# Separated hydrolysis and fermentation of water hyacinth leaves for ethanol production

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## Abstract

In this study, the acid pretreatment and enzymatic hydrolysis were used to evaluate to produce more sugar, to be fermented to ethanol. Separated hydrolysis and fermentation (SHF) studies were carried out to produce ethanol from water hyacinth leaves. The abilities of three different yeast cells, *Saccharomyces cerevisiae* TISTR 5048, *Saccharomyces cerevisiae* KM 1195 and *Saccharomyces cerevisiae* KM 7253, on ethanol production were compared. Dilute sulfuric acid pretreatment and enzymatic hydrolysis were conducted to select the optimum pretreatment conditions for further enzymatic hydrolysis research. The optimum dilute acid pretreatment conditions included T = 135 $^{o}$C, t = 30 min, and sulfuric acid concentration = 0.1 M. The cellulosic residue was enzymatically hydrolyzed in 0.05 M citrate phosphate buffer (pH 5.0) using the mixture of enzymes cellulase, xylanase and pectinase. The maximum enzymatic saccharification of cellulosic material (76.8%) was achieved after 24 h incubation at 50 $^{o}$C. SHF of water hyacinth leaves by *Saccharomyces cerevisiae* KM 7253 resulted lowest yield of ethanol. SHF by monoculture of *Saccharomyces cerevisiae* KM 1195 achieved the highest yields of ethanol. Further improvement in ethanol production was accomplished with the co-culture of *S. cerevisiae* TISTR 5048 and *Candida tropicalis* TISTR 5045 in the ratio of 1:1 which contributed to the highest increase in ethanol production. In this case, the ethanol concentration of 3.39 (g/l), percentage of the theoretical ethanol yield of 96.07%, the ethanol yield of 0.25 g/g and the volumetric productivity of 0.221 g/l-h were obtained.

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# Introduction

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Bioethanol, a renewable fuel is becoming increasingly important as a consequence of major concern for depleting oil reserves, rising crude oil prices and greenhouse effect (Hu et al., 2008). Lignocellulosic feedstock is considered as an attractive raw material because of its availability in large quantities at low cost (Parisi, 1989) not only for the liquid transportation fuel but also for the production of chemicals and materials, i.e. the development of carbohydrate-based biorefineries (Farrell et al., 2006). Besides terrestrial plants, aquatic plants are also promising renewable resource.

The water hyacinth, *Eichhornia crassipes* (Mart.) Solms, is a tropical species belonging to the pickerelweed family (Pontederiaceae). The water hyacinth plant is a free-floating aquatic plant originating from the Amazon River basin in South America and has spread to more than 50 countries on five continents. The plant tolerates extremes in water level fluctuations, seasonal variations in flow velocity, nutrient availability, pH, temperature and toxic substances (Gopal, 1987). It can even grow at salinity levels up to 0.24% as was shown in Indonesia (Kikuchi et al., 1997). Extremely high growth rates of up to 100–140 ton dry material $Ha^{-1}$ $year^{-1}$ (Gunnarsson and Petersen, 2007) were reported, depending on the location and time of the year (Nigam, 2002). The coverage of waterways by water hyacinth has created various problems. Examples are the destruction of ecosystems, irrigation problems and an increase in mosquito populations. These negative effects have pinpointed the water hyacinth as one of the world's weeds and stimulated the search for control measures.

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 (Nigam, 2002). Due to its fast growth and the robustness of its seeds, the water hyacinth has since then caused major problems in the whole area. Attempts to control the weed have caused high costs and labour requirements, leading to nothing but temporary removal of the water hyacinths. Fast growth is a feature valued in crops grown by man. The water hyacinth would, therefore, have a great potential if seen as raw material for industries or if incorporated into agricultural practice. Water hyacinth, which is widely prevalent aquatic weed in Thailand, exceptionally fast growing plant and can provide cellulosic sugars for bioconversion to fuel ethanol. The present study, investigated ethanol production from the defined lab media (i.e.simulated synthetic hydrolysate medium) water hyacinth cellulose acid and enzyme hydrolysate using mono-culture and co-culture of yeasts fermentation.

# Materials and methods

### Substrate preparation

Fresh water hyacinth plant were harvested from the Chaophaya River in Bangkok. The collected leaves were washed manually using tap water to remove adhering dirt, dried at 45 $^{o}$C in a hot-air oven for 4 days, milled, screened to select the fraction of particles with a size of 45-697 μm, homogenized in a single lot and stored until needed.

