FACULTY OF SCIENCE AND TECHNOLOGY

MASTER'S THESIS

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Abstract

In recent years, microalgal biotechnology has experienced significant growth across multiple industry sectors due to the versatility of various microalgae species. Manny applications for both microalgae themselves and microalgae products derived from algal biomass are mentioned in this thesis. However, the economic inefficiency and difficulties in commercial scale-up production arise from the high costs of chemical nutrient media used for algal cultivation. Piggery wastewater offers an alternative to commonly used chemicals, like Bolds basal media, and contain much of the nutrients needed for algal growth.

This thesis explores the possibility of using PWW as a nutrient media when cultivating *Chlorella sp.* microalgae immobilized in sodium alginate. Different concentrations diluted with distilled water were tested in order to find the optimal concentration yielding the most growth. Growth patterns were measured by optical density and nutrient depletion were measured by analyzing the total nitrogen and total phosphorous in the samples.
A 70% depletion of total nitrogen was observed from all variants of immobilized microalgae beads, while a 90% depletion of total phosphorus was observed from all variants of immobilized microalgae.

The thesis also explores microalgae immobilization optimization using sodium alginate. Different sized beads were tested to find the bead yielding the best growth conditions for the immobilized algae inside.

The results from these experiments offer information useful for future applications of microalgae cultivation and sodium alginate immobilization.

Contents

List of figures

Table of Chemicals

Chemical	Supplier
Ethanol	Supelco
Calcium chloride	Merck KGaA
Hydrochloric acid	Merck KGaA
Sodium Alginate	Merck KGaA
Sodium hydroxide	Chemi-Teknik as
Sodium dihydrogen phosphate	UCW
Sodium hydrogen phosphate	UCW
Spectroquant COD cell test	Merck KGaA
Spectroquant Phosphate Cell Test	Merck KGaA
Spectroquant Total Nitrogen Cell Test (	Merck KGaA

Abbreviations

BBM	Bolds Basal Medium
DNA	Deoxyribonucleic acid
COD	Chemical Oxygen Demand
N	Nitrogen
NIVA	Norsk institutt for vannforskning / Norwegian institute for waterscience
OD	Optical Density
P	Phosphorous
PWW	Piggery Wastewater
TN	Total Nitrogen
TP	Total Phosphorus
TSS	Total Suspended Solids
UV	Ultraviolet

1.1 Introduction

The cost of microalgae production is still high and the need to find viable alternate options for current growth medias are therefore sought after.

Piggery wastewater offers an alternative to commonly used chemicals, like Bolds basal medium, and contain much of the nutrients needed for algal growth.

1.1 Scope of work

The main scope of this work to check the feasibility of the piggery wastewater as an alternative source and cost effective medium for the microalgae growth, in specific scope as study in sodium alginate immobilized microalgae

1.2 Objectives

Cost effective media:

Evaluate if PWW media can become a suitable replacement for expensive chemical medias used today.

Propper strategy of immobilization:

Find a good and effective way of creating durable, and penetrable, beads of immobilized microalgae.

Optimization of media with immobilized microalgae:

Find the PWW media concentration best suited for cultivation.

1.3 Thesis outline

Title: **A feasibility study of piggery wastewater as a cost-effective media using sodium alginate immobilized microalgae**

1. Introduction
2. Theory
3. Materials and methods
4. Results and discussion
5. Challenges met during the experimental design

6. Conclusions
7. Future work

2. Theory

2.1 Microalgae

Microalgae are microscopic organisms existing as mono or multicellular forms that include eukaryotic protists and procaryotic cyanobacteria. There are estimated to be between 200 000 – 800 000 species of microalgae and they typically range from 5 µm to 200 µm in size. They are invisible to the naked eye, but can organize themselves into macroscopically visible groups and colonies. They can be found all over the globe inn fresh, marine and brackish water systems growing in both the water column and in sediments. Microalgae can even be found in aerial terrestrial environments like trees, soils and building facades (Fleurence, 2021). Like higher multicellular plants, they grow photoautotrophically, meaning they photosynthesize using sunlight and carbon dioxide(Ferreira de Oliveira & Bragotto, 2022). They do not have leaves, stems or roots like higher plants, but are specially and well adapted to live free in viscous environments (Tan et al., 2020). Microalgae produce more than half of all oxygen required by living beings on earth and synthesize a multitude of bioproducts like proteins, carotenoids, lipids, polysaccharides, pigments and vitamins (Zuccaro et al., 2019).

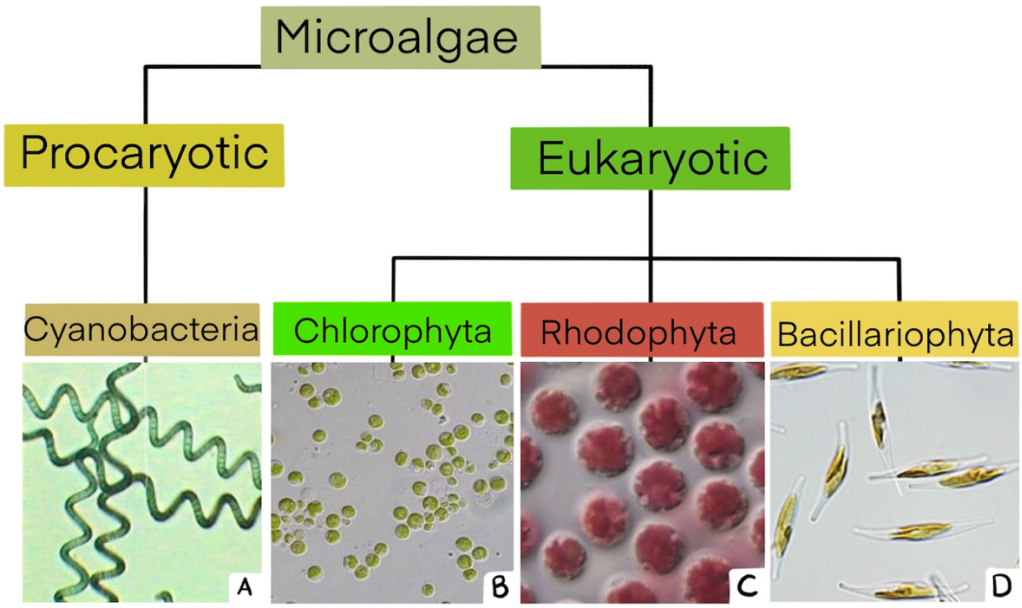


Figure 1: Various prokaryotic and eukaryotic microalgae: (A) Arthrospira platensis, (B) Chlorella sorokiniana, (C) Prophyridium purpureum, and (D) Phaeodactylum tricornutum. Images by (Figueroa-Torres et al., 2020).

Microalgae include a range of different species including, *Chlorophyceae, Rhodophyceae, Bacillariophyceae, and Phaeophyceae.* Eukaryotic microalgae have garnered attention due to their vast application potential in various fields of research. There are many different eukaryotic microalgae belonging to different taxonomic groups with unique characteristics giving huge possibilities for future research (Figueroa-Torres et al., 2020). Some of most common examples of eukaryotic microalgae include *chlorella, spirulina, hematococcus and dunaliella.*

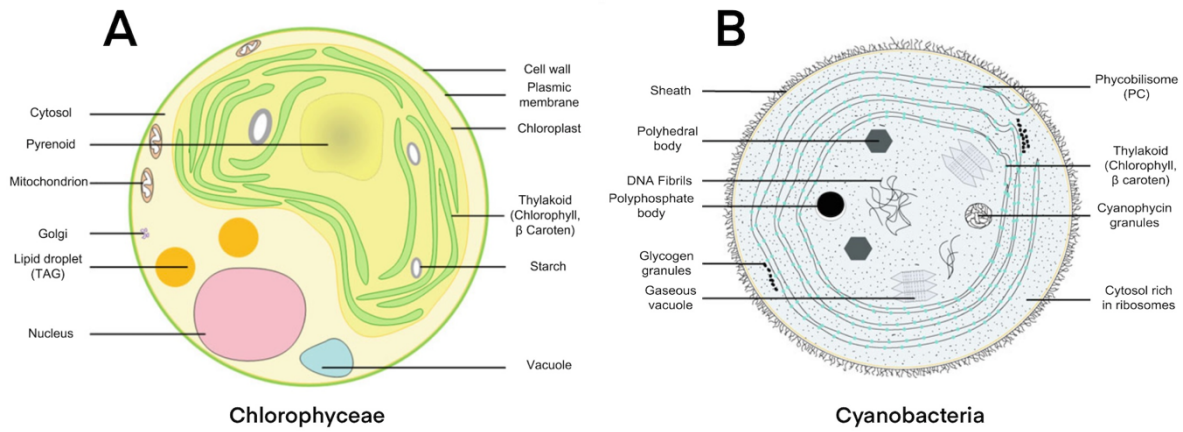


Figure 2: Sketched illustration of the structure of a eukaryotic (A) and a prokaryotic (B) microalgal cell. Based on (Pignolet et al., 2013).

Figure 2, above, displays a general eukaryotic microalgal cell, there can be significant differences between the various species and strains where organelles could be differently placed or completely missing (Bernaerts et al., 2019). The general microalgae anatomy and structure is relatively simple, containing only the most vital components for its own function and survival.

The morphology of eukaryotic microalgal cells, like the *chlorella sp.*, are similar to higher plants. They have a cell wall which is the most important defense against abiotic and biotic factors and can reach a thickness up to 21 nm (Yamamoto et al., 2004). They have mitochondria which carry out the metabolic processes that are most vital for the microalgae to acquire the energy necessary for its growth and maintenance.

They have chloroplast which is necessary for carrying out photosynthesis and within the chloroplast there is the capability of synthesizing starch granules. The thylakoids are also found within the chloroplast which is where the chlorophyll resides. When the chlorophyll is irradiated by light, the cell creates its own energy by photosynthesis (Safi et al., 2014).

The eukaryotic microalgae cell also has a nucleus housing its genetic material in the form of chromosomes. In the microalgal cells cytoplasm, a variety of organelles, like ribosomes and the cytoskeleton, reside. The cytoplasm also contains the cytosol, which is an intercellular gel-like substance that facilitates important cellular processes like protein synthesis, signal transduction, and metabolic reactions. The Golgi apparatus plays a central role in sorting and processing proteins and lipids, as well as in protein glycation and biosynthetic processes (Li et al., 2022). The cytoplasm is separated from the extracellular fluid by a plasma membrane and is found in all eukaryotic microalgal cells. This membrane is protected by the cell wall, which is composed of a phospholipid bilayer containing proteins. The membrane serves vital functions, like cell protection, structural support, selective permeability, cell signaling, and molecule transportation (Li et al., 2022).

Procaryotic microalgae cells are cyanobacteria but are more commonly known as blue-green algae. The prokaryotic microalgae have an outer plasma membrane that enclose the protoplasm containing photosynthetic thylakoids, ribosomes, and DNA fibrils which are not enclosed within a separate membrane. Chlorophyll *a* is the main photosynthetic pigment, and oxygen is evolved during photosynthesis (Lee, 2008).

2.1.1 Chlorella sp.

The green eukaryotic microalgae *Chlorella* is one of the most remarkable and versatile microalgae there is. It is found all over the globe and is characterized by its small and spherical cells. The name *Chlorella* originally comes from the Greek word *chloros*, meaning green, and the Latin suffix *ella* which refers to its microscopic size. The *chlorella sp.* are unicellular microalgae that grows in fresh water and has been present on earth since the pre-Cambrian period 2.5 billion years ago. Its genetic integrity has remained the same since that time (Safi et al., 2014).

The chlorella sp. consists of several strains such as *C. vulgaris, C. pyrenoidosa, C. ellipsoidea,* and *C. sorokiniana,* and possess remarkable resilience to temperature, thriving within a broad range of 15 °C to 40 °C. They also exhibit versatile growth patterns including autotrophic growth in inorganic environments and the ability to flourish under mixotrophic and

heterotrophic conditions by utilizing organic compounds like acetic acid and glucose (Masojídek & Torzillo, 2008).

In the early 1900s, some European scientists became interested in the Chlorella sp. due to its high protein content, considering using it as an unconventional food source. By the 1950s, the Carnegie Institution of Washington took over the research and successfully scaled up the cultivation of this microalga for CO^2 abatement (Safi et al., 2014). In modern times, Japan is the largest consumer of Chlorella worldwide, utilizing it in medical treatments due to its proven immune-modulating and anti-cancer properties (Justo et al., 2001).

Studies involving rats, mice, and rabbits fed with Chlorella powder have revealed protective effects on haematopoiesis, as well as against age-related diseases such as cardiovascular diseases, hypertension, and cataracts. It has been found to reduce the risk of atherosclerosis and promote collagen synthesis for skin health. Additionally, C. vulgaris can accumulate significant amounts of lipids, especially under nitrogen starvation, with a fatty acid profile suitable for biodiesel production. Add the citation

Chlorella stands out as one of the most extensively cultivated eukaryotic algae due to its widespread use as a health supplement and food additive. It is also highly regarded in the pharmaceutical, nutraceutical and cosmetics industries due to its rich composition of proteins, carotenoids, immunostimulators, polysaccharides, vitamins, and minerals (Masojídek & Torzillo, 2008).

