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Application of Grass Juice for Microalgae Cultivation

Master Thesis

In collaboration with Danish Technological Institute

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Abstract

The demand for organic protein resources for feed is increasing due to the fast increase in organic farming. It is a challenge to get organic soy protein that does not contain genetically modified organisms (GMO) to be used as feed. Using soybean as protein feed is not a sustainable and efficient solution as it needs to be transported through a huge distance from one country to another. This results in high cost and emission of CO_2. The concept of green biorefinery provides useful solutions for an unconventional protein feed by using green biomass in forms of plants or grasses that are naturally grown and contain high amounts of proteins. Microalgae is a potential alternative protein-rich source for animal feed and thus, it can be used with these green biomasses to produce high yields of protein. This thesis studies the possibility of using brown juice - fermented and heat coagulated - as a culture media for microalgae cultivation. From the results of the different experiments performed, it is observed that the fermented brown juice is suitable for microalgae growth, under light cultivation with the addition of extra nitrogen and phosphorus to the growth media.

Acknowledgment

The authors would like to thank Malene Fog Lihme Olsen for her experimental advice and assistance in the laboratory.

Hypothesis

The hypothesis of this thesis is the *"Evaluation of two different brown juices from Alfalfa as growth media for microalgae cultivation"*. This hypothesis will be answered during the thesis report. To confirm the above hypothesis, sub-questions are formulated that will be discussed in this report:

1. Is it possible to use fermented brown juice or heat coagulated brown juice for microalgae cultivation?
2. Is it possible to find optimal concentration of the grass juices for microalgae cultivation?
3. Can the addition of extra nitrogen and phosphorus improve the growth media?
4. Does the algae have the ability to grow both in the light and in the dark?

1. Introduction

Organic farming is growing fast and therefore, there is a demand for organic protein resources. The huge concerns and challenges are to reach an effective amino acid profile that can be easily affordable. The difficulty in getting organic soy protein that does not contain genetically modified organisms (GMO) is a challenge as it is the main supply in organic agriculture (Santamaria-Fernandez et al., 2017). The need for organic sources will increase in the future and will result in high cost. Soybeans are transported from Asia to guarantee the sustainability of the products. However, Europe has the resources of producing more agricultural schemes based on the utilization of nearby produced feedstocks (Santamaría-Fernández et al., 2017). A theory to produce biomass locally is the green biorefinery concept". The concept of green biorefinery provides useful solutions for an unconventional protein feed by separating proteins from green biomass, which contain high amounts of proteins. The emphasis is mainly on producing organic feed for monogastric animals. The purpose of this concept is to provide helpful techniques to overcome some challenges, such as low crop yield and limited supply, which restrict the development of organic production. Crops containing high-value proteins are selected as potential feedstocks and they can be cultivated in an organic manner in farms located locally (Santamaría-Fernández et al., 2017). Using organic farming to increase the protein levels of the produce as well as getting pesticide and fertilizer-free crops to animals has not been a productive solution so far. Plants and grasses are mostly used in organic acid fermentation that gives an efficient precipitation of proteins for the effective recovery and harvest. The above-mentioned problems and challenges in terms of using soybean as a protein source as well as transportation

and cost effectiveness can be solved by using microalgae, which is a potential alternative protein-rich source for animal feed.

The aim of this thesis is to test the possibility of using brown juice from Alfalfa for the cultivation of microalgae. The objective is to evaluate two types of brown juices; fermented and heat coagulated for their suitability as growth media for microalgae.

1.1 Background

1.1.1 Alfalfa

The bio-based economy is becoming more developed in Europe and biomass will be used to produce food and feed as well as biochemicals and fuels as a substitute for fossil resources thus making it a valuable resource in the future (Höltinger et al., 2014). Alfalfa (*Medicago sativa* L.) is a perennial crop that is primarily cultivated for use as feed for dairy cows, sheeps, horses, and other livestock in many countries. It is mainly processed and used on the farm in the form of silage, dried hay, and fresh forage or is eaten by animals in pastures. The protein content, minerals, vitamins, and fiber contents make the crop greatly nutritious feed for ruminant animals (Samac et al., 2006). Alfalfa has the ability to produce its own nitrogen fertilizer thereby enriching poor soils (Comis D, 2002). It can potentially serve as a feedstock for the production of feed, fuels, and industrial materials. Although, the refining of Alfalfa for the production of feed, fuels, and industrial materials is still undeveloped. Alfalfa is an attractive crop for biorefining and production of fuels due to its different attributes. It has been grown for a long period of time all over the world (Russelle, 2001). Each year, on average, Alfalfa produces

7.8Mg of dry matter (DM) per hectare and it is the fourth most extensively planted crop in the United States (USDA-NASS, 2004). There are several environmental advantages of using Alfalfa as a biomass crop. In order to reduce contamination of water and erosion, it is highly important to increase the usage of perennial crops in agriculture. Alfalfa can reduce the concentrations of nitrate in the soil and in drainage water, and it can also prevent soil erosion (Huggins et al., 2001). The energy cost for the production of Alfalfa is very low. A study by Kim and Dale, 2008, shows that the energy used for Alfalfa production is much lower compared to the production of soybean and corn. This is because Alfalfa does not need nitrogen fertilizer. Alfalfa is very easy to separate the stems from the leaves to generate co-products. This gives it an additional advantage compared with other crops. The leaves of Alfalfa are components of high value to the crop because they contain a great amount of protein. Based on dry weight, the total herbage of Alfalfa contains 18 - 22% protein, the leaves contain 26 - 30% protein, and the stems contain 10 -12% (Arinze et al., 2003). The refining of Alfalfa can be performed in several different integrated processes. From the dried hay on the field, the leaves can be mechanically separated from the stem. The leaf meal can be used as feed rich in protein while the stems can be used for gasification and electricity conversion (Downing et al., 2005) or it can be fermented to ethanol (Dale, 1983). Conversely, the leaves can be crushed to extract the proteins while the leftover can be utilized for fermentation (Koegel et al., 1999; Sreenath et al., 2001; Weimer et al., 2005). Previous research has focused on developing the leaves of Alfalfa as an alternative source of food protein. This method involves cutting up the harvested leaves, expressing the juice mechanically, coagulating the protein to heat the juice to 55 - 60^{o}C, centrifugation, and freeze-drying of the recovered precipitate. Acid precipitation can also be used rather than

heating and membrane filtration methods. Depending on the separation method used, the dried Alfalfa leaves, the leaf juice, press cake, or precipitate from fermented leaf juice can serve as sources for food or feed protein (Hojilla-Evangelista et al., 2017).

