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Biodegradation of Lignocellulosic Residues of Water Hyacinth (*Eichhornia crassipes*) and Response Surface Methodological Approach to Optimize Bioethanol Production Using Fermenting Yeast *Pachysolen tannophilus* NRRL Y-2460

A. Manivannan, R. T. Narendhirakannan

***Abstract*—**The objective of this research was to investigate biodegradation of water hyacinth (*Eichhornia crassipes*) to produce bioethanol using dilute-acid pretreatment (1% sulfuric acid) results in high hemicellulose decomposition and using yeast (*Pachysolen tannophilus*) as bioethanol producing strain. A maximum ethanol yield of 1.14g/L with coefficient, 0.24g g^{-1}; productivity, 0.015g $l^{-1}h^{-1}$ was comparable to predicted value 32.05g/L obtained by Central Composite Design (CCD). Maximum ethanol yield coefficient was comparable to those obtained through enzymatic saccharification and fermentation of acid hydrolysate using fully equipped fermentor. Although maximum ethanol concentration was low in lab scale, the improvement of lignocellulosic ethanol yield is necessary for large scale production.

***Keywords*—**Acid hydrolysis, Biodegradation, Hemicellulose, *Pachysolen tannophilus*, Water hyacinth.

I. Introduction

WATER hyacinth (*Eicchornia crassipes* (Mart.) Solms), a noxious aquatic weed found in many tropical and sub-tropical fresh water habitats due to its faster growth rate and its utilization as a cheap feed stock for biodegradation into fuel ethanol [1]. Bio-ethanol is an alternative fuel that is produced almost entirely from food crops. It represents an important, renewable liquid fuel for motor vehicles. An important advantage of crop-based ethanol is its Green House Gas (GHG) benefits [2], [3]. With increasing gap between the energy requirement of the industrialized world and inability to replenish such needs from the limited sources of energy like fossil fuels, ever increasing levels of greenhouse pollution from the combustion of fossil fuels in turn aggravate the perils of global warming and energy crisis [4]. There is a growing interest worldwide to find out new and cheap carbohydrate sources for production of bio-ethanol [5]. Production of ethanol from renewable sources of lignocellulosic biomass can improve energy security, decrease urban air pollution, and reduce accumulation of carbon dioxide in the atmosphere [6].

Cellulosic materials are renewable natural biological resources and generation of biobased products and bioenergy from such substances is important for the development of humans [7]. Biological methods for using lignocellulosic biomass in ethanolic fermentation are becoming cost-effective. The most commonly used microorganism, *Saccharomyces cerevisiae*, can only ferment certain mono- and disaccharides (such as glucose, fructose, maltose and sucrose) efficiently into ethanol. It cannot convert pentoses, which are also major components of lignocellulosic biomass [8]. The yeasts *Pichia stipitis*, *Candida shehatae* and *Candida intermedia* can assimilate pentoses into ethanol [8]. The conversion of cellulose from lignocellulosics to ethanol is more challenging than conversion of soluble carbohydrates from food crops [1].

In this study *Pachysolen tannophilus* was used for fermentation of water hyacinth, and obtained highest transport capacity of glucose, pentose and xylose, reflected in the improved yield of ethanol.

II. Materials and Methods

A. Plant Material and Microorganism

Fresh water hyacinth plant with long stem was collected from a natural pond, Periya kullam (Big Lake), in Coimbatore city, Tamil Nadu, India. Water hyacinth *Eicchornia crassipes* (Mart.) Solms has been authenticated by Botanical Survey of India (BSI) BSI/SRC/5/23/2012-13/Tech. 464- TNAU Coimbatore, Tamil Nadu, India. Water hyacinth was thoroughly washed several times with tap water to remove adhering dirt, chopped into small pieces (~1-2cm), blended to small particles (~3-5mm), and finally dried in a hot air oven at 105°C for 6h. Dried material was stored at room temperature until further process.

Phloroglucinol (1,3,5-trihydroxybenzene), absolute ethanol and potassium dichromate were sourced from Merck. All other chemicals and reagents were of analytical grade. *Pachysolen tannophilus* NRRLY-2460 was procured from Agricultural Research Service-New York and made to grow in Sabouraud's Dextrose Agar (SDA: neopeptone, 10; and dextrose, 20g/L; pH 6.5) at 4°C. Subculture was then performed on Sabouraud

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Xylose Agar (SXA) medium containing xylose (20 g/L) prior to fermentation.

