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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(7): 216-220

www.thepharmajournal.com
Received: 23-05-2019
Accepted: 25-06-2019

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Evaluation of composition, characterization and enzymatic hydrolysis of pre-treated agricultural waste for production of bioethanol

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Abstract
Rapid increasing population and industrialization, the demand of energy is also increased. The different agricultural waste which are rich cellulose such as maize, sugarcane etc has been considered as major source of raw material for production of bio-ethanol. The production of bio-ethanol from agro-residues involves four processes of pre-treatment, enzymatic hydrolysis, fermentation and distillation. In present study sugarcane, corn and sorghum were selected as raw material for bioethnol production. The combination of acid and alkaline pretreatment method used to increase the concentrations of fermentable sugars after enzymatic hydrolysis which is followed by the simultaneous enzymatic saccharification and fermentation (SSF) process. The fermentation is carried out under influence of *Saccharomyces cerevisiae*. The result indicate after pretreatment selected biomass is rich cellulose and hemi cellulose, consider as promising material for ethanol production. Among them sugarcane showed highest production comparative to other.

Keywords: Ethanol, SSF, *Saccharomyces cerevisiae*, agro-waste, sugarcane, corn

1. Introduction
Due to rapid growth in population and industrialization, worldwide ethanol demand is increasing continuously. Biofules have been considered as an alternative solution to energy security, rising prices of petroleum based fuels and for reduction in greenhouse gas emissions hence it is one of the recreation drug and renewable product. Ethanol is a non-toxic and renewable form of energy obtained from fermentation of the carbohydrates, oxygen, sugar fractions of plant biomass material [1].

Several inventors has a paid the attention to the conversion of numerous substrates such as molasses, sorghum, corn, cassava, sugar cane, potato, cashew apple juice, fruit juice, pearl millet, rice, wheat etc. to fuel bioethanol [2]. Now a day's microorganism such as yeast, fungi and bacteria are playing a very important role in fermentation industries, pharmaceutical industry, and mining [3]. Although majority of the alcoholic fermentations are being carried out using yeast cells. The main steps of cellulosic ethanol production process are pretreatment, hydrolysis, fermentation and distillation [4].

Ethanol from biomass is produced by fermentation of carbohydrates present in the biomass. Though these carbohydrates are easily generated in corn or wheat, however the complex lignin–cellulose–hemicellulose matrix of biomass has first to be disintegrated, by employing a pretreatment step. Thereafter, the released carbohydrate polymers need to undergo hydrolysis step to yield fermentable sugars [5]. This hydrolysis step is catalyzed by enzymes like alpha amylase and glucoamylase. Thus, commercial production of bioethanol from lignocellulosic biomass requires large quantities of efficient cellulolytic enzymes. One of the major impediments for cost-effective bioethanol production from lignocellulosic material is the higher cost of the enzymes [6].

A cost-effective enzyme technology is essential for degrading polysaccharides into fermentable sugars for producing viable biofuels. Large-scale production of cellulases demands robust cellulase producing microorganisms with improved activity, volumetric productivity and better cellulose hydrolysis efficiencies, in addition to use of cheaper substrates for growth [7]. Considering this scenario, there is a constant search for better cellulolytic microorganisms capable of producing large quantities of cellulase and other components in balanced proportions for efficient saccharification of lignocellulosic biomass [8]. Filamentous fungi have been predominantly exploited for cellulolytic enzyme production

using submerged fermentation [9]. Filamentous fungi like *Penicillium*, *Aspergillus*, *Chrysosporium*, *Acremonium*, etc. have been intensified. These microorganisms utilizes low value substrates, could be a major step towards developing an economically feasible and sustainable process and could be a game changer in the current scenario of lignocellulosic biofuel industry [10].

In the present study *Aspergillus niger* used for the isolation of glucoamylase enzyme. Fermentable sugars i.e. glucose produced by hydrolysis of cellulose using cellulase enzyme can easily be fermented into ethanol using yeast. Simultaneous saccharification and fermentation (SSF) processes are highly adopted technologies for bioethanol production from lignocellulosic biomass. In SSF, hydrolysis and fermentation steps can be carried out sequentially and separately, at optimal conditions of pH and temperature. However, sugar accumulation in hydrolysis step inhibits the activity of cellulases [11].

