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Characterization of the flocculating agent from the spontaneously flocculating microalga *Chlorella vulgaris* JSC-7

Md. Asraful Alam,[1] Chun Wan,[1] Suo-Lian Guo,[1] Xin-Qing Zhao,[1,*] Zih-You Huang,[2] Yu-Liang Yang,[3] Jo-Shu Chang,[4] and Feng-Wu Bai[1]

School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China,[1] Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan,[2] Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan,[3] and Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan[4]

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High cost of biomass recovery is one of the bottlenecks for developing cost-effective processes with microalgae, particularly for the production of biofuels and bio-based chemicals through biorefinery, and microalgal biomass recovery through cell flocculation is a promising strategy. Some microalgae are naturally flocculated whose cells can be harvested by simple sedimentation. However, studies on the flocculating agents synthesized by microalgae cells are still very limited. In this work, the cell flocculation of a spontaneously flocculating microalga *Chlorella vulgaris* JSC-7 was studied, and the flocculating agent was identified to be cell wall polysaccharides whose crude extract supplemented at low dosage of 0.5 mg/L initiated the more than 80% flocculating rate of freely suspended microalgae *C. vulgaris* CNW11 and *Scenedesmus obliquus* FSP. Fourier transform infrared (FTIR) analysis revealed a characteristic absorption band at 1238 cm^{-1}, which might arise from P=O asymmetric stretching vibration of PO_2^- phosphodiester. The unique cell wall-associated polysaccharide with molecular weight of 9.86×10^3 g/mol, and the monomers consist of glucose, mannose and galactose with a molecular ratio of 5:5:2. This is the first time to our knowledge that the flocculating agent from *C. vulgaris* has been characterized, which could provide basis for understanding the cell flocculation of microalgae and breeding of novel flocculating microalgae for cost-effective biomass harvest.

[**Key words:** *Chlorella vulgaris*; Spontaneous flocculation; Biomass harvest; Flocculating agent; Cell wall polysaccharides]

Microalgae are potential biomass sources for biorefinery to produce biofuels and bio-based products due to their advantages, such as not competing for arable land with agricultural production, high efficiency in capturing solar energy, and mitigating CO_2 emissions compared to terrestrial plants (1). In addition to mass cultivation, biomass recovery of tiny microalgal cells presents another challenge, since the biomass density is extremely low in open culture systems that are suitable for microalgae culture at low cost for the biorefinery purpose. For example, dry cell weight (DCW) achieved with raceway ponds is only 0.14 g/L (2), which consequently produces large amount of culture broth.

Currently, filtration using various filters such as plate-and-frame pressure filters, rotary-drum filters and vacuum filters has been applied in microalgae dewatering with a long history, and is suitable for small scale cultures with closed photobioreactors for value-added products, in which much higher biomass density is achieved, and the quantity of culture broth is much smaller. Nowadays, more efficient membrane-based technologies have been developed for microalgae dewatering (3). On the other hand, centrifugation with disk stack centrifuge is applied for microalgae harvest when more culture broth is processed, but significant capital investment on the equipment and energy consumption on its operation are needed (4,5). Apparently, these technologies are not practical for microalgal biomass recovery at larger scales for the purpose of biorefinery.

Biomass recovery by cell sedimentation through the flocculation of microalgal cells does not require significant capital investment, and energy consumption associated with the process is negligible, making it one of the most economically competitive strategies for microalgal biomass harvest at large scales (6). Although microalgal cells can be flocculated by chemicals such as Al^{3+}, Fe^{3+} and polyelectrolytes (6,7), large amount of wastewater discharged from these processes is not suitable for microalgae cultivation, and the chemical flocculation also raises environmental concerns on the wastewater treatment.

To date, several spontaneously flocculating microalgae have been discovered, such as *Ankistrodesmus falcatus*, *Tetraselmis suecica*, *Scenedesmus obliquus* and *Ettlia texensis* (8–10), the cells of which can settle down from culture broth for easy biomass recovery, which enables a novel strategy in microalgae harvesting via cell self-flocculation. It was reported that water-soluble extract of marine microalga *Skeletonema marinoi* induced flocculation of *Nannochloropsis oculata*; however, it was not clear which specific compound might be responsible for the flocculation (11). No doubt, elucidation of the mechanism underlying the spontaneous flocculation of those flocculating microalgal species and studies of the flocculating agents will benefit genetic modifications of non-

* Corresponding author. Tel.: +86 411 8470 6319; fax: +86 411 8470 6329.
E-mail address: xqzhao@dlut.edu.cn (X.-Q. Zhao).

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flocculating microalgal strains with the flocculation phenotype for cost-effective biomass recovery by sedimentation. Unfortunately, flocculating agents produced by microalgae are so far not well characterized.

