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Identification of harmful protozoa in outdoor cultivation of *Chlorella* and the use of ultrasonication to control contamination

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ABSTRACT

Contaminating organisms in mass cultivation present one of the major challenges that must be overcome for successful commercialization of algal biofuels. The present study identified a range of contaminating organisms in *Chlorella* cultures cultivated in outdoor raceway ponds at the Arizona Center for Algae Technology and Innovation (Mesa, AZ). Nineteen organisms or Operational Taxonomic Units (OTU) in the *Chlorella* culture were identified by a combination of microscopic observation and 18S rRNA denaturing gradient gel electrophoresis (DGGE). More detailed analyses identified these contaminating organisms as 2 fungi, 7 flagellates, 3 amoebae, 4 ciliates, 1 rotifer, and 2 large insects. Among them *Poterioochromonas* sp., a small flagellate, appeared to be one of the most harmful causing culture collapse. In order to control *Poterioochromonas* sp., various operational parameters and application strategies of ultrasonic treatment were investigated. During sixteen-day consecutive *Chlorella* cultivation in batch mode, the ultrasonication conditions of 6 L min^{-1} flow rate with the power of 495 W at 100% amplitude and a treatment frequency of once for 1 h every day, was proven to be the most effective in preventing *Poterioochromonas* outbreak in *Chlorella* culture with volume of 60 L. The above ultrasonication method was also effective at destroying an unknown fungus, an amoeba (*Acanthocystis* sp.), and ciliates (a member of the family Orchitophryidae). Our findings can serve as a technical foundation for the application of ultrasonication to control some of the contaminating microorganisms in mass cultivation of microalgae including *Chlorella*.

1. Introduction

Among the many challenges of microalgal cultivation at either a demonstration or commercial scale, contamination control is one of the most problematic [1,2]. As biomass productivity dictates the viability of the microalgae industry, it is essential that the appropriate crop management is developed [2]. However, there are only a limited number of studies on the identification of common contaminating organisms in the mass cultivation of industrially important species such as *Chlorella* [3]. Furthermore, an equally small body of work is present in the literature on management regime to control these contaminating species effectively.

The freshwater microalga *Chlorella* (Chlorophyta) is an industrial species that has been used for the production of nutraceuticals and fish feeds for several decades. Recently it has shown promise as a feedstock for advanced biofuels because of its high productivity and its robustness in various environments [4]. As a result, mass cultivation of *Chlorella* has attracted substantial interest. However, sustained mass cultivation of *Chlorella* is more challenging than with other species, such as *Spirulina* and *Dunaliella*, that are cultivated under extreme conditions such as very high salinity or pH [5,6]. *Chlorella* culture conditions cannot inhibit the growth of other microalgae or grazers effectively.

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The contamination of mass cultivation by various organisms can drastically reduce the overall productivity of algal cultivation, because these organisms can compete for nutrients, graze on algae, and can often cause a culture crash [7–9]. Unfortunately, there are few numbers of systematic studies on how to identify and manage contaminating organisms in algal cultures. Reasons for the paucity of studies on these topics include: 1) the study requires infected cultures; 2) the infection cycle usually cannot be repeated exactly the same as the previous one even under well controlled conditions [10]; 3) crop protection strategies developed by commercial companies may be deemed to be commercially sensitive information and are not in the public domain. As a result, only a limited amount of information on crop protection is available in the literature [1,2].

The successful identification of contaminating organisms is critically important when devising effective control mechanisms that are specifically designed to control the most damaging contaminants. Based on morphological observation, *Chlorella* cultures previously have been found to be susceptible to contamination by copepod [11], the ciliate *Stylonychia* sp. [12], and some amoebae [8]. However, the information from these studies is far from enough to understand the contamination. Molecular biological methods, such as metagenomics, now offer new opportunities for researchers to analyze the structure and species composition of microbial communities in microalgal cultures. Denaturing gradient gel electrophoresis (DGGE) is one of the most commonly used techniques for analyzing complex microbial communities [12,13]. Recently, this technique has been successfully applied to identify the bacterial communities in photo-bioreactor cultures of *Chlorella vulgaris* [14], *Dunaliella tertiolecta* [15], and also eukaryotic organisms, such as *Colpoda* sp. (a ciliate), in the culture of *Chlorella vulgaris* [16]. Since most of the contaminants in microalgal culture are eukaryotes, such as fungi, protozoans, large zooplankton and filter-feeders [9,17,18], universal eukaryotic primers that have been successfully used to study the diversity of picoeukaryotes in natural marine assemblages [19], eukaryotes in microalgal culture, and microeukaryotes in soil [20], can be used to identify most of the contamination in microalgal cultures. Minor changes in the community composition of the contaminants can also be monitored.

