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Full Length Research Paper

Ethanol production from the water hyacinth *Eichhornia crassipes* by yeast isolated from various hydrospheres

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Water hyacinth is a promising plant for the production of bioethanol. The water hyacinth was saccharified with diluted sulfuric acid and fermented to ethanol by yeast obtained from different hydrospheres. We found that the best conditions for water hyacinth hydrolysis were 1% (v/v) sulfuric acid at 121oC for 1 h. In the next step, we searched for yeast that could produce ethanol from the water hyacinth hydrolysate. For this, 624 strains were isolated from 28 bodies of water. Strain 484 produced 22.4 ml of ethanol/kg of dried water hyacinth, which suggests that it may be an efficient producer of bioethanol. Through sequence analysis, we identified strain 484 as Candida intermedia.

Key words: biomass, ethanol, fermentation, hydrospheres, water hyacinth, yeast.

INTRODUCTION

Bioethanol is being considered as a potential liquid fuel due to the limited amount of natural resources. Cellulose biomass is also being investigated as a potential substrate for bioethanol production. The water hyacinth (*Eichhornia crassipes*) is a native plant of Brazil but has been naturalized in many tropical/temperate countries. It is regarded as a nuisance because of its remarkable growth rate. Although the water hyacinth is considered by many as an invasive pest, it could be useful as a source of biomass, because it is abundant and easy to cultivate. Previous studies have addressed the potential use of water hyacinth as biomass (Abraham and Kurup, 1996; Nigam, 2002; Rahman et al., 1986) or as a way to improve water quality because of its capacity to absorb heavy metals and organic compounds (Simeon et al., 1985, 1987; Ingole and Bhole, 2002). These studies indicate that water hyacinth is a promising plant for ethanol production.

Our previous work has identified many fermentative microorganisms that are distributed in hydrospheres (Ueno et al., 2001, 2002, 2003, 2005; Urano et al., 1998, 2001, 2002a, 2002b) . The goal in this study was to produce ethanol from water hyacinth with yeast found in hydrospheres. Using the modified method of sulfuric acid saccharification (Hamelinck et al., 2005), we investigated proper sulfuric acid concentrations and heating times to reduce the amounts of sulfuric acid and heat energy required. And we propose a novel approach for generating ethanol from water hyacinth and fermentative yeast.

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MATERIALS AND METHODS

Hydrolysis of water hyacinth

Water hyacinth was hydrolyzed by a modified procedure with diluted sulfuric acid (Hamelinck et al., 2005) because yeast is able to directly ferment water hyacinth hydrolysate after neutralization. A low sulfuric acid concentration (0.5 - 4%) was chosen to ensure a low ionic strength of the neutralized products which is conductive for high fermentation activity. The water hyacinth was grown in an open aquarium during the summer. Gathered and washed water hyacinth was homogenized in 3-fold volumes of distilled water by using a mixer, and dried at 70^{o} overnight. The crushed water hyacinth powder was passed through a net with a 0.9 mm pore size to remove large debris. A 3 g sample of water hyacinth powder was added to 50 ml of diluted sulfuric acid (0.5 - 4%) in a 100 ml flask and autoclaved at 121^{o} for 0.5 - 1.5 h. The hydrolysate was filtered, neutralized with 10 N NaOH, and re-filtered through filter paper (Advantec No.1, Tokyo, Japan) to remove any sediment. At this point, the volume of the hydrolysate was roughly 38 ml. The effects of the sulfuric acid concentration and hydrolysis time on D-glucose production were investigated. The amount of D-glucose recovered was measured using the F-kit (Roche Co., Ltd., Basel, Switzerland). In this study, the hydrolysis temperature of the autoclave was lower than the 215^{o} used in industrial hydrolysis equipment (Hamelinck et al., 2005). Japanese cedar was hydrolyzed as standard cellulose biomass under the same conditions as for water hyacinth. The resulting amounts of D-glucose and reducing sugars were measured. The reducing sugars

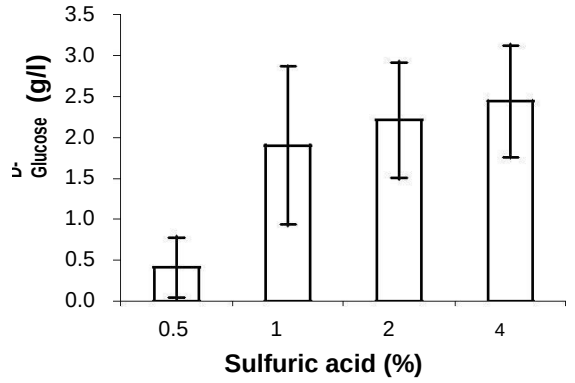

Figure 1. Effect of sulfuric acid on D-glucose production from water hyacinth at 121°C for 1 h. Each value is an average of the results of three experiments. Error bars indicate standard deviations.

