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A. Aman ullah[1*], S. Noreen[1], R.R. Zohra[1,2], S.K. Kazmi[1]

[1]Jinnah University for Women, Karachi, Pakistan
[2]University of Karachi, Karachi, Pakistan
*e-mail: ayisha.aman@gmail.com
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Improved production of bioethanol from alkali-pretreated lignocellulosic feedstock using *Saccharomyces cerevisiae* under simultaneous saccharification and fermentation

Abstract. The most plentiful bioresource on earth is lignocellulosic biomass. The breakdown of lignocellulosic biomass attains different fermentable sugars which are considered to be very significant for the manufacturing of biogas, bioethanol etc. The major composition of lignocellulosic biomass is cellulose, lignin and hemicelluloses which are strongly linked with each other by hydrogen and covalent linkages thus generating a tough and rigid structure. The recalcitrant structure of lignin resists to solubilization thus arresting the degradation of cellulose and hemicelluloses present in bioplymeric structure. This addresses a striking challenge for the production of biofuels. To cope up with these limitations, immense research has been carried out for the development of pretreatment strategies. The present study utilized the alkaline pretreatment approach to digest cellulose, hemicelluloses and lignin content in locally available different agroindustrial wastes. This study employed Simultaneous Saccharification and Fermentation (SSF) process for bioethanol production from selected lignocellulosic biomass. Prior to fermentation all collected biomass was treated with 1% NaOH and then fermentation was carried out using *Saccharomyces cerevisiae.* The SSF was conducted at 30°C for 4-5 days. Along with pretreated biomass, untreated biomass was also subjected to SSF in order to compare the yield of bioethanol. The results of present research elaborate that pretreatment with 1% NaOH significantly degraded the complex polymeric structure of biomass hence induced enhanced production of bioethanol as compared to untreated agrowaste. Among all lignocellulosic biomass, pretreated banana peels attained higher production of bioethanol 118g/L with fermentation efficiency 139.75% and sugar utilization 97.9% than untreated banana peels achieved maximum ethanol production 42.4g/L with fermentation efficiency 53% and sugar utilization 68%. These findings indicate that before fermentation, a suitable pretreatment protocol must be applied to lignocellulosic substrates in order to increase the yield of ethanol and to meet the commercial needs.
Key words: Bioethanol, alkali preatreatment, lignocellulosic biomass, *Saccharomyces cerevisiae,* simultaneous saccharification and fermentation.

Introduction

Pakistan, being an agriculturally-developed country, releases huge quantity of agricultural waste from agriculture practices and food processing indusries. Approximately 56.22 million tons agricultural debris per annum including 19.83million tons from wheat, 12.87 million tons from sugar-cane, 12.46 million tons from cotton, 8.16 million tons from rice and 2.90 million tons from maize are produced. Agricultural waste and by-products have been employed abundantly to produce industrially important products like fuel-grade bioethanol [1]. The major sources of energy utilization are rapid industrialization and quick expansion in world's population; however energy requirement is increasing constantly. The feedstock of first generation biofuels such as wheat, corn, maize potato, sugarcane etc can be utilized for bioethanol production but due to food security problems their use is limited. Thus, second generation biofuels are extensively produced worldwide using non edible lignocellulosic feestock [2]. Utilization of lignocellulosic biomass like sugarcane baggase, corn husk, wheat and rice straw, cotton stalk, peels of vegetables and fruits for the production of biofuels is attractive concern of fuels industries nowadays.

The enhanced production of ethanol becomes necessary due to increased requirement for multiple commercial purposes such as industrial solvents,

alternate energy sources, preservatives, cleansers etc. Moreover, production of ethanol by microbial fermentation suggests a more promising balanced trade and improved security of energy. As compared to gasoline, ethanol is less toxic to animals and humans. Ethanol is appropriate fuel as it declines the risk of smog formation due to its less volatility, photochemical reactivity and least production of ignition products [3].