**Hydrolysate preparation**

Hydrolysate was prepared by autoclaving under 15 lb/inch$^2$ the 1.5 g dried powder of water hyacinth leaves with 100 ml of 0.1 M sulfuric acid, in conical flasks. Then, 250-ml filter-sterilized cellulase (Sumitime C; Shin Nihon Chemical Co. Ltd., Japan) solution (cellulase activity: 20 Filter paper units (FPU) (g substrate)$^{-1}$, α-amylase 100 unit (g substrate)$^{-1}$, amyloglucosidase 100 unit (g substrate)$^{-1}$, xylanase activity: 500 unit (g substrate)$^{-1}$) and pectinase activity: 250 unit (g substrate)$^{-1}$) in 0.1 M sodium phosphate (pH 5.0) was added to the flask and reacted at 50 °C and 120 rpm for 48 h for hydrolysis. After the enzymatic reaction, the hydrolysate was centrifuged at 21,000 x g for 10 min. The supernatant was supplemented with additional nutrients to give a base medium composition of: 1 g/l yeast extract; 2 g/l $(NH_4)SO_4$; 1 g/l $MgSO_4 \cdot 7H_2O$.

**Water hyacinth cellulose hydrolysate medium**

Fermentation medium containing (g/l): yeast extract 1; $(NH_4)SO_4$ 2; $MgSO_4 \cdot 7H_2O$ 1.

**Batch fermentation**

Batch fermentation was conducted in a 250 ml conical flask with a working volume of 100 ml. The fermentation medium was inoculated with 5% v/v inoculum (20 h culture, 1 x 10$^7$ cells/ml). The fermentation temperature was kept constant at 30±0.2°C in an incubation shaker. The broth was kept under agitation at 50 rpm. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol and residual sugars in the broth. All experiments were carried out in duplicate.

**Analytical methods**

Total solids (TSs) moisture and crude protein in water hyacinth were determined according to standards (AOAC, 1975). Cellulose, hemicellulose and lignin contents were determined by the detergent extraction method (Robertson and van Soest, 1981).

**Biomass estimation**

Culture dry weight was measured by centrifugation and drying at 105 °C, until no weight change between consecutive measurements was observed.

**Sugar estimation**

Total reducing sugar was estimated by using dinitrosalicylic acid (DNS) reagent (Miller, 1959). The fermentation was carried out at 30°C for 18 h. The fermentation broths were filtered through a 0.45 mm Millipore filter.

**Ethanol analysis**

Ethanol in the samples was determined by gas chromatograph using a 60:80 Carbopack B: 5% Carbowax 20 M glass column. The injector was operated at 200°C. The flame ionization detector (FID) was kept at 200°C. Nitrogen gas was used as carrier gas at a flow rate of 30 ml/min. The temperature was programmed at 120°C for 1.4 min, from 120°C to 240°C at 30°C/ min, then held 5 min at 240°C.

## Results

The average composition of water hyacinth is summarized in Table 1.

**Table 1.** Chemical analysis of water hyacinths according to different sources.

| Parameter (% on DM basis) | This study | Abdelhamid and Gabr (1991) | Bolenz et al. (1990) | Chanakya et al. (1993) | Patel et al. (1993) | Poddar et al. (1991) |
|---|---|---|---|---|---|---|
| Hemicellulose | 32.69 | 33.4 | 22 | 33.39 | 43.4 | 18.42 |
| Cellulose | 19.02 | 19.5 | 31 | 18 | 17.8 | 25.61 |
| Lignin | 4.37 | 9.27 | 7 | 26.36 | 7.8 | 9.93 |
| Crude protein | 10.20 | 20 | - | - | 11.9 | 16.25 |

