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Appropriate Technology for the Bioconversion of Water Hyacinth (*Eichhornia crassipes*) to Liquid Ethanol: Future Prospects for Community Strengthening and Sustainable Development

Chartchalerm Isarankura-Na-Ayudhya, Tanawut Tantimongcolwat, Thikamporn Kongpanpee, Ponpitak Prabkate, Virapong Prachayasittikul*

Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand. Telephone: 662-849-6318, Fax: 662-849-6330,
E-mail: mtvpr@mucc.mahidol.ac.th (*corresponding author)

ABSTRACT

This article appraises the need for introducing appropriate technology to improve the production of renewable energy, particularly on the community basis and social aspect of sustainability. Using two-sequential steps of acid hydrolysis (10% sulfuric acid) and yeast (*Candida shehatae*; xylose-fermenting yeast) fermentation, bioconversion of water hyacinth (*Eichhornia crassipes*; a noxious weed and fast growing aquatic plant widely distributed in many tropical regions of the world) to liquid ethanol has successfully been performed. The maximum ethanol yield coefficient of 0.19 g g^{-1}_{WH} with the productivity of 0.008 g l^{-1} h^{-1} was achieved. This is as well comparable to those obtained from the enzymatic saccharification and/or the fermentation of acid-pretreated water hyacinth hydrolysate using fully-equipped fermenter reported elsewhere. More importantly, determinations of xylose and ethanol contents can potentially be performed using two reliable colorimetric approaches (Phloroglucinol and Dichromate assays, respectively) in conjunction with home-made portable photometer. The technology presented herein can be transferred and implemented to gain opportunity of becoming self-reliance of community in the third world countries.

Keywords: water hyacinth, ethanol, bioconversion, bioenergy, *Candida shehatae*, fermentation, acid hydrolysis

INTRODUCTION

Global depletion of energy supply due to the continuing over-utilization is being a major problem of the present and future world community. It is estimated that the fossil fuels will be running out by the next few decades (Bentley, 2002; Cavallo, 2002), therefore, attention has currently been dedicated to the conversion of biomass into fuel ethanol. Production of ethanol provides several advantages over gasoline: first, utilization of abundant and inexpensive sources of renewable resources; second, reduction in greenhouse gas emission and toxic substances; third, macroeconomic benefits for rural community and social aspect of sustainability; and fourth, pertaining in national energy security (Knauf and Moniruzzaman, 2004; Lin and Tanaka, 2006). Conversion of biomass-to-ethanol can be performed by i) acid/alkaline hydrolysis or enzymatic saccharification of starchy materials (e.g. cereal grains, potato and cassava etc.) into fermentable sugars and ii) microbial fermentation of these sugars to ethanol (for recent review see Hahn-Hagerdal et al., 2006). In some circumstances, sugar-rich feedstocks including sugarcane, sugar beet and various fruits are converted to ethanol directly and yield high ethanol contents. However, a major drawback from

the high costs of materials becomes an obstacle for large scale expansion. Therefore, production of ethanol from other lignocellulosic wastes (e.g. wood residue, sugarcane bagasse, corn fiber, rice straw, cassava waste etc.) has received widespread interest due to their availability, abundance and relatively low cost (Gonzales et al., 1986; Pessoa Jr et al., 1997). It is important to note that many factors are still needed to be taken into consideration for the large scale production. For examples, multi-steps of separation and breaking down of these lignocellulosic materials (composed of hemicellulose, cellulose and lignin) into soluble sugars are required due to their structural complexity and the presence of toxic-degraded substances. Special attention has to be given to the fermentation of xylose (a major component of hemicellulose), since it is difficult to ferment by common ethanol-producing organisms (Olsson and Hahn-Hagerdal, 1993). Furthermore, the use of high technology and complicated instrument-ations with high operating costs may in turn limit their commercialization and industrial application, especially in the third world countries.

