ENHANCEMENT OF MICROALGAE ADHESION TO SUBSTRATE VIA ALGAL ORGANIC MATTER PRE-TREATMENT

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by
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Project report submitted in partial fulfilment of the requirement for the degree of Bachelor of Chemical Engineering
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ABSTRAK

Kelebihan kultivasi substrat berliang (PSC) berbanding dengan kultivasi suspensi konvensional termasuk penggunaan air dan rintangan pemindahan jisim gas-cecair yang rendah. Dalam kajian ini, tahap lekatan sel-sel alga mikro seperti *Amphora coffeaeformis*, *Cylindrotheca fusiformis* dan *Navicula incerta* pada permukaan jalur-jalur membran polivinilidena fluorida (PVDF) komersial sebelum dan selepas disalut oleh bahan polimer luaran sel larut (sEPS) dari *N. incerta* telah ditentukan. Ketiga-tiga spesies menunjukkan tahap lekatan yang lebih kuat pada permukaan jalur membran PVDF bersalut berbanding dengan membran PVDF dara. Hal ini demikian kerana permukaan jalur membran bersalut telah ada filem penyesuaian manakala biofilem hanya dapat jerap atas permukaan jalur membran dara setelah eksperimen dimulakan. Secara umumnya, sEPS menunjukkan pengeluaran polisakarida yang lebih tinggi daripada protein manakala bagi kes bahan polimer luaran sel terikat (bEPS), produktiviti polisakarida dan protein dibandingkan antara jalur membran bersalut dan yang dara. Bagi alga mikro yang berlekat pada jalur membran dara, ketiga-tiga spesies mengeluarkan bEPS yang mengandungi protein yang lebih tinggi daripada polisakarida manakala bagi alga mikro yang berlekat pada jalur membran bersalut, pengeluaran polisakarida bEPS lebih tinggi daripada protein untuk *A. coffeaeformis* dan *C. fusiformis*. Hal ini berkaitan dengan penyesuaian spesies atas kebolehbasahan permukaan substratum. Namun, *N. incerta* tidak menunjukkan fenomena tersebut dan hal ini mungkin disebabkan oleh kadar metabolik spesies ini yang stabil tapi rendah atas jalur membran bersalut. Oleh itu, enzim luaran sel *N. incerta* dihasilkan untuk memampas kadar metabolik yang rendah lalu meningkatkan pengeluaran protein.

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ABSTRACT

Porous substrate cultivation (PSC) is more advantageous than the conventional suspension cultivation in terms of the reduction of water volume needed and the omission of gas-liquid mass transfer resistance. Thus, the degree of adhesion of *Amphora coffeaeformis*, *Cylindrotheca fusiformis* and *Navicula incerta* on commercial polyvinylidene fluoride (PVDF) membrane strip surface before and after coating with the soluble extracellular polymeric substances (sEPS) of *N. incerta* was determined in this study. All three species showed stronger cell adhesion on the coated PVDF membrane strips than on the pristine ones. This was because the coated PVDF membrane strips already had a conditioning film on its surface after the coating while the pristine membrane strips only had biofilms start developing on them when the experiment started. The sEPS generally had higher productivity of polysaccharide than of protein while for bounded EPS (bEPS), the productivities of polysaccharide and protein between pristine and coated membrane strips were compared. All three microalgal species on pristine membrane strips produced bEPS that contained higher protein content than polysaccharide whereas on coated membrane strips, the productivity of bEPS polysaccharide was higher than that of protein for *A. coffeaeformis* and *C. fusiformis* and this result was related to the species' adaptation to the substrate surface wettability. However, *N. incerta* did not show such relation maybe because it had a stable, yet low metabolic rate on the coated membrane strips, so extracellular enzymes were secreted to compensate the low rate, leading to a higher protein productivity.

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LIST OF ABBREVIATIONS

PSC	Porous substrate cultivation
AOM	Algal organic matter
IOM	Intracellular organic matter
EOM	Extracellular organic matter
EPS	Extracellular polymeric substances
sEPS	Soluble extracellular polymeric substances
bEPS	Bounded extracellular polymeric substances
PVDF	Polyvinylidene fluoride
PSPBR	Porous substrate photobioreactor
TL PBR	Twin-Layer system photobioreactor
VOC	Volatile organic compounds
PAM	Pulse amplitude modulation
LCP	Light compensation point
LSP	Light saturation point
ITO	Indium tin oxide
DLVO	Derjaguin, Landau, Verwey, Overbeek
XDLVO	Extended DLVO
ANOVA	Analysis of variance
ATR-FTIR	Attenuated total reflection-Fourier transform infrared
TEP	Transparent exopolymer particles
SEM	Scanning electron microscope
PSA	Phenol-sulphuric acid
BSA	Bovine serum albumin

CHAPTER 1

INTRODUCTION

1.1 Background

Microalgae are useful in various industries because it can produce a plethora of substances, for instance biofuel and complex polysaccharides, which are value-added products in pharmaceutical and cosmetics industries that have anti-inflammatory and antioxidant properties (Ekelhof and Melkonian, 2017). Wastewater treatment is also one of the sectors that utilises microalgae due to low operating costs, reduced carbon emissions, and safer removal of heavy metals present in wastewater as compared to using chemicals in conventional wastewater treatment processes (Barros, Gonçalves and Simões, 2019).

