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Growth promotion of *Chlorella vulgaris* by modification of nitrogen source composition with symbiotic bacteria, *Microbacterium* sp. HJ1

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ABSTRACT

To increase algal growth in treated livestock waste water, we designed a culture system targeting symbiotic bacteria. *Microbacterium* sp. HJ1 is a symbiotic bacteria associated with *Chlorella vulgaris*, which was found to increase the growth rate when controlled by nitrogen addition. The validated analysis model for nitrogen source mixture was used to analyze the growth and final pH of *Microbacterium* sp. HJ1, in different compositions of nitrogen sources, by elucidating the functions of each nitrogen ions such as NO_3^-, NO_2^-, and NH_4^+. By modifying the growth medium made from treated livestock waste water with additional nitrogen source, we were able to increase dry cell weight (DCW) of *C. vulgaris* by 65.7% and chlorophyll a contents by 78.8%. This is an example of an indirect method to increase algal biomass by changing the population of symbiotic bacteria, and it is the practical application of positive effects from symbiotic bacteria to the host.

1. Introduction

Microalgae has been widely used for the treatment of waste water to improve water quality [1], and it has been well reported for its use in the treated piggery [2,3], domestic [4], and industrial waste waters [5]. There have been a lot of efforts involved in these developments, because algae have remarkable properties for nitrogen reduction and biodiesel

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production. However, the culture of algae is still very difficult and is poor in growth, because they are easily affected by the nutrient conditions such as nitrogen, sulfur, oxygen, or phosphate, and maintaining its pure culture is also very difficult. As a result, selection of good strains and optimization of culture conditions are needed for better utilization of waste water for algal biomass production [3]. In that point, *Chlorella* sp., a microalgal species with a fast growth rate [6], exhibits a great potential for creating enhanced products.

The mixed culture of algae and bacteria have been used for growth-promoting effect [7], extension of lifetime [8], and mutualistic associations between microalgae and heterotrophic bacteria [9]. It has been reported with bacterial uptake of extracellular organic carbon (EOC), which is released during the microalgal photosynthesis, and with microalgal uptake of growth promoting factors produced by heterotrophic bacteria [10]. Most of the previous works tried to explain and achieved either the final effect of symbiotic bacteria to algae or the practical application of the mutualism to algae [6,9,10] *Microbacterium* sp. has N_2 fixing ability and can exchange organic nutrient with other associated cocultured microorganism [10,11]. Based on the previously determined positive effects of this symbiotic bacteria on their host, we monitored the growth of symbiotic bacteria depending on the compositions of nitrogen sources and applied the method to increase biomass of *Chlorella vulgaris*.

In a mixture experiment, the independent factors are proportions of different components in the blend [12]. The purpose of the experiment is to model the blending surface with some form of mathematical equation, so that the responses can be predicted for any mixture or combination of the ingredients. Designs for these experiments are useful, because many product designs and development activities in the industrial settings involve formulations or mixtures [12]. Although people do not know why a composition will be the optimum, it is widely applied to optimization and the change of ratio affects the final results [13].

The waste streams treated by membrane bioreactor contain various nitrogen sources such as NO_3^-, NO_2^-, and NH_4^+ [14]. However, there is no information on the optimal nitrogen composition for the growth of *C. vulgaris*, and finding this will be very helpful for increasing the biomass. In this study, we have examined the effects of nitrogen sources on symbiotic bacteria, and we showed the changes of bacterial growth resulting in the increase of *C. vulgaris*. This indirect way of increasing algae biomass, which is changing symbiotic bacterial population, will increase algal biomass without any further modification to the reactors or other environmental factors and with a simple modification of nitrogen source from the waste streams.

