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Characterization of starch-accumulating duckweeds, *Wolffia globosa*, as renewable carbon source for bioethanol production

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ABSTRACT

The growth and starch accumulation ability of two types of duckweeds (*Wolffia globosa*), designated as duckweeds J and B, respectively, were investigated under different nutrition conditions using HYPONeX. Both duckweeds J and B grew better in rich nutrient condition (5,000-fold diluted HYPONeX solution) than in poor nutrient condition (80,000-fold diluted HYPONeX solution). In terms of starch accumulation, duckweed J accumulated more starch in the rich nutrient condition, whereas duckweed B accumulated more starch in the poor nutrient condition. In the rich nutrient condition, the dry weight of duckweed J increased by about 5.1 folds and the accumulated starch content was about 22% (w/w) of dry duckweed after 1-week cultivation. In the poor nutrient condition, the dry weight of duckweed B increased by about 5.0 folds and the accumulated starch content was about 28% (w/w) of dry duckweed after 1-week cultivation. Furthermore, ethanol production from the duckweeds was investigated using *Saccharomyces cerevisiae* NBRC0224. The most effective pretreatment of duckweeds for ethanol production was treatment with 1% hydrogen peroxide for 1 h, followed by treatment with 1% sodium hydroxide for 1 h. In the case of the duckweed J, 69 g/L ethanol was produced from 30% (w/v) of the pretreated duckweed with 20 mM urea or 0.1% yeast extract and 30 mM ammonium sulfate. In the case of the duckweed B, 30 g/L ethanol was produced from 30% (w/v) of the non-pretreated duckweed without nitrogen source. In conclusion, the duckweeds, *W. globosa*, were found to be a promising renewable carbon source for the production of third-generation bioethanol.

1. Introduction

Nowadays, there is a global increase in the development of techniques that can produce useful materials from renewable biomass. In particular, owing to the depletion of fossil fuels and global warming, various methods that can produce biofuels from renewable biomass are being established. Bioethanol, a representative biofuel, is produced from different kinds of renewable feedstocks such as foodstuffs, including corn and cassava (first generation), cellulose biomass (second generation), and algal biomass (third generation) (Baeyens et al. 2015; Maity et al. 2014). In addition to first-generation bioethanol, various other useful products are also being produced from foodstuffs. Nevertheless, the use of foodstuffs as feedstock may result in the shortage of food for human consumption and an increase in food prices. Moreover, the production of second-generation bioethanol has disadvantages such as high cost owing to degradation difficulties and utilization of more land area, thus, decreasing the cultivable land available for agriculture. Therefore, in the present study, we focused on the use of duckweed (*Wolffia globosa*), a freshwater plant that accumulates starch, as a renewable biomass for the production of third-generation biofuels.

The duckweed is the smallest flowering plant that does not have a root and purifies wastewater (Fujita et al. 1999). It thrives well on the surface of fresh waters and accumulates high amount of starch (> 50% of dry weight). Because of its ability to accumulate high quantity of starch, the duckweed can not only be used in biofuel production but also in the production of foods and other high value-added materials. In the present study, we attempted to develop a bioethanol production system with duckweeds using simultaneous saccharification and fermentation (SSF) and very high gravity (VHG) technology, which involved fermentation of considerably high amounts of mashed duckweed powder

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(> 300 g/L) to yield high concentration of ethanol (Pereira et al. 2010; Srichuwong et al. 2009; Yingling et al. 2011).

2. Materials and methods

2.1. Chemicals

α-Amylase from *Bacillus* sp. was purchased from Sigma–Aldrich (St. Louis, USA). Glucoamylase from *Rhizopus* sp. was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). All of the other chemicals were of analytical grade and were obtained commercially.

2.2. Duckweeds and microorganisms

The duckweeds, *Wolffia globosa*, designated as duckweed B and duckweed J, were obtained from Chitose Laboratory Corp. (Kanagawa, Japan). *Saccharomyces cerevisiae* NBRC0224 was obtained from NITE Biological Resource Center (NBRC, Chiba, Japan).

2.3. Cultivation media

The medium for the cultivation of duckweeds was diluted HYPONeX stock solution 6-10-5 (N (6%), P (10%), K (5%), Mg (0.05%), Mn (0.001%), and B (0.005%)). HYPONeX stock solution 6-10-5 was purchased from HYPONeX JAPAN (Osaka, Japan). The medium for the preculture of *S. cerevisiae* comprised 20 g/L peptone, 10 g/L yeast extract, and 20 g/L glucose.