The focus of much research regarding the cultivation of microalgae previously was on the suspension cultivation until limitations were exhibited during the scaling up process, subsequently, the cost-effectiveness was also a main concern (Grobbelaar, 2010; Chisti, 2013; Olivieri, Salatino and Marzocchella, 2014; Podola, Li and Melkonian, 2017). Thus, cultivating microalgae biomass immobilised on a surface as a biofilm has emerged as a relatively new technology to overcome certain limitations shown by the conventional suspension cultivation, such as extensive usage of water, mass transfer limitations from the gaseous phase containing carbon dioxide to the microalgal cells suspended in liquid phase, and the difficulty to harvest microalgal cells (Podola, Li and Melkonian, 2017).

PSC is one of the configurations of biofilm cultivation, where the microalgal biofilm is attached and grown on porous substrates, i.e., membranes, synthetic non-woven/textile combination, and plain printing papers etc. (Podola, Li and Melkonian, 2017) while the gaseous phase flows directly above the biofilm and the liquid medium

flows on the other side of the porous substrate, so that the ambient light and gas supply are not diluted (**Figure 1.1**). This cultivation mode significantly reduces the volume of water and gas-liquid mass transfer limitation. Adhesion of microalgal cells on the porous substrate is achieved due to the secretion of AOM, or more specifically, EPS, that forms a layer of biofilm matrix. AOM can be divided into IOM – the organic matter inside the cell but will be released to the surroundings after cell death/lysis (Thurman, 1985), or after water treatment process for water containing high amounts of microalgae (Plummer and Edzwald, 2002; Ma *et al.*, 2006) – and EOM, or EPS, which consists of the excreted metabolites of microalgal cells (Paralkar and Edzwald, 1996). EOM can be further divided into sEPS and bEPS, where sEPS is released by the suspended microalgal cells to the culture solution and bEPS is found on cell surfaces (Qu *et al.*, 2012). As compared to EOM, IOM contained higher percentage of total organic nitrogen, in which there were more hydrophobic and higher ratio of components with high molecular weight (> 10 kDa), higher percentage of free amino acids but lower percentage of aliphatic amines (Fang *et al.*, 2010). The main components of EPS contributing toward the biofilm adhesiveness are found to be xylose, fucose, glucuronic acid (Domozych et al., 2005; Domozych, 2014; Xiao & Zheng, 2016), proteins, and DNA (Flemming and Wingender, 2010; Xiao and Zheng, 2016).

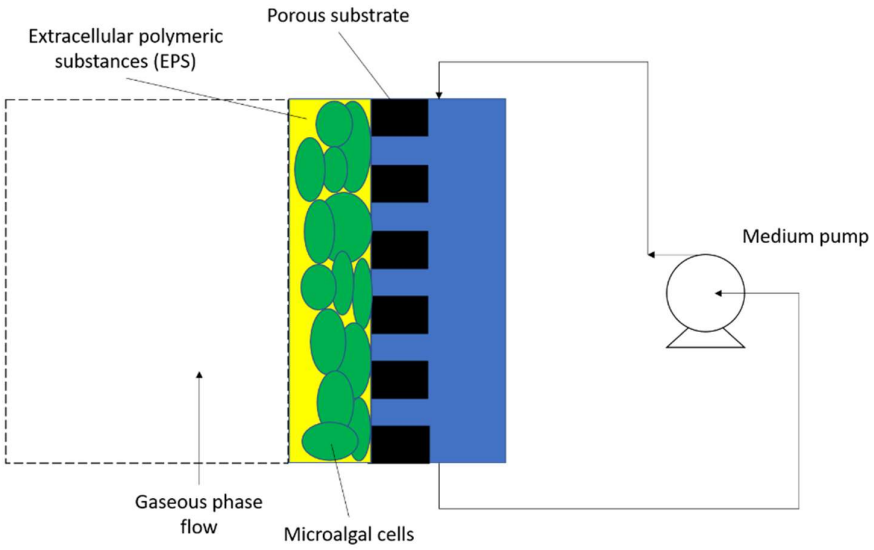


Figure 1.1. Visual representation of PSC.

The three microalgae species used in this study were *Amphora coffeaeformis*, *Cylindrotheca fusiformis* and *Navicula incerta*, which are all classified as diatoms. Diatoms are unicellular, but can exist as either filaments or colonies via aggregation within the EPS matrix (Xiao and Zheng, 2016), and can be found in almost every habitat, from freshwater to brackish waters (Likens, 2009). Diatoms are the only organism on Earth to have transparent-silica-composed (glass-like) cell walls but their motility is limited and can only approach substrates along a channel formed by their secreted EPS (Underwood *et al.*, 2004). Therefore, this study is to determine the adhesiveness of microalgal cells on pre-treated PVDF membrane to provide insight to the feasibility of PSC.

1.2 Problem Statement

Suspension cultivation mode for microalgae has several drawbacks as stated previously, which made it undesirable in large scales. Thus, PSC is seen as a more desirable choice of algae cultivation mode recently. However, not all algae are able to attach to the surface of the porous substrate due to their different nature. In this study,

the sEPS produced by *Navicula incerta* was used to pre-treat the PVDF membrane surface via coating and whether this affects the degree of adhesion of microalgal cells on it.

1.3 Objectives

The objectives of this final year project are as below:

- To determine the degree of adhesion of *A. coffeaeformis*, *C. fusiformis* and *N. incerta* on PVDF membrane surface as the porous substrate, before and after pre-treatment with sEPS of *N. incerta*.
- To characterise the polysaccharide and protein in the sEPS and bEPS of *A. coffeaeformis*, *C. fusiformis* and *N. incerta* grown on both pristine and pre-treated PVDF membrane surfaces.

CHAPTER 2

LITERATURE REVIEW

In the previous chapter, the limitations of conventional suspension cultivation of microalgae, the prospects of PSC as an alternative, and a brief introduction on AOM were established. Thus, in this chapter, previous results and reviews from credible scientific literatures that are related to this final year project title were presented, which includes the overview on the features of EPS, PSPBR, and the factors affecting the growth kinetics of microalgal cells.