1.1.2 Biorefinery

As the global population is increasing, there is increased pressure on supplies of food, mitigation of climate change, and the exhaustion of fossil fuels (Höltinger et al., 2013). These issues require a change in technology and policy in order to use natural resources effectively. Therefore, international countries such as Europe are changing towards a bio-based economy. This initiative aims at effectively utilizing biomass feedstock across the supply food chain in order to minimize the pressures on the environment as well as feedstock and resource competition (Tilman et al., 2009). Many European countries have realized the potential of green biorefineries to create opportunities for business and employment (Mandl et al., 2010). The green biorefinery concept aims at reducing the import of soybean meal feed and preserving cultural landscape and biodiversity. The objective of the green biorefinery is to use green biomass to generate valuable products such as proteins, energy, lactic acids, amino acids, and fibers (Kamm et al., 2010). Alternatively, GBR may also solve the problems associated with monogastric animals in organic farming such as the insufficient supply of organic fertilizer, supply of feed organic protein with the correct amino acid profile and low value of leguminous crops as well as low yields in non-leguminous crops in organic crop rotation. The scope is to use biomass for the production of food and feed, fertilizer and also production of biochemicals and fuels which will replace fossil fuels and manage the increase in energy demand (Höltinger et al.,

2014). Hence, the solution is to develop a biorefinery system that converts biomass into numerous products through efficient and non-waste generating processes. The technique of the green biorefinery concept is to perform mechanical fractionation in order to separate the freshly harvested feedstock into solid, that is press cake, and liquid fraction, green juice. The press cake can be used in a biogas plant as feed for livestock or for fiber application. The green juice undergoes lactic acid fermentation to obtain 2 fractions, protein concentrate and residual brown juice. In the biorefinery concept, protein concentrate is the primary product. The objective is to use the protein concentrate as a substitute for imported soybeans which is currently the main protein source for feeding animals in organic farming.

The press cake and brown juice can undergo anaerobic digestion to produce biogas in the form of methane. The liquid effluent from anaerobic digestion contains nutrients residual organic matter and it can be used as an organic fertilizer (Höltinger, 2014).

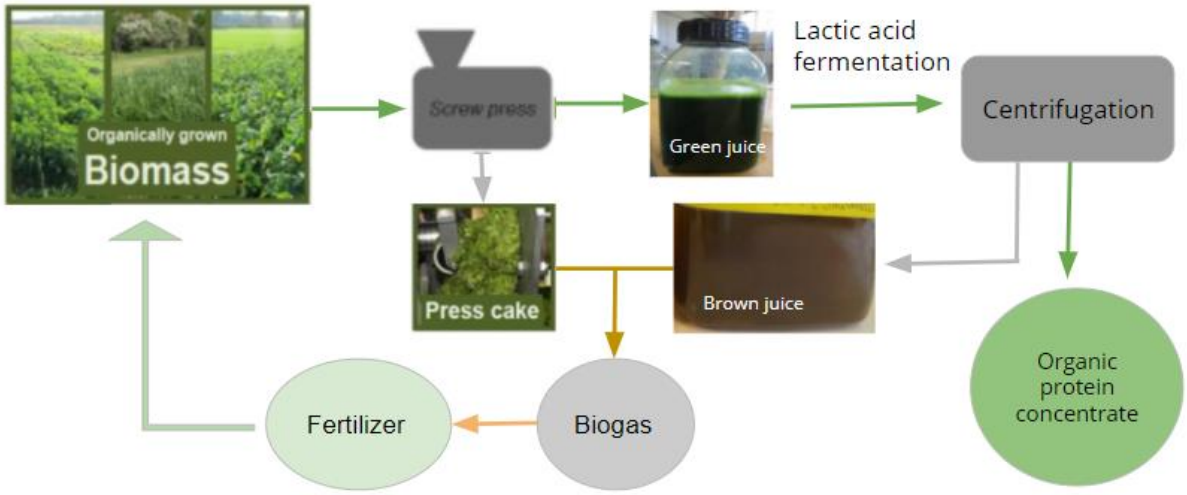


Figure 1: Green Biorefinery concept scheme.

1.2 Microalgae

Microalgae are known as photosynthetic unicellular microorganisms mainly discovered in freshwater, marine, and sediment environments. Microalgae consist of a group of heterogeneous species, which contains eukaryotic and prokaryotic microorganisms (Chew et al., 2017). This group of microorganisms have the ability of converting carbon dioxide and light into biomass using photosynthesis. In this process, the most important nutrients are phosphorus and nitrogen that need to be bioavailable as PO_4^{3-}, NH^{4+} and various micronutrients like vitamins and trace metals. Microalgae have high biochemical diversity and capability of producing various types of metabolic compounds such as vitamins, antioxidants, proteins, and lipids, which makes them an interesting and promising source of metabolites (Chew et al., 2017). For instance, microalgae are mostly used as food or feed additives, pharmaceutical products, and substrates to produce biofuels like biodiesel (Astorg, 1997).

1.2.1 Scenedesmus Species

The genus, *Scenedesmus* is a unicellular green microalga from the genera *Chlorophyceae* and is grown in freshwater (An et al., 1999). *Scenedesmus* species are nonmotile with different cell shapes and contain 2-32 coenobia cells in rows (Komarek et al., 1983). The picture below shows the Scenedesmus algae having more than one cell and formed as clusters (Figure 2). The microalgae are promising for producing high value products such as lipids, proteins and carbohydrates for feed purposes as well as biodiesel and bioethanol (Arbib et al., 2013). Compared to continental plants, the microalgae contain higher rates of biomass (Arbib et al.,

2013). The microalgae have the ability to reduce CO_2 emissions by converting it to chemical energy through photosynthesis. In terms of nutrient, carbon, phosphorus and nitrogen are the most significant nutrients for the production of microalgal biomass (Arbib et al., 2013). The nitrogen content can range between 1-10% depending on the algal species and nutrients available. The phosphorus content is about 1% of the algae biomass while the carbon content is about 50% of the algal biomass (Grobbelaar, 2004).

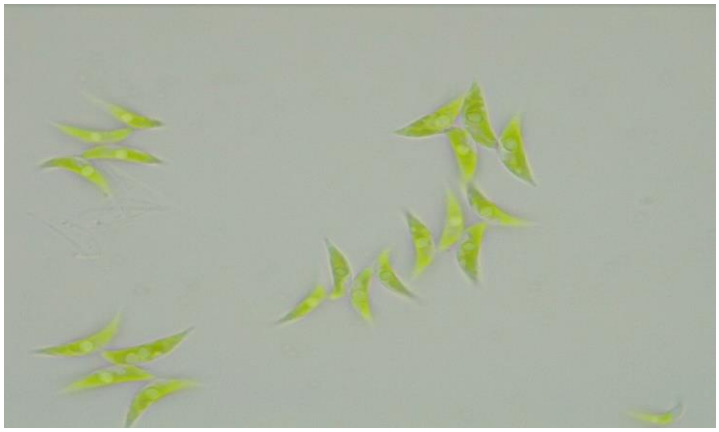


Figure 2: Scenedesmus sp. (DTI lab, microscope).

1.3 Microalgae Cultivation

The conditions for the cultivation of microalgae are based on the composition of the microalgae and the growth characteristics (Chojnacka and Marquez-Rocha, 2004). There are four main types of conditions for microalgae cultivation. They are photoautotrophic, photoheterotrophic, heterotrophic, and mixotrophic cultivation (Chojnacka and Marquez-Rocha, 2004).

1.3.1 Photoautotrophic Cultivation

For this type of cultivation, microalgae use light e.g., sunlight as the source of energy and inorganic carbon such as CO_2 as the carbon source to form chemical energy through photosynthesis (Huang et al., 2010). This method is the most used method of microalgae cultivation (Gouveia et al., 2009; Gouveia & Oliveira, 2009; Illman et al., 2000; Mandal & Mallick, 2009; Yoo et al., 2010). Photoautotrophic cultivation is operated in open ponds or photobioreactors. When compared to other types of cultivation conditions, an advantage of using autotrophic cultivation is that the problem of contamination is reduced. Hence, outdoor upscaled systems for microalgae cultivation, such as open and raceway ponds, are generally operated under phototrophic cultivation conditions (Mata et al., 2010).

