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CO-CULTURE OF *CHLORELLA VULGARIS* AND *ESCHERICHIA COLI* ENHANCE INTRACELLULAR ORGANIC MATTER IN BIOFILM MODE

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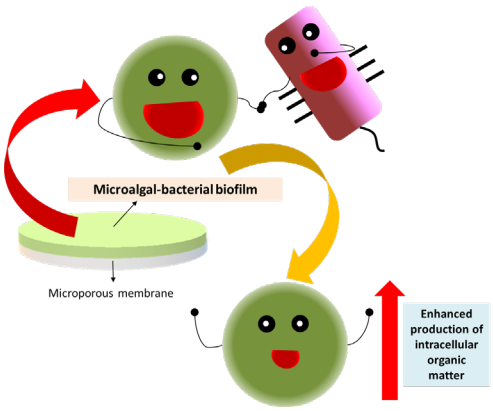
Graphical abstract



Abstract

Microalgal-bacterial biofilm studies over years often highlights challenges in biomass harvesting due to the larger pore sizes, grooves, or pores found on most of the supporting substrates, which trapped the cells easily, hence causing frequent maintenance or replacement with new batch of solid supporting materials. In light of this, the current work addresses this limitation by investigating the use of microporous membranes as a cultivation substrate for co-culture biofilms. A self-designed permeated system was employed to enable the cultivation of biofilms on these membranes throughout 15 days. Findings showed that cell density of *C. vulgaris* in the co-culture biofilm was at least three times higher than the monoculture control, peaking at approximately $49.84 \times 10^{10} \pm 4.44 \times 10^{10}$ cells m^{-2} on day 10. The total chlorophyll yield depicted by co-culture group shown an average of two-fold higher productivity than that of control. Additionally, co-culture system also demonstrated higher extracellular polysaccharide (380% higher at day 5) and protein (870% higher at day 15) levels. The presence of *E. coli* in co-culture undoubtedly stimulated algal metabolism through nutrient recycling or cross-feeding. Intracellularly, permeated biofilm system facilitated a remarkable accumulation of six- to nine-times higher as compared to their respective extracellular counterparts. Insights gained from this work would pave a way to the exploration of more potential co-culture systems in enhancing both the biomass and algal organic matter growth through symbiotic interactions with bacteria.

Keywords: Bacteria, Biofilm, Extracellular, Intracellular, Microalgae

1.0 INTRODUCTION

Microalgae, characterized by their simple unicellular structures, are a versatile group of photoautotrophs that exhibit rapid multiplication and resilience in extreme environments. These microscopic organisms have the inherent ability to attach themselves to solid surfaces, facilitating cell colonization and biofilm formation, with the condition of sufficient nutrient and illumination [1]. Benefits from microalgae and their organic matters in biofuel, food and beverages, pharmaceutical industry, and cosmetic industry have led to the development of an expanding microalgae market, particularly in combination with genetic modification strategies aiming to improve microalgal lipid productivity [2]. In view of this, there is a continuous endeavor to explore different efficient methods for enhancing microalgal growth to facilitate large-scale production of microalgae [3]. Among those potential methods, co-culturing microalgae of interest with their associated growth-promoting bacteria is deemed as the best practice since it allows mutualistic interactions between different species [4]. Such

type of cultures are having strong functional properties of biofilms, increased metabolic diversity, improved resistance to environmental stressors, as well as the production of valuable compounds [5]. It is also well known that biofilm-based cultivation mode always surpasses the conventional suspension-based cultivation mode by offering huge reduction in total production cost, water volume requirement and land area requirement, while enhancing the biomass harvesting efficiency [6].

Realizing this fact, studying the microalgal-bacterial biofilm could offer a novel approach to completely understand the intricate dynamics and ecological significance of these interactions. For example, without the external energy for aeration, coupled microalgal-bacterial biofilm on sponges successfully improved wastewater quality by giving 4-fold COD reduction [7]. Removal of about 94% venlafaxine and 18-51% carbamazepine was acquired with only 5 days of microalgal-bacterial biofilm treatment [8]. During a 30 days trial run for phyco-remediation, co-culture has effectively enhanced the biomass dry weight by 67%, exhibiting a 42% lipid, 55% carbohydrate, and 18.6% protein content enhancement at the same time [9]. Despite extensive researches from the archival literature, biofilm cultivation of microalgae and bacteria was mainly found on solid supports like plastic, sponges, rocks, geotextile patches, cotton ropes and canvas [10-12]. These supporting materials are usually having a rough surface with lots of grooves, non-uniform but big pore sizes, and irregular in their respective particulate size. Such surface properties could somehow hamper the biomass harvesting process as biomass is believed to be easily trapped between the tiny pores or holes, eventually resulting in frequent maintenance or exchange with new solid supports. Therefore, this current study works on the utilization of microporous membranes to perfectly confine the biofilm cells and completely separate the cells from liquid medium in a permeated biofilm system [13].