2.1 Porous Substrate Cultivation

PSC is an emerging biotechnology for cultivation of microalgae in recent years because it can overcome the various drawbacks of conventional suspension cultivation and even enhance the growth performance of microalgal cells. In fact, this cultivation technique was first recorded in the late 19^{th} century where microalgae were cultured as biofilms on solid substrates but was subsequently neglected by researchers due to the shift in focus to suspension cultivation (Podola, Li and Melkonian, 2017).

As briefly described in Section 1.1 and depicted in **Figure 1.1**, PSC consists of a solid porous substrate that has liquid medium flowing on one side while microalgal cells are cultured on the other side, attached on the surface, forming biofilm. The porous substrate must be permeable with respect to the liquid medium and the dissolved nutrients. On the other side, since the microalgal cells are attached on the surface, the cells are directly exposed to air and light source; this reduces resistance to gas transfer and the dilution of light.

Recently, PSC technique is being considered as an alternative after researchers faced a series of issues when scaling up microalgae cultivation using the suspension

mode, including the mass transfer limitation of gases in liquid, the energy requirement for sparger, mixer, or pump to promote mass transfer (especially when more EPS is produced in the suspension with respect to growth, which increases its viscosity), the water efficiency, and the dilution/scattering of light source in liquid phase (Ekelhof and Melkonian, 2017; Podola, Li and Melkonian, 2017). For PSC, all the drawbacks stated above were solved due to the absence of large amount of water as compared to the suspension cultivation. Hence, PSC has been a lucrative alternative for cultivating microalgae and the recent research focus shifted from conventional suspension cultivation to the more effective PSC.

In the recent decade, there were several notable literatures studying on the performance of PSC of microalgae. In 2013, Naumann et al. studied the productivity of a TL PBR for four microalgal species; in 2017, Ekelhof and Melkonian cultivated *Netrium digitus* using PSPBR to produce EPS; in 2019, Do et al. determined the effects of inoculum density and storage time on the growth and productivity of astaxanthin by *Haematococcus pluvialis* in an angled TL PSPBR; lastly, Garbowski et al. (2020) used a natural substrate – pine bark – to cultivate microalgae in raw municipal sewage.

2.1.1 Potential porous substrates

There were several porous substrates that were used for PSC of microalgae in past studies, for example Johnson and Wen (2010) used polystyrene foam, Schultze et al. (2015) used polycarbonate membrane while plain printing paper was used by Naumann et al. (2013) and Schultze et al. (2015). Furthermore, Zhang et al. (2015) cultivated on electrostatic flocking cloth, Gross et al. (2016) concluded that nylon and polypropylene mesh with 0.50-1.25 mm openings showed the best initial attachment and long-term growth among the materials and mesh opening studied. Recently, Poad

and Derek (2019) reported that almost all microalgae species studied showed the highest chlorophyll content after attaching and growing on cellulose acetate membrane whereas the microalgae species showed better attachment on polyamide membranes under microscopic observation; then, microalgae cultivation on the surface of pine bark was experimented by Garbowski et al. (2020) while Tong and Derek (2021) studied polyvinylidene fluoride membrane as porous substrate.

Although the various literatures reviewed successfully used the respective porous substrates to grow microalgae, plain printing paper showed more difficulty in harvesting the attached microalgae due to its fragile nature when wet; one of the literatures reviewed (Naumann *et al.*, 2013) could not harvest one of the microalgae species without imposing damage on the paper substrate. Moreover, most studies reviewed here used membrane as the porous substrate, perhaps this phenomenon is due to the potential of microalgae coupled with the separation ability of membrane in fields like wastewater treatment. For instance, algal biofilm systems are simple and energy-efficient in the absorption of nitrogen and phosphorous from wastewater while the separation of algal biofilm from the wastewater bulk is relatively easy and inexpensive (Miranda *et al.*, 2017).

2.1.2 Porous substrate photobioreactor

A TL system is an extension of PSC where the flow of liquid medium is sandwiched by two layers of porous substrate instead of using single layer (Ekelhof and Melkonian, 2017; Podola, Li and Melkonian, 2017; Do *et al.*, 2019). It is modular, which means the system could be expanded whenever needed simply by adding another TL unit without significant system modifications. This variation was initially introduced as a biosensor to detect VOC such as methanol and formaldehyde in air. The

biosensor response was measured using some parameters from the PAM chlorophyll fluorescence method (Naumann *et al.*, 2013; Podola, Li and Melkonian, 2017).

Naumann et al. (2013) determined the productivity of a TL PBR for *Phaeodactylum tricornutum* (UTEX 642), *Tetraselmis suecica* strain PLY 305, and *Isochrysis* sp. T.ISO while it was unable to determine for *Nannochloropsis* sp. because the surface layer was damaged upon harvesting. In terms of average biomass yield, *T. suecica* and *Isochrysis* sp. T.ISO recorded 14.5 ± 4.9 g/m^2 growth area and 10.5 ± 5.0 g/m^2 growth area, respectively, after being cultivated for 25 days while *P. tricornutum* recorded 12.4 g/m^2 growth area after 14 days. As for water content, it was reported that *T. suecica* had 72.0 ± 1.0 % while *Isochrysis* sp. T.ISO had 83.7 ± 0.8 %; the water contents found were considered acceptable ranges (75-85% water content) that could directly enter downstream processing without pre-treatment such as flocculation and centrifugation (Uduman *et al.*, 2010; Xiao and Zheng, 2016). Hence, it was observed that *Isochrysis* sp. T.ISO had less biomass yield but higher water content than *T. suecica*. Meanwhile, Naumann et al. (2013) did not detect contamination in any form upon observation by light microscopy after harvest. All microalgae species studied experienced a lag phase of 2-4 days, except for *Isochrysis* sp. T.ISO; then, linear growth kinetics were observed for all four species. The reported growth rates were 0.6 g/m^2/day (*Isochrysis* sp. T.ISO), 0.8 g/m^2/day (*Nannochloropsis* sp.), 1.5 g/m^2/day (*T. suecica*), and 1.8 g/m^2/day (*P. tricornutum*). It was also found that production of 1 kg microalgal dry mass required 1 kWh for pumping in PSPBRs (Naumann *et al.*, 2013).

