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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