Study on the Improvement of Lipid and Succinate Productivity on Microalgae and Cyanobacteria by Alginate Immobilization and its Application

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Study on the Improvement of Lipid and Succinate Productivity on Microalgae and Cyanobacteria by Alginate Immobilization and its Application

固定化された微細藻類と藍藻による脂質およびコハク酸生産の強化

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PREFACE

This doctoral dissertation is submitted by the author to Kobe University in partial fulfilment of the requirements for the degree of Doctor of Engineering. The studies were carried out between the period of 2020 and 2023 under supervision of Professor Chiaki Ogino in the Laboratory of Biochemical and Bioprocess Engineering, Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University.

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CHAPTER I
Introduction

I.1. Microalgae and cyanobacteria for biofuels production

Algae are prokaryotic (cyanobacteria) or eukaryotic (green algae and diatoms) photoautotrophs [1], with the ability to grow in various habitats such as freshwater, seawater, wastewater, and brackish water [2]. Several active compounds and biomolecules can be extracted from microalgal biomass such as astaxanthin, lutein and eicosapentaenoic acid (EPA) [3].

Several species of microalgae and cyanobacteria can store significant amounts of energy-rich compounds such as lipids and polysaccharides (starch and glycogen), which can be utilized for the production of biofuels such as bio-diesel and bio-ethanol [4]. The biofuels produced by algal biomass can ensure a stable and sustainable fuel supply, microalgae biodiesel is a promising alternative fuel for use in compression ignition engines and gas turbines for power generation. [5].

The Oleaginous microalgae are the specific species that are able to accumulate lipids over 20 % of their biomass this makes them attractive due to their ability to capture energy from sunlight and convert CO_2 into lipids. The lipids produced are mostly found in the form of triglycerides, and their compositions are similar to those of common plant oils that can be converted into biodiesel [6]. The triglycerides concentrations vary between species, and under optimum growth conditions they represent a fraction of the microalgae biomass, however most microalgae significantly accumulate triglycerides in the cytoplasm as dense lipid bodies in response to environmental stress, temperature, pH,

salinity and CO_2 concentrations affect the lipid concentrations on microalgae cells, the most researched are nitrogen, and silicon for diatom species [7].

The lipids suitable for biodiesel production are subjected to a transesterification process, some of the most commonly used microalga strains for biodiesel production are *Chlorella vulgari*, *Chlorella minutissima*, *Spirulina platensis* and *Botryococcus braunii* [8]. The production of biodiesel from microalgae lipids starts from the microalgae isolation and identification up to the cultivation and harvest followed by lipid extraction and finally conversion to biodiesel, this is a multistep process and each step is specific to a particular microalga strain [9]. The amount of lipids, carbohydrates and proteins vary between species of microalgae and environmental growth conditions [10].

The bottleneck in microalgae for biofuel production involves the cost, which can be 2-3-fold higher than that of production using first- and second-generation biomass [11]. Harvesting, drying, and oil extraction accounted for half of the total production cost. Harvesting alone occupies almost 30% of total production cost, which can amount to as much as US $10/kg [12]. To achieve economic feasibility in biofuel production from microalgae, it is necessary to focus on the most important areas, such as low lipid accumulation, expensive cultivation steps, and unproductive downstream processes [11].

In order to reduce the microalgae cultivation costs, and make the biofuel production more cost-effective, the use of wastewater as nutrient and water source has been proposed [13]. Wastewaters usually contain heavy metals, carbon, nitrogen and phosphorous, which can be utilized by the microalgae to grow [14]. Apart from using the microalgae biomass for biodiesel production, the same biomass can be utilized for the extraction of other high value compounds such as agar, carrageenan and diatomite.

Table 1. Previous studies on the production of lipids for biofuels production of *Chlorella sorokiniana.*

Microalgae	Growth condition	Initial glucose (g/L)	CO_2 (%)	Temp. (°C)	DCW (g/L)	Lipids (% of DCW)	Reference
Chlorella Sorokiniana	Autothropic (Free cell)	0	0.04	37	0.4	44%	[10]
Chlorella Sorokiniana	Autothropic (Free cell)	0	5	37	1.0	10%	[10]
Chlorella Sorokiniana	Autothropic (Free cell)	0	10	37	1.2	11%	[10]
Chlorella Sorokiniana	Autothropic (Free cell)	0	15	37	1.1	20%	[10]
Chlorella Sorokiniana	Autothropic (Free cell)	0	1	25	0.68	8.98%	[10]
Chlorella Sorokiniana	Mixotrophic (Free cell)	2	1	25	1.66	6%	[11]
Chlorella Sorokiniana	Mixotrophic (Free cell)	4	1	25	3.55	7%	[11]
Chlorella Sorokiniana	Mixotrophic (Free cell)	6	1	25	4.57	13%	[11]
Chlorella Sorokiniana	Mixotrophic (Free cell)	8	1	25	5.01	18%	[11]
Chlorella Sorokiniana	Mixotrophic (Free cell)	10	1	25	5.08	22%	[11]
Chlorella Sorokiniana	Mixotrophic (Free cell)	20	1	25	4.91	22%	[11]
Chlorella Sorokiniana	Heterotrophic (Free cell)	2	1	25	0.73	13%	[12]
Chlorella Sorokiniana	Heterotrophic (Free cell)	4	1	25	1.46	21%	[12]
Chlorella Sorokiniana	Heterotrophic (Free cell)	6	1	25	2.78	32%	[12]
Chlorella Sorokiniana	Heterotrophic (Free cell)	8	1	25	3.62	35%	[12]
Chlorella Sorokiniana	Heterotrophic (Free cell)	10	2	25	4.23	32%	[12]
Chlorella Sorokiniana	Heterotrophic (Free cell)	20	1	25	4.12	32%	[12]

Taking into account the biochemical composition, several cyanobacteria species can be utilized for the production of a variety of chemicals, such as biodiesel, bioethanol and colorants, in the case of biodiesel the cyanobacteria biomass can be utilized for its production [15]. Cyanobacteria accumulates several primary metabolites such as carbohydrates, lipids and proteins which can be utilized for the production of useful chemicals; however, they mainly contain carbohydrates in their cell wall as intracellular carbon storage. The carbohydrates present in the cell wall provide structural support to the cell, while the polysaccharides it accumulates become an energy source to the cell or for survival under environmental repressions [16].

The major storage polymer in Cyanobacteria are polysaccharides, such as glycogen, these storage carbohydrates are accumulated as energy storage as a response to unfavorable environments in chloroplasts, these environments can be nitrogen and phosphorous limitation, salt stress, or disproportionate C:N or C:P ratios [17]. Cyanobacteria are attractive because their metabolic pathways can be genetically modified to enhance their photosynthetic activity [18]. Using metabolic engineering techniques, such as gene deletion and overexpression, *Synechocystis* sp. PCC6803 converts CO_2 directly into useful substances, such as cyanophycin, 2,3-butanediol, isobutanol, 3-hydroxypropionic acid, and PHAs [19,20]. Synechocystis sp. PCC6803 can excrete succinate in the absence of sugar via autofermentation under dark anoxic conditions [21].

Table 2. Previous studies on the production succinate by cyanobateria strains.

Cyanobacteria	Conditions	Succinate titer	Succinate productivity	Study
Synechococcus sp. PCC 6803	Dark anaerobic, +sigE, ΔackA	115 mg/L	1.38 mg/L/h (0–96 h)	[18]
Synechococcus sp. PCC 6803	Dark anaerobic, +sigE, ΔackA, 200mM KCl	141.0 mg/L	2 mg/L/h (0–72 h)	[19]
Synechococcus elongatus PCC 7942	Photosynthetic, nitrogen starvation, Δglgc,+gltA, +ppc	435.0 µg/L	9 µg/L/h (0–48 h)	[20]
Synechococcus elongatus PCC 7942	Photosynthetic, nitrogen starvation, repression of glgc, sdhA, sdhB	580–630 µg/L	13 µg/L/h (0–48 h)	[21]
Synechosystis sp. PCC 6803	Dark anoxic, Ppc-ox, NaHCO3, 37 °C	159 mg/L	2.2 mg/L/h (0–72 h)	[21]

I.2. Microalgae immobilization

Immobilization technology has been proposed as an alternative technique to simplify the separation of microalgal biomass from the liquid phase [22]. There are six type of immobilization types available, these are, affinity immobilization, adsorption, covalent coupling, confinement in liquid-liquid emulsion, capture in a semipermeable membrane and entrapment within polymers [23].

- Affinity immobilization: this method is based on complementary biomolecular interactions without drastic reactions or chemical exposure, it is often used for purification or separation of biomolecule mixtures [24].
- Adsorption immobilization: this method is a reversible process in which the cells strongly adhere to the sorbent [23].
- Covalent coupling: this method is a well-known technique for immobilizing enzymes, but it is not used for living cells due to possible cell leakage from cell division [23].
- Confinement in liquid-liquid emulsion: this is an aqueous immobilization method in which the phase separation occurs between two different water-soluble polymers based on their surface properties [23].
- Capture in a semi-permeable membrane: this method consists on the immobilization of the cells into a membrane, it is usually used for biosensor fabrication [23].
- Entrapment within polymers: this is the most commonly utilized immobilization method, in which the cells are captured within a polymeric matrix, the polymers can either be synthetic such as polyurethanes or polyvinyl, or natural polymers such as agars, carrageenan and alginates [25].

Table 3. Polymers utilized for entrapment immobilization of cells [25].

	Polymer	Uses	Properties
Synthetic	Polysulphone and epoxy resin	Immobilization of cyanobacteria for biosorption, metal accumulation	Epoxy resins are toxic for living cells
	Polyvinil and Polyurethane foams	Immobilization of microalgae for polysaccharides production	Entrapment rapidly leads to death of cells due to toxicity of the foams
	Polycarbamoylsulphonate (PCS)	Immobilization of bacteria	Very low toxic hydrogel matrix
	Polyvinil alcohols and glutaraldehyde		Not reccomendable for living cells
	Silica gels (sol-gel)	Immobilization of microalgal cells for metal absorption, immobilization of antibodies	Not reccomendable for living cells
Natural	Carrageenan	Immobilization of microalgae for nutrient removal, immobilization of yeasts for ethanol production, long term storage of immobilized bacteria	Low stability, partially destroyed after one week, leakage of cells and loss of bead structure
	Agar	Immobilization of microalgae cells for storage, and metal biosorption	Thermoreversible gel, only for species able to resist a short thermal shock
	Alginate	Immobilization of microalgae and cyanobacteria cells for biofuel production, nutrient removal, metals absorption, wastewater treatment, toxicity measurement, hydrogen production.	Cells do not suffer extreme physical-chemical condition changes during immobilization, permeability, null toxicity and high transparency

Alginate is one of the most abundant biopolymers of natural origin available after cellulose, with many useful properties such as low toxicity, biodegradability, biocompatibility, and good hydrogel-forming ability by addition of divalent cations such as Ca^{2+} [26]. Entrapment in a polymeric matrix, such as calcium alginate, is one of the most widely used immobilization techniques for microalgae, the microorganisms do not experience extreme physicochemical conditions during the immobilization process [25].

Alginate beads are formed when the solution of sodium alginate and microalgae cells is extruded into a Ca^{2+} solution, at that moment an instantaneous interfacial polymerization occurs, producing the beads with the immobilized microalgae inside, the bead hardness is increased with the permeation of calcium ions through the beads [27].

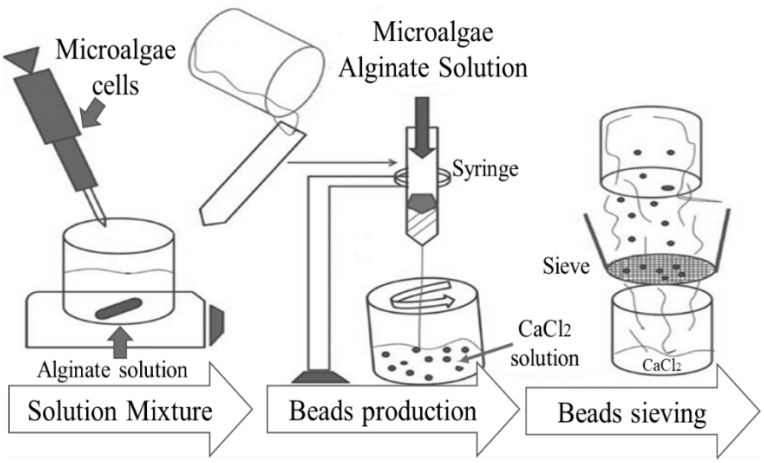

Figure 1. Methodology for the immobilization of microalgae cells into alginate beads

Figure 2. Microalgae cells immobilized in alginate beads

Currently, the most frequent applications of immobilized microalgae include wastewater treatment [28,29], removal of nutrients from aquatic media [30,31], removal of organic or metal pollutants [32,33], toxicity measurement [34,35], and energy production *via* hydrogen [21,22] or electricity [36,37].

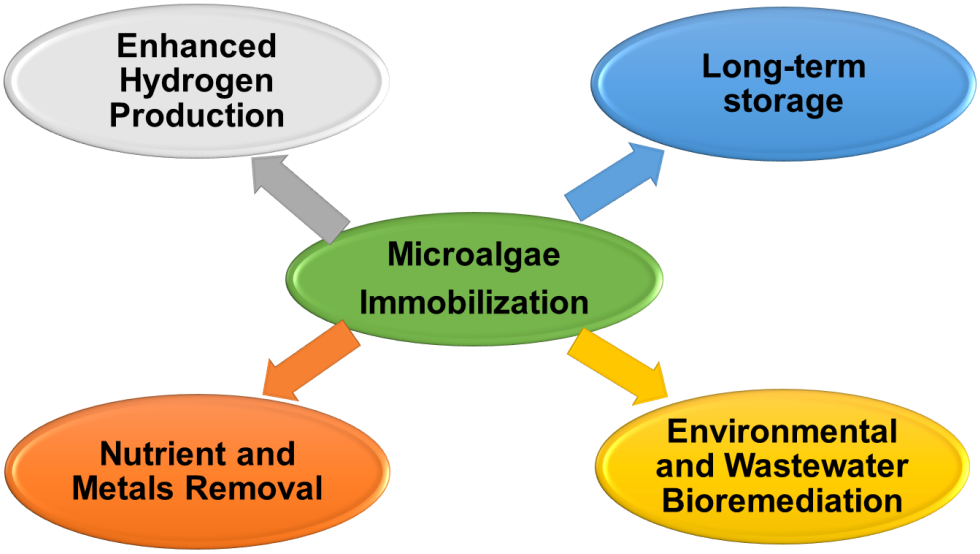

Figure 3. Microalgae immobilization applications.

Microalgae immobilization can improve growth, pigment and lipid content compared with suspension cultures. The growth of *Nannochloropsis* sp. immobilized in alginate gels was studied by Cheirslip et al. (2017) [38], who were able to cultivate the immobilized cells to achieve a comparable growth and lipid production to the free cells by varying the bead volume and inoculum size. Li et al. (2018) [39] found that the optimum immobilization conditions for *Chlorella vulgaris* were of 2.42 % sodium alginate and 2.69 % $CaCl_2$ obtaining a growth 1.97 g/L, Lee et al. (2020) [40] studied the effect of the alginate bead size for optimal growth and nutrient removal of *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, their results showed that the optimal bead size was of 3.5 mm. The growth of free and immobilized cells of *Chlorella vulgaris* was studied by Sepian et al. [41], with a combination of matrices of sodium alginate, calcium alginate, and sodium carboxymethyl cellulose, and nearly identical cell densities were achieved between free and immobilized cells.

Table 4. Advantages that immobilization has over suspension cultures [25].

Drawbacks Free Cell Culture	**Advantages Immobilization**
High-cost downstream processes.	Simple Harvesting.
High energy consumption for stirring.	Simple Stirring.
Uneven light distribution (Photoinhibition).	Protects culture from photoinhibition.
Easy contamination.	Protects from pH variations, salinity.
Costly Harvesting and Dewatering processes.	Provide reactor design flexibility.
Mechanical complexity for supplying CO2.	Avoids cell washouts.

I.3. Palm oil mill effluent utilization for chemicals production

One of the most popular vegetable oils worldwide is palm oil [42, 43]. Palm oil mill effluent (POME) is found in the wastewater of the palm oil industry, when fresh fruit bunches (FFV) from palm oil trees are pressed to make palm oil, palm mill oil effluent (POME) is created. POME requires treatment before it can be discharged into water bodies, and even though it is non-toxic, due to its high organic content, chemical oxygen demand (COD), and biochemical oxygen demand (BOD), the POME effluent is regarded as extremely polluting [43,44]. Fresh POME is released as a hot, acidic liquid waste that is brownish in color and colloidal in consistency. It contains roughly 96% water, 0.7% oil, and 5% total solids. In comparison to municipal sewage, POME has a BOD level of more than 180 mg/L and a high COD content of more than 450 mg/L [45].

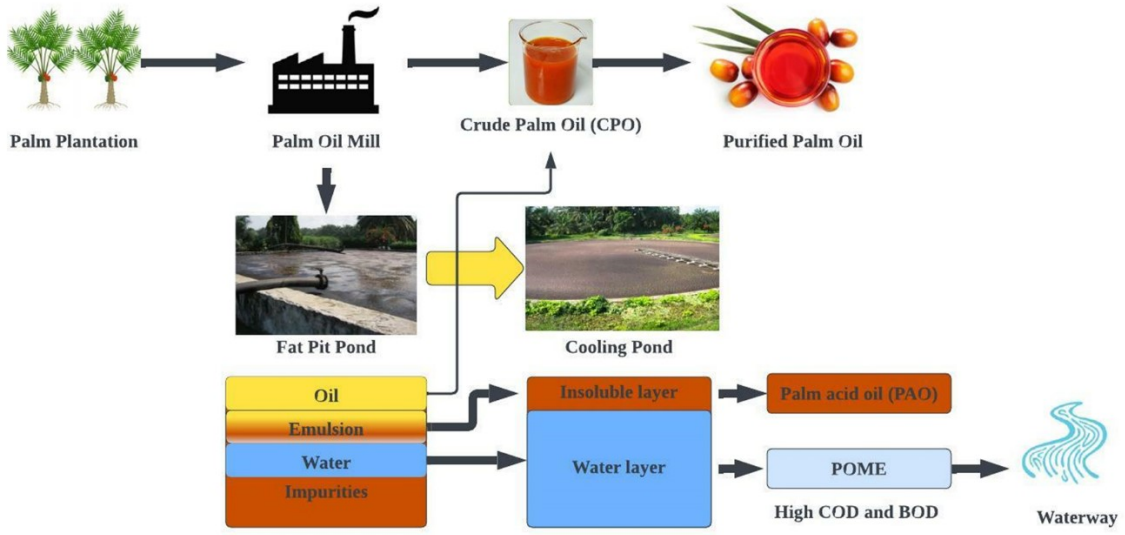

Figure 4. Current scheme for wastewater treatment of POME.

Many processes have been developed for the conversion of POME into several valuable chemicals such as citric acid, biodegradable polymers such as polyhydroxyalkanoate (PHA) copolymers and volatile fatty acids [46-48]. An economically and environmentally attractive method for POME treatment is its use as a raw material for biological processes [49], such as the production of methane, biohydrogen, biodiesel, or polymers [50]. POME contains a high organic matter content of carbohydrates and fermentable sugars [51,52], also significant amounts of nutrients as shown on Table 1, such as nitrogen, potassium, magnesium, calcium, and iron, making it a viable medium for microbial production [53].

Table 5. Characteristics of POME.

Parameters[a]	**Average concentrations**
Total suspended solid (TSS) (mg/L)	47,690 ± 156
Volatile suspended solid (VSS) (mg/L)	30,870 ± 105
Temperature (°C)	80 ± 1
pH	3.4 ± 0.1
COD (mg/L)	69,500 ± 240
BOD_5 (mg/L)	37,750 ± 143
Total nitrogen (mg/L)	692 ± 45
Total protein (% TS)	12.31
Total carbohydrates (mg/L)	22.27×10^{-3}

Photosynthetic microorganisms such as microalgae and cyanobacteria have been used to treat POME and produce raw materials for fuels and chemical products. Microalgae cells are capable of reducing the COD of diluter POME in anaerobic ponds by approximately 2,000–8,700 mg/L [54]. However, for microalgae and cyanobacteria cells to grow in POME, it is necessary to dilute it, also POME is acidic (pH 4) making the treatment by microalgae alone complicated, POME pH must be adjusted to 6.5-8 in order to culture microalgae such as *Chlorella sorokiniana* [55]

POME can also be utilized for the production of biohydrogen and methane through anaerobic digestion. To produce hydrogen through anaerobic digestion, hydrogen-consuming bacteria must be eliminated to avoid the methanogenesis phase. This elimination is performed by establishing a hostile environment for these bacteria [56].

There are certain drawbacks to using POME for anaerobic digestion. One such problem is that the substrates might not be readily available to bacterial cells because of the complexity of POME, and this could increase the time for adaptive phases and result in a lower conversion yield [57]. To achieve higher yields of methane from POME, various pretreatment processes have been evaluated. These include ozonation and ultrasonication [58,59], and both of these methods have been used to increase the methane yield by as much as 7-fold compared with non-pretreated POME.

These pretreatment techniques have also been used to increase the hydrogen yield by increasing the substrate bioavailability of POME. Ultrasonication pretreatment has been used to increase Hydrogen production by 38% [53]. Ozonation pretreatment has increased hydrogen production by 49% [54]. These pretreatment processes break down the complex molecules into simpler forms, which can then be converted into hydrogen.

Palm acid oil (PAO) is a by-product extracted from the POME on the top layer of a pond. Free fatty acids, neutral oil, and moisture make up most of the PAO, making it available for the production of biodiesel [19,20].

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Synopsis

CHAPTER II

Enhanced growth and lipid productivity by living *Chlorella sorokiniana* immobilized in Ca-alginate beads

The bottleneck for the production of biofuels from microalgae consists on costly harvesting processes and low lipid production, immobilization technology could play a part on making the production of biofuels more feasible. The aim of this study was to evaluate the effect of alginate immobilization on the growth and lipid productivity of the microalgae *Chlorella sorokiniana*, so far, the main focus of immobilization technology has been its use for wastewater treatment and nutrient removal from effluents. The microalgae *Chlorella sorokiniana* was cultured in both free and immobilized forms under optimal autotrophic growth conditions. Microalgae were immobilized in calcium alginate beads generated by mixing algal cells with a sodium alginate solution, followed by extrusion into a $CaCl_2$ solution. The results obtained in this study showed that the growth of the microalgae immobilized in alginate beads, was enhanced and achieved a dry cell weight 1.4-fold higher than that of a free cell culture, a higher light transmittance was also achieved in the alginate immobilized culture, and the lipid productivity was increased from 54.21 ± 2.48 mg/L/d in the free cell culture to 82.22 ± 8.48 mg/L/d in the immobilized culture. These results demonstrate the effectiveness of immobilization technology for promoting growth and lipid productivity in the microalgae *Chlorella sorokiniana*.

