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Enhanced acid hydrolysis for bioethanol production from water hyacinth (*Eichhornia crassipes)* using fermentating yeast *Candida intermedia* NRRL *Y-981*

A Manivannan, P Hepsibha Jayarani and R T Narendhirakannan*

Department of Biotechnology, School of Biotechnology & Health Sciences, Karunya University, Coimbatore 641 114, India

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This study presents bioconversion of water hyacinth (*Eichhornia crassipes*) to bioethanol using two-sequential steps of acid hydrolysis (10% sulfuric acid) and yeast (*Candida intermedia*) fermentation. Maximum ethanol yield co-efficient was comparable to those obtained through enzymatic saccharification and fermentation of acid hydrolysate using fully equipped fermentor. A maximum ethanol yield (coefficient, 0.21 g g^{-1}; productivity, 0.010 g $l^{-1}h^{-1}$) was comparable to predicted value (0.23 g g^{-1}) obtained by CCD (Central Composite Design). Two colorimetric methods (phloroglucinol and dichromate assays) were used for determination of xylose and ethanol using UV-Vis spectrophotometer. Although maximum ethanol concentration was low, an economically efficient overall process was carried out to convert a lignocellulosic biomass to bioethanol.

Keywords: Acid hydrolysis, Bioconversion, Bioethanol, *Candida intermedia*, Water hyacinth

Introduction

Bioethanol is considered as an effective liquid fuel produced from cellulosic biomass[1]. Excessive consumption of fossil fuels has led to an increasing demand for alternative sources of fuel[2]. Currently, ethanol is mainly produced from sugar or starch for fuel industry. However, demand for these raw materials, which are also food sources, will not be sufficient to meet the need for fuel ethanol[3]. Cellulosic ethanol is one of the most promising technological approaches available to reduce emission of greenhouse gases from transportation sector[4]. Also, lignocellulosic biomass is a low cost feedstock that is widely available and does not involve ethical concerns associated with the use of potential food resources[5]. Pretreatment is required to alter structural and chemical composition of lignocellulosic biomass to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars[6]. Water hyacinth (*Eichhornia crassipes* Solms; WH) is a fast growing perennial aquatic plant widely distributed throughout the world[7]. Much attention has been focused on the potentials and constrains of using WH for a variety of applications[8] (production of paper, crafts, ropes and furniture). Great emphasis has also been given to WH as a food product due to its high protein content and richness of vitamin A[9]. Bioconversion of WH[10-15] to biogas or fuel ethanol using different fermentation yeasts (*Saccharomyces cerevisiae, Candida shehatae*) is currently established in a number of developing countries, mainly in India.

This study used *Candida intermedia* for fermentation of WH, and obtained highest transport capacity of glucose and xylose, reflected in the improved yield of ethanol.

Experimental Section

Materials

Fresh WH with long stem was collected from a natural pond, Periya kullam (Big Lake), in Coimbatore city, Tamil Nadu, India. WH 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 h. Dried material was stored at room temperature (RT) until used.

Phloroglucinol (1, 3, 5-trihydroxybenzene), absolute ethanol and potassium dichromate were sourced from

*Author for correspondence
E-mail: bionaren_phd@yahoo.co.in

Merck. All other chemicals and reagents were of analytical grade. *C. intermedia* NRRLY-981 was procured from ARS (NRRL) - New York and made to grow in Sabouraud's Dextrose Agar (SDA: neopeptone, 10; and dextrose, 20 g l^{1}; pH 6.5) at 4°C. Subculture was then performed on Sabouraud Xylose Agar (SXA) medium containing xylose (20 g l^{-1}) prior to fermentation.

Detoxification of Hemicellulose Hydrolysate

Hemicellulose acid hydrolysate was heated to 60°C and then basidified with solid NaOH to get pH 9.0-9.5. Solid $Ca(OH)_2$ was added in solution to detoxify harmful materials present in hydrolysate[16]. Insoluble residues were removed by filtration, and supernatant was collected for further use.

