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Bioethanol production from the nutrient stress-induced microalga *Chlorella vulgaris* by enzymatic hydrolysis and immobilized yeast fermentation

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HIGHLIGHTS

- Carbohydrate production was enhanced by nitrogen stress cultivation.
- Pectinase enzyme saccharification was conducted for hydrolyzing microalgal cells.
- Microalgae hydrolysate converted into bioethanol by yeast fermentation.
- Saccharification efficiency (79%) and fermentation efficiency (89%) were obtained.

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ABSTRACT

The microalga *Chlorella vulgaris* is a potential feedstock for bioenergy due to its rapid growth, carbon dioxide fixation efficiency, and high accumulation of lipids and carbohydrates. In particular, the carbohydrates in microalgae make them a candidate for bioethanol feedstock. In this study, nutrient stress cultivation was employed to enhance the carbohydrate content of *C. vulgaris*. Nitrogen limitation increased the carbohydrate content to 22.4% from the normal content of 16.0% on dry weight basis. In addition, several pretreatment methods and enzymes were investigated to increase saccharification yields. Bead-beating pretreatment increased hydrolysis by 25% compared with the processes lacking pretreatment. In the enzymatic hydrolysis process, the pectinase enzyme group was superior for releasing fermentable sugars from carbohydrates in microalgae. In particular, pectinase from *Aspergillus aculeatus* displayed a 79% saccharification yield after 72 h at 50 °C. Using continuous immobilized yeast fermentation, microalgal hydrolysate was converted into ethanol at a yield of 89%.

1. Introduction

The ongoing consumption of limited fossil fuel resources has increased the cost of transportation. Consequently, a serious energy crisis may soon arise when fossil fuels are exhausted in the future. In addition, the emissions of CO_2 from fossil fuels have resulted in environmental pollution, global warming, and climate change problems. The development of a new source of alternative, sustainable, and clean fuel is one of the solutions (Laurens et al., 2012). Biofuels such as biodiesel, bioethanol, and biohydrogen can provide sources of fuel to satisfy future demand due to their great potential (Mussatto et al., 2010). These sources are environmentally friendly when compared with traditional fuels, since they minimize further contribution to emissions at the present time. Therefore, biofuel technology and its related markets are expected to grow rapidly in the near future (Demirbas, 2010).

Starch and lignocellulosic based biomass have been used as main sources in bioethanol production because they can be hydrolyzed into sugar (Mielenz, 2001). However, starch and lignocellulosic biomass (which are normally known as first- and second-generation biomass) have disadvantages for bioethanol production (Cheng and Timilsina, 2011). Starch based biomass competes with human food, which increases the price of crops and negatively impacts the economy (Pimentel et al., 2009). Additionally, lignocellulosic biomass has disadvantages in its complicated processing. Because of its structure and lignin component, which play a significant role in inhibiting degradation, the steps in processing lignocellulosic biomass are far more complex than starch based

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biomass in terms of its pretreatment and hydrolysis steps (Kumar et al., 2009).

Microalgae have attracted particular interest as one of the most promising sources of biomass for biofuel production. Microalgae have a high CO_2 fixation ability, 10 times more efficient than that of terrestrial plants, and can produce 30–100 times more energy per hectare than agricultural crops (Sun and Cheng, 2002; Chisti, 2008). In contrast to lignocellulosic biomass, microalgae can be produced on non arable land, minimizing the impact of biomass production on agriculture and can be produced year-round. Microalgae do not threaten food supplies, and the productive yield is much higher than that of agricultural crops. Moreover, their cell wall structures do not contain lignin, which forms a physical barrier to enzymatic hydrolysis and is not easily removed by pretreatment. This quality is advantageous in the pretreatment and enzymatic hydrolysis steps of the ethanol production process (Sun and Cheng, 2002).

Many studies on microalgae-based fuels have focused on the production of biodiesel rather than bioethanol due to the high lipid content and rather simple process of producing these biofuels. The major components in microalgal biomass are proteins, lipids, and carbohydrates (Alvira et al., 2010). However, due to the relatively low carbohydrate content of microalgae, little research has been conducted on bioethanol production from microalgal biomass. Many microalgal species are rich in lipids and proteins, nevertheless some species such as *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, and *Spirulina* are known for their particularly high carbohydrate content of over 50% of the dry cell weight under specific culture conditions (Ueda et al., 1996; Ho et al., 2012a). In addition, the production of biomass depends on the species of microalga, and the use of a species with a fast growth rate and high carbohydrate content is important for the commercialization of bioethanol production using microalgae (Mielenz, 2001; Wi et al., 2009). In studies on the commercialization of microalgae as a biomass, *Chlorella*, *Scenedesmus*, and *Chlamydomonas* are known as the most appropriate candidates for carbohydrate-based microalgae feedstock in bioethanol production (Brányiková et al., 2011; Chen et al., 2013; Hirano et al., 1997). Moreover, environmental stress is known to change the composition of microalgae and must be accounted for to maximize the carbohydrate content of microalgae. Controlling environmental factors such as nutrients, light, and temperature in cultivation, is known to affect both algal growth and biomass composition. In recent studies, strategies involving the limitation of nutrients (such as sulfur, nitrogen, and phosphate) were employed to increase the accumulation of carbohydrates in microalgae by forcing them to transform protein or peptides into carbohydrates (Dragone et al., 2011; Harun and Danquah, 2011). Microalgae tend to degrade nitrogen-containing macromolecules such as proteins particularly under nitrogen limitation. Therefore, nitrogen starvation leads microalgae to accumulate large amounts of carbohydrates and fats (Kromkamp, 1987).

