ROLE OF MICROALGAL ORGANIC MATTER-DERIVED BIOCOATING TOWARDS CELL DEPOSITION IN NATURAL BIOFILM FORMATION

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by

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PERANAN BIOCOATING TERBITAN BAHAN ORGANIK MICROALGAL TERHADAP PEMENDAPATAN SEL DALAM PEMBENTUKAN BIOFILEM SEMULAJADI

ABSTRAK

Kultivasi alga berasaskan biofilem semakin popular sebagai platform yang berdaya maju untuk pengeluaran alga, rawatan air sisa dan sebagai sumber bahan suapan untuk inisiatif biorefinery berasaskan mikroalga. Walau bagaimanapun, interaksi antara sel dan salutan bio kurang difahami. Dalam kertas kerja ini, tahap lekatan *Navicula incerta* pada permukaan jalur membran microporous polivinilidena fluorida (PVDF) komersial sebelum dan selepas disalut dengan AOM iaitu bahan polimer ekstraselular terikat (bEPS), bahan polimer ekstraselular terlarut (sEPS) dan bahan organik intraselular (IOM) diekstrak daripada Navicula sp. telah dikaji. Tambahan pula, polisakarida, protein dan hidrofobisiti sEPS, bEPS dan IOM *Navicula sp*. pada permukaan membran PVDF yang murni dan pra-rawatan telah disiasat. Penemuan menunjukkan bahawa lekatan sel mikroalgal mempunyai lekatan yang lebih tinggi pada jalur PVDF membran bersalut berbanding membran tulen. Ini adalah kerana bahan polimer ekstraselular (EPS) pada membran pra-bersalut menyediakan rangkaian seperti gel untuk membentuk lekatan biofilm yang kuat pada permukaan. Sudut sentuhan air bagi membran permukaan bersalut adalah lebih tinggi daripada permukaan tulen bermakna permukaan bersalut diklasifikasikan sebagai lebih banyak membran hidrofobik. Telah didapati bahawa permukaan dengan hidrofobisiti yang lebih tinggi dan kekasaran permukaan memberikan lekatan sel yang lebih baik dan pembentukan biofilm yang lebih kuat. Produktiviti polisakarida sEPS adalah lebih tinggi daripada produktiviti protein sEPS. Juga, bEPS mempunyai produktiviti

polisakarida yang lebih tinggi daripada protein. Walau bagaimanapun, protein dalam IOM mempamerkan kepekatan yang konsisten secara praktikal pada empat jalur membran bersalut berbeza. Polisakarida dan protein adalah komponen yang diperlukan untuk pembentukan biofilm. Kebolehbasahan permukaan EPS juga mempengaruhi lampiran sel.

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ABSTRACT

Biofilm-based algal cultivation is gaining popularity as a viable platform for algal production, wastewater treatment and as a feedstock source for microalgae-based biorefinery initiatives. However, the interaction between cells and biocoatings are poorly understood. In this paper, the degree of adhesion *Navicula incerta* on commercial microporous polyvinylidene fluoride (PVDF) membrane strip surface before and after coating with AOM namely bounded extracellular polymeric substances (bEPS), the soluble extracellular polymeric substances (sEPS) and intracellular organic matter (IOM) extracted from *Navicula sp.* was studied. Furthermore, the polysaccharide, protein and hydrophobicity of the sEPS, bEPS and IOM of *Navicula sp.* on pristine and pre-treated PVDF membrane surfaces were investigated. The findings showed that microalgal cell adhesion had higher adhesion on coated membrane PVDF strips than pristine membranes. This was because EPS on the pre-coated membranes provided a gel-like network to form a strong biofilm adhesion to surfaces. It was found out that surfaces with higher hydrophobicity and surface roughness provided better cell adhesion and stronger biofilm formation. The productivity of sEPS polysaccharide was higher than the productivity of sEPS protein. Also, the bEPS had higher productivity of polysaccharide than of protein. However, the protein in IOM exhibited practically consistent concentration on four different coated membrane strips. Carbohydrates and proteins are the required elements for the biofilm formation. Surface wettability of EPS also influences cell attachment.

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LIST OF ABBREVIATIONS

EPS	Extracellular polymeric substances
AOM	Algal organic matter
bEPS	Bounded extracellular polymeric substances
sEPS	Soluble extracellular polymeric substances
IOM	Intracellular organic matter
PVDF	Polyvinylidene fluoride
SEM	Scanning electron microscope
TEP	Transparent exopolymer particles
SAPs	Soluble algal products
EOM	Extracellular organic matter
DOC	Dissolved organic carbon
PZC	Point of zero charge
S_{mr}	Areal material ratio
S_v	Maximum pit height
S_a	Absolute of the ordinate values
PSA	Phenolsulfuric acid
BSA	Bovine serum albumin
RMS	Root-mean-square
AFM	Atomic force microscopy

CHAPTER 1

INTRODUCTION

1.1 Background

With the world's population and energy demand growing, the need for renewable energy sources has become critical. (Sun *et al.*, 2018). There has been renewed interest in using microalgae as a promising source of biofuel as it can reduce CO_2 emission by promoting microalgal growth and high lipid content to produce biodiesel (Sun *et al.*, 2018; Rosmahadi *et al.*, 2021). However, biofuel production which culturing microalgae biomass in the form of suspension culture has not been commercialized due to the poor productivity and high costs associated with suspended cultivation systems (Johnson and Wen, 2010). This is mainly due to the harvesting difficulties and extensive use of energy in separation of cells and media. Thus, attached microalgal cultivation emerges as a viable alternative to overcome the limitations of the conventional method (Caldwell *et al.*, 2021).

