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COMPARISON OF BIOFLOC FORMATION FROM MICROALGAL CULTIVATION BY AUTO- AND BACTERIA-ASSOCIATED TYPES OF FLOCCULATION

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SUMMARY

Microalgal harvesting has still been a challenge to investigators who take their investiment into microalgal production. The cost for microalgal downstream processing is as high as 20% of the total production of biodiesel. Among hundreds of curent methods of biomass harvesting, autoflocculation and bacteria-based aggregation are still being researched and applied in large-scale producuion. This study implemented a comparison of how microalgal cells aggregate large bioflocs according to two types of flocculation. The microalgal autoflocculation was implemented by adding Ca^{2+} or Mg^{2+} with an increase of pH to 11, resulting in cell biomass of 13.7 or 15.5 mg/l, respectively. Meanwhile, the bioflocculation under the support of *Bacillus subtilis* MT300405 and *Escherichia coli* ATCC 85922 could produce large bioflocs with a cell biomass of 1.5 times higher than the autoflocculation case without the influence of pH. Moreover, images from scanning electron microscopy indicated differences between two types of flocculation. With the presence of bacteria, microalgal cells were more tightly bound by a membrane or a layer of extracellular polimeric substance, inducing to form large bioflocs. This was not found under autoflocculation.

Keywords: microalgal harvesting, autoflocculation, bioflocculation, aggregating mechanism, coagulant

INTRODUCTION

Regarding microalgal harvesting demand, several types of biomass collection were studied to improve the cell density in culture with target to enhance the performance of cell removing and facilitate the establishment of downstream processing. The optimization in flocculation combining coagulation and aggregation with bio-flocculants, were used to aggregate and microalgal cells, but the cost of this process had to be considered (Xia *et al.,* 2017). Cetyltrimethylammonium bromide (CTAB) and Al^{3+} with concentration of 60 mg/g dry matter and 40 mg/g dry matter, respectively, were used to float 98.73% microalgae. However, the cost for flocculants put pressure to investigators if they paid objects for microalgal production in

large-scale. Fortunately, many studies have solved this problem by developing techniques of autoflocculation, which aggregated microalgal cells in culture media to form large bioflocs, by changing pH and time of cultivation. *Chlorella vulgaris (C. vulgaris)* is using as one of the most promising microalgal strain for investigating at high performance of cell removing out suspension because it is easy to grow in medium of nitrogen resource. As a result, *C. vulgaris* has been proved that the cell settling efficiency obtained to 90% as combined with an adjustment of Ca^{2+} and Mg^{2+} and alkaline conditions (Vandamme *et al.,* 2012; Nguyen *et al.,* 2014). Likewise, *Nannochloropsis oculata* was harvested up to 90% under the co-coagulant of Ca^{2+} and Mg^{2}+ (Tran *et al.,* 2017) at pH higher than 10.

Meanwhile, various studies have proved the co-existence of bacteria during conventional microalgal cultivation could enhance cell aggregation to form large bio-flocs and called this phenomenon is bioflocculation (Magdouli *et al.,* 2016; Nguyen *et al.,* 2019). In the presence of bacteria, the efficiency of microalgal harvesting significantly increased without chemical adjustment and high pH. For instance, in a xenic cultivation of *C. vulgaris* under the co-existence of bacteria, 94% cells was harvested in comparison to 2% flocculation of the culture without bacterial strains (Lee *et al.,* 2013). However, this bio-flocculation has been favorably investigated in wastewater cultivation of microalgae. Meanwhile, it is neccessary to culture microalgae in pure environment to concentrate on downstream production such as lipid extraction for biofuel, protein extracts for suplementary food, carotenoic products for additives… exception for wastewater treatment.

This study was focused on explaining the formation of bioflocs in pure medium of microalgal cultivation. *C. vulgaris* was choosen as a microalgal strain for a pure cultivation in Bold's Basal Medium (BBM). The formation of bioflocs by the addtion of cation such as Ca^{2+} or Mg^{2+} was examined in comparison with that in the presence of bacterial cells such as *Escherichia coli* or *Bacillus subtilis*.