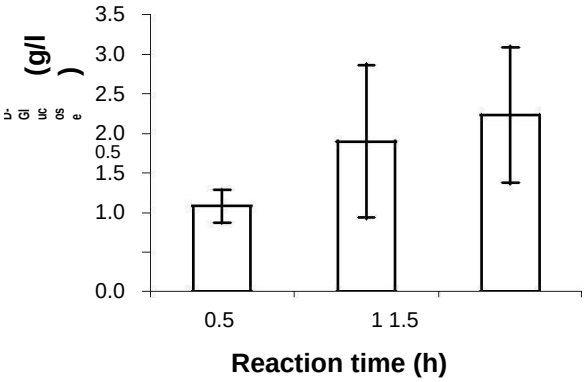

Figure 2. Effect of reaction time on D-glucose production from water hyacinth at 121°C with 1% sulfuric acid. Each value is an average of the results of three experiments. Error bars indicate standard deviations.

were measured by the Somogyi-Nelson method using D-glucose as a standard (Somogyi, 1952).

Isolation of yeast from hydrospheres and screening for fermentation activity

Water samples (500 - 1000 ml) were collected from 28 kinds of river, lake, or coasts in Japan. Screening for fermentative yeast was done according to our previous study (Ueno et al., 2001) at 25°C for 3 days. Colonies were picked, enumerated for identification, and stored at 4°C.

Selection of yeast strains

To select the yeast strains that assimilate the water hyacinth hydrolysate, we inoculated each strain into a water hyacinth solid medium (2% agar in water hyacinth hydrolysate) and cultured for 3 days at 25°C. The strains forming relatively large colonies were selected and tested for their ability to produce ethanol. The ethanol production and fermentation efficiencies after 5 days at 25°C were measured by using a method described in our previous study (Ueno et al., 2001). Water hyacinth hydrolysate or 5% D-glucose solution was used as the fermentation substrate. Each experiment was done in triplicate.

Identification of the selected strains

Identification of the selected strain was carried out by sequencing the 28S rDNA. The D1/D2 domain of 28S rDNA was amplified by PCR with the NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'- GGTCCGTGTTTCAAGACGG-3') primers (Kurtzman and Robnett, 1998). An ABI PRISM 310 Genetic Analyzer was used for sequencing. Other conditions were according to our previous study (Ueno et al., 2005) The BLASTN program (Altschul et al., 1997) was used to search for gene homology. The nucleotide sequence of the selected strain was deposited in DDBJ under accession number AB278599. The physiological properties (assimilation and fermentation of carbons sources) of the strain were determined according to Bernett et al. (1990). For the carbon assimilation tests, yeast nitrogen base culture medium without amino acids (Difco/Becton Dickinson Co., Sparks, MD, USA) was used as the nitrogen source. About 2.5×10^4 cells of strain 484 were counted using a Thoma hemocytometer and added to 10 ml of liquid medium. After 3 weeks of cultivation at 25°C yeast growth was confirmed. The Yeast Identification PC program version 4 was used to identify the selected strain.

RESULTS AND DISCUSSION

Water hyacinth hydrolysis

The effects of sulfuric acid concentration and reaction time on D-glucose production are shown in Figures 1 and 2. We found that the efficient water hyacinth hydrolysis occurred when the plants were incubated in 1% sulfuric acid at 121°C(the maximum temperature of autoclave) for 1 h in an autoclave. We predict that the D-glucose was derived primarily from cellulose and not from storage sugars since the major components of water hyacinth have been reported to be cellulose and cellulose-related polysaccharides (Mukherjee et al., 2004; Nigam, 2002; Ingole and Bhole, 2002; Abraham and Kurup, 1997). The amounts of D-glucose and reducing sugars produced from water hyacinth and Japanese cedar were measured after hydrolysis with 1% sulfuric acid at 121°C for 1 h (Figure 3). The amount of cellulose in Japanese cedar was previously shown to be 49.7% (Sagehashi et al., 2006), which is higher than 18.2 or 21.2% previously measured in water hyacinth (Nigam, 2002; Ingole and Bhole, 2002). However, the amount of D-glucose and reducing sugars produced from the water hyacinth exceeded those from equal starting amounts of Japanese cedar. Under this condition, water hyacinth is more readily hydrolyzed. This may be because these conditions were suboptimal for Japanese cedar. These results may be due to the difference between the amounts of lignin in each plant. The water hyacinth has. been shown to have 4.6% of lignin (Abraham and Kurup, 1997) whereas the lignin concentration of Japanese cedar has been shown to be 32.3% (Sagehashi et al., 2006)