1.3.2 Heterotrophic Cultivation

This is a type of cultivation where microalgae use organic carbon as both energy and carbon source. Some microalgae species, like bacteria, can grow both under phototrophic conditions and dark conditions (Chojnacka & Marquez-Rocha, 2004). Heterotrophic cultivation is independent of light; hence, it can prevent the problems associated with limited light which inhibits high cell density in large scale photobioreactors during phototrophic cultivation (Huang et al., 2010). The production and productivity of biomass can be higher when using heterotrophic cultivation. During heterotrophic growth, some microalgae species show higher lipid content (Xu et al., 2006). Microalgae can incorporate different organic carbon sources for their growth such as glucose, fructose, acetate, lactose, sucrose, galactose, etc. Studies have researched cheaper alternative sources of organic carbon like corn powder hydrolysate which

results in high productivity of lipids and biomass (Xu et al., 2006). The use of heterotrophic cultivation gives much higher lipid productivity than that obtained using phototrophic cultivation. Although, the heterotrophic cultivation system using sugars often results in contamination (Chen et al., 2011).

1.3.3 Photoheterotrophic Cultivation

In this cultivation, microalgae need light as an energy source when utilizing organic compounds as carbon source. In comparing mixotrophic and photoheterotrophic cultivation, phototrophic cultivation uses light as an energy source. Hence, it requires both light and sugars at the same time. On the other hand, mixotrophic cultivation can use organic compounds as energy sources (Chojnacka & Marquez-Rocha, 2004).

1.3.4 Mixotrophic Cultivation

Microalgae uses both inorganic carbon (CO_2) and organic compounds as a carbon source for growth and also undergoes photosynthesis. In other words, microalgae can live under either heterotrophic or photoheterotrophic conditions or they can live under both conditions. Microalgae incorporates CO_2 and organic compounds as a carbon source. The CO_2 released by microalgae through respiration will be trapped and used again under phototrophic cultivation (Mata et al., 2010). The microalgae in this process are mainly grown in bioreactors or open ponds with a media containing phosphorus, nitrogen, and organic carbon in order to repair both inorganic carbon with photosynthesis and use organic carbon through light circumstances

with low intensity (Wang et al., 2014). In comparison with phototrophic and heterotrophic cultivation, mixotrophic cultivation is hardly used in the production of microalgal oil.

1.4 Liquid and Solid Media

For the cultivation of microalgae, two types of culture media can be used, liquid and solid media. The liquid media can be utilized for both batch and continuous cultures. The batch culture is done by cultivating the inoculum for microalgae in a controlled volume of the culture media (Bux and Chisti, 2016). The continuous culture is performed in a photobioreactor, where a fresh stream of the culture media is poured, and the culture is continuously harvested at a constant flow rate. This process allows the culture to stay in an exponential phase for an unlimited time (Wood et al., 2005). For solid media, a streaking technique is mainly used, which can be performed using agar plate in a petri dish (Bux and Chisti, 2016).

2. Materials and Methods

The equipment and materials used in this project are shown in Appendix 1, page 44 and 45.

2.1 Sampling

The brown juices used in this study are obtained from Aalborg University. The brown juice is a by-product of the fractionated Alfalfa grass. The first step after harvesting the grass was to separate the crops through mechanical separation. This separation produces the green juice and press cake. The green juice undergoes Lactic acid fermentation or heat coagulation and also centrifugation to produce 2 fractions, a protein concentrate and residual brown juice. The brown juice is kept in a -20^{o}C freezer for composition analysis and further experiments.

Juice 1: Fermented brown juice.

Juice 2: Heat coagulated brown juice.

2.2 Composition Analysis

The two types of brown juices are analyzed to determine the dry weight and total solids. The nitrogen, phosphorus and protein content in both juices are also measured. The total solids, carbon, and nitrogen content were analyzed at Aalborg University. The analysis is performed using an elemental analyzer (CHNS analyzer). The estimate of crude protein is determined by multiplying the nitrogen content with the factor 6.25 (Fuentes et al., 2000; Becker, 1994). Table 1 shows the result of the analysis.

Table 1: Composition of the two types of brown juice

	Fermented juice	Heat coagulated juice
% Dry weight	2.7	2.2
% T. S	87.4	88.4
% Phosphorus	0.100	0.08
% Carbon	36.09	35.33
% Nitrogen	1.32	1.29
% Crude protein	8.27	8.03

2.3 Microalgal Strain

The strain of microalgae used in this study is *Scenedesmus* sp. obtained from the collection of algae culture used in the ReMAPP project at the Danish Technological Institute. The Inoculum for the experiments is cultivated in an indoor vertical airlift photobioreactor which has a tube diameter of 6 cm. Pure CO_2 is injected into the reactor and artificial light is used. The microalgae are cultured under 16:8 light/dark cycles. After cultivation, the algae is centrifuged and washed before being used for later experiments in order to ensure that extracellular nutrients are not transferred to the new media.

2.4 Centrifugation and Filtration

Aim: To remove solids and sterilize the samples.

The juices are transferred to falcon tubes and centrifuged for 10 minutes at 23°C with a speed of 8300 rpm. After centrifugation, the liquid is separated from the pellet in a beaker for filtration. Filtration is done by first using vacuum filtration and then using a Q-max syringe filter (0.2 μm) in red beaker tubes.

2.5 Characterization of Brown Juices

The brown juices are characterized in terms of pH, total nitrogen, and phosphate.

2.5.1 pH Measurement

Aim: To adjust the pH of the brown juices to 7.

The pH is estimated with a pH-meter by dipping the glass of the electrode in the brown juice. The pH is adjusted by using a 2M NaOH. The heat coagulated brown juice has a starting pH of 5.5 and it is adjusted to 7.4 by adding 10mL of 2M NaOH while the fermented brown juice has a starting pH of 3.8 and adjusted to 7.5 by adding 25mL of 2M NaOH.

2.5.2 HACH Analysis

The phosphate, total nitrogen and ammonia concentration in the treated brown juices are measured using HACH kits. Before performing the HACH analysis using the kits, the nitrate, phosphate, and ammonia test strips are used to measure the amounts of nitrogen, phosphate, and ammonia in the juices in order to find the range needed for the HACH analysis. For the nitrate

test strips, the juices are diluted 10x and 20x. The result is zero for both dilutions. For the phosphate test strip, the juices are diluted 5x and 10x. The result of the test is zero for both juices. For the ammonia strip test, both samples are diluted 2x. Both juices have an ammonia concentration of 45 mg/L. To perform the HACH analysis, the two brown juices are diluted the same as the dilutions made for the measurements using test strips. The HACH analysis is carried out based on the protocol enclosed in the kits.

2.5.3 HACH Kits

2.5.3.1 Total Nitrogen

The total nitrogen TN_b LCK 238 kit is used.

0.5 mL of diluted brown juice, 2.0 mL of solution A and 1 tablet B from the kit are added into a dry reaction tube and closed immediately. The tubes are heated immediately at 120^{o}C for 30 minutes. Afterwards, the tubes are set to cool down at room temperature. After cooling, the tubes are inverted several times. 0.5 mL of samples in the reaction tubes are slowly pipetted into the cuvette test tubes. 0.2 mL of solution D is pipetted into the cuvette. The tubes are immediately closed and inverted a few times until there are no streaks in the cuvette. The tubes are observed to slowly turn pink and after 15 minutes the cuvette tubes are evaluated using a HACH spectrophotometer.

