

Review

Assuaging Microalgal Harvesting Woes via Attached Growth: A Critical Review to Produce Sustainable Microalgal Feedstock

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Abstract: Third-generation biofuels that are derived from microalgal biomass have gained momentum as a way forward in the sustainable production of biodiesel. Such efforts are propelled by the intention to reduce our dependence on fossil fuels as the primary source of energy. Accordingly, growing microalgal biomass in the form of suspended cultivation has been a conventional technique for the past few decades. To overcome the inevitable harvesting shortcomings arising from the excessive energy and time needed to separate the planktonic microalgal cells from water medium, researchers have started to explore attached microalgal cultivation systems. This cultivation mode permits the ease of harvesting mature microalgal biomass, circumventing the need to employ complex harvesting techniques to single out the cells, and is economically attractive. However, the main bottleneck associated with attached microalgal growth is low biomass production due to the difficulties the microalgal cells have in forming attachment and populating thereafter. In this regard, the current review encompasses the novel techniques adopted to promote attached microalgal growth. The physicochemical effects such as the pH of the culture medium, hydrophobicity, as well as the substratum surface properties and abiotic factors that can determine the fate of exponential growth of attached microalgal cells, are critically reviewed. This review aims to unveil the benefits of an attached microalgal cultivation system as a promising harvesting technique to produce sustainable biodiesel for lasting applications.

Keywords: biodiesel; microalgae; attached growth; suspended growth; support material; harvest

1. Introduction

The growing concerns for the global environmental issues such as global climate change, acid rain, the greenhouse effect, and air pollution have intensified due to the

excessive usage of fossil fuels [1], thus pushing scientists and researchers to explore other alternative energy sources. In fact, the majority of researchers had chosen biofuel production as an alternative energy source to reduce fossil fuel energy dependency. Examples include biomasses attained directly or indirectly, i.e., harvested microalgae or plant materials and animal wastes [2]. Naik et al. [3] reported that only 1.25% of the total production of biomasses worldwide—approximately 100 billion tons per annum of organic dry matter from land-based biomasses and 50 billion tons of aquatic biomasses—had been used as food and daily consumption materials. Thus, the remaining unutilized biomasses can be potentially used as the feedstock for chemical productions as well as energy sources, instead of disposing them to the natural recycling system of Earth. In addition, biofuels not only reduce the dependence on fossil fuels but can also help to lower CO₂ emissions in the environment through the photosynthesis of plants, whereby the CO₂ is used as a source of biomass plant growth [3]. Biofuels can also be divided into two different types, which are primary biofuels and secondary biofuels. In general, the examples of primary biofuels are fuelwoods that have been used in an unprocessed form for heating, cooking, and electricity production. Examples of secondary biofuels are bioethanol, biogas, and biodiesel, which are produced from chemical or biological processes and can be used for powering up vehicles and industrial activities [1]. In conjunction with the secondary biofuels, there are three subcategories of biofuels, which are first, second, and third generation. Each generation of biofuels has different origins and yields different final products. The first generation of biofuels is also known as conventional biofuel which uses food crops such as sugarcane, corn, barley, potato wastes, and vegetable oil as a feedstock for biofuel production [4,5]. However, the most common feedstock that has been used is sugarcane, and Brazil is one of the advanced countries that has been using it as a main energy source. Brazil chooses sugarcane as the main feedstock for biofuels production because of its source availability and the simple process of extracting and converting it into ethanol [4]. The sugarcane is initially crushed in a solvent (water) to remove its sucrose and then it is purified in order to extract the ethanol from the sugar. Ethanol is one of the famous first-generation biofuel products. Lee and Lavoie [4] mentioned in their studies that ethanol can be easily processed through the fermentation of corn sugars, which mostly consist of glucose, by using classical or genetically modified organisms (GMO) yeast strains (*Saccharomyces cerevisiae*). The other advantages of first-generation biofuels are that they are able to blend with petroleum-based fuels and combust in existing internal combustion engines. Naik et al. [3] reported that almost 50 billion liters of first-generation biofuels are produced annually for domestic and industrial usage. Nevertheless, the application of first-generation biofuels contributes to a high demand for food crops since the source is also exploited from foods. The consumption will increase the price of the food crops [3], causing severe food shortages and requiring large land areas to grow the biomass sources [1].

As a solution for previous biofuel generation issues, the second generation of biofuels uses non-food feedstock sources [5], i.e., the low-cost and abundant remaining plant-based biomass wastes such as lignocellulosic materials, while also contributing zero carbon to the environment [3]. Lignocellulosic materials are non-edible sources that can be processed through hydrolysis and fermentation to produce advanced biofuels. Furthermore, oilseeds such as *Jatropha curcas*, high erucic mustard, Indian beech (*Pongamia pinnata*), grass, and aquatic biomass are examples of feedstock that can be used in second-generation biofuel production. Naik et al. [3] reported that *Jatropha* seed kernel consists of 35–40% oil and the total amount of oil that could be produced through the whole-crop biorefinery was 1–1.5 tons per ha. Based on Saladini et al. [5], they proved that rapeseed crops were very sustainable feedstock for second-generation biofuel production, which contributed 40% of renewable energy consumed in Brazil. Moreover, the two common mechanisms for producing second-generation biofuels are thermochemical and biochemical processing. Thermochemical processing is a process that employs thermal decay and chemical reformation in order to convert the biomass from plants into a range of biofuels product with the presence of different concentrations of oxygen [3]. Meanwhile, biochemical processing

is defined as a process that depends on microbial activities to convert the biomass source into biofuel products. However, both mechanisms used for producing second-generation biofuels require ample time and this has been one of the limitations in applying these methods. The mechanisms to convert woody biomass into fermentable sugar not only require plenty of time but are also very expensive [1]. The existence of these limitations in second-generation biofuels has led researchers to continue studies on the application of third-generation biofuels [2].

Third-generation biofuels are defined as biofuels that are produced from the microalgal biomass that has a rapid growth rate, serving as a viable alternative energy resource [1] compared with the classical lignocellulosic biomass [4]. This generation can also reduce CO₂ emissions, the greenhouse effect, and global warming. Dragone et al. [1] reported that microalgae could produce 15–300 times more oil than the traditional crops used for biodiesel production. Moreover, microalgae can be harvested within a short period of time (1–10 days depending on the species and cultivation process adopted), which increases the yield for biofuel production. In the case of terrestrial crop plants, they can only be harvested once or twice a year upon reaching maturity [1]. Microalgae are single-cell microscopic organisms that can be found in fresh water as well as in marine environments and have more than 300,000 species that are larger than terrestrial plants [2]. They grow in a nutrient medium with sufficient light and carbon dioxide sources. The microalgal biofilm also can be formed over wastewater flows as a medium culture [6] due to the presence of bacteria and nutrients such as nitrogen and phosphorus that promote microalgal growth [7]. Guzzon et al. [7] mentioned in their studies that complex interactions between microalgae and bacteria in wastewater create a sense of photosynthesis, as the microalgae are producing oxygen for the heterotrophic bacteria in order to minimize the chemical oxygen demand [7]. Meanwhile, the bacteria provide carbon dioxide and mineral nutrients from respiration with the organic matter degradation, which can be changed into biomass when the microalgae are visible to the source of light [7]. Therefore, microalgae can be classified as oxygenic phototrophs such as diatoms and cyanobacteria, which exhibit energy and reduce carbon dioxide in order to produce the organic substrates (biomass) and oxygen [8]. Biofuels that can be produced from microalgal biomass after going through processing procedures are methane, biodiesel, and bio-hydrogen [2]. In general, lipids in the microalgae are vital to produce high-value biofuel, which is biodiesel. The lipid content of different microalgae species is not similar as the composition of the major chemical components such as lipids, proteins, and carbohydrates vary for different microalgal cells [9,10]. Table 1 shows the compositions of different microalgae species [9]. Ananthi et al. [11] reported that microalgae growing in optimum conditions could accumulate 50–70% of lipids that could be later converted into biodiesel. Moreover, *Chlorella vulgaris* is the most employed microalgal species for producing biodiesel due to its high lipid content which is 60–70% and high productivity which is 7.4 g L⁻¹ [4]. Alam et al. [2] stated that microalgae cultivation for third-generation biofuels could produce 60,000–240,000 liters per year of biofuels, or 360–1500 barrels per annum. However, the microalgal biomass harvesting methods for the suspended microalgal cultivation require a high amount of energy to separate the water medium from mature cells before proceeding to the biofuel procedures [4]. Furthermore, the methods that are often used for microalgae harvesting such as centrifugation and flocculants not only require more energy to perform but also lead to cell damage [12] and contamination of the harvested biomass [13]. This is where attached microalgal cultivation becomes the solution for all the issues associated with suspended cultivation to produce third-generation biofuels. Attached microalgal cultivation is developed by introducing substratum into the medium to let the microalgae grow onto the surface of the substratum [4], thereby facilitating the harvesting process once the cultivation reaches maturity. This method of cultivation can reduce the usage of energy during harvesting, reducing the cost of biodiesel production [4]. Furthermore, based on the three generations of biofuels, biodiesel is the only biofuel being produced at the industrial scale due to its high demand [4]. The process of producing biofuel is different from ethanol since it can be categorized as a chemical