2. Methods

2.1. Chemicals and Substrates

All the chemicals and reagents used in the study were of analytical grade and procured from standard manufactures. Raw materials used in the present work were sugarcane, sorghum, corn which acts as a substrate for the fermentation process. These were obtained from nearby area of Vijayapura city, Karnataka, India.

2.2. Pretreated material

The composition of substrate was estimated by calculating three parameters like Neutral detergent fibre (NDF) Acid Detergent Fibre (ADF) and Acid detergent Legnin (Goering and Van Soest 1975).

2.3 Acid pretreatment

About 50 g Chopped dried substrate were suspended in acid solution (1, 3, 5, 7 and 9% Sulfuric acid) in ratio of 1:10 (w/v) biomass and Sulfuric acid. The mixtures were autoclaved at 121°C for 15 min. After that, hydrolysate was pressed through cheese cloth and the amount of reducing sugar in juice was measured by using Luff schoorl method.

2.4 Alkali pre-treatment

About 50 g Chopped (≈2 cm length) dried materials were suspended in 1, 2, 3, 4, 5% NaOH in ratio of 1:10 (w/v) biomass and NaOH. After that the samples were incubated in water bath 85°C for 1 h. Finally, hydrolysate was pressed through cheese cloth. The amount of reducing sugar in juice was measured as above.

2.5 Scanning electron microscopy analysis

The surface morphological changes in native and enzymatic hydrolyzed pretreated material were analyzed by Scanning Electron Microscope (SEM) (JOEL – JSM 6390, Japan). Specimen to be coated were mounted on a conductive tape and coated with gold palladium using a JOEL JFC – 1600 auto fine coater and observed using a voltage 10 to 15 KV.

2.6 FTIR Analysis

Structural and functional group changes in extracted bioethanol from all three sample were subjected to FTIR analysis (Shimadzu Spectrometer, Japan). Sample (3-4% *w/w*) were thoroughly mixed with dry powdered spectroscopic grade KBr and the mixture was pressed with 10,000 psi into a transparent pellet. The spectra were obtained at 4 cm -1 resolution accumulating 25 scans per spectrum over the wave number range 4000 – 400 cm -1.

2.7 Isolation of Enzymes

Alpha amylase and gluco amylase which are required for liquifaction and sacchrification were isolated from potato and glucoamylase from *Aspergillus niger* which was procured from MTCC Chandigarh, India. Enzyme activities were determined by dinitrosalicylic acid (DNS) method (Miller1959).

2.8 Fermentation

2.8.1. Microorganism and growth conditions

Saccharomyces cerevisiae was used for fermentation assays. For inoculum preparation, cells were grown in 100 mL Erlenmeyer flasks containing 0.1 g yeast extract, 0.75 g glucose,0.05 g ammonium sulfate, 0.025 g monobasic potassium phosphate, and 0.0075 g magnesium sulphate heptahydrate, with a total volume of 25 mL. The medium was incubated 12 h at 35 °C in an orbital shaker (150 rpm) to obtain an active culture. Since lower inoculum size reduces cost of production in ethanol fermentation, experiments started with a low initial inoculum of 0.25 g.L−1 of yeast cells.

2.8.2. Simultaneous saccharification and fermentation (SSF)

Enzymes and yeast inoculum were incubated simultaneously since the beginning of experiments. Flasks were incubated in an orbital shaker (150 rpm) at 35 °C during all the reaction. Each material was run in triplicate.

2.9 Downstream processing/Recovery

After 5 days of fermentation period, the sample was filtered using Whatman Filter Paper to separate the ethanol from the residue. The fermentation broth is heated in a flask. Ethanol has a lower boiling point i.e. 70°C than water so it evaporates first. The ethanol vapour is then cooled and condensed inside the condenser to form a pure liquid. When all the ethanol has evaporated from the solution, the temperature rises and the water evaporates.

