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Comparison of red microalgae (*Porphyridium cruentum*) culture conditions for bioethanol production

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HIGHLIGHTS

- *Porphyridium cruentum* (PC) can grow in seawater and freshwater conditions.
- Freshwater *P. cruentum* (FPC) biomass can efficiently produce the bioethanol.
- SSF process was superior to SHF process for bioethanol production from PC.

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ABSTRACT

Microalgae biomass are useful resources in biofuel production. The objective of this study was to evaluate bioethanol production in response to *Porphyridium cruentum* culture conditions. Enzymatic hydrolysis of seawater *P. cruentum* (SPC) and freshwater *P. cruentum* (FPC, 1% substrate loading, w/v) resulted in glucose conversion yields of 89.8 and 85.3%, respectively, without any pretreatment. However, FPC hydrolysate was more efficiently converted to ethanol about 7.1% than SPC hydrolysate. The comparison of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) showed that SSF processing is a superior method for bioethanol production from both SPC and FPC. Though SSF processing (5% substrate loading, w/v) in a 500-mL twin-neck round bottom flask, we achieved ethanol conversion yields of 65.4 and 70.3% from SPC and FPC, respectively, after 9 h. These findings indicate that *P. cruentum* can grow in freshwater conditions and is an efficient candidate for bioethanol production.

1. Introduction

According to the US Department of Energy (DOE, Independent Statistics & Analysis), world biofuel production will increase from approximately 1.3 million barrels per day in 2010 to approximately 3.0 million barrels per day in 2040 (International Energy Outlook, 2014). New, renewable energy sources, such as microalgae and lignocellulosic biomass, have attracted increasing attention and become an important issue related to the generation of alternative energy (Ho et al., 2013; Kim et al., 2012; Mussatto et al., 2010). Specifically, microalgae may become useful resources in biofuel and bio-industrial applications due to their many advantages, including rapid and sustainable growth. Some *Chlamydomanos* species have been reported to double their mass within 6 h, with carbon saving effects, and they have a high CO_2 fixation ability, i.e., more than 10-fold that terrestrial plants (Chen et al., 2009; Mostafa, 2012). Some microalgae have high a carbohydrate content that exceeds 50% of their dry weight (John et al., 2011). For these reasons, many studies have investigated the application of microalgal resources to bioethanol production (Ho et al., 2013; Markou et al., 2013). Many studies have been conducted on the optimal culture conditions of microalgae, investigating the influence of nutrients such as nitrogen, sulfur, and phosphate on microalgal chemical composition (Kim et al., 2014; Razaghi et al., 2014; Wang et al., 2015). For example, in *Chlorella vulgaris* and *Porphyridium cruentum*, nitrogen deficiency in the growth medium can increase carbohydrate content (Kim et al., 2014; Razaghi et al., 2014).

Many studies involving microalgae have focused on their utility in biodiesel production, due to their high lipid content, environmental advantages, and economical production (Hill et al., 2006; Hannon et al., 2010). Recent studies have focused on cost-effective bioethanol production methods, particularly efficient biomass harvesting methods, and low enzyme loading, and rapid

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growth processes (Sathe and Durand, 2015; Kim et al., 2015). Additionally, environmentally sound practices are required in biofuel production. The use of certain chemicals may lead to fermentation inhibition and the production of environmental pollutants (Bensah and Mensah, 2013). Thus, eco-friendly processes may increase fermentation yield and reduce external environmental costs.

The red algae *P. cruentum* (PC) is one of the most promising candidate organisms for producing fatty acids, lipids, carbohydrates, and pigments (Plaza et al., 2009). In a previous study, PC biomass was found to contain up to 57% carbohydrate (Becker, 1994). In this study, we investigated bioethanol production from PC by comparing culture conditions and morphological changes according to the nitrogen concentration. We also evaluated ethanol production of FPC and SPC by compared to the separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) methods.

2. Material and methods

2.1. Microalgae cultivation and growth conditions

P. cruentum (KMMCC-1061) was purchased from the Korea Marine Microalgae Culture Center (Pusan, Korea). The microalgae were pre-cultured in a 100 mL flask at 20 °C with a filtered air pump for aeration (16–8 h, light–dark cycle), and then inoculated into 1 L of Jaworski's medium (JM) consisting of (mg/L) $Ca(NO_3)_2{\cdot}4H_2O$ (20), KH_2PO_4 (12.4), $MgSO_4{\cdot}7H_2O$ (50), $NaHCO_3$ (15.9), FeNa-EDTA (2.25), EDTA-Na_2 (2.25), H_3BO_3 (2.48), $MnCl_2{\cdot}4H_2O$ (1.36), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1), cyanocobalamin (0.04), thiamine HCl (0.04), biotin (0.04), $NaNO_3$ (80), and $Na_2HPO_4{\cdot}12H_2O$ (36) with deionized water and f/2 medium consisting of (mg/L) $NaNO_3$ (75), $NaH_2PO_4{\cdot}H_2O$ (5), $Na_2SiO_3{\cdot}9H_2O$ (30), $FeCl_3{\cdot}6H_2O$ (3.15), $Na_2EDTA{\cdot}2H_2O$ (4.36), $CuSO_4{\cdot}5H_2O$ (0.0098), $Na_2MoO_4{\cdot}2H_2O$ (0.0063), $ZnSO_4{\cdot}7H_2O$ (0.022), $CoCl_2{\cdot}6H_2O$ (0.01), $MnCl_2{\cdot}4H_2O$ (0.18), cyanocobalamin (0.0005), biotin (0.0005), and thiamine HCl (0.0001) with filtered seawater. After 12 days cultivation, the microalgae were harvested by centrifugation for 10 min at 26,000*g* (Avanti J-E, Beckman, Fullerton, CA, USA).