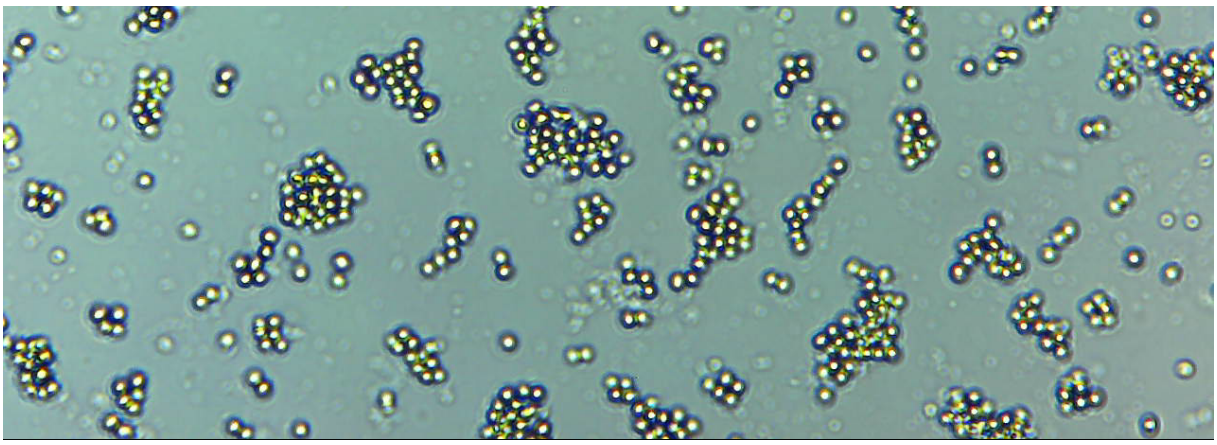


Figure 3: Chlorella sp. algae seen through the microscope in the lab. Photo by Marius Tveter

2.2 Applications for microalgae

As previously mentioned, there are estimated to be thousands of different microalgae species with different properties and characteristics. There is increased commercial interest in microalgae due to the huge variety of applications. Microalgae can be used in aquaculture, wastewater and pollutant remediation, as animal feed and as sources of essential nutrients and chemicals in food production, for cosmetics, biofuels, bioenergy, pharmaceuticals, and coloring. Omega-3 fatty acids and antioxidants can be extracted from microalgae and used in nutraceutical supplements (Figueroa-Torres et al., 2020).

Food and supplements

Even though algae have been used as a source of food and nutrients since ancient times, cultivation of microalgae for consumption began only a few decades ago as a response to the growing population and the fear that there would become a lack of protein-rich food sources (Sathasivam et al., 2019). Most microalgae products till now have been sold as supplements in the form of powder or pills, but new innovative ways of incorporating microalgae into food and drinks are emerging. You can now find microalgae in bread, smoothies, chocolate and crackers (Lafarga, 2019).

The current microalgae products available can be categorized into two main categories. The first one are those that incorporate microalgae biomass for coloring purposes. The second are those that use microalgae as a strategic marketing tool or to enhance the nutritional, physicochemical or the sensory attributes of the final products. There have been limited knowledge and scientific research on the nutritional and sensory properties of microalgae infused food products in the past. Microalgae infused food have long been associated with challenges due to intense coloration or fishy taste and odor. Recent studies have however shown that microalgae can be successfully integrated into food products without compromising physicochemical, nutritional, and sensory quality. The market is currently experiencing a growing interest in, and acceptance for, microalgae induced food products and thereby the number of food products containing microalgae is increasing. Microalgae in becoming not just a trendy ingredient, but as a valuable resource with the potential to become a staple in food products globally (Lafarga, 2019).

Microalgae has the potential to be of great benefit as a supplementary food as well due to the various compounds they produce such as proteins, carbohydrates, lipids and pigments. This makes the microalgae a source that has high nutritional value with the potential of even greater value with further research and development. Optimized growth could result in a higher yield of the desired compounds. It is however important to note that the optimized growth factors must vary depending on the compound you want to produce (Tandon et al., 2017).

Biofuel

Depending on the sources, biofuel could be considered as a renewable substitute for fossil fuels which currently is the most important source of energy and energy production on the planet. With the increasing energy demand due to the growing global population and the increased focus on renewable energy, microalgae have been looked to as an alternative to fossil fuels. Biofuels made by fermenting biological feedstock, like microalgae, is considered one of the renewable energy sources (Mat Aron et al., 2020). Biological feedstock for creation of biofuels have been categorized by Mat Aron et al., (2020) into four separate generations. The first generation is edible feedstock which include amongst other, rice, wheat and vegetable oil. The second generation is non-edible feedstock which forest residues and woody biomass. The third generation is microalgae biomass, and the fourth generation is genetically modified microalgae (Mat Aron et al., 2020).

Ordinary microalgae, the third generation, are considered a great source for the creation of biofuels due to the large concentrations of lipids in the dry cell weight. It is also favorably looked upon due to being eco-friendly, non-toxic and having the potential to contribute to CO_2 reduction in the atmosphere (Mat Aron et al., 2020). Microalgae are estimated to contribute to 40% of the global CO_2 reduction every year (Pierobon et al., 2018).

Similarly to first generation biofuel production, microalgal biofuels begin with cultivating and harvesting the product. The lipids are then extracted and undergo transesterification, which is the technology used to convert extracted oils into usable biofuels (Rios et al., 2013).

The cost of making biofuels using microalgae is however costly due to the large amounts of expensive chemicals, nutrients and fresh water needed to cultivate the microalgae. It can in some cases be ten times as costly to produce biofuel using microalgae than to produce fuels from crude oil (He et al., 2013). There are currently several hurdles to overcome to be able to successfully utilize microalgae for biofuels. These hurdles include the development of cost-effective growth systems, efficient and energy-saving harvesting methods, and environmentally

friendly approaches for oil extraction and conversion. These enhancements are crucial to make microalgal fuels appealing to both consumers and investors (Leite et al., 2013).

Various wastewaters have been considered as a cost effective alternative to conventional lab-made nutrient medias due to its high contents of nutrients suitable for microalgae growth (Wang et al., 2016). Some wastewater nutrient medias does however pose a potential danger due to the hazardous chemicals they may contain that could contaminate the microalgae (Chew et al., 2018). Due to the fact that microalgae biofuels have been found to be less stable than first and second generation biofuels and lipid extraction and biomass drying requires large amounts of energy a fourth generation biofuel production have been gaining more traction lately (Mat Aron et al., 2020).

Fourth generation biofuels involve genetically modified microalgae. The goal is to develop microalgae that can capture large amounts of CO_2 and hopefully be carbon negative. The genetic modification techniques for microalgae have evolved from traditional methods like RNA interference to more advanced methods like metabolic pathway engineering, syntetic engineering and genetic engineering (Mat Aron et al., 2020). Widespread application of these techniques is however hindered by the limited availability of genetic and biological information for many microalgae species. Some progress has been made in understanding the genetic makeup of specific microalgae like *Chlorella variabilis*, and *Chlorella sorokiniana*, but genetic modification of these species is still in development (Arriola et al., 2018; Lin et al., 2019).

Biogas

The continuous global demand for newer and cleaner energy have prompted the exploration of alternative sources. Biogas have emerged as a viable option and research indicates that utilizing microalgae for biogas production offers advantages over traditional fossil fuels (Coronado-Reyes et al., 2020). The cost of production is however heigh, like previously mentioned, due to various challenges associated with sustaining microalgae growth like nutruents, chemicals temperature and more. There is therefore a need to expand the research in this field. The biomass of microalgae, especially the *Chlorella sp*., holds great potential for biofuel and biogas production due to the lipids it generates (Coronado-Reyes et al., 2020; Medipally et al., 2015)

Cosmetology

Aside from the various industrial energy production applications of microalgae already mentioned, some species have great value in health and cosmetology industries as well. The *chlorella sp*., especially, have the ability to synthesize compounds such as lutein, which is a valuable xanthophyll. Due to its antioxidant activities, it has a promising potential in preventing retinal degeneration, various types of cancers, and cardiovascular diseases (Li et al., 2020).

It has also been discovered that some microalgae protect against UV radiation due to compounds they produce such as, mycosporine-like amino acids, sporopollenin and scytonemin (Santiesteban-Romero et al., 2022). Microalgae such as *Chlorella sp*. can also protect the skin against wrinkling and sagging. These microalgae attributes and characteristics stem from the exposure to oxidative stress during growth, compelling them to synthesize protective compounds like carotenoids. Microalgae are incorporated into cosmetic products as thickeners, water binding agents, and antioxidants. However, due to the variations in chemical composition among microalgae species, continuous research on synthesis, characterization, extraction, and purification of active compounds is essential for diverse product applications (Ariede et al., 2017; Coronado-Reyes et al., 2020)

Bioremediation

Manny different species of eukaryotic microalgae can easily adapt to different growth medias and are therefore an excellent contributor in wastewater treatment and bioremediation. Microalgae can be used to biologically purify several different varieties of polluted water such as textile wastewater, petrochemical wastewater, agricultural wastewater and more(Abdelfattah et al., 2023) Wastewater purification using microalgae have been shown to be a promising alternative to conventional wastewater treatment technologies that use large amounts of energy, discharge sludge and emit greenhouse gasses effecting the environment. Microalgae grow fast and proliferate by very efficiently acquiring carbon dioxide and nutrients, like nitrogen and phosphorus from the water it resides in (Wang et al., 2022).

Microalgae play important roles in the pharmaceutical and nutraceutical industries, and are used in cosmetics and biofuel production all due to the valuable compounds they can produce. Microalgae have also been gaining attention for their significant potential in wastewater treatment and renewable energy industries (Kahn et al. 2018). Due to microalgae's ability to grow and thrive in various conditions like polluted water bodies and wastewater, their capability

of reducing pollutants have become greatly appreciated. Microalgae are capable of reducing the amount of pollutants like nitrogen, phosphorus and carbon, as well as toxic compounds like heavy metals and pharmaceuticals, in water bodies in a more cost effective and less energy demanding way (Plöhn et al., 2021).

2.3 Growth conditions and requirements

Microalgal growth is dependent on several parameters, such as nutrients, light intensity, temperature, pH and salinity to be able to thrive, photosynthesize and grow. These conditions vary from species to species and the correct balance between them is required to ensure successful cultivation.

2.3.1 Light

Light is the main energy input for photosynthetic microorganisms and is therefore the most important factor for growth and productivity. Excess light coupled with low temperatures or high levels of oxygen can hurt the organism and yield less growth (Chowdury et al., 2020).

2.3.2 Temperature

Temperature is an environmental factor that influence microalgal growth rate, biochemical composition and nutrient requirements. Most microalgae have an optimal growth range between 20°C to 35°C, but some species can endure higher temperatures without being caused significant stress. Low temperatures result in a lower yield, but too high temperatures will cause cell damage, it is therefore important to keep a stable and suitable temperature to maximize the potential growth (Chowdury et al., 2020).

2.3.3 Nutrients

Nutrient limitation significantly impacts the biochemical composition of microalgae. Microalgae need nutrients like nitrogen, phosphorus, carbon, and even iron for growth. Carbon is an important building block of all organic substances synthesized by cells, and microalgae are no exception. About half of microalgal biomass consist of carbon which is found in

carbohydrates, lipids, proteins, nucleic acids and vitamins. CO2 and bicarbonates are essential for achieving high autotrophic production.

Nitrogen is another macronutrient that play a vital role in the formation of operational and structural proteins making it the most important one after carbon. Nitrogen is vital for the synthesis of vitamins, nucleic acids, proteins and photosynthetic pigments. It is mostly provided as N_2, in inorganic forms like nitrite, nitrate and ammonium or organic forms like urea or amino acids. Phosphorous is also an essential macronutrient needed for microalgal growth. It is involved in metabolic activities like energy transfer and synthesis of DNA (Chowdury et al., 2020).

Additional to the nutrients mentioned above, microalgae need essential micronutrients like; Na, Cl, Ca, Mo, Zn, Co, Cu, B, Mg, S and Fe, for optimal growth. Magnesium, sulfur and iron being the most important of these. Iron is involved in microalgal growth due to contributing to electron transport during photosynthesis (Markou & Georgakakis, 2011). Agricultural fertilizers and salts in wastewater and seawater are common sources of these macro and micronutrients. (Chowdury et al., 2020)

2.3.4 pH

The pH is significant in microalgae cultivation due to the fact that it determines the solubility of minerals in and CO_2 in the growth medium. pH alteration by adding external chemicals in an effort to obtain optimal conditions may hurt the microalgae cells by disrupting cellular processes like pH-sensitive cytoplasm and its enzymes. pH variations during microalgal cultivation can be influenced by the composition, buffer capacity, CO_2 concentration, metabolic activity, and temperature. Optimal pH range normally falls within the range of 6 to 8, but some species can endure pH levels as low as 3 and as high as 10.