process. Even though the process of biofuel production still uses biomass as the feedstock, the process itself involves extracting the oils and converting them into biodiesel through transesterification [4]. In fact, biodiesel is the best alternative to replace petroleum diesel and can be the main alternative in the decarbonization of the transportation sector by 80% of the total biofuel production [14]. In the second generation of biofuels, cooking oil waste has produced advanced biodiesel which has good quality and low production costs [14]. Meanwhile, among the third-generation biofuels, microalgae-based biodiesel production has been more promising than others since it only requires a short period of time and low energy for harvesting attached growth microalgae, whilst producing a high yield of biomass with a good quality of neutral lipids for high-value biodiesel production [11].

Table 1. The compositions of different microalgae species.

Microalgae	Lipid (%)	Protein (%)	Carbohydrate (%)	Reference
<i>Botryococcus braunii</i>	33–86	4–40	20	[15]
<i>Chlamydomonas reinhardtii</i>	18–22	46–48	17	[16]
<i>Chlorella ellipsoidea</i>	10–30	34–35	24–51	[17]
<i>Chlorella pyrenoidosa</i>	8–35	31–47	20–57	[17]
<i>Chlorella vulgaris</i>	10–50	29–58	12–17	[18]
<i>Spirulina platensis</i>	30	38	24	[19]
<i>Dunaliella tertiolecta</i>	3–13	26–61	22	[20]
<i>Euglena gracilis</i>	11	29	32	[21]
<i>Scenedesmus obliquus</i>	18–52	34–41	22–24	[22–24]
<i>Tetraselmis suecica</i>	5–17	37–92	5–24	[25]
<i>Nannochloropsis</i> sp.	15–30	27–43	10–36	[26–30]
<i>Nannochloropsis oceanica</i>	31	15	8	[31,32]
<i>Nannochloropsis granulata</i>	29	46	15	[33]
<i>Haematococcus pluvialis</i>	20–25	21–45	15–74	[34,35]
<i>Nannochloropsis salina</i>	6–26	18–36	18–36	[36]
<i>Nannochloropsis gaditana</i>	17	47	22	[37,38]

2. Suspended Microalgal Cultivation

A suspended microalgal system is the conventional cultivation that has been developed for years, whereby the microalgae flow freely in the culturing medium and the overall solid content in the cultivation system is less than 1% [39,40]. This system can be set up in open ponds and closed photobioreactors [41], which are designed to keep the microalgae in the suspension form [42]. Pal et al. [43] reported that microalgae cultivation in an open system condition such as an open pond is preferable to a closed-system. Open system benefits from utilizing the sunlight and atmospheric air as the surviving source for the microalgae, has cost-effective installation and operation, low energy consumption, and it is not necessary to build an external cooling system for the microalgae cultivation [43]. Indeed, the negative charge on the microalgal cells' surface helps the microalgae be suspended in the culture medium and avoids cell aggregation [44]. The suspended microalgal cells receive energy mainly from the light as well as from external nutrients such as carbon sources in the absence of light [45]. Usually, carbohydrates play role as the external carbon source that can be assimilated by microalgae, influencing the growth of microalgae at different productivities [46]. Studies have been conducted to prove that the usage of glycerol and sodium bicarbonate could increase the productivity of lipid content in the microalgae. On another note, the suspended microalgal growth system has high chlorophyll content, leading to a high level of oxygen released during photosynthesis [40], especially when wastewater is used as the medium culture. The presence of CO₂ from bacteria can be a great source of carbon dioxide for microalgal growth and promote the photosynthesis rate to produce more biomass and oxygen [47]. This will create a mixed consortia of microalgae and bacteria through the synergistic gas exchange between microalgae and bacteria; hence, this system might lower the costs related to aeration in conventional wastewater processes [47]. However, in some cases when the medium culture is not wastewater because of the possibility

of microalgal growth inhibition by contaminants such as heavy metals [48], insufficient supply of carbon dioxide may occur due to the high cost of commercial CO₂ sources [49]. This has reduced the microalgal densities in the system, influencing the productivity of microalgal lipids and biomass. As reported by Davis et al. [50], the density of microalgal could be as low as 0.5 g L⁻¹ in open ponds and 2.6 g L⁻¹ in photobioreactors. The low concentration of microalgae biomass demands more energy for intensive dewatering and biomass harvesting prior to microalgal bioproduct extraction [51]. Katarzyna et al. [39] found that the water content took 99% of culture volume and left another 1% of microalgal biomass for biofuel feedstock.

Recent studies proposed that suspended microalgae can utilize waste organic carbons, replacing the CO₂ carbon source to reduce the cost [52,53]. The organic carbon sources for growing suspended microalgae are often extracted in the liquid form before administering them into the culture medium [54]. This alternative can also help to reduce costs of disposing the waste organic products and mitigate environmental pollution. While carbon sources are of importance to microalgae production due to the various carbon metabolic pathways in microalgae, other macronutrients such as nitrogen, phosphorus, and sulfur are also essential in influencing the starch and lipid accumulation metabolism in microalgae [55]. Thus, combined macronutrient manipulation strategies such as nutrient balancing and starvation techniques are often employed to achieve optimized microalgae biofuel production [56]. A discovery of waste organic products from the edible extracts can improve the microalgal biomass productivity and the lipid accumulation rates, contributing to the development of high value-added products derived from microalgal biomass such as biodiesel, bioethanol, biogas, and biohydrogen [56]. To obtain a good quality of bio-products such as biodiesel, huge amounts of biomass need to be harvested, which is difficult to produce in the suspended cultivation system. Moreover, the high cost of carbon dioxide sources, harvesting via flocculation and centrifugation [57], and dewatering of suspended cultivation also contribute to the high cultivation production costs. The harvesting operations are expensive due to high water content [42] and low concentration of the harvested biomass [57], making the harvesting processes more difficult and requiring intensive energy [58]. Gross and colleagues [42] found in their research that harvesting and dewatering accounted for as much as 30% of the total production cost. Gross et al. [59] also reported that the suspended cultivation of microalgae in an open pond system could consume up to 21% of the total cost of biomass harvesting. Therefore, studies by Rinanti et al. [60] proposed to add microalgae that can easily sediment in the suspension culture and facilitate the harvesting method. For instance, *Scenedesmus obliquus* is one of the microalgae species that has been proven to increase the harvesting rate of the biomass microalgae and act as a bio-flocculant that overcomes the constraints that may occur during the flocculating process [60]. The addition of auto-flocculant microalgae such as *Scenedesmus obliquus* has the potential to influence the sedimentation rate faster than the sedimentation rate of non-flocculant microalgae in order to make the harvesting more effective [60]. However, other harvesting processes such as centrifugation can become a factor in microalgae cell damage. Despite the fact that centrifugation is widely used for research and small-scale operations due to its high reliability and efficiency, Molina-Miras et al. [61] and Xu et al. [62] have proven in their studies that damage to microalgae cells occurs because of the high shear rates and high centrifugal forces between the cells during the harvesting process. The conditions needed to reach complete microalgae cell separation from biomass tend to damage the cells [12]. The cell damage occurs in relation to the hydro dynamic shear forces, the velocity gradients, relative cell-fluid movement during the settlement, and the compressive centrifugal forces to which microalgae cells are subjected in the pellet [12]. Therefore, two significance parameters that determine the cell survival in the centrifugation process are how long the microalgae cells stay in the pellet and the G-force applied in the process [12]. The cell damage happened when the microalgae cells remained in the pellet for a longer time with the compressive forces applied [63,64]. On the other hand, in microalgae harvesting operations through flocculants, it is possible to contaminate the