Generally, mutualistic co-cultivation study has been extensively conducted throughout the years but unfortunately only limited to certain species pairing such as *Chlorella sorokiniana-Azospirillum brasilense* [14], *Chlamydomonas reinhardtii-Azotobacter chroococcum* [15], *Chlorella minutissima-Escherichia coli* [16] and so forth. With these, researchers were in huge interest in exploring more possible mutualistic systems across different species. For example, there were some recent studies successfully demonstrating the positive results for this novel combination group of *C. vulgaris* and *E. coli* [17-20]. Furthermore, the inclusion of *E. coli* in the co-cultivation with *C. vulgaris* could provide a valuable platform for researchers to engineer potential genetic modifications for further algal growth enhancement as *E. coli* is a well-characterized genome in genetic studies, eventually opening avenues for innovative advancements in biotechnology. Therefore, by co-cultivating the suggested symbiosis group on microporous membrane, microalgal growth was monitored together with their respective extra- and intra-cellular organic matter productivity over 15 days of cultivation. Findings from the study could have huge contribution to environmental sustainability, especially towards the energy issues related to clean water and sanitation (SDG6), affordable and clean energy (SDG7) and climate action (SDG13).

2.0 METHODOLOGY

2.1 Establishment of Microalgal and Bacterial Culture

Microalgae strain, *Chlorella vulgaris* ESP 31 was cultivated in sterile BG11 medium (autoclaved at 121℃ for 15 min) at 25℃ under continuous illumination of 1,800 lux. On the other hand, NEB®5-alpha competent *Escherichia coli* bacteria was cultured overnight in liquid SOC medium at 37℃ one day before the experiment. In order to establish a co-culture, two-week old axenic microalgae were intentionally inoculated with bacteria (overnight) at an approximate ratio of 40:1 in terms of cell number.

2.2 Experimental Set-Up

The main solid support used was mildly hydrophilic polyvinylidene fluoride (PVDF, 0.1 μm pore size) flat sheet membrane roll purchased from Merck Milipore, Durapore, USA. Membrane was cut into 47 mm diameter circles and cleaned with 10% ethanol solution. Air-dried membranes were then placed into a self-designed permeated cultivation system with fresh nutrient medium continuously flowing beneath the inoculated membranes. After that, 5 mL of co-culture from Section 2.1 was carefully deposited onto each membrane. The co-culture biofilm cultivation was conducted for 15 days. Triplicates samples were retrieved every five days for observation and quantification.

2.3 Quantification of Algal Cell Growth and Characterization of Biochemical Profile from Microalgal-Bacterial Biofilm

Biofilm was easily scrapped off from the retrieved membranes using a mini spatula into 15 mL of distilled water. Immediate cell counting was performed to avoid cell lysis under osmotic stress. Re-suspended biomass was subsequently centrifuged at 4,000 rpm for 10 min, followed by vacuum filtration to recover the supernatant, named as extracellular organic matter (EOM). Afterwards, the cell pellets residues were re-suspended with 4 mL of Folch solvent and sonicated for 30 min at 40 kHz. The absorbance of the Folch extracts were measured spectrophotometrically at 645, 663, and 750 nm. Subsequently, around 15 mL of 0.9% NaCl was added into the extracts and mixed vigorously. Same operating conditions were adapted by centrifuging the samples and filtering the supernatant to have the filtrate as intracellular organic matter (IOM).

The total polysaccharides content was determined according to phenol-sulfuric acid method using glucose as standard. Meanwhile, proteins content was determined according to the instructions in BCA protein assay kit using bovine serum albumin (BSA) as standard.

2.4 Statistical Analysis

Mean data was reported throughout the entire study with standard deviation (error bars in all the graphs). Analysis of variance was employed to assess the overall significance of the data across different time points for both monoculture and co-culture groups. Following that, post hoc tests were also performed to conduct pairwise comparisons and rank the value in accordance.