2. Materials and methods

2.1. Bacterial strains and growth media

The *C. vulgaris* strain (KCTC AG10002) was provided by the Korean Culture Type Collection (Korea). *Microbacterium* sp. HJ1 was isolated from heterotrophic algal culture by adding yeast extract to BG-11 and deposited to Korean Culture Center of Microorganisms (Deposit number: KCCM 43126). The isolated *Microbacterium* sp. HJ1 was pre-cultured in 5 ml of tryptic soy broth (TSB) for 48 h at 25 °C in 3.3 Hz shaking incubator. The cells were harvested and washed twice with sterilized distilled water, and then was used to inoculate 5 ml of modified BG-11(MBG) medium [15]. MBG medium was prepared as a composition of BG-11 medium without $NaNO_3$, with the addition of 10 g L^{-1} glucose and 5 g L^{-1} Yeast extract. Cell growth was monitored by measuring optical density (OD) at 595 nm, starting from an initial OD595 of 0.05. The cells for high biomass growth experiments were initially grown in 5 mL of modified BG-11 medium in a test tube, which was grown for 48 h at 25 °C. All chemicals including $NaNO_3$, $NaNO_2$, and $(NH_4)_2SO_4$ were purchased from Sigma–Aldrich (Korea).

2.2. Co-cultivation of C. vulgaris and Micorbacterium sp. HJ1

C. vulgaris was pre-cultured in BG-11 medium for 14 days at 25 °C, and *Microbacterium* sp. HJ1 was pre-cultured in tryptic soy broth (TSB) for 48 h at 25 °C. Cells of *C. vulgaris* and *Microbacterium* sp. HJ1 were harvested and washed twice with sterilized distilled water, and then the optical densities (OD658 and OD595) were adjusted to 1.0 for each microalgal and bacterial cells. Approximately 230 ml of modified MBG medium contained 1 g L^{-1} glucose as carbon source, with NO_3^-, NO_2^-, and NH_4^+ as nitrogen sources at the ratios of 0.35:0.15:0.5 (35 mg L^{-1}, 15 mg L^{-1} and 50 mg L^{-1}), 0.2:0.4:0.4 (20 mg L^{-1}, 40 mg L^{-1} and 40 mg L^{-1}), and 0.7:0.2:0.1 (70 mg L^{-1}, 20 mg L^{-1}, 10 mg L^{-1}), respectively. In a 500-ml conical flask, 16 ml and 4 ml of the microalgal and bacterial grown culture were inoculated, which has algal: bacterial cells ratio of 4:1 by volumn. For *Microbacterium* sp. HJ1 cultured in different ratios of nitrogen sources, as shown above, the final pH were presented as 5.09, 5.31, and 5.09, respectively. The co-culture system was operated in a fluorescence light incubator, with illumination intensity of 7000 Lux, for 14 days at a constant temperature of 25 °C in 3 Hz.

2.3. Design of experiments and mixture analysis

To develop a strategy for optimizing cell growth and chlorophyll a production, a mixture analysis model of three nitrogen sources in the MBG medium was developed and populated, by using a standard mixture analysis methodology [16,17] and the Minitab V16 program (http://www.minitab.com). For designing the mixture analysis experiments to populate the model, we used a simplex lattice method augmented by the design of axial points. The degree of lattice for this mixture analysis is 3, therefore, the experimental design contains the set of all 13 combinations, where each value of nitrogen sources are 0, 33, 66, and 100 (Table 1). The experimental data for response trace plots are also shown in Table 1. All experiments were performed by using 5 ml cultures with 100 mg $L^{-1}$of total nitrogen content. The cultures were grown for 48 h in each culture condition and were tested in duplicates. To plot mixture contours, a mixture regression using the model fitting method was applied with full quadratic component terms initially included. In the data analysis, the

Table 1 – Experimental design points chosen through a simplex lattice methodology for the mixture analysis model and their experimental results.

ID#	$NaNO_3$ (mg L^{-1})	$NaNO_2$ (mg L^{-1})	$(NH_4)_2SO_4$ (mg L^{-1})	$O.D_{595nm}$ average	pH average
1	100	0	0	0.349	4.77
2	67	33	0	0.222	5.15
3	67	0	33	0.352	4.83
4	33	67	0	0.110	5.59
5	33	33	33	0.197	5.21
6	33	0	67	0.353	4.90
7	0	100	0	0.084	5.84
8	0	67	33	0.102	5.65
9	0	33	67	0.159	5.31
10	0	0	100	0.343	5.00
11	67	16.5	16.5	0.323	5.08
12	16.5	67	16.5	0.106	5.62
13	16.5	16.5	67	0.278	5.13

coefficients with p value below 0.1 were used as parameters (Online Resource 1). To examine the accuracy of these models, three additional mixture compositions were selected and tested, which are not included in the first set of 13 conditions. The results of these cultures validated the predictive power of the model for CDW and pH change.

