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Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production

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ABSTRACT

The production of ethanol from feedstock other than agriculture materials has been promoted in recent years. Some microalgae can accumulate a high starch content (about 44% of dry base) *via* photosynthesis. Algal biomass, *Chlamydomonas reinhardtii* UTEX 90, was converted into a suitable fermentable feedstock by two commercial hydrolytic enzymes. The results showed that almost all starch was released and converted into glucose without steps for the cell wall disruption. Various conditions in the liquefaction and saccharification processes, such as enzyme concentration, pH, temperature, and residence time, have been investigated to obtain an optimum combination using the orthogonal analysis. As a result, approximately 235 mg of ethanol was produced from 1.0 g of algal biomass by a separate hydrolysis and fermentation (SHF) method. The main advantages of this process include the low cost of chemicals, short residence time, and simple equipment system, all of which promote its large-scale application.

1. Introduction

From a greenhouse standpoint, renewable fuels such as ethanol are considered to be excellent alternative clean-burning fuels to gasoline in the future as the combustion products are environmentally safe (Kito-Borsa et al., 1998). Today, the most common renewable fuel is ethanol, derived mainly from glucose or starch sources of agricultural stock (Gray et al., 2006; Nigam and Singh, 1995). The human demand for food, however, has yet to be met. To solve both the energy and food problem, there has been increasing interest and worldwide studies in producing bioethanol from algal biomass, the alternative to agricultural stock (Baras et al., 2002; Kim and Dale, 2003; Sánchez and Cadona, 2008). *C. reinhardtii*, a unicellular green alga, is well-known as a photoautotrophic microorganism having a great ability to fix CO_2 and accumulate a high content of stored polysaccharides, mainly starch, in complex multilayered cell walls (Hall and Rao, 1994; Hirano et al., 1997). This bears a strong structural and functional resemblance to higher plant storage starch (Libessart et al., 1995). With a high growth rate, the microalgae can be easily cultured at high yields and low costs utilizing an unlimited energy source, sunlight (Hirayama et al., 1998; Sze, 1998). These advantages allow the microalgae to be preferentially selected as a safe and prospective feedstock for bioethanol production by *Saccharomyces cerevisiae*.

Prior to ethanol fermentation, the feedstock needs to be processed by enzymatic or acidic pretreatment technology in order to release fermentable sugars. Since the first application of microbial enzyme in the food industry in the early 1960s, a great deal of effort has been made to replace traditional acid hydrolysis with enzymatic hydrolysis in almost all glucose production due to higher yields under mild conditions, less by-products, and no corrosion issues (Balat et al., 2008). Several hydrolytic enzymes possessing improved activity have been developed and used extensively in the starch processing industry for liquefaction and saccharification (Olsen, 2004; Schäfer et al., 2007). To this end, a commercial amyloglucosidase, AMG 300L, was produced by a genetically modified strain of *Aspergillus*.

The main obstacle of enzymatic hydrolysis is that intercellular starch granules are bound within rigid cell walls (Libessart et al., 1995), thus a biomass pretreatment step is needed to break down the cell wall to release polysaccharides such as starch, structural carbohydrates, and other nutrients, prior enzymatic hydrolysis and fermentation steps. The cell wall of *C. reinhardtii* contains glycoproteins as the predominant constituents in its extracellular matrix (Sze, 1998). A commercial α-amylase derived from *Bacillus licheniformis*, Termamyl 120L, shows a protease activity particular to the degradation of glycoproteins within cell walls (Imam and Snell, 1987). Starch hydrolysis involves liquefaction and saccharification of the starch. The objective of this study was to optimize conditions to apply the starch-degrading enzymes sequentially to pretreatment of the algal biomass. The optimal conditions required for the enzymatic hydrolysis of starch, such as enzyme concentration, temperature, pH, and residence time, were selected by the orthogonal analysis method, which weighed the effects on both hydrolysis and ethanol fermentation; a maximum rate of ethanol

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production could then be achieved. Two-step enzymatic hydrolysis by commercially available α-amylase and amyloglucosidase would yield lower energy consumption, lower content of non-glucosidic impurities, and thus, a much better suitability for ethanol production (Mojović et al., 2006). To the best of the authors' knowledge, this is the first report regarding enzymatic pretreatment of microalgal biomass optimized for ethanol fermentation. The work herein demonstrates that the enzymatic hydrolysate from the microalgae is potentially useful as a feedstock for fermentation by the ethanol-producing yeast, *S. cerevisiae* S288C.

2. Methods

2.1. Algal biomass

The medium used for the culture of the green algae, *C. reinhardtii* UTEX 90, was 1.6 L of tris–acetate–phosphate (TAP) medium, which was prepared as reported in our previous work (Thu et al., 2009). A cell culture was carried out during 96 h at 23 °C and 130 rpm in a 2.5 L photo-bioreactor with 10% inoculation of seed under continuous illumination (450 μE m^{-2} s^{-1}) by white fluorescent lamps. Light intensity in the photo-bioreactor was measured with an Li-250 Li-Cor quantum photometer (Lambda Instrument Corp., Lincoln, USA). The pH of the medium was automatically adjusted to the range of 7.0–7.4 by the feeding of 1.0 M acetic acid. Cell growth of the algae was monitored by measuring optical density (OD) at 600 nm. Dry cell weight (DCW) was determined by an 80 °C oven drying method using filter paper (GC/F, City, England). The cells harvested by centrifugation were characterized and used for pretreatment of the feedstock biomass.

