral composition of white and monochromatic lights greatly influenced pigment composition, metabolism and growth. *Calothrix* PCC 7601 can alter their PBP content to the wavelengths of the incident light available during growth (Liotenberg *et al.*, 1996). This process in which cyanobacteria dramatically alters its' PBP content, composition and colour in response to different wavelength and quality of the light is called complementary chromatic adaptation (Dautania & Singh, 2012). Bercea *et al.* (2012) reported that cyanobacteria generally favour low light intensities as this stimulates PBP synthesis. Furthermore the growth kinetics and physiological responses of cyanobacteria are greatly influenced by the photoperiod (Dautania & Singh, 2012). Changes in light regimes have been shown to bring about differences in pigment and biochemical composition of blue- green algae. Hemlata & Fatma (2009) reported that there was a 58% and 69% decrease in PBPs in complete darkness and continuous light, respectively in *Anabaena* NCCU-9 when compared with 16:8 light-dark cycle.

Since cellular processes of cyanobacteria are temperature dependent, their rates increase rapidly with an increase in temperature with maximum values occurring between 25°C and 40°C (Chaneva *et al.*, 2007). Increases in the temperature causes an increase in the substrate-saturated reaction rate of RUBISCO, therefore, the potential increase in growth rate of cyanbacteria (Fei-Xue *et al.*, 2007). Growth rate and PBP production of *Arthrospira platensis* increased with an increase in temperature with high growth rates observed at a maximum of temperature 35°C (Kumar *et al.*, 2011). Robarts & Zohary, (1987) found that the optimum growth temperatures for *Anabaena, Aphanizomenon, Microcysti Toria* was 25°C or greater. A study by Islam *et al.*, (2003) further reported that the optimal growth temperature for *Arthrospira platensis* was found to be at 32°C and that for *Arthrospira fusiformis* was at 35°C.

pH is an essential factor however, very little attention has been given to the influence of pH on the photosynthetic apparatus of cyanobacteria (Poza-Carrión *et al.*, 2001). Changes in pH affect the solubility and bioavailability of nutrients, transport of substances across the cytoplasmic membranes, and the activity of intra- and extracellular enzymes, as well as photosynthetic electron transport and the osmotic potential of the cytoplasm (Stumm & Morgan, 1981). Cyanobacteria possess different mechanisms for pH homeostasis depending their natural habitats (Bano & Siddiqui, 2004).They are capable of maintaining an internal, constant pH of 7.1 to 7.5 in the range of external pH from 5 to 10. Deshmukh & Puranik (2012) reported that the pH of the medium influenced the growth as well as the production of PBP in *Gloeotrichia natans.*

The objective of this study was to determine the effects of abiotic factors which include light quality and quantity, temperature and pH on the biomass and PC production.

5.2 Materials and Methods

5.2.1 Effect of Photoperiod on Growth and Phycocyanin Production

The effect of the photoperiod was evaluated for four photoperiods (24:0, 18:06 12:12 and 8:16 h light: dark) exposed to 100 µmol.m-2.s-1 for 10 days. *Cyanothece* cultures were cultivated in BG11 media at 27°C at 90 rpm in an orbital shaking incubator to allow uniform light penetration and circulation of air and nutrients.. Cultures were monitored daily using ODs at 637 nm and DCW mentioned in section 3.1.3. After a period of 2 weeks biomass was harvested and the PC was extracted and analysed spectrophotometrically as previously described in section 4.2.4

5.2.2 Effect of Light Quality (Wavelength) and Intensity on Growth and Phycocyanin Production

A Culture of *Cyanothece* sp. in the exponential growth phase was inoculated into 250 mL Erlenmeyer flasks containing 200 mL sterilized BG11 medium. The cultures were grown according to previously described conditions. To study the effects of different light intensities and wavelengths, the cultures were exposed to high (125 µmol.m^{-2}.s^{-1}), medium (75 µmol.m-2.s-1 and low (25 µmol.m^{-2}.s^{-1}) at varying wavelengths i.e. blue, red and green and Grolux light at the respective wavelength bands of 380 to 760 nm, 515 to 540 nm, 460 to 475 nm for a 16:8 light dark cycle. For the purpose of this study 25, 75 and 125 µmol.m^{-2}.s^{-1} was referred to as low, medium and high light intensity respectively.

The desired light intensity was obtained by varying the number and distance of Sylvania Octron T8 fluorescent 8W and Sylvania Grolux 12" T5 8W fluorescent tubes (Osram Sylvania, Germany) from the inoculated flasks. Gro-Lux light provides blue and red light in the proportions needed for photosynthesis. Light intensity was measured using a digital lux meter (Major Tech, South Africa). To establish the different spectral conditions the fluorescent light bulbs were covered with blue (BL), red (RL) and green (GL) cellophane. These different conditions generated complementary chromatic acclimation processes.

5.2.3 Effect of Temperature on Growth and Phycocyanin Production

To observe the influence of temperature on growth and pigment accumulation, the cultures of *Cyanothece* sp. were grown at different temperatures i.e. 20, 25, 30, 34, 40 and 45°C. Each Erlenmyer flask containing 200 mL sterilized BG11 medium was inoculated with a 10% v/v of culture in the exponential growth phase. Thereafter, the cultures were incubated in an orbital shaking incubator (90 rpm) and subjected to various temperatures at a constant light intensity of 100 $\mu mol.m^{-2}.s^{-1}$ with a 16:8 light dark cycle.

5.2.4 Effect of pH on Growth and Phycocyanin Production

The effect of pH on growth and PC was investigated at pH 5, 6, 7, 8, 8.5, 9 and 10. The pH was adjusted with 10 M NaOH and 1N HCl solution. Operating conditions and flasks were prepared as previously described.

5.2.5 Statistical Analysis

Results of the analyses were compared by one way ANOVA at $p< 0.05$ level, to determine if the different culture conditions influenced the production of PC, and to establish if a significant difference existed amongst the conditions.

5.3 Results

By varying temperature, it was ascertained that the *Cyanothece* sp. grew effectively up to 35°C, thereafter, increases in temperature resulted in a decrease in the biomass yield. The highest biomass yield (1.2 g/L) was achieved at 35 °C (Figure 5.1). A significant increase (p< 0.05) of the final biomass was observed from 25°C to 30°C and at 35 °C. From the results presented in Figure 5.2, it can be seen that the PC production changed due to the alterations in cultivation temperatures. Among the different temperature, 35 and 40 °C resulted the highest PC content of 4720 and 4824 µg/mL respectively, however, there was no significant differences (p>0.05) between these values. A decrease in PC was observed when the temperature was increased to 45°C.

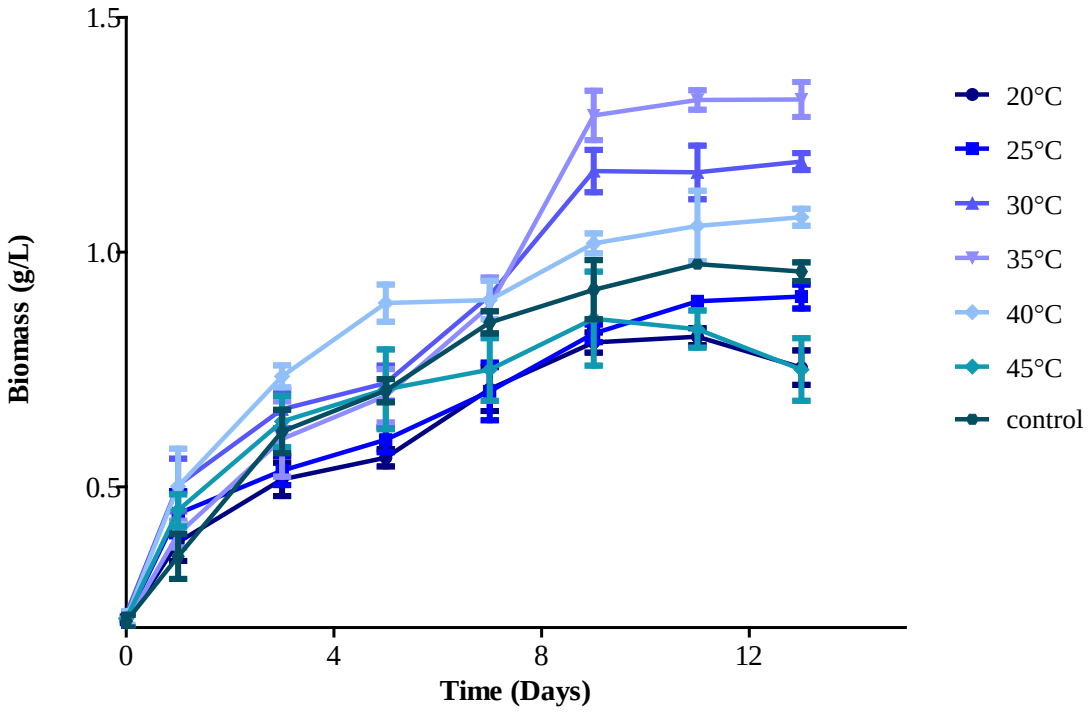

Figure 5.1: The effect of temperature at 20, 25, 30, 35, 40 and 45 ºC on the biomass production after 12 days of cultivation. Each value is the mean value of three trials. Error bars indicate standard deviations.

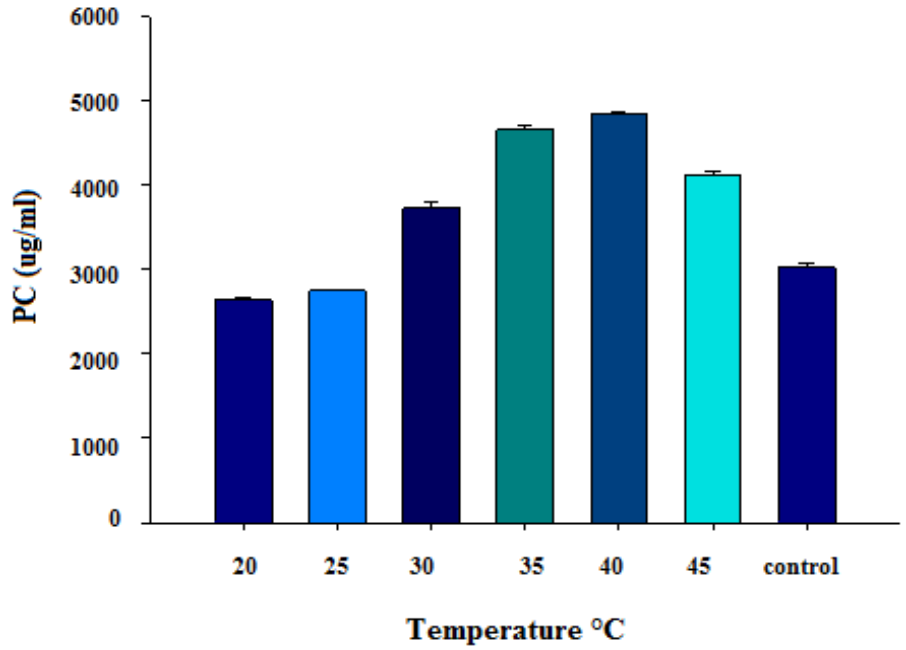

Figure 5.2: Effect of temperature on PC production by *Cyanothece* sp. grown in BG11 medium (pH 7) at 100 µmol.m^{-2}.s^{-1} with varying temperature of 20, 25, 30, 35, 40 and 45°C**.** Each value is the mean value of three trials. Error bars indicate standard deviations.

Among the various pH investigated, the cyanobacterium produced a high quantity of PC at pH 8 and the least at pH 10. A 14% increase in PC was observed when the pH was increased from the control (i.e. pH 7) to pH 8, and a 40% decrease in PC resulted with an increase in Ph from pH 8 to pH 10. Biomass concentrations of the cyanobacterium at different pH conditions are shown in Figure 5.3 and their growth rates in Table 5.2. These results revealed that the *Cyanothece* sp. showed optimum growth at pH 8 with a specific maximum growth rate of 0.254.day^{-1} and biomass production of 1.13 g/L. According to biomass and pigment quantities, *Cyanothece* sp. appeared to have preferred a pH between 7 and 8. Further increase in pH resulted in a significantly lower biomass yield.

Table 5.1: Extracted PC yield from *Cyanothece* sp. grown in BG11 medium at varying pH for 14 days. Data were expressed as the means and standard deviations of 3 replicates.

pH	PC (µg/mL)
5	2312.616±0.92
6	2406.323±0.78
7	3075.033±0.25
8	3577.124±0.14
9	2694.426±0.98
10	2129.013±1.01

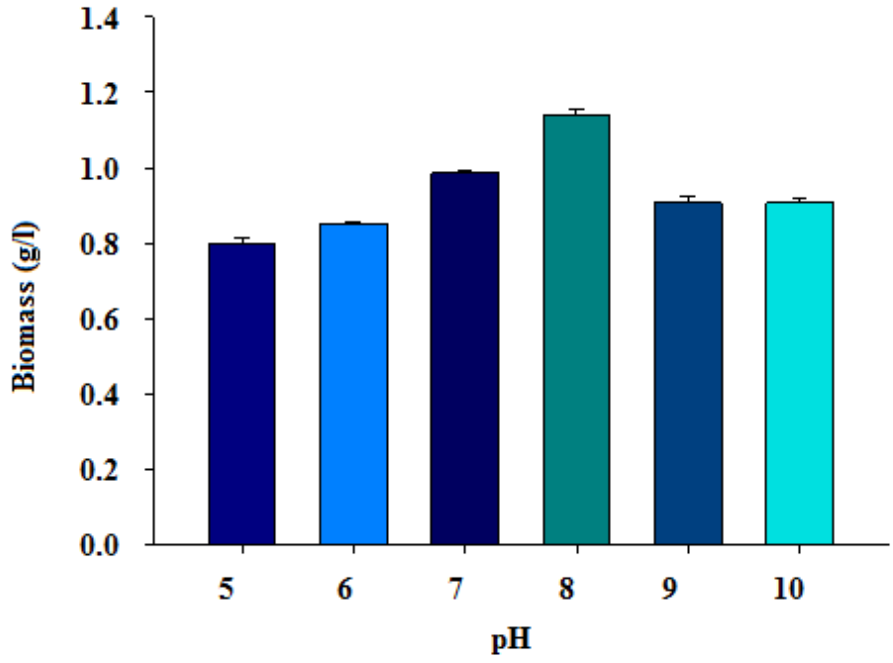

Figure 5.3: Biomass of *Cyanothece* sp. obtained after 14 days of cultivation in BG11 medium under different pH conditions. Each data point is the mean of three replicates and bars are the standard error.

Table 5.2: Specific growth rates of *Cyanothece* sp. cultivated for 14 days in BG11 media at different pH. Data were expressed as the means and standard deviations of 3 replicates

pH	Specific growth rate (day^{-1})
5	0.132
6	0.141
7	0.184
8	0.254
9	0.157
10	0.153

The effect of different photoperiods (8:16, 12:12, 16:8 and 24:00 light/dark) at a light intensity of 100 $\mu mol.m^{-2}.s^{-1}$was investigated. Results showed that a 16:8 light- dark regime was the optimal photo-period for the production of PC and biomass in *Cyanothece* sp. (Table 5.3). There was a 15.43 % decrease at 12:12, 42.35 % decrease at 8:16, and 74.83 % decrease in PC production at continuous light when compared to the optimal photoperiod (16:8). *Cyanothece* cells cultivated under optimal photoperiod i.e. 16:8 light/ dark cycle remained blue-green in colour at day 14 (Figure 5.4), whereas the there was a colour change (from blue green to light green) occurred in cells exposed continuous light. This colour change indicated a decrease and degradation of the pigment.

Table 5.3: Effect of photoperiod on *Cyanothece* sp. biomass accumulation and PC production. Data were expressed as the means and standard deviations of 3 replicates.