2.4. Chlorophyll a content quantification

To analyze the chlorophyll a, 2 ml of algal and bacterial cell suspension was centrifuged at 9800 g for 3 min, and the supernatant was discarded. Hot (60 °C) DMSO (2 ml) was added and the cells were resuspended by vortexing. Samples were incubated at 60 °C for 10 min before centrifuging, with occasional shaking. After centrifugation, the supernatant pigment was separated and diluted with DMSO to an OD of less than one. The OD at 649 and 665 nm were determined by using UV–Vis spectrophotometer, and the pigment content was calculated by using the equations below [18].

$$\text{Chlorophyll a (Chl a) (mg L}^{-1}) = 12.47(OD665) - 3.62(OD649)$$

3. Results and discussion

3.1. Isolation of symbiotic bacteria

During algal culture, some bacteria-like populations were detected on the surface of *C. vulgaris* by scanning electron microscopy image (Fig. 1). This isolated bacteria did not have an autotrophic growth, being unable to grow only with light source and without any nutrient in BG-11. But, they were able to grow in heterotophic media containing yeast extract at 30 °C. The heterotrophic microorganisms were separated from *C. vulgaris* liquid culture and were cultivated on TSB agar plates, and the microorganisms were shown in orange color. The identification of the isolated bacteria was carried out by 16S rDNA analysis. The strain HJ1 showed 99% similarity with *Microbacterium kitamiense*, when analyzed by GENEDOC and phylogenetic tree (Fig. 2). These similar strains were reported to live with the plants such as neem and sugar canes, and these organisms produced both insoluble and soluble exopolysaccharides (EPSs) by utilizing sucrose as their sole carbon source (Fig. 3) [8]. In addition, there are some reports on symbiotic bacteria living with algae [3], which are similar to *Microbacterium* sp. HJ1 being symbiotic bacteria living with *C. vulgaris*.

3.2. Optimizations of mixed nitrogen concentration

The initial experiments were performed to determine the effect of respective nitrogen source concentrations explored in the mixture analysis. Specifically, we examined the growth of *Microbacterium* sp. HJ1 in MBG media containing individual nitrogen source. It was found that NO_3^- have positive effect, but NO_2^- and NH_4^+ have negative effect on growth of the symbiotic bacteria. In particular, when NO_2^- concentration was critically over 200 mg L^{-1}, *Microbacterium* sp. HJ1 appeared to have no growth (Fig. 4). To predict effect of nitrogen source by modification of nitrogen composition, we applied the mixture analysis model for the effect of the three nitrogen sources on cell growth and pH, which affected the growth of *C. vulgaris* in final media. Before we applied mixture analysis, we

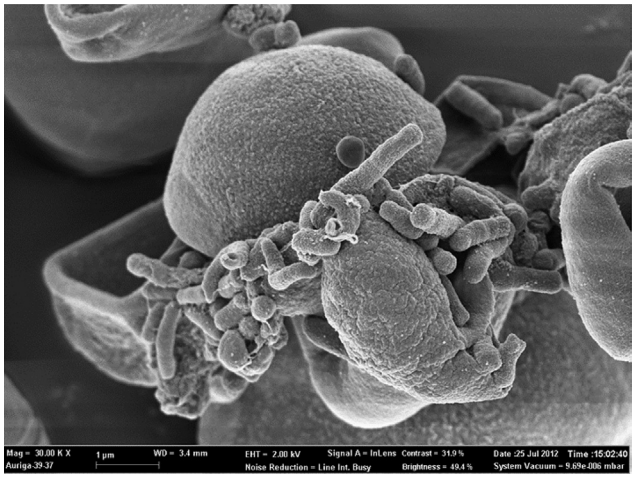

Fig. 1 – Scanning electron microscopy of *C. vulgaris* with *Microbacterium* sp. HJ1.