Water hyacinth (*Eichhornia crassipes*) is a fast growing perennial aquatic plant widely distributed throughout the world (for recent review see Malik, 2007). This tropical plant can cause infestations over large areas of water resources and consequently lead to series of problems. These include reduction of biodiversity, blockage of rivers and drainage system, depletion of dissolved oxygen, alteration on water chemistry, and involvement in environmental pollution. Therefore, on one hand, attempts have been geared towards the use of biological, chemical and mechanical approaches for preventing the spread of, or eradication of, water hyacinth. On the other hand, much attention has been focused on the potentials and constrains of using water hyacinth for a variety of applications (for recent review see Gunnarsson and Petersen, 2007). For instances, production of paper, crafts, ropes and furniture have been reported. Great emphasis has also been taken as food products due to its high protein content and richness of vitamin A (Neogi and Rajagopal, 1949). It can be utilized as green fertilizer, animal fodder and means of metal remediation (Gajalakshmi and Abbasi, 2002; Lu et al., 2004; Al Rmalli et al., 2005; Gunnarsson and Petersen, 2007). The possibility of converting water hyacinth to biogas or fuel ethanol is currently established in a number of developing countries, mainly in India (Abraham and Kurup, 1996; Sharma et al., 1999; Nigam, 2002; Singhal and Rai, 2003). However, these pilot facilities have not yet been transferred and implemented in the community.

Therefore, the present study aims at applying a simple and reliable process of two-sequential steps (acid hydrolysis and yeast fermentation) for the bioconversion of water hyacinth to ethanol (Figure 1). Estimations of xylose (a major fermentable sugar) and ethanol contents can potentially be performed by colorimetric determinations. Such approaches are believed to be simple enough that most things can directly be managed at a local level. Moreover, they are also expected to provide significant positive impacts on the basis of community strengthening and opportunity of becoming self-reliance, once the technology has been brought up throughout the developing countries.

MATERIALS AND METHODS

Chemicals, reagents, and microorganism

Phloroglucinol (1,3,5-trihydroxybenzene) was purchased from Fluka. Absolute ethanol and potassium dichromate were supplied from Merck. All other chemicals and reagents were of analytical grade and commercially available.

Candida shehatae strain TISTR 5843 was obtained from Thailand Institute of Scientific and Technological Research, Thailand. The organism was maintained on