Photoperiod (h)	Biomass (g/L)	PC (µg/mL)
8:16	2.17	2102.157±0.75
12:12	2.85	3087.432±1.02
16:08	3.36	3647.371±0.56
24:0	0.90	917.012±0.89

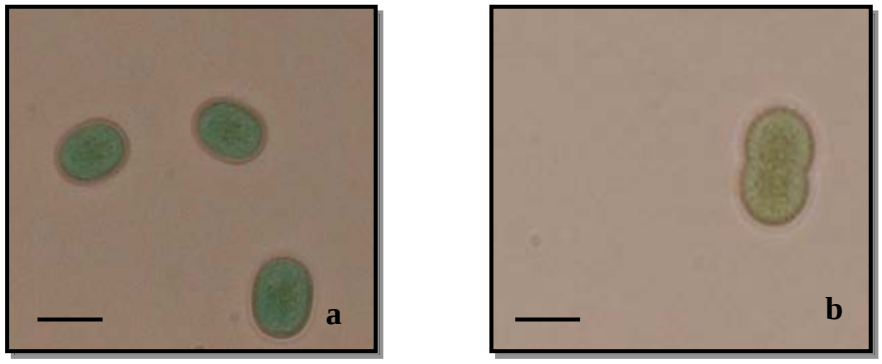

Figure 5.4: Micrograph of *Cyanothece* sp. cultures on day 14 exposed to (a) 16:8 light dark cycle showing blue-green cells and (b) continuous light showing a colour change from blue-green to light green, indicating a loss in PC content.

Absorption spectra indicating PC presence in *Cyanothece* sp. at the different wavelengths is illustrated in Figure 5.5. During Grolux (combination of red and blue light) and red light treatment, PC absorption was the highest, where the other light conditions were considerably lower.

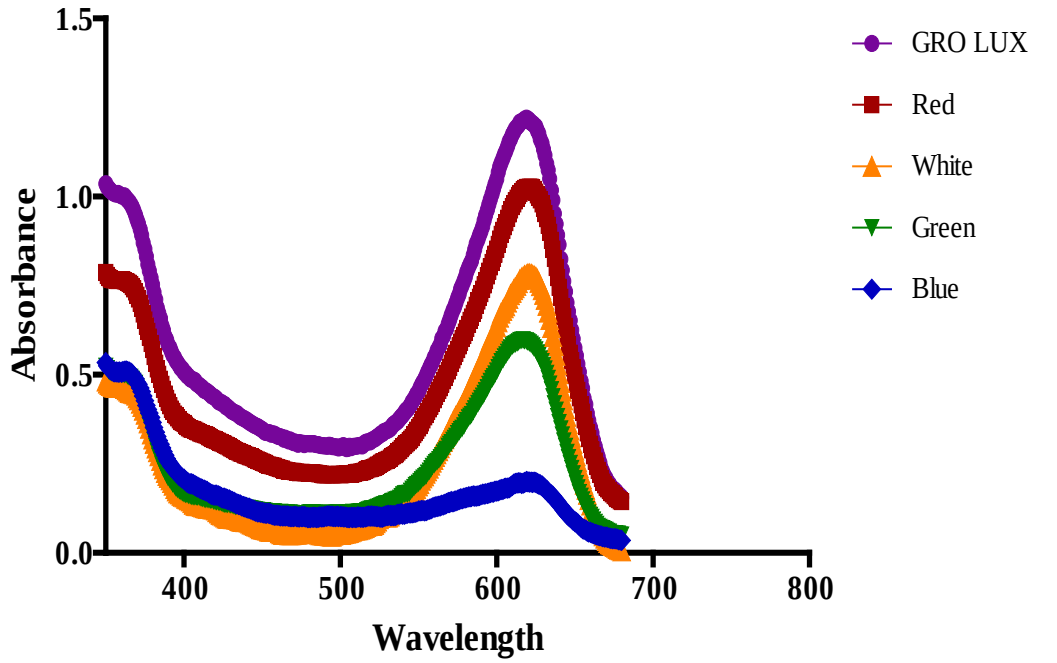

Figure 5.5: Spectrum indicating the varying concentration of PC from *Cyanothece sp.* under different wavelengths at 100 µmol.m^{-2}.s^{-1} of light.

The PC content and biomass was analysed at the various light wavelengths and intensities in order to determine the relationship between PC and light. The following observations were made: a higher biomass quantity was produced at greater light intensity from different light sources (Table 5.4). The only exception was for the red light, where medium light intensity (75µmol.m^{-2}. s^{-1}) produced a higher biomass concentration (1.89 g/L) compared to a higher intensity of 125µmol.m^{-2}.s^{-1} (1.08 g/L). Grolux light at 125µmol.m^{-2}.s^{-1} yielded the highest biomass of 3.3 g/L, while the blue light at the same light intensity produced the lowest biomass of 0.5 g/L. When analyzing the specific growth rate, a similar trend was observed. Under the same light source a higher growth rate was obtained when higher light intensities were used.

Table 5.4: Biomass production and growth rate of *Cyanothece* sp. under various light sources with different levels of light intensity.

Light colour	Light Intensities					
	Low (25 μmol.m-2.s-^{1})		Medium (75 μmol.m-2.s-^{1})		High (125 μmol.m-2.s-^{1})	
	Biomass g/L	Growth rate (day^{-1})	Biomass g/L	Growth rate (day^{-1})	Biomass g/L	Growth rate (day^{-1})
Grolux	1.475	0.632	2.980	0.567	3.307	0.266
Red	0.766	0.124	1.895	0.350	1.082	0.187
Green	0.640	0.199	0.804	0.131	1.142	0.099
Blue	0.455	0.079	0.509	0.073	0.542	0.062
White	1.115	0.296	1.349	0.241	1.627	0.194

A higher PC content was observed at greater light intensity (125μmol.m^{-2}.s^{-1}), when exposed to green, white and Grolux lights. Red light at 75μmol.m^{-2}.s^{-1} yielded a higher PC content when compared to a higher intensity of 125μmol.m^{-2}.s^{-1}. When comparing light quality, relatively high PC was produced by *Cyanothece* sp. when cultures were exposed to Grolux lights at all intensities (Table 5.4). A significant increase (p<0.05) in PC was observed when light intensity increased from 75μmol.m^{-2} s^{-1} to 125μmol.m^{-2} s^{-1}. The highest PC content produced was 3307 μg/mL when culture was exposed to Grolux light at 125μmol.m^{-2} s^{-1}. Overall blue light had a negative effect on PC production producing the least amount of PC, i.e. 475, 578 and 698 μg/mL at low, medium and high light intensities, respectively.

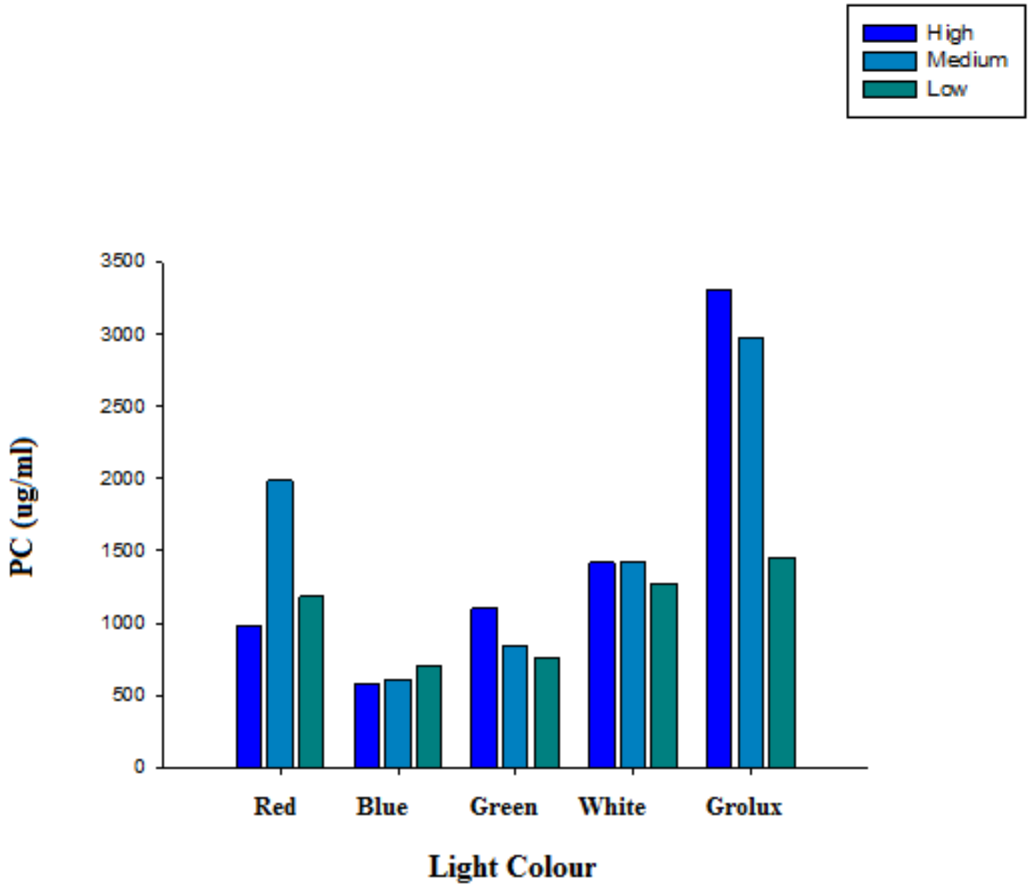

Figure 5.6: Phycocyanin production under different light sources at vary intensities in *Cyanothece* sp.

5.4 Discussion

5.4.1 Temperature

The optimum temperature for PBP synthesis depends on the strain, but usually, extremely high and low temperatures significantly decrease the quantity of the pigments (Gigova *et al.*, 2012). Temperature also influences the growth of cyanobacteria as it impacts on the enzyme kinetics (Islam *et al.*, 2003). Therefore, it is important to determine the optimum temperature range for growth and PC production as well as to identify the unsuitable culture concentrations. The growth curve at various temperatures revealed that *Cyanothece* sp. is tolerant to temperatures ranging from 20 to 40ºC. However growth analysis of cultures grown at different temperatures showed different growth patterns under these conditions. Figure 5.1 revealed that the growth of the *Cyanothece* sp. increased with an increase in temperature. Cultures at 20ºC grew slower, which proved that low temperatures did not favour the growth of *Cyanothece* sp. The highest biomass concentration was found to be at 35°C. This may be attributed to the fact that the partial pressure of CO_2 in the medium is higher at 35°C than at 40°C. This leads to a higher concentration of bicarbonate and subsequently an increased rate of photosynthesis (Colla *et al.*, 2007). Furthermore, Robarts & Zohary (1987) reported that at higher temperatures (i.e. greater than 35°C) there is an increase in dark cycle respiratory activity in which the cells use reserve materials (e.g. carbohydrates) for respiration, resulting in a decrease in cell weight. According to Chaneva *et al.*, (2007) the highest biomass and PC content in *Arthronema africanum* was obtained at 37ºC. Fu *et al*. (2007) reported the maximum biomass with high pigment content in *Arthrospira platensis* was obtained at 35°C. In addition Hemlata & Fatma, (2009) showed results for optimum temperature being 30ºC for *Anabaena NCCU-9.* Boussiba & Richmond, (1980) found that the optimal temperature for growth of a *Arthrospira* sp was between 35 to 37°C, however increasing in temperature up to 40°C was detrimental to the culture.

The optimum temperature range for PC production was between 35- 40°C (Figure 5.2). There was 46.7 % decrease in PC at 20°C, 42.6% at 25°C and 23.3% at 30°C when compared with maximum PC yield of 4837 µg/mL at 35°C. Colla *et al.* (2007) found that a temperature of 35 °C had a negative effect on biomass production of *Arthrospira* s *platensis* but encouraged the production of protein, lipids and phenolics. Similar results were observed in this study at 40°C, where a significant decrease in biomass (p<0.05), and a high PC content was observed. Furthermore, increases in temperature to 45°C resulted in a 38.2 % decrease in PC

production. This decrease in PC could be due to a change in the PBSs organization. This causes an impairment of energy transfer thus decreasing the PC content. Additionally, at high temperatures, a decrease in the PC occurs due to the bleaching of PBPs (Murthy *et al.*, 2004). Jodłowska & Latała, (2013) reported that an increase in temperatures significantly reduced the photoinhibition, caused by the denaturation of the D1 protein.

5.4.2 pH

The influence of pH on the PBP of cyanobacteria has received little attention. The optimum for PC production was pH 8, which yielded significantly higher (p<0.05) PC compared to the other pH values assessed. Results showed that the *Cyanothece* sp. was able to grow in acidic (pH 6.5) and alkaline medium. This suggests that cyanobacteria are able to adapt to variable pH conditions (Lee *et al.*, 2005). Ifeanyi *et al.* (2011) reported the effect of pH on *Aphanocapsa* sp. and noted that the organism grew in a unique manner under a range of different pH values. An increase in pH from 6 to 8 significantly improved the total PBP content in *Anabaena* NCCU-9 (Hemlata & Fatma, 2009) and a pH-8 was found to be optimum for *Synechocystis* (Hong & Lee, 2008). *Cyanothece* sp. showed the highest growth rate (Table 5.1) and the highest biomass generated (Figure 5.3) at pH 8. These findings are in agreement with Bano & Siddiqui (2004), who showed that a pH range of 7.4 to 8.0 was the optimum for growth of marine cyanobacteria, *Katagnymene accurate, Lyngbya contorta, Pseudoanabaena lonchoides* and *Spirulina major.*

5.4.3 Photoperiod

The duration of light exposure to the cyanobacterium is also critical for successful photosynthesis. Growth of cyanobacteria were significantly influenced by the length of the photoperiod and by the ratio of light to dark hours. Foy & Gibson (1993) observed that shorter day length resulted in a decrease in the growth of cyanobacteria. Similar findings were observed for *Cyanothece* sp. were a lower biomass and PC was found when the cyanobacteria were exposed to the shortest light period of 8 h. A 31% and 18% increase in biomass of *Cyanothece* sp. was observed from 8:16 to 12:12 and 12:12 to 16:8 light-dark cycles, respectively. An overall 74 % increase in PC was observed in *Cyanothece* sp. cultivated under 16:8 light-dark cycles. Over saturation of light results in photo inhibition, this is due to the low conversion of light energy into biomass. Photo inhibition most probably

occurred in cultures exposed to continuous light; this was evident by the decrease in pigment synthesis which can be seen in Figure 5.4. Furthermore, a significant decrease (p<0.05) in biomass was also observed from 18:6 to continuous light (Table 5.3). Although light promotes cell division an excess of light could inhibit cell division, this is caused by photo oxidation reactions inside the cell due to excess light that cannot be absorbed. Similar findings were reported by Dautania & Singh (2012), who found that in continuous light growth of *Anacystis nidulans* was reduced due to photo inhibition and photooxidation. Oversaturation of light causes denaturation of protein D1 that carries the binding site for electron Q1; thereby the electron flow from water to PSII becomes inhibited (Jensen & Knusten, 1993). Ibrahim (1993) also reported that continuous stimulated biosynthesis of chlorophyll in cells which leads to an increase in photosynthesis as well as the accumulation of bioproducts such as glucose and carbohydrates. Therefore, exposure to continuous light, results in insufficient time for consumption of these products which eventually leads to a decrease in photosynthetic intensity.