Submerged biofilm system is one of the configurations of microalgal biofilm-based cultivation in which the microalgae are fixed and grown on the surface of the substrate, covered by a thin layer of medium. Flow channels are commonly used to build constant submerged systems (Berner *et al.*, 2015) . To avoid nutrient and CO2 depletion at the biofilm-water interface, the growth media is mixed. (Caldwell *et al.*, 2021). The primary benefits of a submerged biofilm system are increased biomass concentration and the ease of harvesting process (Moreno Osorio *et al.*, 2021). Shen et al. (2014) established a simple submerged biofilm system with *Nannochloropsis oculata* and discovered that the maximum biomass productivity of the attached system was 13.5g m^{-2} d^{-1} which was 2.8-times higher than the suspended system.

Biofilms are largely made up of 10 % microbial cells and 90% extracellular polymeric substances (EPS) with 50 to 90 percent of the total organic carbon in biofilms (Donlan, 2002). Proteins generated from EPS aid in the initial adhesion process and form three-dimensional polymer network that immobilizes biofilm cells (Rosmahadi *et al.*, 2021; Shen *et al.*, 2015). Shen et al. (2015) reported that increasing EPS synthesis promoted biofilm formation. They concluded that the strong biofilm adhesion was due to protein released by microbial cells (51.6%). Furthermore, the biofilm formation is affected by surface, environmental and cellular factors (Moreno Osorio *et al.*, 2021). One of the elements influencing surface adhesion is hydrophobicity. The hydrophobicity of the surface can be increased by increasing the protein component of EPS. Proteins from the solution adsorb on hydrophobic surfaces, making the cells stick to the surface due to a strong adhesive attachment (Tong and Derek, 2021). Also, Kataryzna et al. (2015) reported that hydrophobic substratum can enhance the microalgal cell attachment.

However, in nature, as new biofilms form, the cells tend to clump together into aggregates. Detachment will happen as a result of single cell motility or the sloughing off of large clumps of cells. (Kragh *et al.*, 2016). As a result, the natural biofilm lifecycle may fail leading to biomass loss. Furthermore, not all microalgae are conducive to biofilm development. Thus, a number of researches have started to examine the biocoatings and biocomposites with different microorganisms. Biocoatings and biocomposites have been shown to improve biological processes and performance when compared to suspension cultures (Caldwell *et al.*, 2021). Colloid-based latex biocomposite can increase 500-1000 fold the immobilized biomass (Cortez *et al.*, 2017). However, some cells only existed as flocculates and not coated in thin coatings. Besides, latex coatings were used to immobilize of bacteria, fungi and yeast

in vinyl acetate copolymers as they formed large pores for colonization by microbial colonisation. Low coating porosity, poor mechanical stability, and a lack of cell viability data were key challenges in these early biocoating research. (Cortez *et al.*, 2017). From the study of Bernal et al. (2014), four distinct cyanobacteria strains (*Synechocystis PCC6803*, *Synechococcus PCC7002*, *Synechocystis PCC6308* and *Anabaena PCC7120*) were employed to study the concept of latex coatings of cyanobacteria with a porous paper nonwoven substrate. Cyanobacteria cells and binder are suspended in a 50/50 (V/V) homogenous solution of cyanobacterium cells and latex binder. Droplets of cell formulation are put on delimited 3MM chromatography paper. The final 1.54 cm^2 coating is now ready for transfer into the Balch tube bioreactor. It was found out that latex binder coating can increase the specific photoreactivity of suspended cyanobacteria by 5 to 10-fold. Biocoatings allow for the concentration of enzyme and photopigment activity while utilizing biological hosts to build and maintain these biochemical systems. Furthermore, thin biocoatings enable microorganisms survive in latex film production and rehydrated to restore viability (Flickinger *et al.*, 2017). Hence, the purpose of this work is to look into the effect of microalgal organic matter-derived biocoating in cell deposition in natural biofilm development.

1.2 Problem statement

The biology of nongrowing or slowing growing organisms and the reactivity of microorganisms when contact with different surface chemistries are poorly understood (Flickinger *et al.*, 2017). To date, only few studies revolve around the role of biocoatings towards enhanced biomass productivities and works regarding the association between algal organic matter and biofilm formation were still in scarcity.

Thus, this work will generate another fresh insight into the application of algal organic matter derived from marine microalgae, *Navicula incerta* to enhance its cell attachment on commercial microporous polyvinylidene fluoride membrane. The algal organic matter (AOM), namely bEPS, sEPS and IOM extracted from *Navicula sp.* will be used to pre-treat the PVDF membrane prior to a setting up of submerged biofilm cultivation system. This study will eventually look into the biochemical composition changes of the attached microalgae cultivated on both pristine and EPS pre-coated microporous surfaces coupled with the assessment of substrate surface properties. After cultivation, several analyses will be conducted on the samples collected to study the feasibility on the adhesion degree of microalgae on the membranes. Microscopy with alcian blue staining and scanning electron microscope (SEM) will be carried out to determine the existence of transparent exopolymer particles (TEP) on the substrate surfaces and biofilm thickness. The functional groups in the AOM and the quantification of carbohydrate and protein will be carried out by colorimetric characterisation method.

1.3 Objectives

The objectives of this paper are:

i. To study the degree of attachment and growth rate of *Navicula sp.* on PVDF membrane after pre-treatment with AOM.

ii. To study the polysaccharide, protein and hydrophobicity of the sEPS, bEPS and IOM of *Navicula sp.* on pristine and pre-treated PVDF membrane surfaces.