Fermentation of Water hyacinth (WH) Hydrolysate to Ethanol

For preparation of fermentation medium, neopeptone (10 g) was added to over limed hydrolysate and adjusted solution pH to 5.6. This solution was placed in 2 l Erlenmeyer flask, filled with distilled water up to 1 l, and autoclaved (121°C and 15 lbs) for 15 min. Two plates of *C. intermedia* on SXA were inoculated into fermentation medium and further incubated at 30°C for 3 weeks. For comparison, Sabouraud Dextrose Broth (SDB) and Sabouraud Xylose Broth (SXB) (containing 20 g dextrose and xylose, respectively) were used as control media.

Xylose content was determined using Phloroglucinol assay[17,18]. Color reagent [phloroglucinol, 0.5 g; glacial acetic acid, 100 ml; and conc. hydrochloric acid (HCl), 10 ml] prepared freshly and a stock solution of standard xylose (10 g l^{1}) was prepared by dissolving D-xylose powder in saturated benzoic acid and used in the preparation of calibration curve. Sample (200 µl) was mixed with color reagent (5 ml) and subsequently heated at 100°C for 4 min. Reaction was rapidly cooled down to RT in water and absorbance at 540 nm was recorded in a UV-Vis spectrophotometer (Hitachi U-2910, Japan).

For determination of ethanol content by Dichromate assay[19,20], acid dichromate solution (0.1 M $Cr_2O_7^{2-}$ in 5 M H_2SO_4) was prepared by dissolving of potassium dichromate (7.5 g) in dilute sulfuric acid and final volume was adjusted to 250 ml with deionized water. To prepare calibration curve, ethanol solution (300 µl each) was filled into small plastic cups and placed into beakers containing acid dichromate (3 ml). Beakers were tightly sealed with parafilm and kept at RT for 30 min. Maximum absorbance at 590 nm was recorded in a UV-Vis spectrophotometer.

Table 1—Variables in experimental design

Variables	Coded levels				
	-1.682	-1	0	1	1.682
Time	28.4	36	48	60	67.6
pH	3.377	4	5	6	6.63
Temp., °C	26.84	30	35	40	43.16

Table 2—Central composite design (CCD) matrix employed for ethanol yield

Run no.	X_1	X_2	X_3	Ethanol conc., Y (g/l)	
				Observed	Predicted
1	-1	-1	-1	22.25	21.36
2	-1	-1	-1	12.33	11.25
3	-1	-1	-1	25.86	21.95
4	-1	-1	-1	26.02	25.42
5	-1	-1	1	23.65	22.14
6	-1	-1	1	24.01	23.39
7	-1	-1	1	21.02	19.67
8	-1	-1	1	14.69	12.17
9	-1.682	0	0	30.14	29.64
10	1.682	0	0	15.03	15.73
11	0	-1.682	0	24.32	23.17
12	0	1.682	0	25.12	25.37
13	0	0	-1.682	18.43	16.15
14	0	0	1.682	31.76	29.23
15	0	0	0	28.43	26.21
16	0	0	0	19.18	17.04
17	0	0	0	22.05	20.63
18	0	0	0	22.18	21.38
19	0	0	0	14.54	13.59
20	0	0	0	25.13	23.87

Experimental Design and Optimization

Central composite design (CCD) was used in optimization of ethanol production. Time (X_1, h), pH (X_2), temperature (X_3, °C) were chosen as independent variables (Table 1). Ethanol concentration (Y, g/l) was used as dependent output variables. Experiments (20) were performed to optimize parameters (Table 2). Among them, six replications were at center points ($n_{0=6}$), while axial points were determined to be $\sqrt{3}$. Coefficients of polynomial model were calculated as

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_{i<j}}^{k} \sum_{j}^{k} b_{ij} X_i X_j$$

where Y is predicted response, and i, j are linear, quadratic coefficients, respectively. B and k are regression coefficient and number of factors studied in the experiment, respectively.

