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Optimisation of microalgae upstream and downstream processes for sustainable biodiesel production

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Abstract

The sustainability of microalgae biodiesel production primarily relies on the choice of algae species, carbon sources utilised and mode of cultivation. Careful choices of these features can stimulate the production of commercial and suitable biodiesel of international standards. Optimisation of the key units such as cultivation, harvesting and cell disruption is necessary to cut down excess inputs that will not only circumvent hike in the cost of production but help identify the overall conditions for optimal biodiesel yields. In this study, firstly, two selected oleaginous microalgae species *Chlorella vulgaris* (*C. vulgaris*) and *Nannochloropsis oculata* (*N. oculata*) were cultivated in photobioreactor (PBR) and multi-cultivator (MCR) mixotrophically and photoheterotrophically utilising glycans such as β-glucan, β-mannan and xylan, and folic acidas carbon sources using artificially made wastewater and formulated growth media (BG 11). The aim is to investigate their effects on biomass productivity and growth rate, lipids content, fatty acid composition, biodiesel yields and properties. Biodiesel properties were estimated empirically using equations available in literature. Secondly, microalgae samples were harvested via flocculation. Cell wall disruption and lipid extraction were carried out using a mild and low energy device 'Tissue-lyser II' after cell wall weakening via osmotic shock. Thirdly, key parameters of three microalgae biodiesel production units comprising of photoheterotrophic cultivation, harvesting by flocculation and cell disruption were optimised using the response surface methodology (RSM).

Results obtained from the preliminary growth experiment conducted using wastewater (mixture of distilled water and miracle gro. fertilizer) showed a limited degree of growth (maximum OD of 0.44). Supplementing the medium with folic acid solution inhibited microalgae growth and eventually terminated the growth phase, signifying that the dissociation of folic acid in culture did not make available the much-needed carbon required for algae growth, and therefore is unsuitable for use as a carbon source. Utilising a properly formulated Blue Green (BG 11) growth medium supplemented with organic carbon sources enhanced growth. Results obtained after a period of 8 cultivation days in BG 11 showed that *C. vulgaris* outweighs *N. oculata* in all growth parameters such as biomass concentration, productivity, and growth rate, signifying better nutrient absorption and photosynthetic activities by the former species. Biomass concentration of 0.186, 0.287, 0.267, 0.214 and 0.232 gl^{-1} were obtained from *C. vulgaris* and 0.133, 0.223, 0.238, 0.167 and 0.195 gl^{-1} from

N. oculata when cultivated mixotrophically in culture supplemented with control (culture not supplemented with carbon source), glucose, mannose, β-glucan and β-mannan respectively. Comparatively, these results are higher than 0.184 and 0.218 gl^{-1} obtained from *C. vulgaris*, and 0.165 and 0.184 gl^{-1} from *N. oculata* obtained when cultivated photoheterotrophically, indicating that the act of passing air bubbles into culture (aeration) practiced under mixotrophic mode of cultivation further supplements culture with carbon dioxide (CO_2) which resulted in the upsurge in growth parameters observed. The outcome of optimising the photoheterotrophic mode of cultivation clearly showed that growth is dependent on the combination and interaction of both nutrients: organic carbon (glycans) and organic nitrogen sources(urea). More so, utilising β-glucan and β-mannan from different sources (yeast and barley) and structures as carbon sources did not significantly influence growth parameters when compared. Additionally, the growth performance of both microalgae species cultivated in MCR was also influenced by glycans (β-glucan, β-mannan and xylan) when utilised as carbon sources in such a decreasing order of xylan>β-mannan>β-glucan. The algae species and glycans studied are good candidates for biodiesel production, with maximum biomass and lipid yields from the species cultivated in the culture supplemented with xylan.

Thereafter, the two selected algae species which were cultivated under the same conditions were harvested and flocculated at different times in acidic, alkaline, and neutral media, aimed at investigating and comparing how flocculation parameters can influence algae flocculation efficiencies. Results revealed a high flocculation efficiency (over 80 %) of both species, but at different flocculation conditions. It was observed that *N. oculata* flocculated better than *C. vulgaris* in alkaline culture (pH of 11) whereas *C. vulgaris* is better flocculated in acidic culture (pH of 3). At maximum flocculation, it was observed that *N. oculata* was very resistive to cell loss when compared with *C. vulgaris*. Cell loss of *C. vulgaris* is over 3fold higher than that *N. oculata*.

In another unit process, wet and dry harvested algae species were subjected into cell disruption regime. Results obtained from cell disruption experiments conducted on wet algae samples using the Tissue-lyser showed that owing to the stronger cell wall composition and matrix which amplifies the resistance to shear damage of *N. oculata*, lower disruption efficiency was observed in *N. oculata* (55.9±5.7%) than *C. vulgaris* (84.15±0.95%) under the same disruption conditions. Observably, the effects of treatment time were coherent in the

first 20 minutes, thereafter, the rate of disruption declines. Also revealed was that more cells were disrupted at lower specific energy on agitating culture of higher biomass concentration due to the exposure of more biomass to the grinding beads resulting in the disruption of more cells. Cell disruption efficiency increases with increasing specific energy and decreasing biomass concentration. Energy input as a function of time in the disruption regime was relatively low. However, energy utilisation estimated in form of specific energy (energy input per unit mass) is high due low mass of algae biomass treated. The specific energy that resulted in maximum disruption efficiency and lipids yield is 18 MJ/g and can be further reduced by increasing the mass of biomass in the treated culture. Also, lipid was extracted from dry algae samples subjected to bead shaking using the Tissue-lyser II. Maximum lipid yields of 26, 18, 23.5, 17.5 and 11.5 % by weight of dry *C. vulgaris*, and 23.5, 15, 22.5, 17 and 8.5% by weight of dry *N. oculata* were obtained from culture grown under mixotrophic cultivation using β-glucan, glucose, β-mannan, mannose and control respectively, indicating that glycans are potential carbon sources for sustainable microalgae lipid yields for biodiesel production. For obvious reason, the specific energy consumption is relatively very low (0.9 MJ/g) which is one-twentieth of the 18 MJ/g obtained on treating wet algae utilising the same device.

More so, the influence of various carbon sources on fatty acid composition and biodiesel fuel properties were observed to be significant in this study. The values of key biodiesel properties that can enhance engine performance (cetane number, oxidative stability and viscosity) are within the range of set international standards, signifying that glycans utilised in this study can enhance the volume and quality of biodiesel from microalgae extracts.

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Dedication

I dedicate this research thesis to Almighty God, my parents (Mr. & Mrs Emmanuel Nwachukwu Okoro), wife (Mrs Goodness M. Victor) and children (Newton Victor, Wilson Victor, and Endwell Victor) for their kindness and prayers.

Declaration

I, Okoro Victor Ikenna hereby declare that this thesis represents my work. I can attest that it has not been previously published by any other person except where appropriate references have been made. This thesis contains no material which has been accepted as part of the requirements of any other academic degree or non-degree program, in English or in any other language. It contains over forty-nine thousand two hundred words.

I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

Name: Okoro Victor Ikenna

Date: 6-04-2022

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List of Abbreviations/symbols

1. greenhouse gasses (GHGs)
2. optical density (OD)
3. internal combustion (IC)
4. carbon dioxide (CO_2)
5. open system (OP)
6. raceway pond (ORP)
7. photobioreactor (PBR)
8. multi-cultivator (MCR)
9. Response Surface Methodology (RSM)
10. fatty acid (FA)
11. fatty acid methyl ester (FAME)
12. triacylglyceride (TAGs)
13. tricarboxylic acid (TCA)
14. CO_2-concentrating mechanism (CCM)
15. Pentose Phosphate (PPP)
16. Embden-Meyerhof-Pranas (EMP)
17. 6-phosphate glucose (G6P)
18. ribulose 5-phosphate (Ru5P)
19. glyceraldehyde 3-phosphate (G3P)
20. dihydroxyacetone phosphate (DHAP)
21. High-Rate Algae Pond (HRAP)
22. chamber filter press (CFP)
23. pentose phosphate pathway (PPP)
24. photosynthetic photon flux density (PPFD)

List of publications

1. Okoro V, Azimov U, Munoz J, Hernandez HH, Phan AN. Microalgae cultivation and harvesting: Growth performance and use of flocculants-A review. Renewable and Sustainable Energy Reviews. 2019;115 :109364.
2. Azimov U, Okoro V, Hernandez HH. Recent Progress and Trends in the Development of Microbial Biofuels from Solid Waste—A Review. Energies. 2021; 14:6011.
3. Okoro V, Azimov U, Munoz J. Recent advances in production of bioenergy carrying molecules, microbial fuels, and fuel design - A review. Fuel. 2022; 316:123330.
4. Investigating the feasibility of direct utilisation of complex sugars as carbon sources and Tissue-lyser for low energy cell disruption and lipid extraction of *Chlorella vulgaris* and *Nannochloropsis oculata* for sustainable biodiesel production (Submitted)
5. Statistical optimisation of flocculation parameters on *Chlorella vulgaris* and *Nannochloropsis oculata*: Comparison and effects of these parameters on cell morphology and viability (Submitted)
6. Wet oleaginous microalgae cell disruption and lipid recovery using combined mild disruption techniques (Submitted)

Chapter one

1.0 General introduction

1.1 Introduction

The need for energy is increasing due to surge in human population and industrialization [1, 2]. Maintaining a secure global energy supply while minimizing environmental impact of energy use is one of the most pressing challenges facing humanity [1]. Therefore, replacing conventional fossil fuel with alternative fuels such as biodiesel has become a necessity due to increasing emission of greenhouse gasses (GHGs) and decreasing fossil fuels reserves [3, 4]. Biodiesel is considered as one of the most promising liquid biofuels produced from various feedstocks due to its low carbon emission and can be utilised in the existing internal combustion (IC) engine infrastructure with little or no modification. The sustainability of microalgae biodiesel is tied on the cost of production, quantity, and quality of biodiesel obtainable. Notably, it is only when the properties of biodiesel comply with ASTM (US biodiesel standard); EN14214 (Europe biodiesel standard) and GB25199 (Chinese biodiesel standard) can it be commercialized and used as an alternative source of fuel.

To date, various biomasses (feedstocks) and technologies have been utilized for biofuels production. They are categorized as first, second, third and fourth generations [5]. In the first- and second-generation which have attained mature and demonstrative technological status, biomass was sourced from food and nonfood products and are no longer used due to the food vs. fuel debate. The use of microalgae, a third-generation biofuels source ameliorated the problems associated with biofuels from first and second generations. The emergence of fourth generation biofuels, which involves the application of metabolic and genetic engineering on microbes such as microalgae can improve biofuels yields [6, 7]. The technological status, features, substrate utilized, advantages and disadvantages of each generation is illustrated in Figure 1.

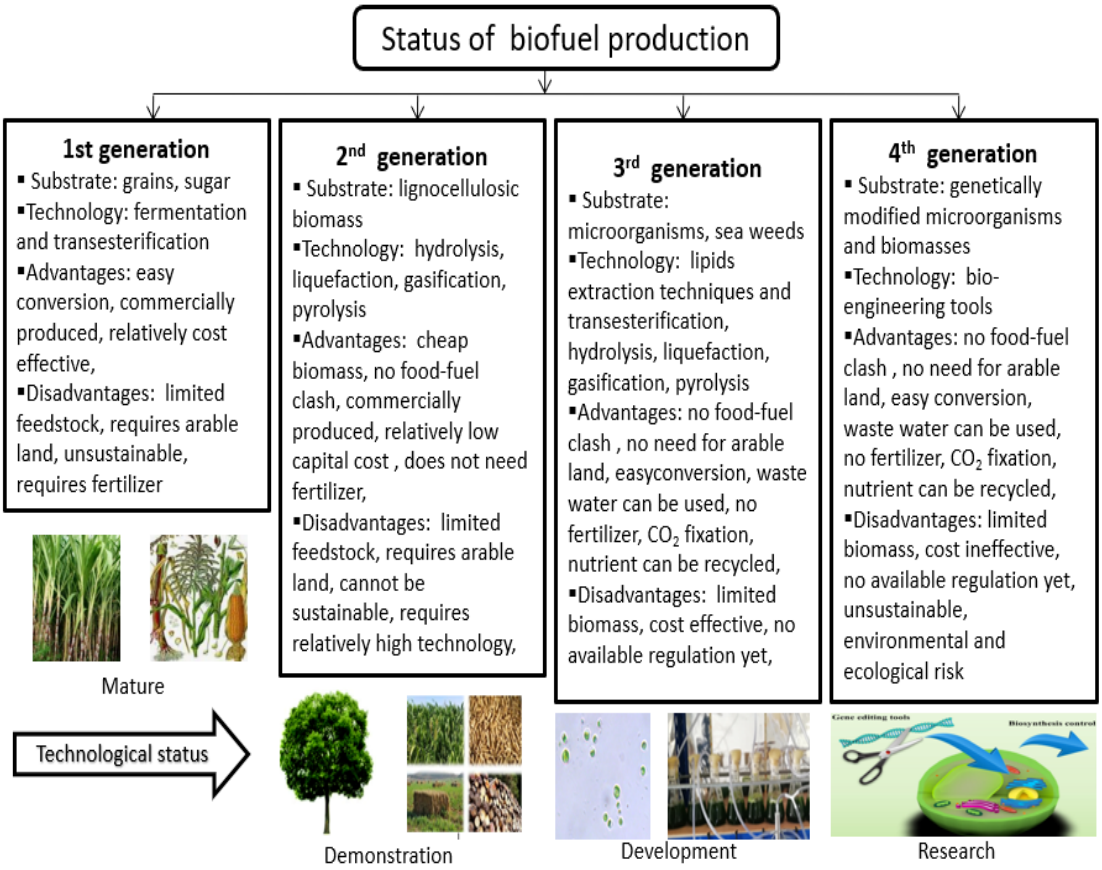


Figure 1: Development of biofuel production and technological status

Amongst the feedstocks available, utilizing microalgae feedstock for biodiesel production is promising due its high lipid accumulation and growth rate, carbon dioxide (CO_2) sequestration or bioremediation capability and photosynthetic efficiency when compared with other feedstocks. Apart from being a feedstock for renewable source of various clean biofuels such as; (bio-char, bioethanol, biodiesel and bio hydrogen), microalgae have been viewed as solutions to various challenges in our environment, agriculture (human and animal feeds) and pharmaceutical (food supplements and medicines) industries [8]. Microalgae biofuels and other value-added products have been displayed in Figure 2.

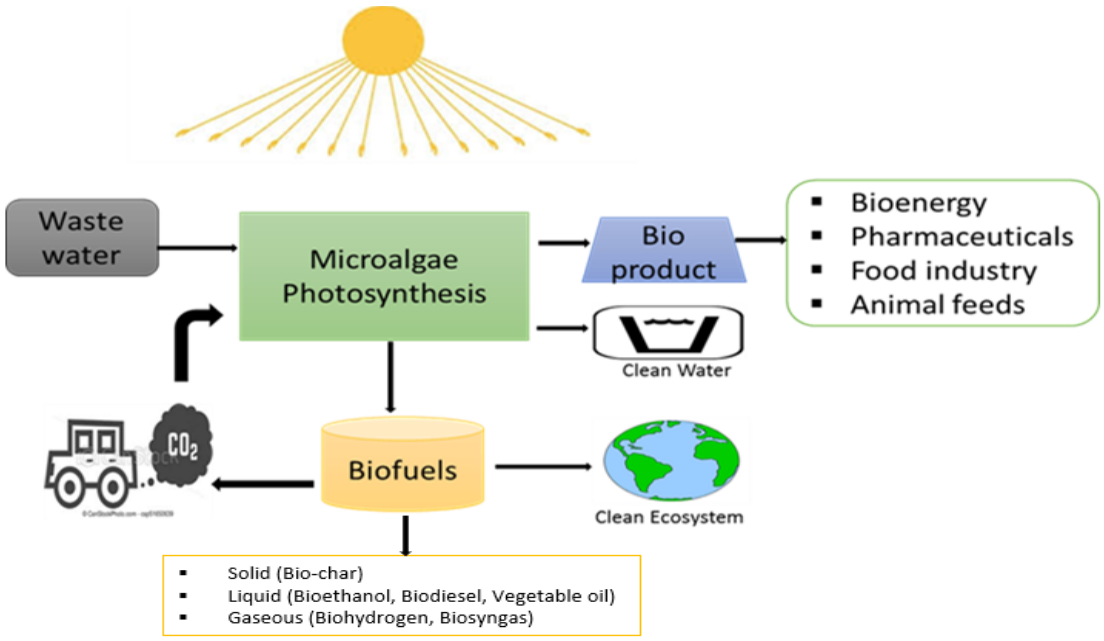


Figure 2: Microalgae biofuels and value-added products

In spite of these environmental and economic benefits of microalgae, various upstream and downstream operations of biodiesel production as illustrated in Figure 3 have been meted with numerous challenges incurred as a result of 1) low biomass and lipid accumulation, arising from the complexity and diversity in the properties of microalgae species/strains, mode of cultivation, nutrients utilised and growth conditions; 2) energy intensive microalgae biomass recovery processes; and 3) rigid cell wall which hinders lipid extraction and engender high energy demand in cell disruption process [6, 9]. These shortcomings created doubt about the viability and profitability of its commercialization and has made its sustainability questionable.

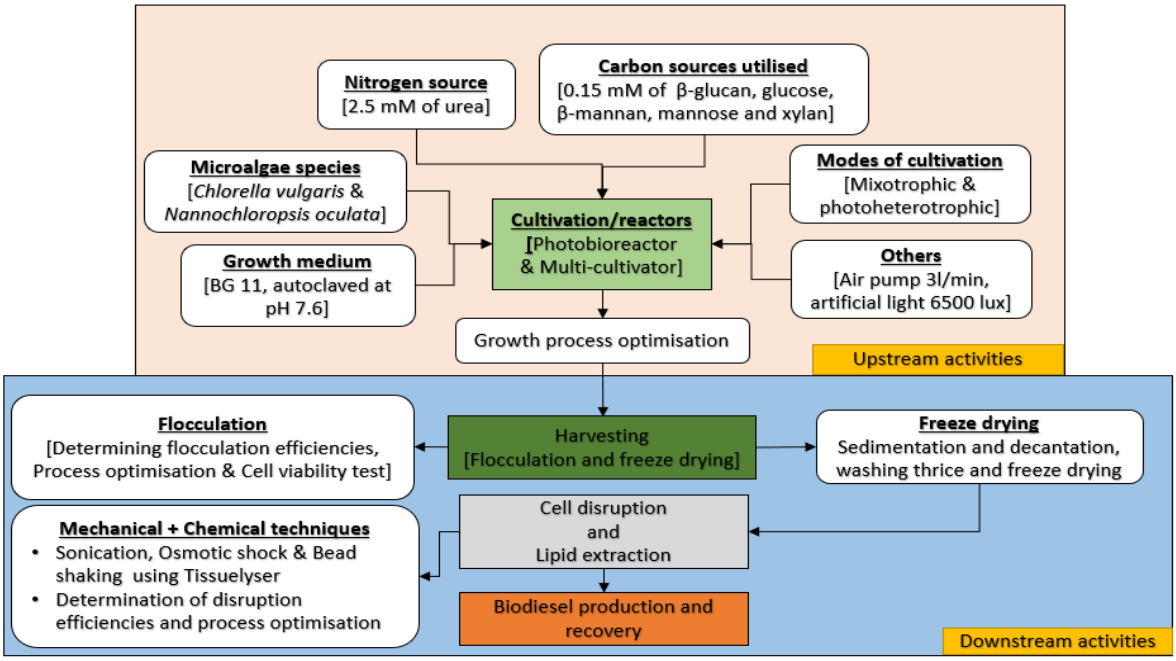


Figure 3: An insight of the breakdown of key upstream and downstream activities. Upstream and downstream activities stated in white rectangular boxes are the key tasks executed in this study. The coloured boxes contain microalgae biodiesel production unit steps

To ameliorate these challenges, several systems, mode and methods of cultivation have been studied and applied. Studies of various cultivation systems such as open system, closed system and hybrid system have been reported [10, 11]. The application of closed system, also known as photobioreactor (PBR) which include but not limited to bubble column, tubular and flat plate are widely studied. This is because, growth conditions in a closed system can be controlled, bacterial contamination can be avoided, and the design permits a more controllable and effective use of light compared to open ponds [10]. Furthermore, more sophisticated photosystem instrument like multi-cultivator (MCR) are being utilised to enhance the synthesis of biomolecules by controlling the environmental factors such as light intensity, illumination area and multi-colour assay, temperature, and method of cultivation. Other factors such as growth medium and salinity, nature (gaseous or liquid state), chemical composition (organic or inorganic) and concentration of nutrients utilised in the cultivation process can greatly influence biomass productivity and lipid accumulation [12-14].

During cultivation, microorganisms including microalgae have the same respiratory metabolic pathway, where carbon is the building block for the biosynthesis of various bioproducts [15].

The source of carbon (inorganic or organic) utilised during algae cultivation can influence biomass and lipids productivities. So far, utilising organic carbon sources has been yielding promising results [16]. The ability of microalgae to utilize organic carbon sources for growth is strain dependent and relies on the availability of a system that transports it into the cell and presence of enzymatic pathways to convert the carbon source into suitable precursors for carbon metabolism [17, 18]. Some microalgae species can change their metabolic pathway, depending on organic substrates (carbohydrates, organic acids, alcohol etc.) utilised [19]. Most organic carbon sources derived from simple sugars or monosaccharides such as glucose, fructose, galactose, arabinose, xylose and mannose have been utilised as carbon sources for microalgae cultivation. Disaccharides such as sucrose, lactose and maltose have also been used [18, 20]. Polysaccharides or complex carbohydrates or glycans whose structure is based on glycosidic bond between monosaccharides, which have been used as carbon sources have shown capacity of improving biomass and lipid productivity [18, 19, 21], but undesirably the process of extracellular enzymatic hydrolysis of these complex carbohydrates to simple sugar prior to culture utilisation incurs some costs and takes some time. For instance, Wei et al. [21] researched on the effects of starch hydrolysate on cell growth and lipid accumulation of *Chlorella protothecoides*. The results showed that cassava starch hydrolysate can be a better alternative carbon source for algae cultivation, depending on its concentration in the medium, but the two steps enzymatic hydrolysis of cassava starch by α-amylase and glucoamylase into glucose was time consuming and incurred more cost in the cultivation process. More so, carbohydrate from lipid-free microalgae biomass used as carbon source in microalgae cultivation enhanced biomass productivity and lipids accumulation. This inference is drawn from the research conducted by Abomohra et al. [22].

More so, it was reported that microalgae species could accumulate high lipid when cultivated with composite carbon sources such as sweet sorghum and yams [23]. Also, the direct use of polysaccharides such as cassava starch, xylan cellulose and maize as exogenous carbon sources in the cultivation of cyanobacterium *Phormidium sp.* showed a better lipid accumulation capability than glucose [20]. But to the best our knowledge, the direct utilisation of some polysaccharides or complex glycans such as β-mannan, β-glucan and xylan as carbon sources in the cultivation of certain species of microalgae seem not to have been reported in literature. The effects of the utilisation of these glycans, as well as cheap and

available folic acid and wastewater on algae growth employing various modes of cultivation will be examined in this study.

The utilisation of cheap and available miracle gro. Fertilizer (described in detail in section 3.1.2), ambient CO_2, and folic acid as a source of nutrient for microalgae growth has not been attempted. Wastewater produced by contaminating distilled water with miracle gro. fertilizer, supplemented with folic acid will serve as artificial growth medium. Folic acid is readily soluble in solution, helps plants grow well as it decomposes rapidly when exposed to light and enables plants growth [24]. It has been found to be present in green vegetables. Interestingly, Esfandiari et al. [25] narrated the positive effects of folic acid on growth and development of seeding and indicated its important role in biochemical and physiological process in plant cells. In addition, a recent work done by Raeisi et al. [26] also showed the efficacy of folic acid in the overall welfare of strawberry. Attempt will be made in this research to examine its effect on the growth of the selected microalgae species. However, one of the major hitches of utilising fertilizers is unknown concentration and inappropriate composition of nutrients therein. Therefore, formulated growth media such as Blue- Green (BG) 11 will be utilised.

Apart from nutrients present in growth medium, as stated earlier, mode of microalgae cultivation can influence biomass and lipid production. The cultivation of microalgae using inorganic carbon dioxide (CO_2) and light as the only carbon and energy sources respectively, known as photoautotrophic mode of cultivation has been broadly studied and major drawbacks usually occasioned by low specific affinity for carbon dioxide identified [27]. Low cell density, long cultivation period and high harvesting cost and energy associated with photoautotrophic mode of cultivation triggered the introduction of heterotrophic, photoheterotrophic and mixotrophic as alternative modes of cultivation. The heterotrophic mode of cultivation utilizes organic carbon from simple sugars, glycerol, acetate etc. as the solo carbon and energy sources, while photoheterotrophic mode of cultivation simultaneously utilises organic carbon and light without the addition of any external inorganic carbon source or via aeration. The mixotrophic mode utilises the trio of organic and inorganic carbon sources as well as light [28]. Under certain growth conditions, depending on microalgae species and strains, biomass productivity and lipid content higher than those obtained under photoautotrophic mode of cultivation have been achieved by the application

of the heterotrophic mode using mono- and disaccharides as carbon sources [29]. In comparison, photoheterotrophic and mixotrophic modes of cultivation using simple organic carbon sources have enhanced the biomass growth and synthesis of valuable metabolites such as lipids [28], but the effects of utilising glycans as carbon sources on microalgae biomass and lipids productivity under photoheterotrophic mode of cultivation have not been widely studied [30, 31].

After cultivation, harvesting follows. The harvesting (separation) of diluted microalgae from the growth medium has continued to be one of the hurdles against the economics of microalgae large scale production due to the small size of microalgae (1–30 μm), low concentration (0.3–5 g/l) and density, and negative charge that scatter and keep cells apart in culture [32]. The techniques of harvesting microalgae include filtration, centrifugation, flotation, flocculation, gravity sedimentation, electrolytic process, electrophoresis and magnetic separation [33]. Apart from energy intensiveness of most of these processes, attention needs to be paid to cell damage, microalgae strain characteristics (like density and size), salt concentration and acceptable level of moisture before selecting an appropriate technique to apply [33, 34]. Notably, easy application, cheap and available flocculants, and high coagulation performance (greater than 80 %) have made flocculation superior to most known methods, depending on some key factors such as microalgae strain, ionic strength and size, flocculant type and concentration, pH, and flocculation time [35]. However, comparative study of the optimisation of these parameters on the species subjected to the same growth and flocculation conditions is scanty. Further studies will deepen the knowledge of the effects of these parameters on various microalgae species.

Cell disruption and lipid extraction regimes arise after harvesting. To enhance lipid extraction from dry or wet samples for biodiesel production, disrupting the cell wall is commonly practiced. This allows extraction solvents passage into the internal component of the cell, enhance solubilisation and mass flow rate of intracellular contents. Techniques such as biological (phage, enzymatic), chemical (acid, alkaline, surfactants, oxidation, osmotic shock) and mechanical (sonication, microwaves, bead mill, homogenization) have been studied, single or combination of various techniques have been applied [36-38]. In mechanical cell disruption using bead mill, cell rupture is due to the agitation, collision and grinding effects of the beads against the cells at high speed [39]. Factors such as biomass concentration, algae species, bead size, bead type, bead filling and operation time can affect the disruption and

lipid extraction efficiencies [40, 41]. It has been presumed that due to greater hardness and density, zirconium oxide beads with a volume fraction of 0.5 can enhance cell disruption, and bead diameter of 0.1 mm is optimal for bacteria and 0.5 mm for yeast and microalgae [40, 42-44]. Although the process has been adjudged being energy intensive, it can be effective with high microalgae concentration (100-200 g/l) [45, 46], but generation of high temperature and production of complex lysates are other key challenges [47]. Thus, a mild bead milling cell disruption process, which may reduce these drawbacks and preserve the cellular biomolecules is essential. Notably, different bead mill cell disrupters such as Mini-Bead Beater BioSpec Products and ECM-Pilot Dyno-Mill have been used for the disruption of cell walls of various microalgae species [40, 41, 48-50] , but to the best of our knowledge, a mild and low energy Tissue-lyser II has not been used for microalgae cell disruption. The reason is rather unclear but may be related to low disruption efficiency. Interestingly, in a research conducted by Balasundaram et al. [51], the cell wall of *Chlorogloeopsis* was completely disrupted using Tissue-lyser II (Qiagen Inc USA) operated with glass beads of diameter 1 mm agitated at 1800 rpm for 30 minutes. The underpinning principle of the working of a Tissue-lyser II is the rapid shaking of the cell suspension in the presence of beads. The beads accelerate in the direction of the motion of the Tissue-lyser (horizontal axis), forming high shear and impact forces that can rupture the cells. It is remarkably to note that, microalgae form tough cell wall structures with estimated tensile strength in the order of 9.5 MPa, which is about the same as that of bacteria or yeast [52, 53]. Thus, Tissue-lyser II may be viable in disrupting microalgae cells especially after suspending culture in sodium chloride solution, allowing for osmotic shock prior to bead beating. Osmotic shock has been adjudged as one of the low energy microalgae cell disruption methods. However, themethod merely damages and hardly rupture microalgae cell wall [54]. Intensifying the cell rupture process by the combination of dual low energy methods can reduce the amount of energy expended during disruption process.

Therefore, in this research, 1) the feasibility of direct utilisation of some polysaccharides or glycans such β-glucan, β-mannan and xylan as carbon sources under mixotrophic and photoheterotrophic modes of cultivation of two microalgae species *C. vulgaris* and *N. oculata*, boycotting the usual enzymatic hydrolysis process of breaking down these glycans into their corresponding utilisable simple forms was studied. The effects of wastewater made from

miracle gro. fertilizer and folic acid solution were examined. Biomass and lipids biosynthetic ability of the two algae species grown in media supplemented with each carbon source in the two modes of cultivation were evaluated; 2) the synergistic effects of each glycan and urea on biomass production was studied by optimising the growth pattern using Response Surface Methodology (RSM) of a software package Minitab 18.1.0 version statistical software; 3) parameters such as flocculants concentration, pH and flocculation time of one of the cheapest harvesting methods (flocculation) were optimised and results compared amongst both selected algae species; 4) the efficiency of bead shaking of microalgae samples using Tissue-lyser II (Qiagen Inc-USA) as a mild and low energy cell disruption and lipid extraction technique was studied. Results were compared with other methods such as sonication and osmotic shock; 5) Fatty acid profile and properties of biodiesel or fatty acid methyl ester (FAME) as a function of carbon sources utilised were evaluated and compared amongst algae species. Critical biodiesel properties such as viscosity, oxidative stability, calorific value, cetane number, iodine value, saponification value, cold filter plugging point and long-chain saturation factor were estimated using equations domicile in literature. Biodiesel properties obtained from each selected microalgae species were compared with respect to the various utilised carbon sources, and consensually accepted optimal biodiesel properties stated in literature as applicable in the existing internal combustion (IC) engines.

1.2 Research motivation

- There is much need to reduce the level and effects of greenhouse gases (GHGs) in our ecosystem by utilising bio-based fuels, hence any research on carbon dioxide sequestration or bioremediation is desired.
- Biodiesel can run in internal combustion engines with a very little or no modification
- While the use of electric vehicles seems to be an ideal solution for short-haul travel, battery-electric vehicles will probably not be a feasible solution for long distance travels and heavy-duty machinery like tractor trailers, cargo ships and passenger jets, which will still rely on diesel.
- Some microalgae species have a very high growth rate and lipid accumulation capabilities. Processes can be recycled.
- Glycans utilised as carbon sources in microbes cell factory can be sourced from food and agricultural waste littered around our environment.

1.3 Research aim

- This research is aimed at optimising microalgae upstream and downstream processes for sustainable biodiesel production. For the upstream activities, the feasibility of utilising varieties of new carbon sources to enhance lipid accumulation will be examined. For the downstream process a mild and low energy cell disruption and lipid extraction technique for biodiesel production will be considered. Biodiesel properties as a function of fatty acid composition will be evaluated to determine the carbon source that is the best for microalgae biodiesel production.

1.4 Research objectives

- **Cultivation:** To cultivate microalgae by direct utilisation of β-glucan, β-mannan and xylan as carbon sources, boycotting the prior enzymatic hydrolysis, thereby saving time and cost accruable by the hydrolysis stage, and affirm the presence of enzymes that can breakdown these glycans in *C. vulgaris* and *N. oculata*.
- To investigate the effects of utilising β-glucan sourced from yeast and barley; β-mannan varieties (galactomannan and mannan) on pigment synthesis, biomass productivity and lipid yields.
- To optimise and compare the effects of utilising these glycans photoheterotrophically on growth of *C. vulgaris* and *N. oculata*.

- **Harvesting**: To optimise and compare the effects of flocculant dose (ferric chloride), pH and flocculation time on the flocculation and morphology of *C. vulgaris and N. oculata.*
- **Cell disruption**: To determine the degree of cell disruption and lipids extraction using Qiagen Tissue-lyser II sample disruptor; results will be compared with those obtained using osmotic shock and MSE soniprep 150 plus ultrasonic disintegrator. The specific energy expended by each method will be evaluated to determine which is more energy efficient.
- **Biodiesel production & Fatty acid profiling**: To trans esterify the extracted lipid to biodiesel and determine the fatty acid profile from both species cultivated using various carbon sources.
- **Biodiesel physicochemical properties:** To evaluate the physicochemical properties of biodiesel produced from various carbon sources using their fatty acid composition.

1.5 Thesis structure

This thesis is structured into six chapters. The first chapter, which is the "introductory chapter" briefly highlights the upstream and downstream activities, and background of each unit process (cultivation, harvesting, cell disruption, lipid extraction and biodiesel production) of utilising microalgae as a feedstock for biodiesel production. The challenges confronting each unit process, attempts made so far, and the proposed aim of the study are also highlighted. In order words, the aim, motivation, and objectives of this study are stated in this chapter. This precedes chapter two ("literature review"), which reviews relevant literatures from published sources in line with the set aim and objectives. It is part of introduction, but in a separate chapter and more elaborate. It provides detailed review of various carbon sources, mode of cultivation, biomass harvesting, cell disruption and lipid extraction techniques. Results obtained from relevant research reported in literature are captured, tabulated, and examined/discussed in furtherance to exposing the gap briefly mentioned in chapter one. Chapter three, otherwise known as "material and methodology chapter" outlines the various materials and method adopted in this study. The chapter provides reason(s) and justification for all the choices considered in this study. For instance, the reasons for the choices of algae samples (*Chlorella vulgaris* and *Nannochloropsis oculata*), cultivation methods (mixotrophic and photoheterotrophic), carbon sources (complex glycans such as β-glucan, β-mannan and xylan), harvesting by flocculation and cell disruption techniques (sonication, Tissue-lyser II and osmotic shock) are outline in subsections in this chapter. Pitfalls in some these choices and their possible remedies were also discussed. For example, the reasons for the selected algae species are clearly stated in section 3.3.1, which seem to be a perfect match for the objective of this study. Tissue-lyser disrupts cell walls in mild and ambient condition, but lipid yields could be low. Resuspending samples in sodium chloride solution to initiate osmotic shock before being treated with the Tissue-lyser may improve lipid yields. Chapter four ("result and discussion") outlines our findings in relation to the research questions or hypotheses. It represents our clearly visualize results in forms of tables, charts and graphs. The discussion section includes our own analysis and interpretation of the data gathered, comments and explanation of our results. In chapter five, we briefly mentioned the achievements or outcome and future prospect of our study in relation with the set objectives.

Chapter two

2.0 Review of related literature linked to this study

2.1 Overview

In microalgae biodiesel production, enhanced biomass and lipid biosynthesis during cultivation are desired. These can be achieved by understanding the effects of some cultivation manoeuvres and use of potent carbon sources in cultivating oleaginous species. Also, to maximize biodiesel production, it is necessary to obtain high volume of saponifiable lipids through cell disruption and lipids extraction processes. Figure 4 itemises the various unit operations towards microalgae biodiesel production and utilisation cycle. Therefore, the review of literature in this thesis will cover microalgae cultivation utilising various complex glycans such as β-glucan, β-mannan and xylan as carbon sources, and urea as organic nitrogen source. Also discussed are harvesting or biomass recovery by flocculation and drying, various methods of microalgae cell disruption including the agitation of zirconium beads originating from the adapter of a Tissue-lyser II, lipid extraction and biodiesel production, recovery and characterisation.

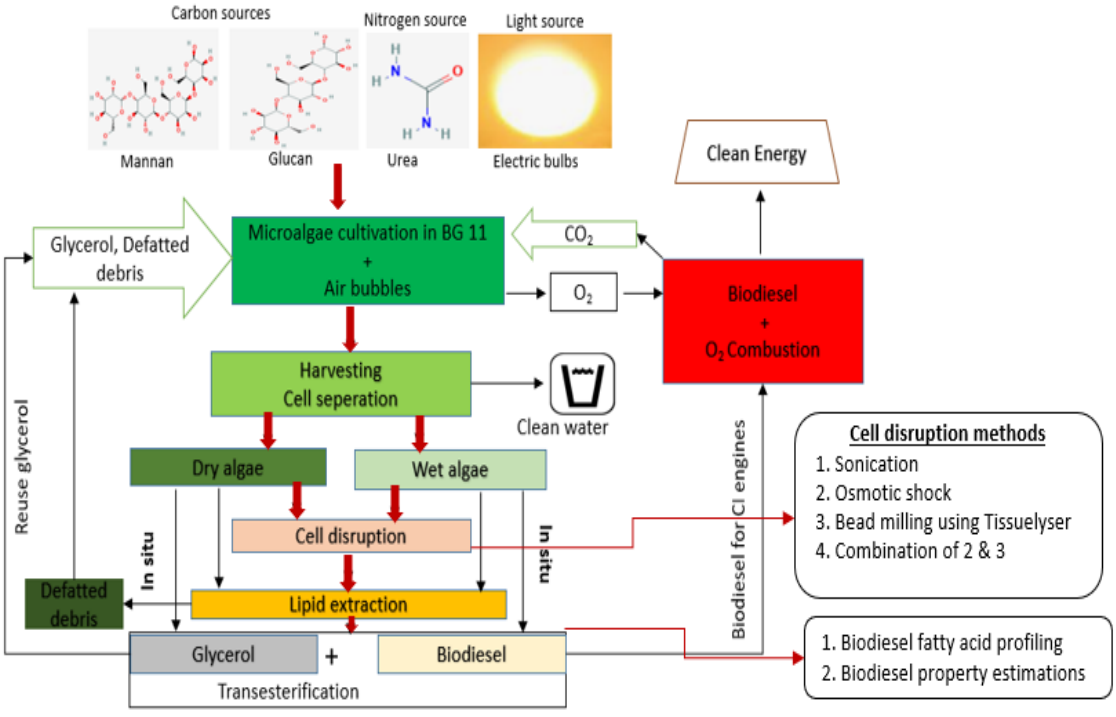


Figure 4: Microalgae biodiesel production and energy cycle.
Each rectangular box carries a biodiesel production step inscribed therein. Arrows in red colour indicate the paths considered in this research

2.2 Microalgae and growth mechanisms

2.2.1 Microalgae

Algae, classified as macroalgae and microalgae have been identified as sources of biofuel and other value-added biomolecules. Algae leave predominantly in aquatic environments employing photosynthetic apparatus for survival. Macroalgae, commonly referred to as 'seaweed' are multicellular plants growing in salt or fresh water. They are being considered for the natural sugars and other carbohydrates they contain, which can be fermented to produce biogas or alcohol-based fuels. However, in their growth pattern, there exists no exponential growth regime, hence, take longer time to grow. Conversely, microalgae are being widely researched as a biofuel source (mainly biodiesel, which is considered in this research) since they can accumulate much lipids than macroalgae with faster growth rate.

Microalgae (singular: microalga) are photosynthetic microscopic organisms with about 50,000 species already described and over 30,000 species identified for potential applications [55]. They are single-celled algae that can exist independently or in colonies. They do not have true stems, leaves, and roots, hence can flourish in diverse ecological conditions such as marine, freshwater, deserts, hot springs and Antarctica. Microalgae cells can be classified as prokaryotic and eukaryotic depending on the cell structure and composition. The prokaryotic microalgae, regarded as cyanobacteria resemble bacteria due to the absence membrane structures (nuclei, mitochondria, plastids, flagella, and Golgi bodies) which regulate cellular activities. Consequently, they do not have the ability to amass a significant amount of neutral lipids used as precursor for biodiesel production [56]. The eukaryotic microalgae have these membrane organelles, and are therefore well studied and can be utilised for biodiesel production [57]. Due to the presence of these organelles, their growth conditions can be manipulated to amass a range of desirable energy yielding biomolecules [58]. Notably, widely studied eukaryotic microalgae with desirable features for ingenious and economic status of CO_2 fixation, wastewater application and lipid synthesis for biodiesel processing are; green algae (Chlorophyceae) and diatoms (Bacillariophyceae) [59]. The Chlorophyceae or green algae exist in dual nature; microscopic or macroscopic. They occur primarily in freshwater, but also in marine terrestrial and sub-terrestrial settings. They primarily store different scales of carbohydrate, lipids, and proteins, depending on species/strains. Some of the commercially exploited microscopic green algae of the Chlorophyceae class are *C. vulgaris*, *N. oculata* and *Dunaliella sp*. [60].

The biodiversity of microalgae is enormous and they represent an almost untapped resource. Several studies have been conducted on diverse species. Some were discovered to be candidates for biodiesel production. The selection of any species/strain suitable for biodiesel production requires the evaluation of some key parameters; including lipid content, growth rate and productivity, strain adaptableness, and ability to withstand various harsh weather conditions such as temperature, salinity and pH, and high CO_2 sinking capacity. For biodiesel production, lipid content is the most critical factor considered, and species capable of synthesizing huge percentage of lipids as lipid (known as oleaginous microalgae) are desirous [61-63]. Some oleaginous microalgae such as *C. vulgaris* and *N. oculata* have shown capacity to positively respond to most of these critical selection criteria. It is also possible to amass other desired metabolite in these microalgae species to a large extent by varying some these key growth conditions [64, 65].

2.2.2 Oleaginous microalgae feedstock and biodiesel production

The word oleaginous means oil/lipid content or biomass that can yield lipids. Microalgae species that can yield lipids are termed oleaginous microalgae. These species commonly are found in marine, fresh and brackish water surroundings. Under optimal cultivation conditions, several microalgae species such as *Chlorella sp*., *Nannochloropsis sp*. and *Botryococcus braunii* are favourable for biodiesel production due to; high growth rates and high lipid accumulation (up to 70%), the capability to metabolise and utilise cheap and readily available classes of sugars as carbon sources [66]. Microalgae can synthesis up to 58,700 lh^{-1} bio-oil which can produce 121,104 lh^{-1} biodiesels [67]. High lipids content has been observed from oleaginous microalgae grown under nutrient (especially nitrogen, phosphorous, or silicon) limitation[68]. Lipids content varies in both quantity and quality with varied growth conditions. While high lipid yields can be obtained under nutrient limitation, it is accompanied by a decline in biomass yields. In this nitrogen-deficient regime, microalgae cells can assimilate abundant carbon but will not be able to proceed to cell division and development, instead, they switch to lipid biosynthesis pathway to amass triacylglyceride (TAGs) [69]. These TAGs cannot be used for synthesizing membranes, thus, are stored as lipid molecules. Also, it has been reported that higher microalgae cell growth and lipid content can be achieved under mixotrophic conditions because of the combination of photosynthesis and aerobic respiration coupled with the catabolism of polysaccharides present in the growth medium[70]. In other

words, microalgae can accumulate more storage lipids under mixotrophic conditions by increasing the availability of carbon therein [71]. Nonstop research has been ongoing, and investments are made to enhance microalgae cultivation for biodiesel production at the industrial level using cost-effective approach and conditions. Growth enhancement techniques and genetic engineering have been deployed to improve their potential as a future source of renewable biodiesel [72, 73].

Notably, microalgae biodiesel has attracted much attention recently due to its features of being renewable, biodegradable, nontoxic and proven to run in four stoke engines with little or no alterations [74, 75]. Many microalgae strains have shown mostly in lab scale to produce up to 50 percent of their biomass as lipid with much of this as TAGs, the anticipated precursor for biodiesel production via transesterification reaction. Transesterification, which is the reaction of these microalgae lipids with excess short chain alcohol in the presence of a catalyst leads to the production of fatty acid methyl ester (FAME), otherwise known as biodiesel [9]. The essence of transesterification of microalgae lipids is to reduce its viscosity and increase fluidity, and the need for excess alcohol is to fasten the rate of reaction and shift equilibrium to the right for increased product (biodiesel) production [76]. Although, in long run utilization of biodiesel, the maintenance problems related to injector coking remains, hence biodiesel is mainly utilized in blends with petroleum diesel resulting in an almost unchanged engine performance and fuel consumption when used in compression ignition (CI) engine [77]. As a renewable fuel, biodiesel combustion has low impact on the global CO_2 emission and when blended with petroleum diesel the emissions of NOx compounds are decreased. In addition, biodiesel contributes to the lubrication properties to the diesel fuel. To ensure high quality and functionality, standard specifications for biodiesel have been established in countries all over the world; ASTM D 6751 (American biodiesel standard) and EN 14214 (European biodiesel standard) are mostly specified.

2.2.3 Biomass biosynthesis and growth mechanisms

Microalgae have carbon, nitrogen and phosphorus absorbing mechanisms. In their growth process, they absorb carbon as HCO_3^-, dissolve nitrogen from water as NH_4^+, NO_3^- , NO_2^- or organic nitrogen and synthesis the absorbed nutrients into biomass composed of proteins, carbohydrate and lipid contents [78]. Therefore, the growth of microalgae is a strategy for improved removal of nitrogen from wastewater. Also, during metabolism, microalgae absorb phosphate in the form of either di hydrogen phosphate ($H_2PO_4^-$) or mono hydrogen

phosphate (HPO_4^-), through a process called phosphorylation [79]. This development releases adenosine di phosphate (ADP) and adenosine tri phosphate (ATP) by either oxidation of substrate in the system or light in case of photosynthesis. Moreover, microalgae leave and grow photosynthetically, hence are necessary for life on earth especially absorption of carbon dioxide (CO_2) and release of oxygen (O_2) [80]. They can convert light energy, simple inorganic salts, nitrogen source and CO_2 to biomass during photosynthesis. The photosynthetic nature of microalgae cells is a key illustration of a naturally occurring biological oxidation-reduction (redox) reaction as shown in Equation 1. It involves the fixation of CO_2 into organics (firstly sugars), requires light of the appropriate quantity (not too low, not too high) and quality (light in the visible spectrum), photosystems to capture photons and convert the energy into chemical energy (ATP and reductant), and also the enzymes of the Calvin cycle (most notably Ribulose bisphosphate carboxylase; RuBisCO)[81, 82].

$$6CO_2 + 6H_2O + Light\ energy \rightarrow C_6H_{12}O_6\ (sugar) + 6O_2 \quad (1)$$

Two reactions are usually involved in the photosynthetic process. Though both reactions are necessary for the existence, growth and reproduction of microalgae, the light reaction precedes the dark reaction. First is the light reaction which executes the oxidative process in the thylakoids, followed by the dark reaction in the stroma [83]. The chlorophyll [coloured pigment] absorbs light, gets excited and loses electrons, which are used to split hydrogen ions from water molecules. These hydrogen ions and electron create ion gradient that generates adenosine triphosphate ATP [energy carrier], nicotinamide adenine dinucleotide phosphate NADPH [electron carrier] and oxygen in the presence of some inorganic elements. In the dark reaction, NADPH and ATP produced in the light reaction enter the stroma for the reaction [84]. The energy and electron provided by NADPH and ATP combine with carbon dioxide to produce monosaccharide. The synthesis of carbohydrates (starch), proteins (amino acid) and lipids (fatty acid) by microalgae is what grows the microalgae and the convertibility of these intracellular substances into biofuel and bio hydrogen, as shown in Figure 5, makes the study of microalgae very desiring, especially now that the world is facing energy and environmental crisis.

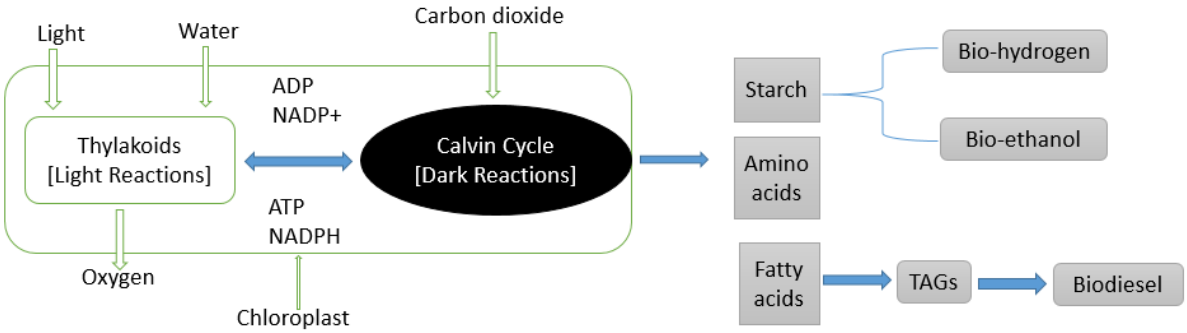


Figure 5: Photosynthetic pathway of microalgae biomass yield for biofuel production [84, 85].

In microalgae growth process, cell is divided by two mechanisms: binary fission and multiple fission. Binary fission is the separation into dual daughter cells, as observed in most eukaryotic organisms. It also signifies a move to several fissions, which will lead to the formation of several daughter cells. The presence of Deoxyribonucleic acid [86] helps in carrying out the genetic instructions needed for growth and development of microalgae. In microalgae biomass reproduction, the mitochondria duplicate their DNA and divide essentially in response to the energy requirements of the cell [87, 88]. Moreover, cell-cycle progression can be simply changed by changing growth conditions, and intracellular growth is light dependent whereas cellular division is light independent [89]. At any optimal growth condition, the microalgae mother cell divides into 2^n, where n is an integer from 1 to 10. Many eukaryotic cells divide by binary fission, but so many microalgae species divide by multiple fission with the value of n ranging from 3 to 4. By implication, the maximum number of daughter cells a microalgae species can produce ranges from 8 to 16 [88].

2.2.4 Lipids biosynthesis and accumulation mechanisms

Lipids are groups of organic compounds that are insoluble in water or polar solvents (due to predominate hydrophobic hydrocarbons) and soluble in organic solvents or non-polar solvents like chloroform, diethyl ether, acetone, benzene, ether and so on [90]. Lipids are very essential structure and functional part of microalgae, which according to the researches done by [91, 92] are biosynthetically produce in the chloroplast and ended in the endoplasmic reticulum [ER] [93]. The knowledge of microalgae lipid biosynthesis is desirous for optimal production of lipid needed for quality biodiesel production. Microalgae lipids biosynthesis ensues in two stages which are fatty acid biosynthesis and triacylglycerol biosynthesis pathways as shown in Figure 6, depending on the nature of carbon source utilised [94]. The

inorganic carbon sources are mainly utilised by microalgae through the CO_2-concentrating mechanisms (CCMs), while the organic carbon sources are absorbed through the Pentose Phosphate (PPP) Pathway and the Embden-Meyerhof-Pranas (EMP) pathway [94, 95], depending on the mode of cultivation. Glucose can be metabolized by microalgae cells through the PPP pathway under heterotrophic conditions, while under mixotrophic condition the EMP pathway is used [96]. Organic carbon sources can be in simple or complex form. The simple organic carbon sources such as glucose, mannose can be directly utilised whereas complex carbohydrates are first degraded enzymatically into simple form before it can be utilised by microalgae. To this study, only mechanism of microalgae lipid synthesis and accumulation through PPP/EMP pathway is briefly discussed on species that can utilise organic carbon for mixotrophic. Microalgae lipid biosynthesis is usually initiated through PPP route. The PPP pathway usually commence by the degradation of hexose via oxidation to yield 6-phosphate glucose (G6P). The G6P is converted into ribulose 5-phosphate (Ru5P) and CO_2, while two molecules of NADPH are generated. Thereafter, Ru5P is either isomerized to R5P or converted into two intermediate metabolites (fructose-6-phosphate and glyceraldehyde-3-phosphate) of EMP pathway by non-oxidation process. The produced substances continue to degrade and absorbed by microalgae cells through the EMP pathway (Kopp and Sunna, 2020) to form glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) which reversibly reacts to produce two G3P under the catalysis of triose phosphate isomerase. As a result, pyruvate, ATP and NADH are produced through a series of oxidation and enzyme catalysis [97, 98]. The produced pyruvate is then decarboxylated to form acetyl-CoA (end product of glycolysis) which is connected to TCA cycle and biochemical reactions resulting in lipids synthesis [99]. Microalgae lipid synthesis pathways can be altered to influence lipid content, depending on cultivation conditions such as stress and mode of cultivation [100].

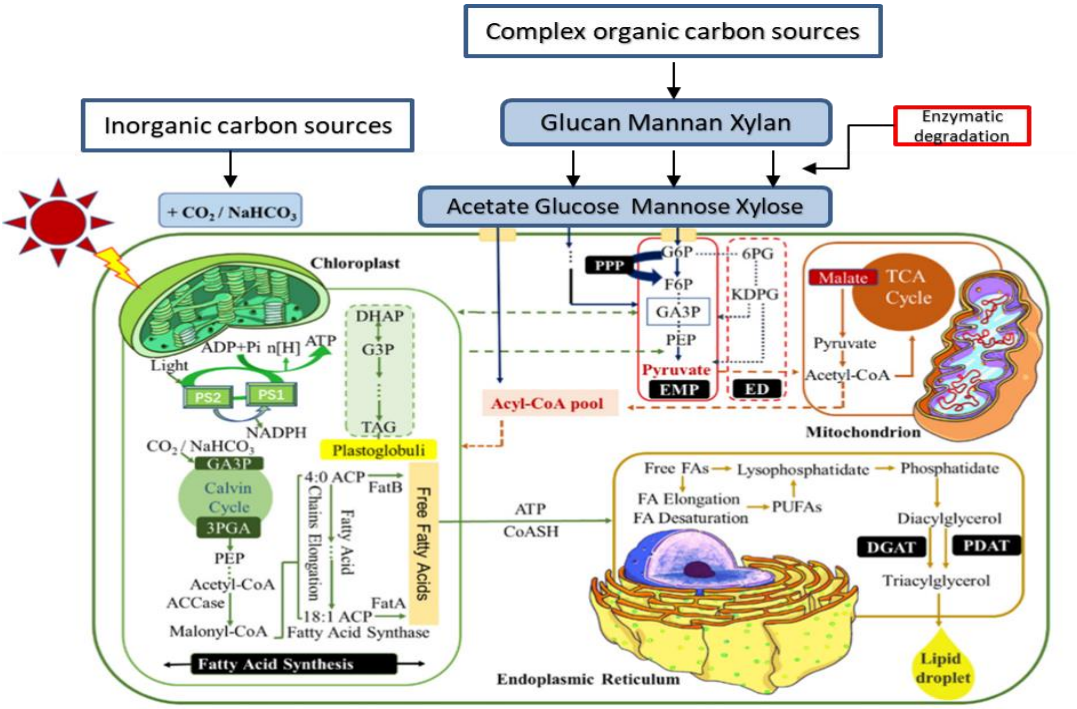


Figure 6: An outline of microalgae lipid biosynthesis pathways, utilising simple sugars from enzymatically degraded glycans. Illustrating different pathways for the utilisation of both inorganic and organic carbon sources [93].

