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Isolation and characterization of autoflocculating mutants of cyanobacterium *Arthrospira platensis*

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Abstract−Harvesting microalgae is a major concern for mass culture in industry. Flocculation is an easy and effective way to harvest microalgae. However, flocculation using chemical flocculants is not feasible for scaling-up due to their toxicity. As an alternative technique, mutation breeding of autoflocculating microalgae strain has been reported in this study. We characterized autoflocculating mutants of *Arthrospira platensis* (*A. platensis*) by ethyl methane sulfonate (EMS). The cells of mutants were aggregated during the culture and dry cell weight increased 1.2- to 1.8-fold compared to the wild type. Autoflocculation was induced highly at an optimal pH level of 9 and the flocculation efficiency reached almost 90%. Mutants showed higher flocculation efficiency irrespective of the addition of chemical flocculants. Thus, it is definitely useful to harvest microalgae using autoflocculating mutants in large-scale culture without any drawbacks of harvested algal biomass.

Key words: Microalgae, *Arthrospira platensis*, Autoflocculation, Ethyl Methane Sulfonate, Mutation

INTRODUCTION

Arthrospira platensis (*A. platensis*) is an economically important filamentous cyanobacterium. Extensive studies on improving its growth condition and cultivation have been carried out for mass production [1,2]. One of the problems in large-scale production of microalgae is harvesting cells due to the high cost of operating during process [3,4]. Flocculation is preferred for harvesting microalgae due to its low cost compared to other methods, such as centrifugation, filtration and sedimentation [5]. The flocculation efficiency of microalgae can be improved by the use of flocculants [6]. It was reported that algae were flocculated using chemical flocculants, such as aluminum sulfate and ferric chloride [7,8]. Chemical flocculants have been used widely due to their effective flocculation efficiency and lower cost [9,10]. However, it is not appropriate for harvesting of microalgae in large-scale culture because excess cationic flocculants need to be removed from the medium [11]. Because toxic alum and iron are left in the biomass, they have negative effect on the quality of the final products.

Sometimes, effective flocculation could be achieved by simply adjusting the pH, especially alkaline condition, of the microalgae culture medium. *Botryococcus braunii* was harvested effectively by adjusting the pH to 11. In this case, flocculation activity was higher than when using aluminum sulfate and microbial flocculant Pestan [12]. However, it is not certain that it is applicable to other microalgae which are grown in alkaline conditions like *A. platensis*.

As alternative ways for replacement to toxic chemicals, bioflocculation of microalgae with bacteria was studied widely because they are biodegradable, nontoxic and without secondary pollution [13]. Others, strain improvement by induced mutation was applied in biotechnological industries [14,15]. It is an active area for research, being possible to develop an appropriate and economical harvesting system for any microalgae species. Little is known about the autoflocculating microalgae strains by mutation. In this study, we described the characteristics of autoflocculating mutants of *A. platensis* derived from EMS.

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EXPERIMENTAL SECTION

1. Algal Strain and Culture Conditions

Arthrospira platensis NIES 39 (KCTC AG30033) was obtained from the Biological Resource Center of Korea Research Institute of Bioscience and Biotechnology (KRIBB). *A. platensis* was grown in SOT medium (pH 9) at 35 °C with 6,000 lux light illumination and constant shaking (120 rpm). The composition of SOT medium is as follows: 16.8 g $NaHCO_3$, 0.5 g K_2HPO_4, 2.5 g $NaNO_3$, 1 g K_2SO_4, 1 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.08 g Na_2EDTA, 0.03 mg H_3BO_3, 0.025 mg $MnSO_4 \cdot 7H_2O$, 0.002 mg $ZnSO_4 \cdot 7H_2O$, 0.0079 mg $CuSO_4 \cdot 5H_2O$ and 0.0021 mg $Na_2MoO_4 \cdot 2H_2O$ in 1 *l* distilled water. The algal growth was monitored by measuring the absorbance at 520 nm using a spectrophotometer.