MATERIAL AND METHODS

Microbial strains and chemicals

C. vulgaris 211-19 SAG was cultured in 350 ml of pure BBM medium in 500-ml Erlenmayer flask until obtaining the absorbance of 0.8 at 680 nm (Nguyen *et al.,* 2014). This culture was used as starter for further experiments. *B. subtilis* MT300405 (Tran *et al.,* 2021) and *E. coli* ATCC 85922 were used for experiments of bacteria-assisted flocculation. The composition of BBM and microalgal culture conditions was referenced from the pulication of Nguyen *et al.,* 2014.

The chemicals used in this research are common and ensured the high purity for each experiment.

Formation of bio-floc

Prior to implement experiments of flocculation, *C. vulgaris* 211-19 was collected from original culture. Microalgae were cultured in BBM medium until death phase. Flocculation experiments were carried out in a 100-ml glass cylinder with 70 ml microalgal culture. $CaCl_2.2H_2O$ with the concentration of Ca^{2+} from 10 to 20 mg/L and KH_2PO_4, or $MgSO_4.7H_2O$ with its concentration from 10 to 20 mg/L was added to the culture for examining the flocculation. Alternatively, 1mL of *B. subtilis* MT300405 or *E. coli* ATCC 85922 with the concentrations 2,5 x10^8 and 1,8 x 10^8 CFU/mL, respectively, was added into the culture for bacteria-assisted flocculation. Large bioflocs were collected to measure the biomass after being completely decanted to the bottom of cylinder.

Analysis

Microlgae growth was determined through absorbance of optical density at 680 nm (OD680) by spectrophotometer (LABOMEC, USA). In addition, cell density of bacteria was counted through a representative regression line to

correlate the number of cell (CFU/ml) with OD680.

To evaluate the settling efficiency, OD680 of microalgal harvesting was recorded before the addition of cation (Ca^{2+} or Mg^{2+}), bacterial culture (*B. subtilis* MT300405 or *E. coli* ATCC 85922) and when the flocculation completed. This efficiency was calculated as follow:

$$SE, \% = \frac{(1 - OD680_2)}{OD680_1}\, x\, 100$$

Where $OD680_1$, $OD680_2$ was absorbance of cell microalgal suspension at 680 before adjusting Ca^{2+} and Mg^{2+}, respectively, *B. subtilis* MT300405, and *E. coli* ATCC 85922, and almost microalgal cells deposited in the bottom of cylinder.

To characterize different behaviors of microalgal aggregation, or to observe how Ca^{2+} and Mg^{2+}, *B. subtilis* MT300405, and *E. coli* ATCC 85922 aggregates with microalgal cells, scanning electron microscopy was was carried out according to method described by Nguyen *et al.,* 2018.

Statistical analysis

All experiments were performed in triplicate, processed by Excel software, and treated by one-component ANOVA at the level of significance $p \leq 0.05$. The results were shown as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Formation of bioflocs

C. vulgaris cell population entered the dead phase on the day 7 during 15-days cultivation. At this time, microalgae cells stopped division, resulting in an decrease of OD680. Cells could not decant and exist in suspension. Each of four choosen coagulants was added into this suspension and the formation of large bioflocs were recorded and shown in Table 1 and Figure 1.

Table 1. Focculation of *C. vulgaris* culture by difference chemicals and bacterial coagulants.