harvested biomass as well as the processed water [65–67]. Coagulation–flocculation may reach a separation efficiency of 86–100% by utilizing the aluminum sulphate with the concentration from 20 to 180 mg Al L⁻¹ [68,69] or ferric chloride with the concentration between 140–400 mg Fe L⁻¹ [69,70]. However, the inevitable release of the chemicals flowing through the processed water is the limitation, causing the harvested biomass to be contaminated as well as inhibiting photosynthesis and microalgae growth due to the residual chemicals [65–67]. In fact, the harvesting process will reduce the possibility of the water recycling to be used as the growth medium and its direct release into the environment [65,66]. Moreover, Leong et al. [71] proposed in their studies that a single processing unit involving the harvesting, cell disruption, and extraction of microalgae products based on Liquid Biphasic systems (LBs) are quite promising, especially for the microalgae bio-refinement and improving the techno-economic feasibility of microalgae cultivation. Liquid Biphasic systems conduct a gentle working environment for the target microalgae products' recovery and can extract the products of interest without damaging the microalgae cell and chemical elements [72,73]. Figure 1 [71] is a schematic diagram of Liquid Biphasic systems distributing the substances and the phase separation. However, these systems still require depth-studies and established research to improve the current suspended microalgae cultivation.

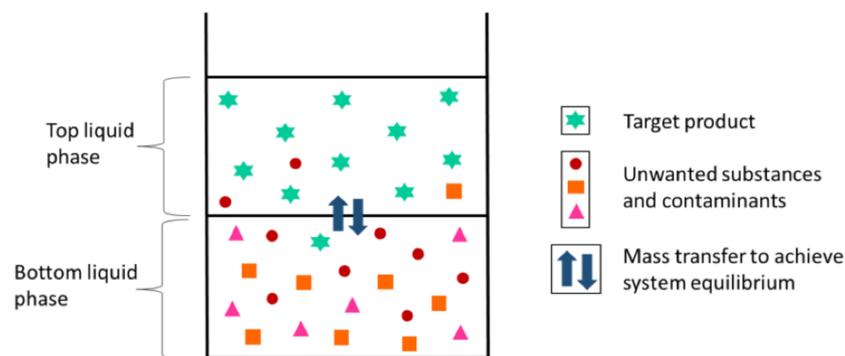


Figure 1. Schematic diagram showing a selective distribution of substances (product of interest, unwanted substances, and contaminants) and phase separation in LBs [71].

3. Attached Microalgal Cultivation

Attached microalgal cultivation is one of the alternatives to overcome the limitation of suspended cultivation [39], especially in terms of operational cost and ease of the harvesting process. A substratum is introduced into the cultivation medium and microalgal biofilm forms on the surface of the substratum [74]. The attached system produces higher solid content of biomass than the suspended system. The mature microalgal biomass can be harvested easily by using a simple mechanical scraping method [51]. The high concentration of biomass in the attached system can be achieved with total solid content between 12–16% (90–150 g L⁻¹) [51,75], which is more than the suspended cultivation system (0.5–4 g L⁻¹) [76]. In some studies, it is reported that *Botryococcus braunii* generated higher biomass concentration of 96.4 g L⁻¹ with 0.71 gm⁻² day⁻¹ productivity in the attached system than the suspended cultivation, which produced 1.02 g L⁻¹ of biomass with the productivity of 25 gm⁻² day⁻¹ [75,77,78]. Johnson and Wen [79] also mentioned that *Chlorella* sp. in an attached microalgae system generated 0.34 g of total biomass produced, more than the suspended system, which generated 0.25 g. The biomass production of other microalgae species is summarized in Table 2 with different attachment materials. The high concentrated biomass also helps to reduce dewatering process. Moreover, Gross [42] mentioned that the attached system achieved huge amount of biomass with high carbohydrate content, but low lipid content as compared with the suspended system. Ozkan et al. [75] mentioned in their studies that *Botryococcus braunii* in the attached cultivation system produced lower lipid content (9.81%) than the suspended growth system (29.6%). This may be due to the inhibition of microalgae lipid or fatty acid

synthesis when high nitrogen presented in the medium culture especially when using manure wastewater [78]. Lipids are important feedstock to produce biofuels, as they have high energy density and are easily converted into biodiesel. Thus, some studies proposed attached cultivation to create a mixotrophic metabolic pathway (using light and waste organic products as carbon source) for microalgae growth since it could produce a high density of microalgal biomass and a high accumulation rate of lipids for later biodiesel production [42]. The synergistic effect of light and organic carbon may be the reason why the mixotrophic pathway has higher productivity than other metabolic growth pathways [80]. The two energy sources in the mixotrophic mode, which are light and organic carbons, are simultaneously exploited and eventually enhance the biomass and lipid productivity under optimum conditions [16,17,81]. Smith et al. [82] and Grama et al. [83] recently reported that mixotrophic cultivation can possibly minimize the gas/liquid exchange process because the O₂ required by aerobic heterotrophic growth can be generated by oxygen from photosynthesis. Meanwhile, the required CO₂ to perform photosynthesis will be covered by the heterotrophic metabolism which will promote the biomass production on substrate [84], practically making the process carbon neutral [81]. Recent studies show that mixotrophic growth can potentially reach 94% of substrate converted to biomass [81]. Marquez et al. [85] also stated in their studies that *Spirulina platensis* generated 1.5 times more biomass in mixotrophic growth than photoautotrophic growth. Moreover, higher lipid productivity of neutral lipids was obtained in the stress phase of the mixotrophic-mixotrophic mode, 284 g/kg (52%), and the mixotrophic-heterotrophic mode, 154.3 g/kg (58%) [86], more than the heterotrophic mode, which is 48% of lipid content [87]. This results from the external carbon source that is supplemented even during the stress phase such as the absence of light intensity or nitrogen starvation that influences more generation of unsaturated fatty acid methyl (FAME) and neutral lipid in the mixotrophic mode [86]. When microalgae are under nitrogen starvation, the limited endogenous nitrogen present in the microalgal cells is drastically depleted negatively, promoting protein involvement in the starch biosynthesis and its accumulation [88]. Thus, this limitation of low starch content during the stress phase will be overcome by the supplemented carbon source to increase starch content that will stimulate its conversion to lipids [86]. Fan et al. [89] reported that lipid accumulation in microalgal cells under nitrogen starvation is increasing simultaneously along with the glucose concentration. Therefore, all the high-cost operations such as expensive concentrating, energy consumption, and dewatering from suspended cultivation are not required in harvesting the attached microalgal biomass [57]. These matters reduce the energy consumption up to 99.7% as opposed to the suspended growth system [75,90,91].

Frequent harvesting also helps to reduce the light limitation, enhance the carbon dioxide mass transfer features [59], extend biomass retention time, and enhance water utilization [41]. The frequent harvesting of attached microalgal biomass can still obtain high solid content biomass as compared with suspension system; hence, frequent harvesting gives the dilute algal density a high amount of water and nutrients to thrive [41]. This frequent harvesting is also helping to overcome the limitation of CO₂ molecules dissolved into the biofilm surface since the CO₂ molecules need to go through interior layers of the biofilms [41]. As the attached microalgal growth conditions are different from suspended cultivation, the physiological properties of attached cultivation are also not similar to the suspended system. The formation of attached cultivation involves two steps, (1) initial adhesion of microalgal cells onto substratum surface and (2) thickening of formed microalgal biofilm, and the main interactions within the steps are cell–substratum and cell–cell interactions [51]. Microalgae cells are introduced onto the surface of substratum by gravitational or hydrodynamic forces in the initial adhesion with various mechanisms of cell attachment to a substrate such as hydrophobic interactions, surface energy, and acid–base interactions [41]. Hydrophobic microalgal cells may form the biofilm better than hydrophilic species [92] and good surface energy between the microalgae and surface substratum may lead to high cell attachment [59]. For the acid–base interaction, the microalgal species determines whether it is suitable to grow in acidic, neutral, or alkaline conditions