2.5.3.2 Orthophosphate

Phosphorus is measured as orthophosphate and the phosphate LCK 349 kit is used.

2.0 mL of the diluted brown juices are carefully pipetted into the cuvette tube and closed immediately. The tubes are shaken vigorously so the powder in the Dosicap can react with the samples. The tubes are heated in the thermostat for 30 minutes at 120^{o}C. The tubes are set to cool down to room temperature and afterwards, the tubes are shaken vigorously. 0.2 mL of reagent B is pipetted into the cooled cuvette tubes and it is closed with a grey Dosicap C. The cuvettes are inverted a few times until the freeze-dried content is completely dissolved. After 10 minutes, the cuvettes are inverted a few more times and evaluated using a HACH spectrophotometer.

2.5.3.3 Ammonia

The Ammonia LCK 303 kit is used.

0.2 mL of brown juice is carefully pipetted into the Dosicap tubes. The tubes are closed and shaken vigorously so the powder in the DosiCaps can react with the juice. After 15 minutes the DosiCap tubes are evaluated using a HACH spectrophotometer.

After performing the HACH analysis, the results obtained are inconclusive. This could be because some of the HACH kits used have expired. Hence, the brown juices were sent to Eurofins for nutrient analysis.

2.6 Preparation of Inoculum

A single colony from a monoculture of the ReMAPP algae *Scenedesmus* sp. is transferred from a petri dish with solid BG11 media to a cultivation flask containing 100 mL BG11 liquid media. The

flask is placed at a light intensity of 100 µE at 23°C for 7 days with manual shaking every second day. The Inoculum is ready when OD (720nm) is between 0.8 - 1.0.

2.7 Multiwell Screening

Aim: To find the optimal concentration of the juice for algae cultivation and to determine if the addition of extra nitrogen (N) and phosphate (P) can improve the media.

First, the two juices are diluted in falcon tubes (concentrated, 2x, 4x, 8x. 16x, 32x, 64x).

From the dilutions made, identical screenings are carried out in multiwell 48 well plates for each of the juices. 4 well plates are prepared for light and dark cultivations. The various media compositions are tested in triplicates (3 wells for each). That is, the wells from 1-7, A-F each contain 785 µL of diluted juice. In column 1-7, A-C, 15 µL of H_2O and 15 µL of microalgae are added to each well. The same dilutions are repeated with the addition of extra N and P in each well (5 mM NH_4NO_3 and 2mM KH_2PO_4). That is, the wells in column 1-7, D-F, 15 µL N and P stock solution and 15 µL of microalgae are added to each well. For column 8, BG11 liquid media is added to well A-C while BG11 media + 1.0 w/v% solution of glucose is added to wells D-F in triplicates as control in all plates (Figure 3). Of the 4 well plates prepared, one plate containing fermented juice and the other containing heat coagulated juice are covered in silver foil. All plates are then cultivated under light/dark conditions for 7 days. After 7 days of cultivation, the optical density at 720 nm is measured and the most promising media composition is selected for cultivation in 250 mL culture flasks. After observing the plates, the fermented juice is selected for the cultivation in 250 mL flasks.

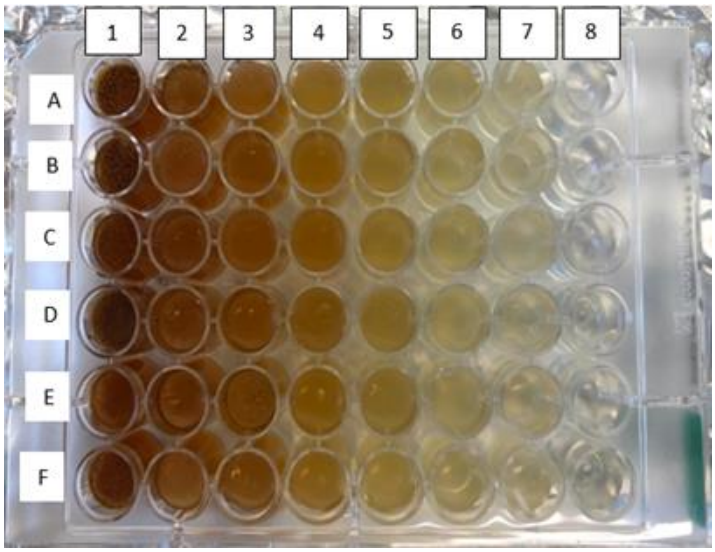


Figure 3: Multiwell screening plate.

1 = Concentrated, 2 = 2x, 3 = 4x, 4 = 8x, 5 = 16x, 6 = 32x, 7 = 64x, 8= BG11 control.

D-F: Addition of extra N and P.

2.8 Cultivation in 250 mL Flasks

Aim: To estimate biomass yield as well as protein content of microalgae cultivated in media based on the juice and also model the growth of microalgae.

The cultivation is carried out only with light exposure. Based on the results obtained from the multiwell screening, the 16x, 32x, and 64x dilutions of fermented brown juice as well as 32x dilution of heat coagulated juice are selected for the cultivation in 250mL flasks. 390 mL of sterile H_2O is added to a measuring cylinder together with 7.8 mL of N and P stock. 4.7 mL of microalgae and the different dilutions of the juices are also added in the measuring cylinder. A set up with a total of 18 cultivation flasks containing 100 mL media is prepared. The flasks are prepared in triplicates. The flasks are cultivated under light at a light intensity of 100 µE at 25°C with the flasks shaken twice a day. 14 mL of samples is taken from each flask on day 0, day 3, day 7 and day 10

and placed in the freezer at -20°C. After 10 days, the cultivation is terminated, and the optical density is measured for all samples.

2.9 Cell Counting

Aim: To estimate the number of cells present in the samples.

The cells in the 16x, 32x, and 64x fermented well plates as well as 32x heat coagulated well plates are counted under a microscope using a counting chamber. The results are calculated to estimate how many cells are present in a 1mL sample and a graph is made. The graph gives a pictorial illustration of how many cells there are in each diluted sample.

2.10 Analysis of Biomass Composition

After cultivation, the algae samples taken from the flasks on day 0,3, 7 and 10 are analyzed. The following analyses are performed: DM measurement, total nitrogen, and crude protein content (i.e., nitrogen content × 6.25) (Fuentes et al., 2000; Becker, 1994).

2.10.1 Dry Matter Measurement (DM)

After 10 days of cultivation in flasks, the DM content in each sample is measured using vacuum filtration. Empty Whatman filters are prepared for each sample and each filter is weighed. All the samples from the 10 days of cultivation are filtered using vacuum filtration. First, the filters are moisturized with H_2O and then 10 mL of each of the samples are filtered. The filters with samples are transferred to a steel tray and placed in the oven at 80°C overnight. Next day, the filters were taken out from the oven and weighed (Appendix 2, page 46-47).

2.10.2 Protein Analysis

For the analysis of protein, the 16x and 32x dilutions of fermented media as well as BG11 from the 250 ml cultivation flasks are chosen for preparing the samples. The media from the flasks are transferred into falcon tubes and centrifuged for 10 minutes at 10.000 rpm. Empty silver cups are weighed using an analytical balance. Afterward, the pellets from the centrifuged tubes are transferred into the silver cups and placed in the oven at 105°C overnight. Next day, the silver cups are weighed on the analytical balance. The samples in the silver cups are ground in a mortar using a pestle to make the samples into powder form and these powdered samples are then transferred into Eppendorf tubes and sent to Aalborg University for the analysis. The analyses are performed using Elemental analyzer (CHNS analyzer).