Several researches have resulted in high lipid accumulation under stress condition (nitrogen, phosphorus limitation) [101]. Under this condition, cells will lose their capacity to divide, shifting metabolic activities towards the production of lipids accompanied with low biomass productivity. Adopting a two – stage cultivation process has assisted in addressing the problem, but further improvement is required. More so, the use of some organic carbon as carbon source has shown the potentials of enhancing microalgae lipids accumulation [102]. For instance, the utilisation of organic carbon sources such as glucose stimulated the enrichment of acetyl CoA/malonyl CoA pool, thus increasing the lipid synthesised [102, 103]. Miao and Wu [104] cultivated *Chlorella protothecoides* heterotrophically utilising glucose as a carbon source. Results obtained showed the lipid content was fourfold when compared with autotrophic growth mode. Although, no specific reason was given by the Author, but lipid content was influenced by supplying different concentrations of organic and inorganic carbons, and nitrogen sources. Other research works conducted using related carbon sources have shown similar observation, with higher lipid yields in adopting mixotrophic mode of cultivation [13, 102]. In this present study, the effects of other various possible organic carbon sources will be examined.

2.3 Upstream activities

This section will provide brief insights into the upstream processing of microalgae earlier outlined in Figure 3 of this thesis. The most important phases of upstream processes in microalgae research include preparation of culture medium, cultivation (modes, systems and methods), control of environmental factors, selection of microalgae species and, growth parameter monitoring and evaluations [105]. In considering microalgae upstream processes, factors affecting algae growth and development (biomass and lipids) must be integrated. These factors are classified as abiotic, biotic and process related as shown in Table 1.

Table 1: Factors affecting microalgae growth and development [106-108]

Abiotic	Biotic	Process related
• Temperature • pH • Light • Concentration of nutrients • Carbon dioxide content • Cultivation medium • Salinity • Presence of toxic chemical in culture	• Bacteria • Fungi • Viruses • Microalgae and strain • Microalgae cell history • Presence of other strain in the culture	• Biomass concentration • Intensity of mixing • Frequency of algae harvesting

2.3.1 Culture media

Wastewater from various sources and fertilizer compositions and formulated media (usually enriched in nitrogen and phosphorus compounds) are frequently used as culture media to supply nutrients for microalgae growth [109]. A few studies have considered the use of certain agricultural fertilizers, domestic and industrial wastewater as cheap nutrient source for microalgae cultivation [110]. Utilising fertilizers or miracle gro. for large-scale cultivation of microalgae production can reduce the burden of the cost of production. Park et al. [111] proposed the utilisation of fertilizer in a large scale hypothetical High Rate Algae Pond (HRAP) to avoid nutrient limitation for microalgae growth, assuming that for a typical microalgae composition of ($C_{106}H_{181}O_{45}N_{16}P$), a fertilizer with N:P formulation of 16N:P (i.e. 7.3gN:1gP) would essentially be required. Miracle gro. of chemical composition of the fertilizer is: 6 % Nitrogen, 3 % Phosphorus, 5 % Potasium, soluble Copper, Chelated by EDTA 0.002%, soluble Iron, Chelated by DTPA 0.03 %, soluble Manganese Chelated by 0.01 %, soluble Molybdenum 0.001 % and soluble Zinc, Chelated by EDTA 0.002 % may also serve as a source of nutrient for

microalgae growth. So far, there is no remarkable difference in terms of the growth rate and biomass concentration between the microalgae cultured with agricultural fertilizers or miracle gro. when compared with the control standardly formulated growth medium. Besides, utilising cheap agricultural grade fertilizers (e.g. Urea 46) may be cost effective but can be a source of heavy metal contamination that can limit the growth of sensitive microalgae strains while posing considerable burden on the net energy balance (NEB) and sustainability of the entire production process, factoring in the energetic costs of fertilizer production [112]. It should be noted that microalgae biomass produced in wastewater cannot be used in food, cosmetics, medicine production etc. for human consumption due to the risk of contamination of biomass produced in wastewater with organic and inorganic pollutants and microbes, which could be detrimental to human health. The biomass can be used for biofuel production.

Unlike wastewater, formulated growth media have recommended concentrations of micro and macro nutrients as outlined in the methodology section of this thesis. These growth media such as Blue Green (BG 11), F/2 and Bold's Basal Medium (BBM) have been widely tried on different freshwater and marine microalgae cultivation. BG11 medium has been adjudged suitable for the cultivation of various freshwater and marine microalgae [113].

Contrary to formulated media, concentrations of nutrients in wastewater are most times unknown, hence the need to utilise formulated growth media such as BG 11 throughout microalgae growth experiments conducted in this study.

2.3.2 Carbon sources and utilisation

Roughly 50% of the dry weight of microalgae biomass is composed of carbon, which is largely derived from various inorganic or organic carbon sources [114]. Inorganic carbon can be sourced from stored CO_2, ambient CO_2, flue gas flared from industrial, transport, energy or power generating, household and agricultural activities and carbon compounds like sodium carbonate [115]. Organic substrates of glycerol, ethanol, sodium acetate, monosaccharides and complex carbohydrates or glycans have been utilised as organic carbon sources with varying remarkable results depending on microalgae species, other growth conditions and mode of cultivation [116]. Both simple sugars and complex glycans are utilised as carbon sources in this research. Simple organic carbon sources such as glucose and mannose metabolized via pentose phosphate pathway (PPP) without further decomposition. Due to

complex nature of β-glucan, β-mannan and xylan, several enzymes are required to enzymatically decompose them into simple status needed for microalgae metabolism. Notably, addition of carbon to microalgae growth media has been broadly reported and observed to have enhanced growth, biomass and lipid content, depending on the source and nature [117]. Growth media with low ` carbon concentration can decrease the rate of synthesing lipids and carbohydrates which have the presence of multiple carbon to carbon bonds. It may also retard the synthesis of vital enzymes involved needed for the synthesis of proteins and pigments. Utilising inorganic and organic carbon sources simultaneously for mixotrophic cultivation has shown capability of enhancing microalgae biomass and lipids yields for biodiesel production. It has been reported that certain microalgae species such as *Chlorella spp.* and *Nannochloropsis* spp. could accumulate high lipid when cultivated with composite carbon sources such as sweet sorghum and yams [23], and the direct use of polysaccharides such as cassava starch, xylan cellulose and maize as exogenous carbon sources in the cultivation of *cyanobacterium Phormidium sp.* showed a better lipid accumulation capability than glucose [20]. Therefore, continuous search for suitable organic carbon sources that may enhance microalgae biomass and lipid productivity for biofuel production is required. The effects of utilising β-glucan (polymer of glucose), β-mannan (polymer of mannose) and xylan (polymer of xylose) is being investigated in this study.

2.3.2.1 β-glucan degradation pathway and utilisation as a carbon source

β-glucan is a water soluble, natural, nontoxic, biodegradable and non-starch polysaccharide of d-glucose monomers linked through β-glycosidic bonds [118]. They can be found in yeast, fungi (including mushrooms), some bacteria, seaweeds, and cereals (oat and barley) [119]. Glucose monomers are linked via β-(1→3) glycosidic bonds in bacteria and microalgae whereas glucose monomers are linked via β-(1→3) and β-(1→6) glycosidic bonds in yeast and mushrooms. In oats and barley, glucose monomers are linked via β-(1→4) and β-(1→3) glycosidic bonds [118, 120]. The structure and description of β-glucan from several sources are shown in Figure 7. The physical properties of β-glucan such as solubility, rheological behaviour and functional effects depend on the structure and molecular weight [121]. It has been reported that β-glucan is soluble in water and can be degraded enzymatically using reactive enzymes (β-glucanase), oxidatively using hydrogen peroxide and acid or alkali induced degradation [122, 123]. Cereal β-glucan and yeast β-glucan are treated in this study. Its degradation initiates the process of production of simple sugar (glucose) metabolised

during microalgae cultivation. The major product of β-glucan degradation using β-glucanase or hydrolases enzymes is glucose [124, 125].

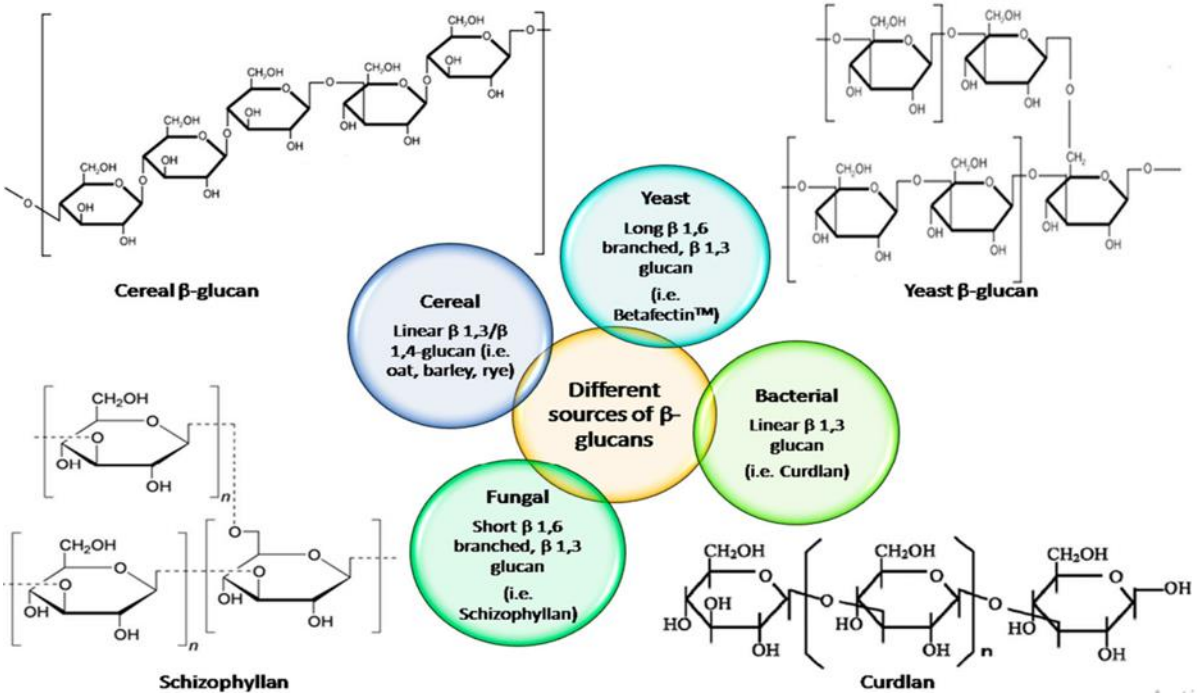


Figure 7: Different sources and molecular structures of β-glucan [119]

β-glucanase is an essential lyase predominant in bacteria, fungi, plants and microbes such as microalgae. It is classified into *exo*-β-1,4-glucanase (EC3.2.1.58) and *endo*-β-1,3(4) -glucanase (EC3.2.1.6 or EC3.2.1.39); both classes are released at the same time and jointly participate in the degradation process. The *exo*-β-1,4-glucanase hydrolyses β-glucan by serially breaking glucose molecules at the non-reducing end to produce glucose whereas *endo* β-1,3(4) -glucanase randomly cleaves or breaks the β-1-4 glycosidic bonds along the β-glucan chain. The final product of hydrolysing β-glucan with *exo*-β-1,4-glucanase enzymes is glucose whereas that of *endo* β-1,3(4) -glucanase yields an intermediate glucosyl residues of (1→3) and (1→4) prior to glucose formation [126]. Figure 8 shows the complete hydrolysis of wall-bound (1→3), (1→4)-β-D-glucans to glucose. In microalgae growth media supplemented with β-glucan, the (1→3), (1→4)- β-D-glucans *endo* β-1,3-glucanase hydrolyses and breaks the (1→4)-β-glycosidic linkages (Left hand side of Figure 8) to produce (1→4)- β-glucosyl and (1→3)-β-D-glucosyl residues, represented as $\bar{G}4$ and $\bar{G}3$ respectively, followed by (1→3), (1→4)-β-D-glucans of higher molecular mass (Right hand side of Figure 8). The red as shown indicates

the reducing end. The (1→3), (1→4)-β-D-glucans of higher molecular mass is further hydrolysed with *endo* β-1,3-glucanase to yield another glucosyl residues of (1→3) and (1→4) followed by *exo*-β-1,4-glucanase hydrolyses to produce glucose from both paths [127].

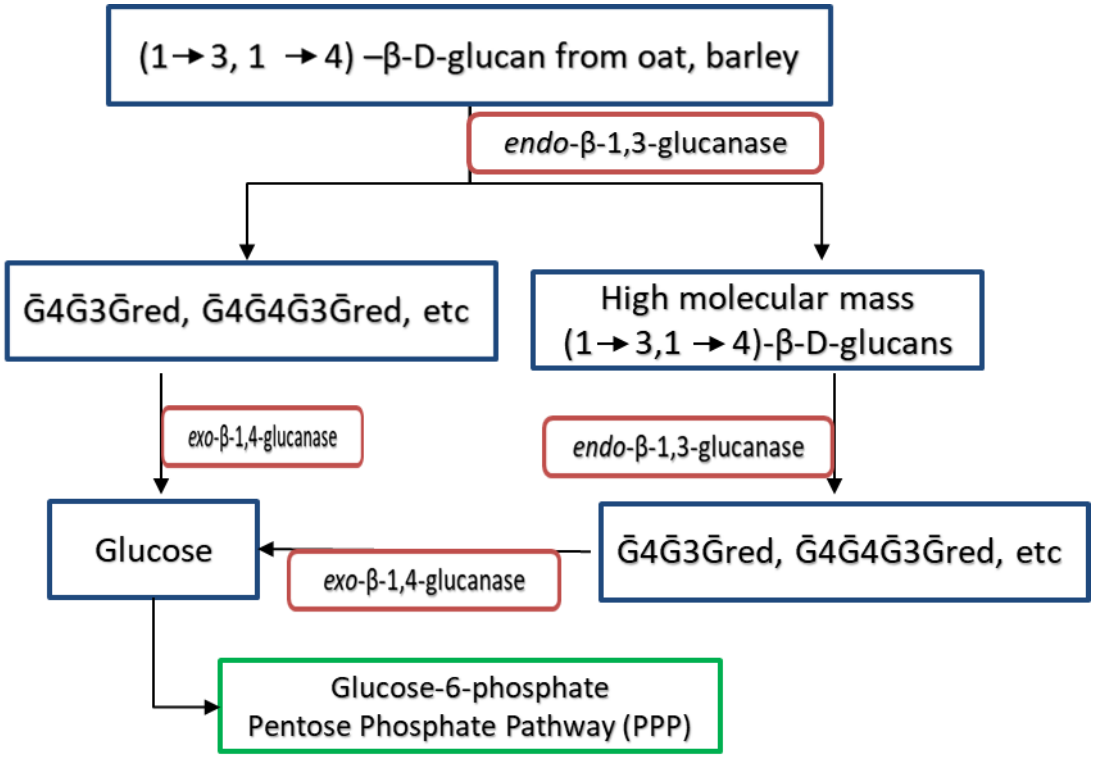


Figure 8: Pathways for the enzymic degradation of β-glucans

The enzyme *endo*-β-1,3-glucanase dislodges the (1,4)-β-glycosidic linkages to produce (1,4)-β-glucosyl (designated as G̅4) and (1-3)-β-glucosyl (designated G̅3) residues, followed by a higher molecular mass of (1,3), (1,4)-β-D-glucans which is further hydrolysed utilising the same enzyme. Thereafter, the intermediates are hydrolysed with *exo*-β-1,4-glucanase to produce glucose. G̅red denotes the reducing end.

2.3.2.2 β-mannan degradation pathway and utilisation as a carbon source utilisation

β-mannan is a natural, nontoxic, biodegradable, and non-starch polysaccharide. Mannan is one of the important members of the hemicellulose family, thus has attracted so much research attention. Naturally, mannan can exist in four diverse forms, each with β-1,4-linked backbone comprising mannose (linear mannan) or a combination of glucose and mannose residues (glucomannan), occasional side chains of α-1,6-linked galactose residues (galactomannan) and galactoglucomannan [128]. Due to its complex and variable nature, glycoside hydrolases (enzymatic degradation) of its substrates are necessary to produce monomers needed for cell factory development. Two kinds of enzyme exo-hydrolases and

endo-hydrolases are involved in the degradation of mannan and its variants. The exo-hydrolases act on the terminal glycosidic linkages and release terminal monosaccharide units from the nonreducing end, and the endo-hydrolases cleave internal glycosidic bonds at random or at specific positions[129, 130]. In all these four forms, *endo* 1,4-β-mannanase enzymes cleave/degrade β-1,4 internal linkages between mannose - mannose (as in linear mannan), and mannose - glucose (as in glucomannan). In galactomannan and galactoglucomannan, α-galactosidase enzymes release galactose residues. In glucomannan and galactoglucomannan, β-glucosidase enzymes cleave glucose residues from the nonreducing ends of oligosaccharides produced by the action of β-mannanase enzymes[131]. Figure 9 shows the various structures of different forms of β-mannan and bond cleaving enzymes, while Figure 10 shows the enzymatic degradation of mannan which is considered in this study.

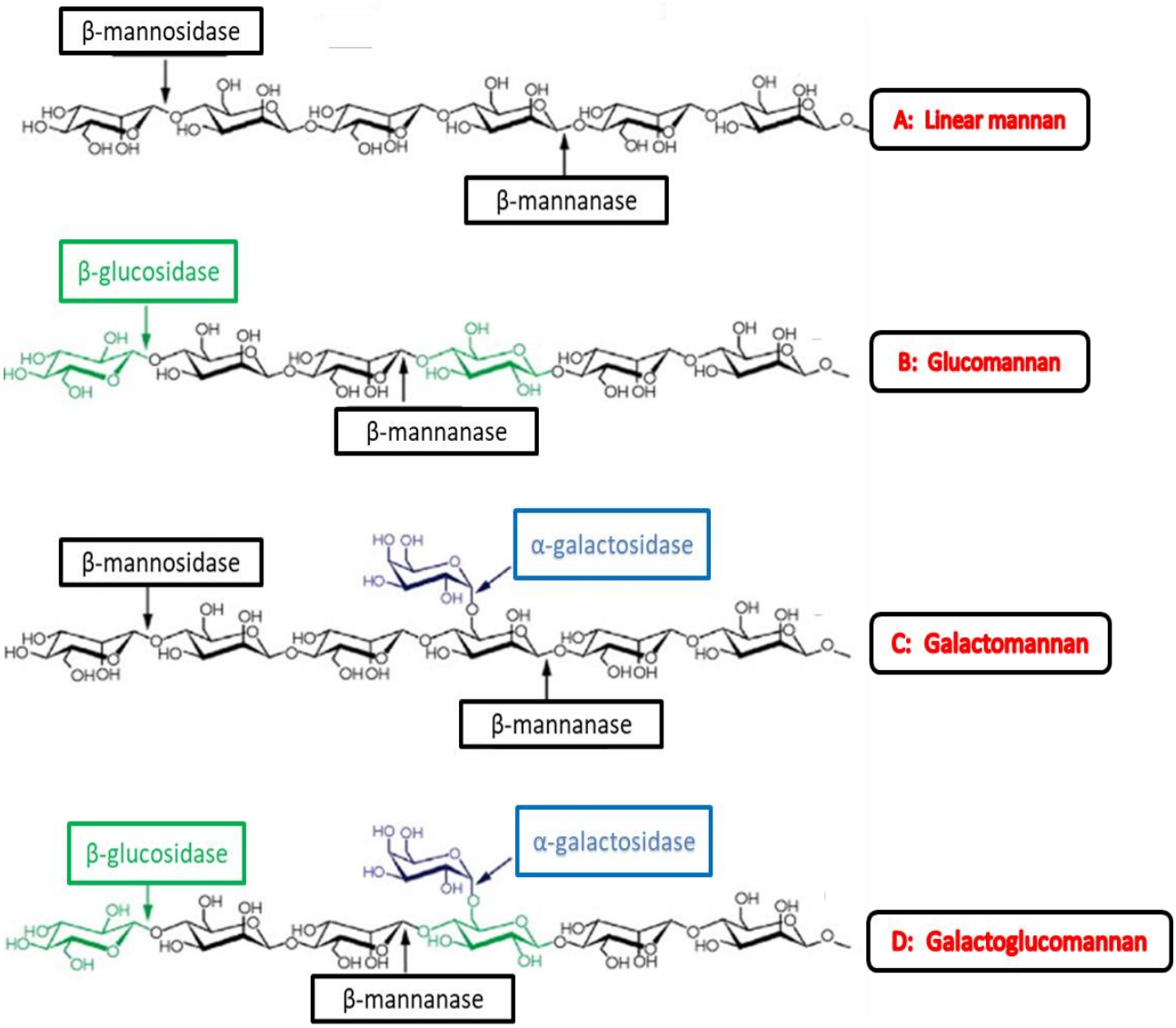


Figure 9: Structures of four different forms of mannan with various bond cleaving enzymes. A) Mannan structure, a main chain of residues; B) Glucomannan, a main chain of β-1,4 linked mannose and glucose residues; C) Galactomannan, an α-1,6 linked galactose residues coupled to some β-1,4 linked mannose and D) Galactoglucomannan, β-1,4 linked mannose and glucose residues with α-1 ,6 linked galactose residues coupled to some mannose residues. Adopted from [128].

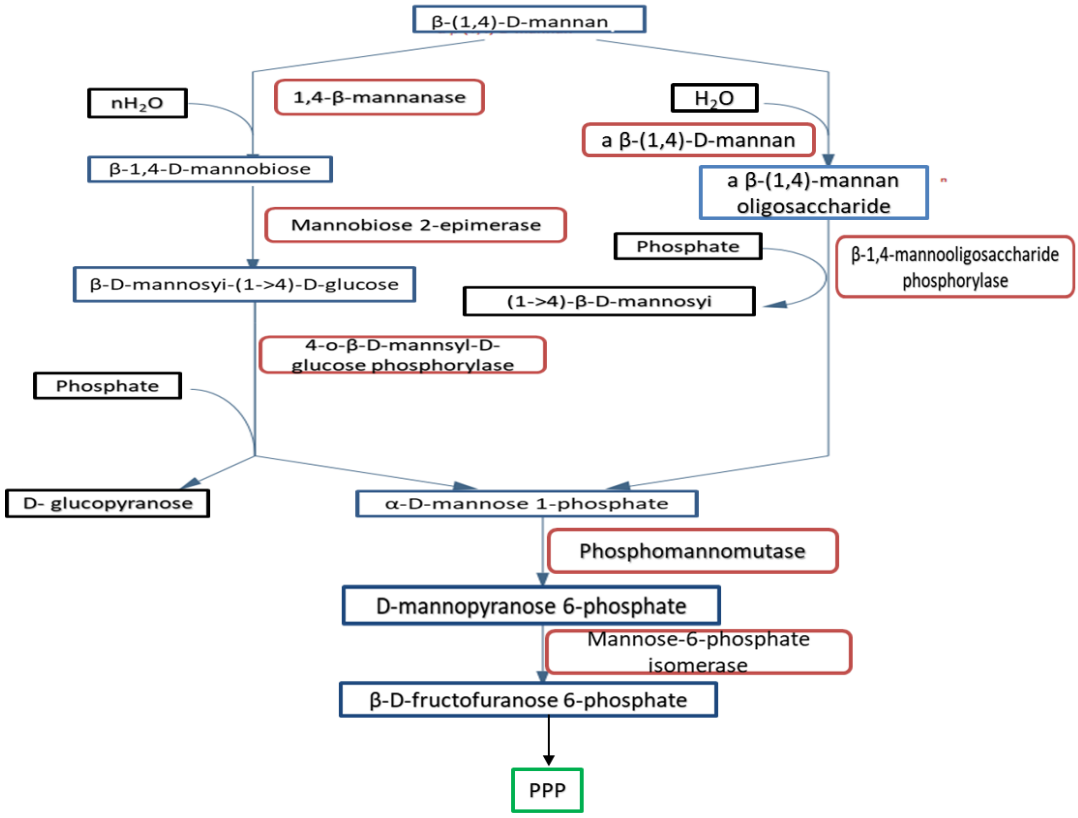


Figure 10: Pathways for the enzymic degradation β-mannan [131, 132].
Contents in red shape outline are enzymes; contents in blue shape outline are products.

2.3.2.3 Xylan degradation pathway and utilisation as a carbon source utilisation

Xylan polymer is composed of a several β-1,4 xylose residue backbone, a reducing end sequence (RES) of xylose, rhamnose and galacturonic acid. The structure of xylan is quite variable, ranging from linear backbones constituted of β-1,4-linked poly-xylose residues denoted as homo-xylans to branched hetero-xylans, whereby the prefix 'hetero' denotes the presence of branching sugar residues other than D-xylose [133-135]. Due to xylans complex nature, variety of enzymes are involved in the enzymatic degradation to xylose. Figure 11 shows a typical enzymatic degradation pathway of xylan. Xylan is degraded by endo-xylanase, which acts randomly on the main xylan backbone to liberate unbranched xylo-oligosaccharides, that are further degraded by β-xylosidase into xylose. Xylose is the main component of xylan, and its production is of great research interest because of its role in the formation of valuable biomolecules and in biofuel production in microbes like microalgae [136].

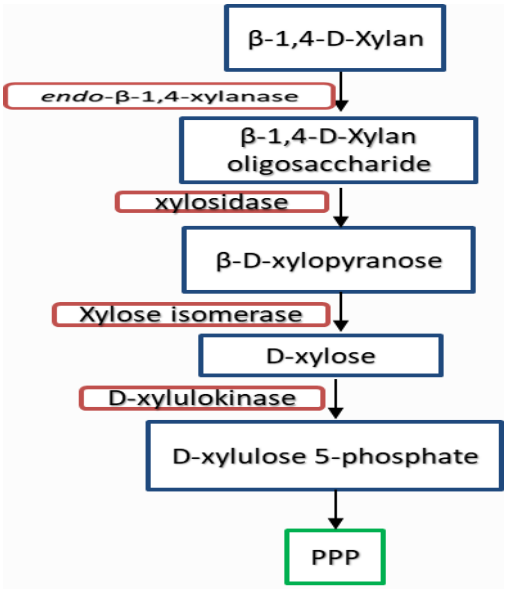


Figure 11: Pathways for the enzymic degradation of xylan [136].
Contents in red shape outline are enzymes; contents in blue shape outline are products.

2.3.3 Other nutrients and environmental factors

Other factors such as nitrogen source, light intensity and colour, temperature, pH, and aeration can affect microalgae biomass and lipid productivities [58, 137]. Nitrogen is an essential macronutrient for microalgal growth and plays an important role in protein, lipid, and carbohydrate synthesis. Microalgae growth media can be supplemented with nitrogen from various sources such as inorganic nitrate and ammonium, and organic urea [137]. The utilisation of various nitrogen depends on microalgae species. Supplementing growth media with organic carbon sources (glucose, mannose, β-glucan, β-mannan and xylan) and organic nitrogen source (urea) as utilised in this study can facilitate nutrient absorption, leading to enhanced biomass and lipid productivity. Apart from heterotrophic mode of cultivation, every other microalga mode of cultivation such as photoautotrophic, photoheterotrophic and mixotrophic must have a light source as a primary energy source. Natural sunlight, and fluorescent and LED lights have been utilized as lighting system for microalgae cultures. Light intensity, wavelength, and photoperiod (lightning time) are three important characteristics of light that can significantly affect the growth of microalgae in photosynthetic cultures [138]. The pH of growth medium is a key factor that influences microalgae biomass and lipid productivity. Its effect can be classified as fatal (extremely low acidic or high basic), tolerable (pH of 5 to 8) and optimum, depending on the species inoculated and type of nutrient used to supplement the medium [139, 140]. Also, the effect of temperature is species dependent, as a few species can tolerate extremely low and high temperatures [141, 142]. Temperature with the ambient 25±5 ^{O}C can significantly boost the growth of most species [143]. For instance, Converti et al [3] investigated the effects of varying temperature on the biomass and lipid productivity of similar microalgae species considered in this research (*C. vulgaris* and *N. oculata*). Results reported showed a maximum biomass and lipid yields at 25^{O}C and 20^{O}C for *C. vulgaris* and *N. oculata* respectively, a decline at higher temperature and cell death seen to have occurred at 38^{O}C.

2.4 Microalgae cultivation

Microalgae can be cultivated under various modes (photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic), systems (open, closed and hybrid systems) and methods (batch, continuous and fed-batch or sei-continuous methods). Each of these modes, systems and methods has got peculiar features which can directly influence microalgae biomass and lipid productivity, depending on growth factors and microalgae species. Regardless of significant variability in the literature and lack of long-term full-scale data, microalgae can be cultivated for sustainable biodiesel production since the quantity and quality of microalgae biodiesel weightily depend on upstream activities and decisions. As a result, merits, demerits, recent developments, and prospects of various forms of modes, systems and methods of microalgae cultivation for biodiesel production had been studied

2.4.1 Microalgae modes of cultivation

The mode of microalgae cultivation can significantly influence biomass productivity and lipid yields, as each mode utilises unique nutrient and energy sources. However, existing technologies and processes seem not to have addressed the inverse proportionate biomass yield and lipid accumulation during cultivation, hence, results till date still show that high biomass yield compensates low lipid accumulation in each batch of cultivation. The four major modes of cultivation are photoautotrophic, heterotrophic, photoheterotrophic, and mixotrophic modes of cultivation. Advantage and disadvantages, type of nutrient sources, energy demand and other conditions for each mode of cultivation are discussed and recorded in [144, 145]. Figure 12 summarises the carbon and energy demand of each mode of cultivation.

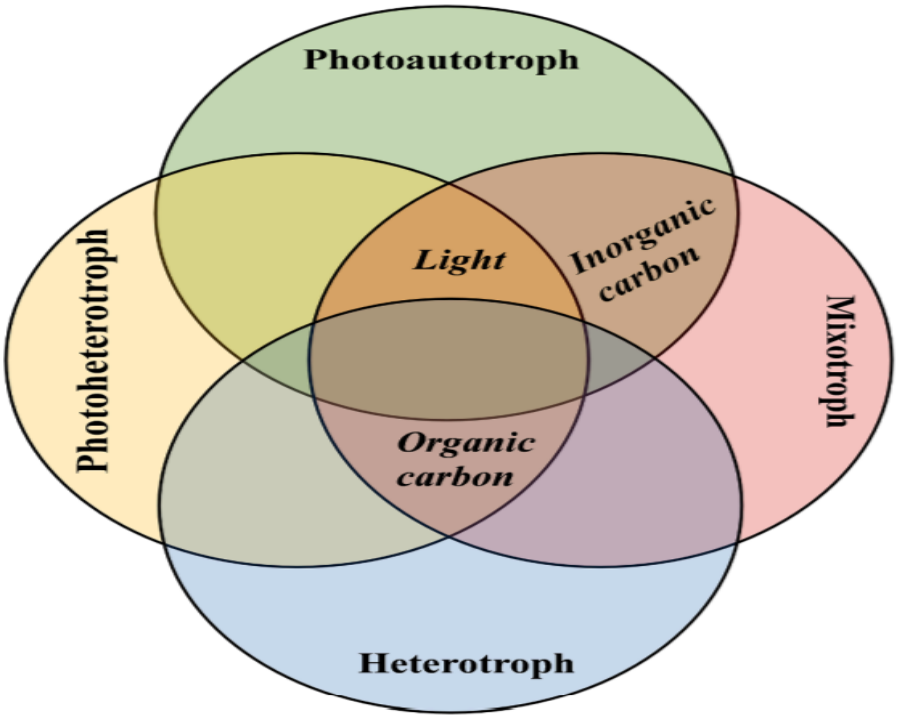


Figure 12: A Venn diagram showing definitions of various microalgae cultivation modes indicating carbon and energy sources.
Photoautotrophic mode (utilises inorganic carbon and light); Heterotrophic mode (utilises organic carbon which serves as both carbon and energy sources); Photoheterotrophic mode (utilises organic carbon and light without additional inorganic carbon source); mixotrophic mode (inorganic carbon, organic carbon, and light). Adopted from [105]. In this study, photoheterotrophic and mixotrophic modes of cultivation were employed.

Cultivation of microalgae using inorganic carbon dioxide (CO_2) and light as carbon and energy sources respectively, known as photoautotrophic mode of cultivation has been widely studied. Utilising atmospheric CO_2 for photoautotrophic cultivation can boost global CO_2 reduction and reduce the effect GHG. The process offers the possibility of sequestering CO_2. Thus, constructing cultivation sites near factories and power plants is encourage, as huge amount of CO_2 will be available for microalgae consumption within the domain. However, some major drawbacks due to low specific affinity for CO_2 have been identified [27]. Low cell density, long cultivation period and high harvesting cost and energy associated with photoautotrophic mode of cultivation triggered the application of heterotrophic, photoheterotrophic and mixotrophic as alternative modes of cultivation.

The heterotrophic mode of cultivation utilizes organic carbon from simple and complex sugars such as (glucose, acetate, mannose, arabinose, xylose, crop flours, and wastewater), glycerol,

acetate as both carbon and energy sources. In this mode, microalgae cell growth does not depend on light, hence, seems to encourage scale-up option because of the small surface to volume ratio of reactor. Some studies have shown that a higher biomass and lipid productivity of microalgae can be obtained by using heterotrophic cultivation condition[146, 147]. Under the condition of a dark heterotrophic system, glucose is mainly metabolized through the Pentose Phosphate pathway (PPP). This process has high cell production and promotes easy harvest due to higher cell density. However, care should be taken as heterotrophic cultivation might utilize more energy than autotrophic cultivation due to need for organic carbon source. Other drawbacks such as; limited types of microalgae strains that can grow heterotrophically, expensive due to the addition of organic substrate (e.g., glucose, nitrogen, phosphorus and trace elements), easily contaminated by another microorganism and, unable to generate light-induced metabolite may be observed.

Photoheterotrophic cultivation, also known as photo-assimilation or photo-metabolism is a cultivation condition where microalgae require light while using organic compounds as the carbon source. The major difference between photoheterotrophic and mixotrophic cultivation is that light must be present during photoheterotrophic growth for energy purposes while mixotrophic cultivation can use organic compounds to serve this purpose [43]. Thus, photoheterotrophic cultivation needs both sugars and a light source at the same time [42]. Although the production of some light-regulated useful metabolites can be enhanced by using photoheterotrophic cultivation, production of biodiesel using this approach is still rarely to be seen [56]. Like mixotrophic and heterotrophic cultivation conditions, contamination problems arise in photoheterotrophic cultivation as sugar based organic compounds are used as carbon sources. Moreover, this mode requires a special design of photobioreactor as the cultivation medium during scale up can increase the capital cost and operating cost of cultivation[148]. Different systems scale-up differently as different cultivation conditions require different operating conditions and environment. While the photoheterotrophic mode of cultivation simultaneously utilises organic carbon source and light without the injection of any external inorganic carbon source or aeration, the mixotrophic mode utilises the trio of organic and inorganic carbon sources as well as light [28]. The process combines the advantages of photoheterotrophic and heterotrophic modes, while overcoming the disadvantages of the duo. Mixotrophic mode of microalgae cultivation supports the utilisation

of complex organic carbon substrates to enhance biomass and lipid synthesis, hence, considered in this study [149].

2.4.2 Microalgae systems of cultivation

The systems of microalgae cultivation are open system (OP), closed system and hybrid system. The closed system, also known as photobioreactor (PBR) is now widely studied because growth conditions can be controlled, bacterial contamination can be avoided, and the design permits a more controllable and effective use of light compared to open ponds [150]. The open raceway pond (ORP) is the commonest type of OP. The energy expended during microalgae cultivation using PBRs is dissimilar with that of ORPs due to unique design and operation devices. The advantages and limitations of each system have been reviewed in [151, 152], and Table 2 shows the design and operation parameters of OP and PBR. Figure 13 shows various types of PBR in use. The advantages and disadvantages of various PBR design have been recorded in literature [153, 154].

Table 2: Comparison of operational parameters of OP and PBR [155-158].

Parameter	Open system	Photobioreactor
Energy consumption	Low	High
Maintenance	Easy	Difficult
Biomass concentration	Low	High
Productivity	Low	High
Costs (capital & operating)	Low	High
Risk of pollution or contamination	High	Low
Controllability limit	Low	High
Carbon dioxide losses	High	Low
Water losses by evaporation	High	Low & preventable
Area to volume ratio required	Large	Small
Overheating problems	Low	High
Weather dependence	High	Low
Species cultivatable	Few & specific species	Variety of species
Scalability	Easy	Difficult
Harvesting efficiency	Low	High
Light utilisation	Poor	High

The major contributors of energy consumption terms in OP are CO_2, water pumping and mixing propellers or paddlewheels, whereas those of PBRs are water pumping, mixing (air pumping and CO_2) [159]. Table 3 indicates the energy input in cultivating microalgae utilising ORP and PBR. The utilisation of flue gas from combusted fossil fuels as carbon source in the

cultivation of microalgae in OP can reduce cost and energy if properly harnessed. High capture cost, energy required to transport and purify it and presence of other gases and particulate matter that can inhibit growth have restricted its usage [160].

Figure 13: Photobioreactors used for microalgae cultivation
a) raceway pond b) vertical column (single) c) vertical column (interconnected) d) flat- plate e) inclined tubular f) horizontal tubular g) helical tubular h) hybrid i)vertical tubular [161].

To utilise raw flue gas as carbon source; 1) pond needs to be located near to the flue gas source 2) gases that inhibit microalgae growth can be isolated or removed 3) Microalgae species and strains that can tolerate these gases can be cultured. It has been reported [162, 163] that PBRs require more energy (from 2-15 times) but produce more biomass than OP due to high mass transfer and the attainment of optimal growth conditions.

In energy balance analysis [164], energy consumption for carbon dioxide injection, water pumping and paddle wheel together with energy spent on construction, urea and phosphate, is equivalent to 3.25 MJ/kg of *Chlorella vulgaris* (14% energy content of dried algae biomass). Previous studies [159, 162, 165, 166] indicate that upscaling of microalgae cultivation process creates more energy disparity between OP and PBR. However, for commercial scale cultivation of microalgae using PBR to be sustainable and economical, designing PBR against

energy requirement and with practicable assumptions has become necessary. Integrating photovoltaic technology and energy efficient devices, building ponds or PBR near a power plant, recycling of process by-products (like medium, glycerol) and the use of wastewater can reduce energy required for microalgae cultivation in both OP and PBR [167].

Table 3: A literature on comparative review of hypothetical energy input in cultivating microalgae utilising ORP and PBR.

Species(process)	Energy input (scale)	Specific energy utilised MJ/kg	Productivity g/m²/d	Ref.
Chlorella vulgaris **(ORP and PBR)**	d = 0.2; m; E_{CO2} = 0.027(flue gas); E_{CO2} = 0.145 (pure); E_P = 0.7; E_{AP} = 0.264; E_{PW} = 0.223; (2.2 kg of dry biomass, 1000 l)	OPR 0.95 PBR 1.11	n/a	[159]
Chlorella vulgaris **(ORP)**	E_{CO2}= 1.14; E_P 0.334; E_{PW} = 0.304; others = 1.473	3.25	10.5	[164]
Nannochloropsis sp **(PBR and ORP)**	d = 0.2 m; E_{PW} = 1.152; E_{AP} = 3.21; E_{CO2} = 0.146 (1 kg of biomass, 125 l in PBR & 2000 l in ORP)	OPR 1.15 PBR 3.356	25	[165, 168]
Chlorella vulgaris **(ORP)**	d = 0.3 m; E_{PW} = 0.72, which is the most energy intensive device used. (1000ton dry algae)	0.72	30	[169]
Scenedesmus dimorphus **(ORP)**	d = 0.3 m; E_{CO2} = 0.18; E_P =1.23; E_{IW} = 0.72; E_{PW} = 1.06; E_{WD} = 0.22;(1 kg of dry biomass, low nutrient condition)	3.41	16	[170]
Chlorella vulgaris **(ORP)**	d = 0.3 m; E_{CO2} = 0.1; E_{PW} = 0.72 (1 kg of algal)	0.82	25	[171]

Hint: d = depth of ORP, E_P = water pumping energy, E_{IW}= internal water pumping, E_{WD} = energy for water desalination, E_{PW}= paddle wheel energy, E_{CO2} = CO_2 pumping energy, E_{AP} = mixing-air pumping energy. For uniformity, Energy (E) utilised in cultivation process recorded in literature were estimated and expressed in MJ.
Specific Energy in (MJ/kg) = 3.6 multiplied by Energy in (kWh) divided by mass (kg)
n/a = not available; only the energy required in the cultivation process is considered in the review. Utilising PBR consumes less energy than ORP (Justifies our choice of PBR in this study)

2.4.3 Microalgae methods of cultivation

Presently, there are three key methods of microalgae cultivation viz; batch, fed batch (semi-continuous) and continuous. In batch cultivation method, microalgae inoculant, growth medium and nutrients are introduced in an enclosed vessel in the beginning of the cultivation process till the end. No additional nutrients are not added during the cultivation. As the cultivation process progresses, the nutrients decrease with presumably increasing biomass production. Factors such as temperature, pH and dissolved oxygen affect microalgae growth in batch cultivation. The main drawbacks are varying irradiance as a result of cell self-shading effect and continuous nutrient consumption by microalgae. In the continuous cultivation, nutrients are regularly added to the system, while the effluent which may consist of product or waste is repetitively discharged. The continuous cultivation system starts with batch cultivation, in which the microalgae growth pattern follows the ordinary growth cycle. Then, new mediums or nutrients are added during the exponential growth phase, which allow the microalgae to reproduce continuously at an indeterminate rate. As a result, the volume of microalgae biomass will be greatly increased. The volumetric flow rates for influent and effluent streams are maintained once the stationary growth phase is achieved. However, the continuous cultivation system is not preferred in industrial scale as the system is easier to be contaminated by bacteria [68]. In addition, the biomass yield produced is low at steady state operation [68]. The advantages of this cultivation method are the nutrient concentration and pH can be easily manipulated [70]. Semi-continuous cultivation Semi-continuous cultivation is a more practicable process than batch cultivation in which part of the cultivation medium is regularly discharged, and the remaining culture is utilized as the seed to continue the cultivation. In addition, high inoculum ratio must be maintained at the moment of introducing a new cultivation cycle. The amount of fresh culture added into the cultivation is known as “renewal rate” and the biomass concentration is known as “blend concentration. Semi-continuous cultivation process can be operated for multiple cycles, depending on the microalgae reproducibility. This will help increase the overall biomass productivity due to the elimination of lag phase, resulting in high biomass yield. Another advantage of using semi-continuous cultivation process is it will maintain the quantity of inoculum in the cultivation and ensure microalgae are always remained at high specific growth rate. Figure 14 shows the photobioreactor of a semi-continuous cultivation system.

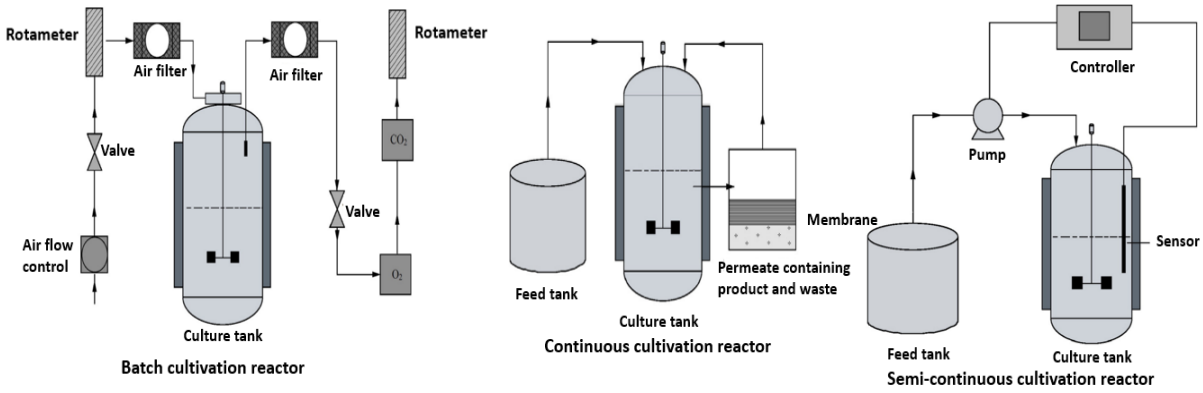


Figure 14: The photobioreactor of a Batch, Continuous and Semi-continuous modes of cultivation [9].

2.4.4 Advanced microalgae cultivation using the Multi-cultivator (MCR)

Microbes such as microalgae utilize light and capable of CO_2 sequestration; thus, they are promising in our bid to curb the menace of global warming occasioned by GHG. However, utilising microalgae in large scale has been hindered by reactor engineering limitations, with the typical OP cultivation being susceptible to the risk of contamination and lack of control of the growth parameters. Contrarily, the closed PBRs guarantee improved control on microalgae growth parameters in terms of maximum exposure to natural light, pH of the growth media, CO_2 and water and nutrient supply. Multi-Cultivator MC-1000-OD (MCR) is a cost-effective small-scale cultivation device developed for cultivation of microbes. MCR consists of 8 cultivation vessels, where up to 85 ml of suspension can be maintained under controlled light, temperature and aeration conditions. The cultivation vessels are immersed in a temperature-controlled water bath. All vessels can be bubbled with air or selected gas of different flow rate through a manually adjustable valve manifold. Each vessel is separately illuminated by an array of cool white LEDs (optionally warm white, red, or blue LEDs) that generate incident photosynthetic photon flux density (PPFD) up to 1,000 $\mu mol.m^{-2}s^{-1}$. The illumination is independently adjustable for each cultivation vessel in intensity, timing and modulation which allows to set unlimited number of user-defined light protocols. It is a multi-colour instrument and can combine up to four different colours within each cultivation slot for definition of specific spectra. The growth of cultivated organisms is automatically monitored by measuring of optical density at two wavelengths of 680 nm and 720 nm and the biomass accumulation can be controlled via the turbidostatic device.

2.5 Downstream activities

2.5.1 Techniques of microalgae biomass harvesting

Several studies have reported that microalgae biomass harvesting cost about 20–30% of total energy needed for microalgae downstream processes [172]. Dilute microalgae solutions (0.3-5 g/l or 0.05-0.075 % dry matter for OP and 0.3-0.4 % for PBRs), high moisture content in the suspension, and the small size of microalgae cells (3-30 µm) are the various contributors of high cost and energy incurred in harvesting process [173-175]. Thus, effective techniques of microalgae biomass recovery have been developed. The techniques of harvesting microalgae include flocculation, filtration, centrifugation, flotation, gravity sedimentation, electrolytic process, electrophoresis and magnetic separation. The advantages and limitations of each method are detailed in [173, 174, 176]. In microalgae harvesting, either thickening, dewatering or both can be applied. Flocculation, flotation and gravity sedimentation processes can thicken microalgae solution to slurry of about 2-7 wt% of total suspended solids (TSS), before dewatering to 15-20 wt% (TSS) by filtration or centrifugation [174]. Attention needs to be paid to cell damage, microalgae strain characteristics (like density and size), salt concentration and acceptable level of moisture before selecting an appropriate technique to apply. A suitable harvesting process should 1) be fit for most microalgae strains 2) allow the realisation of high biomass concentrations, with low costs of operation, energy and maintenance 3) not contaminate or toxify microalgae biomass 4) allow recycling of culture medium [32, 177]. Napan et al. [178] reported that biomass and lipid could be lost due to unexpected delays during harvesting. Also, two or three methods can be combined to obtain a very high biomass concentration, but in most cases, cost and energy are compromised.

Till date, centrifugation is regarded as the commonest and fastest methods of harvesting microalgae, with harvesting efficiency of over 98 % [179]. But the high initial capital cost and energy consumption hinder the adoption of this process. Gravity sedimentation, rated as the simplest, cost and energy effective means of harvesting microalgae takes much time, with low harvesting efficiency. The combination of various methods, especially flocculation and centrifugation can improve harvesting efficiency [180]. Various methods of microalgae harvesting technique consume different amount of specific energy, depending on microalgae species and biomass concentration [172]. Table 4 shows the estimated energy requirement in various harvesting techniques.

Notably, cultivating algae in PBRs achieves over 2.5 times culture concentration than in ORP, resulting in the reduction of the energy required to harvest algae by 62 % [181]. From the analysis done by Weschler et al. [181], 1.44 MJ/kg is the energy required for the harvest of microalgae, when cultivated in PBR and harvested by chamber filter press (CFP). But 3.6 MJ/kg, 2.5 time higher than the former, is required for the harvest of microalgae, when cultivated in ORP and harvested by the same process.

Table 4: Energy input of various microalgae harvesting processes.

Species(scale)	process	Changes in conc.	Energy required MJ/kg	Ref.
***Nannochloropsis sp* (2000 l)**	125 mg coagulant ($FeCl_3.6H_2O$) per litre of culture, ASACF and centrifugation	ASACF & Coagulant: 0.05 to 3% w/w Centrifugation: 3 to 15% w/w	0.377	[168]
***Chlorella vulgaris* (220 l)**	ASACF and centrifugation	ASACF: 0.05 to 1 % w/w; Centrifugation: 1 to 20% w/w	0.23	[159]
***Scenedesmus dimorphus* (Culture containing one metric ton of biomass)**	Bioflocculation, DAF and centrifugation	Bioflocculation & DAF: 0.5 to 50 g/l Centrifugation: 50 to 120 g/l	0.65	[170]
Chlorella vulgaris	Natural settling and centrifugation	Settling: 0.5 to 10 g/l Centrifugation 10 to 50 g/l	0.15	[171]
***Chlorella vulgaris* (n/a)**	Cultivated in PBR, ORP & harvested with chamber filter press	0.05 to 90 % w/w	PBR 1.44 ORP 3.6	[181]
***Nannochloropsis* (n/a)**	Flocculation with $Al_2(SO4)_3$, decantation & centrifugation	n/a	PBR 0.0036 ORP 0.216	[182]
n/a	Flocculation, DAF & centrifugation	0.1 to 200 g/l	5.18	[183]

ASACF = air sparging assisted coagulation flocculation; DAF = dissolved air flotation; only the energy required in the process is considered.

Energy in (MJ/kg) = 3.6 multiplied by energy in (kWh)/mass (kg) of biomass.
Microalgae cultivated in PBR requires less amount harvesting energy those cultivated in OPR.

2.5.2 Harvesting by flocculation technique

Flocculation can be induced by charge neutralisation, bridging and electrostatic patch [174]. Most multivalent metallic ions and organic flocculants interact with microalgae negatively charged cellular surface to form flocs of larger size and then sediment by gravity [184-186]. Safi et al. [152] recorded that it is difficult to neutralise negative surface charge of exponentially growing algae, because at that growth stage, surface charge is very high and remains dispersed. However, at the stationary growth phase, the negative surface charges diminish, thus algae flocculation can be accelerated by increasing the media pH using sodium hydroxide, inducing over 90% flocculation at a pH of 11 with a concentration of 9 mg of sodium hydroxide per gram of dry biomass [187, 188]. Moreover, the significance of co-flocculants has been emphasized by Oh et al. [189], where 6.8 mM of calcium chloride was used to improve flocculation efficiency to 83%. According to the authors, magnesium, calcium, potassium, sodium and iron can also be used as co-flocculants. Flocculation efficiency and percentage of microalgae broth removed at the end of flocculation can be estimated [189-191]. Chatsungnoen and Chisti [190] stated that achieving 95 % flocculation efficiency depends on factors such as ionic strength of the culture medium, type and concentration of flocculants, size of microalgae species and the concentration of the biomass in the broth. Of which, microalgae species characteristics (size, surface charge) are the most influential factor on the flocculation activity of biomass concentrate [192]. The synergistic effects of these parameters can be observed by optimising them.

In an experiment conducted by Vandamme et al. [187], 0.5 M of NaOH concentration was used to vary the pH of *C. vulgaris* to investigate the pH effect on flocculation. The suspension was intensively mixed at 1000 rpm for 10 minutes, then 250 rpm for 20 minutes and left to sediment for 30 minutes. The result showed a recovery efficiency of 98 %. In another experiment conducted by Vandamme et al. [193], the effect of excreted Algae Organic Matter (AOM) on the flocculation of *C. vulgaris* was investigated. The work was done using five methods of flocculation, and results revealed that AOM inhibited flocculation resulting in a need to increase flocculants dosage. Attempts made to remove the AOM by centrifugation failed. In a separate flocculation experiment conducted on *C. vulgaris*, in another flocculation

experiment conducted on *C. vulgaris*, Oh et al. [189] used *Paenibacillus sp. AM49* as a bioflocculant within a pH range of 5 – 11 and 0.5 mM $CaCl_2$ as co-flocculant, obtaining a recovery efficiency of 83 %. The flocculation activity seemed to be deterred by the action of the bioflocculant, compared to 86 % recovery efficiency when bioflocculant was not used. Gerchman et al. [194] flocculated *C. vulgaris* using the polymeric flocculant polydiallylammonium chloride (PDADMAC) and compared the flocculation efficiency to those of other polymeric flocculants [194]. PDADMAC showed a better flocculation efficiency than others in the timespan of one hour. Recently, Zhu et al. [195] compared the recovery efficiency of chitosan to that of alum and observed that both flocculants had the same biomass recovery efficiency 92 % using concentrations of 2.5 g/l and 0.25 g/l for alum and chitosan respectively [195]. Lee et al. [196] flocculated the same *C. vulgaris* using chitosan – Fe_3O_4 nanoparticles and recorded even a higher recovery efficiency of 99% in 2 minutes flocculation time. Surendhiran and Vijay [197], investigated the flocculation capacity of eight inorganic reagents in *N. oculata*: $AlCl_3$, $Al_2(SO_4)_3$, $FeCl_3$, $Fe_2(SO_4)_3$, $ZnSO_4$, $ZnCl_2$, $MgSO_4$ and $MgCl_2$. Their respective flocculation efficiencies were determined at different flocculant concentrations and temperatures. It was observed that 0.4 g/l $FeCl_3$ has the highest flocculation efficiency at 93.8 % in 180 minutes. In the absence of iron compounds and at 35 ^{O}C, $ZnCl_2$ showed a flocculation efficiency of 92 %. It was concluded that chloride salts have better flocculation ability than their corresponding sulphate salts. Higher valence cations, such as Fe^{3+} and Al^{3+} and anions with high electronegativity tend to flocculate better than others. Table 5 shows a review of the degree of flocculation of the two species selected in this study. The knowledge of the optimisation of some parameters and comparative studies on microalgae flocculation are still scanty.

Furthermore, in continuation of the flocculation experiments conducted by Surendhiran and Vijay, a cell wall viability check was carried out to ascertain the structural stability of *N. oculate*. It was discovered that cell was intact with zinc, partially distorted with aluminium chloride and completely ruptured with ferrous salts; while process is independent of temperature[198, 199]. The efficacies of *Moringa oleifera* after oil extraction (MOAE) and *Moringa oleifera* without oil extraction (MOWE) as flocculants was investigated by Baharuddin et al. [200] using jar test method. Media pH, settling time and flocculants dosage were investigated. The highest flocculation efficiency of 93.7% was obtained in 150 minutes,

at a pH of 7 when 5000 mg/l of MOAE was used [200]. The conditions for maximum flocculation efficiencies at a given time for each microalgae species had also been recorded.

