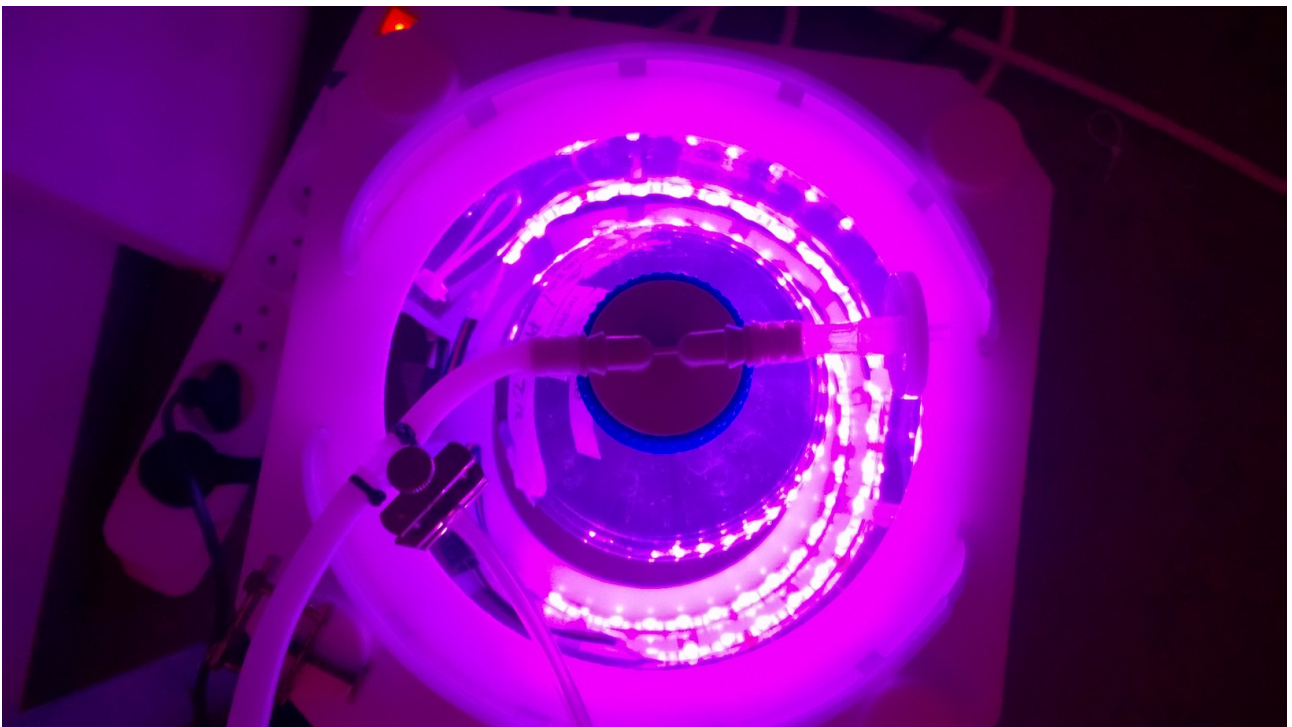




AALBORG UNIVERSITET

Improving the biomass productivity and phycocyanin concentration by mixotrophic cultivation of *Arthrospira platensis*

Tobias Valdemar Rybner
Section for Sustainable Biotechnology
Aalborg University Copenhagen





Semester: 3 and 4

Title: Improving the biomass productivity and phycocyanin concentration by mixotrophic cultivation of *Arthrospira platensis*

Project period: 1 September, 2015 – 10 June, 2016

Supervisor: Peter Westermann (AAU)

Education: Master of Science in Sustainable Biotechnology

ECTS: 60

Department name: Department of Chemistry and Bioscience

Section name: Sustainable Biotechnology

Number of pages: 57

Appendix: 2

Student: Tobias Rybner

Study number: 20112770

Submitted: 10.6.2016

Abstract:

Arthrospira Platensis is a phototrophic organism that naturally contains the pigment phycocyanin. *A. platensis* is cultivated in industrial autotrophic open raceway ponds that have several drawbacks including risk of contamination and low productivity. Many of the disadvantages associated with open ponds cultivation systems can be improved by cultivation in closed photobioreactors. Cultivation in closed systems is still an expensive method mainly associated to a high-energy consumption. Therefore, in order to make it more profitable to cultivate in closed systems, it is necessary to reduce the energy consumption. This can be accomplished by using LED light sources for cultivation and by improving the productivity of *A. platensis* to reduce cultivation time. In this study the effect on productivity of *A. platensis* cultivated mixotrophic with acetate as organic carbon source under different light conditions was investigated. The average biomass productivity was increased with around 20% for the mixotrophic cultures but the phycocyanin content per gram biomass was the same for both the autotrophic and mixotrophic cultures. Lower light intensities seem to improve the phycocyanin content. However, cultivating with only red light did not seem to effect phycocyanin content. It also seems like there is a correlation between high biomass density and low phycocyanin content. The biomass productivity was increased when *A. platensis* was cultivated with acetate and phycocyanin content was increased with low light intensities, suggesting that acetate could be used as a substrate for cultivation of *A. platensis* at low light intensities in order to improve production of phycocyanin and reduce energy consumption.

Acknowledgements

First and foremost, I would like to thank my supervisor Peter Westermann for a good cooperation and valuable guidance doing the master thesis. I have enjoyed our collaboration and the opportunity to develop a project which lies a little outside the sections normal research areas, which have been extremely exciting and educational.

I will also like to thank Gitte Hinz-Berg our head lab technician for helping me in the laboratory and for analysing my HPLC samples.

Finally, I would like to thanks Aalborg University study board of chemistry and environmental engineering for approval of my thesis project.

Contents

1	Introduction.	6
1.1	Surplus production of wind power.....	7
1.2	General <i>Arthrospira platensis</i>	7
1.3	Morphology and taxonomy for <i>Arthrospira</i>	8
1.4	Ecology of <i>Arthrospira</i>	10
1.5	Effect of temperatures	10
1.6	Effect of pH.....	10
1.7	Mixotrophic cultivation	11
1.8	Phycocyanin and the photosynthesis	12
1.9	Marked for phycocyanin.....	13
1.10	Industrial cultivation of <i>photosynthetic organism</i>	14
1.11	Photobioreactor and artificial lighting with Light emitting diode (LED).....	16
2	Project description	18
2.1	Problem statement.....	18
2.2	Objective:	18
2.3	Selection of organism and experiments	19
2.4	Hypotheses:.....	19
3	Material and methods.....	20
3.1	Sterilization.....	20
3.2	Microorganism	20
3.3	Culture media	20
3.4	Artificial LED light sources	23
3.5	High performance liquid chromatography (HPLC)	24
3.6	Biomass production.....	24
3.7	Phycocyanin concentration	26
3.8	<i>Acetobacterium</i> cultivation.	27
3.9	Test of growth kinetic for <i>A. platensis</i>	27
3.10	Test of phycocyanin extraction methods on <i>Arthrospira platensis</i>	28
3.11	Test growth rate on <i>Arthrospira platensis</i> when cultured Auto-, hetero- and mixotrophic.	29
3.12	Test growth for <i>Arthrospira platensis</i> in a medium for <i>Acetobacterium</i> supplied with acetate.....	30
3.13	Mixotrophic cultivation with different concentration of acetate.	30
3.14	Test of low illumination within the red and blue light spectra in a batch Photobioreactor setup.	31
3.15	Test of low illumination within the red light spectra in a batch Photobioreactor setup.	32
4	Results and discussion.....	33
4.1	<i>Acetobacterium sp.</i> cultivation.....	33
4.2	Test of growth kinetic for <i>Arthrospira platensis</i>	33
4.3	Test of phycocyanin extraction methods on <i>Arthrospira platensis</i>	34
4.4	Test growth rate on <i>Arthrospira platensis</i> when cultured Auto-, hetero- and mixotrophic.	36
4.5	Test growth for <i>Arthrospira platensis</i> in a medium used to <i>Acetobacterium</i> supplied with acetic acid.	39
4.6	Mixotrophic cultivation with different concentration of sodium acetate..	40

4.7	Photobioreactor cultivation with low illumination in the red and blue light spectra.....	44
5	Conclusion	49
6	Perspective	50
7	List of references.....	52
8	Appendices	57
8.1	Appendix 1. Extraction protocol for phycocyanin	57
8.2	Appendix 2. Experimental data and calculations	57

List of Figures

Figure 1 Photosynthetic light harvesting complex in cyanobacteria.....	12
Figure 2 Absorbance spectra of different algae pigments.	13
Figure 3, A) Light spectra cultivation light L2, L3 , L4.	24
Figure 4 Standard curve for <i>A. platensis</i> biomass VS. OD	25
Figure 5 Standard curve Phycocyanin concentration VS. Abs	26
Figure 6. Growth curve for <i>Arthrospira platensis</i> cultivated in basal Zarrouk medium.	34
Figure 7 Phycocyanin yield from 1 gram biomass [mg PC/g X] from 5 different extraction conditions.....	34
Figure 8 Growth curve for <i>Arthrospira platensis</i> cultivated autotrophic, mixotrophic, heterotrophic	37
Figure 9 Growth curve for <i>Arthrospira platensis</i> tested in DSMZ <i>Acetobacterium</i>	39
Figure 10 Growth curve for <i>Arthrospira platensis</i> cultivated autotrophic and mixotrophic.	41
Figure 11 Growth curve for <i>Arthrospira platensis</i> cultivated autotrophic mixotrophic.	41
Figure 12 sAcetate consumption by <i>Arthrospira platensis</i>	43
Figure 13 Growth curve for <i>Arthrospira platensis</i> cultivated mixotrophic under different illumination.	45
Figure 14 Acetate concentration in the photobioreactors overtime.	47

List of Tables

Table 1 advantages and disadvantages for open and closed photocultivation systems	16
Table 2 Component for 1 litre Basal Zarrouk medium	21
Table 3 Components to prepare 1 litre A5 micronutrient solution.....	21
Table 4 Components to make 1 litre 1M sodium acetate solution.	21
Table 5 Components used to prepare 1 litre 135a <i>Acetobacterium</i> medium	22
Table 6 Component used to prepare 1 litre trace element solution.....	22
Table 7 Component used to prepare 1 litre vitamin solution	23
Table 8 LED light sources and their light intensity.	23
Table 9 Growth parameters for <i>A. platensis</i> cultivated in basal Zarrouk medium.....	33
Table 10 Result from test of 5 extraction methods.....	36
Table 11 Growth parameters for <i>Arthrospira platensis</i> cultivated autotrophic and mixotrophic.1	38
Table 12 Phycocyanin content in the biomass at day 23. Cultivated autotrophic and mixotrophic 1	38
Table 13 Growth parameters for <i>Arthrospira platensis</i> cultivated autotrophic and mixotrophic 2.	43
Table 14 Phycocyanin content in the biomass at day 20. cultivated autotrophic and mixotrophic 2..	44
Table 15 Growth parameters for cultivated mixotrophic with 1 g/L acetate under different illumination.	46
Table 16 Results from phycocyanin extraction from biomass cultivated mixotrophic with 1 g/L acetate under different illumination at day 30 and day 35.....	48
Table 17 Results from phycocyanin extraction from <i>Arthrospira platensis</i> biomass cultivated mixotrophic with 1 g/L acetate under low illumination red light spectra.....	48

List of Pictures

Picture 1 Filaments of <i>Arthrospira platensis</i> seen through a microscope.....	9
Picture 2 <i>Arthrospira platensis</i> cultivated on solid Zarrouk medium seen through a microscope	9
Picture 3 Open pond raceway track for algae production	14
Picture Three types of closed photobioreactor system	15
Picture 5 . Filament of <i>Arthrospira platensis</i> seen through a microscope after 9 days cultivation in DSMZ 135a Acetobacterium medium..	40
Picture 6 Cultivation of <i>A. platensis</i> with different acetate concentration.....	42
Picture Photobioreactor RH and system used to extract biomass.....	44

1 Introduction.

The term microalgae has no formal taxonomic upright but is often used to embody a highly diverse group of non-cohesive O₂ evolving photosynthetic microorganisms, which are able to live under a variety of different conditions, such as high and low temperatures, light intensities, pH and salinity (Barsanti et al., 2008). The general definition of algae is that they are considered simple photosynthetic microorganisms because they are not organised into organs, like higher plants (Wijffels et al., 2013). Microalgae are an evolutionarily diverse group of unicellular, eukaryotic organisms, although prokaryotic cyanobacteria are often included in the same context. The evolutionary and phylogenetic variety of these photosynthetic organisms also results in a wide variety of chemical compositions within these organisms. The broad diversity in the chemical composition found in microalgae and cyanobacteria has increased the interest for them and therefore makes them very attractive for bioprospecting, whilst also having potential for industrial production of a variety of biological compounds (Borowitzka, 2013). Microalgae already play a key role in the commercial production of high value chemicals such as carotenoid, long chain polyunsaturated fatty acids and phycobilins (Kuddus and Ramteke, 2012). The prokaryotic cyanobacteria, which is often referred to as blue-green algae, and eukaryotic microalgae are all promising to commercial producers of biochemical, biofuels and food additive (Wijffels et al., 2013).

Phototrophic organisms produce sugar and oxygen from carbon dioxide, water, nutrients and sunlight by photosynthesis (Leu and Boussiba, 2014). A big advantage of cultivating Phototrophic organisms is that they can be cultivated in marginal or saline water on unproductive dry land. This means that they do not have to compete with agriculture for arable land and fresh water. These properties make them a very promising source for a sustainable production of various biomaterials, biochemical and biofuels, especially on large unexploited, sunny and dry land areas where other agricultural activities are not suitable because of other factors such as lack of clean water for irrigation and fertile soil (Leu and Boussiba, 2014).

The idea of culturing microalgae in the laboratory was first presented by Warburg in 1919 where he cultured *Chlorella* as part of his work studying photosynthesis. He discovered that some microalgae could daily increase their biomass significantly and that the dry matter from them could contain over 50% of crude protein (Richmond and Soeder, 1986). The first attempt to upscale the culturing of *Chlorella* was performed in a pilot plant in Massachusetts and Tokyo in 1950s. Japan became the first country to produce *Chlorella* biomass as a health food (Richmond and Soeder, 1986; Skjånes et al., 2013).

In the early 1960s the first commercial use of the eukaryotic microalgae *Chlorella* in a large-scale production was started, which was soon followed by the cyanobacteria *Arthrospira* (sometime referred to as Spirulina) in the 1970s. By 1980 the commercial

production of food supplements with the microalgae *Chlorella* was established in Asia, India, USA, Israel and Australia and has since become an important market and are sold as health food all over the world (Wijffels et al., 2013). The algae biomass is very rich in protein and can be used as animal feed or food supplements whilst the extracts from the algae can be used in the production of cosmetics and pharmaceuticals (Skjånes et al., 2013). The interest and research for microalgae biotechnology has increased in the last decade and is now considered to be a very promising technology for biofuels and biochemicals, which means that the research in algae cultivation and processing is not only focusing on improving products but also on new products and applications (Wijffels et al., 2013).

1.1 Surplus production of wind power

As the effects of climate change are becoming more concrete, there has been an increased focus on the problems caused by climate change and the development of renewable energy sources in order to liberate us from fossil energy sources. This has created a growing interest in wind power, especially in Denmark, but also in Germany and several other countries, which has led to massive investment in wind energy projects. This has resulted in a more frequent surplus production of wind power (Neslen, 2015). As Denmark and other north European countries produce a larger amount of their electricity with wind power, there will be days of high winds where electricity production from wind power will exceed the amount of electricity required. During these periods, the electricity is sold at very low prices or it may for short periods even cost money to get rid of the electricity (Wittrup, 2013).

Therefore, there is a need for a method to store or utilize the surplus production of electricity generated. The most well tested storage method for electricity used today is chemical storage in batteries. The energy in a battery is of high quality, however there is great loss of energy associated with storing electricity in this way. In relation to the amount of energy that will need to be stored, it is not possible the energy could be stored in this way and, in addition, batteries are very expensive making it a very uneconomical way to store electricity (Energinet.dk, 2011). Thus, until better technologies for storing surplus production of electricity generated becomes available or the process becomes cheaper there will be a foundation for transforming the energy to other products. For instance, to convert the surplus production of electricity into hydrogen by electrolysis (Mogensen, 2015).

1.2 General *Arthrospira platensis*

Cyanobacteria belong to the kingdom of Eubacteria and constitute one of the largest groups of prokaryotes. They are some of the simplest life forms on earth and the cellular structure is a simple prokaryote, which can perform photosynthesis like plants but without the plant cell walls resembling primitive bacteria (Stanier et al., 1981). Like animals cell they also have complex sugars, such as glycogen, on their cell

membrane (Singh et al., 2005).

However, they are truly prokaryotic lacking nuclear membranes, internal organelles and histone protein associated with chromosomes. Cyanobacteria are able to live autotrophic utilising CO₂ as their sole carbon source using the reductive pentose phosphate pathway or Calvin cycle (Stal and Moezelaar, 1997). They are mainly aquatic and larger than other types of bacteria (Mühling, 2000). Since they are able to photosynthesis and are aquatic, they are often mentioned as blue-green algae, which can be misleading since they are not an algae (eukaryote). Also, they are all unicellular, although many grow in colonies or filaments (Singh et al., 2005).

Some of them are toxic while others are edible. Amongst the edible genera are *Nostoc*, *Arthrospira* and *Aphanizomenon*. *Arthrospira* have been commercially cultivated and sold as a food supplements due to its high protein content (60-70% of its dry weight) (Sánchez et al., 2003).

The cyanobacteria biomass is rich in carotenoid, chlorophyll, phycocyanin, amino acid, minerals and many other bioactive components, which makes it ideal to use as a food additive along with many other applications within biology, biotechnology, food and medicine industry (Eriksen, 2008; Sánchez et al., 2003). The composition of the nutrients in the biomass depends on the growth conditions such as light intensity, temperature, pH, salinity etc., which can have a major affect on the lipid and pigment content in the cells (Stal and Moezelaar, 1997).

