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Contaminations in mass cultivation of cyanobacteria: Highly resilient *Colpoda steinii* leads to rapid crash of *Synechocystis* sp. cultures and is inhibited by partially anoxic conditions

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ABSTRACT

Contaminations in microalgae cultures are one of the major drawbacks when cultivating in an industrial scale. What is often experienced as culture crash has been rarely investigated in detail. Here, we show that the ciliated protozoa *Colpoda steinii* is capable to clear a dense culture of the cyanobacterium *Synechocystis* sp. within 2–3 days. In a 200 L tubular photobioreactor, this ciliate frequently appeared and led to great losses. The ciliate was isolated and characterized in the laboratory. Its grazing rate exceeded the growth rate of three tested *Synechocystis* strains (*Synechocystis* PCC6803, *Synechocystis* CCALA192 and *Synechocystis minuscula* SAG 258.80) by far. Due to its devastating effect on *Synechocystis* sp., its ability to form cysts and its resilience, *Colpoda steinii* was experienced as serious threat for large scale cultures of *Synechocystis* sp. The most common means against contaminations are high salinities and high pH values. Though, cultivating with salinities up to 20‰ and pH values up to 10 did not inhibit *Colpoda steinii*. Other strategies like high ammonia concentration, carbon dioxide (CO_2) asphyxiation and anoxic conditions during the dark period were tested against the ciliate as well. While high ammonia concentration and CO_2 asphyxiation did no inhibit *Colpoda steinii*, partially anoxic conditions inhibited this ciliate effectively and appears to be a promising cultivation method for *Synechocystis* sp. under non-sterile conditions.

1. Introduction

Cyanobacteria are phototrophic prokaryotes playing a substantial role in the global carbon and oxygen cycle [1]. They take up carbon dioxide (CO_2) from the atmosphere and are considered as sustainable biomass producers. From the estimated 6000 cyanobacterial species [2] only *Arthrospira* sp. is produced in a larger scale, mainly for food purposes. However, the small unicellular cyanobacterium *Synechocystis* sp. is a promising production organism for a big variety of products and a lot of research has been published in recent years. The genome of *Synechocystis* PCC6803 has been fully sequenced in 1997 and all basic molecular biology techniques are available for metabolic engineering [3]. Wild type strains are capable to produce the degradable bioplastic polyhydroxybutyrate (PHB) [4–7], giving the opportunity to produce bioplastics directly from CO_2. Another highly interesting cell component is the blue fluorescent pigment phycocyanin, which can be used as food colorant [8–10]. *Synechocystis* PCC6803 has also been reported as promising vehicle for sustainable energy supply, as it can produce energy rich molecules like hydrogen or ethanol via photosynthesis [11–14]. Further promising products from *Synechocystis* sp. are platform chemicals like lactic acid [15] or isoprene [16–19].

For an economic production of most of these products, *Synechocystis* sp. needs to be cultivated in ponds or large photobioreactors. Generally, these systems do not provide the possibility of sterilization and also when closed photobioreactors are used, biological pollutants will inevitably enter the culture [20]. *Synechocystis* sp., as small phytoplankton, is situated at the very beginning of the food chain and is grazed by zooplankton, what makes the establishment of a large-scale culture under non-sterile conditions very difficult. Although there are many possible applications for *Synechocystis* sp., large-scale experiments under non-sterile conditions have been scarcely reported. Touloupakis and colleagues produced hydrogen with *Synechocystis* PCC6803 [12] and had to deal with contaminations as well, as they reported culture crashes due to *Poterioochromonas* sp. [21].

In this study, we report the effect of *Colpoda steinii* on a large-scale culture of *Synechocystis* CCALA192 and a cultivation strategy to inhibit this contaminant. Our attempts to produce polyhydroxybutyrate with *Synechocystis* CCALA192 repeatedly failed due to culture crashes. We

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successfully isolated *Colpoda steinii*, a 30 μm large ciliate, and characterized it in the laboratory. Several strategies against *Colpoda steinii* were tested and the predator – prey relations are discussed.

2. Material and methods

2.1. Organisms and standard culture conditions

Three different *Synechocystis* strains were used for this research. *Synechocystis* CCALA192 was ordered from the Culture Collection of Autotrophic Organisms, Institute of Botany, Trebon, Czech Republic. *Synechocystis* PCC6803 was ordered from the Pasteur Culture Collection of Cyanobacteria, Institute Pasteur, Paris, France. *Synechocystis minuscula* SAG 258.80 was ordered from the Culture Collection of Algae at Göttingen University, Göttingen, Germany. *Synechocystis* CCALA192 and *Synechocystis* PCC6803 were grown in BG11 medium [22] supplemented with 1 g/L sodium hydrogen carbonate ($NaHCO_3$) with a resulting pH of 8.5. *S. minuscula* SAG 258.80, originally isolated from a natron lake, was grown in BG11 medium supplemented with 5 g/L $NaHCO_3$ with a resulting pH of 9.6. All strains were grown in 50 mL shaking flasks under a light intensity of 1000 lx at 25 °C. Light-dark cycle was 16 h/8 h.

The ciliate *Colpoda steinii* was isolated from the photobioreactor and cultivated in the lab in 50 mL shaking flasks with *Synechocystis* CCALA192 as prey. For this, 1 mL of *Colpoda steinii* containing culture was transferred into a well-grown *Synechocystis* CCALA192 culture with above described standard culture conditions. Keeping *Colpoda steinii* for a longer period of time turned out to be simple due to the formation of resting cysts, that excyst 24–48 h after transferring into a fresh, well-grown *Synechocystis* CCALA192 culture.