Presently, production of various categories of biofuels like bioethanol, biohydrogen, biodiesel and biogas methane) is achieved using lignicellulosic substrates rather than food crops. Every year, the agrowastes are released abundantly in environment without proper disposal which eventually contribute pollution. An alternate strategy is to consume agricultural wastes in eco friendly manner that minimizes the human dilemma of "food vs fuel "encountered with the feed stocks of 1st generation biofules [4,5]. Furthermore, this approach can decrease the production cost and regulate a healthy ecosystem by reducing the emission of green house gases (GHG) that ultimately alleviate global warming [6].

It is well recognized that lignocellulosic materials have the potential to be used in the production of bioethanol. However, the utilization of lignocelluloses may face some challenges. The major difficulty is to convert complex polymers in to fermentable reducing sugar as lignocelluloses possess rigid crystalline conformation, the matrix of cellulose and lignin is occupied by hemicellulose molecules that limit the availability to hydrolytic enzymes. To overcome these challenges, a pretreatment of lignocellulosic biomass is recommended. The pretreatment is necessary step that leads to the substantial degradation of lignocellulosic compact structure, delignification and hydrolysis of the hemicellulosic components [7,5]. The pretreatment process can be accomplished either by chemical, physical, physiochemical or biological (enzymatic hydrolysis) treatment [8,9]. In order to attain maximum efficiency, these pretreatment methods can be combined [10]. The enzymatic accessibility towards cellulolytic fractions can be improved by employing efficient pretreatment plan. The goal of chemical pretreatment is to eliminate lignin and hemicellulose content in order to enhance the hydrolysis of cellulose molecules. Among chemical pre treatments, alkali treatment is commonly employed as it solubilizes lignin. It catalyzes the ester and glycosidic side groups present in lignin structure leading to swelling of cellulose with reduced crystallinity. This treatment also allowed the solubilization of hemicellulose [11-14]. Calcium, sodium, potassium and ammonium hydroxide are used in alkaline treatment. However, sodium hydroxide is mostly utilized and has found to be most effective in the catalysis of lignocellulosic biomass [15,16]

Production of bioethanol via separate saccharification and fermentation processes is associated with various problems such as (i) increased levels of reducing sugars may arrest the growth of yeast cells, (ii) two bioreactors are needed for separate hydrolysis and fermentation, (iii) the production process becomes costly as it requires two separate fermentors along with raw materials. So the solution of these limitations is simultaneous saccharification and fermentation (SSF) which allows the conversion of reducing sugars formed from saccharification in to ethanol in the presence of yeast simultaneously [5].

The aim of current research is to utilize alkali pre treated agroindustrial wastes for improved production of bioethanol in cost effective manner by employing simultaneous saccharification and fermentation (SSF) strategy. This study also compares the production of ethanol of alkali-pretreated and untreated lignocellulosic substrates.

Materials and methods

Chemicals and Reagents. Cellulose, DNS, Potassium dichromate, sulphuric acid, magnesium sulphate, Dipotassium hydrogen phosphate

Microorganisms. Microroganisms used in this study include *Saccharomyces cerevisiae* (Baker's yeast) and locally isolated *Bacillus* specie as a source of cellulolytic enzymes. The yeast culture was maintained on potato dextrose agar (PDA). Further, the both microbial cultures were preserved in agar slants and kept at 4°C.

Collection of lignocellulosic agrowaste. Total nine different agroindustrial substrates were locally collected and subjected to simultaneous saccharification and fermentation (SSF). The substrates were peels and husk of the following fruits: *Mangifera indica* (mango peels), *Musa* (Banana peels), *Saccharum officinarum* (sugarcane baggase), *Solanum tuberosum* (potato peels), *Zee mays* (Corn husk), *Malus domestica* (apple peels), *Carica papaya (Papaya peels), Cocos nucifera* (coconut husk), *Manilkara zapota (*sapodilla peels).

Processing of raw substrates. The collected agrowastes were washed thoroughly with water in order to remove dust residues, followed by drying at room temperature. After wards, dried biomass was

ground in electric grinder to obtain fine powder.