Microalgae produce hydroxide ions during photosynthesis which can elevate pH levels in the media by fixating CO_2 and encouraging formation of carbonate. By lowering the pH chemical equilibrium can be achieved which produces CO_2 which is the preferred carbon source of microalgae. pH may also rise when phosphorous and nitrogen in the growth media is depleted and higher pH levels can promote the production of ammonia and precipitation of phosphate.

Maintaining a stable pH in the media is therefore essential for optimized and continuous microalgal growth (Chowdury et al., 2020).

2.3.5 Salinity

Salinity requires attention due to the potential evaporation of water in the growth media. This will cause an increase in concentration of salt and other minerals. Freshwater microalgae have a lower salinity tolerance meaning that increased salinity could cause stress and restrict or inhibit growth (Chowdury et al., 2020).

2.4 Eutrophication of nutrients in water environments

Nitrogen and phosphorus are two of the most vital macronutrients and are essential for biological processes of life in water bodies. They play a crucial role in protein and DNA synthesis, cellular growth, primary production and reproduction. Excessive quantities of nitrogen and phosphorus are however a significant source of pollution in water bodies and a cause of eutrophication. Eutrophication is the process where water bodies are gradually enriched with nutrients and minerals by erosion and runoff bringing organic and inorganic forms of dissolved nitrogen and phosphorus (Shen et al., 2020). Eutrophication is today largely attributed to anthropogenic activities worldwide such as agriculture, mining, and release of industrial waste and is a significantly larger problem than prior to worldwide industrialization (Häder et al., 2020).

Eutrophication triggers algal bloom, anoxic conditions and acidification in bodies of water. These conditions will then lead to reduced water quality, dead zones, fish death, toxin production, a decrease in plant species diversity and altered food chains. Additionally it poses a human health risk and causes recreational and economical losses (Ngatia et al., 2019).

2.4.1 Nitrogen

Nitrogen is present in both living tissue and detrital organic matter and is essential for life on earth. Biologically available forms of nitrogen such as nitrite, nitrate and ammonium are just a tiny part of the nitrogen available on earth making it a limiting factor of primary productivity in aquatic environments. Nitrogen is mostly found in its gaseous form dinitrogen (N_2) in the earths atmosphere, but a small portion of it can be dissolved in water or fixated by

microorganisms into the various nitrogenous molecules previously mentioned (Ngatia et al., 2019).

2.4.2 Phosphorus

Phosphorus is also a nutrient essential for life on earth. It exists as various organic compounds such as polyphosphate, pyrophosphate, metaphosphate and orthophosphate and inorganic compounds coupled crystalline forms of aluminum, copper and iron in soil and aquatic environments. The pH levels play a vital role in controlling the solubility and thereby the bioavailability of phosphorus for organisms dependent on it. Phosphorus fertilizers are not volatile like nitrogen fertilizers and are therefore not as easily distributed into nearby ecosystems. Excess use could however cause pollution of nearby bodies of water where phosphorous will remain in its particulate or dissolved forms (Ngatia et al., 2019).

2.5 Immobilization

Immobilization of microalgae refers to a practice where microalgal cells are fixed, confined or entrapped within a matrix in order to prevent free movement within a liquid medium.
Immobilization can be used for several purposes which includes, enhancing microalgae cultivation, wastewater treatment and bioremediation Han et al. (2023). Immobilization provides stability to the entrapped microalgae and has the potential to improve overall productivity and efficiency in biotechnological processes (Moreno-Garrido, 2008).
Immobilized microalgae have for several years been utilized for biomass and metabolite production, bioremediation of wastewater and bio capture of nutrients and heavy metals. The advantages of immobilizing microalgae, compared to cultivating free in media, is the cells are better protected against unfavorable conditions like high or low temperatures, acidity, and toxication. It also facilitates cultivation, makes biomass harvest simpler and allow for continuous system operation (de-Bashan & Bashan, 2010; Eroglu et al., 2015).

Immobilization can be classified as both passive and active immobilization. Passive immobilization exploits the natural tendencies of microalgae to attach to surfaces, but free algae in the medium is unavoidable in this instance. (Robinson et al., 1986) Active immobilization involves several techniques such as flocculant agents, chemicals attachment and gel

entrapment. Gel entrapment can be performed using either synthetic polymers or natural polysaccharides. The most common and widely used form of microalgal immobilization is gel entrapment in natural polysaccharides. Carrageenan, agar and alginate are the most commonly used polysaccharides for this. (Moreno-Garrido, 2008) While synthetic polymers like polyacrylamide, polyvinyl and polyurethane have been found to be more stable, especially in wastewater experiment, natural polysaccharides will allow for higher nutrient and production diffusion rates while also having a lower environmental impact (de-Bashan & Bashan, 2010). Beads made with natural polysaccharides cultivated in wastewater rich in nutrients could then be recovered, dried and used as fertilizer further enhancing sustainability (Bettani et al., 2019).

Immobilization matrices made by entrapping various cells, like microalgae, is commonly done using sodium alginate due to its ability to form gel beads in the presence of multivalent cation like Ca^{+2}. Alginate is extracted from brown algae and is an anionic polysaccharide. Depending on the source, alginate is composed of β-D-mannuronic (M) acids and α-L-guluronic (G) acid residues covalently linked in different proportions and sequential arrangements (de Jesus et al., 2019). In the process known as the egg-box model, calcium induced gelation of alginate is a result of interactions, that are strong and quite specific, between guluronate blocs and the calcium ions (Cao et al., 2020). In this process the solvent is trapped in the empty gaps of the interstices of a three-dimensional network which is connected by junction zones. These zones involves collaborative association of long segments of polymer chains (Voo et al., 2011).

The process of crosslinking alginate to form gel beads for immobilization is normally performed by external gelation using Ca^{+2} ions and this technique is commonly referred to as the diffusion method (Ma et al., 1994). The method involves dripping a solution of alginate and cell solution into a calcium solution, like $CaCl_2$, to create the beads. The bead formation occurs immediately, as the cationic calcium ions diffuse from the solution into the interior of the alginate droplets creating a gelled alginate matrix (Paques et al., 2014). In this process, calcium ions first diffuse into and thereby crosslink the outer layer of the alginate bead. This pulls the polymer chains closer together crating a surface that is less permeable and leads to the core of the bead being less densely crosslinked. It is important to let the bead stay in the solution until it sinks to the bottom and has been gelled all the way through. Beads removed too early will have a still liquid core (Chan et al., 2006).

Immobilization of microalgae in alginate matrices have several advantages. It is relatively easy to produce, it is not toxics to the cells, there is high retention of cell viability, the beads are translucent meaning light will reach even the innermost cells and it is cost effective (de-Bashan & Bashan, 2010). Alginate immobilization does however have its disadvantages. It is chemically unstable in the presence of cation chelating agents like phosphate, lactate and citrate. It is also unstable cations like sodium and magnesium, which will displace calcium and cause bead disruption or dissolution. Phosphate removal from the medium or presence of calcium can improve bead stability(de Jesus et al., 2019; Voo et al., 2011).

The alginate beads physical properties like durability, permeability, porosity and stability in regards to antigelling cations are affected by its characteristics, its polymer type and concentration of the crosslinking agent (Martinsen et al., 1989). It is therefore important to find the right balance of sodium alginate, water, and cells that in turn will interact with the best concentration of calcium ion solution, for the correct amount of time, to create the most optimal alginate bead.

3. Materials and methods

3.1.1 Pre-culture preparation

All equipment used had to be sterilized before use to avoid potential contamination. Glassware used for algae cultivation were sealed with aluminum and marked with autoclave tape before placed into the TOMY SX-700E autoclave for autoclaving at 120°C for 30 min. Other equipment not suited for the autoclave were washed with 96% ethanol and rinsed with distilled water. All the equipment were then placed in a LAF-cabinet and exposed to UV light for 10-15 min before inoculation and the cultivation experiments.

3.1.2 Algae strain

Taiwan

Chlorella sp. was collected by the coast of Taixi Township in Yunlin County, Taiwan (23°42'51.0"N 120°10'23.3"E) and cultured by Chyi- How Lay at Feng Chia University.

Norway

Chlorella sp. was ordered from NIVA and cultured by Bryan Han Xin Chou at the University of Stavanger.

3.1.3 Bolds Basal Media (BBM)

Bold's Basal Medium, or BBM, is a microalgae growth medium that has been used to grow several species of green microalgae cultures like *Chlorococcum* and *Chlorella* without the need for soil-extract or vitamins. The mostly inorganic nature of BBM makes it an axenic-culture maintenance medium (H.C. Bold, 1964).

Stocks:	**per 400 ml**
1) $NaNO_3$	10,0 g
2) $MgSO_4*7H_2O$	3,0 g
3) NaCl	1,0 g
4) K_2HPO_4	3,0 g

5) KH_2PO_4	7,0 g
6) $CaCl_2*2H_2O$	1,0 g
per 1000 ml or liter	
7) Trace element solution (autoclave to dissolve)	
$ZnSO_4*7H_2O$	8,82 g
$MnCl_2*4H_2O$	1,44 g
MoO_3	0,71 g
$CuSO_4*5H_2O$	1,57 g
$Co(NO_3)_2*6H_2O$	0,49 g
8) H_3BO_3	11,42 g
9) EDTA	50,0 g
KOH	31,0 g
10) $FeSO_4*7H_2O$	4,98 g
H_2SO_4 (conc.)	1,0 ml
Medium:	**per liter**
Stock solutions 1-6	10,0 ml each
Stock solutions 7-10	1,0 ml each

3.1.4 Media (piggery wastewater/ Slurry)

Taiwan

Piggery wastewater, or slurry, was collected from a pig farm in the Dali district in Taichung city, Taiwan (24°05'34.2"N 120°40'15.3"E) in March of 2023 and stored in an airtight container until use.

Initial concentration of total nitrogen concentration was measured to be 1819 mg/L.

Norway

Piggery wastewater, similar to the one collected in Taiwan, was collected from a local farm at Sele in Klepp municipality, Norway in May and September of 2023 and stored at 4°C until use. The sampled slurry contained mostly pig manure and water, but also contained traces of pig hair, wood shavings and hay which were filtered out before use leaving only the liquid piggery

wastewater (PWW). To remove all suspended solids, the PWW was at later stages filtered through 1,2 μm Whatman^{TM} Microfiber filters. The filtered PWW was then autoclaved to sterilize it and eliminate bacteria and pathogens possibly present in the media. After sterilization, the filtered PWW was analysed for several parameters such as Chemical Oxygen Demand (COD), Total Phosphorus (TP), Total Nitrogen (TN) and pH. Initial concentration of TN was measured to be 2100 mg/L, TP was measured to be 321 mg/L and pH was measured to 7,3.

3.1.5 Incubator

Taiwan

An incubator was needed to incubate the algae during the experiment. A metal frame measuring 50 cm high, 100 cm long, and 60 cm deep was constructed, and cardboard was cut to size and used as walls. A 40000lux fluorescent light was mounted to the ceiling and the inside walls were lined with aluminum foil for better light distribution.
Due to fluctuations in temperatures between day and nighttime, all bottles with microalgae were placed in a plastic tub filled with water at a constant temperature, around 29-30°C, during the entirety of the experiment. To keep temperature relatively stable, an electric temperature controller with a heating-rod and a digital thermometer was added as well as a small circulation pump. Additionally, under each bottle, a magnetic stirrer was placed to keep constant movement inside the bottles.

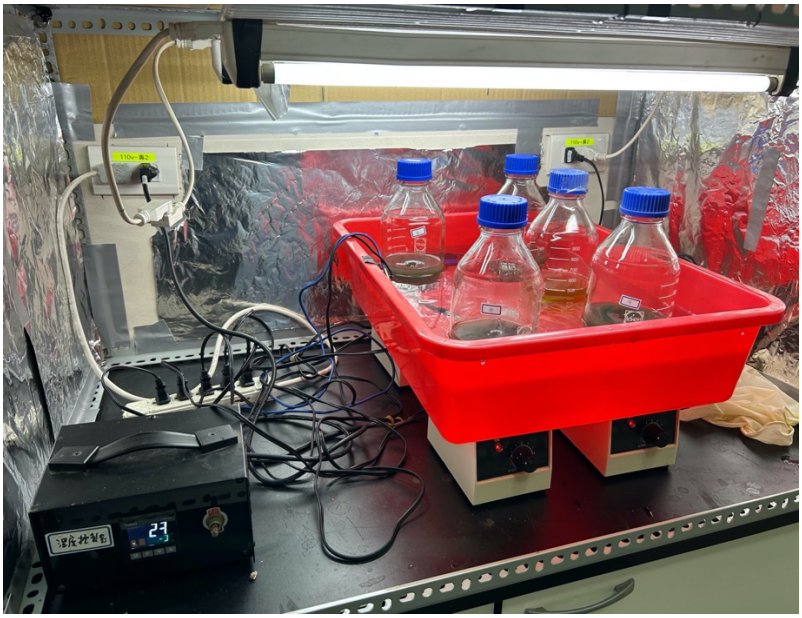


Figure 4: Inside of the Taiwan incubator showing temperature controller, fluorescent light fixture, waterfilled tub and magnetic stirrer setup.