Table 3—Significance of ethanol coefficients of ethanol production model (R^2= 0.95455)

	Regression coefficient	Standard error	t	p
Mean	-141.317	22.05961	-6.40616	0.000016
Time	1.104	0.27446	4.02061	0.001264
Temp.	8.491	1.08473	7.82760	0.000002
Temp. ×Temp.	-0.105	0.01449	-7.24648	0.000004
Time × Temp.	-0.023	0.00779	-2.94020	0.010753
pH × Temp.	-0.099	0.01029	-9.66839	0.000000

Significance of each coefficient was determined using student's value. Results were analyzed by using MINITAB (15.1, PA, USA) software. Three-dimensional plots and their respective contour plots were obtained to study interaction of one parameter with another. Optimum concentration based on hump was identified in three-dimensional plots.

Results and Discussion

WH is an excellent substrate for the production of ethanol through enzymatic or acid/alkali pretreatments followed by fermentation[21,22]. Fermentation of ethanol by *C. intermedia* was successful using acid hydrolysis. Ethanol yield was comparable to that of alkali and enzymatic hydrolysis methods. Hydrolysis of WH by dilute acid yields mixture of sugars with xylose as a major component[12] (~ 60%). Therefore, increasing acid concentration by 10% in saccharification of WH gave rise of 6-10 times higher xylose than when it was 1%. Maximum xylose concentration of up to 134 mg g^{-1} WH was found in acid hydrolysate. Xylose degradation also generates byproducts as a consequence of acid hydrolysis[23]. Acetic acid is produced as one of the principal components of hemicellulose hydrolysate[24]. Therefore, removal/reduction of volatile compounds (furfural and phenol) is performed by over liming with $Ca(OH)_2$ and heating at high temperature. This resulted in better fermentation of hydrolysate[16].

Response Surface Analysis for Optimization of Three Factors

Actual ethanol yield in CCD (Table 2) showed a satisfactory explanation (R^2 = 0.9645), not all the effects of factors and their interactions on ethanol concentration were significant ($p<0.05$). In order to simplify the model as well as to enhance the effect of significant items, the ones which showed trivial effect were eliminated. In new model (Table 3), entire item showed important effect on ethanol concentration ($p<0.05$). Second order polynomial

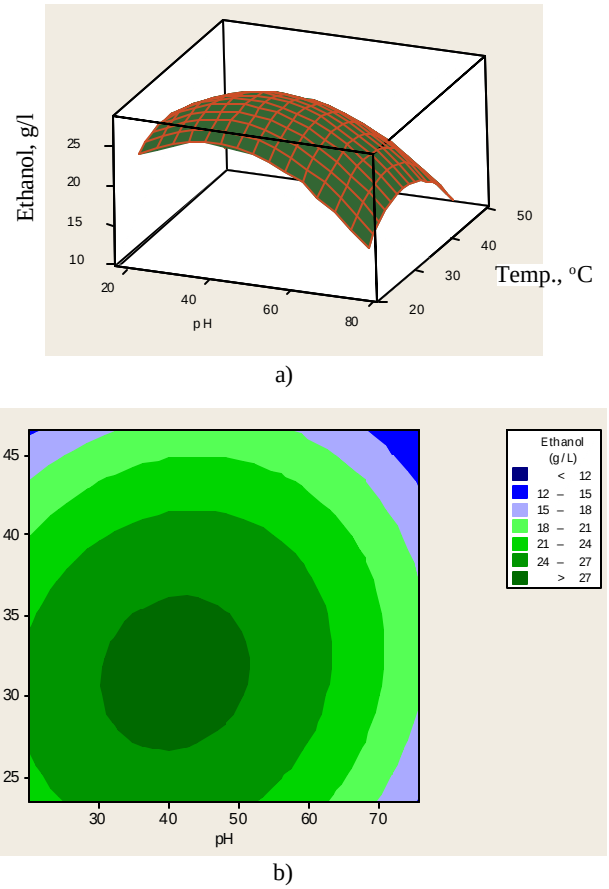

Fig. 1—Interaction effects of temperature and pH on ethanol production: a) Surface plot; b) Contour plot