2.2 Microalgal Biofilm

Microalgal biofilm is an amorphous matrix where microalgae clusters are encapsulated in it and forms a thin film on solid supports (Parker, 2013; Podola, Li and

Melkonian, 2017), which can be observed in nature on tree trunks, rocks, and walls on damp regions; meanwhile, biofilm formation on solid substrate surfaces is induced by the attachment and subsequent growth of microalgal cells on solid substrate surfaces (Huang *et al.*, 2018). Excretion is associated with the growth of any living organisms; for the case of microalgal cells, EPS is excreted into their surroundings, which forms a hydrated biofilm matrix. Besides excretion, diatoms also produce EPS via secretion, sorption and cell lysis (Jin *et al.*, 2018). One of the characteristics of EPS is its ability to retain a stable matrix structure and form a three-dimensional polymer network, which in turn allows interaction between microalgal cells and assist in cell adhesion to surfaces (Xiao and Zheng, 2016). The ability of microalgal cells to attach onto porous substrates is one of the most important factors in ensuring the operability of PSC.

2.2.1 Composition of biofilm

The general composition of biofilm generated by microorganisms include EPS, water, lipids, extracellular DNA, and extracellular vesicles (Wickramasinghe *et al.*, 2020). As stated in Section 1.2, EPS can be further divided into sEPS and bEPS (Qu *et al.*, 2012). Comprehension on the components of EPS is required to facilitate the pre-treatment process in this study. It was found that adhesion and aggregation of microalgal cells were mainly related to polysaccharides, proteins, and DNA in the EPS (Flemming and Wingender, 2010) while sulphates give EPS its hydrophilicity and making it a gel-like substance (Wingender, Neu and Flemming, 1999). Upon further analysis, more specific compounds such as *N*-acetylglucosamine, xanthan (Xiao and Zheng, 2016) and arabinose (Bahat-Samet, Castrosowinski and Okon, 2004) were identified to play a major role in microalgal cell adhesion while Domozych et al. (2005)

and Domozych (2014) reported that xylose, fucose, and glucuronic acid linked cells and substrates.

2.2.2 Functions and characteristics of biofilm

In aquatic ecosystems, biofilms were found to offer protection to algae from external attacks of grazers, toxins, parasites, biocides and lytic enzymes, and physical protection from dehydration, cold and osmotic stress. Furthermore, biofilms also seem to enhance certain algae abilities such as the sequestering of vital organic and inorganic nutrients from the surrounding liquid and serve as a region that boost photosynthesis, respiration, and nutrient transfer (Domozych et al., 2005). However, the formation of biofilms is not always desirable. Biofouling is the term used to describe the undesired attachment and growth of living organisms on man-made surfaces, and biofilm – often caused by bacteria or diatoms – is a type of biofouling called "micro-fouling" (Jin *et al.*, 2018). Biofilms increase the drag of ship hulls and hence, decrease the fuel efficiency; biofilms also decreases the thermal efficiency in cooling-heating systems (Ozkan and Berberoglu, 2013).

2.3 Factors Affecting Microalgae Growth Kinetics

Like in any other organisms, growth can be controlled by a series of actions. From the literature reviewed, the important factors affecting the growth kinetics of photosynthetic microalgae include light intensity and the mass transfer of gases and nutrients.

2.3.1 Light intensity

Several literatures reported relatively high photosynthetic efficiencies in PSPBRs with moderate light intensities (Liu *et al.*, 2013; Schultze *et al.*, 2015). From the works of Liu et al. (2013), *Scenedesmus obliquus* was used and the biomass productivity increased with increasing light intensity, where the LCP – a point at which the biomass productivity is zero – is around 10 µmol photons/m^2/s while the LSP – a point at which the biomass productivity is maximum and upon exceeding, will start to decrease – is at 150 µmol photons/m^2/s; the maximum biomass productivity was found to be around 10 g dry weight/m^2/day. Meanwhile, according to the study by Schultze et al. (2015), the productivity of *Halochlorella rubescens* was found to be 31.2 g dry weight/m^2/day using a light intensity of 1023 µmol photons/m^2/s and a 3% CO_2 level.

2.3.2 Mass transfer of gases and nutrients

Microalgal cells in PSPBR can efficiently capture CO_2 and release O_2 due to the direct exposure of the biofilm to the atmosphere. As a result, the O_2 produced by the microalgal cells via photosynthesis in PSPBR would not accumulate and cause photoinhibition, hence, increasing the photosynthetic efficiency (Murphy and Berberoglu, 2014; Li *et al.*, 2016). A characteristic of PSPBR cultivation is that no mixing is required for the nutrients because the nutrients are dissolved in the medium and are diffused through the substrate surface to the biofilm. In several studies, long diffusion paths associated with thick biofilms do not impose mass transfer limitations on nutrients if the fresh culture medium contains sufficient nutrients (Murphy and Berberoglu, 2014; Li *et al.*, 2016; Li, Podola and Melkonian, 2016).

