International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **7(12),** 8-14, December **(2018)** *Int. Res. J. Biological Sci.*

Bioethanol production from mild alkali pretreated sawdust and groundnut shells

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Available online at: www.isca.in, www.isca.me
Received 10th August 2018, revised 20th November 2018, accepted 5th December 2018

Abstract

Lignocellulosic materials like sawdust (SD) and groundnut shells (GS) are abundant renewable resources exploited for second generation bioethanol production. The present study investigated the production of bioethanol from mild alkaline pre-treated SD and GS. The SD and GS were pre-treated with 1% (w/v) NaOH followed by autoclaving at 121ºC for 45 min. The pre-treated biomass revealed 20% and 32% loss of lignin content, accompanied with a release of 2.97g/100 g and 2.47 g/100 g sugars in the pre-treated hydrolysate of SD and GS. The pre-treated biomass also revealed changes in physical characteristics such as 18.5% and 21.8% weight loss and change in colour intensity. The saccharification of pre-treated SD and GS by commercial cellulase (ex Aspergillus niger) resulted in maximum reducing sugar content of 15.64g/100g and 8.48g/100 g at 48h and 24h of incubation, respectively. The fermentation of hydrolysate (saccharified and pretreated) of SD and GS with Saccharomyces cerevisiae resulted in 5.48g/100g and 2.84g/100g bioethanol at 120h of incubation, respectively.

Keywords: Sawdust, groundnut shells, pre-treatment, saccharification, *saccharomyces cerevisiae,* bioethanol.

Introduction

The upsurge in energy demands and future supplies are the strategic agendas of almost every nation today[1]. The adverse socio-political and environmental impacts of fossil fuels as well as energy security concerns have spurred interest in nonpetroleum energy sources such as bioalcohols (methanol, ethanol, butanol) and biodiesel[2]. Among these alternative energy sources, bioethanol has garnered considerable interest because it is the only renewable primary energy resource that can provide alternative transportation fuels[3]. The Government of India launched the draft of the new policy that promotes the production of bio-ethanol from LCB with an aim of 20 percent ethanol blending in petrol by 2030[4]. The bioethanol can either be used solo with dedicated engines or blended with conventional gasoline requiring no engine modifications until the mix reaches 30%.

According to the type of feedstock, there are four generations of bioethanol i. first generation, where bioethanol is produced from ingredients of human food/animal feed (e.g., soybean, rice, corn, wheat, sugarcane, etc.); ii. second generation bioethanol from lignocellulosic biomass/agro-industrial residues (e.g., wheat straw, corn cobs, sugar cane bagasse etc.), iii. third generation bioethanol produced from aquatic biomass (such as cyanobacteria, macroalgae, and microalgae)[5] and iv. fourth generation, it is a modified form of third generation bioethanol i.e., in this algae are genetically modified. The Second-generation ethanol (lignocellulosic bioethanol) has the ability to use different types of lignocellulosic materials as a source of glucose such as grasses, agricultural residues (groundnut shells, wood chips, and sawdust), Municipal solid waste (paper, cardboards and wood)[6]. The bioethanol production from lignocellulosic biomass (LCB) has many distinctive advantages over fossil fuels like 2nd generation biofuels can be grown on land which has low agricultural value, having less impact on soil quality and low CO_2 emissions as the most prominent making it a clean-burning fuel[7].