5.4.4 Light Quality

The PC content and biomass in *Cyanothece sp* changed considerably, depending on the quality or wavelength of light. Takano *et al.* (1995) reported that red light or blue light was essential for PC production of *Synechococcus* sp. NKBG 042902. Similar results were found in this study, where the PC concentrations were significantly higher (p<0.05) in Grolux and red light compared with the other three light treatments. Grolux lamps provide blue and red light in the proportions needed for photosynthesis (Sylvana). This subsequently results in increased biomass and total PC (Dautania & Singh, 2012; Walter *et al.*, 2011). *Cyanothece* sp. changed PBP colour in response to light quality through CCA. The order of chromatic acclimation for PC production in this study was found to be Red- White- Green-Blue light, which suggested that coloured light played a stimulatory effect on PC production (Figure 5.5). Blue light treatment was the least efficient producing low biomass (0.542 g/L). High biomass (1.082 g/L) was produced when cultures were exposed to red light. Lopez-Figureoa *et al.* (1989) and Ollemuller *et al.* (1988) reported red light stimulated mRNA accumulation which has the genes that encode for PC synthesis. Lee *et al.* (2005) reported that red light increased PC production in *Synechococcus* strains and *Westiellopsisiyengarii.* Similar findings were reported by Walter *et al.* (2011), where PC production was higher under red filtered light, indicating an adaptation to the narrower range of light. It was found that

Pseudanabaena accumulated significantly higher amounts of PC during red light treatment compared to white, green, yellow and blue light conditions (Dautania & Singh, 2012). Under red light *Fremyella diplosipho* accumulated high levels of PC (Kilani & Lebeault, 2007). Wang *et al.* (2007) recorded a high specific growth rate of 0.40 day^{-1} for *Arthrospira platensis* grown under red light using light-emitting diodes (LEDs), and that treatment under blue light was the least efficient. Chen *et al.* (2010) reported the maximum PC of *Arthrospir platensis* when grown using red LEDs, whereas the blue light gave the lowest PC content. It is interesting to note the yield of PC was produced when exposed to high green light and red light. Obtained results from one way ANOVA showed significant differences ($p<0.05$) between both PC concentration under these conditions. These observations are consistent with the findings of Ollemuller *et al.* (1988) where cyanobacteria have two PC gene sets are located on the genome: while transcripts from one (inducible) gene set are only detectable in cells grown in red light; and transcription from a second (constitutive) gene set occurs in both red and green acclimated cyanobacteria.

5.4.5 Light intensity

Light influence metabolic processes, and consequently the growth rate, photosynthetic activity, and pigment composition (Jodłowska & Latała, 2013). The growth rate, biomass and PC results revealed that 125 $\mu mol.m^{-2}.s^{-1}$ was the optimum light intensity for *Cyanothece* sp. The variations in the PC concentrations are likely to reflect the adaptation of the cyanobacterium to the light level. When light intensities were compared at all wavelengths, 25$\mu mol.m^{-2}.s^{-1}$ was the least favoured for PC production (Figure 5.6). Singh *et al.* (2011) reported that 100 $\mu mol.m^{-2}.s^{-1}$ was found to be optimal for *Nostoc muscorum.* Also results described by Carvalho *et al.* (2009) found that the highest PC content in *Pavlova lutheriwith* was obtained at 60–105 $\mu mol.m^{-2}.s^{-1}$ of light. A study conducted by Gantara *et al.* (2012) demonstrated that an irradiance of 120 to 200 $\mu mol.m^{-2}.s^{-1}$of light was also essential for increased pigment production for *Arthrospira* strains. A similar trend was observed for biomass accumulation, where higher biomass was obtained at increased light intensities (Table 5.4).

Temperature, pH and light influenced the growth and pigment composition of *Cyanothece* sp., thus biomass and PC production could be enhanced by changing culture conditions. *Cyanothece* sp produced the highest biomass when cultivated at 35°C in BG11 media (pH 7 to 8), exposed to 125 µmol.m-2.s-1 of Grolux light for a 16:8 cycle.

CHAPTER 6 : STATISTICAL OPTIMIZATION OF NUTRIENT COMPOSITION FOR INCREASED PHYCOCYANIN PRODUCTION USING DESIGN OF EXPERIMENTS

6.1 Introduction

A limited number of cyanobacteria exist in natures that possess the inherent ability to produce high quantities of PC. In recent years the application and use of PC in biotechnology, diagnostics, foods, cosmetics and medicine has increased. Therefore, it is important that cyanobacterial strains are screened by manipulation of culture conditions (Singh *et al.*, 2009). Some cyanobacteria can be grown in simple mineral media requiring only Vitamin B_{12} as a growth enhancing factor, whereas other species require a more complex media containing essential nutrients (nitrogen, phosphorus, sulphur, carbon, iron and trace elements) to sustain growth (Ernst *et al.*, 2005; Mur *et al.*, 1999). It is imperative to select the most appropriate nutrients and their quantities required for growth as this will directly influence biomass yield as well as pigment production. Very little information is currently available in literature regarding the nutritional requirements that promote PC production in *Cyanothece* sp.

Conventional media optimization methods are based on the one-factor-at-a-time approach (OFAT). This is done by varying a single factor while keeping all others at a constant at a specific level (Wu *et al.*, 2007). Over the years, this has proved to be a tedious and time-consuming process, as this approach requires a large number of experiments to be carried out (Dobrev *et al.*, 2007; Mehta *et al.*, 2012). Furthermore the OFAT method does not identify the true optimal conditions since it does not measure interactive effects among the variables into considered (Silveira *et al.*, 2007). In order to overcome the limitations of the traditional optimization method, statistical models are used as tools to improve productivity of various valuable products such as pigments, polysaccharides, proteins, lipids and hydrogen from microalgae, including cyanobacteria (Deshmukh & Puranik, 2012; Hong & Lee, 2008).
Factional factorials are well suited for screening and optimization of growth medium factors. This can be achieved by initial screening experiments using DOE to identify the most important components required in the media, followed by a secondary screening by varying the concentration of important media components. Finally, a CCD is applied to examine the

responses of the significant factors and optimize media (Dautania & Singh, 2012). Response surface methodology is a design method that shows correlations between one or more measured dependent responses with a number of input (independent) factors. This process is designed to identify the optimum values of continuous variables to maximize or minimize a given response by sampling a minimum number of discrete points (Burrows *et al.*, 2008). Unlike the classical OFAT method, using DOE is rapid, economical and time-saving due to a significant reduction in the total number of experiments (Gohel *et al.*, 2006; Mitra *et al.*, 2011). Furthermore, a large number of factors can be varied simultaneously at different levels, and the combined effects of multiple factors involved can be ascertained (Han *et al.*, 2008).

The present chapter, discuss the evaluation of suitable medium components required for PC production. Firstly, various media were compared to determine the best medium for growth of the cyanobacteria. Thereafter, statistical experimental designs; involving (i) FFD used to screen the significant factors and (ii) a CCD was applied to optimize the medium constituents for maximum PC production.

6.1 Materials and Methods

6.1.1 Experimental Design for Media Components Optimization

Following the selection of the best medium, i.e. BG11 for growth of the *Cyanothece* sp., a series of FFD experiments, involving seven nutrient components of the standard medium was designed using the statistical software, Design Expert 8 (Stat-ease, 2008). This program produced a matrix of factors that allowed for screening of media components and identification of these components that affected PC production the most. Results were statistically analysed generating a mathematical model for each experiment. The models, described with mathematical equations, were used to guide the direction in which concentrations of media components should be adjusted. These experiments allowed for screening of significant factors affecting PC production in each round of experimental designs. Once factors were reduced to the three most significant factors, RSM based on CCD, was applied to optimize PC production. The optimized medium was verified in confirmatory experiments and compared to predicted values.

6.1.1.1 Fractional Factorial Design (FFD) using design of experiments

I. Initial Screening

The purpose of the first optimization step was to identify medium components that had a significant effect on PC and biomass production. Fractional factorial design is one of the statistical tools that can be used to screen the significant factors within a medium. Seven media components ($NaNO_3$, K_2HPO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$,NaCl, trace metal and minor nutrients made up of [citric acid, Ferric ammonium citrate, EDTA and $NaCO_3$]) at varying concentrations were used as outlined in Table 6.1. These were selected on the basis of preliminary media experiments. The (-) level means 0.5 times lower than the original component and (+) level means 1.5 times higher than the original component. The concentration and levels of the variables utilized at the preliminary design are listed in Table 6.2. The fractional factorial experimental design was selected with five center points, which is used to estimate errors and provide a quality check for the experimenter's accuracy. Because of the many medium components, a full factorial was not used as this would have led to a remarkable large number of runs excluding center points (128). A resolution IV

design was selected with 16 runs with five center points that for a total of 21 runs. All chemicals required were obtained from Sigma-Alrich® (South Africa).

Table 6.1: Concentrations of variables in the first screening fractional factorial experiment (FF0721) indicating low, high and center points. All chemicals in the medium were in g/L except for minor nutrients and trace metal solutions added in ml/L

Symbol	Factor	Coded Variable		
		-1	0	+1
$\mathbf{X_1}$	$NaNO_3$	1.0	1.5	2
$\mathbf{X_2}$	K_2HPO_4	0.02	0.04	0.06
$\mathbf{X_3}$	$MgSO_4.7H_2O$	0.04	0.075	0.12
$\mathbf{X_4}$	$CaCl_2.2H_2O$	0.01	0.035	0.06
$\mathbf{X_5}$	NaCl	50	100	150
$\mathbf{X_6}$	Minor nutrients	5	10	15
$\mathbf{X_7}$	Trace metals	0.5	1	1.5

Table 6.2: Experimental design of FF0721 with five center points, showing the coded values of the variables and randomized order used to determine the effect of medium components on PC production of *Cyanothece* sp.

Run	Factors						
	X_1	X_2	X_3	X_4	X_5	X_6	X_7
1	2	-1	-1	1	1	1	-1
2	2	1	1	1	1	1	1
3	1	-1	1	-1	1	1	1
4	1.5	0	0	0	0	0	0
5	1	1	-1	1	1	-1	1
6	2	-1	1	1	-1	-1	1
7	2	1	1	-1	1	-1	-1
8	2	-1	1	-1	-1	1	-1
9	1	1	-1	-1	1	1	-1
10	1	-1	-1	-1	-1	-1	-1
11	1	1	1	-1	-1	-1	1
12	1.5	0	0	0	0	0	0
13	1.5	0	0	0	0	0	0
14	1.5	0	0	0	0	0	0
15	1.5	0	0	0	0	0	0
16	2	1	-1	1	-1	-1	-1
17	2	1	-1	-1	-1	1	1
18	1	1	1	1	-1	1	-1
19	1	-1	1	1	1	-1	-1
20	2	-1	-1	-1	1	-1	1
21	1	-1	-1	1	-1	1	1

II. Secondary Screening

Significant factors identified in the primary screening experiment were further investigated to narrow down the choices. A full factorial design was not done as this would have meant the assessment of 32 individual experiments; instead a resolution V design was done with 16 runs and five center points that gave a total of 21 runs. The five factors and their levels are listed in Table 6.3. Table 6.4 shows the randomised experimental design with five center points. Trace metals, sodium nitrate and sodium chloride were kept at low concentrations as indicated in Table 6.2. A resolution V design was selected with sixteen runs and five centre points. Running of five replicates at the central points was important to determine the curvature and to compensate the lack of fit values which indicate the significance of the model (Design Expert 8.0).

Table 6.3: Concentrations of variables of different levels in the second factorial experiment FF0521 indicating low, high and center points

Symbol	Factor	coded variable		
		-1	0	+1
X_1	$NaNO_3$	2	2.5	3
X_2	K_2HPO_4	0.06	0.09	0.12
X_3	$MgSO_4.7H_2O$	0.12	0.15	0.18
X_4	$CaCl_2.2H_2O$	0.06	0.045	0.08
X_5	Minor nutrients	15	17.5	20

Table 6.4: Experimental design for the secondary screening (FF0521) of five media components for PC production by *Cyanothece* sp. Variables are in coded levels randomized run orders are shown.

Run	Factors				
	X_1	X_2	X_3	X_4	X_5
1	1	1	-1	1	-1
2	0	0	0	0	0
3	0	0	0	0	0
4	-1	1	1	1	-1
5	-1	1	-1	1	1
6	0	0	0	0	0
7	-1	1	1	-1	1
8	-1	-1	1	-1	-1
9	0	0	0	0	0
10	0	0	0	0	0
11	-1	-1	-1	-1	1
12	1	1	1	1	1
13	-1	-1	1	1	1
14	1	-1	-1	1	1
15	1	-1	1	-1	1
16	1	1	-1	-1	1
17	1	-1	1	1	-1
18	1	-1	-1	-1	-1
19	1	1	1	-1	-1
20	-1	1	-1	-1	-1
21	-1	-1	-1	1	-1

6.1.1.2 Central Composite Design

Based on the results of the FFD0721, significant variables were subsequently employed to determine their effect on PC production. One widely used method in optimization is the RSM based on the CCD. It is a mathematical and statistical technique which allows one to evaluate the relationship between independent variables and to predict the response in an effective experimental design (Tasharrofia *et al.*, 2011). It is useful for the modelling and analysis of problems in which a response of interest is influenced by several significant variables and the objective is to optimise this response. CCD used to acquire data to fit a polynomial. For fitting a response surfaces consists of a full factorial design with center points, corner points of coded distance ±1, and axial points of a given distance. To achieve rotatability, which means that the variance has the same value on concentric spheres, adding stability to the variance, the axial points have to be of coded distance 1.414 from the center (Burrows *et al.*, 2008).

The central composite experiment was conducted to obtain a predicted maximum PC concentration. Three variables identified as significant for CCD0320 were minor nutrients, $MgSO_4$ and $CaCl_2$. Table 6.5 shows the actual and coded values for the low, center and high values, as well as two additional points at $\pm\alpha = 1.414$.This experiment was conducted with 20 flasks, with varying the three nutrient components. The experiment design is shown in Table 6.6.

Table 6.5: Central Composite design showing low, center and high values, two additional points at $\alpha\pm$ 1.141 were also included

Symbol	Factor	Coded Variable				
		-α	-1	0	+1	+α
X_1	Minor nutrients	10.30	12	14.50	17	18.70
X_2	$MgSO_4.7H_2O$	0.10	0.12	0.15	0.18	0.20
X_3	$CaCl_2.2H_2O$	0.02	0.03	0.05	0.07	0.08

Table 6.6: Experimental design of a three factor central composite (CC0320) experiment showing coded factor levels with randomized run order to determine effect of the variables on PC production

Run Order	X_1	X_2	X_3
1	-1	-1	1
2	1.414	0	0
3	-1	1	1
4	0	0	0
5	1	-1	1
6	-1	-1	-1
7	0	0	0
8	0	0	0
9	1	-1	-1
10	0	-1.414	0
11	0	1.414	0
12	0	0	0
13	0	0	0
14	0	0	1.414
15	1	1	1
16	0	0	0
17	1	1	-1
18	-1	1	-1
19	0	0	-1.414
20	-1.414	0	0

6.1.1.3 Confirmatory Experiment

A point-prediction was made to maximize the amount of PC production while optimizing the three factors tested. The result of the point-prediction was run as a confirmatory experiment. After conducting the two fractional factorial and central composite designs, the media components and levels outlined in Table 6.7 were determined as optimal for the maximum PC production. These were run in triplicate in 100 mL volumes in 250 mL Erlenmeyer flasks. A 10% v/v *Cyanothece* sp. inoculum in an exponential growth phase was used to inoculate the flasks, as described previously.

Table 6.7: Concentration of individual media components used in the confirmatory experiment. All components are in g/L except for minor nutrients and trace metal which are in ml/L

Component	Concentration (g/L)
MgSO4	0.12
CaCl2	0.035
Minor nutrients	13.70
$NaNO_3$	2
K_2HPO_4	0.06
NaCl	100
Trace metals	0.5

6.1.1.4 Statistical Analysis of Experiments

Statistical analysis of the data was performed by design package Design Expert 8.0.7.1 to evaluate the analysis of variance (ANOVA), to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case.

6.1.1.4.1 Validation of the model

After performing the experiment according to the design and recording the results, the data set generated was analysed. Response ratio of the maximum to minimum response is analysed to determine if a transformation was needed. Larger ratios (greater than 10) indicate a higher chance that a transformation is needed, for ratios less than 3, power ratios have no effect.

6.1.1.4.2 Model fit

Thereafter the Design Expert software computes the 'sequential or extra sum of squares'. The highest order polynomial where additional terms are significant is selected, whereas aliased models are not selected. The ideal model for a CCD is a Quadractic model. The suggested model is based on: (i) adding higher order terms to the model add further explanation; i.e. their sequential SS is significant, (ii) the model fit the data better, and (iii) the model has the highest adjusted and predicted R-squares.

The predicted residual sum of squares (PRESS) statistic indicates how well the model fits the data. The PRESS for the chosen model should be small relative to the other models under consideration. The standard deviation of the error in the design should be small.