equation giving ethanol (Y, g/l) as a function of time (X_1, h), pH (X_2) and temperature (X_3, °C) were obtained as

$$Y= 141.37 + 1.104X_1 + 8.491X_3 - 0.105X^2 - 0.023X_1X_3 - 0.099X_2X_3$$

Deviation between observed and predicted ones was less. R^2 of model was found to be 0.95545, implying that model was a good fit that 95.545% of variation could be explained well by the model.

Interactions among Factors

Ethanol production increased with increase in temperature (Fig. 1), but with increase in pH, trend was reversed. In a relative low pH and medium temperature, optimum ethanol production could be attained. Between 28-34°C and at maximum time duration (Fig. 2), optimum ethanol yield could be attained. An increase in time with temperature increased ethanol production, but at high temperature (> 34°C), ethanol production decreased.

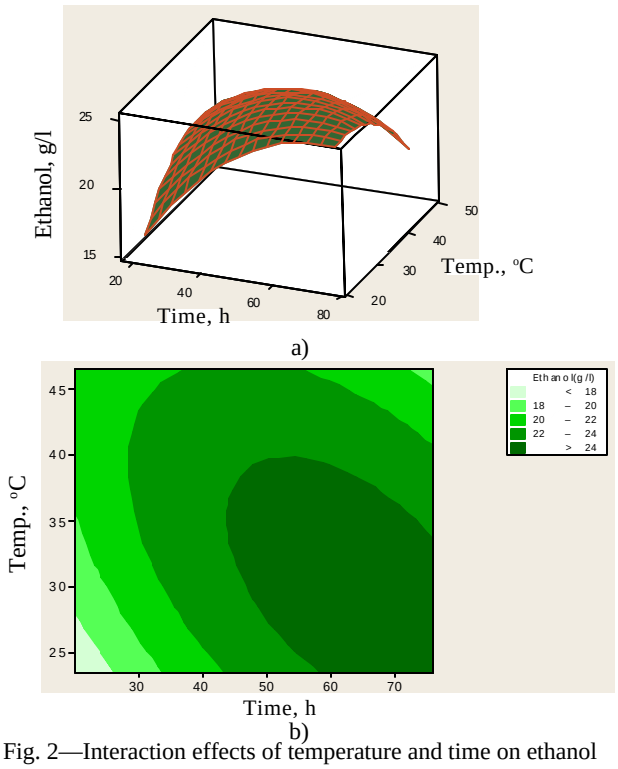

Fig. 2—Interaction effects of temperature and time on ethanol production: a) Surface plot; b) Contour plot

