Growth of Chlamydomonas reinhardtii in acetate-free medium when co-cultured with alginate-encapsulated, acetate-producing strains of Synechococcus sp. PCC 7002

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Abstract

Background: The model alga Chlamydomonas reinhardtii requires acetate as a co-substrate for optimal production of lipids, and the addition of acetate to culture media has practical and economic implications for algal biofuel production. Here we demonstrate the growth of C. reinhardtii on acetate provided by mutant strains of the cyanobacterium Synechococcus sp. PCC 7002.

Results: Optimal growth conditions for co-cultivation of C. reinhardtii with wild-type and mutant strains of Synechococcus sp. 7002 were established. In co-culture, acetate produced by a glycogen synthase knockout mutant of Synechococcus sp. PCC 7002 was able to support the growth of a lipid-accumulating mutant strain of C. reinhardtii defective in starch production. Encapsulation of Synechococcus sp. PCC 7002 using an alginate matrix was successfully employed in co-cultures to limit growth and maintain the stability.

Conclusions: The ability of immobilized strains of the cyanobacterium Synechococcus sp. PCC 7002 to produce acetate at a level adequate to support the growth of lipid-accumulating strains of C. reinhardtii offers a potentially practical, photosynthetic alternative to providing exogenous acetate into growth media.

Keywords: Biofuels, Algae, Cyanobacteria, Lipid production, Acetate production, Co-culture, Alginate immobilization

Background

With the ongoing increase in global energy demand, the development of alternative energy sources has been at the forefront of recent modern research. Of the various alternative energies, bioenergy has been of interest because of its many potential benefits over the currently used petroleum, natural gas, and coal. These benefits include carbon neutrality, renewability, low environmental toxicity, and reduction of dependence on foreign energy sources. Biodiesel and bioethanol, which together currently account for the major proportion of biofuels, are mainly produced from higher plants [1]. These fuels can replace diesel and gasoline, respectively, in conventional engines without modification. Of these two biofuels, biodiesel has been considered the most viable option in the United States [2]. However, the production of biodiesel from crops grown on arable land is controversial due to the potential implications of the competition with agricultural food production [3,4]. One alternative to using food crops as oil sources is to produce biofuels using microalgae and/or cyanobacteria, which can be grown in areas not suitable for crop growth, such as deserts [5,6]. Microalgae can be used to assimilate CO2 and produce multiple fuel molecules, including hydrogen, starch, and lipids [7-9]. Starch can be fermented to hydrogen or ethanol, while lipids can be converted to biodiesel [10-12].

Some lipid-accumulating microalgae, such as Chlamydomonas reinhardtii, are able to grow photoautotrophically on sunlight and CO2, chemotrophically on acetate, or photomixotrophically in a combination of these two growth modes [13,14]. Optimal lipid production by C. reinhardtii
is observed under photomixotrophic conditions in the presence of acetate, which could present economic and practical challenges for large-scale production of algal-based biofuels [15,16]. An alternative is to co-culture lipid-producing *C. reinhardtii* with an acetate-producing cyanobacterium, such as certain *Synechococcus* sp., that can naturally produce acetate during photosynthesis and/or fermentation [17-19].

In this study, we present results of co-culturing wild-type and a lipid-accumulating (*sta6*) strain of *C. reinhardtii*, and an acetate-accumulating strain (*glgA1*) of *Synechococcus* sp. PCC 7002. We demonstrate sustained photomixotrophic growth of *C. reinhardtii* with acetate produced by *Synechococcus* 7002.

**Results and discussion**

**Effect of temperature on growth of *Synechococcus* sp. PCC 7002 and *C. reinhardtii***

To determine conditions under which both organisms could grow well together, the wild-type and mutant strains of *C. reinhardtii* and *Synechococcus* sp. PCC 7002 were grown individually on Tris-Acetate-Phosphate (TAP) medium, a standard medium for *C. reinhardtii*, and A+ medium, a standard medium for *Synechococcus* sp. PCC 7002. Consistent with previous reports, the results of our preliminary experiments showed that the optimum temperature for the growth of *C. reinhardtii* is ~30°C [20-22] and that for *Synechococcus* 7002 ~ 38°C [23]. An increase in temperature from 30°C to 34°C and then to 38°C provided sufficient growth conditions for both organisms.
increased the lag phase in the C. reinhardtii cultures (Figure 1A). In contrast, decreasing the temperature from 38°C to 34°C and finally to 30°C did not significantly affect the growth of either wild-type (Figure 1B) or mutant strains of Synechococcus sp. PCC 7002 under the light intensities and CO₂ concentrations used in this study (data not shown). Therefore subsequent co-culture optimizations were conducted at 30°C.