2.2. Comparison of mass productivity, cell number, and growth according to the cultivation period

After 5, 10, and 15 days cultivation, the FPC and SPC were harvested by centrifugation for 10 min at 26,000*g* (Avanti J-E, Beckman, Fullerton, CA, USA). The harvested FPC and SPC were washed in water several times, and then dried at 105 °C. The cell number of FPC and SPC were counted using a hemocytometer. The change in growth of FPC and SPC were observed by light microcopy after staining with 0.1% Toluidine blue.

2.3. Chemical composition analysis

After organic solvent extraction of SPC and FPC using ethanol-benzene, the neutral sugar contents (glucose, xylose, arabinose, mannose, galactose, and rhamnose) were analyzed using gas chromatography (GC; Choi et al., 2013).

2.4. Enzyme activity and optimization of enzyme

Cellulase (Celluclast 1.5 L) and pectinase (Pectinex SP-L) were purchased from Novozyme A/S (Bagsvaerd, Denmark). The cellulase activity was determined by the National Renewable Energy Laboratory (NREL) method (Adney and Baker, 2008) and the pectinase activity was measured with method described by Kittur et al. (2003). The cellulase and pectinase activities were 0.122 filter paper unit (FPU)/mg protein and 240 international unit (IU)/mg protein, respectively. We carried out the enzymatic hydrolysis of 1% (w/v) dry matter SPC and FPC with varying loadings (2.4, 4.8, 9.6, 14.4, 19.2, and 28.8 mg/g SPC or FPC) of pectinase at 37 °C for confirmation of optimal enzyme loadings. After 24 h, the reducing sugars were measured using a 3,5-dinitrosalicylic acid (DNS) reagent and a standard glucose curve (Miller, 1959).

2.5. Scanning electron microscopy (SEM)

In preparation for SEM analysis, the raw SPC and enzyme-treated SPC samples were fixed with 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The washed samples were dehydrated in a graded ethanol series (50, 70, 90, 95, and 100%), and then lyophilized. Samples were coated with a thin layer of gold (20 nm) and observed via SEM (S2400; Hitachi, Tokyo, Japan).

2.6. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF)

Enzymatic hydrolysis was conducted with SPC and FPC samples in a 5 mL total volume containing 1% (w/v) dry matter, pectinase (4.8 mg/g PC), cellulase (7.2 mg/g PC), 0.1% (w/v) yeast extract, 0.2% (w/v) peptone, and 50 mM citrate buffer (pH 4.8) at 37 °C for 7 h in a 15-mL conical tube. After enzymatic hydrolysis, fermentation was performed using 3 mL hydrolysate with 3 mg dry yeast (*Saccharomyces cerevisiae*, KCTC 7906) at 30 °C for 7 h.

SSF was conducted for SPC and FPC samples in a 5 mL total volume containing 1% (w/v) dry matter, pectinase (4.8 mg/g PC), cellulase (7.2 mg/g PC), 5 mg dry yeast (*S. cerevisiae* KCTC 7906), 0.1% (w/v) yeast extract, 0.2% (w/v) peptone, and 0.05 M citrate buffer (pH 4.8) at 37 °C for 10 h in a 15-mL conical tube. To increase ethanol concentration, SSF was performed in a 100 mL total volume with 5% substrate of SPC and FPC in a 500-mL twin-neck round bottom flask at 37 °C for 12 h. All SHF and SSF processes were conducted with three replicates.

2.7. Sugar and ethanol analysis by high-performance liquid chromatography (HPLC)

After SHF and SSF reactions, the contents of sugars and ethanol were analyzed by HPLC using a refractive index detector (2414; Waters, Milford, MA, USA). The REZEX RPM (Phenomenex, Torrance, CA, USA) column (300 mm × 7.8 mm) was used at 85 °C, and samples were eluted with deionized water at a flow rate of 0.6 mL min^{-1}.

3. Results and discussion

3.1. Culture condition and chemical composition

Sufficient carbohydrate content and efficient biomass harvest are required for economical bioethanol production from microalgae. Therefore, many studies have reported increase of carbohydrate content through the control of nutrient stress, as well as harvesting methods to reduce cost and energy consumption (Morales-Sánchez et al., 2014; Milledge and Heaven, 2013). In one such study, bicarbonate supplementation for microalgae increased biomass and biochemical content (Pancha et al., 2015). In general, PC is known to grow in seawater (Golueke and Oswald, 1962). The neutral sugar content of seawater *P. cruentum* (SPC) and freshwater *P. cruentum* (FPC) was determined by gas chromatography (Table 1). SPC and FPC had total sugar compositions of 27.0 and 28.8%, respectively. Notably, PC showed the low carbohydrate