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Ethanol production from candidate energy crops: Water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes* L.)

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Abstract

Fermentation modes and microorganisms related to two typical free-floating aquatic plants, water hyacinth and water lettuce, were investigated for their use in ethanol production. Except for arabinose, sugar contents in water lettuce resembled those in water hyacinth leaves. Water lettuce had slightly higher starch contents and lower contents of cellulose and hemicellulose. A traditional strain, *Saccharomyces cerevisiae* NBRC 2346, produced 14.4 and 14.9 g l^{-1} ethanol, respectively, from water hyacinth and water lettuce. Moreover, a recombinant strain, *Escherichia coli* KO11, produced 16.9 and 16.2 g l^{-1} ethanol in the simultaneous saccharification and fermentation mode (SSF), which was more effective than the separated hydrolysis and fermentation mode (SHF). The ethanol yield per unit biomass was comparable to those reported for other agricultural biomasses: 0.14–0.17 g g-dry^{-1} for water hyacinth and 0.15–0.16 g g-dry^{-1} for water lettuce.

Keywords: Aquatic plant; Water lettuce (*Pistia stratiotes* L.); Water hyacinth (*Eichhornia crassipes*); Biomass; Ethanol

1. Introduction

Bioethanol has been produced from waste biomass produced by agricultural and forest industries such as corn cobs, sugar cane bagasse, wheat straw, and wood chips (Eklund and Zacchi, 1995; Sreenath et al., 2001; Martín et al., 2002). Instead of terrestrial plants, aquatic plants are the next promising renewable energy resource. Aquatic plants have many advantages such as growing on and in bodies of water without competing against most grains and vegetables for arable land; they are also used for water purification to extract nutrients and heavy metals. Especially, the vegetation form of free-floating aquatic plants will facilitate their movement and harvest. Despite those advantages, no data on bioethanol production from aquatic plants are available except for water hyacinth (*Eichhornia crassipes*) (Kahlon and Kumar, 1987; Nigam, 2002; Abraham and Kurup, 1996). Another free-floating plant that might serve well as a substrate for ethanol production is water lettuce (*Pistia stratiotes* L.) whose growth rate is as high as water hyacinth (60–110 t ha^{-1} yr^{-1}) (Gumbricht, 1993). The soft body of water lettuce would facilitate its milling to increase biochemical responsiveness (Mishima et al., 2006). Compared to water hyacinth (1 m or more from roots to leaves), the smaller body size (around 0.3 m) of water lettuce is suitable for low-labor harvesting.

Previous studies of ethanol production from water hyacinth adopted saccharification with subsequent fermentation of the generated sugars (separated saccharification and fermentation mode: SHF), but the simultaneous saccharification and fermentation mode (SSF) has never been applied even though SSF has a high possibility for improving both the production and economical efficiencies

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through reduction of byproduct inhibition and the number of reaction tanks (Wyman, 1999).

The fermenting microorganisms are also important for ethanol production from lignocellosic biomasses. A traditional fermenting yeast, *Saccharomyces cerevisiae*, produces ethanol from hexoses. Surprisingly, recent genetic engineering has produced microorganisms that can use pentoses in addition to hexoses for ethanol production (Wooley et al., 1999; Dien et al., 2003). The recombinant microorganisms would enhance ethanol production from aquatic plants containing large amounts of hemicellulose, which can be converted into a mixture of pentoses and hexoses using saccharification processes.

In this study, water hyacinth and water lettuce were applied to ethanol production using two fermentation modes, SHF and SSF, with two fermenting microorganisms, *S. cerevisiae* and a recombinant *Escherichia coli*.

2. Methods

2.1. Samples and pretreatment

Water hyacinth and water lettuce leaves were harvested from the Yodo River in Osaka. The collected leaves were washed manually using tap water, dried at 60 °C and then powdered to pass the 0.8 mm-mesh sieve. Alkaline/oxidative pretreatment, which is the best method for the enzymatic hydrolysis of the aquatic plants (Mishima et al., 2006) was applied. Briefly, the samples were reacted in 1% (w/v) NaOH at room temperature for 12 h, then 31% H_2O_2 (w/v) was gently added so that the final concentration of H_2O_2 reached 1% (w/v); the reaction was performed for another 12 h. After the reaction, the pretreated samples were collected and washed with tap water using a 38-μm-mesh sieve until the pH value of the drained water reached neutral. Then the samples were dried at 60 °C and powdered. The pretreated samples were used for the SHF and SSF experiments. Composition of cellulose, hemicellulose and lignin in the samples was determined using detergent method (Van Soest, 1963a,b; Van Soest and Wine, 1967). Starch contents were estimated using F-kit starch (R-Biopharm AG, Germany), a commercial analytical tool using amyloglucosidase.