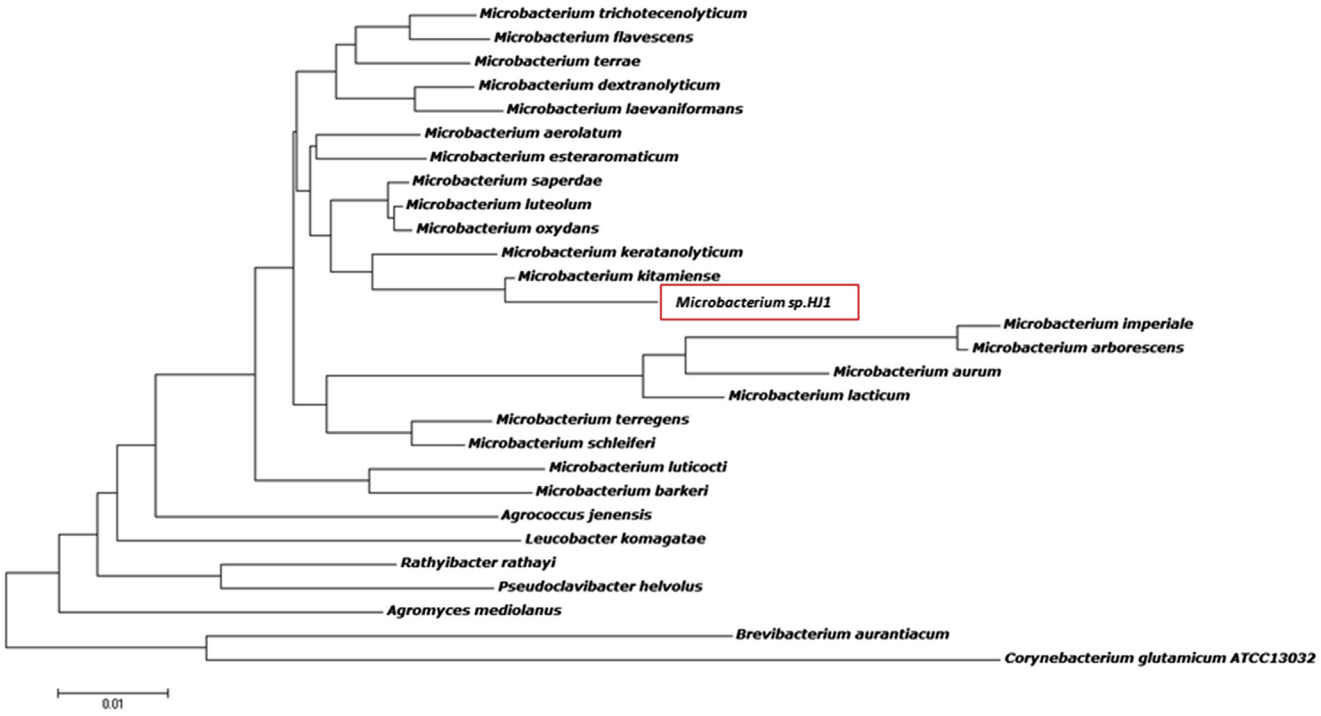

Fig. 2 – Phylogenetic tree based on bacterial 16S rDNA sequence.

examined the nitrogen ratio in treated piggery waste water and found that its composition was 31:68:1 and the maximum growth was observed with 100 mg L^{-1} of the total nitrogen concentration (data not shown). As a result, 100 mg L^{-1} of the total nitrogen was applied to mixture analysis. *Microbacterium* sp. HJ1 was grown with 13 culture compositions of mixed nitrogen sources, as described in "Materials and methods" (Table 1). Contour plots of each variable, generated by Minitab, were used to predict growth and pH of the final media over the range of compositions tested (Fig. 5). It was found out that pH had correlation to NO_2^-, and increasing pH and NO_2^- of the final media clearly had negative effect on the growth of *Microbacterium* sp. HJ1. However, NO_3^- showed positive effect, unlike the respective results, and NH_4^+ showed decreasing effect.