Table 5: A review of the outcome of flocculation experiments conducted on *C. vulgaris* and *N. oculata.*

Algal species	Microalgae dosage	Flocculant(s) used	Flocculant's dosage	Settling time min	pH	Recovery efficiency %	Ref.
C. vulgaris	0.5 g/l	NaOH	0.5 M	30	12	98	[187]
C. vulgaris	50 ml	Paenibacillus Sp. AM49	20 ml/l	10	11	83	[189]
C. vulgaris	1.5 g/l	Cationic Polymer [PDADMAC]	1 g/l	60	10	90	[194]
C. vulgaris	n/a	Chitosan & Alum	0.25 g/l & 2.5 g/l	10	n/a	92	[195]
N. oculata	50 ml	$FeCl_3$	0.4 g/l	180	8	93.8	[197]
N. oculata	100 ml	NaOH and Flopam	30 mM NaOH and 0.5 ppm Flopam	30	8.5	96	[201]
N. oculata	500 ml	MOAE	5000 mg/l	150	7	93.8	[200]
		MOWE	4000 mg/l	90	7	70.6	
N. oculata	500 ml	MOWE $Al_2(SO_4)_3.18H_2O$	2000 mg/l and 5 g of $Al_2(SO_4)_3.18H_2O$ in 100 ml distilled water	30	6	100	[200]

2.6 Overview of microalgae cell disruption techniques

Extracting lipids from microalgae is challenging due to the chemical intricacy and tough nature of the cell walls. Therefore, rupturing the cell wall, aimed at giving extraction solvent access to lipids has become a prerequisite of improving lipid yield [202]. Disrupting algae cell walls and extracting intracellular lipids are different biorefinery steps but can be run simultaneously. Most times, the efficiency of microalgae cell disruption process is quantified by the measure of lipid recovered [203]. Mechanical method has been adjudged the most efficient cell disruption method when compared with chemical and biological methods [199]. The techniques of microalgae cell wall disruption are shown in Figure 15, and Table 6 shows the factors affecting the outcome, advantages, and disadvantages of each mechanical cell disruption techniques.

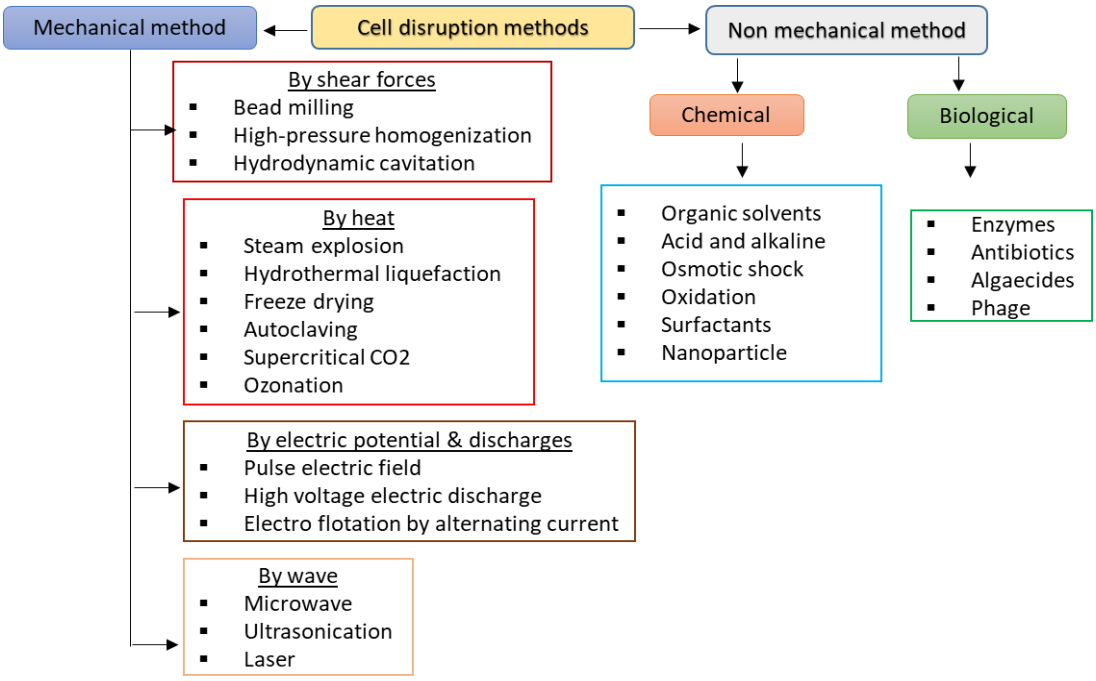


Figure 15: Various Techniques of microalgae cell wall disruption [38, 204]

Several mechanical (sonication, microwaves, bead mill, homogenization), chemical (acid, alkaline, surfactants, oxidation, osmotic shock) and biological (phage, enzymatic) methods of cell disruption have been widely studied singly or in combination of various methods [36-38]. Due to availability, simplicity, energy consumption and reproducibility, soniprep, osmotic shock and Tissue-lyser II would be considered in this study. Usually, results obtained by

utilising several mechanical disruption techniques vary in line with microalgae species, diverse cell wall matrices, configuration, and composition. Insight into the cell wall structure is important in the selection of an appropriate cell wall disruption technique.

Table 6: Independent variables, advantages and disadvantages of cell disruption techniques [47, 205, 206].

Method (mechanism)	Scalability potentials	Independent variables	Advantages	Disadvantages
Bead mills (Beads collide against cell)	High	Bead type; bead size; bead density; (residence time; agitation speed; biomass concentration)	Process is simple; low labour intensive; rapid extraction; effective; not cell dependent	High energy requirement; high maintenance cost; requires extra energy to remove beads
High pressure homogenisation (Cavitation & shear force)	High	Number of passes; biomass concentration; microalgae species; medium flow rate; (pressure; temperature; valve and orifice design)	Disruption cell walls can be at ambient temperature; good for the extraction neutral lipid; not cell dependent.	Energy intensive; not good for the extraction of proteins.
Hydrodynamic cavitation (Cavitation & shear)	High	inlet pressure, temperature and orifice to pipe diameter ratio	Comparatively low energy requirement; simple; can disrupt wet algae suspension; effective	High capital cost; limited cavitation area
Steam explosion (Hot steam)	High	Microalgae species; biomass concentration; (temperature; pressure)	Process is eco-friendly; effective; comparatively low energy requirement; economical	Microalgae species dependent.
Hydrothermal liquefaction (Water at a very high pressure & catalyst)	Low	Particle size; biomass feedstock; solvent density; (pressure; residence time; temperature)	Can disrupt wet biomass.	High energy requirement; requires expensive catalysts

Pulse electric field (Electroporation by electric field)	Low	Type of electrode material and gap; microalgae species; (pulse duration; electric field strength)	Eco-friendly; maintains the quality of extracts	Difficult to scale up; still premature.
Ultrasonication (Cavitation shear force)	Low	Microalgae species; cell concentration; (ultrasonic power, reaction temperature, extraction time)	Eco-friendly; less solvent requirement; easier cell penetration; efficient; comparatively economical	Difficult to scale up; energy intensive; species dependent
Microwaves (Heat from electromagnetic field)	High	Biomass concentration; (temperature; applied energy; exposure time)	Eco-friendly; less solvent requirement; easier cell penetration; efficient; comparatively economical	Energy intensive; high capital cost;

2.6.1 Microalgae cell wall composition, a hitch to lipid extraction

The cell wall of microalgae displays structural diversity and rigidity, complicating the development of efficient downstream processing for the recovery of intracellular bio-molecules [207]. Therefore, an understanding of microalgal cell wall, structure and composition is important for effective cell disruption and comparative studies. The fundamental components of microalgal cell wall consisted of a microfibrillar network within a gel-like protein matrix. In general, the chemical composition of cell wall included celluloses, proteins, glycoproteins, polysaccharides and lipids. However, microalgae cell walls are complex, their thickness and chemical composition change significantly in response to the growth environment and species. The recalcitrance, complexity, and diversity of microalgal cell wall is fundamentally species dependent. Figure 16 shows the cell wall matrices of the two species selected in this study. *N. oculata* cell walls are known to comprise of a thick and cellulose based multilayer polysaccharide and algaenan. This cellulose-based layer is connected to the cell membrane via the strut that constitute the inner layer of the cell wall. *C. vulgaris* cell walls have been identified to be very stable and comprise of fibrillar polysaccharides[207, 208]. Consequently, *N. oculata* will be resistant to mechanical rupture than *C. vulgaris*, resulting in lower disruption degree of the former.

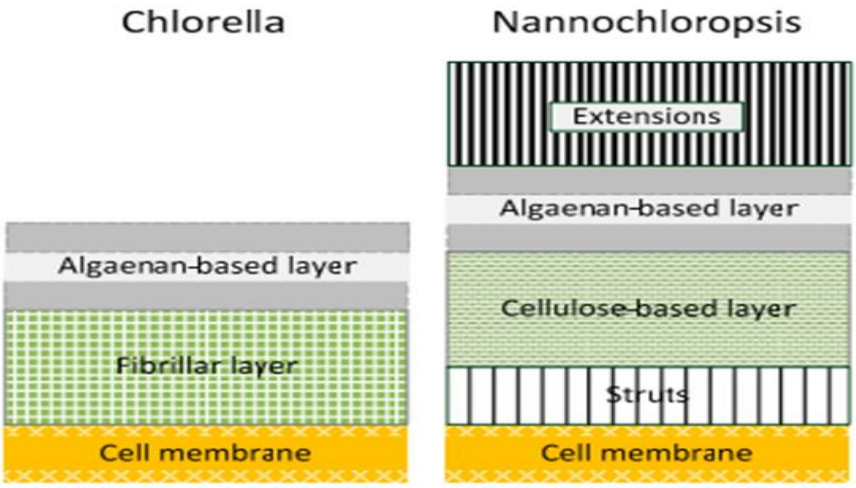


Figure 16: Cell walls of the microalgae selected in this study.

N. oculata shows more layers and extensions than *C. vulgaris*, indicating that the tensile strength of the former is higher. Thus, would have a lower disruption efficiency and by implication lower lipid yields [209, 210].

2.6.2 Cell wall disruption: review of various mechanical options

Bead mills is an efficient method of algae cell wall disruption, especially with high microalgae concentration of 100 – 200 g/l [46]. Notably, microalgae cell wall can be ruptured by a high-speed spinning of the culture vessel with some beads [42, 45, 211]. The rupture is due to agitation, friction, collision and grinding effects of the beads at high speed [212]. The disruption efficiency depends on microalgae species, algae concentration, residence time, beads and agitator speed [40]. Agitating with denser beads like zirconium is preferred in high viscous media whereas beads of less dense beads like glass is preferred in low viscous media [40, 213]. For better lipid yields, a lot of research have shown that optimising factors like; algae concentration and species, bead size, bead type, bead filling and operation time is necessary. Doucha et al.[40] studied *Chlorella* cell wall disruption using different mill types. Results showed that, increase in treatment time, agitator speed, number of cycles, feed rate and bead filling up to 85 % enhanced disruption efficiency whereas increase in dry cell weight and biomass flow rate marred the process. Using ECM-Pilot, Dyno-Mill with installed power of 7.5kW, ZrO_2 bead size of 0.5 mm and 70 % bead filling for 90 min., 98.5 % cell disruption was obtained while with glass beads 0.25-0.5 mm in diameter, a cell disruption efficiency of 95 % was returned.

Sonication process involves both cavitation and shear stress. During the process, the ultrasound produces microbubbles that build up pressure on the cell wall. These bubbles collapse to generate localized heat shock waves jet streams that disrupts the cell membrane by high shear force [214]. However, high ultrasonic frequencies produce low mechanical cavitation and high free radicals from water degradation. These radicals can attack and disrupt microalgae cell wall [215]. The process is quite simple, eco – friendly, cost effective and has high lipid yield efficiency. However, the process has got some pitfalls such as high energy demand, poor product quality due to damages during the process and difficult to be scaled up [204, 216, 217]. Ultrasonic assisted algae bio – refinery process can be influenced by microalgae type, ultrasonic power, reaction temperature, cell concentration and extraction time. Also, in applying high pressure homogenisation (HPH), cell walls are disrupted by the combined effects of turbulence, viscous and high-pressure shear, cavitation and force of cell walls on striking the surfaces of valve and impact ring. The key factor of HPH is the application of pressure on the suspended samples. Other factors are temperature, number of passes, design of valve and orifice, cell concentration, microalgae species and flow rate[218]. The influence of microalgae species on the pressure needed for 50 % cell walls rupture was reported [219].

The pulse electric field (PEF) involves the use of unipolar or bipolar electric pulses generated from electric field. These PEFs initiate the electroporation of microalgae cell membrane and allow for the permeability of extraction solvents into the cell. Consequently, the extraction solvents that infiltrated will enhance the solubilisation and mass transfer of intracellular lipids. Some electro technologies applied in microalgae lipid extraction have been studied [220]. The process is fast, non-thermal, very high efficiency and can curse irreversible electroporation, depending on the electric field strength. Although, the process needs initial investment costs, it has a low running cost and consumes less energy [204]. PEF assisted microalgae cell disruption and lipid extraction can be influenced by factors such as: Electric field strength, type of electrode material and gap, microalgae species and pulse duration. Electric field strength and pulse duration have shown to be the most influential factors on microalgae cell disruption and lipid extraction [221].

Finally, the use of microwave is a simple and very effective method of microalgae downstream process [48, 222]. Microwave generate heat due to the molecular interaction or friction forces from intra and inter molecular movement initiated by electromagnetic field [223]. The generated heat leads to the formation of water vapour, which rises the internal pressure of cells and leads to cell disruption. As a result, intracellular metabolites extraction becomes more efficient than the conventional process. However, this process has got some pros and cons. It is/has cost effective, eco-friendly, minimal processing time, low solvent consumption and better extraction yield. The removal of solid residue by centrifugation and poor microwave efficiency are the limitations of MAE [217]. Microwave assisted cell disruption and lipid recovery can be influenced by temperature, energy consumption, applied energy and time.

2.6.3 Use of a bead shaking Qiagen Tissue-lyser II disruption machine

The Tissue-lyser provides rapid and efficient disruption of biological samples, including animal and human tissues, plant tissues, bacteria, and yeast. Disruption and homogenization are achieved through the beating and grinding effect of beads on the sample material as they are shaken together in the grinding vessels. The Tissue-lyser both disrupts and homogenizes sample material in one simple and reliable step. The advantages include fast disruption of samples in minutes, effective, reproducible disruption and homogenization, compatibility with a wide range of sample specie/types, no cross-contamination of samples and disruption can be conducted with variable parameters and in ambient conditions. The Tissue-lyser is easily

programmed to provide variable speeds from 3 to 30 Hz (180–1800 oscillations/minute) and run times from 10 seconds to 99 minutes. Disrupting cell walls of bacteria and yeast having equal estimated tensile strength in the order of 9.5 MPa like microalgae shows it may be utilised for microalgae cell disruption [52, 53]. The process can be intensified by the adoption of dual cell disruption techniques. Figure 17 shows the picture and features of the Qiagen Tissue-lyser II used in this study.

Figure 17: Picture of Qiagen Tissue-lyser II (Qiagen Inc USA).
A: Closed Tissue-lyser II; B: Tissue-lyser clamps; C: Clamp handle wheel; D: Handle wheel locking pin; E: Clamped adapter; F: "Memory" display, with "PROG" and "SET" keys for storing disruption parameters; G: "Frequency (Hz)" display, with "–" and "+" keys for adjusting oscillation frequency; H: "Time min/sec" display, with "–" and "+" keys for adjusting disruption time; I: D "START" and "STOP" keys for starting and stopping operation of the Tissue-lyser II.

2.6.4 Use of Soniprep 150 plus

The Soniprep 150 plus is an improved digital version of the original Soniprep 150. It is a benchtop ultrasonic disintegrator enclosed in a purpose-built chamber to reduce residual sound from sonication. The rapid pressure produced by the sound waves travelling through the culture causes theses minutes gas filled bubbles to disintegrate implosively during rarefaction phase of the sound wave. This produces intense local shock waves in which the pressure may reach several thousand atmosphere and rapid microstreaming of the liquid around the point of collapse. It measures power in watts being delivered to the probe. There is a range of titanium

probes available to satisfy a wide variety of requirements. The Soniprep 150 plus used in this study and its various views are displayed in Figure 18.

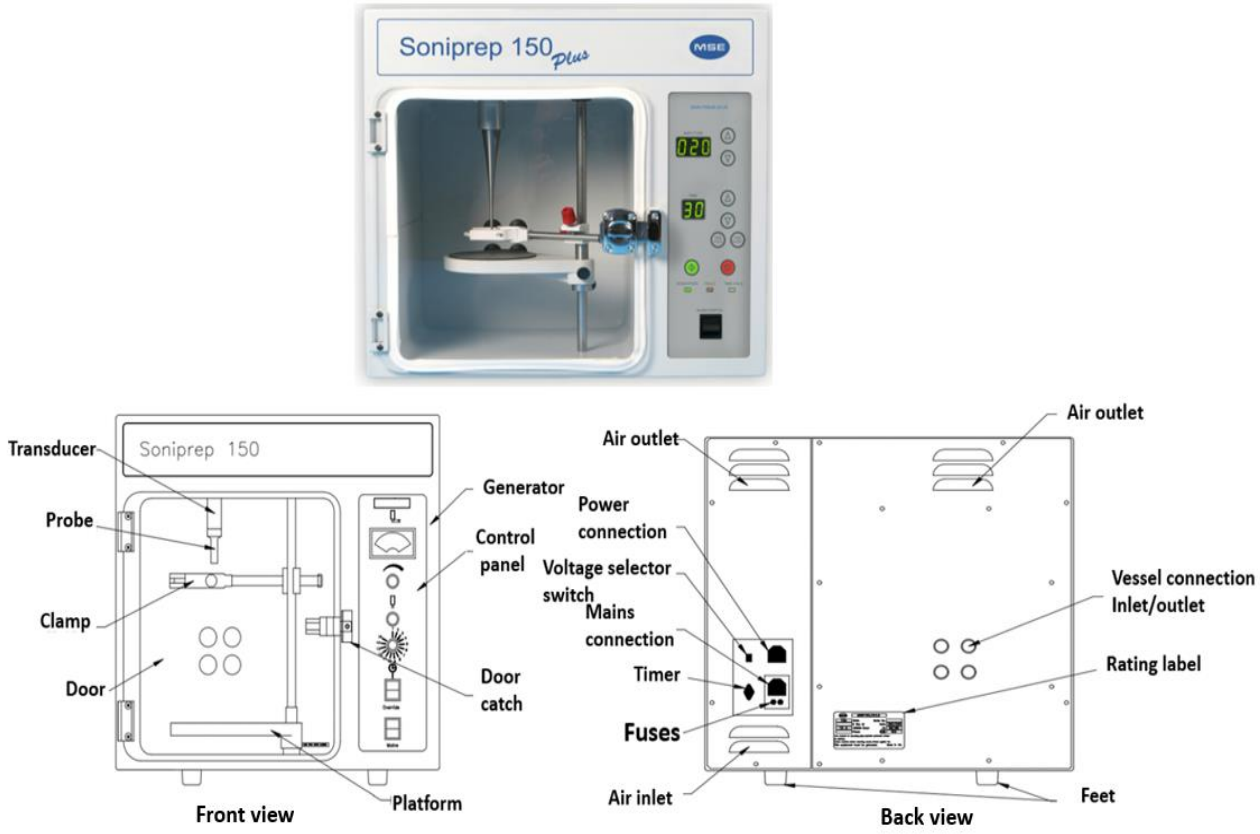


Figure 18: A picture and drawing of soniprep 150 plus utilised in this study

2.6.5 Review of energy requirement of various mechanical cell disruption processes

Most mechanical means of microalgae cells rupture are engrossed with high energy demands depending on the species and biomass concentration of cells disrupted, disruption device utilised and treatment parameters. The energy utilised during cell disruption is proportional to the degree of disruption and lipids extracted. In a research work done by lee et al., different microalgae species of concentration of 5 kg/m^3 were disrupted using bead mill (Bead diameter 0.1 mm, BioSpec Product Inc., USA) at a speed of 2800 rpm for 5 minutes. The power input of 0.84 kW was used for 5 minutes. The process consumed a specific energy of 504 MJ/kg of dry mass [42, 48]. The result showed lipid extraction efficiencies of 21.8 % for *Botryococcus sp* and 8-10 % for *Chlorella sp.* from the 100 ml suspension of each species, indicating that the process

is species dependent [48]. Safi et al. [49] disrupted 100 g/l of *Nannochloropsis gaditana* using 0.5 mm Zirconium oxide bead size. With a percentage bead filling of 65 % v/v, speed of 8 m/s and operation temperature of 35 ^{O}C, over 95 % cell disruption efficiency and 53 % protein extraction were obtained in 2.5 minutes, expending 0.43 kWh/kg of energy: equivalent to 1.548 MJ/kg. This energy is comparatively low, probably because of short bead milling time (2.5 min) and optimal bead size (0.5 mm). In disrupting microalgae cell wall by HPH, studies have shown that a range of values of energy of 0.25 kWh/kg to 147 kWh/kg is required [224]. Process optimisation is necessary to cut the energy requirement near 22 MJkg^{-1}, the estimated energy content of algae biomass. Yap et al. [225] studied the energy consumed for the disruption of *Nannochloropsis sp.* cell wall by HPH. It was observed that with in the feed concentration of 0.25 to 25 wt%, there was no observable difference in the cell rupture across the homogeniser, but power draw and energy consumption vary inversely to concentration. At a concentration above 25 wt% and pressure of 150 MPa, a very low energy consumption of 0.8 MJ/kg degraded the cell with an efficiency of 80 %. It can be concluded that the effect of microalgae feed concentration became very significant [225]. Table 7 shows the factors; disruption conditions and estimated energy demands of various mechanical techniques obtained from literature. Overall sustainability can be improved by employing energy-efficient cell disruption and lipid recovery processes to maximize the extraction of desired and unadulterated lipids from microalgae biomass. From the disruption techniques outlined, bead mills are efficient method of algae cell wall disruption, especially with high microalgae concentration of 100 – 200 g/l, but energy intensiveness, generation of high temperature and production of complex lysates are the key challenges, leading to the consideration of mild and low energy options such as osmotic shock and Tissue-lyser II.

Table 7: Summary of energy consumption of some cell disruption and lipid extraction experiments [42].

Species (scale, conc. phase)	Cell disruption (method); optimal operating conditions	Energy input MJ (Specific energy input MJ/kg dcw)	Disruption efficiency %	Extraction solvents (ratio, method)	Extraction efficiency % w/w (extract)	Ref.
***Botryococcus sp., Chlorella sp., Scenedesmus* (lab, 0.5 g/100 ml, wet)**	• (Bead mills); Bead diameter 0.1 mm, BioSpec Product Inc., USA; rated 0.84 kW; high speed of 2800 rpm for 5 min • (Microwave) microwaves at a temperature of 100^{o}C and 2450 MHz,700 W, 5 min.	0.252 (504) 2.1 (420)	n/a	Chloroform: methanol (1:1, Bligh & dyer)	21.8, 9,9 28.6,10,10 (lipid)	[48]
Chlorella sp. **(158 g/l dcw, wet)**	• (Bead mills); ECM-Pilot, Dyno-Mill; 7.5 kW; ZrO_2 beads 0.5 mm; feed rate of 62 kg/h; 70 % bead filling (v/v); speed of 10 m/s: 90 min.	40.5 (256.3)	98.5	n/a	n/a	[40]
Chlorella vulgaris **(16.2 g/185 ml, wet)**	• (Bead mills); Dyno-Mill; 7.5 kW; ZrO_2 beads 1 mm; 65 % bead filling v/v; speed of 9 m/s: 200 secs.	0.68 (42)	99.8	NaCl solution	36.6 (protein)	[44]
Scenedesmus spp. **(15 g/40 l, wet)**	• (PEF); treatment intensity of 30.6 kWh/m^3, 54 ^{o}C.	4.4 (293.3)	97	Chloroform: methanol (2:1, Bligh & dyer)	34 (lipid)	[226]
Nannochloropsis oculata **(4.3 g/5 ml (1 g dcw), wet)**	• (Microwaves); (1025C, Merry Chef Ltd., UK); 1025 W (IEC 705) and 2450 MHz; 5min	0.3075 (307.5)	92.81	Ethanol: Hexane (1:2, mixing & filtering) Water:	5.2 (lipid)	[227]

Scenedesmus obliquus (1:1 w/w, 84.8 %, wet)	(Microwaves); 1.2 kW; 2.45GHz; 30 min.	2.16 (n/a)	n/a	Hexane (1:2, separating funnel)	77 (lipid)	[228]
***Nannochloropsis salina* (20 g/l, wet)**	• (ultrasonication); CX-750, S&M, US; 0.75 kW. • (Hydrodynamic cavitation); orifice diameter 0.5 mm, 1.27 kW;	0.2 (10) 0.163	n/a	Water: Hexane (1:1, centrifugation)	27 99 (lipid)	[229]
***Nannochloropsis salina* (20 g/200 ml, 90 % w/v, wet)**	• (Steam explosion); steam pressure of 1.8 MPa; 0.1 s pressure for 5 min.	8.15	n/a	Chloroform: methanol (1:1, Bligh & Dyer)	93.4 (lipid)	[230]
Chlorella sp.(1.05 g, dry biomass)	• (Microwave) MAS-II microwave synthesis; sample exposed at 80^{o}C for 10 min.	0.00252(2.52)	n/a	Chloroform: methanol (1:1. Filter paper)	18.7 (lipid)	[231]

Note: where authors did not state the energy consumed in the process, it was calculated using; energy input (MJ) = power (MW) x time (s) = energy density (MJ/m^3) x volume (m^3) and specific energy (MJ/kg) = energy input/ dry weight mass. Only the conditions for maximum lipid yield are considered in this review. Energy requirement for solvent evaporation: E = volume of solvent(ml) X density of solvent(g/ml) X enthalpy increase(J/g)[230].

2.6.6 Process intensification approaches, aimed at enhancing lipid yields

Energy efficient and eco-friendly microalgae biofuel production processes can be improved by the introduction of process intensification (PI) via new process designs, combination of two or more existing techniques or modifying the existing techniques [232]. The PI concept is to reduce the number of steps in a process and size of unit/plant, enhance efficiency of energy and resources and the offshoot (glycerol) as a source of carbon [233, 234].

Park *et al.* [235] intensified lipid extraction process on *Chlorella vulgaris* by combining ultra-sonication and homogenization. It was recorded that the initial fatty acid content of the *C. vulgaris* was 360.2 mg/g cell. Though, the lipid extraction conditions need to be optimised, factors such as reaction time, cell concentration and lipid recovery solvent type showed to have influenced the process. At the end of cell disruption PI process, chloroform/methanol was used as recovery solvent. Result showed that 237.5 mg lipid/g cell of lipid was recovered in 1 h, which amounted to 66 % lipid recovery. Also, using hexane as recovery solvent yielded 152.0 mg lipid/g cell, which amounted to 42 % lipid recovery. These results are lower than that obtained by Wang D. et al. [219], which has been reported earlier. The reason could be that enzymes degrade cell wall/membrane better than homogenization, thus, improve mass transfer, lipid extraction and recovery processes.

In another research conducted by Qv *et al.* [236], lipid was extracted from *Dunaliella tertiolecta* through ultrasound – enhanced and microwave – assisted processes. Experiments were firstly conducted using solo process. The effect of combining the two processes was also investigated. It was observed that, at optimal conditions, 45.94 %, 57.02 % and 50.0 % of lipid recovery were obtained by ultra-sonication, microwaves and the combination of both techniques respectively. This shows that combining the two processes did not actually yield a desired result, as microwave extraction process outwits the ultrasonic extraction process and the combination of both processes under same optimal conditions. It is possible that, in microwave lipid extraction process, the microwave irradiation has a combined effect of heating and electroporation of the microalgae cell membrane, which makes the process more efficient than ultrasounds.

The effects of frequency on lipid recovery from *Scenedesmus dimorphus* and *Nannochloropsis oculata* had been studied by Wang *et al.* [237]. The aim was to evaluate the effectiveness of microalgae cell disruption using high frequency focused ultrasound (HFFU) and compare results with those obtained by low frequency non-focused ultrasound (LFNFU). The effect of

HFFU was observed to be significant, but the combination of both improves cell disruption efficiency, depending on the algae species treated.

Wang et al. [219] intensified the cell wall disruption and lipid recovery processes of *Neochloris oleoabundans* by combining high pressure homogenisation and enzymatic hydrolysis. From their results, a maximum rise in disruption degree of 35.2 % (i.e. from 40 % to 75.2 %) was attained when the homogenisation pressure rose from 40 MPa to 60 MPa. Using cellulase for enzymatic hydrolysis process rose the degree of disruption by 16.2 %. Combining both processes showed the efficacy of PI, as the degree of disruption rose to 95.4% with lipid recovery of 92.2%, which is higher than the use of individual processes. The outcome of the experiment shows that the combination of both processes yielded a better result. However, the estimation of the specific energy consumption was not calculated and the required parameters were not also provided [219]. Pan et. al [238] conducted an experiment on three microalgae species [*Nannochloropsis salina*, *Galdieria sulphuraria* and *Chlorella sorokiniana*], aimed at determining the influence of the combination of microwave and ionic liquid in microalgae lipid extraction process. At the same reaction conditions, *chlorella sorokiniana* had the best lipid yield of 230 mg/g of dry sample. And when compared to ultrasounds, conventional heating and solvent (Soxhlet extraction in hexane), 160 mg/g, 11 mg/g and 10 mg/g were respectively obtained. Results showed that ionic liquid [bmim][HSO4] can efficiently extract more lipids than known solvents. Therefore, the application of microwave and ionic liquid can improve lipid extraction process and increase lipid yield. However, as water content (water to algae mass ratio) increases, lipid yield decreases for all algae. This shows that water negatively affect the microalgae lipid extraction. The efficiency of the combination of bead beating using Tissue-lyser and osmotic shock on selected oleaginous microalgae species with thick cell walls will be tried in this study.

2.7 Lipid extraction, biodiesel production and fatty acids analysis

2.7.1 Lipids and its extraction mechanism

Lipids play vital role in microalgae cell membrane structure, energy storage and protection of microalgae species against osmotic stress [239]. Lipids can be classified as 1) 'neutral or nonpolar lipids', predominantly triacylglycerides (TAGs) and free fatty acids (FFAs), which are for energy purposes and environmental adaptation [240], located in the cell cytoplasm and 2) 'polar lipids' composed of phospholipids and glycolipids, located in the bilayer of the cell membrane[241]. The phospholipids are glycerol based, thus called glycerophospholipids. These glycerophospholipids possess α – structure and L - configuration [242], and the amphiphilic nature of the phospholipids is responsible for the formation of thin polar membrane that consists of two layers of lipid molecules [243]. These bilayers or lipid bilayers consist of two non-charged long fatty acid tails that are hydrophobic and charged phosphate heads that are hydrophilic [244]. Hence, extracting lipids from dry or wet microalgae has some challenges due to complexity of the intracellular biomolecules and structurally robust nature of microalgae cell walls [245], resulting in the development of several methods of lipids extraction. Figure 19 shows the various applied lipid extraction strategies.

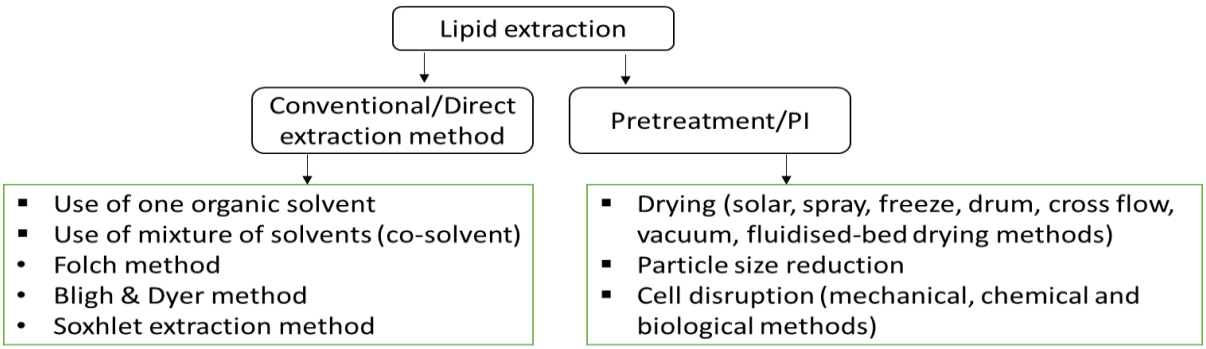


Figure 19: Various methods of microalgae lipid extraction.

It is a common practice to extract microalgae lipid using organic solvents, which are either used directly (traditional or conventional lipid extraction process) or after pre-treatment (drying, particle size reduction or cell disruption) of the microalgae cells. The essence of pre-treatment is to improve the lipid extraction efficiency from ruptured or weakened cell, by allowing the extraction organic solvent infiltrate into the cell at ease, associates with the neutral lipids to form a solvent-lipids complex. This complex flows out of the cell due to

concentration gradient and associates with the bulk solvent outside [246]. A fraction of neutral lipids that form neutral lipids-polar lipids complex, held by proteins via hydrogen bonds are dissociated by the addition of polar solvents. The polar solvent like methanol dissociates the lipid-protein association through the formation of hydrogen bonds with the polar lipids in the complex. Therefore, to completely extract all the neutral lipids needed for biodiesel production, the use of both non-polar and polar organic solvents is preferably, and the lipid extraction efficiency depends on the properties of the chosen solvents and the ratios in which they are applied. The use of polar and nonpolar solvents as co-solvent in microalgae lipid extraction process leads to the solubilisation of more lipids, enhance phase separation, and makes lipid recovery easy. The co-solvents blend with the intracellular neutral lipids and membrane associated polar lipids, through Van der Waals forces and hydrogen bonds respectively. The hydrogen bonds are strong enough to dissociate the lipid-protein complex in the membrane, forming organic solvent-lipid complex [247]. All the organic solvent-lipids complexes that are formed from intracellular and membrane lipids then diffuse across the membrane as shown in Figure 20, to the bulk solvents for lipid recovery.

The interaction between microalgae lipids and the extraction solvents can be assessed by some physical indicators; such as dipole moment (D), solubility parameter (S), polarity index (P) and partition coefficient (logP) [248]. The D is the measure of the polarity of a molecule. It is the net sum of the dipole moments of its polar bonds and depends on the electro negativities of combining elements. The S shows the relationship between the internal energy (addition of energies from dispersion bonds, polar bonds, and hydrogen bonds) of solvents and lipids [249]. The P is a measure of the ability of the solvent to interact with various polar test lipids [90, 250, 251]. P increases with solvent polarity; solvents of higher values of P will be more polar than those of lower values. Also, a solvent with high polarity index such as ethanol (5.2) and methanol (5.1) will tend to have high values of D (1.55) and (1.621) respectively and will therefore have low solubility with nonpolar neutral lipids. On the other hand, hexane with P and D of zero can readily dissolve neutral lipids, but hardly dissolves polar lipids. Partition coefficient is the ratio of the concentrations of complexes in a mixture of two immiscible phases at equilibrium [252]. The experiments done by [253] showed that high values of D and P and lower value of logP resulted in a higher lipid yield.

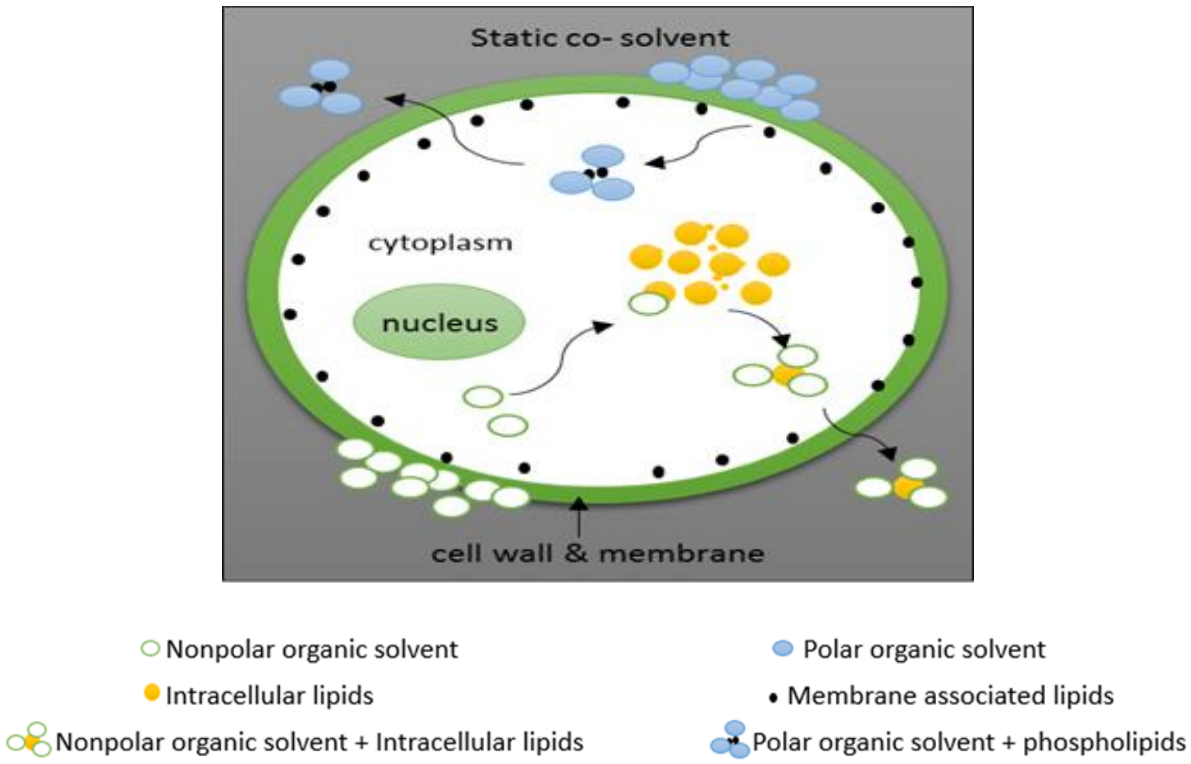
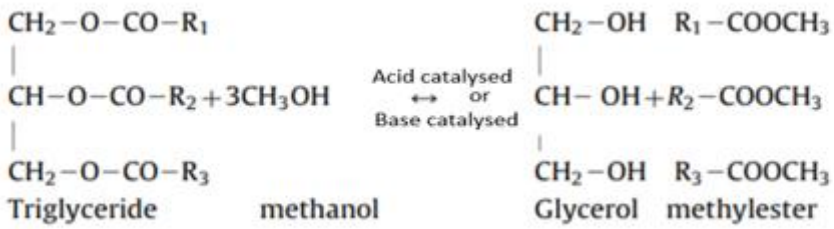


Figure 20: Lipid Extraction Mechanism. Nonpolar and polar solvents interact with intracellular lipids and phospholipids respectively, solubilise and diffuse out of the cell into the bulk solvent for lipids recovery.

More so, in choosing solvents for efficient lipid extraction and recovery, solubility, density and volatility are key factors that permit low-energy phase-separation-evaporation-based lipid recovery process [247]. Extracting lipids with solvent of relatively low boiling point, which is less than 100 ^{O}C, low density and high polarity or Hydropath Index is recommended. These properties will allow for easy evaporation, clear phase separation and good interaction of solvents and lipids respectively, hence enhance recovery efficiency [254, 255].

2.7.2 Biodiesel (FAME) production via transesterification

Biodiesel is produced through transesterification reaction; a reaction of microalgae lipid with excess short chain alcohols with a catalyst (Equation 2) [9]. The process can be influenced by reaction time, type of catalyst, reagent to oil ratio, reaction temperature and pressure [256]. The catalyst used can be acid, alkaline or enzyme. Alkaline (NaOH, KOH) is commonly used because of its high biodiesel conversion efficiency at low temperature and pressure [257]. However, alkaline catalysts are not suitable for high free fatty acid (FFA) feedstock i.e., up to 5 wt.% in algae oil due to the occurrence of saponification reaction. The algae lipid is pre-treated with an acid before the introduction of the base catalysed transesterification. Therefore, excess base will be required to performs two functions; 1) as a catalyst and 2) a fraction neutralises the acid. Acid catalysed transesterification of bio-oil has shown to be unfavourable due to corrosiveness and slow reaction rate (4000 times slower than alkaline process).



(2)

Enzymatic catalysed transesterification offers a biological route for biodiesel production. Enzymes operate under a mild reaction condition, increase biodiesel yield, and requires low energy consumption. Transesterification reaction can also be conducted at supercritical conditions [258]. In supercritical method, a single-phase mixture is formed. This is facilitated by the reduction of dielectric constant because of high temperature and pressure. This is quite different from the usual formation of two phases of oil and alcohol [75, 259, 260]. Although, purification step is not needed, the process is not economical due to high energy demands. At the end of the transesterification process, glycerol by-product is formed and can be reused as carbon source for microalgae cultivation. It can also be converted to biohydrogen through anaerobic fermentation. The microalgae biomass residue, which is rich in carbohydrate and proteins can be a potential source of biohydrogen, bioethanol and biogas through fermentation and anaerobic digestion of the substrate [261]. All these lead to the

maximization of energy production from microalgae. Notably, biodiesel, which is derived from triglycerides (TAGs) has attracted much attention recently as it has been proven to run in IC engines with little or no alterations [74, 75]. The engine performance and emission of biodiesel depend on its physicochemical properties which are functions of its fatty acids composition [262].

2.7.3 Biodiesel (FAME) property estimation

As has been stated earlier in this thesis, the sustainability of microalgae biodiesel businesses does not only depend on a low-cost production system, but on the selection of the species and corresponding nutrients required to produce commercially acceptable biodiesel of satisfactory physicochemical properties. Biodiesel properties depend on the percentage composition of fatty acids, chain length (molecular weight) and the existence of unsaturated carbon bonds, which can be significantly influenced by the algae species and carbon sources utilised during cultivation. The biodiesel properties estimated were viscosity, oxidative stability, calorific value, cetane number, iodine value, saponification value, degree of unsaturation, cold filter plugging point and long-chain saturation factor. These properties were empirically estimated utilising equations available in literature.

2.7.3.1 Saponification value (*SV*)

The saponification value indicates how much potassium hydroxide (KOH) that is needed to saponify 1g fat under a specific condition and use to measure the molecular weight or chain length of fatty acids. This information can be used to calculate how many acids (esters and free acids) are contained in a fat or oil [263, 264]. The greater the number of saponification, the more short- and medium-chain fatty acids the fat contains. It is estimated using.

$$SV = \sum[(560 * F) \div MW] \quad (3)$$

2.7.3.2 Iodine value (***IV***)

Iodine value (IV) is the amount of iodine (I_2) in mg that is consumed by 100 g of substrates in a chemical reaction [264]. It usually measures the addition of double bonds in fatty acids that are related to unsaturation. It is estimated using.

$$IV = \sum[(254 * F * D) \div MW] \quad (4)$$

2.7.3.3 Cetane number (***CN***)

The cetane number (CN) is an indicator of the ignitibility of biodiesel fuels. It provides information about the ignition delay, that is, the speed of self-ignition of biodiesel fuel when injected into hot air through the fuel injector [265]. It is estimated using.

$$CN = 46.3 + \frac{5458}{SV} - (0.225 * IV) \quad (5)$$

2.7.3.4 High Heating value (***HHV***)

The heating energy released during the combustion of the unit value of fuels is considered as the heating value of fuels and it is also known as calorific value or heat of combustion [266]. It is estimated using.

$$HHV = 49.43 - 0.041\ (SV) - 0.015\ (IV) \quad (6)$$

2.7.3.5 Long-chain saturation factor (***LCSF***)

LCSF is also an important property, which determines the behaviour of biodiesel at lower temperatures [267]. It is estimated using.

$$LCSF = (0.1 * C_{16:0}) + (0.5 * C_{18:0}) + (1 * C_{20:0}) + (1.5 * C_{22:0}) + (2 * C_{24:0}) \quad (7)$$

2.7.3.6 Cold flow plugging point (***CFPP***)

Cold filter plugging point (CFPP) is the lowest temperature, expressed in degrees Celsius (°C), at which a given volume of diesel type of fuel still passes through a standardized filtration device in a specified time when cooled under certain condition [268]. It is estimated using;

$$CFPP = (3.1417 * LCSF) - 16.477 \quad (8)$$

2.7.3.7 Oxidative stability (***OS***)

The presence of unsaturated carbon to carbon atoms, and double bond in fatty acid chains of biodiesel are responsible for their interaction with oxygen when being exposed to air. It has been well documented that the degree of unsaturation, location and number of double bonds severely affect the rate of autooxidation[269]. It is estimated using.

$$OS = \frac{117.9295}{[(wt\%\ C_{18:2}) + (wt\%\ C_{18:3})]} + 2.5905 \quad (9)$$

2.7.3.8 Viscosity (***V***)

Kinematic viscosity is an important fuel property of biodiesel that defined by its ability to flow, speed and quality of injected spray in the combustion chamber of the engine. It is estimated using.

$$\ln(v_i) = -12.503 + 2.496 * \ln(M_i) - 0.178 * N \quad (10)$$

2.7.3.9 Density (***d***)

Density is the mass per unit volume of biodiesel. It plays a crucial role in fuel injection and spray properties, which can directly influence other parameters such as cetane number, heating value and engine performance. It is estimated using.

$$d = 0.8463 + \frac{4.9}{MW} + 0.018 * D \quad (11)$$

$Where\ ; F = \%tage\ composition\ of\ each\ fatty\ acid\ obtained\ from\ table\ 8$

$MW = molecular\ weight\ of\ each\ fatty\ acid\ as\ shown\ in\ table\ 8$

$D = number\ of\ double\ bonds\ present\ in\ the\ fatty\ acid$ [270, 271]

Chapter three

3.0 Material and method

This chapter describes all the methods employed to achieve the objectives of this study. Two microalgae samples *C. vulgaris* and *N. oculata* were selected, cultivated mixotrophically and photoheterotrophically in closed systems (PBR and MCR) for biodiesel production. Growth parameters were determined and biomass from each species was harvested via flocculation and drying processes. Wet and dry cells of harvested biomass were disrupted utilising three cell disruption techniques, followed by lipid extraction. Alkaline based transesterification was carried out to convert the extracted lipid to biodiesel (FAME). Thereafter, the FA composition is determined, and biodiesel characterised using empirical.

3.1 Selection of microalgae species, growth media, modes, and method of cultivation

In this study, *C. vulgaris* and *N. oculata* (pictures shown in Figure 21) were selected and first cultivated in wastewater artificially formulated by blending distilled water with miracle gro. fertilizer. Also, formulated BG 11 growth media supplemented with various carbon sources was utilised. Species were cultured mixotrophically and photoheterotrophically in PBR and MCR using BG 11 growth media. Understandably, identifying and selecting microalgae species, growth media and cultivation mode that can boost biomass and lipid productivity is desirous.

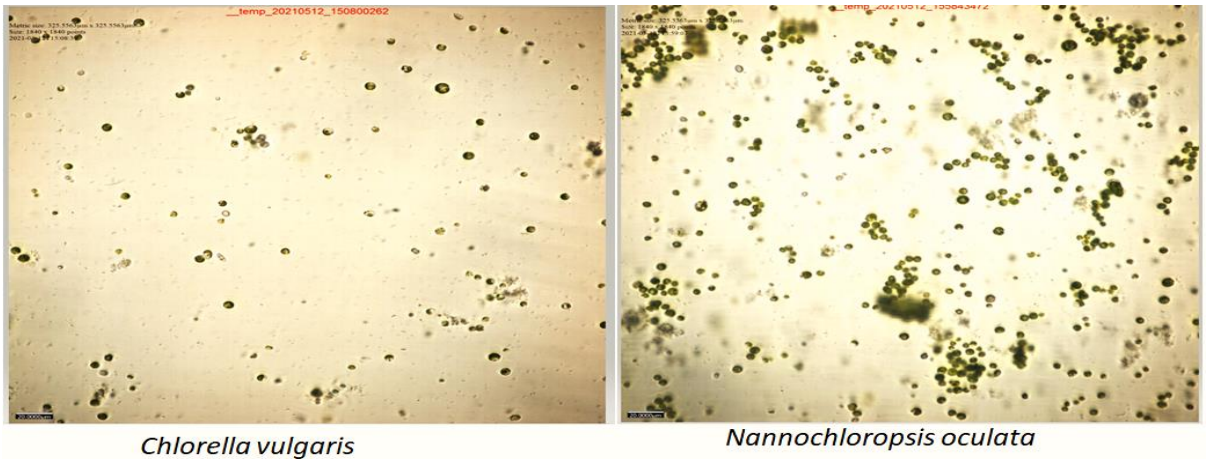


Figure 21: Cells of the selected species cultivated in PBR and viewed under an Alcona 3D microscope at (X50).

3.1.1 Microalgae selection

Both species (*C. vulgaris* and *N. oculata*) were selected because they have rigid cell walls and can survive in harsh growth conditions while accumulating high biomass and lipids fractions. Their features are perfect match for the purpose of this study. The use of organic carbon sources to cultivate microalgae can be problematic, since some microalgae species/strains that lack transporters or tricarboxylic acid (TCA) cycle enzymes are unable to utilize organic carbon as energy source, while only certain microalgal species can utilise organic carbon. Interestingly, both selected species have been reported to have utilised organic carbon sources in several literatures [272-274]. *C. vulgaris* is one of the well-known green eukaryotic microalgae, which can be cultured in fresh water and belongs *a genus Chlorella* [275]. *C. vulgaris* has an enzyme-digestible cell wall, spherical microscopic cell with 2–10 µm diameter, cell wall thickness from early growth to maturity range of 17 - 21 nm and is capable of accumulating significant volume of lipids (up to 58 % dwt) that can be used for the production of biodiesel [152, 276].

The cell rigidity of *C. vulgaris* is due to the presence of thin unilaminar layer which increases in thickness as the cell grows up to maturity, forming a microfibrillar which is composed of monosaccharide glucosamine layer [276, 277]. They tend to respond very effectively to photosynthetic process due to the presence of chlorophyll in the chloroplast. It reproduces asexually, and in optimal growth conditions, a mother cell can reproduce in quadruple by rupturing after maturity. The remains of the ruptured mother cell will be consumed by those newly formed daughter cells as food [152]. This accounts to the rapid growth of *C. vulgaris*.
N. oculata remains a class of a genus *Nannochloropsis*. It is a single cell microalga that can survive in both freshwater and marine. It has spherical or slightly ovoid cells with a diameter of 2–5 µm, the presence of *chlorophyll a* only with violaxanthin pigment and absence of chlorophyll b and cellular xanthophyll pigment. *N. oculata* has a very thick cell walls of average thickness 63 - 119 nm, made up of two constituents: the fibrillar and the amorphous [278, 279]. This species of microalgae has been one of the most promising species due to its high lipid productivity, high growth rate because of high photosynthetic efficiency, good environmental adaptability, contamination resistance, proper fatty acid composition, and ability of being genetically and technologically scalable. Table 8 provides a summary of the characteristics of the two microalgae species studied in this work.

Table 8: Selected microalgae cell description and lipid content [280]

Microalgae species	Description	Lipid content % dcw
C. vulgaris	• Freshwater • Eukaryotic [has linear DNA] • Unicellular [single-celled] • Cell wall thickness 17 – 21 nm • Thin cell wall • Presence of Enzyme – digestible cell wall • Very high growth rate • Spherical shape	28-58
N. oculata	• Marine and freshwater • Eukaryotic [has linear DNA] • Unicellular [single-celled] • Cell wall thickness 63 – 119 nm • Rigid and thick cell wall • Cell wall made of fibrillary and mucilaginous materials • High growth rate • Spherical or slightly oval shape	23-30

3.1.2 Growth media with supplemented carbon sources

Selected microalgae were firstly grown using artificial wastewater prepared with miracle gro. Fertilizer produced by Scotts Miracle-Gro Company UK. The chemical composition of the fertilizer is: 6 % Nitrogen, 3 % Phosphorus, 5 % Potassium, soluble Copper, Chelated by EDTA 0.002%, soluble Iron, Chelated by DTPA 0.03 %, soluble Manganese Chelated by 0.01 %, soluble Molybdenum 0.001 % and soluble Zinc, Chelated by EDTA 0.002 %. The growth medium was prepared by adding and blending 5 ml of miracle gro. with 1 L of distilled water. 10 ml of *C. vulgaris* and *N. oculata* were put into the medium in different vessels. The growth medium was kept at a pH of 8±0.5 using a pH regulator and temperature kept at 21±1^{O}C. Light source of intensity 4000 lux was utilized with light: dark ratio of 18:6. A mixture of CO_2 and air was passed, leading to the agitation of culture by aeration of 3 l/min using (Hailea Adjustable Air Pump ACO9610-10L/min). The cultivation was carried out simultaneously with other vessels containing culture supplemented with 1 g/l folic acid solution. Folic acid has been known to boast reproduction in both plants and animals. Growth was monitored via spectrophotmeter for 11 days.

Also, samples were grown in 250 ml Erlenmeyer flasks containing 150 ml a more formulated BG 11 medium with the following chemical composition (per liter of distilled water):0.1 g $Na_2MGEDTA$; 0.6 g ferric ammonium citrate; 0.6 g citric acid. $1H_2O$; 3.6 g $CaCl_2$. $2H_2O$; 7.5 g $MgSO_4$.$7H_2O$; 3.05 g K_2HPO_4; 2.86 g H_3BO_3; 1.81 $MnCl_2$. $4H_2O$; 0.222 g $ZnSO_4$. $7H_2O$; 0.079 g $CuSO_4.5H_2O$; 0.05 g $CoCl_2.6H_2O$; 0.391 g $NaMoO_4.2H_2O$. The pH of the medium was adjusted to 7.6 using 2 M of HCl and NaOH and autoclaved at 121 ^{o}C for 30 minutes. The growth medium in all the flasks was supplemented with 2.5 mM of urea which was used as nitrogen source and 0.15 mM each of the simple and complex organic carbon sources glucose, β-glucan, mannose, β-mannan and xylan at various batch of experiments. The BG 11 contained in 250 ml Erlenmeyer flasks was roofed with foam plug that allows exchange of gas in the cabinet at a temperature of 22±2^{o}C. Inoculants of *C. vulgaris* and *N. oculata* were dropped in 150 ml of the growth medium. Cultures were illuminated with light intensity of 6500±50 lux, which is equivalent to 120±1 $\mu molm^{-2}s^{-1}$ using cool-white lamps (Phillips) that were fixed on the cabinet cover. The light intensity on the flasks was measured using a LUX meter (MLX-3809 LED light meter) and set at a photoperiod of 18:6 light/dark using a time switch. In

another batch of growth experiments, β-glucan sourced from yeast and barley, β-mannan and galactomannan were utilised as carbon sources minding similar growth conditions.