CHAPTER 2

LITERATURE REVIEW

2.1 Microalgal biofilm

Algal biofilms may grow on any surface that receives enough light and moisture. Biofilms contain diverse populations of autotrophic and heterotrophic species. As a result, these biofilms may include a variety of microorganisms, including cyanobacteria, bacteria or protozoa, as well as diatoms (Moreno Osorio *et al.*, 2021). Microalgal biofilms, which are composed of cyanobacteria and/or green microalgae, may be found in almost all photic aquatic settings. Biofilms are differentiated by the fact that they both produce and are functionally governed by energy and chemical product gradients. Algal biofilms are classified into two categories. The first is a biofilm made up of a single species of microalga (axenic culture). For this type of biofilm, the concentrated algal biomass inoculum is adhered on support material which will initiate the attached cultivation. The biofilm diversity remains steady during culture in this situation and no visible contamination occurs. The second type of biofilm is associated to biofouling (Moreno Osorio *et al.*, 2021).

The formation of microalgal biofilm is dependent on the initial adhesion of the microalgal cell to the surface of the substratum. The biofilm begins to expand and develop by consuming the nutrients from soluble algal products (SAPs) and EPS once the initial attachment has occurred. Cellular community attachment can be enhanced by the release of EPS from the microorganisms of the biofilm. EPS are high molecular weight compound composed of polysaccharide, protein, nucleic acids, humic substances, and ionizable functional groups such as carboxylic, phosphoric amino, and hydroxyl groups (Shen *et al.*, 2015). Hydrogen bonding, electrostatic interactions, and

van der Waals forces between functional groups all contribute to the binding strength of EPS found in AOM (Moreno Osorio *et al.*, 2021). EPS give biofilms mechanical stability, facilitates their attachment to surfaces, and forms a cohesive, three-dimensional polymer network that interconnects and immobilises biofilm cells (Shen *et al.*, 2015). Furthermore, the surface physicochemical characteristics and surface properties of the support materials affect the initial adhesion of microalgae (Rosmahadi *et al.*, 2021).

2.2 Attached mircoalgal growth system

Biofilm cultivation methods are classified into three basic groups based on the location of the culture medium and microalgae on the cultivation surface. These cultivation systems are fully submerged, partially submerged and permeated systems. Figure 2.1 below shows the three types of biofilm cultivation systems. The microalgae in the first two categories are immersed in a layer of medium all of the time (constantly submerged systems) or a portion of the time (intermittently submerged systems). For permeated systems, microalgae grow on the porous substrate that provides moisture and nutrients and are exposed to the surrounding gas phase (Berner *et al.*, 2015).

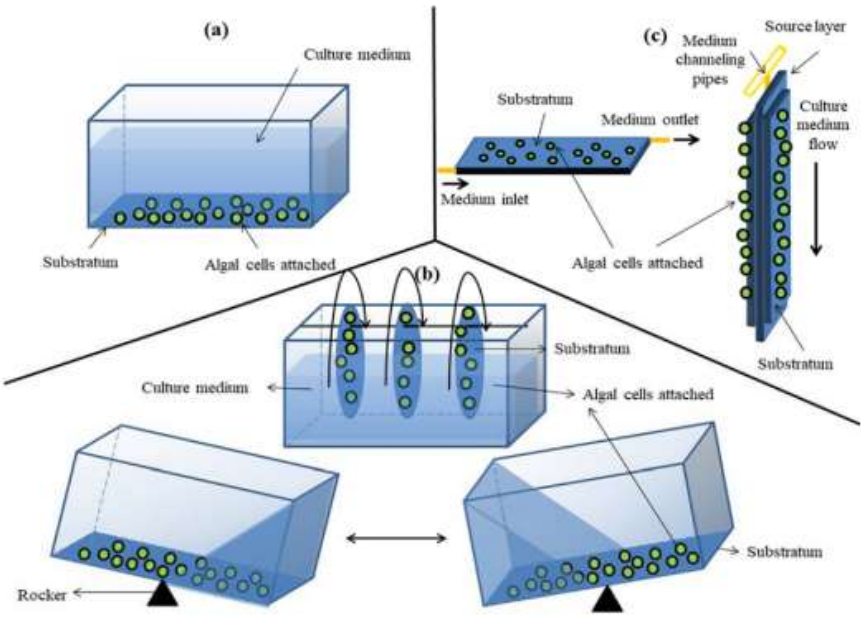

Figure 2.1. Schematic designs of the microalgal biofilm systems: (a) constantly submerged biofilms, (b) partially submerged biofilms and (c) permeated biofilms (Cheah and Chan, 2021)

Martin et al. (2015) reported that high biomass productivity of 46.8 g m^{-2} d^{-1} was obtained using a trough-based revolving algal biofilm system. Furthermore, higher biomass productivity of 50–80 g m^{-2} d^{-1} was achieved when the attached culture of *Scenedesmus obliquus* was carried out outdoors and less water consumption was used, only 7% of the control pond (Shen *et al.*, 2016). A considerable amount of literature has been published on the use of microalgal biofilms for wastewater treatment (Moreno Osorio *et al.*, 2021). However, there is currently a significant lack of large-scale practical uses of microalgal cultivation systems (Sukačová, Vícha and Dušek, 2020). The feasibility of an outdoor pilot-scale biofilm reactor for nutrient removal from municipal wastewater was investigated by Boelee et al. (2014). However the findings showed limited efficacy, with the average phosphorus removal efficiency being just 14 percent (Boelee *et al.*, 2014).

2.3 Microalgae attachment mechanisms

Microalgae attachment typically consists of two steps: initial attachment of microalgal cells to substratum surfaces (adsoption) and the production of EPS results in irreversible attachment. The two stages' primary interactions are cell-substratum and cell-cell interactions (Moreno Osorio *et al.*, 2021).