The cyanobacterium *Arthrospira platensis* is gaining more attention on key biotechnology research because of its economical, ecological and nutritional importance. *A. platensis* has a great potential to provide the industry with biological produced ingredients that can be used for food production and related nutritional materials, such as colouring agents, vitamins, γ -linolenic acid and enzymes etc. (Borowitzka, 2013). There are many different proteins present in *A. platensis* including phycobilin proteins, which are a family of brilliantly coloured, hydrophilic and stable fluorescent pigment proteins that are classified into three main groups: phycocyanin (C-PC), phycoerythrin (C-PE) and allophycocyanin (C-APC) with different inherent colour and absorbance properties, which will be examined later in the report. Phycocyanin is the main protein in the phycobilin protein and can be used for more than just a nutritive ingredient and natural dye in foods and cosmetics. It can also be used as a potential therapeutic agent in oxidative diseases and as a fluorescent marker in biomedical research (Antelo et al., 2010).

1.3 Morphology and taxonomy for *Arthrospira*

Arthrospira genus are multicellular filamentous cyanobacteria, which if seen through a microscope appear as blue-green filaments (picture 1) (Ciferri, 1983). The blue colour comes from phycocyanin and the green colour is from chlorophyll, however the two pigments cover a third group of pigment, the carotenoids, which normal appears as a red, orange or yellow colour (Richmond and Soeder, 1986).



Picture 1 show the filaments of *Arthrospira platensis* seen through a microscope (40x). (photo by T. Rybner)

The filaments are composed of cylindrical cells arranged in unbranched helicoidal trichomes that are a characteristic for the genus. The helical shape of the trichome is characteristic for the genus. However, the helical structure can differ in length, pitch and the helix dimension from different species and even within the same species (Richmond and Soeder, 1986). Their filaments are motile gliding along their axis and do not have heterocysts. They only have the helical shape when grown in a liquid medium. When grown on a solid medium the filaments shape takes the form of a flat spiral (Picture 2) (Ciferri, 1983; Sánchez et al., 2003).



Picture 2 shows *Arthrospira platensis* cultivated on solid Zarrouk medium seen through a microscope (40x). (photo by T. Rybner)

The diameter of the cells varies from 1 to 3 μm in the smaller species and up to 12 μm in the larger species, such as *A. platensis* and *A. maxima*. The larger species have a granular cytoplasm that usually contains a gas vacuoles and a easily visible septa (Richmond and Soeder, 1986).

The cell wall in *Arthrospira* is composed of four layers numbered from the innermost and outwards as LI, LII, LIII, and LIV. It is the LII layer that is composed of

peptidoglycan substances that gives the cell wall its rigidity whilst the three other layers are very weak and provide only a little structural support (Ciferri, 1983; Sánchez et al., 2003).

1.4 Ecology of *Arthrospira*

Arthrospira can be found in many different environments such as soil, marshes, fresh water, brackish water and seawater. *Arthrospira* colonize many extreme environments where other organisms have difficulties to live. An example of this can be found in some alkaline lakes in Africa and Mexico where *A. platensis* and *A. maxima* are the dominant organisms (Vonshak and Richmond, 1988). This was extensively studied by Iltis in 1967 in the African alkaline lakes within the Chad region where he classified these different bodies of water into three categories according to their salt concentration, which mainly consisted of carbonate and bicarbonate (Ciferri, 1983). The categories are:

1. Lakes with salt concentration below 2.5 g/L.
2. Mesohaline lakes, salt concentration ranging from 2.5 to 30 g/L.
3. Alkaline lakes, salt concentration above 30 g/L.

In the first category with salt concentration below 2.5 g/L he found a varied population of microorganisms belonging to *Chlorophyceae*, *cyanobacteria* and *diatoms*. In the second category where the salt concentration was ranging from 2.5 to 30 g/L he found that the cyanobacterial population was predominantly comprised of many different species such as, *Synechocystis*, *Oscillatoria*, *Arthrospira* and *Annabaenopsis*. In the third category with salt concentration above 30 g/L the cyanobacterial population was almost monospecific with *Arthrospira* the only microorganism present. *A. platensis* has been isolated from water with salt concentration reaching 270 g/L, however, growth seems to be optimal with salt concentration ranging from 20 to 70 g/L (Ciferri, 1983; Richmond and Soeder, 1986).

1.5 Effect of temperatures

Arthrospira is a mesophile microorganism that can tolerate and still grow in temperatures ranging from up to around 40°C and down to around 15°C, although this may deviate between strains (Richmond and Soeder, 1986).

The optimal temperature for *A. platensis* growth is 30°C, which has been shown by Ogbonda who investigated the effect of temperature and pH on biomass production for *Arthrospira* species (Ogbonda et al., 2007).

1.6 Effect of pH

Cyanobacteria can be found in many different environments and conditions. For example, *Arthrospira platensis* thrives in extreme alkaline environments. However, even among cyanobacteria, *A. platensis*'s ability to adapt to high pH values is very unique because even though most cyanobacteria can tolerate alkaline conditions,

they still have optimal growth in a neutral medium (pH 7) (Ciferri, 1983). This is not the case for *A. platensis*, which fails to grow at pH 7 or lower and the optimal growth is obtained around pH 9 to 10 (Ogbonda et al., 2007; Schlesinger et al., 1996). Even at pH 11.5 the growth rate for *A. platensis* has been approximately 80% of the optimal growth rate and it can therefore clearly be defined as an obligate alkaliphile microorganism (Schlesinger et al., 1996).

1.7 Mixotrophic cultivation

The main carbon fixation route in *Arthrospira* is by photosynthesis. When both light and an organic carbon source are available during the cultivation, *Arthrospira* have the ability to combine autotrophic photosynthesis with heterotrophic assimilation of an organic carbon source in a combined process known as mixotrophic (Andrade and Costa, 2007). The photosynthetic fixation of carbon dioxide (CO₂) is regulated by the light intensity and the heterotrophic assimilation of organic carbon is regulated by the accessibility to the organic carbon source (Zhang et al., 1999). Culturing *Arthrospira* under a mixotrophic condition then the growth will not strictly depend on the photosynthesis, which means that light stops being the only limiting growth factor (Andrade and Costa, 2007; Chojnacka and Noworyta, 2004). Therefore, mixotrophic culturing becomes a dual limitation process in which a too high concentration of the organic carbon source or too high light intensities can be limiting for the growth. However, too low concentration of organic carbon or too low light intensities can also have an inhibiting effect on cell growth and therefore light intensity and organic carbon substrate become the two most important growth factors (Chojnacka and Noworyta, 2004; Zhang et al., 1999). Mixotrophic cultivation of *Arthrospira* could increase the biomass concentration, whilst the on-going improvement in closed photobioreactors for mass cultivation of phototrophic microorganism could also contribute to make the mixotrophic growth of cyanobacteria economical feasible (Chen, 1996; Chen and Zhang, 1997; Marquez et al., 1995). Experiments with the filamentous cyanobacteria *A. platensis* and glucose as an organic carbon source showed that the biomass and photosynthetic pigment produced when growing mixotrophic was increased by 1.5 - 2 fold when compared to cultures grown photoautotrophic. However, the pigment concentration per gram biomass was almost the same in the mixotrophic and photoautotrophic cultures (Marquez et al., 1995; Vonshak et al., 2000).

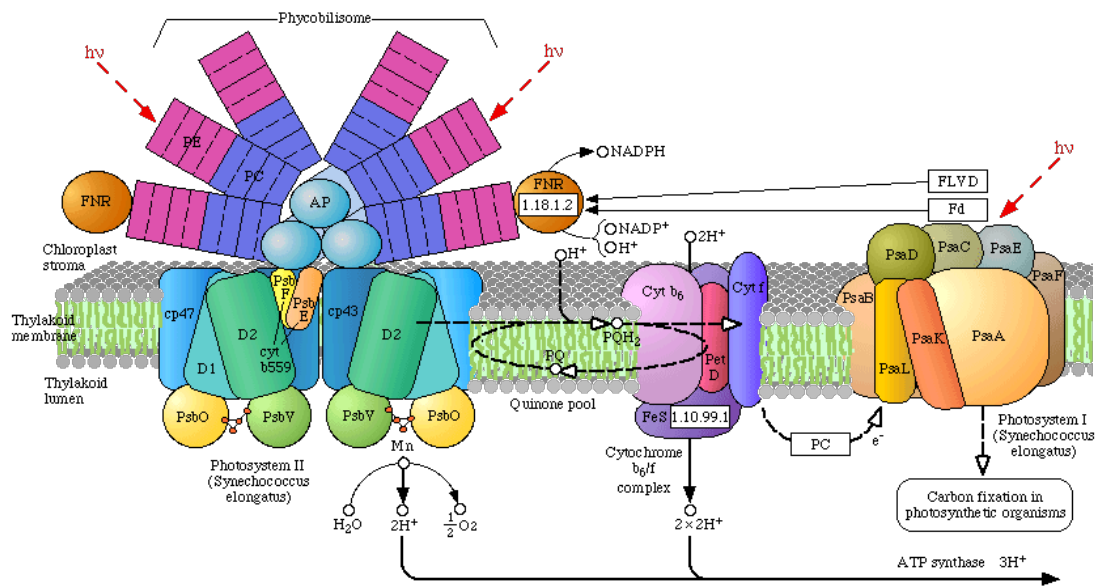


Figure 1 Shows the photosynthetic light harvesting complex in cyanobacteria. The photosynthetic electron flow and ATP synthesis in the thylakoid membrane (Johnson, 2006).

1.8 Phycocyanin and the photosynthesis

In Cyanobacteria three multi complex light harvesting systems exist: photosystem I, photosystem II and phycobilisomes, which are used to capture light energy from the visible light spectra (400nm to 700nm) and convert it to chemical energy by the photosynthesis (Figure 1) (Gao et al., 2016; Ting et al., 2002). The three common types of phycobiliproteins have the major biological role of photosynthetic light harvesting: the red phycoerythrin that absorbs most strongly at wavelength around 550 nm, the blue phycocyanin which absorbs maximum around 620 nm and allophycocyanin that absorbs around 650 nm, which is located at wave lengths in the visible light spectra where chlorophylls have low extinction coefficients (absorbs poorly) (Figure 2) (Madigan et al., 2012). The phycobilinproteins are combined into groups called phycobilisomes, which is attach to the thylakoid membrane. Allophycocyanin are located in the centre of the phycobilisomes complex and surrounded by either phycocyanin or phycoerythrin or both depending on the organism (Figure 1) (Johnson, 2006; Madigan et al., 2012). The phycobilisomes are organised so phycocyanin absorbs at higher energies (shorter wavelength) then allophycocyanin, which is placed closer to the reaction centre chlorophyll that absorbs at lower energies (longer wavelengths) than allophycocyanin. So the energy flow is from phycocyanin → allophycocyanin → chlorophyll of photosystem II (Eriksen, 2008; Madigan et al., 2012). Therefore, phycobilisomes facilitate the energy transfer that allows cyanobacteria to grow at very low light intensities (Madigan et al., 2012).

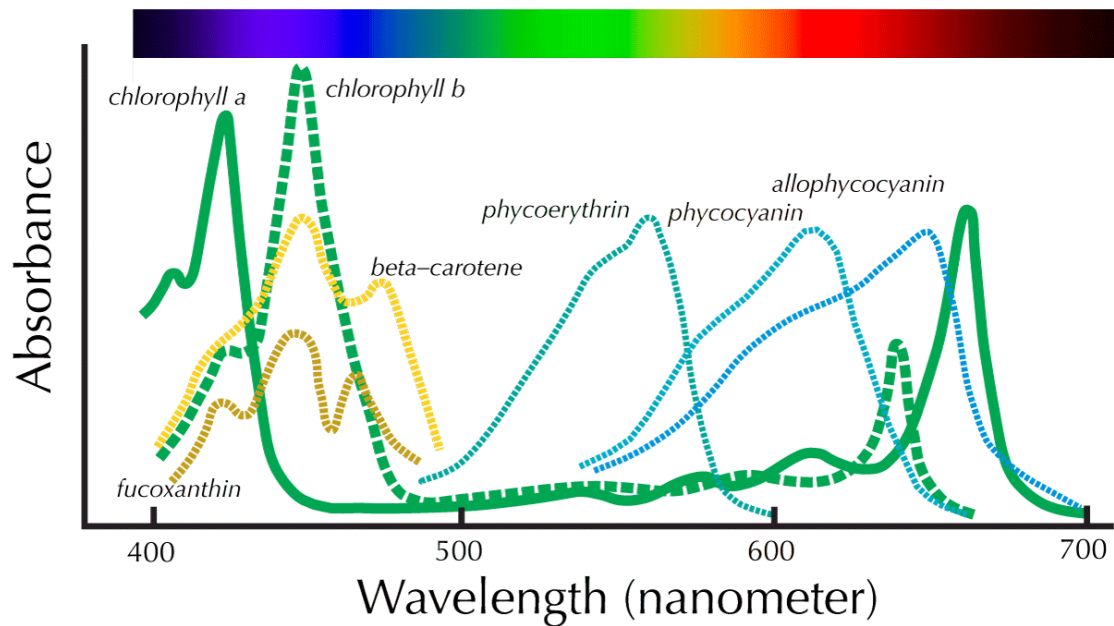


Figure 2 shows the absorbance spectra of different algae pigments (WIKI, 2014).

1.9 Marked for phycocyanin

Phycocyanin is a commercially promising biochemical present in *A. platensis*, however, the majority of the 3000 tons of dry weight of *A. platensis* biomass that is produced every year are sold as health food and animal feed, which has a low economic value (around 36 € per kilo) relative to phycocyanin, which is considered as a high value product (Borch, 2011; Eriksen, 2008). Dependent of the purity of the C-phycocyanin, the sell price lies from around 500 US\$/kg up to 100,000 US\$/kg, with the current estimated market value for phycobiliprotein products as greater than 60 million US \$ (Borowitzka, 2013), whilst the annual market for phycocyanin lies between 5 to 10 million US\$ (Yaakob et al., 2014). The purity of C-phycocyanin is divided into three classes: food grade (purity of 0.7, defined as the ratio between the absorbance of 620 nm to 280 nm), reactive grade (purity of 3.9) and analytical grade (purity greater than 4.0). The commercial value of food grade C-phycocyanin is around 0.13 US \$/mg whilst the reactive grade is around 1 to 5 US \$/mg and the analytical grade can go as high as 15 US \$/mg (Eriksen, 2008; Rito-Palomares et al., 2001).



Picture 3 shows an open pond raceway track for algae production located in Western Australia (Watson, 2013).

1.10 Industrial cultivation of *photosynthetic organism*

There are two commonly used systems for cultivating photosynthetic microorganism: The open raceway ponds system (Picture 3) and closed photobioreactor system (Picture 4)(Harun et al., 2010).The commercial cultivation of *Arthrospira* is mainly carried out in open raceway ponds where the solar energy absorbed by the cyanobacteria is used to fix inorganic carbon (CO_2) (Picture 3) (Vonshak et al., 2000). The main drawback of the open ponds cultivation system is the low cell concentration that is around 0.4-0.8 g/l, which means that relatively large cultivation systems are required in order to produce an efficient amount of phycocyanin (Chen et al., 2006). Other disadvantages of cultivation in an open ponds system is the risk of contamination from other microorganism, insufficient stirring/mixing and the lack of control of environmental conditions such as temperature, evaporation and light intensity (Chen and Zhang, 1997). However, the risk of contamination can be solved by cultivation under extreme culture conditions (high salinity or alkalinity), which means that only a small amount of organisms are suited for this cultivation method (Brennan and Owende, 2010). However, these are factors that are undesirable when producing a product (phycocyanin) for the chemical and pharmaceutical industries since the cost for product recovery and purification can become costly, especially due to the low cell concentration in the open pond systems (Chen and Zhang, 1997).



Picture 4 shows three types of closed photobioreactor system (A) Column photobioreactor (Yeomans, 2013). (B) Horizontal tubular photobioreactor (Bennekom, 2014). (C) Flat plate photobioreactor (AGICAL, 2013).

Some of the drawbacks for open pond systems can be avoided by culturing in a closed photobioreactor, which provides a system that can control and even manipulate cultivation parameter such as the nutrient supply for growth, temperature, pH, dissolved CO₂, light intensities and specific wavelength of light and cultures can be maintained axenic (Eriksen, 2008; Harun et al., 2010; Ugwu et al., 2008). Closed photobioreactor systems include the tubular, flat plate and column photobioreactors (Picture 4).

Several studies have indicated that the type of photobioreactor and especially culture condition such as: pH of the medium, type of media, CO₂ supply, nitrogen concentration and light intensity significantly affect the biomass production and phycocyanin accumulation in *Arthrospira* cultures (Chen et al., 2013; Leema et al., 2010; Zeng et al., 2012). These condition can be controlled in a closed photobioreactor and it has been shown that the productivity of *Arthrospira* cultures is increased when they are cultured in closed photobioreactor (Chen et al., 2013). It has been reported that volumetric biomass productivity in closed photobioreactors can be 5 to 20 times above what can be obtain in an open raceway pond and that the volumetric productivity of phycocyanin is therefore also increased when *A. platensis* is cultured in a closed photobioreactor system (Eriksen, 2008). This may favour using a closed photobioreactor system for commercial production of C-phycocyanin but the cost of producing phycocyanin in closed photobioreactor still needs to be reduced considerably in order to make it economically feasible on an industrial scale (Xie et al., 2015). The cost involved for establishing, running and maintaining a closed photobioreactor system is much higher than that of an open

raceway pond system, which is mainly caused by the equipment requirement and energy consumption involved in the closed photobioreactor system (Harun et al., 2010). Some of the advantages and disadvantages for culturing in open ponds and closed photobioreactor systems are outlined below (Table 1).