Coagulant	pH	Flocculation time (hour)	Concentration of biofloc (mg/l)	Settling efficiency (%)
Ca^{2+}	11.0 ± 0.5	20 ± 1.5	13.7 ± 1.05	78.5 ± 1.05
Mg^{2+}	10.5 ± 0.52	21 ± 0.5	15.5 ± 1.5	80.2 ± 1.05
***B. subtilis* MT300405**	7.02 ± 0.2	23 ± 0.2	21.5 ± 0.5	94.5 ± 1.05
***E. coli* ATCC 85922**	7.05 ± 0.1	24 ± 0.5	19.5 ± 0.76	95.1 ± 1.05

Based on Table 1, it was found that the microalgal settling efficiency was higher than 90% with the addition of either *B. subtilis* MT300405 or *E. coli* ATCC 85922 compared to less than 80% with cation adjustments in *C. vulgaris* suspension. Certainly, if the settling efficiency is high, the biomass or concentration of bioflocs is high, i.e. most of the microalgae were separated from the suspension and settled at the bottom of the inner cylinder with the addition of bacteria (Figure 1). Moreover, the concentrations of the bioflocs were almost doubled after the addition of bacteria to a suspension containing microalgal cells.

Although the microalgal settling efficiency with the support of cations was lower than that of bacteria, flocculation time of the former showed more advantage than the latter. In just 20 hours the microalgal cells were removed from the suspension after adding salts containing metal cations and increasing the pH. Meanwhile, it took up to 24 hours for these cells to settle almost completely at the bottom of the cylinder after adding bacteria. According to Tran *et al.* (Tran *et al.,* 2017), *Nannochloropsis oculata* was harvested at pH 10.5 with a flocculation efficiency of 90% by calcium carbonate and calcium phosphate, but the settling time was not

reported. Likewise, in the range of pH from 8 – 12 and using Ca^{2+} and PO_4^{3-} maximal flocculation efficiency was optimized to 98% with the strain of *C. Vulgaris* CCALA 256 cultured in fresh medium, but flocculation time was not mentioned neither. Specially, our results were similar with those reported by Nguyen et al. (2014) with a microalgal settling efficiency of 90% in 8 h by the addition of $CaCl_2.2H_2O$ and KH_2PO_4, or by $MgSO_4.7H_2O$ under alkaline condition. Meanwhile, the role of bacteria in microalgal harvesting was well-mentioned in various studies even in fresh culture media as well as wastewater. Lee *et al.* (2013) reported a flocculating activity of 94% when *C. vulgaris* was co-cultured with *Flavobacterium* sp., *Terrimonas* sp., *Sphingobacterium* sp., *Rhizobium* sp., and *Hyphomonas* sp. In our this study, the addition of *B. subtilis* MT300405 or *E. coli* ATCC 85922 into *C. Vulgaris* CCALA 256 suspension resulted in a flocculation efficiency of 94% or 95%, respectively. But one significant difference this study did not implement the microalgal growth under xenic condition. Particularly, one of our results indicated that strain of *C. vulgaris* 211-19 was harvested to 80% in suspension of sterilizing seafood wastewater with presence of *E. coli* (Nguyen *et al.,* 2018). Although there was a difference of two microalgal culture media, obtained flocculation efficiencies were higher than 80%.