2. Ethyl Methane Sulfonate (EMS) Mutagenesis

Exponentially grown culture of *A. platensis* cells was harvested by centrifugation at 2,000 g for 10 min. The cells were washed with phosphate buffered saline (PBS) and treated with 1% EMS (Sigma, USA) in the dark with moderate shaking for 1 h at 35 °C. After incubating, the cells were washed with distilled water to remove the remaining EMS and kept in the dark for 24 h at 4 °C. Cells were diluted with SOT medium and plated on SOT solid medium, then incubated at 35 °C about three weeks until the colonies came out.

The resulting colonies were isolated and further cultured in liquid medium.

3. Measurement of Flocculating Efficiency

Measurement of flocculating efficiency was modified following protocol described by Lee et al. [12]. The flocculation efficiency was evaluated by comparing the remaining cell density in the clear region with the concentration before flocculation. The algae culture suspension (10 ml) was placed in a 50 ml falcon tube. The cell suspension was stirred for 1 min and left to settle for 1 h. After the flocculation of algae cells, an aliquot of cells in the upper region was withdrawn and measured the absorbance at 520 nm.

To determine the effect of pH on flocculation, each containing 10 ml culture medium in 50 ml conical tube was adjusted with pH 4, 7, 9 and 12 by adding 0.1 M HCl or 6 N NaOH. The algae cells were inoculated in the different pH medium. After incubation for three days, the cells were harvested. The effect of chemical flocculants was tested by adding ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$) and zinc sulfate ($ZnSO_4$). Different dosage of chemical flocculants, 50, 100, 200, 300, 400 and 500 mg/*l*, was added to the aliquots of culture, followed with mixing and allowing flocculation. Flocculation efficiency (%) was calculated by the following equation:

Flocculation efficiency (%)=1/A− 1/B×100,

where A is absorbance of sample, B is absorbance of reference.

4. Chlorophyll Extraction and Biomass Analysis

Algal cells were centrifuged at 2,000 g for 10 min, rinsed with distilled water and pellet was extracted with methanol at 60 °C until the green color faded out. After centrifugation, the supernatant was measured with spectrophotometer at 665 nm and 650 nm for chlorophyll determination [15].

Biomass concentration in the culture suspension was determined as dry cell weight [13]. The dry cell weight was measured by filtering an aliquot (1 ml) of culture suspension on a pre-weighed Whatman filter. The filters were dried at 105 °C for 3 h and reweighed.

RESULTS AND DISCUSSION

1. Isolation of *A. platensis* Mutants

A. platensis was mutagenized with the chemical mutagen, EMS. Roughly 70 colonies were screened and selected mutants based on the amount of flocs they produced (small, medium and large). They were designated E3K, E33K and E41K, respectively, and those strains were used for further analysis. The culture medium of the wild type of *A. platensis* showed homogeneity, but several flocs were observed in the medium of mutant cells after two weeks of culture (Fig. 1(a)). Microscopic examination of wild type indicated that the cells formed long and loosely coiled shapes as a single cell. However, they stuck together in massive clumps and aggregation occurred in the mutant cells (Fig. 1(b)). Culturing of these clumps in fresh medium showed growth as usual, indicating the cells were alive even after flocculation and capable of reproduction and growth.

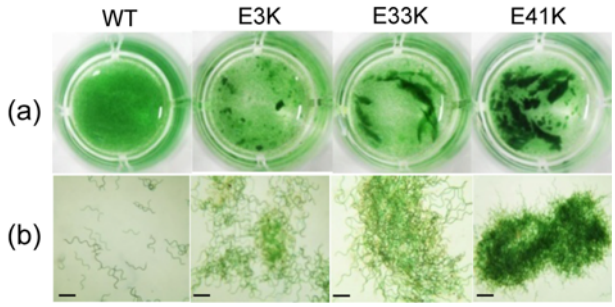

Fig. 1. Selection of autoflocculating mutants of *A. platensis*. (a) Phenotype of wild type and autoflocculating mutants of *A. platensis*. The algae cells from the stationary phase were taken from the culture medium and replated in 24-well plate. (b) Microscopic image of wild type and mutants. The formed flocs were observed and photographed under a Leica microscope (Wetzlar, Germany) equipped with digital camera system (Canon, USA). The scale bar represents 200 μm.