The most integral step of bioethanol production, irrespective of the feedstock employed, is to disintegrate the recalcitrant meshwork of LCB (cellulose, hemicellulose and lignin) that helps to make cellulose more accessible to cellulolytic enzymes and generate fermentable sugars[8-10]. The pre-treatment involves various procedures like physico-chemical pre-treatment (uncatalyzed steam explosion, liquid hot water pre-treatment (LHW), mechanical comminution, and high energy radiation); chemical pre-treatment (catalyzed steam-explosion, acid, alkaline, ammonia fiber/freeze explosion, organosolv, pH-controlled liquid hot water, ionic liquids pre-treatments), and biological pre-treatment (wood degrading microorganisms, chiefly white-, brown-, soft-rot fungi, and bacteria). The key advantage of chemical pre-treatment using dilute acids (sulphuric, nitric acid) is the greater rate of solubilisation of hemicelluloses and lignin in acidic medium resulting in high glucose recovery[11]. Similarly, alkali pre-treatment disintegrates the structure of lignocellulosic biomass by dissolving hemicelluloses and lignin, by hydrolyzing uronic and acetic esters, which leads to cellulose swelling that aids in digestion of lignin matrix[12]. The present research work is focused at

establishing the fact that chemical pre-treated LCB can be used as a veritable resource for bioethanol production. The specific objectives of the work are to pre-treat sawdust (SD) and groundnut shells (GS) using dilute sodium hydroxide for enhanced saccharification and fermentation of hydrolysates to ethanol.

Materials and methods

Chemicals: The chemicals and reagents used were of analytical grade and purchased from HiMedia Pvt. Ltd., Mumbai, India. Freshly prepared distilled water was used for all the experiments.

Commercial Cellulase: This Cellulase extracted from *Aspergillus niger* (Meicellase) was purchased from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai.

Yeast Culture: The lyophilized culture of *Saccharomyces cerevisiae* (MTCC 173) was procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh.

Collection of sample: The Sawdust and Groundnut shells were collected from local market in Punjab.

Physical pre-treatment of SD and GS: The biomass (SD and GS) was washed extensively with tap water to remove dust and debris. The biomass was ground in a mixer-grinder followed by sieving through 15 mesh size (≈ 1000µ). The samples were sun dried and then dried in a hot air oven at 60ºC for 12 hours to remove the moisture content. The samples were packed in plastic bags until further analysis.

Chemical pre-treatment: The 15 mesh biomass (SD and GS) were soaked in 1% NaOH in the ratio of 1:10 (substrate: alkali) for 2hrs followed by steaming at 121ºC for 45 min[13,14]. After the steam pre-treatment, the contents of the flask were filtered with the help of double layered muslin cloth. The filtrate was stored in glass vials for the estimation of reducing sugars and fermentation. The pre-treated biomass was washed extensively with tap water to neutralize it and to make it free from the chemicals. The neutralized biomass was oven dried in a hot air oven at 60°C for 12h and was weighed to determine the weight loss. Later it was stored in clean poly bags for lignin determination and saccharification.

Saccharification of pre-treated biomass: The experiment was carried out in a 100mL sterile Erlenmeyer flask, with 2 g of pre-treated samples, added with Meicellase @10 FPU/g of substrate, 0.2% Tween 20, and 30µL Gentamycin (to control the growth of the microorganisms and to prevent the consumption of liberated sugars). The final volume of reaction mixture was made to 40mL with 0.1M sodium citrate buffer (pH 4.8). The flasks were incubated in a shaking water bath incubator at 50ºC (100rpm). The aliquots were periodically withdrawn after every 24 hours and centrifuged at 2000rpm for 10 min. The pellet was discarded and the clear supernatant was analyzed for reducing sugars.

Inoculum preparation for fermentation: The yeast culture, *Saccharomyces cerevisiae* (MTCC 173) was cultured in Glucose Yeast Extract (GYE) broth (pH 5) and incubated at 30ºC in a BOD incubator.

Fermentation of pre-treated and saccharified hydrolysates: The fermentation was carried out by inoculating the pretreated and saccharified hydrolysates with *S. cerevisiae* at 10% (v/v). The flasks were incubated at 28±2ºC in a BOD incubator and aliquots were periodically withdrawn after every 24h. The aliquots were centrifuged at 2000 rpm for 10 min and clear supernatants were used for the estimation of residual reducing sugars and ethanol.

Analytical methods: All the experiments were performed in triplicates and a control was also used along with each experiment.