Alkali-pretreatment of feed stock. Fine powder of each substrate (12.5 gm) was allowed to soak in 1% NaOH (100 ml) for 60 minutes. Then the each flask was autoclaved at 121 °C for 1 hour. Thereafter, the alkali- heat treated sample was filtered and solid particles rinsed with distilled water till pH reaches to neutrality. Solid residues were kept at 70°C for 24 hours for drying.

Simultaneous saccharification and fermentation (SSF). For SSF, 8 gm of each substrate was soaked in 100ml nutrient solution containing $MgSO_4$ (0.5g L^{-1}) and K_2HPO_4 (1.0g L^{-1}) and sterilized at 121°C for 15 minutes at 15 psi. After autoclaving, each flask with different agro –substrates was provided with 1.0 ml cellulose enzyme followed by inoculation with 1% yeast cells and incubated at 30°C for 4-5 days under static conditions. Control experiment was also run in which glucose was used as a sole source of carbon substrate. The medium of control experiment constituted glucose 80g L^{-1}, $(NH_4)_2.SO_4$ 4.0 g L^{-1}, $MgSO_4.\ 7H_2O$ (1.0g L^{-1}) and $KH_2\ PO_4$ (2.0g L^{-1}).

Extraction of cell free fluid. After incubation, each flask was filtered in order to harvest fermented liquid from biomass. For the separation of cell free fluid (CFF) from fermented broth, each sample was allowed to centrifuge at 10,000 rpm for 15 minutes at 1°C. Then CFF was subjected to ethanol and enzyme estimation.

Analytical Procedures. Ethanol estimation was done by taking 1 ml cell free fluid in test tube in which 9 ml distilled water and 1ml potassium dichromate was added. The mixture was boiled for 10 minutes that led to color change from orange to green. This suggests that during heating, ethanol is oxidized in the presence of acidified oxidizing agent (potassium dichromate in diluted sulphuric acid) leading to the reduction of oxidizing agent to green colored chromium (III) sulphate. The optical density of resulting mixture was observed at 600 nm. The cellulase enzyme assay was also carried out to measure total enzyme units produced during fermentation. 100 µl enzyme extract was reacted with 100 µl substrate (1% cellulose). Then reaction mixture was kept in incubation at 50°C for 15 minutes. Afterwards, 3 ml Dinitrosalicylic Acid Reagent was added and solution was boiled for 10 minutes. The final colored product was analyzed spectrophotometrically at 545 nm. Glucose was used as a standard.

Ethanol yield and fermentation efficiency. Following theoretical equations were used to calculate sugar utilization, concentration of ethanol produced, ethanol yield and productivity and fermentation efficiency.

$$\text{Sugar Utilization (\%)} = \frac{\text{amount of original sugar content} - \text{amount of residual sugar content}}{\text{amount of original sugar content}} \times 100$$

$$\text{Ethanol Yield } \left({}^{g}/_{g}\right) = \frac{\text{Maximum Ethanol Production}}{\text{Substrate Utilized}}$$

$$\text{Ethanol Productivity } \left({}^{g}/_{L.\,h}\right) = \frac{\text{Maximum Ethanol Concentration}}{\text{Fermentation Time}}$$

$$\text{Fermentation Efficiency (\%)} = \frac{\text{Experimental Ethanol Recovery (g/L)}}{\text{Theoretical Ethanol Recovery (g/L)}} \times 100$$

Results and discussion

The bioconversion of agroindustrial waste in to ethanol requires pretreatment. Pretreatment is crucial tool for conversion process of lignocellulosic material. Without pretreatment, the complex lignocellulosic biomass cannot be degraded by enzymes alone, as lignin content in plant material possess resistant against enzymatic cleavage [17]. However, prior to saccharification pretreatment is critical step in order to deplete lignin content and enhance the surface area of lignocellulosic substrate for better enzyme action. Along with this property, the efficacy of pretreatment and improvement in hydrolysis process has been associated with the elimination of lignin and hemicellulose and the alleviation of cellulose crystallinity [18].