to attain optimum growth, which is a dominant mechanism for microalgal attachment. The variation in the normalized net photosynthesis rate with pH can be fitted to the cardinal model development for microalgae, which will determine the microalgae growth with the optimum pH [93,94]. The cardinal model is defined as a simple function such as net photosynthesis depending on a maximum, a minimum, and optimum value, with different values of the variables such as pH and temperature that only can be found in between the maximum and minimum tolerable values [94,95]. Interestingly, Ippoliti et al. [95] projected in their studies that temperature and pH demonstrated similar behavior when the net photosynthesis rate is zero under 12 °C and pH is 3, rising to a maximum value with 36 °C and pH 7.5, but decreasing to zero when it reached maximal values of 45 °C and pH 10. The optimum temperature to obtain the highest photosynthesis rate was around 36 °C. As for the pH parameter, the tolerance varied for every strain [95]. Most microalgae are reported to have optimum growth at pH values ranging from 7.0 (neutral) to 8.0 [96], but others such as the blue green microalgae, namely *Spirulina platensis*, require extremely high pH (alkaline) between 8.5–10 to produce high biomass yields [95,97]. In addition, the net photosynthesis rate is maximum at a dissolved oxygen concentration from zero to 11 mg L⁻¹, but then exponentially reduced to zero at 20 mg L⁻¹ because of the oxygen inhibition [95].

Table 2. Biomass production of different microalgae species and attachment materials in attached cultivation.

Algal Species	Attachment Material	Biomass (gm ⁻²)	Productivity (gm ⁻² day ⁻¹)	Reference
<i>Botryococcus braunii</i>	Concrete	25	0.71	[75]
<i>Chlorella</i> sp.	Polystyrene	25.65	2.59	[79]
<i>Scenedesmus obliquus</i>	Glass	29.4	2.10	[98]
<i>Nitzschia palea</i>	Glass	39.2	2.80	[98]
<i>Scenedesmus obliquus</i> , <i>Chlorella vulgaris</i> , <i>Coccomyxa</i> sp., <i>Nannochloris</i> sp., <i>Nitzschia palea</i> , <i>Oocystis</i> sp., <i>Oocystis polymorpha</i>	Polycarbonate	1.58	1.25	[99]
<i>Isochrysis</i> sp.	Printing paper	10	0.6	[100]
<i>Tetraselmis suecica</i>	Printing paper	15	1.5	[100]
<i>Phaeodactylum</i> <i>tricornutum</i>	Printing paper	12.4	1.8	[100]
<i>Chlorella vulgaris</i>	Cotton duct	25	3.51	[101]
<i>Scenedesmus obliquus</i>	Filter paper	10.6–83.7	1.33–10.46	[102]
<i>Botryococcus braunii</i>	Cellulose acetate	10–51	1–6.45	[103]

Soluble algal products (SAPs) and extracellular polymeric substances (EPS) are derived from biochemical reactions that occur within cells to generate protein and help bind the microalgal cell onto the substratum surface for the initial adhesion process, as shown in Figure 2 [51]. When the initial adhesion has successfully occurred, the formation of microalgal biofilm starts to thicken by utilizing the nutrients from extracellular SAPs and EPS. Microalgal cells proliferate, improving the biofilm strength [39]. Wang et al. [51] mentioned that microalgal biofilm could be thickened from 22 to 2 µm. In addition, the initial adhesion of microalgae is also influenced by the surface physicochemical properties and surface textures of the materials. The substratum with suitable surface texture can act as a shelter to the attachment for microalgal cells, preventing the sloughing of attached cells from happening [59,92]. Years ago, there was a study conducted on a smooth material as the substratum to attach the microalgae and the results showed that the smooth material did not have a high attachment rate of microalgae, while rough surfaces could enhance the microalgal attachment [59]. Attached cultivation can be categorized based on the substratum orientations in Figure 3 [51], which are horizontal, vertical, rotating, and radial.

Substratum in horizontal systems incline less than 10 degrees to allow the medium to flow through the whole microalgal biofilm [75] and the area of the microalgal attachment is very small. Meanwhile, vertical systems arrange the substratum vertically and maximize the area for the microalgal attachment. In rotating systems, on the other hand, the attachment cells of microalgae have limited access to the liquid culturing medium and are mostly exposed to the atmosphere to undergo the gas exchange process [104]. Radial systems, also known as “suspended-attached systems”, have small-sized substratum submerged in the cultivation medium for both suspended and attached cultivation [104,105].

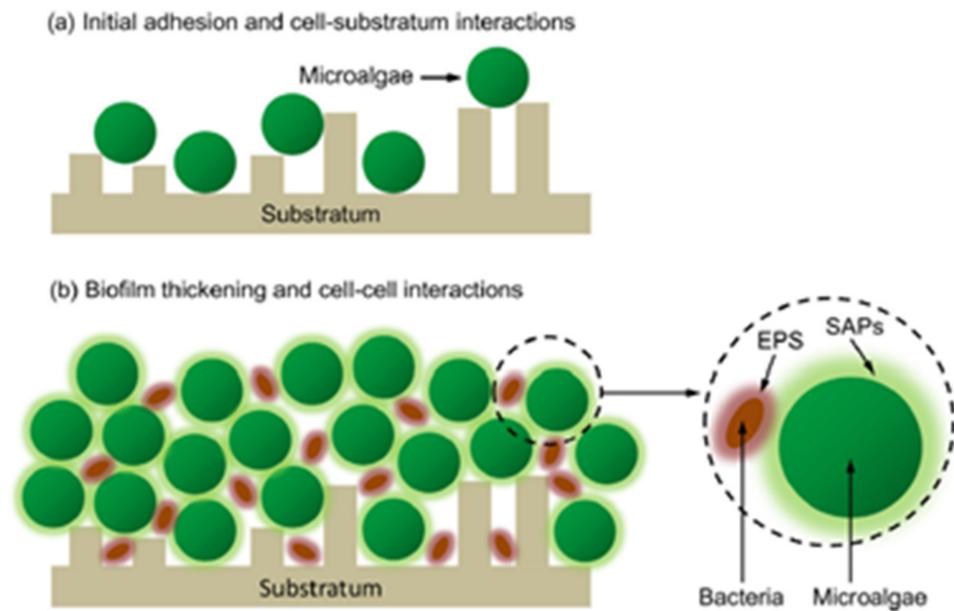


Figure 2. Mechanisms of microalgal attachment: (a) initial adhesion and (b) biofilm thickening [51].

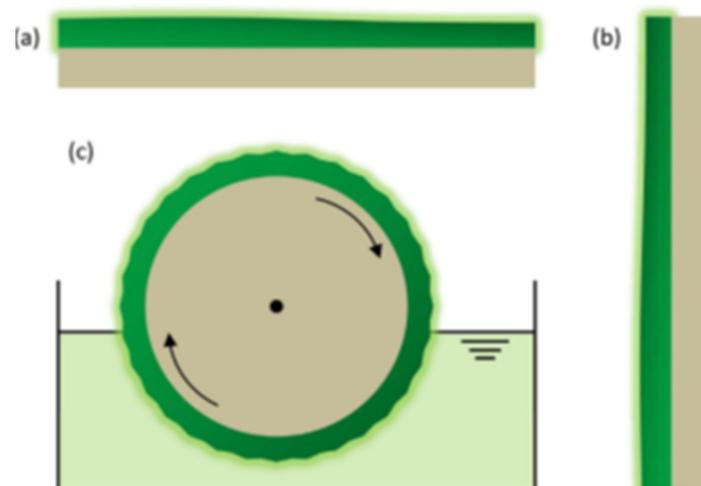


Figure 3. Cont.

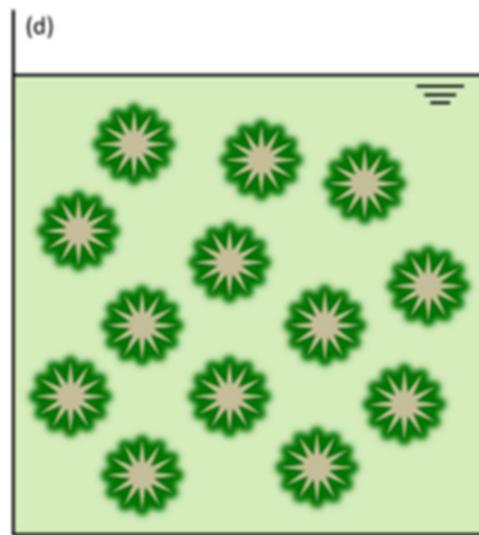


Figure 3. Classification of attached microalgae systems based on substratum orientation. (a) Horizontal system; (b) vertical system; (c) rotating system; (d) radial (suspended-attached) system. Light brown color indicates attachment substratum; green color indicates attached microalgal biofilm [51].