B. Preparation and Detoxification of Hemicellulose Hydrolysate

About 1000 mL of 1% dilute sulfuric acid was prepared in an Erlenmeyer flasks. The flasks were added with 100g of water hyacinth and autoclaved at 121°C, 15 lbs for 15 minutes. The hydrolysate was filtered using whatman paper No. 1 to remove the unhydrolysed material. Then 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 [9]. Insoluble residues were removed by filtration, and supernatant was collected for further use.

C. Fermentation of Water Hyacinth Hydrolysate to Ethanol

For preparation of fermentation medium, neopeptone (10g) was added to over limed hydrolysate and adjusted solution pH to 6.5. This solution was placed in a 2L Erlenmeyer flask, filled with distilled water up to 1L, and autoclaved (121°C and 15lbs) for 15min. Two plates of *P. tannophilus* 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 20g dextrose and xylose, respectively) were used as control media.

Xylose content was determined using Phloroglucinol assay [10], [11]. Color reagent [phloroglucinol, 0.5g; glacial acetic acid, 100mL; and conc. hydrochloric acid (HCl), 10mL] prepared freshly and a stock solution of standard xylose (10g/L) was prepared by dissolving D-xylose powder in saturated benzoic acid and used in the preparation of calibration curve. Sample (200µL) was mixed with color reagent (5mL) and subsequently heated at 100°C for 4min. Reaction was rapidly cooled down to room temperature in water and absorbance at 540nm was recorded in a UV-Vis spectrophotometer (Hitachi U-2910, Japan).

For determination of ethanol content by Dichromate assay [12], [13] acid dichromate solution (0.1M $Cr_2O_7^{2-}$ in 5M H_2SO_4) was prepared by dissolving of potassium dichromate (7.5g) in dilute sulfuric acid and final volume was adjusted to 250mL with deionized water. To prepare calibration curve, ethanol solution (300µL each) was filled into small plastic cups and placed into beakers containing acid dichromate (3mL). Beakers were tightly sealed with parafilm and kept at room temperature for 30 min. Maximum absorbance at 590 nm was recorded in a UV-Vis spectrophotometer.

D. Central Composite Design

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 I). Ethanol concentration (Y, g/L) was used as dependent output variables. 20 experiments were performed to optimize parameters. 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.

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.

III. Results and Discussion

The production of ethanol from Water hyacinth by *P. tannophilus* through acid pretreatment followed by fermentation was successful. Ethanol yield was comparable to that of alkali and enzymatic hydrolysis methods. Therefore, 1% acid concentration in saccharification of water hyacinth gave rise of 6-10 times higher xylose. Maximum xylose concentration of up to 134 mg/g water hyacinth was found in acid hydrolysate. Xylose degradation also generates byproducts as a consequence of acid hydrolysis [14]. Acetic acid is produced as one of the principal components of hemicellulose hydrolysate [15]. 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 [9]. The concentration of ethanol increased with the increase of fermentation time and yeast biomass. The viable cell numbers increased from 3 x 10^8 CFU/g substrate (0 h) to 18.5 x 10^9 CFU/g substrate (67 h) after which it decreased drastically at 96 h (1 x 10^8 CFU/g substrate). The decline in biomass concentration could be due to reduced substrate availability and the inhibitory effect of ethanol on yeast cells [16], [17].

A. Response Surface Analysis for Optimization of Three Factors

The experimental results associated to the processing set up of each independent variable are listed in Table I five level central composite design matrix and experimental responses of the dependent variable (ethanol concentration) are listed in Table II Second order polynomial 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

$$Y = -147.239 + 1.136X_1 + 8.371X_3 - 0.107X_3^2 - 0.028X_1X_3 - 0.114X_2X_3$$

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 III), entire item showed important effect on ethanol concentration ($p<0.05$). 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.