Cultivation system	Advantages	Disadvantages
Tubular photobioreactor	Large surface area → light utilization. No need for agricultural land. Good biomass productivity. Suitable for outdoor mass culturing.	Wall growth → fouling. Large area of land required. Limitation on length of tubes. Maintenance.
Flat plate photobioreactor	High biomass productivity. Easy to sterilise. Low accumulation of dissolved O ₂ . High photosynthetic efficiency. Large surface area → light utilization.	Difficult to scale up. Temperature control. Wall growth → fouling.
Column photobioreactor	Control of growth conditions. Efficient mixing. High volumetric mass transfer rates. Low cost compared to the other closed systems. Compact and easy to operate.	Small illumination area. Expensive compared to open ponds.
Open raceway ponds	Relatively cheap to construct. Easy to clean. Low energy input. No need for agricultural land. Low energy input. Easy maintenance.	Low biomass productivity. Large area of land required. Few phototrophic organisms are suited. Insufficient mixing → light and CO ₂ utilization. Risk of contamination.

Table 1 outlines some of the advantages and disadvantages for open and closed cultivation systems for phototrophic organism (Brennan and Owende, 2010).

1.11 Photobioreactor and artificial lighting with Light emitting diode (LED).

A major obstacle to make it economical efficient and environmental friendly to use artificial light for cultivating phototrophic microorganism such as *A. platensis* in order to produce biofuels, biochemical etc. is the high energy input associated with traditional agricultural lighting systems used for photosynthetic cultivation (Faunce et al., 2013). The majority of closed photobioreactor systems used today are tubular photobioreactors with tubes in different sizes and shapes depending on the reactor design. Regardless of the reactor design used, the biotechnological objective is to ensure optimal cultivation conditions. The two important

parameters to ensuring for optimal growth conditions for the phototrophic organism are turbulence (mixing) and illumination (Pulz, 2001). These two parameters are important to control in order to insure an efficient cultivation process. The control of the illumination is probably the most important parameter when designing a closed photobioreactor for a phototrophic cultivation process that operates 24 hours per day. This is because most of the energy used in a photobioreactor is for lighting and light wavelength may also influence the composition of the cells in relation to pigments, protein, polysaccharides and lipids (Koc et al., 2013; Rivkin, 1989). Therefore, the design of a photobioreactor will have to be economical, robust, reliable and have an effective light source in order to make it suitable for industrial production (Beardall and Raven, 2013). When choosing a suitable light source for the photobioreactor it is important that the spectral characteristics of the light flux and the wavelength range are right because different types of phototrophic microorganism may require different light intensity (photon flux density), duration and wavelength of light. Too high a light intensity may lead to photooxidation and photoinhibition, whilst too low light intensity may become growth limiting (Carvalho et al., 2011). A suitable light source for photobioreactors could be light emitting diode (LED) because they have a high luminous efficiency, low energy consumption, cool emitting temperature, the option to select specific wavelength and they are very robust, and have a long lifetime compared to other agricultural light sources (Massa et al., 2008). The LED technology has relatively narrow wavelength bands that makes it possible to produce specific wavelength, which in addition to making it possible to illuminate the microbes with light in the specific wavelength suited for optimal growth and desired product, LED also has a positive effect on the energy consumption, and thus making the process more energy efficient. LED can reduce stress conditions associated to excessive illumination of the microbes, which often appears with other agricultural light systems because with the LED systems the light intensity can easily be regulated (Koc et al., 2013; Yam and Hassan, 2005). The LED technology also has the advantages of a low head conduction compared to other light systems, which prevent overheating of the growth medium and thereby reducing the energy input for stabilising the temperature in the photobioreactor. Finally, LED are small in structure and easy to install (Koc et al., 2013). All these advantages make LEDs very suitable as a light source for a photobioreactor, whilst also more environmentally friendly than other agricultural light systems. Therefore, in order to justify (in a sustainable perspective) using artificial light to cultivate photosynthetic organism, the utilization of the energy input should be as effective as possible. This can be achieved by using LED technology that has a high energy efficiency and is able to provide light in the specific wavelength and intensity that is required for the phototrophic organism to carry out their photosynthesis (Fauce et al., 2013).

2 Project description

The increasing international focus on sustainability and utilization of biological and renewable resources has resulted in an increasing interest in finding alternative solutions to the crude oil based production of energy, fuels and chemicals.

Phycocyanin is a high value product with many applications that exist naturally in *A. platensis*. Phycocyanin is produced by cultivation of *A. platensis* in open raceway ponds that have drawbacks of risk of contamination and low productivity. This could be significantly improved if *A. platensis* was cultivated in closed photobioreactor systems, however, this is still a very expensive solution. Therefore, if the productivity could be increased more by cultivation *A. platensis* mixotrophic with a cheap and renewable carbon source it could help make it more economical feasible to cultivate in closed photobioreactors.

Thus, the idea behind this project is that the surplus production from wind turbines could be converted to hydrogen (H₂) by electrolysis, and subsequently the H₂ is used to produce acetate with homoacetogenic bacteria by anaerobic fermentation with H₂ plus CO₂. The effluent containing acetate could be used for mixotrophic cultivation of *A. platensis* to increase productivity of cultivation. After the cultivation the phycocyanin can be extracted from the biomass and thereby the surplus wind power production could be used to increase the productivity of phycocyanin production.

Based on this acetate was used as organic carbon source in this study to test mixotrophic cultivation of *A. platensis* with a view to extract phycocyanin.

2.1 Problem statement

Can the productivity of *Arthrospira platensis* cultivated at low light intensities be increased if acetate is added to the growth medium?

Can the phycocyanin concentration per gram of biomass be increased if *Arthrospira platensis*, under cultivation, is illuminated only with light in the red light spectra?

2.2 Objective:

The overall objective for this 60-ECTS master thesis is to investigate the effect on biomass production and phycocyanin concentration in *A. platensis* when cultivated mixotrophic with acetate as an organic carbon source. Also to investigate whether it is possible to cultivate *A. platensis* in the fermentation broth from anaerobic acetate production by *Acetobacterium sp.*

Furthermore, the objective is to upscale the cultivation of *S. platensis* in a 2-litre batch photobioreactor setup under red and blue illumination with different light intensities.

2.3 Selection of organism and experiments

A. platensis can be cultivated mixotrophic, and has already been tested with a number of different organic carbon sources, which have shown to increase the biomass growth rate (Andrade and Costa, 2007; Chojnacka and Noworyta, 2004; Marquez et al., 1993; Vonshak et al., 2000).

2.4 Hypotheses:

1. The overall hypothesis is that *A. platensis* can increase its grow rate when cultivated mixotrophic with acetate.
2. Fermentation broth from anaerobic fermentation of acetate by *Acetobacterium sp.* can be used to cultivate *Spirulina platensis* mixotrophic.
3. Also when cultivated mixotrophic with low light intensities in the red and blue light spectra, *S. platensis* will still grow well and the concentration phycocyanin per gram of biomass will still be high, since phycocyanin absorbs light in the red spectra.
4. The process can become more energy effective because we only use light in the relevant light spectra's for maintaining growth (red and blue light) and by using LED technology for illumination.

3 Material and methods

3.1 Sterilization

The growth medium and glassware used for culturing *Arthrospira platensis* were steam sterilized in an autoclave at 121 °C and a steam pressure of 1 bar for 15-20 min. Trace metal, micronutrient and vitamin solutions were filter sterilized through a 0,2 µm cellulose acetate membrane filter and added to the mediums after autoclaving to avoid precipitation.

3.2 Microorganism

The microorganism used in this study was *Arthrospira platensis*. The pure culture of *A. platensis* was procured from Experimental Phycology and Culture Collection of Algae at the university of Göttingen (EPSAG), strain number 21.99. The strain was maintained in a basal Zarrouk medium, but from a recipe found in R. Gupta Ph.D. thesis (Gupta, 2007; Zarrouk, 1966). Maintaining and culturing of starter cultures was carried out under illumination of L3 and L4 LED light bolts 403 lux (Table 8).

3.3 Culture media

Autotrophic cultivation of *A. platensis* was carried out in a basal Zarrouk (BZ) medium (Table 2 and 3). The pH of the medium was adjusted to a pH value around 9 to 9,5 with potassium hydroxide (KOH). Cultures were incubated in a culture room at 30 ±3°C and illuminated with artificial light in the red and blue spectra (Table 8). For the mixotrophic cultivation of *A. platensis* the BZ medium was supplied with and organic carbon source from either acetic acid 99.8-100.5 % obtained from Sigma Aldrich or acetate from a 1 M sodium acetate solution (Table 4).

For the acetate production with homoacetogenic bacteria by anaerobic fermentation with H₂ plus CO₂. *Acetobacterium sp.* were cultivated in DSMZ *Acetobacterium* medium 135a and the composition of it is shown below in table 5, 6 and 7.

Basal Zarrouk medium	
Solution I	
Sodium bicarbonate (NaHCO ₃)	18.00 g
Potassium phosphate dibasic (K ₂ HPO ₄)	0.50 g
De-ionized water (dH ₂ O)	500 ml
Solution II	
Sodium nitrate (NaNO ₃)	2.50 g
Potassium sulphate (K ₂ SO ₄)	1.00 g
Sodium chloride (NaCl)	1.00 g
Magnesium sulphate (MgSO ₄)	0.20 g
Calcium chloride (CaCl ₂)	0.04 g
Iron sulphate (FeSO ₄)	0.01 g
Ethylene diamine tetra acetate (C ₁₀ H ₁₆ N ₂ O ₈)	0.08 g
De-ionized water (dH ₂ O)	500 ml
A5 micronutrient solution*	1 ml
<i>Autoclave solution I and II separately, assemble after cooling and add solution A5 in sterile solution.</i>	

Table 2 shows the component for 1 litre Basal Zarrouk medium (Gupta, 2007; Zarrouk, 1966).

* Preparation of A5 micronutrient solution	
Boric acid (H ₃ BO ₃)	2.860 g
Manganese(II)chloride (MnCl ₂)	1.810 g
Zinc sulphate (ZnSO ₄)	0.222 g
Sodium molybdate (Na ₂ MoO ₄)	0.0177 g
Copper(II)sulphate (CuSO ₄)	0.079 g
De-ionized water (dH ₂ O)	1000 ml
<i>Filter sterile if sterile condition is required.</i>	

Table 3 shows the components to prepare 1 litre A5 micronutrient solution used to prepare the basal Zarrouk medium (Gupta, 2007; Zarrouk, 1966).

1M Sodium acetate solution	
Sodium acetate (C ₂ H ₃ O ₂ Na)	82 g
De-ionized water (dH ₂ O)	1000 ml

Table 4 shows the components to make 1 litre 1M sodium acetate solution.

DSMZ 135a <i>Acetobacterium</i> medium	
Sodium chloride (NaCl)	20.00 g
Ammonium chloride (NH ₄ Cl)	1.00 g
Monopotassium phosphate (KH ₂ PO ₄)	0.33 g
Dipotassium phosphate (K ₂ HPO ₄)	0.45 g
Magnesium sulphate heptahydrate (MgSO ₄ x 7 H ₂ O)	0.10 g
Trace element solution*	20.00 mL
Yeast extract	2.00 g
Sodium bicarbonate (NaHCO ₃)	5.00 g
Resazurin	1.00 mg
Vitamin solution**	10.00 mL
Ethylene glycol	1.25 g
L-Cysteine-HCL x H ₂ O	0.50 g
Sodium sulphide nonahydrate (Na ₂ S x 9 H ₂ O)	0.50 g
De-ionized water (dH ₂ O)	1000.00 mL
<i>The medium was prepared anaerobically with N₂ and CO₂ gas mixture.</i>	

Table 5 shows the components used to prepare 1 litre 135a *Acetobacterium* medium (DSMZ, 2016).

* Preparation of trace element solution	
Nitrilotriacetic acid	1.50 g
MgSO ₄ x 7 H ₂ O	3.00 g
MnSO ₄ x H ₂ O	0.50 g
NaCl	1.00 g
FeSO ₄ x 7 H ₂ O	0.10 g
CoSO ₄ x 7 H ₂ O	0.18 g
CaCl ₂ x 2 H ₂ O	0.10 g
ZnSO ₄ x 7 H ₂ O	0.18 g
CuSO ₄ x 5 H ₂ O	0.01 g
KAl(SO ₄) ₂ x 12 H ₂ O	0.02 g
H ₃ BO ₃	0.01 g
Na ₂ MoO ₄ x 2 H ₂ O	0.01 g
NiCl ₂ x 6 H ₂ O	0.03 g
Na ₂ SeO ₃ x 5 H ₂ O	0.30 mg
Na ₂ WO ₄ x 2 H ₂ O	0.40 mg
Distilled water	1000.00 mL

Table 6 Show the component used to prepare 1 litre trace element solution used in the 135a *Acetobacterium* medium (DSMZ, 2016).

** Preparation of vitamin solution	
Biotin	2.00 mg
Folic acid	2.00 mg
Pyridoxine-HCL	10.00 mg
Thiamine-HCL x 2 H ₂ O	5.00 mg
Riboflavin	5.00 mg
Nicotinic acid	5.00 mg
D-Ca-pantothenate	5.00 mg
Vitamine B ₁₂	0.10 mg
p-Aminobenzoic acid	5.00 mg
Lipoic acid	5.00 mg
Distilled water	1000.00 mL

Table 7 shows the component used to prepare 1 litre vitamin solution used in the 135a *Acetobacterium* medium (DSMZ, 2016).

3.4 Artificial LED light sources

The illumination for the different experimental setups was provided with artificial LED light in the red and blue spectra, which is illustrated below (Table 8).

Unit	Description	Spectra	Light intensity E(lux)
L1	Photobioreactor lighting platform	Red, blue	349
L1	Photobioreactor lighting platform	red	175
L2	LED light panels	Red, blue	839
L3	LED light bolt	Red, blue	403
L4	LED light bolt	Red, blue	403

Table 8 Shows the different LED light sources and their light intensity in lux.

The wavelength and light intensity for L2, L3, and L4 was measured with a spectral meter (Metrue SIM-2 plus), which was provided from Statens Bygge Institute (SBI)(Figure 3). The light intensity of L1 was measured with a Lux meter app (LuxLightMeter) on an iPhone SE (Table 8).

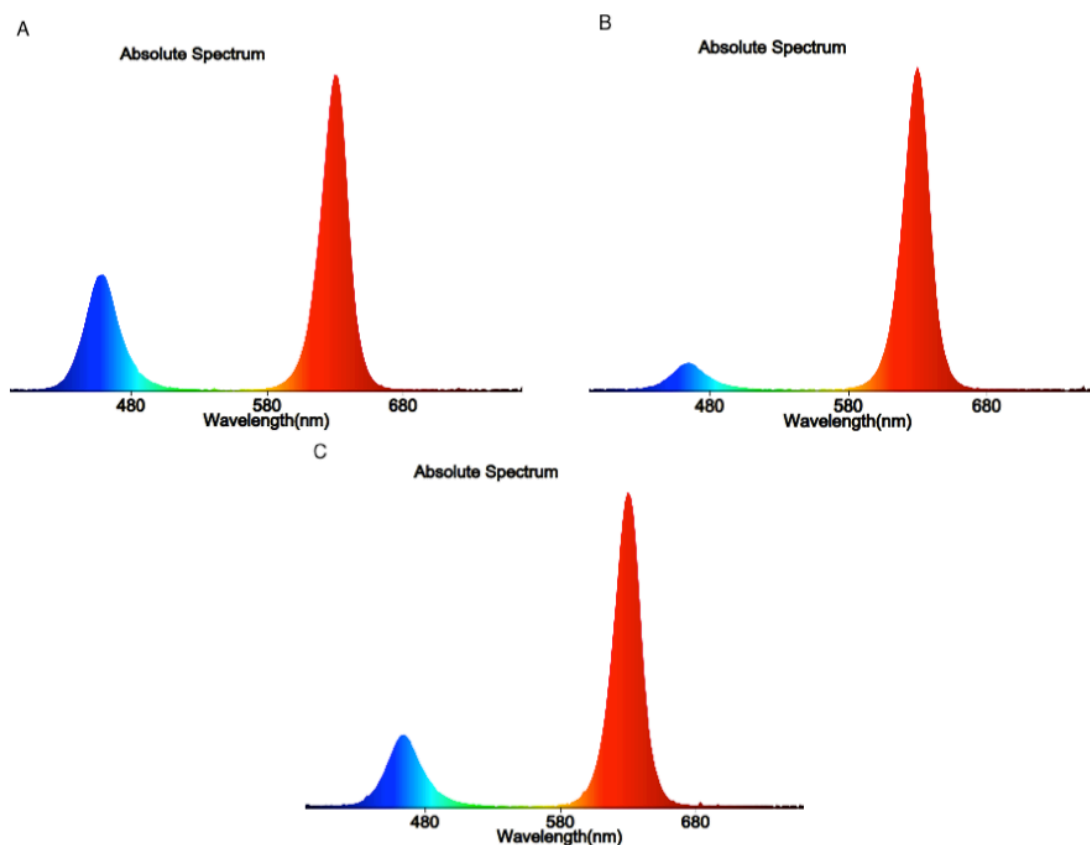


Figure 3, A) Shows the light spectra for L2, B) shows the light spectra for L3 and C) shows the light spectra for L4.

3.5 High performance liquid chromatography (HPLC)

HPLC analysis was used to monitor and determine the concentration of acetate in the BZ medium in order to determine the residual acetate concentration in the medium. 1 mL sample was extracted simultaneous with optical density (OD) measurement for biomass determination and stored in the -20 °C for later analysis. The sample was analysed in a Dionex Ultimate 3000 with RI (refractive index) detector, eluent 4 mM sulphuric acid, column BIORAD Aminex HPX-87H at 60 °C.

3.6 Biomass production

The biomass concentration for *A. platensis* was evaluated by measuring the optical density (OD) of the sample at a wavelength of 675 nm, which was found by performing a wavelength scan of a biomass sample on a spectrophotometer (model DR 3800™ Benchtop Spectrophotometer). A standard calibration curve of optical density versus *A. platensis* biomass concentration was made by performing a dilution series in triplets and measuring the optical density of the sample and plotting the agents of the biomass concentration of the sample, which was found by drying in triplets 3 ml of the diluted biomass samples in an oven at 104 °C until the weight was stable (Figure 4).

