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Integrated grazer management mediated by chemicals for sustainable cultivation of algae in open ponds

Saranya Karuppasamy[1], Amar S. Musale[1], Badrish Soni, Bhaskar Bhadra, Ninad Gujarathi, Meenakshi Sundaram, Ajit Sapre, Santanu Dasgupta, Chitranshu Kumar*

Reliance Research & Development Centre, Reliance Corporate Park, Thane-Belapur Road, Ghansoli, Navi Mumbai 400 701, India

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ABSTRACT

Several challenges need to be overcome for algal biofuels to become economically viable alternative to fossil fuels. The major bottleneck is that it requires continuous production of large quantities of algal biomass at a very low cost. To achieve this algal crops have to be stably cultivated round the year in open ponds at a sufficiently high density. Currently, it is highly difficult as algae crops cultivated in the open ponds are prone to numerous abiotic & biotic stresses, which lead to frequent culture crashes and productivity losses. Abiotic stress can be managed largely through bio prospecting, screening and selection of right kind of algal strains for that geographic location & season. Managing contaminants in open ponds along with maintaining productivity of algal crop to match the economics of biofuel production has so far proved to be an insurmountable barrier worldwide. The technologies successfully used till date to manage competition or grazing in open ponds have proved unsuccessful as these methods either limit desired productivity or are incompatible for prospective candidate algal strains ideal for fuel production.

In the present study, we have taken a holistic approach starting from isolation, identification and characterization of major grazers associated with our algal crop and in parallel screening of several chemicals those could limit grazers in ponds. This was followed by testing the identified chemicals on the grazers, determining the sensitivity of our production strain to the chemicals found effective against grazers and finally providing chemistries that are able to eradicate the pests in open ponds with no adverse effects on our crop. Importantly this study reports 40 chemical candidates that are effective against grazers and can be utilized to prevent grazing during algae cultivation depending upon their compatibility with the algae strain grown.

1. Introduction

The third generation of biofuels is being developed through the production of biomass from the farming of algae and cyanobacteria [1,2]. The process to be economically viable considering the price of present day crude requires production of copious amounts of biomass at the lowest possible cost. For this, the algae needs to be cultivated in open ponds because despite their below par productivities they avoid the high costs associated with the photo-bioreactors [3]. However, algal crop cultivated in the open ponds is highly susceptible to contamination by organisms that graze upon and feed on the crop or the culture is taken over by a foreign alga instead of the desired crop. These phenomena, grazing and dominance by contaminating microorganisms lead to frequent culture crashes of the desired algal crop being cultivated in the open ponds and huge losses in productivity, as occurs with agricultural crops [1,4]. The grazers & contaminants encountered during algal cultivation in the open ponds include protozoans (ciliates, amoeba, dinoflagellates, etc.), viruses, fungi, bacteria & other algae [5–8]. Although grazing is a major problem associated with algal crops cultivated outdoors, little has been published in this regard. Algal productivity losses have been reported because of insect larvae grazing in *Spirulina* ponds [9,10]. Predation by rotifers, metazoan zooplanktons, amoeba and ciliates has been described during mass culturing of *Dunaliella* [11–13]. Grazing by ciliates has been observed to crash cultures of *Dunaliella salina* grown outdoors within few days. Such losses on a commercial scale are disastrous and crop protection approaches to limit grazers and contaminants need to be worked out, if biofuels from algae has to become a tangible economic reality [14].

A few strategies have been tried to alleviate the influence of grazers and contaminants on the algal crop. Selective growth conditions like

* Corresponding author.
E-mail address: chitranshu.kumar@ril.com (C. Kumar).
[1] Both authors contributed equally to this work.

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maintaining high salinity, high alkalinity and high nutrition have been successfully used to cultivate different algal strains at a commercial scale [15]. *Dunaliella* has been grown for considerably long time in open ponds in highly saline conditions, while *Spirulina* is cultivated under conditions of higher alkalinity [16]. Maintaining high nutrition in open ponds has been used to grow *Chlorella* [16]. Providing selective growth conditions is an effective strategy for managing grazers and contaminants, but it limits the desired high productivity, hence is not recommended for biofuel production [17]. Chemical crop control is another alternative strategy for preventing contaminants and predators of algae. Hypochlorite has been used to control protozoans in *Nannochloropsis* mass cultures [18]. Ammonia and acidic pH shock have been used successfully to control rotifers in open ponds [19,20]. *Nannochloropsis* cultures have been shown to be cultivated outdoors, aided by the use of glyphosate and ozone [21,22]. Control of zooplankton in laboratory cultures of *Chlorella* has been achieved through the use of pesticides like dipterex, parathion, and dichlorodiphenyltrichloroethane (DDT) [23]. Several botanical pesticides such as celangulin, matrine and toosendanin have been used to control rotifers in laboratory cultures of *Chlorella* and *Nannochloropsis* [24]. Another chemical strategy, that has met with some success, is the use of the fungicide, Headline to control the fungal pathogen in *Scenedesmus dimorphus* cultures cultivated outdoors [25]. In a recently published review, Day et al. [26] have nicely summarised all the different grazer types encountered during cultivation of different microalgae along with the various crop protection approaches that have been tried so far in lab or during outdoor cultivation of microalgae.