In the present study, the spontaneous flocculation of *Chlorella vulgaris* JSC-7 was studied, and the flocculating agent was characterized. Experimental results indicated that cell wall polysaccharide was responsible for the flocculation of the species, which lay a basis for further understanding of their biosynthetic pathways and identifying genes encoding key enzymes for the biosynthesis of the flocculating agents in order to engineer non-flocculating microalgal strains with the flocculation phenotype for cost-effect biomass recovery.

MATERIALS AND METHODS

Microalgal strains and culture conditions The self-flocculating microalgal strain *C. vulgaris* JSC-7 used in this work was isolated from fresh water in Southern Taiwan. The modified Bold's Basal Medium with 3-fold nitrogen supplementation (3N-BBM) was used for the growth of *C. vulgaris* JSC-7. The composition of the 3N-BBM medium (g/L) was: $NaNO_3$, 0.75; $CaCl_2$, 0.025; $MgSO_4{\cdot}7H_2O$, 0.075; $K_2HPO_4{\cdot}3H_2O$, 0.075; KH_2PO_4, 0.175, NaCl, 0.025; EDTA, 0.0045; $FeCl_3$, 0.00058; $MnCl_2{\cdot}4H_2O$, 0.00046; $ZnCl_2$, 0.00003; $CoCl_2{\cdot}6H_2O$, 0.00004; Na_2MoO_4, 0.0003. The pH of the medium was adjusted to 6.9. Non-flocculating microalgae strains *C. vulgaris* CNW11 and *S. obliquus* FSP were selected and cultivated with BG11 medium (12) and DM medium (13), respectively, to test the function of the flocculating agents isolated from *C. vulgaris* JSC-7.

All the algal strains were cultivated at 28°C under a 13/11 h light/dark cycle with an average illumination of 25.0 μmol/m^2/s.

Biomass density measurement and morphology of *C. vulgaris* JSC-7 Microalgal biomass density was measured by optical density at 690 nm with a spectrophotometer (Varioskan Flash, ThermoFisher, USA). Scanning electron microscopy (SEM, Quanta 450, FEI, USA) was used to observe the morphology of the flocculation of *C. vulgaris* JSC-7.

Evaluation of microalgal flocculation After two weeks cultivation, the cultures of the three microalgal strains were harvested and centrifuged at 6000 rpm for 10 min. The cell pellets were washed with distilled water and re-suspended to the final OD_{690} of ~0.9. Three experiments were performed to evaluate the flocculation efficiency of microalgae: the flocculating *C. vulgaris* JSC-7 (experiment A), the non-flocculating *C. vulgaris* CNW11 and *S. obliquus* FSP (experiment B), and the mixture of *C. vulgaris* JSC-7 with *C. vulgaris* CNW11 and *S. obliquus* FSP (experiment C), respectively, at volumetric ratios of 1:5 and 1:2. All these experiments were performed in duplicate.

For the flocculation assay, microalgae cultures were shaken to disperse the cells, and 10 mL cell suspension was transferred into a test tube to measure OD_{690} by sampling 200 μL cell suspensions at the depth of 4 cm from the top surface, which was designated as *A*. After the test tubes were rested for 30 min, another 200 μL cell suspension was sampled to measure OD_{690}, which was designated as *B*. The flocculation efficiency *F* was calculated by the equation

$$F = (1 - B/A) \times 100\% \quad (1)$$

(14) Evaluation of microalgal flocculation was performed at room temperature, and no metal ion was added during flocculation experiment unless otherwise specified. The same method was also used to measure the flocculating effect of the purified flocculating agents from *C. vulgaris* JSC-7 on the flocculation of *C. vulgaris* CNW11 and *S. obliquus* FSP. To obtain better understanding of flocculating characteristics of flocculating agents, effects of metal ions, pH, and temperature on flocculation efficiency of freely suspended strain *C. vulgaris* CNW11 were investigated. The pH value was adjusted to the range of 4–10 using 5 mM HCl or NaOH. The temperature was controlled in water bath from 15 to 50°C and 120°C in autoclave for 20 min. Metal salts of $FeCl_3$, $AlCl_3$, $CaCl_2$ and KCl at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg/L were tested as cationic coagulants, while $FeCl_3$, $AlCl_3$, and KCl at concentrations of 5–25 mM were tested with the addition of 0.5 mg/L purified flocculating agent.

Purification and characterization of the flocculating agents from *C. vulgaris* JSC-7 The culture of *C. vulgaris* JSC-7 was centrifuged at 6000 ×*g* for 10 min to separate cells from the supernatant. While cell wall-bound polysaccharides were extracted by pronase treatment and ethanol precipitation following the methods described previously (15), two volumes of ethanol were added into the supernatant to precipitate the flocculating agents in the culture broth.