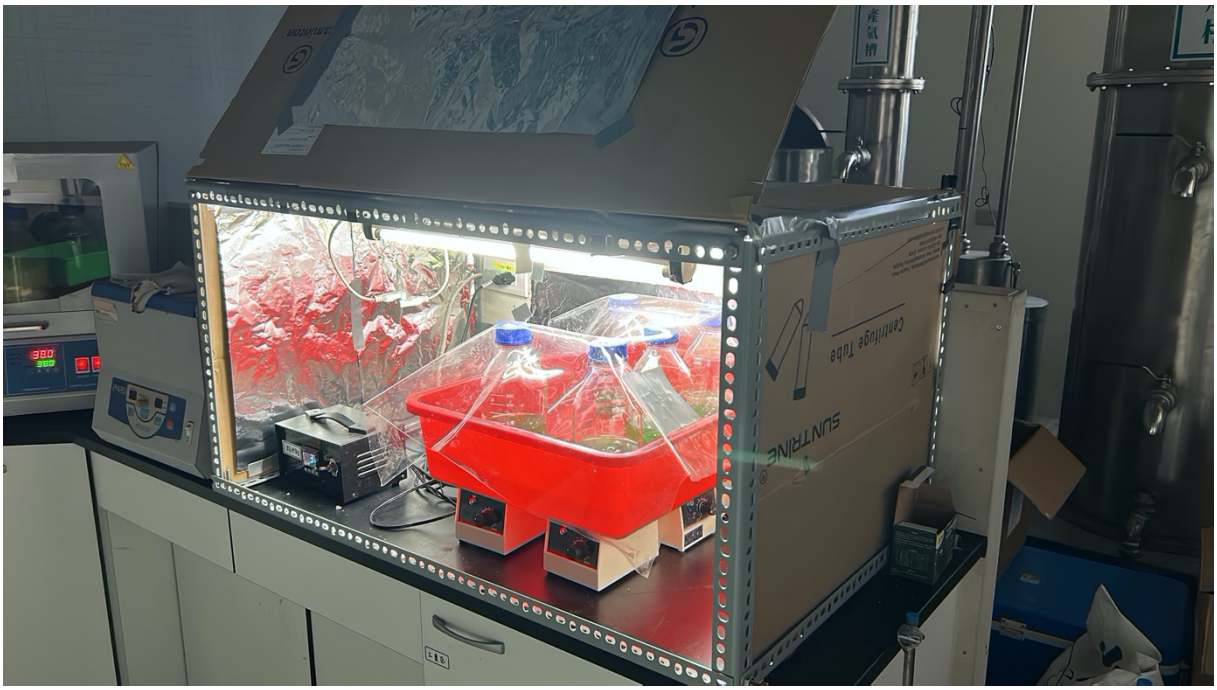


Figure 5: Taiwan incubator

Norway

When arriving back in Norway, a new incubator was built with a wooden frame measuring 65 cm high, 130 cm long, and 60 cm deep. Cardboard was cut to size and used as walls which then were lined with aluminum foil. A semi-translucent plexiglass, acrylic plate, was used as roof

and 10 m of LED-strips were glued to it to give artificial light inside. The LED-strips gave an average light intensity of 97,5 ± 3,2 µmol/m^2/s.
All flasks containing algae used during the experiments, were placed on a magnetic stirrer plate. Aeration was attempted, but after initial experiments decided against due to large loss of liquid media inside the bottles.

Figure 6: Norway incubator with magnetic stirrer plate, algae cultures and LED-strip ceiling.

3.1.6 Biomass / Total suspended solids (TSS) determination

Biomass/ TSS can be determined as dry cell weight. This was done by first drying aluminum weighing boats and Whatman™ filter paper in an incubator overnight and noting the weight. A select amount of biomass was then extracted from the culture and added to the filter paper on a Buchner funnel and vacuum flask to remove most of the water present. The filter was then put back in the aluminum weighing boat and dried overnight again. The whole thing was then weight again and the initial weight subtracted to find the dry cell weight.
TS is calculated as follows:

$$TSS\left(\frac{mg}{l}\right) = \frac{Weight_{Dry\ sample\ with\ filter\ and\ weighingboat} - Weight_{filter\ and\ weighingboat}}{Voume_{sample}}$$

3.1.7 Chemical oxygen demand (COD) determination

The spectroquant® COD cell test kits (Hg-free) with a measuring range of 100-1500 mg/l and serial number 1.09773 were used. These kits measure the concentration of organic and inorganic oxidized compounds in the samples. The test uses hot sulfuric acid solution with potassium dichromate to oxidize the samples and the reaction is catalyzed by silver sulfate. The resulting concentration of yellow $Cr_2O_7^{2-}$ or green Cr^{3+} -ions can then be determined photometrically.

Firstly, 2 ml sample was carefully added to the reaction cell and the cell was shaken vigorously to ensure a that all the contents were well mixed. The reaction cell was then placed in a thermoreactor at 148°C for 120 min for digestion. The cell was then removed from the thermoreactor and cooled to room temperature before being measured in a photometer.

3.1.8 Total Nitrogen (TN) determination

The spectroquant® Nitrogen (total) cell test kits were used for this determination. The kit is based on Koroleff's method where organic and inorganic nitrogen compounds in water samples are oxidized into nitrate using a reaction agent and heath. The reaction agent in the kit is 2,6-dimethylphenol (DMP) which acidifies nitrate when of sulfuric and phosphoric acids are present and results in the formation of 4-nitro-2,6-dimethylphenol. The pink-colored solution can then be measured photometrically to determine TN.

Two different kits were utilized for TN determination. The first, one with serial number 1.14764.0001, measured TN between the range of 10-150 mg/l and the second, with serial number 1.4537.0001, measured TN between the range of 0,5-15 mg/l. The first kit required 1 ml sample along with 9 ml distilled water to be added to a clean empty cell while the second kit required 10 ml sample to be added to a clean empty cell. The next steps prior to digestion where then the same. 1 level microspoon of N-1K was added to the cell and mixed, then 6 drops of N-2K were added to the cell and mixed. The cell was then placed in a thermoreactor at 120°C for 60 min for digestion. The cell was then removed, shaken and cooled to room temperature. The first kit then required 1 ml of digested sample to be transferred to a reaction cell before 1

ml N-3K were added. The reaction cell was then shaken to mix the contents and left for 10 min to fully react before being measured in a photometer.
The second kit required 1 level microspoon N-3K to be added to the reaction cell and vigorously shaken for 1 min before 1,5 ml digested sample was added. Like the first one, the reaction cell was then left for 10 min to fully react before being measured in a photometer.

3.1.9 Total Phosphorus (TP) determination

The spectroquant® Phosphate (total) cell test kits were used for this determination. This kit uses ascorbic acid to reduce molybdophosphoric acid, formed by orthophosphate ions reacting with molybdate ions in a sulfuric solution, to phosphomolybdenum blue (PMb) which can be measured photometrically.

Two different kits were also utilized for TP determination. The first, one with serial number 1.00673.0001, measured PO_4-P between the range of 3,0-100,0 mg/l and the second, with serial number 1.4729.0001, measured PO_4-P between the range of 0,5-25,0 mg/l.
The accuracy of these tests were dependent on the COD levels of the samples. This had to be considered before proceeding with the following steps and dilutions had to be made if necessary. The first kit required 0,2 ml sample, while the second required 1 ml sample to be added to a reaction cell together with one dose of P-1K. The cell was then mixed before being placed in a thermoreactor at 120°C for 60 min for digestion. The cell was then removed and cooled to room temperature before 5 drops of P-2K and one dose of P-3K was added. The cell was then shaken vigorously and left for 5 min to fully react before being measured in a photometer.

3.1.10 Optical density (OD) measurements

Optical density, commonly shortened to OD, is a quick and simple way of measuring or estimating cell density in a medium. It is however important to avoid contaminations that could interfere with the absorbance of microalgal pigments and give false readings (Griffiths et al., 2011). OD should only be considered as an estimation of biomass concentration due to microalgae strains physiological states tendency to greatly vary during growth (Thiviyanathan et al., 2023). Cell concentrations in samples are estimated by the amount of light absorbed by

the microalgal cells. Wavelengths of 680 nm and 750 nm are most commonly used when measuring absorbance (Griffiths et al., 2011).

These two wavelengths were also utilized in this thesis. OD analysis was performed by collecting 1 ml homogenous sample suspension with a pipette and transferring to a cuvette together with 1 ml distilled water. OD measurements were performed once per day using a spectrophotometer. The results were noted and used for growth rate calculations.

$$\mu\left[\frac{1}{day}\right] = \frac{(\ln N(f) - \ln N(i))}{t(f) - t\ (i)}$$

3.1.11 PWW treatment efficiency

The PWW treatment efficiency was estimated by measuring TP, TN and COD. The media was analyzed for these three parameters before the start of the experiment and every second day of the immobilized microalgae cultivation process like described in section 3.1.7-3.1.9.

Due to limited availability of analytical equipment and test kits as well as the small volume in each cultivation batch, a limited number of samples were collected each time, for each cultivation condition, and analyzed. For every media concentration variant, nutrient depletion charts were created.

The nutrient removal efficiencies for TN, TP and COD were determined using the following formula:

$$Removal\ efficiency\ (\%) = \frac{Ci - Cf}{Ci} \times 100$$

3.2 Experiments in Taiwan

Growth of chlorella in PWW with different pH conditions.

One-liter bottles were used and filled with 450 ml media, containing 99% distilled water and 1% PWW, and 50 ml *chlorella sp.* algae.

Five bottles with pH 6-10 were made and pH was adjusted using 1M HCl and NaOH.

Other than the evolution of pH, no significant data was obtained from this first experiment. None of the microalgae seemed to grow in any of the bottles.

Creating immobilized algae using sodium alginate

The procedure for immobilizing microalgae was adapted from methods performed by (Wu et al., 2020) and (Wu et al., 2021). A 30 g/L sodium alginate solution was made by slowly adding sodium alginate powder to lukewarm water under constant stirring on a hotplate. The solution was then autoclaved for 10 min at 110°C to sterilize it, fully dissolve the sodium alginate and expel any bubbles formed in the viscous solution. When cooled, 100 ml of the sodium alginate solution was mixed with 50 ml microalgae culture. The alginate/algae mixture was then added to a 50 ml syringe and slowly, drop by drop, pressed into a 40g/L $CaCl_2$ solution forming beads with diameters of 4±0,3 mm. The beads were then rinsed and stored in distilled water until further use.

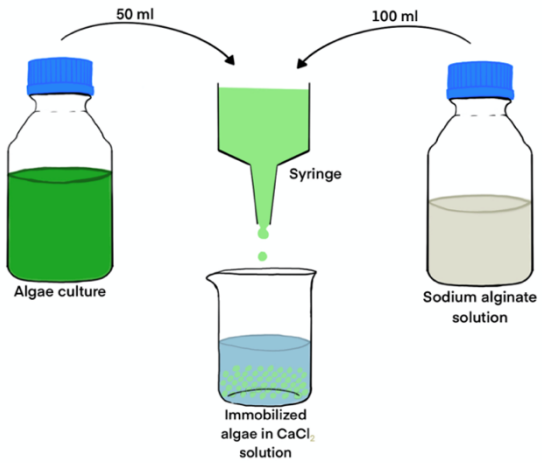


Figure 7: Sketch of creation of immobilized beads. And bead and PWW mix. By Marius Tveter

Creating the optimal bead

The formula for immobilization of microalgae using sodium alginate drew largely from previous research by (Wu et al., 2020), but a series of trials and errors were still necessary to achieve the best possible bead. Adding sodium alginate to lukewarm distilled water on a hotplate required caution. Rapid addition led to alginate clumps, yielding a non-homogenous solution containing bits of undissolved sodium alginate. Too much water created an overly thin solution, resulting in fragile beads that could break or dissolve during cultivation.

Sodium alginate solution was mixed 2:1 with algae culture (containing mostly water) resulting in a less viscous mixture. This was found to be the optimal viscosity for this experiment (and for creating the bead when pumping through the tube using a peristaltic pump). A 40 g/l CaCl2 solution served as the optimal medium for dripping alginate/algae ixture into, forming beads. Experimentation with syringes of varying sizes revealed that 50 ml syringes with 4 mm openings were the most effective. Peristaltic pumps utilized tubes with 4 mm inner diameter for bead production. For smaller beads, tubes were inserted into pipette tips with 1,5 mm openings.

Determining the appropriate flow rate was challenging. The droplets had to fall slowly, one by one, as rapid dripping caused elongated, thread-like structures. Continuous stirring of the $CaCl_2$ solution, using a magnetic stirrer, was crucial. Otherwise, beads adhered or clumped together. Dripping from too great a height above the surface of the $CaCl_2$ solution resulted a splash that would break the alginate/algae droplet and cause the formation of a smaller bead, less than 1 mm in diameter, unsuited for further use and somewhat deform the larger one.

Each of these factors had to be carefully considered to achieve the optimal bead formation.

Growth of immobilized chlorella in PWW with different pH conditions.