However, none of the above chemical strategies have been successful at commercial scale or for cultivating algae for protracted periods in outdoors. Thus, there is an urgent requirement for methodologies capable of controlling different grazer types for sustainable cultivation of algae in open ponds. In the present study, we undertook isolation, identification and characterization of major grazers in open outdoor ponds cultivated with *Chlorella vulgaris* as the production strain. This was followed by screening of a large number of varied chemicals for their efficiency in controlling the isolated ciliates & dinoflagellates. Using this screening we are able to develop control mechanisms against the grazers that are not only low cost but are also compatible with *C. vulgaris*. Finally, we demonstrate the successful application of one of these chemicals in recovering a crashed pond of *Chlorella vulgaris* infected with ciliates.

2. Materials and methods

2.1. Crop protection chemicals

A list of probable chemicals with potential for anti-grazer activity was prepared (Table 1). We tried to cover most of the chemicals that are used to control different pests, bacteria, viruses, fungi, parasites, protozoans, in agriculture, aquaculture and veterinary field. Hence, the final list included various chemicals in the category of anti-microbials, insecticides, pesticides, oxidants, natural compounds, dyes, anti-fungals, antiparasitics, etc.

2.2. Isolation of grazers from the ponds

The ciliates were isolated from the samples collected from the crashed culture of *Chlorella vulgaris* grown in open pond located at 22.3925° N, 69.8173° E near Jamnagar, Gujarat, India. The samples were filtered through 100 μm nylon mesh to reduce major clumps usual symptoms of ciliate infection, followed by filtration through 20 μm and 11 μm nylon mesh. The filtrate was collected and serially diluted using commercial media prepared in sea water (urea-3.33 mM, phosphoric acid-0.21 mM, and trace element mix (Fe-EDTA-23.39 μM, $Na_2EDTA.2H_2O$-10 μM, $CuSO_4.5H_2O$-78.6 nM, $Na_2MoO_4.2H_2O$-52.0 nM, $ZnSO_4.7H_2O$-0.153 μM, $CoCl_2.6H_2O$-84.0 nM, $MnCl_2.4H_2O$-1.82 μM)) used in open pond cultivation system to bring down the ciliate load. Using a micropipette, individual ciliates were picked up under 8× magnification of stereo microscope (ZEISS stereo Discovery.V8, Carl Zeiss, Germany) and five ciliates of same morphology were put together in 3 ml of commercial media along with the log phase culture of *C. vulgaris* in 24 well plate. Ciliates are known not to multiply if cultured from a single cell [27]. The plate was incubated on algae culture racks with 12 h light/12 h dark cycles at 22 °C under light intensity of 100 μmoles/m^2/s. After a few days, ciliates started to multiply and their number increased. At this point the sample was collected from the 24 well plates and transferred to 100 ml conical flask with 20 ml of commercial media and exponential phase culture of *C. vulgaris* as feed for maintenance and/or for further experiments.

In the same way, the dinoflagellates were also isolated from the crashed pond culture of *Chlorella vulgaris*. The dinoflagellates have the ability to multiply from a single cell, so it was easy to isolate a monoclonal population from a serially diluted sample. Once the number of dinoflagellates increased, when grown with *C. vulgaris* as feed, they were sub-cultured similarly to ciliates using fresh exponential culture of *C. vulgaris* for maintenance and/or for further experiments.

Table 1
List of chemicals along with functional categories for testing on grazers.

Category	Chemical name
Antimicrobial compounds	Quaternary ammonium compounds: benzalkonium chloride, benzethonium chloride, cetyl trimethylammonium bromide, tetraethylammonium chloride, tetraethylammonium iodide, tetraethylammonium bromide, cetylpyridinium chloride Others: chlorohexidine gluconate, imidazolidinyl urea, methylisothiazolinone
Fungicides	Pyraclostrobin, fluoxastrobin, captan, propiconazole
Vital dyes	Methylene blue, toluidine blue, Lugol's iodine
Herbicides	Microtubule inhibitors: pendimethalin, ethalfluralin ALS inhibitors: chlorimuron, iodosulfuron Organosulphur: sodium dimethyldithiocarbamate PSII inhibitors: atrazine, isoproturon, metribuzin, bromacil
Oxidants	Sodium percarbonate, sodium perborate, peracetic acid, potassium permanganate
Pesticides	Neurotoxins: cypermethrin, indoxacarb, carbaryl Organophosphates: profenofos, triazophos, methylparathion, malathion, fenthion
Natural compounds	Gallic acid, saponin, salicylic acid, phloroglucinol, rotenone
Antiparasitic compounds	Ivermectin, abamectin, niclosamide
Flavanol	Quercetin
Antifeeding agent	Benzyl isothiocyanate
Anesthetic compounds	Ethyl 3-aminobenzoate methanesulfonate, benzocaine

2.3. Microscopy and phylogenetic analysis of grazers

Identification of the individual grazer types was done by observing their body shape, other morphological features, movements and behaviour under 10×, 40× and 100× magnification of ZEISS Primo star microscope (Carl Zeiss, Germany).

Actively grown axenic culture of *C. vulgaris* (100 ml each) were mixed separately with 10 ml of suspension containing ciliates or dinoflagellates (10^2–10^3 cells/ml). The resultant mix cultures were incubated at 22–25 °C without shaking until they completely crashed (5–7 days). Genomic DNA was extracted from 10 ml of crashed culture [28]. Approximately, 1400 bp of partial 18S rRNA gene were PCR amplified using primer pairs 82F (5′-GTGAAACTGCGAATGGCTCAT-3′) and 690R (5′-AGAATTTCACCTCTG-3′) [29]. Fusion® Taq polymerase master mix (ThermoFischer) used for amplification of the 18S rRNA gene following manufacturer's instruction. PCR products were subjected to agarose gel electrophoresis, and the single PCR amplicon of