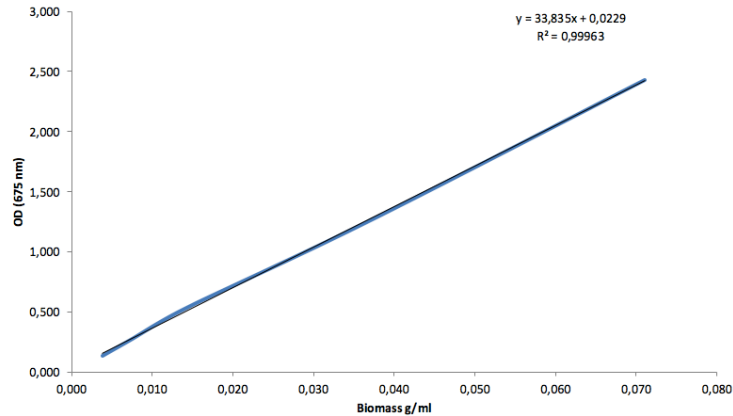


Figure 4 shows the standard curve for *A. platensis* used to determine the biomass concentration with optical density (OD) measurement.

The OD_{675nm} values were converted to biomass concentration by the formula obtain from the calibration curve:

$$Biomass(g/L) = \frac{OD_{675} - 0.0229}{33.835} \times 10^3, R = 0.99$$

The specific growth rate ($\mu = \text{day}^{-1}$) for *A. platensis* cultures was determent for the growth period by the equation:

$$\mu(\text{day}^{-1}) = \frac{\ln(X)}{t}$$

I.e. the maximum slope of a plot of $\ln X$ versus time. Where X is the biomass concentration (gram/litre) and t is the cultivation time (days) (Hill and Robinson, 1974; Xie et al., 2015).

The productivity is the ratio between the differences in biomass concentration for a period of time, which was calculated by the equation:

$$P = \frac{(X_i - X_0)}{t_i}$$

Where P = productivity (gram per Litre/day), X_i = biomass concentration at time i (gram/litre), X_0 = the initial cell concentration and t_i = is the time range (days) between X_i and X_0 (Andrade and Costa, 2007; Walter et al., 2011).

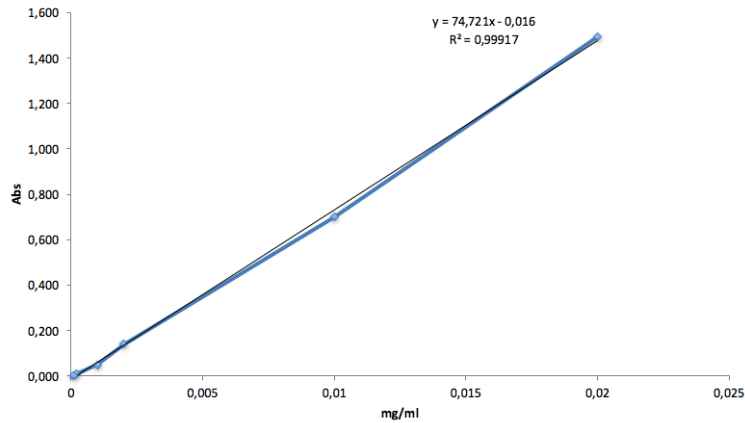


Figure 5 shows the Standard curve made to determine phycocyanin concentration from measuring maximal absorbance (Abs).

3.7 Phycocyanin concentration

The absorbance of Phycocyanin samples was measured with UV/Vis spectrophotometer (Mecasys Optizen POP BIO). The determination of phycocyanin concentration was carried out by making a dilution series of a PC sample purchase from Sigma Aldrich and using it to calibrate the absorbance curve at 620 nm of diluted samples (Figure 5). The maximum absorbance (Abs) of phycocyanin was found by conducting a wavelength scan. The Abs of extraction samples were calculated by the equation obtain from the calibration curve:

$$Phycocyanin(mg/mL) = \frac{Abs_{620} + 0.016}{74.721} \times \frac{V_{sample}}{V_{buffer}}, R = 0,99$$

Where Abs_{620} is the absorbance at 620 nm and V_{sample} is the volume of the biomass sample and V_{buffer} is the volume of the buffer added in the extraction. To evaluate the yield of phycocyanin accumulated per gram of *A. platensis* biomass the following equation was used:

$$Y_{Phycocyanin}(PC\ g/Bio\ g) = \frac{phycocyanin\ (g/l)}{biomass\ (g/l)}$$

The purity of the phycocyanin fraction after extraction was evaluated based on the ratio between absorbance of phycocyanin measured at 620 nm and the absorbance of some of the amino acids present in the proteins in the sample at 280 nm (Walter et al., 2011).

$$PC_{purity} = \frac{Abs_{620}}{Abs_{280}}$$

The phycocyanin samples with an Abs_{620}/Abs_{280} ratio higher than 0.7 are considered as food grade while and ratio higher than 4 are considered as analytical grade (Eriksen, 2008). The measurement of Absorbance for Phycocyanin was conducted on a Mecasys Optizen POP BIO spectrometer.

3.8 *Acetobacterium* cultivation.

Objective of experiment

The objective of performing this experiment was to try to cultivate *Acetobacterium sp.* (DSMZ: 2396) in a DSMZ 135a medium supplied with hydrogen (H_2) and carbon dioxide (CO_2) to see if it will produce acetate. The idea is that the fermentation broth containing acetate could later be used as substrate for mixotrophic cultivation of *A. platensis*.

Experimental setup

The test was carried out in duplets in 20 ml vials with 10 ml DSMZ 135a *Acetobacterium* medium under anaerobic conditions.

The vials were started with an *Acetobacterium sp.* freeze culture (DSMZ: 2396) obtained from DSMZ culture collection. The vials were installed in an incubator at 30 °C. After starting the cultures, the vial was feed with hydrogen (H_2) two to three times a week.

3.9 Test of growth kinetic for *A. platensis*

Objective of experiment

This experiment was performed in order to make a growth curve for *A. platensis*. The growth curve was used to determine when starter cultures of *A. platensis* are in the exponential phase of growth and ready for inoculation and to determine how often I have to measure growth in my later experiment.

Experimental setup

The experiment was carried out in 250 mL blue cap bottles in triplets with 100 ml basal Zarrouk medium in each bottle. The Bottles was inoculated with 5 mL *A. platensis* (21.99) starter culture provided from EPSAG. After inoculation the bottles were installed in an incubator room (temperature $30\pm 2^\circ C$) with agitation (180 RPM) and under constant artificial lighting (L2 -LED light panels 839 lux). Biomass accumulation was monitored by OD_{675nm} measurement and plotted in an excel sheet VS time to form a graph of the growth. 1 mL biomass samples for OD_{675nm} measurement was extracted every 6 hours in the beginning but was later changed to every 8 and then 12 hours by the end. The experiment ran until OD_{675nm} measurement was decreasing for all three bottles.

3.10 Test of phycocyanin extraction methods on *Arthrospira platensis*.

Objective of experiment

The objective for performing this experiment was to find the most suitable procedure for harvesting and extracting the phycocyanin pigment from the *A. platensis* biomass and optimize it for my experiment. This was done in order to evaluate the phycocyanin concentration per gram of biomass and the purity of the extracted phycocyanin. The results and experience from the experiment were used to conduct a protocol for extracting the phycocyanin, which was used in my later work (appendix 1).

Experimental setup

Based on a literature review, five procedures were developed and tested on *A. platensis* biomass (Horváth et al., 2013; Lawrenz et al., 2011). They were named method 1, 2, 3, 4 and 5. The experiment was performed in triplets and the *A. platensis* biomass came from the same culture which had a $OD_{675nm}=1.562$ which is equal to a cell density of 45.49 g/L.

Method 1

Extract by filtration. 10 mL of *A. platensis* biomass sample was harvested and the water was removed by vacuum filtration through a GF/F filter. After filtration the filters were transferred to a 50 mL falcon tube and immediately frozen at -80 °C until further processing. The filters were then subjected to two freeze-thaw cycles of 24 hours each at -80 °C and 5 °C. After the freeze-thaw cycles the filters were mechanically disrupted by grinding the filters with an ice cooled mortar and pestle until the filter was completely disintegrated to a homogeneous slurry. During the grinding 0.5 ml 0,1 M phosphate buffer pH 6 was added. The slurry was then transferred to a 50 mL falcon tube and 4 mL phosphate buffer was added to the falcon tubes. In order to remove all the filter particles, the tubes were first centrifuged for 30 min at 10000 g and 4°C. After the centrifugation the supernatant containing the phycocyanin was vacuum filtered through a clean GF/F filter followed by filtration through a syringe filter with a 0.45µm cellulose acetate membrane filter. The phycocyanin concentration and purity of the sample was then determined with the method described above.

Method 2

Extraction by centrifugation and one freeze-thaw cycle. 10 mL of *A. platensis* biomass sample was harvested and centrifuged in a 15 mL falcon tube for 60 min at 10000 g and 4°C. After the centrifugation the supernatant was decanted away and the cells were re-suspended in 4 mL 0,1M phosphate buffer pH 6 and immediately frozen at -80°C. the samples were exposed to one freeze-thaw cycle of 48 hours each. After the freeze-thaw treatment the samples were centrifuged for 30 min at 10000 g and 4°C and the phycocyanin containing supernatant was extracted and filtered through a syringe filter with a 0.45µm cellulose acetate membrane filter to remove impurities. The phycocyanin concentration and purity of the sample was determined with the method described above.

Method 3

Extraction by centrifugation and two freeze-thaw cycle. The procedure was the same as in method 2 but with two freeze-thaw cycles of 48 hours instead of one.

Method 4

Extraction by centrifugation and three freeze-thaw cycle. Same procedure as in method 2 but with three freeze-thaw cycles of 48 hours instead of one.

Method 5

Extraction by centrifugation and sonication. 10 mL of *A. platensis* biomass sample was harvested and centrifuged in a 15 mL falcon tube for 60 min at 10000 g and 4°C. After the centrifugation the supernatant was decanted away and the cells were re-suspended in 4 mL 0.1 M phosphate buffer pH 6 and sonicated for 60 seconds at 50 W. The samples were immediately frozen at -80°C and exposed to one freeze-thaw cycle of 48 hours. The determination of phycocyanin concentration and purity of the samples were carried out with the same procedure as described above.

3.11 Test growth rate on *Arthrospira platensis* when cultured Auto-, hetero- and mixotrophic.

Objective of experiment

The objective of performing this experiment was to test the effect that acetic acid has on the growth rate of *A. platensis*. To test this *A. platensis* was cultivated autotrophic, heterotrophic and mixotrophic with acetic acid available as organic carbon source for hetero- and mixotrophic cultures.

Experimental setup

The experiment was performed in triplets in 250 mL blue cap bottles under different conditions: (A) autotrophic cultivation with 100 mL BZ medium in each bottle. (B) mixotrophic cultivation 100 mL BZ medium with a concentration of 2 g/L acetic acid in each bottle. (C) heterotrophic cultivation 100 mL BZ medium with 2 g/L acetic acid concentration. The pH was adjusted to around pH 9-9,5 with a 1 M potassium hydroxide (KOH) solution.

They started with 5 mL inoculum with a cell density of 41.14g/L and grown as batch cultures. Culture A and B was installed in an incubator room (temperature 30±2°C) with agitation (180 RPM) and under constant artificial lighting (L2 -LED light panels 839 lux). Culture C was wrapped with aluminium foil and cultivated without light in the incubator room (temperature 30±2°C) with agitation (180 RPM).

They were cultivated for 25 days and cell concentration was monitored every day by measuring the OD_{675 nm} with the method described earlier. At the end of the experiment the growth rate and the phycocyanin concentration was determined in an excel sheet as described earlier.

3.12 Test growth for *Arthrospira platensis* in a medium for *Acetobacterium* supplied with acetate.

Objective of experiment

The objective of this experiment was to test whether *A. platensis* can grow in DSMZ 135a *Acetobacterium* medium supplied with acetic acid in different concentrations (2 g/L, 3 g/L and 4 g/L). This was conducted to simulate the fermentation broth from anaerobic fermentation with *Acetobacterium sp.* (DSM: 2396) and to test whether *A. platensis* can grow in it or not.

The lowest concentration of acetic acid (2 g/L) in this experiment was chosen because *A. platensis* has previously shown to grow well at that concentration. Also I have previously tried to cultivate *Acetobacterium sp.* in DSMZ a135 medium and reach an acetate concentration of 1.9 g/L.

Experimental setup

The experiment was carried out as batch cultivation in 250 mL blue cap bottles and performed in triplets. The medium used in the experiment was DSMZ *Acetobacterium* medium 135a, except resazurin (used to indicate if O₂ is present), Ethylene glycerol (carbon source and I assume that I will be used up in the fermentation broth) and Sodium sulphate (used to help keep the medium anaerobic). The composition of DSMZ 135a medium can be found in table 5, 6 and 7. Each bottle had 100 ml DSMZ 135a medium with different acetic acid concentration: (A) 2 g/L acetic acid, (B) 3 g/L acetic acid and (C) 4 g/L acetic acid. The pH was adjusted to pH 9 with 1 M potassium hydroxide (KOH). 10 mL from three starter cultures was used to start the experiment with cell density: (A) starter culture 45.07 g/L. (B) starter culture 38.25 g/L. (C) starter culture 26.04 g/L.

After inoculation the bottles were placed in an incubator room (temperature 30±2°C) with agitation (180 RPM) and under constant artificial lighting (L2 -LED light panels 839 lux). Growth was monitored by OD_{675 nm} measurement as described earlier.

3.13 Mixotrophic cultivation with different concentration of acetate.

Objective of experiment

The objective of this experiment was to investigate what effect different concentration of acetate will have on the biomass production.

In order to do this, I will try four different concentration of acetate (1 g/L, 2 g/L, 3 g/L and 4 g/L) to find the acetate concentration best suited for mixotrophic cultivation of *A. platensis*.

Experimental setup

The BZ medium was prepared as described in the section culture media but instead of using acetic acid in the medium, 1 M sodium acetate solution was used instead to supply the BZ medium with an organic carbon source for mixotrophic cultivation. This was conducted based on experience from the auto-, hetero and

mixotrophic cultivation experiment with *A. platensis* where acetic acid was used as organic carbon source.

The experiment was performed in triplets and cultivated as batch in 250 mL blue cap bottles with different acetate concentration in the medium and one without acetate: (A) autotrophic culture with 100 mL BZ medium. (B) mixotrophic culture, 100 mL BZ medium with an acetate concentration of 1 g/L. (C) mixotrophic culture, 100 mL BZ medium with a acetate concentration of 2 g/L. (D) mixotrophic culture, 100 mL BZ medium with a acetate concentration of 3 g/L. (E) mixotrophic culture, 100 mL BZ medium with a acetate concentration of 4 g/L. They all started with 10 mL inoculum with a cell density of 52.70 g/L and placed in an incubator room (temperature $30\pm 2^{\circ}\text{C}$) with agitation (230 RPM) and under constant artificial lighting (L2 -LED light panels 839 lux). Growth was monitored by $\text{OD}_{675\text{ nm}}$ measurement as described earlier. At the end of the experiment phycoyanin content from the biomass was determined.

3.14 Test of low illumination within the red and blue light spectra in a batch Photobioreactor setup.

Objective of experiment

The objective of performing this experiment was to test *A. platensis* in BZ medium supplemented with acetate at low light intensities with red and blue light. This was conducted to see what affect mixotrophic cultivation will have on the phycoyanin concentration and the cell density when cultivated at low light intensities.

Experimental setup

The experiment was carried out as batch cultivation in a 2 litre blue cap bottle with 1500 mL BZ medium and an acetate concentration of 1 g/L. The photobioreactor was started with 150 mL starter culture with a cell density of 39.37 g/L. After the inoculation the reactor was placed in an incubator room (temperature $30\pm 2^{\circ}\text{C}$) under constant illumination with red and blue light by L1 lighting platform (349 lux). The photobioreactor was mixed / agitated by pumping atmospheric air through the aquatic phase. The evaporation of water from the aquatic phase in the photobioreactor was adjusted by the addition of sterile water, which was corrected according to marks placed on the side of the reactor. The addition of sterile dH_2O was carried out every day. Liquid samples were collected from the culture broth with a time interval of 48 hours to determine biomass concentration, pH and residual acetate concentration. The experiment ran for 35 days and at the end of the experiment the phycoyanin concentration was determined.

3.15 Test of low illumination within the red light spectra in a batch Photobioreactor setup.

Objective of experiment

This experiment was carried out to test *A. platensis* in BZ medium supplemented with acetate at low light intensities with only red light. This was conducted to see what affect mixotrophic cultivation will have on the phycoerythrin concentration and the cell density when cultivated mixotrophic with only red light and low illumination.

Experimental setup

The experiment was carried out under the same condition as for the batch photobioreactor with red and blue illumination except that in this experimental setup the photobioreactor was illuminated only with red light by L1 lighting platform (175 lux). The photobioreactor was started with a starter culture with cell density 44.34 g/L.

4 Results and discussion

In this section the experimental results and experiences made from the experimental work will be commented on and discussed and will form the basis for the conclusion. All the experimental results and calculations can be found in appendix 2.

4.1 *Acetobacterium sp.* cultivation

The *Acetobacterium sp.* started from a freeze culture and was subsequently cultivated for about 1 month. The purpose of this was to use them as a starter culture for acetic acid production in a larger volume where the effluent from the fermentation could be used to test whether it was suitable as a substrate for a mixotrophic cultivation of *A. platensis*.

Due to many other experimental setups and a bounded amount of time, it was decided to first test *A. platensis* in the 135a medium enriched with acetic acid to see if the media would be inhibitory to *A. platensis* before a fermentation of acetate in a larger volume would be carried out. Therefore, an HPLC analysis from the starter culture was carried out to determine the acetate concentration in the starter cultures. The HPLC analysis shows that the *Acetobacterium* have produced acetate, and that the acetate concentration was 1.9 g/L. This acetate concentration (1.9≈2 g/L) was used to design other experimental setups for mixotrophic cultivation of *A. platensis*.