Lignin determination: Klason lignin contents were determined using a modified version of National Renewable Energy Laboratory's (NREL) method[15]. For primary hydrolysis, pretreated samples (1g) were taken in beakers, and 8.57mL of 72% (v/v) sulfuric acid was subsequently added. The beakers were placed in the water bath incubator at 30°C and stirred every 3 to 5min for 1h. The samples were diluted to 42mL while minimizing Klason lignin losses. The secondary hydrolysis involved the autoclaving of diluted samples at 121°C for 45 min. The autoclaved samples were cooled to room temperature and final volume of the samples was made to 50mL. The samples were filtered through G2 Gooch crucibles and the solids obtained after filtration were heated at 100±2°C for 12 h. The crucibles were allowed to cool and then weighed to determine Klason lignin content by following formula:

$$\text{Lignin } \% = \frac{w2-w1}{W} \times 100$$

w_2 – stands for weight of crucible + sample; w_1 – stands for weight of empty crucible and W-weight of the sample.

Estimation of reducing sugars: The reducing sugars were estimated by DNS method as described by Miller[16]. A standard glucose curve was prepared using glucose from 100 to 500µg/mL with ascending 100µg intervals.

Estimation of filter paper activity: The Filter paper activity of commercial enzyme was determined by the method of Mandels et al.[17].

Estimation of ethanol: The ethanol content was estimated by the method of Caputi and Wright[18]. The percent ethanol was determined from the standard curve. A standard ethanol curve was prepared using analytical grade ethanol from 1% to 10% (v/v) with ascending 2% intervals.

Statistical analysis: All the experiments were carried out in triplicates. The results of the experiments were statistically analysed for standard deviation using MS Excel.

Results and discussion

SD and GS as substrates: Millions of tons of different agricultural wastes are produced annually across the country, but unfortunately a major fraction is burnt or left unattended, leading to environmental pollution[19]. However, in recent years, the wastes have been chemically or biologically treated to obtain useful products before the final disposal. The sawdust and groundnut shells, being rich in large quantities of cellulose that can be converted to fermentable sugars serve as a cheap substrate for bioethanol production. The chemical composition of SD and GS is presented in Table-1[20-24].

Table-1: Chemical composition of SD and GS

Substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)
SD	31–64	71–89	14–34
GS	22–37	12–36	16–36

Pre-treatment of SD and GS: Natural cellulose is a crystalline polymer generally associated with hemicellulose and lignin which is found to be highly resistant and disrupts the bioconversion of cellulose to fermentable sugars. Therefore, pre-treatment is a prerequisite to achieve the maximum yield of ethanol. The pre-treatment of lignocelluloses with alkali coupled with steam pre-treatment has been well reported in previous literature[13,14]. Therefore, the substrates (GS and SD) were pre-treated with 1% NaOH (w/v) immersed for 2h followed by autoclaving at 15psi for 45min. The pre-treatment resulted in lignin reduction of 24% and 32% in SD and GS, respectively (Figure-1). Dai et al.[25] reported reduction in lignin content to 5.1–11.8% from 12.7% in NaOH/Urea treated rice straw. In previous studies the delignification of lignocelluloses have been reported which are similar or even more than the percent delignification in the present study. Kim et al.[26] reasoned that high percentage of lignin removal was not essential for effective conversion of lignocellulosic biomass to fermentable sugars.

The pre-treated biomass samples revealed changes in physical characteristics, like a change of colour in pretreated biomass to dark brown from light brown and yellow colour for SD and GS respectively (Figure-2a and-2b) and change in colour of hydrolysate to deep brown colour. The colour changes can be attributed to solubilisation of lignin or they may also be the result of degradation of sugars, which are transformed to brownish colour at high temperature[27]. A weight loss of 18.5% in SD and 21.8% in GS was also observed after pre-treatment. The weight loss observed following chemical pre-treatment is generally due to lignin removal[28].

