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Potential of using sodium bicarbonate as external carbon source to cultivate microalga in non-sterile condition

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ABSTRACT

In this study, a saline-alkaline tolerant microalgal strain was isolated and identified as *Chlorella* sp. LPF. This strain was able to grow at pH values up to 10 and at salinities up to 5%, and tolerated to 80 g L^{-1} of sodium bicarbonate. The utilization of bicarbonate as carbon source significantly promoted microalgal growth and lipid production. In the non-sterile cultivation supplying with 80 g L^{-1} of sodium bicarbonate, the microalgal growth had no difference with their growth in the sterile medium; however, the bacterial growth was suppressed and the cell number decreased to low levels after six days cultivation. This study gives an insight into the potential that using high concentration of sodium bicarbonate as external carbon source to cultivate microalga in non-sterile condition, and suggests a possibility of using bicarbonate as growth promoter and antibacterial agent for the microalgal outdoor cultivation.

1. Introduction

Global warming has become an increasingly serious problem due to the emission of large quantities of greenhouse gases from the overuse of fossil fuels. The increasing demand for carbon emission reduction makes renewable biofuels as attractive sources of energy. In the past decade, biodiesel from microalgae has been an area of considerable interest, since they have high growth rate, accumulate large amounts of lipid, not require agricultural land to grow biomass, and are easy to transport (Scott et al., 2010). It is widely recognized that microalgal biomass is the most important feedstock for producing the third-generation biofuel, which could provide significant complement to the fossil fuels (Chen et al., 2011). However, commercialization of microalgal biodiesel is still facing significant obstacles because of its high production costs and poor efficiency.

To develop cost-effective technologies that make microalgal biodiesel economically viable, many studies are being carried out to improve microalgal biomass production and lipid accumulation. Heterotrophic or mixotrophic cultivations that supplied with organics as carbon sources have been suggested to be more considerable and efficient than photoautotrophic cultivation (Lin et al., 2017; Mondal et al., 2017). However, utilization of organics in the microalgal cultivation results in high initial operating cost and makes microalgal cultures get contaminated very easily especially in the outdoor cultivation (Chen et al., 2011). After a long time exploration, many researchers believe that the photoautotrophic growth has the most possibility to scale up as an outdoor cultivation. Unfortunately, the kinetic of CO_2 dissolution in open ponds is very slow, which results in the majority of CO_2 pumped into the pond escape into the atmosphere (Lohman et al., 2015). Then, how to improve microalgae CO_2 utilization rate or find substitutive inorganic carbon source is urgent for the development of large-scale outdoor cultivation.

In some diatoms and green algae, the CO_2 concentrating mechanism (CCM) is elevated by active acquisition of bicarbonate from the surrounding environment and accumulation within the cell (Hopkinson et al., 2016; Tsuji et al., 2017). Bicarbonate can act as a kind of substitutive inorganic carbon source of CO_2 that used for microalgal cultivation. For example, addition of 50 mM bicarbonate was reported to have 27% increase of biomass productivity and 7.7% increase of biodiesel productivity in cultures of *Chlorella vulgaris* (Lohman et al., 2015). Bicarbonate supplement with optimum concentration at 0.6 g/L in the cultures of *Scenedesmus* sp. resulted in 21% increase of total lipid production and 23% increase of biomass production (Pancha et al., 2015). Actually, supply of bicarbonate to microalgal cultures not only enhances biomass and lipid production, but also prevents bacterial contamination in outdoor cultivation when the bicarbonate in very high concentrations. A Trebouxiophyte strain *Nannochloris* sp. JB17 that isolated from an extreme saline-alkali soil has been reported to have

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optimal growth at 400 mM sodium bicarbonate and survived at 1000 mM sodium bicarbonate (Qiao et al., 2015). However, there was no report concerning the bacterial contamination in microalgal cultures that cultivated in high concentration of bicarbonate.