The next experiment was similar, but some parameters were changed. The microalgae were now immobilized in sodium alginate beads and the concentration of PWW in the media was increased to 10%.

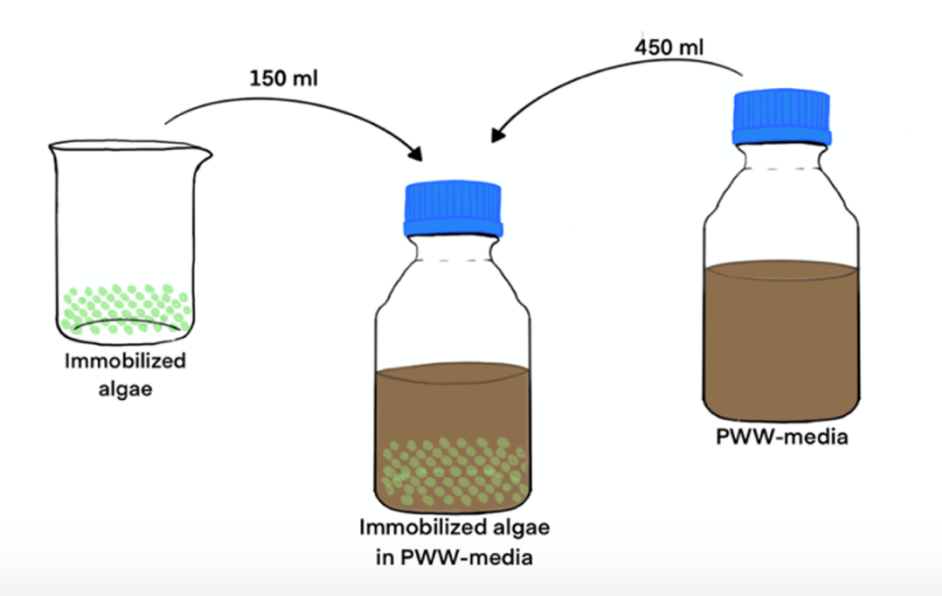


Figure 8: Immobilized algae in PWW media

When checking on the batches the following morning, the beads in the pH 7, 8 and 9 bottle had completely dissolved. Only the beads in the pH 6 batch were still intact. It was then hypothesized that the pH buffers were to blame, and it was discovered that alginate dissolve in basic phosphate buffered solutions due to calcium-ion release from the alginate beads.(Kikuchi et al., 1997) (Voo et al., 2011)

3.3 Experiments in Norway

Growth of immobilized chlorella algae with different PWW concentrations.

New baches of immobilized microalgae now had to be made. The syringe was substituted with a peristaltic pump for both faster and easier creation of the immobilized beads.

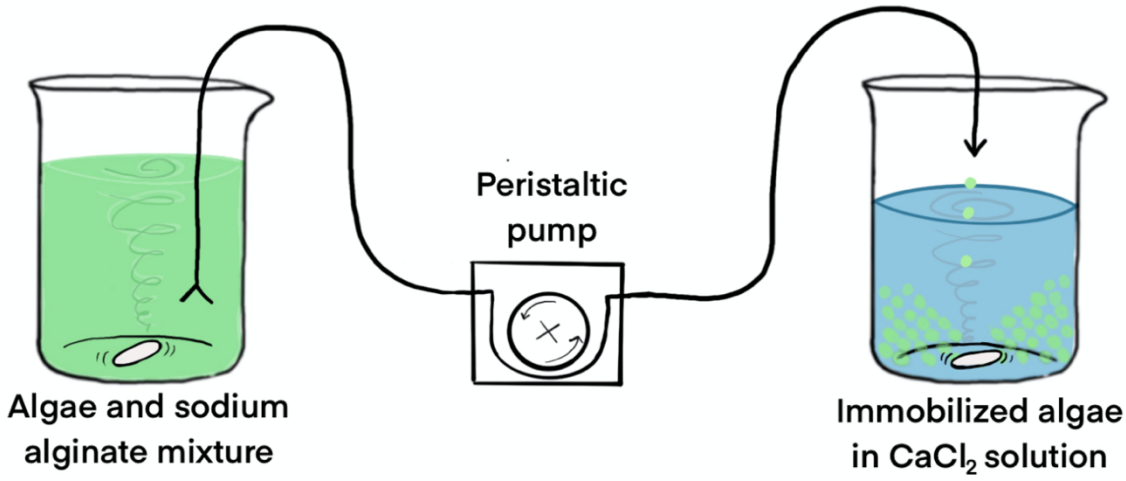


Figure 9: Creation of alginate beads using a peristaltic pump. Scetch by Marius Tveter

Note: Low concentration of algae in these beads, 25 ml chlorella algae culture mixed with 75 ml distilled water was mixed with 200 ml sodium alginate. Initial TS of mother culture measured to be 440 mg/L.
The beads where then left in BBM for 4 days to grow.

Using the pig manure-slurry obtained from Sele, a test experiment was performed. PWW-media was made using 10% pig manure-slurry and 90% distilled water to make it as similar as possible to the PWW used in Taiwan.

Three batches were made, containing 1%, 10% and 100% PWW-media as well as one control containing BBM instead of PWW-media. The flasks were under constant aeration
Microalgae growth and nutrient depletion over time was observed…

Final experimental setup and cultivation conditions

The final setup involved cultivating the immobilized microalgae in pure PWW-meda and its dilutions. These consisted of 100% PWW, 50% PWW + 50% distilled water and 25% + 75 % distilled water. Pure BBM was used as control medium. Light intensity was measured to be 97,5 ± 3,2 µmol/m^2/s and the temperature was measured to be 24 ± 1 °C during the entire cultivation period. OD values were measured daily at wavelengths 680 nm and 750 nm, while TN, TP and COD was measured every other day. There were no significant differences between OD values at the two different wavelengths.

The batches consisted of two parallels of each concentration with 25 ml alginate beads and 225 ml media making each flask contain 10% immobilized microalgae alginate beads and 90% liquid media. All flasks were placed on a magnetic stirrer plate set to a magnetic stirring speed of 120 rpm.

Immobilized beads were regularly extracted from the various batches and microscopically investigated to assess status and growth as well as look for contaminations.

The first batches contained only large alginate beads measuring 4±0,3 mm in diameter. The final batches contained flasks with both large and small beads, measuring 2,5±0,5 mm in diameter. This was done due to the discovery that the microalgae in the center of the large beads did not grow optimally. Smaller beads was therefore created in an effort to remedy this. The

growth and nutrient depletion of the two bead types was then compared. No significant differences were found in the growth between them this time.

4. Results and discussion

4.1 Experiments in Taiwan

The first experiment explored the effect of pH on the growth of the algae and the evolution of pH over time.

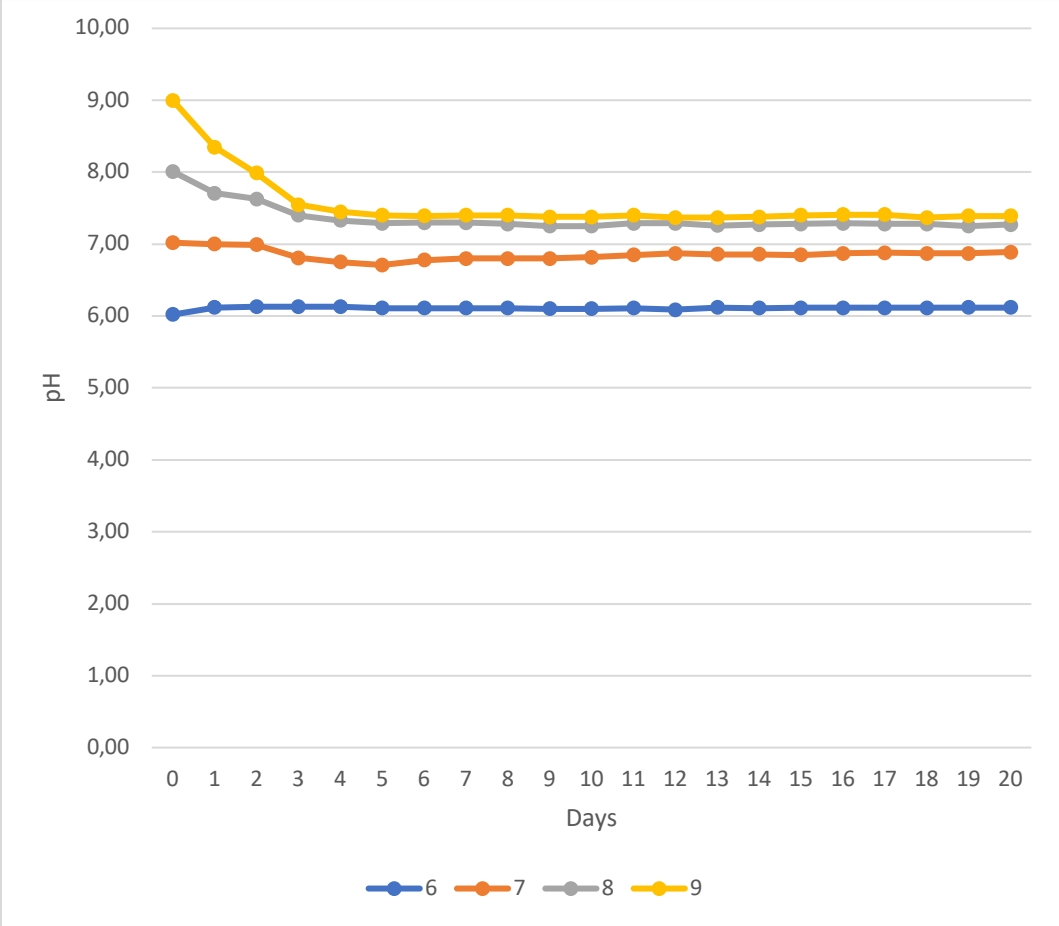


Figure 10: Evolution of pH in batches over time

The bottles cultivating algae in pH 9, 8 and 7 quickly sank before stabilizing and remaining constant throughout the experiment. pH 6 remained more or less the same the entire time.

The bottles quickly got contaminated due to the media being unsterilized and unfiltered. This made it impossible to accurately measure OD…. could not assess growth, TSS was contaminated.

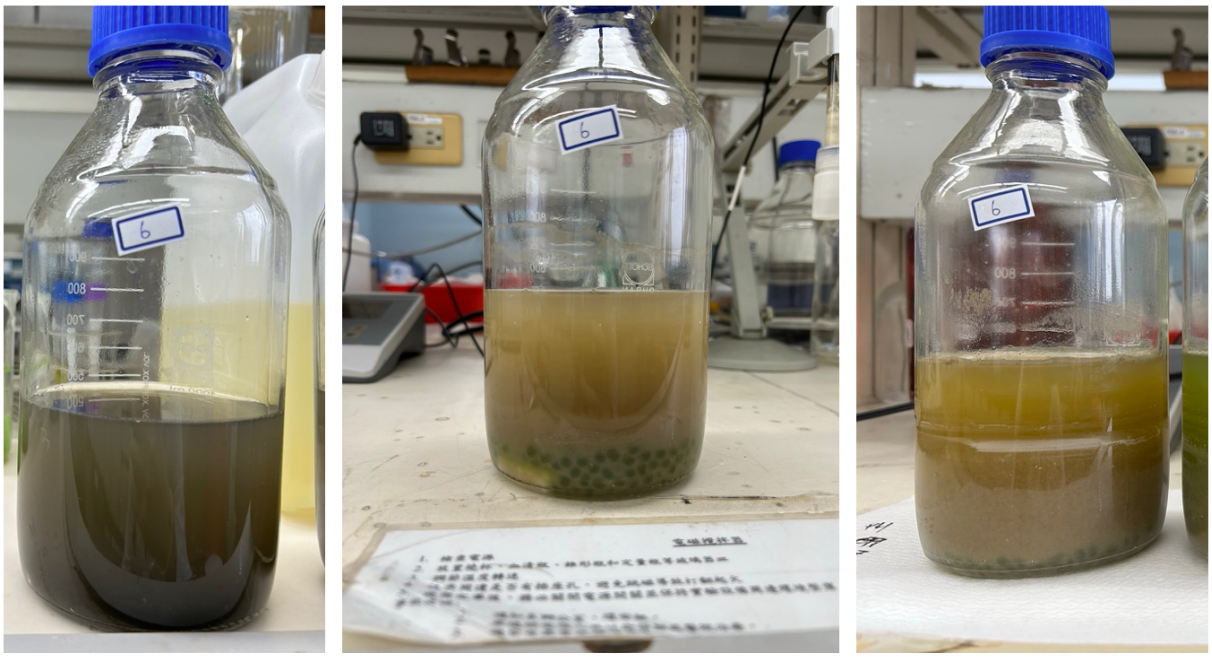


Figure 11: Color change over time. Day 1,4 and 10

Biggest change in colour at the beginning, but slowed down over time.

NH3-H measurement

Ammonia in pig manure measured to be 1819 mg/L . The media used was diluted 1:10 and should then contain 181,9 mg/L.

200-210 mg/L was measured from pH 6-9 at start for each batch.