Keywords: *Chlorella sorokiniana;* biomass*;* lipids; alginate; immobilized cell

CHAPTER III

Alginate Immobilization as a Strategy for Improving Succinate Production During Autofermentation Using Cyanobacteria *Synechocystis* sp. PCC6803

This study focused on immobilization of a cyanobacterial strain for succinate production. Recombinant cyanobacterium *Synechocystis* sp. PCC6803, with an overexpressed endogenous PEPC gene, was immobilized on calcium alginate beads. This immobilization technology improved succinate production during a continuous autofermentation cycle compared with that produced using a free-cell culture. In addition, the immobilized cells achieved a higher dry cell weight than the free cells. Furthermore, this process permitted cultivation within a shorter timeframe than in a free-cell culture. During continuous succinate production, the immobilized cells increased the succinate yield by 1.4-fold, whereas the use of free-cells resulted in a 50 % decrease in the succinate yield. Continuous batch culture offers a convenient strategy for the use of immobilized cyanobacteria, resulting in a simpler and faster process that achieves a higher rate of succinate production with each cycle.

Keywords: *Alginate Immobilization; Cyanobacteria; Autofermentation; Succinate.*

CHAPTER IV

Palm Oil Mill Effluent (POME) Utilization for Succinate Production from Cyanobacteria *Synechocystis* sp. PCC6803

This study focused on the utilization of POME as a nutrient source for the growth of the cyanobacterium *Synechocystis* sp. PCC6803 and subsequent succinate production during autofermentation. The results showed the potential that this wastewater has to produce valuable chemicals such as succinate, when the cyanobacteria cells were cultured in a medium composed of only POME they achieved a growth similar to when the cells were cultured in a BG11 medium, however, the growth on POME medium, increased the cells glycogen accumulation from 39 % in the BG11 medium to 42 % in the POME medium, while also increasing its succinate yield from 146 ± 9 mg/L in the BG11 medium to 227 ± 10 mg/L in the POME medium which is 1.5-fold higher.

Keywords: *POME; Cyanobacteria; Autofermentation; Succinate.*

CHAPTER V

Bioconversion strategies to alter the bio-circular economic perspective for palm oil mill effluent

Palm oil mill effluent (POME) is found in wastewater from the palm oil industry. POME contains palm acid oil (PAO), which consists of free fatty acids that can be converted to biodiesel fuel via enzymatic activity. In this review, the consistency of POME is defined and characterized, and its treatment by several bioconversion technologies is summarized. Following anaerobic digestion that generates hydrogen and methane gas, palm oil wastewater is also available in microalgae cultivation for oil and pigment production. POME is becoming a feedstock candidate for bio-based chemical production. Thus, via the integration of anaerobic and aerobic fermentation, wastewater quality is being improved with a reduction in the release of greenhouse gas (GHG). In addition, these sub-products represent a positive impact on the realization of a bio-circular economy as well as on sustainability of the palm oil industry.

Keywords: *Bio-circular economy; Palm oil mill effluent; Biofuel: Biochemicals; Integrated biorefinery*

CHAPTER II

Enhanced growth and lipid productivity by living *Chlorella sorokiniana* immobilized in Ca-alginate beads

II.1. Introduction

Energy is the primary commodity in the world for the growth of any nation; thus far, this necessity has been fulfilled through the use of fossil fuels. However, this reliance on fossil fuels has resulted in negative effects such as climatic change [1].

Microalgae are fast-growing microorganisms with many advantages over terrestrial plants. These advantages include higher rates of carbon fixation and the ability to grow without the need for arable land to produce high biomass yields [2,3]. Various species of microalgae can store considerable amounts of energy-rich compounds, specifically lipids and polysaccharides, which can be utilized to produce biofuels such as biodiesel and bioethanol [4,5]. Microalgae cells can double the biomass of these energy-rich compounds by 1 to 3 times in 24 h, and under specific conditions, they can store 40 to 60 % of their dry weight in fatty acids [6].

A bottleneck in the use of microalgae for biofuel production involves the cost, which can be 2 to 3-fold higher than when using first- and second-generation biomass [7]. Harvesting, drying, and oil extraction account for half of the total production cost. Harvesting alone occupies almost 30% of total production cost, which can amount to as much as US $10/kg [8]. To achieve economic feasibility in biofuel production from microalgae, it is necessary to focus on the most important areas, such as low lipid

accumulation, expensive cultivation steps, and unproductive downstream processes [7].

Alginate is one of the most abundant biopolymers of natural origin available after cellulose, with many useful properties such as low toxicity, biodegradability, biocompatibility, and good hydrogel-forming ability upon the addition of divalent cations such as Ca^{2+} [9].

Immobilization technology has been proposed as an alternative technique to facilitate the extraction of microalgal cells in the aqueous phase, from which immobilized cells can be harvested through simple filtration without the need for large amounts of energy [10]. Immobilization can be either physical (adsorption) or chemical (entrapment, cross-linkage, or covalent bonding) [11], entrapment in a polymeric matrix, such as calcium alginate, is one of the most widely used immobilization techniques for microalgae [12]. Alginate beads are formed when a solution of sodium alginate and microalgae cells is extruded into a Ca^{2+} solution, at that moment an instantaneous interfacial polymerization occurs, producing the beads with the immobilized microalgae inside, the bead hardness is increased with the permeation of calcium ions through the beads [11].

It has been reported that the cost of harvesting suspended cells by centrifugation is approximately US $1.8 per cubic meter of culture volume, however, the cost of alginate for cell immobilization is about US $0.2 per cubic meter of culture volume [13], taking into account that the cost of the alginate used for immobilization is much lower than harvesting by centrifugation, makes the use of alginate-immobilized cells more economically viable and attractive.

The chlorophyll-a content, which is considered to be a cell activity indicator, is higher in immobilized cultures [14], immobilized cells have also shown a higher level of

chloroplast formation and higher oxygen production rates than free microalgae cells [15]. Immobilized microalgae cells are able to retain 90 % of their chlorophyll even after 3 months, whereas in a free cell culture, the occurrence of pheophytinization occurs just after 7 days [15].

Currently, the most frequent applications of immobilized microalgae include wastewater treatment [16,17], removal of nutrients from aquatic media [14,18], removal of organic or metal pollutants [19,20], toxicity measurement [21,22], and energy production *via* hydrogen [23,24] or electricity [12,25]. In the previously mentioned applications of immobilized microalgae, their main focus has not been the improvement of lipid accumulation, which has not been developed to the same level, making the information regarding this topic to remain limited and scarce, with most studies achieving a similar growth between free and immobilized cells.

Cheirslip et al. (2017) [13] evaluated the growth of *Nannochloropsis* sp. immobilized on alginate beads in a secondary effluent of palm oil mill effluent, they were able to cultivate the immobilized cells to achieve a comparable growth and lipid production to the free cells by varying the bead volume and inoculum size. Li et al. (2018) [26] found that the optimum immobilization conditions for *Chlorella vulgaris* were of 2.42 % sodium alginate and 2.69 % $CaCl_2$ to treat fracturing flowback fluids, achieving a higher COD removal compared to the free cells, and obtained a growth of 1.97 g/L. Lee et al. (2020) [14] studied the effect of the alginate bead size for optimal growth and nutrient removal of *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, their results showed that the optimal bead size was of 3.5 mm. The growth of free and immobilized cells of *Chlorella vulgaris* was studied by Sepian et al. [27], with a combination of matrices of sodium alginate, calcium alginate, and sodium carboxymethyl cellulose, and

nearly identical cell densities were achieved between free and immobilized cells.

This study focused on the immobilization of *Chlorella sorokiniana* for lipid accumulation. It is a robust industrial species that tolerates high temperatures and high levels of intense light [28]. This strain possesses levels of lipid accumulation that can reach >30 % of its dry cell weight, *Chlorella* strains has been studied for commercial production of biodiesel because of their high growth rates, with some species being able to double their biomass and lipid yield within 24 h [29].

The aim of the present study was to demonstrate the potential of immobilization not only as a tool to simplify the harvesting process of microalgal cells but also as a method to increase the lipid productivity of the cells, addressing two of the disadvantages for achieving biodiesel economic feasibility: lipid accumulation and simplification of downstream processes.

II.2. Materials and Methods

II.2.1. Strain and culture conditions

Chlorella sorokiniana UTEX 1230 cells, as described by Amoah et al. [28], were precultured for 96 h at 30 ± 2 °C in 250 mL of modified Bold 6 N medium, supplied with CO_2 (2 %) at 0.5 vvm, under continuous light irradiation of approximately 250 µmol photons/m^2/s. The optimal growth conditions for *C. sorokiniana* are approximately 30–35 °C and light irradiation above 100 µmol photons/m^2/s [29], the optimum temperature for high fatty acid production in *C. sorokiniana* is 30 °C [27].

II.2.2. Optimization of alginate concentration for beads production

To produce stable beads, it was necessary to adjust the concentration of alginate utilized for bead production and the concentration of the gelling solution, $CaCl_2$ was utilized as the gelling solution in this study. Alginate and $CaCl_2$ concentrations were varied between 1% and 4% in order to produce stable and cylindrical beads.

II.2.3. Alginate immobilization of *C. sorokiniana* cells

An aliquot of 0.1 OD_{680} cells was gathered by centrifugation at 7,600 xg for 5 min and washed twice with 50 ml of Milli-Q water [28]. The Microalgae cells were resuspended and mixed with a 2% (w/v) sodium alginate solution (Nacalai Tesque, Japan). The solution was then dripped with a syringe into a 100 ml sterile solution of 2% (w/v) $CaCl_2$ to form spherical beads with a diameter of approximately 4.0 mm as shown on Figure 1, 1 ml of beads contained approximately 40 beads. The beads were cured for 1 h and then washed with distilled water.

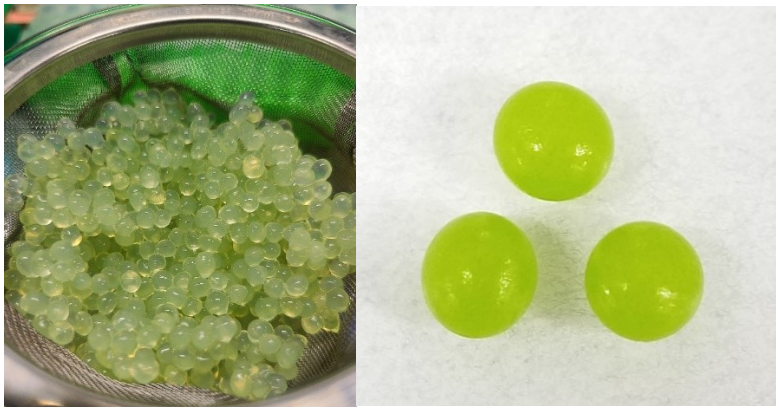


Figure 1. *Chlorella sorokiniana* cells immobilized in alginate beads.

II.2.4. Immobilized culture of *C. sorokiniana* in alginate beads

The immobilized cells of *C.* sorokiniana were cultured in 500 ml cylindrical flasks for a period of 9 days utilizing a volume of 20% beads (100 ml) and 400 ml of Bold 3 N

medium, at 30 ± 2 °C supplied with CO_2 (2 %) at 0.5 vvm under continuous light irradiation of approximately 250 µmol photons/m^2/s. A free-cell culture with an 0.1 OD_{680} was also performed as a control under the same culture conditions as shown on Figure 2. An LI-250A light meter (LI-COR, NE, USA) was used to measure light intensity. The light intensity was measured at both the back and front of the culture flask to calculate transmittance. Three flasks of immobilized beads and three flasks of free *C. sorokiniana* cells were used.

Figure 2. Experimental setup for the culture of immobilized cells (left flask) and free cells (right flask) of *Chlorella sorokiniana*.

II.2.5. Dry cell weight measurement

To measure the dry cell weight, 10 ml of alginate beads (approximately 400 beads)

were taken from the culture, dissolved in a 2 % (w/v) Na_2CO_3 solution, and freeze dried to obtain dry cells that could then be weighed. The biomass produced is shown as the mass of dry cell weight per volume of culture.

II.2.6. Lipid content analysis

Lipid analysis was performed by crushing 3 mg of dried *C. sorokiniana* cells in a multi-bead shocker (Yasui Kikai, Japan) with 0.5 mm glass beads. Using a fatty acid methylation kit (Nacalai Tesque, Japan), the lipids were esterified and extracted, followed by quantification using gas chromatography-mass spectrometry (GCMS-QP2010 Plus, Shimadzu, Japan), as described by Amoah et al. [28]. Purified FAMEs were identified by their retention times, using margaric acid as the internal standard.

II.2.7. Evaluation of *C. sorokiniana* cells growth and distribution inside the alginate beads

To observe the growth and condition of the cells inside the alginate beads, they were analyzed using a Keyence BZ-X810 All-in-one Fluorescence Microscope, for this 1 ml of the alginate beads was taken, followed by degradation of the beads using a 2 % Na_2CO_3 solution; after extracting the cells from the alginate beads, they were resuspended in 1 ml of Milli-Q water, 100 µL of the suspended cells were washed with 100 µL of PBS, followed by a resuspension in 45 µL of PBS and 5 µL of BODIPY (Invitrogen, Thermo Fisher Scientific, Japan) and left staining for 12 h. To observe their lipid accumulation, 1 ml of the free cultured cells was also submitted to staining and observed under the fluorescence microscope as a control.

Analysis of *C. sorokiniana* cell distribution inside the alginate beads was

performed using an Olympus Fluoview FV31S-SW confocal laser scanning microscope (CLSM). One bead was taken from the culture, followed by cell staining with BODIPY (Invitrogen, Thermo Fisher Scientific, Japan) as previously described, and a cross-section cut of the bead was observed using CLSM.

II.2.8. Statistical Analysis

For the statistical analysis, the results data was subjected to a one-way ANOVA using JMP Pro 16 to evaluate significant differences, where $p \leq 0.05$. The data presented in this study are the average of triplicate measurements, and the values were expressed as mean ± standard deviation. The experiments were conducted three times to further verify the results.

II.3. Results

II.3.1. Alginate concentration optimization for stable alginate beads production.

In this study, the beads produced with different concentrations of alginate and $CaCl_2$ were evaluated, the concentrations ranged from 1 % to 4 %. The alginate beads prepared with alginate and $CaCl_2$ concentrations below 2 % did not have a well-defined shape and they broke or dissolved easily due to their low stability.

The beads produced with 2% alginate and 2% $CaCl_2$ had a well-formed spherical shape, as shown in Figure 3, 4 mm size, good stability, not breaking after submitting them to vortex, and were easily degraded by a 2 % Na_2CO_3 solution.

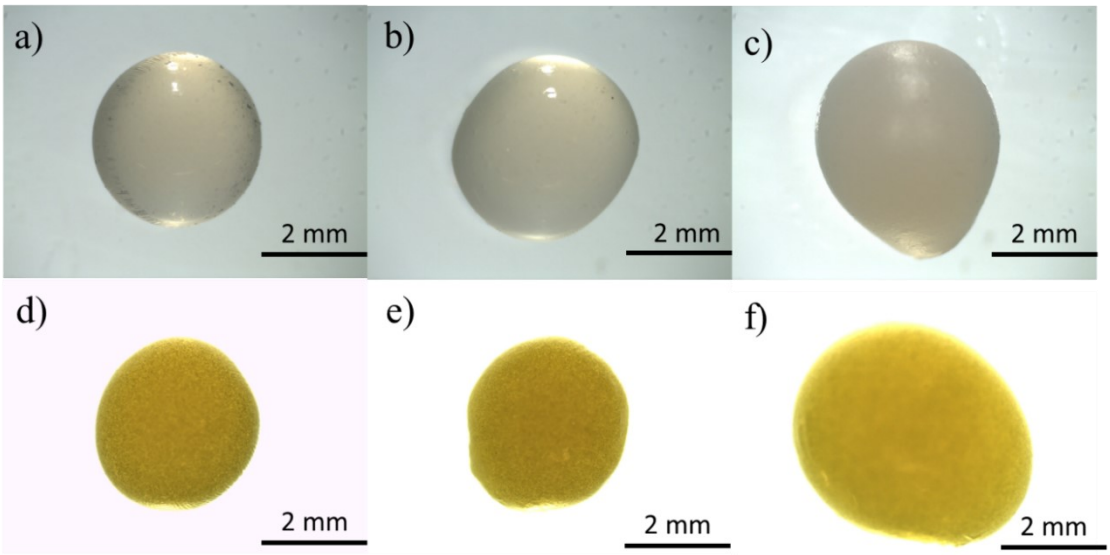


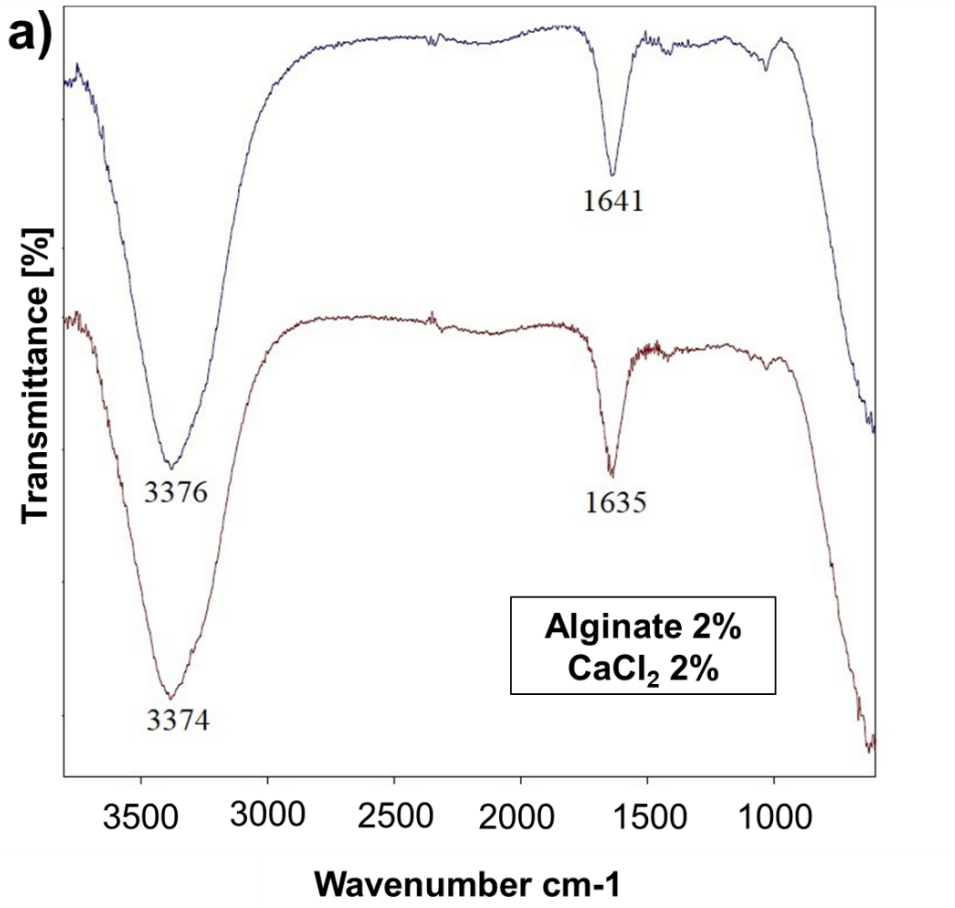
Figure 3. Alginate beads images at different concentrations. (a) Blank alginate bead (2 % alginate, 2 % $CaCl_2$), (b) Blank alginate bead (2 % Alginate, 4% $CaCl_2$), (c) Blank alginate bead (4 % Alginate, 2 % $CaCl_2$), (d) Immobilized cells in alginate bead (2 % Alginate, 2 % $CaCl_2$), (e) Immobilized cells in alginate bead (2 % Alginate, 4 % $CaCl_2$), (f) Immobilized cells in alginate bead (4 % Alginate, 2 % $CaCl_2$).

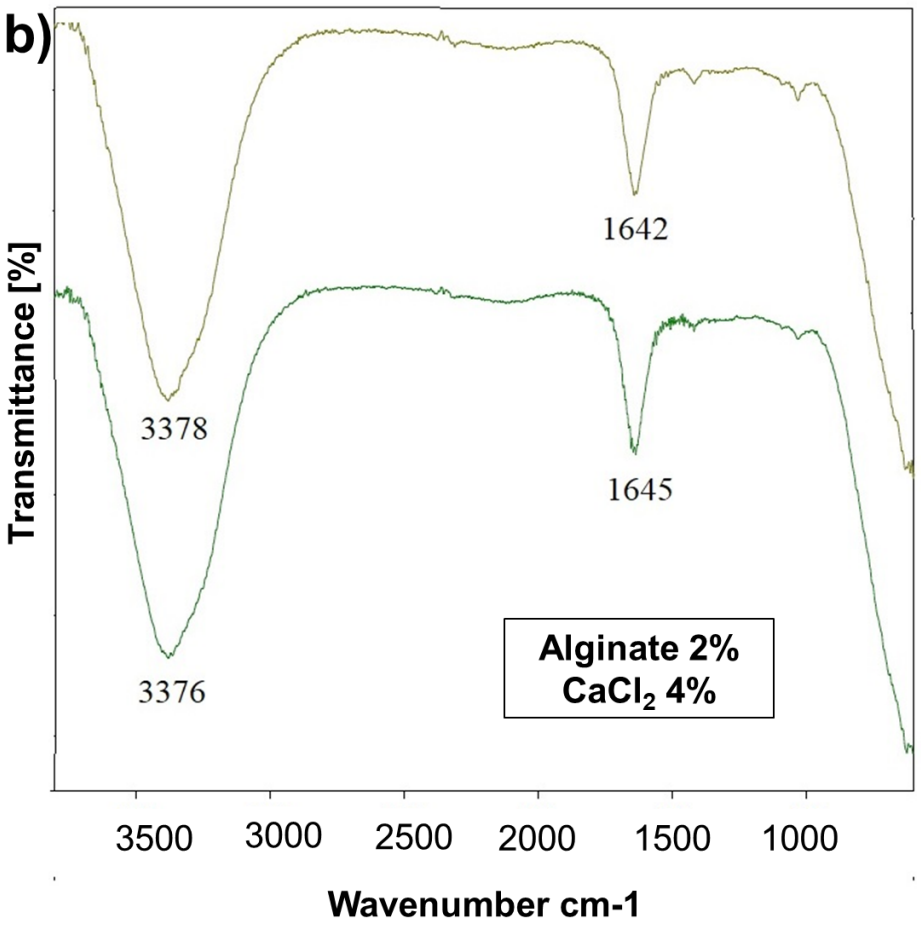
By utilizing alginate concentrations higher than 2 %, the alginate solution became very viscous, making it harder to achieve a good mix with the microalgae cells. Moreover, because of its high viscosity at the time of dropping the alginate solution into the $CaCl_2$ to form the beads, the drops could not form completely and the bead shape was not spherical, their size was bigger compared to the beads produced with lower alginate concentrations, and the degradation of the beads by a 2 % Na_2CO_3 solution was not possible; a higher concentration of Na_2CO_3 solution was needed, and the time it took for the beads to dissolve was three times higher than of the beads produced by a 2 % alginate solution. For these reasons in the following experiments, the concentrations used to produce the beads were of 2 % alginate and a 2 % $CaCl_2$.