3.1.3 Batch cultivation in photobioreactor (PBR)

A small pilot scale photobioreactor (PBR) designed and built by Northumbria University was used to culture the selected microalgae species in this research. Our choice of this bioreactor is due to availability, and it has satisfactorily been utilised for the cultivation of various algae species in the past. The bioreactor was designed to accommodate up to 38 conical flasks or cylindrical growth vessels of variable sizes. Figure 22 shows a model of the photobioreactor created using solid works. The photobioreactor was designed to incorporate the delivery of air and CO_2 for culture agitation. The PBR carries a pH control system that monitors and regulates pH in the process of CO_2 fixation by photosynthesis, as microalgae form OH^- radicals which increases the pH of the growth medium with time. The device regulates and brings back the culture pH to a set value by opening and closing the CO_2 canister valve, depending on the pH value. The lighting system is composed of twenty-four cool-white lamps (Phillips) that were fixed on the cabinet cover. The two selected algae species were grown using this PBR with the conditions outlined in section 3.1.3.

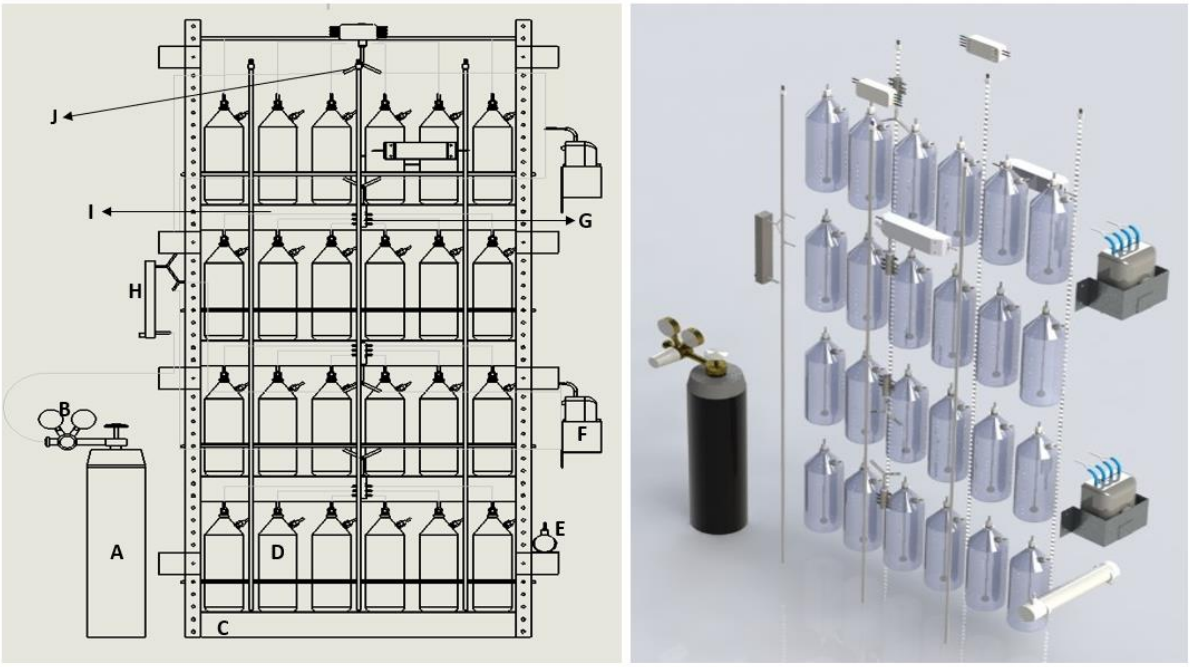


Figure 22: A model of PBR created in solid works (Developed by Northumbria University MSc students)

Bioreactor components; A: CO_2 canister; B: Gas regulator; C: Basement/frame; D: Growth vessels; E: Heater; F: Air pump; G: Manifold; H: Flow meter; I: Air hose tubes; J: Splitter

3.1.4 Batch culture in multi-cultivator (MRC)

A multi-cultivator (MC 1000-OD, Photon Systems Instruments) with eight separate cultivation tubes was used. This unit has the advantage of providing uniform light intensities up to 1000 $\mu molm^{-2}s^{-1}$ and precise temperature control for all tubes. A schematic picture of the whole setup while in use is shown in Figure 23. Prior to the cultivation experiment, the eight tubes, glass aeration tubes and BG 11 growth medium were autoclaved at 121 ^{o}C for 30 minutes, and stoppers and casing sterilised with 70 % v/v ethanol. 80 ml of the growth medium supplemented with 2.5 mM of urea used as nitrogen source and 0.15 mM each of each glycan as organic carbon sources (glucose, β-glucan, β-mannan and xylan). Inoculants of *C. vulgaris* and *N. oculata* were dropped in the 80 ml growth medium at different cultivation batches. Cultures were illuminated at set light intensity of 100 $\mu molm^{-2}s^{-1}$ at an ambient temperature of 20±2^{O}C throughout the experiment. Growth was at various times.

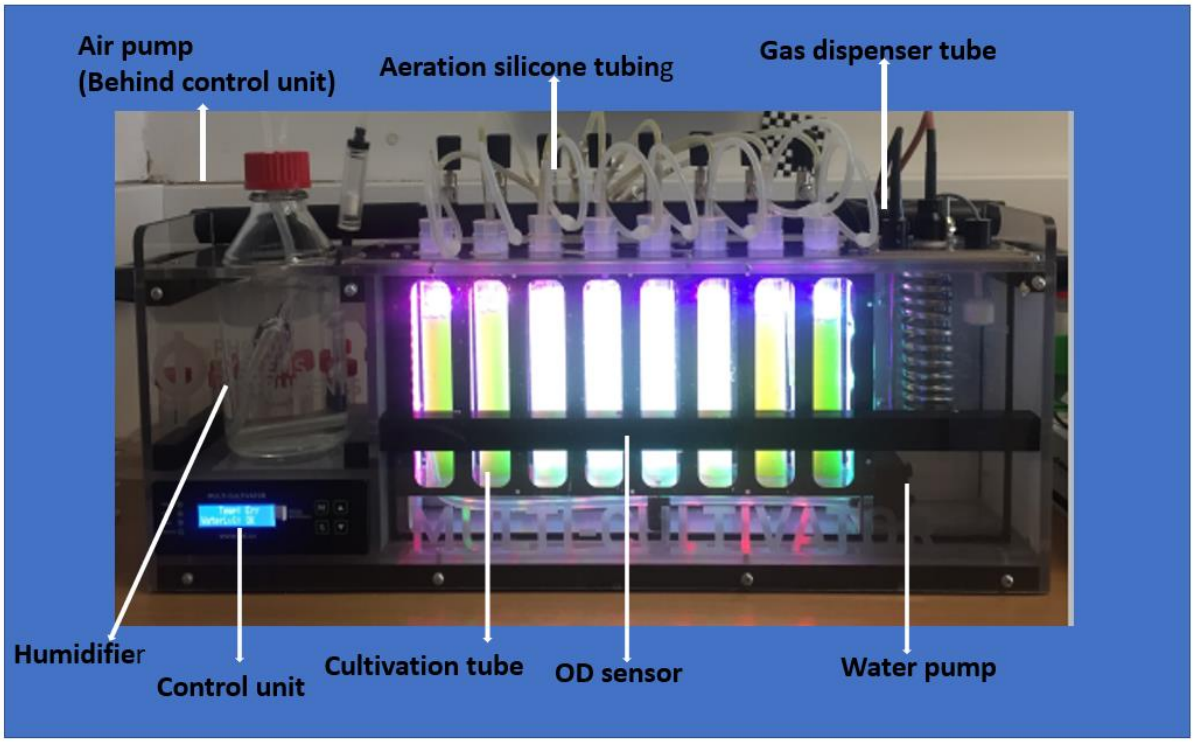


Figure 23: A picture of MCR used in this study

3.2 Analytical methods for growth parameter estimation

3.2.1 Measurement of optical density (OD)

The trend in daily growth is observed by a continuous culture colour change (Figure 24), indicating increase in biomass and its metabolites. Growth was monitored by daily measurements of optical density at 680 nm using a UV–vis spectrophotometer (Jenway 7305 UV/Visible spectrophotometer) from day 0 to day 8.

Figure 24: Growth trend as a function of daily colour change

3.2.2 Evaluation of growth parameters

Biomass concentration was determined by the approach adopted by [281]. Aliquots of known volume and measured OD were obtained, washed twice, dried and weighed daily to estimate the biomass concentration. The relationship between the biomass concentration and OD observed while growing algae in PBR and MCR were plotted in the standard curve as shown in Figures 25 and 26 for *C. vulgaris* and *N. oculata* respectively. The biomass concentration y in (g/l) in each microalgae species was related to the optical density (OD) by the Equations 12 and 13 for species cultured in PBR and Equations 14 and 15 for species cultured in MCR. Other parameters such as biomass productivity and specific growth of both systems (PBR and MCR) were estimated using the standard Equations 16 and 17 obtained from literature, where t is the cultivation time in days.

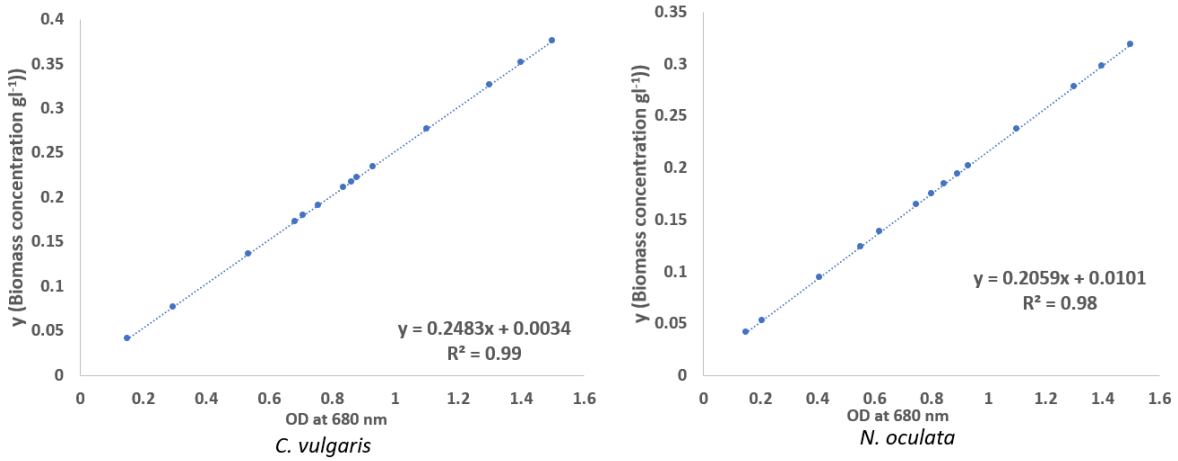


Figure 25: Calibration graph obtained from algae species grown in PBR

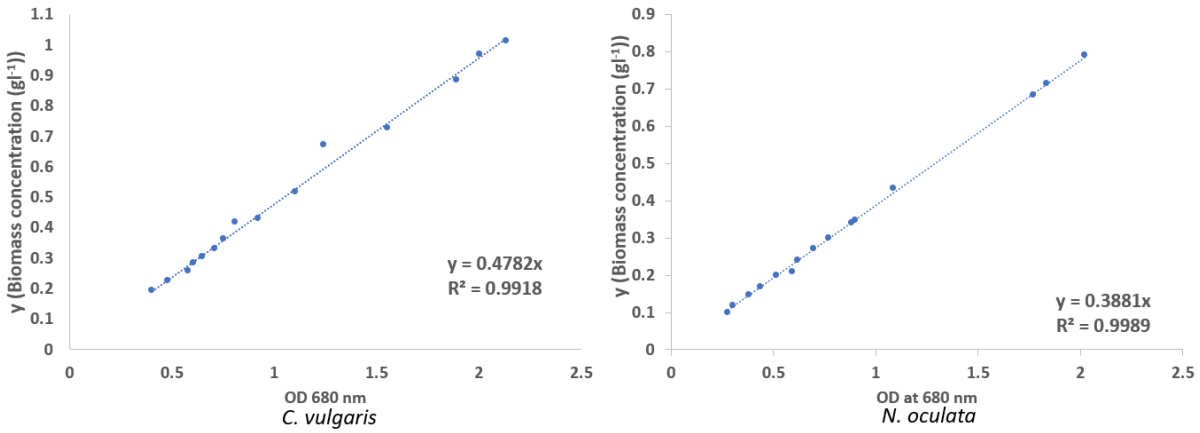


Figure 26: Calibration graph obtained from algae species grown in MCR

After 8 days of each batch of cultivation, harvested culture was subjected to gravitational settling for 24 hrs to allow for the sedimentation of biomass to reduce moisture content. To remove residual medium and other chemicals, distilled water was added, and the suspension was centrifuged at 4000 rpm for 15 minutes using (Sigma 3-18K centrifuge machine). Then biomass slurry was frozen overnight at -82 ^{o}C (Haier Biomedical Ultra Low Energy Freezer ULTDW-86L829BP) and dried using a dryer (Sentry 2.0 Virtis SP Scientific). The freeze-dried biomass was stored for cell disruption and lipid extraction processes.

$$Biomass\ concentration\ y\ (gl^{-1}) = 0.248\ OD_{(680\ nm)}\ for\ C.vulgaris \quad (12)$$

$$Biomass\ concentration\ y\ (gl^{-1}) = 0.206\ OD_{(680\ nm)}\ for\ for\ N.oculata \quad (13)$$

$$Biomass\ concentration\ y\ (gl^{-1}) = 0.4788\ OD_{(680\ nm)}\ for\ C.vulgaris \quad (14)$$

$$Biomass\ concentration\ y\ (gl^{-1}) = 0.3881\ OD_{(680\ nm)}\ for\ N.oculata \quad (15)$$

$$Biomass\ productivity\ \mathrm{P}\ (gl^{-1}d^{-1}) = \frac{y_{2-}\ y_1}{t_{2-}\ t_1} \quad (16)$$

$$Specific\ growth\ rate\ \mu\ (d^{-1}) = \frac{\ln\left(\frac{P_2}{P_1}\right)}{t_{2-}\ t_1} \quad (17)$$

3.2.3 Experimental design for optimisation and statistical analysis of algae growth under photoheterotrophic mode of cultivation

RSM was used for the optimisation of photoheterotrophic cultivation of each of the microalgae species selected. Two input parameters concentration of glycans and nitrogen coded as A and B were considered. To study the effects of these two input parameters on the optimal biomass yield of *C. vulgaris* and *N. oculata*, Central Composite Design (CCD) was created. The CCD involves three-level full factorial (3^k) design based on central level (0) between the minimum (-1) and maximum (+1) levels of the normalized values; (-1, 0, 1 as minimum, zero, maximum) distributed as shown in Tables 9. The effects of the concentration of the independent input variables such as; glycans (0.1, 0.15, 0.2 mM) and urea (1.5, 2.5, 3.5 mM) were studied and optimised.

Table 9: Three level input variables used for the design of experiment

Input variables	Codes	-1	0	1
Concentration of glycans (mM)	A	0.1	0.15	0.2
Concentration of nitrogen (mM)	B	1.5	2.5	3.5

From these different levels of variables and based on CCD in two replicates, 13 photoheterotrophic cultivation experiments were generated, through which experimental values of each independent variable was compared with the values obtained from statistical regression equations, which as well can be regarded as predicted value in this thesis. All the experiments as described above were repeated twice and results obtained were used in the optimisation regime. The interactions amongst various variables were displayed in contour graphs. Experimental results were fitted to a second-order polynomial equation described in Equation 18 by non-linear regression analysis.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_1\beta_2 AB + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 \quad (18)$$

Where Y is the predicted value. A and B are the coded independent cultivation variables. A two-dimensional surface plot for each response and a merged contour plot of both responses were generated to facilitate model interpretation. Models fitting and plots were performed using software package Minitab 18.1.0 version statistical software. Table 10 provides a full factor table for the two variables (concentrations of carbon and nitrogen sources) investigated in the cultivation process and shows a comparison of individual experimental readings and statistical regression models. The statistical regression models generated could allow for the prediction of outcome of the synergistic effects of various inputs with relatively small number of errors.

Table 10: Experimental and predicted optical densities under two input variables. A detailed RSM design matrix for optimising photoheterotrophic mode of cultivation using two input variables at three levels for 13 runs

Run	***C. vulgaris*** **cultivated in glucan**				***C. vulgaris*** **cultivated in mannan**				***N. oculata*** **cultivated in glucan**				***N. oculata*** **cultivated in mannan**			
	A	B	ODexp	ODpre	A	B	ODexp	ODpre	A	B	ODexp	ODpre	A	B	ODexp	ODpre
1	0.10	1.5	0.669	0.645377	0.10	1.5	0.693	0.699342	0.10	1.5	0.589	0.585308	0.10	1.5	0.629	0.615168
2	0.15	2.5	0.718	0.711348	0.15	2.5	0.856	0.849418	0.15	2.5	0.796	0.781600	0.15	2.5	0.887	0.847941
3	0.20	3.5	0.592	0.572960	0.20	3.5	0.661	0.667025	0.20	3.5	0.528	0.534246	0.20	3.5	0.578	0.581568
4	0.10	1.5	0.623	0.645377	0.10	1.5	0.715	0.699342	0.10	2.5	0.612	0.613792	0.10	1.5	0.608	0.615168
5	0.15	1.5	0.701	0.697917	0.10	2.5	0.785	0.806291	0.20	1.5	0.667	0.679846	0.20	1.5	0.589	0.592468
6	0.20	2.5	0.612	0.646667	0.20	1.5	0.695	0.685488	0.20	2.5	0.624	0.644408	0.20	2.5	0.711	0.746929
7	0.10	2.5	0.694	0.665985	0.10	2.5	0.821	0.806291	0.20	2.5	0.665	0.644408	0.10	2.5	0.756	0.759129
8	0.15	3.5	0.672	0.644818	0.15	3.5	0.669	0.672621	0.10	2.5	0.601	0.613792	0.15	3.5	0.668	0.677329
9	0.20	3.5	0.556	0.572960	0.15	3.5	0.685	0.672621	0.15	3.5	0.689	0.703400	0.20	3.5	0.598	0.581568
10	0.20	1.5	0.673	0.640412	0.10	3.5	0.624	0.612367	0.20	3.5	0.544	0.534246	0.10	3.5	0.564	0.583268
11	0.10	3.5	0.602	0.606631	0.10	3.5	0.598	0.612367	0.10	3.5	0.555	0.567554	0.10	3.5	0.599	0.583268
12	0.15	1.5	0.661	0.697917	0.15	1.5	0.710	0.725340	0.10	3.5	0.591	0.567554	0.15	1.5	0.669	0.698729
13	0.10	3.5	0.582	0.606631	0.20	1.5	0.682	0.685488	0.20	1.5	0.689	0.679846	0.20	1.5	0.619	0.592468

3.2.4 Evaluation of pigment concentration

Chlorophyll a and carotenoids concentrations were obtained after cultivation following the method reported in [282]. For each replica, 2 ml of samples were centrifuged at 4500 rpm for 20 min. The pellet was suspended in equal volume of methanol, incubated in the dark at 4 °C for 45 to 180 min to allow complete extraction. Cell fragments were then separated by centrifuging at 4500 rpm for 10 min, and the optical density of the supernatant containing the pigments dissolved in methanol was measured with a spectrophotometer at 480, 652, 665 and 750 nm (Jenway, England or Safas MC2, Monaco). Pigment concentrations in mg/l were determined using Equations 19 and 20 respectively.

$$C_{chla} = 16.5169(OD_{665} - OD_{750}) - 8.0912(OD_{652} - OD_{750}) \quad (19)$$

$$Carotenoid = 4(OD_{480} - OD_{750}) \quad (20)$$

3.2.5 Measurement of dissolved oxygen concentration

The concentration of dissolved oxygen C_{O2} was determined using calibrated Cole-Parmer traceable dissolved oxygen tester. The device was first calibrated and set in display mode expressed in mg/l. It is reliable, waterproof and measures dissolved oxygen within the range of 0.0 to 20.0 mg/l with accuracy of ±0.4 mg/l. At the end of algae growth regime, 20 ml of the culture was poured into a 50 ml beaker. The probe of the calibrated Cole-Parmer traceable dissolved oxygen tester is immersed and used to stir the solution before taking the desired measurement. Figure 27 shows a picture of Cole-Parmer traceable dissolved oxygen tester while taking measurement.

Figure 27: Picture of Cole-Parmer traceable dissolved oxygen tester while taking measurement.

3.2.6 Fluorescence microscopy

The pre-screening of neutral lipid was carried out using Nile Red (Sigma) stained according to White et al. [283]. Cells were suspended in Nile Red solution (0.25 mg/ml in acetone) for 10 min. Samples were agitated in a vortex mixer (SLS Basics vortex mixer) for one minute and viewed under a Leica DM500 microscope operated with **LASV4.3** software equipped with a digital camera linked to a desktop where images were acquired. Blue light was used for excitation in I3 filter with a drop of type F immersion liquid on the cover slip. A 450-490 nm excitation filter, 510 nm diachronic mirror and 515 nm barrier filter with 63 X objective lens were used to visualize the samples. Under these conditions, samples of *C. vulgaris* cells appeared red, indicating the presence of chlorophyll, whereas the cellular lipids fluoresced in yellow-gold [284]. At the end cell disruption by the various techniques in this research, aliquots obtained from disruption technique is viewed under the same microscope at a magnification of 40x to ascertain the extent of cell rupture, as shown in section 4.5.1 of this thesis.

3.3 Microalgae biomass recovery

3.3.1 Harvesting by flocculation

Flocculation is a convenient method for algal harvesting, utilising inorganic salts, organic and microbial flocculants. Determining optimal conditions can aid in material waste and energy reduction. Prior to flocculation experiment after 8 days of cultivation period, the microalgae growth process was terminated. Culture was subjected to gravitational settling for 24 hrs to allow for the sedimentation of biomass to reduce moisture content. The residual medium and other chemicals were removed, washed twice in distilled water to avoid interference of other chemicals during flocculation. Broths were further diluted and raised to equal initial biomass concentration of 0.5 g/l corresponding to optical densities of 2.432 and 2.014 for *N. oculata* and *C. vulgaris* when measured at 680 nm before the commencement of flocculation experiment.

Thereafter, a non-toxic, inexpensive, and readily available flocculant ferric chloride was utilised for the flocculation. A hydrated stock solution of ferric chloride was prepared by dissolving 2 g in distilled water and was made up to 50 ml. Droplets of HCl and/or NaOH were used to alter the pH until the desired value emerged. Three borosilicate 100 ml capacity measuring cylinder were simultaneously used at each batch of the experiment.

For the flocculation tests, 100 mL scooped suspension was dosed with a predetermined concentration of the flocculant using a freshly prepared stock solution, mixed in a magnetic stirrer to disperse the flocculant and adjust suspension to the desired pH via droplets of 2 M solutions of NaOH and HCl. Thereafter, the initial optical density of the treated suspension was measured and recorded, then suspension was poured into the measuring cylinder up to the 100 ml mark and flocs could settle. About 2 ml was taken from the 80 ml mark (20 ml from the top of the cylinder) for the measurement of optical density (680 nm) to investigate the degree of decolouration of suspension at 3 minutes interval. Experiments were repeated twice and were plotted as mean± standard deviation. The flocculation efficiency $F\ (\%)$ was determined following the equation 21;

$$F\ (\%) = \left(1 - \frac{OD_f}{OD_i}\right) x\ 100 \qquad (21)$$

Where OD_f and OD_i represent final and initial optical density respectively

3.3.2 Experimental design for optimisation and statistical analysis of flocculation process using RSM

Three input parameters coded as A, B and C are considered in the flocculation of processes. To study the effects of these three parameters on the flocculation efficiency of *C. vulgaris* and *N. oculata*, Central Composite Design (CCD) was created. The CCD involves three-level full factorial (3^k) design based on central level (0) between the minimum (-1) and maximum(+1) levels of the normalized values; (-1, 0, 1 as minimum, zero, maximum) distributed as shown in Tables 11, for all the independent input variables such as; flocculant dosage (0.2, 0.4, 0.6 g), flocculation time (3, 9, 15 minutes) and pH indicating acidic, neutral and alkaline culture (3, 7, 11).

Table 11: A three level input variables used for the design of flocculation experiment

Input variables	**Codes**	**-1**	**0**	**1**
Flocculant dosage (gl^{-1})	A	0.2	0.4	0.6
pH	B	3	7	11
Time (minutes)	C	3	9	15

From these different levels of variables and based on CCD in two replicates, 40 flocculation experiments were conducted, through which experimental value of each independent variable was determined and compared with that of predicted value. All the experiments as described above were repeated twice and results obtained were used in the optimisation regime. Thus, the interactions amongst various variables were displayed in contour graphs and each variable optimized value were examined. Experimental results were fitted to a second-order polynomial equation described in Equation 22 by non-linear regression analysis.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1\beta_2 AB + \beta_1\beta_3 AC + \beta_2\beta_3 BC + \beta_1\beta_1 A^2 + \beta_2\beta_2 B^2 + \beta_3\beta_3 C^2 \quad (22)$$

Where Y is the predicted value; A, B, C are the coded independent variables representing (flocculant dose, pH and time for each species being compared), β_{ij} are the interactive coefficients, β_{ii} are quadratic coefficients, β_i are linear coefficients, whereas β_0 is a constant. A three-dimensional surface plot for each response and a merged contour plot of both responses were generated to facilitate model interpretation. Models fitting and plots were performed using software package Minitab 18.1.0 version statistical software. Table 12 provides a full factor table for the CCD of three variables engaged in the flocculation experiment, with the experimental (actual) and predicted responses.

Table 12: Experimental and predicted flocculation efficiency under three flocculation input variables in three levels

Runs	Flocculant Dose (gl^{-1})	pH	Time (min)	F (%) actual (*C. vulgaris*)	F (%) predict (*C. vulgaris*)	F (%) actual (*N. oculata*)	F (%) predict (*N. oculata*)
1	0.4	11	15	80.7	79.5	92.2	93.9
2	0.2	3	3	62.1	59.2	59.1	58.4
3	0.6	3	15	89.4	89.5	88.1	84.7
4	0.6	7	3	58.1	56.9	59.7	57.9
5	0.6	7	9	80.5	79.4	74.0	77.0
6	0.2	7	9	81.1	78.6	72.4	75.2
7	0.2	7	15	87.6	86.0	80.8	81.8
8	0.4	7	15	89.0	88.4	85.3	83.1
9	0.4	11	3	51.9	51.4	64.8	66.5
10	0.4	11	9	79.4	74.2	88.4	86.6
11	0.6	3	3	60.5	60.0	61.9	61.0
12	0.4	3	15	92.8	94.7	88.7	84.4
13	0.4	7	3	52.2	58.4	60.0	57.4
14	0.4	7	9	85.0	82.2	73.5	76.7
15	0.2	11	3	49.4	45.3	62.5	65.1
16	0.2	11	9	62.2	69.4	90.2	85.4
17	0.6	11	15	75.0	76.7	94.0	93.7
18	0.4	3	3	60.9	62.7	56.1	60.3
19	0.6	3	9	80.9	83.5	74.7	79.3
20	0.2	3	9	86.7	85.1	77.2	77.0
21	0.2	3	15	91.6	93.5	82.3	82.8
22	0.2	7	3	50.7	53.6	60.4	55.7
23	0.4	3	9	90.5	87.5	79.6	78.7
24	0.6	7	15	85.8	84.5	82.0	83.2
25	0.6	11	3	53.4	51.0	64.7	66.8
26	0.6	11	9	71.0	72.7	88.7	86.7
27	0.2	11	15	79.6	75.8	92.0	92.8
28	0.6	11	15	75.1	76.7	94.2	93.7
29	0.4	3	9	90.0	87.5	78.0	78.7
30	0.2	11	3	49.0	45.3	61.3	65.1
31	0.4	11	3	52.0	51.4	65.1	66.5
32	0.6	11	9	71.0	72.7	89.4	86.7
33	0.2	7	9	81.0	78.6	73.0	75.2
34	0.4	7	3	52.0	58.4	61.0	57.4
35	0.6	3	3	61.0	60.0	62.0	61.0
36	0.2	11	9	62.0	69.4	90.0	85.4
37	0.6	7	15	86.1	84.5	81.0	83.2
38	0.4	11	15	80.0	79.5	92.0	93.9
39	0.2	3	15	91.0	93.5	82.1	82.8
40	0.6	7	3	58.0	56.9	59.0	57.9

3.4 Wet microalgae cell wall disruption and lipid extraction protocol

The disruption of tough cell wall of wet oleaginous microalgae can be energy consuming, especially using mechanical options. High temperature generated during this process can alter the chemical composition and structure of the bioproducts. The Tissue-lyser II (Qiagen Inc-USA) is easy to use, hardly generates heat and can operate mildly in ambient conditions but has been underutilised for microalgae cell disruption. In this study, algae cells would be disrupted using this mechanical means via Tissue-lyser II. Parameters such as operation or treatment time, biomass concentration and frequency would be varied. Cells to be disrupted are both species that were cultivated in this study in PBR and MCR utilising BG 11.

3.4.1 Cell disruption efficiency and lipid content as a function of Tissue-lyser operation time

Both species were concentrated into by centrifugation (4000 rpm for 20 minutes) using (Sigma 3-18K centrifuge machine) to obtain biomass slurries. Thereafter, 10 mg of the recovered pastes were resuspended in 3 ml of 10 % sodium chloride solution (forming a culture of biomass concentration of 10 mg/3ml, equivalent to 3.33 mg/ml or 3.33 g/l), observed to be equivalent to initial biomass concentration of 1.8×10^7 and 1.71×10^7 cells per ml of *C. vulgaris* and *N. oculata* respectively when read via automated cell counter as described in section 3.4.4 of this thesis. The diluted culture was vortexed for 2 minutes and incubated for 48 hrs at room temperature to engender osmotic shock. Thereafter, culture was poured into the Tissue-lyser grinding chamber for mechanical disruption. The usual Tissue-lyser grinding chamber (Eppendorf tube) was replaced with bigger polypropylene tubes of diameter and height 1.5 cm and 4.5 cm respectively in order to slightly upscale the volume of microalgae to be disrupted. Grinding chamber was 50 % filled with zirconium oxide beads of diameter 0.5 mm and density 5500 kgm^{-3} purchased from Sigma-Aldrich. The tubes were sealed and screw-capped to avoid loss of content during rigorous horizontal shaking of the Tissue-lyser which operated within 5 to 30 minutes inclusive at 5 minutes intervals (5, 10,15, 20, 25 and 30 minutes) with content treated at a frequency of 30 Hz. To estimate disruption efficiency at each batch, the biomass concentration (in cells ml^{-1}) was measured before and after disruption process using a flexible and easy to use automated cell counter (Invitrogen countess 3 purchased from Thermo Fisher). Thereafter, lipids were extracted and quantified. The experiments repeated twice and in room temperature.

3.4.2 Cell disruption efficiency and lipid content as a function of biomass concentration

After attaining stationary cultivation stage, biomass could sediment and the growth medium decanted. The harvested samples were washed and centrifuged to obtain working masses of algae slurries of 30 mg, 18 mg, and 6 mg. To each weighed slurry, 6 ml of 10 % sodium chloride solution was mixed to obtain algae slurry solution of biomass concentration of a) 30 mg/6 ml, equivalent to 5 mg/ml or 5 g/l; b)18 mg/6ml, equivalent to 3 mg/ml or 3 g/l ; and c) 6 mg/6ml, equivalent to 1 mg/ml or 1 g/l. The solutions were incubated for 48 hrs to initiate osmotic shock. Thereafter, the solutions were divided into three equal working volume of 2 ml each which was treated with shaking bead using the Tissue-lyser. Prior to cell disruption utilising the Tissue-lyser, the corresponding biomass concentrations of 5, 3 and 1 gl^{-1} were measured before and after disruption process (in cells ml^{-1}) using a flexible and easy to use automated cell counter. The equivalents of equivalent of 3.28 x 10^7, 2.2 x 10^7 and 1.47 x 10^7 cells/ml; and 2.11 x 10^7, 1.47 x 10^7 and 1.4 x 10^7 cells/ml were obtained for *C. vulgaris* and *N. oculata* respectively. Thereafter, cell disruption and lipid extraction experiments were conducted at these biomass concentrations and treatment/operation time of 10, 15 and 20 minutes. The cell disruption and lipid extraction protocols adopted in the previous section was utilised. The experiments were carried out twice and in room temperature.

3.4.3 Cell disruption efficiency and lipid content as a function of Tissue-lyser frequency

To further study the impact of bead shaking using the Tissue-lyser on microalgae cell wall disruption and lipid extraction, culture concentrated up to 2 gl^{-1} was treated at different frequencies (10, 20 and 30 Hz) for 20 minutes. The objective is to evaluate the efficiency of cell wall disruption and subsequently, lipid extraction after shaking the two selected microalgae species on Tissue-lyser at different frequencies. The same method of determining the disruption efficiency and lipid content previously utilised was adopted. From the slurry of each species diluted up to 12 mg/6ml, 2 ml was poured in the grinding chamber for mechanical disruption via bead shaking. The chambers were sealed and screw-capped to avoid loss of content during rigorous horizontal shaking of the Tissue-lyser which operated at frequencies at the time mentioned earlier. To estimate disruption efficiency at each batch, the biomass concentration (in cells ml^{-1}) was measured before and after disruption process

utilising the same flexible and easy to use automated cell counter (Invitrogen countess 3 purchased from Thermo Fisher). Thereafter, lipids were extracted and quantified. The experiments were carried out twice and in room temperature.

3.4.4 Determination of disruption efficiency using trypan blue

The cell disruption efficiency was determined using a rapid response automated cell counter. Cell counts were performed before and after treatment using countess 3 automated cell counter device. 10 µl of sample is mixed with 10 µl of trypan blue. 4 µl is pipetted into a cell counting chamber slide which is placed in an adapter and inserted into the counter machine. Each slide has two separate usable enclosed chambers. The cell counter only identifies and counts whole or intact cells whether live or dead and jettisons disrupted or damaged cells which would have been penetrated or reacted by the dye molecules. All the tests were performed twice and plotted as mean± standard deviation. The disruption rate D_r and disruption efficiency η_{eff} (%) are given in Equations 13 and 14.

$$D_r = \left(1 - \frac{C_i}{C_0}\right) \tag{22}$$

$where\ \ C_0\ and\ C_i\ are\ No.of\ intact\ cells\ \ before\ and\ after\ disruption$

$$\eta_{eff}(\%) = \ 100D_r \tag{23}$$

3.4.5 Protocol for simultaneous cell disruption and lipid extraction from dry microalgae cultured in PBR

Experiments conducted in this subsection were designed to simultaneously examine the effects of carbon sources and the impact of various disruption techniques on lipid yields. Sonication, osmotic shock and shaking bead Tissue-lyser were the cell disruption techniques adopted in line with the procedure suggested by [285]. Dried biomass (100 mg) mixed with hexane and ethanol in a ratio of 3:2 was homogenized using an ultrasonic disintegrator (soniprep 150 plus MSE UK) coupled with a probe of 9.5 mm diameter at 100 % amplitude (16 microns), giving 20 kHz ultrasonic wave frequency for 10 minutes. The samples were kept on ice during the sonication process to avoid overheating. Hexane and water were added to get the mixture to a ratio of (2:1:1), centrifuged 4000 rpm for 10 minutes. The process was repeated two more times on the residue and supernatant was transferred to centrifuge tube and evaporated using rotary evaporator set at a temperature of 40 ^{O}C for 30 minutes. In the osmotic shock protocol, 100 mg of dry biomass was treated with 20 ml of 10% NaCl solution

and vortexed for 2 min. The contents were further incubated for 48 h at room temperature, followed by extraction.

Shaking bead mills like Tissue-lyser is restricted to a 2 ml polypropylene micro tubes. In this study the micro tubes are replaced with bigger polypropylene tubes of diameter and height 1.5 cm and 4.5 cm respectively. The tubes are screw-capped to avoid loss of content during rigorous horizontal shaking of the Tissue-lyser. Similarly, 100 mg of dried biomass mixed with hexane and ethanol in a ratio of 3:2 in the tube 50 % filled with zirconium oxide beads of diameter 0.5 mm was homogenized using the Tissue-lyser II at a frequency of 20 Hz for 10 minutes. The treated sample was poured into a different centrifuge tube and the beads were washed thrice using hexane and water and pooled into the same tube, centrifuged 4000 rpm for 10 minutes. The supernatant was collected, and extraction solvent evaporated. Furthermore, the effect of combining osmotic shock and shaking beads was investigated, where culture subjected to osmotic shock is further homogenised in Tissue-lyser. Outcome from the two selected algae species was compared. In all, algae lipid was weighed using electronic scale and lipid concentration expressed in gram per algal dry weight before the transesterification process. The process was repeated twice, and the mean and standard deviation of both measurements were determined.

3.4.6 Protocol for simultaneous cell disruption and lipid extraction from dry microalgae cultured in MCR

Inoculants of *C. vulgaris* and *N. oculata* were cultured in MCR as described earlier. Cells containing growth medium without carbon source and those supplemented with glucose served as controls. After cultivation, biomass was harvested, dried, and subjected to lipid extraction and FAME production to further study the effects of these glycans on lipid accumulation of both algae species cultivated. Dried biomass (50 mg) mixed with hexane and ethanol in a ratio of 3:2 was homogenized using an ultrasonic disintegrator (soniprep 150 plus MSE UK) coupled with a probe of 9.5 mm diameter at 100 % amplitude (16 microns), giving 20 kHz ultrasonic wave frequency for 10 minutes. The samples were kept on ice during the sonication process to avoid overheating. Hexane and water were added to get the mixture to a ratio of (2:1:1), centrifuged 4000 rpm for 10 minutes. The process was repeated two more times on the residue and supernatant was transferred to centrifuge tube and evaporated using rotary evaporator set at a temperature of 40 ^{O}C for 30 minutes.

3.4.7 Determination lipid content

Lipid was extracted after disruption process. A mixture of hexane and ethanol (3:2) was added to the culture and centrifuged 4000 rpm for 10 minutes for lipid extraction. The supernatant was collected, and extraction solvent evaporated via vacuum device. In each cell disruption process, the corresponding lipid contents was determined using Equation 24.

$$Lipid\ content\ (by\ weight\ of\ biomass\ slurries\ \%) = \frac{mass\ of\ lipid\ extracted}{mass\ of\ microalgae\ biomassslurries}\ X\ 100 \qquad (24)$$

3.4.8 Scanning electron microscopy (SEM)

The SEM examination was carried out with the same procedure recorded in Mahmood et al. [286]. Briefly, the treated and untreated biomass were dried for overnight to remove the moisture content that can interfere with the analysis. The dried biomass was coated with thin carbon and silver paint to increase conductivity. SEM images were viewed and acquired using a **MIRA3 TESCAN** microscope. All SEM images were acquired at an acceleration voltage of 5 kV and view field of 30 µm.

3.4.9 Transesterification

A base-catalysed transesterification reaction was employed [286]. Briefly, extracted crude lipids were suspended in 2 ml of hexane. Then 1 ml of freshly prepared 2 M methanolic potassium hydroxide was added. The transesterification reaction was performed at a temperature of 45 ^{O}C and a stirring rate of 50 rpm for 40 minutes using a shaking incubator (New Brunswick scientific incubator shaker Innova 44R). Then 1 ml distilled water was added to dissolve the unreacted methanolic potassium hydroxide. The mixture is centrifuge 4000 rpm for 10 minutes and allowed for four hours to promote phase separation. The upper layer which contains a mixture of lower density hexane and fatty acid methyl ester (FAME) was collected using a pipette and hexane was evaporated using a rotary evaporator. The FAME can be expressed in percentage of lipids extracted or biomass produced. In terms of biomass, FAME percentage yield is the ratio of mass of FAME and mass of biomass, whereas in terms lipids extracted, it is the ratio of mass of FAME and corresponding mass of lipids extracted.

3.4.10 GC-MS

The produced FAME is suspended in 1 ml of hexane for gas chromatography – mass spectrometric analysis. The analysis by gas chromatography and detection by mass spectrometry (Agilent- model 6890N gas chromatograph and model 5975model mass spectrometer, USA) were performed in an HP-5MS column (30 m length x 0.25 mm I.D x 0.25 µm stationary phase) with split injection of 10:1 and injection volume of 1µL. The initial temperature of the oven was 20°C, which was increased until 300°C at a temperature gradient of 10°C min^{-1}. Helium was used as the carrier gas with flow of 1 mL min^{-1}. Helium was used as the carrier gas with flow of 1 mL min^{-1}.

3.4.11 Biodiesel property evaluation using fatty acid composition

In this research, vital properties of biodiesel were estimated using empirical formulas obtained from [270, 271]. Biodiesel properties depend completely on the percentage composition of fatty acids, chain length (molecular weight) and the existence of unsaturated carbon bonds. It is vital for biodiesel to meet the criteria stipulated by international standards like ASTM 6751, EN 14214 and GB 25199. To access the standard and quality of FAME produced per microalgae species per carbon sources, fuel properties such as Saponification value (SV), Iodine value (IV), Cetane number (CN), High Heating Value (HHV), Long chain saturation factor (LCSF), Cold Filter Plugging Point (CFPP), Oxidative Stability (OS) Viscosity (v) and density (d) were determined from the equations outlined in section 2.7.3 of this thesis.

Chapter four

4.0 Results and discussions

4.1 Microalgae cultivation using the PBR

4.1.1 Experimental investigation of the effects of miracle gro. fertiliser and folic acid on microalgae growth pattern

The procedure of the experiment on the use of miracle gro., CO_2 and folic acid was described in section 3.1.2 of this thesis. Firstly, *C. vulgaris* and *N. oculata* were cultivated in wastewater formed by blending known volume of miracle gro. fertilizer with distilled water, aerated with a mixture of air and CO_2. Secondly, in other vessels the species were cultured in wastewater supplemented with folic acid and aerated with a mixture of air and CO_2. The aim was to observe the effect of utilising miracle gro. and folic acid as nitrogen and carbon sources in microalgae growth regime. After eleven cultivation days, growth was observed in the culture of wastewater and CO_2 as shown in Figure 28. Maximum optical densities (ODs) of 0.45 and 0.34, corresponding to biomass concentration of 0.0927 and 0.08432 gl^{-1} (the unit gram per litre gl^{-1} can also be expressed as g/l) were obtained from *N. oculata* and *C. vulgaris* respectively in the 11th cultivation day. The low biomass concentration observed shows that the growth medium is probably deficient of adequate proportion of necessary nutrients in the artificially formulated wastewater utilised as growth medium.

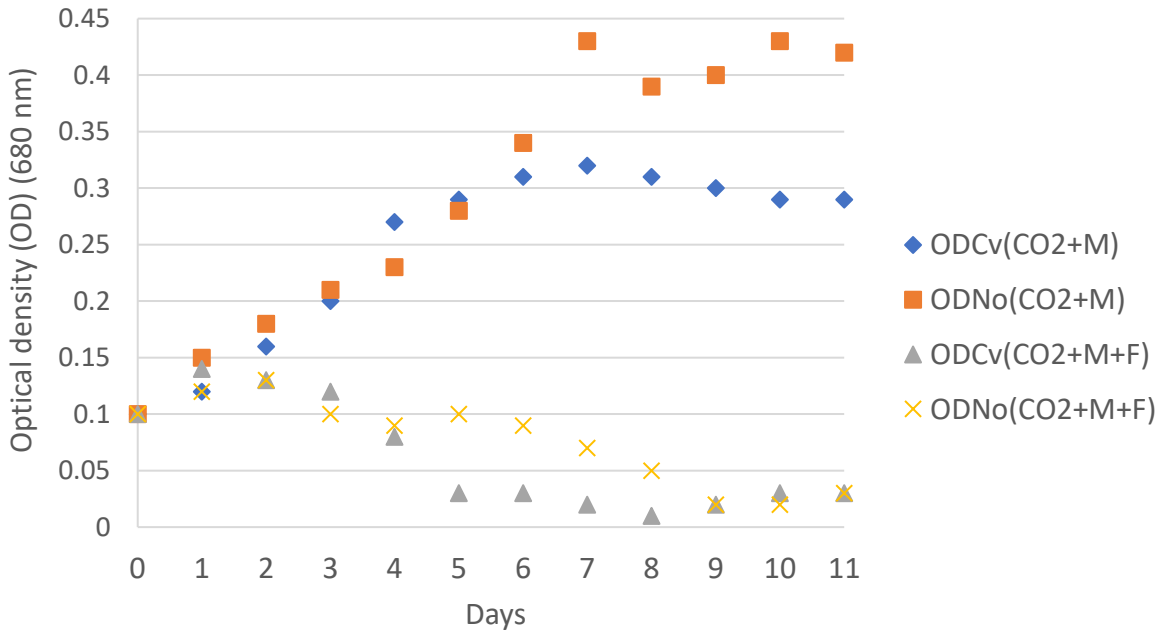


Figure 28: Growth pattern of *C. vulgaris* (Cv) and *N. oculata* (No) cultivated in wastewater (Distilled water + miracle gro. (M)+ folic acid solution (F))

- OD Cv (CO_2 + M): Optical densities of ***C. vulgaris*** when cultivated with fertilizer contaminated water and CO_2
- OD No (CO_2 + M): Optical densities of ***N. oculata*** when cultivated with fertilizer contaminated water and CO_2
- OD Cv (CO_2 + M + F): Optical densities of ***C. vulgaris*** when cultivated with fertilizer contaminated water, folic acid, and CO_2
- OD No (CO_2 + M + F): Optical densities of ***N. oculata*** when cultivated with fertilizer contaminated water, folic acid, and CO_2

Conversely, no growth of both species was obtained in the medium containing miracle gro., CO_2 and folic acid, indicating that folic acid inhibits growth of these algae species. The result suggests that folic acid may have dissociated in algae culture solution, but during growth process, there seems to be a resultant build-up of toxic substances that are responsible for numerous adverse side effects, leading to the death of algae samples in the culture.

4.1.2 Experimental investigation of the effects of monosaccharides (glucose and mannose) concentration of on microalgae growth pattern

C. vulgaris was cultivated under mixotrophic mode in a growth medium BG 11 supplemented with urea as a nitrogen source and simple sugars (glucose and mannose) as carbon sources using PBR. Urea concentration of 2.5mM and various concentrations of glucose and mannose [0, 0.05, 0.1, 0.15, 0.2mM] were used. The aim was to identify the concentration with which maximum growth can be obtained under specified growth conditions. The experiments were carried out in the conditions outlined in section 3.1.2. Results obtained showed growth in all the concentrations of both simple sugars utilized as carbon sources as well as the control medium (Figures 29 and 30). Furthermore, all the media supplemented with glucose and mannose outgrown those of control, indicating that the addition of simple sugar enhanced growth. It could be seen that at sugar concentration of 0.15 mM, growth is maximum. The OD readings on the 11^{th} day of cultivation obtained were 2.24 and 2.11, corresponding to biomass concentration of 0.555 and 0.434 gl^{-1} from *C. vulgaris* and *N. oculata* respectively.

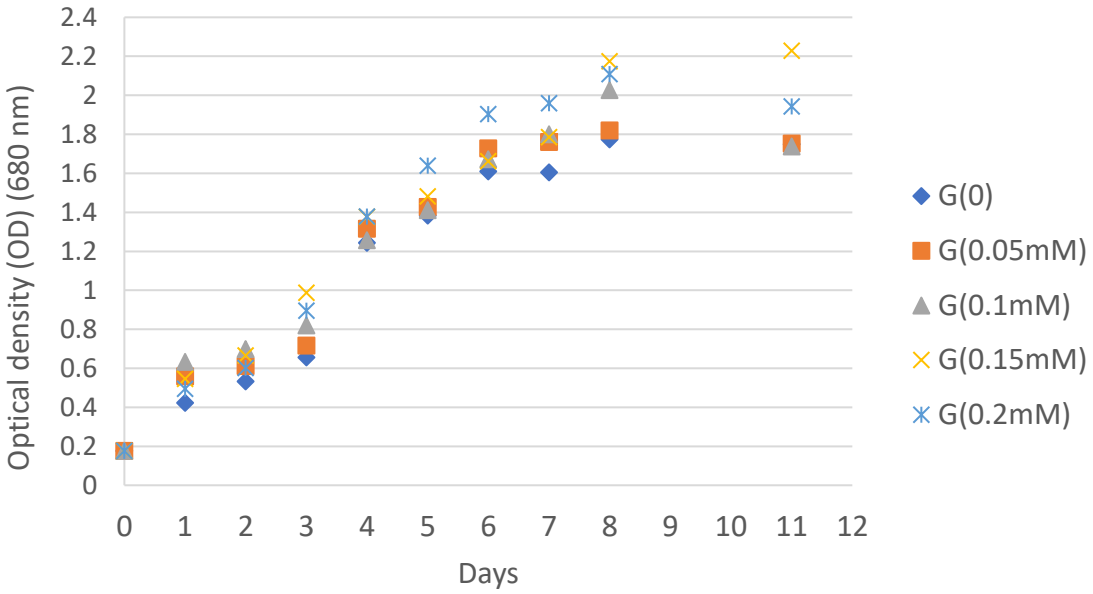


Figure 29: Growth pattern of *C. vulgaris* cultivated in a medium supplemented with glucose (G) of different concentration.

C. vulgaris grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and glucose of different concentrations. Maximum growth was observed in the culture supplemented with glucose concentration of 0.15 mM.

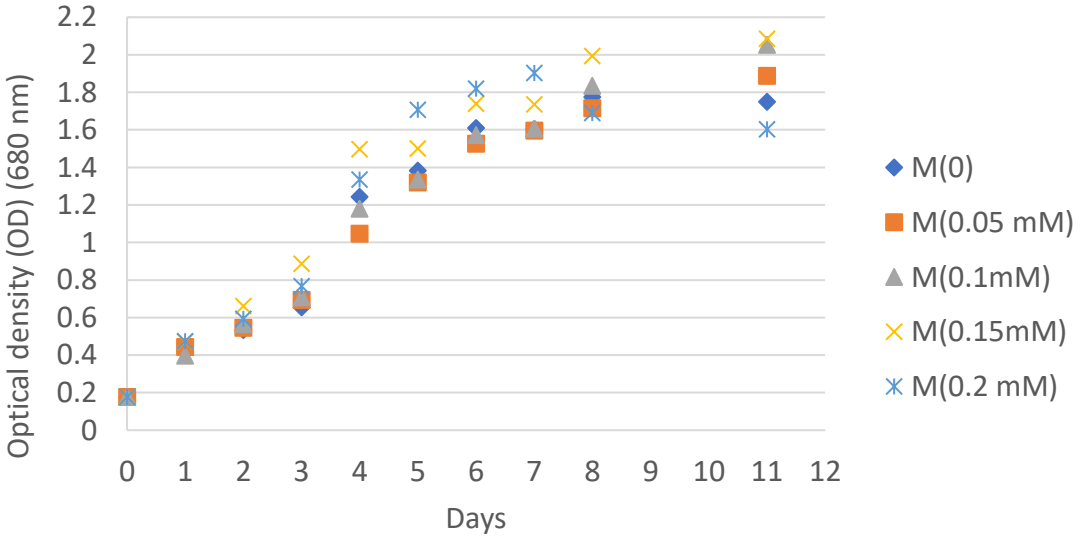


Figure 30: Growth pattern of *C. vulgaris* cultivated in a medium supplemented with mannose (M) of different concentrations.

C. vulgaris grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and mannose of different concentrations. Optimal growth was observed in the culture of mannose concentration of 0.15 mM.

4.1.3 Experimental investigation of the effects of complex glycans (β-glucan and β-mannan) concentration of on microalgae growth pattern

Similar experiments were conducted using glycans such as β-glucan and β-mannan under the same growth conditions and glucose utilised as control. The aim is to likewise ascertain the glycan concentration required for maximum algae growth. The choice of glucose as a positive control is because it is a simple hexose monosaccharide considered as a raw material for photosynthesis and can trigger anaerobic glycolysis process. Results obtained showed maximum ODs, biomass concentration and biomass productivity were found at the same concentration of 0.15mM for both β-glucan and β-mannan at the 7^{th} day of cultivation. Looking at the obtained results displayed in Figures 31 and 32, it can be deducedthat the medium supplemented with glucose produced the highest while that supplemented with β-glucan has the least growth parameters. Biomass concentration of 0.422, 0.273 and 0.372 gl^{-1}, and productivities of 0.068.7, 0.041.6 and 0.058.7 $gl^{-1}d^{-1}$ (which can also be expressed in g/l/d) were observed on media supplemented with glucose, β-glucan and β-mannan respectively. The result also indicates that *C. vulgaris* may have contained some enzymes (possibly glucanase and mannanase) that can hydrolyze β-glucan and β-mannan to simple sugars for algae absorption. The low biomass production observed with β-glucan and β-mannan supplemented media may be because of their complex structures, in which the need to be hydrolyzed into simple structures before entering the glycolysis pathway became necessary. These results as shown in Figures 31 and 32 agree with our previous finding that glucose is an efficient carbon source for high microalgae biomass production and in tandem with the results previously reported in literature [287-290].

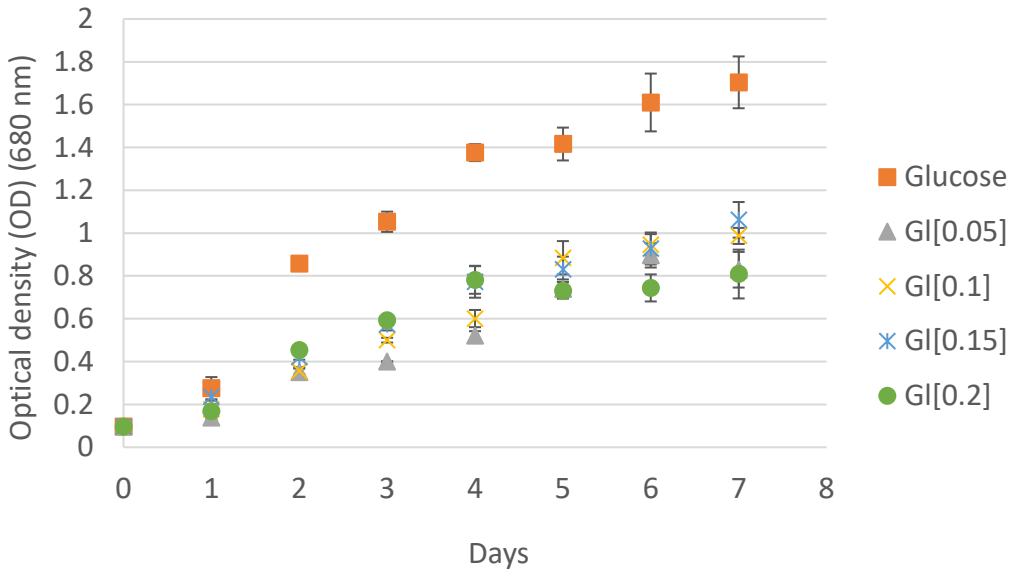


Figure 31: Growth pattern of *C. vulgaris* cultivated in a growth medium supplemented with β-glucan (Gl) of different concentrations.

C. vulgaris grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and β-glucan of different concentrations [0.05 to 0.2 mM]. Maximum growth was observed in the culture of glucan concentration of 0.15 mM.