The goodness of fit of the model is checked by the determination coefficient (R^2) The R-squared value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of one represents the ideal case at which 100% of the variation in the observed value can be explained by the model. Predicted values are the response predicted by the model. This is determined by substituting the coded factor levels into the coded model. The residual is the difference when actual and predicted response are compared

6.1.1.4.3 ANOVA

ANOVA is a statistical method based on the F-test that assesses the significance of the experimental results. It is used to evaluate the adequacy of the fitted model. The *p*-values were used as a tool to check the significance of each of the coefficients. This is essential to understand the pattern of the mutual interactions between the factors (Somnath & Smita, 2010). The F-test with a low *p*-value for response indicates a high significance for the corresponding coefficient and/or regression model. P values less than 0.05 indicate that the model terms are significant, whereas values greater than 0.1 are not significant (Boddy & Smith, 2009).

6.1.1.4.4 Diagnostics

Half normal plot, the normal distribution folded over to right of the zero value by taking the absolute value of the data based on the positive half of the full normal curve. Absolute values are a more sensitive scale for detection of significant outcomes. The half normal plot of allows one to easily see what is significant; however, results must be verified by doing ANOVA

Curvature is the measure of the offset at the centre point of actual versus predicted values from the model. If this is significant a quadratic model may be fitted to the data from a response surface design.

6.1.1.4.5 Graphs

For CCD, the fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. The optimal concentrations of the critical variables were obtained by analyzing 3D plots.

A Pareto Chart ranked the effects of the factors according to the greatest effect on the final response. Positive effects are represented by orange bars and negative effects by blue bars. There were two different t limits plotted on the graph, based on the Bonferroni corrected *t* and a standard *t*. When effects are selected, the effects that are above the Bonferroni Limit are definitely significant. The effects that were above the *t*-value Limit are possibly significant and should be added if they are not already selected. The effects that are below the *t*-value limit are not likely to be significant.

Cube plots are useful for representing the effects of three factors at a time. They show the predicted values from the coded model for the combinations of the low (–1) and high (+1) levels of any three factors that you select. The contour and 3D plots are a two- dimensional representation of the response for selected factors. They are used to help predict the areas of highest PC production in screening and optimization experiments.

6.2 Results

6.2.1 DOE Primary Screening

The maximum PC produced (2795.802 µg/mL) was in run 14 (Table 6.8).The predicted values were calculated using the mathematical model equation; the residue is the difference between the actual and the predicted value.

Table 6.8: Experimental design for FF0721 with the coded values of the variables tested showing actual, predicted and residual values.

Std	Run	Factors							Actual	Predicted	Residual
		X_1	X_2	X_3	X_4	X_5	X_6	X_7			
1	6	-1	-1	-1	-1	-1	-1	-1	671.133	729.634	-58.501
2	16	1	-1	-1	-1	1	-1	1	1030.886	972.385	58.50102
3	11	-1	1	-1	-1	1	1	-1	1448.363	1389.862	58.50102
4	17	1	1	-1	-1	-1	1	1	1088.436	1146.937	-58.501
5	9	1-	-1	1	-1	1	1	1	1976.995	1967.71	9.284214
6	14	1	-1	1	-1	-1	1	-1	2795.802	2805.086	-9.28421
7	7	-1	1	1	-1	-1	-1	1	952.336	961.62	-9.28421
8	12	1	1	1	-1	1	-1	-1	2293.957	2284.672	9.284214
9	19	-1	-1	-1	1	-1	1	1	1503.12	1440.823	62.30183
10	5	1	-1	-1	1	1	1	-1	1592.47	1654.776	-62.3018
11	-1	-1	1	-1	1	1	-1	1	1875.35	1937.651	-62.3018
12	18	1	1	-1	1	-1	-1	-1	1728.23	1665.929	62.30183
13	13	-1	-1	1	1	1	-1	-1	1474.371	1479.855	-5.4834
14	8	1	-1	1	1	-1	-1	1	1178.238	1172.755	5.483401
15	2	-1	1	1	1	-1	1	-1	1625.886	1620.403	5.483401
16	10	1	1	1	1	1	1	1	1793.496	1798.979	-5.4834
17	20	0	0	0	0	0	0	0	2041.21	1961.887	79.32275
18	3	0	0	0	0	0	0	0	2023.656	1961.887	61.76858
19	21	0	0	0	0	0	0	0	1963.65	1961.887	1.76316
20	1	0	0	0	0	0	0	0	2049.51	1961.887	87.62275
21	4	0	0	0	0	0	0	0	1731.41	1961.887	-230.477

The half-normal probability plot (Figure 6.1) used the ordered estimate to select which factors are important and unimportant. The remaining unselected factors were subjected to a Shapiro-Wilk test to determine whether they are normally distributed. The value of the Shapiro-Wilk test was 0.797, and the w-value was 0.989. All effects that lie along the line is negligible, whereas the large effects were far from the probability line thus were selected to be included in the mathematical model.

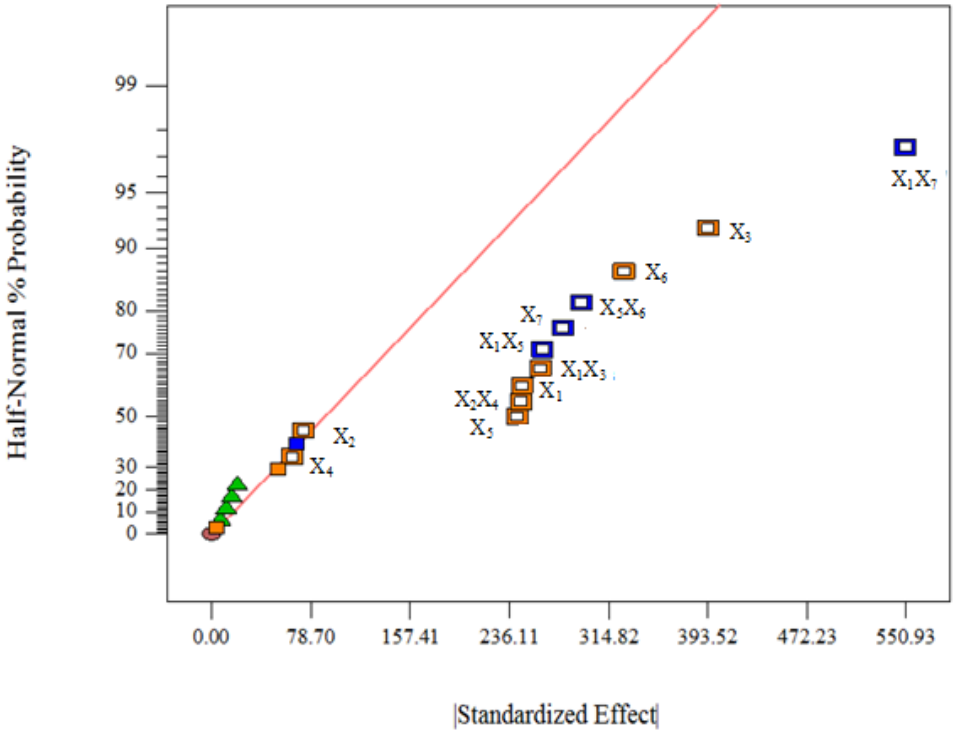

Figure 6.1: The Half-normal probability plot for PC produced by *Cyanothece* sp. Blue squares are negative and orange squares positive effects. The green triangle points are pure errors.

The Box Cox plot helps determine the power of transformation required. The blue line indicated the current transformation; the green line showed the best model and the red lines represent the confidence intervals. A lambda value of 1 indicated that no transformation was required. This is indicated in the plot (Figure 6.2), where the blue line fell between the two red lines (95% confidence intervals).

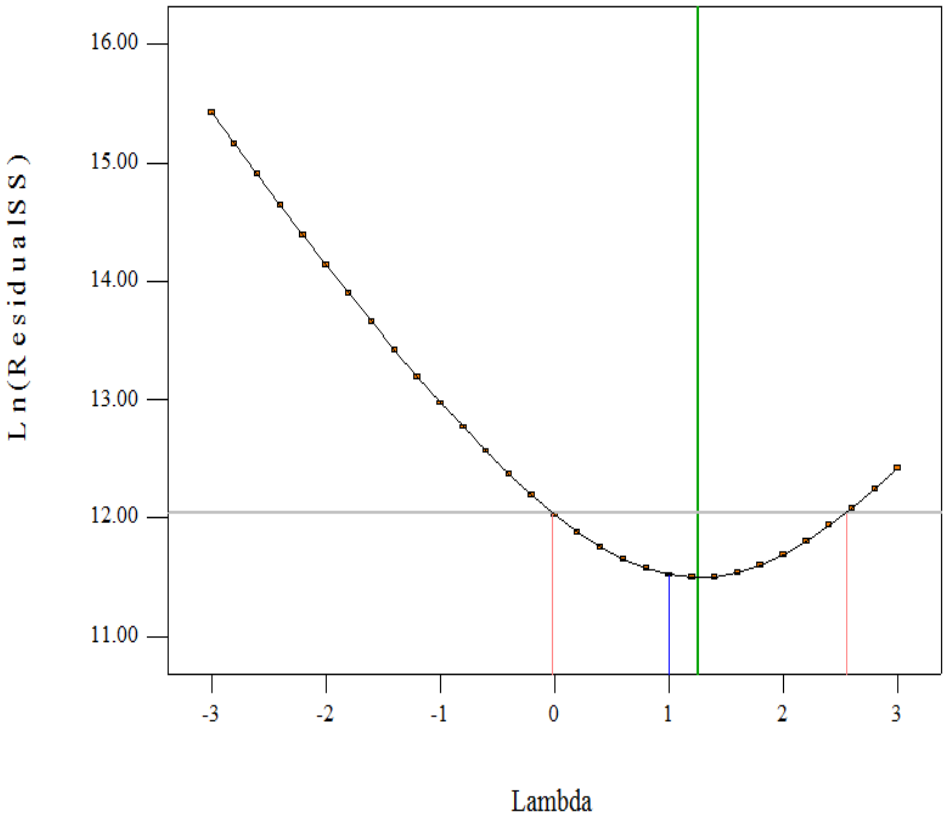

Figure 6.2: Box-Cox for power transformation for FF0721, indicating the position of lambda.

The magnitudes of the effects of individual nutrient components as well as the interaction between the components are shown in a Figure 6.3. Factors that were above the t-limit are significant and those above the Bonferroni limit are most certainly significant and had to be included. The effects are ranked according to the greatest effect, on the production of PC. The largest positive responses were individual factors X_3 ($MgSO_4$) and X_6 (minor nutrients). Individual factors X_1 (NaNO3) and X_3 ($MgSO_4$) were positive, but did not fall above the t-limit. However, the interaction between $NaNO_3$ and $MgSO_4$ was found to be on the bonferroni limit, thus indicating the interaction was definitely significant. The other interaction to be considered were X_2X_4 (K_2HPO_4 and $CaCl_2$) which resulted in a positive effect on total PC yield. The interaction between X_1 ($NaNO_3$) and X_7 (trace metal) resulted in significantly negative effects on PC production. The other negative interaction that effect PC production was the combination of X_5 ($NaCl_2$) and X_6 (minor nutrients). Considering the above, factors X_2, X_3, X_4 and X_6 were then identified for further screening in a second experiment.

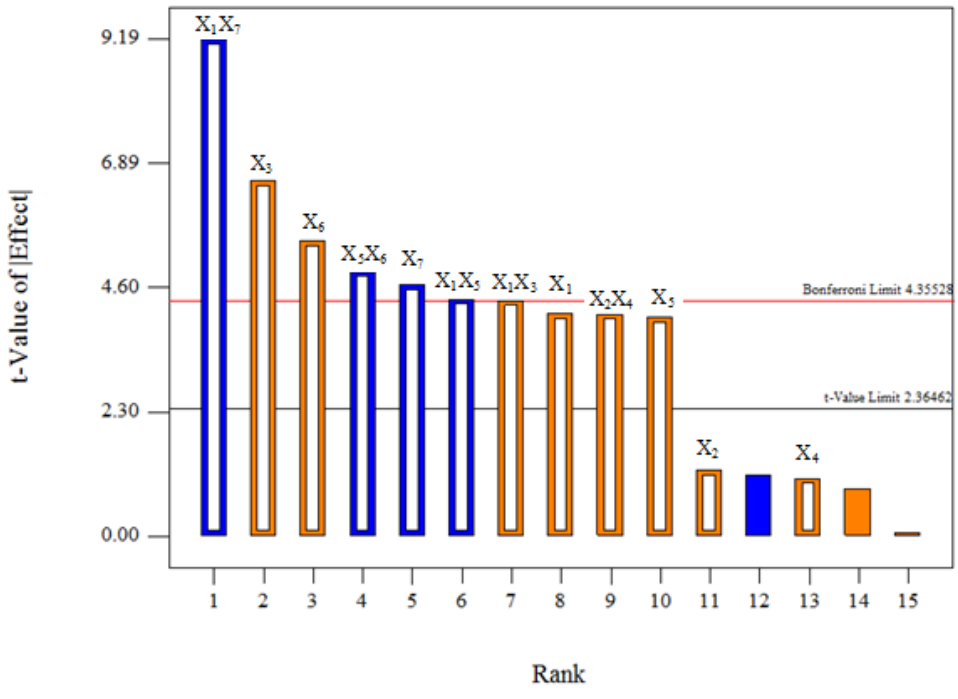

Figure 6.3: Pareto chart showing the most significant effects factors of the 7 factor factorial design on PC production. The Bonferroni limit is 4.35 and *t*-value limit is 2.3.The orange bars indicate the positive effect and the blue bar shows the negative effects. The selected effects are shown by the clear bars.

Examining the ANOVA (Table 6.9) it was observed that the main effects of X_4, X_6 and the combination X_1 X_7, were the only terms with p-values less than 0.05 indicating that they were significant at the probability level of 95%. The Model F-value of 4.01 implied the model is significant. There is only a 2.86% chance that a "Model F-Value" this large could occur due to noise. The lack of fit (p<0.05) is significant indicating that the variation in the mean of the replicates are less than that of the design points. This meant the runs replicated well and their variances were negligible, or the model does not predict their influence on PC production well or a combination of both. The "Curvature F-value" of 6.02E+05 implies that there is significant curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space. There is only a 0.01% chance that a "Curvature F-value" this large could occur due to noise. Curvature implies that a point of optimisation can be reached based on the model.

Table 6.9: Analysis of variance for the seven factor factorial model

Source	Sum of squares	Df	Mean Square	F value	p-value Prob>F	Significance
Model	4.23E+06	12	3.52E+05	3.924	0.0305	Significant
X_1	2.44E+05	1	2.44E+05	2.712	0.1382	
X_2	2.12E+04	1	2.12E+04	0.237	0.6397	
X_3	6.21E+05	1	6.21E+05	6.920	0.0302	Significant
X_4	1.65E+04	1	1.65E+04	0.183	0.6798	
X_5	2.36E+05	1	2.36E+05	2.627	0.1437	
X_6	4.29E+05	1	4.29E+05	4.778	0.0503	Significant
X_7	3.11E+05	1	3.11E+05	3.465	0.0997	
X_1X_3	2.73E+05	1	2.73E+05	3.040	0.1194	
X_1X_4	3.44E+05	1	3.44E+05	3.833	0.0860	
X_1X_5	2.76E+05	1	2.76E+05	3.077	0.1175	
X_1X_7	1.21E+06	1	1.21E+06	13.521	0.0062	Significant
X_2X_4	2.42E+05	1	2.42E+05	2.692	0.1395	
Residual	7.18E+05	8	8.98E+04			
Lack of Fit	6.44E+05	4	1.61E+05	8.660	0.0299	Significant
Pure Error	7.44E+04	4	1.86E+04			
	4.95E+06	20				

From the positive significant factors found (X_1, X_3, X_6 and X_7), there were two pairs of positive interactions X_1X_3 (Figure 6.4) as well as X_2X_4. An increase in $MgSO_4$, while keeping $NaNO_3$ at a concentration of 2g/L resulted in a high PC content of 2110.99 µg/mL. When 1g/L of $NaNO_3$ is used neither and increase nor a decrease in $MgSO_4$ had an effect.

