

BIOETHANOL PRODUCTION FROM WATER HYACINTH BIOMASS USING ISOLATED FUNGAL STRAIN FROM LOCAL ENVIRONMENT

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ABSTRACT

Fermentation of sugar released by saccharification of lignocellulosic materials produce bioethanol which is a renewable energy source. There are some technological barriers such as pretreatment, saccharification of cellulose and hemicellulose matrix, and simultaneous fermentation of hexose and pentose sugars which needs to be addressed for efficient conversion of lignocellulosic biomass to bioethanol. The present study evaluated water hyacinth as feedstock for bioethanol production, with the possible strategies by which the conversion to sugars from water hyacinth can be maximized. Effect of alkali pretreatment on WHB was studied. In this study, suitability of the feedstock for production of fermentable sugars using crude enzymes produced on site from isolated fungal strain (F13) from the local environment at CSIR-CMERI, Durgapur (Latitude: 23.55^o; Longitude: 87.31^o) was demonstrated where in the highest xylose yield obtained was 236.56 mg/g. Cellulase and xylanase activity was determined for biomass saccharification process. Finally, fermentation of the hydrolysate using pentose fermenting yeast, *Pichia stipitis* yielded an ethanol concentration of 3.193g/L.

Key words : Water Hyacinth, Pretreatment, Saccharification, Fermentation, Bioethanol.

INTRODUCTION

Due to continuous depletion of fossil fuel reserves, there is a need to utilize renewable efficient, sustainable, cost effective and safe alternative energy source. (Chum et al,2001). Lignocellulosic biomass can be used as an alternative to the automobile fuels due to its availability, abundance and relatively low cost. Extensive research has been completed on conversion of lignocellulosic materials to ethanol in the last two decades (Dale et al., 1984).

Overuse of fossil fuel is increasing the carbon dioxide level in the atmosphere and significantly contributing to global warming. Thus, there is pressing need to adapt to the use of bioethanol as a renewable and clean energy source. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol has a much higher latent heat of vaporization (855 MJ/kg) than petrol (293 MJ/kg) as well as a higher octane number (99) than petrol (80–100) and, as a result, pre-ignition does not occur when ethanol is used (Ganguly et al, 2012).

Water Hyacinth (*Eichhornia crassipes*), a lignocellulosic biomass is a freshwater aquatic plant in India whose high hemicelluloses content (30-55% of dry weight) can provide hemicellulosic sugars for bioconversion to ethanol fuel. The biomass of water hyacinth has about 48% hemicelluloses, 18% cellulose 3.5% lignin (Nigam, 2002). Also being an aquatic plant it has an added advantage of not being a competition to food crops for arable land resources (Mishima et al, 2008). The biomass can be used to produce bioethanol by decomposition of fermentable saccharides and the byproducts can be used as organic manure.

In the present work, xylose and reducing sugar obtained after pretreatment and saccharification processes were estimated. Biomass saccharification was carried out by enzymes produced onsite by isolated fungal strain F-13. Finally fermentation was performed using *Pichia stipitis* and ethanol content was measured using potassium dichromate method.

MATERIALS AND METHODS

Preparation of Water Hyacinth Biomass:

From the ponds of CSIR-C.M.E.R.I campus fresh water hyacinth biomass (WHB) was collected. The samples were washed carefully and thoroughly to remove small bits of dirt and dust from them and were further chopped to very small particles (~2 mm) and finally dried at 106 °C for approximately six hours. At room temperature the dried biomass was stored in air tight containers.

FTIR Analysis:

Fourier Transform Infrared spectra were studied on treated and untreated WHB using a Shimadzu spectrometer (Japan). For this, 3.0 mg of the sample was dispersed in 300 mg of spectroscopic grade KBr and subsequently pressed into disks at 10 MPa for 3 min. The spectra were obtained with an average of 25 scans and a resolution of 4 cm⁻¹ in the range of 4000–400 cm⁻¹.

Scanning Electron Microscopy (SEM):

The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. In most applications, data are collected over a selected area of the surface of the sample, and a 2-dimensional image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20X to approximately 30,000X, spatial resolution of 50 to 100 nm). A large fraction of the xylan and lignin was removed by pre-treatment. For this purpose, SEM pictures of the treated and untreated biomass samples were taken for examining the physical changes in the biomass. Scanning electron micrographs (SEM) were taken at magnification 1,5009 for both native and pretreated water hyacinth biomass (particle size 2 mm) using a JEOL JSM-5600 scanning electron microscope.