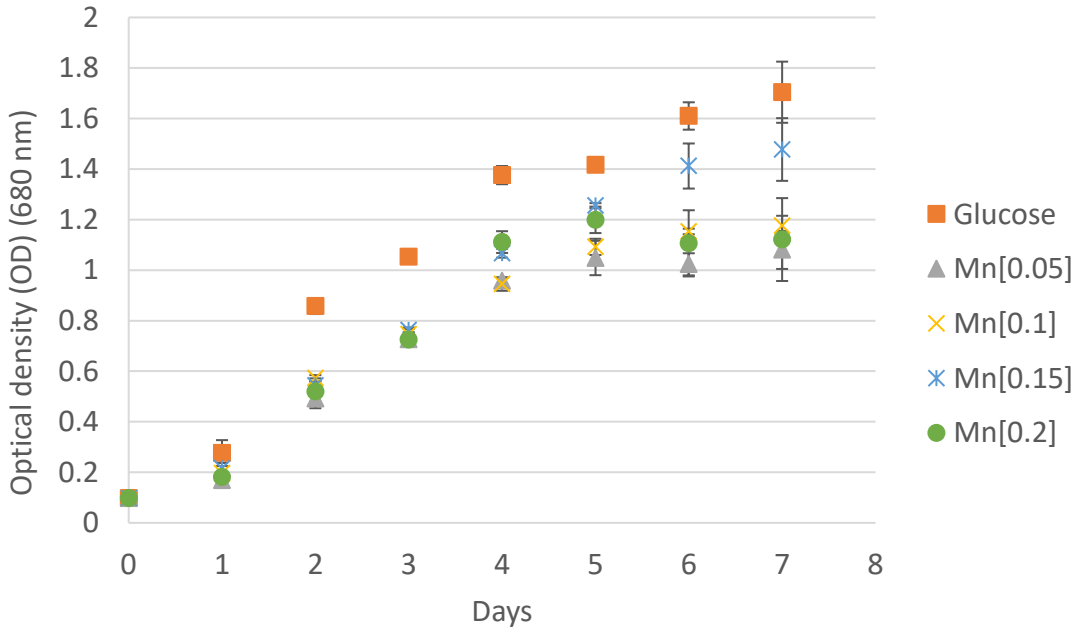


Figure 32: Growth pattern of *C. vulgaris* cultivated in a growth medium supplemented with β-mannan (Mn) of different concentrations.

C. vulgaris grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and mannan of different concentrations. Maximum growth was observed in the culture of mannan concentration of 0.15 mM.

4.1.4 Experiment to compare the effects of various organic carbon sources and modes of cultivation on growth parameters of two algae species cultivated under the same conditions

The two selected algae species were simultaneously cultivated utilising 0.15mM of glucose, β-glucan, mannose and β-mannan as carbon sources and 2.5 mM urea as nitrogen source under mixotrophic and photoheterotrophic conditions. Figure 24 displayed earlier shows the significant colour changes on days zero and eight of cultivation of both species. The utilisation of these organic carbon sources enhanced microalgae biomass productivities. The differences in growth pattern observed in the measurement of optical densities are due to unique carbon sources utilised, as every other growth condition remained unaltered when cultivated mixotrophically and photoheterotrophically. The effects of different carbon sources on the growth of *C. vulgaris* and *N. oculata* cultivated in mixotrophic and photoheterotrophic condition estimated on the 8th day of cultivation are shown in Figures 33, 34 and 35 respectively. The ability of microalgae to grow under any mode of cultivation depends mainly on microalgal species, type and concentration of organic carbon source, and environmental factors. The results obtained indicated that growth was slightly delayed on utilising glycans as carbon sources when juxtapose with simple sugars. Although, the mechanism of glycans hydrolysis by green algae is still not clear, but the growth delay observed could be because of the delays incurred during enzymatic hydrolysis of these glycans to utilisable simple sugar.

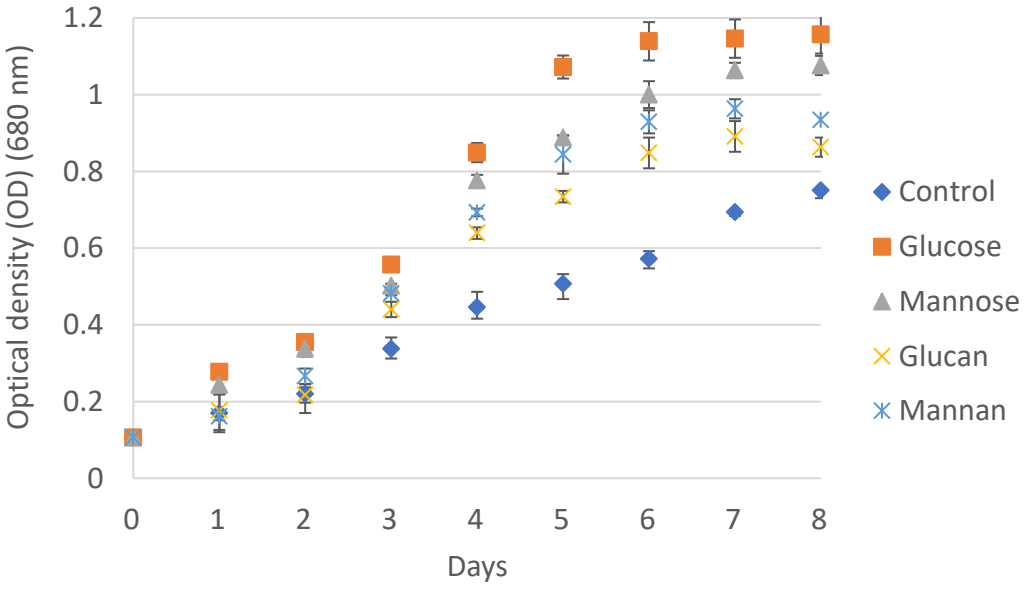


Figure 33: Growth curves of *C. vulgaris* cultured under the mixotrophic condition.

C. vulgaris grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and simple sugars and complex glycans of same concentration (0.15 mM).

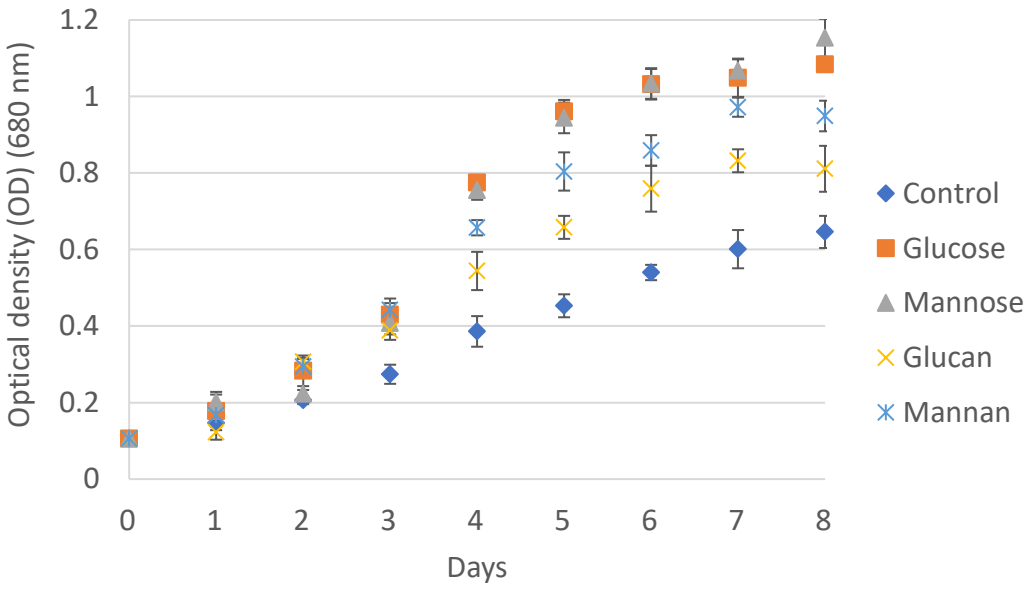


Figure 34: Growth curves of *N. oculata* cultured under the mixotrophic condition.
N. oculata grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and simple sugars and complex glycans of same concentration (0.15 mM).

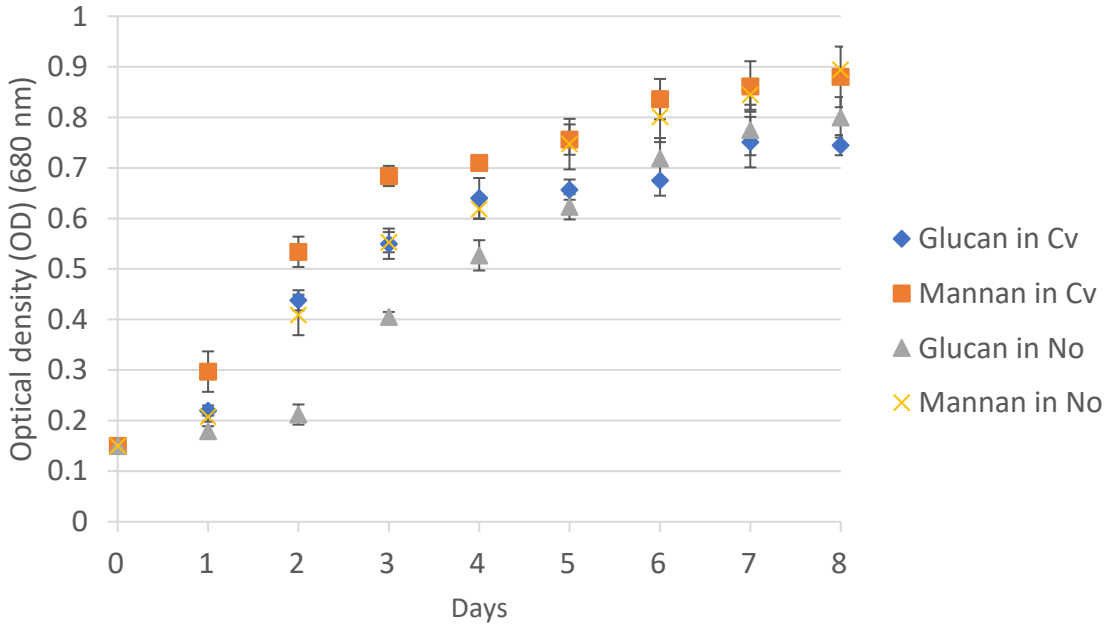


Figure 35: Growth curves of *C. vulgaris* (*C.v*) and *N. oculata* (*N.o*) cultured under the photoheterotrophic condition utilising β-glucan and β-mannan

Algae grown in various Erlenmeyer flasks containing BG 11 growth medium supplemented with 2.5 mM of urea and 0.15mM of glycans. Higher growth was seen in the media supplemented with mannan. No significant growth difference was observed in both species on the 8^{th} day of cultivation.

Comparatively, results (as displayed in Table 13) showed that mixotrophic mode yielded higher biomass than photoheterotrophic mode of cultivation on cultivating both microalgae species, indicating that passing air bubbles into culture (aeration) under mixotrophic mode further supplements culture with carbon dioxide (CO_2) which resulted in the slight increase in biomass concentration observed. Firstly, looking at the results obtained in cultivating *C. vulgaris*, optical densities of 1.157,0.863, 1.076 and 0.934, corresponding to biomass concentrations of 0.287,0.214, 0.267 and 0.232 gl^{-1} were obtained when glucose, β-glucan, mannose and β-mannan were respectively utilised. On evaluating other growth parameters using equations16 and 17, results of biomass productivities and growth rates for both modes of cultivation displayed in Table 13 were obtained. Biomass productivities of 0.0323, 0.0234, 0.0303 and 0.0256 $gl^{-1}d^{-1}$, and growth rate of 0.306, 0.269,0.297 and 0.279 d^{-1}were obtained by cultivating *C. vulgaris* in BG 11 supplemented with glucose, β-glucan, mannose and β-mannan respectively under mixotrophic mode of cultivation. These results suggest that simple sugars were better absorbed by algae in producing biomass than complex glycans when utilised as carbon sources. On biomass productivity, *C. vulgaris* slightly showed better biomass productivity when cultivated in the media supplemented with β-mannan than β-glucan.

Table 13: Biomass production of *C. vulgaris* and *N. oculata* at the 8th cultivation day under photoheterotrophic and mixotrophic modes of cultivation using various carbon sources

Microalgae species Carbon source	Mode of cultivation	OD (680 nm)	Biomass concentration (gl^{-1})	Biomass productivity ($gl^{-1}d^{-1}$)	Specific Growth (d^{-1})
C. vulgaris					
Glucan	▪ Mixotrophic	0.863±0.05	0.214±0.012	0.0234	0.269
	▪ Photoheterotrophic	0.745±0.04	0.184±0.01	0.0196	0.251
Glucose	▪ Mixotrophic	1.157±0.1	0.287±0.025	0.0323	0.306
C. vulgaris					
Mannan	▪ Mixotrophic	0.934±0.05	0.232±0.012	0.0256	0.279
	▪ Photoheterotrophic	0.88±0.06	0.218±0.015	0.0239	0.272
Mannose	▪ Mixotrophic	1.076±0.05	0.267±0.012	0.0303	0.297
C. vulgaris					
Control	▪ Mixotrophic	0.75±0.04	0.186±0.01	0.0202	0.252
N. oculata					
Glucan	▪ Mixotrophic	0.811±0.08	0.167±0.016	0.0175	0.238
	▪ Photoheterotrophic	0.80±0.05	0.165±0.01	0.0173	0.222
Glucose	▪ Mixotrophic	1.084±0.12	0.223±0.025	0.0253	0.275
N. oculata					
Mannan	▪ Mixotrophic	0.949±0.1	0.195±0.02	0.021	0.258
	▪ Photoheterotrophic	0.894±0.15	0.184±0.031	0.0196	0.251
Mannose	▪ Mixotrophic	1.153±0.15	0.238±0.031	0.0272	0.283
N. oculata					
Control	▪ Mixotrophic	0.646±0.05	0.133±0.01	0.0141	0.210

Secondly, on analysing the growth parameters of *N. oculata*, similar trends result with β-glucan and β-mannan, but mannose slightly performed better than glucose. Optical densities of 1.084, 1.153, 0.811 and 0.949, corresponding to biomass concentration of 0.2233, 0.2375, 0.167 and 0.1955 gl^{-1} were obtained with glucose, mannose, β-glucan and β-mannan respectively. Biomass productivity and growth rate of 0.0253, 0.0272, 0.0175 0.021 $gl^{-1}d^{-1}$ and 0.275, 0.283, 0.238, 0.0258 d^{-1} were obtained, respectively. Overall, the two microalgae species studied in this research can synthesize microalgae biomass when cultivated under mixotrophic and photoheterotrophic modes utilising simple sugars or monosaccharides (glucose and mannose) and complex glycans (β-glucan and β-mannan) as shown from the results shown in Figures 36, 37 and 38. The higher results obtained under mixotrophic mode shows the presence of more available carbon in the mixotrophic than photoheterotrophic culture. From these results, it can be deduced that *C. vulgaris* and *N. oculata* can metabolise

β-glucan and β-mannan into glucose and mannose molecules in the growth culture. The metabolism of these complex glycans shows the species capability to carryout enzymatic degradation, cell permeability, membrane diffusion and active transport during cultivation. This result is in consonance with the results of some research previously conducted and recorded in various literatures [28, 95, 291].

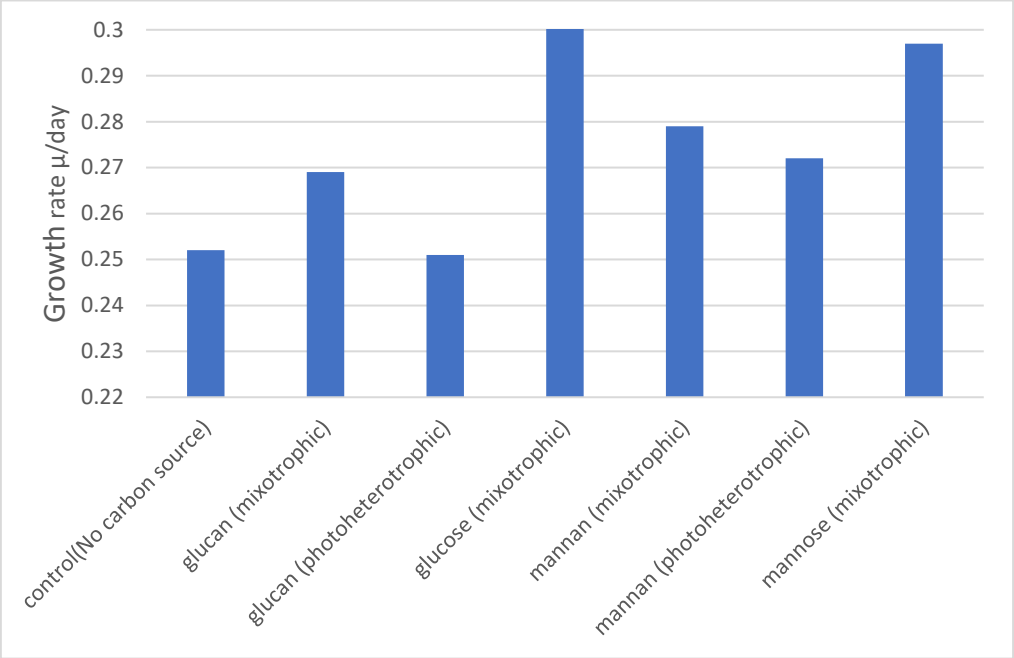


Figure 36: Comparison of specific growth rate of C. vulgaris cultivated under mixotrophic and photoheterotrophic regimes.

Cultures supplemented with simple sugars and nurtured mixotrophically showed higher growth rate than those supplemented with complex glycans. Growth rate obtained from mixotrophic mode of cultivation outweighed that of photoheterotrophic. Higher growth was observed in the media supplemented with various carbon sources than media without carbon source which serves as control.

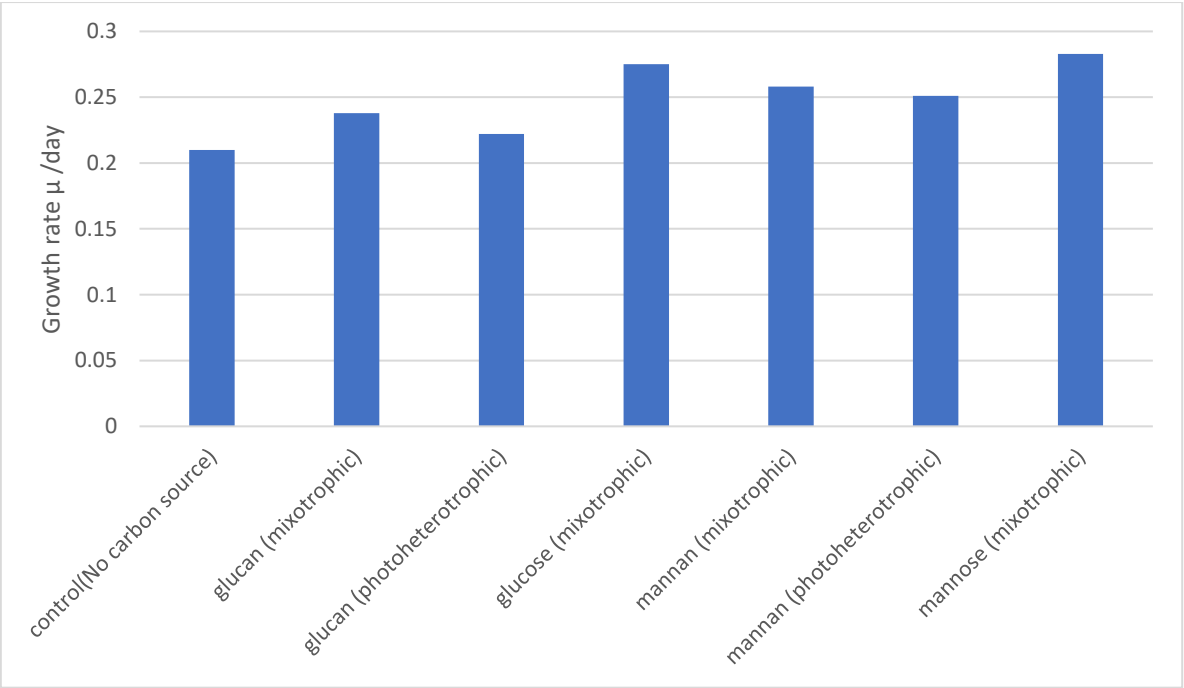


Figure 37: Comparison of specific growth rate of *N. oculata* cultivated under mixotrophic and photoheterotrophic regimes.

Cultures supplemented with simple sugars and nurtured mixotrophically showed higher growth rate than those supplemented with complex glycans. Growth rate obtained from mixotrophic mode of cultivation outweighed that of photoheterotrophic. Higher growth was observed in the media supplemented with various carbon sources than media without carbon source which serves as control.

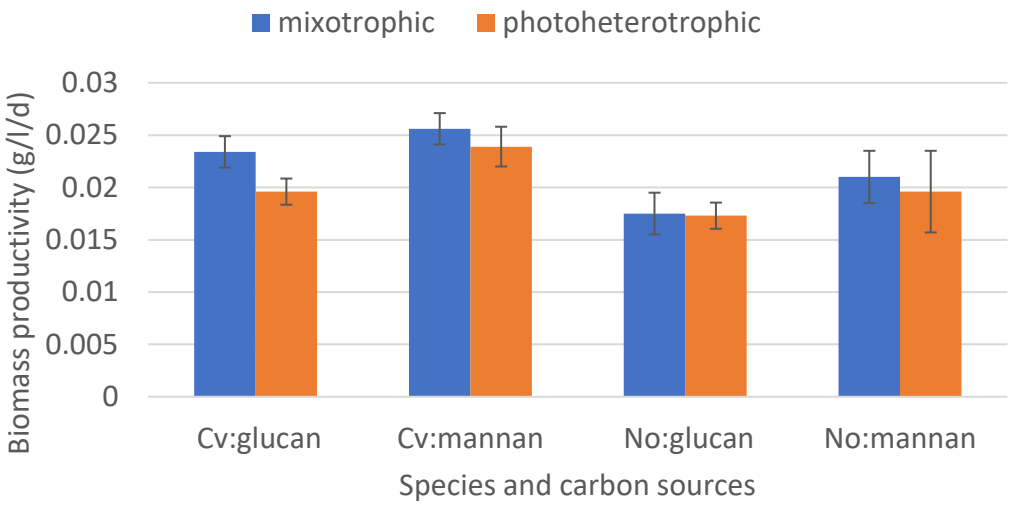


Figure 38: Comparison of biomass productivities of ***C. vulgaris*** and ***N. oculata*** cultivated under mixotrophic and photoheterotrophic modes of cultivation. (Mixotrophic mode outwits photoheterotrophic), indicating that culture agitation by aeration enhanced photosynthetic process.

4.1.5 Experiment to compare the effects of different sources and structures of complex glycans on growth parameters and dissolve oxygen concentration (DOC)

β-glucan derived from yeast and barley, and mannan and galactomannan are structurally dissimilar. The aim of this section is to identify and compare the effects of their structural differences on biomass concentration, productivity, and growth rate of both microalgae species. The experimental protocol had been described and the outcome of the experiment is displayed in Figures 39 and 40. Media supplemented with glucose yielded the highest biomass concentration whereas that without any carbon source yielded the least on both species. For *C. vulgaris*, the optimal biomass concentrations were observed on the 6th day of cultivation. Optical densities of 0.952±0.05 and 0.919±0.02, corresponding to biomass concentrations of 0.024 and 0.023 gl^{-1} were obtained using β-glucan sourced from barley and yeast respectively.

On *N. oculata*, the optimal biomass concentrations were also observed on the 6th day of cultivation. Optical densities of 0.638±0.03 and 0.688±0.03, corresponding to biomass concentrations of 0.0131 and 0.0142 gl^{-1} were obtained using glucan sourced from barley and yeast respectively. Overall, it can be deduced that the effect of utilising β-glucan sourced from barley and yeast is insignificant.

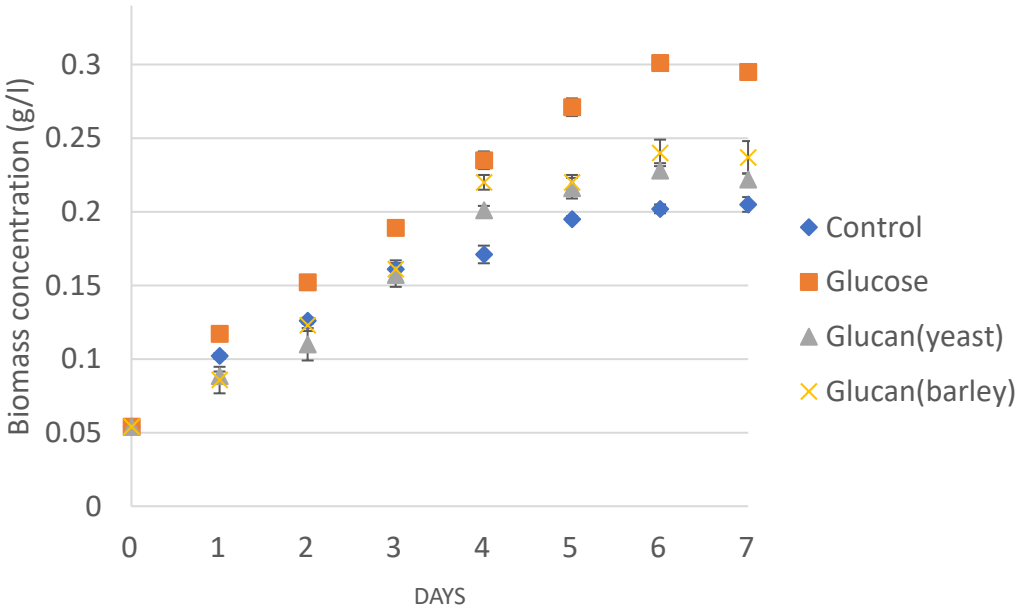


Figure 39: Biomass concentration of *C. vulgaris* grown in a medium supplemented with β-glucan sourced from yeast and barley.

Culture supplemented with glucose accumulated more biomass. The difference in biomass concentration acquired from culture supplemented with β-glucan sourced from yeast and barley is non-significant, indicating that structural isomers of carbon sources may not influence *C. vulgaris* biomass production.

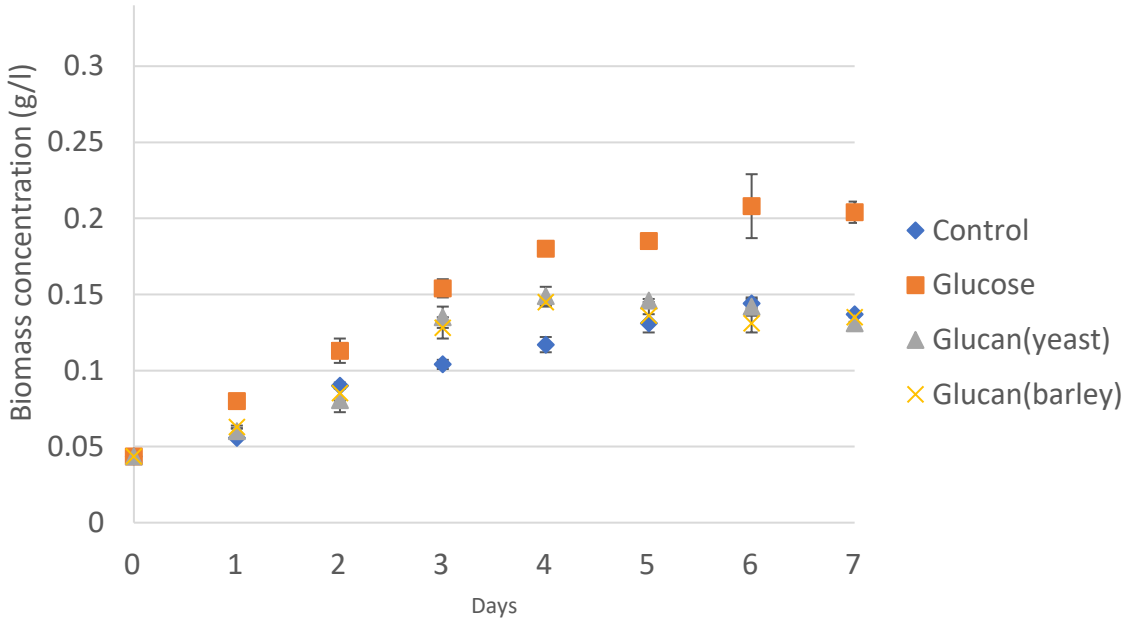


Figure 40: Biomass concentration of *N. oculata* grown in a medium supplemented with β-glucan sourced from yeast and barley.

Culture supplemented with glucose acquired more biomass. The difference in biomass concentration acquired from culture supplemented with β-glucan sourced from yeast and barley is non-significant, indicating that structural isomers of carbon sources may not influence on *N. oculata* biomass production.

Figures 41 and 42 display the outcome of utilising galactomannan and mannan as carbon sources on both species. Again, glucose yielded the highest biomass. However, biomass concentration of 0.264 and 0.232 gl^{-1}, and 0.208 and 0.21 gl^{-1} were obtained on utilising medium supplemented with mannan and galactomannan in culturing *C. vulgaris* and *N. oculata* respectively. This indicates that manna has a significant effect on the growth of *C. vulgaris* when juxtaposed with galactomannan, where the effect on *N. oculata* is insignificant.

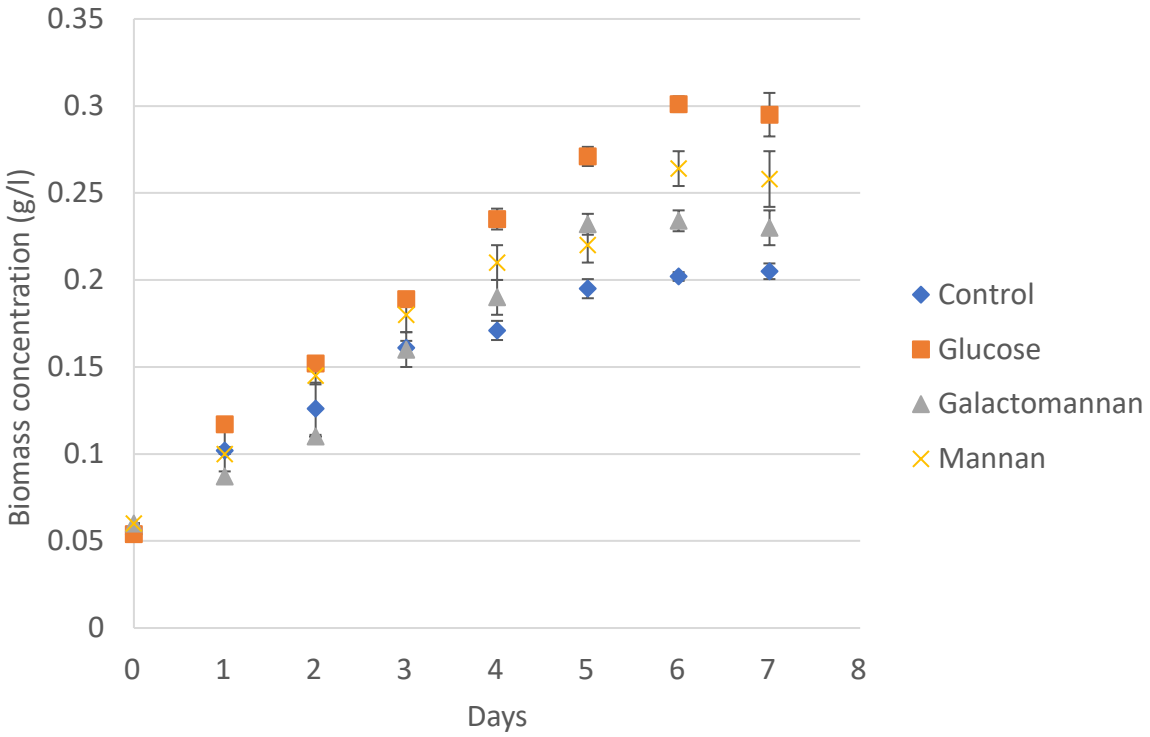


Figure 41: Biomass concentration of C. vulgaris grown in a medium supplemented galactomannan and mannan. Comparatively, growth parameters of *C. vulgaris* grown in the medium supplemented with β-mannan outwitted galactomannan

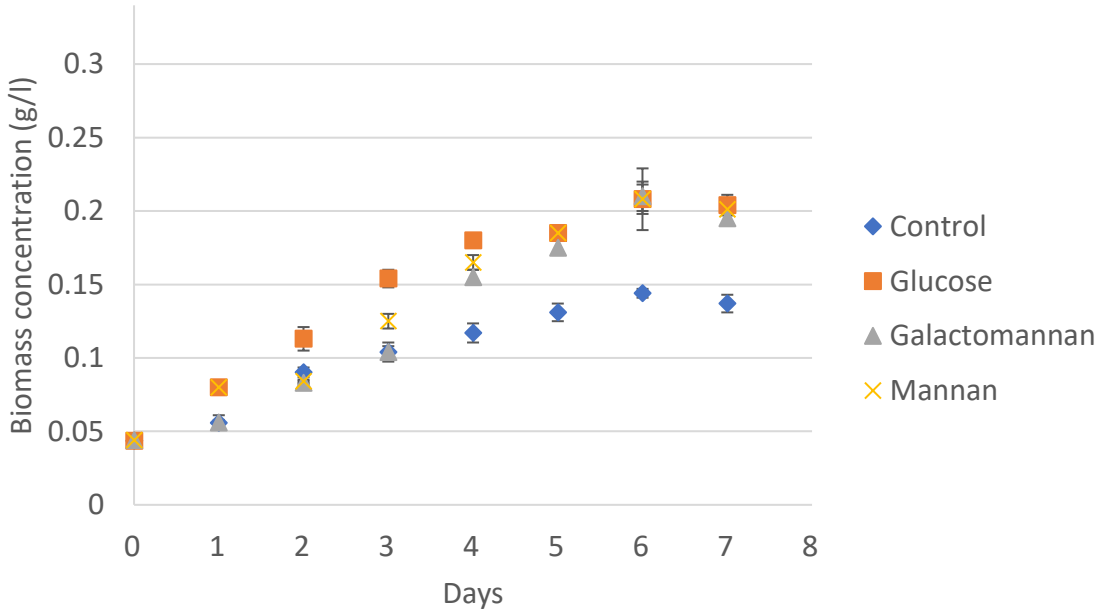
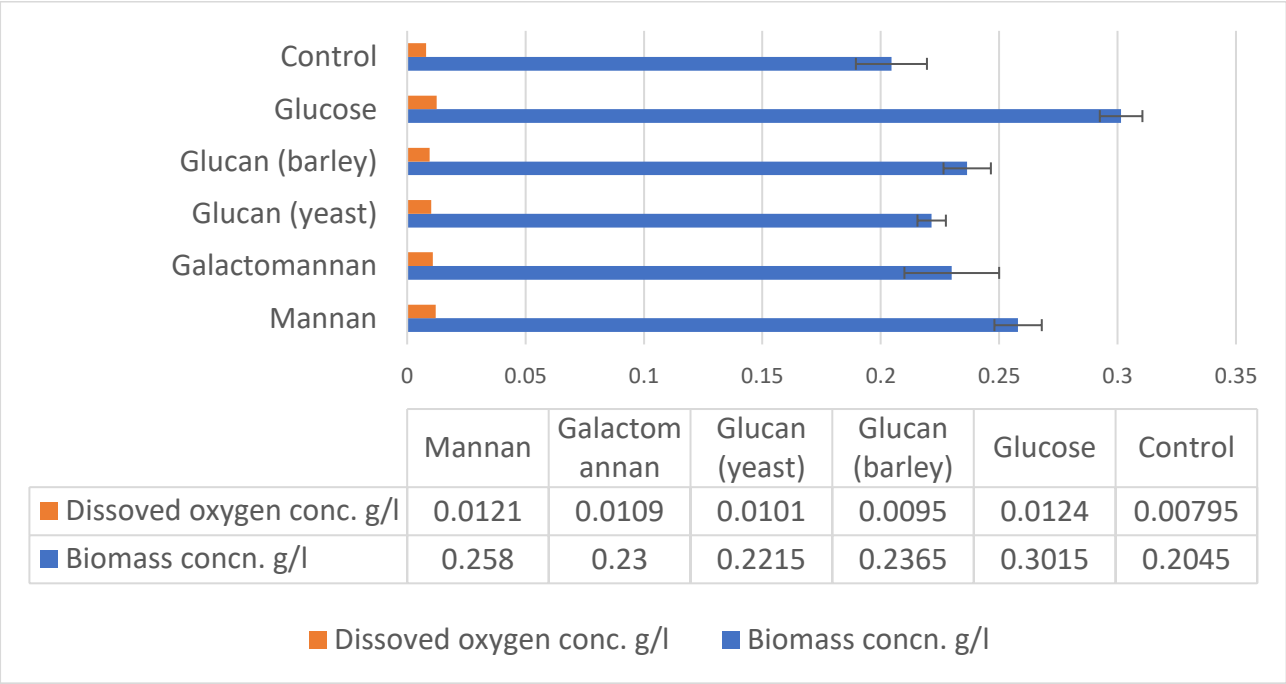


Figure 42: Biomass concentration of *N. oculata* grown in a medium supplemented galactomannan and mannan. No significant difference was observed in *N. oculata* grown in the medium supplemented with β-mannan and galactomannan

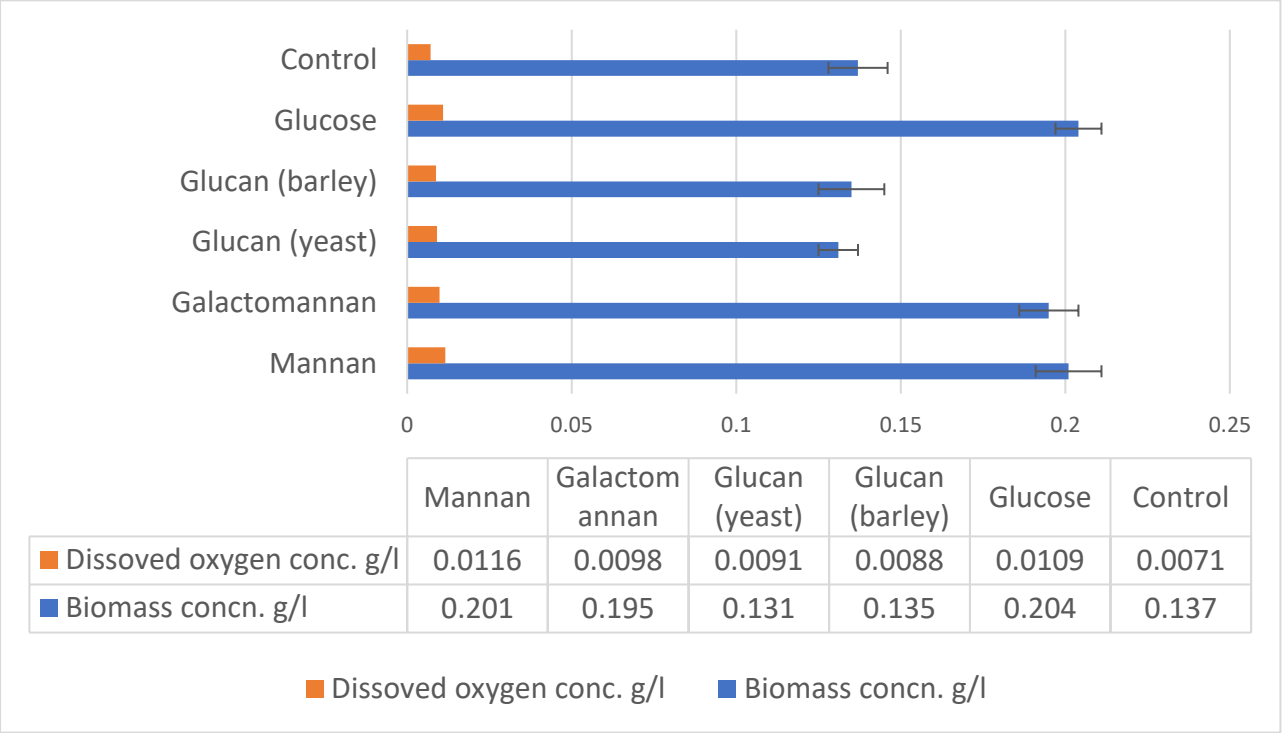
4.1.6 Experiment to compare the effects of β-glucan sourced from yeast and barley, β-mannan and galactomannan on growth parameters and dissolved oxygen concentration (DOC)

The effects β-glucan sourced from yeast and barley, β-mannan and galactomannan on growth parameter and dissolved oxygen concentration (DOC) of the two species when cultivated at the same time were examined. On the 6th cultivation day, DOC was measured and biomass concentration, biomass productivity and growth rate were estimated across board. Figures 43 and 44 display the charts of the results obtained. The evolved oxygen built up to high concentrations in PBRs could negatively affect biomass synthesis, trigger growth inhibition regime, and engender loss in biomass. In this study, the effect of DOC is insignificant on both species since it (DOC) is less than 0.020 g/l, according to Kazbar et al [282]. In their experiment, biomass loses were observed at DOC concentration greater than 30 mg/l (0.03 g/l), which signifies no loses in biomass of both cultivated species and reported in this thesis. Comparatively, carbon sources utilised seem to have influence on DOC as in growth parameters too.



	Mannan	Galactomannan	Glucan (yeast)	Glucan (barley)	Glucose	Control
Dissoved oxygen conc. g/l	0.0121	0.0109	0.0101	0.0095	0.0124	0.00795
Biomass concn. g/l	0.258	0.23	0.2215	0.2365	0.3015	0.2045

Figure 43: Comparison of *C. vulgaris* biomass concentration and DOC at different carbon sources



	Mannan	Galactomannan	Glucan (yeast)	Glucan (barley)	Glucose	Control
Dissoved oxygen conc. g/l	0.0116	0.0098	0.0091	0.0088	0.0109	0.0071
Biomass concn. g/l	0.201	0.195	0.131	0.135	0.204	0.137

Figure 44: Comparison of *N. oculata* biomass concentration and DOC at different carbon sources

From the results obtained, the control cultures (cultures not supplemented with carbon source) harboured the least biomass concentration as well as DOC in both species. There seems to be a relationship between biomass concentration and DOC, as cultures with high biomass concentration trapped high mass oxygen. Overall, the nature and structure of carbon sources utilised in microalgae cultivation may have any noticeable effect on DOC.

Biomass productivity and growth rate were estimated from Equations 16 and 17. The difference in the biomass productivities of *C. vulgaris* grown in a culture supplemented with β-glucan sourced from yeast and barley; and β-mannan and galactomannan are 0.0022 and 0.004 g/l/d ($gl^{-1}d^{-1}$), whereas those obtained from *N. oculata* are 0.0005 and 0.0008 $gl^{-1}d^{-1}$ respectively. It can be deduced that the effect of β-glucan sourced from yeast and barley, β-mannan and galactomannan on growth rate and biomass productivity are more significant in *C. vulgaris* and less significant in *N. oculata*. Figures 45 and 46 represent the chart of the differences of these growth parameters with respect to carbon source utilised.

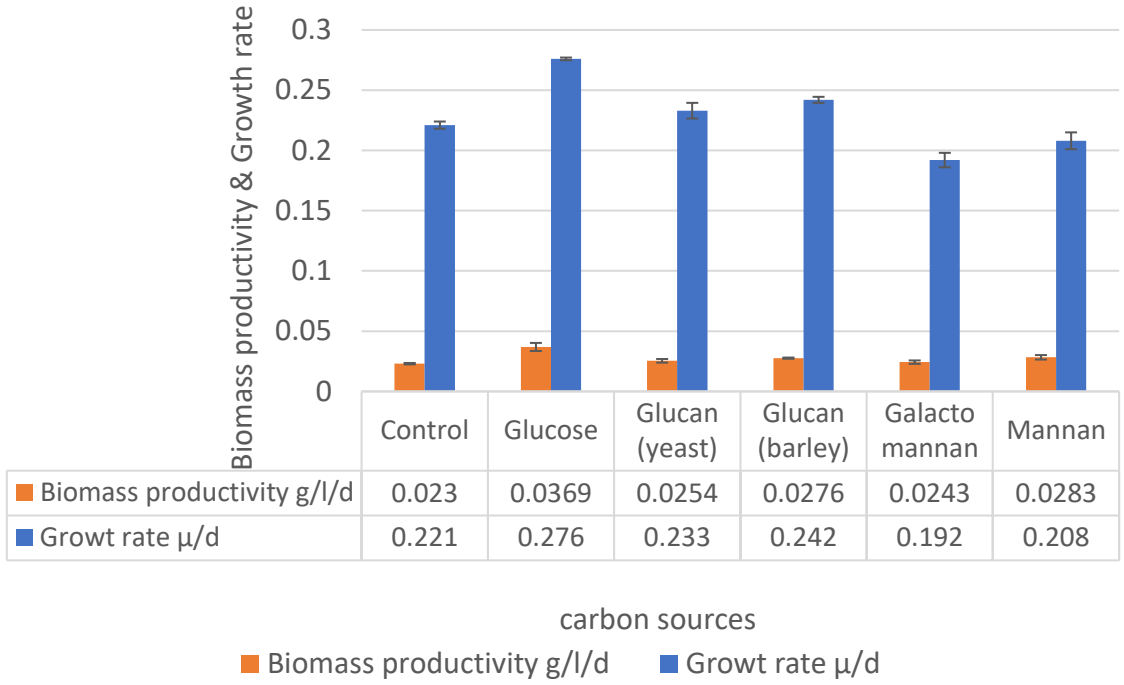


Figure 45: Comparison of *C. vulgaris* biomass productivity and growth rate at different carbon sources. Effect of utilising media supplemented with β-glucan sourced from yeast and barley is relatively nonsignificant, but significant with mannan when compared with galactomannan

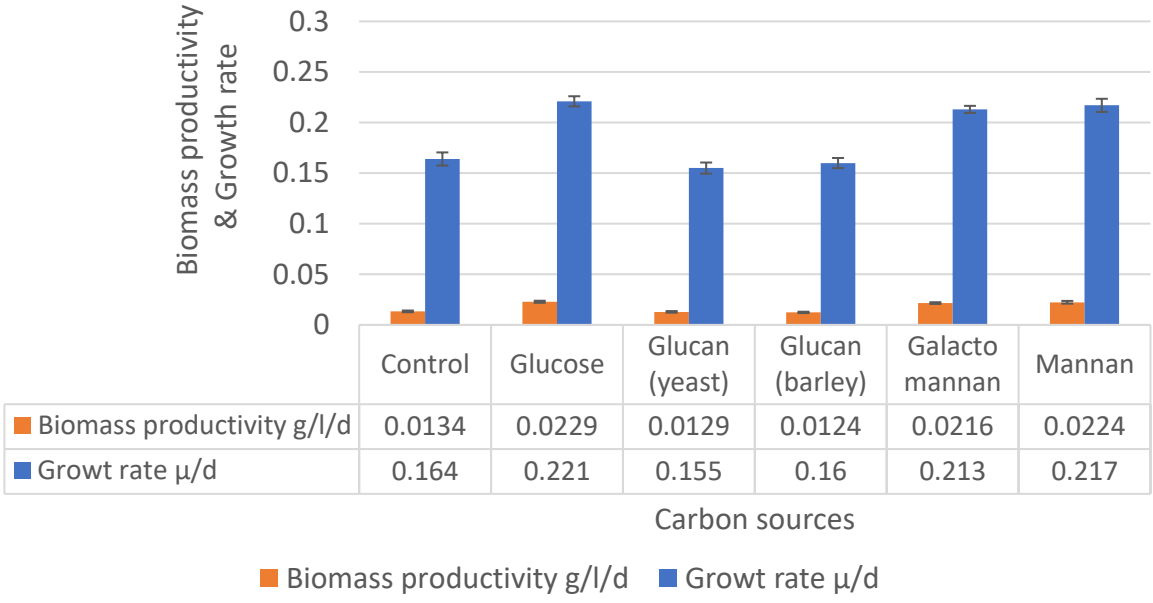
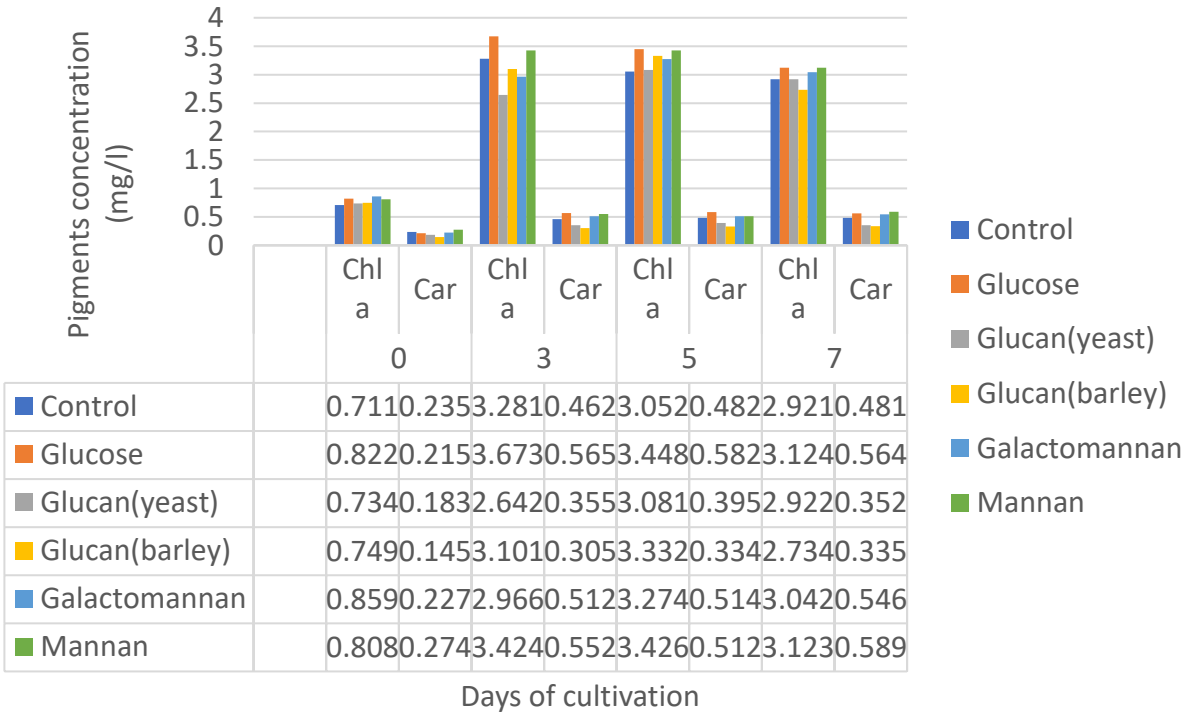


Figure 46: Comparison of *N. oculata* biomass productivity and growth rate at different carbon sources. Effect of utilising media supplemented with glucan sourced from yeast and barley, mannan and galactomannan is relatively nonsignificant on *N. oculata* growth parameters.

4.1.7 Experiment to compare the effects of carbon sources on pigment concentration

The most important chlorophyll a and carotenoids were extracted from *C. vulgaris* and *N. oculata* cultivated in growth media supplemented with different carbon sources and urea as nitrogen source, and their concentrations were evaluated in mg/l following Equations 19 and 20. Figures 47 and 48 show variations in chlorophyll a and carotenoids concentrations extracted from microalgae species cultivated in BG 11 supplemented with diverse carbon sources.

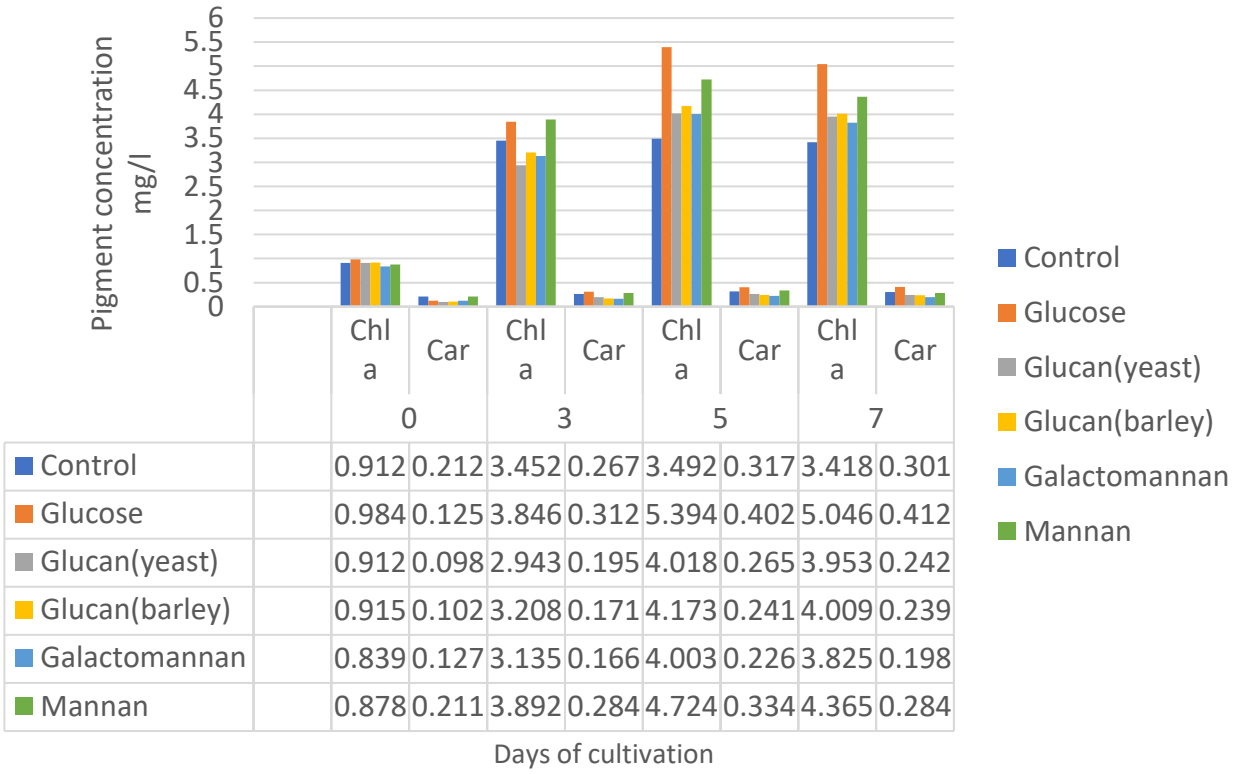
Figure 47 reveals a sharp surge in chlorophyll a concentration during cultivation of *C. vulgaris* from day zero to three. Thereafter, a decline was observed through day five to seven in the culture medium supplemented with glucose, indicating an increase in photosynthetic process and high nitrogen absorption till day three, followed by slight decline thereafter. In the growth medium supplemented with glycans, the surge in the concentration of chlorophyll a continued till the fifth day before a decline was observed. It could be deduced that the use of glycans as carbon sources possibly retards photosynthesis process during microalgae cultivation.



	0		3		5		7	
	Chl a	Car	Chl a	Car	Chl a	Car	Chl a	Car
Control	0.711	0.235	3.281	0.462	3.052	0.482	2.921	0.481
Glucose	0.822	0.215	3.673	0.565	3.448	0.582	3.124	0.564
Glucan(yeast)	0.734	0.183	2.642	0.355	3.081	0.395	2.922	0.352
Glucan(barley)	0.749	0.145	3.101	0.305	3.332	0.334	2.734	0.335
Galactomannan	0.859	0.227	2.966	0.512	3.274	0.514	3.042	0.546
Mannan	0.808	0.274	3.424	0.552	3.426	0.512	3.123	0.589

Figure 47: Concentration of chlorophyll a (Chl a) and carotenoids (Car) extracted from *C. vulgaris* cultivated in growth medium supplemented with different carbon sources.

