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Effective control of *Poterioochromonas malhamensis* in pilot-scale culture of *Chlorella sorokiniana* GT-1 by maintaining CO_2-mediated low culture pH

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ABSTRACT

Although predators in microalgal culture can often be protozoa reducing biomass productivity and culture stability, there are few effective approaches to control them. This study investigated the effect of culture pH (i.e., 6.0, 6.5, 7.0 and 7.5) maintained by supply of compressed air bubbles containing various concentrations of CO_2 on death of the flagellate *Poterioochromonas malhamensis* and several other protozoa in the culture of the green microalgae *Chlorella sorokiniana* GT-1. *C. sorokiniana* GT-1 grew well at pH 6.0 and 6.5 and a sustainable biomass concentration of 1.61 g L^{-1} was obtained from the cultures maintained at pH 6.5. The cultures maintained at pH 7.0 and 7.5 collapsed on days 7 and 4 of culture, respectively, as a result of contamination by *P. malhamensis* and to less extent by other protozoa (e.g., ciliates and amoebae). Further experiments revealed that it was the actual dissolved CO_2, not the low pH itself, or reduced dissolved oxygen in the culture medium that prevented the occurrence of *P. malhamensis*. It is speculated that increased CO_2 partial pressure in the culture media may enhance diffusion of CO_2 into the cytoplasm of *P. malhamensis* that lowers the intercellular pH, and thus results in cell death. The method developed in this study can be effective in protozoan control in pilot-scale *Chlorella* culture in an open raceway pond. It is suggested that a low pH maintained temporarily or constantly by supply of CO_2 may be a promising approach to control *P. malhamensis* and alike in microalgal culture.

1. Introduction

Many advantages of the use of microalgae as biomass for fuels and other valuable products have been extensively reviewed [1–3]. However, there has been limited success in applying microalgal biomass to biofuel production on a large industrial scale, mainly as a result of high costs [4]. Among the various factors that contribute to the high production costs of microalgal biomass, the frequent crash of microalgal cultures has devastating effects on their productivity [5]. The contamination of the cultivation system by predators such as protozoa [6] is one of the leading causes of such crashes. Once certain protozoa have invaded the microalgal culture and proliferated, the biomass productivity can be reduced to zero within a few weeks to a few days [7]. Researchers have, therefore, been exploring various control methods for protozoan predators because their presence in large-scale cultivation systems is inevitable [8,9]. However, only a limited number of approaches have been effective in controlling or killing protozoa without affecting the growth of the microalgae [10]. Becker [11] observed that lowering algal culture pH to an acidic level (pH 3.0) for 1–2 h reduced zooplankton contamination. However, this method also impaired the growth of the targeted microalga in practice. It is therefore important to find a crop protection method that can reduce a predator population while at the same time maintaining a healthy culture of microalgae.

Chlorella is considered as one of the best microalgae for producing proteins for food and fish feed, and lipids for biofuels and non-fuel oils [12]. During five years of *Chlorella* cultivation in the laboratory and outdoors from 2011 to 2016, however, we found that *Chlorella* cultures often suffered from crashes caused by the flagellate *Poterioochromonas malhamensis*. *P. malhamensis* is a mixotrophic flagellate [13] that lives either by photosynthesis or by grazing on bacteria and microalgae. It can rapidly phagocytose a large number of *Chlorella* cells and the cell

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volume of *P. malhamensis* increases by 10–30 times after feeding on microalgae [14]. *P. malhamensis* rapidly grazed *Chlorella* cells and the color of the culture suspension turned from green to yellow brown and deep brown within a few days after *P. malhamensis* occurred in the culture. Although no research has been reported on the destruction of mass cultivation of *Chlorella* by *P. malhamensis*, the contamination of large-scale culture of *Synechocystis* by *Poterioochromonas* was reported [15]. No safe and effective method has been developed to control *P. malhamensis*. Touloupakis [15] reported that a *Synechocystis* culture maintained at pH > 11.0 avoided contamination by *Poterioochromonas*, but the productivity of the culture decreased by 32%.

The main objective of this study was to develop a method to control *P. malhamensis* in *Chlorella* culture. The plausible cause of inhibitory and lethal effect of CO_2 on *P. malhamensis* was investigated and potential application of industrial flue gas CO_2 to microalgal culture for contamination control was discussed.

2. Materials and methods

2.1. Microalgae strain and culture medium

Chlorella sorokiniana GT-1 was originally isolated from the SDIC Microalgae Biotechnology Center R & D facilities in Hebei, China. The alga is preserved in the China General Microbiological Culture Collection Center (No. 11801). The culture medium BG-11 [16] was used to cultivate *C. sorokiniana* GT-1.

2.2. Experimental design

The pH of *C. sorokiniana* GT-1 cultures were maintained at four different levels (pH 6.0, 6.5, 7.0 and 7.5) by adjusting the rate of delivery of CO_2 during a 10-day cultivation period. The experiments were conducted in a 12-L vertical flat-plate photobioreactor (PBR) (JLCTVS-12S, Shanghai Joylab, China), which was made from a rectangular body of polymethyl methacrylate. *C. sorokiniana* seeds from a log-phase culture was obtained from glass columns (φ = 5 cm) with a working volume of 750 mL. The optical density (OD_{730}) at inoculation was 0.4. The culture temperature was 23 ± 1 °C and light was provided continuously at a light intensity of 140 μmol photons $m^{-2} s^{-1}$. All experiments were conducted in duplicate. Four parameters, i.e., cell count, dry weight, OD_{730} and chlorophyll *a* concentration, were used to measure algal growth. Occurrence and population dynamics of protozoan in algal cultures were monitored daily.