**Composition of water hyacinth**

Water hyacinth leaves is considered to be a potential biomass source of cellulose and hemicellulose for conversion to useful products (Sharma, 1971). In this study, the average composition of water hyacinth leaves was; total solids (TSs): 8.7–11.5 (% of wet weight), moisture 40–45 (% of wet weight), hemicellulose (as % of TSs): 32.69 ± 0.024, cellulose (as % of TSs): 19.02 ± 0.017, lignin (as % of TSs): 4.37 ± 0.027, crude protein (as % of TSs): 10.20 ± 0.032 and starch (as % of TSs): 4.16 ± 0.043. Several studies on the chemical composition of the water hyacinths have been reported. In Table 1 the chemical composition of the water hyacinths from different sources is summarised. Due to its high moisture content (93–95%), it was dehydrated before use (Table 1). These results agreed fairly well with the data reported by other investigators (Klass and Ghosh, 1981; Wolverton and McDonald, 1981). Hemicellulose content of water hyacinth was relatively high compared to that of cellulose, which is a rare case among plant biomass. Klass and Ghosh (1981) reported hemicellulose content as high as 55% of TS in water hyacinth. The result indicated that water hyacinth leaves could be a source of cellulose for bioconversion. Water hyacinth also contains appreciable amounts of crude protein, which could provide nitrogen source in any bioconversion process.

**Water hyacinth cellulose acid and enzyme hydrolysate preparation**

Dilute sulfuric acid hydrolysis (0.1 M) autoclaved under pressure of 15 lb/inch$^2$ at 135°C for 10 min and enzyme hydrolysis as described in materials and methods was very effective in releasing a good amount of sugar from water hyacinth leaves (Table 2). Higher temperature, higher yield of glucose and reducing sugars were released. Approximately 14% and 21% of glucose and reducing sugars were released at 120°C less than that of at 135°C, respectively. So, temperature at 135°C was suitable to hydrolyse the water hyacinth leaves for sugar production. Approximately 40% of the reducing sugars were released in the first 10 min of autoclaving and enzyme hydrolysis, at 30 min of autoclaving and enzyme hydrolysis, 99% of the reducing sugars were released after which only slight increase was observed. After 30 min of autoclaving, sugar yield was 100% of dry biomass of D-glucose, 5.81% (Table 3). These sugars were derived primarily from cellulose component. The glucose yield (5.81%) was rather high, showing that cellulose almost practically hydrolyzed.

**Table 2.** Effect of temperature on average sugar composition of water hyacinth cellulose acid and enzyme hydrolysate.

| Temperature (°C) | Components % weight (TSs) | |
|---|---|---|
| | Glucose | Reducing sugars |
| 120 | 3.82 ± 0.09 | 4.05 ± 0.04 |
| 125 | 4.20 ± 0.05 | 4.33 ± 0.07 |
| 135 | 4.44 ± 0.02 | 5.14 ± 0.06 |

**Table 3.** Effect of time on average sugar composition of water hyacinth cellulose acid and enzyme hydrolysate.

| Time (min) | Components % weight (TSs) | |
|---|---|---|
| | Glucose | Reducing sugars |
| 10 | 4.44 ± 0.02 | 5.14 ± 0.06 |
| 30 | 5.81 ± 0.07 | 8.52 ± 0.06 |
| 60 | 5.80 ± 0.04 | 8.51 ± 0.11 |
| 90 | 5.84 ± 0.04 | 8.56 ± 0.12 |