In the present study, a microalgal strain *Chlorella* sp. LPF was isolated and its capabilities growing at high levels of pH and salinity as well as high concentrations of sodium bicarbonate were studied. After that, the effects of bicarbonate on promoting microalgal growth and lipid production, and the potential of using high salinity and high concentration of bicarbonate to minimize bacterial contamination in the microalgal non-sterile cultivation were evaluated.

2. Materials and methods

2.1. Isolation and molecular identification of microalgal strain

Microalgal strain LPF was isolated from the culture of *Nannochloropsis* sp. GY-H14 that obtained from the Shanghai Guangyu Biological Technology Company (SGBT, http://www.leadingtec.cn). Genomic DNA was extracted and purified using a TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa, Dalian, China). The 18S rRNA gene was obtained by PCR amplification by using universal fungal primer pair NS1/NS8 (White et al., 1990); the RuBisCO gene was obtained by using the Form 1AB *rbcL* primer that specific for Chlorophyta (Ghosh and Love, 2011). PCR products were cloned with Mighty TA-cloning kit (TaKaRa, Dalian, China) and sequenced on a MiSeq 300 sequencer (Illumina, San Diego, USA) by IGE Biotechnology (Guangzhou, China). Taxonomic classification of 18S rRNA gene and the translated amino acid sequence of *rbcL* gene were compared with known sequences in GenBank. Phylogenetic trees were constructed by the MEGA program using neighbor-joining algorithms after computing the evolutionary distances via Tajima-Nei method for nucleotide sequences and Poisson correction method for amino acid sequences (Tamura et al., 2011).

2.2. Cultivation of microalga

Microalgal cells were cultivated in 250-mL glass flasks filled up with 100 mL of modified f/2 medium (Lin et al., 2017). Phototrophic growth was maintained in an illumination incubator (ZQLY-180, Zhichu Instruments Co., Ltd, Shanghai, China) at 30 °C and a light density of 100 μmol s^{-1} m^{-2} was adjusted using a 12/12-h light/dark photoperiod. To determine the saline and alkaline tolerances, the f/2 media were adjusted to pHs of 6–10 and salinities of 1–5%, respectively. To determine the tolerance to sodium bicarbonate, a serial concentration of sodium bicarbonate (0, 0.1, 0.5, 1, 5, 10, 20, 40, 60 and 80 g L^{-1}) were added into the f/2 media; the addition of sodium bicarbonate resulted in the initial pH and salinity of culture media ranged from 7.45 to 8.71 and from 2.15 to 4.29%, respectively. To determine the effects of bicarbonate and nitrogen sources on microalgal growth and lipid production, 14 mg L^{-1} N of ammonium, nitrate or urea was supplied as sole nitrogen source and 1 g L^{-1} of sodium bicarbonate was supplied as carbon source.

2.3. Non-sterile cultivation of microalga

To simulate outdoor cultivations, natural water and tap water were used to prepare non-sterile f/2 media. 10 L of natural water were obtained from the Pearl River (23°04′N, 113°41′E) and Centre Lake (23°03′N, 113°23′E) on September 10, 2017. Before being used to prepare culture media, the natural water was allowed to deposit particles by standing settling for 2 days. To reveal the effect of high concentration of bicarbonate on suppressing bacterial contamination in microalgal outdoor cultivation, 80 g L^{-1} sodium bicarbonate (*Chlorella* sp. LPF could tolerate this concentration) was added into the f/2 media, resulting in the initial salinities ~4.2% and pHs ~8.5.