In this study, nine different agroindustal substrates (peels and husks) were collected locally and subjected to alkaline treatment in order to partially hydrolyze cellulosic and hemicellulosic content in agrowastes. This study focuses on the effect of pretreatment of

lignocellulosic biomass on percent yield of ethanol. However, the simultaneous saccharification and fermentation were conducted for ethanol production using both untreated and alkali-treated biomass. Additionally, a standard experiment was also conducted in which soluble glucose was added as a sole source of carbon to compare the productivity of ethanol with the treated biomass. The fermentation process was carried out at 30°C for 96 hours. The pretreated biomass was allowed for enzymatic hydrolysis by cellulase, extracted from indigenously isolated cellulolytic *Bacillus* specie. Furthermore, 1% innoculum of *Saccharomyces cerevisiae* was added in media along with cellulase enzyme to facilitate the conversion of free sugar residues in to ethanol. It was noticed that pretreatment with alkali (sodium hydroxide) significantly delignified the complex polysaccharides in lignocellulosic biomass, hence enhanced the production of ethanol if compare with the untreated samples (Figure 1 and 2).

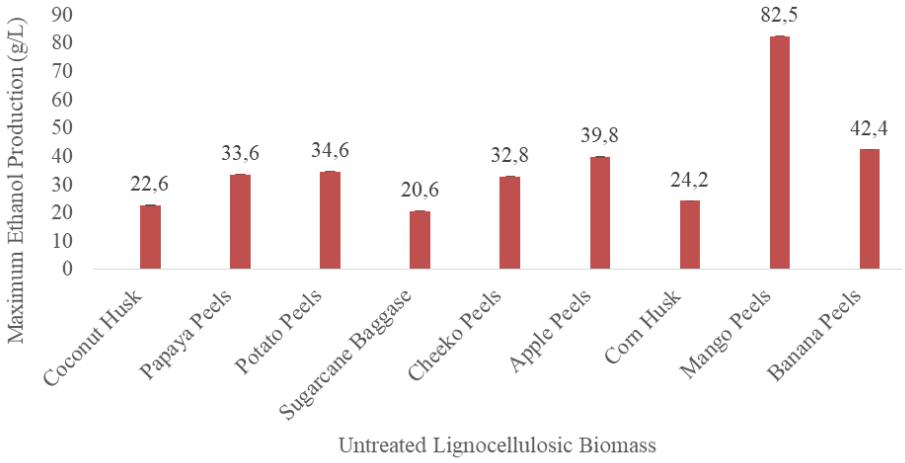

Figure 1 – Ethanol production by untreated lignocellulosic material

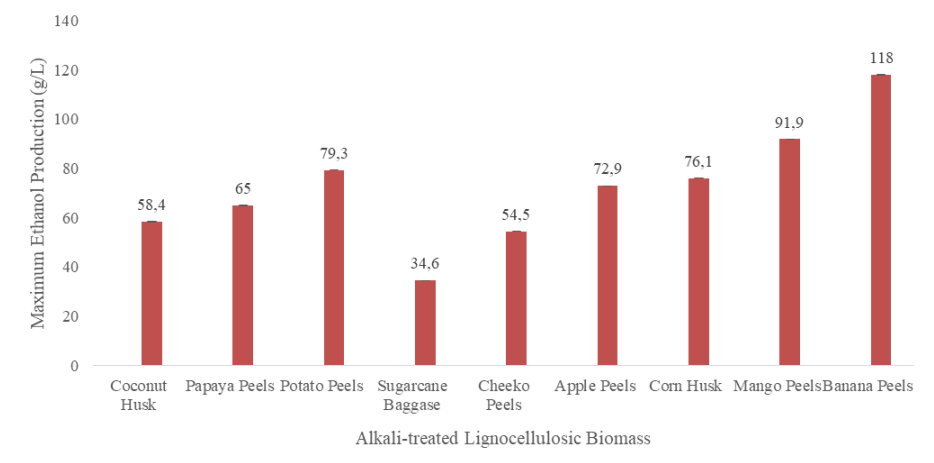

Figure 2 – Ethanol production by alkali-treated lignocellulosic material

Among all pretreated substrates, banana peels gave maximum ethanol production which was 118g/L with fermentation efficiency 139.75% and sugar utilization 97.9%. Although untreated banana peels also provoked notable ethanol production but alkali-pretreatment further improved the production of ethanol. Several previous studies have been reported that indicate the numerous strategies of pretreatment and importance of pretreatment of agro substrate prior to fermentation. Many reports also suggest