FTIR was used to characterize the beads produced in this study, the results of the spectra obtained are shown on Figure 4. Mid-infrared spectral data (4000-500 cm^{-1}) were

used to identify the functional groups of organic and inorganic bonding.

The functional peaks indicate similar spectra for the 3 different configurations of beads, having similar absorption bands. The alginate beads showed important absorption bands regarding hydroxyl, and carboxylic functional groups. The stretching vibration of O-H bonds of alginate appeared in the range of 3000–3600 cm^{-1}. Observed bands in 1645 and 1460 cm^{-1} were attributed to asymmetric and symmetric stretching vibrations of carboxylate salt ion, respectively.




b)
Transmittance [%]
1642
3378
1645
3376
Alginate 2%
CaCl2 4%
3500
3000
2500
2000
1500
1000
Wavenumber cm-1

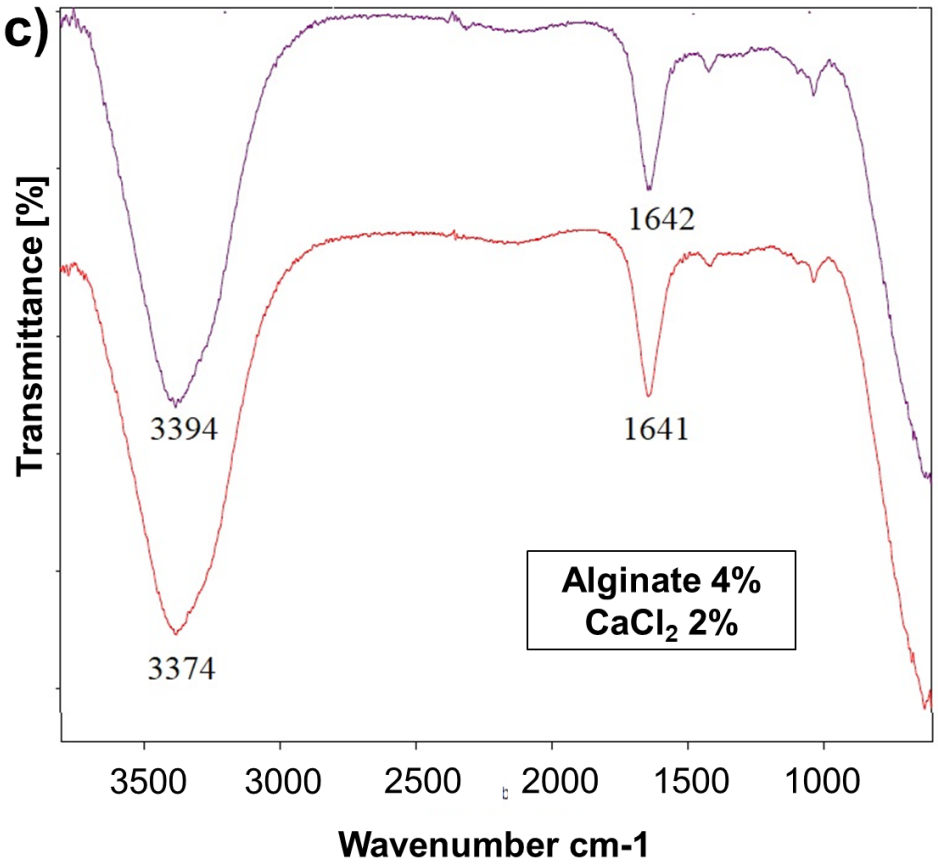


Figure 4. FTIR spectra of alginate beads. (a) Alginate beads (2 % Alginate, 2% $CaCl_2$), (b) Alginate beads (2 % Alginate, 4% $CaCl_2$), (c) Alginate beads (4 % Alginate, 2% $CaCl_2$).

II.3.2. Immobilized *Chlorella sorokiniana* growth

The time course of the increase in the dry cell weight of the free and immobilized cells is shown in Figure 5. Compared to the free-cell culture, the immobilized cells showed a higher dry cell weight that began on day 1, and this tendency continued until day 9, revealing that the immobilized culture had a positive effect on the cell growth compared to the suspended culture. On day 9 the dry cell weight for the free cell culture was of 2.09 ± 0.03 g/L, compared with 2.87 ± 0.24 g/L in the immobilized culture, which was about 1.4-fold higher.

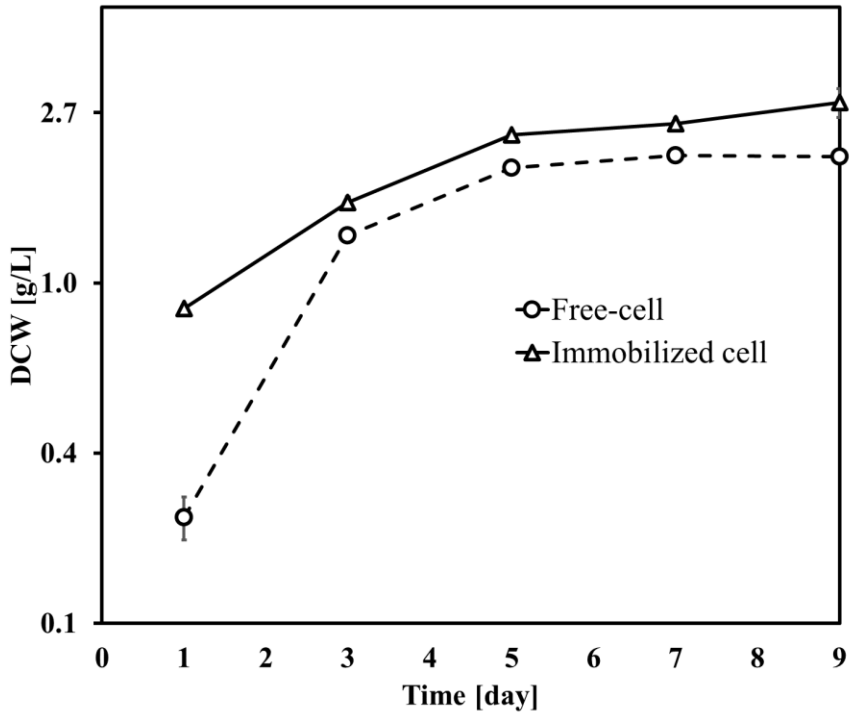


Figure 5. Dry cell weight (DCW) of free-cell and alginate-immobilized culture of *C. sorokiniana*. The error bars show the standard deviation of triplicate values.

II.3.3. Light transmittance in an immobilized culture

The variation in light transmittance during the growth of both the free and immobilized cell cultures is shown in Figure 6. On day 1 the light transmittance was higher in the free cell culture than in the immobilized culture, this was because the number of cells inside the culture medium was still relatively low, for this reason the medium remained transparent, however by day 3 the number of cells had already increased, causing the culture medium to become darker and decreased the light transmittance in the culture medium from 58 % on day 1 to 6% on day 9.

In the immobilized culture, the cells grew inside the alginate beads but not in the

culture medium, and the leaching of the cells into the culture medium was minimal. The alginate beads maintained their stability, and they did not break or dissolve, allowing the medium to remain transparent during the nine days of culture; only the beads became darker. In the immobilized culture, the light transmittance experienced a lower decrease compared to that of free cell culture, going from 23 % on day 1 to 15 % on day 9.

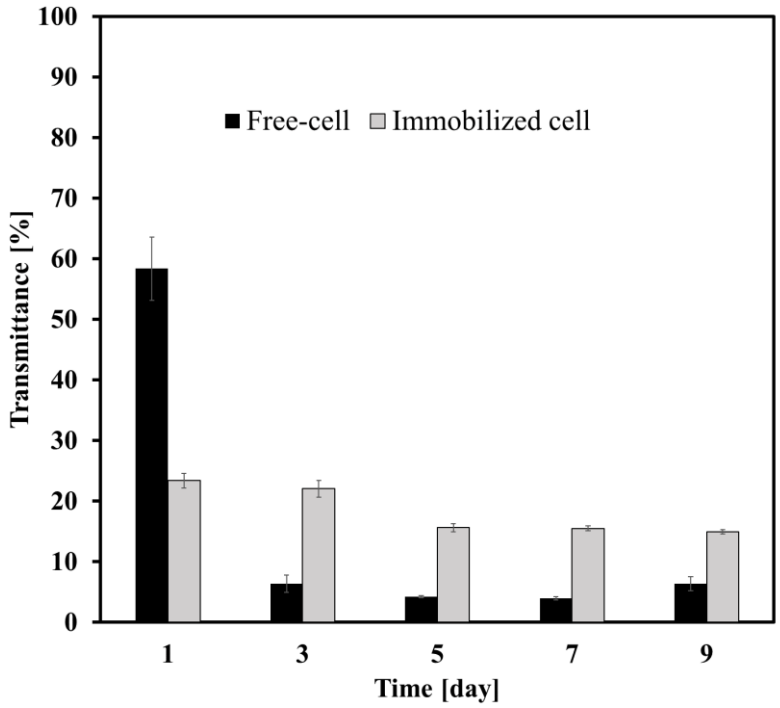


Figure 6. Light transmittance in a free-cell *C. sorokiniana* culture and in an alginate-immobilized culture. The error bars show the standard deviation of triplicate values.

II.3.4. Lipid productivity of immobilized *C. sorokiniana*

The time courses for the increase in lipid content and lipid productivity are shown in Figure 7a for free cells and in Figure 7b for immobilized cells. The lipid content in the immobilized culture was higher than that in the free-cell culture, reaching a 23.3% in the suspension culture and 25.8% in the immobilized cells. Lipid productivity in the

immobilized culture was higher than that in the free-cell culture; this tendency remained during the nine days of culture, and the difference was especially noticeable on days 7 and 9, where it achieved 1.3- and 1.5-fold higher lipid productivity than that in the free-cell culture.

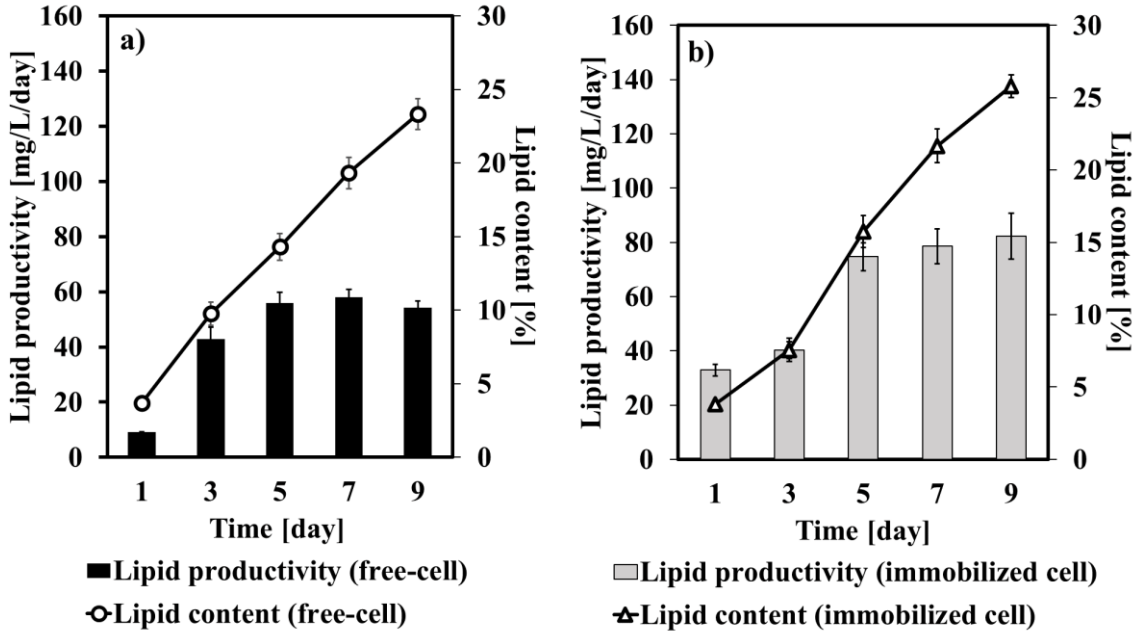


Figure 7. Lipid content and lipid productivity in a) free-cell culture *C. sorokiniana* and b) alginate immobilized culture *C. sorokiniana*. The error bars show the standard deviation of triplicate values.

The highest lipid productivity was obtained on day 9 at 82.22 ± 8.48 mg/L/d for the immobilized alginate culture and 54.21 ± 2.48 mg/L/d for the free-cell culture, this clearly shows how immobilization enhances the microalgae growth and is beneficial for both lipid content and lipid productivity.

The fatty acid profiles of free and immobilized cultures are shown in Table 1. In both configurations, the fatty acids consisted of long-chain fatty acids; the predominant

fatty acid was palmitic acid, followed by linoleic acid and oleic acid. The composition of the fatty acids in the immobilized culture included concentrations of oleic acid (21.6 %) and linoleic acid (25.1 %), which were slightly higher than those found in the free-cell culture (20.4 % and 24.9 %, respectively). The fatty acid profile of palm oil, which is one of the most utilized feedstocks for biodiesel production, contains mainly oleic and palmitic acids [30], which is similar to the fatty acid profile obtained by immobilized *C. sorokiniana*, indicating that these lipids have the potential to be used as biodiesel feedstock.

Table 1
Fatty acid profile of lipids from free and immobilized *C. sorokiniana*.

FAME composition	Free cells (%)	Immobilized cells (%)
Palmitic Acid (C16:0)	34.9	34.6
Palmitoleic Acid (C16:1)	1.9	2.3
Stearic Acid (C18:0)	7.7	7.8
Oleic Acid (C18:1)	20.4	21.6
Linoleic Acid (C18:2)	24.9	25.1
Linolenic Acid (C18:3)	9.7	8.3
Eicosanoic Acid (C20:0)	0.6	0.3

II.3.5. *C. sorokiniana* cells condition and distribution inside of the alginate beads

The alginate immobilized cells were stained and observed in a fluorescence microscope, for both culture forms, the highest lipid productivity occurred between day 5 and 9 of the culture. Fluoroscopic images of the cells grown in suspension culture and cells grown in immobilized culture are shown on Figure 8, it can be observed that the cell density of the immobilized cells was higher than that of the free cells.

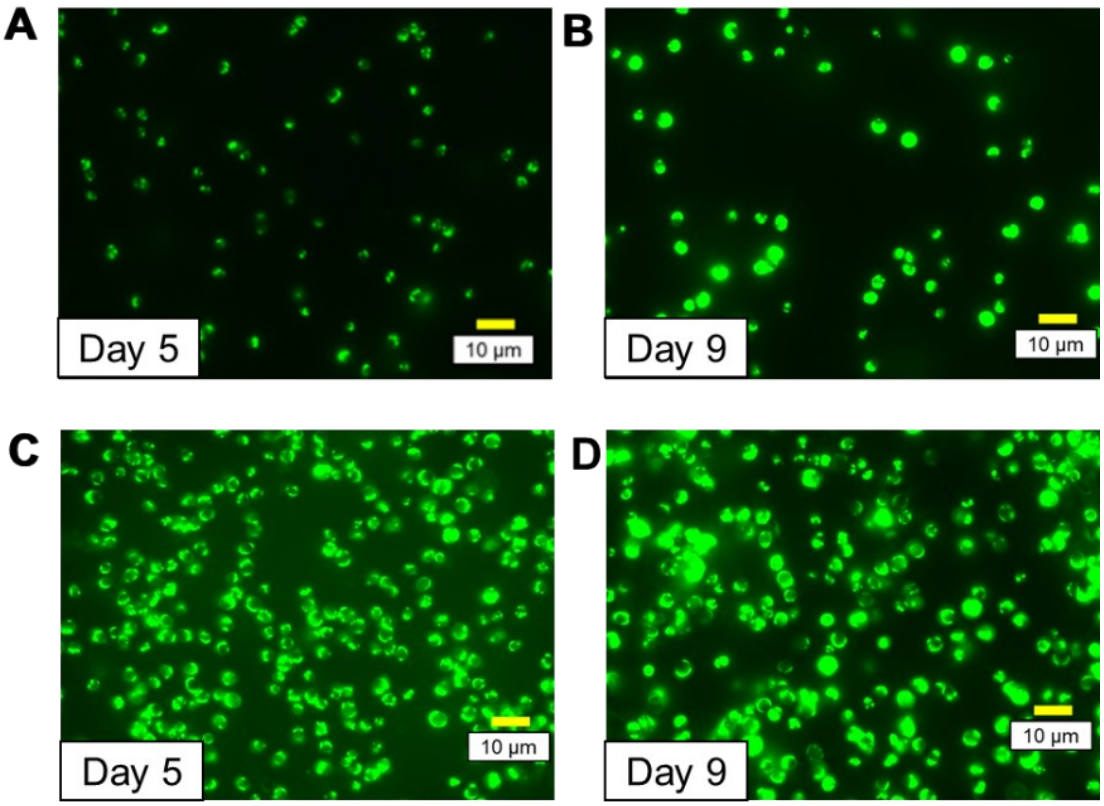


Figure 8. Fluoroscopic images of *C. sorokiniana cells,* (A) Suspension culture day 5, (B) suspension culture on day 9, (C) Immobilized culture on day 5, (D) Immobilized culture on day 9.

The CLSM images of the alginate beads are shown in Figure 9, where it can be observed that on day 5, the cells were mostly around the edge of the bead, and there was a small number of cells in the center; however, by day 9, the total cell number increased, and they were distributed all over the bead.

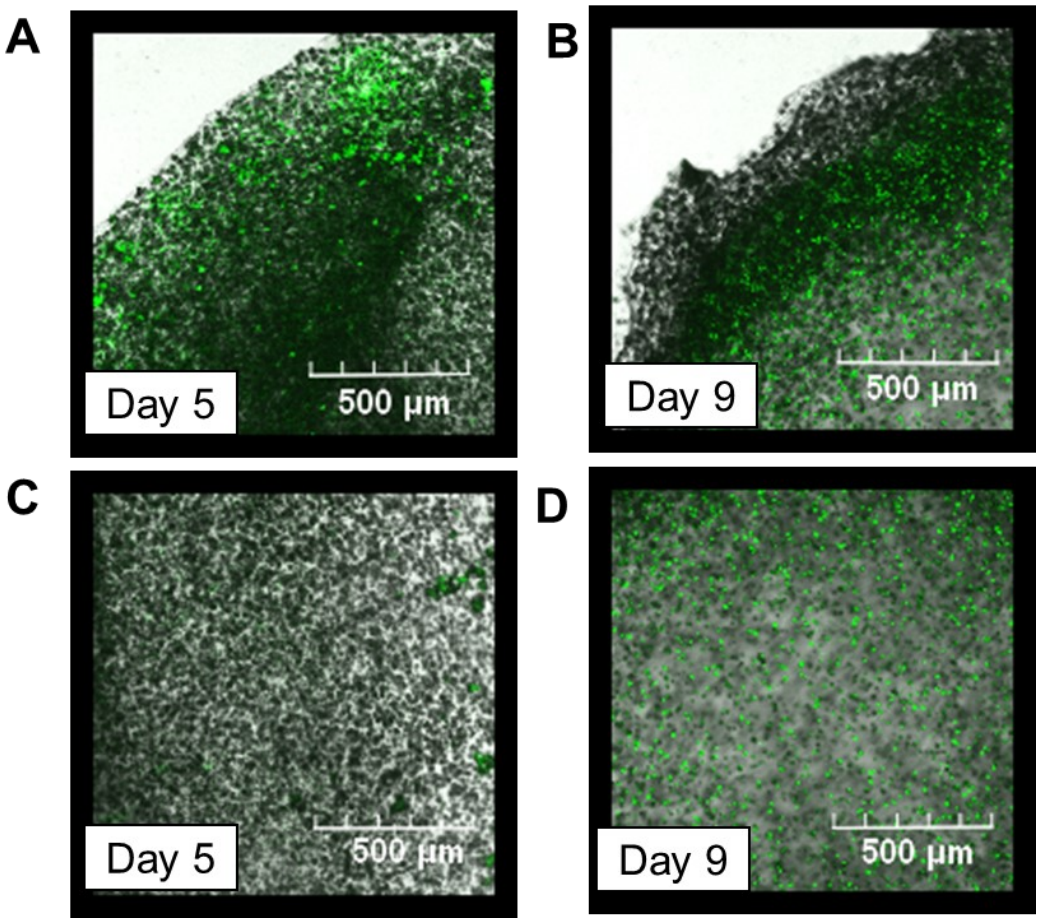


Figure 9. CLSM images of *C. sorokiniana* cells entrapped in an alginate bead, (A) side of alginate bead on day 5, (B) center of alginate bead on day 5, (C) side of alginate bead on day 9, (D) center of alginate bead on day 9.

II.4. Discussion

The results of the experiments conducted in this study showed an overall positive effect on the growth and lipid productivity by the immobilized microalgae *C. sorokiniana*. Previous studies focused on the utilization of alginate immobilized microalgae for the treatment of wastewater or nutrient removal, in each of them different optimized parameters were found, such as the optimum bead volume of 25 % to obtain a comparable growth to that of free cells [11], optimum concentrations for alginate 2.42 % and calcium

chloride 2.69 % [24], and optimum bead size of 3.5 mm [12], the optimized conditions found in each of these studies were applied to this research which allowed the growth and lipid productivity of *C. sorokiniana* to be enhanced.

A comparison between the results obtained in this study and previous studies regarding the use of *C. sorokiniana* for lipid accumulation is shown in Table 2, where it can be seen that the dry cell weight and lipid content obtained by the immobilized culture under autotrophic conditions are higher than those obtained by a free cell culture in other studies, and it is comparable to the growth and lipid content obtained by utilizing heterotrophic or mixotrophic growth conditions, showing the potential that immobilization has to increase the lipid productivity of this microalgae strain.

One of the reasons for this increase in growth could be that in the immobilized system, a more efficient mixing was achieved compared with that of a free-cell culture, this is because the microalgae cells grew inside the alginate beads in the immobilized culture, rather than in the culture medium as in the free cell culture [12,13], allowing the beads to remain suspended and in constant movement inside the culture medium by simple stirring or CO_2 bubbling, without the need for expensive stirring methods.

Mixing is a very important parameter in microalgae cultivation, and its function is to homogenize the culture medium and prevent the clumping of cells or settling inside the reactor [35]. Mixing also allows for a more uniform distribution of nutrients in the medium and it is critical to enhance the biofixation of CO_2. Poor mixing can lead to undesirable pH and nutrients gradients [36]. As immobilized cells remain in constant movement inside the system, they have better access to nutrients. However, it is difficult to achieve perfect cell mixing in suspended cultures.

Table 2

Previous studies of *C. sorokiniana* for biodiesel production.