4. Effect of pH on Attached Microalgal Growth

The pH of the cultivation medium [51] and the substratum surface [92] play an essential role in influencing microalgal growth and biofilm establishment [39]. Microalgae can also create a new whole medium condition that is different from the surrounding by changing the microalgal layer pH during biofilm structuring [39]. In attached growth systems, the pH of the cultivation medium affects the biofilm structuring even more than the nutrients. Indeed, both cultivation systems, attached and suspended, have the same effect from pH. Rosli et al. [96] reported that *Chlorella vulgaris* had the highest dry weight biomass in a cultivation medium of pH 6 for both attachment and suspension systems. Therefore, different species of microalgae can adapt in different pH levels of cultivation mediums and substratum surfaces. Most microalgae species can adapt in neutral and alkaline conditions for optimum growth [106]; only certain species prefer to grow in acidic conditions [96]. For instance, *Nitzschia* had the highest growth rate in neutral pH medium conditions [39], while green microalgal species such as *Dunaliella* thrive well in an acidic cultivation medium of pH 1 [106].

Moreover, Ozkan and Berberoglu [92] confirmed that the effect of pH on microalgal growth and biofilm establishment have a relationship with the cell–substrata and cell–cell interactions, specifically for attached cultivation systems. The presence of ionizable functional groups such as hydroxyl (-OH), carboxyl (-COOH), and amine (-NH₂) groups could be protonated or deprotonated based on medium conditions [92]. In addition, this matter created surface charge as well as surface potential for microalgae and substrata. Therefore, the amine and carboxyl groups on the microalgal surface were protonated at low pH, and the -COOH and -NH₃⁺ created a net positive surface charge throughout the microalgae cells [92]. Meanwhile, those functional groups were deprotonated at high pH and they created a net negative surface charge through the presence of -COO⁻ and -NH₂ on the microalgal surface [92,107]. However, the carboxyl groups would undergo deprotonation (-COO⁻) while the amine groups underwent protonation (-NH₃⁺) in the intermediate pH condition, neutralizing the surface charge of microalgae, known as the point of zero charge (PZC) [92]. Indeed, the PZC could create electrostatic repulsion or attraction for the cell to substratum and cell to cell interactions that influenced the microalgae attachment. A study conducted by Ozkan and Berberoglu [92] proved that the electrostatic energy barrier between cells and substrata between *Chlorella vulgaris* and glass reduced from 2.8×10^3 to 4.0×10^2 kT when the pH environment reduced from pH 8 to

4. However, the energy barrier increased when the pH system decreased from pH 4 to 2 because of the reversal change that increased the magnitude of surface charge [92]. Hence, the pH system of this species must be maintained close to PZC, which was 2.9, to enhance the attachment growth on the substratum [92]. Zhuang et al. [74] reported that when the negative surface charge of the microalgae was weakened at low pH, i.e., pH 6 and below, the cell–cell interaction between microalgae increased and they excreted EPS to protect from extreme pH conditions. The species of *Nitzschia amphibia* on the glass substrata had a higher attachment growth rate at pH 9 than pH 7, even though both were in the same alkaline conditions [108].

In addition, an appropriate pH condition for microalgae not only increases the microalgal growth and biomass production of green microalgae in continuous cultivation [46], but it also reduces the total amount of structural lipids [96]. A high amount of lipid content was significant in order to enhance the productivity of biodiesel production. The use of pH adjustment in microalgal cultivation is to harvest maximum lipid content from the microalgae. In Rosli et al.'s [96] studies, petroleum ether had been used to harvest the non-polar lipid from microalgae and the results showed the lipid content increased in pH 2 to 6, but reduction occurred when the pH increased from 7 to 9. The reason of the lipid content reduction when pH was greater than 6 was because of the mechanism of internal pH regulation inside the microalgae cells [96]. Rosli et al. [96] also proved that in alkaline conditions, *Chlorella vulgaris* could attain the highest weight of attached microalgal biomass due to high microalgal growth, but a low lipid content was extracted. Thus, they concluded that pH 6 was the most preferable cultivation medium for both suspended and attached growths of *Chlorella vulgaris* with the highest lipid content [96]. On the industry scale, when the flue gases are used to cultivate microalgae, the selected microalgal species must be able to adapt the inconsistency of CO₂ concentration in the flue gases that can change the pH environment [106]. However, other studies mentioned that the CO₂ was commonly used in the culture system to maintain a constant pH [42,109]. This could happen, as when CO₂ was absorbed into a culture medium, it was converted into carbonic acid, thereby helping to lower the pH in the system. The pH increased when the microalgae utilized the carbonic acid at higher rate, and the CO₂ levels must be effectively controlled in order to maintain the pH at a steady level [42].

5. Effect of Hydrophobicity and Hydrophilicity on Attached Microalgae Growth

The hydrophobicity and hydrophilicity of microalgae and substratum are also part of the physico-chemical properties that can significantly increase the growth of attached microalgae in the cultivation system. In many studies, the hydrophobicity and hydrophilicity are measured in categorizing the species of microalgae from the similarity of surface interactions at various pH and ionic strengths [59,92,99]. Ozkan and Berberoglu [92] reported that the hydrophobic microalgae could form biofilm faster than hydrophilic microalgae. Meanwhile, Genin et al. [99] stated in their studies that the hydrophobic substratum surface could initiate the primary adhesion of microalgal cells; conversely, the hydrophilic substratum surface could strengthen the microalgal attachment. Hence, the degrees of hydrophobicity and hydrophilicity for both microalgae species and type of media attachment are very important in enhancing the attached microalgal growth. Furthermore, to narrow the effect of hydrophobicity and hydrophilicity on microalgae and substratum, these properties play a consequential role in initial microalgal colonization and adhesion [75], especially for the attached microalgal cultivation system [59]. Biofilm growth of *Chlorella vulgaris* on metals and glass was not affected by the water–material contact angle between the microalgae and substratum [110], but it showed a positive reaction towards the hydrophobicity of the microalgae through the initial attachment of *Chlorella vulgaris* [108]. M. Gross et al. [59] also mentioned in their study that one of the important factors that affected the microalgal attachment was hydrophobic interaction. For green microalgae such as *Botryococcus sudeticus* and *Chlorella vulgaris*, it was significant to have at least one hydrophobic interacting surface for initial adhesion to strengthen the microalgal

cell adhesion without any energy barrier [92]. Hydrophobicity and hydrophilicity of the microalgae and substratum contribute the most to initial adhesion for the microalgal attachment through the cell–cell interaction and cell–substratum interaction [51], similar to the pH of the cultivation medium and substratum. Moreover, the cell–cell interaction between the microalgae cells that is formed by the hydrophobic microalgae may hasten the biofilm thickening process as well as increase the productivity of microalgal biomass production.