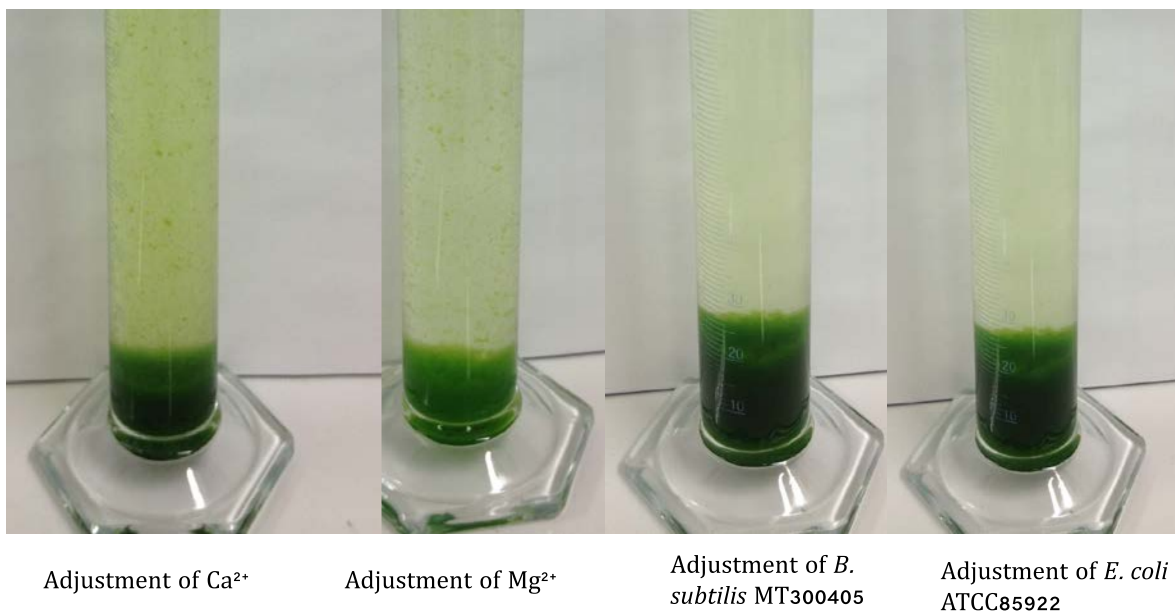

Figure 1. Comparison of flocculation based on chemical and bacterial adjustment.

Biofloc property

To understand how bioflocs were formed, image analysis was performed using SEM. These analysis indicated the differences of cell aggregation of coagulants added in fresh media. Figure 2 and Figure 3 showed significant differences of chemical and bacteria-associated coagulants, respectively. Although, many reports have also published the mechanisms of forming bioflocs, autoflocculation has received little attention because of difficulty of establishing microalgal cultivation processes under non-ideal culture conditions. In this study, bioflocs were harvested in both autoflocculations proved that microalgal cells were aggregated in the same way. The hypothesis of cation precipitations under alkaline conditions induced the cells in

suspension aggregating together (Huo *et al.,* 2014). According to Nguyen *et al.,* 2014 (Nguyen *et al.,* 2014), the precipitation of hydroxyapatite and amorphous tricalcium diphosphate was predicted to microalgal flocculation. However, alkaline conditions in this study have reduced the intensity of the negative surface charge of microalgal cells, promoting their self-aggregation. The result was consistent with the previous one as using Ca^{2+} and Mg^{2+} for flocculating microalgae in culture media, but obtained the settling efficiency. However, this process was favorable as the aggregation enhanced by less time of flocculation.

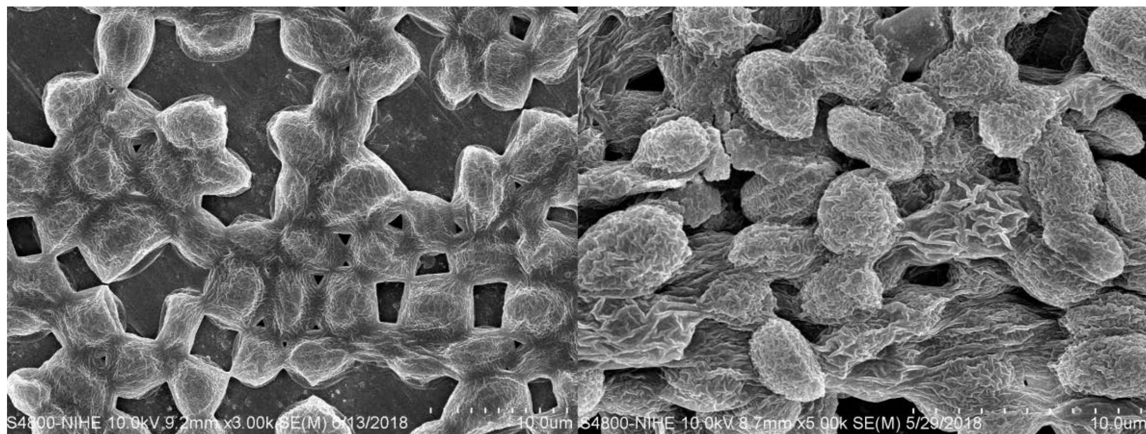

Figure 2. SEM visualization of microalgal aggregation under support of chemical coagulants.

