Algal Research 33 (2018) 345–351

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Enhanced microalgae growth through stimulated secretion of indole acetic acid by symbiotic bacteria

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ARTICLE INFO

Keywords:
Microalgae
Bacteria
Synergistic mutualism
Indole acetic acid
Soluble algal products

ABSTRACT

In many microalgal cultivation systems, microalgae co-exist with bacteria, while little is known about the characteristics of their symbiotic relationships. In this study, twenty-six microalgae growth-promoting bacteria were isolated from a culture system of *Scenedesmus* sp. LX1 cultivated in the secondary effluent from domestic wastewater by using the high-throughput multiple well plate screening method. Ten strains were found to produce and secrete indole acetic acid (IAA), promoting the growth of microalgae. Meanwhile, the microalgae might have secreted signal substances to induce IAA production in bacteria, which was amplified in the tryptophan abundant environment. This indicates that bacteria may mainly promote the growth of the co-existing microalgae through secreting IAA, and microalgae would selectively enhance IAA secretion in turn. Microalgae cultured with microalgal growth-promoting bacteria would be a new potential strategy for improving large-scale microalgal cultivation in an economic and environmentally-friendly way.

1. Introduction

In the commonly used open ponds and closed photobioreactors for the large-scale culture of microalgae, bacterial contamination often occurs. This may accompany the entire microalgal culture process and influence microalgae growth [1–3]. The possible interactions between bacteria and microalgae are synergistic, competitive, parasitic and horizontal gene transfer in the culture system [4,5]. Among these, synergistic mutualism can promote the growth of microalgae. Thus, the synergistic mutualism in microalgae culturing systems may be used to improve biomass productivity and reduce the culturing cost for large-scale microalgal biomass production [6–8].

There are two types of synergistic mutualism between microalgae and bacteria. One is by exchanging materials and resources, and the other is by signal communication. Early studies were mainly focused on material and resource exchange and obtained some important results. Foster et al. [9] showed that nitrogen-fixing cyanobacteria could fix nitrogen for microalgae, likely in the form of ammonia or dissolved organic nitrogen. In addition to cyanobacteria, *Azospirillum*, which is a common N_2-fixing bacterium, has been implicated in promoting the growth of *Chlorella vulgaris* [6]. Some microalgae lack a gene encoding vitamin-B12 independent methionine synthase (MetE) and thus require an exogenous source of vitamin-B12 to synthesize essential methionine. In the aquatic environment, many bacteria are able to produce vitamin-B12, thus providing for microalgae [10]. In return, the microalgal dissolved organic matter (DOM) can serve as the carbon, nitrogen and energy sources for the co-existing bacteria. In these cases, the growth of microalgae and bacteria could be significantly enhanced. However, the synergistic mutualism of exchanging materials and resources is a non-specific interaction.

Signal exchange is another important method of synergistic mutualism between microalgae and bacteria. The substances are used for communication, not as nutrients. They can activate or inhibit gene expression or biological activity, resulting in changes in the growth and metabolism of cells. Over the past few years, the signal interactions between microalgae and bacteria have received increasing attention. A symbiotic relationship between a marine bacterium (*Phaeobacter inhibens*) and marine microalgae (*Emiliania huxleyi*) was reported whereby the bacteria produced growth hormones and antibiotics to influence the growth of microalgae [11–13]. *Chlorella vulgaris* secretes a

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https://doi.org/10.1016/j.algal.2018.06.006
Received 26 February 2018; Received in revised form 3 June 2018; Accepted 9 June 2018

certain signal substance that inactivates the signal substances of the bacterial acyl-homoserine lactones (AHLs), thereby inhibiting the production of bacterial toxins [14], while *Azospirillum* could promote the growth of microalgae by secreting some hormones, such as IAA [6,15]. The study of signal exchange between microalgae and bacteria was mainly focused on the ecological environment. However, little attention has been paid to the process of microalgae production. Moreover, the early study of bacterial isolates was limited to traditional methods that required tedious work and were less efficient [1]. There is less understanding of bacterial species and the signal exchange form in this process, especially regarding how microalgae affect bacteria based on their own growth.

The aim of this study was to investigate the relationship between microalgae and symbiotic bacteria by signal exchange in the microalgae culture process. The method for highly efficient microalgae growth promoting bacteria screening was established. The characteristics of the isolated bacteria were studied, especially their secretion of small molecules. The hypothesis that IAA played an important role as a signaling molecule of the interaction between bacteria and microalgae was proposed and proved. The results provided a strategy to improve the productivity of microalgal biomass in large-scale microalgae cultures.

2. Materials and methods

2.1. Culturing conditions and medium

Scenedesmus sp. LX1 (Collection No. CGMCC 3036 at the China General Microbiological Culture Collection Center) isolated from tap water was used as the microalgae inoculum. *Scenedesmus* sp. LX1 was grown in modified BG11 (mBG11) medium [16] at 25 °C under a light intensity of 6000 lx, with light/dark periods of 14 h/10 h.

Bacterial colonies were obtained using the gradient dilution plate method. First, *Scenedesmus* sp. LX1 was cultivated in the secondary effluent until the end of logarithmic growth. Then, the microalgae-bacteria mixed culture was diluted in a 10 times gradient using phosphate buffer saline (PBS) (pH = 7.3). Next, the diluted solution was spread on Reasoner's 2A (R2A) medium (0.5 g starch, 0.5 g yeast extract, 0.5 g tryptic peptone, 0.5 g glucose, 0.3 g K_2HPO_4, 0.05 g $MgSO_4$, 0.25 g succinate, and 0.5 g casamino acids in 1000 mL distilled water) agar plates and cultured at 25 °C until single colonies appeared.