4.2 Test of growth kinetic for *Arthrospira platensis*

In figure 6, we can see that the growth curve is divided in two by a red dotted line. This is to indicate when an extra LED light source was added to the experimental setup and to indicate two different growth trends, one up to day 16 and one after day 16. The extra light source was added to the experimental setup because after 15 days of cultivation the growth curve resembled a zero order reaction, which could indicate that something was inhibiting the process.

The growth rate increased significantly after day 16 and the extra light source was applied to the experimental setup, which shows that light was the limiting factor and that the L2-LED light panel does not provide enough energy (839 lux) to support *A. platensis* full photosynthetic potential (table. 9).

Sample	$X_{\max 1}$ [g/L]	$X_{\max 2}$ [g/L]	P_1 [g L ⁻¹ /d]	P_2 [g L ⁻¹ /d]	$\mu_{\max 1}$ [d ⁻¹]	$\mu_{\max 2}$ [d ⁻¹]
<i>A. platensis</i>	18.47±1.63	69.88±4.91	1.01±0.09	7.91±1.00	2.87±0.08	3.80±0.08

Table 9 shows the growth parameters for *A. platensis* cultivated in a basal Zarrouk medium.

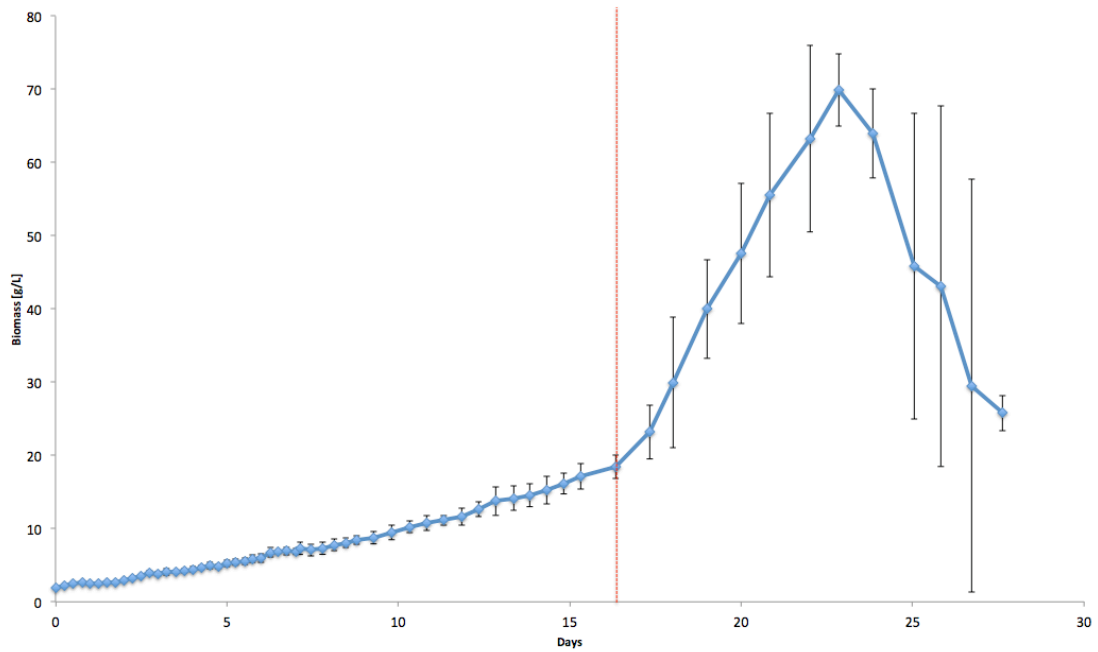


Figure 6. shows the biomass production of *Arthrospira platensis* cultivated in basal Zarrouk medium. The dotted red line indicate day 16 where and extra light source was added.

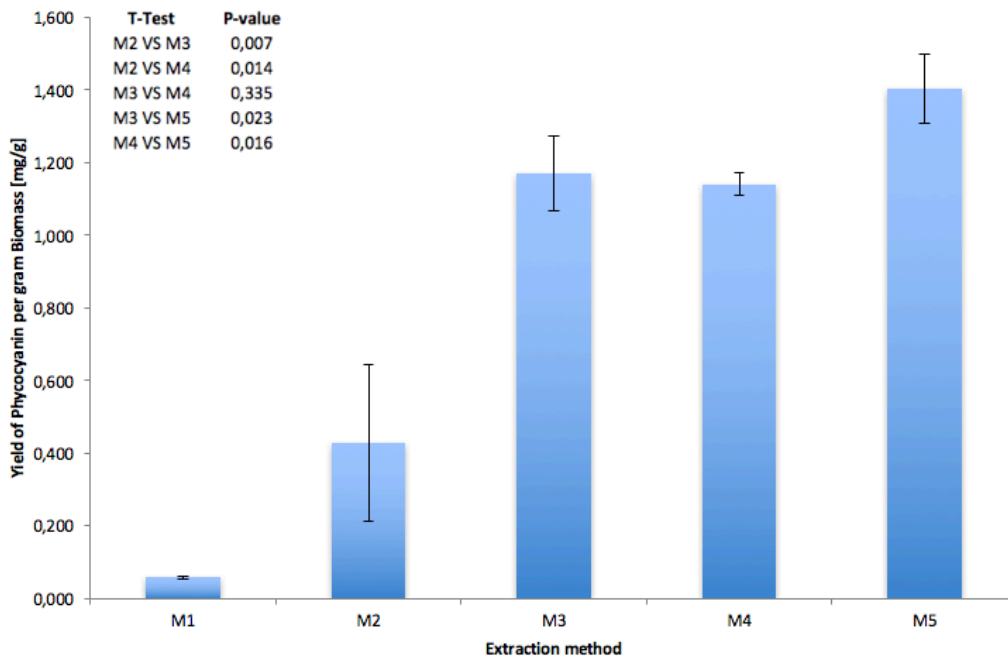


Figure 7 shows the phycocyanin yield from 1 gram biomass [mg PC/g X] from 5 different extraction conditions.

4.3 Test of phycocyanin extraction methods on *Arthrospira platensis*.

During the testing of different extraction methods of phycocyanin from *Arthrospira platensis*, several obstacles emerge along the way and will be discussed in this section. This was carried out to find the most suited method for extracting

phycocyanin from *A. platensis* that would be used to determine the phycocyanin content in the biomass throughout this work.

In figure 7 it can be seen that method M5 (sonication with one freeze-thaw cycle) gave the highest phycocyanin yield per gram biomass (1.402 ± 0.096 [mg/g]) of all the five extraction methods tested, and with a strong statistical evidence (T-Test: $0.01 < p < 0.05$) when tested against method M3 (two freeze-thaw cycles) and M4 (three freeze-thaw cycles) which are the ones closest to M5 (Figure 7). Extraction method M5 had the best results within the 3 parameters (concentration, yield and purity) used to evaluate the 5 extraction methods and was calculated based on the Abs (620 nm and 280 nm) measurement of the extracted phycocyanin samples, which are shown in table 10.

There is no statistical evidence (T-Test: $p > 0.10$) that could support that Method M3 is more efficient than M4 because their results are very close to each other and method M3 has a very large standard deviation (figure 7). However, the results obtain from M3 and M4 could indicate that the third freeze-thaw cycle doesn't have any significant effect on the extraction of phycocyanin and therefore are redundant for the extraction process. The large standard deviations observed for the centrifuged samples is properly caused by difficulty of getting the biomass samples separated into two perfect phases (solid and liquid) by centrifugation and therefore could easily lose some of the biomass when the liquid phase is decanted away. Extraction method M2 had the lowest phycocyanin yield of the centrifuged samples and also a large standard deviation. However, it can be seen from the results that there is a positive effect on the amount of extracted phycocyanin if the biomass samples are exposed to two freeze-thaw cycles instead of one freeze-thaw cycle and with good statistic evidence (T-Test: $0.01 < p < 0.05$)(table 10). However, there is no statistical evidence indicating whether two or three freeze-thaw cycles has the best effect on the amount of extracted phycocyanin (T-Test; $p > 0.10$).

It is clear to see from figure 7 and the results shown in table 10 that extraction of phycocyanin by filtrating (method M1) the biomass samples is not as efficient as centrifuging the samples. This may be caused by the way the filtrated samples are stored under the freeze-thaw cycles. When the biomass samples were filtered through a GF/F filter paper they were subjected to freeze-thaw cycles without a phosphate buffer, which could have caused a degradation of the phycocyanin pigment during the thaw period in the freeze-thaw cycle. A second factor that has a negative effect on the concentration of phycocyanin extracted in method M1 is due to the loss of biomass in the mortar when transferring the slurry (biomass and filter paper) to a centrifuge tube after the mechanical disruption of the GF/F filter papers in the mortar, thereby also contributing to a lower yield.

Sample	Variable	Phycocyanin [mg/mL]	Yield [mg PC/g X]	Purity [A _{620nm} /A _{280nm}]
M1	Filtration	0.003±0.000	0.058±0.004	0.18±0.03
M2	One freeze-thaw cycle	0.019±0.010	0.428±0.217	4.44±0.83
M3	Two freeze-thaw cycles	0.053±0.005	1.171±0.103	7.57±1.57
M4	Three freeze-thaw cycles	0.052±0.001	1.141±0.031	7.33±3.36
M5	Sonication, one freeze-thaw cycle	0.064±0.004	1.402±0.096	8.94±4.82

Table 10 shows the result from the test of 5 extraction methods from a 10 ml biomass samples with a cell density of 45.49 g/L.

Based on the results of the test of extraction methods it can be seen that sonication followed by one freeze-thaw cycle is the most effective method to disrupt the cell and extract the phycocyanin pigment from the biomass (table 10). These results correspond well with the observations from Lawrenz and Horvatha studies where both suggested that one freeze-thaw cycle followed by sonication was the most efficient extraction method for phycocyanin extraction from filamentous cyanobacteria (Horváth et al., 2013; Lawrenz et al., 2011).

The experience and results obtained doing this test of extraction methods were used to construct a protocol for extraction phycocyanin pigments from the *A. platensis* biomass (appendix 1). The extraction protocol was used to extract phycocyanin pigment from the *A. platensis* biomass throughout this project.

4.4 Test growth rate on *Arthrospira platensis* when cultured Auto-, hetero- and mixotrophic.

The growth curve of *Arthrospira platensis* cultivated under three conditions: (A) autotrophic, (B) mixotrophic and (C) heterotrophic are shown in Figure 8.

From the growth curve (Figure 8) it can be seen that the mixotrophic (B) cultures had a lack phase in the beginning of the cultivation (day 0 to day 4), which is most likely due to the acetic acid in the medium and metabolic adjustment to the new substrate. The heterotrophic (C) cultures showed negative or no growth and the small variations on the growth curve (Figure 8) are most probably caused by cell debris floating around in the growth medium. The results for the heterotrophic and mixotrophic cultures correspond well with Richmond and Soeder who write that *A. platensis* is an obligate photoautotroph and cannot grow in the dark with organic carbon sources (heterotroph) but can utilize organic carbon sources when cultivated with light (mixotrophic) (Richmond and Soeder, 1986). The results are also in agreement with Haxthausen who showed that when *Chrorella sorokiniana* was cultivated heterotrophic in the dark with acetate as an organic carbon source, the growth was negative or equal to zero (Haxthausen, 2015). The results do not fit with Marquez results, which showed that *A. platensis* can be cultivated heterotrophic in the dark in a nutrient medium enriched with glucose as organic carbon source

(Marquez et al., 1993). This could indicate that *A. platensis* is able to use glucose (sugar) as carbon source to support growth when cultivated heterotrophic in the dark but not acetate (carboxylic acid) when cultivated without light. As *A. platensis* was able to grow with acetate as a carbon source when cultivated mixotrophic with light but not heterotrophic without light could indicate that *A. platensis* can metabolise sugars into energy but not acetate into energy, and instead store it as cellular carbon instead of metabolising it to CO₂. This is only a presumed conclusion and will require further research in order to provide an answer.

As can be seen from the growth curve (Figure 8) none of the cultures reached a stable growth trend (exponential phase), which is properly caused by the low illumination (839 lux) that also was discussed in test for growth kinetic of *A. platensis*. Therefore, the specific growth rate was calculated by linear regression of the data plots (Figure 8).

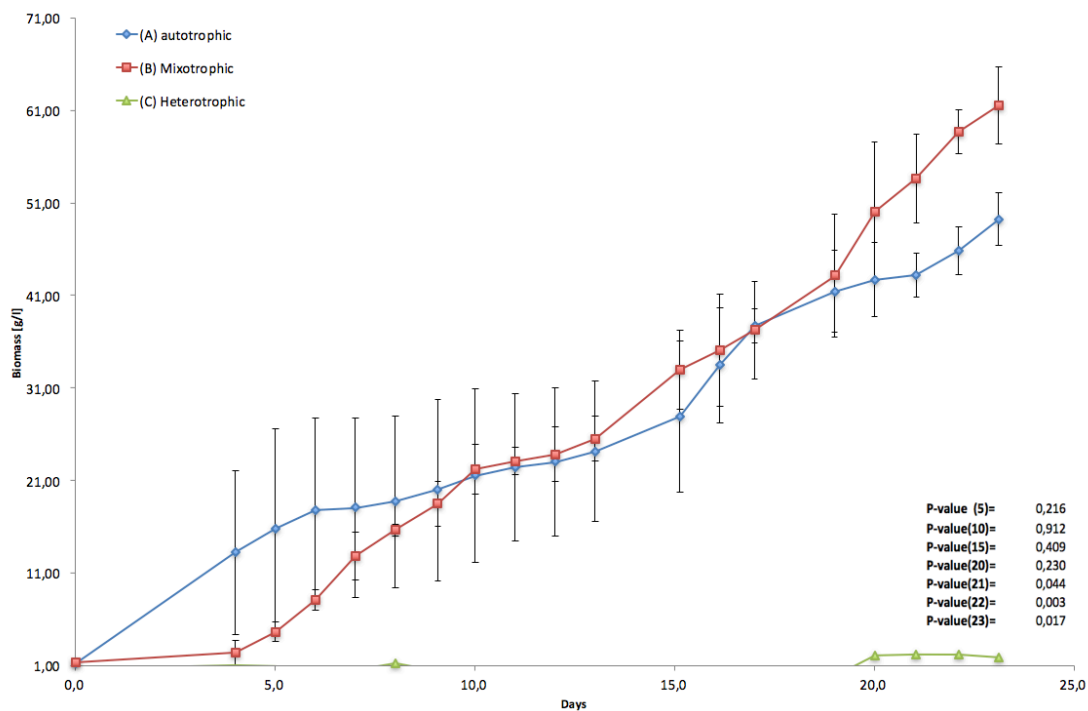


Figure 8 shows biomass production of *Arthrospira platensis* cultivated A) autotrophic, B) mixotrophic (2 g/L acetic acid) and C) heterotrophic (2 g/L acetic acid).

Sample	X_{\max} [g/L]	P [g L ⁻¹ /d]	μ [d ⁻¹]
A	49.23±2.84	2.08±0.12	1.91±0.29
B	61.59±4.16	2.61±0.16	2.78±0.32

Table 11 shows the growth parameters for *Arthrospira platensis* cultivated A) autotrophic and B) mixotrophic. X_{\max} =maximum biomass concentration, μ = specific growth rate, P= productivity.

The mixotrophic (B) cultures had a higher specific growth rate than the autotrophic (A) cultures and with good statistical evidence for the results (T-Test; $p=0,03$) (table 11). These results are in agreement with those from Vonshak who also found that mixotrophic cultivation of *A. platensis* with 2 g/L glucose in the growth medium had a higher grow rate than those cultivated autotrophic. Vonshak also suggested that the mixotrophic cultures required less light for growth, although mixotrophic cultures do not utilize light energy more effectively at low light levels but are able to use more light energy than the autotrophic cultures (Vonshak et al., 2000). As can be seen from the p -values on the growth curve (Figure 8) there is no statistical evidence for the biomass concentration between the autotrophic and mixotrophic cultures before day 21. After 23 days of cultivation the biomass concentration for the mixotrophic (B) cultures was 61.59±4.16 g/L and the autotrophic (A) cultures 49.23 g/L (T-Test; $p=0,02$) (table 11). The average acetate consumption rate for the mixotrophic cultures was 0.021 g/L.

The mixotrophic (B) cultures had a productivity of 2.61±0.16 [g L⁻¹/d], which is approximately 20% higher than the autotrophic (A) cultures and with strong statistical evidence for the result (T-Test; $p=0,01$) (table 11).

In table 12 the results of phycocyanin extraction from the autotrophic and mixotrophic cultures are shown. From the results shown in table 12 it does not seem that mixotrophic cultivation with acetate has an effect on the phycocyanin concentration per gram of biomass and the purity of the phycocyanin is also very high for both samples (table 12). However, the mixotrophic (B) samples have a relatively high standard deviation, although this is probably caused by loss of biomass during the extraction process.

Sample	Phycocyanin [mg/mL]	Yield [mg Pc/g X]	Purity [A_{620nm}/A_{280nm}]
A	0.039±0.002	0.801±0.006	7.89±1.22
B	0.049±0.009	0.798±0.199	10.53±4.73

Table 12 Shows the phycocyanin content in the biomass at day 23. A) autotrophic cultivated in basal Zarrouk medium, B) mixotrophic cultivated in basal Zarrouk medium with 2 g/L acetic acid.

From the results it does not seem as though mixotrophic cultivation with acetate has an effect on phycocyanin content per gram of biomass. However, it does have a significant effect on biomass productivity.

Based on the results it cannot be said whether mixotrophic cultivation will have any effect on the maximum biomass concentration as the experiment only ran for 23 days and therefore it is not known whether the mixotrophic cultures would get a higher biomass concentration than the autotrophic cultures over time.