2. Growth and Flocculation Rate of *A. platensis* Mutants

To assess the influence of the mutants on the growth, we measured time-dependent changes of optical density (Fig. 2(a)). The cultures of wild type and mutants were collected at 3, 6, 9 and 12 days. The growth pattern of mutants was similar to wild type, but high flocculating strain E41K showed lower growth rate.

The aggregated cells making a clump easily fell to the bottom of the medium to be harvested [16]. Measurement of flocculating efficiency was modified following the protocol described by Lee et al. [12]. The flocculating activity of wild type increased rapidly with

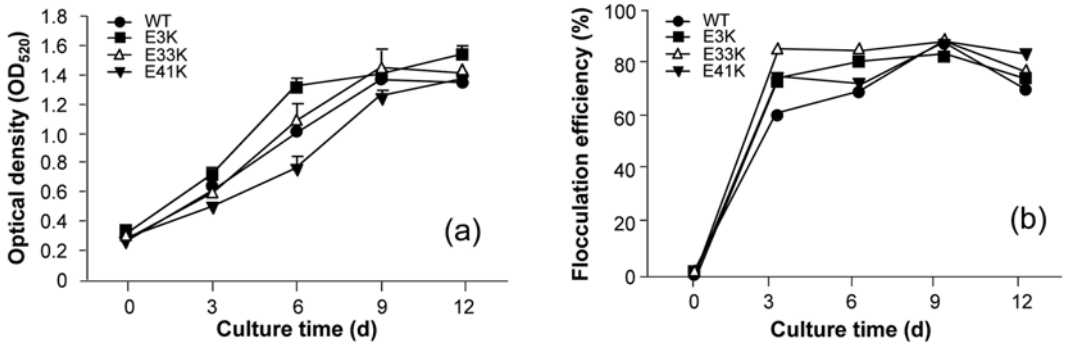

Fig. 2. Growth and flocculation efficiency of *A. platensis*. (a) Growth pattern of wild type and mutants of *A. platensis*. Wild type (●) and mutants (E3K-■, E33K-△, E41K-▼) were cultivated in SOT medium at 35°C for several days. Cell density (OD_{520}) was determined by spectrophotometry at 3-day intervals. (b) Flocculation efficiency of *A. platensis* strains at different growth stages. The cells were taken from each growth stage and measured for flocculation activity. The data represents the average ±SD from two independent experiments.

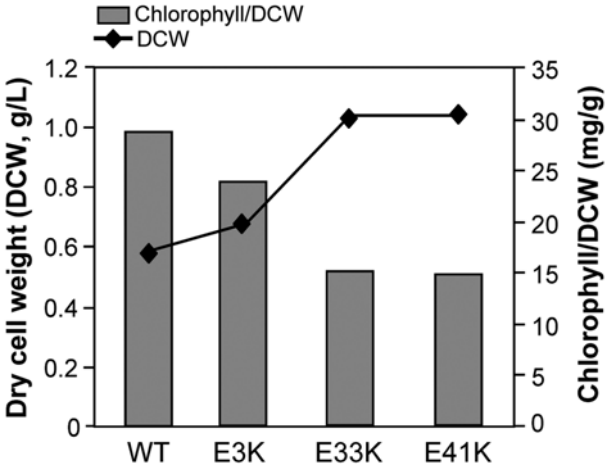

Fig. 3. Biomass and chlorophyll content of wild type and mutants of *A. platensis*. After 12 days of culture, the cells were harvested and measured dry cell weight (DCW) and chlorophyll content per DCW.