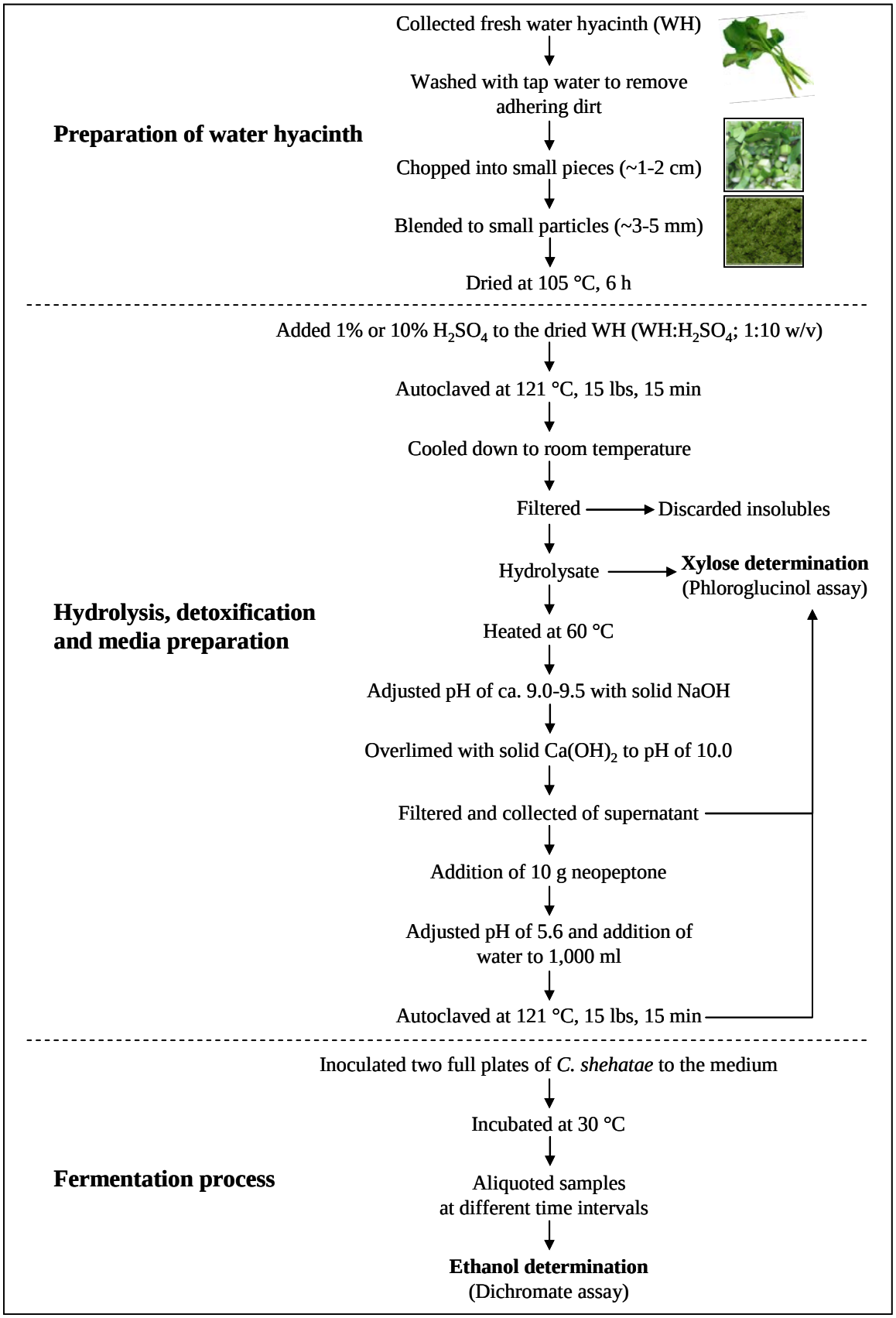

Figure 1: Schematic representation of the bioconversion process of water hyacinth to ethanol.

Sabouraud Dextrose Agar (10 g l^{-1} neo-peptone, 20 g l^{-1} dextrose, pH 6.5; SDA) at 4°C. Subculture was then performed on the SXA medium containing 20 g l^{-1} xylose prior to fermentation.

Preparation of water hyacinth

Fresh water hyacinth (WH) with long stem was collected from a natural pond in Klongmai community, Nakornpathom province, Thailand in May 2006. The water hyacinth was thoroughly washed several times with tap water to remove adhering dirt, chopped into small pieces (~1-2 cm), blended to small particles (~3-5 mm), and finally dried in a hot air oven at 105 °C for 6 hour. The dried material was stored at room temperature until used.

Preparation of hemicellulose acid hydrolysate

One hundred grams of dried water hyacinth were mixed with 1% or 10% of sulfuric acid to a final volume of 1,000 ml. The mixture was autoclaved at 121 °C, 15 lbs for 15 min and further cooled down to room temperature. The hydrolysate was filtered using washman paper No. 1 to remove the unhydrolysed material. The filtrate was collected and subjected to analyze the xylose content.

Detoxification of hemicellulose hydrolysate

The hemicellulose acid hydrolysate was heated to 60 °C and then basidified with solid NaOH until the pH reached 9.0-9.5. Solid $Ca(OH)_2$ was added to the solution in order to detoxify harmful materials presented in the hydrolysate (Martinez et al., 2000). Insoluble residues were removed by filtration, and the supernatant was collected for further used as fermentable sugars.

Preparation of fermentation medium

Ten grams of neopeptone (Difco Laboratories, Detroit, USA) was added to the overlimed hydrolysate and the pH of the solution was adjusted to 5.6. This solution was placed in 2-L Erlenmeyer flask, filled up with distilled water up to 1 L, and autoclaved at 121 °C, 15 lbs for 15 min.

Fermentation of water hyacinth hydrolysate to alcohol

Two full plates of *C. shehatae* on SXA were inoculated into the fermentation medium and further incubated at 30 °C for 3 wks. Samples were aliquoted at different time intervals and assayed for ethanol content. For comparison, Sabouraud Dextrose Broth (SDB) and Sabouraud Xylose Broth (SXB) (containing 20 g dextrose and xylose, respectively) were used as control media.

Determination of xylose content by Phloroglucinol assay

Xylose content was determined using the Phloroglucinol assay (Eberts et al., 1979; Johnson et al., 1984) with minor modifications. Briefly, the color reagent consisting of 0.5 g of phloroglucinol, 100 ml of glacial acetic acid, and 10 ml of conc. HCl was freshly prepared and used within 4 days. Stock standard xylose (10 g L^{-1}) was prepared by dissolving D-xylose powder in saturated benzoic acid and used for preparation of the calibration curve (Figure 2A). To the procedure herein, two hundred microliters of sample was mixed with 5 ml color reagent and subsequently heated at 100 °C for 4 min. The reaction was rapidly cooled down to room temperature in water and the absorbance at 540 nm was recorded.

