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# CHEMICAL CHARACTERIZATION OF BIOMASS FOR THE PRODUCTION OF HIGHVALUE, ENVIRONMENTALLY FRIENDLY BIO FUEL

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## ABSTRACT

Second generation cellulosic biofuels offers a solution to reduce carbon emissions of traffic as well as generation of energy for domestic and commercial uses. A study was conducted to developa approach for the management of agriculture as well as other organic wastes utilization for production of alcoholic fuels like bioethanol and biobutanol. Therefore cellulosic materials like wheat , cotton and rice straws, corn stover , fruit wastes and cogon grass were used in this study Biological and chemical pretreatments were compared for each substrates. Efficiency of bacterial enzymes for saccharification of agricultural substrates was evaluated. It is concluded that these bacterial enzymes have the potential to hydrolyze not only pure substrates but can also degrade agricultural wastes. It is expected that outcome of this study will help to increase production of biofuels and to reduce burden of foreigner exchange that is currently being utilize to import fossil fuel from other countries.

**Key words**; Bioethanol, Biomass, Green house gases, Climatic changes

## Introduction

Exploration of sources for alternate energy have been increasedbecause of increasing concerns about energy security and climate change. The transportation sector plays a significant role for emission of greenhouse gases due to uses of fossil fuels, However, replacement of oil derived fuels such as ethanol could reduce environmental impacts and give advantages on social as well as economical levels (Humbird et al,2011). Various alternatives to generate sustainable biofuels are being investigated. Biological energy resources arelike bioelectricity, biogases, biodiesel and bioalcohols. Among these sources, bioalcohol shows a great potential to reduce the emission of

greenhouse gases, decrease the dependence on fossil fuel and act as a chemical feedstock and fuel for transport (Dhamole et al., 2015).The production of bioethanol has been improved extremely because many countries are trying to reduce the import of oil, improving the quality of air and growingrural economics. The global ethanol production is 51,000 million liters(Renewable Fuels Association, 2007). Ethyl alcohol has some advantages as a fuel as it has higher oxygen contents. The higher oxygen level permits improved oxidation of hydrocarbons with successive reduction in aromatic compounds and carbon monoxide emission. Ethanol has greater octane rating properties (Thomas and Kwong, 2001).

Biomass is a vital energy resource in Pakistan because ofagricultural based country. The biomass produced in livestock and agriculture sector in the form of animal waste and crop remaining as sugarcane bagasse and rice husk (Amiri et al. 2014; Chaudhry *et al*., 2009). Second generation biomass is mainly composed of lignocellulosic material. Lignocellulosic biomass is more plentiful organic substance on earth and consists of cellulose (35-50%), hemicellulose (20-35%) and lignin (5-30%)(Huber et al., 2006).Various renewable energy resources include different agricultural substances like green leaves, fruit shells, straws, nut shells and fruit seeds (Demirbas, 2001). Most commonly used feedstocks are wheat straw, wheat bran, corn stover, corn steep liquor and apple pomace (Ejezi*et al*., 2006).Now a day, agricultural waste is used for the production of biofuels like biodiesel, bioethanol, biohydrogen and methane as compared to energy crops because they have competition with food crops. As huge amount of agrowaste is available and have discarding problem so, alternate option is the utilization of lignocellulosic biomass in order to reduce the competition between fuel and food (Mahro and Timm, 2007). The grasses are considered as reliable substance for extraction of

ethanol. The utilization of perennial grasses is advantageous and possibly it further decreases the cost for the production of ethanol and its use as fuel (Gomez et al., 2008).