3. Results and Discussion

With the aim of investigating the possibility of utilizing brown juice for microalgae cultivation, 2 types of brown juices are used for microalgae growth under identical growth conditions.

3.1 Multiwell screening

Screening for microalgae cultivation is performed using multiwell plates. 4 well plates are prepared for the filtered fermented and heat coagulated brown juices and are cultivated under light and dark conditions for 7 days. Figure 4 below shows images of the well plates after 7 days of cultivation in light/dark.

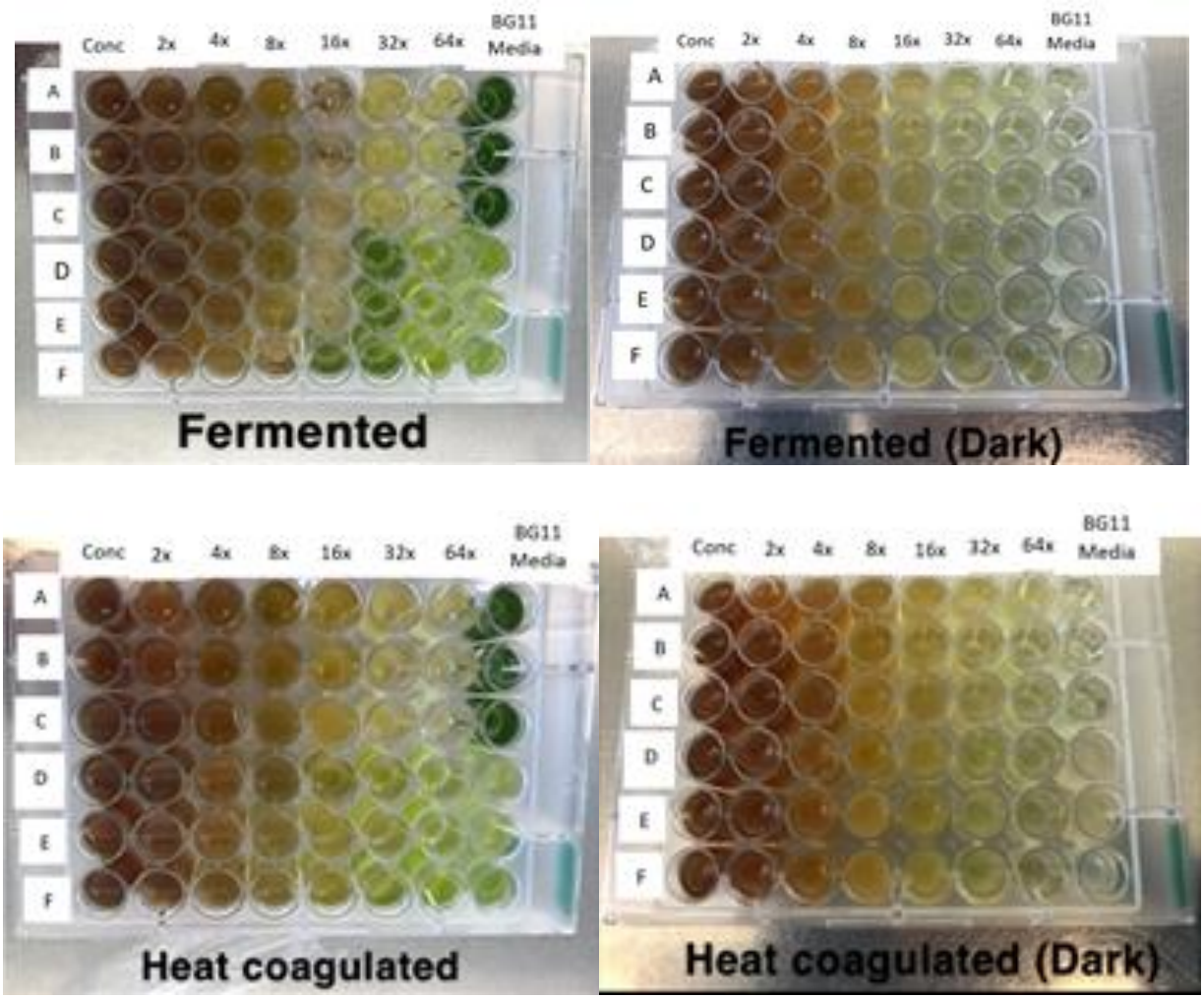


Figure 4: Multiwell plates for the two types of brown juice cultivated under light and dark.

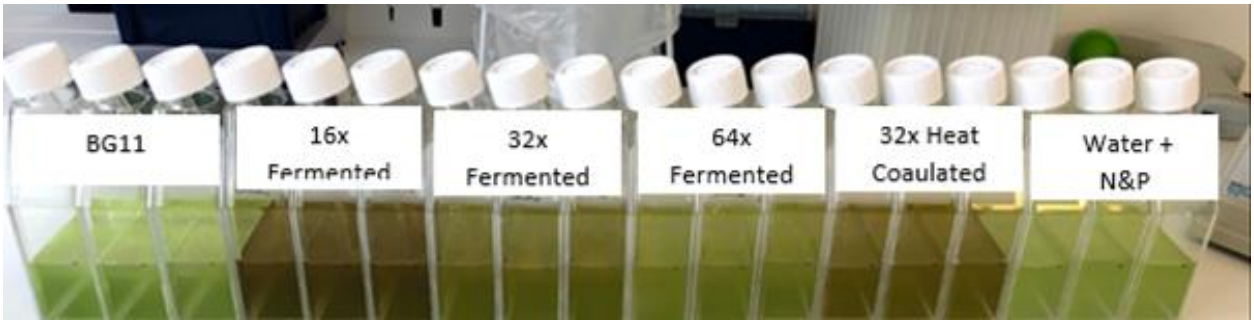
The observation from each cultivation showed that microalgae is able to grow at a certain concentration of the brown juice media. The microalgae is cultivated in well plates under different conditions to evaluate the effect of light and addition of extra N and P on microalgae growth. The algae presented different growth in the fermented and heat coagulated brown juice media under light cultivation. On the other hand, the algal growth is hindered in the absence of light as the plates containing the fermented and heat coagulated juice grown in the dark yielded no result. This is expected because microalgae require light for growth. The result

shows that the addition of extra N and P has an influence on the growth of microalgae. It also shows that the algae is able to uptake the nutrients of brown juice at a certain concentration with the addition of extra N and P. For the fermented plate cultivated under light, it can be seen that BG11 showed the best result followed by the 16x, 32x and 64x dilutions with the addition of extra N and P. For the plate containing heat coagulated juice grown under light, it can be seen that the BG11 control yielded the best result. BG11 is the standard medium used for microalgae cultivation, hence, the result was expected. However, some growth can also be seen in the 32x dilution with the addition of extra N and P. Under dark conditions, it can be seen the standard BG11 media yielded no growth. A reason for this could be because the *Scenedesmus* sp. used for this experiment is obtained from the ReMAPP experiment which is cultivated strictly under light conditions. Therefore, it could not adapt to changes in growth parameters, that is, dark cultivation.