Determination of ethanol content by Dichromate assay *(Adopt from Bennett, 1971; Pilone, 1985)*

Acid dichromate solution (0.1 M $Cr_2O_7^{2-}$ in 5 M H_2SO_4) was prepared by dissolving 7.5 g of potassium dichromate in dilute sulfuric acid and the final volume was adjusted to 250 ml with deionized water. To prepare the calibration curve, 300 µl of ethanol solutions were filled into small plastic caps and placed into beakers containing 3 ml of acid dichromate. The beakers were tightly sealed with parafilm and kept at room temperature for 30 min. The

maximum absorbance at 590 nm was recorded (Figure 2B).

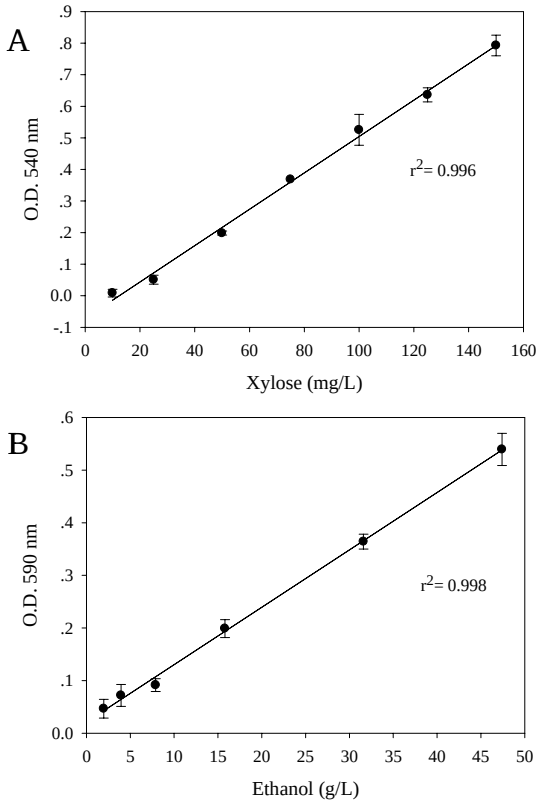

Figure 2: Calibration curves of xylose (A) and ethanol (B) contents obtained from Phloroglucinol and Dichromate assays determined by UV/Vis Spectrophotometer (Shimadzu model 1601).

Assay of xylose and ethanol contents by a portable photometer

The xylose and ethanol contents were also determined by using a portable multi-wavelengths photometer developed by the Faculty of Medical Technology, Mahidol University in collaboration with the Q'nic Company (Figure 3). The dimensions of the device are 137 (W) × 92 (D) × 44 (H) mm. The main components of the photometer consist of ultra bright spotted LEDs (light sources), highly sensitive photodetector, liquid crystal display, rechargeable battery, and data acquisition interface (RS-485). Calibration curves of standard xylose and ethanol (measured at 560 and 600 nm, respectively) were prepared (Figure 4).

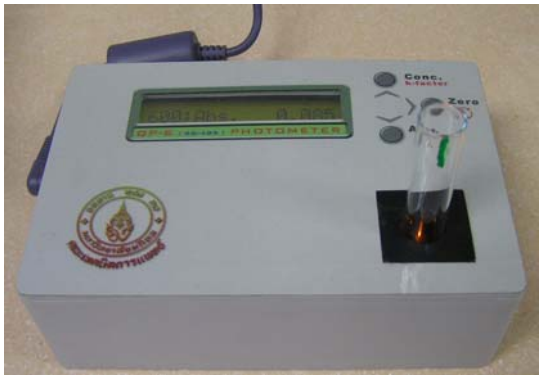

Figure 3: Portable MT photometer used for measurement of xylose and ethanol contents.