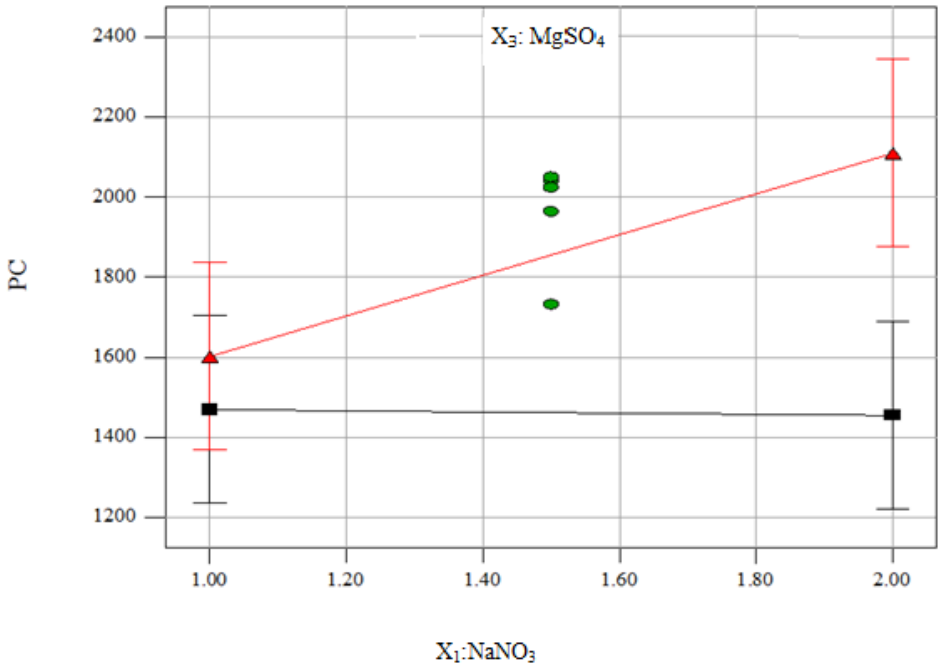

Figure 6.4: Interactions of the two factors, magnesium sulphate and sodium nitrate showing an increase in PC production.

In order to approach the proximity of the optimum values, a model which included the linear and interaction terms (Eq 6.1), was fitted to the data obtained from the fractional factorial design experiments. The values of the regression coefficients were calculated and the following equation was derived using the coefficients of the coded factors:

PC (µg/mL) = +1659.93+123.37 * X1+36.44* X2+197.07 * X3+32.08* X4+121.42* X5+163.75 * X6-139.46 * X7+130.62* X1 * X3-131.41* X1 * X5-275.47* X1 * X7+122.90* X2 * X4 146.66 * X5 * X6 **(Eq 6.1)**

6.2.2 Secondary Screening Experiment

The second screening to optimize media components for PC production consisted of five factors with five center points. The five factors were as follows: X_1: NaNO3 (low-2 g/L; high-3 g/L), X_2: K_2HPO_4 (low-0.06 g/L, high-0.12 g/L), X_3: MgSO4.7H2O, (low-0.18 g/L; high-0.12 g/L), X_4: $CaCl_2$.2H2O (low 0.06 g/L; high 0.08 g/L) and X_5: minor nutrients (low-15 ml/L; high-20 ml/L). Table 6.10 shows the low and high values of the component, as well as the predicted and actual values after the experiment was conducted.

Table 6.10: Secondary screening experiment with five center points together with the actual, predicted and residual PC yields after 16 days

Std	Run	Factors					Actual	Predicted	Residual
		X_1	X_2	X_3	X_4	X_5			
1	11	-1	-1	-1	-1	1	90.44173	200.7841	-110.342
2	18	1	-1	-1	-1	-1	104.5191	96.80569	7.713415
3	20	-1	1	-1	-1	-1	1955.215	1939.265	15.95041
4	16	1	1	-1	-1	1	72.67751	264.835	-192.158
5	8	-1	-1	1	-1	-1	1331.756	1384.496	-52.7395
6	15	1	-1	1	-1	1	358.5289	203.1604	155.3684
7	7	-1	1	1	-1	1	1000.709	853.5774	147.1314
8	19	1	1	1	-1	-1	1271.769	1242.693	29.07568
9	21	-1	-1	-1	1	-1	886.6999	884.0695	2.630352
10	14	1	-1	-1	1	1	1380.788	1280.79	99.99858
11	5	-1	1	-1	1	1	668.9759	487.1621	181.8138
12	1	1	1	-1	1	-1	82.71897	88.32561	-5.60664
13	13	-1	-1	1	1	1	2028.404	2173.428	-145.025
14	17	1	-1	1	1	-1	1323.893	1281.497	42.39573
15	4	-1	1	1	1	-1	242.597	282.0165	-39.4194
16	12	1	1	1	1	1	48.85474	185.6424	-136.788
17	0	0	0	0	0	0	1067.31	1070.272	-2.96184
18	0	0	0	0	0	0	1077.428	1070.272	7.156618
19	0	0	0	0	0	0	1047.728	1070.272	-22.5439
20	0	0	0	0	0	0	1156.451	1070.272	86.17938
21	0	0	0	0	0	0	1002.442	1070.272	-67.8302

The half-normal plot (Figure 6.5) shows the selection of all factors that were included in a mathematical model for the production of PC. The only positive effects were X_3 ($MgSO_4$) and the interaction between X_4 ($CaCl_2$) and X_5 (minor nutrients). In the previous experiment FF0721, X_2 (KH_2PO_4), resulted in a minor positive effect and strong positive interaction with X_4 (CaCl2); however, in FF0521, a negative interaction was present. An increase in both factors X_2 and X_4 caused an overall negative effect on the PC production.

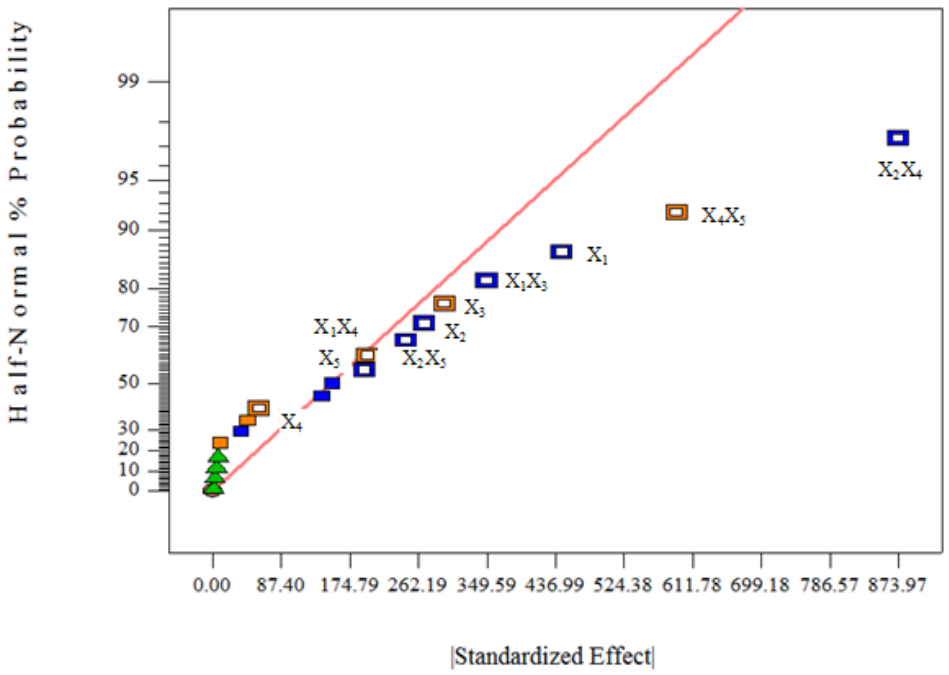

Figure 6.5: Half-normal probability plot to indicate which of the factors were selected to be included in the model. The orange squares indicate the positive effect and the blue squares shows the negative effects. The selected effects are shown by the clear centred squares.

In the FF0721, X_1 ($NaNO_3$) had a positive effect but an increase in FF0521 resulted in a significant negative effect, as well as a negative interaction between X_1 and X_3 ($MgSO_4$) (Figure 6.6). It is clear from the results that factors X_1 and X_2 were in excess. Factors above the Bonferroni limit were significant and those that fell above the t-value were possibly significant in the production of PC and were taken into consideration when choosing factors. Although X_4 fell below both the Bonferroni limit and the *t*-value, it was involved in a major positive interaction with X_5.

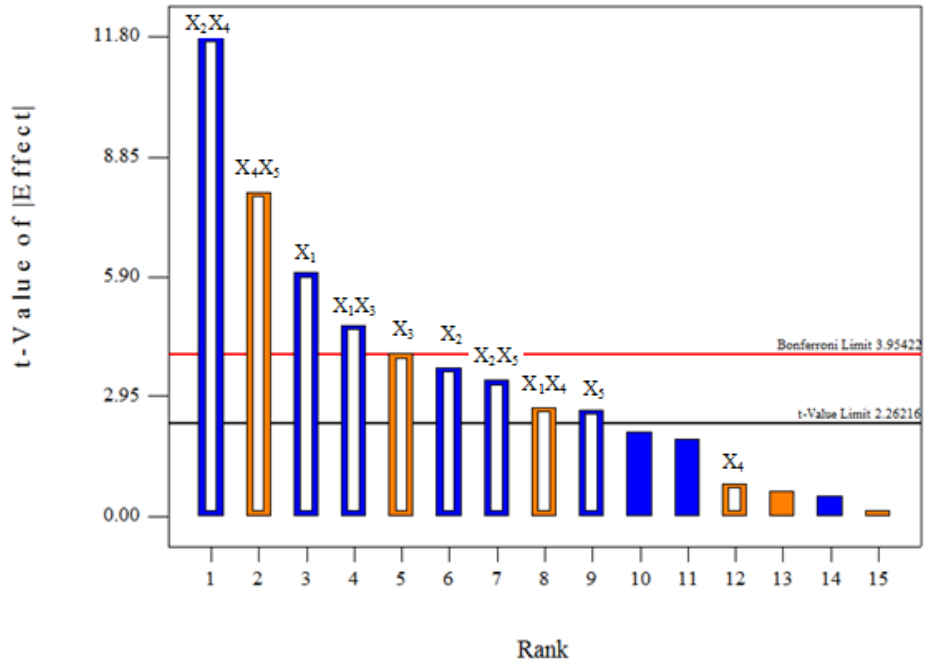

Figure 6.6: Pareto chart showing the most significant effects factors of the 5 factor factorial design on PC production. The Bonferroni limit was 3.95 and *t*-value limit is 2.26.The orange bars indicated the positive effect and the blue bars showed the negative effect. The selected effects are shown by the clear bars.

The interaction between X_4 ($CaCl_2$), and X_5 (minor nutrients) resulted in a strong positive effect. However, an increase in $CaCl_2$ while minor nutrients were kept at a low (15mL), results in a decrease from 1299 to 697 ug/mL of PC (Figure 6.7). Also the increase in minor nutrients from 15 mL to 20 mL results in a decrease in PC production.

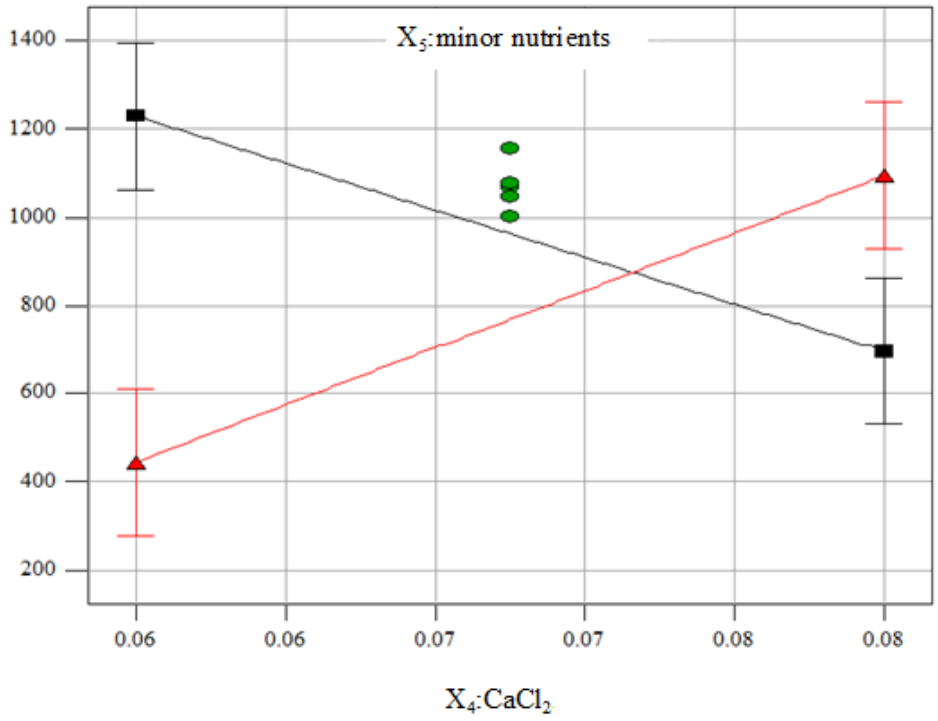

Figure 6.7: Positive interactions between calcium chorine and minor nutrient stock

All factors, except X_4 were significant (p<0.05) and the model itself was found to significantly fit the data (p<0.0001). Although $CaCl_2$ was not significant, it contributed to overall PC production as a strong positive interaction with minor nutrients. Therefore it was considered in further decisions. The lack of fit was significant indicating that not all the data points fitted the design space, this means the experimental runs replicate well and their variances are negligible, or the model does not predict well or a combination of both. There was also significant curvature in this mode (Table 6.11).

Table 6.11: Analysis of variance for five factor factorial model indicated the significance of one- and two-factor interactions applied to the model. Also shown, are curvature and lack of fit results for the applied model

Source	Sum of Squares	df	Mean square	F- value	p-value Prob>F	Significance
Model	6940883	10	694088	31.6114	0.0001	Significant
X_1	792567	1	792567	36.0964	0.0002	Significant
X_2	292009	1	292009	13.2992	0.0053	Significant
X_3	349421	1	349421	15.9139	0.0032	Significant
X_4	14239.4	1	14239.4	0.64851	0.4414	
X_5	150115	1	150115	6.83681	0.0281	Significant
X_1X_4	156066	1	156066	7.10781	0.0258	Significant
X_2X_3	488528	1	488528	22.2494	0.0011	Significant
X_2X_4	3055297	1	3055297	139.15	< 0.0001	Significant
X_2X_5	243142	1	243142	11.0736	0.0088	Significant
X_4X_5	1399499	1	1399499	63.7384	< 0.0001	Significant
Curvature	272061	1	272061	12.3907	0.0065	Significant
Residual	197612	9	21956.9			
Lack of Fit	185016	5	37003.3	11.7508	0.0167	Significant
Pure Error	12596	4	3149.01			
Cor Total	7410556	20				

$$PC\ (\mu g/mL) = +866.66 - 222.57 * X_1 - 135.09 * X_2 + 147.78 * X_3 + 29.83 * X_4 - 96.86 * X_5 + 98.76 * X_1 * X_4 - 174.74 * X_2 * X_3 - 436.99 * X_2 * X_4 - 123.27 * X_2 * X_5 + 295.75 * X_4 * X_5 \quad \textbf{(Eq 6.2)}$$

6.2.3 Central Composite Design

Response Surface Methodology helps in evaluation of relationship between dependent (PC production) variable and independent variable (media components). The experiments consisted of twenty one experiments and two levels of concentration for each factor. In order to study the combined effect of these variables, experiments were performed using different parameter combinations. Table 6.12 summarizes the central composite experimental design along with the predicted and actual response for each individual experiment. It shows the production of PC (µg/mL) corresponding to the combined effect of three components in the specified ranges. The optimum levels of the selected variables were obtained by solving the regression equation and by analysing the response surface contour and surface plots (Singh *et al.*, 2009). The regression equation obtained after the analysis of variance provides an estimate of the level of the PC concentration.