Rapid rise in the concentration of pigments within the 3rd day, followed by slight decline on the 7th day of cultivation.



	Chl a	Car	Chl a	Car	Chl a	Car	Chl a	Car
	0		3		5		7	
Control	0.912	0.212	3.452	0.267	3.492	0.317	3.418	0.301
Glucose	0.984	0.125	3.846	0.312	5.394	0.402	5.046	0.412
Glucan(yeast)	0.912	0.098	2.943	0.195	4.018	0.265	3.953	0.242
Glucan(barley)	0.915	0.102	3.208	0.171	4.173	0.241	4.009	0.239
Galactomannan	0.839	0.127	3.135	0.166	4.003	0.226	3.825	0.198
Mannan	0.878	0.211	3.892	0.284	4.724	0.334	4.365	0.284

Figure 48: Concentration of chlorophyll a (Chl a) and (Car) carotenoids extracted from *N. oculata* cultivated in growth medium supplemented with different carbon sources. Rapid rise in the concentration of pigments up to the 5th day, followed by slight decline on the 7th day of cultivation

The concentration of carotenoids also increases in the first three cultivation days across board, thereafter, insignificant changes were observed. Similar trend was observed with *N. oculata* as shown in Figure 48. Notably, chlorophyll is a nitrogen-rich compound and is easily accessible and utilized as an intracellular nitrogen pool to support further cell growth and biomass production as the nitrogen in the media becomes depleted [292]. The utilisation of this intracellular nitrogen reduces the chlorophyll concentration over time. Comparatively, under the same growth and pigment extraction conditions, *N. oculata* showed a higher content of chlorophyll a than *C. vulgaris*, indicating that chlorophyll a synthesis can be species dependent. However, there are many parameters that can influence the chlorophyll biosynthesis and concentration [293]. Nitrogen concentration in growth media, culture irradiance and photoperiod, cultivation days or growth stage and extraction solvents are

some of those. The variations observed in chlorophyll a and carotenoids across cultivation days in each species are relatively not significant. Indicating that carbon sources may not have strong effect on pigment yields in both species [294]. The influence of carbon sources on pigments and carotenoids concentration extracted from microalgae has not been reported in literature. Therefore, the variations observed in the results could be due to the different degrees of nitrogen absorption with respect to the various utilised carbon sources during cultivation. However, results showed that growth media supplemented with β-glucans and glucose have the least and highest chlorophyll a yield in both species on the 7th cultivation day. Conversely, *C. vulgaris* showed a higher content of carotenoids than *N. oculata*. The reason is not clear, but we understand that some microalgae species can exhibit unique productivity of biomolecules under distinct sets of operating conditions, where they may accumulate different bioproducts to high levels.

4.1.8 Growth optimisation using RSM on both species cultivated under photoheterotrophic mode

The effects of different concentrations of β- glucan and β- mannan when utilised as carbon sources and their interaction with organic nitrogen source 'urea' on microalgae growth pattern have been optimised using one of the most widely used statistical software MINITAB 18. The software provides for a simple and effective way to input and manage data, identify trends, and pattern, and extrapolate results to the end user. The application of this software has been explained in in section 3.2.3 of this thesis. A CCD with three coded levels for two factors or input variables which are concentrations of complex glycans (coded as A) and urea (coded as B) were considered for this purpose. The levels of the variables for the CCD were chosen within the range of the concentration of nutrients (0.1, 0.15 and 0.2mM for glycans and 1.5, 2.5 and 3.5 mM for urea) which were the experimental inputs. The range of the variables, experimental designs, and results (experimental and predicted) obtained for microalgae growth are shown in Tables 9 and 10. The outcome of the optimisation process on individual species clearly showed that growth is dependent on the combination and interaction of both nutrients. This can be observed in the Pareto charts displayed in Figure 49. The Pareto chart is utilised to determine the magnitude and the importance of the effects of variables in single and synergy. On the Pareto chart, bars that cross the reference line marked 2.36 are statistically significant, hence are considered as critical factors that have great influence on the response. The length of bars indicates the degree of significance of

various variables. Thus, it can be deduced that the responses obtained depend purely on microalgae species and the type of carbon source utilised.

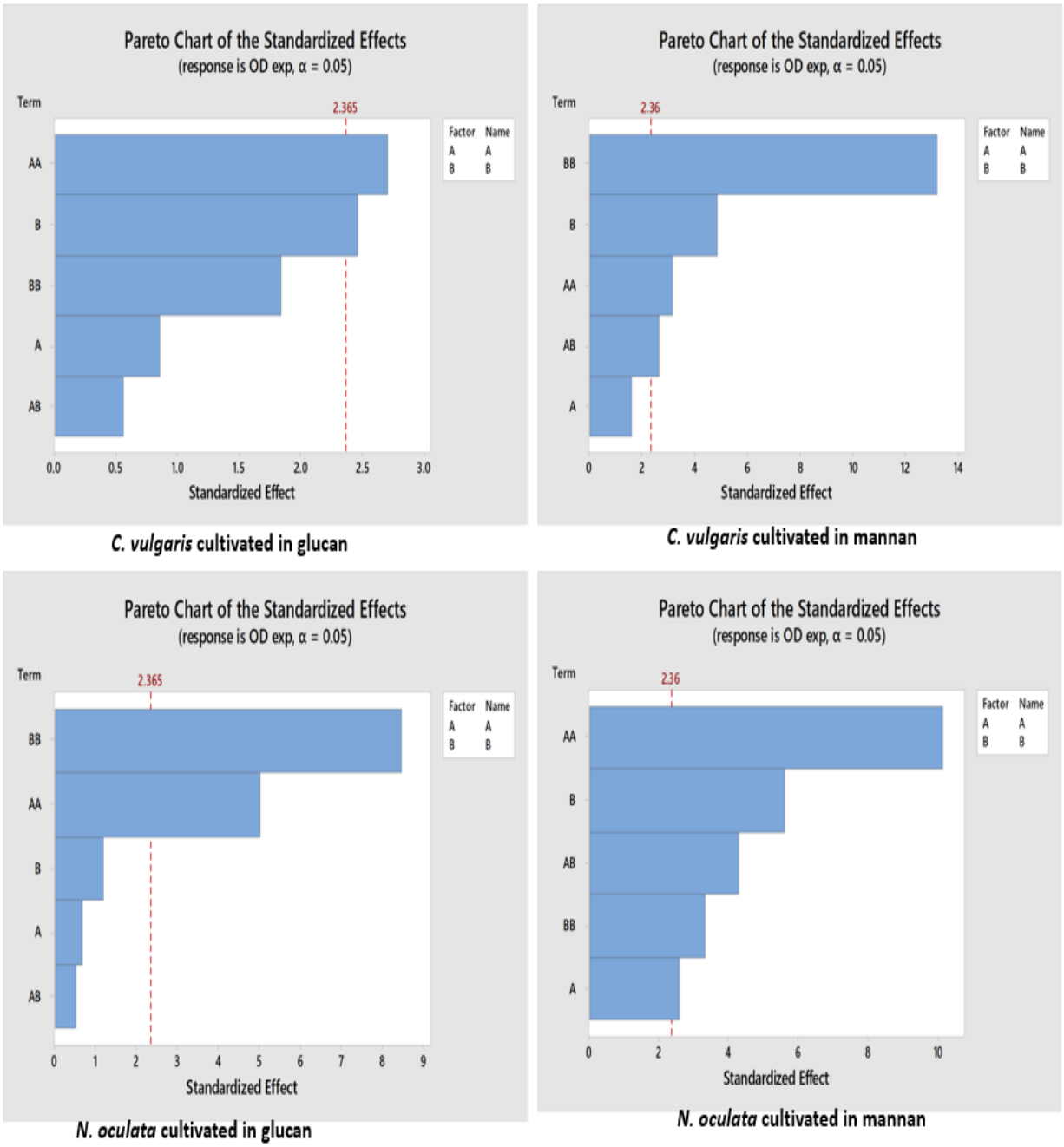


Figure 49: Pareto chart showing the degree of the effects of independent variables coded A and B, and the synergistic effects AA, BB, AB

Furthermore, the optimization of the results obtained by utilising β-glucan and β-mannan as carbon sources in the cultivation of both algae species for 8 days resulted in the generation of unique analysis of variance (ANOVA) and regression models obtained directly from Minitab software for each microalgae species and their corresponding carbon sources, as shown in

Tables 14 and 15. Although, predicting cell growth of microorganisms like bacteria and microalgae can be tasking because experiments can be affected by factors such as biotic, abiotic, and other process related factors, R- squared values obtained from the ANOVA and regression model are employed in this analysis. R-Squared is a statistical measure of fit that indicates how much variation of a dependent variable is explained by the independent variable(s) in a regression model. Having R^2 and R^2 (adjusted) far greater than 0.5 (0.759,0.971, 0.941 and 0.961) and (0.587, 0.969, 0.936, 0.958) indicate the suitability of models to predict these responses in varying independent variables. Furthermore, with these high values of R^2, having p values of less than 0.05 indicates strong evidence of high significance of the regression models.

Overall, it can be observed that the regression model is significant as F-value is greater than 3.0 in all cases considered in this study. Here, F-values of *C. vulgaris* cultivated in β-glucan and β-mannan are (4.0 and 47.08) and *N. oculata* (22.49 and 34.52), indicating that the use of these carbon sources is significant to the overall cell growth in photoheterotrophic cultivation of these selected species. Correspondingly, in RSM, p-value of lack of fit greater than 0.05, indicates that the model fits well. Thus, having various lack of fit p-values of greater than 0.05 (0.21, 0.513, 0.117 and 0.503) further affirm the significance and adequacy of the models.

Table 14: ANOVA model summary of photoheterotrophic growth regime

Source	***C. vulgaris* grown with glucan**	***C. vulgaris* grown with mannan**	***N. oculata* grown with glucan**	***N. oculata* grown with mannan**
P	0.039	0.00	0.00	0.00
F	4.0	47.08	22.49	34.52
P_{lf}	0.21	0.513	0.117	0.503
R^2	0.759	0.971	0.941	0.961
$R^2_{(adj)}$	0.587	0.951	0.90	0.933

P_{lf}: p-value for lack of fit

Table 15: Summary of regression analysis of photoheterotrophic growth regime

Source	***C. vulgaris* grown with glucan**	***C. vulgaris* grown with mannan**	***N. oculata* grown with glucan**	***N. oculata* grown with mannan**
P	0.00	0.00	0.00	0.00
F	34.6	369.94	176.7	271.26
R^2	0.759	0.971	0.941	0.961
$R^2_{(adj)}$	0.737	0.969	0.936	0.958

The final mathematical second order polynomial model related to the responses obtained on the 8th cultivation day and expressed in coded terms A and B are shown in Equations 25 and 26 for *C. vulgaris* and Equations 27 and 28 for *N. oculata* cultivated in β-glucan and β-mannan respectively. The predicted and experimental values indicate that there exists a good agreement amongst the experimental and predicted values as could be seen in the data presented earlier in Table 2 and plots in Figure 50.

$$OD_{680\,nm} = 0.008 + 6.77A + 0.195B - 22.01A * A - 0.04B * B - 0.144A * B;\ R^2 = 0.759 \quad (25)$$

$$OD_{680\,nm} = -0.223 + 3.3A + 0.674B - 13.17A * A - 0.15B * B - 0.343A * B;\ R^2 = 0.971\ (26)$$

$$OD_{680\,nm} = -0.921 + 11A + 0.773B - 39.96A * A - 0.1599B * B - 0.105A * B; R^2 = 0.941 (27)$$

$$OD_{680\,nm} = -1.008 + 20.2A + 0.242B - 61A * A - 0.0374B * B - 0.639A * B;\ R^2 = 0.961\ (28)$$

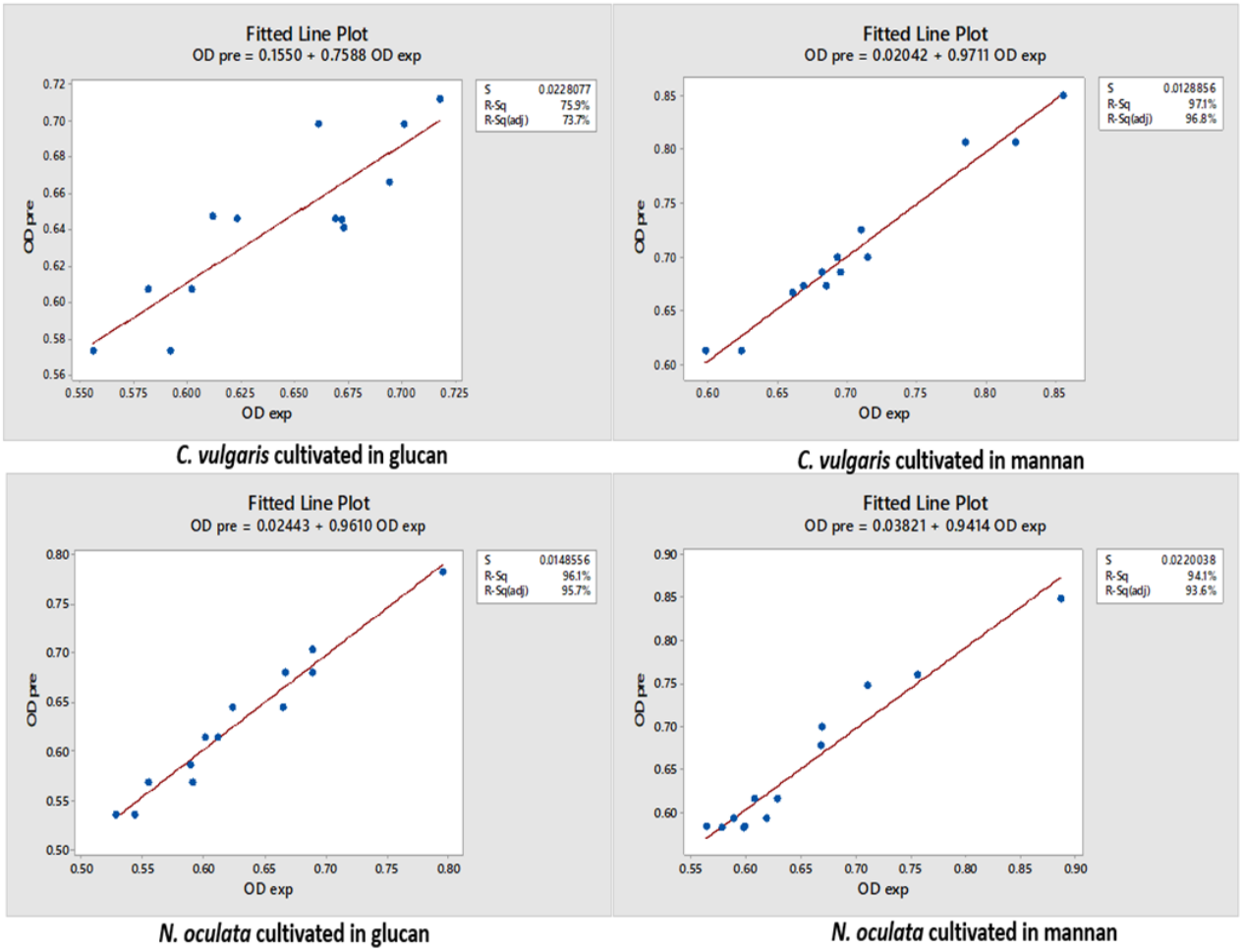


Figure 50: Relationship between the predicted (OD pre in vertical axis) and experimental values OD exp in horizontal axis). R-squared of over 90 %.

Indicating the suitability of models to predict responses in varying the independent variables.

Contours show the relationship between concentrations of carbon and nitrogen sources coded as A and B on microalgae growth. The contours are curved because the models contain quadratic terms that are statistically significant. Darker green regions indicate the area of higher growth, from where the domain optimal growth can be determined.

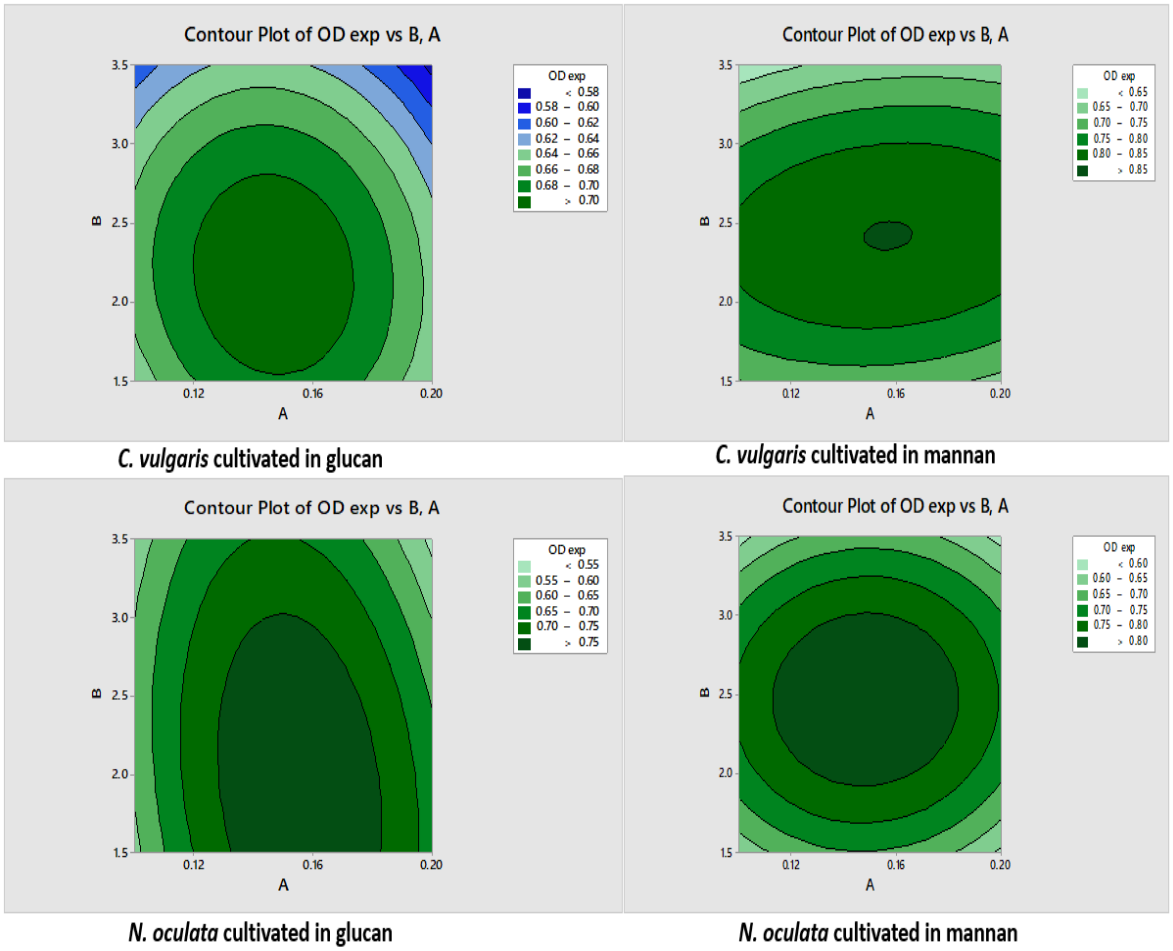


Figure 51: Contour plots showing the synergic effects of two independent variables coded A and B.

4.1.9 Fluorescence microscopy

After the cultivation regime, a pre-screening test was carried out using Nile Red as described in section 3.2.6. Cells were suspended in Nile Red solution (0.25 mg/ml in acetone) for 10 min. Samples were agitated in a vortex mixer (SLS Basics vortex mixer) for one minute and viewed under a Leica DM500 microscope operated with **LASV4.3** software equipped with a digital camera linked to a desktop where images were acquired. Pictures taken showed the presence of intracellular lipids droplets across all media supplemented with various carbon sources (Figure 52), indicative of lipids synthesis during cultivation.

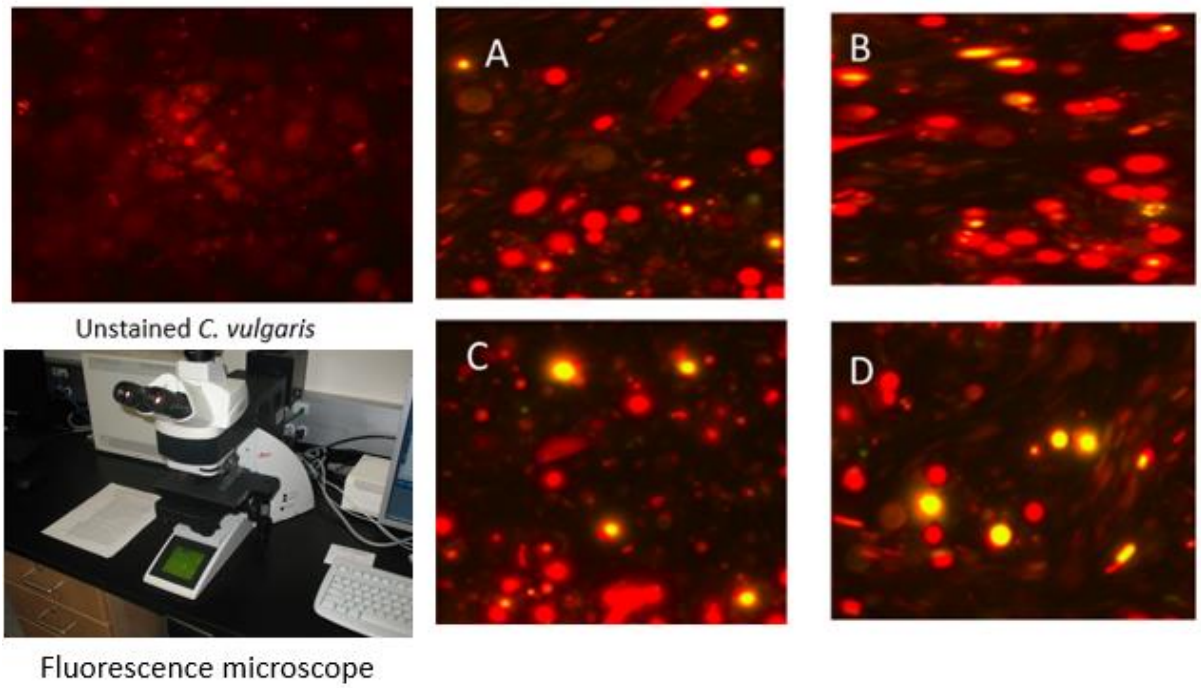


Figure 52: Nile red fluorescence of *C. vulgaris* samples cultured in medium supplemented with various carbon sources.

The unstained culture appeared red, indicating the presence of chlorophyll. The bright yellow fluorescence shows on stained samples designates lipids bodies, indicating that simple sugars and glycans can synthesis lipids when utilised as carbon sources for the cultivation of *C. vulgaris*: A: Control B: Glucose C: Glucan and D: Mannan

4.2 Microalgae cultivation using the MCR

A multi-cultivator (MC 1000-OD, Photon Systems Instruments) with eight separate cultivation tubes was used for batch cultivation of both species selected in this study. The aim is to explore the flexibility and applicability of its wide range of variable growth conditions. Utilising this device does not really call for comparison between MCR and PBR, due to dissimilar growth conditions and environments. However, results obtained from each microalgae species will be compared along with various carbon sources utilised.

4.2.1 Experiment to compare the effects of β-glucan, β-mannan and xylan on biomass growth parameters

The protocol used was briefly described in section 3.1.4 of this thesis. 80 ml of the growth medium supplemented with 2.5 mM of urea was used as nitrogen source and 0.15 mM each of the organic carbon sources (glucose, glucan, mannan and xylan). Inoculants of *C. vulgaris* and *N. oculata* were dropped in the 80 ml growth medium. Growth was monitored daily. Growth parameters such as biomass concentration, biomass productivity and growth rate were evaluated and plotted against days of cultivation. In all, growth was observed to be speedy as shown in Figures 53 and 54, leading to the attainment of early stationary stage. This may have been Probably triggered by culture exposure to optimal environmental factor which led to the intake large fraction nutrients.

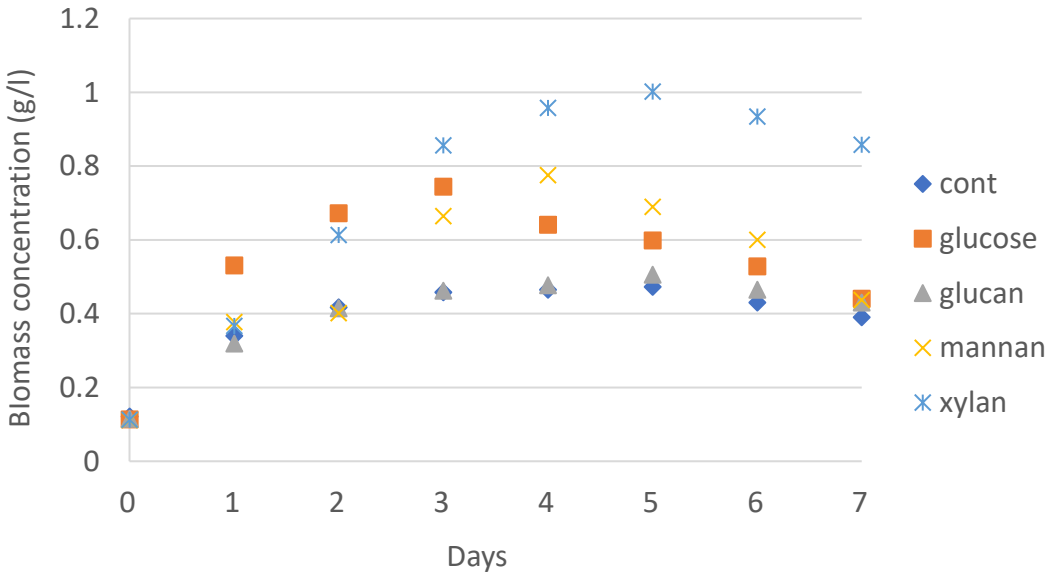


Figure 53: Biomass concentration of *C. vulgaris* cultivated in MCR utilising various glycans.

C. vulgaris cultivated in the medium supplemented with glucose experienced a rapid growth up to the third day of cultivation, followed by a sharp decline thereafter.

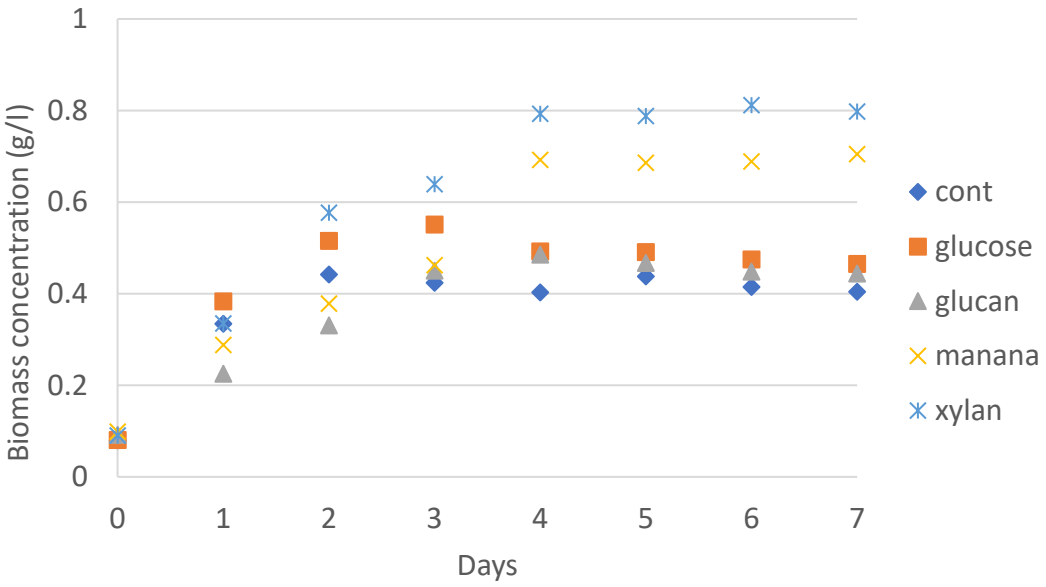
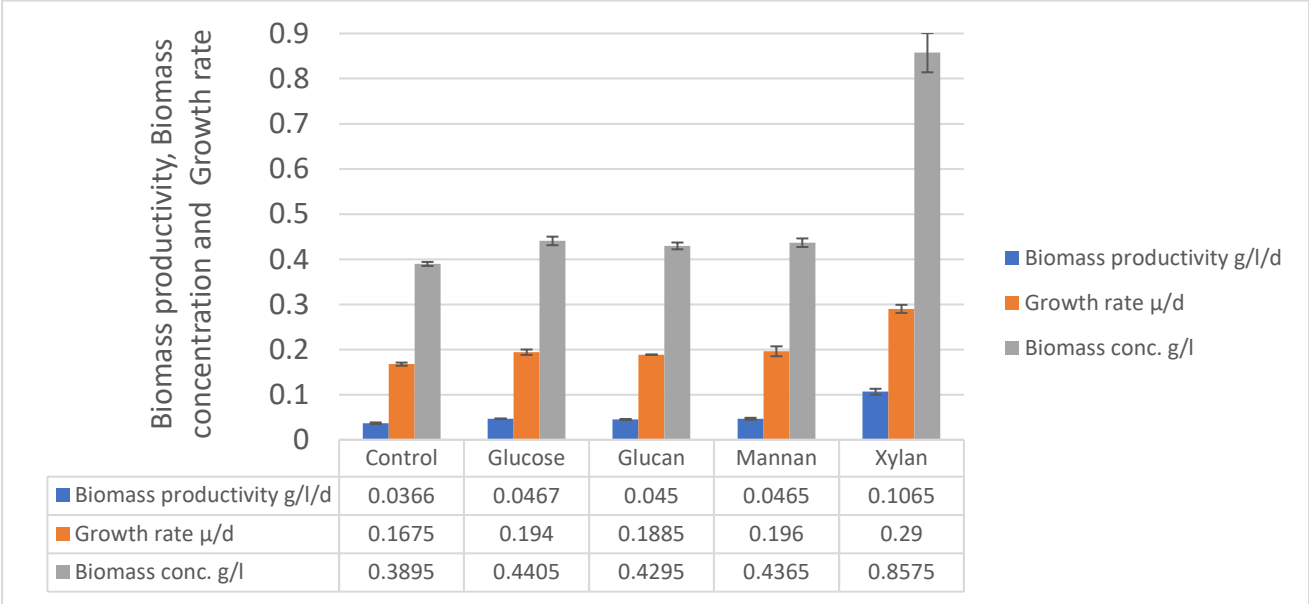


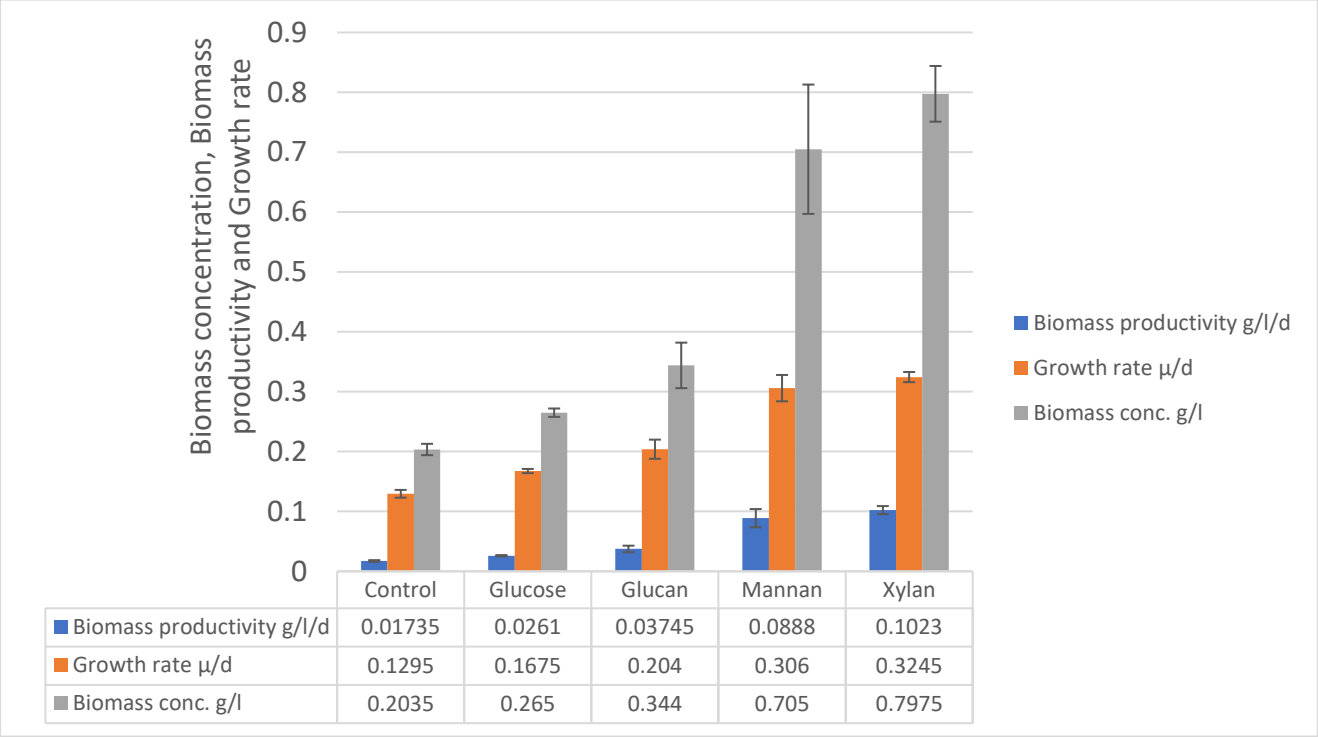
Figure 54: Biomass concentration of *N. oculata* cultivated in MCR utilising various glycans. *N. oculata* cultivated in the medium supplemented with glucose experienced a rapid growth up to the third day of cultivation, followed by a sharp decline thereafter.

Results as shown in Figures 55 and 56 showed that higher growth parameters were observed in *C. vulgaris* than *N. oculata*. Media supplemented with xylan amass the maximum biomass concentration, productivity and growth rate 0.86, 0.11 and 0.29 gl^{-1} and 0.8, 0.32 and 0.1 respectively after seven days of cultivation.



	Control	Glucose	Glucan	Mannan	Xylan
Biomass productivity g/l/d	0.0366	0.0467	0.045	0.0465	0.1065
Growth rate µ/d	0.1675	0.194	0.1885	0.196	0.29
Biomass conc. g/l	0.3895	0.4405	0.4295	0.4365	0.8575

Figure 55: Compares biomass productivities, biomass concentration and growth rate of *C. vulgaris* cultivated in MCR utilising various glycans.



	Control	Glucose	Glucan	Mannan	Xylan
Biomass productivity g/l/d	0.01735	0.0261	0.03745	0.0888	0.1023
Growth rate µ/d	0.1295	0.1675	0.204	0.306	0.3245
Biomass conc. g/l	0.2035	0.265	0.344	0.705	0.7975

Figure 56: Compares biomass productivities, biomass concentration and growth rate of *N. oculata* cultivated in MCR utilising various glycans

4.3 Flocculation results

4.3.1 Effects of flocculation parameters

After the cultivation regimes of both species under the same growth medium and conditions, experiments on flocculation were conducted using microalgae cultures each containing 0.5 g/l of *C. vulgaris* and *N. oculata* obtained after cultivation. The flocculation efficiency F (%) of each species was calculated from Equation 21. The experimental results obtained were used to optimise the critical flocculation parameters of each selected species and results obtained after the treatment of both species were compared. The flocculation efficiency of *C. vulgaris* as a function of flocculation time at different flocculant dosages and culture pH are presented in Figures 57, 58 and 59.

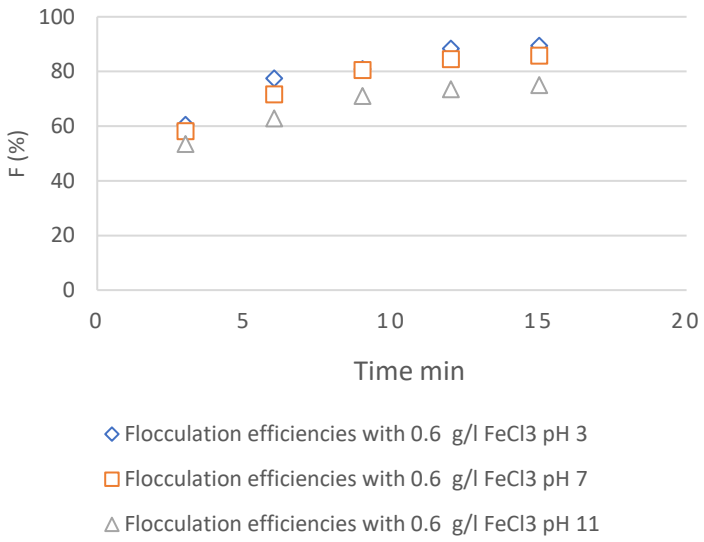


Figure 57: Flocculation efficiency of *C. vulgaris* as a function of time at different pH level and 0.6 g/l flocculant dose

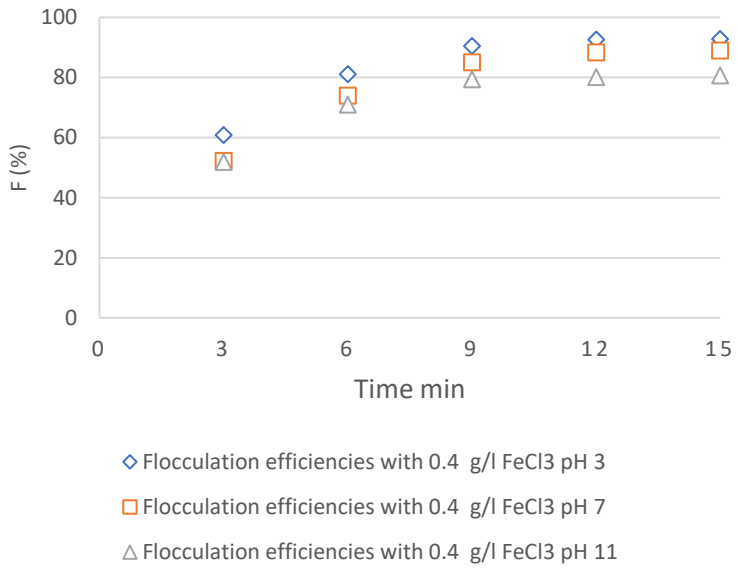


Figure 58: Flocculation efficiency of *C. vulgaris* as a function of time at different pH level and 0.4 g/l flocculant dose

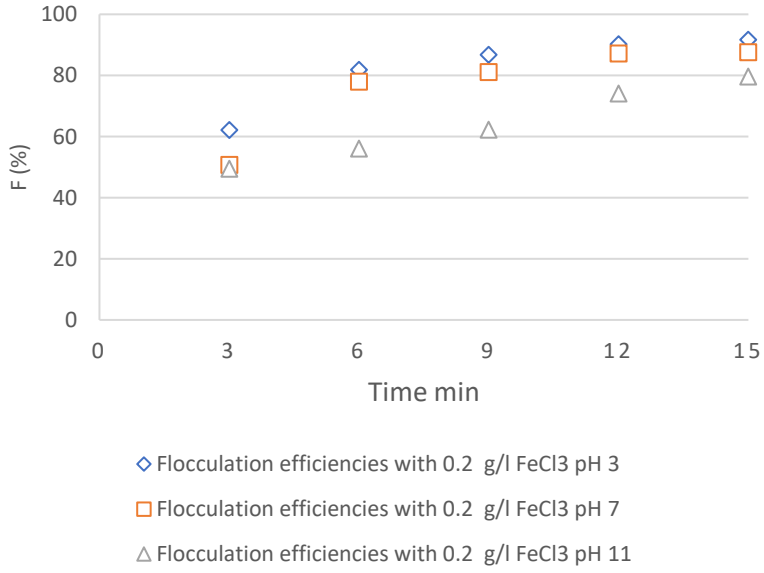


Figure 59: Flocculation efficiency of *C. vulgaris* as a function of time at different pH level and 0.2 g/l flocculant dose

Flocculating *N. oculata* under the same conditions yielded different flocculation efficiencies as shown in Figures 60, 61 and 62, confirming flocculation dependency of algae species. However, both treated microalgae species flocculated well, as flocs were seen to have settled at the bottom as shown in Figures 63 and 64, but at different marks on the translucent measuring cylinder after 15 minutes of the flocculation time. The effect of flocculant concentration was minimal whereas time and pH predominantly influenced flocculation efficiencies of both species. Comparatively, in a flocculation process time of 15 minutes, the results clearly showed that *C. vulgaris* flocculate better in acidic suspension (pH of 3) whereas *N. oculata* flocculate better in alkaline suspension (pH of 11). Optimal flocculation efficiencies of 92.8 and 94.2 % were observed in *C. vulgaris* and *N. oculata* when treated with flocculant doses of 0.4 and 0.6 gl^{-1} at pHs of 3 and 11 respectively. The optimum flocculation occurred in 12 to 15 minutes on both species. Process optimisation will unfold the solo and synergistic effects of all the flocculation parameters considered in this study.

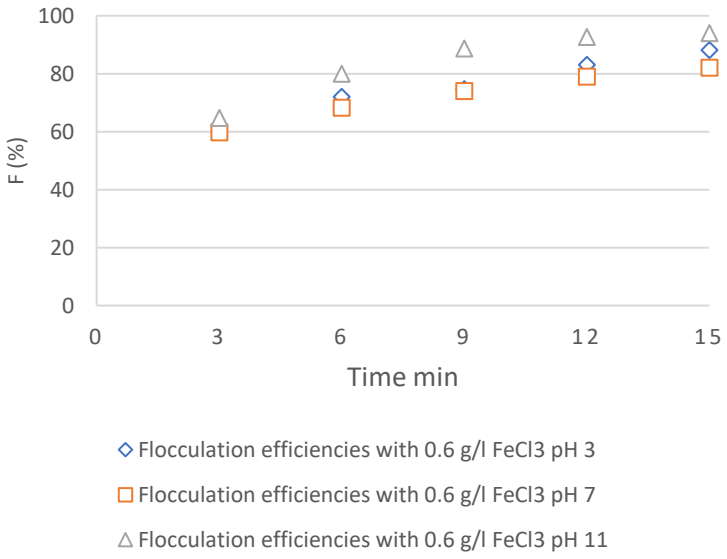


Figure 60: Flocculation efficiency of *N. oculata* as a function of time at different pH level and 0.6 g/l flocculant dose

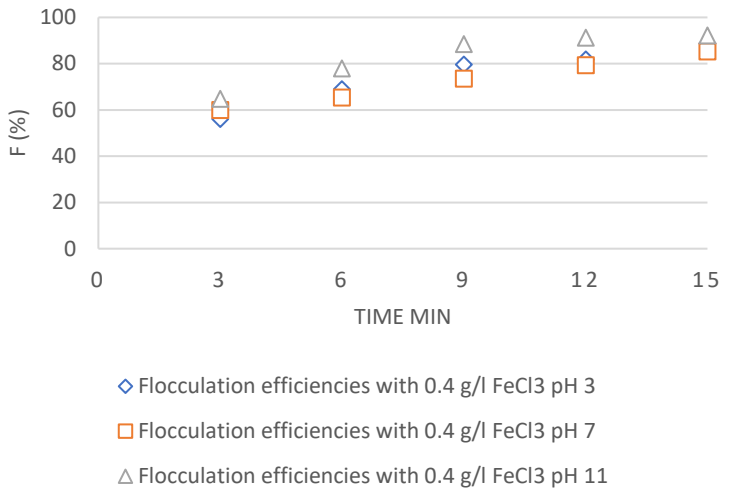


Figure 61: Flocculation efficiency of *N. oculata* as a function of time at different pH level and 0.4 g/l flocculant dose

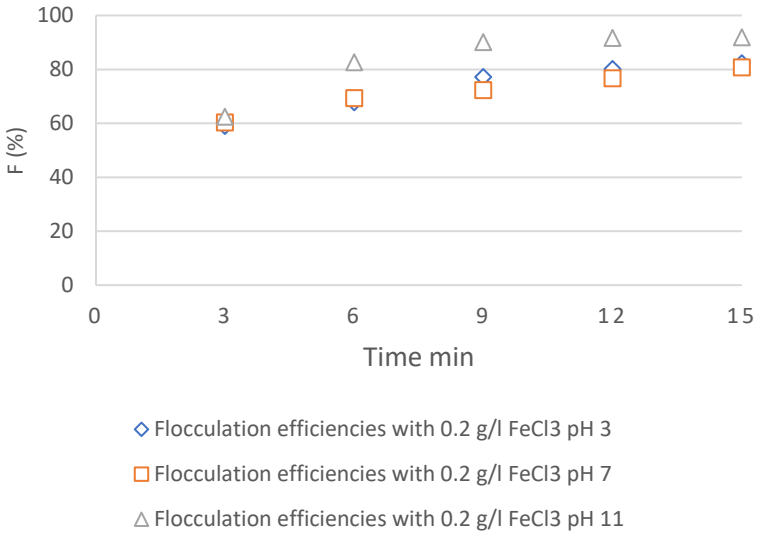


Figure 62: Flocculation efficiency of *N. oculata* as a function of time at different pH level and 0.2 g/l flocculant dose

These results agree with the findings in research conducted by [295-298]. The reason is usually connected to the strength of the negative surface charges caused by the ionisation of hydroxyl (-OH), carboxyl (-COOH) and amine ($-NH_2$) functional groups of different microalgae species and magnitude of zeta potential [35, 298, 299]. Different microalgae species have different concentration of functional groups. Higher concentration can enhance their ionisation and release of much charge in the culture which could result in high flocculation efficiency. The magnitude of the zeta potential (positive or negative) indicates the degree of

electrostatic repulsion between adjacent similar negative surface charges, hence brings about dispersion of microalgae cells. Higher value of zeta potential engenders cell stability thereby resist floc formation. Zeta potential is majorly affected by the culture pH, species, flocculant concentration, temperature, ionic strength, and growth phase [300-302]. In flocculating *C. vulgaris* using ferric chloride, zeta potential increases with pH, thus, cells become more stable and resist flocs formation [300, 303]. In low pH of 3, zeta potential decreases, leading to the aggregation of large flocs that resulted in high flocculation efficiency. Increasing the pH of *N. oculata* makes more positive charge available in the culture as the zeta potential decreases. These positive charges will neutralise the negative surface charge of the cells present in the culture to form flocs.

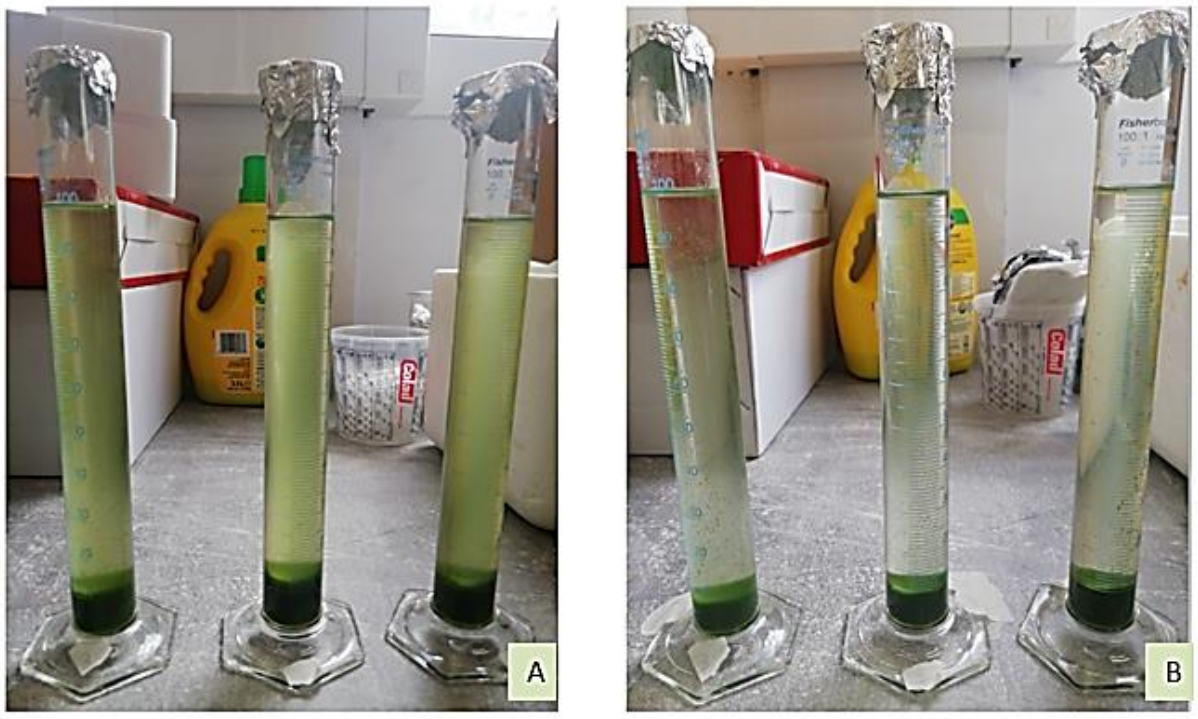


Figure 63: Flocculation of C. vulgaris and N. oculata in alkaline culture

A: ***C. vulgaris*** after 15 minutes flocculation period at a pH of 11 (Alkaline culture)
B: ***N. oculata*** after 15 minutes flocculation period at a pH of 11 (Alkaline culture)

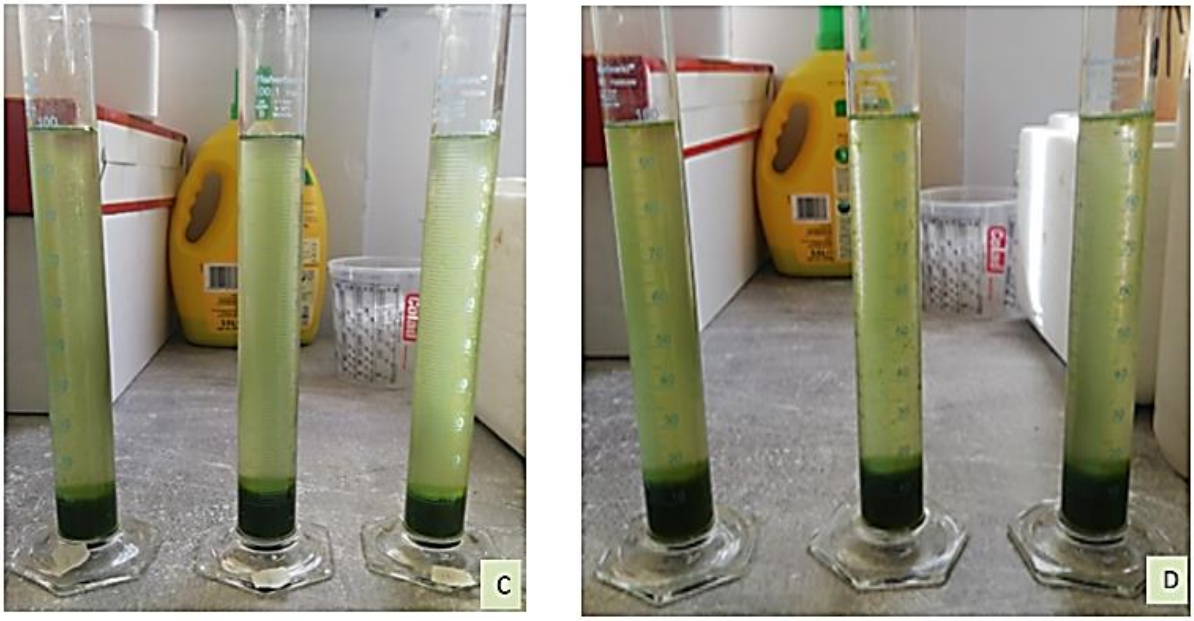


Figure 64: Flocculation of *C. vulgaris* and *N. oculata* in acidic culture

C: ***C. vulgaris*** after 15 minutes flocculation period at a pH of 3 (Acidic culture)
D: ***N. oculata*** after 15 minutes flocculation period at a pH of 3 (Acidic culture)

4.3.2 Microscopic observation

In order to observe the effect of flocculation on the cell morphology and aggregation, samples were viewed under Alcona 3D microscope. The images obtained at optimum flocculation conditions were viewed at different pH levels. Treated and untreated species were displayed in Figure 65. It was observed that cells were separated and wholly spherical when untreated, indicating non-flocculated cells with very few or no aggregates or flocs formation. The treated cells of both species showed flocs formation of different sizes and levels of cell wall distortion. Flocs of *C. vulgaris* were seen to be decreasing with increasing pH levels. Cell wall distortion was observed to be most severe in acid medium (at a pH of 3). Severity decreases with increasing pH. Conversely, flocs of *N. oculata* formed under similar conditions increases with increasing pH. But cell walls seem to have slightly increase in size with a little or no distortion. Similar trends were observed with 0.2 and 0.6 gl^{-1} flocculation concentrations. This could be attributed to the fact that cells of *N. oculata* are tougher than those of *C. vulgaris* due to the presences of hard cell wall components such as strut, cellulose based layer and extensions in *N. oculata* cell wall matrix [304].

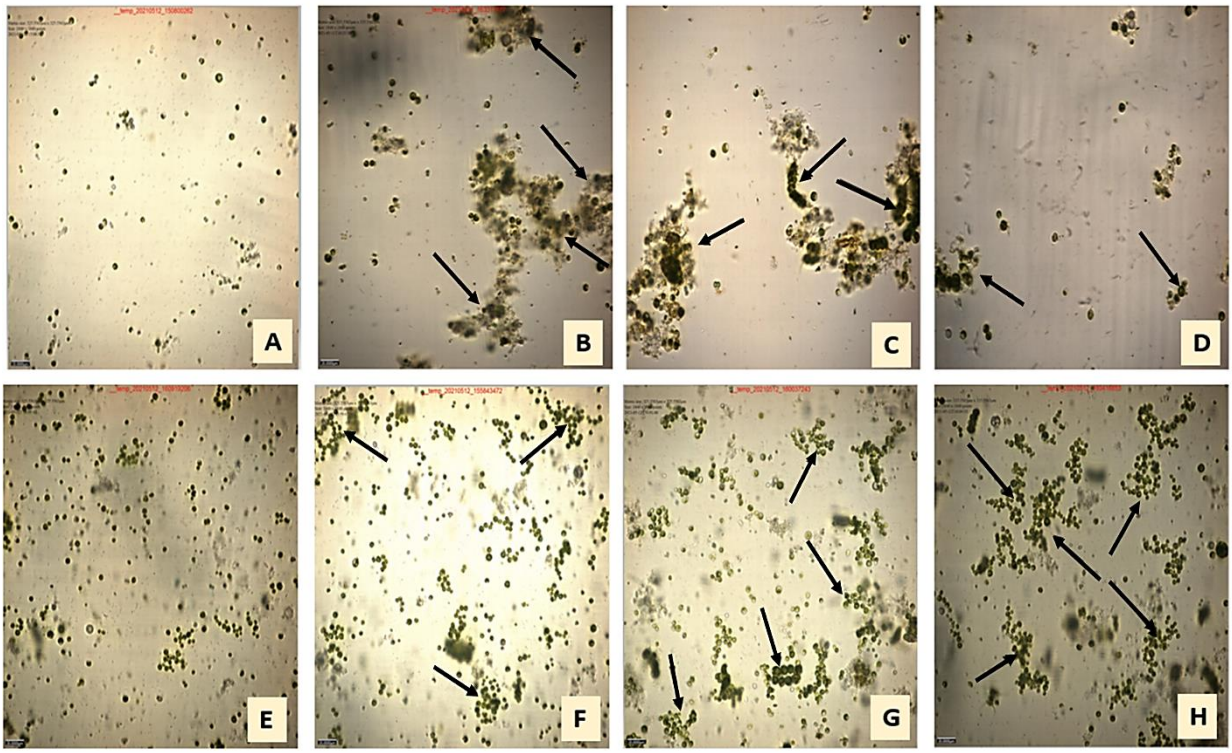


Figure 65: Visual images (X50) of flocs formation and cell walls morphology *C. vulgaris* (A (untreated), B, C and D are treated) and *N. oculata* (E (untreated), F, G and H are treated). Arrows indicate some regions of flocs formed. Arrows indicate floc formation in the treated culture. More flocs of *C. vulgaris* aggregate in acidic culture (B) and less in alkali culture(D). Whereas more flocs of *N. oculata* aggregate in alkali culture (H) and less in acidic culture (F).