However, based on the experiment it is concluded that *A. platensis* when cultivated mixotrophically with acetic acid can achieve a high biomass concentration faster than by autotrophic cultivation. These results indicate the potential of acetic acid as an organic substrate for mixotrophic cultivation of *S. platensis* to increase biomass productivity.

4.5 Test growth for *Arthrospira platensis* in a medium used to *Acetobacterium* supplied with acetic acid.

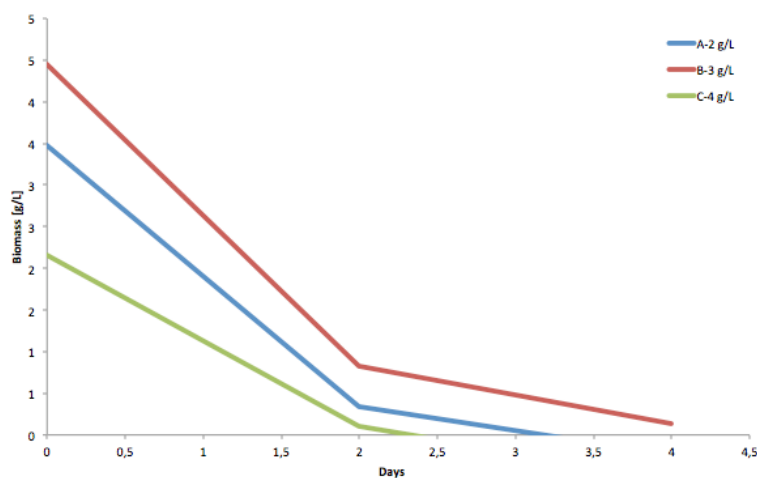


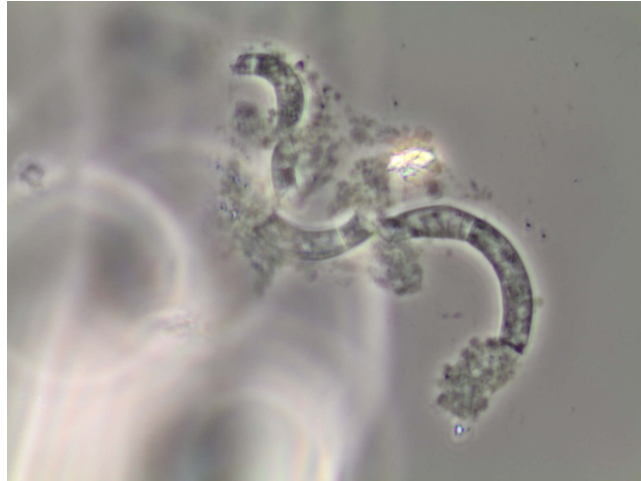
Figure 9 Shows *Arthrospira platensis* tested in DSMZ *Acetobacterium* medium 135a mixed with acetic acid. The figure shows the biomass concentration in 3 different acetic acid concentration.

Figure 9 shows that the *Arthrospira platensis* were unable to grow in DSMZ 135a medium. This could indicate that something in the DSMZ medium has an inhibitory effect on *A. platensis*, since previous experiments with acetic acid in the basal Zarrouk medium have shown that adding acetic acid to the growth medium has a positive effect on growth for *A. platensis* (figure 8).

Therefore, it must be something in the DSMZ medium that *A. platensis* can't tolerate or an absence in the medium since *A. platensis* can't grow. Cysteine-HCl could be the inhibiting factor in the DSMZ medium as it is a relatively strong reductant, however, this would require further investigations to determine whether this is true.

After inoculation the cell concentration dropped. Then after only 2 days of incubation the cells concentration was almost zero for all bottles, which shows that all the cells were dead (figure 9). This can also be seen from picture 5 which shows what believed to be a dead *A. platensis* cell the picture is from one of culture A bottles after 9 days of incubation in DSMZ 135a medium.

Based on this experiment it can be concluded that *A. platensis* cannot grow in the DSMZ Acetobacterium (135a) medium. In order to use the combination to produce acetate with *Acetobacterium st.* and use the effluent to increase the biomass productivity of *A. platensis* the medium used for producing acetate must be modified or a new medium should be used before the effluent can be used to cultivate *A. platensis*.



Picture 5 . Show a filament of *Arthrospira platensis* seen through a microscope (40x) from bottle A (2 g/L acetate) after 9 days cultivation in DSMZ 135a Acetobacterium medium. (photo by T. Rybner).

4.6 Mixotrophic cultivation with different concentration of sodium acetate.

The objective of this experiment was to test four concentrations of acetate (1, 2, 3, 4 [g/L]) in Zarrouk's growth medium in order to find the concentration best suited for mixotrophic cultivation of *A. platensis* measured by growth rate, productivity and maximal biomass concentration. A sodium acetate solution was used instead of acetic acid as it makes it easier to control pH when preparing the growth medium. Due to an error made in the laboratory, the acetate concentration became only one tenth of what was planned so instead of 1 g/L it became 0.1 g/L acetate and so on. Therefore, this experiment cannot be used to determine which acetate concentration was best suited for mixotrophic cultivation of *A. platensis*. Instead the experiment will be used to look at phycocyanin content in the biomass, autotrophic and mixotrophic cultivation and the acetate consumption rate for the mixotrophic cultures.

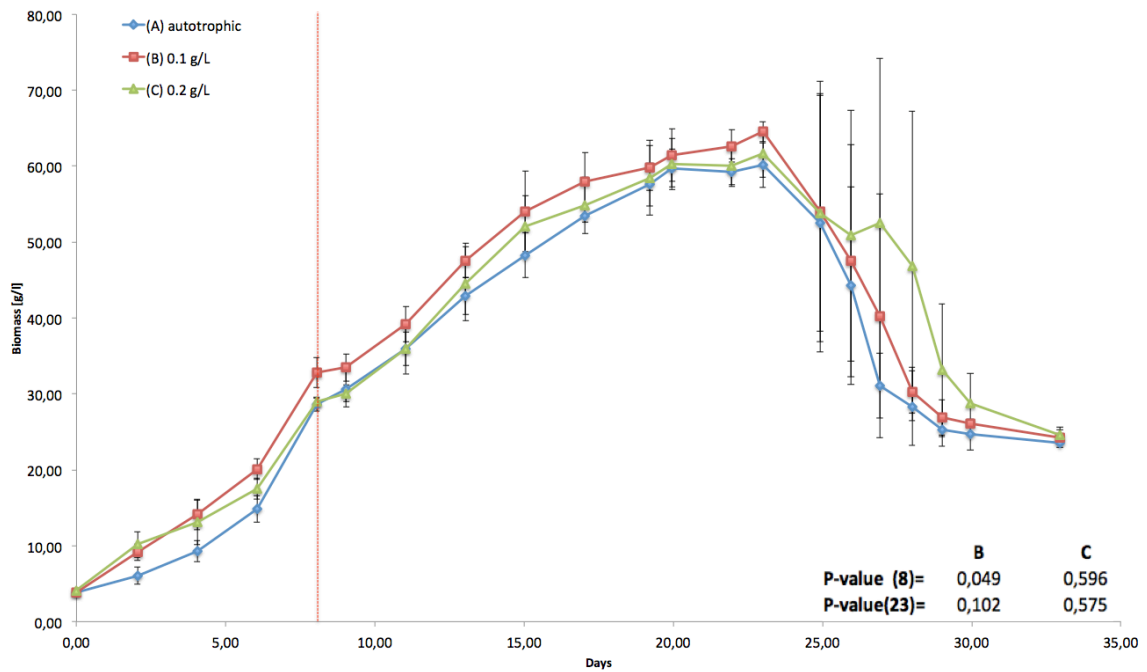


Figure 10 shows the growth curve for *Arthrospira platensis* cultivated autotrophic (A) and mixotrophic (B and C). The vertical red line indicate day 8. The P-values for biomass concentration at day 8 and 23 are shown down in the right corner.

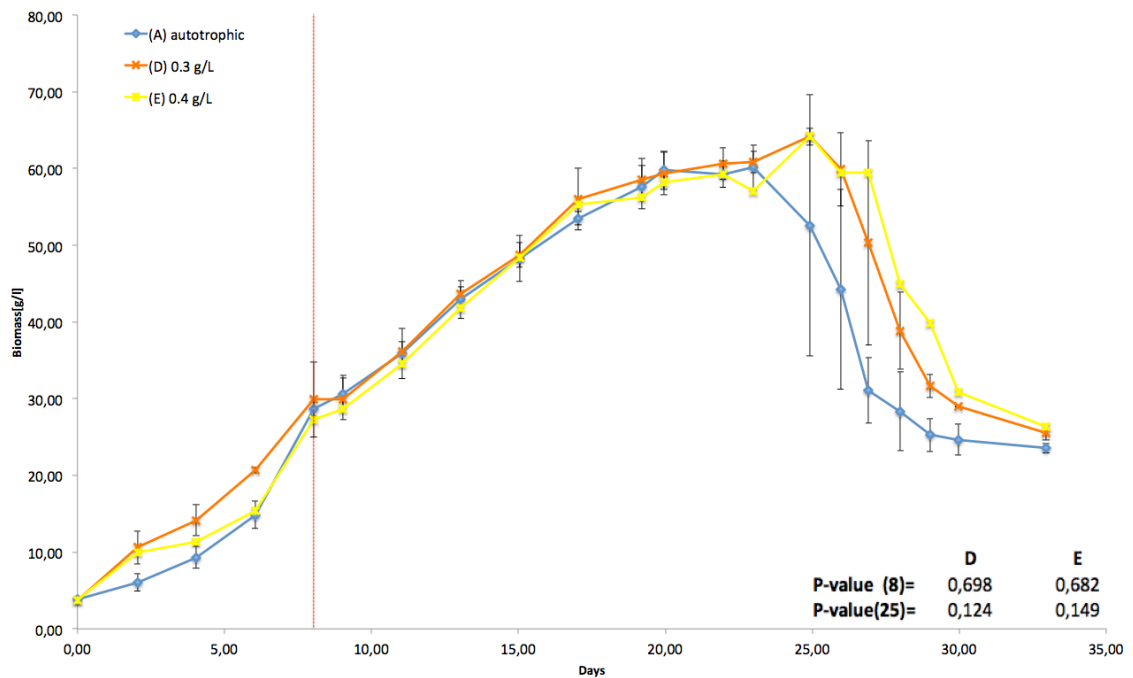


Figure 11 shows the growth curve for *Arthrospira platensis* cultivated autotrophic (A) and mixotrophic (D and E). The vertical red line indicate day 8. The p-values for biomass concentration at day 8 and 25 are shown down in the right corner.

The autotrophic cultures (A) had the lowest growth rate, productivity and achieved the lowest biomass concentration, although the difference to the mixotrophic cultures (B, C, D, E) are very small and the results are all very close (table 13). This is probably because the mixotrophic cultures had too little acetate and therefore the acetate concentration in the growth medium was not enough to make a significant difference on growth.

There was a stop in the growth for all cultures on around day 8, which is indicated by a red dotted line on the growth curves in figure 10 and 11. This is probably caused by difficulty taking an entirely homogeneous sample from the culture bottles and that the samples were taken with only one day apart instead of two days.

Cultures A, B and C had maximum biomass concentrations around day 23 and D and E around day 25 (figure 10 and 11). The biomass concentrations in the cultures differ considerably, which is also reflected in error bars and p -values in figure 10 and 11. This is probably due to difference in growth rate between triplet cultures. The difference in growth rate between samples could be due to an unequal distribution of light between samples because of how they were placed in the shaker during incubation (picture 6). Therefore, the bottles were moved around during cultivation to try to compensate for the unequal light distribution in the shaker.



Picture 6 shows how the bottles were placed in the shaker during cultivation of *A. platensis* with different acetate concentration.

As there was no clear exponential growth phase probably due to low illumination (839 lux) (figure 10 and 11). The specific growth rate was calculated by linear regression of the datasets from day 0 to 20 for the autotrophic cultures (A) and from day 0 to 17 for the mixotrophic cultures (B, C, D, E) (table 13).

Sample	X_{\max} [g/L]	P [g L ⁻¹ /d]	μ [d ⁻¹]
A	60.12±2.91	2.80±0.12	3.04±0.16
B	64.55±1.31	3.18 ±0.23	3.36±0.25
C	61.63±3.13	2.98±0.23	3.15±0.31
D	64.18±1.12	3.07 ±0.25	3.05±0.18
E	64.22±2.70	3.02±0.10	3.09±0.09

Table 13 shows the growth parameters for *Arthrospira platensis* cultivated autotrophic and mixotrophic. X_{\max} =maximum biomass concentration, μ = specific growth rate, P= productivity

The acetate consumption rate for the mixotrophic cultures is shown in figure 12 and was determined by linear regression of acetate concentration VS time. The acetate consumption rate is almost the same for all cultures, respectively B=0.017, C=0.017, D=0.018, E=0.019 day⁻¹. These results could indicate that the consumption rate of acetate for *A. platensis* cultivated mixotrophic depends on the illumination and not the acetate concentration in the medium. However, this will require further investigation.

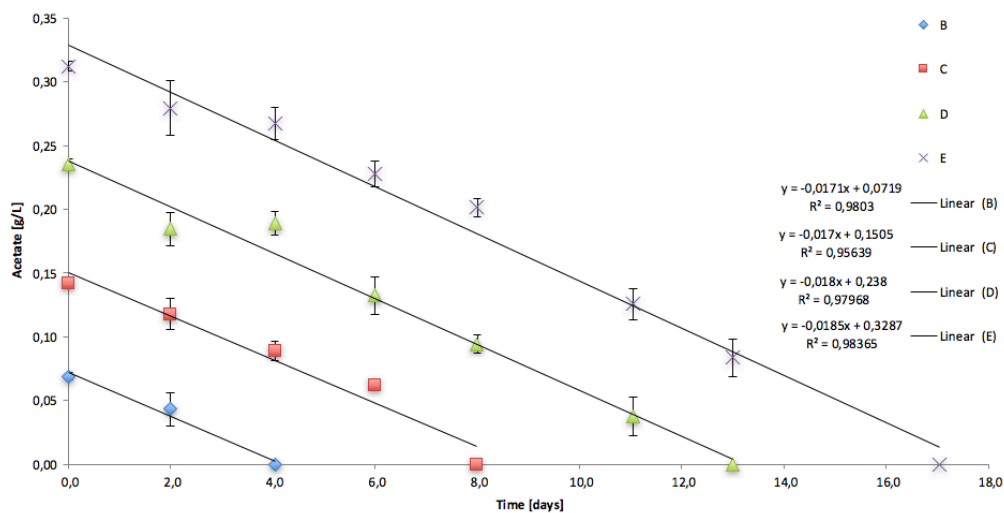


Figure 12 show the rate of acetate consumption by *Arthrospira platensis*

Phycocyanin yield is more or less the same for all cultures, and purity of the phycocyanin samples is also high for all cultures (table 14). The phycocyanin yield was much lower for *A. platensis* in this cultivation than in previous cultivations. The biomass samples were by a mistake only sonicated for 30 seconds at 30 W instead of 60 seconds by 50 W, as they should have been therefore they were sonicated again for 30 seconds at 50 W which may have influenced the results and, therefore the results cannot be used to compare with previous experiments. The difference in phycocyanin concentration per gram biomass and the purity of the samples is more likely due to factor associated to the extraction process than the cultivation conditions.

Sample	Phycocyanin [mg/mL]	Yield [mg Pc/g X]	Purity [A _{620nm} /A _{280nm}]
A	0.020±0.001	0.342±0.022	5.96±0.94
B	0.021±0.001	0.344±0.028	6.84±2.27
C	0.022±0.001	0.359±0.037	6.02±1.75
D	0.023±0.001	0.386±0.009	10.37±5.54
E	0.019±0.001	0.318±0.008	8.55±0.22

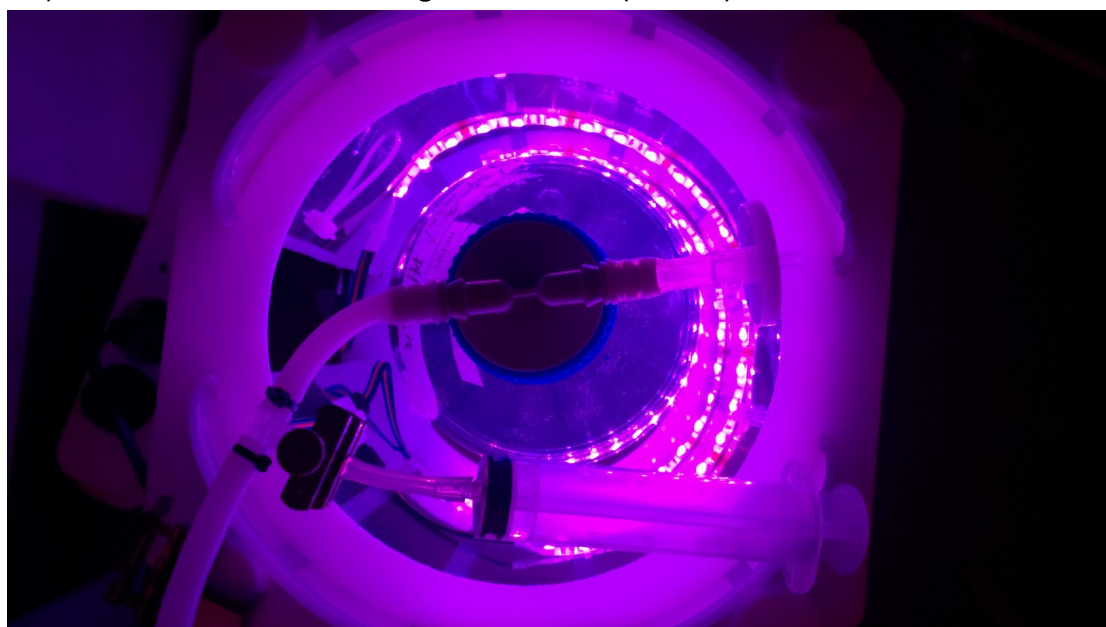
Table 14 Shows the phycocyanin content in the biomass at day 20.