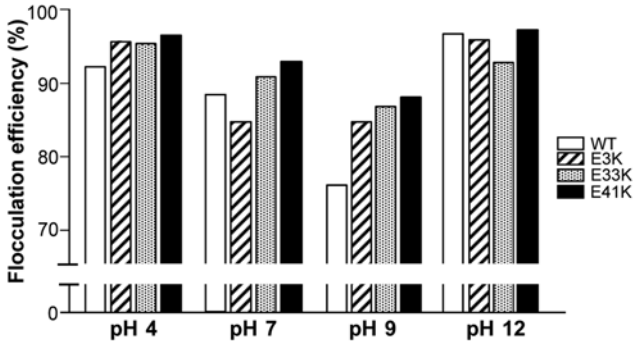

Fig. 4. Effect of pH on flocculation in wild type and mutants of *A. platensis*. The algae cells were inoculated the medium pre-adjusted with pH levels of 4, 7, 9 and 12. After culture for three days, the flocculation activity was measured.

increasing time and reached a maximum value after nine days. The mutants showed higher flocculation efficiency than wild type throughout the growth stage except the stationary phase. The induction rate of flocculation in mutants compared to wild type was highest at three days, relatively at early time, 15%, 24% and 14% increased in E3K, E33K and E41K, respectively (Fig. 2(b)).

3. Chlorophyll Content and Biomass of *A. platensis* Mutants

The amount of chlorophyll per dry cell weight (DCW) in the mutants, E3K (23.7 mg/g), E33K (15.1 mg/g) and E41K (14.6 mg/g), was reduced compared to the wild type (28.6 mg/g) (Fig. 3). Flocculating cells can cause shading, which prevents cells from being exposed to light in the culture medium. Because the light is rapidly attenuated in dense mass culture, the cells can acclimate to light-limited conditions [17]. This represents a limitation in the microalgae mass cultivation when high cell density is required. For this reason, it was reported that chlorophyll antenna size of microalgae was reduced to improve microalgal productivity in mass culture [18]. The mutants having low chlorophyll content could be grown at higher light density and reach higher biomass concentration without low light acclimation. Moreover, dry cell weight of mutants, E3K, E33K and E41K, increased 1.2-, 1.8- and 1.8-fold compared to wild type, respectively, at 12 days after culture. Thus, mutant strains could be grown for high density cultivation without low light acclimation effect.

4. Effects of pH on the Flocculation Efficiency of *A. platensis* Mutants

Some reports showed that the pH of the culture condition affected the flocculation efficiency [3,19]. The optimum pH for growth of *A. platensis* is 9. To determine the effects of pH on the flocculation activity of mutants, we examined these strains at various pH values. Fig. 4 shows the flocculation efficiency of wild type and mutants of *A. platensis* cultures with pH adjustment prior to flocculation. A flocculation of *A. platensis* was induced by changing the culture conditions by applying extreme pH values, such as pH 4 or pH 12. However, in this condition, harvesting of the *A. platensis* at large-scale culture was not desirable, because maximum biomass production was obtained under pH 9 [20,21]. Induction rate of flocculation was 14% (E3K), 15% (E33K) and 16% (E41K) at optimal pH.

It was known to be difficult to harvest *Arthrospira* by sedimentation and centrifugation, because they use their gas vesicles mainly composed of hydrophobic protein [22]. In the report of Yoo et al. [23], mutant strains of *A. platensis* by 0.24% EMS were demonstrated. The flotation activity of mutants was increased 2-fold compared to parental strain. In our results, autoflocculating mutants by 1% EMS had higher flocculation efficiency than wild type and reached around 90%. Depending on the EMS concentration, the characteristics of *A. platensis* mutants were different.

5. Effects of Chemical Flocculants on Flocculation of *A. platensis* Mutants

Microalgae cells have negative charges on the surface that prevent the cells from aggregating. To reduce or neutralize the charge, the addition of cations is preferred [24]. Chemical flocculants are known to be effective, but they are toxic and pH adjustment is needed to obtain a maximum flocculating activity. We analyzed the effect of cationic salts on the flocculation activity in wild type and mutants (Fig. 5). $FeCl_3$, $Al_3(SO_4)_2$, or $ZnSO_4$ was added to a final concentration of 50, 100, 200, 300, 400 and 500 mg/*l* in the culture medium. Chemical flocculants were added and caused large microalgal flocs which settled rapidly to the bottom of the container. The flocculation efficiency of the wild type was increased gradually with an increasing dosage of $FeCl_3$ and $Al_3(SO_4)_2$. The flocculation efficiency by adding of $FeCl_3$ and $Al_3(SO_4)_2$ at 500 mg/*l* was 66% and 76%, and the induction rate was 18%, 28%, respectively (Fig. 5(a), (b)). A maximum value of flocculation efficiency was observed at 200 mg/*l* of $ZnSO_4$, and the induction rate was 27% compared to the untreated cells. There was no increase in the higher concentration (Fig. 5(c)). Among three chemicals, Zn^{2+} was more effective for flocculation of *A. platensis*. These results are in agreement with previous study reporting that bivalent cations (i.e. Zn^{2+}) significantly improved flocculating activity. However, addition of monovalent and trivalent cations (i.e. Fe^{3+}, Al^{3+}) resulted in marginal improvement [25]. According to the report of Kim et al. [26], *A. platensis* cells with Al^{3+} and Fe^{3+} were aggregated as our results, but not Na^{+} and Mg^{2+}. Mutants showed higher flocculation efficiency irrespective of addition of chemical flocculants. These results indicated that it was not needed to add chemical flocculants to mutants and this could prevent undesired contamination.