2.2. Fermenting strains

A yeast strain, *S. cerevisiae* NBRC 2346, and a recombinant bacterial strain *E. coli* KO11 (Ohta et al., 1991) were used for this study. In fact, *E. coli* KO11 contains the *Zymomonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) for enhancing ethanol production. Before the fermentation test, *S. cerevisiae* NBRC 2346 was cultured on solid YM medium (5 g l^{-1} peptone, 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract) with 20 g l^{-1} agar and 20 g l^{-1} glucose. *E. coli* KO11 was cultured on solid Luria Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} sodium chloride) with 20 g l^{-1} agar supplemented with 20 g l^{-1} glucose and 40 mg l^{-1} chloramphenicol. They were incubated at 28 °C for 24 h. Then large colonies were transferred to 300-ml Erlenmeyer flasks containing 100 ml of each liquid medium, and incubated at 28 °C and 120 rpm for 24 h (*S. cerevisiae* NBRC 2346) or 8 h (*E. coli* KO11) on a rotary shaker before their use as inocula.

2.3. Ethanol production from the pretreated biomass

Two different modes, SHF and SSF, were used for enzymatic hydrolysis and fermentation of the pretreated biomass for ethanol production. At the enzymatic hydrolysis step in SHF process, Erlenmeyer flasks (500 ml), each containing 25 g of the pretreated sample, were autoclaved at 121 °C for 20 min. Then, 250-ml filter-sterilized cellulase (Sumitime C; Shin Nihon Chemical Co. Ltd., Japan) solution (cellulase activity: 20 Filter paper units (FPU) (g substrate)$^{-1}$, xylanase activity: 615 unit (g substrate)$^{-1}$) in 0.1 M sodium phosphate (pH 5.0) was added to the flask and reacted at 45 °C and 120 rpm for 96 h for hydrolysis. After the enzymatic reaction, the hydrolysate was centrifuged at 21,000×*g* for 10 min. The supernatant was supplemented with additional nutrients to give a base medium composition of: 2.0 g l^{-1} yeast extract, 0.2 g l^{-1} $(NH_4)_2HPO_4$, 0.02 g l^{-1} $MgSO_4 \cdot 7H_2O$ for *S. cerevisiae* NBRC 2346, or LB medium and 40 mg l^{-1} chloramphenicol for *E. coli* KO11. The initial pH was adjusted, respectively, to 5.0 and 6.8 for *S. cerevisiae* NBRC 2346 and *E. coli* KO11. The 80 ml of hydrolysate was transferred to a 125 ml Erlenmeyer flask with a rubber cap and sampling needle, then autoclaved again to stop the enzymatic reaction, and finally sterilized. For fermentation, 4 ml of each preculture was inoculated aseptically into the flask. Fermentation was carried out for 96 h at 30 °C and at 120 rpm on a rotary shaker. Samples were withdrawn periodically for HPLC analysis of sugars and ethanol.

The SSF reaction mixtures consisted of 8 g of the pretreated aquatic plant samples (previously autoclaved for 20 min at 121 °C), filter-sterilized cellulase (20 FPU (g substrate)$^{-1}$) solubilized in 0.1 M sodium phosphate, the basal medium and 5 ml microbial inoculum to give the same concentration as that of the SHF experiment. The initial pH of the SSF mixture was adjusted to 5.0 for *S. cerevisiae* NBRC 2346 or 6.0 for *E. coli* KO11. The SSF reaction was carried out at 37 °C in 125 ml conical flasks with 80 ml working volume. The flasks were sealed with rubber stoppers through which hypodermic needles had been inserted for exhaust of the produced CO_2 and for sampling. Samples were withdrawn through the needle, and analyzed for contents of ethanol, sugars and degradation products of sugars.