Different species of microalgae may have different indicators in determining whether hydrophobic or hydrophilic substratum is suitable for the microalgal attachment to transpire. Lin-Lan et al. [40] reported that hydrophilic substratum such as cellulose acetate, nitrate membrane, polycarbonate, and cotton were able to form the initial microalgal biofilm more easily than the hydrophobic substratum. On the other hand, Kataryzna et al. [39] suggested that in order to promote the microalgal attachment, it was better to choose hydrophobic substratum for the microalgal cell attachment. Free energy of cohesion (ΔG_{coh}) is an indicator to determine the degrees of hydrophobicity and hydrophilicity of microalgae. A cohesion free energy with a negative sign ($-\Delta G_{\text{coh}}$) represents hydrophobicity where the surface–surface interactions of substratum are stronger than surface–water interactions [92]. On the other hand, the cohesion free energy value with a positive sign ($+\Delta G_{\text{coh}}$) indicates hydrophilicity [92]. Furthermore, the value of cohesion free energy with the respective sign can also be used to determine if either the relative adhesion strength of microalgae or substratum is larger to influence the attractive acid–base interaction energy between the microalgal and substratum surfaces [92]. Acid–base interactions between these surfaces are the driving force of microalgal cells attachment [75]. The positive value of cohesion energy free (hydrophilic species) creates a smaller magnitude of attractive acid–base interaction between microalgae and substratum, which leads to weaker adhesion strength than hydrophobic substratum. Thus, a lower attachment rate occurs [92]. For most hydrophilic green microalgae such as *Nannochloris* sp., previous research predicted that this species could not have adhesion interactions with any substratum due to large electron donor parameters in this species that created a strong acid–base repulsion towards substratum [92], thus contributing to very low microalgal growth in the attached system. Fundamentally, Wang et al. [51] proposed that the hydrophobic substratum contributes to better microalgal attachment than hydrophilic substratum. This was proven when the initial layers of microalgae cells preferred to form on the hydrophobic substratum in a short period of time than the hydrophilic materials [51]. Therefore, the usage of hydrophobic substrate can enhance the attached microalgal growth, which may increase the production of microalgal bioproducts such as biodiesel.

6. Effect of Substratum Surface Properties on Attached Microalgal Growth

Suspended microalgae cultivation is facing major challenges in terms of productivity of microalgal biomass as well as harvesting of mature microalgal biomass which represent almost 21% of the production cost [41]. In fact, the current harvesting method of suspended biomass requires a huge amount of energy due to the complicated process to separate the low weight of microalgal biomass from the culture medium. Therefore, attached microalgal cultivation serves as an alternative to overcome the limitation by introducing substratum into the culture medium. The microalgae then grow on substratum surfaces, easing the harvesting process once reaching maturity [51]. Thus, it is significant to choose a suitable material for the substratum in that it must have appropriate surface texture and area, since these are the factors affecting the initial adhesion of microalgal cells onto the substratum surface [59]. As mentioned by Lin-Lan et al. [40], cotton rope was selected to be the most optimum substrate for initiating microalgae attachment over the other materials such as polyester, cotton (low thread), cotton (high thread), and acrylic based on the highest weight of microalgal biomass production. Kataryzna et al. [39] also reported that the physical properties of substrate surfaces were one of the main factors in impacting the microalgal growth. Certain properties of substratum surfaces can increase the water storage supplying the attached microalgae, owing to the presence of porous material surfaces [59].

Aeroterrestrial microalgae have higher biomass growth on rough and porous materials than a smooth flat surface [39,111]. Moreover, substratum with a good surface texture can serve as shelter for the attached microalgal cells, preventing the attached cells from sloughing issues [59]. Indeed, every substratum material has different textures. Wang et al. [51] reported in their study that the substratum surface roughness plays an essential role in the initial microalgal adhesion, similar to the hydrophobicity of the microalgae. The substratum with a rough surface could increase the productivity of microalgal biomass growth compared to a smooth one. Furthermore, the surface roughness can be intensified by using sandpaper scratching [58], meshed substratum materials, and micro-structured surfaces [112]. However, it was suggested in the previous study to use meshed substratum materials, since they could be more easily prepared than sandpaper scratching, which is labor-intensive and may form uneven surface roughness. On the other hand, the application of micro-structured surfaces machined by laser could be expensive for a large cultivation scale [51]. Nylon and polypropylene were selected as the best materials for substratum with a mesh opening of 0.5–1.25 mm. Accordingly, their use managed to increase 73% of microalgal biomass density to the highest tune of 4.2 gm^{-2} on day 1 as opposed to cotton duct [59]. Conversely, Cui et al. [112] reported that the substratum surfaces with mesh openings smaller than 0.5 mm tended to increase the microalgal growth rate as compared with bigger mesh openings. Kataryzna et al. [39] also stated in their research that a rough surface was crucial to enhance the microalgal growth and particle deposition. The microalgal biomass density of red algae, *Halosaccion glandiforme*, on a rough surface such as cotton increased by 35 times as opposed to the microalgae on a smooth surface [39], leading to higher production of biodiesel from the attached microalgae. However, there is one study by Lin-Lan et al. [40] reporting that the microalgae were best grown on a smooth substrate surface such as glass, since it was easy to harvest using the mechanical scrapping method.

Moreover, the suitable size of the substrate surface can also enhance the microalgal attached growth and increase the microalgal biomass production. The attachment of cells increases when the size of the substrate surface is bigger than the size of microalgae cell in assisting the cell deposition [39]. Zou et al. [113] reported that the *Scenedesmus obliquus* and *Chlorella vulgaris* achieved maximum biomass densities at 97.43 and 70.49 gm^{-2} , respectively, when the size of a walnut shell substratum was optimized with sufficient light intensity and dissolved CO_2 concentration provided. Hence, it is important to identify the best material size of substratum that can be used to enhance the attachment process of microalgal cultivation.

7. Effect of Photoperiod and Light Intensity on Attached Microalgal Growth

Generally, the growth of attached microalgae is influenced by conditions including the photoperiod length and light intensity [114]. This is particularly applicable to the metabolic pathways of microalgae that involve the exploitation of light as the energy source, such as autotrophic and mixotrophic cultivations. The attached microalgal cultivation has different light adsorption mechanisms as compared with the suspended microalgal cultivation due to the concentrated attached microalgal cells that are growing on the substratum and may become thicker over time [115]. Wang et al. [116] concluded in their studies that the efficiency of light penetration between a suspended open pond system and an attached culture system are influenced by the nitrogen replete condition, proven when the immobilized cells of *Scenedesmus dimorphus* were 100% received the light intensity of $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ along with the increment in the biomass production from 8.8 to 107.6 gm^{-2} in 10 days of cultivation [115]. Therefore, the biofilm that became thicker in a continuous cultivation will experience a reduction in nitrate concentration which reduces the efficiency of the light penetration inside the biofilm [115]. Different culture conditions and microalgal culture densities for various species will develop different requirements on the ratio of the light and dark cycle (photoperiod) during the cultivation process [117]. However, most of the microalgal species had shown excellent growth

rates and biomass productivities when the photoperiod lengths were increased [118]. For instance, Shen et al. [115] proved in their study that the biofilm productivity and lipid content of the microalgal biomass were enhanced by changing the illumination time of the light:dark cycle from 8 h to 16 h. In this regard, the optimum photoperiod length for *Desmodesmus* sp. was a 16:8 h light:dark cycle with the maximum biofilm and lipid productivity of $35.67 \pm 0.75 \text{ gm}^{-2}\text{d}^{-1}$ and $7.83 \pm 0.12 \text{ gm}^{-2}\text{d}^{-1}$, respectively, could be achieved [115]. Other than that, the growth and lipid productivity of *Nannochloropsis* sp. [119] as well as *Chlorella protothecoides* sp. [118] increased when the illumination time was increased up to 16:8 h light:dark cycle. The highest final biomass of *Chlorella protothecoides* sp. was recorded at 3.0 g L^{-1} [118]. Cheng et al. [117] also found that *Botryococcus braunii* produced the highest biomass productivity which was $6.0 \text{ gm}^{-2} \text{ day}^{-1}$ when it was under the continuous illumination of a 24:0 h cycle, and slowly decreasing to $0.9 \text{ gm}^{-2} \text{ day}^{-1}$ when the light:dark cycle was changed to 4:20 h.