The Cogon grass (*Imperatacylindrica*) can be grown all over the yearworldwide, particularly in subtropical as well as tropical countries. Cogon grass has been exploited to rise the soil stability and as fodder, it is recognized as worst weed and it is known as pest by almost 73 countries in all over 35 crops. The roots of cogon grass have secondary metabolites which have medical importance. It is known as perennial grass and could be cultivated in any soil which usually considered as unfit for production of crops. The cogon grass could be utilized as a raw material for renewable source of energy (Lin and Lee, 2011).The *Cynodondactylon* (Coastal Bermuda grass) is perennial grass that has the higher cellulosic content and can also be used for ethanol production. The excellent raw material for yield of ethanol is coastal Bermuda grass as it is either sold at a very cheap price or is wasted in most of the cases. Comparison of corn and Bermuda grass has shown that most potential source for production of ethanol is Bermuda grass because of higher contents of biomass and conversion of whole carbohydrates into bioethanol. Bermuda grass is predominantly present in tropical and subtropical part of the world. It reaches 1-30 cm in height and have deep roots up to 2m into the ground, however various roots

penetrated less than 60 cm under-ground( Sun and Cheng, 2005).Even though some species of Bermuda grass can grow up to 15-20 cm, others may reach a height of greater than 1m long. Bermuda grass can naturally grow in many continents such as North Africa, southern Europe, Asia and Australia (Sluiteret al., 2008).

In the present study various agrowaste samples such as wheat, cotton and rice straws as well as corn stover, cogon grass and peels of fruit wastes were selected as these are main organic wastes in Pakistan. Other than wheat and rice the thirdvital cereal is corn. Its production is 4.695 million tons annually and grown in 1130thousand hector. The second major crop is cotton and it is cultivated annually. The thirdimportant crop is rice and it is cultivated at 2891 thousand hector and it is produced 7005thousand tons annually (PES, 2014-15). Various studies reported that 2.7 million tonswaste such as rice straw, rice husk, canola straw, wheat straw, cotton stalks, cotton bagasse and sugarcane remains are cultivatedin Sanghar which is known as agricultural area in Sindh region of Pakistan. Almost 75-85 percent of these feedstocks are not utilized are burnt away. So, these materials could be used for production of energy and having noeffect on food or other domestic resources (UNEP, 2011).Maize (*Zea mays*) is the best of the cereal stovers and very abundant livestock feed. It can be grazed off otherwise, it is mostly burned in fields in many areas of Pakistan before next crop to sow and all parts of maize are usable for different purposes (Kim and Dale, 2004). Termite gut has one of the highest microbe densities on earth.

Termites depend on the microbes in their gut or digestive tract to digest the complex sugars in wood into simpler molecules . Cellulose is a major sugar in wood , it is broken down by bacteria available in gut of termite and finally converted into various products including fatty acids and alcohol like ethanol etc.( Kim and Haltzapple.2005).*Clostridium* **,**genus of rod shaped gram positive bacteria member of which found in soil, water and intestinal tract. *Clostridium acetobutylicum* ferments sugar to a mixture of organic solvents like acetone , butanol and ethanol.***Saccharomyces cerevisiae*** ( known as baker s years) single celled eukaryotes which is frequently used in fermentation process for production ethanol and other alcoholic products.

## MATERIAL AND METHODS

### Collection of Agricultural Substrates

Various samples of wheat and rice straws as well as, peel wastes were collected from various areas . About 1 Kg samples of each samples were collected in fine plastic bags. The samples were shad followed by sun and oven dried for overnight at 55 °C. The samples were converted into fine powder form by electric grinder and passed through 40 mesh standard size sieve. The powder form of samples were saved in fine plastic bags duly labeled with the name and were stored in refrigerator at 4°C till further uses.

### Proximate Analysis of Samples

All samples were analyzed for ash contents, volatile matter, crude protein, crude fiber, crude fat and wet as well as dry weight (AOAC,1990). The standard methods were used for the estimation of total solids and moisture contents by drying at 105 ºC to remove moisture from the samples (Sluiter, 2005).

### Chemical analysis of raw biomass

The cellulose content of sample was estimated by using reported method. The hemicellulose was determined by computing ADF (Acid Detergent Fiber) and NDF (Neutral Detergent Fiber) differences .The lignin contents were determined by standard method as reported by AOAC (1990).