2.4. Analytical procedures

Before sugar component analysis, the plant biomasses (200 mg) were hydrolyzed using 2 ml of 72% H_2SO_4 for

1 h at 30 °C. After addition of 56 ml of water, the sample was autoclaved for 1 h at 120 °C and analyzed for sugar compositions using HPLC (Puls et al., 1985). The cell density was measured spectrophotometrically at 660 nm. An OD of 1.0 at 660 nm was, respectively, equivalent to 0.50 mg and 0.62 mg dry weight of *S. cerevisiae* NBRC 2346 and *E. coli* KO11 cells ml^{-1}. In SSF, the cell density was not measured because of the high turbidity by substrate. Ethanol, sugars and byproducts were measured using HPLC (liquid chromatograph LC-10AT; Shimadzu Corp.) with a refractive index (RI) detector (refractive index detector RID-10A; Shimadzu Corp.) using exclusion column (300 × 7.8 mm, Bio-Rad Aminexion HPX-87H; Bio-Rad Laboratories Inc., USA) maintained at 65 °C. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. Mannose, xylose and galactose (man/xyl/gal) were not separable using this column, but the sum of these sugars was approximately quantified. Because the calibration area for each peak on the analysis varied by less than ±5%, the concentration of the total sugar was estimated from the man/xyl/gal peak using a standard containing xylose. The cellulase activity was estimated on filter paper as FPU (Ghose, 1987). The xylanase activity was assayed using Birchwood xylan as a substrate (Bailey et al., 1992). Units of cellulase and xylanase were expressed as the amount of enzyme producing 1 μmol of reducing sugars (glucose or xylose equivalent) per minute. All experiments were at least duplicated and the mean values were shown as a result. The figures do not include standard error bars because relative errors were dwarfed by graphical symbols, except for cases when the standard deviation was greater ±5% of the value. The conversion efficiency was defined as the ratio of the produced ethanol yield to the theoretical yield calculated on the assumption that the entire glucose component in the biomass was convertible into ethanol.

3. Results and discussion

3.1. Sugar and carbohydrate polymer components in aquatic plants

Table 1 shows sugar and carbohydrate polymer contents of the aquatic plants and typical agricultural wastes. The sugar contents, except for arabinose, in water lettuce were similar to those in water hyacinth leaves. Water lettuce had slightly higher contents of starch and lower contents of cellulose and hemicellulose. After the alkaline/oxidative pretreatment, the percentage of the carbohydrate components increased, probably because of the removal of small particles and soluble components, which mainly consisted of non-carbohydrate residues (Mishima et al., 2006). The sugar contents of the aquatic plants were lower than for wheat straw, but comparable to the cotton gin waste.

3.2. Ethanol production from aquatic plants by SHF

In the enzymatic hydrolysis stage of SHF, around 60% of the enzymatically degradable sugars in the biomass were hydrolyzed in 24 h; the hydrolysis was almost completed in 96 h. The glucose, man/xyl/gal, and arabinose concentrations in the resultant enzymatic hydrolysate of water hyacinth were 30.1, 2.2, and 0.8 g l^{-1} and those of water lettuce were 33.3, 2.3, and 0.3 g l^{-1}, respectively. The glucose concentration in the enzymatic hydrolysate from each aquatic plant was comparable to or a little higher than that obtained using acid hydrolysis (Table 1), suggesting that the cellulose and starch in the biomass were sufficiently hydrolyzed by cellulase in this enzymatic hydrolysis stage. The concentrations of the other sugars derived from hemicellulose hydrolysis were less than half of those in the acid hydrolysis.

Figs. 1 and 2 show the consumption of the sugars and production of ethanol from the aquatic plants in the

Table 1
Main sugar compositions and carbohydrate polymer compositions of aquatic plants and typical agricultural wastes[a]

	Aquatic plants				Water hyacinth		Typical agricultural wastes	
	Water hyacinth leaves		Water lettuce leaves				Wheat straw	Cotton gin waste
	Raw	Pretreated	Raw	Pretreated				
Sugars								
Glucose	17.2	25.7	19.5	27.0			36.6	20.0
Man/Gal/Xyl	5.1	5.6	5.0	5.1			22.4	6.8
Arabinose	2.8	3.9	N.D.[b]	N.D.[b]			2.4	2.3
Carbohydrate polymers								
Cellulose	19.7	34.2	16.5	28.4	35.0	18.2	30	
Hemicellulose	27.1	27.0	17.3	18.7	18.3	48.7	50	
Starch	4.1	4.1	6.4	7.4	1.9			
References	This study[c]				Abraham and Kurup (1996)	Nigam (2002)	Lee (1997); Sun and Cheng (2002)	Lee (1997)

[a] % of sugar equivalent.
[b] N.D.: not detected.
[c] The values of sugars were in acid hydrolysates of each biomass.