On the other hand, some studies showed that the continuous illumination such as a 24 h light:dark cycle may contribute to the damage of the microalgal photosystem, forcing the microalgae to reach the stationary phase earlier [115]. This led to photoinhibition, reducing the microalgal biomass productivity [118]. The short timescale of photoperiods can be efficient to enhance the microalgal biofilm formation in relation to the photosynthetic electron transfer chain reaction, influencing the microalgal photosynthetic performance [120]. Zhang et al. [120] and Martín-Girela et al. [121] proved in their research that *Nannochloris oculata*, *Chlorella pyrenoidosa*, and *Chlorella* sp. under the photoperiods of 3:3 s and 5:5 s could produce 11–24% and 7–22% higher biomass yield and lipid content, respectively, than under the continuous lighting photoperiods of 30:30 min and 12:12 h. Indeed, the attached microalgal cultivation showed the highest photosynthetic capacity when it was under the photoperiods of 3:3 s and 5:5 s by increasing the rate of photosynthetic electron transfer chain reaction [120]. Moreover, Toninelli et al. [122] also demonstrated that the formation of *Scenedesmus dimorphus* biofilm under the photoperiods of 1:1 s and 5:5 s was more effective than producing it under the photoperiods with the scales of minutes and continuous illumination. The continuous illumination with the photoperiod of 24:0 h could also be a factor to lower the nitrate concentration, leading to the pH increment in the microalgal cultivation medium. Shen et al. [115] stated that the nitrate concentration was reduced to 0.07 g L^{-1} at the third day of cultivation, which influenced the pH to increase until 10.89. If the pH of the culture rose over 10, it may contribute to the higher inhibitory effect towards the microalgae growth whilst reducing the biomass productivity [112]. In fact, the continuous lighting may as well affect the photochemical quantum yield of microalgal cells, increasing the conversion of optical energy of the photoperiod chemical energy, thereby increasing the attached microalgal cells' growth [117].

Regarding light intensity, the attached microalgal cells are more sensitive to the light intensity fluctuation than the suspended cells, since the immobilized cells that are attached onto the substratum are exposed directly to the light [115]. However, the attached microalgal cultivation can ameliorate the light intensity usage better than the suspended cultivation by absorbing a lower light intensity to energy ratio. Accordingly, the maximum biomass productions of *Desmodesmus* sp. in the attached cultivation system were achieved at lower light intensities, i.e., $700 \text{ mol m}^{-2} \text{ s}^{-1}$ [115], as opposed to the suspended cultivation at $750 \text{ mol m}^{-2} \text{ s}^{-1}$ [123]. The optimum light intensity with an appropriate photoperiod condition is an essential factor in ensuring the maximum growth of attached microalgal cultivation, especially for the microalgae following the autotrophic metabolic pathway, since these species absorb light as its only energy source [118,120,124]. Other research found that the light intensity requirements for each type of microalgal species depended on the growth stages [115]. Indeed, every stage of microalgal growth attained its respective biofilm thicknesses. Hence, the light switching technique is an effective way to illuminate the sufficient light for the growing microalgae in the form of biofilm formation. For instance, Shen et al. [115] proved that the maximum attached microalgal lipid production was obtained at 53.62 gm^{-2} on the eighth day of cultivation when the

light intensity was switched from 700 to 1134 mol m⁻² s⁻¹ at day 3. The low light intensity is usually needed for the initial lag phase of microalgal growth in order to prevent the photoinhibition, since the newly formed cells are still weak and the young biofilm is still thin. During the exponential phase of growth, higher light intensity is required to avoid the attached microalgal cell shading, which is usually caused by the photo-limitation [115]. Wang et al. [116] reported that once the upper layer of the attached microalgal biomass increased, the specific growth rate of the inner layer tended to dwindle due to the reduction in the light penetrating from the upper layer to the inner part of the biofilm. Moreover, Murphy et al. [125] also demonstrated that the productivity of biofilm increased from 8.2 to 11.9 gm⁻²d⁻¹ when the biofilm thickness increased from 20 to 100 µm. However, it started to reduce when the biofilm thickness exceeded 200 µm because the efficiency of light penetration reduced exponentially with the increment in biofilm thickness [125].

In addition, high intensity of light during the exponential growth phase might be preferable for the microalgae to store high hydrocarbon content in producing high weight of biomass and lipid accumulation [117,126]. However, at some points of the exponential phase, the attached microalgal growth rate starts to decline, whilst producing less biomass as well as lipid content [115,127]. This is plausibly due to photo-oxidative cell damage, inhibiting further growth of microalgae [115]. The phenomenon is known as the light saturation point (LSP), and thus, the light intensity employed for growing the attached microalgal cells should be equal to or lower than LSP in order to achieve the high rate of photosynthesis [76,127]. Wan et al. [128] proved in their study that the optimum LSP for *Haematococcus pluvialis* was 160 mol m⁻² s⁻¹, since the high rates of biomass production were attained when light intensities ranging from 90 to 160 mol m⁻² s⁻¹ were adopted. It started to reduce when the light intensity was set beyond 160 mol m⁻² s⁻¹. Other than that, Zhang et al. [129] also mentioned that the biomass productivity of *Spirulina platensis* started to show a constant rate when the light intensity was above 200 mol m⁻² s⁻¹. Hence, the light intensity value was concluded to be the threshold of LSP for the *Spirulina platensis* species. In fact, *Spirulina platensis* is categorized as having the strong resistance to irradiation among the microalgae species due to its higher LSP adaptability than other microalgal species such as *Aucutodesmus obliquus*, *Pseudochlorococcum* sp., and *Botryococcus braunii*, which possess LSP values of about 150 mol m⁻² s⁻¹ [102], 100 mol m⁻² s⁻¹ [130], and 150 mol m⁻² s⁻¹ [117], respectively.

On another note, the application of the same light intensity but different light colors can also influence the formation of the biofilm structure, later impacting the growth of attached microalgae [131]. Each color of the light sources has different spectra as well as illumination value [131] and this phenomenon can be observed based on the cell-cell interactions of the cell's physicochemical properties [132]. The attached microalgal cells that are cultured under white light illumination with an optimum photoperiod can form a heterogeneous biofilm with many voids, giving rise to high biofilm porosity and roughness. In this regard, it leads to a low cell-to-cell repulsive interaction as well as low $\Delta G_{\text{co-adh}}$, receiving more uniform light and CO₂ distributions within the biofilm. Meanwhile, the attached microalgal cells that are growing under blue and red lights tend to form a homogenous biofilm with less biofilm porosity and roughness, engendering the high cell-cell repulsive interaction and high $\Delta G_{\text{co-adh}}$ among the microalgal cells [132]. Therefore, the attached microalgae illuminated with white light will produce higher weight of biomass than those exposed to the blue and red lights. This is because of the low biofilm porosity structure when the blue and red lights are used, making the diffusion coefficient within the biofilm low. In contrast, studies reported that the red light spectrum proved to be the most effective in accumulating lipids through the cultivation of *Nannochloropsis* sp. and *Botryococcus braunii*. [133,134]. Kumar et al. [131] also reported that red light spectrum enhanced the microalgal growth of *Ourococcus multisporus* and *Micractinium pusillum*, which were 2.6 and 2.85 g L⁻¹, respectively, compared to the white light spectrum (2.23 and 2.4 g L⁻¹, respectively). Moreover, the lipid content (27%) and productivity (31 mg L⁻¹d⁻¹) of both microalgae under the red light were higher than the white light spectrum (26% and

26 mg L⁻¹d⁻¹), which shows that red light can promote lipid production better than white light [131]. Accordingly, the cells receive inadequate light energy and CO₂ as well as other nutrient sources needed to grow [132,135]. Nevertheless, the impact of light intensity on attached microalgal cultivation still requires further study for a better understanding of the mechanisms of biofilm structure formation under different light conditions.

8. Ways Forward for Sustainable Cultivation of Attached Microalgae

Despite the various advantages associated with the production of microalgal biomass via the attached cultivation system, several improvements need in-depth research in order to further increase the microalgal growth rate by leveraging the employment of the attached cultivation mode. First, the exploration of pretreatment processes the support materials need to undergo is deemed essential to enhance the attachment rate of microalgal biomass onto modified materials. Owing to the fact that the attached cultivation needs to be feasible for the implementation in the commercial scale, the modified materials are anticipated to be reusable whilst reducing the production cost [136]. At the current stage, the recent study conducted by Rosli et al. [137] demonstrated that the reusability of unmodified polyurethane foam support material to grow attached microalgae led to the decline in attached microalgal biomass. The decline was noticed even at the first cycle of reusing the spent polyurethane foam support material and the recyclable study was terminated after merely four cycles of reusing to grow attached microalgal biomass. The declining cause stemmed from the bottom layer of microalgal biofilm that had undergone growth retardation and cell attachment viability was impoverished after the spent support material was used many times. This inevitably resulted in the facile detachment of microalgal biomass as triggered by the shear force from the medium turbulence [137]. Furthermore, Wang et al. [51] also found in their study that the substratum rotation of the biofilm panel and nutritional impoverished microalgal biofilm may lead to the strong shear stress towards attached microalgal cells rising from the medium turbulence. On another note, the research exploring the vital presence of specialty compounds such as extracellular polymeric substances (EPS) and soluble algal products (SAPs) in the biofilm-derived microalgal biomass is also crucial to scale up the applications [41,51]. Indeed, current research on this matter is still scarce, even though this foundation could be the key factor that enhances attached microalgal growth. The EPS are the carbohydrate- and protein-based polymers that can strengthen the microalgal cells' adhesion to the support materials' surfaces while holding the adjacent cells altogether. Tian et al. [138] confirmed that some of the microalgal strains tended to form biofilm easily, particularly when the species could produce more EPS during the cultivation. Other than that, the EPS can also act as a storage compartment for water and nutrients that are significant for the microalgal growth as well as to protect the attached microalgal cells from grazers. All these EPS contributors are favoring fast microalgal initial adhesion [39]. Meanwhile, SAPs secretion may provide the protein and polysaccharides that promote microalgal cell binding with the substratum materials' surfaces [139]. In addition, the structure of thickening microalgal biofilm can be maintained by strengthening the bonding among microalgal cells by exploiting the nutrients from the culture medium and SAPs and EPS from the production of new microalgal cells [39].