For calculation of ethanol yield the following equation was applied:

$$\text{Ethanol. Yield} = \frac{\text{Measured. Ethanol. in. Sample (g)}}{\text{Theoretical. Ethanol (g)}}$$

Theoretical ethanol (g) = Amount of initial sugar content (g) in fermentation solution × 0.5

All the measurements were duplicated and the data reported are average of two replications.

3. Results and discussion

3.1 Effect of acid pre-treatment methods on sugar content

Increasing the acid concentration showed reverse effect on sugar concentration in sample. This is maybe because of degradation of monomeric sugars (xylose, glucose) in furfural and hydroxymethyl furfural. The highest sugar up to 21.45% sugar (sugarcane) could be obtained using 1% sulfuric acid.

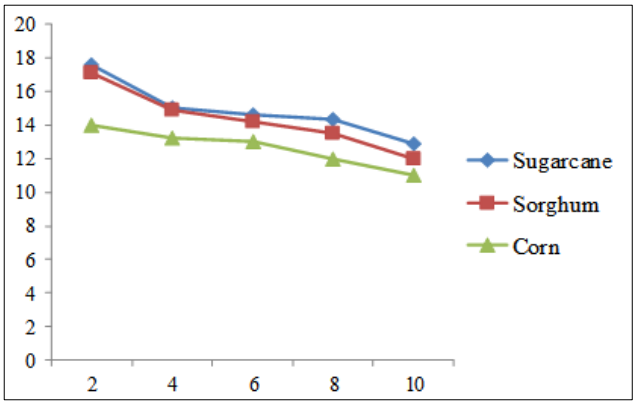

Fig 1: Sugar content in acid pre-treated substrates.

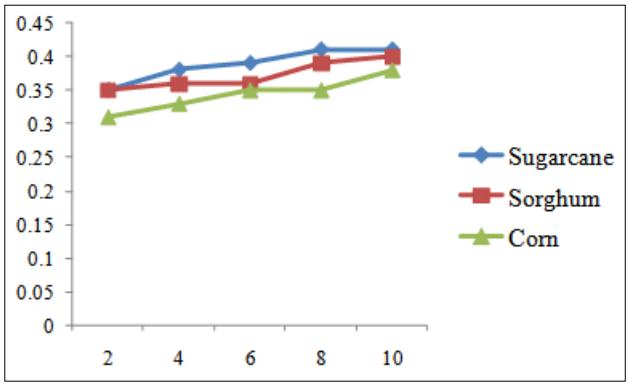

Fig 2: Sugar content in alkali pre-treated substrates.

3.2 Effect of alkali pre-treatment methods on sugar content

Higher concentration of alkali leads to slight increasing the sugar in sample. Generally, very low sugar content of ≤0.30 in case of corn and high in sugar.

The substrate were used in this study mainly contained cellulose, hemicellulose and lignin. The compositions of the bagasse after the pretreatments were analyzed and results are presented in (Table 1). Cellulose was the dominant component in all materials. The structural change in the substarte was followed by FTIR and SEM analysis.

Table 1: Chemical composition of untreated substrate.

Substrate	% Cellulose	% Hemi cellulose	% Lignin
Sugarcane bagasse	81	34	16
Sorghum	47	23	50
Corn	65	11	3

Images obtained after acid treatment by scanning electron microscopy on the surfaces of milled raw sugarcane bagasses revealed the morphological analysis of bagasse samples showed that the amount of pith was reduced considerably, which indicates that pith is less resistant to acid degradation than the fiber structures. The morphology of corn husk fiber shows a longitudinal section of corn husk fibers, it is possible to see three ribs on the adaxial surface (white asterisks) and a large number of microfibrils randomly distributed (white arrows). In case of sorghum the image revealed that the external and internal layers were removed during the pretreatment and also there was total removal of external surface and exposure of cellulose fibers.