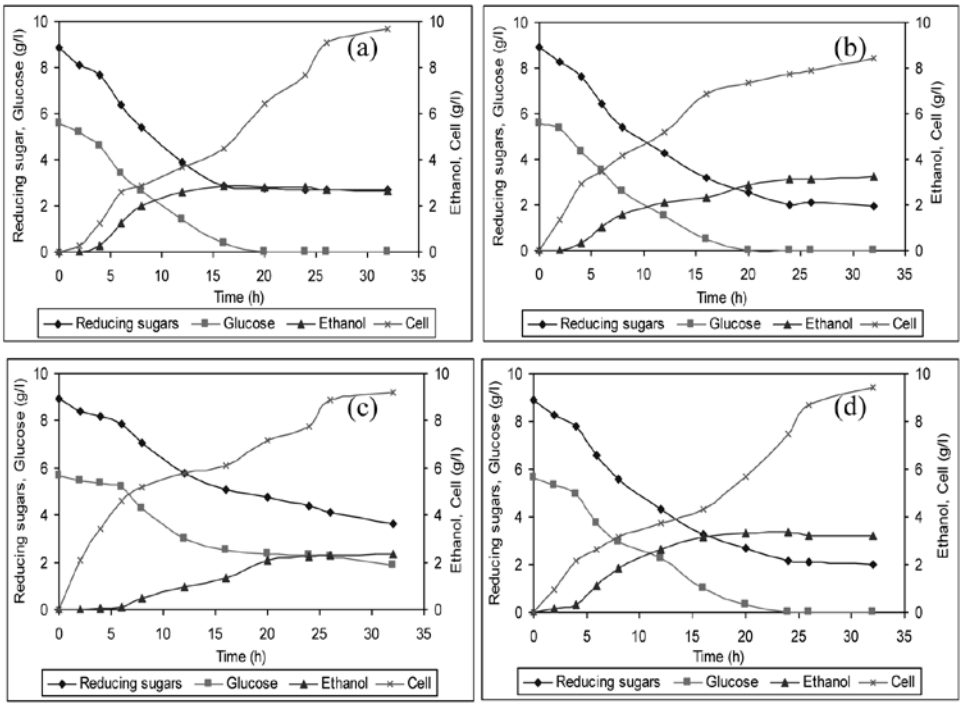


**Figure 1.** The time course of growth, utilization of reducing sugars and glucose and ethanol concentration by *S. cerevisiae* TISTR 5048 (a), *S. cerevisiae* KM 1195 (b), *S. cerevisiae* KM 7253 (c) and co-culture of *S.cerevisiae* TISTR 5048 and *C. tropicalis* TISTR 5045 (d) at 30 ± 0.2 °C and pH 5.0 ± 0.2 using simulated synthetic hydrolysate medium.

**Table 4.** Fermentation parameters of the mono-culture and co-culture of yeasts when grown in the water hyacinth cellulose acid and enzyme hydrolysate and simulated synthetic hydrolysate medium.

| Strain | $C_E$ | $P_{max}$ | $Q_E$ | $Y_{p/s}$ |
|---|---|---|---|---|
| SHF with mono-culture | | | | |
| *S. cerevisiae* TISTR 5048 | 0.19 | 2.84 | 0.21 | 0.45 |
| *S. cerevisiae* KM 1195 | 0.24 | 3.29 | 0.17 | 0.47 |
| *S. cerevisiae* KM 7253 | 0.16 | 2.27 | 0.09 | 0.41 |
| SHF with co-culture Incubation of | | | | |
| *S. cerevisiae* TISTR 5048 with *C. tropicalis* TISTR 5045 | 0.27 | 3.42 | 0.22 | 0.51 |

$C_E$ Ethanol yield per unit biomass (g (g-biomass)$^{-1}$)
$P_{max}$ Maximum ethanol production (g/l)
$Q_E$ Ethanol production rate (g/l/hour)
$Y_{p/s}$ Product (ethanol) yield coefficient (g (g-total sugar)$^{-1}$)

**Table 5.** Ethanol yields per unit biomass from various biomasses.

| Biomass | Pretreatment | Fermentation mode | Fermentation strain | $C_E$ | Reference |
|---|---|---|---|---|---|
| Water hyacinth Leaves | Diluted acid Steam pretreatment | SHF | *S. cerevisiae* TISTR 5048 | 0.19 | This study |
| Water hyacinth Leaves | Diluted acid Steam pretreatment | SHF | *S. cerevisiae* KM 1195 | 0.24 | This study |
| Water hyacinth Leaves | Diluted acid Steam pretreatment | SHF | *S. cerevisiae* KM 7253 | 0.16 | This study |
| Water hyacinth Leaves | Diluted acid Steam pretreatment | SHF | *S. cerevisiae* TISTR 5048 with *C. tropicalis* TISTR 5045 | 0.27 | This study |
| 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 | TMB3001 | 0.18 | Martin et al. (2002) |

$C_E$ Ethanol yield per unit biomass (g (g-biomass)$^{-1}$)

**Fermentation**

The amounts of ethanol yield of water hyacinth leaves obtained by diluted acid steam pretreatment with enzymatic saccharification are given in Table 5. As summarized in Table 5, the ethanol yields per unit biomass were comparable to those reported for other agricultural biomasses, i.e., 0.16–0.27 g g-dry$^{-1}$ of water hyacinth leaves. It was found that different microorganism strain produced different amount of ethanol. The maximum amount of ethanol was obtained when of co-culture of *S. cerevisiae* TISTR 5048 with *C. tropicalis* TISTR 5045 was used, followed by *S. cerevisiae* KM 1195, *S. cerevisiae* TISTR 5048, and *S. cerevisiae* KM 7253. It was also found that mono-culture of *S. cerevisiae* KM 1195 was suitable mono-culture for fermentation of the water hyacinth leaves. The results indicated that ethanol production depended on microorganism stain.