that banana waste is excellent substrate for ethanol production. ChongKhong and Doromae [19] used 2M NaOH for the pre-treatment of banana waste that produced hydrolyzed product with 4.29g/L glucose content. Improved production of ethanol (26.84 g/L) was recorded when pre-treated kinnow and banana peels (25g) were utilized as substrate [20]. According to one study, alkali treatment causes saponification reaction that improves the interaction of enzyme towards its substrate [21]. Alkaline treated (0.5-2.0 M NaOH, 121 °C, 60 minutes) empty palm fruit bunch fibre induced the delignification with ethanol yield 21 g/L with in 28 h [22]. Other researches indicated the combined pre-treatment processes for efficient yields. Production of ethanol was improved by employing two-step process of alkali and hydrothermal pretreatments of wheat straw that effectively enhanced enzymatic saccharification [23].

Similarly, alkali treatment with 1% NaOH of rice straw greatly reduced the lignin and hemicelluloses content [24]. Optimization of alkaline pretreatment conditions of sugarcane bagasse and filter mud enhanced biomethanation with 86.27% delignification and 82.20% methane production in the presence of 1% NaOH [25]. Likewise, pretreatment with sodium hydroxide has been proved an effective to improve anaerobic digestion for enhanced yield of methane from pretreated vinegar residue (VR) with 3% NaOH if compare with untreated VR [26]. Studies on alkali pretreatment with calcium hydroxide (lime) have also been carried out and showed effectiveness in the degradation of complex cellulosic components in agricultural wastes. Shah and Tabassum [27] used CaOH pretreatment on corn cob for efficient production of biogas. The lime pretreatment provoked the digestion of lignin leading to 2 times higher yield of biogass than untreated corn cob residue. The investigation on the effects of lime pretreatment on date palm leaves under non aerated and aerated conditions was conducted. It was revealed that oxidative treatment (at 40◦C with 0.2 g g−1 lime) stimulated more digestion of lignin as compared to non oxidative condition [28]. Sakuragi et al. (2018) [29] used ammonia based alkaline treatment on six various species of hardwood due to non- toxic, non-corrosive nature and convenient recovery of residual ammonia.

In present research pretreated mango peels were observed to be a good inducer of ethanol after banana peels as they induced maximum ethanol concentration 91.9g/L with fermentation efficiency 114.8% and sugar utilization 92%. The ethanol production from alkali treated (1% NaOH) feedstock was recorded as follows, Banana peels> Mango peels> Potato peels> Corn husk> Apple peels> Papaya peels> Coconut husk> Cheeko peels> Sugarcane baggase (Table 1).

The results of standard experiment highlight that soluble glucose in media produced 41.6g/L ethanol with fermentation efficiency 81.25% and sugar utilization 77.5% (Table 2)

Table 1– Effect of alkali-treated lignocellulosic substrates on bioethanol production and fermentation efficiency from *Saccharomyces cerevisiae*

Substrate Used	Alkali- Treated Substrates				
	Sugar Utilization (%)	Maximum Ethanol Production g/L	Ethanol Yield g/g	Ethanol Productivity g/L.hr	Fermentation Efficiency (%)
Coconut Husk	77.6	58.4	0.467	0.486	73
Papaya Peels	77.6	65	0.52	0.541	81.25
Potato Peels	74.4	79.3	0.634	0.660	99.12
Sugarcane Baggase	84	34.6	0.276	0.288	43.25
Cheeko Peels	92	54.5	0.436	0.454	68.1
Apple Peels	92	72.9	0.583	0.607	91.12
Corn Husk	82.4	76.1	0.608	0.634	95.12
Mango Peels	92	91.9	0.73	0.76	114.8
Banana Peels	97.9	118	0.894	0.931	139.75