2.4. Cultivation of duckweeds

To prepare the stock culture of duckweeds, duckweed B was cultivated in 80,000-fold diluted HYPONeX solution and duckweed J was cultivated in 5,000-fold diluted HYPONeX solution. Subsequently, these stock cultures were transferred to freshly diluted HYPONeX solution every 2 weeks, and contaminants, including algae, in the culture were removed using a mesh sieve. For starch accumulation test, 200 mg of wet duckweed J (dry weight, 12 mg) or duckweed B (dry weight, 8 mg) from the stock culture were inoculated into 100 mL of 5,000-fold diluted HYPONeX solution, 80,000-fold diluted HYPONeX solution, or Milli-Q water and cultivated at 30 °C for 1 week.

2.5. Harvest and pretreatment of duckweeds

For starch accumulation analysis, the duckweeds cultivated in 80,000-fold diluted HYPONeX solution for 2 weeks were harvested using 212-μm mesh sieve. The collected duckweeds were washed using tap water, dried at 60 °C, and powdered using mortar. The powdered duckweeds were treated with 1% sodium hydroxide and 1% hydrogen peroxide for 12 h (Mishima et al. 2008; Mishima et al. 2006; Srichuwong et al. 2010). Subsequently, the treated duckweed powder was collected, washed with tap water using 38-μm mesh sieve until the pH of the lavage fluid became neutral, dried at 60 °C, and then powdered using mortar.

2.6. Comparison of pretreatment conditions

Fermentation tests were performed with duckweed J pretreated under different conditions. The pretreated duckweed J (2% (w/v)) was glycated using α-amylase and glucoamylase (Choi et al. 2010). The reaction mixtures comprised filter-sterilized α-amylase (18 U/g duckweed powder), glucoamylase (100 U/g duckweed powder), and 50 mM sodium citrate buffer (pH 5.0). The reaction was conducted at 60 °C for 2 h. Subsequently, the reaction mixtures were cooled to room temperature and *S. cerevisiae* preculture was added to them. The preculture of *S. cerevisiae* was prepared by cultivating the organism in YPD medium at 28 °C for 18 h and then washing the culture twice with 0.9% (w/v) NaCl.

2.7. SSF

Non-pretreated or pretreated dry duckweed powder with high amount of accumulated starch (about 30% (w/w)) was dispensed into 50 mM sodium citrate buffer (pH 5.0) containing 20 mM urea, 30 mM ammonium sulfate, or 0.1% yeast extract and 30 mM ammonium sulfate. To gelatinize the starch accumulated in the duckweeds, filter-sterilized α-amylase (18 U/g duckweed powder) was added to the mixture. The reaction was conducted at 60 °C for 2 h. After the reaction, *S. cerevisiae* preculture (prepared as described above) and glucoamylase (100 U/g duckweed powder) were added to the mixture to commence SSF performed at 30 °C.

2.8. Analytical techniques

The ethanol and sugar contents were measured using Shimadzu (Kyoto, Japan) HPLC equipped with an Aminex Fermentation Monitoring Column (Bio-Rad Laboratories, Hercules, CA, USA) and refractive-index detector. Elution was achieved at 60 °C with 5 mM hydrogen sulfate at a flow rate of 0.6 mL/min. The concentrations of ethanol and glucose were calculated from the peak areas.

3. Results and discussion

3.1. Growth of duckweeds and their starch accumulation ability

The effects of nutritional condition on the growth of duckweeds and their starch accumulation ability were investigated using diluted HYPONeX solution and Milli-Q water (Fig. 1). Both duckweeds J and B exhibited high growth rate when cultivated in rich nutrient condition (5,000-fold diluted HYPONeX solution). In case of duckweed J, the total starch accumulation depended on the growth rate, and higher amount of starch (13.5 mg) was noted in samples cultured in 5,000-fold diluted HYPONeX solution than that noted in samples cultivated in 80,000-fold diluted HYPONeX solution and Milli-Q water (Fig. 1A). Conversely, in case of duckweed B, the total starch accumulation did not depend on the growth rate, and higher amount of starch (11.4 mg) was observed in samples cultured in 80,000-fold diluted HYPONeX solution than that observed in samples incubated in 5,000-fold diluted HYPONeX solution and Milli-Q water (Fig. 1B). This could be probably because of the harvesting of duckweed B cultured in 5,000-fold diluted HYPONeX solution before starch accumulation caused by nitrogen depletion. It is believed that duckweeds J and B exposed to poor nutrient condition could have accumulated starch as a survival strategy via a mechanism similar to glycogen accumulation in *Arthrospira platensis* under condition of nitrogen depletion (Hasunuma et al. 2013). Thus, it can be concluded that duckweed B is more suitable for starch production than duckweed J owing to the higher amount of starch accumulation under nitrogen depletion condition (80,000-fold diluted HYPONeX solution).

3.2. Comparison of pretreatment conditions

The pretreatment conditions for duckweeds were examined using sodium hydroxide and hydrogen peroxide (Mishima et al. 2008; Mishima et al. 2006). The effects of pretreatment on the enzymatic hydrolysis are shown in Fig. 2. When the duckweeds