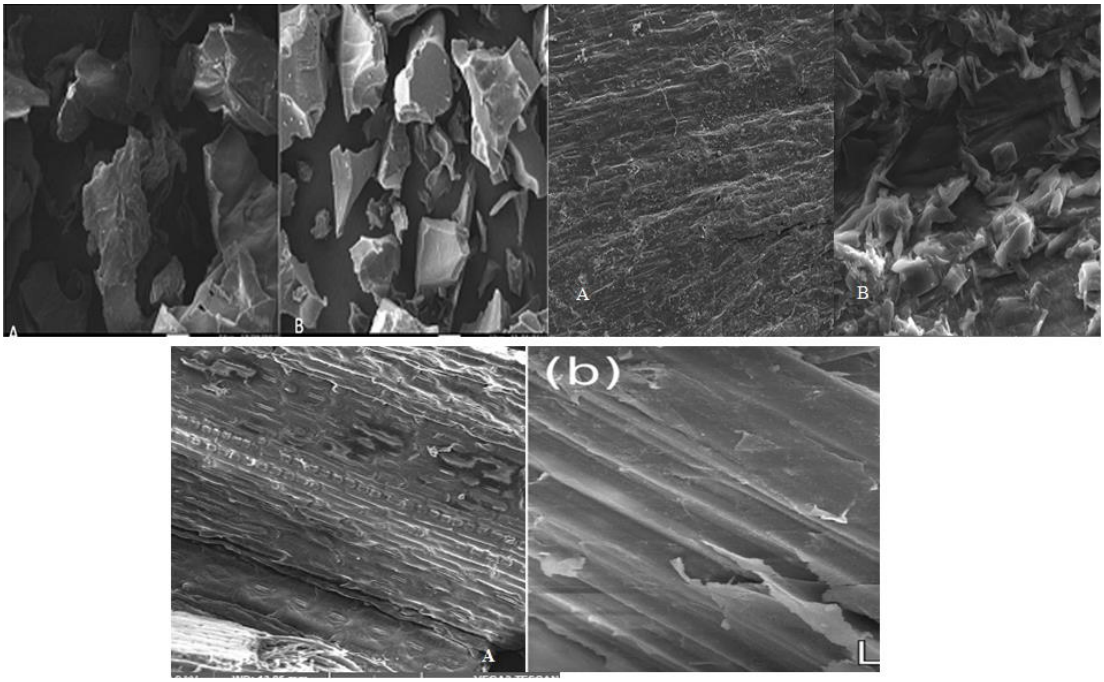

Fig 3: SEM analysis of untreated and pretrated 1) Sugarcane bagasess, 2) corn, 3) sorghum.

The FTIR analysis of selected substrate showed that in sugarcane bagasses, wave number 3333 cm-1 shows the presence of OH compound like Ethanol, Xylitol, Alcoholic Lignin, Sugars from Cellulose and Hemicellulose. While graph of sorghum showed that 3336 cm-1 and in corn 3401 represent presence of OH compound like ethanol, alcoholic lignin.