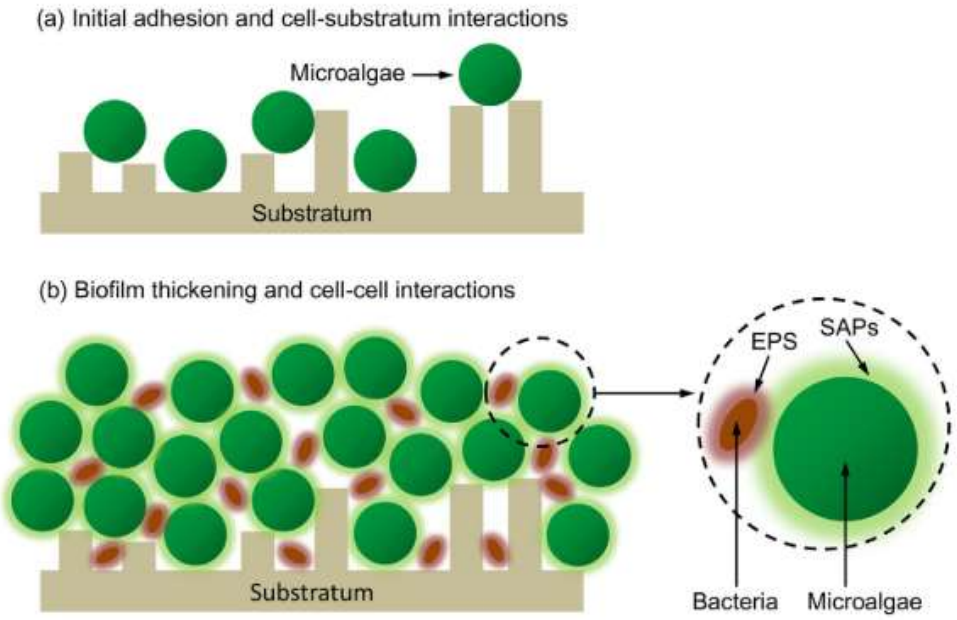

Figure 2.2. Mechanisms of microalgae attachment. (a) Initial attachment; (b) Irreversible attachment (Wang et al., 2018)

2.3.1 Initial cell attachment

The initial adhesion of microalgae cells to the surface of a substrate is primarily governed by cell-substratum interactions depending on the physicochemical properties of the interacting surfaces i.e. hydrophobicity and surface roughness of substratum. Microalgal cells adhere to the surface of substratum by gravitational or hydrodynamic forces. Once the microalgal cells have been retained at the substratum surfaces, cells will secret soluble algal product (SAP), forming of proteins, polysaccharides, and other substances that aid in the irreversible cell attachment to the substratum surfaces. SAP including polysaccharides, proteins, and phospholipids may enhance the

characteristics of substratum surfaces and thereby promote microalgal cell attachment. Furthermore, EPS may act as a storage compartment for nutrients and water required for microalgal growth, as well as protect attached cells from grazers, both of which encourage rapid microalgal initial adhesion (Moreno Osorio *et al.*, 2021; Wang *et al.*, 2018).

2.3.2 Irreversible cell attachment

Irreversible cell attachment involves biofilm thickening through the production of EPS. Microalgal cells multiply by consuming nutrients from the ambient liquid media. Extracellular SAPs and EPS from co-existing bacteria would promote cell attachment and hence contribute in the preservation of a thickening microalgal biofilm's structure. Hydrogen bonding, electrostatic interactions, and van der Waals forces between functional groups all contribute to the binding strength of EPS. This attachment is based on cell-surface interactions which has resulted in numerous adaptation strategies once the coating and the first biofilm layer are developed; for example, surface charge and hydrophobicity of membranes. However, biofilm sloughing will occur when the biofilm becomes thicker as light and nutrients are only available at the surface of the developed biofilm with bottom layer of attached cells lacking in light or nutrition and lose viability. Moreover, turbulence in the liquid medium creates shear stress on attached cells which may cause the cells to detach from microalgal biofilms if the thickness of the attached microalgal biofilm is not properly controlled (Moreno Osorio *et al.*, 2021; Wang *et al.*, 2018).

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2.4 Algal organic matter

Extracellular polymeric substances (EPS) are macromolecular molecules that are released outside of the cell. EPS functions include the production of a gel-like matrix that strengthen biofilm formation, the control of biofilm adhesion to surfaces, infection establishment, and the defense from harmful environmental impacts. Extracellular polysaccharides are key structural constituents of the EPS matrix, regulating the mechanical stability of biofilms via noncovalent interactions between polysaccharide chains or indirectly via multivalent cation bridges. (Salama *et al.*, 2016).

Polysaccharides (40–95%), proteins (PN) (1–60%), microcells (2–5%), and other substances are the main components of EPS. The species, culture age, development phase, and environmental circumstances will change these component proportions (Tong and Derek, 2021). In biological wastewater treatment plant, around 20 % humic compounds could be a significant constituent of EPS in biological wastewater treatment reactor sludge. Furthermore, EPS is also composed of nucleic acids, lipids, uronic acids, and certain inorganic compounds. The extraction procedures and sludge sources will influence their fractions in EPS (Sheng et al., 2010).

Proteins are crucial element of microalgal EPS. EPS matrix is maintained and stabilized by non-enzymatic structural proteins, as well as enzymatic proteins that degrade exopolysaccharides, which can serve as a carbon and energy source for organisms. Furthermore, proteins also involve in hydrophobic bonding inside the EPS matrix. Extracellular proteins in biofilms, on the other hand, serve as enzymes in the surroundings of immobilised cells, digesting foreign macromolecules and particle debris. As a result, they supply low-molecular-weight nutrients that are easily absorbed and processed by cells. Polysaccharide breakdown in EPS may be caused by

enzymes inside the biofilm matrix, resulting in the release of biofilm microorganisms into the environment. (Salama *et al.*, 2016).