The results of this experiment could indicate that acetate added to the growth medium increases the growth rate, productivity and maximal biomass concentration of *A. platensis*. However, there is no statistical evidence of this statement from these results (T-Test: $p > 0.10$). The results could also indicate that there is a correlation between acetate consumption and light intensity, although it is only circumstantial evidence and will require further investigation.

4.7 Photobioreactor cultivation with low illumination in the red and blue light spectra.

For the photobioreactor experiment the cultivation of *A. platensis* was performed in two experimental setups with different illumination levels, respectively 175 lux at only red light and 349 lux by a combination of both red and blue light.

In this discussion, they will be referred to as RL for reactor at low illumination (175 lux) and RH for reactor at the high illumination (349 lux).



Picture 7 shows the photobioreactor RH and system used to extract biomass samples during cultivation. RH=Reactor high illumination (349 lux) red and blue light spectra. (photo by T. Rybner)

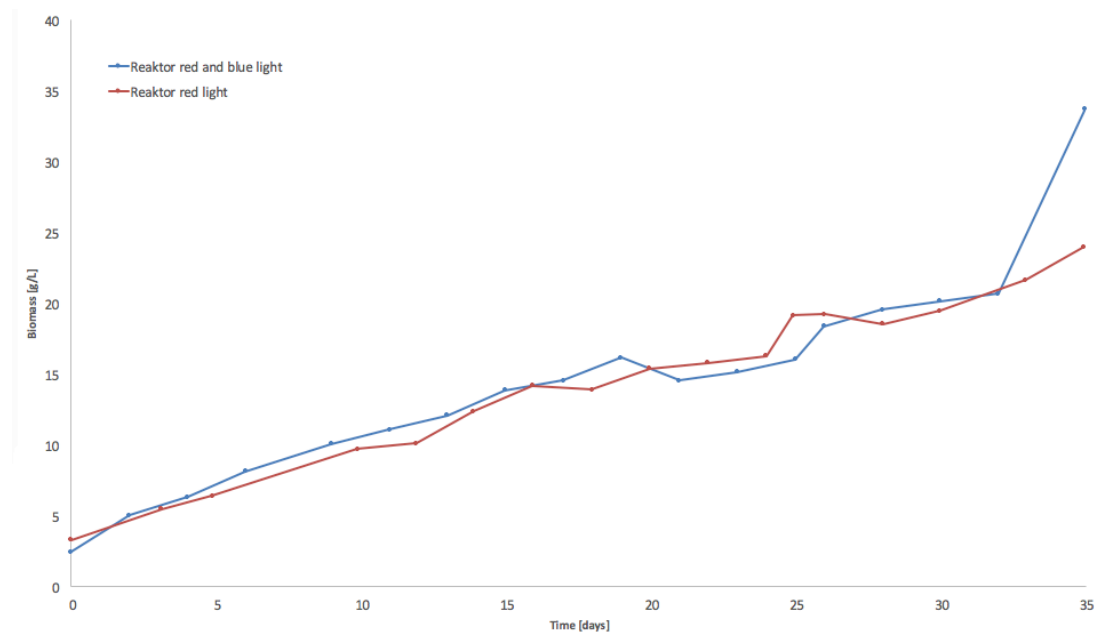


Figure 13 shows the growth curve for *Arthrospira platensis* cultivated mixotrophic with 1 g/L acetate under different illumination. RH=Reaktor high illumination (349 lux) red and blue light spectra, RL=Reaktor low illumination (175 lux) red light spectra.

From the growth curve (figure 13) of the two mixotrophic cultivations of *A. platensis* that is used to illustrate their growth patterns it can be seen that growth was almost identical for both processes.

From the growth curve it can also be seen that by day 33 and until the end of the experiment RH increased suddenly (figure 13). It is difficult to say what the sudden growth in RH was caused by; however, one reason could be the way biomass samples were extracted during cultivation. During cultivation the biomass samples were taken aseptically with a 60 mL syringe through a tube in the lid of the photobioreactor (Picture 7). At the end of the experiment the lid was removed and the biomass sample were taken directly from the photobioreactor. Extraction of samples through the tube may have caused problems of getting a homogenous sample as the biomass density became higher causing the biomass (cells) to clump together and thereby making bigger clumps of biomass that were unable to be extracted through the tube. A large clump of biomass in the sample taken at the end of the experiment can therefore be the reason for the sudden increase in the biomass concentration. Generally it has been difficult throughout this study of *A. platensis* to determine the exact biomass concentration because *A. platensis* tend to clump together making it difficult to get a completely homogenous sample taken. The problem of *A. platensis* clumping together also means that when the biomass concentration is determined by OD measurements in a spectrophotometer, the measurements deviate a lot and also contributes to the uncertainty of the biomass concentration. This could perhaps of been solved by disrupting the cells first by sonication before the OD measurement to ensure a homogeneous sample.

These problems also emphasize the necessity of having an effective circulation in a photobioreactor, which will also ensure a better distribution of nutrients and light energy (photons) to the phototrophic microorganism cultivated.

The importance of circulation and homogeneity in the photobioreactor is also one of the things that Pulz highlights in his review of production systems for phototrophic microorganisms as a very important factor to ensure optimal cultivation conditions in a photobioreactor (Pulz, 2001).

Sample	X_{\max} [g/L]	P [g L ⁻¹ /d]	μ [d ⁻¹]
RH	33.75	0.89	0.62
RL	24.00	0.59	0.56

Table 15 shows the growth parameters for *Arthrospira platensis* cultivated mixotrophic with 1 g/L acetate under different illumination. RH=Reactor high illumination (349 lux) red and blue light spectra, RL=Reactor low illumination (175 lux) red light spectra. X_{\max} =maximum biomass concentration, μ = specific growth rate, P= productivity.

RH achieved the highest biomass concentration of 33.75 g/L where as RL only reached 24.00 g/L (table 15). The highest specific growth rate and productivity was also observed in RH (table 15).

These results are perhaps a little misleading since RH and RL were almost identical until day 33 as seen from biomass concentration measurements (figure 13). However, the actual biomass concentration in RH and RL may have been higher for the same reason as just discussed above about homogeneity of the biomass samples from the photobioreactor. As the photobioreactor experiments were not performed in doublets or triplets, there is no statistical evidence to say whether the results are significant.

Compared with the other cultivation experiments of *A. platensis* performed in this study, the RH and RL experiments achieved only approximately 50% of the biomass concentration that was achieved in the previous autotrophic and mixotrophic cultivation of *A. platensis* at a light intensity of 839 lux (table 9, 11 and 13).

During the experiment, the pH increased in both RH and RL and at the end of the experiment (day 35) the pH value in RH was pH 10.3 and pH 10.4 in RL.

In figure 14, the acetate concentration in RH and RL are shown over time. The acetate consumption rate was determined by linear regression, which is illustrated by the red and blue dotted line in figure14.

The acetate consumption rate is almost the same for *A. platensis* cultivated in RH red and blue light (349 lux) and RL only red light (175 lux). However, the acetate consumption rate was slightly lower at RL (175 lux) than at RH (349 lux), respectively around 0.004 day⁻¹ in RL and 0.005 day⁻¹ in RH (figure 14).

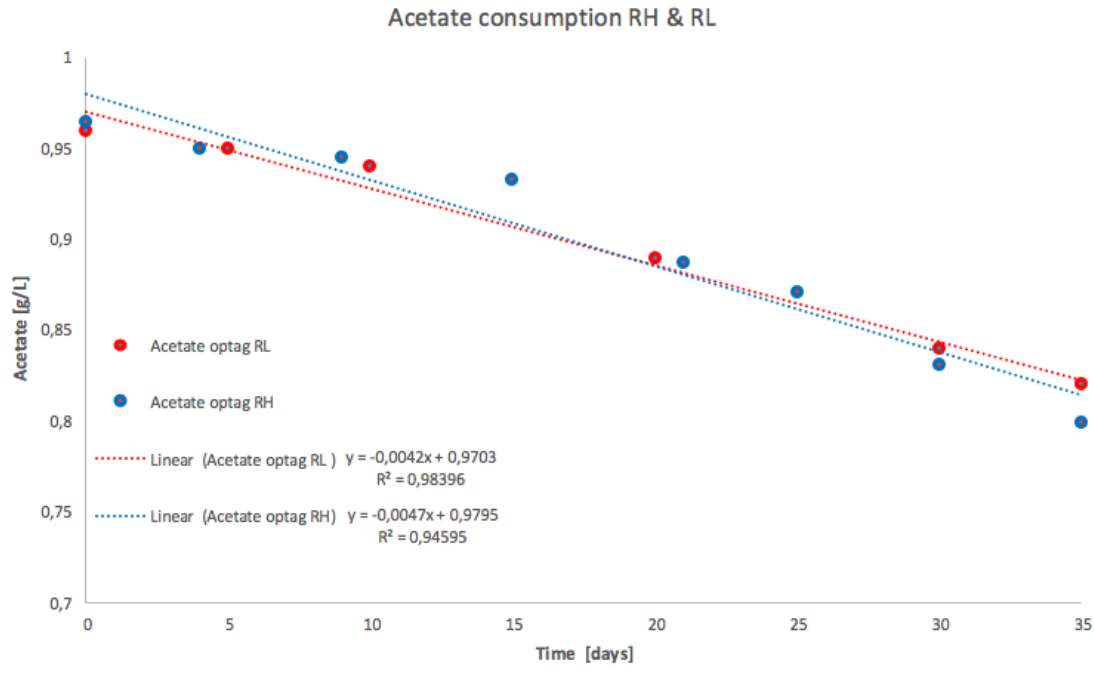


Figure 14 shows the acetate concentration in the photobioreactor overtime. RH=Reactor high illumination (349 lux) red and blue light spectra, RL=Reactor low illumination (175 lux) red light spectra.

Based on the results of acetate consumption rate, maximal biomass concentration and light intensity from the mixotrophic and heterotrophic cultivation experiments conducted in this study, it could indicate that the rate *A. platensis* utilize acetate depends in the light intensity. However, this is just an observation based on acetate concentration in the medium and the light intensity used during the cultivation of *A. platensis* and will therefore require further investigation in order to determine whether this is the case.

As can be seen from table 16, the yield of phycocyanin in mg per gram of biomass is good for both RH and RL compared to previous phycocyanin yield, yet the purity of the phycocyanin samples is not as good as the previous phycocyanin samples (table 12 and 14).

From the experimental results of RH and RL it seems unlikely that there are any positive effects on the phycocyanin content in *A. platensis* when cultivated with only red light as the RL samples is lower than RH samples for both day 30 and day 35 (table 16). According to T-Test performed on the phycocyanin yield results for RH and RL from day 30 and day 35 there is very strong statistical evidence against this observation at day 30 (T-Test: $p < 0.01$) and strong statistical evidence against this observation at day 35 (T-Test: $0.01 < p < 0.05$).

However, based on the results from this experiment it cannot be said whether this is caused by the red light or the difference in light intensity. Although research conducted by Danesi with *A. platensis* showed that lower light intensities increased the pigment content (Danesi et al., 2004). Thus, by making the assumption that the pigment content should be higher at lower light intensities and that red light should

favour phycocyanin I do not think that there is a significant effect on the phycocyanin content, cultivating *A. platensis* with only red light, however, this will require further experiment to confirm.

Sample	Phycocyanin [mg/mL]	Yield [mg PC/g X]	Purity [A _{620nm} /A _{280nm}]
RH (day 30)	0.022±0.000	1.093±0.021	3.25±2.50
RL (day 30)	0.016±0.001	0.822±0.032	2.61±0.60
RH (day 35)	0.030±0.002	0.945±0.065	3.22±0.75
RL (day 35)	0.017±0.000	0.705±0.004	2.68±0.60

Table 16 Shows the results from phycocyanin extraction from *Arthrospira platensis* biomass cultivated mixotrophic with 1 g/L acetate under different illumination at day 30 and day 35. RH=Reactor high illumination (349 lux) red and blue light spectra, RL=Reactor low illumination (175 lux) red light spectra.

The biomass samples were taken from RL to determine the phycocyanin content in the biomass at day 20, 25, 30 and 35. This was conducted to see if there is a change in phycocyanin content in *A. platensis* during cultivation (table 17). As can be seen from table 17, there is no significant change in the phycocyanin content per gram biomass, which is around 0.8 mg phycocyanin per gram biomass for all samples. The difference between the samples is very small and is probably caused by varying efficiency of the extraction process (table 17).

This is also supported by a T-Test performed on the phycocyanin yield of *A. platensis* from RL, which showed that the phycocyanin yield was not significantly (T-Test: $p>0.10$) influenced over time except for day 35 (T-Test: $p<0.01$). These results indicate that the phycocyanin content is constant or only change little in *A. platensis* during cultivation.

However, this observation conflicts with Xie who reported that by fed-batch cultivation of *A. platensis* the phycocyanin content increased over time but after a while decreased again, which was attributed to the lower cell viability associated with the extended cultivation time (Xie et al., 2015). This could explain why phycocyanin yield is higher for both RH and RL at day 30 than at day 35, even though the biomass concentration is higher at day 35 for both cultures. Whether this is the case or whether it is due to other factors, such as loss of biomass doing extraction is not known.

Sample	Phycocyanin [mg/mL]	Yield [mg PC/g X]	Purity [A _{620nm} /A _{280nm}]
RL (day 20)	0.012±0.000	0.806±0.017	1.89±0.53
RL (day 25)	0.016±0.001	0.835±0.036	2.48±0.48
RL (day 30)	0.016±0.001	0.822±0.032	2.61±0.60
RL (day 35)	0.017±0.000	0.705±0.004	2.68±0.60

Table 17 Shows the results from phycocyanin extraction from *Arthrospira platensis* biomass cultivated mixotrophic with 1 g/L acetate under low illumination (175 lux) red light spectra.

5 Conclusion

From this study of mixotrophic cultivation of *A. platensis* using acetate as organic carbon source it seems unlikely that acetate has an effect on the phycocyanin content in the biomass. The results in the study also indicate that the phycocyanin content in *A. platensis* is constant or only changes little during cultivation, yet does change with different light intensities.

Furthermore, this study shows that there is no or little effect on the phycocyanin content when cultivating *A. platensis* with only red light, however, it would seem that there is a correlation between a high cell density and lower phycocyanin content. Although, this it is not possible to determine from this study, as there are many uncertain factors that may be affecting the results.

The best results for extraction of phycocyanin were achieved by disrupting the cell with sonication followed by one freeze-thaw cycle, which is in agreement with what similar studies have shown.

Results from the mixotrophic cultivation of *A. platensis* with acetate in the growth medium showed that when cultivated at light intensities of 839 lux or lower *A. platensis* didn't reach a stable growth pattern, indicating that light intensities are still a key limiting factor for growth of *A. platensis*. The study also indicated that *A. platensis*'s ability to utilize acetate as organic carbon source is dependent on light energy, since when cultivated at lower light intensities the acetate consumption rate decreased. Another factor contributing to this observation was that *A. platensis* wasn't able to grow heterotrophic with acetate. However, *A. platensis* in similar studies with glucose and amylase in the growth medium have been shown to grow heterotrophic.

It was not possible to cultivate *A. platensis* in an *Acetobacterium* medium enriched with acetate. If this had been possible, it could be used for converting surplus output electricity from wind turbines to high value products such as phycocyanin if the electricity was converted to hydrogen, which could be used to feed *Acetobacterium* to produce acetate and use the effluent for mixotrophic cultivation of *A. platensis*. In this way, surplus production from wind turbines could be used to produce a high value product like phycocyanin. However, based on this study a new media for *Acetobacterium* would have to be used in order to fulfil this idea.

From this study it cannot be said whether mixotrophic cultivation with acetate can increase the maximum biomass concentration of *A. platensis*.

However, mixotrophic cultivation of *A. platensis* with acetate can increase productivity and in this way contribute to make it more profitable to produce phycocyanin by reducing cultivation time.

The economy of phycocyanin production with *A. platensis* could further be improved by selling the solid fraction (cell debris) that is left after the phycocyanin is extracted. The solid fraction containing cell debris is very rich on protein and therefore could be sold as food supplements, animal feed, substrate for biogas plants or used on an

onsite biogas plant to produce electricity, which could lower energy cost for the production facility.

Finally, the results from this study indicate acetate potential as an organic carbon source for mixotrophic cultivation of *A. platensis* and that acetate is suitable as a substrate for production of high value products such as phycocyanin.

6 Perspective

For future work with *A. platensis* it could be interesting to look at fed-batch and continuous cultivation since this is an area where it might be possible to increase the productivity and maximal biomass concentration even more (Xie et al., 2015).

Subsequently, it could also be interesting to upscale the process and look more into photobioreactor designs since shape, circulation, and illumination seem to have a huge impact on all growth parameters involved in a successful cultivation and that the parameters will properly change when upscaling to a higher volume. Therefore, this is an area where it really is possible to make improvements that could really make a difference and make phycocyanin production more economical feasible (Carvalho et al., 2011; Pulz, 2001).

Based on the experience obtained doing this study it could also be interesting to look more into extraction methods as it ultimately comes down to how much phycocyanin can be extracted from the biomass, which could make it easier in future work to determine the actual phycocyanin content in the biomass. A major challenge in the extraction of phycocyanin in this study has been to isolate the biomass without loss. This could perhaps be improved by using a membrane instead of centrifugation to separate the biomass from the liquid where it has been difficult by centrifugation to establish a clear phase separation between the liquid and biomass that has resulted in a loss of biomass. If a membrane could be used to separate the liquid from the biomass and subsequently washing the biomass off the membrane with the phosphate buffer used in the extraction process of phycocyanin, the loss of biomass would be minimized ensuring a homogeneity between samples. It could also be interesting to further optimize the efficiency of the sonication by testing different sonication time and the intensity (watt) of the sonication in order to find the optimal combination.