Microalgae	Growth condition	Initial glucose (g/L)	CO_2 (%)	Temperature (°C)	DCW (g/L)	Lipids (%)	Reference
C. sorokiniana	Autothropic (Free cell)	0	5	37	1.0	10%	[31]
C. sorokiniana	Autotrophic (Free cell)	0	10	37	1.2	11%	[31]
C. sorokiniana	Autotrophic (Free cell)	0	15	37	1.1	20%	[31]
C. sorokiniana	Heterotrophic (Free cell)	6	1	25	2.8	12%	[32]
C. sorokiniana	Mixotrophic (Free cell)	4	1	25	3.6	7%	[32]
C. sorokiniana	Autotrophic (Free cell)	0	2	30	2.1	23%	Present study
C. sorokiniana	Autotrophic (Alginate immobilized)	0	2	30	2.9	26%	Present study

As shown in Figure 6, the microalgae cells were able to grow and disperse inside the alginate beads and their movement was not restrained. Lee et al. [14] reported that the optimum bead size for cell growth was approximately 3.5 mm, if the bead size was bigger, there would be empty spaces inside the bead, caused by the cells inside the alginate beads migrating from the inside of the beads to the edge to prevent limited substrate diffusion and to find the optimal light conditions [14]. However, the bead size utilized in this study seemed to be appropriate because by day 9 of culture, the cells were localized both at the edge and in the center of the bead, allowing them to receive light and nutrients efficiently.

Light is important for microalgal growth; this basic energy source affects cell growth, carbon fixation ability, and productivity [37]. In an immobilized culture, the cells grow inside the beads rather than in the culture medium, which allows for better light

penetration than that in the free cell culture. The light transmittance results shown in Figure 3 indicate that the immobilized culture had a higher level of light transmittance than the free cell culture, which is beneficial for the cells. This could have been one of the reasons for the improvement in dry cell weight because it prevents the process of self-shading which usually occurs in free cell cultures, where the cells are unable to receive the same amount of light, and cells closest to the surface receive most of the light [38]. In an immobilized system, on the other hand, the cells grow inside a matrix rather than in the culture medium, which remains transparent and allows the cells to receive an equal amount of light.

From the results in Figure 2, it can be seen that by day 7, the free cell culture had already reached its stationary phase and was approaching its death phase. However, for the immobilized culture, even after day 7, it continued to grow, resulting in a higher dry cell weight and a higher lipid productivity than that of the free cell. In addition, the immobilization strategy offers a shorter lag phase and acclimatization period than free cell culture [14], allowing lipid productivity to begin sooner.

II.5. Conclusions

The results of the present study showed the potential for immobilization to improve the growth and lipid productivity of the microalgae *C. sorokoniana* by achieving a growth that was 1.4-fold higher than that of a free cell culture, leading also to an increase in lipid content and productivity. Immobilization also simplifies the harvesting process, and its application could lower the cost associated with the harvesting of suspension cultures, which should promote the economic feasibility of biofuels production from microalgal biomass.

A limitation of this technology is the need to separate the cells from the alginate beads, in order to extract the lipids accumulated inside the cells, however, this could be avoided by utilizing a strain that secretes the desired chemical to the medium, future studies should apply the immobilization technology to these kinds of strains, to take advantage of the increase in growth by the immobilization and the simplification of harvesting, an example of these types of strains would be the cyanobacteria *Synechocystis* sp. PCC 6803 which secretes succinate and lactate into its fermentation medium [39].

II.6. Statistical analysis data Oneway Anova

Dry cell weight data (Free-cell)

Table 3. Oneway Anova Summary of Fit Dry cell weight data (Free-cell)

Rsquare	0.996063
Adj Rsquare	0.994488
Root Mean Square Error	0.054432
Mean of Response	1.544853
Observations (or Sum Wgts)	15

Table 4. Analysis of Variance Dry cell weight data (Free-cell)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	4	7.4957011	1.87393	632.4829	<.0001*
Error	10	0.0296281	0.00296		
C. Total	14	7.5253292			

Table 5. Means for Oneway Anova for Dry cell weight data (Free-cell)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	0.25480	0.03143	0.1848	0.3248
3	3	1.31667	0.03143	1.2466	1.3867
5	3	1.95493	0.03143	1.8849	2.0250
7	3	2.10547	0.03143	2.0354	2.1755
9	3	2.09240	0.03143	2.0224	2.1624

Std Error uses a pooled estimate of error variance

Dry cell weight data (Immobilized cell)

Table 6. Oneway Anova Summary of Fit Dry cell weight data (Immobilized cell)

Rsquare	0.974955
Adj Rsquare	0.964937
Root Mean Square Error	0.142028
Mean of Response	2.04628
Observations (or Sum Wgts)	15

Table 7. Analysis of Variance Dry cell weight data (Immobilized cell)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	4	7.8527294	1.96318	97.3217	<.0001*
Error	10	0.2017209	0.02017		
C. Total	14	8.0544502			

Table 8. Means for Oneway Anova for Dry cell weight data (Immobilized cell)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	0.86087	0.08200	0.6782	1.0436
3	3	1.60053	0.08200	1.4178	1.7832
5	3	2.37080	0.08200	2.1881	2.5535
7	3	2.53420	0.08200	2.3515	2.7169
9	3	2.86500	0.08200	2.6823	3.0477

Std Error uses a pooled estimate of error variance

Lipid content data (free-cell)

Table 9. Oneway Anova Summary of Fit Lipid content data (free-cell)

Rsquare	0.984278
Adj Rsquare	0.97799
Root Mean Square Error	0.010723
Mean of Response	0.140733
Observations (or Sum Wgts)	15

Table 10. Analysis of Variance Lipid content data (free-cell)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	4	0.07198079	0.017995	156.5172	<.0001*
Error	10	0.00114973	0.000115		
C. Total	14	0.07313051			

Table 11. Means for Oneway Anova for Lipid content data (free-cell)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	0.036700	0.00619	0.02291	0.05049
3	3	0.097567	0.00619	0.08377	0.11136
5	3	0.143033	0.00619	0.12924	0.15683
7	3	0.193133	0.00619	0.17934	0.20693
9	3	0.233233	0.00619	0.21944	0.24703

Std Error uses a pooled estimate of error variance

Lipid content data (Immobilized cell)

Table 12. Oneway Anova Summary of Fit Lipid content data (Immobilized cell)

Rsquare	0.989445
Adj Rsquare	0.985222
Root Mean Square Error	0.010461
Mean of Response	0.14916
Observations (or Sum Wgts)	15

Table 13. Analysis of Variance Lipid content data (Immobilized cell)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	4	0.10257383	0.025643	234.3438	<.0001*
Error	10	0.00109427	0.000109		
C. Total	14	0.10366810			

Table 14. Means for Oneway Anova Lipid content data (Immobilized cell)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	0.038267	0.00604	0.02481	0.05172
3	3	0.075400	0.00604	0.06194	0.08886
5	3	0.157533	0.00604	0.14408	0.17099
7	3	0.216733	0.00604	0.20328	0.23019
9	3	0.257867	0.00604	0.24441	0.27132

Std Error uses a pooled estimate of error variance

Lipid Productivity data (Free-cell)

Table 15. Oneway Anova Summary of Fit Lipid Productivity data (Free-cell)

Rsquare	0.972313
Adj Rsquare	0.961238
Root Mean Square Error	3.761922
Mean of Response	44.05355
Observations (or Sum Wgts)	15

Table 16. Analysis of Variance Lipid Productivity data (Free-cell)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	4	4969.8755	1242.47	87.7942	<.0001*
Error	10	141.5206	14.15		
C. Total	14	5111.3961			

Table 17. Means for Oneway Anova for Lipid Productivity data (Free-cell)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	9.1975	2.1719	4.358	14.037
3	3	42.8735	2.1719	38.034	47.713
5	3	55.9161	2.1719	51.077	60.756
7	3	58.0627	2.1719	53.223	62.902
9	3	54.2178	2.1719	49.378	59.057

Std Error uses a pooled estimate of error variance

Lipid Productivity data (Immobilized cell)

Table 18. Oneway Anova Summary of Fit Lipid Productivity data (Immobilized cell)

Rsquare	0.929988
Adj Rsquare	0.901983
Root Mean Square Error	6.983209
Mean of Response	61.74045
Observations (or Sum Wgts)	15

Table 19. Analysis of Variance Lipid Productivity data (Immobilized cell)

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Day	4	6477.5975	1619.40	33.2081	<.0001*
Error	10	487.6521	48.77		
C. Total	14	6965.2496			

Table 20. Means for Oneway Anova for Lipid Productivity data (Immobilized cell)

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
1	3	32.9263	4.0318	23.943	41.910
3	3	40.3125	4.0318	31.329	49.296
5	3	74.6885	4.0318	65.705	83.672
7	3	78.5563	4.0318	69.573	87.540
9	3	82.2187	4.0318	73.235	91.202

Std Error uses a pooled estimate of error variance

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CHAPTER III

Alginate Immobilization as a Strategy for Improving Succinate Production During Autofermentation Using Cyanobacteria *Synechocystis* sp. PCC6803

III.1. Introduction

Cyanobacteria and microalgae are known for their ability to store high amounts of useful compounds, such as proteins, lipids, and carbohydrates, which can be used to produce biochemicals and biofuels [1]. The cyanobacterial strain, *Synechocystis* sp. PCC 6803 can produce organic acids such as succinate and D-lactate by catabolism of glycogen, which is accumulated during photosynthesis as a primary storage polysaccharide [2].

In 1996, the sequencing of the genome of *Synechocystis sp. PCC6803* was developed by Kaneko et al. [3], allowing genetic modifications of its metabolic pathways to enhance its photosynthetic activity [4]. By performing metabolic engineering techniques, such as gene deletion and overexpression, *Synechocystis* sp. PCC6803 converts CO_2 directly into useful substances like cyanophycin, 2,3-butanediol, isobutanol, 3-hydroxypropionic acid, and PHAs [5,6], and the overexpression of phosphoenolpyruvate (PEP) carboxylase genes (ppc) improve its succinate production [2].

Succinic acid, a four-carbon (C4) dicarboxylic acid, is a significant platform chemical that can be used in the pharmaceutical and agricultural industries [7] and has the potential to displace the maleic anhydride platform as a versatile basic chemical. Succinate can be used as a polymer monomer, surfactant, and ion chelator [8] it also

serves as a precursor for numerous useful chemicals utilized in industry, including 1,4-butanediol, dimethyl succinate, tetrahydrofuran, and γ-butyrolactone [9]. The succinate market has expanded over time, in recent years the annual global production volume has been 58500 tons. The succinic acid market size was $157.2 million in 2015, and it is anticipated to increase up to $75000 million [9].

The generation of succinate has been achieved by utilizing native producers like *Actinobacillus succinogenes* and certain fungi [10–13]. These are heterotrophic microorganisms; however, require carbon sources, such as sugars for succinate production, which generates a bottleneck for the production of biochemicals. Through autofermentation under dark anoxic conditions, the cyanobacterium *Synechocystis* sp. PCC6803 can secrete succinate without the need for sugar [14].

Several researches have focused on improving the succinate yield achieved by *Synechocystis* sp. PCC6803 by genetic modifications such as the deletion of succinate dehydrogenase (SDH) which increased its succinate accumulation while also decreasing its cell numbers [15], the introduction of a glycogen shunt which resulted in a higher extracellular succinate accumulation compared to a wild type strain [16], and the overexpression of malate dehydrogenase that enhanced the production of succinate [17].

However the use of cell immobilization to increase succinate production has yet to be evaluated, previous studies on hydrogen production from immobilized *Synechocystis* sp. PCC6803 [18] showed that immobilization technology increased hydrogen production in immobilized cells compared to that in free cells. Immobilization also simplifies both the cell separation and two-phase H_2 production processes [18], based on these results it is plausible to assume that immobilization could also support succinate production, as it does for hydrogen production.

Succinate production by *Synechocystis* sp. PCC6803 is yet to be optimized and downstream separation of the cells from both the culture medium and the secreted succinate continues to require costly processing. Immobilization technology has been proposed as an alternative technique to facilitate the extraction of microalgal cells in an aqueous phase, from which immobilized cells can be harvested through simple filtration without the need for large amounts of energy [19]. It has been reported that the cost of harvesting suspended cells by centrifugation is approximately US $1.8 per cubic meter of culture volume, however, the cost of alginate for cell immobilization is about US $0.2 per cubic meter of culture volume [20] because the cost of the alginate used for immobilization is much lower than harvesting by centrifugation, it makes the use of alginate-immobilized cells more economically viable and attractive.

Immobilized cells show a higher level of chloroplast formation and higher oxygen production rates [21]. Entrapment in alginate gels is the preferred strategy for immobilization because of its low toxicity and high transparency [22,23]. Currently, the most common applications of immobilized cyanobacteria include wastewater treatment [24,25], removal of toxic chemicals, such as hexavalent chromium [26], production of molecular hydrogen [18], long-term storage, removal of heavy metals from industrial effluents [27,28], and metal recovery [29].

In this study, we evaluated the potential of immobilization technology to increase succinate production by autofermentation of recombinant *Synechocystis* sp. PCC6803. Cyanobacterium cells were cultured under free and immobilized conditions and were later subjected to a dark anoxic fermentation stage for the production of organic acids such as succinate, lactate, and acetate.

III.2. Materials and Methods

III.2.1 Cyanobacterium strain and culture conditions

In this study, a recombinant *Synechocystis* sp. PCC6803 strain with an overexpressed endogenous PEPC gene (ppc, sll0920) was used, as described by Hasunuma et al. [2]. The cells were pre-cultured in 150 mL BG-11 medium containing 20 mM HEPES-KOH for 4 days at 30 °C in an NC350-HC plant chamber (Nippon Medical and Chemical Instruments, Japan) with light irradiation of 50 μmol $m^{-2}s^{-1}$ white fluorescence light and 100 rpm agitation.

III.2.2 Immobilization of the cyanobacterium

An aliquot of 0.1 OD_{750} cells was centrifuged at 7,600 x*g* for 5 min, washed twice with distilled water, the cyanobacteria cells were resuspended in a modified BG-11 medium (containing 5 mM NH_4Cl and 50 mM HEPES-KOH) mixed with a 3% (w/v) sodium alginate solution in a 1:2 proportion. The solution was then dripped with a syringe into a 100 ml sterile solution of 2% (w/v) calcium chloride to form spherical beads with a diameter of approximately 4.0 mm. The beads were allowed to stand for 1 h for curing, and then washed with distilled water.

III.2.3 Immobilized culture

The immobilized culture (the main culture stage) of *Synechocystis* sp. PCC6803 was performed in a closed two-stage flask. With 50 mL of 2 M $NaHCO_3/Na_2CO_3$ in the lower stage to produce 1% (v/v) CO_2, and 70 mL of modified-BG-11 medium in the upper stage as shown on Figure 1. A 14 ml volume of beads at 0.4 OD_{750} was used, and cultivation was performed for 3 days at 30 °C with a light irradiation of approximately

100 µmol photons and m^{-2} s^{-1} and 100 rpm agitation. A free-cell culture was used as a control with a cell concentration of 0.4 OD_{750}.

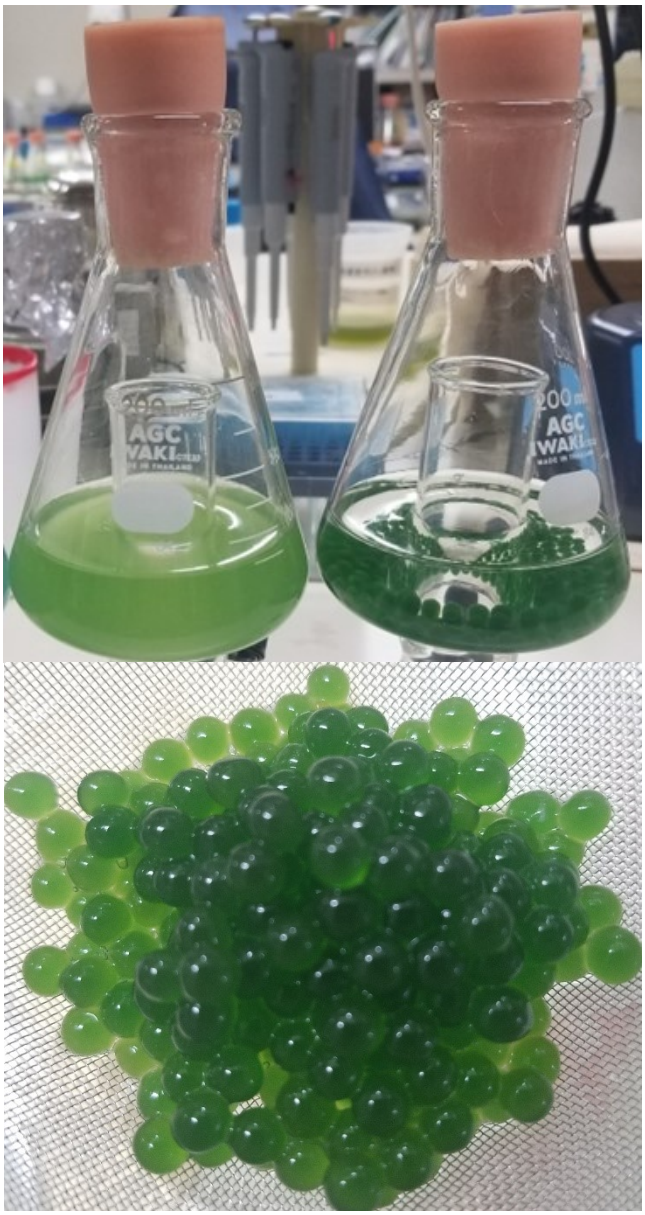


Figure 1. Culture of free and immobilized *Synechocystis* sp. PCC6803 in alginate beads.

III.2.4 Dry-cell weight measurement

For the dry-cell weight measurement, 2 ml of alginate beads were taken from the culture and dissolved in a 2 % (w/v) Na_2CO_3 solution. After the cells were lyophilized to obtain dry cells, they were weighed. Biomass production was expressed as the weight in grams of dry cell weight per litre of culture.

III.2.5 Batch fermentation of free and immobilized cells

After a phototrophic cultivation period of 3 days, *Synechocystis* sp. PCC6803 cells were subjected to anaerobic fermentation. For this, cells at a concentration of 20 OD_{750} were transferred into a 30 mL closed bottle containing a solution of 100 mM HEPES-KOH (pH 7.8), followed by cultivation for 3 days at 37.5 °C with 170 rpm agitation.

III.2.6 Organic acid measurement from the *Synechocystis* sp. PCC 6803 cells

The organic acids in the fermentation medium were analyzed using an HPLC system (Shimadzu) with an Aminex HPX-87H column (300 mm x 7.8 mm; Bio-Rad, CA) and an RID-10A (Shimadzu). The operating conditions were 50 °C and a flow rate of 0.6 mL min^{-1} with 5 mM H_2SO_4 as the mobile phase.

III.2.7. Intracellular metabolites Analysis.

Following a previously described method by Hasunuma et al., [30], briefly, 5 mg of DCW cells were filtrated into PTFE disks of 1µm pore size, followed by washing with a solution 20 mM ammonium carbonate, the cells were placed in a methanol solution containing 37.38 µM PIPES and 37.38 µM L-methionine sulfone used as internal standards. The solubilized protein was removed by filtration through a Millipore 3kDa

membrane. The intracellular metabolites were analyzed by CE-MS system (CE, Agilent G7100; MS, Agilent G6224AA LC/MSD TOF; Agilent Technologies) controlled by MassHunter Workstation Data Acquisition software.

III.2.8 Continuous-batch culture strategy

Continuous fermentation was accomplished following the fermentation stage by subjecting both free and immobilized cells to a main culture stage for continuous growth and glycogen accumulation, followed by anaerobic fermentation and production of succinate. This cycle shown on Figure 2 was repeated for continuous succinate production by the same cells.

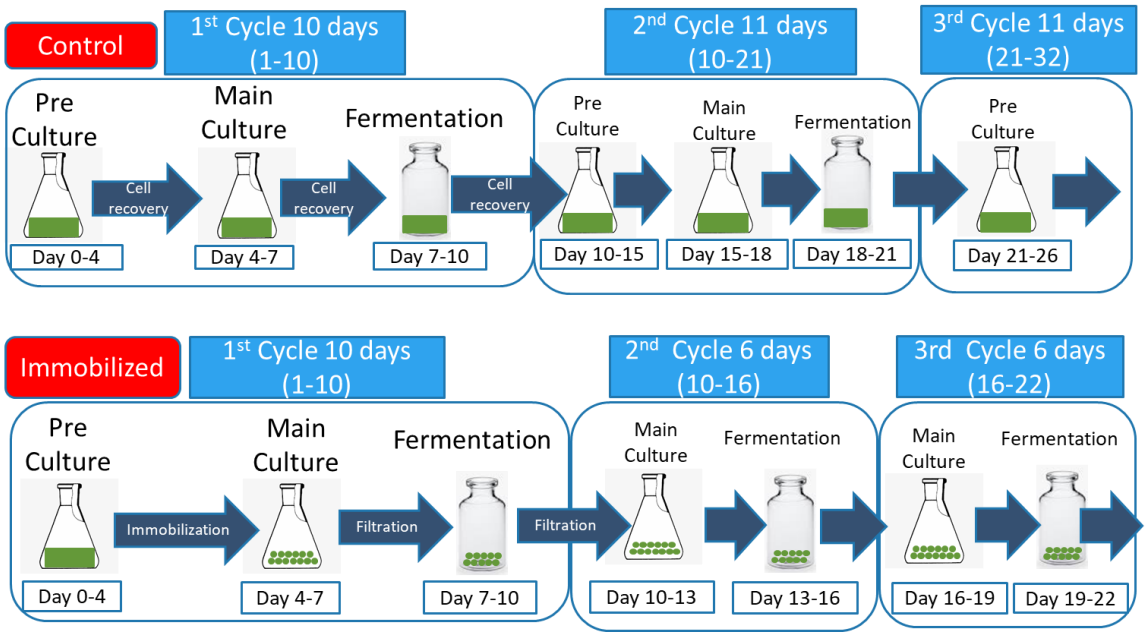


Figure 2. Methodology followed for the continuous fermentation culture of free and immobilized *Synechocystis* sp. PCC6803

III.2.9 Succinate production optimization of immobilized *Synechocystis* sp. PCC 6803

$NaHCO_3$ addition has the potential to increase the succinate yield of free-cell *Synechocystis* sp. PCC 6803 [2]. The effect on immobilized cells was evaluated by adding four different concentrations of $NaHCO_3$ to the medium at the fermentation stage: 0, 50, 100, and 200 mM.

III.3. Results

IV.3.1 Immobilized *Synechocystis* sp. PCC6803 growth

Compared with the free-cell culture, after 3 days the alginate immobilized culture of the cyanobacterium *Synechocystis* sp. PCC 6803 showed greater cell growth compared with that of the free-cell culture, it increased from 1.7 ± 0.1 for the free-cell to 2.2 ± 0.1 g/L in the immobilized cell. Growth in the immobilized cell culture was 1.3-fold greater than that in the free-cell culture.