2.3.3 Nutrient source and concentration

The effects of nitrogen source and concentration on the growth of microalgae species *Botryococcus braunii* on cellulose acetate membranes were studied by Cheng et al. (2014). For the first study, they treated modified Chu 13 culture medium with five nitrogen sources, which were 1.98 mM of KNO_3, equimolar nitrogen concentration of $NaNO_3$, urea, $(NH_4)_2CO_3$ and NH_4NO_3. Then, for the study on the concentration effect, both $NaNO_3$ and KNO_3 were identified as the most favourable nitrogen sources in the first study due to high growth rate results, but $NaNO_3$ was selected to study the effect due to a lower cost. Hence, 0.40, 0.99, 1.49 and 1.98 mM $NaNO_3$ were added to the modified Chu 13 medium; results show that under circulating model, the optimum nitrogen concentration was 1.49 mM while it was 0.99 mM under non-circulating model.

As a conclusion, the major controlling factors of the photosynthetic efficiency of PSPBRs are the light intensity, and the concentrations of nutrients and inorganic carbon source (usually CO_2). This indicates that the growth rates of microalgae, and subsequently, the lipid content to produce biodiesel could be optimised either experimentally or using mathematical modelling. However, it is very difficult to compare the parameters currently because different microalgae strains were used in different studies, which means that the parameters used to obtain promising results reported for one strain, may not work for another. This imposes challenges for the operation of a large-scale plant because engineers could not determine the most suitable microalgae species to be used according to the verdicts on current studies.

2.4 Factors Affecting Microalgae Adhesion on Substrates

Besides microalgae species, the strength of microalgae adhesion on substrates can be influenced by several factors, i.e., acid base interaction between microalgal cells and substrate, the material of substrate, surface wettability, and light, as suggested by several literatures.

2.4.1 Acid base interaction between microalgal cells and substrate

According to Ozkan and Berberoglu (2013), *Chlorella vulgaris* (planktonic cell) and *Botryococcus sudeticus* (benthic cell) attached on ITO much stronger than on glass. The cells of both species retained fully on ITO even after being flushed for 5 minutes at a wall shear rate of 10 s^{-1} whereas the respective cells on glass were able to be flushed, although *C. vulgaris* cells barely retained on glass surface and only a small portion of *B. sudeticus* cells were flushed. It was identified that the larger acid-base attraction and lower electrostatic repulsion of ITO than that of glass helped the cells of both species to adhere stronger.

The authors also used three models to predict and later compare with the experimental results, which are the (1) thermodynamic model, (2) DLVO model, and (3) XDLVO model. The thermodynamic model calculates the difference between the total interfacial free energy of a substratum, microorganism, and liquid system before and after cell adhesion on the substratum; this difference is termed "ΔG_{adh}". This model says that cell adhesion is thermodynamically favourable if $\Delta G_{adh} < 0$, which means that the total interfacial free energy decreases after attachment. The total interfacial free energy of adhesion is also the sum of its Lifshitz-van der Waals and acid-base components. Next, the DLVO model describes cell adhesion as a balance between van der Waals (often attractive forces) and electrostatic (usually repulsive forces due to

negatively charged algal cells and substratum) interactions. The indicator in this model is the total interaction energy, ΔG_{tot}, where $\Delta G_{tot} < 0$ indicates attractive interaction between cells and substratum while $\Delta G_{tot} > 0$ indicates repulsive interaction; the magnitude of ΔG_{tot} and the respective separation distance indicates the reversibility of attachment. Lastly, the XDLVO model weighs in the contribution of acid-base interactions and the van der Waals-electrostatic interactions in the DLVO model. Based on the surface wettability of substratum, acid-base interactions can be either attractive (hydrophobic attraction) or repulsive (hydrophilic repulsion).

After the authors compared the experimental results with the predictive models, all three models successfully predicted the cell attachment density. However, it was reported that only the thermodynamic and XDLVO models, which considered the acid-base interactions between cells and substratum in the calculations, were more accurate in predicting the cell adhesion strength than the DLVO model. This result shows that the acid-base interaction between cells and the substratum was critical for the cell attachment rate and adhesion strength.

2.4.2 Substrate material and surface wettability

C. vulgaris was also studied in another literature (Sekar *et al.*, 2004) along with *Nitzschia amphibia* (diatom) and *Chroococcus minutus* (cyanobacteria) where the substrates used included admiralty brass, aluminium brass, copper, glass, Perspex, stainless steel 316-L, and titanium. *C. vulgaris* attached the most on stainless steel while *N. amphibia* and *C. minutus* adhered maximally on titanium. It was through ANOVA that the authors found that the substrate material, time, and the interaction of both were significant to the cell adhesion for all three species. In addition, by using Pearson's

correlation test, it was found that surface wettability was correlated to the adhesion of *N. amphibia* and *C. minutus* cells, but not of *C. vulgaris* cells.

2.4.3 Light

The adhesion of *Chlamydomonas reinhardtii* cell to substrates via flagella was studied by Kreis et al. (2018) using *in vivo* force spectroscopy and it was found that the adhesion can be switched on and off by varying the visible light spectrum from white to red. The response of *Chlamydomonas* microalgae to the change in light was rapid, which only happened within seconds. They suggested that the phenomenon was due to the redistribution of flagella membrane proteins that promote adhesion. The adhesion force exhibited by the cell was consistently stronger under white light and it was not affected by the light intensity. However, upon further varying the wavelength of the light source, the authors observed a peak in adhesion force under blue light; this means that the blue-light receptor in the flagella of *Chlamydomonas reinhardtii* plays a major role for the adhesiveness of the cell. The results exhibited the same trend as for white light, but there was a threshold for light intensity (2 to 5×10^8 photons/m^2/s) where the flagella do not adhere to the substrate anymore.