3.2 Cultivation in 250 mL Flasks

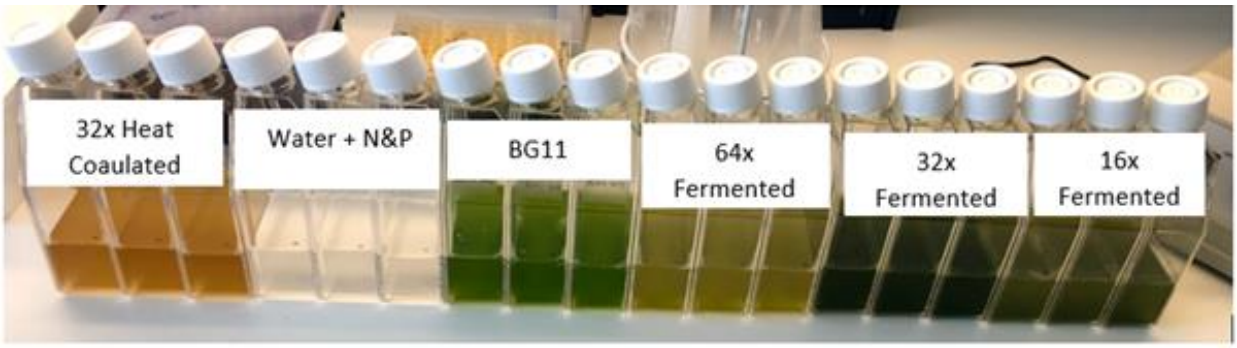
For the cultivation of microalgae in flasks, the most promising results obtained from the multiwell screening, 16x, 32x and 64x dilution of fermented juice as well as the 32x dilution of heat coagulated juice are used. BG11 media is also used as a control. The microalgae is cultivated for a total of 10 days and samples are taken out on days 0,3,7 and 10. The pictures below show the cultivation of the different dilutions for different days:

Day 0:



Day 3:

Day 7:



Day 10:

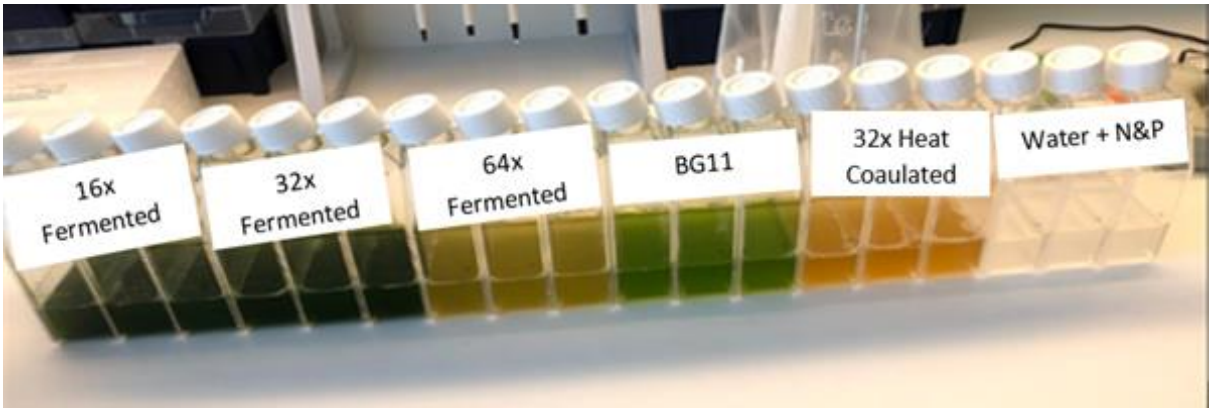


Figure 5: 0,3,7 and 10 days cultivation of microalgae in 250 mL cultivation flasks.

From these pictures on day 10 of cultivation, it can be clearly seen that fermented juice for the 16x and 32x dilutions have more growth compared to the other flasks. This confirmed the results obtained from the multiwell screening. It can also be observed that the 32x dilution containing heat coagulated juice has a brownish color which means that there is no growth of microalgae. The media containing N and P and water is observed to be colorless which means there is no growth of microalgae. A reason for this could be that too much water might have been added to the media which resulted in the algae dying. Before the experiment, the microalgae was washed before use, which could have resulted in the removal of some vitamins needed for algal growth. Figure 6 below shows the results obtained after 10 days of cultivation and growth curves for each cultivation are constructed using DM.

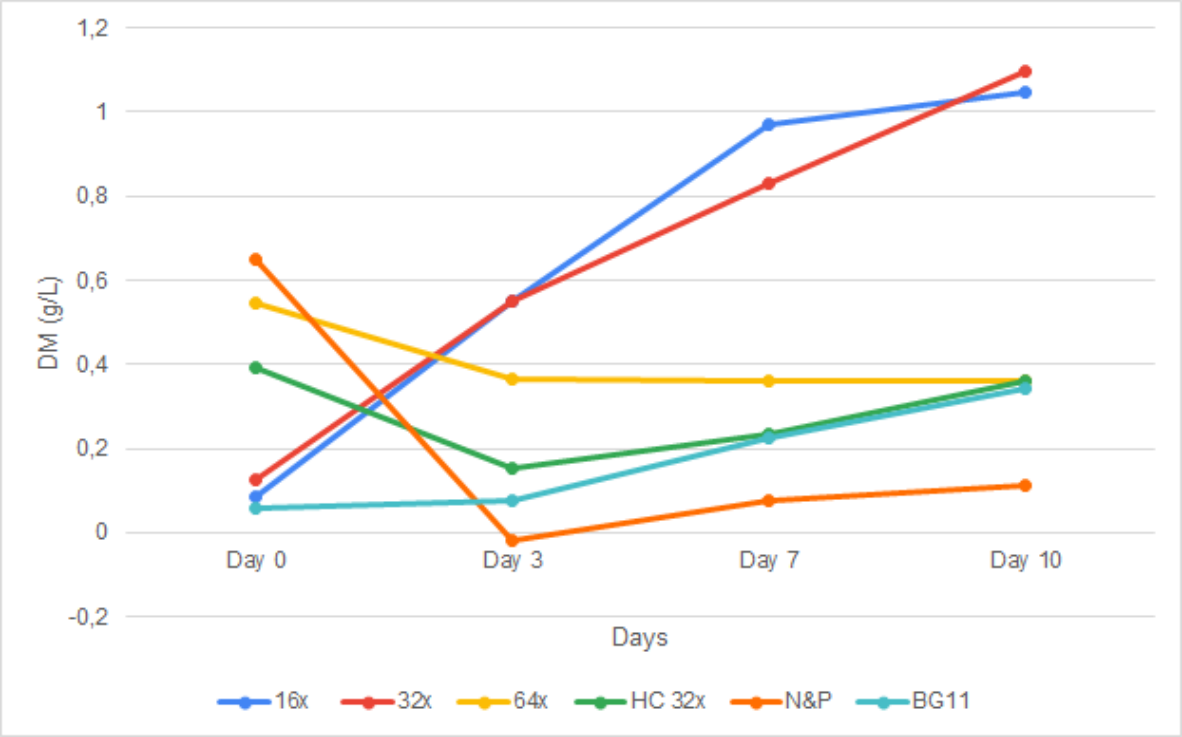


Figure 6: Growth curve of microalgae cultivation from Day 0- Day 10.