Effect of media composition on growth of C. reinhardtii and Synechococcus sp. PCC 7002

The main difference between TAP medium (C. reinhardtii) and A⁺ medium (Synechococcus sp. PCC 7002) is that medium A⁺ is a marine medium that contains 300 mM sodium chloride. Other differences include the use of ammonium chloride as the nitrogen source in TAP medium versus sodium nitrate in A⁺ medium, the presence of acetate in TAP, and the presence of vitamin B₁₂ in A⁺ medium. To accommodate the growth of both C. reinhardtii and Synechococcus sp. PCC 7002 in co-culture, both the TAP and A⁺ media were modified (henceforth referred to as modified TAP and modified A⁺, respectively). Modified TAP medium consists of TAP medium supplemented with sodium nitrate and vitamin B₁₂; modified A⁺ medium consists of standard A⁺ medium supplemented with ammonium chloride and acetic acid, with the sodium chloride concentration reduced to 150 mM. (Additional file 1: Table S1). The results of our experiments showed that C. reinhardtii cultures are unable to grow on A⁺ medium and/or modified A⁺ medium at 30°C (Figure 2, and insert in Figure 2) and 38°C (data not shown). However, Synechococcus sp. PCC 7002 was able to grow on both TAP and modified TAP media at 30°C (Figure 2).

Effect of acetate on growth of C. reinhardtii and Synechococcus sp. PCC 7002

To investigate the effects of acetate on the growth of both wild-type and mutant cultures of C. reinhardtii and Synechococcus sp. PCC 7002, cultures were grown on TAP medium containing acetate and on a modified form of TAP medium that lacked acetate (termed co-culture medium). The results of these experiments showed that the growth rates of wild-type and mutant cultures of C. reinhardtii were higher in the presence of acetate when compared to cultures grown in the absence of acetate. Cultures of C. reinhardtii grown in acetate-free medium showed little growth under these conditions (Figure 3A). Although Synechococcus sp. PCC 7002 cultures grown without acetate had slightly lower final cell densities than cultures grown in the presence of acetate, the growth rates with and without acetate were similar for both wild-type and mutant strains (Figure 3B). During the growth of wild-type and sta₆ mutant strains of C. reinhardtii, acetate consumption correlated with an increase in cell number (Figure 4A). Acetate production by the glgA₁ mutant of Synechococcus 7002 was higher than that of the wild type (Figure 4B).

Optimizing co-cultures

Based on the initial experiments, the sta₆ mutant of C. reinhardtii was grown on co-culture medium at pH 7.0 and 30°C with the glgA₁ mutant of Synechococcus sp. PCC 7002. To investigate the effect of initial cell number
in the inoculum during co-culturing of free cells, different initial ratios, 1:1 and 1:10, of *C. reinhardtii* to *Synechococcus* sp. PCC 7002 cells were tested. The ratio 1:10 was chosen because *Synechococcus* sp. PCC 7002 appeared to produce less acetate than required to produce optimal growth of *C. reinhardtii*, and it was rationalized that a larger number of *Synechococcus* sp. PCC 7002 cells might be advantageous in the co-cultures. However, the results of the co-culturing experiments with free cells revealed that *Synechococcus* sp. PCC 7002 cells grew faster than *C. reinhardtii* at both initial cell ratios and eventually took over the co-culture (data not shown). To avoid the problem of overgrowth by *Synechococcus* sp. PCC 7002, the cells were encapsulated in alginate beads to slow their growth (see Methods section for details). The growth of the *C. reinhardtii* *sta6* mutant in the presence of control (empty) beads in modified TAP media showed that the alginate encapsulating matrix had no effect on the growth of *C. reinhardtii* cells (data not shown). The results of the experiments using alginate-encapsulated *Synechococcus* sp. PCC 7002 cells showed that the growth of the lipid-accumulating *C. reinhardtii* *sta6* strain could be supported by the presence of the acetate-producing *Synechococcus* sp. PCC 7002 *glgA1* gene.

**Figure 3** Effect of acetate on growth of *C. reinhardtii* and *Synechococcus* sp. PCC 7002. The cultures of *C. reinhardtii* (A) and *Synechococcus* sp. PCC 7002 (B) were grown in the presence (closed symbols) and absence (open symbols) of acetate. Both *C. reinhardtii* and *Synechococcus* sp. PCC 7002 wild-type cultures are shown in circles. The *sta6* mutant of *C. reinhardtii* and the *glgA* mutant of *Synechococcus* sp. PCC 7002 are shown in squares.
strain (Figure 5) and that immobilization controlled the growth of *Synechococcus* sp. PCC 7002 and kept the co-cultures from being overgrown.