Alkali Pretreatment:

By using 5% NaOH the pretreatment process was carried out (1 g sample + 10 mL of 5% NaOH). After mixing the biomass and alkali it was allowed to soak for 1:00 hour followed by the treatment time of 10:00 min inside the furnace at 150 °C. By filtration method the hydrolysate was collected.

Crude enzyme extraction and production:

For individual enzyme assay of cellulase and xylanase, the fungi were grown on water hyacinth under solid state fermentation conditions. In this, properly sporulated fungal plate cultures of the isolated fungal strain from local environment of CSIR-CMERI (F-13) was taken and alkali treated dried WHB was used as the only carbon source. 5 g of alkali treated WHB was taken and the rest media

components were supplemented with Mandel media's mineral components. The moisture content was adjusted to 6.0% (12 mL). The incubation was done at 28 °C for 5-6 days.

For extraction of crude enzyme, the contents in each flask were suspended in 100 mL cold citrate buffer (0.05M, pH- 4.8) and put on a rotary shaker for 10:00 min. The contents were then centrifuged at 10,000 rpm at 4 °C for 10:00 min to separate the biomass. The supernatant was collected and four parts of ice cold acetone was added. The mixture was again centrifuged at 10,000 rpm at 4 °C for 15:00 min. The supernatant was discarded and the aqueous layer of crude enzyme obtained was collected. This crude enzyme was then suspended in citrate buffer (0.05M, pH- 4.8) and stored at 4 °C for further use. Cellulase was measured according to the IUPAC methods (Ghose, 1987) using Whatmann filter paper no.1 as substrate and glucose as standard. Xylanase was also estimated (Somogyi,1952) in which xylan was used as substrate for xylanase and xylose as standard. All the values were expressed in terms of IU/min/mL.

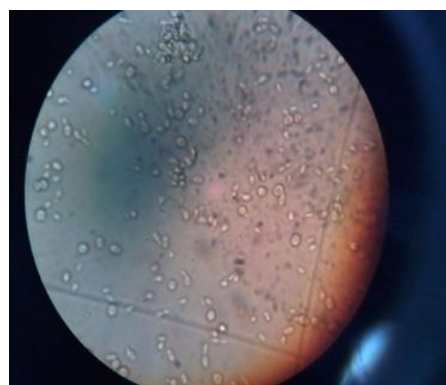
Biomass Saccharification:

The treated biomass was exposed to enzymatic saccharification for better production of reducing sugar. In reaction mixture containing treated biomass, enzymatic saccharification of alkali treated WHB was carried out using Mandel media (Mandels et al, 1976). Enzymes produced onsite was dissolved in citrate buffer and 1 mL was added to reaction mixture. After that the reaction mixture was incubated on a rotary shaker at 50 °C and 125 rpm. For determining the reducing sugar and xylose content the samples were withdrawn at intervals of 12:00 h up to 60:00 h. After complete saccharification, for deactivating the enzymes the reaction mixture was heated slightly. Finally, using filter paper the saccharified biomass was filtered and the hydrolysate was obtained. The hydrolysate obtained was used for fermentation.

Fermentation:

To carry out fermentation, the yeast *Pichia stipitis* was used. A loop full of culture from fully cultured plates of each strain was transferred into autoclaved broth medium [broth medium for the culture of *Pichia stipitis* was prepared with the following composition (g/L) : Malt extract, 3g; Glucose, 5g; D-xylose, 50g; Yeast extract, 3g; Peptone, 5g; pH, 5.0 under laminar air flow and was cultured in shaker incubator for about 20:00 hrs. The broth was then centrifuged for 10:00-15:00 min at about 10,000 rpm. The pellets containing the yeast cells were collected and supernatant was discarded. Pellets were then dissolved in 15mL distilled water. The spore count was around 7.2×10^8 which was measured using a haemocytometer as shown in Figure-1.