Table 6.12: Central composite experimental design showing the actual and predicted PC concentrations

Std	Run	Factors			Actual	Predicted	Residual
		X_1	X_2	X_3			
1	6	-1	-1	-1	4685.98	4755.29	-69.31
2	9	1	-1	-1	4082.16	3964.12	118.036
3	18	-1	1	-1	4245.26	4058.21	187.048
4	17	1	1	-1	3092.76	3140.83	-48.066
5	1	-1	-1	1	4002.13	3947.69	54.4394
6	5	1	-1	1	3202.63	3383.3	-180.67
7	3	-1	1	1	3005.39	3117.05	-111.66
8	15	1	1	1	2502.13	2426.45	75.6828
9	2	+α	0	0	4503.18	4536.09	-32.91
10	20	-α	0	0	3313.97	3290.07	23.8968
11	10	0	-α	0	4482.06	4432.9	49.1595
12	11	0	+α	0	2983.94	3042.11	-58.172
13	19	0	0	-α	3806.34	3914.88	-108.54
14	14	0	0	+α	2734.58	2635.05	99.5265
15	12	0	0	0	4320.34	4346.32	-25.977
16	16	0	0	0	4344.04	4346.32	-2.2773
17	4	0	0	0	4363.17	4346.32	16.8527
18	8	0	0	0	4346.24	4346.32	-0.0773
19	7	0	0	0	4353.12	4346.32	6.80273
20	13	0	0	0	4352.54	4346.32	6.22273

Table 6.13 provides the ANOVA for PC production for the CCD0320. The model was significant with F value of 70.63. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The coefficient estimates and their corresponding P values suggest that all the variables are significant. Values of "Prob > F" less than 0.05 indicate model terms are significant. The data had a statistically significant lack of fit; this is due to the six center points on the graph which were close, therefore, they overlap and some are hidden below the response. The fit of the models were controlled by the coefficient of determination R^2. Based on the ANOVA results, the models report high R^2 value of 0.9845. Moreover, an acceptable agreement with the adjusted determination coefficient is necessary. In this study, the Adj-R^2 value of 0.9706 was found. The values of R^2 and Adj-R^2 are close to 1.0, which indicates a high correlation between the experimental values and the predicted values.

Table 6.13: Analysis of variance of the quadratic model for CC0320,

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	8901031	9	989003.5	70.62931	< 0.0001	Significant
X_1	1874102	1	1874102	133.8383	< 0.0001	Significant
X_2	2334896	1	2334896	166.7457	< 0.0001	Significant
X_3	1977186	1	1977186	141.2	< 0.0001	Significant
X_1X_2	7965.744	1	7965.744	0.568871	0.4681	
X_1X_3	25714.58	1	25714.58	1.836397	0.2052	
X_2X_3	8919.137	1	8919.137	0.636957	0.4434	
X_1^2	338112.8	1	338112.8	24.1462	0.0006	Significant
X_2^2	667694.2	1	667694.2	47.68313	< 0.0001	Significant
X_3^2	2067646	1	2067646	147.6602	< 0.0001	Significant
Residual	140027.3	10	14002.73			
Lack of Fit	138978.7	5	27795.74	132.5341	< 0.0001	Significant
Pure Error	1048.626	5	209.7252			
Cor Total	9041058	19				

According to small *p*-value for the quadractic terms, a second order model for the PC response was fitted. Based on Eq 6.3 and confirmed by contour and 3D plot, all three variables negatively influenced the PC production indicating that lower concentrations of these components in the medium would result in higher production of PC.

The production of PC may be best predicted by the following quadratic equation in terms of coded factors

PC (µg/mL) = 4346.317271-370.4429116 * X1-413.4837622* X2-380.494526 * X3-31.555* X1 * X2 56.695* X1 * X3-33.39* X2 * X3-153.1719882* X12-215.2471247* B2-378.77971* C2 **(Eq 6.3)**

Response surface and contour plot figures obtained by the analysis of the experimental data of CCD showed a relationship between the two variables at a time while maintaining third variable at a fixed level. Figures 6.8a and b indicated a two and three-dimensional contour plot respectively of the interaction between $MgSO_4$ and $CaCl_2$. The contours converging on a point i.e the red region (Figure 6.8a) could be predicted using equation 5. A typical rising ridge to indicate that the factors can be optimized numerically (Figure 6.8b). The response surface and contour plots showing the interaction for $MgSO_4$ and minor nutrients, and $CaCl_2$ and minor nutrients are shown in Figures 6.9 and 6.10 respectively.

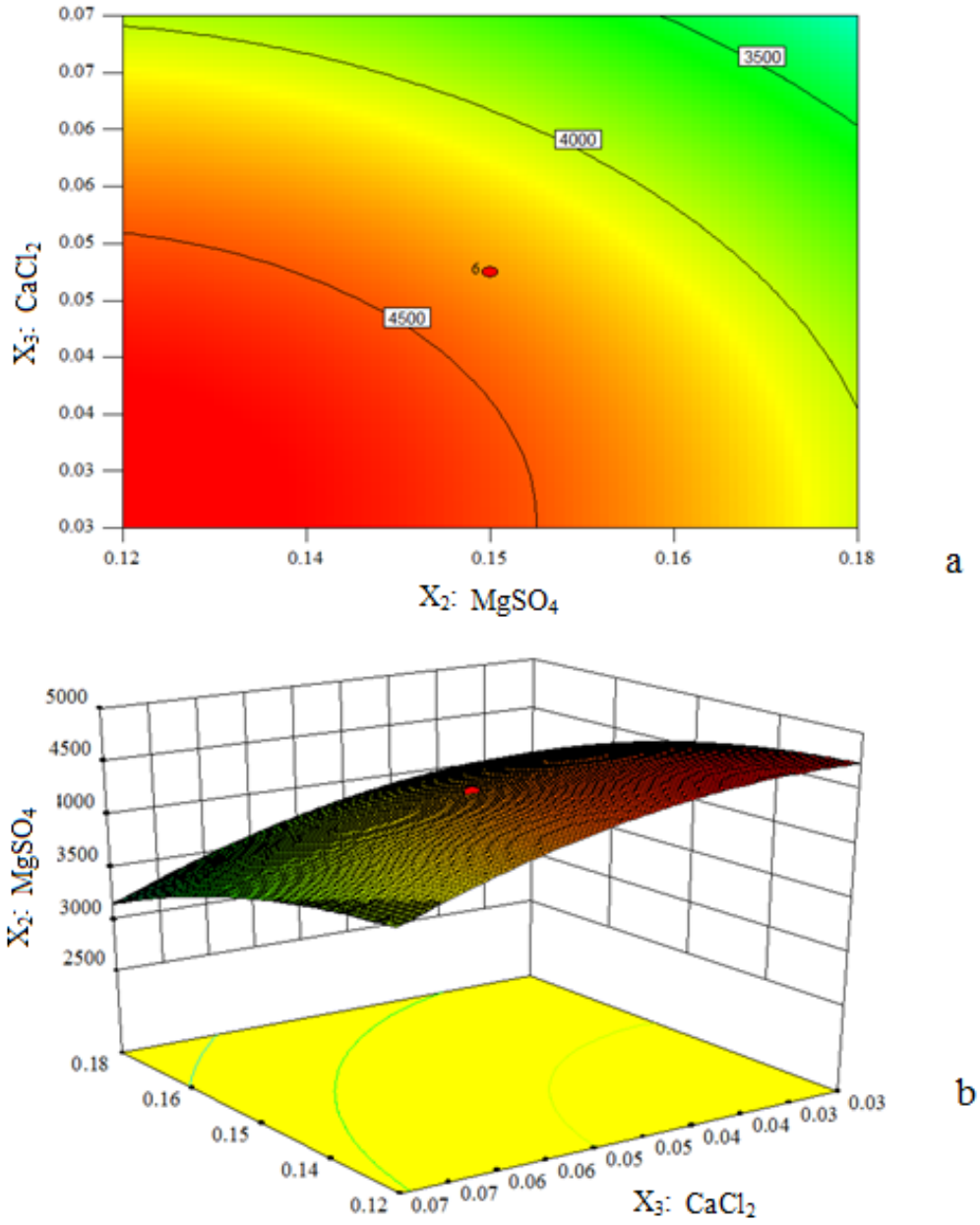

Figure 6.8: Response surface plots (a) contour plot and three-dimensional plot (b) of PC production by *Cyanothece* sp. showing the effects of two factors, $MgSO_4$ and $CaCl_2$.

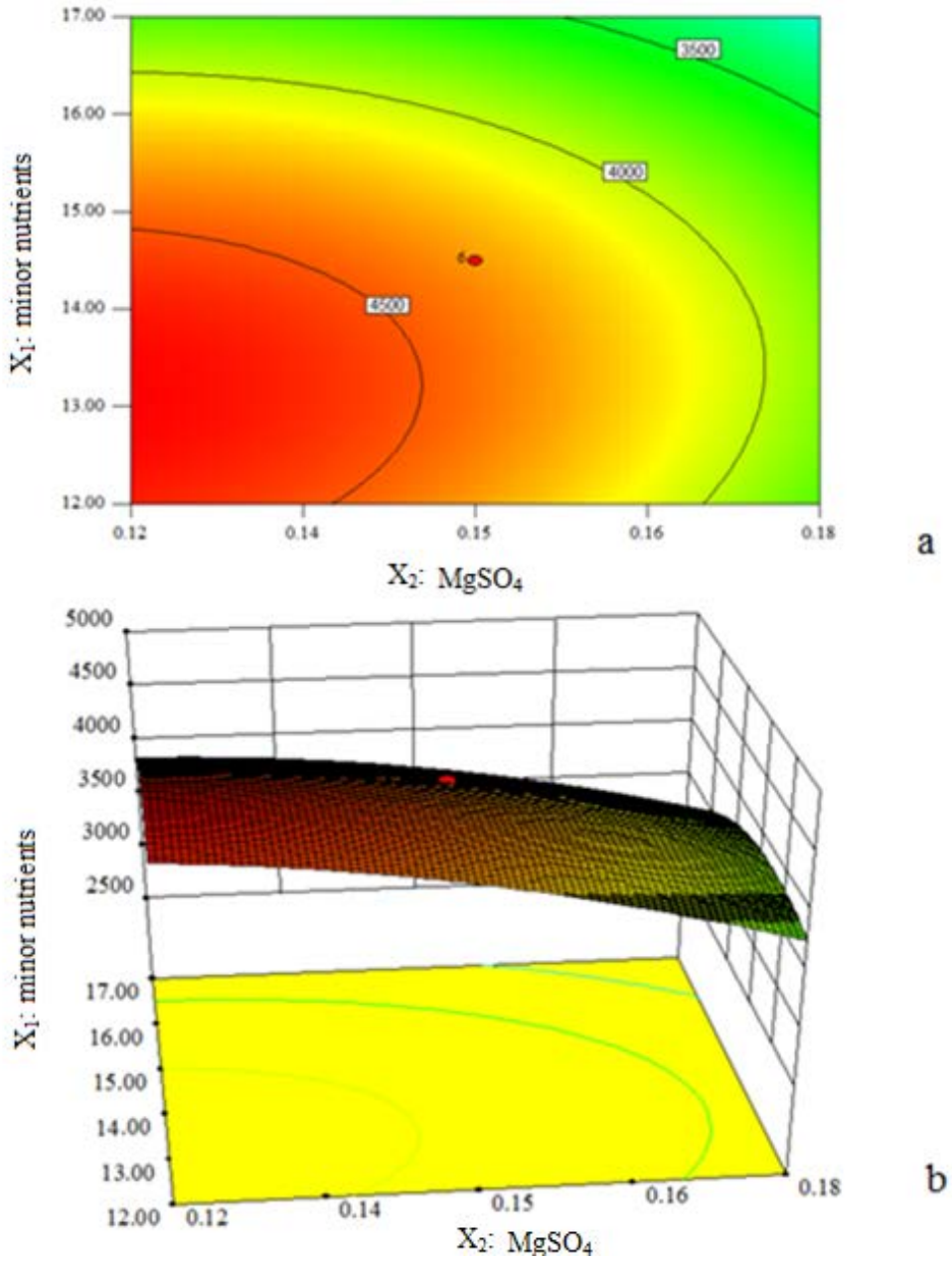

Figure 6.9: Contour plot (a) and three-dimensional plot (b) of PC production by *Cyanothece* sp. showing the effects of two factors, $MgSO_4$ and minor nutrients.

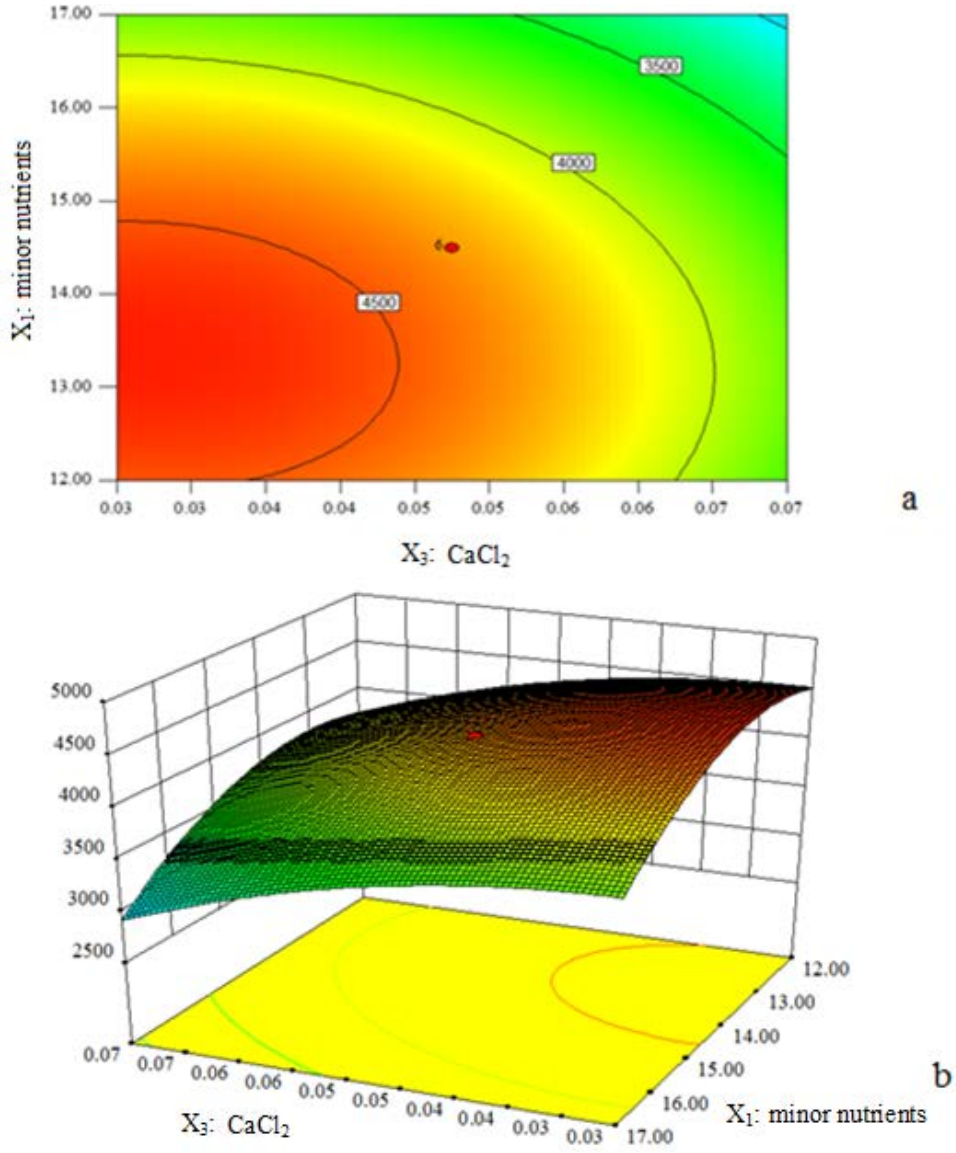

Figure 6.10:Contour plot (a) and three-dimensional plot (b) of PC production by *Cyanothece* sp. showing the effects of two factors, $CaCl_2$ and minor nutrients.