Several processing steps are involved in the production of bioethanol from biomass including pretreatment, saccharification, and fermentation. The main challenge in bioethanol production from microalgal biomass is to efficiently release fermentable sugars from microalgal cells. The carbohydrates of microalgae are mainly from the inner cell wall and the plastid polysaccharide entrapped inside the cell. Therefore, to release sugars, it is necessary to weaken the cell walls for enzymes to be accessible.

In this study, the bioethanol production process was investigated using the nutrient stress-induced microalga *Chlorella vulgaris*, with milling pretreatment, a pectinase enzyme for saccharification, and immobilized yeast in fermentation. The effects of various pretreatment methods, enzymes, and conditions on the saccharification step were examined, including enzyme composition, loading quantity, hydrolysis time, and microalgae loading volume. Batch and continuous type fermentation was conducted using immobilized yeast, converting hydrolyzed microalgal biomass into bioethanol.

2. Methods

2.1. Microalgae cultivation and growth conditions

C. vulgaris (KMMCC-9; UTEX 26) was purchased from the Korean Marine Microalgae Culture Center (Daejeon, Korea). The algae were precultured in a 500-mL flask at 20 °C, with a 16–8 h light–dark cycle and a filtered air pump for aeration. To prevent contamination, autoclaved bold basal medium (BBM) was used consisting of (g/L) $NaNO_3$ (0.25), K_2HPO_4 (0.075), KH_2PO_4 (0.175), NaCl (0.025), $CaCl_2{\cdot}2H_2O$ (0.025), $MgSO_4{\cdot}7H_2O$ (0.075), EDTA·2Na (0.05), KOH (0.031), $FeSO_4{\cdot}7H_2O$ (0.005), H_3BO_3 (0.008), $ZnSO_4{\cdot}7H_2O$ (0.0015), $MnCl_2{\cdot}4H_2O$ (0.0003), MoO_3 (0.00025), $CuSO_4{\cdot}5H_2O$ (0.0003), $Co(NO_3)_2{\cdot}6H_2O$ (0.0001). After pre-cultivation, the medium was transferred to 20-L transparent plastic containers at a working volume of 15-L BBM. The microalgae were cultured for 14 days using an air pump with a 16–8 h light–dark cycle, temperatures of 20–22 °C, and a photon flux of approximately 150 μmol m^{-2} s^{-1}. Microalgae were harvested by centrifugation for 20 min at 3500 rpm and 3000*g* (Union 5KR, Hanil, Korea). For the quantification of nitrogen and sulfur, harvested microalgae were cultivated in BBM without sources of either nitrogen or sulfur for 3 days. The $NaNO_3$ was removed for nitrogen limitation conditions, while $MgSO_4{\cdot}7H_2O$, and $FeSO_4{\cdot}7H_2O$ were replaced with $MgCl_2$ and $FeCl_2{\cdot}4H_2O$, respectively, under sulfur-limited conditions. For following experiments, algal samples were freeze-dried after harvesting.

2.2. Analytical methods

2.2.1. Carbohydrate analysis

Gas chromatography (GC) was used for the identification and quantification of monosaccharides in the microalga *C. vulgaris* (Wi et al., 2009). The samples were hydrolyzed with 72% sulfuric acid for 45 min at room temperature and diluted with distilled water to 4% sulfuric acid followed by autoclaving at 121 °C for 1 h. To neutralize the samples, ammonia solution was added until pH 7 was reached, and myo-inositol was used as an internal standard. Dimethyl sulfoxide (0.1 mL) containing 2% sodium tetrahydroborate ($NaBH_4$) was added to each test tube and incubated at 70 °C for 30 min. Acetic acid (18 M, 0.1 mL) was added to decompose the sodium tetrahydroborate, and to ensure that the acetylating process was complete; 0.2 mL of methylimidazol and 2 mL of anhydrous acetic acid were also added, and then the tubes were vortexed and held for 10 min at room temperature. Dichloromethane (2 mL) and water (5 mL) were added, and the tubes were vortexed. The dichloromethane layer was transferred to a small tube and then evaporated completely in a stream of nitrogen. Approximately 1 μL of each sample was injected into a GC apparatus (GC-2010; Shimadzu, Kyoto, Japan) with a J&W DB-225 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent, Santa Clara, CA) and helium as a carrier gas. The column temperature was 100 °C for 1.5 min and increased at 5 °C/min up to 220 °C. The injector temperature was 220 °C and that of the flame ionization detector (FID) was 300 °C.

2.2.2. Fourier transform infrared (FTIR) spectroscopy

Microalgal cells cultivated under nutrient-rich and nitrogen-limited conditions were freeze-dried after being harvested and prepared for FTIR spectroscopy. Spectra were collected using a Spectrum 400 FTIR spectrometer (Perkin Elmer, Waltham, MA) in