3.0 RESULT AND DISCUSSION

3.1 Microalgal Growth Within The Co-Culture Biofilm

As shown in Figure 1, monoculture cell growth was remarkably lower than that of co-culture throughout 15 days of cultivation. Cell density of *C. vulgaris* in co-culture biofilm was at least three times higher than the monoculture control group, achieving its maximum value at day 10 with approximately $49.84\times 10^{10} \pm 4.44\times 10^{10}$ cells m^{-2}. In the contrary, monoculture group depicts a gradual increase albeit slow in the microalgal growth from day 5 to day 15, which its value peaks around $16.91\times 10^{10} \pm 1.03\times 10^{10}$ cells m^{-2}. Throughout 15 days of cultivation, both monoculture (df=8, F=19.981, p<0.005) and co-culture (df=8, F=8.403, p<0.05) group exhibit significant variation in the microalgal growth.

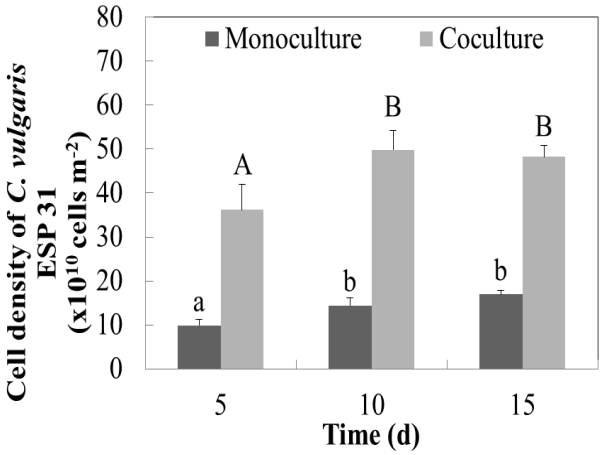


Figure 1 Cell density of *C. vulgaris* ESP31 co-cultured with bacteria *E. coli* on microporous support throughout 15 days, n=3.

A similar trend was observed in Figure 1 showing that regardless of monoculture or co-culture, cell growth increased exponentially from day 5 to day 10. It was due to the exponential phase of the microalgal growth cycle, whereby this specific phase is always characterized by rapid cell division and population growth. At this stage, immobilized microalgae tend to maximize the nutrient uptake efficiency or carbon bio-fixation rate for photosynthesis, resulting in exponential biomass increase. Data was supported by a previous finding demonstrating that carbon bio-fixation rate increased initially and peaked during the exponential growth of *Chlorella vulgaris*, but then gradually declined for the rest of the cultivation period [21]. In a co-culture of microalgae and bacteria, microalgae primarily rely on bacteria to obtain energy and carbon dioxide for photosynthesis, while bacteria often rely on organic carbon sources or by-products from microalgae in return for cell growth [22]. In an enclosed system, nutrients such as nitrates, ammonium, or organic compounds in growth medium are typically shared between two for essential cellular processes. Consequently, there are chances for two to compete for the sources to fulfill their growth requirement, possibly leading to insignificant algal growth changes from day 10 to day 15 [5, 23].

3.2 Extra- And Intra-Cellular Organic Matter Yield Within The Co-Culture Biofilm

In Figure 2, total chlorophyll yield was displayed for both studied group. The monoculture group shows a gradual rising trend whereas co-culture group started with a high total chlorophyll yield and drop towards the experimental time course. In co-culture, chlorophyll yield peaks at day 10 with a value around 36.70 ± 2.46 mg m^{-2} and starts declining afterwards to 17.97 ± 1.56 mg m^{-2} at day 15. Generally, the increase in total chlorophyll content within microalgae is attributed to several key factors, including light availability, nutrient availability, growth stage, and stress response. In line with the cell growth data, total chlorophyll yield maximum at day 10, directly implying the most active growth phase of co-culture at that specific day, as well as verifying the suitability of using chlorophyll content as an indicator for microalgal growth [24]. However, the results demonstrate that total chlorophyll yield did not increase linearly as the presence of bacteria could somehow inhibit the production of chlorophyll via nutrient competition as described in Section 3.1. This photosynthetic pigment is also a sensitive bio-compound for various environmental stresses as intracellular chlorophyll synthesis is served as a cellular protective mechanism to mitigate the reactive oxygen species-induced damage to the photosynthetic system [25].