TABLE I
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 II
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	24.19	23.27
2	-1	-1	-1	15.43	13.48
3	-1	-1	-1	27.92	25.33
4	-1	-1	-1	28.04	26.82
5	-1	-1	1	25.98	23.17
6	-1	-1	1	27.00	24.78
7	-1	-1	1	23.09	21.03
8	-1	-1	1	17.89	15.42
9	-1.682	0	0	31.18	29.76
10	1.682	0	0	17.09	15.93
11	0	-1.682	0	26.58	24.21
12	0	1.682	0	27.02	25.66
13	0	0	-1.682	20.09	19.48
14	0	0	1.682	33.59	31.25
15	0	0	0	30.49	28.16
16	0	0	0	22.21	20.76
17	0	0	0	24.31	22.91
18	0	0	0	16.67	14.45
19	0	0	0	27.09	25.75
20	0	0	0	25.48	23.84

TABLE III
SIGNIFICANCE OF ETHANOL COEFFICIENTS OF ETHANOL PRODUCTION MODEL (R^2= 0.95455)

	Regression coefficient	Standard error	t	P
Mean	-147.239	22.05961	-6.40616	0.000021
Time	1.136	0.27446	4.02061	0.001285
Temp.	8.371	1.08473	7.82760	0.000003
Temp. ×Temp.	-0.107	0.01449	-7.24648	0.000006
Time × Temp.	-0.028	0.00779	-2.94020	0.010782
pH × Temp.	-0.114	0.01029	-9.66839	0.000000

B. Interactions among Factors

Surface and contour plots demonstrating the effects of different process parameters, two parameters varied at a time while keeping the third at middle level, on the ethanol concentration were shown in Figs. 1-3. The stationary points were examined by analyzing these plots. Generally, circular contour plots indicate that the interactions between parameters are negligible. On the contrary, elliptical ones indicate the evidence of the interactions [18].

Fig. 1 showed the effect of temperature and pH on the ethanol concentration. The convex response surface suggested well-defined optimum variables (temperature and pH) and that the ethanol concentration increased to the peak with the increase of temperature and pH up to 42ºC and 6, respectively; then declined with the further increase of these two parameters. This result demonstrated that the response surface had a maximum point for ethanol yield. Similar results have been obtained by Wilkins et al., [19] who reported that ethanol production from simultaneous saccharification and fermentation of citrus peel waste by *S. cerevisiae* was greatest when the fermentation temperature and pH were adjusted to 37ºC and 6.0, respectively. 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. Thus interaction between pH and time showed little significance. Only low pH and long incubation times were found beneficial for ethanol production (Fig. 3). Besides the increase in temperature accelerates the inhibition effect of ethanol on the cell activities, thereby lowering both cell and ethanol yields [20]. Therefore, for optimum ethanol production (33.28g/L), optimum parameters were found to be: time, 67.60h; pH, 6.45; and temp., 34°C. To validate optimum concentration, an experiment with specified condition was performed. Resultant concentration (32.05g/L) showed that the model was useful to predict concentration as well as the optimization of experimental conditions.

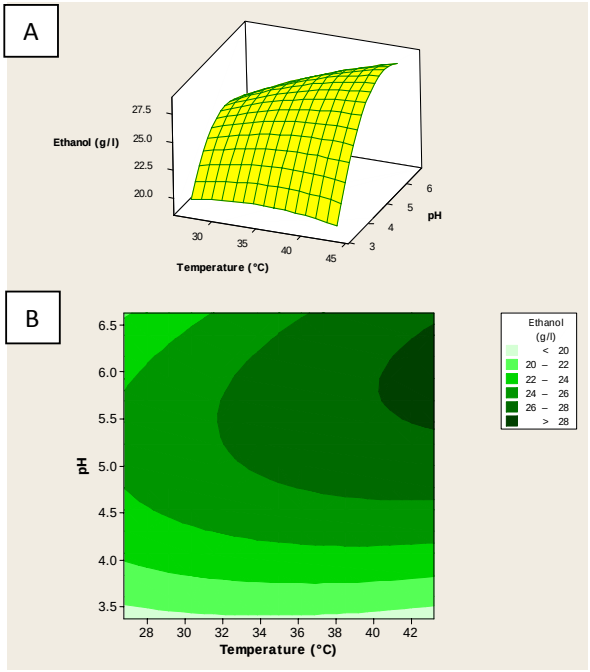

Fig. 1 Interaction effects of Temperature and pH on ethanol production: A Surface plot; B Contour plot