2.4.1 sEPS, bEPS and IOM

EPS and intracellular organic matter (IOM) are the specific groups of algal organic matter (AOM). EPS can be further divided in sEPS – secreted into medium and weakly bounded with cells and bEPS- which is bounded with cell. Polysaccharides, proteins, lipids, and nucleic acids are the primary compositions of EPS (Babiak and Krzemińska, 2021). High quantity of phycocyanin was found in the IOM extract solution while the EOM extract contained extracellular metabolites such as polysaccharides, amino acids, enzymes, hormone compounds, and inhibitors. Furthermore, the dissolved organic carbon (DOC) (100.5 mg L^{-1}) and algal toxin MC-LR (506.1 mg L^{-1}) concentrations in IOM extract solution were much greater than in EOM (29.7 mg L^{-1} DOC and 141.2 mg L^{-1} MC-LR). IOM had 3.2mg L^{-1} chlorophyll-a, which accounted for 3.15 percent of the DOC (Li *et al.*, 2012).

Cheah et al. (2021) employed *Navicula incerta*, *Cylindrotheca fusiformis*, *Amphora coffeaeformis* and to evaluate the effect of high temperature on microalgal organic matter and its effects on membrane fouling. The results showed that *A. coffeaeformis* induced the most severe membrane fouling. Among the three diatoms in this investigation, sEPS accounted for the majority of the total AOM (60–70%), thus sEPS had a stronger relationship with membrane fouling than bEPS. Furthermore, IOM was primarily responsible for the protein from AOM released by diatoms at lower temperatures. As the temperature increased, the quantity of polysaccharide in the sEPS and bEPS increased linearly (Cheah *et al.*, 2021).

2.5 Factors affecting EPS synthesis process

2.5.1 Effect of light

Light regime and light are two critical elements affecting microalgal development and metabolic pathways. Light is required for the generation of EPS by microalgal cells. Several literatures reported EPS production can be enhanced by increasing light intensity (Babiak and Krzemińska, 2021). From the works of Liqin et al. (2008), the highest EPS production level was achieved 0.95 g L^{-1} at the light intensity of 80 µ m^{-2} s^{-1} and started to decline after the light saturation point. *Nostoc sp.* produced more EPS at 80 µE m^{-2} s^{-1} (206.20 mg g^{-1} DW) than at 40 µE m^{-2} s^{-1} (155.49 mg g^{-1} DW). According to the authors, the rate of carbon dioxide fixing, nitrate absorption, and metabolism in the cells can be increased by increasing light intensity (Cheah and Chan, 2021).

Attached microalgal culture has distinct light adsorption methods than suspended microalgal growth as the concentrated attached microalgae grow and expand on the substratum. (Rosmahadi *et al.*, 2021). According to Shen et al. (2018), when the light intensity was increased from 700 to 1134 mol m^{-2} s^{-1} at day 3, the maximum attached microalgal lipid production was obtained at 53.62 g m^{-2} on the eighth day of cultivation. However, *Desmodesmus sp.* achieved the maximum biomass production at lower light intensities (700 mol m^{-2} s^{-1}) than suspended cultivation at 750 mol m^{-2} s^{-1} (Rosmahadi *et al.*, 2021). Furthermore, Wang et al. (2015) studied the relationship between the efficiency of light penetration and the nitrogen replete condition. They concluded that the biomass production of *Scenedesmus dimorphus* increased from 8.8 to 107.6 g m^{-2} in 10 days of cultivation when the immobilized cells received the light intensity of 100 µmol m^{-2} s^{-1} (Rosmahadi *et al.*, 2021).

Light intensity has a different influence on EPS synthesis depending on the microalgae and growth circumstances. *Botryococcus braunni CCALA 778*, for example, demonstrated the highest EPS productivity in outdoor cultivation at 0.29 g L^{-1} day^{-1} under 2000 µE m^{-2} s^{-1} (16:8 light-dark cycle) in hot weather, whereas *Porphyridium cruentum* produced 0.095 g L^{-1} day^{-1} of EPS polysaccharide in flat plate photobioreactors at 80 µE m^{-2} s^{-1} (18:6 light-dark cycle) (Cheah and Chan, 2021).

Furthermore, the light wavelength is another component that influences microalgal EPS composition and yield. Blue light and red light were found to promote extracellular polysaccharide synthesis (Babiak and Krzemińska, 2021). In the study of *Porphyridium cruentum*, blue (400–500 nm) and red (600–700 nm) lights efficiently boosted the synthesis of polysaccharide. *Chlorella sp.* biofilm also showed the same results that blue, green, and red lighting, which promoted greater accumulation of polysaccharide than white light (Cheah and Chan, 2021).

2.5.2 Effect of temperature

One of the most critical elements governing metabolic activity in microalgal cells is temperature. Temperature can also influence EPS production, and the ideal temperature for EPS synthesis depends on microalgae species intensity (Babiak and Krzemińska, 2021). At 25°C, *Cylindrotheca closterium*, for example, had collected a total EPS of roughly 15 g mL^{-1}. Furthermore, different microalgal species e.g. *Isochrysis galbana, Skeletonema marinoi, Thalassiosira pseudonana, Pseudonitzschia fraudulenta and I. aff. galbana* produced more sticky exopolysaccharides with increasing temperature then decreased at higher temperatures (Cheah and Chan, 2021).

In the study of Kumar et al. (2017), they investigated *Dictyosphaerium chlorelloides* and found out that the greatest amount of EPS was produced at a moderate temperature (25.7 C) with a light intensity of 50.3 mol m^{-2} s^{-1}. The specific growth rate of *D. chlorelloides* decreased at the ideal temperature for maximum EPS generation.