Furthermore, addition of external bacteria was used to assess the suppression of bacterial contamination by high concentration of bicarbonate. In this experiment, *Escherichia coli* was used as the external bacteria. To prepare bacterial cells, the *E. coli* that cultivated in the Luria-Bertani medium were harvested by centrifugation at 10,000 rpm and washed for three times by using culture media; about 2×10^7 cells mL^{-1} bacterial cells in final concentration were inoculated into the microalgal cultures. Non-sterile cultivations were carried out in 1 L beakers containing with 500 mL of culture medium and maintained in an illumination incubator at 30 °C and a light density of 100 μmol s^{-1} m^{-2} was adjusted using a 12/12-h light/dark photoperiod. The microalgal cultures were mixed by magnetic stirrers with a speed of 50 rpm. The water lost by evaporation in beakers was made up by distilled water.

2.4. Analytical methods

Microalgal growth in cultures was determined by cell counting performed with a hemocytometer or at an optical density at 680 nm using spectrophotometer. Bacterial cell number in the non-sterile cultivation was determined by counting of colony forming units on the Luria-Bertani agar plates.

Neutral lipid in microalgal cells was strained with Nile Red and analyzed on an Infinite M200 Multimode Reader (Tecan, Mannedorf, Switzerland) at the selected excitation (480 nm) and emission (580 nm) wavelengths.

Chlorophyll content in microalgal cells was determined as previously reported (Luo et al., 2013). Briefly, microalgal cells from cultures were collected by centrifugation at 1500*g*; pellets were resuspended in 95% (v/v) ethanol and incubated at 75 °C for 5 min; the supernatant was collected by centrifugation at 10,000*g* for 5 min and used for absorbance determination at 649, 664 and 750 nm with a spectrophotometer (Unico UV2802S, Shanghai, China). Chlorophyll content was calculated according to the following equation: $Chl = 5.24 \times (A_{664}\text{-}A_{750}) + 22.24 \times (A_{649}\text{-}A_{750})$.

The total lipids was extracted by a modified method according to the Bligh and Dyer (1959). Briefly, microalgal cells were harvested by centrifugation at 4000*g* for 5 min; the pellet was frozen in liquid nitrogen and then freeze-dried overnight under vacuum; lipid was extracted by vortexing of dried biomass with methanol:chloroform:water (1:1:1) mixture for 15 min at room temperature; the supernatant was separated by centrifugation at 6000*g* for 5 min and the lower phase was collected; lipid was obtained after vacuum evaporation at 50 °C. The crude lipid was converted to their fatty acid methyl esters (FAME) by acid-catalysed transmethylation as described previously (Goold et al., 2016). The FAME mixture was extracted into hexane and analyzed by gas chromatography (7890A, Agilent Technologies) equipped with a flame ionization detector. The sample was passed through DIKMA CP SIL column (DIKMA, Beijing, China) (60 m × 0.25 mm × 0.2 μm) using nitrogen as carrier gas flowing at a rate of 45 mL min^{-1}. The inlet temperature was set to 250 °C while detector temperature was maintained at 280 °C. The initial oven temperature of 140 °C was held for 5 min before being ramped first to 180 °C at a rate of 4 °C min^{-1}, to 200 °C at 2 °C min^{-1}, and finally to 280 °C at 5 °C min^{-1}.

2.5. Relative quantity of gene expression by real-time PCR

Microalgal cells from an exponential culture grown in the modified f/2 medium (882 μM nitrate) were collected, washed twice with nitrogen-free medium and then inoculated into fresh nitrogen-free f/2 medium. After nitrogen starvation for five hours, cultures then received additions of either (1) 42.5 mg L^{-1} $NaNO_3$ + 1 g L^{-1} $NaHCO_3$, (2) 42.5 mg L^{-1} $NaNO_3$ + 1 g L^{-1} NaCl, (3) 26.5 mg L^{-1} NH_4Cl + 1 g L^{-1} $NaHCO_3$, (4) 26.5 mg L^{-1} NH_4Cl + 1 g L^{-1} NaCl, (5) 30.0 mg L^{-1} urea + 1 g L^{-1} $NaHCO_3$, or (6) 30.0 mg L^{-1} urea + 1 g L^{-1} NaCl. All cultivations were maintained at 30 °C and a light density of

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