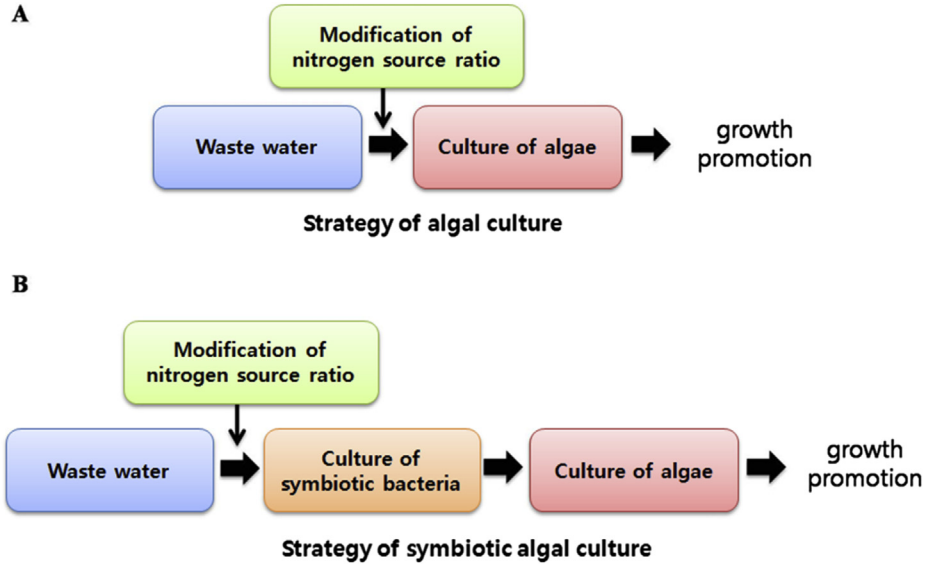

Fig. 3 – Scheme of two strategies for general algal culture (A) and symbiotic culture of *C. vulgaris* and *Microbacterium* sp. HJ1 (B) by modification of nitrogen source ratio.

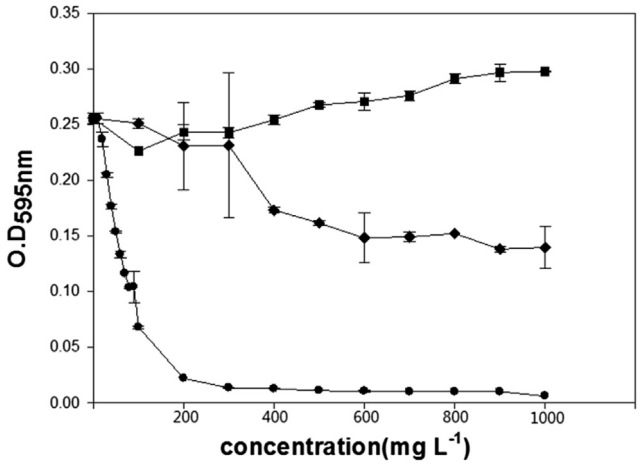

Fig. 4 – Growth of *Microbacterium* sp. HJ1 in liquid minimal medium with $NaNO_3$ (square closed), $NaNO_2$ (circle closed), or $(NH_4)_2SO_4$ (diamond closed) as the additional nitrogen source. Cultures were inoculated to an initial OD_{595} of 0.05, and cultured for 2 days.