2.5.3 Effect of nutrient

As the extracellular matrix includes several metabolites generated by algal cells, EPS may be involved in nutrition distribution (Babiak and Krzemińska, 2021). From the study of Arce et al. (2004), the macromolecular specificity of diatom EPS influenced the adhesion of *Navicula sp.* to surfaces with varying physicochemical properties. Diatoms secrete hydrophobic and hydrophilic EPS (Moreno Osorio *et al.*, 2021). When nitrogen (1 mg/L) and silicon (0.01 mg/L) were flushed from the solution, the biofilms sloughed off the substrates and failed to form. In contrast, a non-starved culture of *S. obliquus* biofilm, on the other hand, developed well for 26 days without sloughing. Nitrogen and silicon are important as nitrogen creates critical metabolic components such as amino acids, nucleic acids, and proteins for cell reproduction whereas silicon creates the frustule exoskeleton. Thus the biofilm growth was stopped when the cells were under nitrogen starvation (Cheah and Chan, 2021).

2.5.3.1 Nitrogen

Several studies have revealed that one of the variables that promotes greater EPS generation in microalgal cells is nutritional constraint. The effect of nitrogen constraint on the microalgae *Parachlorella sp.* BX1.5 was studied by Sasaki et al. (2020). With the nitrogen-deficient conditions (BG11 without NaNO3; 2%CO2), EPS was

produced in excess. Furthermore, bEPS from nitrogen and phosphorus-depleted *Chlorella sp.* ADE4 showed larger carbohydrate and protein than control circumstances which had enough phosphorus and nitrogen. Undesirable growth environments, such as low nitrogen-to-phosphorus ratios in wastewater, induce algae to produce EPS to aid in nutrient absorption from the environment, resulting in an increase in the protein proportion of EPS. (Cheah and Chan, 2021).

2.5.3.2 Phosphorus

The effect of phosphorus and nitrogen depletion on the synthesis and molecular composition of extracellular carbohydrates generated in axial batch culture by the diatom Cylindrotheca fusiformis was examined by Magaletti et al. (2004). It was to investigate the effect of phosphate limitation on the growth and EPS production of the diatom *Cylindrotheca fusiformis*. The lack of inorganic phosphorus resulted in a greater release of organic carbon per cell when compared to a nutrient-rich and nitrogen-limited environment. The results show that reduced phosphate concentration affects the increase in total EPS production and the composition of dissolved polysaccharides (Magaletti *et al.*, 2004).

2.5.3.3 Carbon

Carbon in the solution helps to enhance the formation of intracellular and soluble extracellular polysaccharides under mixotrophic and heterotrophic growth conditions. This was supported by Zhang et al. (2019), who found that *Chlorella vulgaris* produces more EPS under mixotrophic condition, i.e., 364.3 mg L^{-1}, compared to 235 mg L^{-1} in autotrophic conditions. Furthermore, as the glucose content in the growth medium was

raised from 0.2 percent to 1 percent (w/v), *Chlorella sp.* produced more EPS (Babiak and Krzemińska, 2021).

2.5.4 Effect of pH

The pH of the culturing medium and the substratum surface have a significant impact on microalgal development and biofilm formation. Microalgae can produce a new overall medium state that differs from the surrounding environment by altering pH during biofilm structuring. (Rosmahadi *et al.,* 2021). According to Ozkan and Berberoglu (2013), when the system was reduced from pH 4 to 2, the energy barrier rose because the reversed shift increased the magnitude of the surface change. To promote the growth of attachment to the substrate, the pH system of this species must be kept at the point of zero charge (PZC), which is 2.9. PZCs can cause electrostatic repulsion or attraction in cell-matrix and cell-cell interactions, affecting microalgae adhesion. It was discovered that depending on the medium conditions, the surface charge and surface potential of microalgae and substrates are formed by the protonation and deprotonation of ionizable functional groups (Rosmahadi *et al.,* 2021).

2.6 Substrate surface properties affecting cell adhesion

2.6.1 Effect of hydrophobicity and hydrophilicity

Hydrophilicity is one of the physicochemical features that can significantly boost the development of attached microalgae in the culture system. The initial adhesion for microalgal attachment is affected by the hydrophobicity and hydrophilicity of the microalgae (Moreno Osorio *et al.,* 2021). The adhesion can be achieved by removing water films on the surfaces. Higher hydrophobicity microalgae have larger adhesion forces (Donlan, 2002). For biofilm growth of green microalgae like *Botryococcus*

sudeticus and *Chlorella* vulgaris on metal and glass, hydrophobic interaction surface is important for initial attachment to increase microalgal cell adherence without any energy barrier (Rosmahadi *et al.*, 2021).

The degree of hydrophobicity and hydrophilicity of microalgae are determined by free energy of cohesion (ΔG_{coh}). A cohesive free energy with a negative sign (-ΔGcoh) indicates hydrophobicity, which occurs when substratum surface–surface contacts are higher than surface–water interactions while cohesion free energy value with a positive sign (+ΔGcoh) indicates hydrophilicity (Rosmahadi *et al.*, 2021). According to Wang et al. (2018), they concluded that hydrophobic substratum such as polyethylene aids microalgal adhesion more than a hydrophilic substratum i.e. glass. This was demonstrated that the microalgae cells developed faster on the hydrophobic substratum rather than the hydrophilic substratum. As a result, using a hydrophobic substrate can boost associated microalgal development, potentially increasing the generation of microalgal bioproducts such as biodiesel.

2.6.2 Effect of roughness

The surface roughness of the substrates influences the degree of cell colonisation on the surfaces. Rougher surfaces, such as pine sawdust, were shown to promote algal cell adhesion, resulting in higher biomass productivity. (Tong and Derek, 2021). From the study of Tong and Derek (2021), biofilm formation of *C. fusiformis* was observed on different substrata i.e. plain drawing paper, polypropylene fabric, commercial polyvinylidene fluoride flat sheet membrane and polyethylene plastic. Polypropylene fabric and plain drawing paper developed stronger biofilm adhesion because of their rougher surfaces. The deposition of microalgae onto the surfaces of substrates and the production of algal biofilm can be enhanced by surface roughness (Zhang *et al.*,

2020a). This can be supported by the study of Zhang et al. (2020) that pine sawdust (0.420 to 0.595 mm) achieved the productivity of algal biofilm 14.75–15.75 g $m^{-2}day^{-1}$. Lignocelluosic materials in pine sawdust and plain drawing paper have various nature micro- patterns which can provide sufficient space for the biofilm growth (Zhang *et al.*, 2020a).