The three factors represented in the cube plot had a significant impact on the production of PC. A high concentration of 4775.29 ug/mL of PC (Figure 6.11), was predicted when these components are used viz, X_1: $MgSO_3$ at a low 12.0 g/L, X_2: $CaCl_2$ at a low (0.03 g/L) and X_3:(Minor nutrients) at a low (12 ml/L)

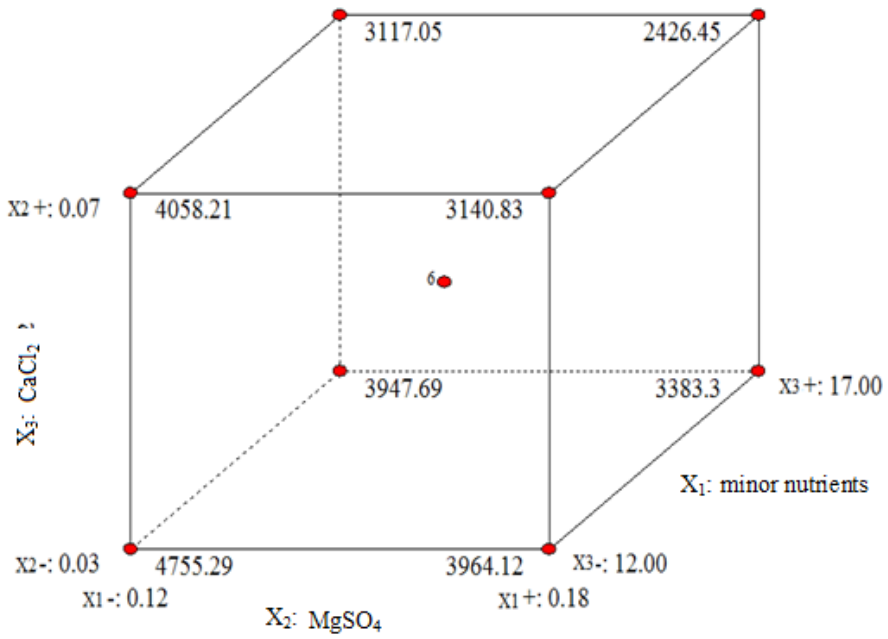

Figure 6.11: Cube plot showing the interactions between X_1: minor nutrients, X_2:$MgSO_4$ and X_3:$CaCl_2$

Point prediction was done using Design Expert 8, a total of 39 solutions were generated, and the best five are shown in Table 28. $MgSO_4$, $CaCl_2$ and minor nutrients were set in range and the goal was to optimize PC. To obtain the maximum optimum activity, the factor levels and response were set at the desired goal desirability level equal to one. Of the solutions, the suggested solution was 0.12g/L $MgSO_4$, 0.035 g/L $CaCl_2$ and 13.7 ml/L minor nutrients. It has a relative low standard error, which can result in of 4814.08 ug/mL for the PC. The interaction between MgSO4 and CaCl2 showing the peak PC production is shown in Figure 6.14. This represented an overall increase from the start of experimentation. The result correlates well with the predicted value and the model was proven to be adequate.

Table 6.14: Optimized solutions from the model generated for the two remaining factors, to maximize PC production

Solution	$MgSO_4$	$CaCl_2$	Minor nutrients	PC Prediction	SEM	95% CI Low	95% CI High	
1	0.12	0.035	13.70	4814.08	59.88	4680.66	4947.50	Suggested
2	0.12	0.033	12.91	4831.06	69.19	4676.90	4985.22	
3	0.12	0.032	13.49	4819.94	64.14	4677.02	4962.86	
4	0.12	0.038	12.90	4819.35	63.61	4677.61	4961.10	
5	0.13	0.035	13.80	4786.84	54.60	4665.18	4908.50	

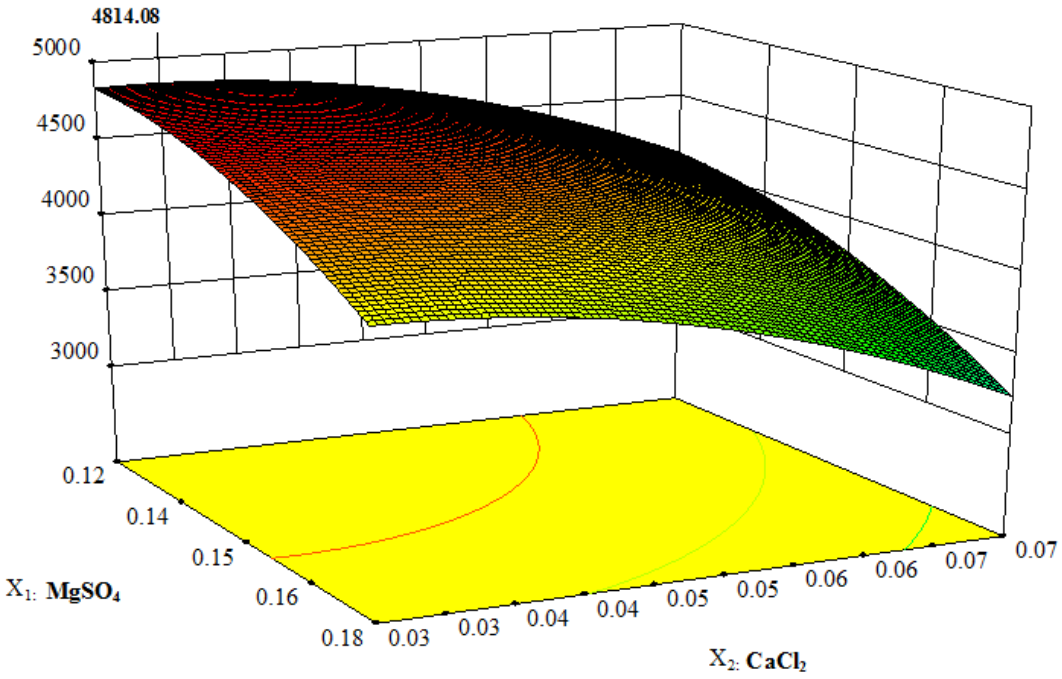

Figure 6.12: Interaction between $MgSO_4$ and $CaCl_2$ to show a peak PC production.

In order to validate the experimental model, a verification experiment was performed using the statistically optimized medium. The three replicate experiments yielded an average maximum concentration of 4877.56 µg/mL, which was 79% higher than the PC yield from the original BG11 medium (2730 µg/mL), respectively (Table 6.15). The good agreement between the predicted and the experimental results verified the validity of the model, and the improvement of PC production.

Table 6.15: Predicted and actual PC yield extracted from *Cyanothece* sp. Concentration of actual PC is an average of three replicates,

Media Components			PC Production (µg/mL)	
$MgSO_4$	$CaCl_2$	Minor Nutrients	Predicted	Actual
0.12	0.035	13.70	4814.08	4887.56

A significant increase in PC production by *Cyanothece* sp. was observed when comparing the yield of PC produced when the cyanobacterium was cultivated in the original BG11 medium to the modified medium (Figure 6.13).

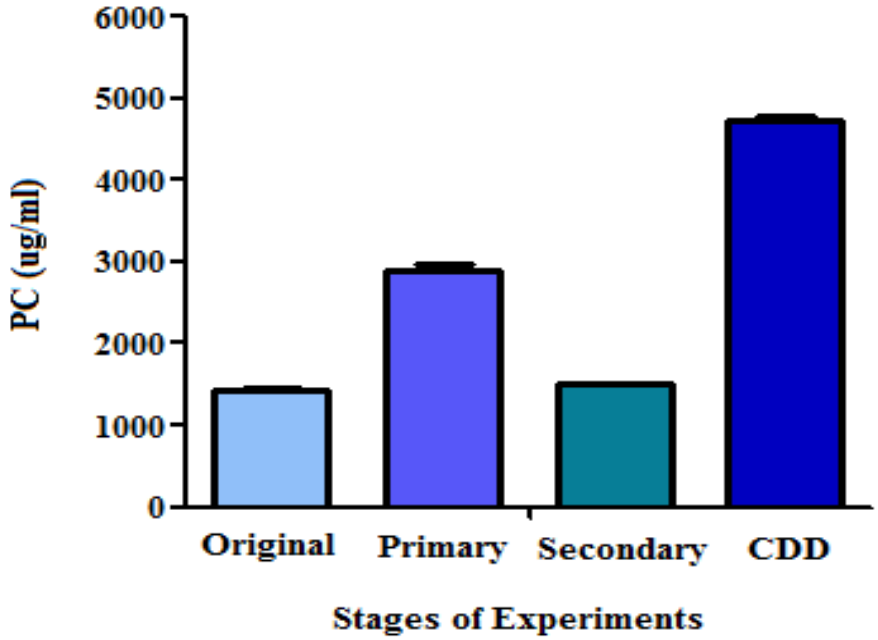

Figure 6.13: Comparison of PC production by *Cyanothece* sp., (a) in the original media, (b) primary screening, (c) secondary screening and (d) central composite

6.3 Discussion

6.3.1 Design of experiments

The first requirement for any process development is to look for a high yielding strain, thereafter, identify the variables which influence the yield and to optimize conditions for a higher yield. Since every cyanobacteria strain has its own nutrient requirements, there isn't a single medium for used for PC production. Therefore this requires optimization of nutrient media, however, it is difficult to determine all the main nutrient factors and obtain their optimum levels in a process. In our study, a statistically based experimental design proved to be a valuable tool in optimizing the medium for PC production. Optimization through statistical experimental design is a common practice in biotechnology (Singh *et al.*, 2009).

6.3.1.1 Primary Screening experiment

The optimization process was started by performing a screening fractional factorial experiment, the first of which was a primary screening (FF0721) experiment including five centre points. Actual and predicted values shown in Table 6.12 did not vary greatly from each other, as evidenced by the residual column. *Cyanothece* sp. produced the highest PC of 2795.8 µg/mL when grown in media containing ($NaNO_3$, 2.00g; K_2HPO_4, 0.02g; $MgSO_4$, 0.12; $CaCl_2$,0.01g; NaCl ,50.00 g/L; minor nutrients,15 mL and trace metal 0.5 mL).

Figure 6.1, a half-normal plot, showed the selected factors after completing the experiment and both single and two-factor interactions were selected. The Pareto chart, indicated the most significant factors and rank them based on the greatest effect on PC production (Figure 6.3). The largest effect, being a significantly negative effect was the interaction between $NaNO_3$ and trace metals. Magnesium sulphate followed by minor nutrient stock had the largest positive effect. The Box Cox plot indicated that the predicted model fell between the 95% confidence interval and therefore a transformation was not required (Figure 6.2). An ANOVA of the model fitted to the data on FF0721 experiment (Table 6.9) revealed that the model significantly fit the data (p<0.05). Sodium nitrate (X_1), magnesium sulphate $(X)_3$, sodium chloride (X_5) and minor nutrients (X_6) were significant factors.

The interaction between trace metal and $NaNO_3$ resulted in a large negative effect. An increase in trace metal from 0.5 to 1.5mL did not have a significant effect (Figure 6.3). This could be due to the fact that the cyanobacterium did not require large amounts of complex trace metals, therefore, trace metal concentration was set at a low of 0.5 ml/L for next experiment. Cyanobacteria may have special requirements regarding nitrogen sources such as NO_3 or NH_4 (Chaneva *et al.*, 2007). Nitrogen is required for synthesis of the amino acids, which make up proteins and other cellular components such as PC (Colla *et al.*, 2007). The use of nitrate in cultivation caused an increase in biomass and pigment production (Urek & Tarhan, 2012). It was found that higher PC content when the culture was supplement with a nitrate, and under limiting nitrogen conditions, significantly lower PC was present. Hong & Lee, (2008) also reported that the growth and PBPs content of cyanobacterium *Nostoc* and *Oscillatoria* decreased under nitrogen limitation conditions. This degradation of PBPs is possibly due to the loss of a major Rubisco isoenzyme. Phosphate seems to play an important roles in many cellular processes, such as the biosynthesis of nucleic acids, energy transfer, etc., while maintaining pH of culture media by its buffering capacity.

Although NaCl had a positive effect, the interactions between itself and $NaNO_3$, as well as with minor nurients resulted in a high negative effect. Furthermore from preliminary studies, *Cyanothece* sp. preferred a NaCl concentration between 100-120 g/L. It was able to grow in NaCl up to 200 g/L, but a decrease in PC concentration occurred. Furthermore a decrease of NaCl below 75 g/L resulted in a decline in PC content. Since the cyanobacterium could tolerate a range of salt concentrations it is assumed to be euryahaline.

6.3.1.2 Second Fraction Factorial Experiment

In the second screening experiment, NaCl and trace metal was kept to a minimum of 50 g/L and 0.5 mL and 0.5 mL, respectively. This was done to reduce the number of factors from seven to five, expecting to produce higher amounts of PC. Table 6.10 shows the design for FF0521, including the level of each chemical component and amount of PC produced in runs from one to twenty one. Run 13, standard 13 produced the highest PC yield 2028.404 µg/mL when the culture was grown in 2g/L $NaNO_3$, 0.06g/L K_2PO4, 0.18 g/L $MgSO_4$, 0.8g/L $CaCl_2$ and 20mL minor nutrients. The highest amount of PC in this set of experiments was lower than the previous experiment 2978ug/mL. This decrease in PC is possibly related to the

excess of nitrate and phosphate in the medium. It should be noted, that one should be very careful in selecting concentration ranges for such medium components as even very slight variations may result in quite different effects.

The interaction between $CaCl_2$ and minor nutrients showed the largest positive effect on PC production as seen in the pareto chart (Figure 6.4). The largest negative effect was due to the interaction between K_2HPO_4 and $CaCl_2$. The other significant effects were a strong negative effect by $NaNO_3$ and a positive effect by $MgSO_4$. Analysis of variance (Table 6.11) showed that the model was significant and indicated that all the individual factors, as well as their interaction, except for $CaCl_2$ were significant. Although nitrate was found to be an essential factor in the FF0721 experiment, influencing the PC produced, a further increase from 2 to 3 g/L resulted in a negative effect. This could be due to a decrease in the rate of consumption. An increase in K_2HPO_4 from 0.06 to 0.12g/L also resulted in a negative effect. Although these nutrients are crucial for biomass and PC production an excess becomes detrimental thus decreasing the pigment concentration.

6.3.1.3 Central Composite Design

Using the primary (FF0721) and secondary (FF0521) screening experiments, a central composite experiment was designed. The actual and predicted PC concentrations are shown in Table 6.12, with most values were close to each other as demonstrated by small residual values. An ANOVA of the model fitted to the data on CC0317 shows the model is significant ($p< 0.0001$) (Table 6.13). This means the fit for the model was good. Since the mathematical model for the central composite designs is a quadratic one, it includes the squares of the individual factors. All other factors were found to be significant ($p< 0.05$). The three-dimensional response surface curves are plotted to understand the interactions of the factors and find the optimum concentration ranges of components required for maximum PC concentration. Figure 6.8a and b are the response surface and contour plots showing variation in PC concentration, for the two factors ($CaCl_2$ and $MgSO_4$) with the other being at their constant. The response for the interactions between minor nutrients and $MgSO_4$ and $CaCl_2$ and minor nutrients are shown in Figure 6.9a and b and 6.10a and b, respectively.

From the data, it was observed that minor nutrients containing (citric acid, ferric ammonium citrate, EDTA disodium salt and $NaCO_3$) were found to be crucial for the production of PC. Swingley *et al.* (2005) reported that iron (Fe) plays an important role in photosynthetic electron transport, in the synthesis and regulation of PC, nitrate assimilation and nitrogen fixation. Pandey & Pandey, (2008) reported that maximum biomass accumulations and 26% increase PC were observed in cultures supplemented with Fe, and suggested that iron should be applied at 10 mg/L for the growth of *Nostochopsis lobatus*. It is thought that Fe is absorbed in two steps, chelating of inorganic iron by siderophores, followed by transport of membrane-bound Fe into the cells (Wakte *et al.*, 2011). Iron is a key component of chromophore synthesis, and biosynthesis of chlorophyll and phycobilin pigments have iron dependent steps even though neither of them contain iron.