Pre-treatment usually results in a considerable mass loss of plant biomass components, depending on the type of pre-treatment method, the applied experimental conditions, and the type of biomass used for conversion[29-32]. Sahare et al.[28] found that there was a weight loss (21%) when corn cobs were pre-treated under alkaline conditions (1% NaOH at 50°C).

Saccharification of pre-treated SD and GS: Enzymatic hydrolysis of NaOH pre-treated SD and GS was carried out for depolymerisation of carbohydrate fraction into fermentable sugars using commercial cellulase (Meicellase) ex. from *Aspergillus niger*. The saccharification of pre-treated SD and GS resulted in maximum reducing sugars of 15.64 g/100 g at 48h and 8.48g/100 at 24 h of incubation, respectively (Figure-3).

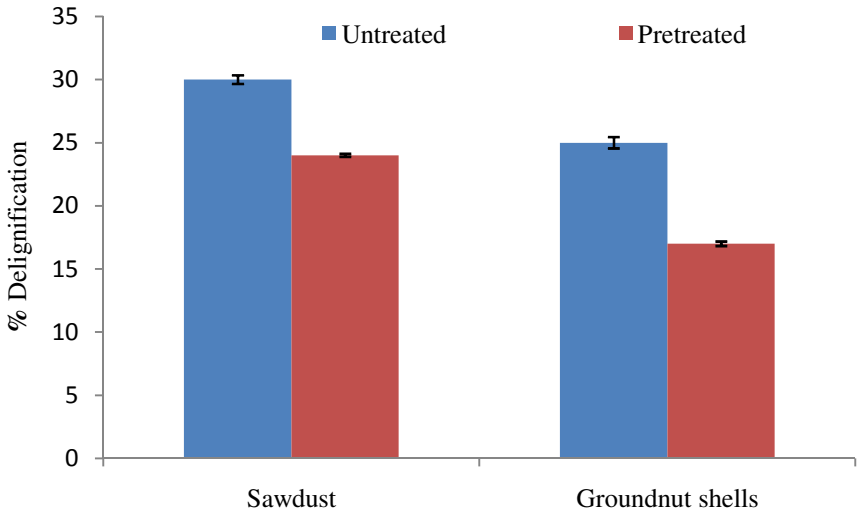
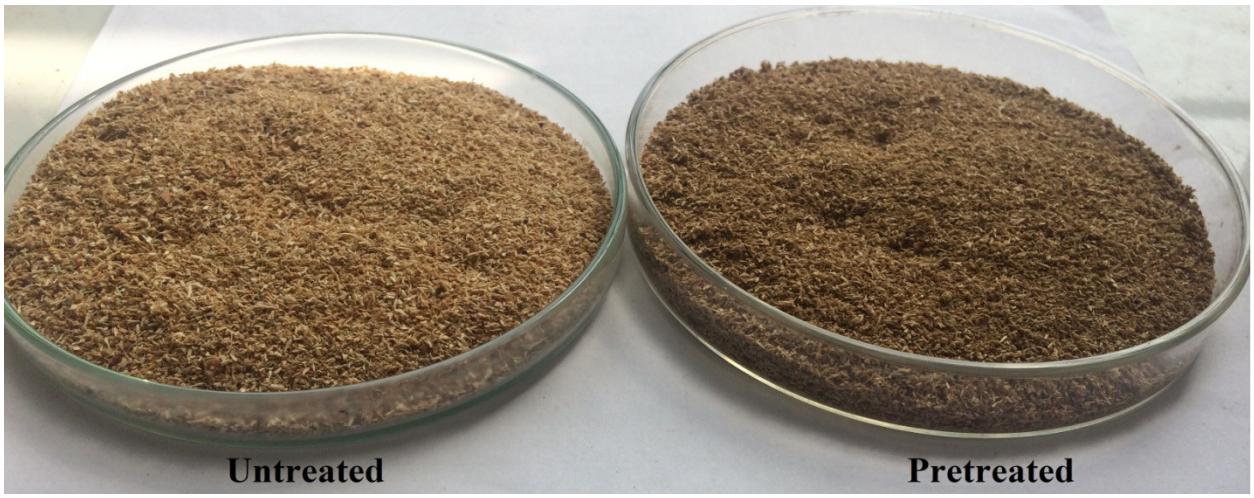
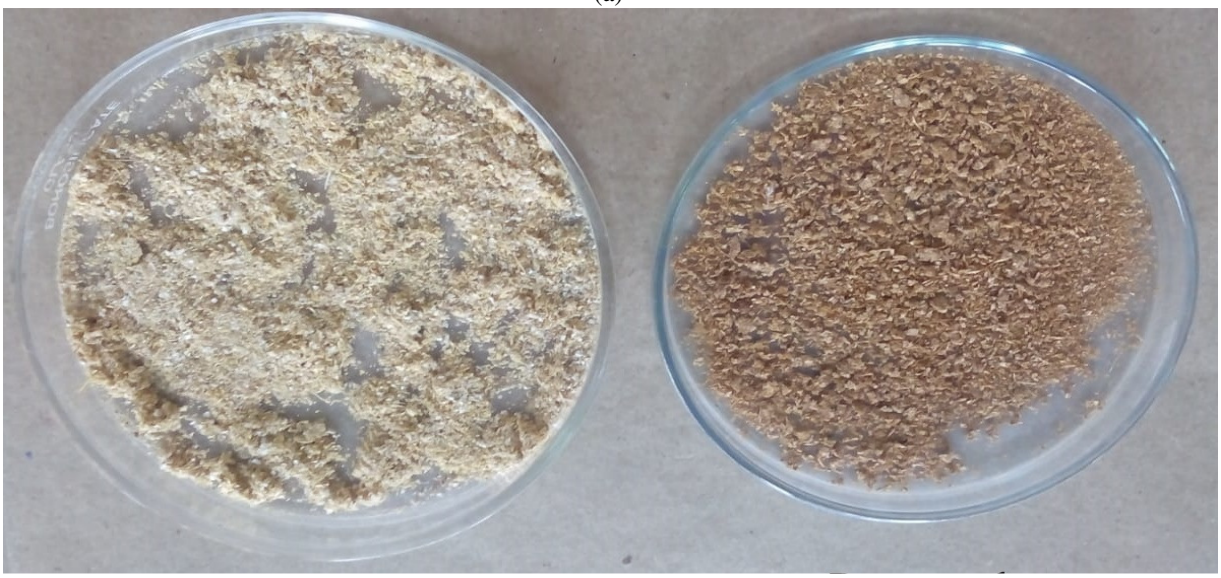


