Vietnam Journal of Biotechnology **20**(3): 487-494, 2022

INVESTIGATION OF MICROALGAE CULTURE BY AUTOFLOCCULATION METHODOLOGIES

Le Thi Van Anh[1,3], Tran Thi Ngoc Thu[2,✉], Nguyen Thi Dong Phuong[2]

[1]*Publishing House for Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[2]*The University of Danang, University of Technology and Education, 48 Cao Thang Street, Hai Chau District, Da Nang City, Vietnam*
[3]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*

✉To whom correspondence should be addressed. E-mail: thuttn@gmail.com

Received: 09.12.2021
Accepted: 23.04.2022

SUMMARY

Harvesting of microalgae from their different cultivation media has pointed out challenges in resolving the problems of flocculation. These challenges must be faced with a suitable method for inducing flocculation that avoid or limit the microalgae's contamination. This study developed the fundamental experiments with a support of chemicals and some bacteria strains inducing the flocculation of *Chlorella vulgaris* SAG 211-19. Particularly, the determination of minimum content of Mg^{2+}, Ca^{2+}, *E. coli* ATCC 85922 and *Bacillus subtilis* MT300405 was effectuated with co-cultivation of microalgae and set up in batch culture in Bold's Basal Medium. As a result, the adjustment in 25 minutes of 199.2 mg/L $CaCl_2.2H_2O$, 50 mg/L KH_2PO_4, and of 141 mg/L $MgSO_4.7H_2O$ induced a microalgal settling efficiency of 81% and 70%, respectively. Meanwhile, the perfomance of microalgal removing reached up to 83.6% and 84% by the inoculation into microalgal culture media of a minimum initial cell density of 8.1×10^5 CFU/mL of *Bacillus subtilis* MT300405 and 12×10^5 CFU/mL of *E. coli* ATCC 85922, respectively. The flocculation of microalgal cells by bacterial inoculation did not require a high pH adjustment as in the case of salt addition.

Keywords*:* Microalgal cultivation, microalgal harvesting, flocculation, autoflocculation

INTRODUCTION

Microalgae flocculation can also be obtained by the precipitation of salts such as calcium carbonate or phosphate, which have high enough concentrations in corresponding ions in the culture medium (Wang *et al.*, 2022; Vandamme *et al.*, 2013). Compared to flocculation by coagulation with hydrolyzing metal salts, precipitation occurs at lower pH, typically 8.5-9.0 for calcium phosphate and 9.5-10.0 for carbonate calcium, but with lower sedimentation efficiency (Smith, Davis, 2012). Various mechanisms have been proposed, including charge neutralization by a positively charged calcium phosphate precipitate at the cells surface (Sukenik, Shelef, 1984), weighting effects and sweep flocculation (Smith, Davis, 2012; Semerjian, Ayoub, 2003). Weighting effects are related to the deposition of a dense precipitate at the particle surface that acts as a 'weighting agent' by increasing the apparent density of cells leading to their decantation (Semerjian, Ayoub, 2003). Chemistry of the solution has a great influence on flocculation efficiency, in particular the presence of anions

according to their capacity of aggregating aluminum in place of the hydroxyl anion. The presence of sulfate ion in the solution can reduce significantly the positive charge of aluminum hydrolysis products, and sulfate, bicarbonate or chloride ions have a great influence on the pH range for which $Mg(OH)_2$ precipitate can aggregate to settleable flocs.

Basic principles governing the coagulation of colloidal particles by hydroxides of hydrolyzing trivalent metal Al^{3+} or Fe^{3+} are quite well-understood, even if some uncertainties remain regarding the detailed mechanisms (Duan, Gregory, 2003; Wang *et al.,* 2022). As a consequence, charge neutralization of negatively charged particles can be achieved only at pH values below the isoelectric point (IEP) of the precipitated oxide. This is not a problem when a microalgae suspension is destabilized by $Mg(OH)_2$ precipitation because its IEP is above 11.5 (various values have been reported in the literature: 11.5 (Li, Somasundaran, 1991), 11.9 (Liu *et al.,* 2008), 12.4 (Parks, 1967)). It is known that IEP vary according to the conditions of precipitation and the anions present, and maybe with the age of the precipitate as Luo *et al.,* 2004 reported that an aged $Mg(OH)_2$ had an IEP around pH 10. It seem be routine with these salt methods in microalgal harvesting because of the emergence of those with bacteria. Up to day, the flocculation of microalgal cells has been investigated with various bacteria strains or bacteria community while microalgae were cultured in traditional media as well as wastewater media (Schryver *et al.,* 2008; Nguyen *et al.,* 2018). Various studies proved that bacteria helped suspended cells into an aggregation by forming a biofilm (Syafri *et al.,* 2017; Zhang *et al.,* 2019; Schryver *et al.,* 2008). This enhances the attachment of microalgal cells into bioflocs, which resolve the difficulty in harvesting of suspension. According Nguyen *et al.* (2018), *Chlorella vulgaris* was cultured in seafood wastewater in 14 days, then cells were flocculated under the presence of bacteria in media, resulting in 90% cell removing by forming bioflocs (Nguyen *et al.,* 2018).