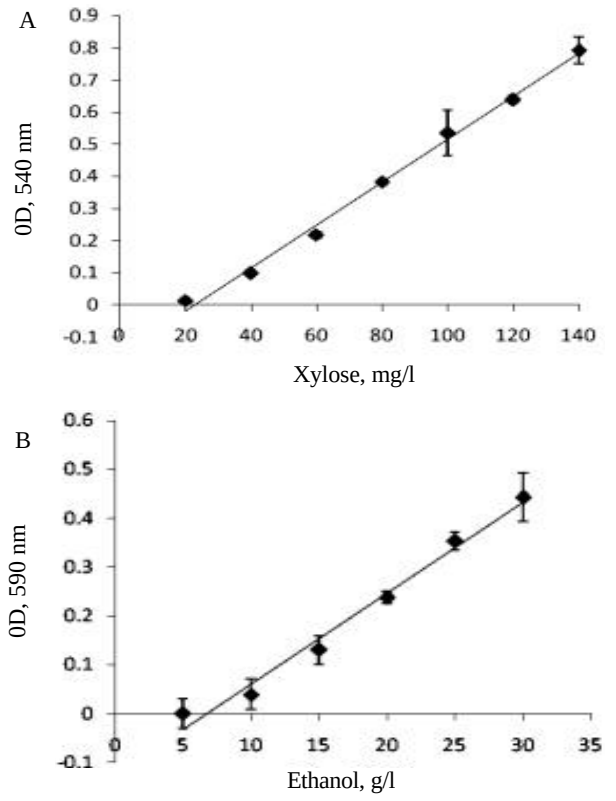

Fig. 4—Calibration curves of standard xylose (A) and ethanol (B) contents obtained from phloroglucinol and Dichromate assays determined by UV/vis spectrophotometer

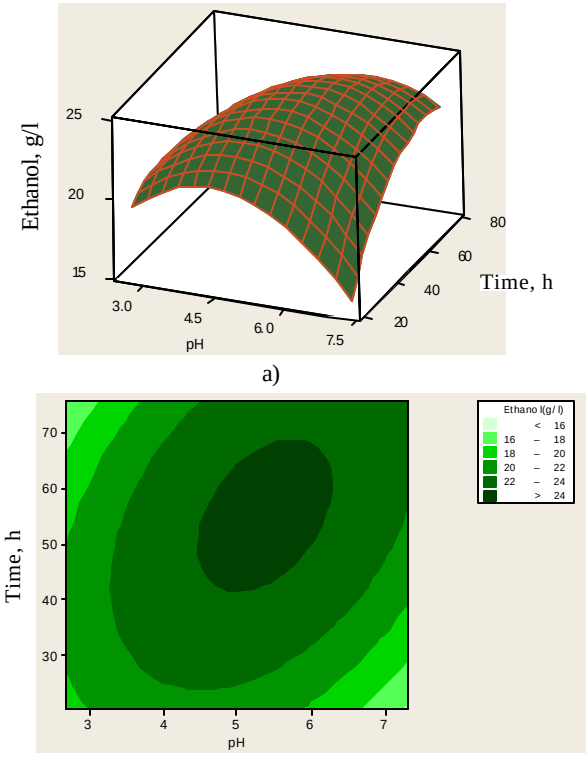

Fig. 3—Interaction effects of time and pH on ethanol production: a) Surface plot; b) Contour plot

Thus interaction between pH and time showed little significance. Only low pH and long incubation times were found beneficial for ethanol production (Fig. 3). Therefore, for optimum ethanol production (33.04 g/l), optimum parameters were found to be: time, 67.60 h; pH, 4.18; and temp., 35°C. To validate optimum concentration, an experiment with specified condition was performed. Resultant concentration (34.21 g/l) showed that the model was useful to predict concentration as well as the optimization of experimental conditions.

Determination of Xylose and Ethanol contents by UV- Vis Spectrophotometer

Xylose and ethanol contents were also determined by using a UV-Vis Spectrophotometer. Calibration curves (measured at 560 and 600 nm, respectively) were prepared of standard xylose and ethanol (Fig. 4) and obtained xylose and ethanol (Fig. 5). Industrial yeast strains (*S. cerevisiae*) normally ferment hexoses (glucose, fructose and sucrose), but not pentoses (xylose and arabinose). Therefore, *C. intermedia* were selected in this study. Total yield of ethanol production as well as the rate of fermentation was determined on the dextrose and xylose-containing media. Ethanol (1.0-1.5 g l^{-1}) was