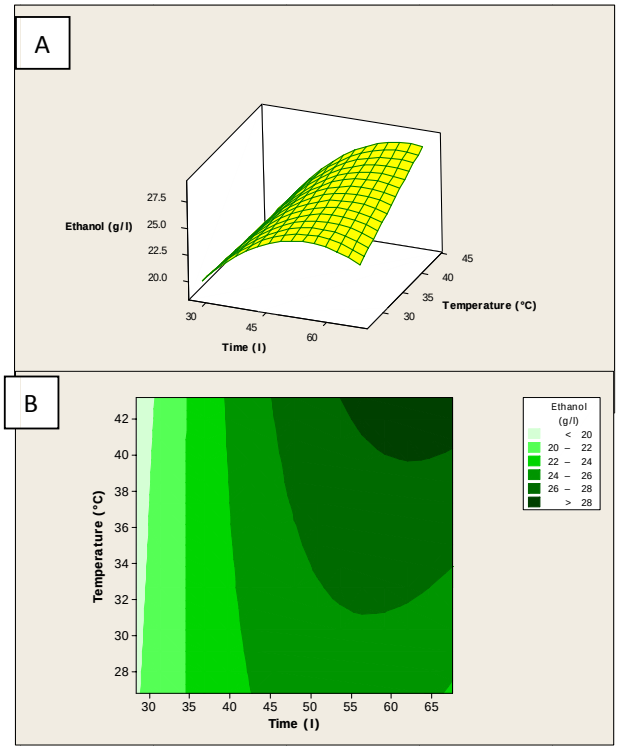

Fig. 2 Interaction effects of Temperature and Time on ethanol production: A Surface plot; B Contour plot

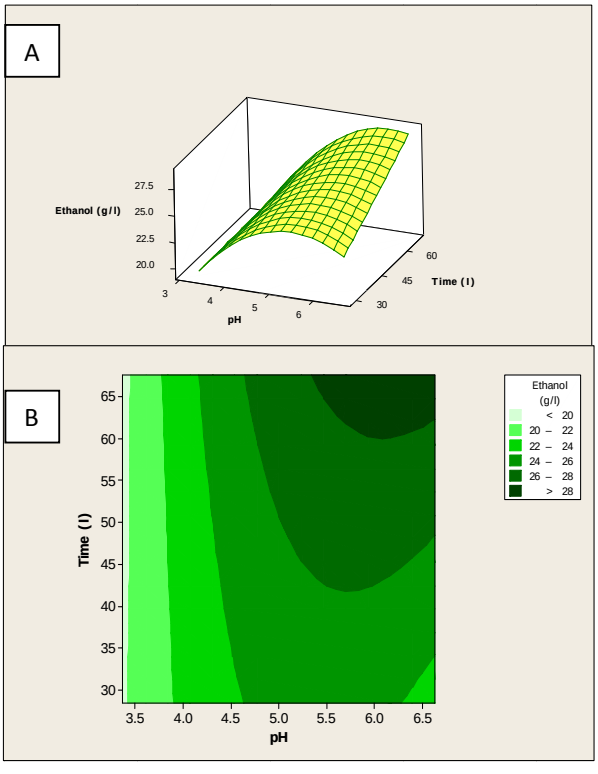

Fig. 3 Interaction effects of Time and pH on ethanol production: A) Surface plot; B) Contour plot

C. 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). In our previous study we have used *Candida intermedia* for fermentation of water hyacinth and obtained highest transport capacity of glucose and xylose [21] where industrial yeast strain *Saccharomyces cervisiae* normally ferment hexoses (glucose, fructose and sucrose), but not pentoses (xylose and arabinose). Therefore, *P. tannophilus* was selected in this study for its preferential utilization of pentose and hexose sugar. Total yield of ethanol production as well as the rate of fermentation was determined on the dextrose and xylose-containing media. Ethanol (1.0-1.5g/L) was achieved when yeast was grown in xylose-fermenting medium (SXB) up to 3 weeks. This implies that detoxification procedure potentially reduces significant amount of toxic elements.