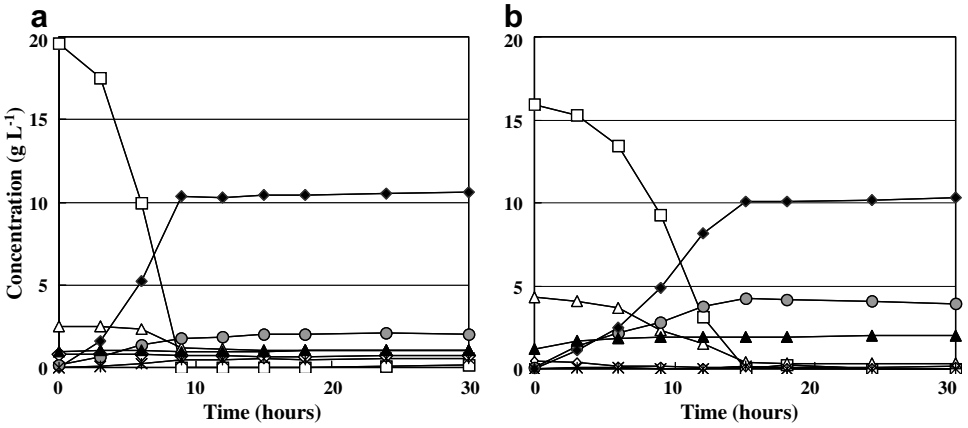

Fig. 1. Ethanol production in SHF from water hyacinth hydrolysate by *S. cerevisiae* NBRC 2346 (a) and *E. coli* KO11 (b). Symbols: □, glucose; △, xylose/mannose/galactose; ◊, arabinose; ∗, glycerol; ▲, acetic acid; ●, biomass; ♦, ethanol.

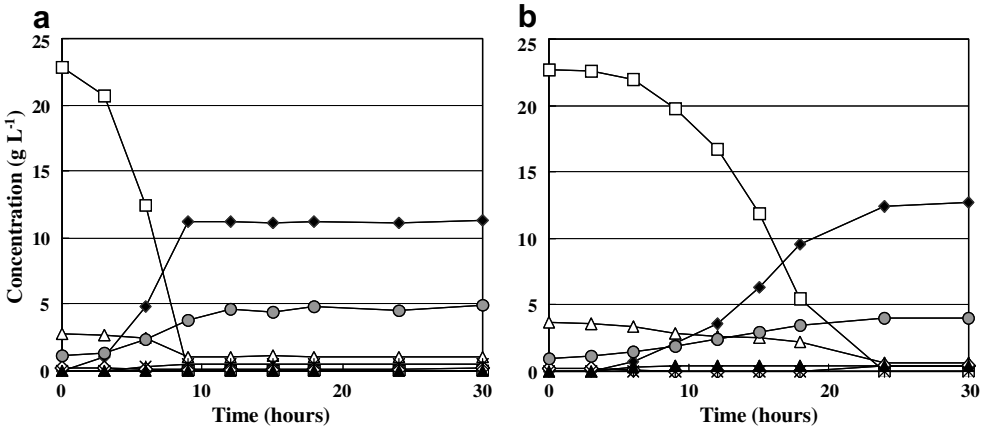

Fig. 2. Ethanol production in SHF from water lettuce hydrolysate by *S. cerevisiae* NBRC 2346 (a) and *E. coli* KO11 (b). Symbols: □, glucose; △, xylose/mannose/galactose; ◊, arabinose; ∗, glycerol; ▲, acetic acid; ●, biomass; ♦, ethanol.