Therefore one possible way by which iron and magnesium influence the production of PC is that they are correlated in the regulation of the PCB. These are linear tetrapyrrole molecules that function as the direct precursors of the chromophores of the light-harvesting phycobiliproteins. Studies indicate that the biosynthesis of phytobilins shares mutual intermediates with heme and chlorophyll biosynthetic pathways with protoporphyrin IX (PIX) as the last common precursor, (Wang *et al.*, 2007). The branched metabolic pathways diverge by metalation with iron or magnesium. In the magnesium branch magnesium catalyzes the chelation of Mg in PIX, while the iron branch insertion of Fe^{2+} into PIX by ferrochelatase leads to the formation of protoheme, there by directing tetrapyrroles into chlophyll synthesis. Cyanobacteria and plants accumulate various tetrapyrrole species in different quantities in the cell.

6.3.1.4 Validation experiment

To confirm these optimal media obtained, a validation experiment was performed. Under this optimized nutrient concentration, the predicted response for PC production was 4814.50 µg/mL, and the observed experimental value was 4887.56µg/mL, which was higher than the predicted value. The agreement between the observed and predicted values confirms the validity and precision of the model.

Using statically methods the media composition was successfully optimized increasing the overall PC production.

CHAPTER 7 : CONCLUSIONS AND RECOMMENDATIONS

7.1 Overview of the Chapter

As stated in Chapter One, this study aimed to increase the recovery of PC by optimizing the extraction procedure, thereafter increase the production of the protein by manipulation of culture conditions of the cyanobacterium, as well determining the effect of physical factors which includes temperature, light and pH on the PC synthesis. The findings have indicated extraction method used is highly crucial in order to obtain maximum PC. The research has also found that the *Cyanothece* sp. has the ability to produced relatively high quantities of PC when exposed to optimal conditions. Also, the findings have suggested temperature, light and pH effect the overall PC production. This chapter provides conclusions and recommendations on the basis of findings, sets out some significant findings in relation to the research objectives, and enlists suggestions and implications for future research.

7.2 Significant Conclusions from the Study

- Morphology results revealed the cells were Gram negative, unicellular, round to widely oval, ranging from 3 to 5 µm. The cyanobacterium phylotype recovered from this study had a high affinity to the *Cyanothece* clade and a sister group composed of *Euhalothece sp.* Based on the morphology and phylogenetic analysis the cyanobacterium, it was identified as *Cyanothece* sp., belonging to the genus Chroococcales.

- The findings of this research have shown that sodium phosphate buffer (10 mM; pH-7.5) was a suitable buffer yielding 810.710 µg/mL phycobiliproteins during extraction. Supplementation of 0.15M NaCl and lysozyme enhanced the total PC extraction capacity. Repeated freezing at –20 ºC and thawing method at 4ºC method using the optimized buffer gave maximum PC content (1490.170 ug/mL). Phycocyanin showed gradual increase in its purity from 0.25 – 1.2 after passing through fractional precipitation and dialysis. Absorbance and fluorescence studies confirmed that the PBP isolated from *Cyanothece* sp. was PC. A band of 18.5 kDa observed on SDS-PAGE corresponds to its alpha subunit of pure PC.

- Abiotic factors have an influence on the PC production. The findings have also shown that extremely low and high temperatures caused changes which deteriorated the biomass and also decreased the PC content. *Cyanothece* sp. has a high potential to adapt to various environments as it can grow in a wide temperature range (20°C to 45°C).

- Light quality treatment influenced the growth and pigment composition. Results showed that the optimal conditions for PC and biomass production were *Cyanothece* sp. grown under Grolux light at high intensities of 125 $\mu molm^{-2}. s^{-1}$ for 16:8 light: dark regimes. Overall blue light caused a negative effect on PC production. The use of light quality treatments including spectral composition in cultivation of this cyanobacterium can be used to manipulate pigment composition.

- The production of PC was increased by optimizing the growth media. Fractional factorial and central composite designs CCD and response surface methodology were useful to determine the optimum concentration levels of medium components that significantly influence the yield of PC from *Cyanothece* sp. According to the RSM results $MgSO_4$ and minor nutrients are involved in PC synthesis. PC production was increase by a 3.5 fold; using the optimized medium, the highest PC yield obtained was 4887.56 ug/mL. Thus by using DOE one can determine the factors and/or combination of factors that have a significant effect on PC

7.3 Significance of the Research

Phycocyanin is an expensive pigment with high value and applications in various biotechnology sectors. To date there are no published reports indicating *Cyanothece* sp. as a high phycocyanin producer. This thesis provided valuable information regarding the potential use of *Cyanothece* sp. for PC production. At the same time, it also provided some insight into the environmental parameters and nutrients that most likely influence PC accumulation and growth. Comparisons of biotic and abiotic factors provided a good basis for additional research towards enhancing PC and biomass production. This research can provide a framework for such work. The study revealed insight on the characteristics and abilities of the cyanobacterium. This indigenous, unicellular *Cyanothece* sp. isolated from a hyper saline environment has a hardy cell wall with a mucilaginous layer which allowed the cyanobacteria to grow under extreme conditions. It was capable of tolerating high salt concentrations i.e 120g/L. Since the *Cyanothece* sp. was able to flourish under extreme conditions including a range of temperatures between 20 to 45°C; a pure culture can be cultivated in large open ponds for mass production without being subjected to contamination.

The cultivation of this strain is cost effective due to the low amounts of nitrogen and phosphorous required for biomass production. This study established that the trace metals, iron and magnesium significantly influence the production of PC, since these nutrients are associated with regulation of the PCB. Furthermore it was found that no definite relationship existed between biomass and PC production. Even at a high biomass yield, a lower PC quantity was noted; owing to degradation of PBS. This indicates that there is no correlation between an increase in biomass and an increase in PC production. This research has further revealed that high quantities of PC can be easily extracted from the isolated *Cyanothece* sp. using a simple phosphate buffer. The pure compounds obtained thereafter, show much promise in biotechnological applications.

7.4 Recommendations

The following are suggestions and implications for further research:

- This study focused on optimising the extraction procedure using wet biomass, however wet biomass cannot be stored for a longer time and can be utilized by bacteria which causes degradation of its chemical composition. Therefore a suitable and efficient drying method to obtain maximum yield and purity ratio should be investigated
- It is also crucial to investigate the effect of temperature and pH on the stability of extracted PC and to determine which preservatives are suitable for maintaining the stability of the protein.
- It is essential to test the antibacterial property of the extracted phycobiliprotein against human pathogens. This gives another way to exploit the protein for food industry for colorant
- Thirdly, in order to determine the application of the protein, the purity of the PC is critical. Purity can be further improved by optimization the purification process and addition of more pre-treatment techniques or chromatographic methods.
- Toxicological studies must be carried out to assess the PC feasibility for commercial production.
- In order to study the relationship between the abiotic factors i.e. light , temperature and pH; experimentation using DOE should be conducted.

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APPENDICES

Appendix 1: Media Recipes

Bold's Basal (BB) Medium

Nutrients	1 litre stock solution g/L	Quantity (ml/L)
$NaNO_3$	25.0	10.0
$MgSO_4 \cdot 7H_2O$	7.5	10.0
K_2HPO_4	7.5	10.0
KH_2PO_4	17.5	10.0
$CaCl_2 \cdot 2H_2O$	2.5	10.0
H_3BO_3	11.42	1.0
Trace elements solution		1.0
EDTA stock (EDTA 50g/L & 31g KOH)		1.0
Fe solution ($FeSO_4 \cdot 7H_2O$ 4.98 g & 1mL H_2SO_4)		1.0

Add each component to seawater (100g/L NaCl). Autoclave at 15 psi for 15 minutes. After sterilization add trace elements.

Trace elements solution:

Components	g/L
$ZnSO_4 \cdot 7H_2O$	8.82
$MnCl_2 \cdot 4H_2O$	1.44
MoO_3	0.71
$CuSO_4 \cdot 5H_2O$	1.57
$Co(NO_3)_2 \cdot 6H_2O$	0.49

Each trace element was added separately to ~800 mL of dH_2O and fully dissolved between addition of each component. Then make up to 1l. Autoclave at 15 psi for 15 minutes.

BG-11 Medium for Blue Green Algae

	1 liter stock solution g/L	Quantity (ml/L)
$NaNO_3$	15.0	100.0
K_2HPO_4	4.0	10.0
$MgSO_4 \cdot 7H_2O$	7.5	10.0
$CaCl_2 \cdot 2H_2O$	3.6	10.0
Citric acid	0.06	10.0
Ferric ammonium citrate	0.06	10.0
EDTA (disodium salt)	0.01	10.0
$NaCO_3$	0.2	10.0
Trace metal solution		1.0

Add components to 1l seawater (containing 100g/L NaCl). The pH should be 7.1 after sterilization

Trace metal solution:

Components	g/L
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$NaMoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg

Each element was dissolved in 1l dH2O, thereafter sterilized

Artificial seawater (ASW)

	1 liter stock solution g/L	Quantity (per liter medium)
Anhydrous salts		
NaCl		100.00g
KCl		0.78g
$NaHCO_3$		0.40g
H_3BO_3		0.12g
Hydrous Salts		
$MgCl_2.6H_2O$		5.2g
$CaCl_2 \cdot 2H_2O$		1.54g
$MgSO_4.7H_2O$		7.12g
Major Nutrients		
$NaNO_3$	8149	5.0mL
NaH_2PO_4	8.8	1.0mL
$Na_2SiO_3.9H_2O$	60.82	0.5mL
Minor Nutrients		
Ferric ammonium citrate	0.06	10.0mL
EDTA (disodium salt)	0.01	10.0mL
$NaCO_3$	0.2	10.0mL
Trace metal solution		1.0mL
Vitamin		1.0mL

Anhydrous salts were added individually and dissolved completely by continuously mixing with a magnetic stirrer. Thereafter the hydrous salts were added and dissolved in the solution. The pH of the medium was adjusted to 7.2 using 1M HCl to avoid excessive precipitation of salts. Mmedium was sterilized for 20 minutes at 121ºC and 100 kPa pressure. Vitamin stock was added after sterilization to avoid degradation of the vitamins.

Trace metal:

Components	g/200mL
$CuSO_4.5H_2O$	3.92
$MnCl_2 \cdot 4H_2O$	72.0
$ZnSO_4 \cdot 7H_2O$	8.80
$NaMoO_4 \cdot 2H_2O$	2.52
$CoCl_2.6H_2O$	4.00
$Na_2EDTA.2H_2O$	0.14

Vitamin:

	mg/100mL
Thiamine-HCl	20
Biotin ($10.4mg.100mL^{-1}$)	1.0
B_{12} ($11.4mg.10mL^{-1}$)	1.0

Primary stocks of B-12 and biotin were initially prepared. The biotin mixture was dissolved by adjusting the pH to 10. All stocks were adjusted to pH 4.5 – 5.0, made up to a final volume and stored at 4°C. The vitamin stock was prepared by each in 100mL dH_2O.

F_2-Si (Guillard, 1975)

Compound	Stock solution (g/L)	Quantity (ml/L)
$NaNO_3$	75.0	1.0
$NaH_2PO_4.H_2O$	5.0	1.0
Trace metal solution		1.0
vitamin solution		1.0

All components were aseptically added 996mL of sterile seawater.

Trace metal solution

Compound	Quantity (g/L)
$FeCl_3.6H_2O$	3.15
$Na_2EDTA.2H_2O$	4.36
$CuSO_4.5H_2O$	0.0098
$Na_2MoO_4.2H_2O$	0.0063
$ZnSO_4.7H_2O$	0.022
$CoCl_2.6H_2O$	0.012
$MnCl_2.4H_2O$	0.178

Each compound was add to 950mL distilled H_2O, and fully dissolved thereafter made up to 1 litre with distilled H_2O, sterilize (autoclave or filter) and store in fridge.

Vitamin solution

Compound	Quantity
B_{12} **(0.5g/L)**	1.0mL
Biotin **(5mg.l)**	1.0mL
Thiamine HCl	100.0 mg

Make up to 1 litre with distilled H2O, filter sterilize into plastic vials and store in freezer.

ASN-III

Component	g/L
NaCl	100.0
$MgSO_4 \cdot 7H_2O$	3.5
$MgCl_2 \cdot 6H_2O$	2.0
$NaNO_3$	0.75
$K_2HPO_4 \cdot 3H_2O$	0.75
$CaCl_2 \cdot 2H_2O$	0.5
KCl	0.5
$NaCO_3$	0.02
Citric acid	0.003
Ferric ammonium citrate	0.003
EDTA	0.005
Vitamin B_{12} (10µg/mL)	1.0 mL
Trace metal solution	1.0 mL

Trace metal solution:

Component	g/L
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$NaMoO_4 \cdot 2H_2O$	0.39
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.0494 mg

Allen and Arnon Medium (ATTC Medium 1142)

Compound	Quantity
NaCl	100.0 g
KNO_3	0.253 g
$NaNO_3$	0.212 g
K_2HPO_2 (56g/L)	6.25 mL
Solution A	25.0 mL

Each of the above is added to 969.0 mL of filtered seawater. K_2HPO_2 solution was aseptically added after sterilization.

Solution A:

Components	g/L	Per litre solution (mL)
4% $MgSO_4 \cdot 7H_2O$		500.0
1.2% $CaCl_2 \cdot 2H_2O$		500.0
Microelements stock		500.0
$MnCl_2 \cdot 4H_2O$	0.36	
MoO_3	0.036	
$ZnSO_2 \cdot 7H_2O$	0.044	
$CuSO_2 \cdot 5H_2O$	0.0158	
H_3BO_3	0.572	
NH_4VO_3	0.0046	
$CoCl_2 \cdot 6H_2O$	0.008	
FeEDTA solution**	160.0 mL	

** Firstly 5.2 g KOH is dissolved in 186 mL dH_2O, thereafter 20.4 g $Na_2EDTA \cdot 2H_2O$ is added to this solution. $FeSO_4 \cdot 7H_2O$ (13.7 g) is added to 364 mL of dH_2O. Finally both solutions are mixed. Filtered air is bubbled through solution until colour change. Final pH ~3.5

Appendix 2: Sequence

GGTGTTCTTCCCGATCTCTACGCATTTCACCGCTACACTGGGAATTCCCTCTACCC
CTGCCGCCCTCTAGTTAACCAGTTTCCAGTGCTCTACCGGAGTTAAGCTCCGACC
TTTAACAGCAGACTTGATGCGCCCCCTGCGGACGCTTTACGCCCAATAATTCCGG
ATAACGCTTGCCTCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAGGC
TGATTCCTCAGGTACCGTCAGTTCTTCTTCCCTGAGAAAAGTGGTTGACAACCCA
AGAGCCTTCTTCCCACACGCGGCGTTGCTCCGTCAGGCTTTCGCCCATTGCGGAA
AATTCCCAACG

Appendix 3: Analysis of PC

Extraction of PC

- Culture was centrifuged in a sterile 50mL tube at 3000 rpm for 30 min; thereafter supernatant removed and washed with sterile distilled water to remove excess salts.
- The pellet was transferred to a 2mL microtube and microfuge for 10 min.
- Supernatant was discarded, and the pellet left to air dry then placed in a -20°C freezer overnight
- The pellet is then thawed and re-suspended in selected buffer
- The suspension was vortexed and incubated at 37°C for 2h in a water bath then left in the fridge 4 °C overnight
- The suspension is then mixed by vortexing and centrifuged for 30 min at and the supernatant is transferred to a new sterile microtube

Preparation of dialysis tubing

Solution 1 (make 2l): 0.2 M Na_2CO_3 & 0.01 M EDTA

Solution (make 1l): 0.01 M N-ethylmaleimide in solution 1

Solution 3 (make 1.5 l) : 25% of EtOH

- Dialysis tubing (40 cm) was cut using a sterile blade
- Tube pieces were placed in 2l beaker containing pre-heat dH_2O.A beaker was put on top of tubes for weight. Boil for 30 min thereafter drain.
- Tubes boiled in solution 1 for 30 min, thereafter drained and rinsed twice using dH_2O
- Tubes were then soaked in solution 2 for 2 hr, after which were drained and rinsed using dH_2O several time
- Tubes were once more boiled for 30 min in dH_2O, then drained and rinse using dH_2O
- Finally rinsed in Solution 3, drained and stored in solution 3 at 4°C.