From an operational point of view, flocs settling rates, flocs strength and ions consumption are of great importance from an economic point of view as they can have a great impact either on CAPEX (Capital expenditures) or OPEX (Operating expenses). Harvesting microalgae by autoflocculation under the support of Mg^{2+}, and Ca^{2+} in high pH media is one of the best efficacy methods that have been carried out. Besides, some bacteria strains was also added in culture media of microalgae for cell flocculation without increasing pH. In this study, the autoflocculation of microalgae was investigated in simple experimental set-up which was suitable for laboratory conditions in Vietnam. Microalgae was cultured in batch under the white light with a standard environment. During this cultivation, the microalgal growth was monitored by recording alive cells until the death phase appeared. At this moment, cations of salt and bacteria were separately supplied into the culture, and their aggregation with microalgal cells were observed. The performance of cell flocculation or settling efficiency was calculated to determine the ability of salt cations, and bacteria in microalgal harvesting through auto-flocculation.

MATERIALS AND METHODS

Strain and growth conditions

For starter culture, *Chlorella vulgaris* SAG 211-19 was cultured in 250 mL of BBM medium with ingredients as follows: 1151.98 mg/L $NaNO_3$, 140.00 mg/L $MgSO_4$. $7H_2O$, 100.00 mg/L KH_2PO_4, 840.00 mg/L $NaHCO_3$, 25.00 mg/L $CaCl_2.2H_2O$, and 0.5 mL Hutner solution; LED white light at a flux of 150 μmoL/m^2/s. It is easy to access further manipulations with a correlation between the absorbance at 680 nm of microalgal growth and its chlorophyll-a (Nguyen *et al.,* 2014).

Bacillus subtilis MT300405 and *E. coli* ACTT 85922 (isolated and identified by Tran *et al.,* 2021) were grown as described in this article. For bacterial cells, the correlation between cell absorbance at 600 nm and number of cell per mL

(CFU/mL) was drawn by experimental spreading colonies on LBA for *B. subtilis* MT300405 and TSA for *E. coli* ACTT 85922, respectively (Tran *et al.,* 2021).

Experimental setup

A starter culture with microalgal cell density (OD_{680}) of 0.8 was collected for a mass culture in an Erlenmeyer of 2.0 L with standard media of BBM while bacterial strains was grown in LB broth and TSB for *B. subtilis* MT300405 and *E. coli* ATCC 85922, respectively. The miccroalgae growth was recorded daily in OD_{680} for evaluating final biomass before harvesting. After 9-day when microalgal population reached the death phase, cells flocculation was carried out by the addition of Ca^{2+}, Mg^{2+} with adjustment of pH to 9, and *E. coli* ATCC 85922, *B. subtilis* MTT300405. The screening for flocculation of minimal concentrations of these two ions was performed by increasing pH with solution of NaOH 0.1M. The autoflocculation process was stopped when the flocs increase ended.

Settling efficiency

The settling efficiency of microalgal cell which evaluates the performance of cell flocculation or cell removing out of media is estimated according to the Beer-Lamber law as follows:

$$E,\% = (1 - \frac{A_f}{A_o}) \times 100$$

Where A_f, A_o was absorbance of cell microalgal suspension at 680 nm at the final and initial harvesting suspension, respectively.

Analysis

Biomass dry matter (DM) was obtained by cell filtration on GF/F filters, dried and weighed. Pigments were extracted from cells using methanol for 4 h. Total chlorophyll a content was determined according to Ritchie, 2006. Analysis data were recorded as the average of triplicate measurements and barre error was calculated.