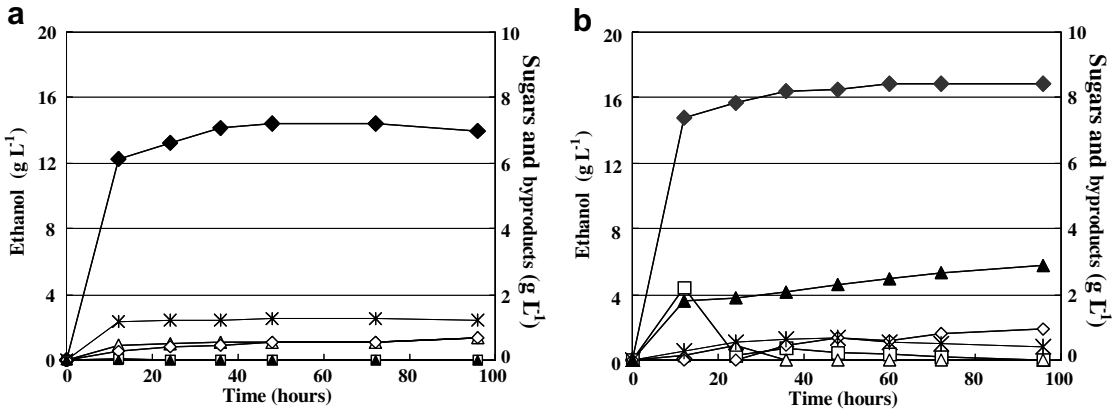

Fig. 3. Ethanol and other byproduct concentrations in SSF fermentation of water hyacinth leaves using *S. cerevisiae* NBRC 2346 (a) and *E. coli* KO11 (b). Symbols: □, glucose; △, xylose/mannose/galactose; ◊, arabinose; ∗, glycerol; ▲, acetic acid; ♦, ethanol.

fermentation stage of SHF. In 9 h, *S. cerevisiae* NBRC 2346 smoothly converted glucose in the hydrolysates of both biomasses to ethanol. Xylose and arabinose were somewhat reduced in the fermentation. The respective conversion efficiencies for ethanol from water hyacinth and water lettuce were 80.9% and 86.2%. The final ethanol concentrations in SHF of water hyacinth and water lettuce were 10.1 and 11.3 g l^{-1}, respectively.

Actually, *E. coli* KO11 showed a slightly longer lag phase and slower fermentation than *S. cerevisiae* NBRC 2346. Despite those disadvantages, *E. coli* KO11 consumed not only glucose but also xylose and arabinose in the hydrolysates completely, and produced 10.3 g l^{-1} and 12.7 g l^{-1} ethanol from water hyacinth and water lettuce in 30 h, respectively. Although these values for the ethanol concentration were slightly higher than those of *S. cerevisiae* NBRC 2346, the difference was not so remarkable. The difference might be attributable to the lower contents of the pentose in the aquatic plants and incomplete degradation of the hemicellulose component. The conversion efficiencies were, respectively, 82.5% and 96.8% for water hyacinth and water lettuce leaves.

In addition to *E. coli* KO11 used in this study, many recombinant microorganisms including *S. cerevisiae* NBRC 2346, which can utilize xylose/arabinose (Dien et al., 2003) and convert starch to ethanol directly (Kondo et al., 2002; Shigechi et al., 2002) have been developed recently. Effective ethanol production demands selection of suitable fermenting strains from among such diverse microorganisms depending on the aquatic plants' chemical composition.

3.3. Ethanol production from aquatic plants by SSF

Figs. 3 and 4 show the occurrence and consumption of the sugars and production of ethanol from the aquatic plants in SSF. Ethanol production by the fermenting microorganisms proceeded smoothly; it was almost finished within 36 h. *S. cerevisiae* NBRC 2346 and *E. coli* KO11 accumulated glycerol and acetic acid as the main by-products, respectively, reflecting their different metabolic pathways.