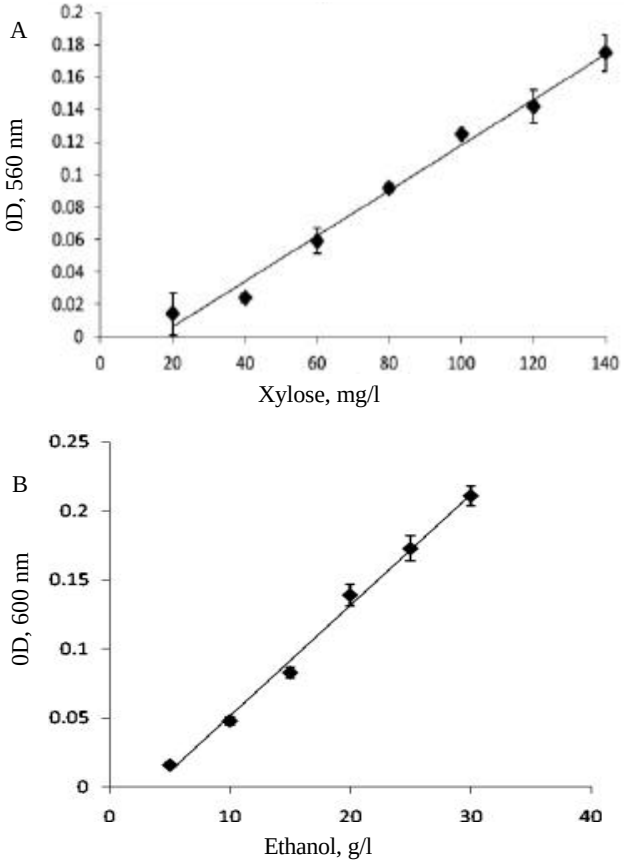

Fig. 5—Calibration curves of obtained xylose (A) and ethanol (B) contents obtained from phloroglucinol and Dichromate assays determined by UV/vis spectrophotometer

achieved when yeast was grown in xylose-fermenting medium (SXB) up to 3 weeks (Fig. 6). This implies that detoxification procedure potentially reduces significant amount of toxic elements.

Results reveal that using sulfuric acid hydrolysis followed by bioconversion of *C. intermedia* yielded maximum ethanol (1.03 g/l) with 173 maximum yield coefficient (0.21 g g^{-1}) and productivity (0.010 g $l^{-1}h^{-1}$). These values are well comparable to those obtained from phenol-tolerant strain of xylose fermenting bacterium[26]. This coefficient is greater than the results reported elsewhere using acid hydrolysis (0.14 g g^{-1}) and cellulase catalysis reaction (0.18 g g^{-1})[10]. This study showed 2% increase in the yield compared to earlier study[15]. It showed only a 1.8 fold lesser coefficient yield than those obtained from using a fully-equipped fermentor[12].

Conclusions

RSM based on CCD established a high similarity between the observed value and predicted ones. Optimum ethanol production (0.23 g g^{-1}) from WH was determined to be 33.05 g/l (incubation time, 67.60 h; pH, 4.18; and temp., 35°C). Integration of low cost pretreatments followed by fermentation with improved ethanol-producing strains of microorganisms may play a crucial role in lowering the cost of biomass bioconversion processes. This study may lead to focus on improving methodologies for lignocellulosic bioconversion and can help in turning what is considered a noxious weed into a resource.

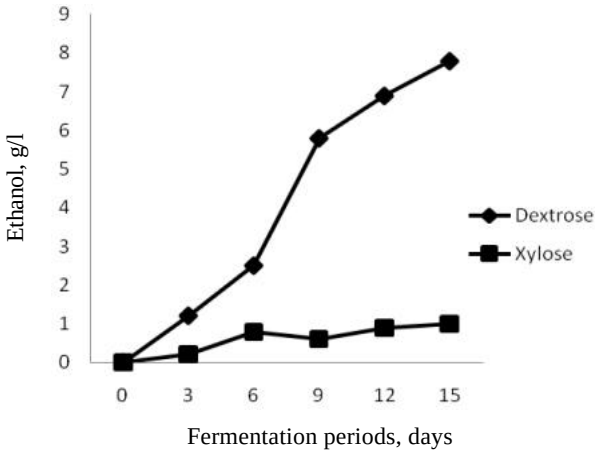

Fig. 6—Efficiency of *C. intermedia* on bioconversion of dextrose and xylose to ethanol at different time intervals

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