III.3.2 Organic acids production by immobilized *Synechocystis* sp. PCC6803

The time courses of the organic acids produced by the free and immobilized cultures of the cyanobacterium *Synechocystis* sp. PCC 6803 are shown in Figures 3A, 3B and 3C, for succinate, lactate, and acetate, respectively. Regarding differences in succinate production between the free-cell and immobilized cultures, the succinate yield measured after 3 days of fermentation was slightly higher for the free-cell culture, achieving 96 ± 5 mg/L compared with 78 ± 4 mg/L for the immobilized cell, but this pattern was repeated in the lactate and acetate yield, where the free cell achieved higher lactate and acetate production compared with that of the immobilized cell.

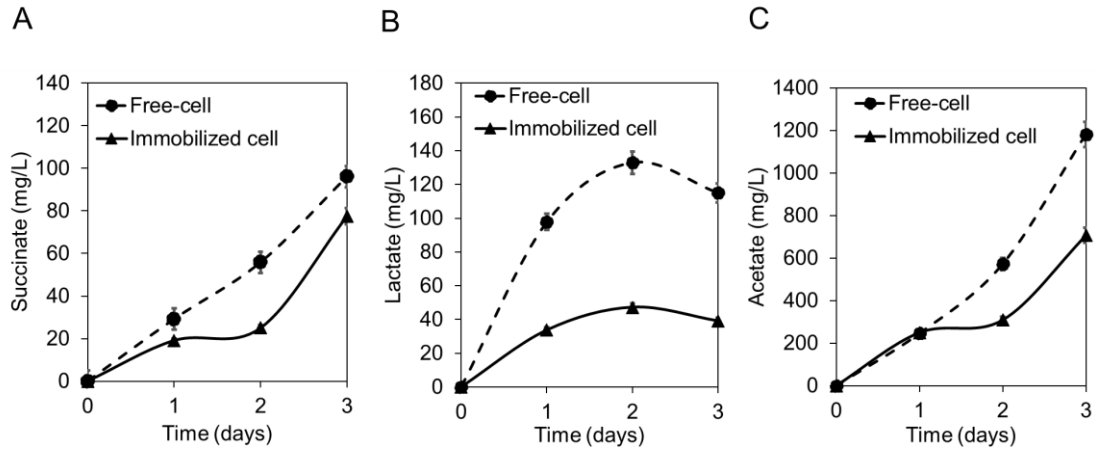
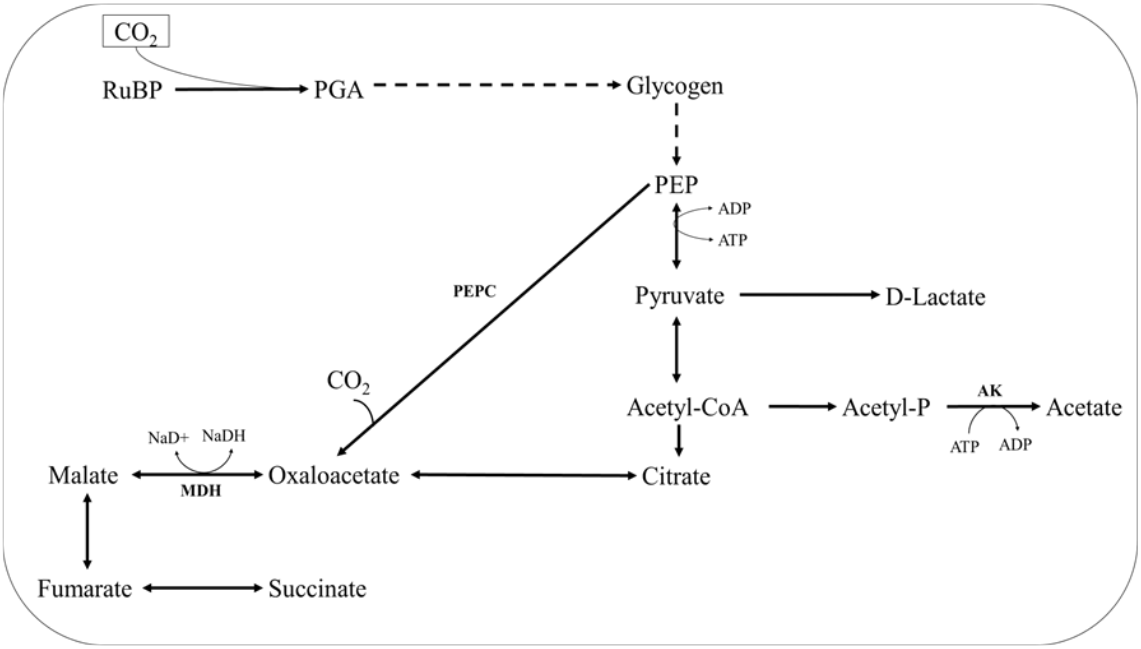


Figure 3. Time course for the organic acids production by free and immobilized *Synechocystis* sp. PCC6803. (A) Succinate yield, (B) Lactate yield, (C) Acetate yield. The error bars show the standard deviation of triplicate values.

III.3.3 Intracellular metabolite production on *Synechocystis* sp. PCC6803

The time course of intracellular metabolite production by both free and immobilized cells is shown in Figure 4, and the upper panel shows the succinate pathway proposed by Hasunuma et al. [29]. Pyruvate levels followed a similar pattern during the three days of fermentation. Intracellular malate and fumarate were higher in the free cells; however, citrate was higher in the immobilized cells, the intracellular succinate and lactate levels were similar between the free cells and immobilized cells on day 3.



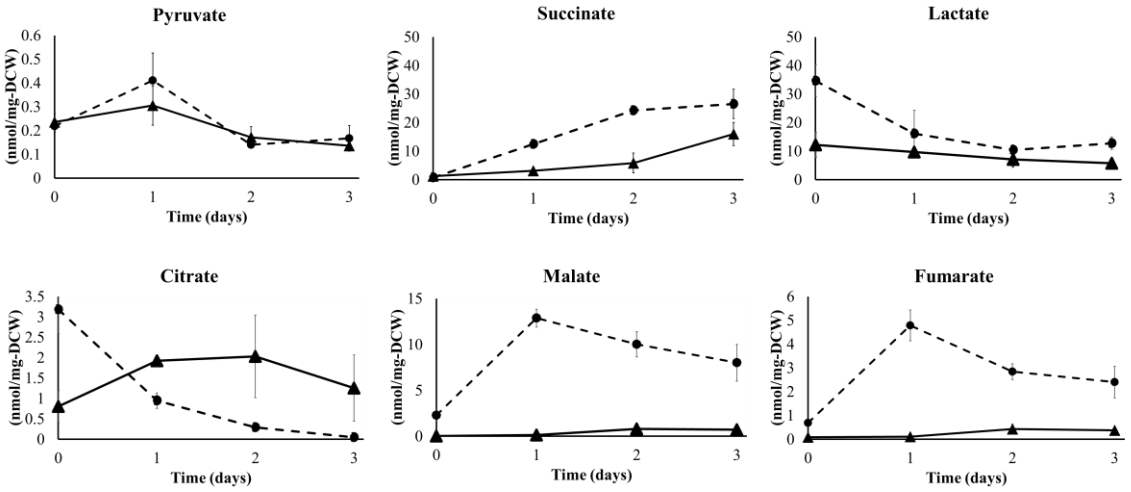


Figure 4. Time course of intracellular metabolites for free-cell (closed circles) and immobilized cell (closed triangles). The error bars show the standard deviation of triplicate values.

III.3.4 Continuous-batch culture of immobilized *Synechocystis* sp. PCC6803

The time course for succinate production in the free and immobilized cultures of

Synechocystis sp. PCC6803 are shown in Figure 5. Each cycle represents the time from when the cells grew in the main culture stage until the cells finished 3 days of the fermentation stage wherein succinate was produced. As shown in Figure 3, the length of the cycles was reduced for the immobilized culture compared with that of the free-cell culture. This was because the free-cell culture required an extra stage of pre-culture following the fermentation stage to continue in this process, which allowed faster and greater yields of succinate by the immobilized culture.

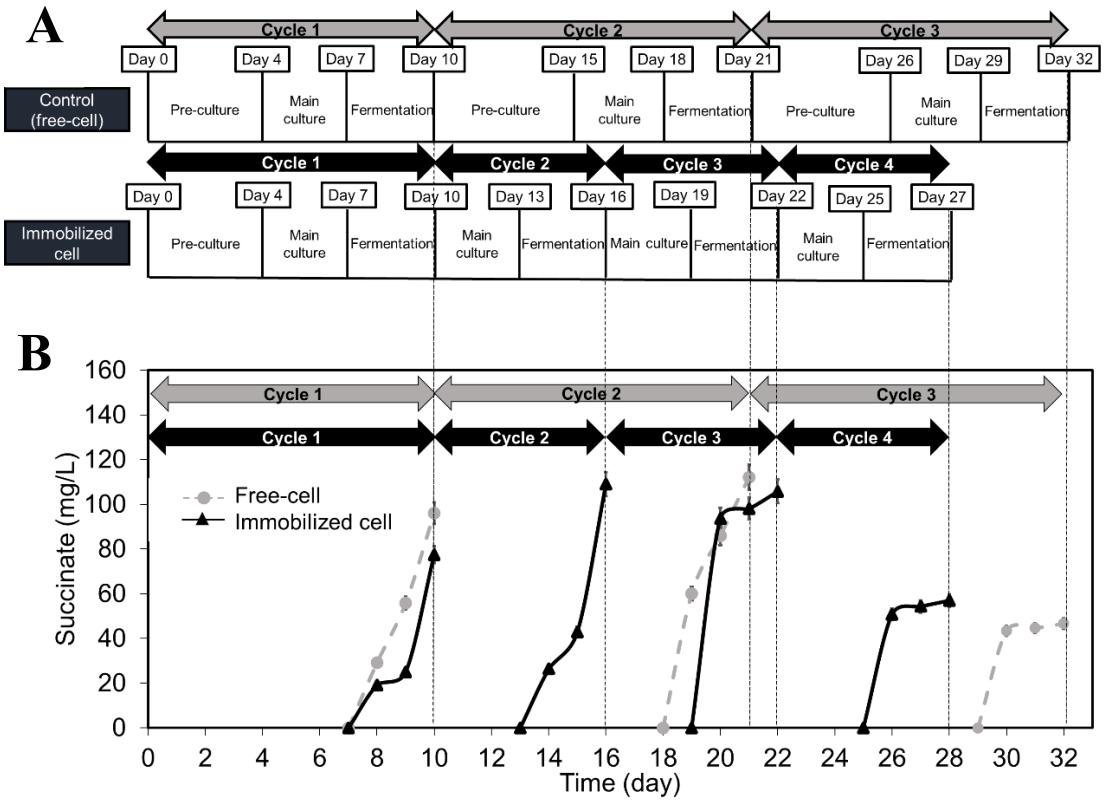


Figure 5. Continuous succinate production by free and immobilized *Synechocystis* sp. PCC6803. (A) Schematic diagram of the continuous production cycles, (B) Time course for the continuous succinate production. The error bars show the standard deviation of triplicate values.

Table 1 shows the maximum succinate, lactate, and acetate yields achieved in each cycle for both the free and immobilized cultures. In the first cycle, the succinate yield was higher in the free-cell culture than in the immobilized culture. By the second cycle, however, the succinate yield was almost the same between the two culture methods:112 ± 5 mg/L for the free cells and 109 ± 5 mg/L for the immobilized cells. By the third cycle, the succinate yield was higher in the immobilized culture (106 ± 5 mg/L) than in the free-cell culture (46 ± 2 mg/L) as shown on Figure 6. The maximum lactate and acetate yields of the free cell and immobilized cell fermentations are also shown on Table 1; the free cell produced a higher amount of lactate and acetate in the first and third cycles than the immobilized cell.

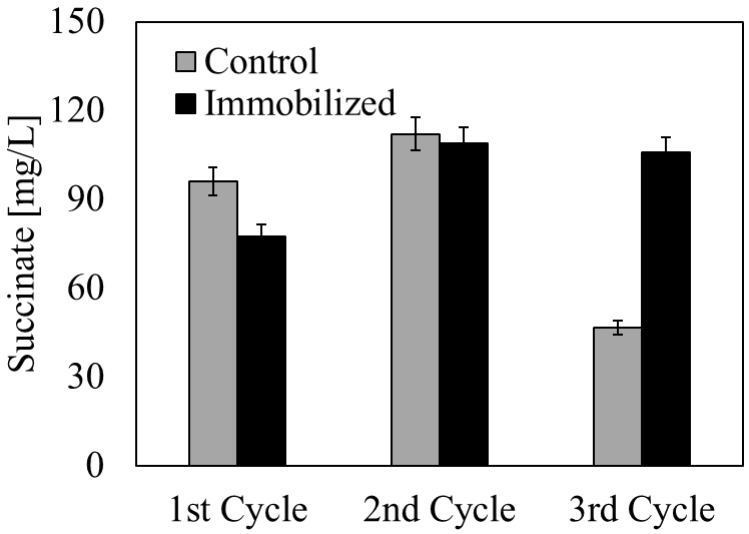


Figure 6. Succinate produced on each cycle for control (free cell) and immobilized cultures.

Table 1. Maximum succinate, lactate and acetate production by free and immobilized *Synechocystis* sp. PCC6803 on day 3 for each of the continuous succinate production cycles.

		Succinate yield (mg/L)	Lactate yield (mg/L)	Acetate yield (mg/L)
Free-cell	Cycle 1	96 ± 5	115 ± 5	1180 ± 59
	Cycle 2	112 ± 5	52 ± 2	788 ± 39
	Cycle 3	46 ± 2	91 ± 5	954 ± 47
Immobilized cell	Cycle 1	78 ± 4	39 ± 2	707 ± 35
	Cycle 2	109 ± 5	96 ± 5	818 ± 41
	Cycle 3	106 ± 5	26 ± 1	608 ± 30
	Cycle 4	57 ± 3	37 ± 2	557 ± 26

Figure 7 shows the productivity of the total amount of succinate accumulated by both free and immobilized cells. During the first cycle of continuous succinate production, succinate productivity from free cells was greater than that from immobilized cells. Starting with the second cycle, however, succinate productivity increased in the immobilized cells and remained at a higher level. The immobilized cell achieved its highest level of productivity during cycle 3 (day 22) at 13 ± 1 mg/L/day, whereas the free cell achieved its highest-level during cycle 2 (day 21) at 10 ± 1 mg/L/day.

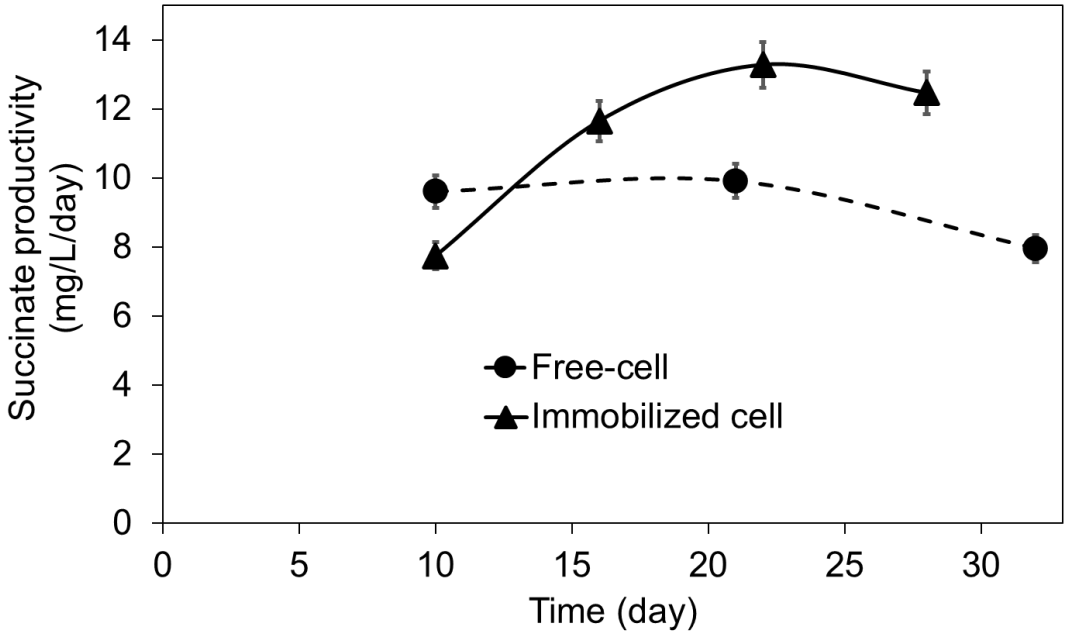


Figure 7. Accumulated Succinate productivity by free-cell and immobilized *Synechocystis* sp. PCC6803 during continuous succinate production cycles. The error bars show the standard deviation of triplicate values.

III.3.5 $NaHCO_3$ addition effect on succinate and lactate production from immobilized *Synechocystis* sp. PCC 6803

The effect that the addition of $NaHCO_3$ has on the succinate and lactate production by immobilized *Synechocystis* sp. PCC 6803 are shown in Figure 8. Succinate and lactate yields were clearly affected by the addition of $NaHCO_3$ to the fermentation medium of the immobilized cells. The highest succinate yield was obtained by the addition of 200 mM $NaHCO_3$. Production reached 202 ± 9 mg/L, which was 2.6-fold higher than the yield obtained when fermentation was performed without the addition of $NaHCO_3$, but at this concentration, the beads were dissolved. The second highest succinate yield of 199 ± 10 mg/L was achieved with the addition of 50 mM $NaHCO_3$.

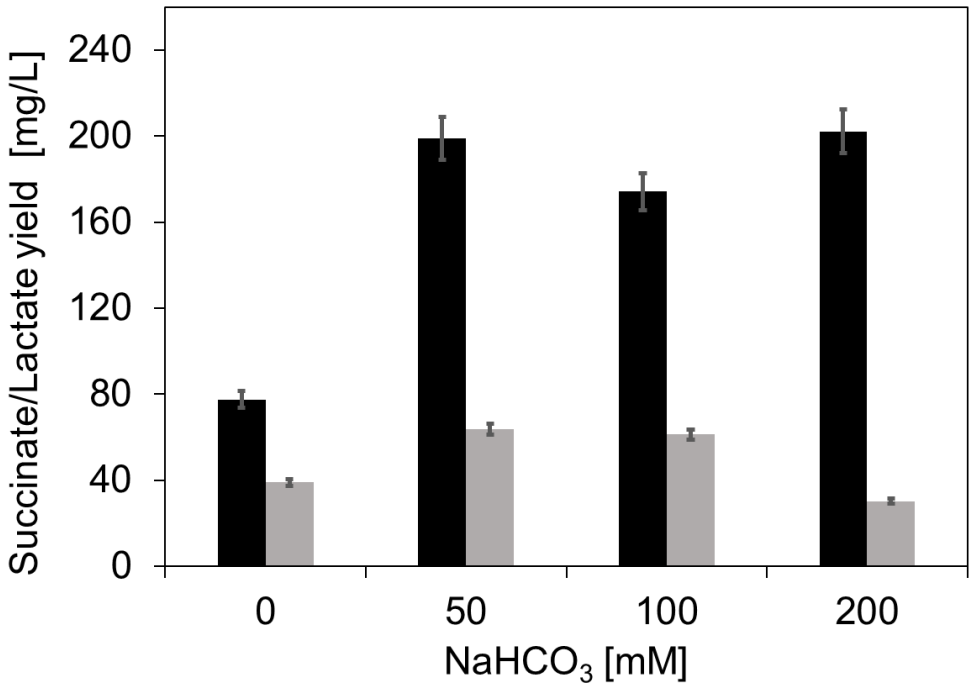


Figure 8. Succinate and lactate production by immobilized *Synechocystis* sp. PCC6803 at different $NaHCO_3$ concentrations on day 3 of autofermentation. The error bars show the standard deviation of triplicate values.

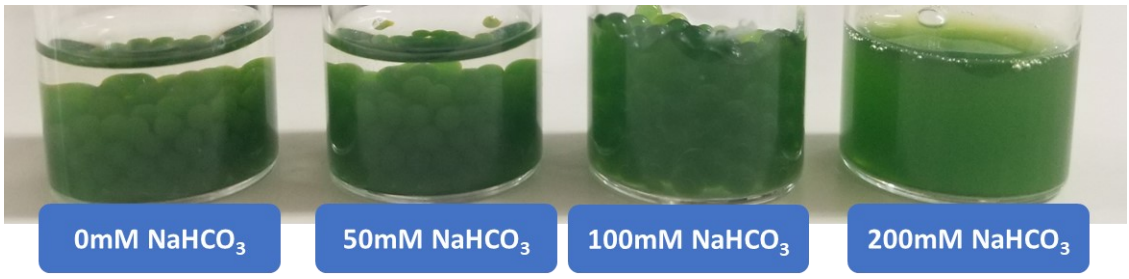


Figure 9. Alginate beads after fermentation with different concentrations of $NaHCO_3$.

III.4. Discussion

The results obtained in this study show the potential of immobilization to improve the growth and succinate production from the cyanobacterium *Synechocystis* sp. PCC 6803. The immobilization strategy achieved a higher dry cell weight than that by using a

free-cell culture, and the yield of succinate was also improved. Immobilization showed no negative effects on succinate production because the immobilized cells produced an amount of succinate that was similar to that of free cells after 3 days.

The conditions for cell growth were among the reasons for the higher dry cell weights obtained in the immobilized culture. In the immobilized state, cells grow inside the alginate beads rather than in the culture medium [20,31], which allows for more efficient mixing inside the system. In addition, light penetration was improved because the culture medium remained transparent in the immobilized culture. In a suspended culture, the cells grow in the culture medium, which causes it to become darker and decreases the ability of light to penetrate the medium [32].

Succinate production from immobilized *Synechocystis* sp. PCC6803 was more efficient because less lactate and acetate were produced compared to free cells, as shown in Figure 3. In the fermentation stage, organic acids are converted from intercellular glycogen that accumulates during the main culture stage [33] where free cells produce a higher amount of succinate compared to that produced by immobilized cells. During this initial stage, however, free cells also produced greater amounts of lactate (2.9-fold higher) and acetate (1.7-fold higher).

The results obtained from the intracellular metabolite analysis showed that the free and immobilized cells follow a similar metabolic pathway to produce succinate, under anaerobic conditions the TCA cycle runs backwards in cyanobacteria, from oxaloacetate to succinate [30]; however, it seems that the immobilized cells suffered a slight delay to begin the production of succinate, and the immobilization decreased the TCA intermediates to succinate. On the first day, the intracellular and extracellular succinate produced were lower in the immobilized cells compared to the free cells; however, by

day 3, the succinate secreted achieved a similar value as seen in Figure 3.