Based on our previous laboratory experience of the naturally occurring contamination of *C. sorokiniana* GT-1 by *P. malhamensis*, we experimentally created contamination of the culture by simply letting the culture grow for 10 days. The PBRs with cover-lids were first pretreated with sodium hypochlorite (75 ppm) and then washed at least four times with distilled water before inoculating the seed culture. However, the PBRs were exposed to the environment as the lids cannot be sealed completely, which resulted in spontaneous contamination in the laboratory setting. Therefore, our results were obtained from *C. sorokiniana* GT-1 cultures that were spontaneously contaminated by *P. malhamensis*, not from cultures pre-inoculated with a given number of *P. malhamensis*. Our experiments were designed with the intention of reproducing the natural initiation and progression of contamination as closely as possible. We also rationalized that our experimental design would allow us to gain additional insights into how *C. sorokiniana* GT-1 population responds to the progression of contamination by *P. malhamensis*.

2.3. Control of culture pH by supply of CO_2 at different flow rates

To maintain a desired pH in the culture media, the rate of delivery of CO_2 was adjusted and recorded four times each day after monitoring with a pH meter (Model FE20, Mettler Toledo, USA). This approach was shown to be effective in maintaining the pH with minor fluctuations. The rate of delivery of CO_2 was adjusted using an air flowmeter (Model LZM-6T, Cheng Xin, China). CO_2/air rates of 10–30%, 6–15%, 1–6% and 0.5–2% were used to maintain pH levels of 6.0, 6.5, 7.0 and 7.5, respectively.

2.4. Monitoring the growth status of Chlorella sorokiniana

The growth parameters, including cell number density, cell dry weight, OD_{730} and chlorophyll *a* concentration, were applied to *Chlorella* cultures maintained at different pH values. Each parameter was measured in triplicate for each sample.

2.4.1. Cell number density of Chlorella sorokiniana

Cell number of *C. sorokiniana* GT-1 in a culture was counted by microscope with a hemacytometer (Improved Neubauer, USA).

2.4.2. Dry cell weight of Chlorella sorokiniana

Dry weight of *C. sorokiniana* GT-1 cells was determined gravimetrically, using a method adopted from [17]. An aliquot (5–10 mL) of culture suspension, depending on cell density, was filtered through a preheated (105 °C, 24 h), pre-weighed glass microfiber filter (Whatman GF/C, 47 mm, UK). The filter was washed twice each with 20 mL of 0.5 M ammonium bicarbonate. The filter was weighed after drying at 105 °C for 24 h to reach a constant weight. Dry weight (DW, g L^{-1}) was calculated using Eq. (1):

$$\mathrm{DW} = \frac{W_1 - W_0}{V} \tag{1}$$

where W_1 and W_0 are the weight of the filters at the end and start of cultivation, respectively, and V is the volume of the microalgae suspension filtered.

2.4.3. Determination of chlorophyll a

Chlorophyll a was determined using the method of [18] with a slight modification. An aliquot (5 mL) of culture suspension was filtered through a glass microfiber filter (Whatman GF/C, 47 mm). The filter containing algae pellets was transferred to a 15-mL centrifuge tube covered by silver paper. Pure methanol (10 mL) was added to the centrifuge tube, which was then placed in a water-bath at 75 °C for 20 min. Afterward the sample was centrifuged (4000 × *g*, 10 min, 4 °C) and the supernatant was transferred to a cuvette for measurement of optical density at 653 nm (OD_{653}) and 666 nm (OD_{666}). The amount of chlorophyll *a* (*Chl*-a, mg L^{-1}) was calculated using Eq. (2):

$$\mathit{Chl}\text{-a} = \frac{(15.65 \times \mathrm{OD}_{666} - 7.34 \times \mathrm{OD}_{653}) \times V_{\mathrm{MeOH}}}{V_{\mathrm{algae}}} \tag{2}$$

where V_{MeOH} is the volume of methanol and V_{algae} is the volume of microalgae suspension used for the extraction of pigments.

2.4.4. OD_{730} of Chlorella sorokiniana

Cell density of *C. sorokiniana* culture was measured as optical density at 730 nm (OD_{730}) using a fluorescence spectrophotometer (DR6000, HACH, USA). Samples were diluted to a concentration that gave a final OD_{730} reading between 0.2 and 0.8. BG-11 medium was used as the blank solution.

2.4.5. Observation of morphology of Chlorella sorokiniana

The color and turbidity of the *C. sorokiniana* cultures were observed daily and a camera (Canon EOS 60D, Japan) was used to record the macro-morphological changes. The samples, in 50 mL colorimetric tubes, were photographed everyday under the same conditions with fixed camera parameters. The integrity and agglomeration of the cells were monitored every day using a microscope (BX53, Olympus, Japan) to record the micro-morphological changes in *C. sorokiniana* during cultivation. Appearances and colors of *C. sorokiniana* cultures at