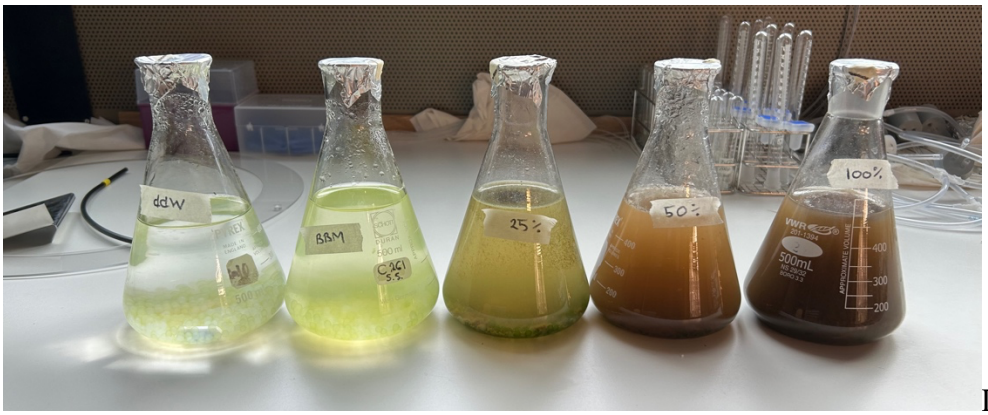
After 5 days it was measured again, still showing 200mg/L

After 12 days, it was measured again, all said 0 mg/L, exccept ph 8 which said 1mg/L

4.2 Experiments in Norway

Color change of bottles over time:

Day 0



Day 3


Day 9


Day 12

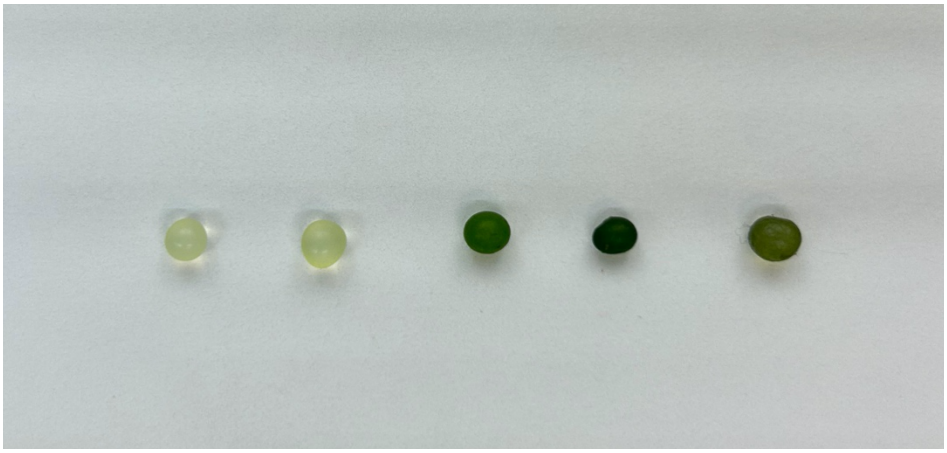


Figure 12: Immobilized beads from each flask after 12 days. Distilled water, BBM, 25% PWW media, 50% PWW media and 100% PWW media. (PWW media consists of 90% distilled water and 10% pig manure slurry.)

The first attempts at cultivating immobilized microalgae gave good growth results with 25% PWW media and 50% PWW media yielding best growth. 100% media became too dark and not enough light penetrated resulting in slightly less growth compared to the other two.

It was also discovered, when the beads were cut in half, that only the microalgae cells in the outermost part of the alginate bead grew and multiplied.

For following experiments smaller beads with a diameter of 2,5±0,5 mm were created in hopes of media reaching/penetrating further into the bead.

TSS

Culture used had a microalgae concentration of 960,0 mg/l.

125 ml culture was mixed 1:1 with 125 ml distilled water making the concentration 480,0 mg/l. This new 250 ml mixture now contained approximately 120 mg microalgae.

The 250 ml mixture was them mixed with 500 ml sodium alginate solution. This created a 750 ml sodium alginate and algae mixture with an approximate microalgal concentration of 40,0 mg/l.

Average size of the large beads was 4 mm diameter meaning beads had a volume of 18,85 mm^3.

Small beads had an average diameter of 2,5 meaning small beads had a volume of 12,63 mm^3.

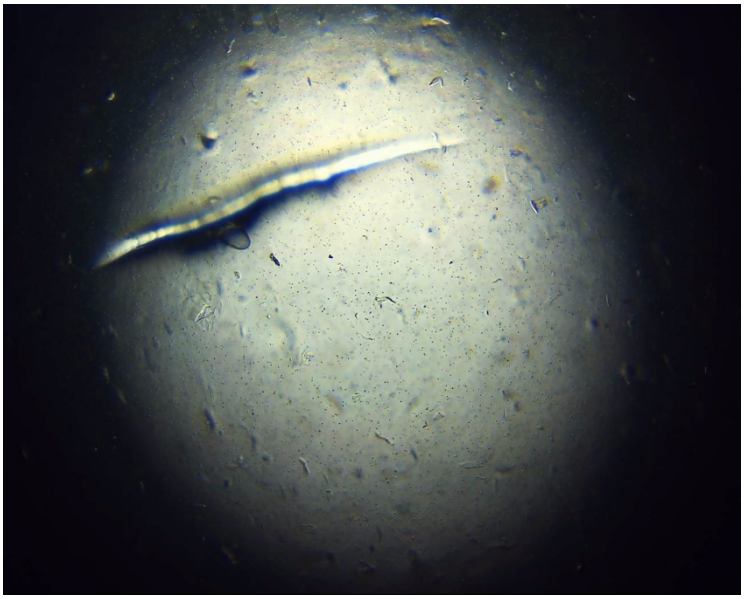


Figure 13: Alginate bead day 0, first cultivation

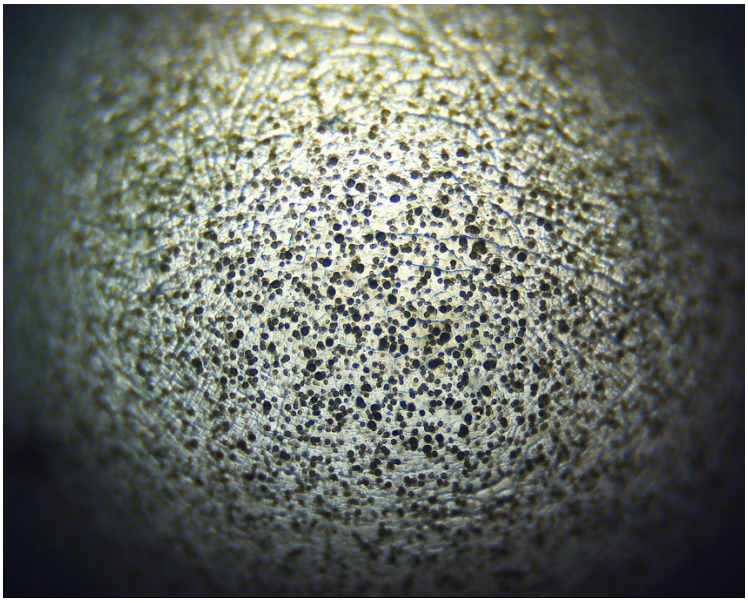


Figure 14: Alginate bead Day 9, first cultivation

In the initial attempts to replicate conditions of the experiments performed in Taiwan, collected pig manure was diluted with distilled water to create PWW containing only 10% manure. This was then filtered through a sieve to remove most of the hay, wood shavings, pig hair, bugs and other debris. This resulted in PWW that was still quite dark and contained some amount of particles.

A new version of PWW was then created by filtering the previous PWW through 1,2µm Whatman™ filter paper using a Buchner funnel creating a new PWW media free from particles.

This media had a brown-yellow coloration and faint smell compared to the previous media which had a dark brown coloration and stronger smell.

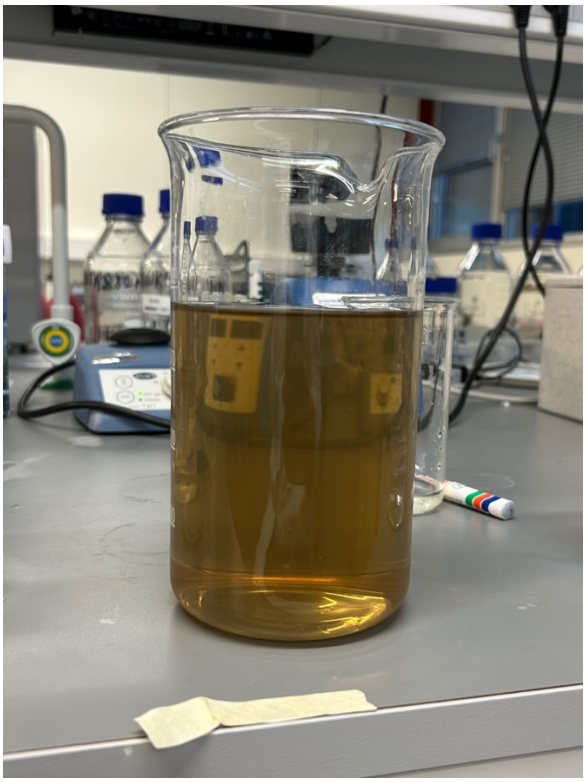
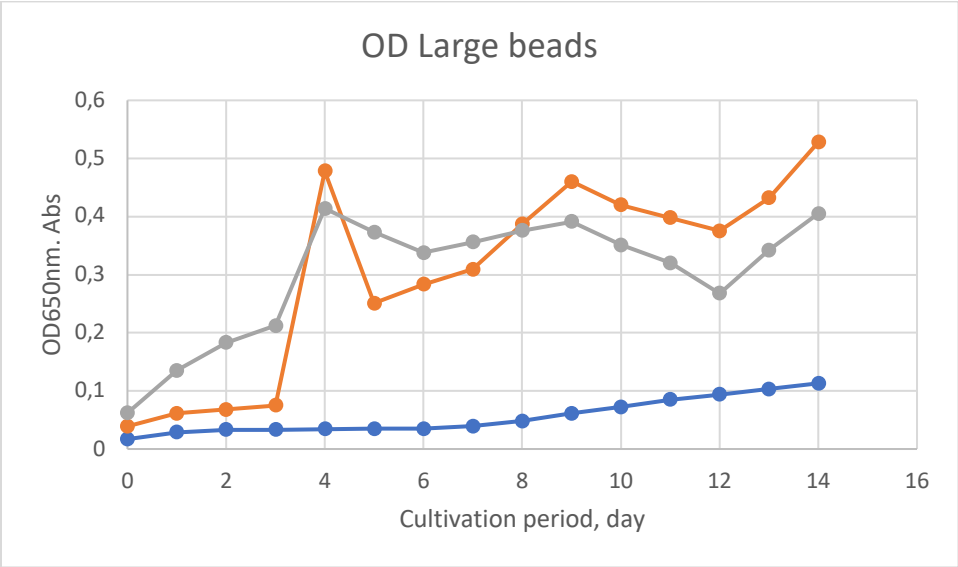
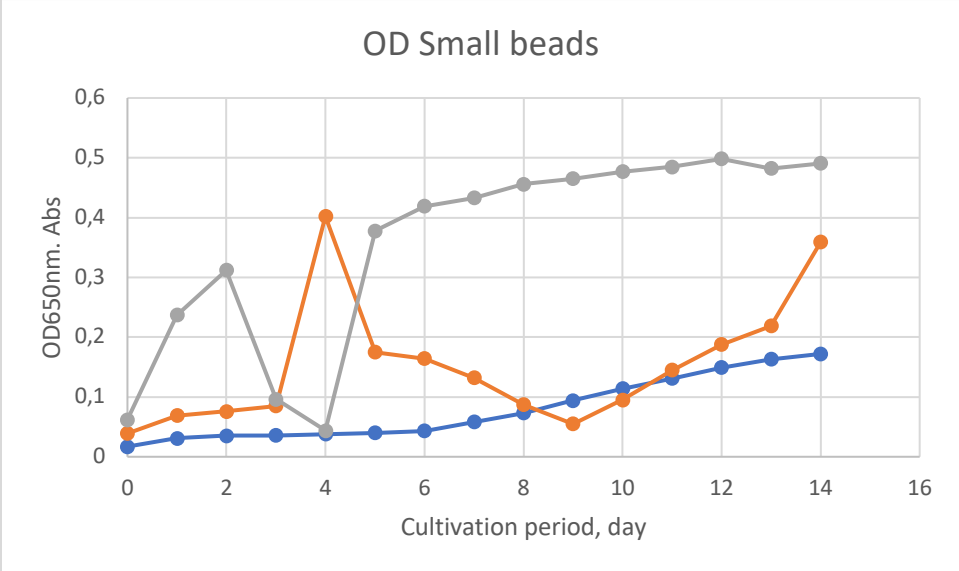