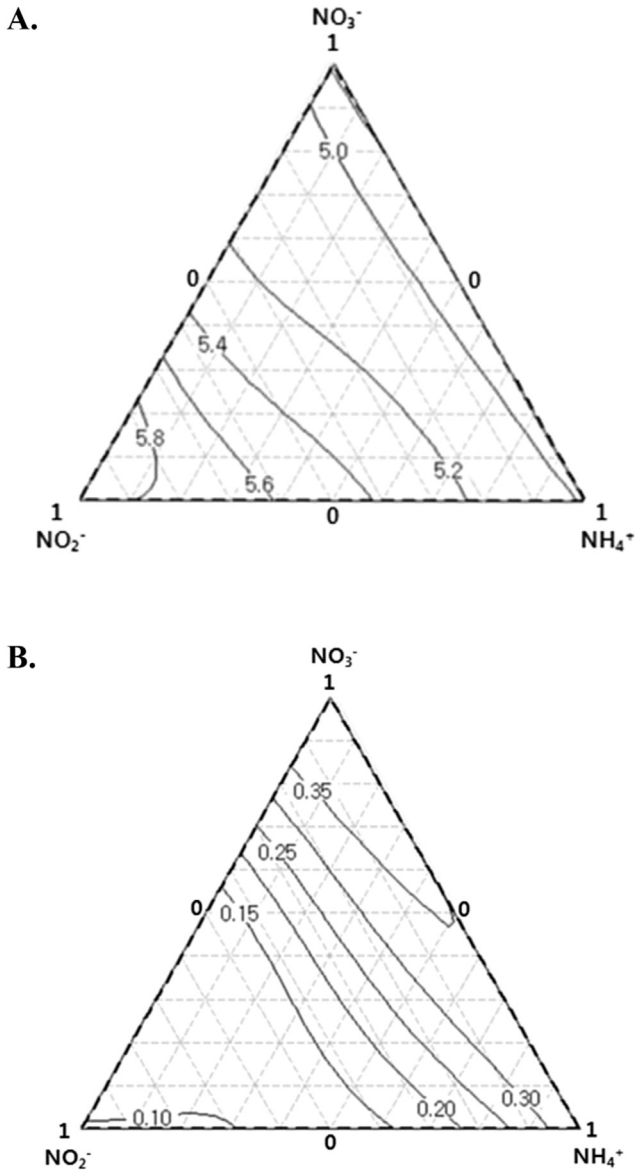

Fig. 5 – Mixture analysis of $NaNO_3$, $NaNO_2$, or $(NH_4)_2SO_4$ as nitrogen source for final pH (A) and growth (B).

Based on the mixture analysis, we were able to predict the results of growth and pH in every different composition of nitrogen source for *Microbacterium* sp. HJ1. The maximum growth point was expected to be at 92:7:1 (NO_3^-:NO_2^-:NH_4^+). In overall, the validated mixture optimization model led to the finding of trends on growth and pH, with very small number of experiments. Therefore, we selected some candidates based on the mixture analysis model.

3.3. *Application of one pot system and comparison of algal culture and co-culture in MBG medium*

The three conditions used for growth-promoting effect on *C. vulgaris* were selected from 35:15:50, 20:40:40, and 70:20:10 (NO_3^-:NO_2^-:NH_4^+) points, which are expected to show different growths and final pH of 5.09, 5.31, and 5.09, respectively. *Microbacterium* sp. HJ1 was cultured with suggested nitrogen sources in MBG for 2 days, and *C. vulgaris* were inoculated for autotrophic growth (Symbiotic culture) for 14 days in culture. General algal culture was also performed with different nitrogen sources in BG-11 medium, without any addition of inoculation or pre-culture of *Microbacterium* sp. HJ1 (Algal culture). Although these ratios are expected to promote the growth of *Microbacterium* sp. HJ1, 20:40:40 (NO_3^-:NO_2^-:NH_4^+) only showed positive effect on the growth of *C. vulgaris* among applied composition (data not shown). The compositions of 35:15:50 and 70:20:10 (NO_3^-:NO_2^-:NH_4^+) caused aggregation of algal biomass because of the bacterial cells and pH, and they showed different phenotypes of algae suggesting that the ratios of symbiotic bacteria and algae have changed. When we compared symbiotic culture with algal culture by using 20:40:40 (NO_3^-:NO_2^-:NH_4^+) of nitrogen source in MBG media (optimized) and 31:68:1 (NO_3^-:NO_2^-:NH_4^+) of nitrogen source in MBG media (control), respectively, the medium optimization slightly affected on DCW (5.8%) and more chlorophyll a contents (18%) were in algal culture.