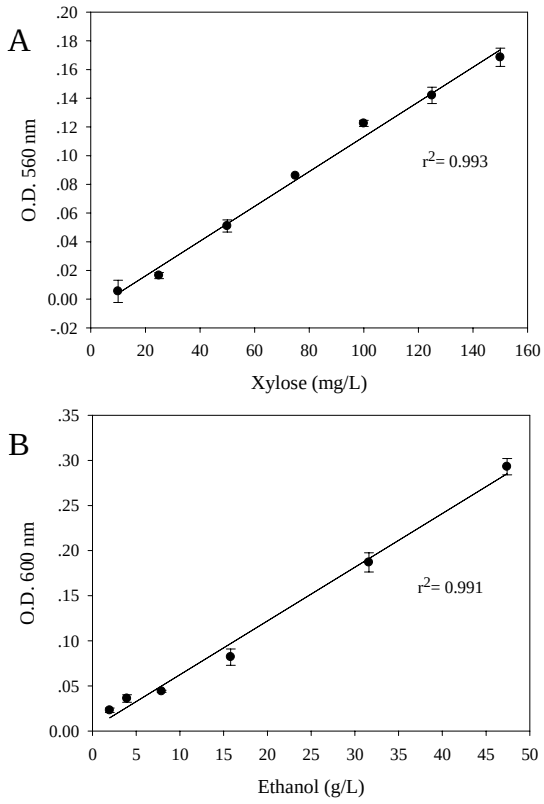

Figure 4: Calibration curves of xylose (A) and ethanol (B) contents derived from the portable MT photometer.

RESULTS AND DISCUSSION

Successful bioconversion of lignocellulosic from local material to liquid fuel have been achieved by using two-sequential steps (acid hydrolysis and yeast fermentation). Since the fast growing and the high hemicellulose content (30-55% of dry

weight) (Malik, 2007; Nigam, 2002), water hyacinth has increasingly been recognized as a potential target for production of ethanol and methane (Abraham and Kurup, 1996; Sharma et al., 1999; Nigam, 2002; Singhal and Rai, 2003). Hydrolysis of water hyacinth by dilute acid yields mixture of sugars with xylose as a major component (~ 60%) (Nigam, 2002). Herein, increasing of acid by 10% on the saccharification of water hyacinth gave rise approximately 6-10 times higher of xylose than 1%. The maximum xylose of up to 134 mg g^{-1} water hyacinth was found in the acid hydrolysate (Table 1). Our results are in good agreements with previous reports on the acid treatment of hemicellulose (Roberto et al., 1994; Elander and Hsu, 1995; Pessoa Jr et al., 1997). Notification has to be made that furfural, a by-product of xylose degradation, is also generated as a consequence of acid hydrolysis (Ackerson et al., 1981). The rate of degradation depends on temperature and concentration of sulfuric acid (Gonzales et al., 1986). Additionally, acetic acid is produced as one of the principal components of the hemicellulose hydrolysate (Grohman et al., 1985). Therefore, overliming with $Ca(OH)_2$ and heating at high temperature are required for removal/reduction of volatile compounds (e.g. furfural and phenol), acetic acid and tannic acid, which is generally resulting in better fermentability of the hydrolysate (Martinez et al., 2000). However, diminutions of approximately 35% and 66% of total xylose content were observed upon overliming with $Ca(OH)_2$ and autoclave sterilization of the fermentation medium, respectively (Table 1).

Table 1: Xylose contents of water hyacinth acid hydrolysate determined at different steps of preparation.

Samples	Xylose* (mg/g WH)
Hydrolysate	134 ± 8.5
Hydrolysate overlimed with $CaOH_2$	86.5 ± 19
Hydrolysate broth for cultivation	45 ± 7.1

*Results are means of two independent experiments.

It should be mentioned that industrial yeast strains (e.g. *Saccharomyces cerevisiae*) normally ferment hexoses (glucose, fructose and sucrose), but not pentoses (xylose and arabinose). Therefore, *Candida shehatae* was selected in this study. The total yield of ethanol production as well as the rate of fermentation was determined on the dextrose and xylose-containing media (Figure 5). Using 20 grams of sugars, the total yield of up to 7 g L^{-1} ethanol with a production rate of 0.5-1 gram per day was found upon supplementation with dextrose (SDB). Meanwhile, the alcohol content of approximately 1-1.5 g L^{-1} ethanol was achieved when the yeast was grown in the xylose-fermenting medium (SXB) up to 3 wks. More importantly, the rates of ethanol production between the SXB and the water hyacinth hydrolysate were almost identical (data not shown). This implies that detoxification procedure mentioned above potentially reduces significant amount of toxic elements.