2.6.3 Substrate topography

According to the study of Khoshkhoo et al. (2019), the adhesion of algae to natural rock substrata was investigated using a variety of surface topography characteristics. i.e. areal material ratio (S_{mr}), maximum pit height (S_v) and arithmetical mean of the absolute of the ordinate values (S_a). They discovered greater topographic parameter values of substrata boosted biomass accrual rate. Two level of substratum designs were used to determine the total algal biomass accumulated. The results showed that the total algal biomass accrued for level 2 (1.86 ± 0.40 mg cm^{-2}) was 3.7 times higher than level 1 (0.50 ± 0.16 mg cm^{-2}). The higher average roughness (a greater departure from the mean surface plane) of level 2 can enhance cell entrapment and hydrodynamic characteristics hence increase the rates of early colonization. Furthermore, level 2 with steeper hills and deeper dales on average, which may aid to enhance the possibility of entangling and therefore trapping algae cells in flowing water (Khoshkhoo *et al.*, 2019).

CHAPTER 3

METHODOLOGY

3.1 Flowchart methodology

Figure 3.1 below depicts the flow diagram of research activities.

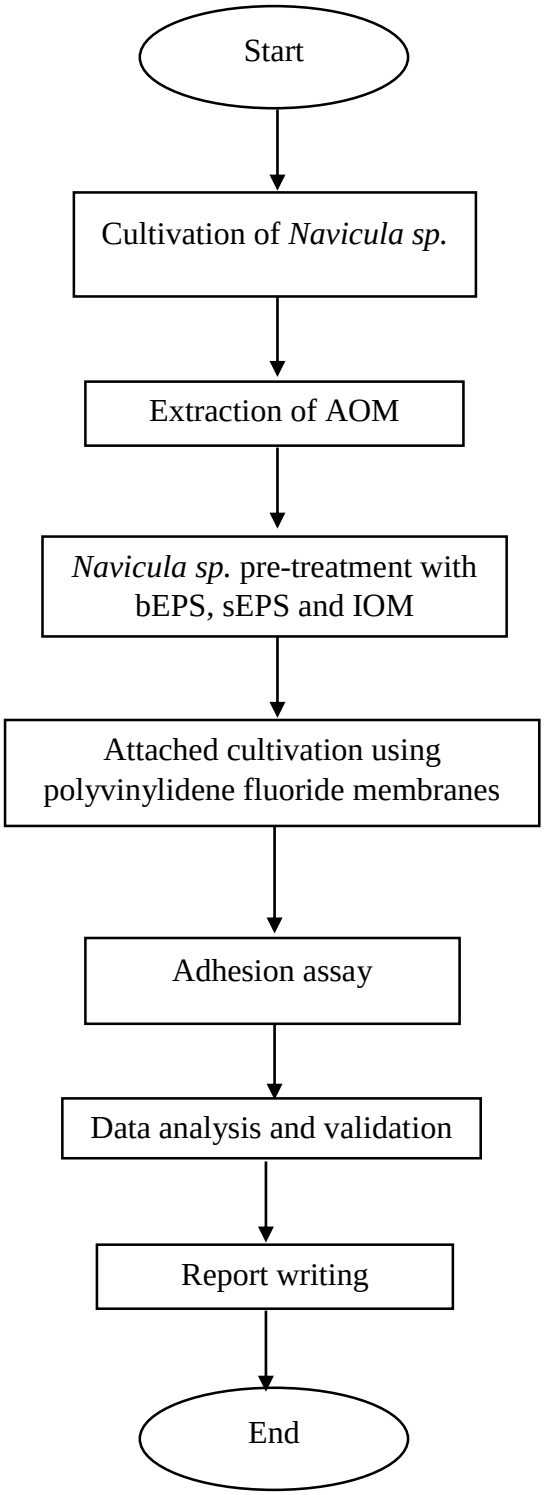

Figure 3.1. Proposed workflow for project.

3.2 Materials and methods

3.2.1 Microalgae strain and cultivation

Navicula sp. (UTEX 2044) was cultivated in sterilised f/2 + Si medium in 1.6 litre batch cultures and autoclaved at 121°C for 15 min. Table 3.1 shows the composition of sterilised f/2 + Si medium. The cultivation medium was incubated at 25 ± 2°C under fluorescent tubes with a 12-hour light-dark photoperiod. Neubauer chamber and a light microsope (BX-51, Olympus, Japan) were used to monitor the average microalgal cell concentration by counting the cells. The microalgae were collected after 21 days post-inoculation. This cultivation was a preliminary step in producing EPS from algae.

Table 3.1. The composition of sterilised f/2 + Si medium

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

3.2.2 Extraction of bEPS, sEPS and IOM

To remove the tightly bound EPS from the cells, the cells were scraped off from the membrane and extracted with 14 ml of 1.5 M NaCl at 30°C for 1 hour. The sEPS

was extracted from the algal suspension in the petri dish. Both obtained samples were centrifuged for 15 minutes and then bEPS and sEPS supernatant were filtered through cellulose acetate membrane filter. To extract IOM, the precipitated cells were washed with salt water and resuspended in 20 ml of distilled water. When cells were subjected to sonication at 37 kHz for 30 minutes, intercellular materials were released. The suspension was centrifuged and the IOM supernatant was collected. Colorimetric detection method was used to evaluate the total carbohydrate and protein of EPS solution.