Figure-1: Percent delignification of pretreated SD and GS.



(a)



(b)

Figure-2: Pre-treated biomass samples.

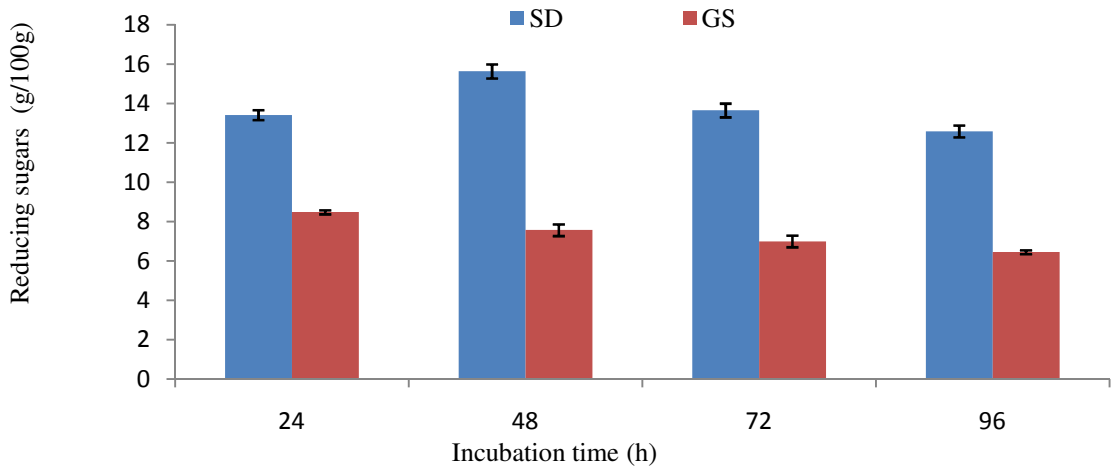


Figure-3: Reducing sugars yield at different incubation periods.

After which the concentration of sugars started to decline off which could be explained on the basis of accumulation of glucose and cellobiose during hydrolysis that reduces the efficiency of cellulase enzyme[33]. Trevorah et al.[34] reported, highest glucose yield of 16.2±1.4%, produced from 10% NaOH pretreated sawdust for 0.5hr at 60°C with a cellulase (Accellerase) dosage of 9.1 FPU/g_{TS} and 325±63 pNP G U/g cellulose after 70 hr of saccharification. Gajula et al.[35] reported that 670 mg/g of sugars were released upon the enzymatic hydrolysis of the dry pretreated substrate of GS [15% (w/v) sodium sulfite and autoclaved at 121°C for 15 min] using commercial enzymes (Dyadic® Xylanase PLUS (Cellulase 45000–55000 U/g, β-glucanase 12000–15000 U/g and xylanase 34000 – 41000 U/g)) 25 FPU/g at 50°C (100rpm and pH 5.0).

Fermentation of pre-treated and saccharified hydrolysates: The fermentation of enzymatic and pre-treated hydrolysates was carried out with 10% (v/v) *S. cerevisiae*. The fermentation of enzymatic hydrolysates of SD and GS resulted in maximum ethanol concentration of 5.47g/100 g and 2.8g/100 g with residual reducing sugar concentration of 2.10 g/100 g and 1.7 g/100 g at 120 h of incubation, respectively. After 120 h of incubation, no noticeable change in the concentration of ethanol was observed. Similarly, the pre-treated hydrolysate of SD resulted in maximum ethanol concentration of 0.01 g/100 g and residual reducing sugars of 0.83g/100 g at 24h of incubation. Whereas, inappreciable amount of ethanol (>0.01) in the hydrolysate of pre-treated GS was produced. The final ethanol production from SD and GS was observed to be 5.48g/100 g and 2.8 g/100 g, respectively with fermentation efficiency (79%). It was observed that there was a sharp decrease in residual reducing sugars during first 24h of incubation, followed by a gradual decrease in reducing sugars and a gradual increase in ethanol production until 120 h of incubation (Figure-4). The increase in ethanol production and decrease in the amount of reducing sugars is due to the fact that, during fermentation *S. cerevisiae* utilized the hexoses as a source of carbon and energy, as a result of which ethanol is produced[36]. Nyachaka et al.[14] reported ethanol yield to be 6.2 mL and 7.9 mL on the first and the seventh day from 420 g of substrate inoculated with 15 mL and 4 mL of *S. cerevesiae* incubated at 30°C and 300 rpm. Shide et al.[37] reported 6.6 mg/mL ethanol production from 0.1 M HCl pre-treated wood sawdust.

Various types of lignocellulosic substrates have been used for bioethanol production with different pre-treatment methods. Table-2 summarizes the reducing sugars yield and amount of ethanol produced from lignocellulosic feedstocks[38-43].