For detecting IAA secretion ability, bacteria were cultured under six conditions. Two conditions with microalgae cells (containing 10 mg L^{-1} L-tryptophan (Trp) and Trp free, respectively) were used to examine the effect of microalgae cells. Two conditions with microalgal soluble algal products (SAP) which removed microalgae cells (containing 10 mg L^{-1} Trp and Trp free) were used to investigate the effect of SAP. Two conditions with R2A medium (containing 10 mg L^{-1} Trp and Trp free) were used as the control. The same dissolved organic carbon (DOC) concentration of 52 ± 2 mg L^{-1} was used in all six conditions before inoculating the bacteria. Bacteria were inoculated in the six conditions with the inoculation 10 times that of microalgal cells (1×10^6 cells mL^{-1}). There were three replicates for each culture condition. The mixture was incubated for 48 h at 25 °C and 180 rpm (ZQZY-70CS, Zhichu, China).

2.2. High throughput screening method

In this study, the high-throughput multiple-well plate method was developed for efficient isolation of bacteria from microalgae (*Scenedesmus* sp. LX1) cultivation (Fig. 1).

First, the mixed culture of microalgae-bacteria was spread on R2A agar plates for picking up single colonies of bacteria. Single colonies were picked up with sterile toothpicks and then inoculated in 2-mL 96-well plates containing the R2A liquid medium. The 2-mL 96-well plates were sealed with a breathable sealing film and cultured at 25 °C and 180 rpm until the OD_{600} value reached 1.0. Then, 20 μL of the fresh microalgae culture (a microalgae density of 1×10^6 cell mL^{-1}) and 2 μL of various bacterial colony cultures (the bacteria density was OD_{600} = 1.0) were added to a new transparent 96-well plate together. Each well was filled with mBG11 medium to 100 μL. In addition, two types of control cultures were used for each batch of 96-well plates, i.e., non-bacterial culture and only culture medium, respectively. The transparent 96-well plate was sealed with a breathable sealing film and rotationally cultured at 25 °C with a 14 h/10 h light and dark cycle. The density of the microalgae was measured at 650 nm using a microplate reader (SpectraMax M5, Molecular Devices, USA).

2.3. Analytical methods

2.3.1. Microalgae/bacteria growth property

The microalgal density was determined by microscopic counting using hemocytometer and absorbance. The bacterial density was determined by the plate counting method.

2.3.2. Identification of bacteria and the phylogenetic relationship

Genome extraction from the isolated bacteria was carried out using the TIANamp Bacteria DNA Kit (TIANGEN, China).

The 16 s rDNA genes of the extracted genome were amplified by PCR with the primers 27F (5′-AGAGTTTGATCCTGGCTCGA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The amplified products were sequenced and then BLAST (Basic Local Alignment Search Tool) was used to compare to the NCBI 16S ribosomal RNA sequences database. The phylogenetic trees of the isolates were reconstructed using neighbor joining with MEGA 5.0 [17,18]. Bootstrap support for the neighbor joining tree was determined using 1000.

2.3.3. Qualitative and quantitative analysis of IAA molecules

The production of IAA by the isolates was determined according to Glickman and Dessaux [19]. First, the isolates were cultured in 50-mL flasks containing 20 mL R2A supplemented with L-Trp (200 mg L^{-1}) for 48 h on a rotary shaker at 120 rpm and 28 °C. Then, 50 μL of Salkowsky reagent (50 mL 35% $HClO_4$ + 1 mL 0.5 M $FeCl_3$) was added to 50 μL of culture, and was allowed to react under dark conditions for 30 min at room temperature. For the positive control, 50 mg L^{-1} of IAA solution was added instead of cultures. Finally, a red color appeared indicating the presence of IAA and confirming that the bacteria could secrete IAA.

An HPLC (LC-20 AT, Shimadzu, Japan) tandem with a photodiode detector (Shimadzu, Japan) was used to measure the IAA concentration. The samples were filtered using a syringe filter with a pore size of 0.22 μm. The filtrate was collected in a 1.5 mL vial. An ODS-C18 column (5 μm particle size, 150 × 4.2 mm, J&K Chemical Co., China) was used to quantify IAA concentration with an elution phase of methanol:water:acetic acid (550:450:1 v/v). The wavelength for detection was 280 nm. The elution rate of the mobile phase was 1.0 mL min^{-1}. The injection volume was 10 μL for the IAA detection calibrated with standard IAA (Sigma, USA).

2.3.4. Extraction of microalgal soluble algal products

The microalgae culture solution (at the end of logarithmic growth) was filtered using a 0.45-μm filtration membrane. The microalgal cells were held on the 0.45-μm filtration membrane and the soluble algal products (SAP) were obtained in the liquid phase.

2.3.5. SEM observation of symbiotic distribution of microalgae and bacteria

The sample were harvested by centrifugation at 12000 *g* for 10 min at 15 °C and the supernatants were removed. The sample were fixed 12 h (4 °C) in the fixative containing 0.1 M phosphate-buffered solution (pH = 7.3), 2% glutaraldehyde, and 4% paraformaldehyde and then were washed with deionized water. Sample was then dehydrated with increasing concentrations of an ethanol solution (50%, 70%, 90% and 100%) and dried in 100% *tert*-butyl alcohol. Finally, the microcosmic structure of microalgae-bacteria symbiosis was observed using scanning