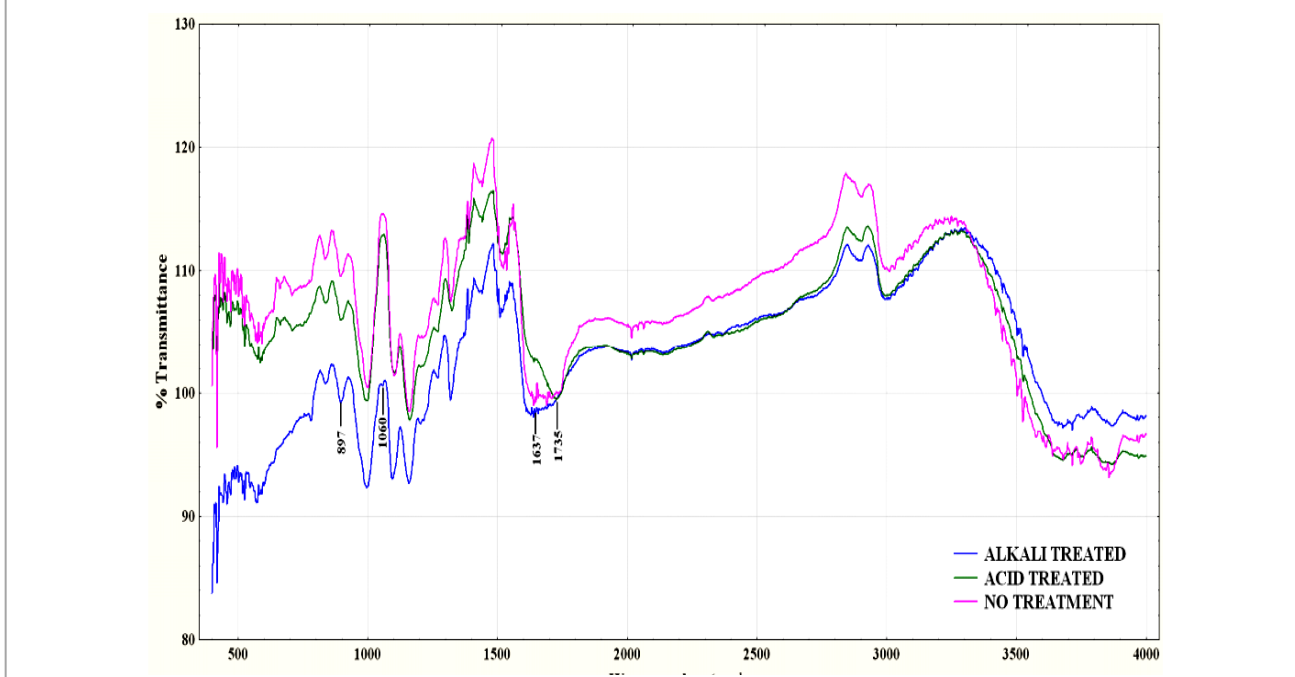
Fig. 1: Spores of *Pichia stipitis*



This suspension was then added to the sterilized hydrolysate obtained after pretreatment and saccharification process. From each flask sample were collected and estimated for production of ethanol at a regular time interval of 90:00 min. This process continued till there was steady production of ethanol.

Tollen's test (for xylose) (Bartos,1979) was performed for determination of different sugar contents and DNSA assay (for reducing sugar) (Miller, 1959). The dichromate assay (Archer et al, 2007) was performed for the determination of ethanol.

Fig. 2: FTIR spectra of untreated, acid treated and alkali treated biomass



Analytical Methods:

Estimation of total reducing sugar in the enzymatic hydrolysate of biomass was done by DNS method and the estimation of xylose by phloroglucinol method (Trinder, 1975).

RESULTS AND DISCUSSION

FTIR analysis:

FTIR spectra of the untreated and treated samples show structural changes in the biomass upon pretreatment and saccharification (Figure-2). Bands at 1000 to 1200 cm^{-1} were related to structural features of cellulose and hemicelluloses (Marimuthu et. al. 2012). It can be seen in Figure-1 that there are changes in the peaks in the given region, suggesting that there is increase in absorbance in these regions. The peak at 1735 cm^{-1} was observed due to either the acetyl and uronic ester linkage of carboxylic group or the ferulic and p-coumeric acids of lignin or/and hemicelluloses (Sun et. al. 2011). A sharp band at 896 cm^{-1} , corresponding to the C1 group frequency or ring frequency, is attributed to the β -glycosidic linkages (1 \rightarrow 4) between xylose units in hemicelluloses (Miller 1959) . These peaks in the alkali pretreated sample have the highest absorbance suggesting increase in cellulose and

hemicellulose content. The peak at 1637 cm^{-1} represents the adsorbed water and this peak was enhanced in the acid and alkali treated WHB. In the FTIR spectrum, the peaks observed at 1060 and 896 cm^{-1} were attributed to C–O stretching and C–H rocking vibration of cellulose structure. At these peaks, the alkali treated sample has the maximum absorbance, suggesting increase in the cellulose content.