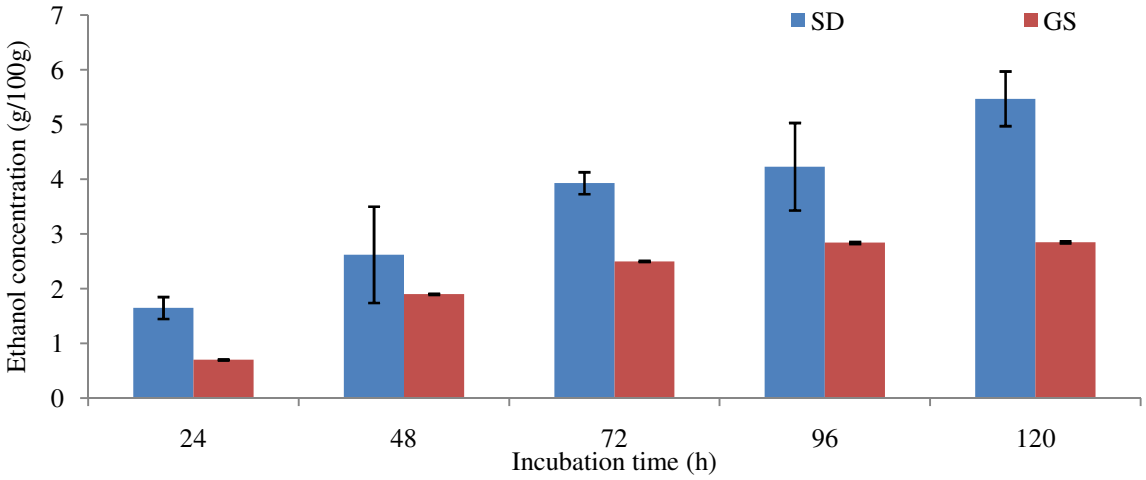


Figure-4: Ethanol production at different incubation periods.

Table-2: Reducing sugar yield and ethanol production from varied types of lignocelluloses.

Lignocellulosic biomass	Pre-treatment	Reducing sugars	Ethanol concentration
Sawdust	10.90% (v/v) HNO_3	99.2 g/100 g	17.1g/L
Sawdust	1% H_2O_2	347.20 mg/g	12.73g/L
Sugarcane bagasse	2%(v/v) H_2SO_4 steam pre-treatment at121ºC for 2hr	8600 µg	11.90 g/L
Peanut shells	Diethyl ether (1:3) substrate: chemical	18 mg/100mL	4.5%
Waste Paper	1.5% (v/v) H_2SO_4 steam pre-treatment at 121°C for 45 min.	0.14 g/g	6.84%
Banana peels	1N NaOH	72%	31.8%

Conclusion

Ethanol production from agricultural waste material is widely explored alternative nowadays. Lignocellulosic biomass is gaining popularity as it is easily available and cheap source of fermentable sugars for biofuel production. In the present study, an attempt was made to use lignocellulosic wastes particularly, SD and GS for bioethanol production. The alkali/ steam pre-treatment of the SD and GS resulted in an ethanol concentration of 5.48 g/100 g and 2.8 g/100 g from SD and GS, respectively with fermentation efficiency of 79%. The results of present study are promising considering mild concentration of alkali used for pre-treatment. Though the lab and pilot scale studies have been reported successful for bioethanol production, still there is a huge gap exists between the projected and actual bioethanol production at industrial scale.

The two main problems are reported to be responsible for the commercialization of this technology which are as follows i. low yield of ethanol ii. presence of inhibitors in hydrolysates. Various optimization techniques can be used such as over liming, solvent and membrane extractions and adsorption with activated charcoal to potentially reduce inhibitors and to increase ethanol titer. Another strategy for optimization of production process in low cost manner is immobilization of yeast cells to cheap supporting material. Reduction in cost of saccharfication enzyme can be achieved by using on-site/in-house enzyme preparations instead of commercially available enzymes. Fermentation of pentose and hexose sugars released from the lignocellulosic feedstock need adapted microorganisms like *Zymomonas mobilis* as it can utilize both C_5 and C_6 sugars and pentose fermenting yeast such as *Pachysolen tannophilus* using co-fermentation technology. Hence, further research needs to be done in all the stages of the process to increase the overall efficiency of production and decrease the costs.

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