Continuous succinate production showed the potential for immobilization, starting from a fresh culture of *Synechocystis* sp. PCC 6803 cells, a normal succinate production cycle, requires at least 10 days. The goal of continuous succinate production was to optimize the cycles and skip the pre-culture stage. This was not possible when working with free cells; because after the fermentation stage, the cells were unable to grow in the main culture stage.

As shown in Figure 5, an extra pre-culture stage was required because of the stress suffered by the cells during the fermentation stage [2]. We were able to overcome this limitation by utilizing an immobilized system. The immobilized cells were able to resume the main culture stage immediately following the production of succinate in the fermentation stage, which reduced the cycle from 10 to 6 days and allowed a faster production of succinate.

When comparing the maximum production of succinate for each of the cycles under both culture conditions, it was apparent that by cycle 3, the succinate yield for the free cells decreased by almost 50% compared with that measured in cycle 1. The disparity could have been due to the stress the cells suffered during the continuous fermentation stages, but could also have been due to damage to the chloroplast from pheophytinization [34]. In an immobilized culture, the microalgae cells retain 90% of their chlorophyll even after 3 months, whereas in a free-cell culture, the occurrence of pheophytinization occurs just after 7 days [34]. For the immobilized cells, the succinate yield increased from cycle 1 to cycle 3, by cycle 4, however, it had decreased as a result of the reduced stability of the alginate beads, which started breaking. This result demonstrates the need to strengthen the beads following the third cycle, which can be accomplished by curing the beads in a $CaCl_2$

solution.

Figure 8 shows that the succinate production by immobilized *Synechocystis* sp. PCC 6803 cells was optimized by the addition of $NaHCO_3$. At the fermentation stage the succinate yield was increased by as much as 2.6-fold over the yield with no addition of $NaHCO_3$. The highest succinate yield was obtained by the addition of 200 mM $NaHCO_3$, but the beads were dissolved at this concentration. That was to be expected because a solution of Na_2CO_3 or $NaHCO_3$ is usually utilized to dissolve calcium alginate beads. We found that at a 50 mM concentration of $NaHCO_3$ the succinate yield is increased by approximately the same amount, but at this low concentration the beads are not dissolved. For Hasunuma et al. [30] the highest succinate yield for the free-cell *Synechocystis* sp. PCC6803, was obtained by the addition of 100 mM of $NaHCO_3$, also Tomita et al. [35] showed the increase of succinate production from *Euglena Gracilis* by the addition of 100 mM of $NaHCO_3$.

III.5. Conclusions

The results obtained in the present study showed the overall positive effects of immobilization on the production of succinate from the cyanobacterium *Synechocystis* sp. PCC 6803. The immobilization strategy not only enhances growth, but also increases the rate at which succinate can be produced by shortening the time required for each cycle of continuous succinate production. This process also simplifies the harvesting of cells, which decreases cost. The application of this technology should increase the feasibility of succinate production by this strain.

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CHAPTER IV

Palm Oil Mill Effluent (POME) Utilization for Succinate Production from Cyanobacteria *Synechocystis* sp. PCC6803

IV.1. Introduction

When fresh fruit bunches (FFV) from palm oil trees are pressed to make palm oil, palm mill oil effluent (POME) is created. One of the most popular vegetable oils worldwide is palm oil [1, 2]. Due to its high organic content, chemical oxygen demand (COD), and biochemical oxygen demand (BOD), the POME effluent is regarded as extremely polluting [3,4].

Fresh POME is discharged as a hot, acidic liquid waste of brownish color and colloidal consistency. It contains roughly 96% water, 0.7% oil, and 5% total solids. In comparison to municipal sewage, POME has a BOD of more than 180 mg/L and a COD content of more than 450 mg/L. Additionally, POME is a feasible medium for microbial growth since it contains important nutrients such nitrogen, potassium, magnesium, calcium, and iron [5]. POME is not a hazardous waste because no chemicals are added during its production; nonetheless, dumping it into aquatic systems could have an negative effect on the environment due to its high capacity to deplete oxygen as well as its high organic and nutritive content [6].

Cyanobacteria and microalgae, which are oxygenic photosynthetic microorganisms, have been utilized to treat POME and provide the basic ingredients needed to make fuel and chemicals. Some reports [7,8] use microalgae isolated from POME (Chlamydomonas sp. and Chlorella sp.), whereas earlier reports have primarily used cyanobacteria and microalgae that were purchased commercially. To sustain high cyanobacteria and

microalgae growth, POME must be diluted below 30% regardless of COD values. This is because the tannic acid in POME has a light-shading effect [9–11] and because substances like ammonium ions directly hinder growth [12].

Succinic acid, a four carbon dicarboxylic acid, is an important platform chemical capable of replacing maleic anhydride as a flexible basic chemical. It can be employed in the pharmaceutical and agricultural industries [13]. Succinate is a precursor of many valuable chemicals used in industry, such as 1,4-butanediol, dimethyl succinate, tetrahydrofuran, and -butyrolactone [15]. It can be employed as a polymer monomer, surfactant, and ion chelator [14]. The succinate market has grown through time, recently reaching 58500 tons of succinate produced annually worldwide. In 2015 the market for succinic acid was of $157.2 million, and it's expected to reach $75,000 million [15].

Using native producers like Actinobacillus succinogenes and certain fungi, succinate has previously been produced [16–19]. These bacteria are heterotrophic, but because they need carbon sources, like sugars for succinate generation, they become a bottleneck for the production of biochemicals. The cyanobacterium Synechocystis sp. PCC6803 can secrete succinate without the need for sugar through autofermentation in dark, anoxic conditions [20]. Synechocystis sp. PCC 6803 is a type of cyanobacterium that can break down glycogen, a major storage polysaccharide that builds up during photosynthesis, to create organic acids such succinate and D-lactate [21]. Synechocystis sp. PCC6803 converts CO2 directly into useful substances like cyanophycin, 2,3-butanediol, 3-hydroxypropionic acid, and PHAs [22,23] through metabolic engineering techniques like gene deletion and overexpression, and the overexpression of phosphoenolpyruvate (PEP) carboxylase genes improves its succinate production [21].

The potential of immobilization for enhancing the growth of cyanobacteria and its

succinate productivity has been shown, previous studies on hydrogen production from immobilized *Synechocystis* sp. PCC6803 [18] showed that immobilization technology increased hydrogen production in immobilized cells compared to that in free cells. Immobilization also simplifies both the cell separation and two-phase H_2 production processes [18].

For this study the growth and succinate production of the cyanobacteria *Synechocystis* sp. PCC6803 in a medium containing POME was evaluated, to the authors knowledge there has not been previous researches focusing on this topic, also the growth of alginate immobilized cells in POME was evaluated.

IV.2. Materials and Methods

IV.2.1. Cyanobacterium strain and culture conditions

A recombinant *Synechocystis* sp. PCC6803 strain with an overexpressed endogenous PEPC gene was utilized for this study. The cells were pre-cultured in a 150 mL BG-11 medium containing 20 mM HEPES-KOH for 4 days at 30 °C in an NC350-HC plant chamber as previously described by Alfaro-Sayes et al. [24] with white light irradiation of 50 μmol $m^{-2}s^{-1}$ and 100 rpm agitation.

IV.2.2. POME pretreatment

POME was obtained from PT. Agricinal (Bengkulu, Indonesia) with an initial pH of 6.5. For its use in this experiment this POME was subjected to pretreatment to avoid any contamination during the culture of the cyanobacteria, the POME was autoclaved at 121 °C followed by a membrane filtration through a 0.1μm membrane and a 0.22 μm membrane.

IV.2.3. *Synechocystis* sp. PCC6803 culture in a POME medium

The main culture stage of *Synechocystis* sp. PCC6803 was performed in a closed two-stage flask. With 50 mL of 2 M $NaHCO_3/Na_2CO_3$ in the lower stage to produce 1% (v/v) CO_2, and 70 mL of modified-BG-11 medium in the upper stage. A culture 0.4 OD_{750} was used, and cultivation was performed for 3 days at 30 °C with a light irradiation of approximately 100 μmol photons and m^{-2} s^{-1} and 100 rpm agitation.

Three different configurations were performed: first mixing the BG-11 medium with POME at different concentrations (0-50 % POME), second keeping the BG-11 nutrient concentration and adding different POME concentrations to the medium (0-100% POME) in free and immobilized conditions, lastly culturing the cyanobacteria utilizing only POME as a culture medium.

IV.2.4. Immobilization of *Synechocystis* sp. PCC 6803

Briefly, as previously described by Alfaro-Sayes et al. [24] an aliquot of 0.1 OD_{750} cells was mixed with a 3 % (w/v) solution of sodium alginate, followed by extrusion through a syringe into a 2 % (w/v) $CaCl_2$ solution, for the formation of the alginate beads.

IV.2.5. Batch fermentation of *Synechocystis* cells

After a phototrophic cultivation period of 3 days, *Synechocystis* sp. PCC6803 cells were subjected to anaerobic fermentation. For this, cells at a concentration of 20 OD_{750} were transferred into a 30 mL closed bottle containing a solution of 100 mM HEPES-KOH (pH 7.8), followed by cultivation for 3 days at 37.5 °C with 170 rpm agitation.

IV.2.6. Organic acid measurement from the *Synechocystis* sp. PCC 6803 cells

The organic acids in the fermentation medium were analyzed using an HPLC system (Shimadzu) with an Aminex HPX-87H column (300 mm x 7.8 mm; Bio-Rad, CA) and an RID-10A (Shimadzu). The operating conditions were 50 °C and a flow rate of 0.6 mL min^{-1} with 5 mM H_2SO_4 as the mobile phase.

IV.2.7. Analysis of intracellular glycogen

Following a previously described method by Hasunuma et al., [21], briefly, glycogen was extracted from 5 mg dry weight cells with 100 µL aqueous KOH (30%, w/v) by incubation in a 90 °C heat block for 90 min, followed by placement on ice. The dried cells were washed three times with cold ethanol, then dried for 30 min at 80 °C. The cells were resuspended in 100 µL of water and centrifugated to obtain the glycogen extract. The glycogen concentration was determined by measuring the glucose released from glycogen by enzymatic hydrolysis. The amount of glucose hydrolyzed from glycogen was measured by HPLC system. The HPLC system was equipped with an Aminex HPX-87 h column and an RID-10A refractive index detector, as described above.

IV.3. Results

IV.3.1. Growth of the cyanobacteria in POME-BG11 culture medium

The results for the growth of the cyanobacteria *Synechocystis* sp. PCC 6803 are shown on Figure 1. The highest growth was obtained when the culture medium consisted of 80 % BG-11 and 20 % POME, reaching a dry cell weight of 2.14 ± 0.1 g/L, this was higher than the growth obtained by utilizing only a BG-11 medium (0 % POME) which was of 1.79 ± 0.1 g/L. We can also observe that the dry cell weight from the mixed culture

medium up to 40 % POME was higher than the growth obtained by the BG-11 medium. At 50 % POME concentration the growth was slightly lower than the one obtained at 0 % POME.

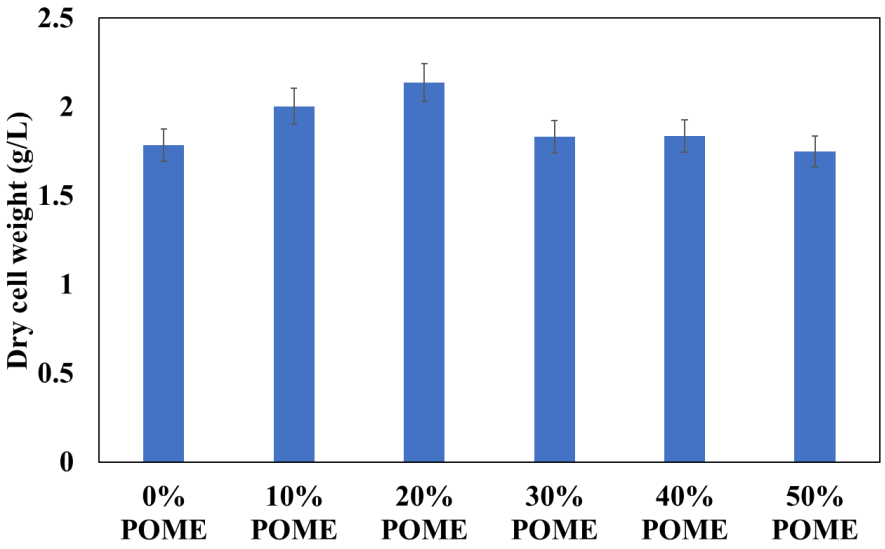

Figure 1. Dry cell weight of *Synechocystis* cells when cultured at different POME-BG11 concentrations. The error bars show the standard deviation of triplicate values.

IV.3.2. Organic acid production on a mixed culture medium of BG11 and POME

The organic acids secreted by the cyanobacteria cells during their autofermentation stage, after being cultured in a medium consisting of mixing BG11 and POME are shown on Figure 2. It can be seen how the addition of POME to the culture medium affects the organic acid production of the cyanobacteria cells, the highest succinate yield of 215 ± 10 mg/L is obtained at 20 % POME 80 % BG11, the succinate yield by the cells in a BG11 medium (0 % POME) was of 146 ± 7 mg/L. The lactate production was higher in the POME mixed medium (50 % POME, 50 % BG11) than in the BG11 medium (0 % POME). The acetate production decreased as the POME concentration was higher.

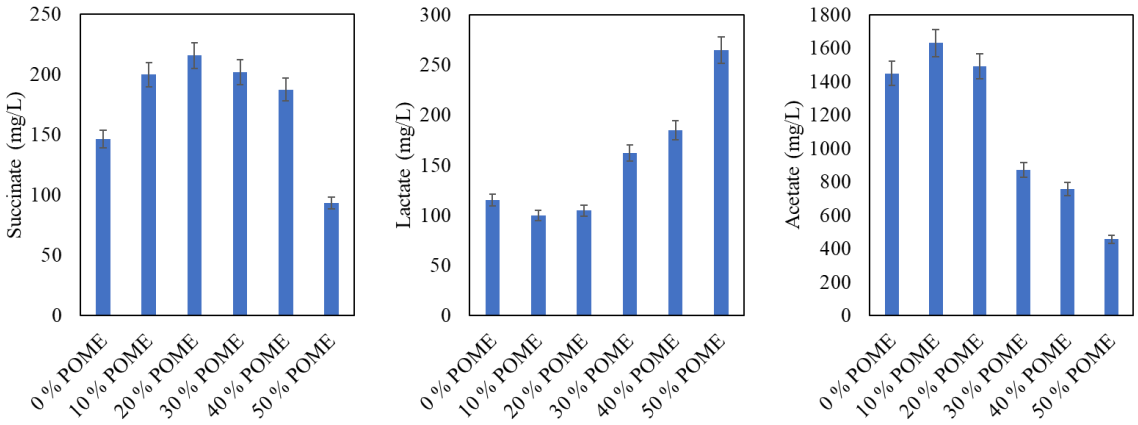

Figure 2. Organic acid production of *Synechocystis* cells when cultured at different POME-BG11 concentrations. The error bars show the standard deviation of triplicate values.

IV.3.3. Effect of POME in the growth of free and immobilized *Synechocystis* sp. PCC 6803

The effect of POME addition to the culture medium while keeping the BG11 nutrient concentration constant is shown on Figure 3. For the free-cells the highest growth was obtained when utilizing a 20 % POME concentration in the culture medium, it achieved a dry cell weight of 2.15 ± 0.1 g/L, which is 1.2 times higher than the growth obtained at 0 % POME. The lowest dry cell weight of 1.45 ± 0.1 g/L was obtained when culturing the cells with a culture medium consisting of 100 % POME. For the immobilized cells the highest growth of 2.3 ± 0.1 g/L was obtained at 0 % POME, however, the addition of POME only decreased the growth of the immobilized cyanobacteria cells.

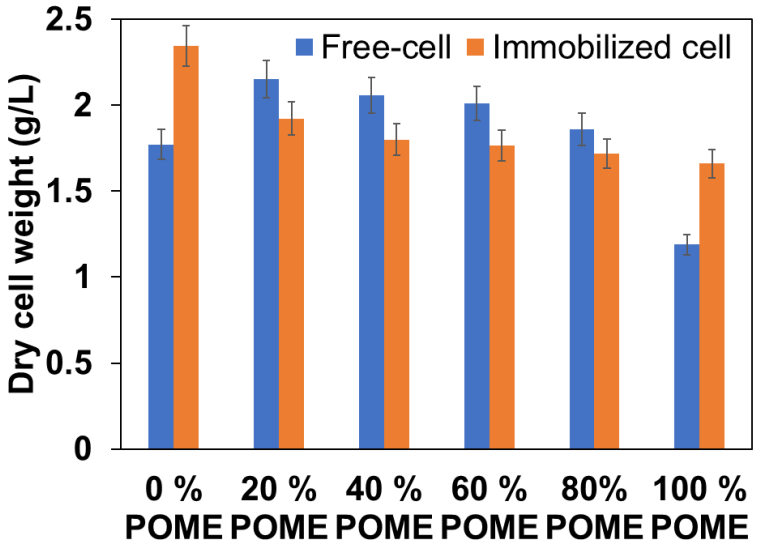

Figure 3. Free and Immobilized *Synechocystis* sp. PCC 6803 growth at different POME concentrations. The error bars show the standard deviation of triplicate values.

IV.3.4. Organic acid production and Glycogen accumulation of *Synechocystis* cells at different POME concentrations

The succinate, lactate and acetate produced by the free *Synechocystis* cells during the fermentation stage, after being cultured at different POME concentrations is shown on Figure 4. The succinate yield increased as the POME concentration increased, reaching the highest production of 245 ± 12 mg/L at 60 % POME. The lowest succinate yield was obtained at 0 % POME. When culturing the cells in a medium consisting only of POME (100 % POME) they were able to secrete 227 ± 10 mg/L of succinate. The lactate and acetate yield decreased with the POME addition. For the immobilized cells the organic acid production was affected by the addition of POME the highest succinate yield was obtained at 0 % POME and the lowest was obtained when cultured in a 100 % POME medium.

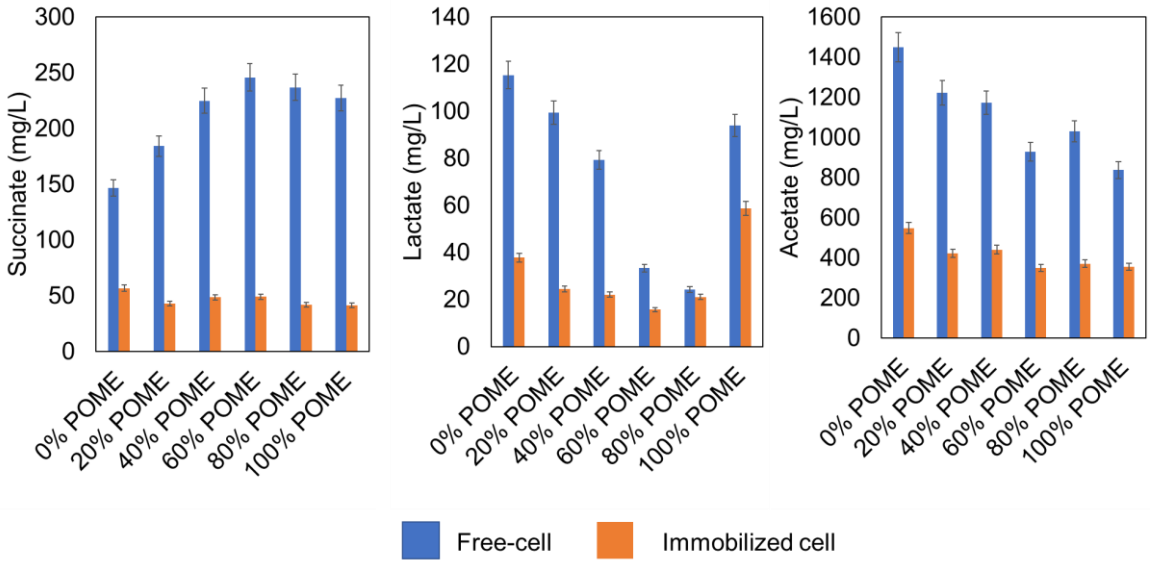

Figure 4. Organic acid production by free and immobilized *Synechocystis* cells when cultured at different POME concentrations. The error bars show the standard deviation of triplicate values.

The glycogen accumulated during the main culture of *Synechocystis* sp. PCC 6803 is shown on Figure 5. It can be seen that the highest accumulation of glycogen was obtained when culturing the cells with a 20 % POME concentration. The glycogen accumulation of the cells cultured in a medium consisting only of POME (100 % POME) was higher than when the cells were cultured in a BG11 medium (0 % POME).

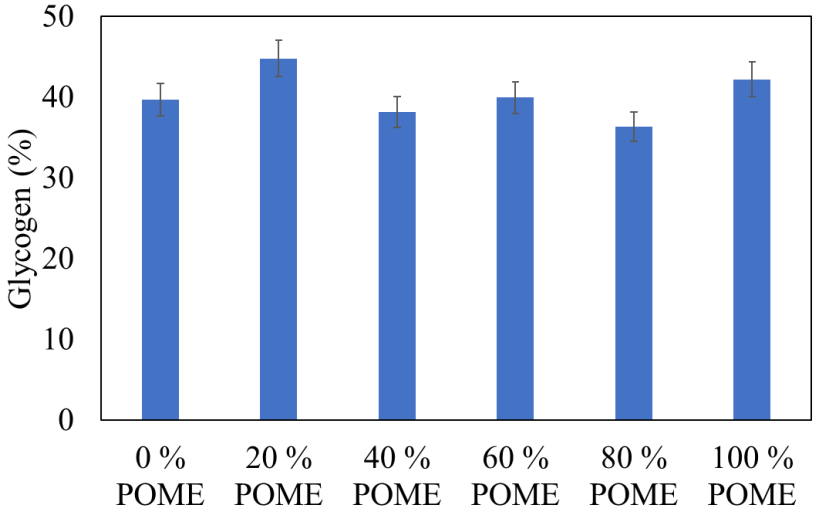

Figure 5. Glycogen accumulation of *Synechocystis* cells when cultured at different POME concentrations. The error bars show the standard deviation of triplicate values.

IV.3.5. Effect of addition of NH_4Cl to POME medium

The effect that the addition of NH_4Cl to the 100 % POME medium has on the growth of the cyanobacteria cells is shown on Figure 6. The addition of NH_4Cl improved the growth of the cyanobacteria from 1.45 ± 0.1 g/L to when no NH_4Cl was added to 1.69 ± 0.1 g/L when 1.25 mM of NH_4Cl was added. When higher concentrations of NH_4Cl are added the dry cell weight is increased, but remains lower than when the cells are cultured in a BG11 medium (0 % POME).