CHAPTER 3

METHODOLOGY

3.1 Overview of Research Methodology

The focus of this project is on determining the degree of adhesion and growth kinetics of microalgal cells on the PVDF membrane surface before and after pre-treatment with sEPS produced by *N. incerta* through experiment. **Figure 3.1** depicts the flow diagram of research activities.

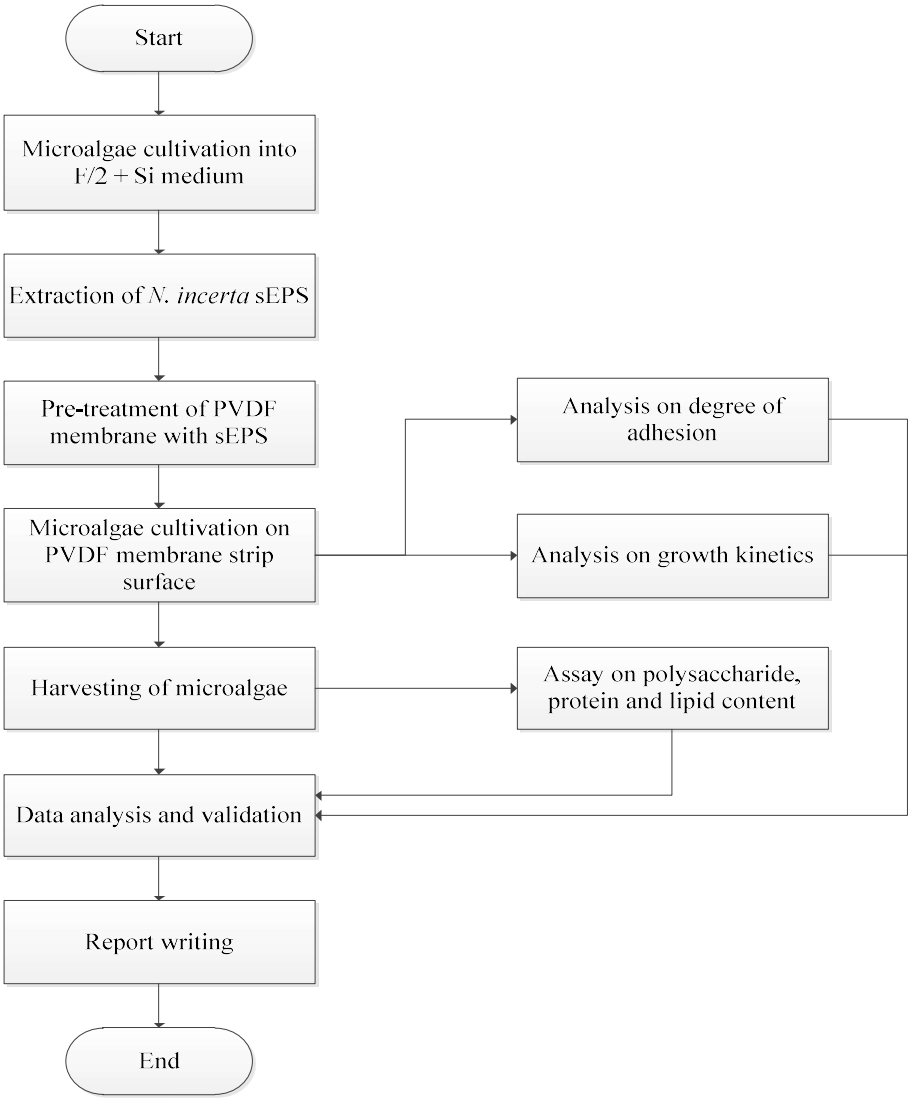


Figure 3.1. Flow of research activities for this project.

3.2 Preparation Procedures

3.2.1 Microalgae strains and cultivation

The microalgae strains used in this study were *Amphora coffeaeformis* (UTEX 2983), *Cylindrotheca fusiformis* (UTEX 2085) and *Navicula incerta* (UTEX 2044). The medium used for the cultivation of the three microalgae is F/2 + Si medium in artificial seawater, where **Table 3.1** shows its composition that needs to be added into distilled water. Fresh medium was prepared and distributed to several conical flasks, where each flask contains 250 mL of the medium. Then, the flasks were autoclaved at 121°C for 15 minutes. After the contents in the autoclave were cooled overnight, the microalgal cells of each species were inoculated into the mediums in a sterilised laminar flow chamber. The cultures were placed under 1500 lx fluorescence light tubes with a 12-hour light-dark photoperiod over the span of 14 days in a culture room; the temperature of the culture room was regulated at 25 ± 2°C.

Table 3.1. Chemical composition of F/2 + Si medium in artificial seawater (Tong and Derek, 2021).

Component	Concentration (µM)
$NaNO_3$	880
$NaH_2PO_4•H_2O$	36
$Na_2SiO_3•9H_2O$	106
$ZnSO_4•7H_2O$	0.08
$MnSO_4•H_2O$	0.9
$Na_2MoO_4•2H_2O$	0.03
$CoSO_4•7H_2O$	0.05
$CuCl_2•2H_2O$	0.04
$Fe(NH_4)_2(SO_4)_2•6H_2O$	11.7
$Na_2EDTA•2H_2O$	11.7
HEPES buffer pH 7.8	50.36
Vitamin B_{12}	0.0996
Thiamine	1.262
Seawater salt	3.3% w/v

At the end of day 14, the microalgal cells of *A. coffeaeformis*, *C. fusiformis* and *N. incerta* were harvested in a laminar flow chamber for cell count to ensure that the cultures had achieved a cell density of 1.5×10^6 cells/mL. 0.1 mL of sample was extracted for cell count, which used a Neubauer counting chamber and a light microscope (BX-51, Olympus, Japan). If the cell density is below 1.5×10^6 cells/mL, the culture will be placed back into the culture room for further cultivation, else the culture will be diluted with fresh F/2 + Si medium.