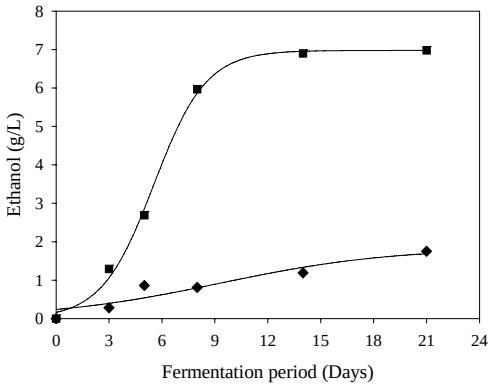

Figure 5: Efficiency of *C. shehatae* on bioconversion of dextrose (square) and xylose (diamond) to ethanol at different time intervals.

The production yields of ethanol obtained from the acid hydrolysis or enzymatic saccharification of water hyacinth using different approaches are summarized in Table 2. Our results revealed that using the sulfuric hydrolysis following by the bioconversion of *C. shehatae* yielded ethanol with the maximum content of 1.01 g/L, the

maximum yield coefficient of 0.19 g g^{-1} and the productivity of 0.008 g l^{-1} h^{-1}. These values are as well comparable to those obtained from the phenol-tolerant strain of xylose fermenting bacterium (Asli et al., 2002). Although, the maximum ethanol produced by the hydrolysate is 2.5 fold lesser than that of the SDB, however, its maximum ethanol yield coefficient (0.19 g g^{-1}) is remarkably higher (up to 1.5- and 4.75-folds) than those of the SDB and SXB substrates. This coefficient is greater than the results reported elsewhere using acid hydrolysis (0.12 g g^{-1}) and cellulase catalysis reaction (0.16 g g^{-1}) (Abraham and Kurup, 1996). Our report herein showed only 1.8 fold lesser in the coefficient yield than those obtained from using the fully-equipped fermentor (Nigam, 2002). Therefore, further maximizations on the development of a versatile tool for ethanol production are now taken as an ongoing research in our lab.

In conclusion, we explored a high feasibility of using an appropriate technology (acid hydrolysis and yeast fermentation) for the bioconversion from water hyacinth to ethanol. As previously mentioned, the water hyacinth is one of the worst weeds that causing the major problem to the global aquatic or terrestrial particularly in the tropics. Although control managements have been widely applied to keep the plant at a low level using herbicides and mechanical removal, in most of the cases, it remains ineffective due to the pernicious invasive growing of the aquatic hyacinth. The technique herein, more or less helps lowering the plant while provides the simple and low-cost process that is suit to the developing countries.

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Table 2: Efficiency of acid/enzymatic saccharification and fermentation processes on the conversion of water hyacinth to ethanol.

Samples	Hydrolysis/ Enzymatic saccharification	Organism used in the fermentation	Initial Xylose conc. ($g\ l^{-1}$)	Initial Glucose conc. ($g\ l^{-1}$)	Maximum ethanol conc. ($g\ l^{-1}$)	Maximum ethanol yield coefficient ($g\ g^{-1}$)	Productivity ($g\ l^{-1}h^{-1}$)	Cultivation time (h)	Reference
Sabouraud dextrose broth (SDB)	-	*Candida shehatae* TISTR 5843	-	20	2.69	0.13	0.022	120	This study
Sabouraud xylose broth (SXB)	-	*Candida shehatae* TISTR 5843	20	-	0.86	0.04	0.007	120	This study
Water hyacinth hydrolysate	10 % H_2SO_4, 121°C, 15 lbs, 15 min, Overliming with $Ca(OH)_2$	*Candida shehatae* TISTR 5843	5.37	n.d.	1.01	0.19	0.008	120	This study
Water hyacinth hydrolysate	1% H_2SO_4, 7 h	*Pichia stipitis* NRRL Y-7124	54.68	n.d.	10.39	0.19	0.098	106	Nigam 2002
Water hyacinth hydrolysate	1% H_2SO_4, 7 h, Boiling 100°C, 15 min, Overliming with $Ca(OH)_2$	*Pichia stipitis* NRRL Y-7124	54.68	n.d.	19.14	0.35	0.18	106	Nigam 2002
Water hyacinth hydrolysate	10 % H_2SO_4, 121°C, 10 lbs, 30 min	*Saccharomyces cerevisiae*	Reducing sugars 0.375 g g^{-1}		-	0.12	-	72	Abraham 1996
Water hyacinth solution	Enzymatic saccharification (Cellulase from *Myrothecium verrucaria*)	*Saccharomyces cerevisiae*	Reducing sugars 0.43 g g^{-1}		-	0.16	-	72	Abraham 1996

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