From the growth curve, it can be seen that the 16x and 32x dilutions of fermented juice are able to grow acceptably in the culture media. Both dilutions start to grow exponentially without showing any lag phase. For the 16x dilution, it can be observed that on day 7 of cultivation, microalgae growth reaches the maximum and then it tends towards a stationary phase. For the 32x dilution, there is a slight change in the growth pattern on day 3, however, the growth continues exponentially. For the BG11 media, the growth first starts at a stationary phase from day 0-3, but on day 3, the growth is exponential. Therefore, comparing the standard BG11 media, the 16x and 32x dilution of fermented juice for microalgae cultivation, it can be observed that the 32x dilution has a faster growth. The highest cell number is observed in the 32x fermented juice media. Hence, it is the most promising media for microalgae cultivation followed by the 16x dilution.

From the results obtained in the flask cultivation, the algal biomass productivity is calculated in the 16x, 32x fermented and BG11 media.

Table 2: Biomass productivity from the DM for the 16x, 32x and BG11. The biomass productivity is calculated using the mean average values of DM for day 3 and 10 for the fermented juice (Appendix 3, page 48).

Media	Biomass productivity (mg/L/d)
16x	71
32x	78.14
BG11	38

From the result, the highest biomass productivity for *Scenedesmus* sp. is 78.14 $mgL^{-1}d^{-1}$ in 32x dilution of fermented media. Since microalgae is light dependent, a higher biomass productivity is achieved with an increase in light transmittance in the brown juice (Santana et al., 2017). Thus, a higher light transmittance is achieved in the 32x dilution because it has been diluted to be less concentrated. Therefore, light is able to penetrate through the flask easily and provide the algae with the needed light in order to grow.

3.2.1 Comparison with Unfiltered Brown Juice

The multiwell screening and cultivation in 250 mL flasks is also carried out on unfiltered brown juices in order to compare the results from the filtered juices. However, the attempt to perform cultivation in flasks failed due to the growth of contaminants. Therefore, the experiment was stopped.

3.3 Cell Counting

In order to estimate the number of microalgae cells present in the cultivation media, the 16x, 32x, and 64x dilutions of fermented well plates are used for counting. The well plates of 16x and 32x heat coagulated dilutions are also counted in order to compare the two juices and BG11 was used as a control. From the figure below, it can be clearly seen that 32x containing additional N and P in well B of fermented juice has the highest number of cells compared to the other wells A and C of the same dilution. A reason for this difference could be that the samples in wells A and C were not properly mixed before being taken up for counting. It can also be seen that for the 32x dilution of heat coagulated plate, only well C has a very high number of cells compared to the other wells A and B. This could have been as a result of more nutrients added by mistake into well C which could have favored the growth of algae.

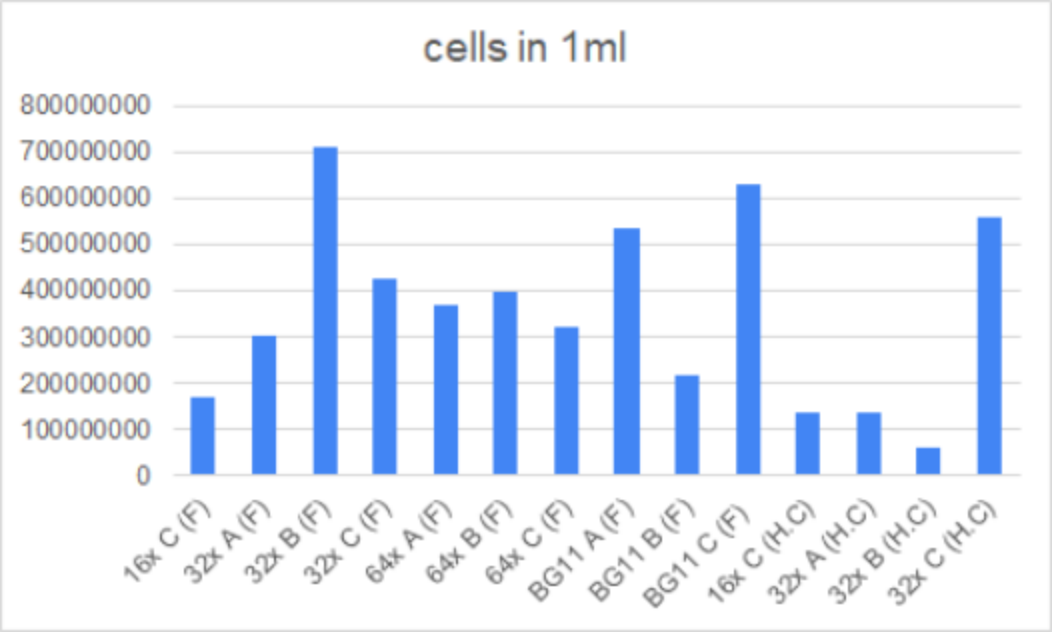


Figure 7: Number of cells in 1 mL sample.

3.4 Nutrient and Protein Analysis

From the results obtained in the well screening and flask cultivation, the fermented brown juice was observed to be the most suitable. The DM and the nutrient composition of the fermented brown juice is shown in the table below.

Table 3: DM and nutrient composition of the fermented juice.

	Fermented juice (g/L)
DM	27
Nitrogen N	3.663
Ammonium-Nitrogen	3.663
Total phosphorus	1.002
Magnesium	1.002
Potassium	16.579
Copper	0.037

The estimate of crude protein present in the fermented juice is obtained by multiplying the nitrogen content of the juice by factor 6.25. Therefore, the potential yield of algal protein from 1 L of concentrated fermented brown juice is about 22.9 g/Liter. From the multiwell screening plate, the 16x dilution has a nitrogen content of 0.369 g/L and an algae protein yield of 2.306 g/L while the 32x dilution has a nitrogen content of 0.254 g/L and a protein yield of 1.59 g/L.

Table 4: Data based on CN-analysis of the different samples in powder form.

Samples	% Carbon	% Nitrogen	% Crude Protein	% T.S	% Protein of DM	g Protein in sample
16x	45.59	4.68	29.26	95.5	30.63	306.3
32x	46.67	3.95	24.72	95.1	25.99	259.9
BG11	49.92	7.74	48.37	92.8	52.13	521.3

From the table above, the control BG11 shows to have the highest protein content which is 52.13 % while the 16x dilution of fermented samples has a DM protein content of 30.6%. The 16x dilution is shown to have a higher protein content than the 32x dilution. A reason for this could be the rate of dilution as the less diluted sample tends to have a higher protein content. It could be because the 32x is diluted more than the 16x sample which makes it less concentrated, there is a loss of proteins in the sample.

4. Conclusion

The aim of this project is to investigate the possibility of using brown juice for the cultivation of microalgae. Brown juice is used to cultivate *Scenedesmus* sp. under different conditions.

The brown juice has a promising nutrient composition that makes it suitable for microalgae growth. The use of the Alfalfa brown juice can eliminate the extracted juice as a waste stream within a green biorefinery concept and thus make the concept more environmentally friendly and economically feasible. To determine the suitability of brown juice as growth media for microalgae, two different juices- fermented and heat coagulated- were prepared in order to find the best media for growth. The results show that the filtered, fermented brown juice is a suitable media for microalgae growth. To determine the optimal concentration of the brown juice for microalgae cultivation, different dilutions were made. From the results, it is observed that the 16x and 32x dilutions of fermented juice is the most suitable media for microalgae growth with the addition of extra N and P. Therefore, the addition of the extra nutrient improved the growth media. The ability for the microalgae to grow both in light and dark is determined by cultivating the algae in light/dark conditions. Microalgae growth is observed from the light cultivation. This shows that the microalgae can only grow best under light cultivation in both the juices. After performing microalgae cultivation on the filtered and unfiltered types of brown juices in order to compare the results, it is observed that microalgae is able to grow only on the filtered juices. High cell density growth is observed in the 16x and 32x fermented brown juice media. For algal biomass productivity, 32x dilution of the fermented media yielded a higher biomass than the BG11 media. This indicates that fermented brown juice with less concentration is able to supply

the microalgae with the necessary nutrients for growth and thus suitable for the cultivation of microalgae. Therefore, juice extracts from naturally grown plants are a great potential as an alternative growth media for microalgae.