Subsequently, the cultures were transferred to three glass bottles with respect to each species and fresh, autoclaved F/2 + Si medium was added to each bottle to bring the final volume to 2 L. Meanwhile, extra *N. incerta* cells were inoculated in conical flasks for its sEPS, which will be used to pre-treat the PVDF membrane strips later. All cultures were incubated in the same culture room for another 14 days at the same conditions.

3.2.2 Extraction of sEPS for the pre-treatment of porous substrate

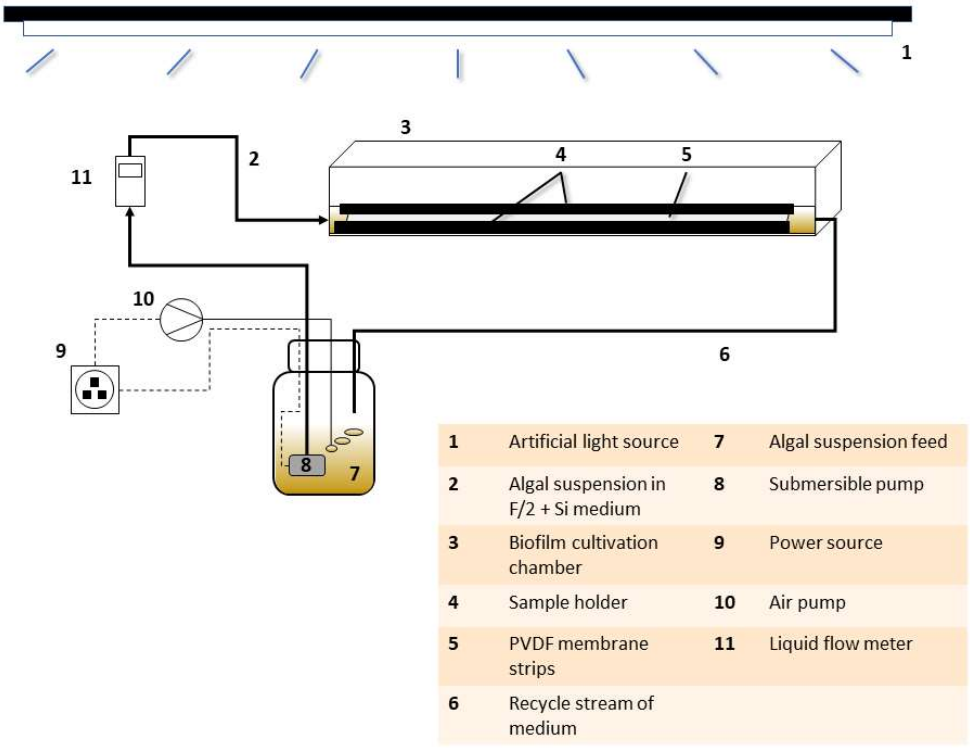
After 14 days, the *N. incerta* cultures from conical flasks were extracted and centrifuged at 4000 rpm for 10 minutes. The supernatant was extracted as sEPS and filtered with a 0.45 μm cellulose acetate membrane filter (47 mm diameter, Sartorius, Germany) under -20 kPa to ensure a cell-free sEPS solution. Meanwhile, the commercial PVDF membrane sheet (Durapore 0.1 μm pore size, Merck, Ireland) was cut into 7.5 cm (length) × 1.5 cm (width) strips. The membrane strips were then soaked in 10% ethanol for 15 minutes, rinsed with distilled water to remove any impurities on the surface and air-dried before pre-treatment. The pre-treatment, or coating, of the PVDF membrane strips was done by filtering 30 mL of 15 mg/L sEPS with the

membrane strips under -20 kPa and air-dried; the procedure was repeated for three cycles to ensure that the sEPS was coated firmly on the surface of membrane strips.

3.3 Microalgae Cultivation on Pre-treated Porous Substrates

Three cultivation lanes were sterilised in laminar flow chamber and set up as depicted in **Figure 3.2** to cultivate *A. coffeaeformis*, *C. fusiformis* and *N. incerta* in respective lanes on the coated PVDF membrane surfaces. Each lane has a marking at the midpoint and each side respectively holds pristine and coated membrane strips. Pristine membrane strips were used as control. The lanes were covered by custom-made glass covers to avoid contamination and to allow light to pass through for photosynthesis. The experimental set up was placed in the culture room with the same conditions as stated earlier.

Triplicate samples of membrane strip from each side of the cultivation lanes were retrieved at the 3^{rd}, 6^{th}, 24^{th}, 48^{th} and 72^{nd} hour after the fresh medium level in the lanes reached steady state. The samples were retrieved carefully using clean forceps to avoid serious detachment of biofilm from the membrane strips. The liquid flow rate was maintained at 100 LPM as indicated by the liquid flow meter.



1	Artificial light source	7	Algal suspension feed
2	Algal suspension in F/2 + Si medium	8	Submersible pump
3	Biofilm cultivation chamber	9	Power source
4	Sample holder	10	Air pump
5	PVDF membrane strips	11	Liquid flow meter
6	Recycle stream of medium		

Figure 3.2. Experimental set up for the cultivation lane.