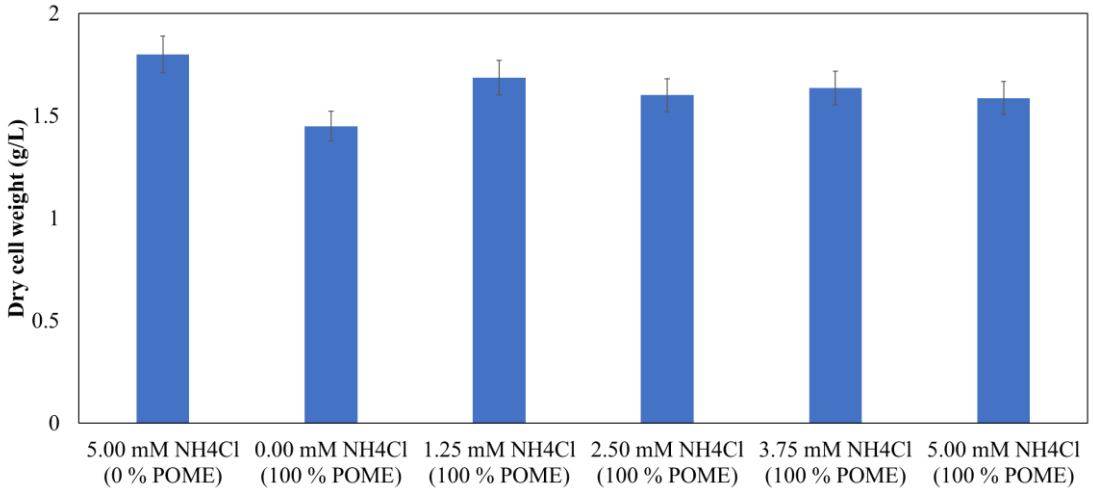

Figure 6. Growth of *Synechocystis* sp. PCC6803 on a POME medium with different concentrations of NH_4Cl. The error bars show the standard deviation of triplicate values.

The production of organic acids from *Synechocystis* sp. PCC 6803 when cultured in a POME medium at different NH_4Cl concentrations is shown on Figure 7. The addition of NH_4Cl in the culture medium also affects the organic acid production from the cells, the highest succinate yield was obtained when 2.50 mM of NH_4Cl were added, however when a concentration higher than that was added it affected negatively the succinate production. At high NH_4Cl concentrations the lactate production is increased in the cells.

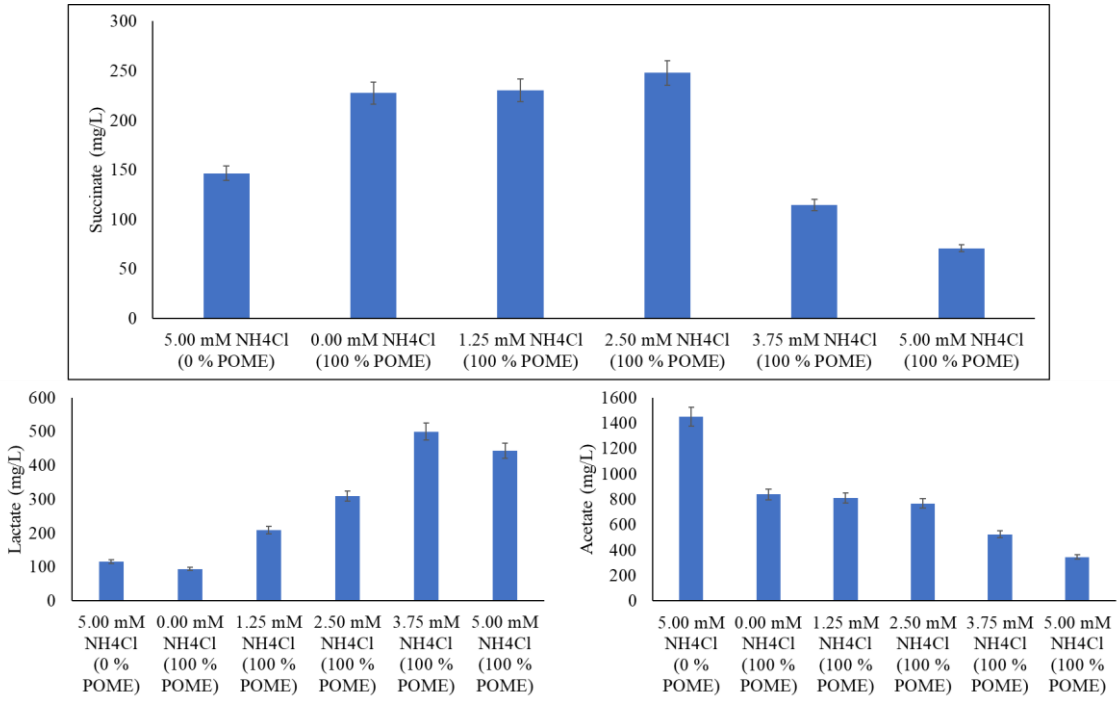

Figure 7. Organic acid production of *Synechocystis* sp. PCC6803 on a POME medium with different concentrations of NH_4Cl. The error bars show the standard deviation of triplicate values.

IV.4. Discussion

From the results obtained in this study it can be seen that the utilization of POME as a nutrient source for the production of succinate by the cyanobacteria *Synechocystis* sp. PCC6803 is possible. By mixing the POME and BG11 medium at different concentrations the growth and succinate production was increased, however the highest dry cell weight was obtained then the POME-BG11 mix was of 80-20, indicating that the nutrients needed for growth and succinate production are not sufficient when the nutrient concentration of BG11 is decreased.

When the nutrient concentration of BG11 remained constant and the only variant was POME, on figure 3 we could observe how the addition of POME improves the growth

of the cyanobacteria, and up to 80% POME addition resulted in an increase in its dry cell weight, this was due to the extra nutrients that the cells can take from the POME in the culture medium. When comparing the results obtained by culturing free and immobilized cells in culture mediums containing POME, we can observe that even though the immobilized cells were able to achieve a higher growth than the free cells, even when cultured in a medium consisting of only POME, the production of organic acids such as succinate was greatly affected by the presence of POME in the immobilized cells.

The POME concentration in the culture medium also improved the glycogen accumulation of the cells, and it even showed an increase in glycogen accumulation when they were cultured on a medium that consisted of only POME (100 % POME), showing the potential that POME has to be used as a culture medium for the production of succinate. This increase in glycogen accumulation was also visible in the increase in succinate production from the cells cultured in mediums containing POME.

However, the growth by POME could be improved as seen on figure 3, even though the glycogen accumulation was higher in the cells cultured on a POME medium, the dry cell weight was still lower than when utilizing a BG11 medium, this could be due to a lower concentration of nutrients for the cells in the culture medium, since nitrogen is one of the most important nutrients for cyanobacteria growth it was chosen to evaluate its effect by the addition of NH_4Cl to the culture medium.

The addition of NH_4Cl to the POME culture medium resulted in an increase of its growth and succinate production, however its growth was not able to overcome the growth of the cells when cultured in the BG11 medium, this lower growth could be due to the lack another nutrient needed by the cyanobacteria.

IV.5. Conclusions

The use of POME as a culture medium for the cyanobacteria *Synechocystis* sp. PCC 6803 showed promising results, even allowing the cells to grow in a medium composed only of POME, this could be beneficial to the industry, because higher amounts of POME could be used to produce valuable chemicals such as succinate, without needing to be diluted first, increasing productivity while also treating the POME and reducing its nutrients that could be damaging for the environment.

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CHAPTER V

Bioconversion strategies to alter the bio-circular economic perspective for palm oil mill effluent

V.1. Introduction

Palm oil is the most widely used vegetable oil and fat in the world, which technically makes it an essential part of our daily lives. In the years 2020 and 2021, 73 million tons of crude palm oil (CPO) were produced worldwide. Malaysia and Indonesia's two largest producers have recently produced 43.5 and 17.9 million tons of CPO, respectively [1]. Palm oil mill effluent (POME) includes palm acid oil (PAO) and is produced during the pressing of fresh fruit bunches (FFB) from palm oil trees. Almost four tons (2.3 to 3.8 tons) of CPO are produced per ton of FFB [2]. POME requires treatment before it can be discharged into water bodies, although it is non-toxic, because the POME effluent complex is considered highly polluting due to a high organic content, chemical oxygen demand (COD), and biochemical oxygen demand (BOD) [3, 4]. An open-lagoon system of ponds is generally used for POME and long-term anaerobic treatment. However, this anaerobic treatment generates large amounts of methane, which has recorded a 28-fold greenhouse effect from CO_2 released into the atmosphere. Yacob et al. (2006) [5] found that 1 ton of POME could produce 12.4 kg of methanePOME is also treated via non-biological methods such as coagulation-flocculation, or adsorption [3, 6], but these methods are economically ineffective for the industry. A more economically and environmentally attractive method for POME treatment is its use as a raw material for biological processes [7] such as in the production of methane, biohydrogen, biodiesel, or polymers [4]. Recently, bio-circular economies have gained importance in achieving

sustainable development [8]. For instance, the sugarcane industry produces ethanol and solid fuels from the molasses subproduct and solid residue generated by industrial sugarcane processing [9]. Likewise, the palm oil industry should pursue technological innovations in utilizing by-products, one of which could be the efficient conversion of POME. Herein, we review and discuss the biological conversion technologies of POME to biodiesel, biogas, and biopolymers, as presented in Figure 1.

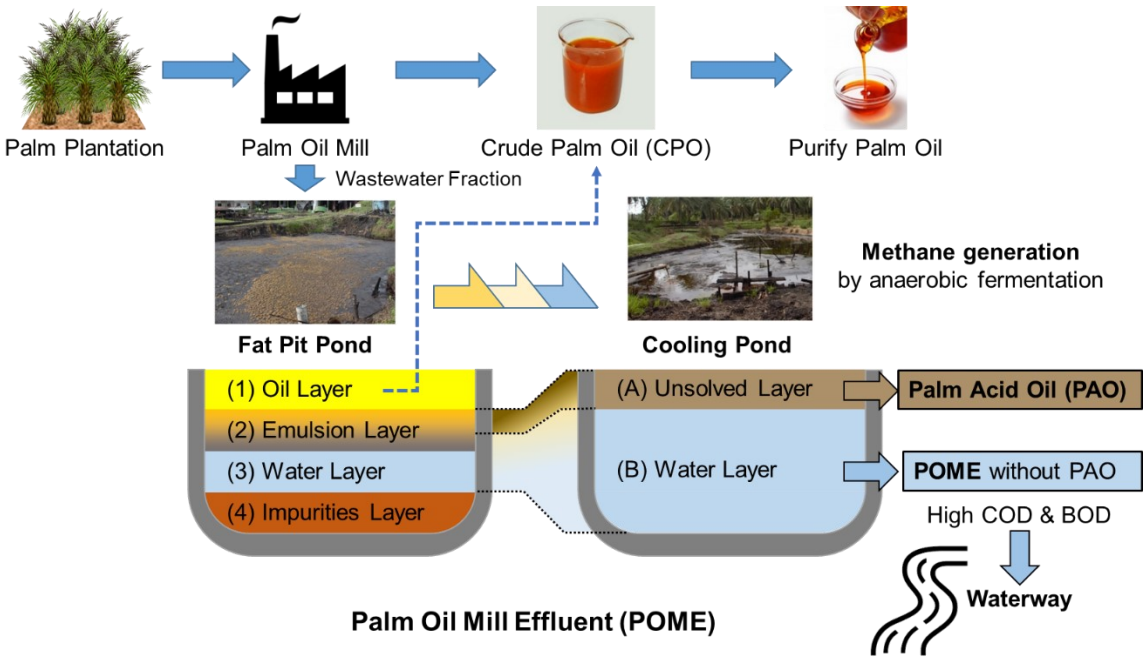

Figure 1. Current scheme for the wastewater treatment of POME by the palm oil industry.

V.2. POME characteristics and its disposal limitations

The difficulties in determining the characteristics of POME reflect a dependence on the milling processes of the raw material that are used by palm oil mills. Fresh POME is discharged as a brownish colloidal suspension of liquid waste that is hot (usually at around 90 °C) and acidic, containing ~96% water, ~0.7% oil, and ~5% total solids. As shown in Table 1, POME has a BOD content that is more than 180 mg/L and a high COD content that is more than 450 mg/L, which is 100-times higher than municipal sewage.

POME also contains a high organic matter content of carbohydrates and fermentable sugars such as arabinose, galactose, glucose, mannose and xylose [10, 11]. Furthermore, POME contains significant nutrients such as nitrogen, potassium, magnesium, calcium, copper, a trace amount of chromium, and iron, making it a viable medium for microbial production [12].

Table 1. Characteristics of POME.

Parameters[a]	Average concentrations
Physical characteristics	
Total suspended solid (TSS)	47,690 ± 156
Volatile suspended solid (VSS)	30,870 ± 105
Temperature (°C)[a]	80 ± 1
pH[a]	3.4 ± 0.1
Chemical property	
COD	69,500 ± 240
BOD_5	37,750 ± 143
Total nitrogen	692 ± 45
Total protein[b]	12.31
Total fat[b]	12.95
Total carbohydrates	22.27×10^{-3}
Reducing sugar	11.26×10^{-3}
Non-reducing sugar	37.75×10^{-3}

[a]Except for pH and temperature, all other parameters are in mg/L.
[b]Unit in % TS.

The majority of POME is made by a milling process that consists of three steps: sterilizing (0.25 tons), clarity (0.45 tons), and hydrocyclone (0.05 tons) in the proportion of 6:15:1. During the process of extracting oil from FFB, one metric ton of processed FFB requires a significant quantity of water [13]. POME is not a dangerous waste because the processing does not include the addition of any chemicals; nonetheless, discharging it into aquatic systems might create environmental problems due to its high oxygen-depleting capabilities as well as its high organic and nutritional content [14].

POME is anaerobically treated to reduce such compounds before being disposed of in aquatic systems. Unfortunately, existing mill plant technologies do not allow for rapid POME treatment onsite; therefore, a substantial amount of POME remains in each pond where it is held and treated, which severely limits its progressive disposal. On the other hand, a succession of such open ponds is becoming a substantial source of greenhouse gas (GHG) emissions, mainly methane (CH_4), which has a 28-fold potential for global warming than carbon dioxide (CO_2) by trapping heat in the atmosphere [15-17].

V.3. Bioconversion of palm acid oil (PAO) to biodiesel

Palm acid oil (PAO) is a by-product extracted from the POME on the top layer of a pond. Free fatty acids (FFA), neutral oil, and moisture make up most of the PAO. In their analysis of the specifications for PAO, Kuntom et al. (1994) noted the following requirements: above 50% of FFA, below 5 meq/kg of peroxide value, 0.15 – 2.16% of moisture content, 39.2 – 57.6 of iodine value, 172 - 197 of saponification value, and 0.04 - 1.67 of unsaponifiable matter. The FFA value of PAO is affected by variations in the palm oil feedstock and the alkaline refining process. PAO has generally been employed in manufacturing distilled fatty acids, soap, and animal feed. Nevertheless, PAO also is

available for the production of biodiesel [18, 19].

Biodiesel is a biodegradable, non-toxic, and non-polluting substance that substantially lowers overall carbon emissions, which thwarts climate change [20]. Biodiesel is produced from various sources that include waste oils such as pig tallow, swine fat, or microalgae, non-edible oils such as jatropha, Karanja, or castor oil, and edible oils such as soybean, palm, sunflower, or rice bran oil. Using edible oils as a substrate for biodiesel, however, would create competition with the food supply [21, 22]. This section reviews the oily waste fraction from POME as a potential feedstock for biodiesel production.

Numerous technologies have been developed for the production of biodiesel from POME. Figure 2 illustrates the most widely used method for manufacturing biodiesel, which involves the transesterification of oils with an acyl acceptor in the presence of a catalyst [23]. The catalysts in the transesterification process are generally assigned to one of three general classifications: bases, acids, and enzymes.

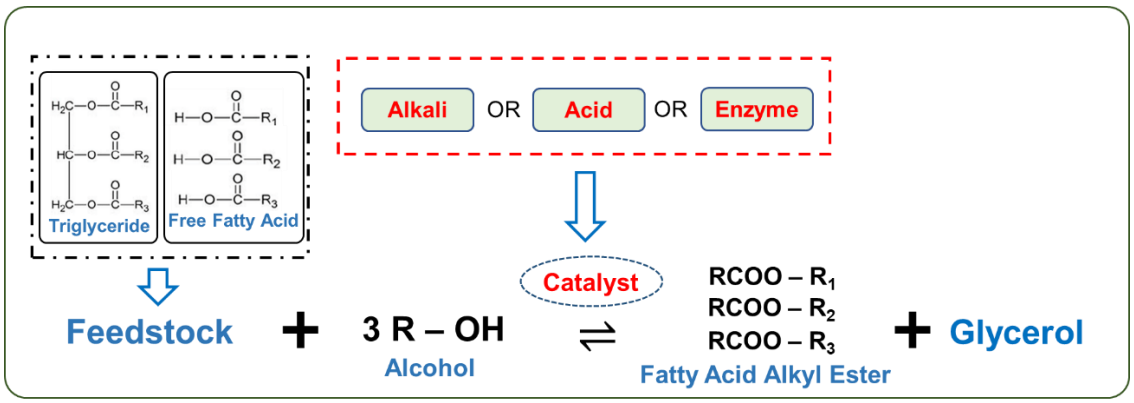

Figure 2. Reaction mechanism for transesterification to biodiesel.

Biodiesel is commonly produced via base-catalyzed transesterification due to several advantages. In 2017, Abdullah et al. created biodiesel using palm oil waste that had been collected as sludge. The ideal conditions for the loading of 1.5 wt% of KOH led to the highest yield of 93% and included a 20:1 methanol-to-oil ratio and a 300 rpm agitation speed at 60 ºC for a one-hour reaction. With reaction conditions of 60 ºC at 400 rpm for 1 hour, Hayyan et al. (2010) [24] produced biodiesel from sludge palm oil using 1 wt % KOH, which was a lower yield than that produced by Abdullah et al. at 76.6%. Suwanno et al. (2017) [25] investigated and compared biodiesel synthesis from the residual oil of POME and compared the biodiesel production from a NaOH catalyst with that from an enzyme catalyst.

In that study, equal parameters of processing showed that the NaOH catalyst achieved a lower yield than the enzyme catalyst. Base-catalyzed processes have shown limitations such as a high sensitivity to water and FFAs in the feedstock, which is problematic because PAO contains high amounts of water and FFAs. These limitations result in soap formation, which reduces the ester yield, complicates the separation and purification of glycerol and ester, and produces biodiesel that is more viscous. To overcome these limitations, an esterification reaction to reduce the FFAs was combined with the transesterification process. Hayyan et al. (2011) [26] used H_2SO_4 in the pre-treatment of sludge from POME for biodiesel production via the esterification process, followed by transesterification, which reduced FFAs from 23.2% to less than 2% when using 0.75 wt % of H_2SO_4 at 60 ºC with a molar ratio of methanol-to-oil of 8:1. The ester yielded 83.7% following transesterification.

Enzymatic transesterification of biodiesel is currently considered to be a green-energy process. The use of biocatalysts for biodiesel generation requires a lower operating

temperature and eliminates the need for downstream processes such as separation and purification. Using lipase from *Litopenaeus vannamei* hepatopancreas, Rakkan et al. (2017) [27] improved the synthesis technique for the transesterification of residual oil from POME. In that study, the greatest biodiesel production of 91.45% was achieved with an enzyme loading of 40 kU, a methanol-to-oil molar ratio of 6:1, an agitation speed of 250 rpm, a reaction temperature of 40 °C, and a reaction period of 12 h.

A quicker procedure saves energy and is more appropriate for industrial-scale processing. Rachmadona et al., investigated the use of bioethanol as an acyl acceptor for biodiesel production from POME [19]. That study integrated PAO as a feedstock and palm sap as bioethanol in the production of biodiesel. Two days of soaking in bioethanol (63% v/v) followed by the distillation of synthetic palm sap with 500 mg of *Thermomyces lanuginosus* lipase with 35 rpm of agitation speed at 40 ºC was sufficient to produce a fatty acid ethyl ester yield of 98.1%. This method has a number of limitations, however, particularly when used for production on a commercial scale. Table 2 summarizes the studies on POME as a feedstock for biodiesel production.

Table 2. Biodiesel production using POME as feedstock.

Acyl Acceptor	Catalyst	Concentration	Condition	FAAE Content (%)	References
Methanol	NaOH	1 wt%	60ºC, 800 rpm, 1 h	96.5	[25]
Methanol	KOH	1 wt%	60ºC, 400 rpm, 1 h	76.6	[24]
Methanol	KOH	1.5 wt%	60ºC, 300 rpm, 1 h	93.0	[28]
Methanol	CaO	6 wt%	45ºC, 700 rpm, 3 h	79.0	[29]
Methanol	-	-	150ºC, 15 min	89.0	[30]
Methanol	H_2SO_4	0.75 wt%	60ºC, 400 rpm, 1 h	83.7	[26]
Methanol	ZrO_2/SO_4^{2-} - Rice Husk Ash	10 wt%	60ºC, 500 rpm, 4 h	83.1	[31]
Methanol	*Pacific white shrimp*	40 kU	40ºC, 250 rpm, 12 h	96.5	[27]
Methanol	Crude lipase from oil palm fruit	36 U	35ºC, 200 rpm, 36 h	96.5	[25]
Methanol	Immobilized palm lipase	6 g	35ºC, 200 rpm, 24 h	93.5	[32]
Methanol	Immobilized *Candida rugosa*	2 g	40ºC, 300 rpm, 5 h	85.0	[33]
Methanol	*Yarrowia lipolytica*	610 U	60ºC, 300 rpm, 72 h	97.0	[34]
Ethanol	*Thermomyces lanuginosus*	2100 U	40°C; 500 rpm; 24 h	97.4	[35]
Ethanol	Immobilizes *Candida antartica* lipase B	446 U	40°C; 35 rpm; 96 h	97.5	[36]

V.4. Bioconversion of POME to biogas

For the anaerobic digestion of complex materials such as POME, anaerobic sludge is utilized, this takes advantage of assorted organisms that act together to improve consumption of the materials [37, 38]. Degradation occurs in four phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [39].

Table 3. Methane production using POME as feedstock.

Species	POME					Methane		References
	Pretreatment	COD (mg/L)	TN (mg/L)	TP (mg/L)	pH	Productivity (mL_{CH4}/L_{POME}/h)	Yield (mL $_{CH4}$/g)	
Mixed culture	pH 6.8	42500-55700	500-700	n.d.	3.8-4.4	247.22	356	[40]
Mixed culture	No	58144	n.d.	n.d.	4.5	145.41	61.5	[41]
Mixed culture	Ozonation	58144	n.d.	n.d.	4.5	1083.7	646.1	[41]
Mixed culture	No	70000	980	608	4.5	700	240	[42]
Mixed culture	n.d.	106934	1402		4.8	833.33	n.d.	[43]
Mixed culture	No	56500	960	110	n.d.	133.3	320	[44]
Mixed culture	Ultrasonication	98250	n.d.	n.d.	4.85-5.6	n,d.,	139.59	[45]
Mixed culture	No	63925	n.d.	n.d.	4.42	n.d.	293	[46]
Mixed culture	Fungal pretreatment	n.d.	n,d.	n.d.	7.8	n.d.	307.7	[47]

The production of methane from biomass, as presented in Table 3, is carried out via chemical or biochemical processes, and anaerobic digestion is a well-known and commonly applied method that is performed under atmospheric pressure and room temperature to produce high yields of methane [43]. During anaerobic digestion, methane production follows two metabolic pathways: the dismutation of acetic acid, and the reduction of hydrogen by methanogenic bacteria [48]. These pathways are affected by the conditions in which the reactor is operated; if conditions are mesophilic, most of the methane will be produced by the first pathway, but if the conditions are thermophilic the second pathway will occur [49].