When we used symbiotic method, the symbiotic culture itself increased DCW by 54% and chlorophyll a contents by 29% in algal culture of the control media. Moreover, when the optimized media was applied to symbiotic method, it showed 65.7% increase in DCW and 78.76% increase in chlorophyll a contents in algal culture of the control media (Fig. 6). Overall, it showed the beneficial effect of symbiotic culture and optimization of nitrogen composition by simple modification of media. In symbiotic associations, the microalgae generally provide O_2 and photosynthetic products to bacteria, and bacteria provide CO_2 by degrading photosynthetic compounds. Moreover, the bacteria also act as algal growth-promoters [19]. In addition, some microorganisms in natural ecosystems coexist and live in symbiotic relationships with plant roots

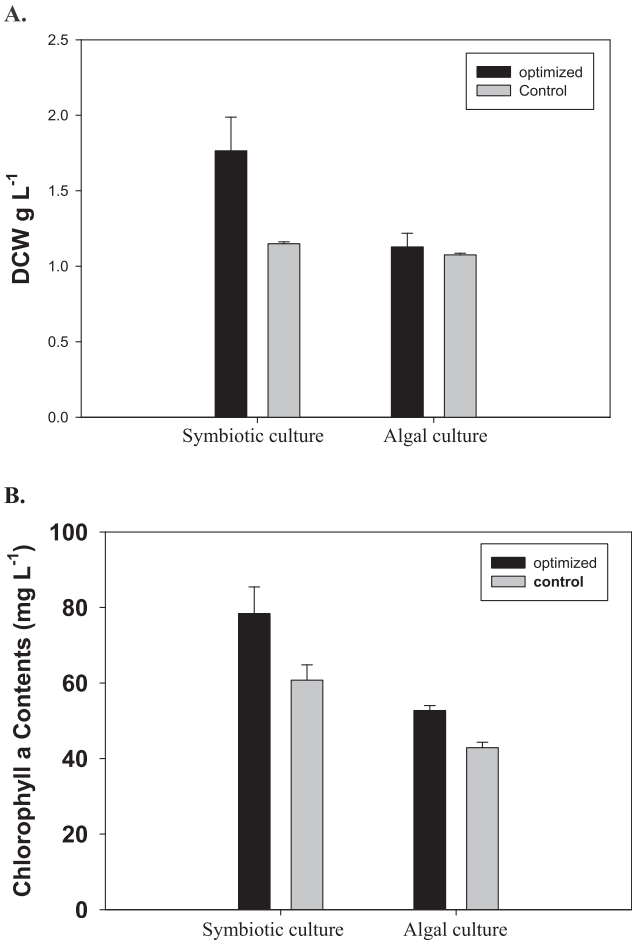

Fig. 6 – Comparison of dry cell weight (DCW) (A) and chlorophyll a (B) by optimized and controlled media with symbiotic culture and algal culture.

[14]. The natural symbiotic relationships of termite, enterobacterium [6,18], and lichens [18] are usually very stable for long periods of time, and our approach to control the symbiotic bacteria for the growth of the host seemed to work.

4. Conclusions

To promote the growth of *C. vulgaris*, the symbiotic association with *Microbacterium* sp. HJ1. and the effect of additional nitrogen sources were examined. There are varity of available algae species that can open doors to new applications and products. Presently, it appears that the production of biofuels and polysaccharides from algae requires a leap in the processing technology. Finding the new symbiotic bacteria and analyzing their growth makes it possible, with the help of mixture analysis. In co-culture with *C. vulgaris* and *Microbacterium* sp. HJ1, increments of algal biomass and chlorophyll a contents were shown. Although major factors for algal growth in symbiotic systems are needed to be studied further, microbial consortial approach may be helpful to increase the algal biomass without any further modification to the reactors or other environmental factors instead, it can be done with simple modification to nutrient source and microbial composition.

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