RESULTS AND DISCUSSION

Cell growth evaluation

The correlation between microalgal absorbance at 680 nm and chlorophyll a as well as number of cells was drawn to the following equation y = 0.1056 x with y as absorbance at 680 nm, x as chlorophyll a (mg/L), and R^2 = 0.9971. The growth of microalgae for 14 days was shown in figure 1. Results in Figure 1 illustrated the cell growth curve in 9 days of cultivation. After 9-day culture, microalgae induced the phase death, and this time was chosen for cell harvesting.

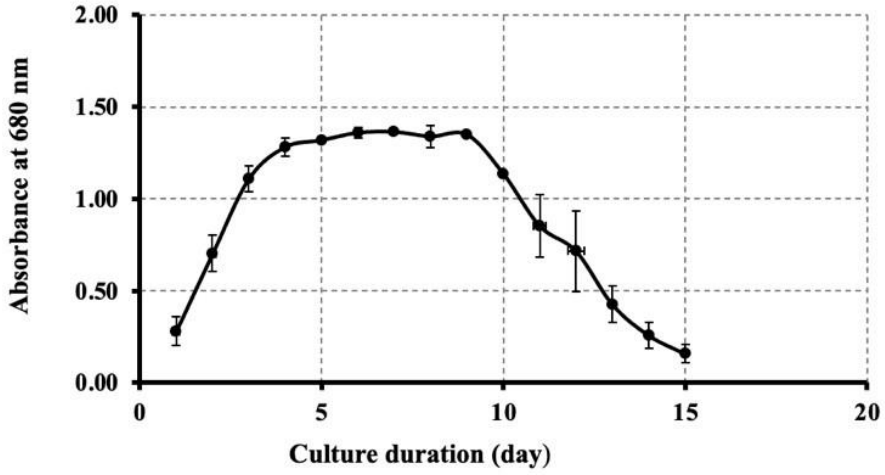

Figure 1. Growth curve of microalgal cultivaiton in Bold's Basal Media.

For bacterial cells, the correlation between cell absorbance at 600 nm and number of cell per mL (CFU/mL) was drawn to a line for *B. subtilis* MT300405 with equation y = 11.612x-1.220 with y as CFU × 10^7/mL, x as absorbance at 600 nm, and R^2 = 0.9955. For *E. coli* ACTT 85922, the equation was y = 14.534x + 0.2797 with y as CFU x 10^7/mL, x as absorbance at 600 nm, and R^2 = 0.9924. Based on these equations, bacterial cells could be determined by recorded absorbance at 600 nm.

Determination of Mg^{2+} minimum concentration for cell harvesting

In order to establish the required minimum Mg^{2+} concentration to allow flocculation, cells were harvested after cell dry matter reached 0.8 g DM/L, then cells were resuspended in a volume of sterile and distilled water so that the final concentration was 0.4 g DM/L. The concentration of control sample is carried out by measuring the pigments and the pigment/cell concentration following calibration curves.

Increasing volumes of $MgSO_4.7H_2O$ stock solution were added to test tubes containing microalgae cell suspension and cell settling was recorded. The settling curves obtained after adding 1 N sodium hydroxide to pH 11.8 on Figure 2 showed that it is possible to obtain a clarification of close to 100% in the supernatant located above the flocs for a Mg^{2+} concentration of 505 mg/L $MgSO_4$. $7H_2O$ (the efficiency at 750 nm was 95%). The minimum quantity of Mg^{2+} necessary to obtain an efficiency E greater than 80% after 20 minutes, the value that we have chosen as a criterion for comparison between the different experiments, was approximately 14 mg/L (0.58 mM) corresponding to 121 mg/L $MgSO_4.7H_2O$. Maximum lightening, under the various conditions tested, was achieved after about twenty minutes.