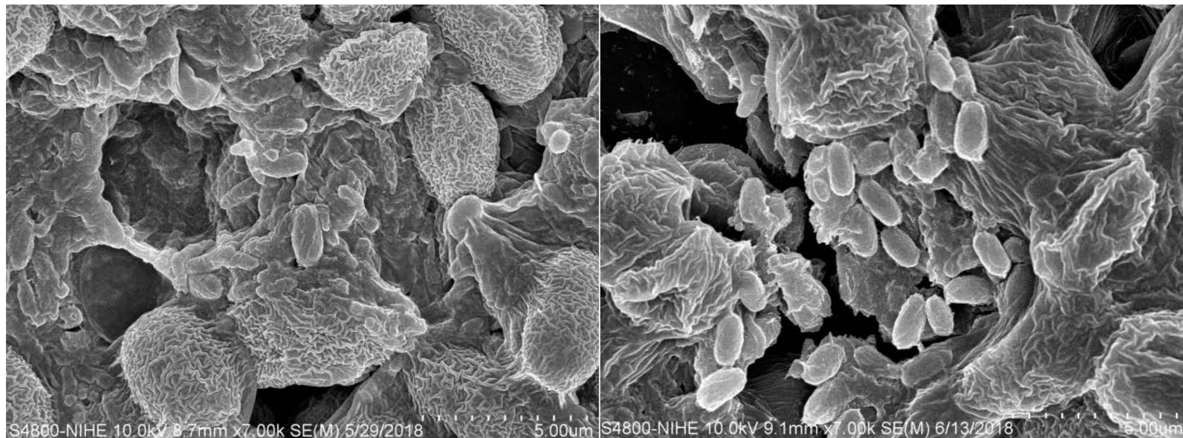

Figure 3. Behavior of microalgal cells and bacteria in matrix of biofloc.

Compared to the results of the autoflocculation, bacteria-associated flocculation, the result in this study is more clearly explained by the presence of bacteria in the matrix of bioflocs. Results from SEM indicated the co-existence of bacteria and *C. vulgaris* cell in the matrix of bioflocs (Figure 3).

Through images taken by SEM, the difference in the way of forming bioflocs between two type of coagulants was obviously shown in biofloc, in the layer of extracellular polimeric substance (EPS). It can be seen that the microalgal cells were surrounded by a solid membrane and inhabited by bacteria. This membrane was proved by various studies, in which bacteria played important role in the harvesting of microalgae because of the contribution of EPS (Nguyen *et al.,* 2018; Guo *et al.,* 2013). The difference between autoflocculation and bioflocculation was shown by EPS layers that induced the settling efficiency of the latter higher than the former. Almost *C. vulgaris* cells were deposited to the bottom of cylinder after 24-hrs adding *B. subtilis* MT300405 or *E. coli* ATCC 85922, followed by the settling efficiency of 94.5% and 95%, respectively. These results were consistent with previous studies, although microalgal cells were cultured in different media such as wastewater, rich nutrient media. Moreover, an important point indicated that microalgae biomass is increased by 1.5 times when using bacteria without the need to accrete pH. Compared to cell autoflocculation by Ca^{2+} and Mg^{2+} with support of pH, bacteria-based bioflocculation gently aggregated microalgal cells to produce large bioflocs, enhanced the settling process without changing the culture conditions.

CONCLUSION

This study was a part of our previous series of studies aiming at microalgal harvesting. However, what set this study apart from other studies is that it offered a comparison of how coagulants aggregate microalgal grown in BBM medium, not in wastewater. Despite wastewater or fresh culture media, bacteria also play an important role in connecting microalgal cells. The microalgal setlling efficiency or flocculation activity obtained over 90%, and biomass of microalgal biofloculation was 1.5 times higher than that of autoflocculation for almost the same time of flocculation of 24 hrs.

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