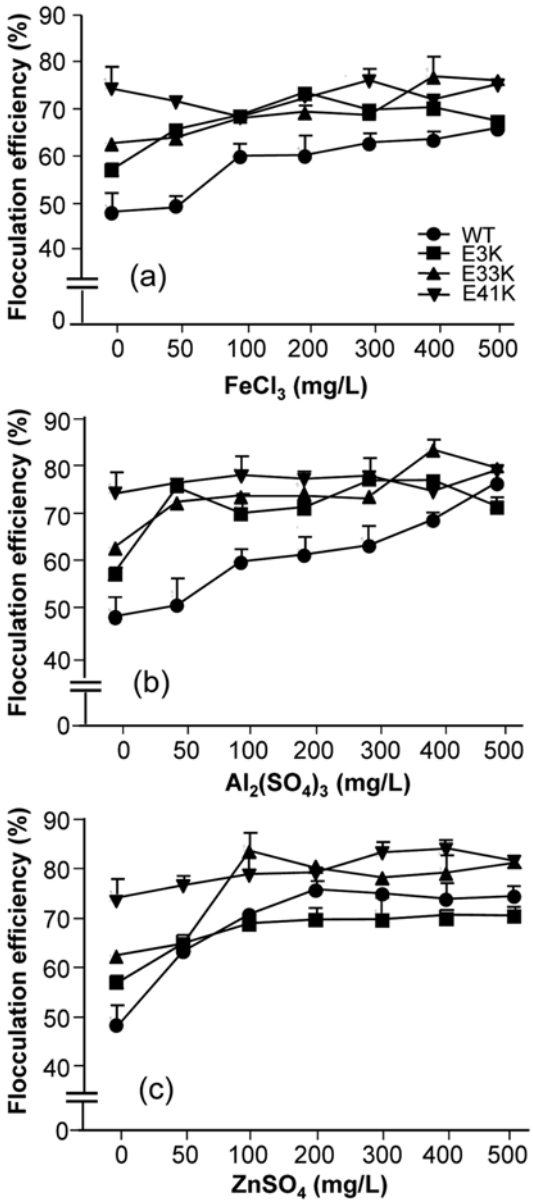

Fig. 5. Flocculation activity of wild type and mutants of *A. platensis* in presence of chemical flocculants. The cells were treated the chemical flocculants, such as ferric chloride, aluminum sulfate and zinc sulfate in a concentration of 50, 100, 200, 300, 400 and 500 mg/*l*. After culture for three days, the flocculation activity was measured. The data represents the average ±SD from two independent experiments.

CONCLUSIONS

The breeding of *A. platensis* mutants high flocculating activity was reported. The autoflocculating three mutant strains, E3K, E33K and E41K, made flocs during culture, and settling rates were enhanced. The biomass of mutants increased although the lower light exposure by shading. Moreover, there was no need to require different culture conditions, because flocculation of mutants was more efficiently induced than that of wild type at optimal pH at 9. Mutants showed higher flocculation efficiency regardless of addition of chemical flocculants. In addition, no extra operational costs are involved for treatment of sediment for further downstream processing. Thus, it is feasible to provide a reliable and safe harvesting process using autoflocculating microalgae strains in large-scale culture.

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