SMS Result

The cell structure of water hyacinth after the pretreatment by 5% NaOH with different parametric variants (temperature 150°C,

Figure-3a: SEM pictures of partial degradation of lignin during pretreatment process (Dried Water Hyacinth)

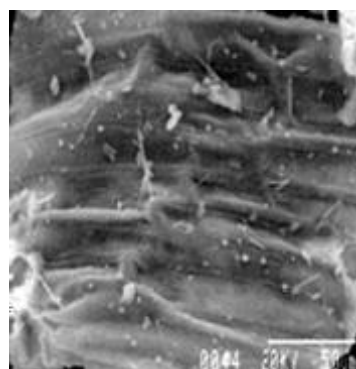
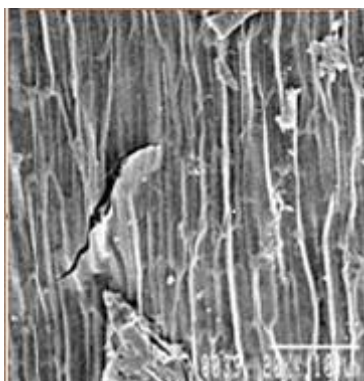


Figure-3b: SEM pictures of partial degradation of lignin during pretreatment process (150 Degrees, 5% NaOH)

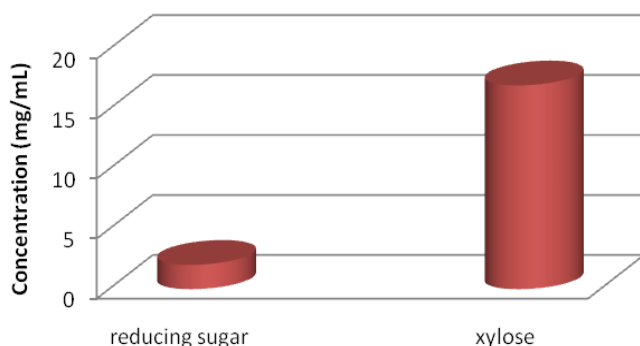


concentration of alkali 5%, treatment time 10min and soaking time 60min) was studied though analysis of photographs taken at various parametric conditions as shown in Figure-3a & 3b. The difference in the cell wall structure confirm the pronounced effect of pretreatment on the water hyacinth biomass.

Effect of Alkaline pretreatment on Water Hyacinth Biomass

Successful bioconversion of water hyacinth to xylose has been achieved by using alkali hydrolysis. Hydrolysis of water hyacinth by NaOH yields mixture of sugars with xylose as a major component as is shown in Figure-4 . The pretreatment achieved high reaction rates and significantly improved the hemicelluloses hydrolysis.

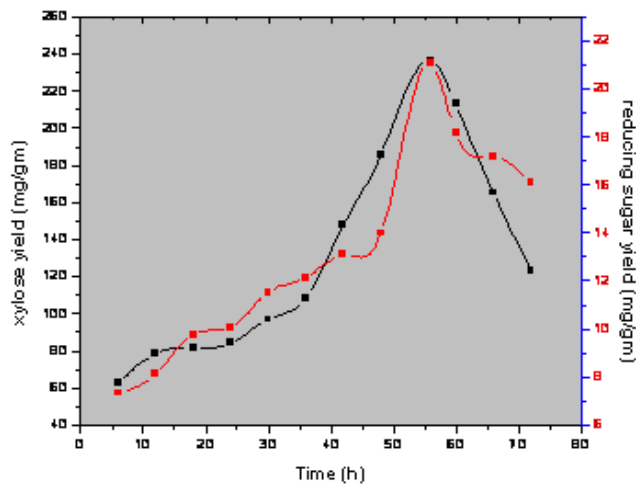
Figure- 4: Alkaline pretreatment



This is because pretreatment causes swelling, leading to decreased degree of polymerization and crystallinity, The pretreatment increased

internal surface area and disruption of the lignin structure, and separation of structural linkage between lignin and carbohydrates which exposed the hemicelluloses and cellulose embedded within it for enhancing the saccharification process.