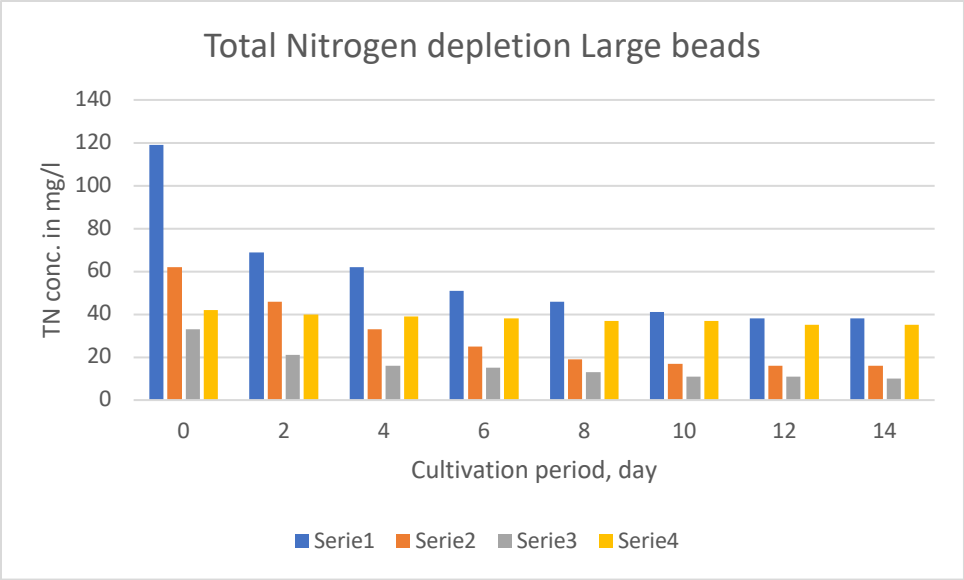
Figure 15: Filtrated PWW media

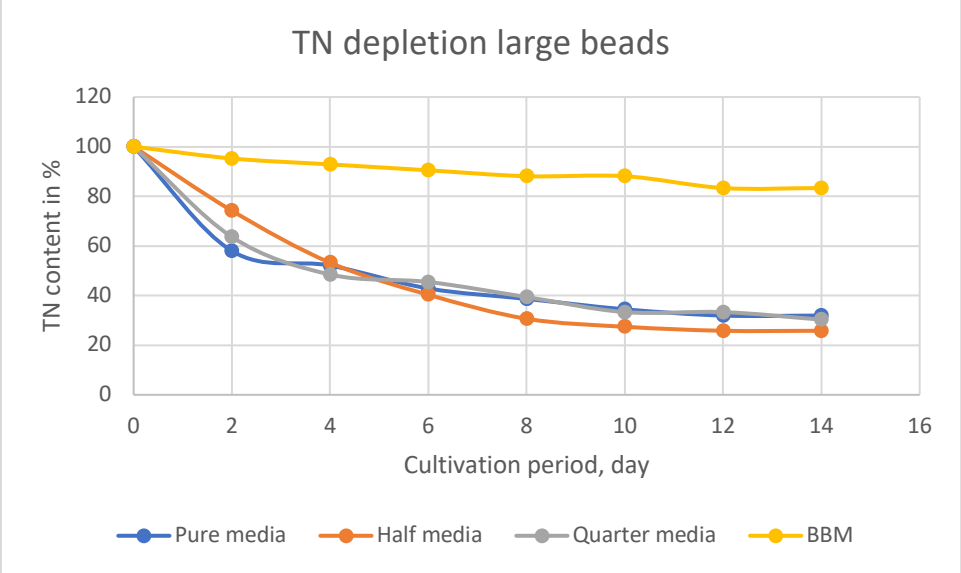
Large/ normal size beads cultivated in PWW media

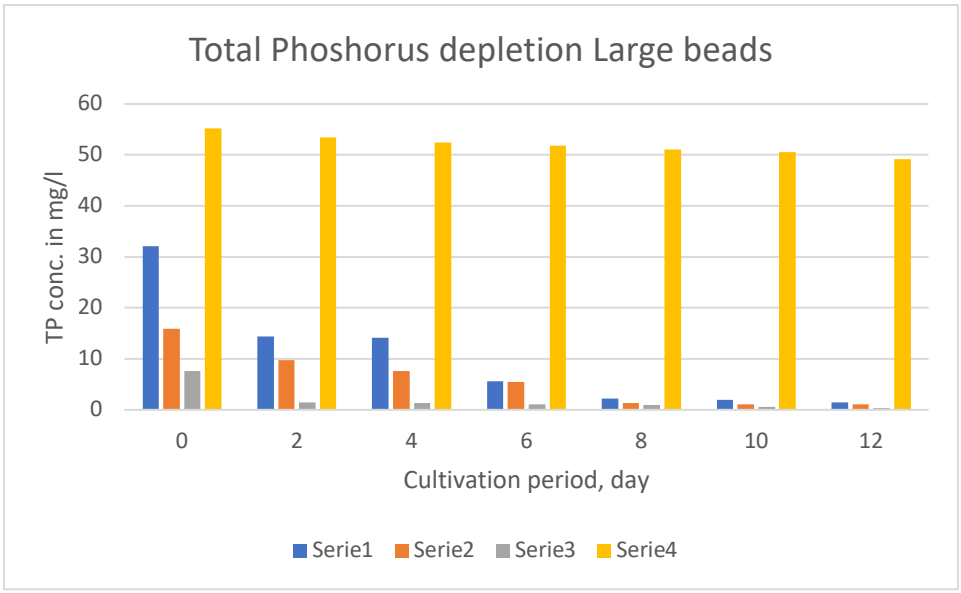


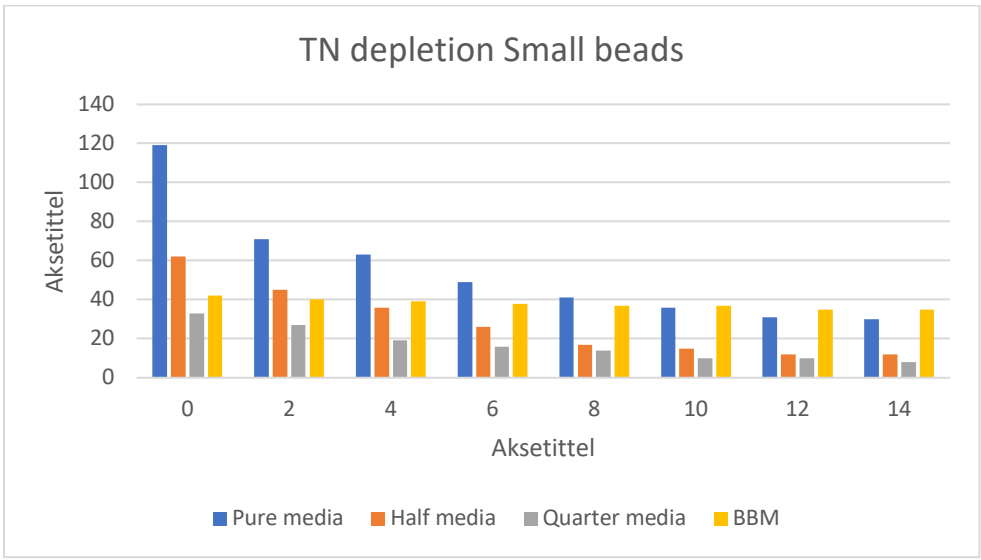
Small beads cultivated in PWW media




Total Nitrogen depletion Large beads
TN conc. in mg/l
140
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Cultivation period, day
Serie1
Serie2
Serie3
Serie4


TN depletion large beads
TN content in %
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Cultivation period, day
Pure media
Half media
Quarter media
BBM


Total Phoshorus depletion Large beads
TP conc. in mg/l
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Cultivation period, day
Serie1
Serie2
Serie3
Serie4


TN depletion Small beads
Aksetittel
140
120
100
80
60
40
20
0
0
2
4
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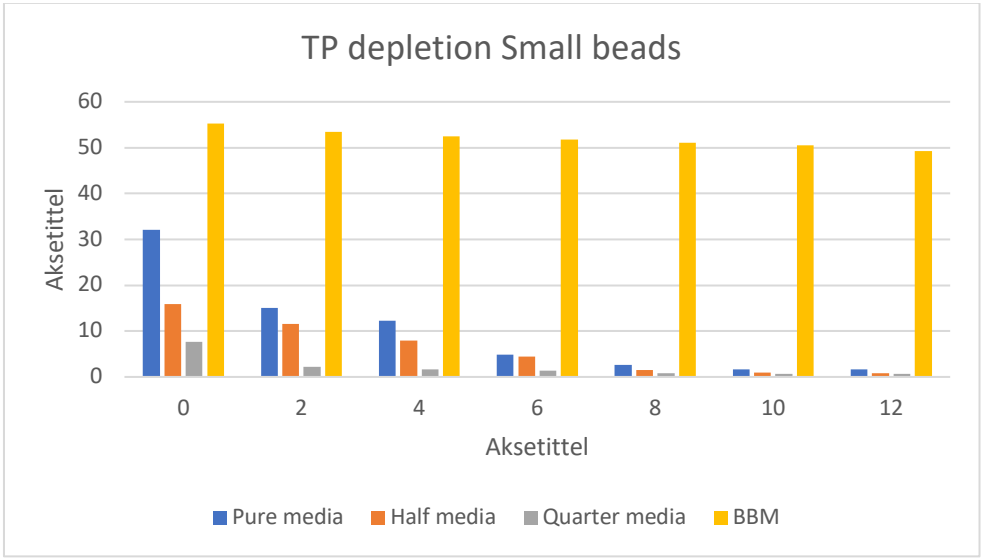
10
12
14
Aksetittel
Pure media
Half media
Quarter media
BBM


TP depletion Small beads
Aksetittel
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Aksetittel
Pure media
Half media
Quarter media
BBM

COD values were also measured every other day, but the values varied greatly from day to day and even between parallels. The measured values could therefore not be used. It was first assumed that this was due to human errors when sampling or performing the method described in the test-kit manual, but it was later hypostasized kit itself could be faulty.

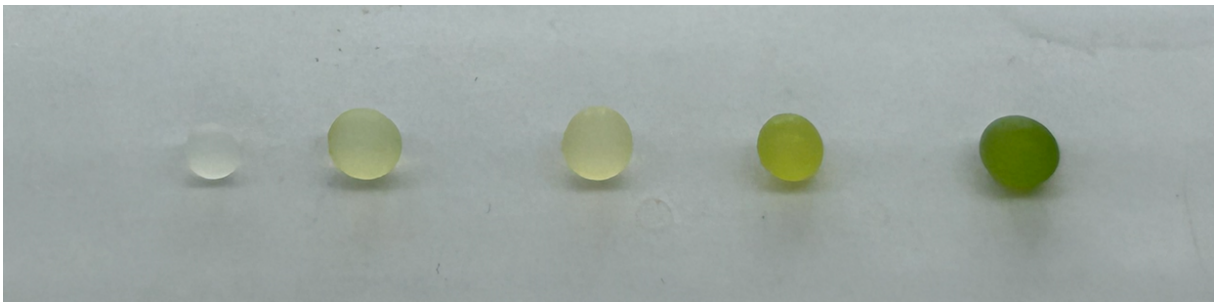


Figure 16: Large beads after 14 days of cultivation

Beads cultivated in distilled water, BBM, 25% PWW, 50% PWW and 100% PWW.

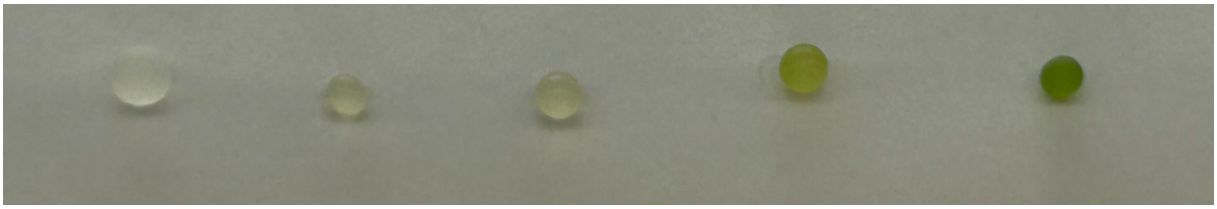


Figure 17: Small beads after 14 days of cultivation

When these beads both large and small were cut open, microalgae far into the bead were found to have grown and multiplied.