Since the chemical composition of cellulose acid hydrolysate is complex and could be expected to contain unknown, potentially toxic elements (Fein et al., 1984; Nishikawa et al., 1988; Frazer and McCaskey, 1989), the fermentation performance of the *S. cerevisiae* and *C. tropicalis* were evaluated in a simulated synthetic hydrolysate medium, the composition of which was designed and formulated to mimic the water hyacinth cellulose acid hydrolysate with respect to reducing sugars. The time-course for growth, sequential utilization of sugars, and ethanol production is shown (Figure. 1). *S. cerevisiae* and *C. tropicalis* consumed glucose and mannose. After hexoses were exhausted, xylose assimilation began. Xylose consumption was total, since no increase in the ethanol concentration occurred during its utilization. Sequential utilization of sugars have been reported by many authors (Jeffries and Sreenath, 1988; Delgenes et al., 1989; Bjorling and Lindman, 1989; Ferrari et al., 1992). The fermentation parameters for both yield and productivity are summarized (Table 4).

The highest values of ethanol yield per unit biomass ($C_E$), the maximum ethanol production ($P_{max}$), ethanol production rate ($Q_E$) and product (ethanol) yield coefficient ($Y_{p/s}$) were found to be 0.27 g (g-biomass)$^{-1}$, 3.42, 0.22 g/l/hour and 0.51 g (g-total sugar)$^{-1}$, respectively, by the fermentation of co-culture of *S.cerevisiae* 5048 and *C. tropicalis* TISTR 5045. The lowest values of ethanol yield per unit biomass ($C_E$), the maximum ethanol production ($P_{max}$), ethanol production rate ($Q_E$) and product (ethanol) yield coefficient ($Y_{p/s}$) were found to be 0.16 g (g-biomass)$^{-1}$, 2.27, 0.09 g/l/hour and 0.41 g (g-total sugar)$^{-1}$, respectively, by the fermentation of mono-culture of *S. cerevisiae* 7253. It was found that mono-culture of *S. cerevisiae* 1195 could produce relatively high ethanol yield almost equal to that of the co-culture (Table 4).

Figure 1 (d) shows the time-course for growth, sugar utilization and ethanol concentration in the cellulose acid hydrolysate medium at initial pH 5.0 ± 0.2 of co-culture of *S. cerevisiae* and *C. tropicalis.* The fermentation parameters are summarized in Table 4. The yield ($C_E$) and productivities ($P_{max}$, $Q_E$ and $Y_{p/s}$) increased 1.42-, 1.2-, 1.05 and 1.13-fold, respectively, when mono-culture of *S. cerevisiae* TISTR 5048 was grown in a medium cellulose acid hydrolysate as compared with the co-culture of *S. cerevisiae* and *C. tropicalis.* This showed that co-culture of *S. cerevisiae* and *C. tropicalis* fermentation employed for the treatment of cellulose acid hydrolysate has partially used reducing sugars as substrate and improved the fermentability.

The fermentation period of co-culture comparing to mono-culture of *S. cerevisia*e KM 1195 or *S. cerevisiae* KM 7253 was also reduced to about 33%. However, the fermentation period of co-culture for the cellulose acid hydrolysate was rather similar to that obtained with *S. cerevisia*e TISTR 5048 (Table 4). This shows that there might be some leftover reducing

sugars in the treated cellulose acid hydrolysate that are not used in the fermentation performance of *S. cerevisiae* TISTR 5048.

## Conclusions

The maximum values of ethanol yield ($C_E$), productivity ($P_{max}$, $Q_E$ and $Y_{p/s}$) and percent sugar utilization were 0.27 g (g-biomass)$^{-1}$, 3.42, 0.22 g/l/hour and 0.51 g (g-total sugar)$^{-1}$, and 77.4%, respectively, at temperature 30 ± 0.2°C and pH 5.0 ± 0.2, when co-culture of *S. cerevisiae* TISTR 5048 and *C. tropicalis* TISTR 5045 was grown in treated cellulose hydrolysate medium. Fermentation by mono-culture of *S. cerevisiae* KM 1195 at the same condition improves the ethanol yield and productivity compared to the other mono-culture of *S. cerevisiae* TISTR 5048 or *S. cerevisiae* KM 7253. Therefore, the fermentation of water hyacinth leaves for ethanol production was carried out in a high yield by optimum treatment and appropriated yeast strain.

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