In connection with this, it would also be relevant to find a better method to extract more homogenous biomass samples and measuring the biomass concentration with optical density (OD). A better circulation/ stirring in the growth medium might ensure more homogeneity and extraction of a larger volume of biomass that could help achieve a more accurate sample. The problems associated with OD measurements could perhaps be reduced if the biomass samples were sonicated before OD measurements is performed in order to insure more stable results.

Based on the results from mixotrophic cultivation of *A. platensis* with acetate in the growth medium, it could be relevant to look further into how *A. platensis* utilize acetate under different light conditions as the results from this study indicate that there was a connection between acetate consumption and light intensities. In addition, further work in this context could also look at how acetate is incorporated in the TCA-cycle and how *A. platensis* utilize different carbon sources because it was not possible from this study to explain why *A. platensis* could not be cultivated heterotrophic with acetate as organic carbon source but in similar studies have shown to growth heterotrophic with glucose ([Marquez et al., 1993](#)). It could also be interesting to further investigate how the surplus production from wind turbines could be converted to acetate that could support a mixotrophic cultivation of *A. platensis*. It will be necessary to investigate why *A. platensis* did not grow in the *Acetobacterium* medium and how this inhibition could be managed.

Finally, it would also be interesting to look further into the effect of different light spectres and light intensities and the effect that this has on the pigment content in *A. platensis*. In order to see how different wavelengths and intensities of light would affect the pigment content of the pigments involved in light harvesting and not only on the phycocyanin content.

7 List of references

- AGICAL** (2013). AGICAL. URL: <http://www.agical.eu/technology.php>, Data Accessed:15.5.2016.
- Andrade, M.R., and Costa, J.A.V.** (2007). Mixotrophic cultivation of microalga *Spirulina platensis* using molasses as organic substrate. *Aquaculture* **264**, 130–134.
- Antelo, F.S., Anschau, A., Costa, J. a V, and Kalil, S.J.** (2010). Extraction and Purification of C-phycoyanin from. *Journal of the Brazilian Chemical Society* **21**, 921–926.
- Barsanti, L., Coltelli, P., Evangelista, V., Frassanito, A.M., Passarelli, V., Vesentini, N., and Gualtieri, P.** (2008). Oddities and Curiosities in the Algal World. In NATO Security through Science Series C: Environmental Security, pp. 353–391.
- Beardall, J., and Raven, J.A.** (2013). Limits to Phototrophic Growth in Dense Culture: CO₂ Supply and Light. In *Algae for Biofuels and Energy*, M.A. Borowitzka, and N.R. Moheimani, eds. (Dordrecht: Springer Netherlands), pp. 91–97.
- Bennekom** (2014). Bennekom, AlgaePARC. URL: <http://www.solartours.nu/2014/algaeparc/>, Data Accessed:15.5.2016.
- Borch, M.M.** (2011). Screening and optimization of case specific sustainable mixotrophic microalgae medium. Technical university of Denmark – DTU.
- Borowitzka, M.A.** (2013). High-value products from microalgae—their development and commercialisation. *Journal of Applied Phycology* **25**, 743–756.
- Brennan, L., and Owende, P.** (2010). Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **14**, 557–577.
- Carvalho, A.P., Silva, S.O., Baptista, J.M., and Malcata, F.X.** (2011). Light requirements in microalgal photobioreactors: An overview of biophotonic aspects. *Applied Microbiology and Biotechnology* **89**, 1275–1288.
- Chen, F.** (1996). High cell density culture of microalgae in heterotrophic growth. *Trends in Biotechnology* **14**, 421–426.
- Chen, F., and Zhang, Y.** (1997). High cell density mixotrophic culture of *Spirulina platensis* on glucose for phycocyanin production using a fed-batch system. *Enzyme and Microbial Technology* **20**, 221–224.
- Chen, C.Y., Kao, P.C., Tsai, C.J., Lee, D.J., and Chang, J.S.** (2013). Engineering strategies for simultaneous enhancement of C-phycoyanin production and CO₂ fixation with *Spirulina platensis*. *Bioresource Technology* **145**, 307–312.
- Chen, T., Zheng, W., Yang, F., Bai, Y., and Wong, Y.S.** (2006). Mixotrophic culture of high selenium-enriched *Spirulina platensis* on acetate and the enhanced production of photosynthetic pigments. *Enzyme and Microbial Technology* **39**, 103–107.

- Chojnacka, K., and Noworyta, A.** (2004). Evaluation of *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. *Enzyme and Microbial Technology* **34**, 461–465.
- Ciferri, O.** (1983). *Spirulina*, the edible microorganism. *Microbiological Reviews* **47**, 551–578.
- Danesi, E.D.G., Rangel-Yagui, C.O., Carvalho, J.C.M., and Sato, S.** (2004). Effect of reducing the light intensity on the growth and production of chlorophyll by *Spirulina platensis*. *Biomass and Bioenergy* **26**, 329–335.
- DSMZ, Z.D. sammlung von mikroorganismen und** (2016). Details: DSM-2396. URL: https://www.dsmz.de/catalogues/details/culture/DSM-2396.html?tx_dsmzresources_pi5%5BreturnPid%5D=304, Date Accessed: 9.6.2016.
- Energinet.dk** (2011). Status for lagringsteknologier i Danmark. URL: <http://www.energinet.dk/SiteCollectionDocuments/Danske dokumenter/Forskning - PSO-projekter/Lagring af energi.pdf>, Date Accessed: 6.6.2016.
- Eriksen, N.T.** (2008). Production of phycocyanin - A pigment with applications in biology, biotechnology, foods and medicine. *Applied Microbiology and Biotechnology* **80**, 1–14.
- Faunce, T. a., Lubitz, W., Rutherford, a. W. (Bill), MacFarlane, D., Moore, G.F., Yang, P., Nocera, D.G., Moore, T. a., Gregory, D.H., Fukuzumi, S., Yoon, K.B., Armstrong, F. a., Wasielewski, M.R., and Styring, S.** (2013). Energy and environment policy case for a global project on artificial photosynthesis. *Energy & Environmental Science* **6**, 695.
- Gao, X., Sun, T., Pei, G., Chen, L., and Zhang, W.** (2016). Cyanobacterial chassis engineering for enhancing production of biofuels and chemicals. *Applied Microbiology and Biotechnology* **34**, 3401–3413.
- Gupta, R.** (2007). Biotechnology of mass production of *Spirulina* with special reference to the biological compounds. Bundelkhand University.
- Harun, R., Singh, M., Forde, G.M., and Danquah, M.K.** (2010). Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews* **14**, 1037–1047.
- Haxthausen, K.R.** (2015). Optimizing culturing parameters of microalgae , for increased yield of target. Technical university of Denmark – DTU.
- Hill, G.A., and Robinson, C.W.** (1974). Measurement of aerobic batch culture maximum specific growth rate and respiration coefficient using a dissolved oxygen probe. *Biotechnology and Bioengineering* **16**, 531–538.
- Horváth, H., Kovács, A.W., Riddick, C., and Présing, M.** (2013). Extraction methods for phycocyanin determination in freshwater filamentous cyanobacteria and their application in a shallow lake. *European Journal of Phycology* **48**, 278–286.

- Johnson, J.D.** (2006). The Origin of Life - the rise of the Oxygen Evolving Complex | Florida State University. URL: <http://www.chm.bris.ac.uk/motm/oec/motm.htm>, Date Accessed: 11.5.2016.
- Koc, C., Anderson, G.A., and Kommareddy, A.** (2013). Use of Red and Blue LEDs and Fluorescent Lamps as Light Sources to Grow Microalgae in a Photobioreactor (PBR). *The Israeli Journal of Aquaculture* 8.
- Kuddus, M., and Ramteke, P.W.** (2012). Recent developments in production and biotechnological applications of cold-active microbial proteases. *Critical Reviews in Microbiology* 38, 330–338.
- Lawrenz, E., Fedewa, E.J., and Richardson, T.L.** (2011). Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extracts. *Journal of Applied Phycology* 23, 865–871.
- Leema, J.T.M., Kirubakaran, R., Vinithkumar, N. V, Dheenan, P.S., and Karthikayulu, S.** (2010). High value pigment production from *Arthrospira* (*Spirulina*) *platensis* cultured in seawater. *Bioresource Technology* 101, 9221–9227.
- Leu, S., and Boussiba, S.** (2014). Advances in the Production of High-Value Products by Microalgae. *Industrial Biotechnology* 10, 169–183.
- Madigan, M.T., Martinko, J.M., Stahl, D.A., and Clark, D.P.** (2012). Brock Biology of Microorganisms, 13th Edition (Pearson Education, Inc). ISBN: 0-321-73551-x
- Marquez, F.J., Sasaki, K., Kakizono, T., Nishio, N., and Nagai, S.** (1993). Growth characteristics of *Spirulina platensis* in mixotrophic and heterotrophic conditions. *Journal of Fermentation and Bioengineering* 76, 408–410.
- Marquez, F.J., Nishio, N., Nagai, S., and Sasaki, K.** (1995). Enhancement of biomass and pigment production during growth of *Spirulina platensis* in mixotrophic culture. *Journal of Chemical Technology AND Biotechnology* 62, 159–164.
- Massa, G.D., Kim, H.H., Wheeler, R.M., and Mitchell, C.A.** (2008). Plant productivity in response to LED lighting. *HortScience* 43, 1951–1956.
- Mogensen, M.B.** (2015). Elektrolyse gør al energi fra vindmøller værdifuld | Videnskab.dk. URL: <http://videnskab.dk/miljo-naturvidenskab/elektrolyse-gor-al-energi-fra-vindmoller-vaerdifuld>, Date Accessed: 6.6.2016.
- Mühling, M.** (2000). CHARACTERIZATION OF ARTHROSPIRA (SPIRULINA) STRAINS. University of Durham.
- Neslen, A.** (2015). Wind power generates 140% of Denmark’s electricity demand | Environment | The Guardian. URL: <http://www.theguardian.com/environment/2015/jul/10/denmark-wind-windfarm-power-exceed-electricity-demand>, Date Accessed: 6.6.2016.
- Ogbonda, K.H., Aminigo, R.E., and Abu, G.O.** (2007). Influence of temperature and pH on biomass production and protein biosynthesis in a putative *Spirulina* sp. *Bioresource Technology* 98, 2207–2211.

- Pulz, O.** (2001). Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology* **57**, 287–293.
- Richmond, A.E., and Soeder, C.J.** (1986). Microalgaculture. *Critical Reviews in Biotechnology* **4**, 369–438.
- Rito-Palomares, M., Nuñez, L., and Amador, D.** (2001). Practical application of aqueous two-phase systems for the development of a prototype process for c-phycocyanin recovery from *Spirulina maxima*. *Journal of Chemical Technology & Biotechnology* **76**, 1273–1280.
- Rivkin, R.B.** (1989). Influence of irradiance and spectral quality on the carbon metabolism of phytoplankton. I. Photosynthesis, chemical composition and growth. *Marine Ecology Progress Series* **55**.
- Sánchez, M., Bernal-Castillo, J., Rozo, C., and Rodríguez, I.** (2003). *Spirulina* (arthrospira): an edible microorganism: a review. *Universitas Scientiarum* **8**, 7–24.
- Schlesinger, P., Belkin, S., and Boussiba, S.** (1996). SODIUM DEPRIVATION UNDER ALKALINE CONDITIONS CAUSES RAPID DEATH OF THE FILAMENTOUS CYANOBACTERIUM *SPIRULINA PLATENSIS*1. *Journal of Phycology* **32**, 608–613.
- Singh, S., Kate, B.N., and Banerjee, U.C.** (2005). Bioactive compounds from cyanobacteria and microalgae: an overview. *Critical Reviews in Biotechnology* **25**, 73–95.
- Skjånes, K., Rebours, C., and Lindblad, P.** (2013). Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. *Critical Reviews in Biotechnology* **33**, 172–215.
- Stal, L.J., and Moezelaar, R.** (1997). Fermentation in cyanobacteria. *FEMS Microbiology Reviews* **21**, 179–211.
- Stanier, R.Y., Pfennig, N., and Trüper, H.G.** (1981). Introduction to the Phototrophic Prokaryotes. In *The Prokaryotes*, (Berlin, Heidelberg: Springer Berlin Heidelberg), pp. 197–211.
- Ting, C.S., Rocap, G., King, J., and Chisholm, S.W.** (2002). Cyanobacterial photosynthesis in the oceans: the origins and significance of divergent light-harvesting strategies. *Trends in Microbiology* **10**, 134–142.
- Ugwu, C.U., Aoyagi, H., and Uchiyama, H.** (2008). Photobioreactors for mass cultivation of algae. *Bioresource Technology* **99**, 4021–4028.
- Vonshak, A., and Richmond, A.** (1988). Mass production of the blue-green alga *Spirulina*: An overview. *Biomass* **15**, 233–247.
- Vonshak, A., Cheung, S.M., and Chen, F.** (2000). MIXOTROPHIC GROWTH MODIFIES THE RESPONSE OF *SPIRULINA* (*ARTHROSPIRA*) *PLATENSIS* (*CYANOBACTERIA*) CELLS TO LIGHT. *Journal of Phycology* **36**, 675–679.

- Walter, A., Carvalho, J.C. de, Soccol, V.T., Faria, A.B.B. de, Ghiggi, V., and Soccol, C.R.** (2011). Study of phycocyanin production from *Spirulina platensis* under different light spectra. *Brazilian Archives of Biology and Technology* **54**, 675–682.
- Watson, E.** (2013). Could algae be the next big thing in the protein market? Part two: Aurora Algae. URL: <http://www.foodnavigator-usa.com/Markets/Could-algae-be-the-next-big-thing-in-the-protein-market-Part-two-Aurora-Algae>, Date Accessed: 15.5.2016.
- Wijffels, R.H., Kruse, O., and Hellingwerf, K.J.** (2013). Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. *Current Opinion in Biotechnology* **24**, 405–413.
- WIKI, B.** (2014). Main Page/BIOL 4160/Absorbing Light - Biology Wiki. URL: http://biologywiki.apps01.yorku.ca/index.php?title=Main_Page/BIOL_4160/Absorbing_Light, Date Accessed: 13.5.2016.
- Wittrup, S.** (2013). Glem lagring af vindmøllestrøm: Brint via elektrolyse er fremtiden | Ingeniøren. URL: <https://ing.dk/artikel/glem-lagring-af-vindmoellestroem-brint-elektrolyse-er-fremtiden-136632>, Date Accessed: 6.6.2016.
- Xie, Y., Jin, Y., Zeng, X., Chen, J., Lu, Y., and Jing, K.** (2015). Fed-batch strategy for enhancing cell growth and C-phycocyanin production of *Arthrospira* (*Spirulina*) *platensis* under phototrophic cultivation. *Bioresource Technology* **180**, 281–287.
- Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., and Takriff, M.** (2014). An overview: biomolecules from microalgae for animal feed and aquaculture. *Journal of Biological Research-Thessaloniki* **21**, 6.
- Yam, F.K., and Hassan, Z.** (2005). Innovative advances in LED technology. *Microelectronics Journal* **36**, 129–137.
- Yeomans, M.** (2013). New photobioreactor makes microalgae cultivation easier for the industry. URL: <http://www.cosmeticsdesign-europe.com/Formulation-Science/New-photobioreactor-makes-microalgae-cultivation-easier-for-the-industry>, Date Accessed: 15.5.2016.
- Zarrouk, C.** (1966). Contribution a l etude d une cyanophycee influence de divers facteurs physiques et chimiques sur la croissance et la photosynthese de *Spirulina maxima* (Setch et Gardner) Geitler. Universite de Paris.
- Zeng, X., Danquah, M.K., Zhang, S., Zhang, X., Wu, M., Chen, X.D., Ng, I.S., Jing, K., and Lu, Y.** (2012). Autotrophic cultivation of *Spirulina platensis* for CO₂ fixation and phycocyanin production. *Chemical Engineering Journal* **183**, 192–197.
- Zhang, X.-W., Zhang, Y.-M., and Chen, F.** (1999). Application of mathematical models to the determination optimal glucose concentration and light intensity for mixotrophic culture of *Spirulina platensis*. *Process Biochemistry* **34**, 477–481.

8 Appendices

8.1 Appendix 1. Extraction protocol for phycocyanin

Extraction of phycocyanin with centrifugation and sonication:

1. extract 5-10 mL biomass and transfer to 15 ml falcon tubes and centrifuge them for 60 min at 10,000 g(RCF) at 4 °C and remove the supernatant (use a 1000 µL pipet to remove the floating cells from the supernatant and pour the rest of the supernatant away)
2. Resuspend the biomass in 5 mL phosphate buffer pH 6
3. Sonicate the biomass in 60 second at 50 watt
4. Freeze at -80°C until further treatment (min 2 hours)
5. Perform one freeze-thaw cycle of 48 hours at 5°C
6. Centrifuge again for 60 min at 10,000 g(RCF) at 4 °C and remove the supernatant containing the phycocyanin
7. Remove remaining impurities by filtrating the supernatant through a clean syringe filter (pore size 0.45 µm cellulose acetate membrane)
8. The phycocyanin concentration in the supernatant can now be determined using spectrophotometer and the formula below

Spectrophotometric quantification:

1. Absorbance (Abs) from the purified extracts is measured at 620 nm in a photospectrometer in 1 cm quartz glass cuvettes against the phosphate buffer as blank.
2. Purity of the extraction sample is determined from the ratio between the absorbance measured at 620 nm and 280 nm (purity=Abs_{620nm}/Abs_{280nm})
3. The phycocyanin concentration can be calculated with the following equation which was found by making a calibration curve from a purchased phycocyanin sample from Sigma Aldrich

4.
$$\text{Phycocyanin}(mg/mL) = \frac{\text{Abs}_{620} + 0.016}{74.721} \times \frac{V_{\text{sample}}}{V_{\text{buffer}}}, R = 0,99$$

8.2 Appendix 2. Experimental data and calculations

As this is an education with focus on sustainability the excel sheet was not printed on paper but instead available electronically on a USB-Drive attached to the report or downloaded from the link below:

<https://www.dropbox.com/sh/64a0r67itv92q6v/AADiavI6-ICPK7FvnBbMFZkua?dl=0>