Table 2 – Effect of soluble glucose on bioethanol production and fermentation efficiency from *Saccharomyces cerevisiae*

	Sugar Utilization (%)	Maximum Ethanol Production g/L	Ethanol Yield g/g	Ethanol Productivity g/L.hr	Fermentation Efficiency (%)
Standard (medium with glucose)	77.5	41.6	0.52	0.34	81.25

Therefore, on the basis of these outcomes, it is suggested that pre-treated agrowaste like banana, mango and potato peels are better choices for carbon substrate than soluble glucose. The consumption of soluble glucose along with other content of media makes the fermentation process costly. However, the utilization of agroindustrial waste does not only make the production process economical but also manages the environmental wastes in eco friendly manner.

Although untreated biomass produced considerable amount of ethanol (Table 3) but pre-treatment with alkali induced efficient and enhanced production of ethanol.

Table 3 – Effect of untreated lignocellulosic substrates on bioethanol production and fermentation efficiency from *Saccharomyces cerevisiae*

Substrate Used	Untreated Substrates				
	Sugar Utilization (%)	Maximum Ethanol Production g/L	Ethanol Yield g/g	Ethanol Productivity g/L.hr	Fermentation Efficiency (%)
Coconut Husk	77.6	22.6	0.180	0.188	28.25
Papaya Peels	84	33.6	0.268	0.28	42
Potato Peels	71.2	34.6	0.276	0.288	43.25
Sugarcane Baggase	76	20.6	0.164	0.171	25.75
Cheeko Peels	80	32.8	0.262	0.273	41
Apple Peels	72.8	39.8	0.318	0.331	49.75
Corn Husk	80.8	24.2	0.193	0.201	30.25
Mango Peels	69.6	82.5	0.66	0.68	103.1
Banana Peels	68	42.4	0.339	0.353	53

The selection of an appropriate pretreatment process is crucial step as it is dependent upon the amount of hemicelluloses, lignin and cellulosic components present in the biomass. Therefore, before yeast fermentation pretreatment is necessary to hydrolyze lignin and hemicellulose content. Pretreatment should improve the amount of cellulose in agro industrial waste. Conventional chemical pretreatment processes like acidic, alkaline, or sequential alkaline-acidic treatments, combined with high pressure or temperature have been reported in different researches. Pretreatment of lignecellulosic biomass with acid is effective in decreasing hemicelluloses content. Similarly, various researches have indicated alkaline pretreatment as simple and easy process for the digestion of lignin in agrowastes in mild conditions with least sugar hydrolysis and no formation of inhibitory substances [30]. By alkali treatment, the complex structure of substrate becomes less rigid hence it becomes more attainable for enzyme action that liberates more reducing sugars. The consequences of present research are in favor of all highlighted previous studies as in this work 1% sodium hydroxide greatly delignified the selected lignocellulosic wastes hence enhanced the production of bioethanol.

Conclusion

Currently, biofuels are in great favor as various environmental challenges are associated with fossil fuels. The attention of fuel industry has been shifted towards the manufacturing of biofuels using 2nd generation biofuel raw material which is lignocellulosic biomass. Utilization of agrowaste for bioethanol production encourages environmental safety and cost effective production process. In order to fulfill the industrial demand, different strategies are required that can produce ethanol in sufficient quantities with low cost. However, consumption of agroindustrial wastes for biofuel production makes the production process cheap plus proper pretreatment of lignocellulosic biomass can produce ethanol in bulk amount. The findings of this study prove that pretreatment of agrowaste is essential step before fermentation in order to get higher amount of biofuel. Moreover, this research recommends the usage of agroindustrial wastes which are cheap and easily accessible raw material, as all of them has great potential to produce bioethanol and among them banana peels induced maximum ethanol production. Prior to industrial production of biofuels, optimization studies for ethanol production are needed.

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