In 48 h, from water hyacinth and water lettuce, *S. cerevisiae* NBRC 2346 produced 14.4 and 14.9 g l^{-1} ethanol, respectively, without remarkable accumulation of glucose. Xylose and arabinose gradually increased as the reaction progressed. The respective conversion efficiencies were 109.1% from water hyacinth and 108.2% from water lettuce leaves. The high conversion efficiencies of more than 100% resulted from the incomplete degradation of

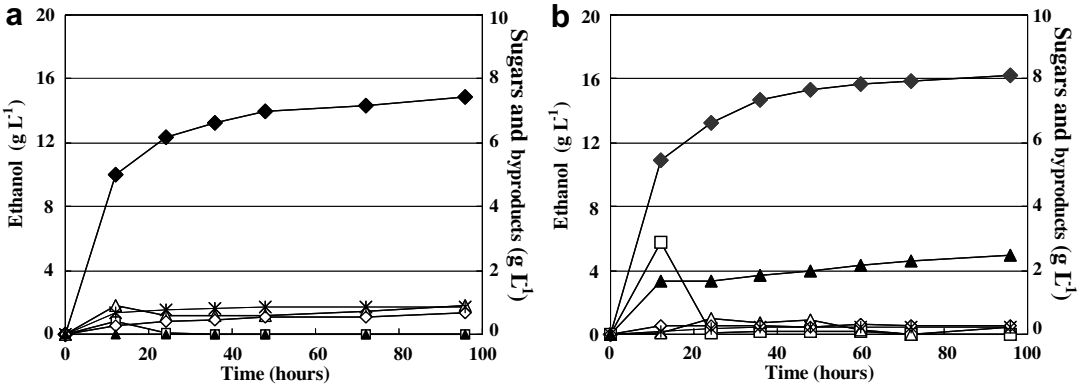

Fig. 4. Ethanol and other byproduct concentrations in SSF fermentation of water lettuce leaves using *S. cerevisiae* NBRC 2346 (a) and *E. coli* KO11 (b). Symbols: □, glucose; △, xylose/mannose/galactose; ◇, arabinose; ∗, glycerol; ▲, acetic acid; ♦, ethanol.

Table 2
Ethanol yields per unit biomass from various biomasses

Biomass	Pretreatment	Fermentation mode	Fermentation strain	Ethanol yield per unit biomass (g (g-biomass)$^{-1}$)	Reference
Water hyacinth leaves	Alkaline/oxidative pretreatment	SSF	*S. cerevisiae* NBRC 2346	0.14	This study
			E. coli KO11	0.17	
Water lettuce leaves			*S. cerevisiae* NBRC 2346	0.15	
			E. coli KO11	0.16	
Willow	SO_2-impregnated steam pretreatment	SSF	*S. cerevisiae*	0.29	Eklund and Zacchi (1995)
Alfalfa fiber (raffinate)	Liquid hot water pretreatment	SSF	*Candida shehatae* FPL-702	0.18	Sreenath et al. (2001)
Sugar cane bagasse	Steam pretreatment	SHF	Recombinant *S. cerevisiae* TMB3001	0.18	Martín et al. (2002)

the aquatic plants in the component analysis even though the acid hydrolysis conditions had been optimized.

In 96 h, *E. coli* KO11 produced 16.9 and 16.2 g l^{-1} ethanol, respectively, from water hyacinth and water lettuce. Although the transient accumulation of glucose was observed in the early stage of fermentation, no lag phase was recognized. The respective conversion efficiencies were 126.1% and 115.1% for water hyacinth and water lettuce.

The total time required for SSF was shorter than that for SHF because the SHF required 96 h for the enzymatic hydrolysis stage before the fermentation stage. The maximum concentrations of ethanol produced from the aquatic plants in SSF were higher than those in SHF. As summarized in Table 2, the ethanol yields per unit biomass in SSF were comparable to those reported for other agricultural biomasses, i.e., 0.14–0.17 g g-dry^{-1} for water hyacinth and 0.15–0.16 g g-dry^{-1} for water lettuce.

4. Conclusions

Water hyacinth and water lettuce were evaluated in this study as substrates for ethanol production. The sugar contents, aside from arabinose, in water lettuce resembled those in water hyacinth leaves. Water lettuce had slightly higher contents of starch and lower contents of cellulose and hemicellulose. The yields and maximum concentration of ethanol from the aquatic plants in SSF were higher than those in SHF. SSF shortened the total period for the ethanol production. In addition, *E. coli* KO11 produced slightly higher concentrations of ethanol from both aquatic plants than *S. cerevisiae* NBRC 2346. The ethanol yields per unit biomass from the two aquatic plants were comparable to those from the other agricultural wastes. It can be concluded that aquatic plants are a promising biomass for ethanol production when the fermentation process is fully optimized.

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