As the contamination can drastically impact the commercial production of microalgal biomass [6–8], a number of methods have been investigated for controlling the contaminants, but with limited success. Early attempts at control have been performed by means of heating, centrifugation, filtration [21], electrical field [22], changes in pH [23,24] or salinity [25,26], and the use of chemicals such as ammonium hydroxide [6], formalin [27], ivermectin [28], quinine sulfate [29], toosendanin [30], and rotenone [31]. As *Chlorella* is a very small green alga (2–4 μm) and its optimal culture pH (7.5–8.5) and salinity (freshwater) are comparatively common among potential contaminating organisms, it is very susceptible to different kinds of contamination, and it is very difficult to use regular methods, such as filtration or changing pH or salinity, to prevent contamination. Moreover, to reduce potential environmental impacts, the use of chemicals is inappropriate over a long period of time. Therefore, it is very important to find methods that are both effective and safe for controlling diverse contamination in cultures of small freshwater uni-cellular algae.

Ultrasound (sound waves of a frequency at or above 20 kHz), generates hydro-mechanical shear forces and sono-chemical effects and has been widely used for the inactivation of microorganisms [32]. The efficacy of ultrasonic treatment varies with the size of the test organism [33]. Difference in tolerance level to ultrasound makes it possible for the selective inactivation of zooplankton. A recent finding, however, has suggested that ultrasonication has minimal impact on the growth of *Chlorella* sp. [32]. Additionally, either by directly immerging the probe in culture or transferring the culture into an ultrasonic device, ultrasonication can be used to kill grazers in in the algal cultures in photo-bioreactors and does not have any known environmental impacts when compared to chemicals. Therefore, ultrasonication could be a potential method to hinder the growth and outbreak of contaminants in *Chlorella* cultures. Although ultrasound cannot impact all the contaminants in *Chlorella* sp. cultures, as the control method has size constraints, it may be possible to protect *Chlorella* cultures by destroying key harmful contaminants.

The main objectives of this study, therefore, were to set up a sensitive method to monitor and identify contaminants in *Chlorella* cultures and, meanwhile, to develop an effective and 'environmentally-friendly' method to protect *Chlorella* cultures from contamination with *Poterioochromonas*, a flagellate protozoan that can be a fatal predator to *Chlorella* cultures.

2. Materials and methods

2.1. Microalgal strain and culture conditions

Chlorella sp. LRB-AZ-1201 was obtained from the Arizona Center for Algae Technology and Innovation (Mesa, AZ). All our algal culture systems were located in the polytechnic campus of Arizona State University (33°18′15″ N, 111°40′23″ W) in the southwestern United States. The climate is primarily dry and hot, with very low rainfall throughout the year and extremely hot in the summers. All the experiments in our study were performed during April to May of 2013. According to the Arizona Meteorological Network (http://ag.arizona.edu/azmet/22.htm), the air temperature ranged from 8.3 to 36.1 °C (47–97 °F).

The initial seed culture was kept at 21 °C with continuous light of 5 μmol $m^{-2} s^{-1}$ in 250 mL Erlenmeyer flasks containing the medium BG11 [34,35]. The pH was maintained at 7.0 with PBS buffer. Cell proliferation was developed in several phases, and the initial OD of every proliferation stage was set as 0.15. The first phase was to inoculate *Chlorella* sp. from the flask into a glass column (0.8 L with a diameter of 46 mm), maintained at 26 °C with continuous light of 135 μmol $m^{-2} s^{-1}$ in a clean room. During the exponential growth phase, the culture was inoculated from the glass column into a flat panel photo-bioreactor (15 L with 40 mm thickness of panel wall) under the same cultivation conditions. After about 7 days, the algae were transferred into a large flat panel (110 L with 38 mm thickness of panel wall) in the greenhouse under natural light with average solar radiation of 22.8 MJ m^{-2} during the experiments. All the cultures in columns or flat panels were aerated using compressed air with 1.5% CO_2 to adjust and maintain the pH within the range 6.5 to 7.5 during the whole cultivation. The algal culture, thus prepared, was then used as seed in our experiments. During the preparation of seed cultures, a contamination survey was conducted every day with a microscope to make sure that seed cultures were free of contaminants.

The predator *Poterioochromonas* sp. has often been found to contaminate *Chlorella* cultures at our facility and cause the cultures to collapse. For the purposes of this study, *Poterioochromonas* sp. cells were collected specifically from *Chlorella* sp. cultures that were heavily contaminated with *Poterioochromonas*. In order to evaluate the effect of the grazing of *Poterioochromonas* sp. on the growth of *Chlorella* sp., the collected *Poterioochromonas* cells were concentrated by centrifuging at 1500*g* for 5 min, and then added to pure *Chlorella* culture (1.0×10^7/mL) to give a concentration ratio of 1:10 (*Poterioochromonas*:*Chlorella*). The cultures were cultivated in 100 mL flask (working volume 30 mL) for 24 h at 26 °C with continuous light of 135 μmol $m^{-2} s^{-1}$ in a clean room. For the control, the same concentration of *Chlorella* sp. was cultivated in the same culture system, but without adding any *Poterioochromonas*. The experiment was conducted in triplicate. Every 6 h, one centrifuge tube with 1 mL culture was collected and preserved with Lugol's solution to a final concentration of 1.0% for quantitative analysis [36]. Cell numbers of *Poterioochromonas* and *Chlorella* were counted with a hemocytometer.