3.2.3 Pre-treatment of PVDF membrane with AOM

Before pre-treatment, 10% ethanol was used to soak the membrane strips for 15 minutes. In order to remove any contaminants on the surfaces, membrane strips were washed with distilled water and air-dried. The PVDF membrane strips were pre-treated, or coated, by filtering 30 mL of 15 mg L^{-1} sEPS through the membrane strips under -20 kPa and air-drying. The method was repeated 3 times to make sure the AOM was firmly coated on the surface of the membrane strips. The pre-treatment of PVDF membrane was repeated with using bEPS and IOM.

3.2.4 Surface contact angle measurement

LAUDA surface analyzer LSA 200 (LaudaKönigshofen, Germany), a video-based optical contact angle measurement tool was used to automatically compute contact angles between deionised water droplet and membrane surfaces. Prior to culture, pristine and coated PVDF membrane strips were fixed to corresponding glass slides and put on the LSA 200. The images were obtained after 2 to 3 seconds of contact

after 10 L of deionised water was sprayed onto the membrane surface as a single water droplet.

3.2.5 Microalgae cultivation in attached cultivation system

Navicula sp. (UTEX 2044) was cultivated on the coated PVDF membrane surface in the cultivation lane. The cultivation lane was sterilised in laminar flow chamber and the set-up was shown in Figure 3.2. As a control, pristine membrane strips were employed. The coated PVDF membrane and pristine membrane strips were fixed on the bottom of the container with a sample holder and immersed in flowing algal suspension. The medium was circulated at flow rate of 100mL h^{-1}. Triplicate membrane strip samples were collected randomly from different spots of the culture lanes at the 3rd, 6th, 24th, 48th, and 72nd hour. To minimise major biofilm separation from the membrane strips, the samples were carefully extracted using clean forceps.

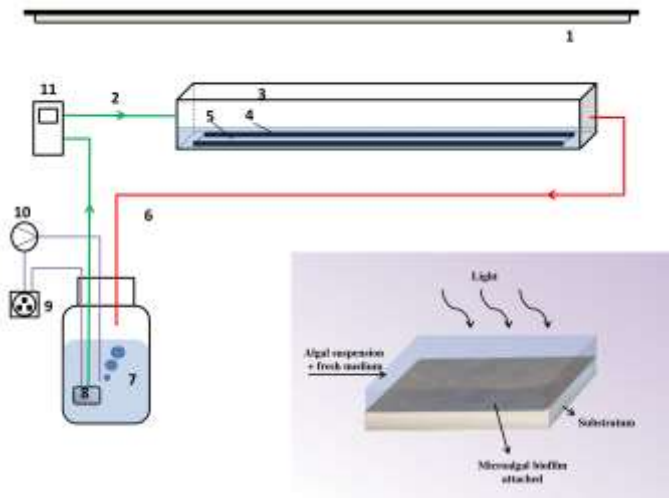

Figure 3.2. Experimental set up for the cultivation lane. (Tong and Derek, 2021)

3.2.6 Determination of the cell density

The algal biomass was scraped off the substrate surfaces using a micro spatula and resuspended in 5 mL of 1.5 M NaCl. To avoid increased cell growth over time, direct

cell counting was performed at least three times on the cell suspension immediately after the sample collection using a Neubauer chamber. EPS extraction was re-performed again as previously described in section 3.2.2, followed by biochemical profile analysis.

3.2.7 Microscopy with alcian blue staining and scanning electron microscope (SEM)

Small sections of pristine and coated PVDF membrane were stained with alcian blue before being examined under a microscope. The purpose of alcian blue staining is to confirm the existence of transparent exopolymer particles (TEP) on the substrate surfaces. Alcian blue 8GX stock was prepared in 0.03 percent acetic acid to a concentration of 0.01 percent. To properly stain the carboxylated transparent exopolymer particles (TEP) and sulphated compounds, alcian blue solution (about 1-1.5mL) was dropped onto the sections to completely cover them. After 10 minutes, distilled water was used to remove the excess dye of the sections before being placed on glass slides. The thickness of the biofilm EPS was observed by SEM (TM3000 Tabletop Microscope, Hitachi, Japan) with 15kV and at least five photos were captured at various locations on the membranes.

3.2.8 Colorimetric characterisation of sEPS, bEPS and IOM

The carbohydrate contents of the sEPS, bEPS and IOM solution were quantified using a previously established phenolsulfuric acid (PSA) technique. The analyses for protein determination were carried out in accordance with the test methods provided by the BCA protein assay kit (Novagen, Merck, Germany), with bovine serum albumin (BSA) serving as the standard. The quantification of carbohydrate and protein

were measured with a UV–Vis spectrophotometer (Cary 60, Agilent Technologies, USA) at wavelength 490 nm and 562 nm respectively.

3.2.9 Hydrophobicity test

The DAX-8 resin column was prepared in accordance with the supplier's (Supelite^{TM}) instructions. Before resin treatment, the pH of the EPS solution was adjusted to 2 with 0.1 M HCl (known as EPS_{before}). Supelite DAX-8 was then used to filter it using a Spin-X centrifuge tube filter (0.45 m cellulose acetate, Corning, USA). All the obtained EPS fraction from the column was collected and named EPS_{after}. Using the procedures described above, the polysaccharide and protein contents of all EPS samples were determined. The following formulae were used to calculate the hydrophobicity of EPS:

$$EPS\ hydrophobicity = \frac{EPS_{before} - EPS_{after}}{EPS_{after}} \times 100\%$$

3.2.10 Statistical analysis

To highlight the potential of data variation, all tests were performed in triplicate, and the average values were provided together with the standard deviation. Error bars in the plots represented the standard deviation between three-category experiments.