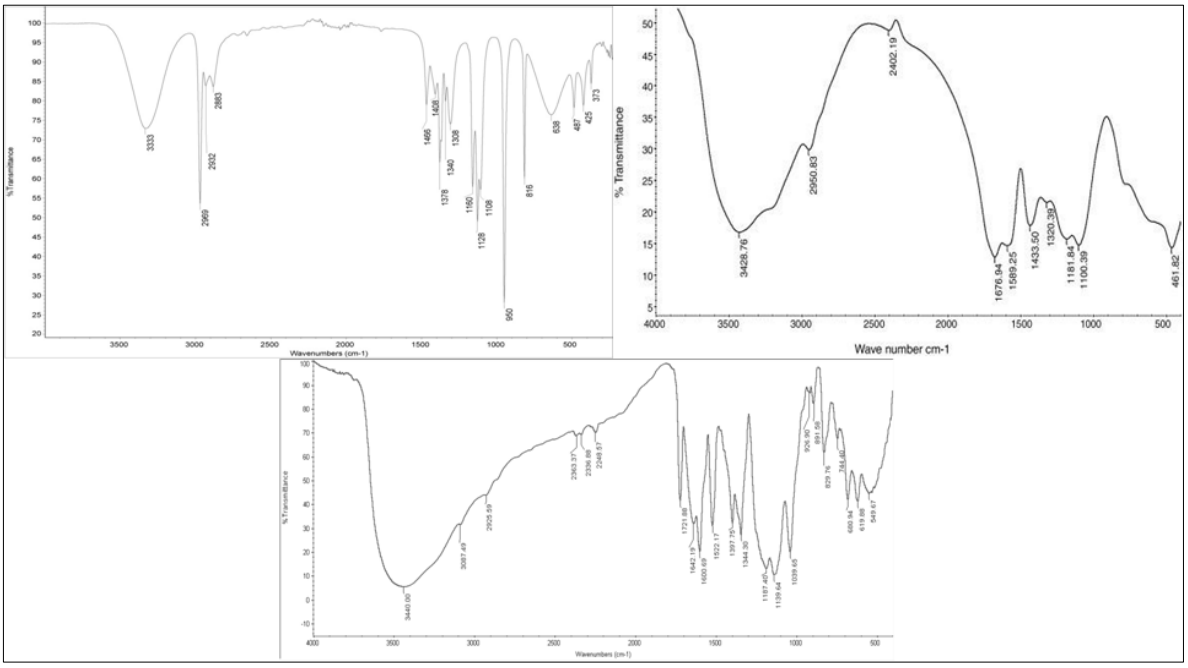

Fig 4: FTIR analysis of sugarcane bagasse, sorghum and corn respectively.

3.3 Enzymatic hydrolysis

Enzymatic hydrolysis of all three substarte were subjected to 72 h enzymatic hydrolysis by addition of cellulase and glucosidase. The results are presented as percentages of theoretical sugar yield in Table 2. The yield of hydrolysis of native bagasse was effectively improved after pretreatments. The sugar content in the hydrolyzates increased sharply in the first 12 h and gradually continued until 72 h. Hydrolysis of the bagasse, sorghum and corn resulted in 42%, 79% and 65% conversion after 12 h and 72 h, respectively.

Table 3: Percentage of Sugar after enzymatic hydrolysis

Substrate	Yield of enzymatic hydrolysis		
	12 hr	24 hr	72 hr
Sugarcane bagasse	42	44	65
Sorghum	35	40	57
Corn	32	37	51

3.4 Fermentation

Hydrolysate obtained by hydrolysis of pre-treated of substrates using in-house cellulase was supplemented with nutrients and was further employed for ethanol production by *S. cerevisiae* shows ethanol production and sugar consumption during SSF. The sugarcane bagasse showed higher percentage of ethanol (78%) production than corn.

Table 4: % of ethanol produced from three substrate.

Substrate	% ethanol on 5th day
Sugarcane	78
Sorghum	70
Corn	75

4. Conclusions

As the price of current ethanol feedstocks is estimated to increase, lignocellulosic materials is good option for ethanol production on the field or wasted otherwise which can be used as low value row material for ethanol production. Even though advances in ethanol production from agricultural waste over last few decades the price of the second generation ethanol is still high (Kumar *et al*., 2009a, b). Sugarcane bagasse, sorghum and corn were chosen as a substrate for the production of bioethanol. These are cheap in price and found abundantly in nature. It is rich in sugars, vitamins, and minerals. Culture medium composition using biomass could be suitable for yeast cell growth and the cost fermentation medium can also be reduced by replacing pure sugars like glucose, sucrose, maltose, fructose and xylose with in large scale industrial bioethanol productions. Initial optimization of physico-chemical and nutritional parameters using response surface methodology for the bioethanol production such as substrate concentration, pH, temperature, agitation, fermentation time, inoculum volume and inoculum age were found to have significant effect on bioethanol production. The present SSF process is rapid with statistically optimum fermentative conditions. Sugarcane biomass produce maximum bioethanol then sorghum and corn.

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