Figure- 5: Biomass saccharification



The results were then analyzed for sugar yield for enzymatic saccharification using enzyme extracted from F13 fungal strain in combination with alkali treated water hyacinth biomass. When 1ml of crude enzyme extracted from F-13 strain was administered, the reducing sugar yield was observed to be **21.08mg/g** while xylose yield of **236.56 mg/g** using alkali treated WHB. This was because the crude enzyme extracted from strain F-13 has more xylanase activity than cellulase. It is evident from Fig.5, that the amount of sugar released increases with time which may be due to the increase in the production of extracellular enzymes (Hu et.al.2011). Thus, it substantiates that there is production of cellulolytic and xylanolytic enzymes which are responsible for saccharification of water hyacinth biomass. Also it was observed that xylose is much more than that of reducing sugars. It may be because of the fact the water hyacinth is richer in hemicelluloses content than that of cellulose content.

Enzyme activity results:

Enzyme activity is expressed in terms of IU/mL.(TABLE 1). *values were too less to be expressed as FPU/ml. Hence, one unit of enzyme

activity is one μ mole of glucose released per minute per ml of enzyme. Thus, it can be concluded that the fungal isolate F-13 produces more xylanase enzyme than cellulase.

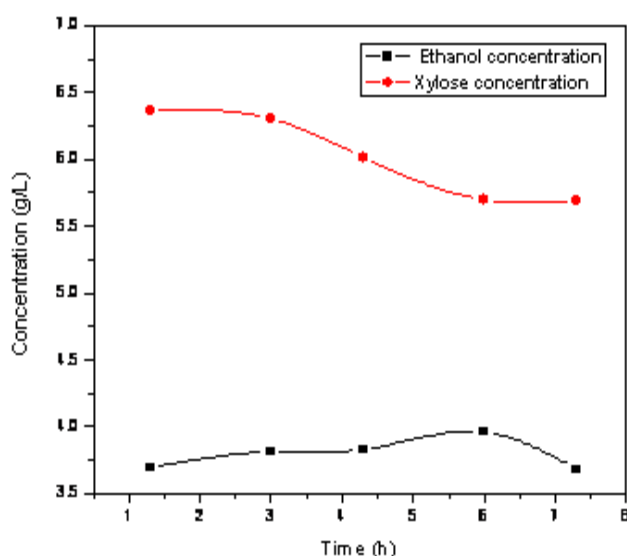
Table-1 Enzyme activity

Strain	Cellulase*	Xylanase
F13	2.21	6.39

Estimation of Ethanol:

The fermentation process was carried out with the saccharified hydrolysate of WHB that yielded highest amount of sugar. The hydrolysate was separately inoculated with *Pichia stipitis*. The process was carried out for seven and half hours. From Figure-6, it can be observed that maximum concentration of ethanol was obtained after 6 hours after which the increase was almost insignificant.

Figure-6: Ethanol and residual xylose concentration at different time intervals using *Pichia stipitis*



Evidently from the above graph, it can be concluded that *Pichia stipitis* gave the maximum yield of ethanol at 6th hour of the process with decrease in xylose concentration over time indicating the utilization of xylose by yeast for fermentation (Bothast et al., 1996; McMillan, 1996). The overall low ethanol production may have aroused due the fact that the pretreatment of lignocellulosic biomass might have produced degradation products with

an inhibitory effect on the fermentation process (Mussatto and Roberto, 2004).

CONCLUSION

This technique is effective in reducing the population of water hyacinth and value addition to the waste, using low cost method, which will be feasible for the developing countries. It was observed based on diverse analyses of sugar content that alkali pretreatment is mainly used for delignification of the water hyacinth biomass and solubilisation of reducing sugars during saccharification process. Therefore major part of the sugar can be utilized for saccharification. During enzymatic saccharification process, maximum yield of reducing sugar and xylose was found to be **21.08mg/g** and **236.56 mg/g** respectively. Also it was demonstrated that the hydrolysate of WHB can be fermented using *Pichia stipitis* which resulted in ethanol concentration of **3.913 g/L**. This was because it can degrade pentoses in addition to hexoses unlike others. Thus, the technologies employed in production of bioethanol from WHB, a noxious weed, is less technically intensive and may be operated by non-skilled workers making the strategy suitable for fuel ethanol production as an alternative biofuel in near future.

ACKNOWLEDGEMENT

The authors are grateful to Director, CSIR-Central Mechanical Engineering Research Institute, Durgapur for constant support, encouragement and permission to publish this paper.

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DOI:

<https://dx.doi.org/10.5281/zenodo.7205744>

Received: 5 April 2014;

Accepted: 21 May 2014;

Available online : 13 June 2014