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Prospective technology on bioethanol production from photofermentation

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HIGHLIGHTS

- *C. reinhardtii* and *R. capsulatus* were utilized to the ethanol production.
- Ethanol was produced from sulfur deprivation and mixotrophic carbon source.
- Hybrid system and co-cultivation increase the ethanol production by photofermentation.

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ABSTRACT

The most important global demand is the energy supply from alternative source. Ethanol may be considered an environmental friendly fuel that has been produced by feedstock. The production of ethanol by microalgae represent a process with reduced environmental impact with efficient CO_2 fixation and requiring less arable land. This work studied the production of ethanol from green alga *Chlamydomonas reinhardtii* through the cellular metabolism in a light/dark cycle at 25 °C in a TAP medium with sulfur depletion. The parameters evaluated were inoculum concentration and the medium supplementation with mixotrophic carbon sources. The combination of *C. reinhardtii* and *Rhodobacter capsulatus* through a hybrid or co-culture systems was also investigated as well. *C. reinhardtii* maintained in TAP-S produced 19.25 ± 4.16 g/L (ethanol). In addition, in a hybrid system, with medium initially supplemented with milk whey permeated and the algal effluent used by *R. capsulatus*, the ethanol production achieved 19.94 ± 2.67 g/L.

1. Introduction

The use of ethanol to replace oil is the most viable way to ensure a sustainable future. However, up to now more emphasis has been given on the yeast performance in reactors for bioethanol production (Andrietta et al., 2008). On the other hand, considering the advantages of microalgae culture such as rapid growth rate and productivity (Li et al., 2008) and their use to minimize contamination, since microalgae may to applied in wastewater treatment from inorganic salts (NH_4^+, NO_3^-, PO_4^{3-}) using them as nutrient materials (Mata et al., 2010), *Chlamydomonas reinhardtii* could be preferentially selected as a prospective biological system for bioethanol production instead of yeast.

Concerning on the application of microalgae, recent research have shown that a diversity of strains may be cultivated to produce biodiesel, hydrogen, methane and ethanol. Specifically to the ethanol production from microalgae, it can be directly synthesized by the cellular metabolism or from the fermentation of microalgae biomass, mainly cellulose and starch that are readily converted to fermentable products by enzymatic or acidic pretreatment technology (Oncel, 2013; Chen et al., 2013). Microalgae based ethanol may be considered as part of integrated process and a promising environmentally friendly alternative, since they could be present a potential for fixing CO_2, high growth at high yields and low costs utilizing light as the energy source. Furthermore, they do not require fertile land and portable water as feedstock based biofuel (Chen et al., 2013; John et al., 2011; Hirano et al., 1997).

The unicellular green alga *C. reinhardtii* uses light to grow photoautotrophically or mixotrophically in the presence of small organic substrates (Goff et al., 2009). *C. reinhardtii* is widely studied for hydrogen production by biophotolysis of water. This method uses the same processes found in plants photosynthesis. Photosynthesis involves the absorption of light by two distinct

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photosynthetic systems operating in series: a water splitting and O_2 evolving system (“photosystem II” or PSII) and a second photosystem (PSI), which generates the reductant used for CO_2 reduction (Das and Veziroglu, 2001). It is important to note that green algae could produce hydrogen not only under light conditions, but under dark anaerobic conditions (Gaffron and Rubin, 1942). Nevertheless, if dark and anaerobic conditions are established on the microalgae cultures, hydrogen yield is quite low corresponding to about one-sixth of direct biophotolysis production (Kosourov et al., 2002). Besides hydrogen, the oxidative reaction of starch become incomplete and depending on the type of the microalga, carbon dioxide, ethanol, lactic acid, formic acid, acetic acid, malic acid, glycerol and other compounds are produced in varying proportions (John et al., 2011; Gfeller and Gibbs, 1984). In addition, the medium composition may be altered to induce anaerobic condition. Hemschemeier and Happe (2004) reported that, under sulfur depletion, *C. reinhardtii* stops growing and accumulates starch. The absence of sulfur forces the algae to reorganize the whole metabolism. Anaerobiosis is established and hydrogen and ethanol could be produced. These authors discussed that the accumulation of ethanol already indicate the activity of pyruvate formate-lyase (PFL). PFL cleaves pyruvate into acetyl-CoA and it can further be reduced to acetaldehyde by acetaldehyde dehydrogenase. Furthermore, ethanol can be formed from cleavage of pyruvate by pyruvate decarboxylase (PDC) producing acetaldehyde as intermediate that is converted to ethanol by alcohol dehydrogenase (ADH).

Previous work suggested it is possible to produce ethanol from metabolism of *C. reinhardtii* maintained in a basal medium supplemented with the mixotrophic carbon source (Costa et al., 2014). And, the purpose of this work was at first to verify the possibility of *C. reinhardtii* produce ethanol using a basal medium with sulfur depletion added mixotrophic carbon source such as milk whey permeate (rich in lactose) and sodium acetate. In the second step, the algae association with the purple non sulfur bacterium, a *Rhodobacter capsulatus*, to improve ethanol content into the medium was also evaluated by hybrid system (two stages) and co-culture. The hybrid systems and co-cultures approaches are strategies used in order to improve the yield. The purpose is to integrate microorganisms with distinct biological routes. Thus, the metabolites produced by one type of microorganism may be the substrate to the second type.