Results reveal that using sulfuric acid hydrolysis followed by bioconversion of *P. tannophilus* yielded maximum ethanol (1.14g/L) with 176 maximum yield coefficient (0.24g g^{-1}) and productivity (0.015g $L^{-1}h^{-1}$). These values are well comparable to those obtained from phenol-tolerant strain of xylose fermenting bacterium [22]. This coefficient is greater than the results reported elsewhere using acid hydrolysis (0.14g g^{-1}) and cellulase catalysis reaction (0.18g g^{-1}) [23]. Corresponding to the ethanol concentration of 32.05 g/L, the ethanol yield was calculated as 0.42g ethanol/g reducing sugar accounting for as high as 74% of the stoichiometric value.

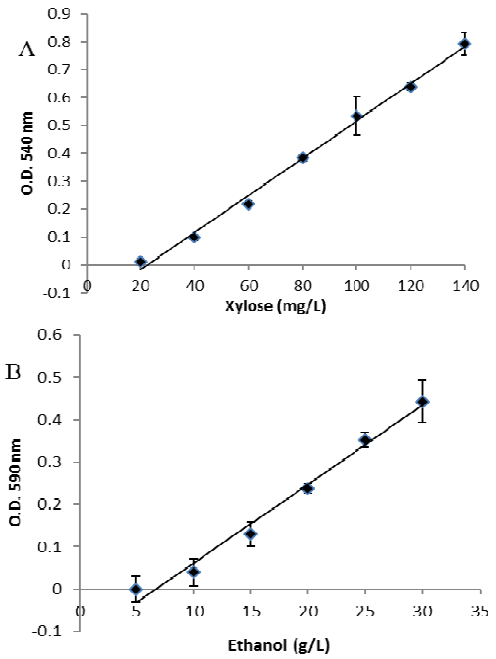

Fig. 4 Calibration curves of standard xylose (A) and ethanol (B) contents obtained from phloroglucinol and Dichromate assays determined by UV/vis Spectrophotometer

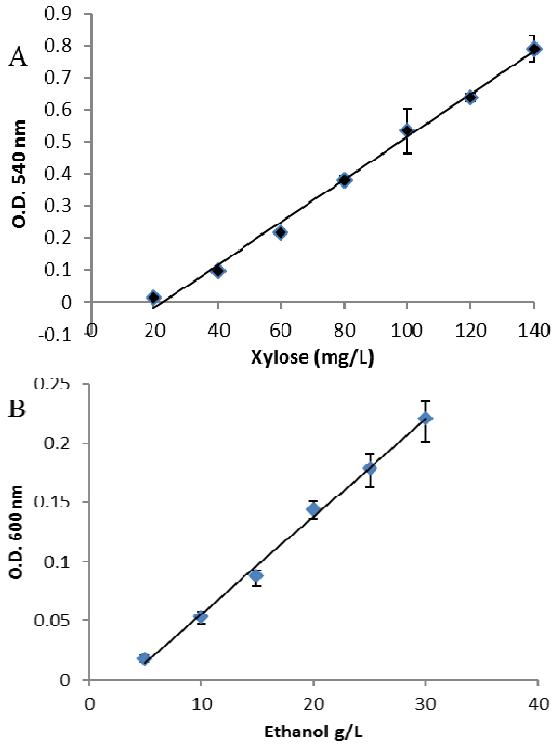

Fig. 5 Calibration curves of obtained xylose (A) and ethanol (B) contents obtained from phloroglucinol and Dichromate assays determined by UV/vis Spectrophotometer

IV. Conclusion

RSM based on CCD established a high similarity between the observed value and predicted ones. Optimum ethanol production (0.24g g^{-1}) from water hyacinth was determined to be 32.05g/L (incubation time, 67.60h; pH, 6.45; and temp., 34°C) using *Pachysolen tannophilus* which utilized both pentose and hexose showed better ethanol yield. The results from the investigation showed that Water hyacinth can be used as an alternative substrate for ethanol production, in comparison to energy-rich food crops, if sterilized suitably prior to fermentation by some low cost energy sources such as solar energy. Further research is, however, warranted to examine the economical viability of the process. 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.

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