4.3.3 Cell viability test with trypan blue

Viability of microalgae cells was determined by the trypan blue assay. The automated cell counter analysis of both species after flocculation showed that flocculation process resulted in about 10 % *C. vulgaris* cell loss whereas an insignificant 3 % cell loss was associated with *N. oculata*.

4.3.4 Flocculation process optimisation using RSM

The effects of the considered flocculation variables; flocculant concentration, pH and flocculation time observed experimentally were optimised and compared amongst species. The design of experiment leading to the optimisation of these effects has been discussed in Section 2.5, and the experimental and model predicted results displayed in Table 3 of this report. The outcome of the optimisation process on each species showed that flocculation is a function of these variables and interaction amongst some of them, as shown in the Pareto

charts displayed in Figure 66. From the Pareto charts, it can be deduced that the synergistic effect of pH and flocculation time (BC) is more significant, as the bars of C and B are lengthier than others. Bars CC and AA projected across the red vertical line marked 2.04 are also significant to the responses observed in *C. vulgaris* and *N. oculata* respectively and therefore are classified as critical variables and interactions. Flocculant's concentration (A) seems not have significant effect on both microalgae species. ANOVA obtained from Minitab software for each species are presented in Table 16 and values therein could be used to predict the adequacy of the models of each species.

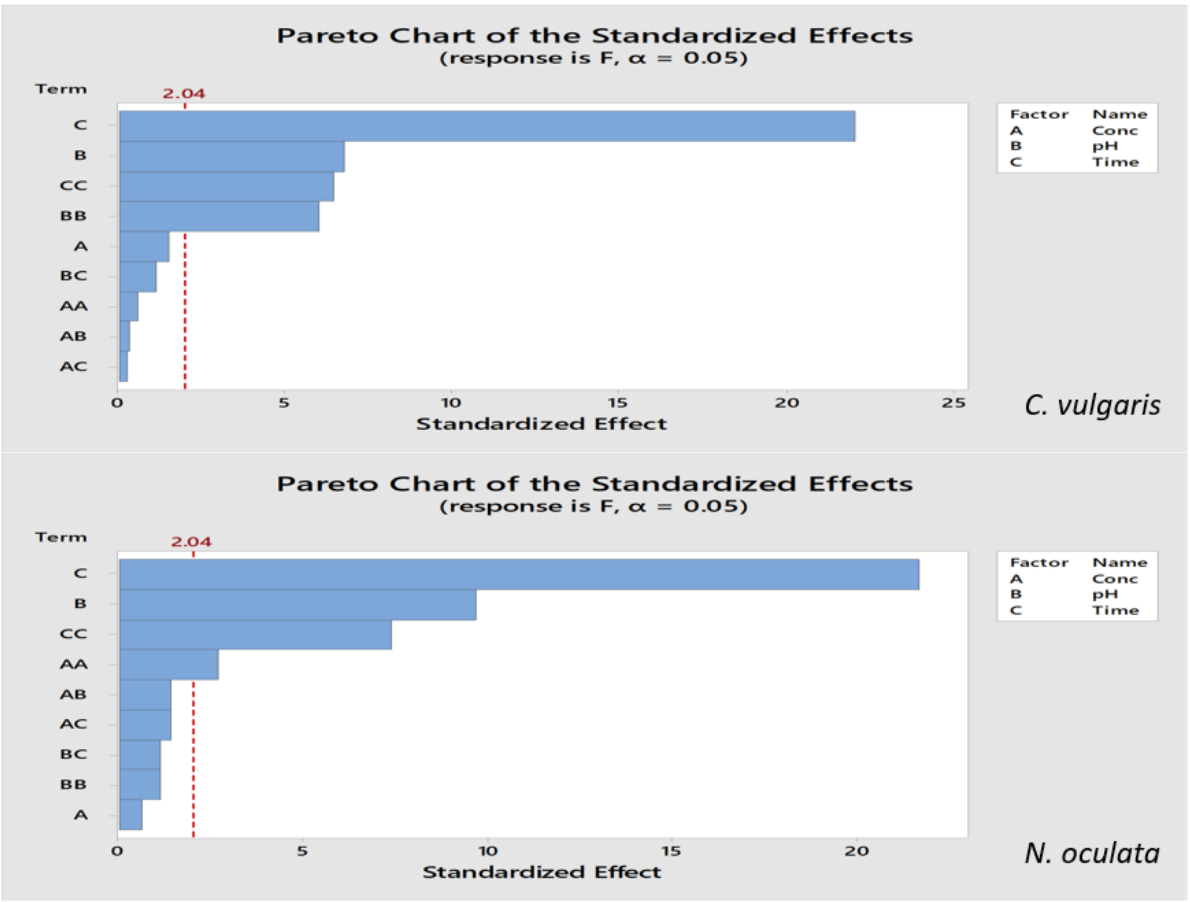


Figure 66: Pareto chart showing single and synergistic effects of parameters coded as A, B, C on the responses.

The effects of time (C) and pH (B) are very significant considering the length of the bar.

Table 16: ANOVA model summary of flocculation experiment

Samples	*P*	F	T	R^2	R^2(adjust)	R^2 (predict)
C. vulgaris	0.00**	74.22	54	0.957	0.9441	0.9261
N. oculata	0.00**	74.29	59.81	0.9571	0.9442	0.9243

P<0.005

From the constant statistical values of P, F, T and R^2 obtained from the ANOVA analysis as itemised in Table 16, it can clearly be deduced that the two microalgae species demonstrate high significance and adequacy of the models. The final mathematical second order polynomial model related to the responses expressed in coded terms A, B and C are shown in Equations 4 and 5 for *C. vulgaris* cultivated and *N. oculata* respectively. The fitted line plots for *C. vulgaris* and *N. oculata* of predicted and experimental values as displayed in Figure 67 indicates that the models for each species can reliably predict the outcome of input variables.

$$F = 32.07 + 64.0A - 0.73B + 7.567C - 79.4A * A - 0.0846B * B - 0.2435C * C + 1.53A * B - A * C - 0.0402B * C \quad (29)$$

$$F = 51.15 + 19.7A - 4.48B + 5.177C - 14.8\,A * A + 0.3765B * B - 0.1783\,C * C - 0.292\,A * B - 0.155\,A * C + 0.0339B * C \quad (30)$$

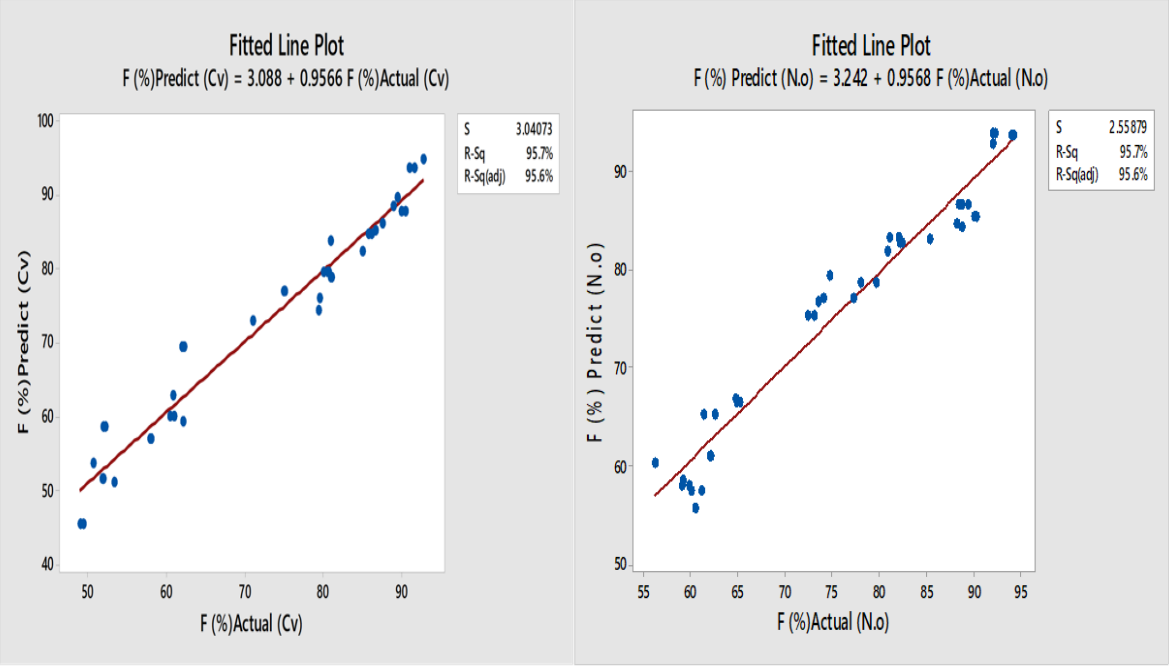


Figure 67: Fitted line plots for actual (experimental) and predictive responses on *C. vulgaris* and *N. oculata*.

Describing the synergistic effects and optimal range of input variables of the three independent factors on the responses, contour and surface plots were presented as shown in Figures 68 and 69. In the contour plots, higher responses are represented in the darkest green portion of the graph and decreases as the darkest green colour fades. Contour plots of Figures 68 A, B & C and Figures 69 A, B & C showed that both species floc across various flocculants concentration. Optimally, flocs of *C. vulgaris* and *N. oculata* were formed at pH less than 5 and pH greater than 10.5 respectively, and within the same flocculation time range of 12.5 to 15. The surface plots in Figures 68D and 69D further indicate the region of maximumflocculation for both microalgae species considered in this study.

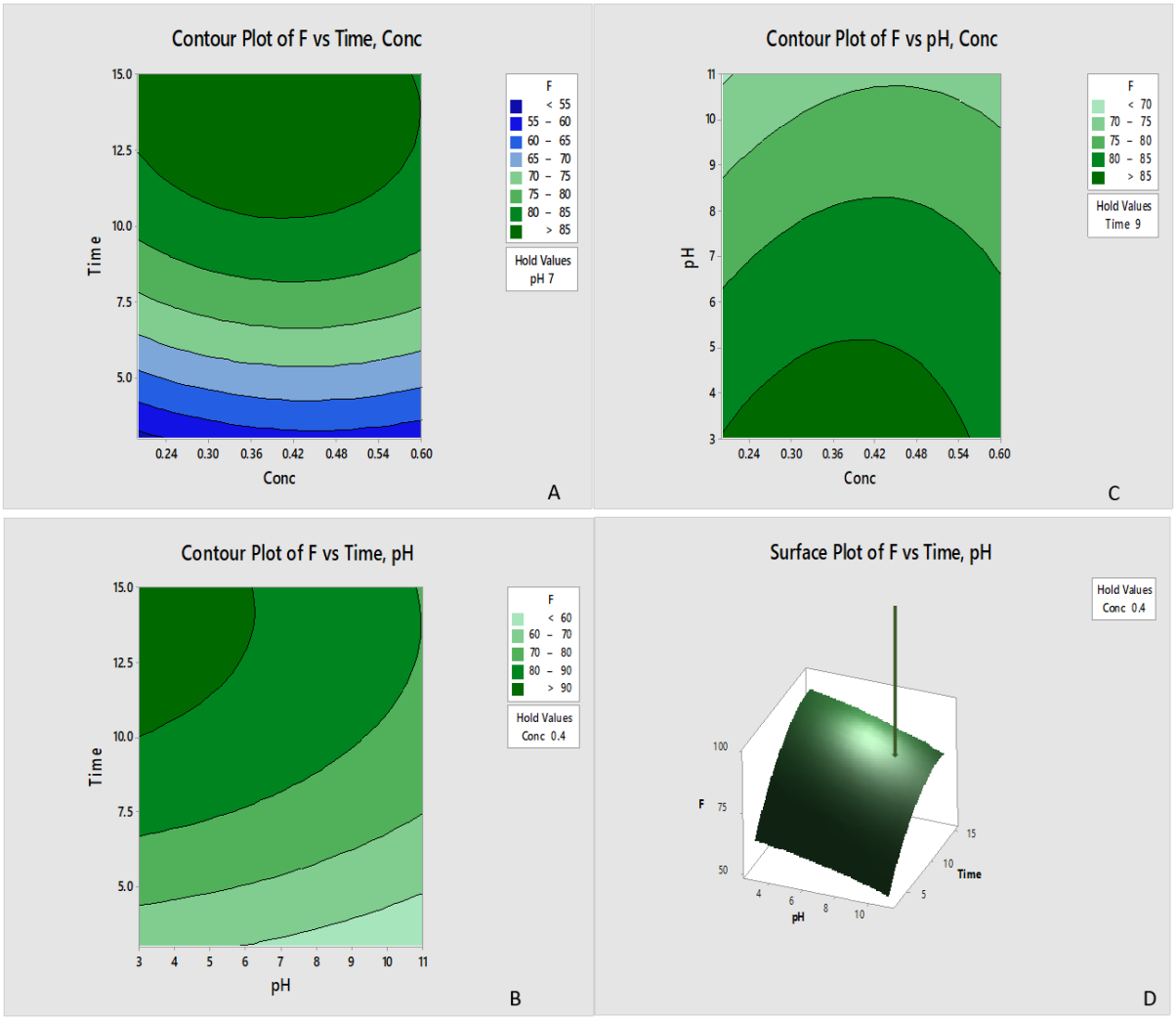


Figure 68: Contour and surface plots showing the synergic effects of independent factors on the responses of *C. vulgaris*.

The dark green area on the contour and the arrow on the surface plots show the domain of maximum flocculation

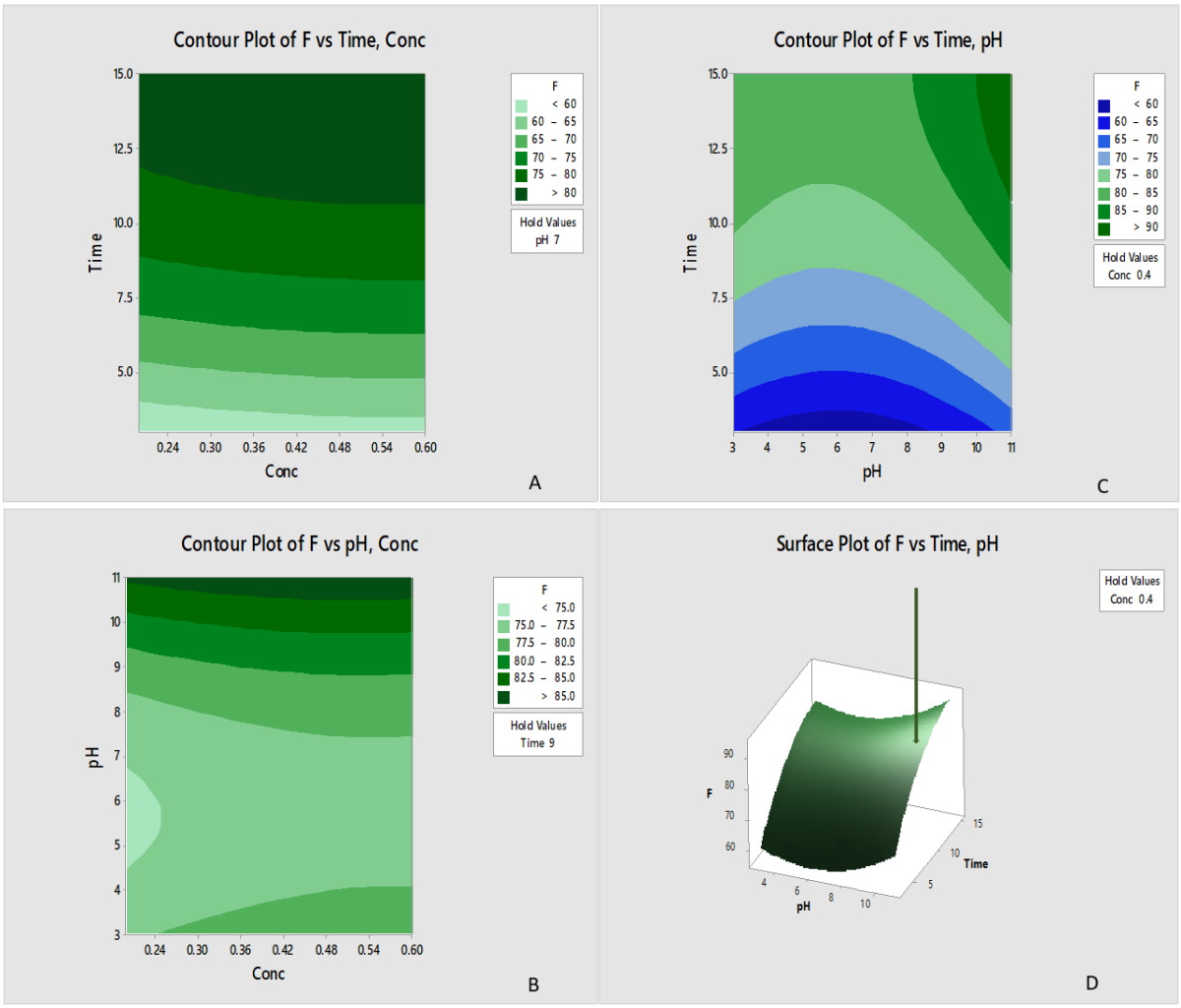


Figure 69: Contour and surface plots showing the synergic effects of independent factors on the responses of *N. oculata*.

The dark green area on the contour and the arrow on the surface plots show the domain of maximum flocculation

4.4 Disruption and lipid extraction from wet algae samples cultured in PBR

High temperature generated during algae cell disruption using mechanical options can alter the chemical composition and structure of the bioproducts. The Tissue-lyser II is easy to use and can operate mildly in ambient conditions but has been underutilised for microalgae cell disruption. This section is aimed at investigating and comparing the effects of algae cell rupture and subsequently lipid extraction of two selected wet microalgae slurries; *C. vulgaris* and *N. oculata* using Tissue-lyser II after cell wall weakening via osmotic shock. The protocol employed was detailed in sections 3.4.1 to section 3.4.4.

4.4.1 Effects of Tissue-lyser operation time on disruption efficiency and lipid yields

10 mg of microalgae slurries suspended in 3 ml of 10 % salt solution was rigorously homogenised between 5 to 30 minutes inclusive at 5 minutes intervals using the Tissue-lyser II. Since the same agitation frequency and volumetric bead filling was used at each time, the total kinetic energy for each bead should be equal, under the assumption that all beads acquire the same agitation speed [305]. Keeping other parameters constant, the effect of operation time on disruption rate was observed to be significant. Results obtained after bead shaking were fitted into polynomial functions as shown in Figure 70, resulting in R^2 values over 0.99 in both species, expressing the suitability of the model. It can be observed that *C. vulgaris* disrupts more than the *N. oculata*. Both species considered in this study had already exhibited structural and morphological dissimilarities presented in literature. For instance, strut and cell wall extensions which are found present in *N. oculata* are absent in *C. vulgaris*, suggesting a stronger cell wall structure and higher resistance to shear damage. Results showed that in both species, the trend of disruption efficiency increased more in the first 20 minutes before a decrease in steep in 25 and 30 minutes. This could be because at a certain stress intensity exacted on microalgae cell wall via agitation, additional increase in stress intensity cannot yield much disruption and lipid extraction measured earlier. This is synonymous to what happens in the rate of a chemical reaction that is rapid in early reaction stage and depreciates with time. After 30 minutes of treatment, disruption efficiencies of 82.5±0.55 % and 60.6±3.75 % were obtained from *C. vulgaris* and *N. oculata* respectively. For the lipid extraction process, result showed that homogenising osmotically shocked microalgae culture enhanced lipid yields. The lipid extracted at different treatment time

intervals using the Tissue-lyser were all higher than that extracted via osmotic shock only which served as control (Figure 71). However, more lipids were extracted from *C. vulgaris* (27.7±0.6 %) than *N. oculata* (18.2±0.6 %) after 30 minutes of agitation, suggesting higher lipid accumulation under the same growth conditions and/or less compacted cell wall matrix leading to weaker cell wall of the former.

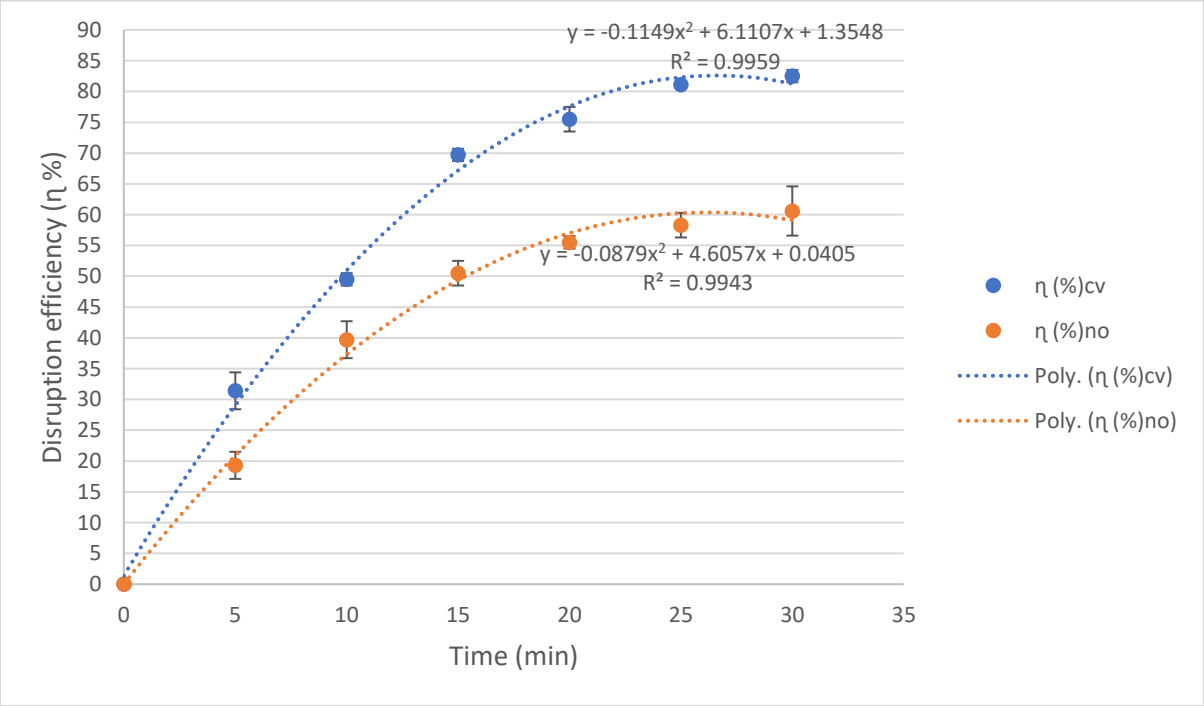


Figure 70: Comparison of cell wall disruption efficiencies of wet *C. vulgaris* and *N. oculata* homogenised at different times using Tissue-lyser II.

The results show *N. oculata* to be more resistant to mechanical rupture than *C. vulgaris*, which is consistent with findings reported in [210, 306]

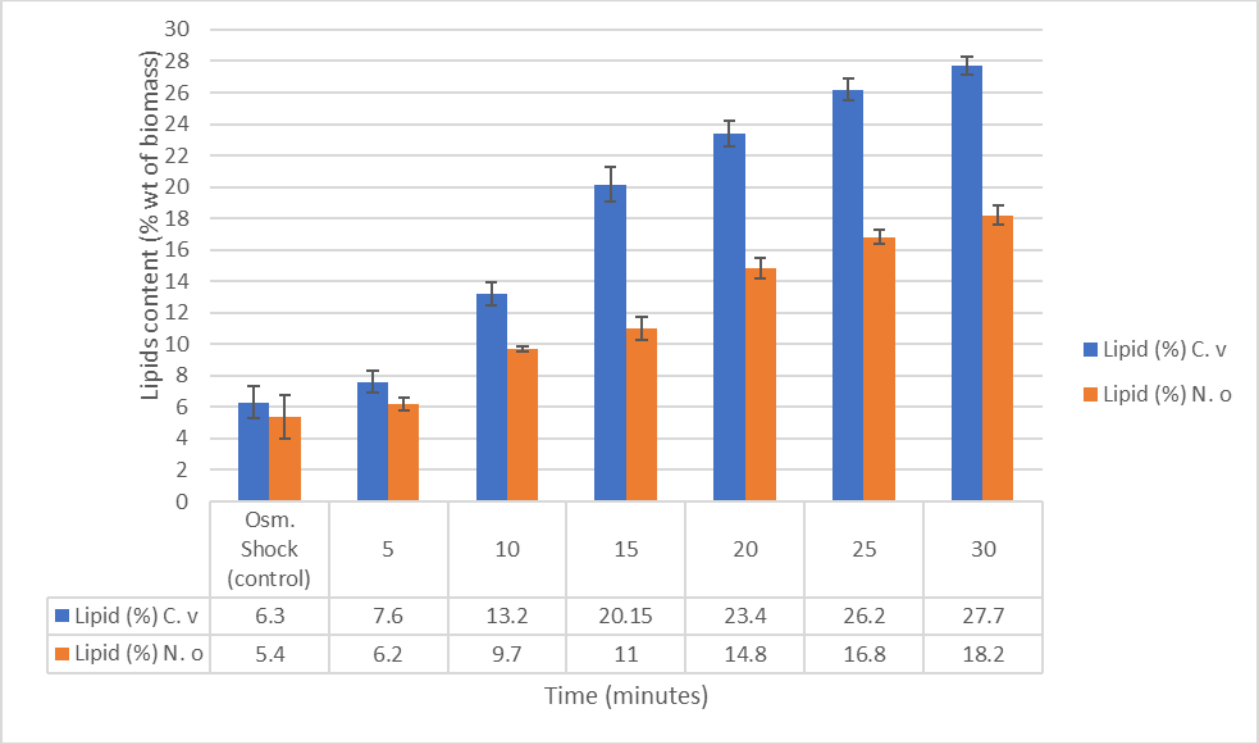


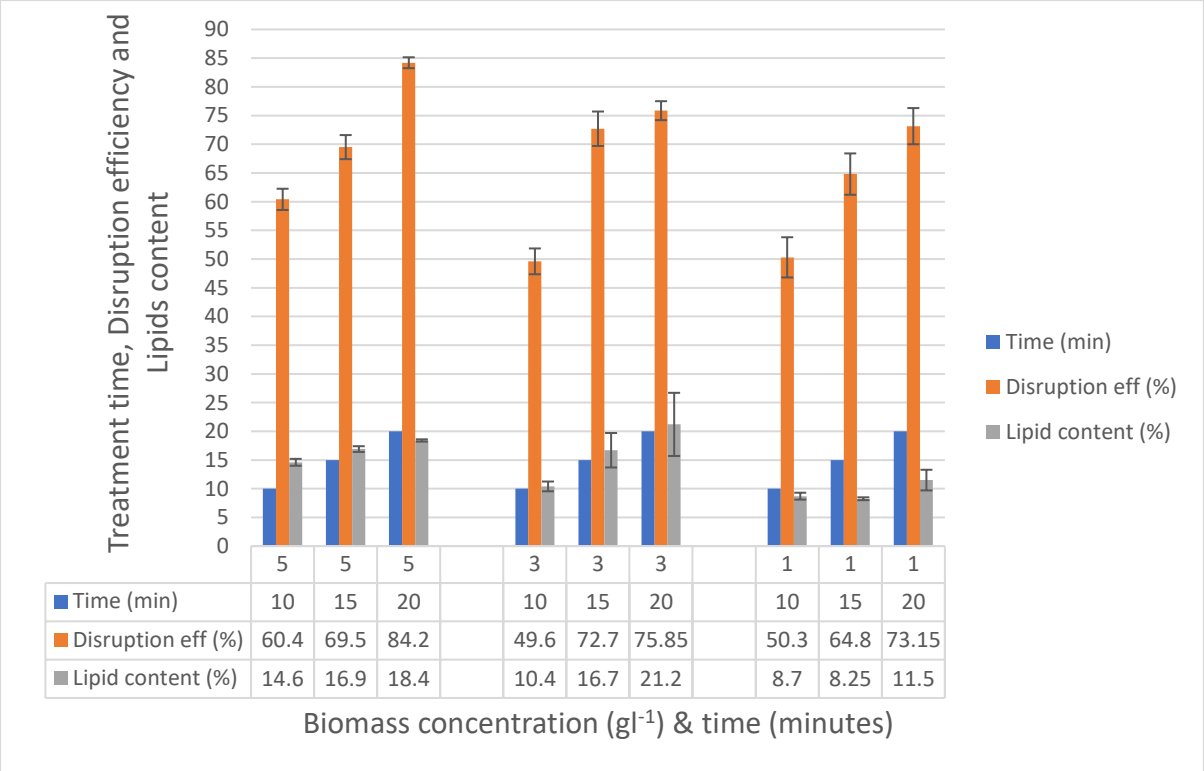
Figure 71: Comparison of lipid extracted from wet *C. vulgaris* and *N. oculata* homogenised at various times 5, 10, 15, 20, 25 and 30 minutes via Tissue-lyser II after cell wall weakening by osmotic shock.

Lipid extracted under osmotic shock serves as control. Error bars represent the standard deviation of duplicate experiments.

4.4.2 Effect of biomass concentration on disruption efficiency and lipid extraction

The effects of biomass concentration on cell disruption and lipid extraction were examined. Different concentrates/slurries (5, 3 and 1 gl^{-1}prepared as described earlier) of the two selected microalgae species were homogenised in batches for 10, 15 and 20 minutes, and results obtained from both species are displayed in Figures 72 and 73 for *C. vulgaris* and *N. oculata* respectively. It was observed that disruption efficiency and percentage of lipids extracted are species dependent, as both species considered in this study showed degrees of diverse results when subjected to the same treatment. This obviously could be attributed to differences in cell wall matrices, morphology, cell tensile strength and lipid accumulation during cultivation. Overall, the effects of biomass concentration and treatment time are significant in both species. High values of biomass concentration resulted in high disruption efficiency, followed by higher lipid yields. More so, the volume of lipids extracted was seen to be a function of the disruption efficiency, as higher value of disruption efficiency reflected in the lipid yields. Observably, in all the biomass concentration considered in this study, there

was a proportionate increase in disruption efficiency and lipid extracted with operation time. Thus, optimal cell disruption and lipid extraction were observed on treating various slurries for 20 minutes. Results obtained from *C. vulgaris* after 20 minutes of agitations revealed that; the maximum disruption efficiencies of 84.2±1.85 %, 75.85±2.1 % and 73.15±0.3 %, followed by lipid yields of 28.4±0.2 %, 21.2±5.5 % and 11.5±1.8 % were obtained by agitating biomass concentration equivalent to 5 gl^{-1}, 3 gl^{-1} and 1 gl^{-1} respectively. For *N. oculata*, the maximum disruption efficiencies of 55.9±5.7 %, 54.9±3.2 % and 44.9±3.2 %, followed by lipid content of 19.4±0.2 %, 13.2±5.5% and 9±1.8 % respectively.



	5	5	5		3	3	3		1	1	1
Time (min)	10	15	20		10	15	20		10	15	20
Disruption eff (%)	60.4	69.5	84.2		49.6	72.7	75.85		50.3	64.8	73.15
Lipid content (%)	14.6	16.9	18.4		10.4	16.7	21.2		8.7	8.25	11.5

Figure 72: Comparison of disruption efficiency and lipid yields from *C. vulgaris*
The blue bars represent the time variables of 10, 15 and 20 minutes.
Increasing treatment time increases energy inputs. Orange and ash bars represent disruption efficiency and lipid content corresponding to time.

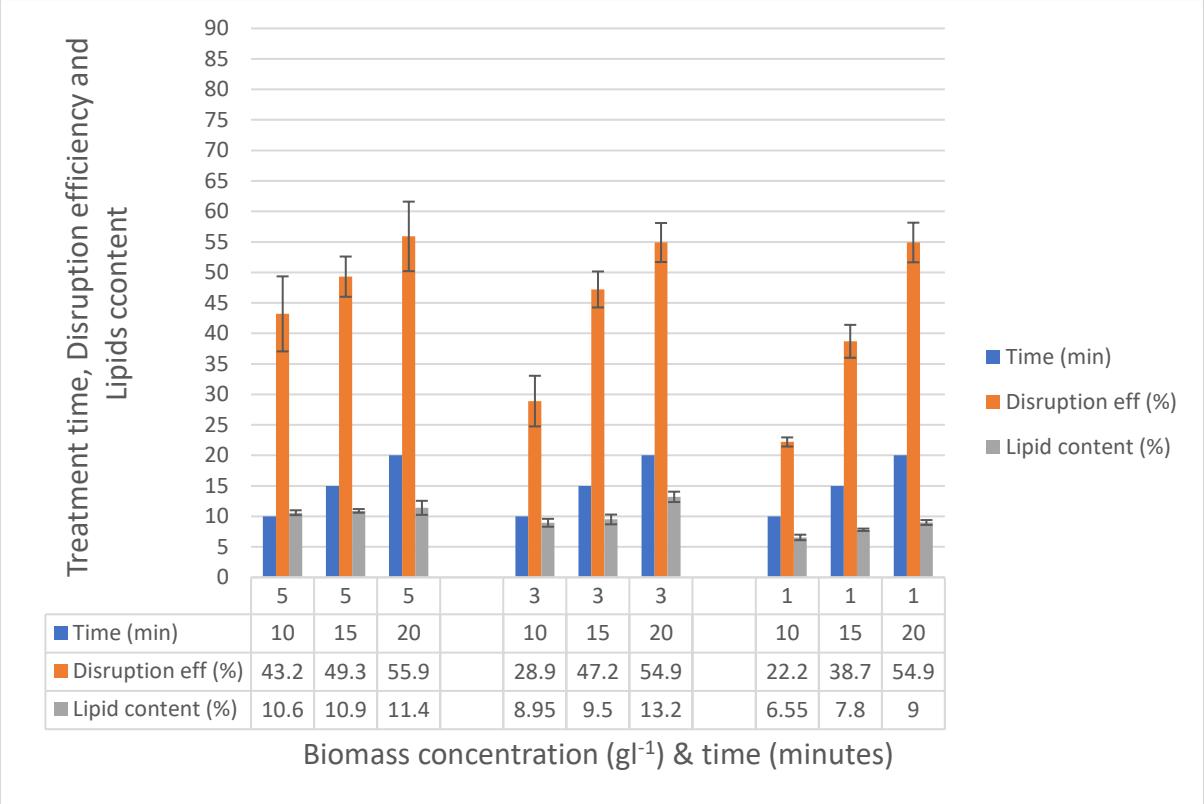


Figure 73: Comparison of disruption efficiency and lipid yields from wet *N. oculata*
The blue bars represent the time variables of 10, 15 and 20 minutes.
Increasing treatment time increases energy inputs. Orange and ash bars represent disruption efficiency and lipid content corresponding to time. The chart indicates increasing disruption efficiency and lipid extracted with increasing operation time. The influence of biomass id insignificant after 20 minutes operation time.

4.4.3 Effects of Tissue-lyser frequency on disruption efficiency and lipid yields

The effects of Tissue-lyser oscillatory frequency on microalgae cell wall disruption and lipid extraction were also examined. Results revealed a significant effect of frequency on the parameters examined. Cell disruption and lipid extraction efficiencies increase with increasing frequency (10, 20 and 30 Hz). This effect can be explained by the increase of the impact forces and speed associated with increasing frequency. The optimal cell disruption efficiency and lipid extraction can be observed at a highest frequency of 30 Hz as shown in Figure 74. At the optimal conditions, disruption efficiencies and lipid yield of 74.6±2.4 %, 18.4±0.1 % and 62.45±1.85 %, 11.4±0.8 % were derived from *C. vulgaris* and *N. oculata* respectively. However, no significant difference was found for lipid extraction on agitating culture at frequencies of 20 and 30 Hz.

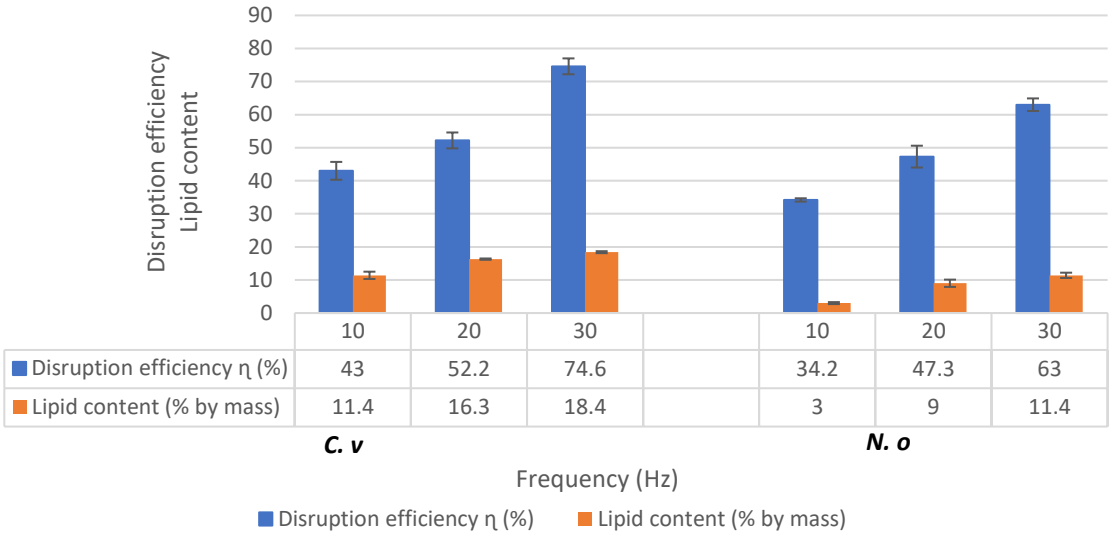


Figure 74: Showing the effects of frequency on disruption efficiency and lipid extracted from wet *C. vulgaris* and *N. oculata*

4.4.4 Specific energy consumption

The energy required in rupturing microalgae cells and extracting intracellular lipids can significantly contribute to the cost of biodiesel production. Also, most mechanical techniques of cell disruption are prone to heat dissipation, hence cooling became necessary. But in combining osmotic shock and Tissue-lyser, no temperature issues were observed as all disruption experiments were conducted in ambient conditions, and disruption efficiency followed by percentage lipid yields were derived using Equations 1,2 and 3. Observably, treatments yielding high disruption efficiency tend to release more lipids, signifying the influence of cell wall disruption on lipid yields. Since disruption efficiency varies proportionately with lipid extraction, both disruption and lipid extraction can be assumed to concur to similar energy trend. Table 17 provides the overview of the influence of biomass concentration and operation time on disruption efficiencies of *C. vulgaris* and *N. oculata*.

The estimated energy consumed is simply the product of Tissue-lyser power rating and treatment/operation time. The power rating of Tissue-lyser from the technical data is 150 w which is multiplied by various treatment/operation times (10, 15 and 20 minutes) to evaluate the energy consumed in each batch of disruption experiment with respect to biomass concentration. Thus, a linear increase in the energy consumption was observed with respect to treatment time. Specific energy which is the energy consumed in disrupting a unit mass of microalgae biomass was estimated for both species considered in this study.

Table 17: Overview of the influence of biomass concentration and operation time on disruption efficiencies of *C. vulgaris* and *N. oculata*

Algae	Biomass concentration m(g/l)	Operation time (min)	Energy consumed E = Pt (MJ)	Specific energy E/m (MJ/g)	Disruption efficiency η (%)
C. vulgaris	5	10	0.09	9	60.35±1.85
		15	0.135	13.5	69.5±2.1
		20	0.18	18	84.15±0.95
	3	10	0.09	15	50±2.25
		15	0.135	22.5	73±3
		20	0.18	30	76±1.65
	1	10	0.09	45	50.3±3.5
		15	0.135	67.5	65.4±3.6
		20	0.18	90	73.15±3.15
N. oculata	5	10	0.09	9	43.15±6.15
		15	0.135	13.5	49.3±3.3
		20	0.18	18	55.9±5.7
	3	10	0.09	15	22±4.15
		15	0.135	22.5	48±3
		20	0.18	30	55±3.2
	1	10	0.09	45	22.15±0.75
		15	0.135	67.5	40±2.7
		20	0.18	90	44.9±3.3

P = Tissue-lyser power rating 150 w gotten from Tissue-lyser II technical data sheet.
The working volume of culture throughout the experiment is 2 ml, representing biomass concentrations and its equivalent of 5 gl^{-1} (10 mg/2ml), 3 gl^{-1} (6 mg/2ml) and 2 gl^{-1} (2 mg/2ml)
Energy E = Power multiplied by treatment time. Specific energy is the energy E per unit mass present in the working volume.

Figure 75 compares the disruption efficiencies on agitating equal mass of both algae species. It shows the influence of biomass concentration on disruption efficiency as a function of specific energy. Results showed that 1) specific energy increases with decreasing biomass concentration, which agrees with a study reported by Postma et. al [305] 2) effect of biomass concentration was observably insignificant on agitating 1 gl^{-1} *N. oculata* for 20 minutes 3) energy utilisation is higher in *C. vulgaris* than *N. oculata*, as higher disruption efficiency was observed

at the same specific energy across board, indicating that under similar disruption process, disruption efficiency is species dependent.

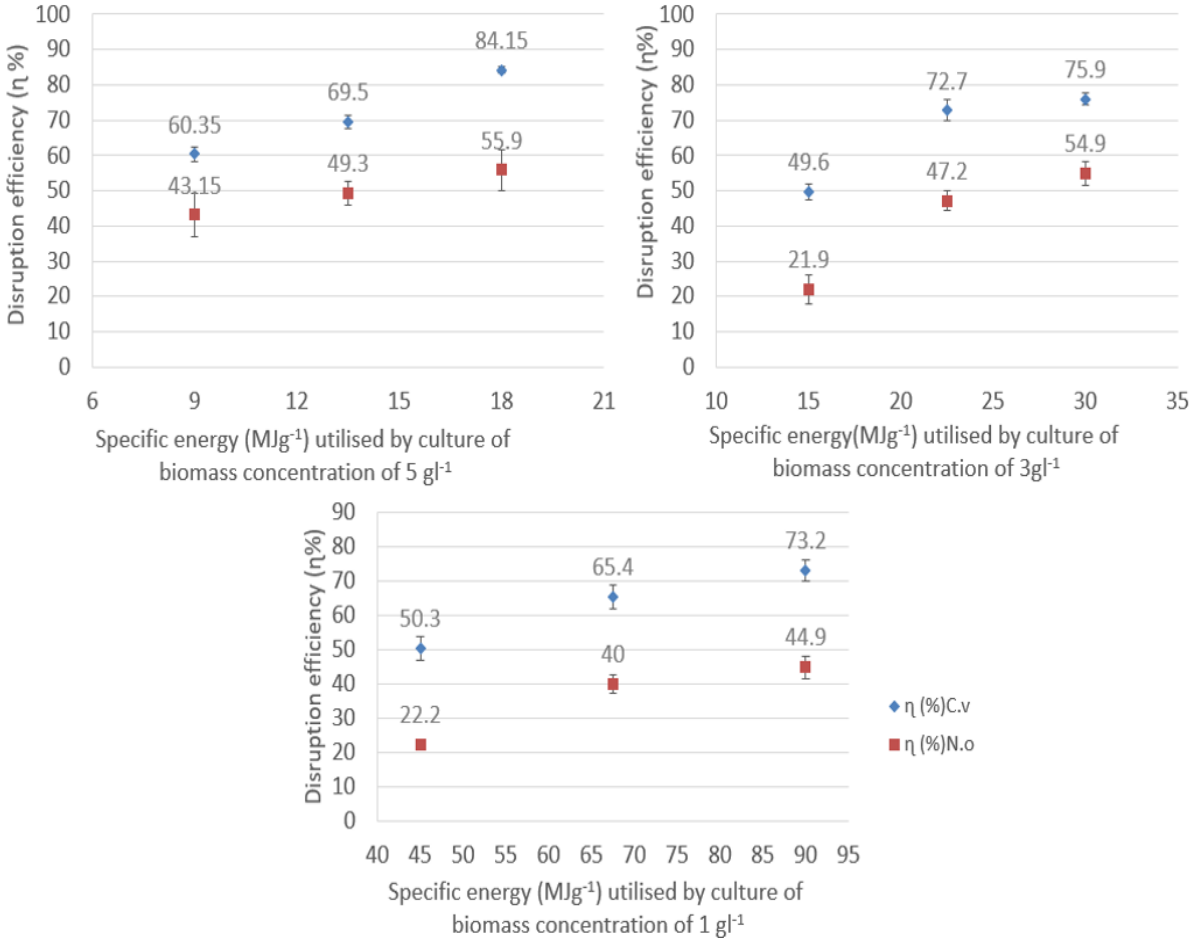


Figure 75: Comparative plot of disruption efficiency as a function of specific energy at different biomass concentrations of both algae species.

Graphs show that in each biomass concentration 1) *C. vulgaris* utilised more energy resulting in higher disruption efficiency than *N. oculata* 2) disruption efficiency increases with increasing specific energy 3) The optimal disruption efficiency of over 80 % was observed in the culture with the highest biomass concentration of $5gl^{-1}$ 4) Specific energy increases with decreasing biomass concentration.

4.5 Disruption and lipid extraction from dry algae samples cultured in PBR

4.5.1 Synergistic effects of cell disruption techniques and carbon sources on lipid yields from dry algae samples

Although, studies showed that microalgae lipid content range between 4 to 75 % dry weight [307], and the ranges of lipid contents of *C. vulgaris* (28-58% wt of dry biomass) and *N. oculata* (23 – 30% wt of dry biomass) have been recorded in literature [308], microalgae lipid content/yield depends on microalgae species, mode of cultivation, type of nutrients utilised, and cell disruption method applied. Various cell disruption techniques were employed on dry algae samples to identify the most effective and energy efficient method. Figure 76 shows a microscopic view of the cell walls of the untreated and treated algae using sonicator and Tissue-lyser. The untreated biomass cells show a definite shape, circular and uniform distribution, whereas the treated biomass appeared to be shrunken, crumpled and tattered; this indicates that biomass cells and the intracellular matter have been exposed to harsh conditions which damaged the cell wall to facilitate lipid yield. More cell rupture was observed in the cells treated using sonicator than Tissue-lyser. Disrupted cells in tissuelyser treated biomass seem to be more dispersed because of additional water used in washing the beads after treatment, which provided a more diluted suspension.

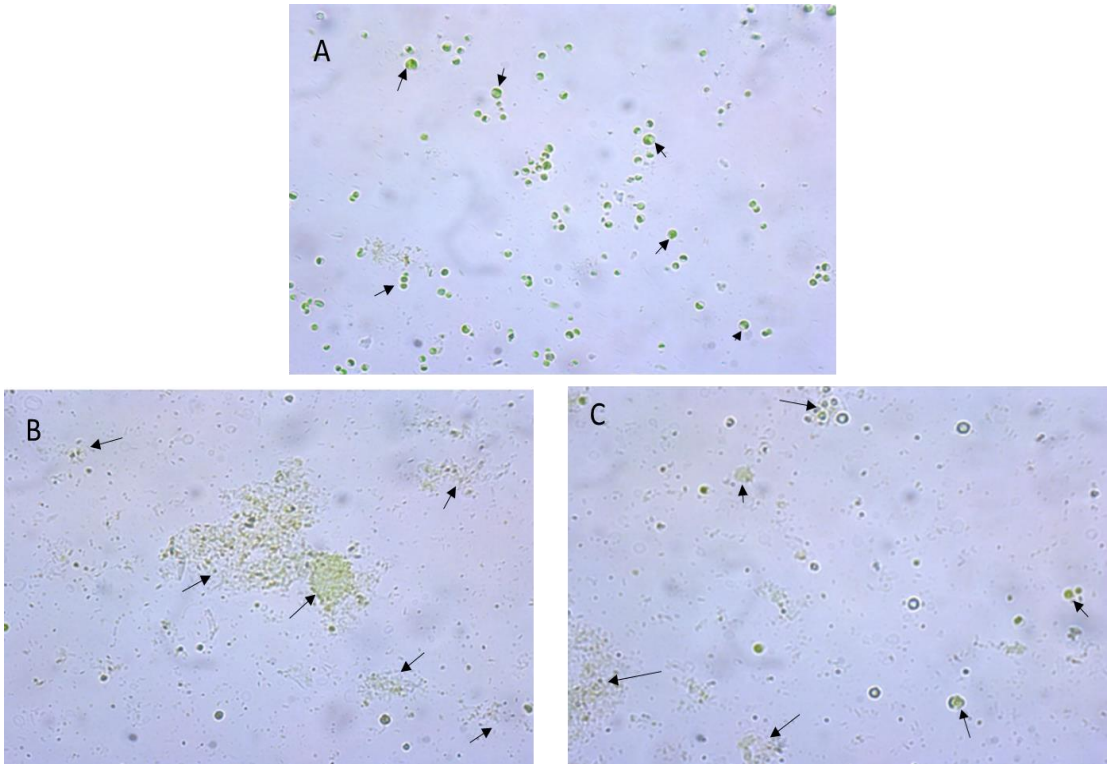
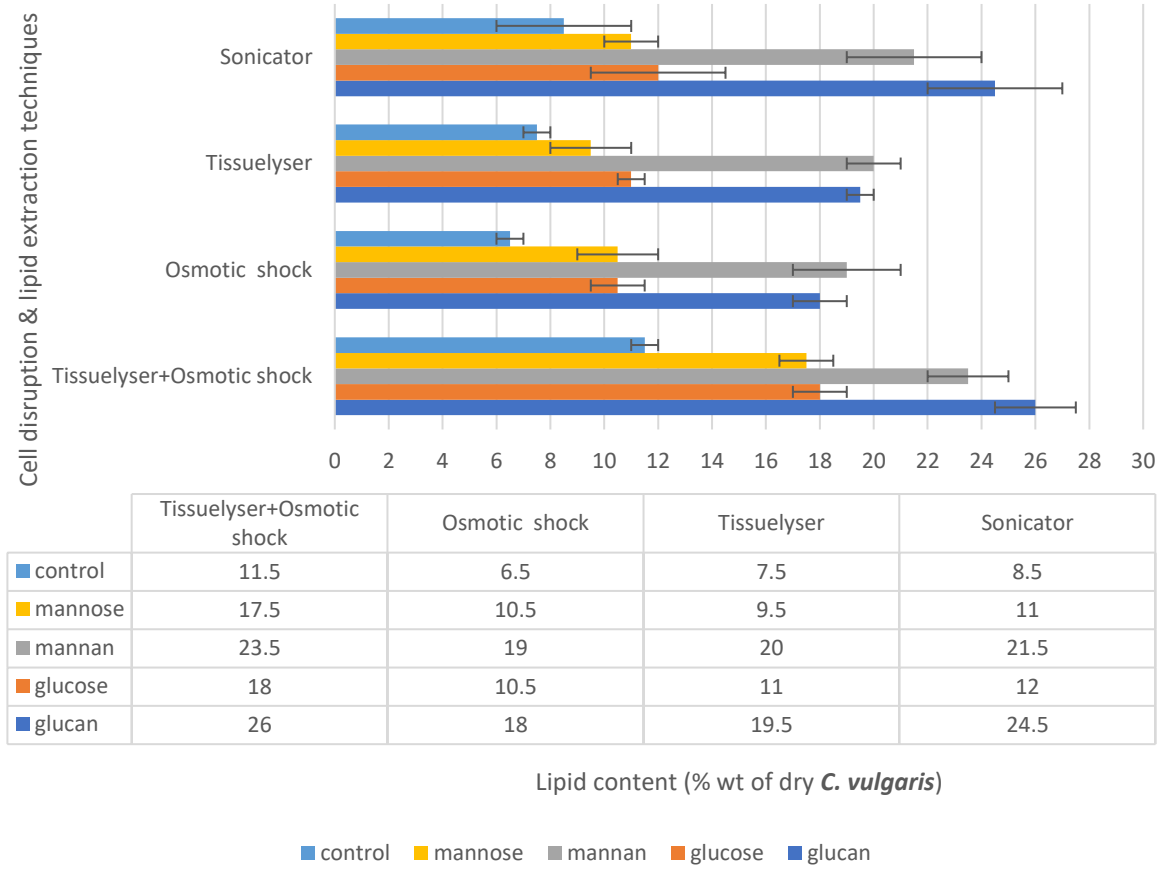


Figure 76: Cell morphology before and after disruption
(A) Intact (untreated) cells (B) Disrupted cells by sonication utilising Soniprep 150 plus (C) Disrupted cells by agitating zirconium oxides beads using the Tissue-lyser II. Arrows in A are pointing whole cells whereas arrows in B and C are pointing at disrupted cells.

Different cell disruption methods used in the study were able to disrupt macroalgae cells, as lipid yields varied with disruption methods utilised. Figures 77, 78 and 79 show varying lipid contents with respect to the carbon sources utilised and cell disruption method applied. Also, Table 18 shows a numerical display of the comparison of lipid content based on parameters. All statistics are reported in a 95 % confidence level ($p < 0.05$). Mean values were estimated, and error bars represent the standard deviation from duplicate measurements obtained on repeating experiments twice.