**Conclusions**

We have developed media formulations and growth conditions that support co-cultures of wild-type and the acetate-producing *glgA1* strain of the *Synechococcus* sp. PCC 7002 and wild-type and lipid-accumulating *sta6* mutant of *C. reinhardtii* (Additional file 2: Figure S1). A temperature of 30°C supported the growth of both organisms; however, *Synechococcus* sp. PCC 7002 overtook the culture when both organisms were present as free cells. Alginate encapsulation was found to be an effective way to slow the growth of *Synechococcus* sp. PCC 7002 while at the same time allowing the production of sufficient acetate to stimulate growth and lipid storage of *C. reinhardtii*. The encapsulation of *Synechococcus* sp. PCC 7002 also provides a potential mechanism for separating and harvesting *C. reinhardtii* for lipids and recycling of the *Synechococcus* sp. PCC 7002 cells. The results reported here provide a strong proof of concept for supplanting the provision of costly medium components through co-culturing that can be optimized through metabolic engineering and implementation of enhanced acetate-producing cyanobacteria.
Methods

Growth of microorganisms

Inoculum cultures of both _Synechococcus_ sp. PCC 7002 and _C. reinhardtii_ (25 ml) were prepared in standard growth media for each organism in 50-ml Erlenmeyer flasks oscillating at 100 rpm at a constant illumination of 100 μmol photons m⁻² s⁻¹ with warm white fluorescent light. Wild-type and glgA1 knockout mutant strains of _Synechococcus_ sp. PCC 7002 were grown in medium A⁺ (pH = 8.0) [24] (Additional file 1: Table S1) and were incubated at 38°C while sparging with 1% v/v CO₂ in air. Wild-type _C. reinhardtii_ and sta6 mutant strains were grown in TAP media (pH = 7.0) incubated at 30°C. The optical density was monitored at 750 nm (ThermoSpectronic Bio Mate 3 spectrophotometer) for _Synechococcus_ sp. PCC 7002 and _C. reinhardtii_ strains. Cultures were harvested by centrifugation for 10 min at 8,000 × g, washed in the medium to be used, centrifuged again, and finally resuspended in the appropriate medium and used to inoculate different culture conditions for coculturing. Optimal co-culturing conditions were determined by first growing wild-type _Synechococcus_ sp. PCC 7002 and _C. reinhardtii_ strains individually at 30°C, 34°C, and 38°C on TAP medium (standard _C. reinhardtii_ growth medium) or A⁺ medium (standard _Synechococcus_ sp. PCC 7002 growth medium). The formulation of the TAP media, A⁺ medium, and modified derivatives of each can be found in the supplemental data (Additional file 1: Table S1). For the study, standard TAP medium was modified by eliminating acetic acid and supplementing with 1 g NaNO₃ L⁻¹ and 4 μg vitamin B₁₂ L⁻¹, and this formulation was termed co-culture medium. A⁺ medium was supplemented with 7.5 g NH₄Cl L⁻¹ and 1 ml glacial acetic acid L⁻¹, and the concentration of NaNO₃ and NaCl was reduced by 50%. Co-cultures were inoculated with a mixture of seed cultures grown in the appropriate culture media, and the optical densities were monitored at both 600 nm and 750 nm. The cultures were monitored for bacterial contamination by microscopic examination, and cell numbers were determined by direct cell counting with a hemocytometer.

Cell immobilization

_Synechococcus_ sp. PCC 7002 cultures were harvested at an OD₆₅₀ nm ranging from 0.8-1.0 (ThermoSpectronic Bio Mate 3 spectrophotometer) via centrifugation and resuspended in 1/5 the original media volume, yielding a concentrated cell suspension. Alginate beads were made by dissolving 3 g of sodium alginate directly in 100 ml of concentrated culture media and adding it dropwise with a syringe and needle into a 1% (w/v) solution of CaCl₂ in air. The bead size was controlled by needle gauge: 22 G yields beads of approximately 2 mm in diameter and 18 G yields beads of 3 mm, while 4-mm beads are made directly from a 10-ml syringe without a needle. Beads were formed immediately and were allowed to harden further in the CaCl₂ solution for 15 min. After hardening, the beads were removed from the CaCl₂ solution and added to fresh growth media. To prevent leakage of cells, a cell-free layer
of alginate was used to coat the beads. To produce the coating, the beads were submerged in a 3% (w/v) alginate solution in growth medium followed by transfer to a 1% (w/v) solution of CaCl₂ in growth medium to allow hardening of the cell-free alginate layer.

Acetate determination

Acetate concentrations were determined by 1H-NMR [26]. Samples were centrifuged for 10 min at 14,000 x g and 500 μl of the supernatant was transferred to an NMR tube to which 50 μl of D₂O was added. Spectra were collected on a Bruker DRX500 NMR spectrometer operating at a frequency of 500.13 MHz with a 1D NOESY pulse sequence and a 100-ms mixing time at 32 K. Typically, 64 4.28-s scans were collected for each sample with a pre-saturation pulse to suppress the water signal.

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