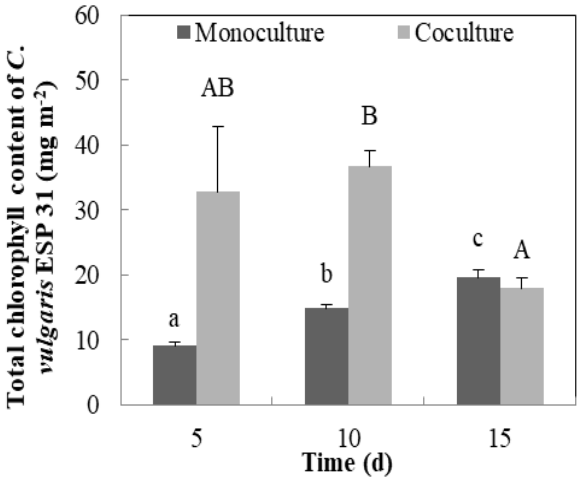


Figure 2 Total chlorophyll yield of *C. vulgaris* ESP31 co-cultured with bacteria *E. coli* on microporous support throughout 15 days, n=3

Aside from total chlorophyll content quantification, both extra- and intracellular organic matter contents were also determined to examine the biochemical profile between monoculture and co-culture groups. Typically, polysaccharides and proteins are the primary components studied due to their essential roles in biofilm development [26]. In Figure 3(a), extracellular polysaccharide isolated from co-culture was about 3.8 times higher than that of monoculture control group at day 5, but experienced a huge decline afterwards. Compared to monoculture group, extracellular polysaccharide yield exhibited a gradual increase towards the end of the experiment, albeit reaching a level approximately half of that observed in the co-

culture group. When cultivating mutualistic consortium in biofilm mode, embedded cells tend to accumulate more intracellular organic matters as shown in Figure 3(b). The intra-polysaccharide yield from both monoculture and co-culture has marked down a substantial six-fold increase as compared to their respective extracellular matter yield.

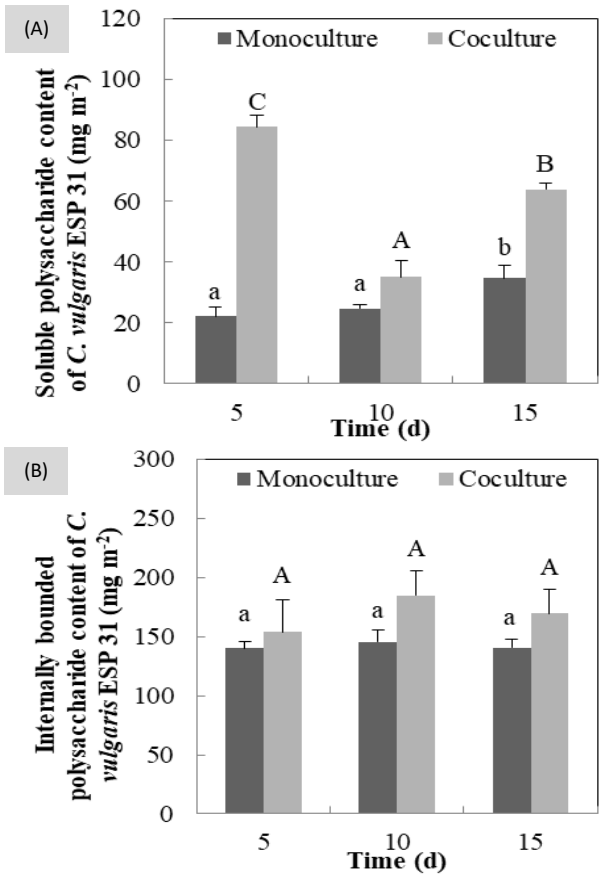


Figure 3 Total (a) extracellular and (b) intracellular polysaccharides profile of *C. vulgaris* ESP 31 co-cultured with bacteria *E. coli* on microporous support throughout 15 days, n=3.

It is important to note that bacterial enzymes within a biofilm are able to degrade and consume surrounding microalgal polysaccharides. They often have strong enzymatic activities to break down complex polysaccharides into simpler mono-based sugars [27]. These bacterial enzymes, such as carbohydrases normally target bio-compounds present in the extracellular matrix of biofilm where they are more accessible. It provides a direct explanation on the fluctuating trend as discovered in Figure 3(a) for co-culture group, suggesting the bio-molecules exchange between microalgae and bacteria. Conversely, no notable effect ($p>0.05$) was found on the buildup of intracellular polysaccharides as this specific organic matter is often predominantly secreted extracellularly, serving to enhance physical stability and promote cell adhesion to the surfaces, instead of becoming an internal storage compounds within the cells [23].