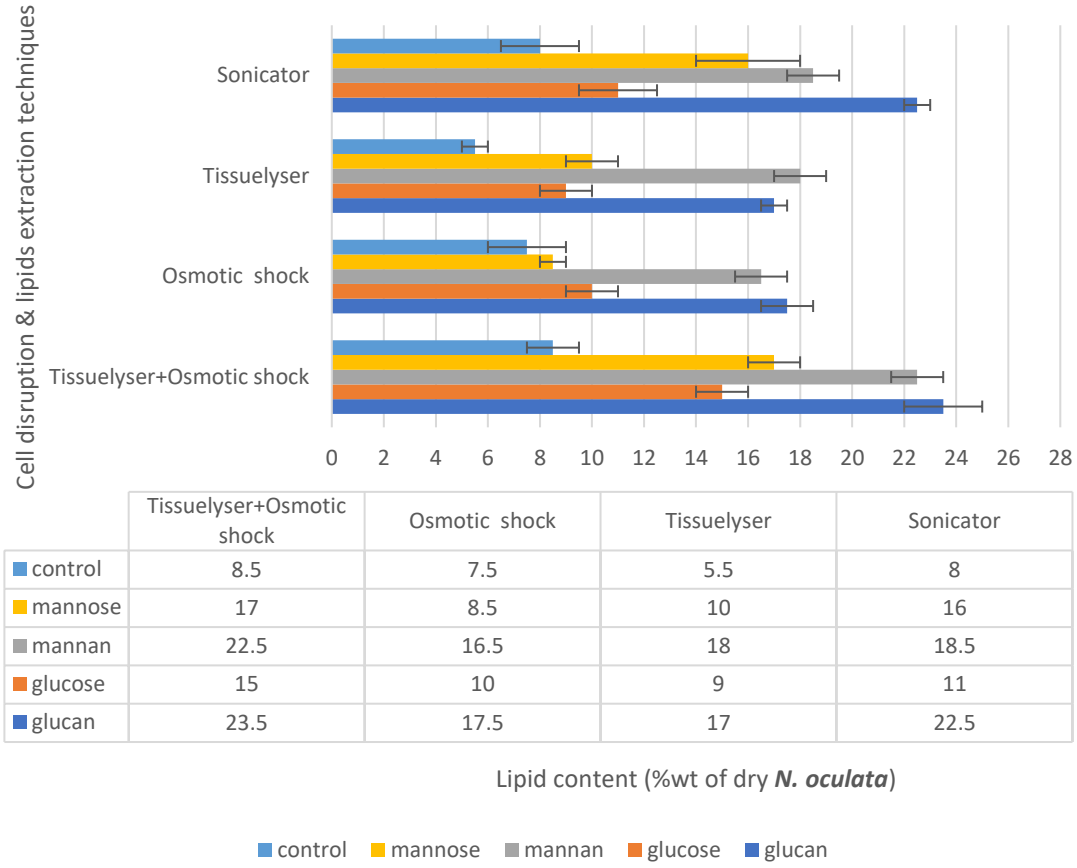


	Tissuelyser+Osmotic shock	Osmotic shock	Tissuelyser	Sonicator
control	11.5	6.5	7.5	8.5
mannose	17.5	10.5	9.5	11
mannan	23.5	19	20	21.5
glucose	18	10.5	11	12
glucan	26	18	19.5	24.5

Figure 77: Lipid content of dry biomass of *C. vulgaris* cultivated under mixotrophic mode of cultivation

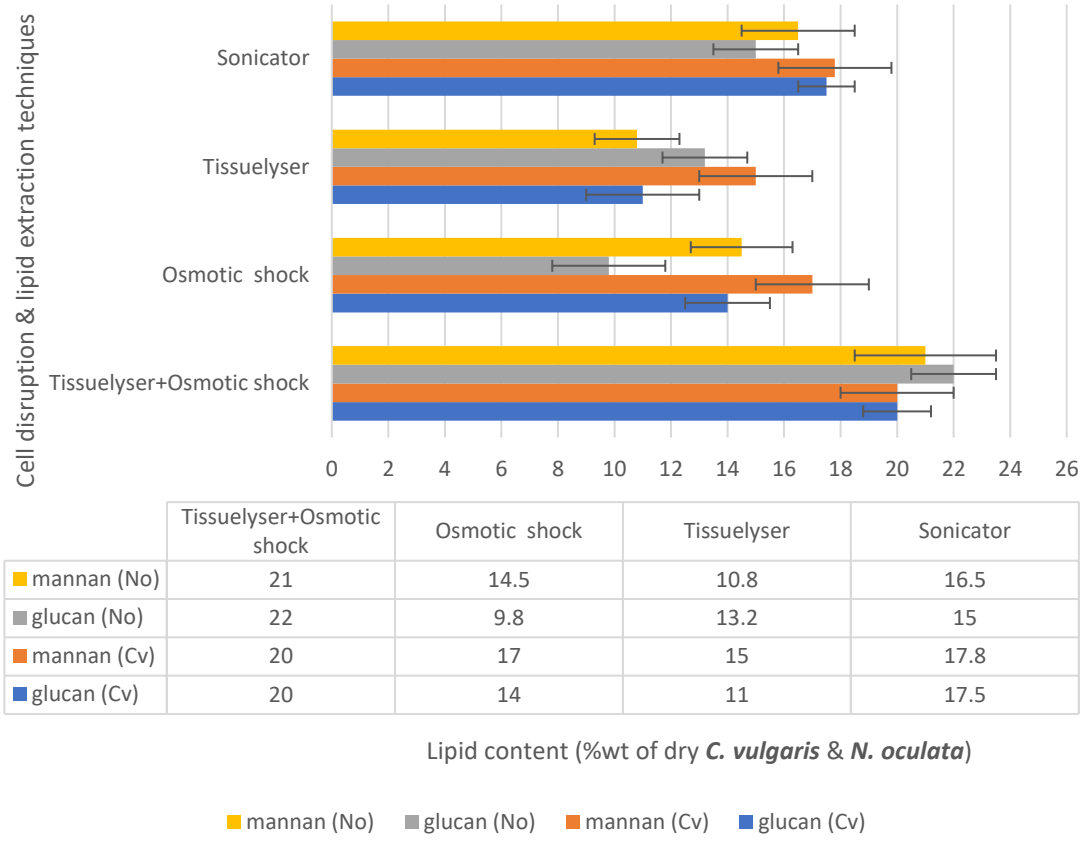
Dissimilar percentages of lipids were extracted because 1) cells were grown in media supplemented with different carbon sources and 2) cells were disrupted/treated with different techniques. Experiments were repeated twice, and Readings were plotted as mean± standard deviation. The mean is the average whereas standard deviation is the variation of the mean value (error bars) of both readings.

The outcome of the lipid extraction process has been briefly discussed in section 3.1 of this report. Sonication and osmotic shock plus Tissue-lyser generated high lipid yields from samples grown under mixotrophic cultivation using β-glucan as carbon source. Results revealed that 24.5±1.5 % and 26±3 % by weight of dry *C. vulgaris* and 22.5± 1.5 % and 23.5±0.5 % by weight of dry *N. oculata* respectively were obtained on treating cells with both techniques. Figure 80 shows a schematic picture of some of the lipids extracted in this study.



	Tissuelyser+Osmotic shock	Osmotic shock	Tissuelyser	Sonicator
control	8.5	7.5	5.5	8
mannose	17	8.5	10	16
mannan	22.5	16.5	18	18.5
glucose	15	10	9	11
glucan	23.5	17.5	17	22.5

Figure 78: Lipid content of *N. oculata* dry biomass grown under mixotrophic mode of cultivation. Readings are represented as mean ± standard deviation



	Tissuelyser+Osmotic shock	Osmotic shock	Tissuelyser	Sonicator
mannan (No)	21	14.5	10.8	16.5
glucan (No)	22	9.8	13.2	15
mannan (Cv)	20	17	15	17.8
glucan (Cv)	20	14	11	17.5

Figure 79: Comparison of Lipid content of *N. oculata* and *C. vulgaris* dry biomasses grown under photoheterotrophic mode of cultivation. Readings are represented as mean ± standard deviation

Table 18: Lipid content (%wt of dry biomass) of ***C. vulgaris*** and ***N. oculata*** under various cell disruption methods, and photoheterotrophic and mixotrophic modes of cultivation using various carbon sources

Microalgae species Carbon sources	Mode of cultivation	Sonication	Osmotic shock	Tissuelyser	Osmotic shock + Tissuelyser
C. vulgaris					
β-glucan	Mixotrophic	24.5±1.5	18 ±1	19.5 ±1.5	26±3
	Photoheterotrophic	17.5±1.2	14±2	11±1.5	20 ±1
Glucose	Mixotrophic	12±1	10.5± 0.5	11±1	18±2.5
C. vulgaris					
β-mannan	Mixotrophic	21.5±1.5	19 ±1	20 ±2	23.5±2.5
	Photoheterotrophic	17.8±1.6	17±1.5	15± 2	20±3.5
Mannose	Mixotrophic	11±1	10.5±1.5	9.5 ±1.5	17.5±1
C. vulgaris					
Control	Mixotrophic	8.5±0.5	6.5±0.5	7.5 ±0.5	11.5±2.5
N. oculata					
β-glucan	Mixotrophic	22.5±1.5	17.5±0.5	17 ±1	23.5±0.5
	Photoheterotrophic	15±1.5	9.8±1.5	13.2±2	22.5±1.5
Glucose	Mixotrophic	11±1	10 ±1	9 ±1	15±1.5
N. oculata					
β-mannan	Mixotrophic	18.5±0.5	16.5±0.5	18 ±2	22.5±1.5
	Photoheterotrophic	16.5±2.5	14.5±1.5	10.8± 1.8	21±2
Mannose	Mixotrophic	16±1	10.5± 0.5	10 ±1	17±2
N. oculata					
Control	Mixotrophic	8±1	7.5±0.5	5.5 ±1.5	8.5±1.5

4.5.2 Specific energy analysis of various cell disruption techniques

Energy (product of power rating and operation time) consumed by each cell disruption methods (sonication, osmotic shock, Tissue-lyser and osmotic shock plus Tissue-lyser) was studied. The comparative estimated energy consumption and processing times of each method is presented in Table 19. Specific energy (energy consumed per unit mass of biomass) was introduced to access the relative merit of each treatment. Applying the sonication method using an ultrasonic processor Soniprep 150, MSE, UK) rated 240 V AC, 5A, 50–60 Hz, giving 20 kHz ultrasonic wave frequency for 10 minutes, consumes 0.72 MJ of energy. Homogenising suspended microalgae using a Tissue-lyser (Qiagen Inc-USA rated 240 V AC, 50–60 Hz, 150 W; see operational manual) attached with a tube 60 % filled with zirconium oxide beads of diameter 0.5 mm at a frequency of 20 Hz for 10 minutes consumes estimated energy of 0.09 MJ. Osmotic shock has been adjudged a moderate energy consumption

method (4.8 $MJkg^{-1}$) [309]. The combination of (osmotic shock + Tissue-lyser) is estimated to have consumed 0.09 MJ, which is quite low when compare with energy expended by sonication. Comparing the energy expended in each process at the same operation time, the energy consumed by Soniprep 150 plus is 8-folds higher than that consumed by the Tissue - lyser plus osmotic shock. This energy margin is comparatively high. Overall, energy estimates showed that the use of osmotic shock plus Tissue-lyser for microalgae cell wall disruption is more energy efficient and cost effective than Soniprep 150 plus. The application of all these techniques will not 1) change the quality of lipid fraction, 2) reduce the amount of extraction solvent used and 3) operate at a controllable or room temperature [44].

Table 19: Energy consumption comparison of various cell disruption methods

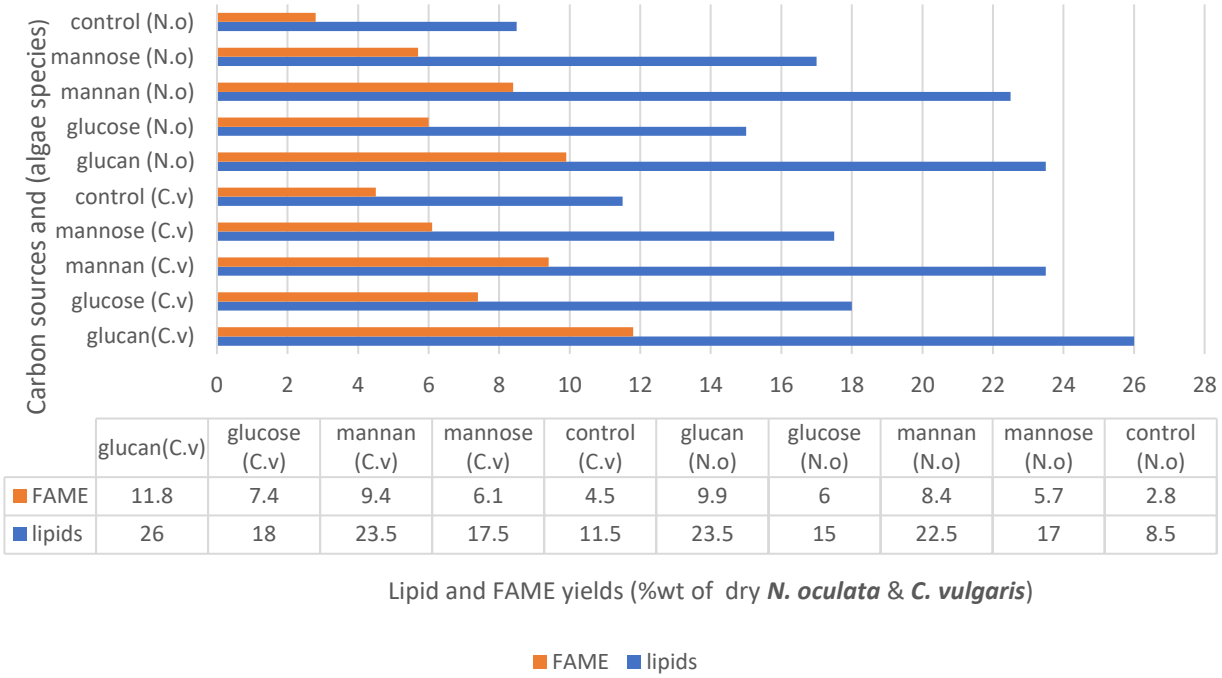
Microalgae disruptor	Disruption mechanism	Power ratings	Processing Time(mins)	Energy Consumed (MJ)	Specific energy (MJ/g of dry biomass)	Optimal lipids extracted %wt biomass
Sonication Soniprep 150	Shock waves resulting from microbubble implosion	240 v, 5A (1200 w)	10	0.72	7.2	24.5
Tissue-lyser	Collision and grinding effects of zirconium oxide beads 0.5 mm diameter	150 w	10	0.09	0.9	20
Osmotic shock	Osmotic pressure from lysate solution during diffusion across cell walls	n/r	2,880 (48 hrs)	0.00048	0.0048	19
Osmotic shock + Tissue-lyser	Descriptions as above	n/r + 150w	48 hrs + 10 mins	0.09048	0.9048	26

Energy = power rating (Wat) multiplied by time (seconds).
Specific energy = energy consumed per unit mass of dry biomass utilised.
Osmotic shock energy rating was determined from Morse equation as used in [309, 310]
n/r: not rated in watt

4.5.3 Experiment to compare the effects of carbon sources on biodiesel (FAME) production

Crude lipids extracted from microalgae consist of neutral, polar, and non-fatty acid, other biomolecules, and contaminants. Only neutral or nonpolar lipids such as TAGs and free fatty acids are saponifiable, that is, can be converted into biodiesel (FAME) via the transesterification process, as the non-saponifiable biomolecules remain in solution. The catalyst used for transesterification can be acid, base, or enzyme. Base/alkaline (NaOH, KOH) is commonly used because of its high biodiesel conversion efficiency at low temperature and pressure [286]. The FAME yield (% wt of dry biomass) and percentage of saponifiable lipids from dry biomass cultivated using different carbon sources are shown in Figure 80 and Table 20. The results indicate that maximum FAME yields were obtained from the extracts of both microalgae species cultivated in the BG 11 supplemented using complex sugars as carbon sources. For *C. vulgaris*, a maximum FAME yield of 11.8 ± 1.2 and 9.4 ± 1.6 %wt, and saponifiable lipid of 45 and 40% were obtained from dry biomass cultivated mixotrophically using beta-glucan and beta-mannan respectively. Likewise, a maximum FAME yield of 9.9 ± 0.9 and 8.4 ± 1.2 %wt of dry *N. oculata* biomass, and saponifiable lipid of 42 and 37% were obtained under the same condition as stated above. This result clearly indicates that over 50% of the total lipids extracted is non-saponifiable. This may be due to the dominance of other non-saponifiable biomolecules like polar lipids, non-fatty acid (ketones, chlorophyll pigments, proteins) and some contaminants in the biomass. Further studies of metabolic pathways of complex sugar and application of genetic engineering may be deployed to enhance the yield of saponifiable lipid (non-polar or neutral lipid) which is a biodiesel precursor. Also, it was observed that complex sugars synthesize more saponifiable lipid when juxtaposed with simple sugar, hence can be better carbon sources for the cultivation of microalgae for biodiesel production.

Notably, microalgae biomass growth rate and lipid accumulation are not only species dependant but can be influenced by so many other factors such as growth conditions and lipid extraction methods. Hence comparing results or outcomes of a research to others is difficult. However, results obtained (biomass productivity up to 0.3 $gl^{-1}d^{-1}$ and lipid yields over 20 % per dry biomass) fall within the range seen in most literatures.



	glucan(C.v)	glucose (C.v)	mannan (C.v)	mannose (C.v)	control (C.v)	glucan (N.o)	glucose (N.o)	mannan (N.o)	mannose (N.o)	control (N.o)
FAME	11.8	7.4	9.4	6.1	4.5	9.9	6	8.4	5.7	2.8
lipids	26	18	23.5	17.5	11.5	23.5	15	22.5	17	8.5

Figure 80: Comparison of lipids extracted and FAME yields from *N. oculata* and *C. vulgaris* biomasses cultivated in growth media supplemented with simple sugars and glycans as carbon sources

Table 20: Total lipid content, FAME yield and saponifiable lipid fraction of ***C. vulgaris*** and ***N. oculata*** under mixotrophic modes of cultivation using various carbon sources

Microalgae	Parameters	Control	Glucan	Glucose	Mannan	Mannose
C. vulgaris	Lipid content (%wt of dry biomass)	11.5±2.5	26±3	18±2.5	23.5±2.5	17.5±1
	FAME (%wt of dry biomass)	4.5±0.6	11.8±1.2	7.4±0.8	9.4±1.6	6.1±1.2
	Saponifiable (%wt of lipid)	39	45	36	40	35
N. oculata	Lipid content (%wt of dry biomass)	8.5±1.5	23.5±0.5	15±1.5	22.5±1.5	17±2
	FAME (%wt of dry biomass)	2.8±0.6	9.9±0.9	6±1	8.4±1.2	5.7±0.8
	Saponifiable lipid (%wt of lipid)	33	42	40	37	34

Saponifiable lipid = (ratio of the mass of FAME produced to the mass of lipid content) X 100

4.5.4 Effects of carbon sources on Fatty acid profile (composition)

In this study, the fatty acid (FA) profiles of the investigated microalgae species cultivated using various carbon sources were identified and quantified using gas chromatography with a mass spectrometry detector. Table 21, Figures 81 and 82 show a numerical distribution of percentage and comparison of FA compositions for *C. vulgaris* and *N. oculata* cultivated. Fatty acids of C16 and C18 are typically seen as the foremost components of microalgae biodiesel and are therefore identified within the retention time of 17 – 21 minutes, as shown in the chromatographs displayed in Figure 83 [311].

The influence of carbon sources and microalgae species on FAs is observable. Palmitic acid C16:0 (31 – 53%) and linoleic acid C18:2 (5 – 23%) of different percentage distributions were identified by both algae species cultured in all the carbon sources considered in this study. Palmitoleic acid C16:1 and oleic acid C18:1 was also obtained in different percentages in all species except in *N. oculata* cultivated using mannose and β-glucan as carbon source. Stearic acid C18:0 was only not obtained in *C. vulgaris* cultivated in growth medium supplemented with β-glucan. Noticeably, saturated fatty acids dominated the FAME produced from each species cultivated in simple sugars, whereas the unsaturated fatty acids responsible for FAME fuel properties are found more in the species cultivated using glycans. For *C. vulgaris*, 52 and 61% of saturated fatty acids were obtained from the biomass cultivated in glucose and mannose, meaning that 48 and 39% were unsaturated fatty acids. Culture supplemented with β-glucan and β-mannan yielded 32 and 36% saturated, 68 and 64% unsaturated fatty acids, respectively. For *N. oculata*, 61 and 53% of saturated fatty acids were obtained from biomass cultivated in the media supplemented with glucose and mannose, hence only 39 and 47% are unsaturated. While culture supplemented with β-glucan and β-mannan yielded 55 and 41% saturated fatty acids, 45 and 59% unsaturated fatty acids respectively. This suggests the utilisation of glycans such as β-glucan and β-mannan as better carbon sources for microalgae cultivation for biodiesel production.

Table 21: Fatty acid composition under different microalgae species and carbon sources (%)

Fatty acids	**Molecular weight**	***C. vulgaris*** **Glucose**	**Glucan**	**Mannose**	**Mannan**	***N. oculata*** **Glucose**	**Glucan**	**Mannose**	**Mannan**
C16:0	270	49.2	31.50	52.63	28.95	52.03	44.28	44.26	35.85
C16:1	268	7.55	4.61	9.27	10.42	4.83	4.64	-	9.22
C16:2	266	-	-	-	-	-	19.36	-	-
C18:0	298	3.26	-	8.26	4.02	9.01	10.47	8.69	3.35
C18:1	296	16.36	51.02	10.86	37.12	20.36	-	19.14	32.5
C18:2	294	9.85	5.93	7.76	13.32	13.77	21.26	22.58	10.75
C18:3	292	7.76	4.63	-	1.95	-	-	-	2.16
C20:0	326	-	-	-	2.97	-	-	-	-
C20:2	322	6.03	2.3	11.21	1.23	-	-	5.33	-
C20:4	318	-	-	-	-	-	-	-	1.91
ΣSFAs		52.46	31.5	60.89	35.94	61.01	54.75	52.95	41.11
ΣMUFAs		23.91	55.63	20.13	47.54	25.19	4.64	19.14	45.98
ΣPUFA		23.64	12.86	18.97	16.5	13.77	40.62	27.91	12.91

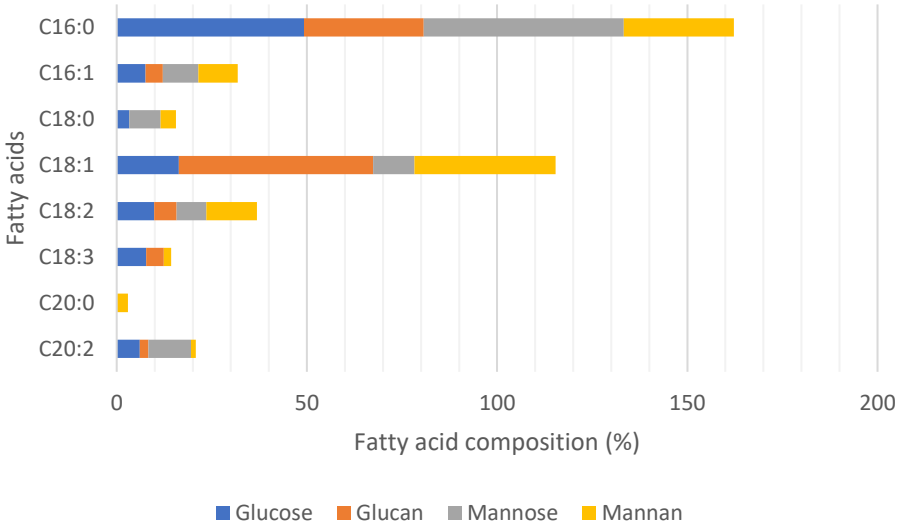


Figure 81: Influence of carbon sources on fatty acid composition in *C. vulgaris* grown in BG 11 medium

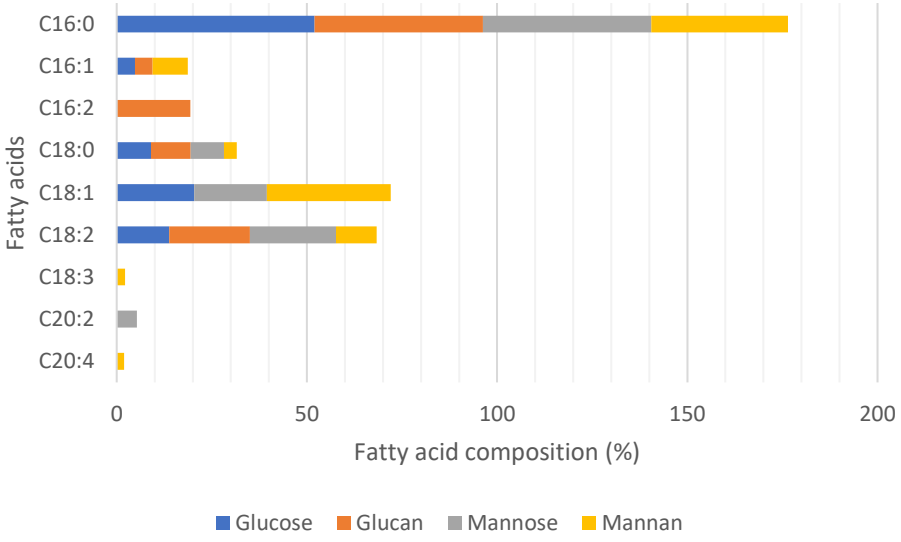


Figure 82: Influence of carbon sources on fatty acid composition in *N. oculata* grown in BG 11 medium

4.5.5 Effects of fatty acids on biodiesel properties

Fatty acid composition has a significant effect on the fuel properties of biodiesel. For instance, a high content of unsaturated fatty acid is beneficial against cold temperature properties (cloud point, cold filter plugging point, and pour point). This would lead to the possible usage of the fuel in cold countries. In this research, vital properties of biodiesel were estimated using empirical formulas obtained from [270, 271]. Biodiesel properties depend completely on the

percentage composition of fatty acids, chain length (molecular weight) and the existence of unsaturated carbon bonds. It is vital for biodiesel to meet the criteria stipulated by international standards like ASTM 6751, EN 14214 and GB 25199. The results obtained for *C. vulgaris* and *N. oculata* are outlined Tables 9. CN is one of the most important properties of biodiesel as it is related to ignition delay, which is the time interval between the start of injection and combustion. Higher value of CN will decrease ignition delay, noise, pollutants emission and enhance heat release during combustion.

Results obtained in this study showed that although FAME produced from the extract of the two the species grown using various carbon sources meet the CN criteria of biodiesel standards (CN≥51) as outlined in Table 22, CN was varied by various carbon sources and microalgae species. This assertion is similar to what was obtained in a research conducted by Madhumanti et al [271]. Values of other important fuel properties of FAME produced in this study that can influence the performance of an internal combustion (IC) engines such as oxidative stability and viscosity (which determine fuel interaction with oxygen and spray conditions respectively) were also compared with those of established international standards. Results showed that these properties met criteria of European, United States and Chinese standards, and are amongst other factors dependent on algae species and carbon sources utilised. CFPP is the lowest temperature in which biodiesel gels or crystallises to form clog in fuel filter and line, hence not regarded as a strong determinant of biodiesel quality. It is directly proportional to LCSF. Values of CFPP obtained in this study are off the set of international standards. However, high values of CFPP as were obtained in biodiesel from *N. oculata* are seen not to be efficient but can be recommended for use in countries of sunny or moderate ambient temperatures, as it may contribute to clogging along fuel filter and line when used in winter season.

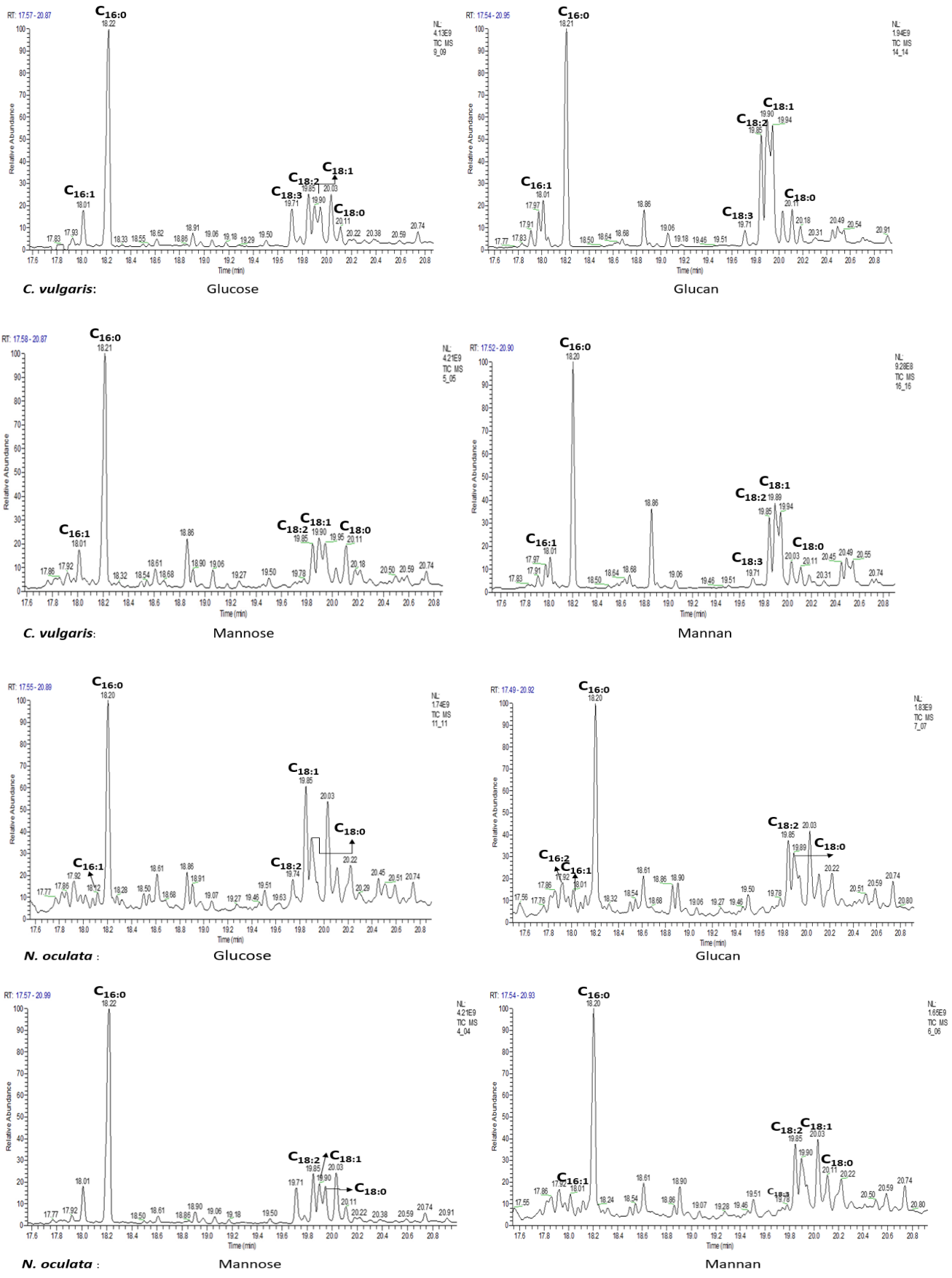


Figure 83: GCMS chromatographs of identified fatty acids.

Table 22: Comparison of biodiesel properties estimated from Fatty acid profiles of *C. vulgaris* and *N. oculata* cultivated from different carbon sources with commercial biodiesel and international standards

Properties	**Diesel fuel**	**ASTM D 6751**	**EN 14214**	**GB 2519**	***C. v***				***N. o***			
					Gs	**Gc**	**Ms**	**Mn**	**Gs**	**Gc**	**Ms**	**Mn**
Saponification value ($mgKOHg^{-1}$)	-	-	-	-	199	196	199	196	200	202	195	195
Iodine value (I_2100g^{-1}lipid)	130	-	-	-	68	74	49	72	46	41	40	40
Cetane number	≥51	≥47	≥51	≥51	58	57	63	58	63	64	65	65
High Heating Value ($MJkg^{-1}$)	42.5	-	-	-	40.2	40.2	40.5	40.3	40.6	40.5	40.8	40.8
Long chain saturation factor (% wt)	-	-	-	-	6.55	3.15	9.39	4.91	9.54	9.66	8.77	8.77
Cold Filter Plugging Point (^OC)	-5	-5 to - -	-2	-	4.1	-6.58	13	-1.1	13.49	13.87	11.08	11.08
Oxidative Stability (h)	≤2.5	15	≥8.0	≥6.0	7.76	11.6	8.81	9.74	11.15	5.49	6.82	6.82
Viscosity (mm^2s^{-1})	3.0-8.0	≥3	3.5-5.0	1.9-6.0	4.2	4.2	4.2	4.58	4.37	4.04	4.6	4.6
Density (gcm^{-1})	0.81-0.85	1.9-6.0	0.86-0.9	0.82-0.9	0.877	0.877	0.877	0.875	0.873	0.876	0.875	0.875

Gs: glucose Gc: glucan Ms: mannose Mn: mannan

ASTM D 6751-2015 US biodiesel standard; EN14214-2013 Europe biodiesel standard; GB25199-2017 Chinese biodiesel standard [271, 312]

4.5.6 Optimal biodiesel properties

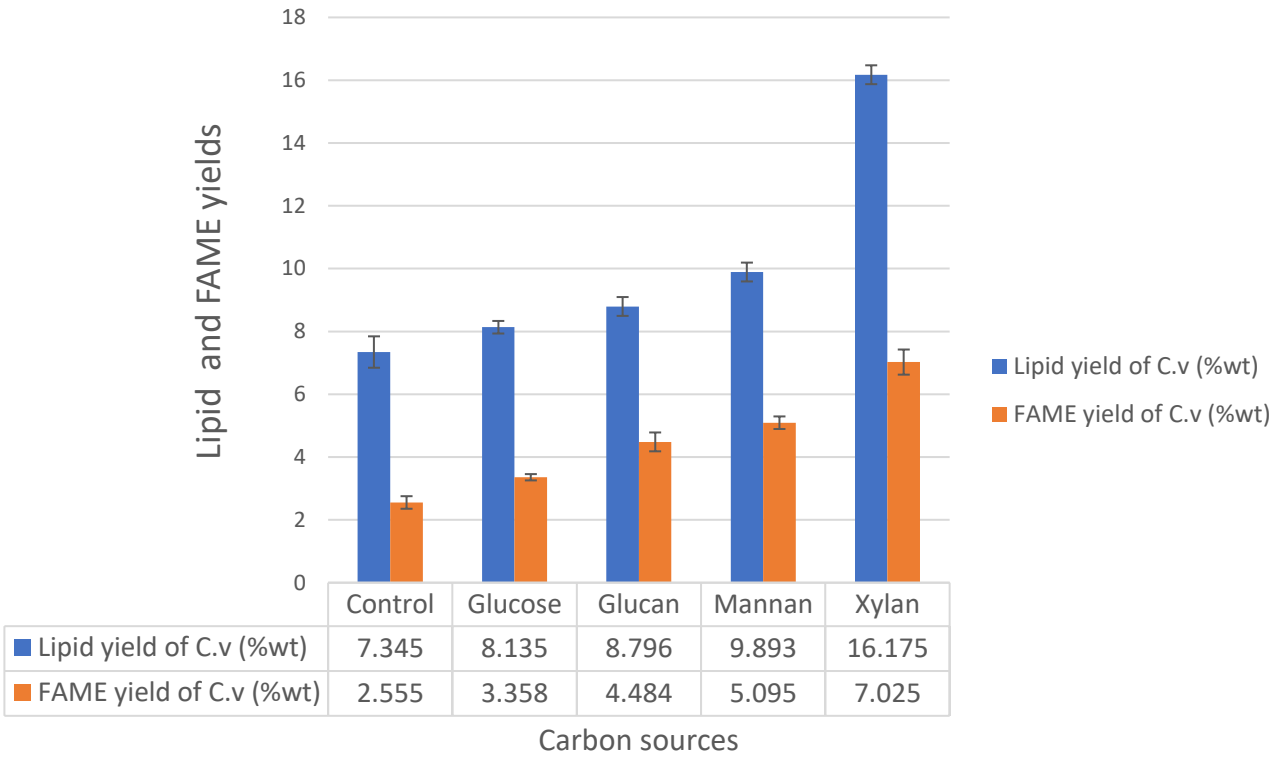
As can be seen from the data and discussion above, fatty acid composition has a significant and observable effects on the fuel physicochemical properties of biodiesel. Various fatty acid characteristics like degree of saturation, chain length (molecular weight) and percentage composition have produced both desirable and undesirable fuel properties. At times, the effects of a property on another can be unavoidable. For instance, high saturation/low unsaturation that favours oxidative stability leads to poor low temperature performance [313]. In practical terms, cetane number, viscosity, cold flow, and oxidative stability directly influence fuel/engine performance. But researchers have a consensus view that cold flow and oxidative stability, which are direct function of degree of saturation are the most critical[264, 314]. Therefore, the assertion that biodiesel of optimum quality would have comparatively low levels of saturated (to minimize cold flow problems) and poly-unsaturated (to minimize oxidative instability), and high levels of mono-unsaturated fatty acids [314, 315]. As a result, some researchers have concluded that palmitoleic acid (16:1) and oleic acid (18:1) provide the best compromise between oxidative stability and cold flow, without much reduction of cetane number [315, 316]. From the results obtained in this study and the assertion made concerning biodiesel optimal quality, it can be deduced that *C. vulgaris* and *N. oculata* cultivated in glucan and mannan respectively under mixotrophic mode have the potential of yielding biodiesel of optimal quality. Optimising these most critical properties will unfold their solo and synergistic effects on engine performance. The application of genetic engineering towards enhancing the accumulation of palmitoleic acid (16:1) and oleic acid (18:1) is desirous for the production microalgae biodiesel of optimal quality.

4.6 Disruption and lipid extraction from dry algae cultured in MCR

Earlier, we reported that same selected species were cultured in 80 ml of the growth medium supplemented with 2.5 mM of urea used as nitrogen source and 0.15 mM each of complex glycan β-glucan, β-mannan and xylan utilised as carbon sources where glucose serves as positive control. The essence of this section is to examine biomass, lipid yields, and properties of FAME produced from both selected algae species cultivated in MCR. Dissimilar growth conditions of the two unique bioreactors (PBR and MCR) utilised in this research do not give room for comparison amongst the reactors. Results obtained from different microalgae species and diverse utilised carbon sources will be compared.

4.6.1 Effects of complex glycans such as β-glucan, β-mannan and xylan on lipid and FAME yields of algae cultured in MCR

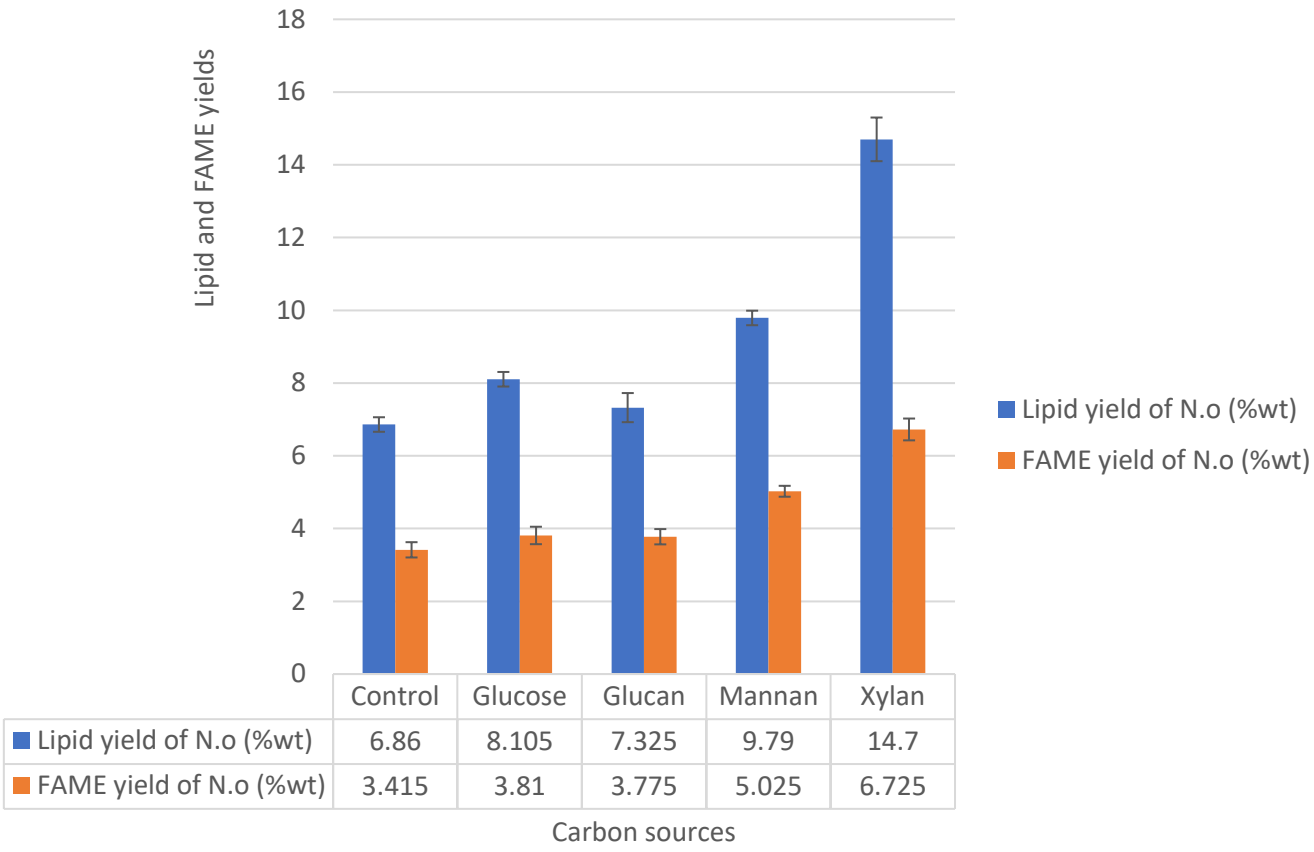
After growing selected algae species as described in section 3.1.4, the effects of three selected glycans on growth parameters were examined and results have been explained in section 4.2.1 of this thesis. Samples were harvested and dried as stated earlier. 50 mg of Dried biomass mixed with hexane and ethanol in a ratio of 3:2 was treated utilising ultrasonic disintegrator (Soniprep 150 plus MSE UK) as described in section 3.4.6. Results obtained and displayed in Figures 84 and 85 showed more lipid yields from samples cultivated in medium supplemented with glycans than control and glucose. This indicates upon the application of photoautotrophic cultivation mode, *C. vulgaris* and *N. oculata* can degrade these glycans to yield simple sugars utilised for lipid accumulation during cultivation.



	Control	Glucose	Glucan	Mannan	Xylan
Lipid yield of C.v (%wt)	7.345	8.135	8.796	9.893	16.175
FAME yield of C.v (%wt)	2.555	3.358	4.484	5.095	7.025

Carbon sources

Figure 84: Relative Lipid and FAME yields from *C. vulgaris* cultivated in MCR using BG 11 growth medium supplemented with glycans utilised as carbon sources



	Control	Glucose	Glucan	Mannan	Xylan
Lipid yield of N.o (%wt)	6.86	8.105	7.325	9.79	14.7
FAME yield of N.o (%wt)	3.415	3.81	3.775	5.025	6.725

Figure 85: Relative Lipid and FAME yields from *N. oculata* cultivated in MCR using BG11 growth medium supplemented with glycans utilised as carbon sources

Both figures again show the suitability of these glycans to serve as carbon sources for the cultivation of algae species in MCR. Results of lipid and FAME yields obtained are presented in the table data of the Figures 84 and 85. Amongst the carbon sources utilised in this study, results showed that maximum lipid and FAME yields were obtained with the medium supplemented with xylan. Although, the metabolic pathways leading to the enzymatic degradation of these glycans has not been well studied, some carbon sources can trigger acetyl CoA/malonyl CoA pool – which represents the central carbon donor for fatty acid synthesis, thereby increasing lipid synthesis [317]. Figure 86 shows a comparison of lipid yields in *C. vulgaris* and *N. oculata* when subjected to the same growth and lipid extraction conditions. The former outwitted the later at different margins across various carbon sources utilised. These observations indicate that the addition complex organic carbon sources lead to a noticeable stimulation of lipid accumulation in both species, and that the ideal organic carbon source to produce lipids is xylan.

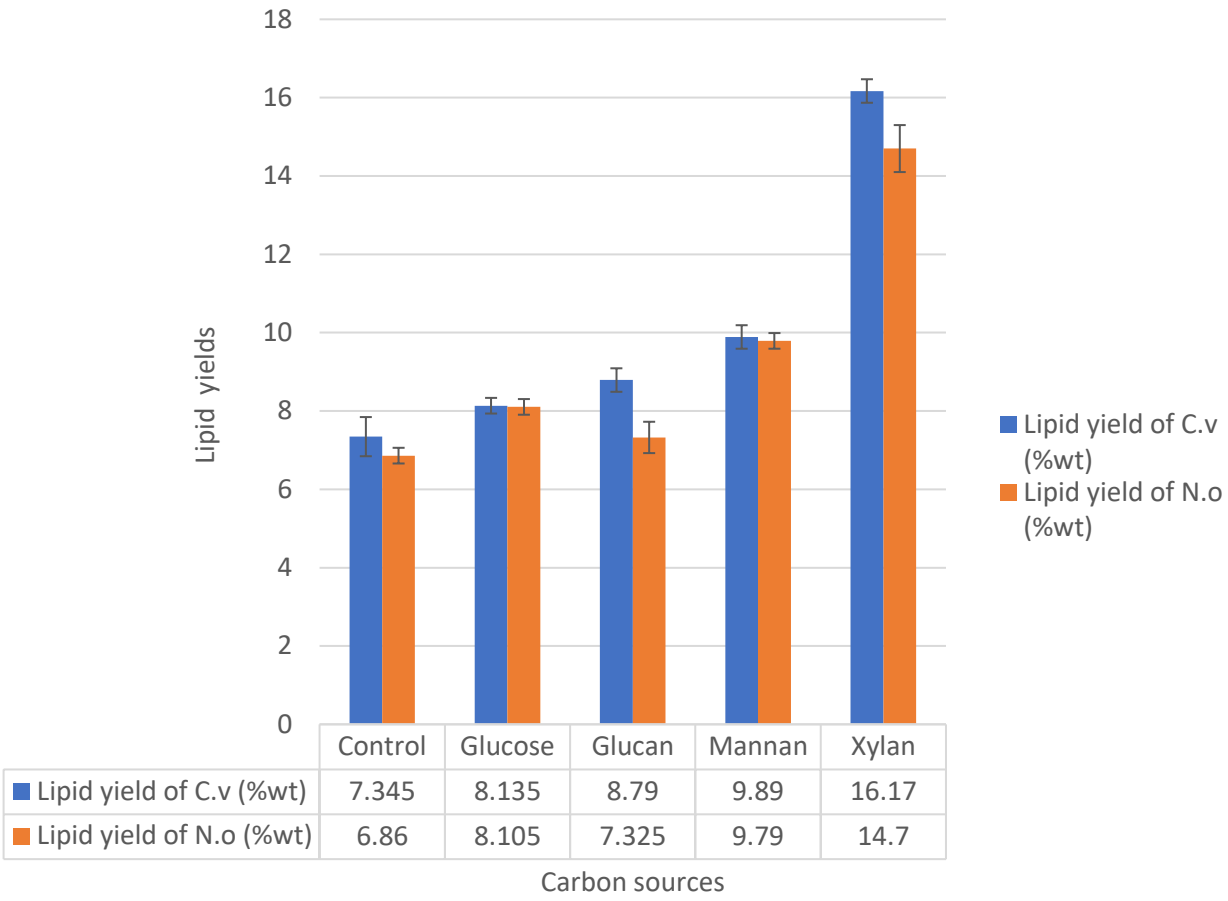


Figure 86: Relative Lipid yields from *C. vulgaris* and *N. oculata* cultivated in MCR using BG 11 growth medium supplemented with glycans utilised as carbon sources

Chapter five

5 Conclusion and prospects

5.1 Conclusion

Microalgae upstream processes such as cultivation, and downstream processes such as flocculation, cell disruption, lipid extraction and biodiesel production were considered in this study. Firstly, wastewater made by blending distilled water with miracle gro. fertilizer, supplemented with folic acid was utilized as growth medium for the cultivation of microalgae mixotrophically utilizing the PBR. Furthermore, glycans such as β- glucan, β-mannan and xylan were also considered as organic carbon sources, while organic urea was utilized as nitrogen source in cultivating two oleaginous microalgae species *C. vulgaris* and *N. oculata* using a well formulated BG 11 adopting both mixotrophic and photoheterotrophic modes of cultivation. The various outcome obtained from microalgae species cultivated showed that pigment concentration, biomass productivity, lipid accumulation, fatty acid composition and biodiesel fuel property were dependent and significantly influenced by microalgae species/strain, mode of cultivation and carbon sources utilised. Indeed, from the results obtained, the metabolism of these glycans indicates that *C. vulgaris* and *N. oculata* have the capability to carryout enzymatic processes, cell permeability, membrane diffusion and active transport during cultivation. Secondly, microalgae degree of flocculation is species' dependent. However, the pH level and flocculation time have significant effect on the flocculation efficiencies of each species. On cell disruption utilising the Tissue-lyser, factors such as biomass concentration, frequency and operation time influenced microalgae disruption efficiency, and by extension, lipid yields and specific energy consumption. In summary, at the end of this study, we arrived at the following conclusions:

- Microalgae growth was inhibited and eventually terminated when cultivated in wastewater supplemented with folic acid solution, signifying that the dissociation of folic acid in culture did not make available the much-needed carbon required for algae growth, and therefore is unsuitable for use as a carbon source.
- Other carbon sources of glycan (β-glucan and β-mannan) substrates utilised in this study have direct and significant impact on biomass productivity, lipids yield, fatty acid composition and biodiesel (FAME) properties/quality. The growth was found to be optimal on supplementing growth media with various carbon sources of

concentration of 0.15mM. Generally, under the same growth conditions, *C. vulgaris* outwits *N. oculata* in bio synthetisation of growth parameters, as mximumbiomass concentration of (0.287 and 0.223 gl^{-1}) and (0.267 and 0.238 gl^{-1}) were obtained on mixotrophically growing both species in media supplemented with glucose and mannose respectively, suggesting better nutrient absorption and photosynthetic activities by the former species. Also, due to the absorption of extra carbon during aeration, mixotrophic mode outwits photoheterotrophic mode of cultivation. Investigating and quantifying the effects of utilising β-glucan from different sources such as yeast and barley showed no significant differences.

- The results of photoheterotrophic mode of cultivation showed that these selected algae species can grow without culture aeration. The outcome revealed that growth is dependent on the combination and interaction of both nutrients (organic carbon and nitrogen sources). Overall, having R^2 greater than 0.5; that is (0.759 and 0.971 for *C. vulgaris*; and 0.941 and 0.961 for *N. oculata* using β-glucan and β-mannan respectively), low p-values (less than 0.05) and high p-values of lack of fit (greater than 0.05) across board indicate the suitability of these models to successfully predict responses (growth).

- Additionally, the growth performance of both microalgae species cultivated in MCR was also influenced by glycans (β-glucan, β-mannan and xylan) utilised as carbon sources in such a decreasing order of xylan>β-mannan>β-glucan. Though the utilisation of all these glycans enhanced biomass and lipids accumulation, culture supplemented with xylan yielded the maximum biomass and lipid of 0.85 gl^{-1} and 16.2 % wt. and 0.8 gl^{-1} and 14.7 %wt. for *C. vulgaris* and *N. oculata* respectively. More so, the utilisation of these glycans synthesized more saponifiable lipid when juxtaposed with glucose and mannose, hence can be promising carbon sources for the cultivation of microalgae for sustainable biodiesel production.
- RSM results obtained from flocculation experiments showed that pH and flocculation time have the most significant synergistic effect on flocculation efficiency of both species whereas flocculant dosage has minimal effect. However, *N. oculata* flocculated better than *C. vulgaris* in alkaline culture (pH of 11) whereas *C. vulgaris* is better flocculated in acidic culture (pH of 3) with flocculation efficiencies of 93% and

86 % respectively. Due to less number cell wall layers and matrices, *C. vulgaris* cell lysis was observed after flocculation when viewed under the microscope, but little or none occurred in *N. oculata,* and at optimum flocculation, *N. oculata* was very resistive to cell loss when compared with *C. vulgaris*.

- All the cell disruption and lipid extraction methods considered in this study (sonication, osmotic shock, use of Tissue-lyser, and osmotic shock + Tissue-lyser) showed variable and substantial degrees of outcomes. The combination of osmotic shock and Tissue-lyser II resulted in the cell wall maximum disruption efficiency, and consequently, optimal lipids yield. However, outcome can be significantly influenced by biomass concentration, frequency, and treatment time. This process can be sustainable, scalable and does not alter the composition of the intracellular biomolecules. With high sums of monounsaturated fatty acids (over 45 % of FA composition) observed from algae cultured in the media supplemented with glycans in this study, it can be deduced that *C. vulgaris* and *N. oculata* cultivated in glucan and mannan respectively under mixotrophic mode have the potential of yielding biodiesel of optimal quality.
- Overall, both microalgae species and growth conditions favour the production of key quality biodiesel markers such as Cetane number (CN), Viscosity (v) and Oxidative Stability (OS), as the values of these parameters fall within range of international standards.

5.2 Future prospect

The effects of utilizing several carbon sources for microalgae cultivation, aimed at enhancing lipid accumulation for sustainable biodiesel production have been widely studied. Carbon sources utilized have shown direct influence on the volume of microalgae biodiesel produced, physicochemical properties and engine performance. Thus, extensive, and in-depth research is needed in this direction, targeted at utilizing more glycans derived from wastes obtained from foods and agricultural industries. Further study is required to unravel algae species and strains of high oleaginous nature capable of synthesizing high percentage of saponifiable lipid. This will be accompanied with the optimisation of biomass and lipid productivity from algae cultivated in wastewater, formulated growth media, nutrients including the concentration of carbon sources and other necessary key growth conditions to cut down excess inputs that will not only circumvent hike in the cost of production but help identify the overall conditions for optimal and sustainable production. Boosting the production of biomass and lipid productivity using cutting edge technology is still desirable.

Further studies of metabolic pathways of complex carbohydrates or glycans assimilation with application of genetic engineering may be deployed to enhance the yield of saponifiable lipid (non-polar or neutral lipid) which is a biodiesel precursor. Furthermore, the application of genetic and metabolic engineering towards enhancing the accumulation of palmitoleic acid (16:1) and oleic acid (18:1) is desirous for the production microalgae biodiesel of optimal quality.

Injecting new ideas into microalgae downstream processes such as harvesting, and cell disruption is necessary to sustain biomass recovery and lipid yields. In lieu of this, the extracellular substances secreted from microalgae during the growth phase portrays a positive effect on microalgae harvest. Additionally, the co-cultivation of microalgae with other biological microbes such as fungi, bacteria, or yeast can improve the efficiency of biomass recovery and lipid accumulation of microalgae [318, 319]. It is important to integrate the cell wall composition and matrix in the modelling and optimisation of cell disruption and lipid extraction of microalgae. Focusing future research on comprehending the relationship between microalgae cell wall matrix and disruption techniques will aid the development of an innovative cell wall disruption system that can enhance lipid yields. Overall, cost-energy environment relationship should be measured and optimized in detail to achieve the cost-

effectiveness of commercial mass production. Full application of process intensification across microalgae biodiesel production chain can enhance the quantity and quality of the obtainable biodiesel.

Determining the final concentration, presence and effects of metals, metalloids and other possible impurities on biodiesel properties is necessary, as it can alter or modify the efficiency and stability of biodiesel. Although, their concentration is usually low (expressed μg/ml), some metals and metalloids can be carcinogenic and generate environmental concerns, others can be utilised as additives. The low concentration of these metal makes its determination very sensitive and challenging. So far, utilising Inductive Couple Plasma Optical Emission Spectrometry (ICP-OES) and Mass Spectrometer (ICP-MS) with Atomic Absorption methods have shown some difficulties, possible remedies towards overcoming these difficulties such as developing a more sensitive device is desirable.

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