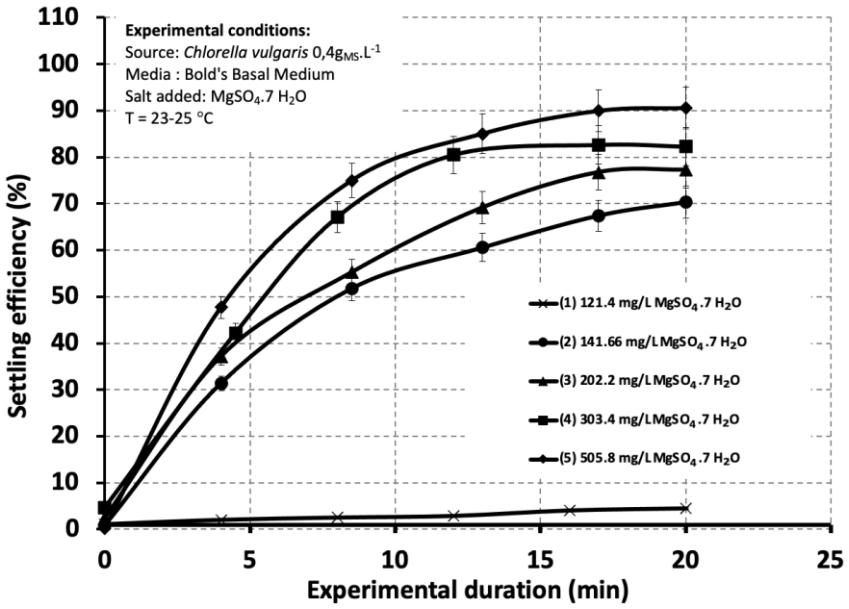

Figure 2. Determination of minimum content of Mg^{2+} for cell autoflocculation.

Determination of Ca^{2+} minimum concentration for cell harvesting

Similarly, the minimum Ca^{2+} and PO_4^{3-} concentrations required to allow cell flocculation by calcium phosphate were examined. The tests were carried out for 3 different concentrations of both Ca^{2+} and PO_4^{3-}. However, unlike the flocculation induced by magnesium, maximum clarification could not be achieved, even at high

Ca^{2+} and PO_4^{3-}concentrations.

Regarding magnesium compounds, only magnesium hydroxide has been assumed to contribute to microalgae flocculation (Vandamme *et al.,* 2015). As the magnesium hydration enthalpy is much higher than that of calcium, the incorporation of Mg into a crystal is much difficult and it has been estimated that magnesite ($MgCO_3$) precipitation rates were at least three to four orders of magnitude lower than calcite ($CaCO_3$) (Arvidson, Mackenzie, 2000). According to this explanation, it can be hypothesized that magnesium phosphates would also precipitate slower than calcium phosphates. However, Wright *et al.* (2011) reported that in presence of magnesium, a substituted form of β tricalcium phosphate can precipitate from low temperature aqueous solutions as whitlockite ($Ca_{18}Mg_2H_2(PO_4)_{14}$) (Wright *et al.*, 2011). In fact, concentrations of 199.2 mg/L $CaCl_2$. $2H_2O$ and 50 mg/L KH_2PO_4 were sufficient to achieve an efficient flocculation (E = 81%). By doubling the concentration of phosphate ion, it is necessary to increase the concentration of Ca^{2+} to 500 mg/L $CaCl_2$ to have cell flocculation, that is to say a molar Ca/PO_4 ratio higher than the previous case (4.6 against 3.7). Then, as the test at 500 mg/L $CaCl_2$ with 300 mg/L, and 50 mg/L KH_2PO_4 showed a increase so high in level of metal concentrations, the ratio of Ca/PO_4 dropped sharply to 1.55. In the first two cases, this could be explained by differences in precipitating compounds. In the latter, the concentrations are such that whatever the compound involved, the precipitation of cations entrains the microalgal cells very quickly. However, the higher the content of salt in culture media, the lower the acception in application of cell harvesting because of involving environmental factors and salt-adapted cell.