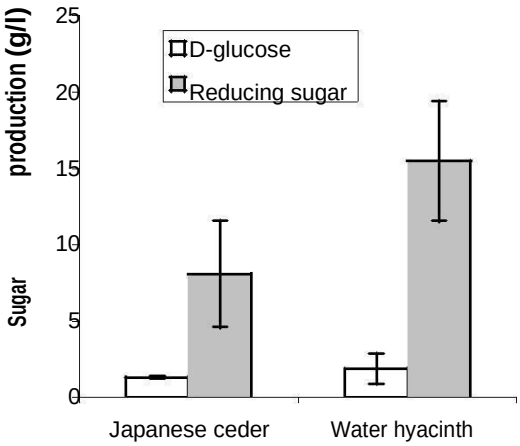

Figure 3. Sugar produced from the hydrolyzed water hyacinth or Japanese cedar with 1% sulfuric acid at 121^{O}C for 1 h. Each value is an average of the results of three experiments. Error bars indicate standard deviations.

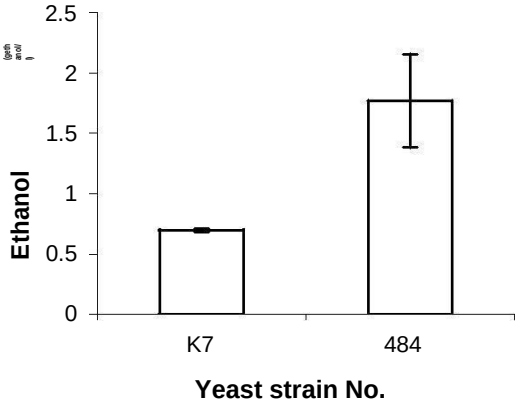

Figure 4. Ethanol production by the yeast strains. K7: *S. cerevisiae* in sake industry. 484: selected yeast in this study and isolated from the Katsuragawa River. Each value is an average of the results of three experiments. Error bars indicate standard deviations.

Isolation of high fermentative yeast

We isolated 624 strains of yeast from 28 hydrospheres in Japan. Approximately one third of these yeasts were found to be fermentative. Next, 210 yeasts were selected on the basis of their CO_2 production. From this selection process, we observed that fermentative yeasts are commonly distributed throughout various hydrospheric environments. From these 210 yeasts, 13 yeasts were selected based on their ability to form relatively large colonies on water hyacinth hydrolysate agar plates. Their ability to grow rapidly under these conditions suggested that the nutrients in the hydrolysate were suitable for these strains. Strain 484 produced 1.77 ± 0.4 g/l ethanol which was more than the other 12 strains tested (1.3 ± 0.4 g/l average ± standard deviation of 12 strains). Its ability to produce ethanol was compared to that of *Saccharomyces cerevisiae* strain K7 (Figure 4). The *S. cerevisiae* strain K7 has been widely used in the sake industry and has been preserved in the brewing society of Japan. We have used the K7 strain as a high fermentative yeast standard in previous studies (Urano et al., 1998, 2002b). Strain 484 produced about 2.5-fold more ethanol than strain K7. We calculated from the results that the amount of ethanol produced from 1 kg of dried water hyacinth using strain 484 would be 22.4 ml. On the other hand, the ethanol fermentation efficiencies of strain K7 and strain 484 on D-glucose were 87.8 ± 9.4%, and 72.1 ± 13.7%, respectively. Although the efficiency of strain 484 was lower than that of the K7 strain, strain 484 produced more ethanol than the k7 strain from the water hyacinth hydrolysate. The ethanol productivity of strain 484 may be related to the high fermentation of the hydrolyzed hemicellulose-related polysaccharides. Unlike strain K7, strain 484 has been shown to ferment arabinose and galactose in water hyacinth hemicellulose hydrolysate on the basis of physiological testing (Nigam, 2002).

Identification of strain 484

The D1/D2 domain sequence of strain 484 is 97% similar to *Candida intermedia* and *C. pseudointermedia*. Accordingly, strain 484 was identified by the physiological method according to YEASTS (Bernett et al., 1990) as *C. intermedia* with 99% probability using The Yeast Identification PC program version 4. These physiological properties are consistent with those described by Bernett et al. (1990). *C. intermedia* lives in marine and soil environments (Bernett et al., 1990). We have found that *C. intermedia* are also present in the Katsuragawa River, a freshwater environment in Chiba prefecture, Japan.

Conclusions

Using the water hyacinth as a cellulose biomass would be a low cost method for ethanol production. The saccharification of water hyacinth was carried out by hydrolyzing it in 1% sulfuric acid at 121^{O} for 1 h. The addition of yeasts from hydrospheric environments could constitute a cost efficient way of producing ethanol from the hydrolysate. The yeast strain 484 produced 22.4 ml of ethanol/kg of dried water hyacinth.

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