From the perspective of protein yield, Figure 4 showcases that co-culture group marked a substantial elevation every five days, with its highest extra- and intracellular protein productivity of 227.89±18.45 mg m^{-2} at day 15 and 1157.67±8.93 mg m^{-2} at day 10 respectively. For monoculture, no significant difference was revealed across the cultivation period, with its average protein productivity lower than that of co-culture group as expected.

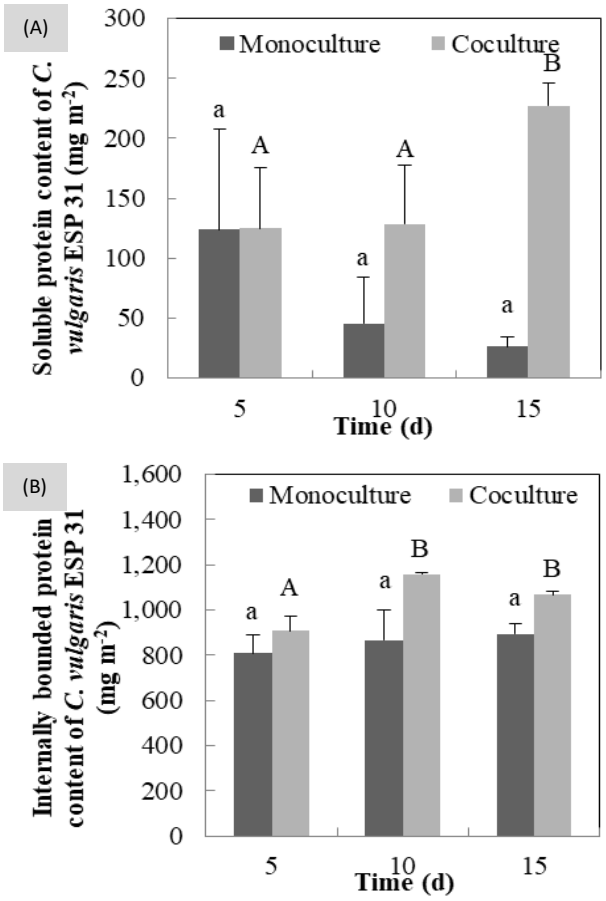


Figure 4 Total (a) extracellular and (b) intracellular protein profile of *C. vulgaris* ESP 31 co-cultured with bacteria *E. coli* on microporous support throughout 15 days, n=3.

When establishing a mutualistic system between the microalgae and bacteria, bacteria could effectively enhance the production of microalgal proteins through nutrient recycling. Nitrogen and phosphorus are broken down by bacteria to make these nutrients more accessible to microalgae [5]. Moreover, cross-feeding, or sometimes known as exchange of metabolic by-products between two, would have stimulated microalgal metabolism. For instances, thiamine, biotin, and cobalamin produced from bacteria are cofactors for enzymes involved in the biosynthesis of amino acids, stimulating the photosynthetic processes of microalgae [28]. Phytohormones such as auxins, cytokinins, gibberellins, and indole-acetic-acid from *Azosipirillum brasilense* increased both cell density and bio-compounds accumulation simultaneously, mainly polysaccharides and proteins, in both *Chlorella vulgaris*, and *Tetradesmus obliquus* [29].

4.0 CONCLUSION

The present work presents advantages of immobilizing microalgal-bacterial co-culture on a microporous membrane in a permeated system. Results have shown that the attached cells grown on solid support were able to render higher intracellular organic matter yield, by marking down a substantial six-fold increase as compared to that of their extracellular organic matter yield. Mutualistic interactions were established between two to enhance biomass productivity, microalgal organic matter yield and overall system efficiency. Coexistence of both actively growing *C. vulgaris* and *E. coli* would easily induce nutrient competition, thereby causing consumption of dissolved extracellular microalgal organic matters, namely polysaccharides and proteins by the existing bacteria. In current set-up, co-culture biofilm cells tend to accumulate higher amount of intracellular polysaccharides and proteins due to zero concern of biomass wash-off and direct contact with atmospheric air that facilitate air diffusion to the grown biofilm. Overall, co-culturing microalgae and bacteria on reusable and durable microporous membranes holds significant promise as a sustainable approach for biomass production, wastewater treatment, and the synthesis of valuable bio-products. More detailed investigations should be planned in future to reveal the mechanisms of molecule exchange involved in such a cultivation system.

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Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper

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