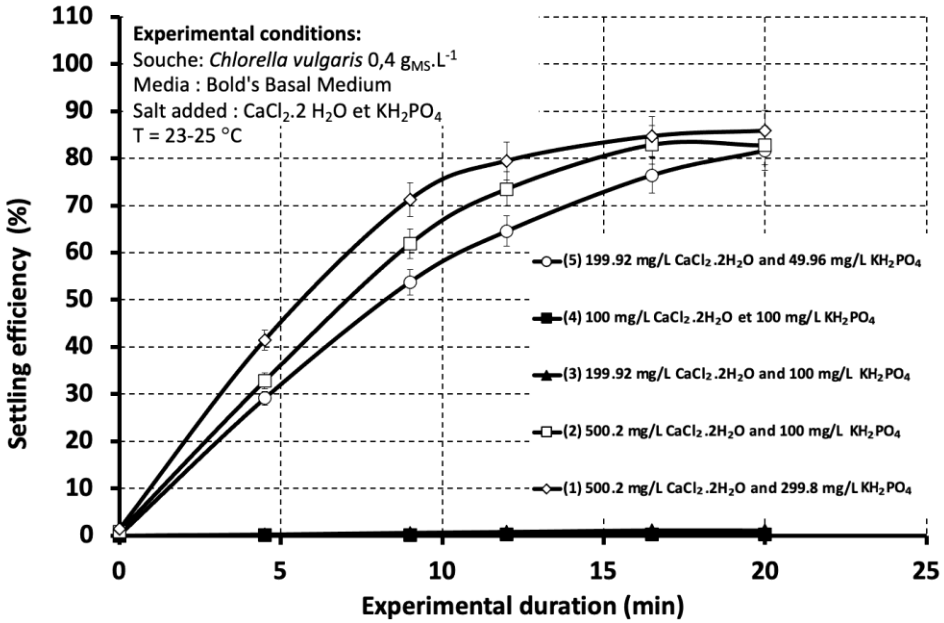

Figure 3. Determination of Ca^{2+} minimum concentration for cell autoflocculation.

The literature highlights the importance of the composition of the medium on its ability and efficiency to flocculate microalgae. Thus, Cao *et al.* (2007) showed that the precipitation of calcium phosphate compounds (in particular hydroxyapatite HAP $Ca_5(PO_4)_3OH$) was inhibited by the presence of several types of compounds, in particular the magnesium ion Mg^{2+} or humic acids, and in to a lesser extent, SO_4^{2-} ions or silica (Cao *et al.,* 2007). Their

incorporation into the precipitate of PAHs may prevent the formation of a clean crystal structure, or these compounds (especially humic acid) may adsorb onto the crystalline nuclein formation and limit their growth (Metsoviti, Papapolymerou, Karapanagiotidis, Katsoulas, 2019).

Determination of minimum content of bacterial cell for microalgal harvesting

Unlike microalgal harvesting by salt cation that flocs of microalgae were collected in 25 minutes, experiments with bacterial strains were lasted up to 24 hours. Results of microalgal flocculation by bacteria in Figure 4 showed that both of bacterial strains enhanced the aggregation of microalgal cells, leading to increase the elimination of microalgal cells out of culture suspension. In fact, microalgal cells were settled to the bottom of Erlenmeyer with the highest settling efficiency of 93% under the presence of *E. coli* ACTT 85922 over night. Comparing with harvesting methods of adding salt cations in culture media, the efficiency was much higher than those of salt cations.

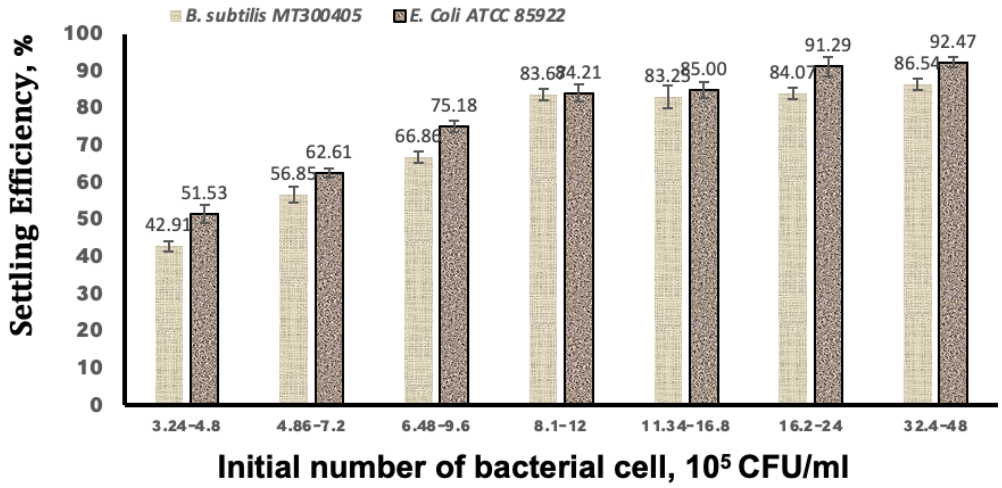

Figure 4. Microalgal autoflocculation - function of initial cell density of bacteria strains as *E. coli* and *B. Subtilis.*