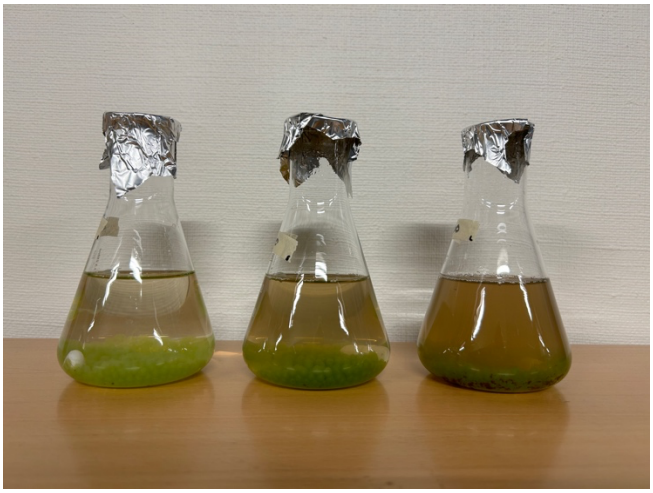


Figure 18: Growth day 0. Flasks from left to right are 25%, 50% and 100% media

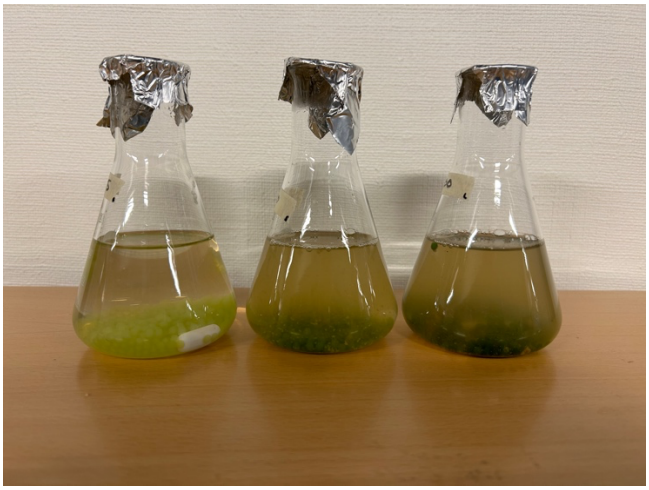


Figure 19: Growth day 7. Flasks from left to right are 25%, 50% and 100% media

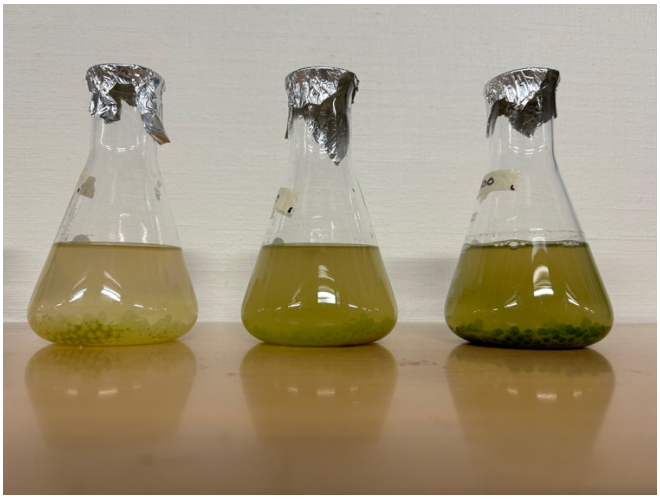


Figure 20: Growth day 14. Flasks from left to right are 25%, 50% and 100% media

Microalgal growth can be clearly observed. Coloration disappears form the pure media and microbial growth is present in the 25% media.

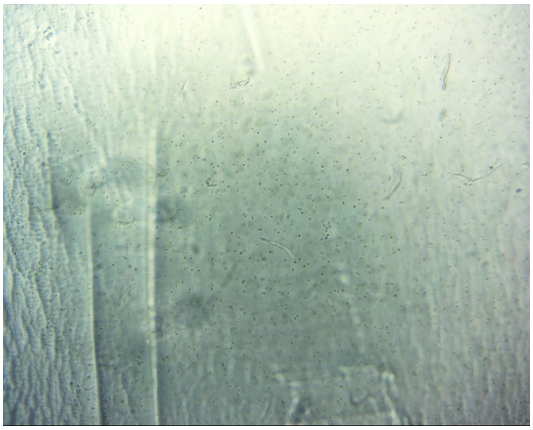


Figure 21: Large bead day 1, zoom 10x.

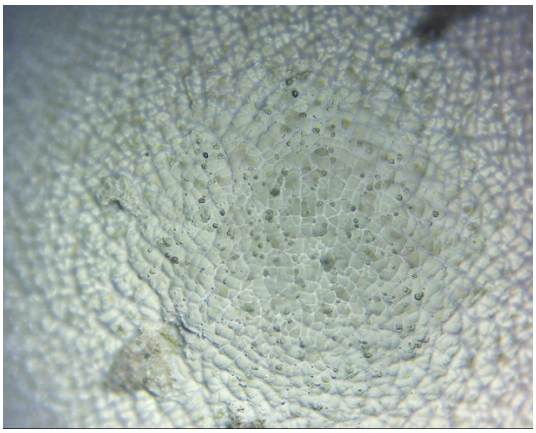


Figure 22: Large bead day 14, zoom 10x, grown in 25% media

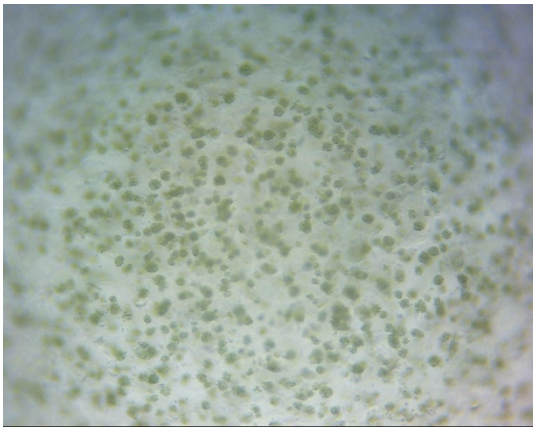


Figure 23: Large bead day 14, zoom 10x, grown in 50% media

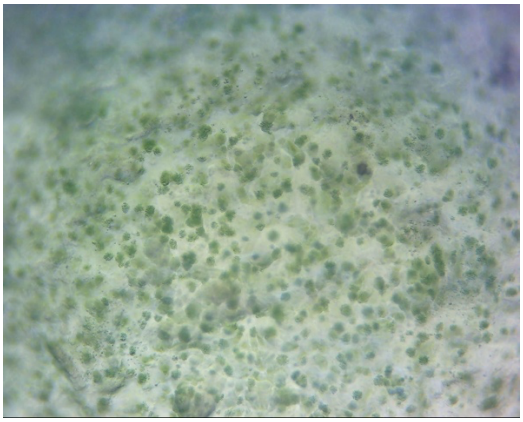


Figure 24: Large bead day 14, zoom 10x, grown in 100% media

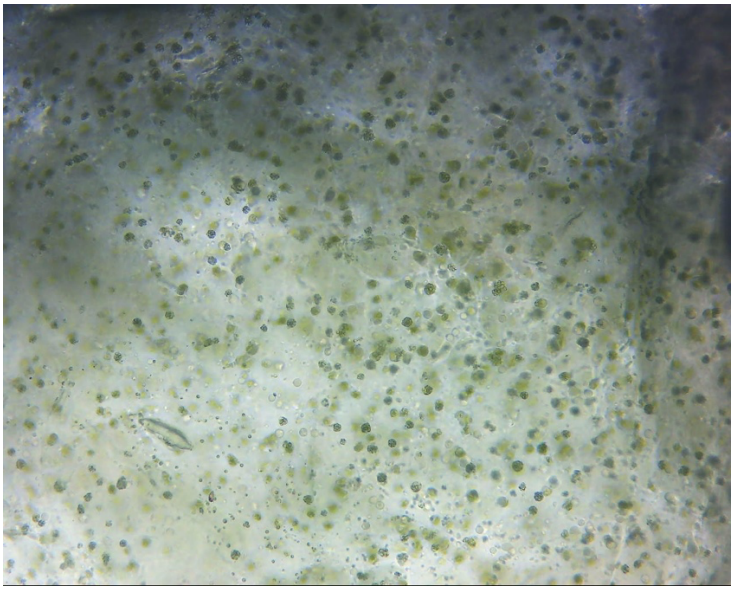


Figure 25: Large bead cultivated in 100% PWW-mediaon day 14 cut in half. Growth was now observed within the center of the bead.

5. Challenges met during the experimental designs

During the experimental setup, multiple hurdles were met and new unforeseen challenges arose along the way.

Baseic pH caused alginate to dissolve

When attempting to cultivate microalgae immobilized in sodium alginate with PWW at different pH levels, sodium hydrogen phosphate and sodium dihydrogen phosphate were used to adjust to the desired pH levels. The alginate beads in the bottles with pH 6 held up fine, but the alginate beads in the bottles with pH 7, 8 and 9 did not. They completely dissolved within 12 hours. It was hypothesized that the pH buffers were to blame, and it was discovered that alginate dissolve in basic phosphate buffered solutions due to calcium-ion release from the alginate beads.(Kikuchi et al., 1997)

Aeration caused loss of liquid and other problems

Initial tests of cultivating immobilized microalgae were done with aeration. This proved successful in terms of growth. It seemed to grow faster and denser than subsequent batches, but it also caused significant problems. The aeration tube had to be lowered all the way to the bottom of the flasks to ensure circulation and movement of the immobilized algae beads. The airstream was not powerful enough to create significant movement. This problem could however have been fixed by using a magnetic stirrer, but that was not available at that point. The other problem caused by aeration was that it caused evaporation of the liquid media. The volume had noticeably decreased after just three days of constant aeration. Some flasks had lost just a few ml liquid, while others had lost 15 to 20 ml compared to the starting volume. Continuing with this would have made OD measurements unreliable and increased the concentration of nutrients in the media, due to water evaporating and nutrients remaining. Maintaining consistent airflow into every single flask was also a big challenge. Air sparging rate varied considerably between flasks. Every tube had to be set to the exact same height and the slightest shift would disrupt the air sparging rate and cause inconsistent airflow into the flasks.

Particles in media caused problems

The first attempts at cultivating immobilized micro algae, utilized PWW-media with too much particles causing disturbance when measuring OD as well as limiting the amount of light that reached the immobilized algae beads at the bottom causing less growth.

Equipment

A limiting factor was the lack of equipment and resources. Magnetic stirrers were in high demand at the university and only a certain number of stirrers were available to each student. The COD, TN and TP test-kits were quite expensive limiting number of tests to work with, this resulted in a lower frequency of nutrient-depletion analysis and parallels than initially intended.

Microbial contamination

Microbial contamination also became an issue an issue during cultivation of the various batches. After a few days, microbial contamination was observed in some of the batches. It is believed that the contaminations were introduced during sampling of media and culture for OD and nutrient measurements. (Too high levels of microbial contamination could cause interference when analyzing OD) Sterile pipette tips were used for sampling, but aseptic conditions were not maintained at every sampling causing the introduction of microbes. (During the tests in Taiwan this was not considered an issue due to PWW media not being autoclaved). To avoid contamination, better aseptic conditions and routines must be kept. It is however near impossible to keep aseptic conditions when cultivating algae like this. Microbial contamination did however not seem to have any effect on the growth of the microalgae free in the media nor the microalgae incapsulated in the alginate beads.

6. Conclusions

The work presented in this thesis was plagued by multiple setbacks and failures, but some conclusions can still be drawn from it. The results of the piggery wastewater characterization showed that it has potential to be used as an alternative to chemical nutrient medias and as a more cost-effective media for microalgae cultivation. The study has shown that piggery wastewater, when adequately diluted, contains the physical and chemical properties, as well as the necessary nutrients, for microalgal growth. Pretreatment of the media was shown to be important to avoid particles and other contaminations in the media. Filtering it through a fiberglass filter significantly reduced the particles in the media making it less turbid and allowed more light to penetrate. In addition, it was vital to have a media free from particles to get a more accurate optical density reading. Sterilization, by autoclaving, was also an important pretreatment step to avoid microbial contamination. Most batches were however contaminated eventually, but this was most likely due to unsterile sampling techniques.

Immobilization of microalgae using sodium alginate proved to work eventually after some trial and error. Maintaining stable beads proved to be difficult due to the fact that basic pH environments would dissolve the beads completely. Basic pH environments without added buffers also made the structural integrity of the bead weaker.

A 30 g/L mixture of sodium alginate and distilled water worked best when mixed 2:1 with non-centrifuged algae culture. Growing microalgae were then shown to be present within the entire bead for both the large and small bead sizes. It is however worth noting that most of the growth still occurred within the outermost layers of the beads.

In conclusion, piggery wastewater seems to be a viable cost-effective alternative to other growth medias. Immobilized microalgae simultaneously serve as a relatively effective wastewater treatment technology with the pros of being more cost-effective and leaving less of a carbon footprint compared to other more conventional treatment technologies.

7. Future work

Microalgae have been gaining more interest and attention over the years due to their properties, abilities and vast potential that is useful in various industries. With continued research and optimization more useful properties could be found in all areas of the microalgae industry.
There are several factors to be considered if this work is to be continued in the future.
Due to limited budget and time, as well as the many setbacks and restarts of the experiments, the most optimal cultivation conditions were not explored. Different strategies and conditions such as light intensity and light cycles as well as temperature conditions were not altered but could have been optimized for better cultivation conditions. Perhaps most importantly, sparging rates with air or CO_2 were not performed due to the various complications it introduced. This would most likely have significantly improved the growth rate of the immobilized microalgae. Given more time, a more optimal alginate bead could be made to ensure better nutrient uptake for all microalgae present in the bead. Be this a smaller sized bead or a structurally different bead with larger pore size for more effective nutrient permeability.

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