To produce hydrogen through anaerobic digestion, hydrogen-consuming bacteria must be eliminated to avoid the methanogenesis phase. This elimination is performed by establishing a hostile environment for these bacteria [50]. It is also possible to produce

hydrogen by isolating some species of bacteria such as *Enterobacter* sp. and *Clostridium* sp., which have an endogenous ability to generate high yields of hydrogen [37, 50].

There are certain drawbacks to using POME for anaerobic digestion. One such problem is that the substrates might not be readily available to bacterial cells because of the complexity of POME, and this could increase the time for adaptive phases and result in a lower conversion yield [51].

Several pretreatment methods have been proposed in order to avoid this drawback, some of the pretreatments are intended to break down the lignin, which are long lipid and protein chains that could affect or inhibit digestion [52]. A proposed pretreatment is the thermal pretreatment of POME, which is known to have many advantages such as increasing the process performance, improving stability, reducing hydrophilicity, and increasing the digestion efficiency [53].

To achieve higher yields of methane from POME, various pretreatment processes have been evaluated. These include ozonation and ultrasonication [41, 54, 55], and both of these POME pretreatment methods have been used to increase the methane yield by as much as 7-fold compared with non-pretreated POME. Table 3 lists a compilation of different research methods used to produce methane by utilizing POME as a substrate, and shows how submitting POME to a pretreatment method can increase the methane productivity compared with the use of non-pretreated POME.

Table 4. Biohydrogen production using POME as feedstock.

Species	POME					Biohydrogen		References
	Pretreatment	COD (mg/L)	TN (mg/L)	TP (mg/L)	pH	Productivity (mL/L/d)	Yield (mL/g)	
Mixed culture	No	49000	227	74.5	4	22.6	10	[56]
Mixed culture	No	40000-80000	n.d.	n.d.	n.d.	144	n.d	[57]
Mixed culture	Ultrasonication	38400	n.d.	n.d.	4.63	n.d.	17.1	[58]
Mixed culture	No	56300	1330	220	4.57	76	27.1	[59]
Mixed culture	No	45200-56300	1100-1500	97-125	4.2-4.5	n.d.	n.d	[47]
Immobilized *Clostridium butyricum* EB6	No	55100-86300	820-910	95-120	4.0-5.0	n.d.	n.d	[60]
Mixed culture	Ozonation	n.d.	n.d.	n.d.	4.4-4.7	11	77.1	[61]
Mixed culture	Ozonation	n.d.	n.d.	n.d.	4.4-4.7	n.d.	182.3	[62]
Mixed culture	No	56500	960	110	n.d.	80	215	[44]
Mixed culture	n.d	56500	960	110	5.1	n.d.	n.d	[44]
Mixed culture	No	48000	n.d.	n.d.	4.5	101.2	n.d	[51]
Mixed culture	n.d	48000	n.d.	n.d.	4.5	229.3	57.3	[51]

These pretreatment techniques have also been used to increase the hydrogen yield by increasing the substrate bioavailability of POME. Ultrasonication pretreatment has been used to increase Hydrogen production by 38% [58]. Ozonation pretreatment has increased hydrogen production by 49% [62]. These pretreatment processes break down the complex molecules into simpler forms, which can then be converted into hydrogen. Table 4 shows the results found in the literature wherein POME was used as a raw material for hydrogen production as part of a mixed culture. It is obvious that the use of pretreatment methods such as ultrasonication and ozonation improve the hydrogen yield compared with that gained by using non-pretreated POME.

V.5. Bioconversion of POME to various value-added products

Several techniques have been developed to convert POME into a variety of value-added commodities, such as volatile fatty acids (VFA) and citric acid, biodegradable polymers such as polyhydroxybutyrate (PHB) homopolymer and polyhydroxyalkanoates (PHA) copolymers, in order to solve the disposal issue for POME. These technologies involve the use of sequencing batch reactors (SBR) [63], agglomeration/flocculation [64, 65], membrane technology [66, 67], and advanced oxidation processes [64, 68]. PHA may be produced by bacteria from POME, and it is then changed into VFA [69, 70]. The high COD of POME has made it a highly sought-after raw material that is widely employed as a feedstock for microbial growth. The process of transforming a waste such as POME via biological pathways with the aid of microbes or other biological agents has enormous value since bacteria make up a numerous and important resource.

A few examples of microbes that have the ability to utilize POME as their sole carbon source to produce such high-quality chemicals and bioproducts are *Aspergillus niger*, *Cupriavidus necator*, and *Rhodobacter sphaeroide* (Table 1). The method for this production makes use of locally sourced microorganisms and POME that is available at low cost, which contributes to its viability and lessens environmental issues while also generating greater revenue for the palm oil milling industry.

The materials made from POME are available for a wide range of applications. Citric acid is used as an emulsifier in items such as ice cream, household goods, cosmetics, and more. PHA is an umbrella term for a group of polymers that are biodegradable and biocompatible. PHA-based materials are expected to eventually replace traditional polymers made from petroleum since they possess characteristics that are similar to those of thermoplastics. The industries most interested in the potential benefits of PHAs and

their derivatives include agriculture, food, and medicine [71].

PHA price is heavily influenced by substrate costs, which account for around 40% of total production costs [72]. Over the last two decades, several low-cost carbon substrates, such as starch, tapioca hydrolysate, whey, and molasses, have been explored for PHA production in an effort to reduce its production costs. POME might be thought of as a cost-free substitute for its use in the PHA production.

Table 5 depicts the synthesis of VFA and PHA using POME as a substrate. According to Ali et al. (1997), the minimal cost for generating PHA from POME is less than USD2/kg, with a content of 50 wt% PHA in dried cells and 2 wt% dissolved in chloroform. The price of PHA might be reduced to less than USD1/kg by increasing the content of soluble PHA in chloroform from 2 to 5 wt%. PHA might be made less costly by increasing its polymer content in the cell from 50 to 80 wt%. The aforementioned pricing is comparable with recent market prices announced by one of the biggest volume producers (Mirel^{TM}) and are estimated to be around USD2/kg [73]. This price was substantially higher than that of starch polymers and other bio-based polyesters due to high raw material prices (30 to 40% of total cost), limited production volumes, and high processing costs (especially for purification) [73].

Table 5. A variety of value-added products from bioprocesses using POME as carbon source.

Microorganism	Product	Yield ($g\ l^{-1}$)	Condition	References
Mixed cultures	VFA	7.8	POME + palm oil sludge. 30 ºC, pH was controlled at 7, SBR (sequenced batch reactor), 24 h	[74]
Mixed cultures	VFA	10 - 14	POME + palm oil sludge with a ratio of 1:1, 300 rpm, 30 ºC, pH was controlled at 7, stirred tank bioreactor fermentation, 84 h	[75]
Mixed cultures	VFA	10.27	POME + palm oil sludge, pH was controlled at 7, SBR, 96 h	[76]
Aspergillus niger (A 103)	Citric acid	5.2	POME: 2% (w w^{-1}) Wheat flour: 4% (w w^{-1}); Glucose: 4% (w w^{-1}); Ammonium nitrate: 0% (w v^{-1}) Time: 5 days	[77]
Aspergillus niger ATCC 9642	Citric acid	1.02	POME: 75% Methanol: 3% Time: 7 days	[78]
Cupriavidus necator	PHB	3.12	POME: 180 ml Sugar: 18 $g\ l^{-1}$ Time: 60 h	[70]
Rhodobacter sphaeroides IFO 12203	PHA	4.0	Synthetic waste based on organic acid profiles obtained during POME treatment. 30 ºC, pH was controlled at 7, photobioreactor fermentation, ≈ 200 h	[74]
Mixed cultures	PHA	24.24	High concentration of POME with 490 COD/N ratio (g COD/ g N) and 160 COD/P ratio (g COD/ g P), 1000 rpm with aeration rate of 1.5L/min, 30 °C. pH was controlled at 7, SBR	[72]

Activated sludge is typically used as a sole substrate for the production of PHA and other biodegradable polymers from waste through a following process: first, waste is acidogenic fermented to generate volatile fatty acids (FFA), then, PHA-accumulating organisms are cultivated and enriched within the sludge, and finally, the sludge is used for PHA production. Researchers have shown that by adding POME to sludge, PHA may be produced [72, 79, 80]. Din et al. (2012) [79] examined the PHA and COD/N/P components. From the results of this analysis, it was determined that a ratio of 180:0.7:1 for COD, N, and P yielded the highest PHA concentration (44.5 wt%) per dry weight of

sludge. According to research by Salmiati et al. (2007), the amount of PHA in a reactor may be raised to 40 wt% by adding more fermented POME. Using a microaerophilic environment, Din et al. (2012) [79] produced a product with a relatively high PHA concentration of 74 wt%, however it required around 40 h of culture time to achieve this. Because of this, it is vital to have a deeper understanding of how to efficiently synthesis PHA from POME. Accelerating production of PHA while simultaneously increasing output would be remarkable.

PHA may be manufactured from any agro-industrial waste, including POME, as long as the quantity and content of the agro-industrial waste utilized to make it are maintained. To optimize the bioconversion process and minimize related energy costs, modern mills for the synthesis of raw materials such as VFAs from POME must incorporate bacterial PHA production into their operational units. In order to achieve zero discharge in the palm oil industry, Hassan et al. (2002) [81] created a scheme to integrate the current treatment system of POME within PHA production. This approach entails stopping methanogenic activity from occurring at any time in order to get rid of the organic acids produced during the anaerobic phase. Clarified organic acids were obtained by further separation and purification processes, which may be employed as a substrate for PHA-producing bacteria [82, 83].

V.6. POME treatment by microalgae and cyanobacteria

Oxygenic photosynthetic microorganisms such as microalgae and cyanobacteria have been used to treat POME and produce raw materials for fuels and chemical products. For this review, we extracted key data from previous reports, and this is summarized in Table 6. Microalgae isolated from POME (*Chlamydomonas* sp. and *Chlorella* sp.) are

used in some reports [84, 85], but previous reports have mainly used commercially obtained microalgae and cyanobacteria. Among them, *Chlorella vulgaris* and *Chlorella sorokiniana*, which are used as supplements and feed, have been widely reported, as well as *Haematococcus pluvialis*, which can produce astaxanthin as supplements, and the food additive phycocyanin-producing cyanobacterium *Arthrospira platensis* (Table 6). Although the salinity of POME is quite lower than that of seawater, the genera *Isochrysis* and *Pavlova*, marine microalgae for feed on rotifers, have also been examined [86, 87]. POME from anaerobic treatment ponds with COD values in the thousands to tens of thousands and POME from aerobic treatment ponds with COD values in the hundreds have been used as medium components (Table 6).

Regardless of COD values, however, POME must be diluted below 30% to maintain high microalgae and cyanobacteria growth. This is due to the light-shading effect of tannic acid in POME [88-90] and to a direct inhibition of growth caused by components such as ammonium ions [91].

Microalgae has a high level of POME treatment activity, which reduces the COD value of diluted POME in anaerobic ponds by approximately 2,000 to 8,700 mg/L [86, 89, 92]. In addition, microalgae and cyanobacteria are able to remove COD from POME in aerobic ponds with low COD values to levels below the effluent standards of Indonesia and Malaysia, which are the major producing countries of palm oil [84, 88, 93-95].

Table 6. COD reduction of POME in anaerobic pond and aerobic pond by microalgae producing useful materials.

Species	POME content	COD in POME medium (mg/L)	COD Removal efficiency	Cell density (g/L)	Product/ product yield (mg/L)	References
Anaerobic pond						
Chlorella vulgaris ESP-31	5%	1385	n.d.	3.5	Lipid/640	[96]
C. vulgaris	20%	4246	96%[a]	0.8	Feed for Moina/-[a]	[89]
	10%	2180	92%[a]	1.0	Feed for Moina/-[a]	[89]
Chlorella sorokiniana CY-1	10%	2770	n.d.	3.2	Lipid/640	[96]
	30%	8310	45%	1.8	Lipid/209	[96]
Isochrysis galbana	15%	8947	77%	-(14×10^6 cell)	Lipid/ (20%)[b]	[86]
	20%	11129	75%	-(12×10^6 cell)	Lipid/ (19%)[b]	[86]
Pavlova lutheri	15%	8947	80%	-(14×10^6 cell)	Lipid/ (35%)[b]	[86]
	20%	11129	78%	-(12×10^6 cell)	Lipid/ (26%)[b]	[86]
Aerobic pond						
Haematococcus pluvialis	7.5%	217	51%	0.5	Astaxanthin/22	[88]
Chlamydomonas sp.[c]	17%	341	12%	0.9	n.d.	[84]
Chlamydomonas incerta	12-19%	250	67%	n.d.	n.d.	[93]
C. vulgaris	10%	152	73%	0.2	n.d.	[95]
Dunaliella salina	10%	152	66%	0.2	n.d.	[95]
Spirulina platensis	10%	152	58%	0.2	n.d.	[95]

Since the dilution of POME is essential for microalgae and cyanobacteria growth, it is difficult to treat POME with microalgae alone, but microalgae could contribute to POME treatment by integrating with other biological processes such as anaerobic fermentation. On the other hand, the maximum cell density reported thus far is 3.5 g/L, which is low for the growth capacity in POME and comparable to synthetic media [96]. Fatty acid production for use as a biofuel feedstock has been attempted in previous reports, but the cell density obtained from POME makes it more amenable to the

production of high value-added pigments and polyunsaturated fatty acids (PUFAs). If the goal is to increase biofuel production, it may be useful to search for microalgae strains that can utilize the free fatty acids in POME, since POME contain few carbon sources that can be used by industrial microalgae and cyanobacteria. In addition, since POME is acidic (pH 4), it must be adjusted to pH 6.5-8 in order to cultivate microalgae such as *C. sorokiniana*, which are best suited to a neutral pH [86, 88, 92, 95, 96]. The use of acid-tolerant microalgae that do not require neutralization treatment should also be considered.

V.7. Conclusions and Future perspective

Currently, POME is commonly applied in anaerobic digestion to produce methane gas, but this causes an increase in greenhouse gases and river pollution. For the palm oil industry to be sustainable, it is necessary to establish technologies that have a low environmental impact. Several technologies were discussed in this review, including enzymatic conversion from POME and fermentation by microalgae, bacteria, and yeast (Figure 3). Enzymatic conversion of PAO by lipase, oil production by microalgae, and fermentation of methane and hydrogen gas will likely be used as a supply of energy for mill plants. Biodegradable polymers such as PHA, are often produced from POME via the use of bacteria or yeast, which the food industry is likely to utilize as plastic material for bottles, cover sheets, and bags. Microalgae fermentation could produce several oil-related chemicals such as triglycerides, lipids, PUFAs, and pigments as well as being used to simultaneously restore wastewater quality. The oil produced could serve as biodiesel feedstock, while the pigments and PUFAs would be used as food additives. Through proposed sequential bio-based conversion, wastewater could be re-generated by sequential fermentations of microalgae and bacteria and could be returned to the land of

palm plantations as a source of freshwater. In addition, each fermentation's waste residue, such as cell pellets, could also be returned to the land for use as fertilizer.

The use of these technologies must become more commonplace in order to convert waste from palm oil mills into energy, value-added materials, and water resources. Tailoring the use of these technologies for individual local sites in the palm oil industry will require combinations that are based on economic and environmental issues. The palm oil industry must find ways to contribute to zero emissions, to a bio-circular economy, and to sustainable development goals (SDGs), as established in previous reviews [16].

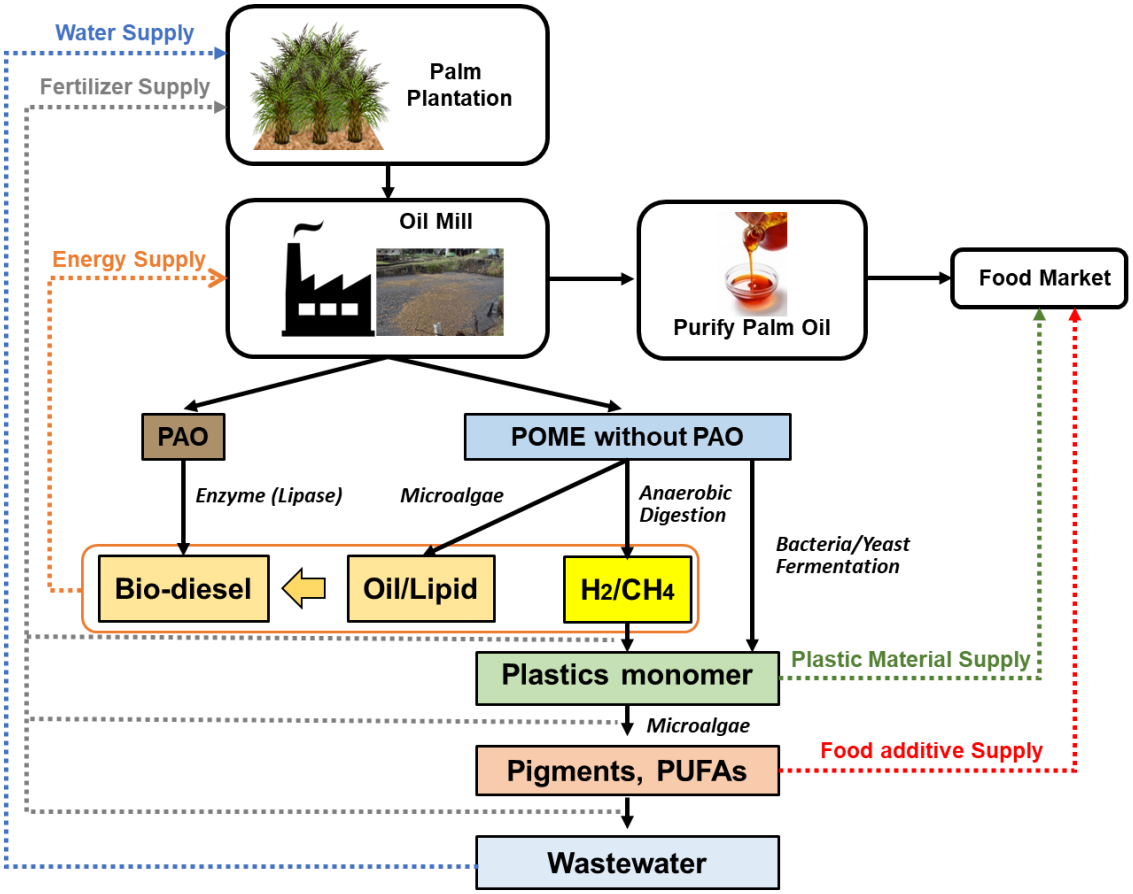

Figure 3. Proposed "bio-circular economy system" in the palm oil industry with sustainability.

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CHAPTER VI
General Conclusion and Future Research

The use of immobilization has been proposed as a way to simplify the downstream processes from the microalgae culture, which are one of the costliest parts for the production of valuable chemicals from microalgae, in this study we showed the potential of alginate immobilization to improve the growth and lipid productivity of the microalgae *Chlorella sorokiniana*, this technology could also be applied to the POME culture to increase its growth and possibly increase its productivity.

The growth and succinate production from *Synechocystis* sp. PCC 6803 was improved when immobilized in alginate beads, especially when utilized in continuous succinate production cycles, in which it increased the succinate productivity by 1.4-fold compared to the free cell, resulting in a convenient strategy for the use of immobilized cyanobacteria, resulting in a simpler and faster process that achieves a higher rate of succinate production with each cycle.

The utilization of a low-cost feedstock such as POME for the production of valuable chemicals such as succinate or biodiesel is very attractive due to its potential to be used as a culture medium, because of the nutrients it contains that are able to be utilized by microorganisms such as microalgae and cyanobacteria. However, one of the limitations of POME utilization for the growth of such microorganisms is the low growth achieved by them, usually lower than when cultured in an artificial medium with the nutrients needed, even though the glycogen accumulation and the succinate yield was increased in the cyanobacteria *Synechocystis* sp. PCC 6803 when it was cultured in a POME medium, the dry cell weight remained low, limiting the total product that could be obtained. It becomes necessary to find an alternative to increase its growth while

keeping its higher succinate yield.

Taking into account the results obtained from the immobilized cyanobacteria, the possibility for improving the growth and succinate yield when culturing the cells in a POME medium is increased, a valuable future experiment should consist on the optimization of succinate production of alginate immobilized *Synechocystis* sp. PCC 6803 cultured in a POME medium in a continuous culture strategy.

LIST OF PUBLICATIONS

Chapter II

D.A. Alfaro-Sayes, J. Amoah, N. Rachmadona, S. Hama, T. Hasunuma, A. Kondo, C. Ogino, Enhanced growth and lipid productivity by living *Chlorella sorokiniana* immobilized in Ca-alginate beads. J. Phys. Energy (2023) in press https://doi.org/10.1088/2515-7655/acb383.

Chapter III

D.A. Alfaro-Sayes, J. Amoah, S. Aikawa, M. Matsuda, T. Hasunuma, A. Kondo, C. Ogino, Alginate immobilization as a strategy for improving succinate production during autofermentation using cyanobacteria Synechocystis sp. PCC 6803, Biochem. Eng. J. 188 (2022) 108681. https://doi.org/10.1016/j.bej.2022.108681.

Chapter IV

D.A. Alfaro-Sayes, S. Aikawa, N. Rachmadona, T. Hasunuma, A. Kondo, C. Ogino, Palm Oil Mill Effluent (POME) Utilization for Succinate Production from Cyanobacteria *Synechocystis* sp. PCC6803 (Under preparation for submission)

Chapter V

S. Aikawa, D.A. Alfaro-Sayes, N. Rachmadona, P. Kahar, I. Rahayu, A. Kosugi, A. Kondo, C. Ogino, Bioconversion strategies to alter the bio-circular economic perspective for palm oil mill effluent. (Under preparation for submission).

Co-authored Publications

N. Rachmadona, E. Quayson, J. Amoah, D.A. Alfaro-Sayes, S. Hama, M. Aznury, A. Kondo, C. Ogino, Utilizing palm oil mill effluent (POME) for the immobilization of *Aspergillus oryzae* whole-cell lipase strains for biodiesel synthesis, Biofuels, Bioprod. Biorefining. (2021) 1–11. https://doi.org/10.1002/bbb.2202.

Doctoral Dissertation, Kobe University

"Study on the Improvement of Lipid and Succinate Productivity on Microalgae and Cyanobacteria by Alginate Immobilization and its Application" (135 pages)

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