In figure 4, results also indicated that type strain of bacteria did not involve the settling efficiency, which was over 80% with the adjustment of *E. coli* and *B. subtlilis* in microalgal culture media at initial cell density of 12×10^5 CFU/mL, and 8.1×10^5 CFU/mL, respectively. Various studies proved that using technologies of microalgal-bacterial aggregation enhanced the removing of nutrients in wastewater treatment. According to Arcila and Buitrón, (2016), the formation of microalgal-bacterial bioflocs (MABAs) increased the settling velocity (V_s) up to 8.3 m/h in the treatment of municipal wastewater as compared to an average V_s of 2 m/h (Gardes *et al.,* 2011). Otherwise, microalgal biomass forming MABAs enable the downstream processing facilitate to large scale as compared to chemical harvesting (Hende *et al.*, 2016; Chen *et al*., 2011). In their research, MABAs were able to be dewatered in a filter press with average large pores of 200 μm , which was once a huge obstacle in harvesting microalgae. A recent study proposed a promising method to obtain better performance in nutrient removal by establishing stable algal-bacterial aerobic granular sludge in wastewater treatment system (Zhang *et al.,* 2019). However, most of previous studies did not discuss about the initial cell density of bacteria, which was adjusted in the treating media to provoke the bioflocs. Meanwhile, our study did not only resulted in the high settling efficiency of microalgae cultured in

conventional media but also indicated minimum number of bacterial cell to conduct microalgae-bacteia aggregates.

CONCLUSION

In this study, *C. vulgaris* 211-19 was investigated following a new method to rapidly establish flocculation in harvesting. Normally, chemical flocculation has once proved to have better microalgal settling efficiency in microalgal harvesting than other ones. Likewise, bacterial strain was also an important object to conduct MABAs, which led to rapidly settle microalgae and nutrients. This work proposed a promising method to establish stable microalgal-bacterial flocs in their own media with minimum cell density of *B. subtilis* and *E. coli* at 12×10^5 CFU/mL, and 8.1×10^5 CFU/mL, respectively. With chemical flocculation, the microalgal settling efficiency was recorded up to 81% for the adjustment of minimum 199.2 mg/L $CaCl_2.2H_2O$, 50 mg/L KH_2PO_4, and to 70% for adding of minimum 141 mg/L $MgSO_4.7H_2O$.

Acknowledgement*: This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106.04-2019.54.*

REFERENCES

Arvidson RS, Mackenzie FT (2000) Temperature dependence of mineral precipitation rates along the CaCO3-MgCO3 join. *Aquat Geochem* 6(2), 249–256. https://doi.org/10.1023/A:1009619426406

Cao X, Harris WG, Josan MS, Nair VD (2007) Inhibition of calcium phosphate precipitation under environmentally-relevant conditions. *Sci Tot Environ* 383(1–3), 205–215. https://doi.org/10.1016/j.scitotenv.2007.05.012

Chen CY, Yeh KL, Aisyah R, Lee DJ, Chang JS (2011) Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresour Technol* 102(1), 71–81. https://doi.org/10.1016/j.biortech.2010.06.159

Duan J, Gregory J (2003) Coagulation by hydrolysing metal salts. *Adv Colloid Interface Sci*, 100–102(SUPPL.), 475–502. https://doi.org/10.1016/S0001-8686(02)00067-2

Gardes A, Iversen MH, Grossart H-P, Passow U, Matthias S Ullrich (2011) Diatom-associated bacteria are required for aggregation of Thalassiosira weissflogii. *ISME J* (5, 436–445. https://doi.org/10.1038/ismej.2010.145

Hende S Van Den, Beelen V, Julien L, Lefoulon A, Vanhoucke T, Coolsaet C, Sonnenholzner S, Vervaereb H, Rousseau DPL (2016) Technical potential of microalgal bacterial floc raceway ponds treating food-industry effluents while producing microalgal bacterial biomass: An outdoor pilot-scale study. *Bioresour Technol*, 218, 969–979. https://doi.org/10.1016/j.biortech.2016.07.065

Li C, Somasundaran P (1991) Reversal of bubble charge in multivalent inorganic salt solutions-Effect of magnesium. *J Colloid Interface Sci* 146(1), 215–218. https://doi.org/10.1016/0021-9797(91)90018-4

Liu W, Huang F, Liao Y, Zhang J, Ren G, Zhuang Z, Zhen J, Lin Z, Wang C (2008) Treatment of CrVI-containing Mg(OH)2 nanowaste. *Angew Chem Int Ed*, *47*(30), 5619–5622. https://doi.org/10.1002/anie.200800172