2.2. Enzymes

Two enzymes for liquefaction and saccharification, thermostable α-amylase of *B. licheniformis* origin (EC 3.2.1.1, Termamyl 120L) and amyloglucosidase from *Aspergillus niger* (EC 3.2.1. 3, AMG 300L), were purchased from Novo Nordisk (Gentafte, Denmark). The enzymatic activities of these enzymes were 120 KNU g^{-1} and 300 AGU mL^{-1}, respectively, as defined by Novo Nordisk. The Kilo Novo α-amylase Unit (KNU) is defined as the amount of enzyme that can hydrolyze 5.26 g of soluble starch per hour at pH 5.6 and 37 °C. The Amyloglucosidase Novo Unit (AGU) is the amount of enzyme which cleaves 1.0 μmol of maltose/min at pH 4.3 and 25 °C.

2.3. Enzyme pretreatment of algae

Enzyme pretreatment consisted of two parts, liquefaction of algal biomass by α-amylase (Termamyl 120L) and saccharification by amyloglucosidase (AMG 300L). The harvested algal biomass were washed once and slurried in water at a 5% solid to liquid ratio (w/v), mixed with Termamyl 120L at a final concentration between 0.0001% and 0.02% (v/w), and pretreated as follows. Hydrolysis reactions were performed in capped flasks in a thermostated water bath. The pH of the mixture was adjusted to pH 6.0, a value known as the optimum (Richardson et al., 2002), with 10% H_2SO_4. The protease activity of the Termamyl 120L, the optimum temperature of which is in the range of 50–60 °C, was activated by increasing the incubation temperature from 25 °C to different temperatures (70, 80, 90, 100 °C) for 30 min, and thenceforth the algal biomass was hydrolyzed by the α-amylase activity of Termamyl 120L by maintaining it at the optimal temperature for 10–60 min. For subsequent saccharification after liquefaction, The pH of the mixture was adjusted to a range of 4.5–5.5, the temperature was reduced to a range of 50–65 °C, and AMG 300L at concentrations of 0.001–0.3% (v/w) was supplied for 5–60 min. By analyzing parameters such as temperature, residence time, enzyme concentration, and pH by orthogonal arrays, two processes of the enzymatic hydrolysis were optimized. Duplicate batches were run to verify results.

2.4. Ethanol fermentation by yeast

To test fermentability of the pretreated algal biomass, separate hydrolysis and fermentation (SHF) was performed using ordinary compressed baker's yeast, *S. cerevisiae* S288C as reported in our previous work (Thu et al., 2009). The solid fraction of the pretreated algal biomass was removed by centrifugation (4000*g*, 10 min). After the aerobically pre-cultured yeast cells were inoculated at 10% of volume size to the liquid fraction of pretreated algal biomass, the yeast cells were cultured anaerobically at 30 °C for 40 h at a rotation of 160 rpm. The concentration of the produced ethanol was analyzed by HPLC under the same conditions as the analysis of monomeric sugars in the pretreatment.

2.5. Analytical methods

The cells were disrupted by vortexing with glass beads in a methanol solution. The methanol was then vaporized by placing the cell lysates in the oven at 80 °C for 6 h. After the addition of 72% perchloric acid, the starch was extracted at 0 °C for 12 h and filtered through a 0.45 μm filter. The contents of the starches in the filtrate were determined by an iodo-starch reaction method (Hirokawa et al., 1982). The total cell carbohydrate was analyzed by a colorimetric method using an anthrone reagent. The algal cell pellets were mixed with 67% sulfuric acid by vortexing for 30 min and then reacted with the anthrone reagent for 5 min at 100 °C. The OD of the resulting solution was measured at 630 nm.

The intracellular monomeric sugar content of the *C. reinhardtii* was determined by high performance anion-exchange chromatography (HPAEC; DX-300 series chromatography system, Dionex, USA). The effluent was monitored with pulsed amperometric detection detector (PAD, Dionex, CA, USA). The cell pellets were washed two times with autoclaved distilled water and freeze-dried. Sample of 2.5 mg dry cell was dissolved in 1 ml trifluoroacetic acid (TFA; Sigma, USA). After hydrolysis at 100 °C for 4 h, the sample was cooled to room temperature and the volatile acid was removed by centrifugal evaporation (Speed-Vac; SPD1010-115, Savant Instruments, USA) under a stream of nitrogen gas. The dried sample was dissolved in 10.0 ml of water and filtered through 0.2 μm syringe filter. The standard sugars were also treated in the same way as described for the samples in order to identify and quantify. The 10 μl of filtered samples were injected into a CarboPac PA-1 anion-exchange column (0.4 × 250 mm, Dionex, CA, USA) that was pre-equilibrated in 18 mM NaOH. Chromatographic separation of the monomeric sugars from the samples was achieved in the isocratic mode with 18 mM NaOH at a flow rate of 1.0 ml/min in 20 min.

The content of the intracellular proteins was determined by the Bradford method (1976). The cells were disrupted by sonication in PBS buffer (pH 7.3), the supernatant was colored by mixing with the Bradford reagent (Sigma Chemical Co., St. Louis, MO, USA), and the OD of it was measured at 595 nm.

The concentrations of the oligosaccharide (dextrin) and monomeric sugar (glucose) in the liquid fraction of the enzymatic hydrolysate and ethanol produced by yeast were quantified by an Agilent 1200 high-pressure liquid chromatography (HPLC) system equipped with a quaternary solvent delivery system (Agilent Technologies, Palo Alto, CA, USA), an autosampler (No. 60044; Spark, Holland, Emmen, Netherlands), a refractive index detector (Acme