2.2. Photobioreactor

The photobioreactor used in this research is located in a small greenhouse at the coal power plant site in Dürnrohr, Austria. It is a tubular photobioreactor with the system of the Schott AG, Germany, with a volume of 200 L. For additional artificial light, six gas-discharge lamps were used with light-dark cycle of 16 h/8 h. A degasser with pressurized air served for oxygen removal and a centrifugal pump circulated the algal culture. The degasser could be operated with nitrogen gas instead of pressurized air as well. The pH value and oxygen concentration were continuously measured with probes (CPS11D and COS51D, both Endress + Hauser GmbH, Austria) and pH was controlled with injection of pure CO_2. Photosynthetically active radiation (PAR) was measured with a PAR sensor (Theodor Friedrichs & Co., Germany). Temperature in the glasshouse was controlled with an air conditioning system (Daikin Industries) and held at 25 °C ± 2 °C.

2.3. Measurement of biomass dry weight and optical density

For biomass dry weight determination, 30 mL of the algal culture were centrifuged. The pellet was washed with deionized water, centrifuged again and dried at 105 °C overnight. Optical density was measured with a plate reader (Tecan Infinite M200 pro) at 680 nm.

2.4. Strategies against Colpoda steinii in the photobioreactor

The photobioreactor was operated with *Synechocystis* CCALA192. After the first culture crashes appeared, several different strategies were tested to inhibit the ciliate and establish a stable culture. The main focus was to remove or inhibit the ciliate. Possible growth rate reduction of *Synechocystis* CCALA192 was not studied. Following strategies were conducted in the photobioreactor.

Sanitizing the photobioreactor: The photobioreactor was thoroughly cleaned after culture crash and sanitized with a 0.1% sodium hypochlorite (NaClO) solution. In another attempt, the photobioreactor was sanitized with 0.5% ammonia solution.

High salinity: As a brackish water species, *Synechocystis* CCALA192 is able to grow with salt concentration up to about 20 g/L without growth inhibition. BG11 medium was supplemented with 20 g/L NaCl to test if this higher salinity inhibited the ciliate.

High pH value: BG11 medium was supplemented with 0.5 g/L sodium carbonate (Na_2CO_3) and then adjusted to a pH value of 10 with pure CO_2.

High ammonia concentration: Nitrate-free BG11 medium was supplemented with 0.6 g/L NH_4Cl (≙0.2 g/L NH_4^+) as sole nitrogen source and adjusted with $NaHCO_3$ and pure CO_2 to pH 8.5 to test ammonia toxicity.

CO_2 asphyxiation: BG11 medium was supplemented with 1 g/L $NaHCO_3$ and pH value was adjusted to 6.35 with pure CO_2 leading to a calculated dissolved CO_2 concentration of 262 mg/L.

Partially anoxic conditions: BG11 medium was supplemented with 1 g/L $NaHCO_3$. Pure CO_2 was used to keep the pH at a maximum value of pH 9. The photobioreactor was degassed with nitrogen gas at a constant flow rate of 500 mL/min leading to anoxic conditions during the dark period.

2.5. Effect of Colpoda steinii to different Synechocystis strains

The effect of *Colpoda steinii* to other *Synechocystis* strains than CCALA192 was tested in the laboratory. Those were *Synechocystis* PCC6803, the well-studied model organism, and *S. minuscula* SAG 258.80, cultivated with above described standard culture conditions. *Synechocystis* CCALA192 was also tested for comparison. 50 mL shaking flasks were inoculated to a final cell number of 10^7 *Synechocystis* cells per mL and after eight days of growth, 1 mL containing approximately 10^4 ciliates were added to the cultures. Each experiment was performed in triplicate.

2.6. Toxicity test of quinine sulphate on Synechocystis and Colpoda steinii

Moreno-Garrido et al. reported the successful application of 10 mg/L quinine sulphate against a not determined ciliate with a size of around 100 μm in *Dunaliella* cultures [23]. Therefore, we tested this substance against *Colpoda steinii*. Quinine sulphate was ordered from Alfa Aesar. First, toxicity of quinine sulphate was tested on *Synechocystis* CCALA192 in concentrations ranging from 5 to 50 mg/L in 50 mL shaking flasks (standard conditions, pH 8.6) and OD680 was measured daily. The cultures were started with an initial OD680 of 0.4 and every concentration was tested in triplicate. The areas under the growth curves for 120 h cultivation time were calculated. The control group without quinine sulphate represented 100%. For the toxicity test against the ciliate, 100 μL of a *Colpoda steinii* culture (three days after transferring) were placed into 96-well plates. Quinine sulphate was added to final concentrations ranging from 5 to 50 mg/L. After 24 h, mobility of the ciliates was checked in the microscope as criterion of death. Every concentration was tested in triplicate.

2.7. Microscopy

Microscopic images were made with an Olympus AHBT3 VANOX microscope in differential-interference contrast mode. Samsung Galaxy S6 was used with an ocular adapter for imaging.

3. Results and discussion

3.1. Ciliate isolation and identification

After first installation of the photobioreactor, *Synechocystis* CCALA192 was grown succesfully for approximately 18 months, when first culture crashes were experienced. Those were first noticed through (i) brownish foam in the degasser, (ii) decreasing oxygen concentration