Metsoviti MN, Papapolymerou G, Karapanagiotidis IT, Katsoulas N (2019) Comparison of growth rate and nutrient content of five microalgae species cultivated in greenhouses. *Plants* 8(8), 1–13. https://doi.org/10.3390/plants8080279

Nguyen TDP, Frappart M, Jaouen P, Pruvost J, Bourseau P (2014) Harvesting Chlorella vulgaris by natural increase in pH: Effect of medium composition. *Environ Technol* (United Kingdom), 35(11), 1378–1388. https://doi.org/10.1080/09593330.2013.868531

Nguyen TDP, Le TVA, Show PL, Nguyen TT, Tran MH, Tran TNT, Lee SY (2018) Bioflocculation formation of microalgae-bacteria in enhancing microalgae harvesting and nutrient removal from wastewater effluent. *Bioresour Technol* 272(October 2018), 34–39. https://doi.org/10.1016/j.biortech.2018.09.146

Parks G (1967) *Equilibrium Concepts in Natural Water Systems*. (W. Stumm, Ed.) (Vol. 67). WASHINGTON, D.C.: AMERICAN CHEMICAL SOCIETY. https://doi.org/10.1021/ba-1967-0067

Ritchie RJ (2006) Consistent sets of spectrophotometric chlorophyll equations for

acetone, methanol and ethanol solvents, *Photosynth Res* 89, 27–41. https://doi.org/10.1007/s11120-006-9065-9

Schryver P De, Crab R, Defoirdt T, Boon N, Verstraete W (2008) The basics of bio- flocs technology : The added value for aquaculture. *Aquaculture* 277, 125–137. https://doi.org/10.1016/j.aquaculture.2008.02.019

Semerjian L, Ayoub GM (2003) High-pH-magnesium coagulation-flocculation in wastewater treatment. *Adv Environ Res* 7(2), 389–403. https://doi.org/10.1016/S1093-0191(02)00009-6

Smith BT, Davis RH (2012) Sedimentation of algae flocculated using naturally-available, magnesium-based flocculants. *Algal Res* 1(1), 32–39. https://doi.org/10.1016/j.algal.2011.12.002

Sukenik A, Shelef G (1984) Algal autoflocculation—verification and proposed mechanism. *Biotechnol Bioeng* 26(2), 142–147. https://doi.org/10.1002/bit.260260206

Syafri J, Ahmad M, Cai W, Zhao Z, Zhang Z, Lei Z, Lee D (2017) Stability of algal-bacterial granules in continuous-flow reactors to treat varying strength domestic wastewater. *Bioresour Technol* 244, 225–233. https://doi.org/10.1016/j.biortech.2017.07.134

Tran TNT, Nguyen TDP, Dinh HT, Bui TT, Ho LH, Nguyen PTX, Khoo KS, Chew KW, Show PL (2021) Characterization of bacteria type strain Bacillus spp. isolated from extracellular polymeric substance harvested in seafood wastewater. *J Chem Technol Biotechnol*, (July). https://doi.org/10.1002/jctb.6870

Vandamme D, Beuckels A, Markou G, Foubert I, Muylaert K (2015) Reversible Flocculation of Microalgae using Magnesium Hydroxide. *Bioener Res* 8(2), 716–725. https://doi.org/10.1007/s12155-014-9554-1

Vandamme D, Foubert I, Muylaert K (2013, April). Flocculation as a low-cost method for harvesting microalgae for bulk biomass production. *Trends Biotechnol*. https://doi.org/10.1016/j.tibtech.2012.12.005

Wang Q, Li H, Shen Q, Wang J, Chen X, Zhang Z, Fang LZ, Yuan T, Liu Y, Lee D-J (2022) Biogranulation process facilitates cost-efficient resources recovery from microalgae-based wastewater treatment systems and the creation of a circular bioeconomy. *Sci Tot Environ* 828. https://doi.org/10.1016/j.scitotenv.2022.154471

Wright KE, Hartmann T, Fujita Y (2011) Inducing mineral precipitation in groundwater by addition of phosphate. *Geochem Trans* 12. https://doi.org/10.1186/1467-4866-12-8

Zhang Y, Dong X, Liu S, Lei Z, Shimizu K, Zhang Z, Adachi Y, Lee DJ (2019) Rapid establishment and stable performance of a new algal-bacterial granule system from conventional bacterial aerobic granular sludge and preliminary analysis of mechanisms involved. *J Water Process Engin* (November), 101073. https://doi.org/10.1016/j.jwpe.2019.101073