Both fractions were dialyzed overnight with MD34 (GE, USA), after which they were dissolved in TE buffer (pH 8.0) and upload to a DE52 anion exchange cellulose column (Whatman, UK) previously equilibrated with TE buffer (pH 8.0), and then eluted with 0.1 M NaCl solution at a flow rate of 0.5 mL/min. The eluted fractions were lyophilized to obtain the purified compound for further characterization.

The total sugar content of the flocculating agents was determined by the phenol-sulfuric acid method using glucose as the standard (16), and their molecular weight was analyzed by gel permeation chromatography (GPC, 1200 Series, Agilent Technology, USA) with the eluent of ddH_2O at the rate of 0.5 ml min^{-1} and chitosan as the standard. Elemental analysis was carried out using an elemental analyzer (varioEL III, Elementar, Germany). The monomer composition of the polysaccharides was analyzed using gas chromatography/mass spectrometry (GC/MS) according to the procedure described elsewhere (17). The FTIR spectra of the flocculating agent were obtained with a FTIR spectrometer (ThermoFisher NEXUS) in the frequency range of 400–4000 cm^{-1}. Phosphate ion was detected and concentrated using ion chromatography (ICS-5000, Dionex, USA) with the eluent of KOH at the rate of 1.0 ml min^{-1} using PO_4^{3-} solution as the standard (18). EPS sample was diluted in ddH_2O before detecting the phosphate ion.

RESULTS AND DISCUSSION

Flocculation of *C. vulgaris* JSC-7 In shake flask culture, *C. vulgaris* JSC-7 cells settled down for biomass recovery due to their spontaneous flocculation. Therefore, the flocculation efficiency of *C. vulgaris* JSC-7 and its ability of flocculating freely suspended *C. vulgaris* CNW11 and *S. obliquus* FSP cells were further studied (Table 1).

Compared to the non-flocculating *C. vulgaris* CNW11 and *S. obliquus* FSP, *C. vulgaris* JSC-7 exhibited much higher flocculation efficiency of 76%. On the other hand, the supplementation of *C. vulgaris* JSC-7 culture into that of *C. vulgaris* CNW11 and *S. obliquus* FSP facilitated their flocculation, making their flocculation efficiencies increased to 68.3% and 62.7%, respectively, from 25.6% and 28.1% observed with the original cultures of the microalgal strains. Moreover, it was found that the flocculation efficiency of the non-flocculating microalgae was higher when more *C. vulgaris* JSC-7 culture was supplemented. Similar observations were also reported elsewhere (9,19) using the flocculating *E. texensis*, *A. falcatus* and *S. obliquus* to harvest the non-flocculating *C. vulgaris*.

Many studies reported that increasing pH could induce the flocculation of various microalgae cells (20). We thus adjusted the pH value ranging from 6 to 10 using HCl and NaOH to investigate the flocculation efficiency of *C. vulgaris* JSC-7, but no significant difference was observed, since slightly higher flocculation efficiency of 76% was at pH 7.0, while 74% and 75% were observed at pH 6.0 and 10.0, respectively, suggesting that the naturally occurred flocculation of *C. vulgaris* JSC-7 was not affected by the pH variations. It has been generally accepted that the mechanisms of yeast flocculation involve the binding of cell surface flocculins (glycoproteins) with sugar residues of the flocculins on the surface of surrounding cells (21,22). We thus further investigated the cell surface components for flocculating agents produced by the flocculating microalga.

Purification and characterization of the flocculating agent of *C. vulgaris* JSC-7 Scanning electron microscopy (SEM) visualization revealed that *C. vulgaris* JSC-7 cells were attached to each other (Fig. 1), while no attachment was observed in the freely-suspended *C. vulgaris* CNW11 and *S. obliquus* FSP. The aggregation observed

TABLE 1. Flocculation rate of self-flocculation and freely suspended microalgae.

Algae strains	OD at 690 nm		Flocculation efficiency (%)
	A	B	
C. vulgaris JSC-7	0.891	0.221	76.3
C. vulgaris CNW11	0.880	0.654	25.6
S. obliquus FSP	0.890	0.639	28.1
C. vulgaris JSC-7: *C. vulgaris* CNW11 at 1:5	0.862	0.562	34.8
C. vulgaris JSC-7: *C. vulgaris* CNW11 at 1:2	0.885	0.280	68.3
C. vulgaris JSC-7: *S. obliquus* FSP at 1:5	0.921	0.542	41.2
C. vulgaris JSC-7: *S. obliquus* FSP at 1:2	0.889	0.332	62.7

A, homogenized cell suspension; B, sedimentation after 30 min. Samples were withdrawn from a depth of 4 cm at the top surface to measure the optical density at 690 nm.