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6. Appendix

Appendix 1: Materials and equipment used in the different experiments:

Equipment / Materials	**Experiment**
Centrifuge (Eppendorf Centrifuge 5804 R)	Centrifugation
Q-max syringe filter	Filtration
Vacuum filter	Filtration
VWR Phenomenal mu6100 L pH meter	pH Measurement
NaOH	pH Measurement
Nitrate test strips	HACH Analysis
Phosphate test strips	HACH Analysis
Ammonia test strips	HACH Analysis
Whatman filters	Dry Matter Measurement
HACH kits	HACH Analysis
HACH spectrophotometer (DR 3900, HACH LANGE)	HACH Analysis

Heater (LT 200 HACH LANGE)	HACH Analysis
Multiwell plates	Multiwell Screening
250 mL flasks	Cultivation
Oven (DRY-Line, VWR)	Dry Matter Measurement
Analytical balance (RADWAG)	Dry Matter Measurement
Microscope (Leica ICC50 W)	Cell Counting
Counting chamber	Cell Counting

Appendix 2: Dry matter measurement of samples in the different media.

After weight - Before weight = Weight 10 ml				Dry matter (100 x weight) g dm/L			
Day 0	Day 3	Day 7	Day 10	Day 0	Day 3	Day 7	Day 10
0.0011	0.0061	0.0087	0.0062	0.11	0.61	0.87	0.62
0.0007	0.0045	0.0124	0.0141	0.07	0.45	1.24	1.41
0.0007	0.0059	0.008	0.0111	0.07	0.59	0.8	1.11
0.0015	0.0066	0.0088	0.0138	0.15	0.66	0.88	1.38
0.0014	0.0042	0.007	0.0092	0.14	0.42	0.7	0.92
0.0009	0.0057	0.0091	0.0099	0.09	0.57	0.91	0.99
0.0055	0.0061	0.0043	0.0011	0.55	0.61	0.43	0.11
0.0075	0.0021	0.007	0.0053	0.75	0.21	0.7	0.53
0.0034	0.0027	-0.0005	0.0044	0.34	0.27	-0.05	0.44
0.0057	0.001	0.002	0.0039	0.57	0.1	0.2	0.39
0.0046	0.0023	0.002	0.0015	0.46	0.23	0.2	0.15
0.0015	0.0013	0.0031	0.0054	0.15	0.13	0.31	0.54
0.0061	-0.0004	0.003	0.003	0.61	-0.04	0.3	0.3
0.0085	0.0002	0.0008	0.0025	0.85	0.02	0.08	0.25
0.0049	-0.0004	-0.0015	-0.0021	0.49	-0.04	-0.15	-0.21
0.0007	0.0006	0.0036	0.0055	0.07	0.06	0.36	0.55
0.0005	0.0009	0.0023	0.0039	0.05	0.09	0.23	0.39
0.0005	0.0008	0.0009	0.0009	0.05	0.08	0.09	0.09

The dry matter is calculated by subtracting the weight of the filter before filtration from the weight after filtration.

After weight - Before weight = Weight 10 ml				Dry matter (100 x weight) g dm/L			
Day 0	Day 3	Day 7	Day 10	Day 0	Day 3	Day 7	Day 10
0.0011	0.0061	0.0087	0.0062	0.11	0.61	0.87	0.62
0.0007	0.0045	0.0124	0.0141	0.07	0.45	1.24	1.41
0.0007	0.0059	0.008	0.0111	0.07	0.59	0.8	1.11
0.0015	0.0066	0.0088	0.0138	0.15	0.66	0.88	1.38
0.0014	0.0042	0.007	0.0092	0.14	0.42	0.7	0.92
0.0009	0.0057	0.0091	0.0099	0.09	0.57	0.91	0.99
0.0055	0.0061	0.0043	0.0011	0.55	0.61	0.43	0.11
0.0075	0.0021	0.007	0.0053	0.75	0.21	0.7	0.53
0.0034	0.0027	-0.0005	0.0044	0.34	0.27	-0.05	0.44
0.0057	0.001	0.002	0.0039	0.57	0.1	0.2	0.39
0.0046	0.0023	0.002	0.0015	0.46	0.23	0.2	0.15
0.0015	0.0013	0.0031	0.0054	0.15	0.13	0.31	0.54
0.0061	-0.0004	0.003	0.003	0.61	-0.04	0.3	0.3
0.0085	0.0002	0.0008	0.0025	0.85	0.02	0.08	0.25
0.0049	-0.0004	-0.0015	-0.0021	0.49	-0.04	-0.15	-0.21
0.0007	0.0006	0.0036	0.0055	0.07	0.06	0.36	0.55
0.0005	0.0009	0.0023	0.0039	0.05	0.09	0.23	0.39
0.0005	0.0008	0.0009	0.0009	0.05	0.08	0.09	0.09

Appendix 3: Mean average values of the DM for the different media on day 3, 7 and 10. The biomass productivity is calculated from the DM analysis of microalgae cultivation.

	Dry Matter (DM(g/L))				
	Day 0	Day 3	Day 7	Day 10	Biomass productivity (g/L/d)
16x	0.08	0.55	0.97	1.047	0.071
32x	0.126	0.55	0.83	1.097	0.078
64x	0.55	0.363	0.36	0.36	-0.0004
H.C 32x	0.39	0.153	0.24	0.36	0.029
N & P	0.65	-0.02	0.077	0.113	0.019
BG11	0.057	0.077	0.227	0.343	0.038

Appendix 4: Cell counting

Sheet below shows the cell numbers for the different samples and these numbers are used to calculate the cells in 1 mL sample and plot the graph (Figure 7, page 33).

Fermentation									
16x C	1	2	3	4	5	Average	Multiply by 10x d	amount of cells i	no of cells in 1ml
	80	32	64	64	96	67.2	672	168000	168000000
Fermentation	32x								
32x					sum				
A	192	80	144	144	48	121.6	1216	304000	304000000
B	192	304	352	288	288	284.8	2848	712000	712000000
C	128	144	160	224	192	169.6	1696	424000	424000000
Fermentation	64x								
A	208	112	160	48	208	147.2	1472	368000	368000000
B	144	64	176	192	224	160	1600	400000	400000000
C	160	96	144	128	112	128	1280	320000	320000000
BG11									
A	144	144	224	288	272	214.4	2144	536000	536000000
B	80	64	112	80	96	86.4	864	216000	216000000
C	128	368	304	288	176	252.8	2528	632000	632000000
Heat Coagulatec	16x								
C	32	32	16	96	96	54.4	544	136000	136000000
Heat Coaguated	32x								
A	464	480	544	592	656	547.2	547.2	136800	136800000
B	224	400	208	160	208	240	240	60000	60000000
C	224	240	272	192	192	224	2240	560000	560000000