2. Methods

2.1. Algal biomass

C. reinhardtii CC-124 was purchased from the Canadian Culture Collection, the *Chlamydomonas* Resource Center. The green alga was maintained in the basal medium Tris Acetate Phosphate (TAP) (Andersen, 2005) at initial pH of 7.0. In order to guarantee enough amounts of cells for the fermentation assays, the algal inoculum was subcultured with the addition of 250 mL of fresh TAP medium in 250 mL of growing culture. The alga was kept in Erlenmeyer (500 mL) at 25 °C under light cycle (night/day) of 12 h at 30 $\mu E\ m^{-2}\ s^{-1}$.

2.2. Photosynthetic bacterial biomass

R. capsulatus was purchased from DSMZ German Collection of Microorganisms and Cell Culture. The strain was cultivated anaerobically in Erlenmeyers (500 mL) maintained at 30 °C using RCV medium (Weaver et al., 1975), at initial pH of 6.8, under photosynthetic conditions of 30 $\mu E\ m^{-2}\ s^{-1}$ (light-grown cells). Sodium glutamate was used as nitrogen source instead of $(NH_4)_2SO_4$.

2.3. Biological strategies to ethanol production by photofermentation

For ethanol production by fermentation, anaerobic condition was used in all strategies: by *C. reinhardtii* cultures, hybrid system and co-culture approach. Thus, 50 mL bioreactors were flushed with Ar gas (99.999%) for 3 min. The inoculum was 10 days age for green algae culture and five days age for the purple non sulfur bacteria culture. Five days of time fermentation was used in all assays. The photoperiod of 12 h (12 h dark/12 h light) was used in all experiments with algae. In the hybrid system, in the second stage by *R. capsulatus*, the assays were carried out under light continuously.

2.3.1. C. reinhardtii under sulfur depletion

In the first step of the current work, assays were carried out to investigate the ethanol production by *C. reinhardtii* in TAP-S (Tris–acetatephosphate-minus-sulfur) medium. The evaluated variables were the initial cell concentration (0.05, 0.10 and 0.20 g/L) of green alga and the type of carbon source in a mixotrophic pathway. In these latter trials, besides acetic acid, as usually present in the TAP medium, it was added more 0.1 or 1.0 g/L of sodium acetate and milk whey permeate (MWP), individually or simultaneously. As the manufacturer, *Sooro Concentrado Industria de Produtos Lácteos* Ltda from Brazil, milk whey permeate contain lactose (93%), proteins (1.2%), ashes (4.6%), among others traces compounds.

2.3.2. Hybrid system

The hybrid system was evaluated as an alternative to increment the biofuel accumulation. In the first stage, *C. reinhardtii* was cultivated in TAP-S medium and the effluent, free of cell and rich in soluble metabolites, was used as substrate by *R. capsulatus*, since these photosynthetic bacteria can convert the organic acids and the non-consumed original carbon source for additional ethanol production.

Initially the effects of supplementing *R. capsulatus*'s medium with sodium glutamate, malic acid, milk whey permeate and micronutrient solution, according Table 1a, were evaluated. Subsequently, similar assays were carried out to verify the influence of mixotrophic carbon source on the ethanol production by *C. reinhardtii* adding to the TAP-S medium different concentrations of sodium acetate and milk whey permeate. In these assays, only the crude effluent was used in the second stage of hybrid system (Table 1b). For all assays, the initial cell concentration was 0.1 g/L and they were performed in duplicate.

2.3.3. Co-culture of algae and bacteria

Co-cultures of *C. reinhardtii* and *R. capsulatus*, being both cultured in TAP-S medium and RCV medium, were evaluated. The initial cell densities were 0.1 g/L, mixed together at variant ratios (from 0% to 100%). Co-culture grew in bioreactor of 50 mL (working volume of 37.5 mL) at 25 °C using 30 $\mu E\ m^{-2}\ s^{-1}$ of light intensity.

2.4. Analytical methods

The growth of cells was measured via spectrophotometry (UVmini-1240, Shimadzu) and biomass dry weight. One milliliter of sample was appropriately diluted with deionizer water and the absorbance of the sample was read at 665 nm (algae) and 660 nm (bacteria). The concentrations of metabolites were determined by HPLC (Shimadzu MODEL LC-20A Prominence, Supelcogel, column C-610H), equipped by ultra-violet and refractive detectors. The UV–VIS was used to determine organic acid concentrations at a wave length of 210 nm and the RID detector quantified the contents of lactose and ethanol. The column temperature was kept at 32 °C and an aqueous solution of 0.1% M H_3PO_4 was used for elution at 0.5 mL/min. The sample volume injected into the HPLC