Interestingly, the proposed idea of employing solid organic wastes as the substratum materials and low-cost carbon sources for growing attached microalgal biomass in developing the biofilm system is novel, yet requiring verification from prior upscaling applications [113]. Several researchers demonstrated that the support materials used in the attached microalgal cultivation could be replaced with solid organic wastes. For instance, Zou et al. [113] proved in their study that the maximum biomass yields from *Scenedesmus obliquus* and *Chlorella vulgaris* could reach up to 97.43 and 70.49 gm⁻², respectively, while utilizing walnut shell as the substratum material for the microalgal attachment formation. This was a breakthrough achievement as compared with the typical biomass yield while using a non-organic waste such as polystyrene foam, attaining the maximum yield of merely 18.2 gm⁻² from *Chlorella vulgaris* [140]. However, some solid carbon sources are

costly for growing certain microalgal species, thus increasing the total cost of attached microalgal cultivation. In this case, further studies are needed to identify the best approach to adopt economical carbon sources such as by pretreating organic wastes and using the materials that contain high carbon contents, such as sugarcane bagasse and spent coffee ground, in growing attached microalgae. Nevertheless, the presence of these organic carbon sources in the microalgal cultivation medium would introduce possible growth of other microorganisms, i.e., yeast, fungi, and bacteria. Thus, these solubilized organic carbon substrates could very well be mineralized into inorganic CO₂ by the aerobic bacteria, which could introduce symbiotic associations between the microalgae and bacteria in the mixed consortia [141]. Studies have also exploited the synergistic relationship between the photosynthetic microalgae and aerobic bacteria, which could eliminate any need for conventional aeration supply in bioremediating wastewaters and thus reduce the upstream cultivation costs [141,142].

Moreover, the importance of attached cultivation parameters for this application also needs to be specifically investigated, depending on microalgal species and the type of support materials used, in order to enhance the microalgal attachment and obtain high biomass yield. Other than that, the competitions and compensations among different microalgal species and even with the presence of bacteria within a multi-species attached cultivation system also need in-depth research [51]. The interactions among a myriad of microorganisms may help to promote microalgal cell adhesion, biofilm formation or degradation, and biofilm structure strength. Meanwhile, the presence of multi-species microalgae is often observed specifically when wastewater acts as the culture medium [143,144]. This multi-species cultivation system is also known as a non-axenic system, which means the microalgae are growing together with other microorganisms such as bacteria, yeasts, and fungi. The presence of bacteria can be significant for the formation of microalgal biofilm. In fact, the bacteria can stimulate the initial adhesion of microalgal cells, frequently on the substratum materials' surfaces [145,146]. The microalgae may have positive interactions with the bacteria which consist of all possible forms of symbiotic relationships [147]. For instance, in the most famous research on the interaction of *Emiliania Huxleyi*, known as single cell marine microalgae, with *Roseobacter*, the mutual interactions were formed between the specific species since the microenvironment of each microalgal cell was not similar; thus, the nutrient exchange between the microalgae and bacteria was the main contributor to spur microalgal growth [148]. Micronutrients such as vitamins [149,150] and macronutrients such as nitrogen and carbon [150–153] are frequently exchanged between the microalgae and bacteria. Bacteria also excrete EPS, similar to the SAPs from microalgae and plant hormones [150], which can increase the growth rate of microalgal cells in forming attachment [154]. Some studies introduced wastewater or sludge into the microalgal culture medium in order to benefit from the promoting effect for microalgae forming attachment via the bacterial excretates [58,98], thus reducing the time taken for the initial adhesion formation. In this regard, both microalgal and bacterial cells will tune their metabolisms to fulfill each other's necessities in symbiotic association [148].

Furthermore, the recent development of oxygenic photogranules (OPG) as an improvement for the activated sludge was proposed by Milferstedt et al. [155] and Qui-jano et al. [156] through their studies on the wastewater process, which can be a great application for sustainability of the biofilm formation. The OPG process is also known as a light-driven process for wastewater treatment, and it was produced based on the photogranulation of filamentous cyanobacteria, non-phototrophic bacteria, and microalgae [157]. Therefore, it is similar to the aerobic granular sludge whereby the phototrophic granular process gives potential to an effective biomass separation from water and promotes the system operation with a small footprint [158,159]. The basin for secondary treatment and the absence of secondary settlers are the differences in the plant layout between the conventional activated sludge system (CAS) and the OPG system [160]. The secondary treatment in the OPG was performed in sequencing batch reactors (SBR) and since the OPG settling took place in the SBR during the settling phase, the secondary settlers are not needed in the process [160].

The photo-trophic biomass in the OPG generates oxygen through photosynthesis for organic oxidation or nitrification and utilizes the carbon dioxide for its growth [160] and can be operated in stirred-tank reactors without aeration [157]. Accordingly, due to photoautotrophic utilization of CO₂, OPG managed to generate biomass 1.17–1.26 g COD/g COD that are 3–4 times more than the CAG which is 0.3–0.5 g COD/g COD [161,162]. The electricity consumption of the OPG system is 359 Wh m⁻³ and 269 Wh m⁻³ is supplied by the combustion of the produced biogas and 90 Wh m⁻³ by electricity from the grid [160]. Meanwhile, the CAS system required 400 Wh m⁻³ of overall electricity consumption, 263 Wh m⁻³ came from the grid, and only 137 Wh m⁻³ is covered by the biogas combustion, which is lower compared to the OPG system [160]. In terms of nitrogen removal, the OPG system is different from the COD as the system is sensitive to the nitrogen loading rate and organic loading rate [157]. In fact, some levels of nitrite present effluents during ammonia removal to influence the occurrence of active nitrification. Abouhend et al. [157] reported in their studies that ammonia removal reached 90–96% efficiency compared to CAS with a high level of nitrate in effluents, which shows that stable nitrification was executed in the OPG system. Moreover, the OPG system has an environmental impact that is inferior compared to the CAS impact ranging from a 4% difference for freshwater eutrophication to 61% for ionizing radiation [160]. However, there are two notable exceptions for the impact categories, terrestrial eutrophication, and acidification, which are 2 and 3 times higher, respectively, than in the CAS system [160]. These represent the limitations to OPG, and it is very important to ensure the environmental benefits increase in generating OPG biomass [160]. Further research is needed in order to overcome these exceptions since there are few studies covering the OPG applications and high-quality biomass.

9. Conclusions

The current employment of suspended microalgal cultivation is beset by the harvesting disadvantages in producing third-generation biofuels, leading to high commercial production costs and energy demands for large-scale microalgal biomass dewatering. Thus, attached microalgal cultivation is being adopted of late due to the simple harvesting mode that allows time and energy savings, while producing more microalgal biomass at a lower cost than the suspended cultivation method. The only challenge that may arise in the attached microalgal cultivation system is identifying the optimum conditions for the selected microalgal species to thrive on the selected support materials. The optimization depends on the physicochemical and abiotic factors, studies of which are still limited. Therefore, improving the growing conditions to enhance the attached microalgal cultivation systems is the way forward in producing microalgae-based biodiesel, dethroning fossil fuels as the primary energy source.

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