3.4 Analytical Procedures

3.4.1 ATR-FTIR spectroscopy

In this study, ATR-FTIR spectrometer (Nicolet iS10, Thermo Scientific, USA) was used to determine the functional groups available on cell-free pristine and coated PVDF membrane strips based on infrared absorption. The wave number range of the ATR-FTIR spectrometer was 4000-600 cm^{-1} and the procedure was conducted at room temperature. This procedure is to confirm the presence of an additional sEPS coating on the membrane surface by identifying any difference in peaks between the spectrum of pristine and coated PVDF membrane samples.

3.4.2 Contact angle measurement

The contact angle between deionised water droplet and the membrane surface was measured with a video-based optical contact angle measuring instrument, LAUDA surface analyser LSA 200 (Lauda-Königshofen, Germany) to identify the change in hydrophobicity of the membrane surface. Pristine and coated PVDF membrane strips prior to cultivation were fixed at respective glass slides and placed onto the LSA 200. Then, 10 μL of deionised water was dispensed onto the membrane surface as a single water droplet and the images were captured within 2 to 3 seconds after contact. Readings were taken on at least four different spots of the membrane strip surface to ensure consistent results.

3.4.3 Alcian blue staining

Cell-free pristine and coated PVDF membrane strips were stained with aqueous alcian blue solution to identify if the sEPS, or more specifically, a subgroup of sEPS called TEP, was successfully coated onto the membrane surface. Besides, the cell-attached membrane strips retrieved at each time point were also stained for analysis after rinsing with distilled water. Aqueous alcian blue solution was prepared by adding 0.01% of alcian blue 8GX stock into 0.03% acetic acid. Then, the alcian blue solution was dropped onto the membrane strips until they are fully covered (about 1 to 1.5 mL) and at pH 2.5 to ensure that the sulphated and carboxylated TEP components were properly stained. Then, the samples were left for 10 minutes and rinsed gently with distilled water to wash off excess dye. After placing the sample on a glass slide, it was quickly observed using light microscope.

3.4.4 Scanning electron microscopy

SEM (TM3000 Tabletop Microscope, Hitachi, Japan) with 15 kV of accelerated voltage was used to observe the morphology of EPS and the attached cells on the PVDF membrane strips at the end of the experiment. For a more consistent observation, a minimum of five images were taken at different spots of the sample.

3.4.5 Harvesting of microalgal cells

The samples obtained were rinsed with distilled water to remove cells that were loosely bounded from the membrane surface. Then, pictures of the membrane strips were taken and are presented in **Figure 4.4**. Next, 5 mL of 1.5 M NaCl solution was used to wash off the biofilm from the membrane surface while the strongly attached cells were scraped using mini spatula in a petri dish. The NaCl solution plays a role in maintaining the osmotic equilibrium to avoid cell lysis. The cell suspension in NaCl solution was then transferred to a glass vial using filter funnel to avoid spillage and labelled with respect to the membrane strips that the cells were scraped off from so that a more accurate result can be obtained in bEPS extraction later.

3.4.6 Determination of cell density

Before conducting the cell count, a Neubauer counting chamber and a glass slide cover were sterilised with 95% ethanol to avoid contamination of the sample, which affects the process of cell count. 0.1 mL sample was extracted from the cell suspension in NaCl solution and was placed onto the Neubauer counting chamber. Cell count was done immediately after cell scraping to ensure that the cell density does not increase with time.

3.4.7 Extraction of sEPS and bEPS

sEPS and bEPS were analysed in this study. 30 mL of sEPS was extracted from the medium suspension in each cultivation lane at the 3rd, 6th, 24th, 48th and 72nd hour. The sEPS was extracted at the front, middle and end of each cultivation lane to ensure consistency in results. Meanwhile, bEPS was extracted from the attached cells on the PVDF membrane strips via the following procedures. The cells that were scraped off were previously suspended in 5 mL of 1.5 M NaCl solution for cell count, so, upon completion of cell count, 10 mL of 1.5 M NaCl solution was added to the 5 mL cell suspension and the associated membrane strips were fully soaked in the 15 mL cell suspension in a centrifuge tube. Subsequently, the centrifuges tubes were subjected to water bath at 30°C for 1 hour to extract the bEPS from the microalgal cells and from the PVDF membrane strips, where some bEPS might be bounded to the membrane surface. Next, the extracted sEPS and bEPS solutions were centrifuged at 4000 rpm for 10 minutes and the supernatants were filtered with 0.45 μm cellulose acetate membrane filter to obtain cell-free sEPS and bEPS solutions. The extracted sEPS and bEPS solutions were then analysed via colorimetric characterisation to quantify the polysaccharide and protein productivities.

3.4.8 Colorimetric characterisation of sEPS and bEPS

PSA method by DuBois et al. (1956) was used in this study to quantify polysaccharide present in sEPS and bEPS solutions extracted earlier. Different concentrations of glucose solutions were used as standard. The wavelength of the UV-vis instrument (Cary 60, Agilent Technologies, USA) was set to 490 nm for this assay. As for the quantification of protein, the procedures were given along with the BCA

protein assay kit (Novagen, Merck, Germany). The standard for protein was different concentration of BSA. In this case, the wavelength of the UV-vis instrument was set to 562 nm.

3.4.9 ANOVA

ANOVA was performed using Design-Expert 13 on the results from (1) contact angle measurement to determine if the sEPS coating influenced the PVDF membrane strip surface wettability and (2) cell count to see if the parameter of time, presence of coating, or microalgae species had significant influences on the cell adhesion. The significance of the parameters is deduced from the p-value. The parameters will be deemed significant if p-value < 0.05 in this study.