### Analytical procedures

The fermentation products like monomer sugars ( hexoses and pentoses ) acetone-butanol and ethanol as well as their bio

products were determined by using reported method (Haifeng et al., 2015).

**Figure 1. Biomass**

**Chemical Pretreatment**

For chemical pretreatment two chemicals were used such as acid ($H_2SO_4$) and alkali (NaOH). Pretreatment experiment was performed by using $H_2SO_4$ and NaOH (1.0, 1.5 and 2%) at diverse temperatures such as 100 °C , 110 °C and 120 °C for different times durations (15, 30, and 45 minutes). Solid sample ( 10 %) (w/v) in reagent bottle was utilized during experiment. After pretreatment, the vacuum filtration assembly was used for filtration of sample in eachbottle and the contents were emptied on filterpaper. After filtration, the solid washedaway with 300 ml distilled water in order to neutralize the pH. The filter paper was than dried at 105 °C and weighed.

**Enzymatic Hydrolysis**

The biomass after pretreatment 5% (w/v) was hydrolyzed with cellulose and β-glucosidases at 50 °C and 160 rpm for 72 hours in a water bath shaker with 0.05 M buffer (sodium citrate) at 4.8 pH. Cellulases having activity of (30FPU g-1). The samples were withdrawn from reagent bottle after every 12 hours to determine the concentration of sugar. After enzymatic hydrolysis, $H_2SO_4$ (µl) was added. Un-hydrolyzed sample was separated by centrifuging for 10 minutes at 13,500g. Supernatant was collected by means of syringe filters for sugar analysis by dinitrosalicylic acid (DNS) method .The amount of sugar was analyzed by p-hydroxybenzoic acid hydrazide (PAHBAH) method. By using the concentration 1Mm-

25mM of xylose the standard curve was drawn. Then by comparing the standard sugar concentration, the amount of sugar in pretreated sample was determined. The best pretreatment condition was selected after enzymatic hydrolysis process. The sample containing higher amount of released sugar was further selected for fermentation process. The solid biomass was stored at 4 °C which was then used for fermentation process .

*Saccharomyces cerevisiae* strain was maintained on YPD (yeast extract 1% (w/v), peptone 2% (w/v) and glucose 2% (w/v)]agar medium at 4°C. Culturing of yeast cellswas carried out in a 5-mL tube of YPD medium containing NaCl 0.9% (w/v) at 30°Cfor 16 h on a rotary shaker (100 r.p.m.) according to (Alfenoro, 2002)

**Separate Hydrolysis and Fermentation**

Fermentation experiment was carried out by using *C. thermocellum*grown in glucose yeast extract broth medium for 48 hours and 10% inoculum was inoculated into 50 mL fermentation medium containing previously saccharified solution and kept for 3 days at room temperature (Jiang et al., 2015).Fermentation experiment was performed at $50^0$C and 120 rpm for 72 hours under anaerobic conditions. After completion of fermentation reaction, the obtained mixture contains methanol, butanol, ethanol and acetone were removed by fractional distillation process in a fractional distillation apparatus on the basis of boiling point. As butanol has higher boiling point (118 $^0$C) than water (100 $^0$C) Butanol can be condensed then separated. The boiling point of ethanol is lower (78.3 $^0$C) in comparison with water that's why it can be condensed earlier than water (Kathleem et al., 2018).

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**Statistical analysis**

Data generated through various analysis were statically analyzed for mean, standard deviation etc.,

## Results and Discussion

Results regarding isolation of bacteria , chemical analysis of biomass samples as well as fermentation of sugars into acetone-butanol- ethanol are given in the following

sections.Termites are considered as good source of various useful bacteria isolates those have industrial applications . These isolates are found to have good potential for conversion of various sugars into alcoholic products . Therefore in current study acetone-butanol - ethanol ( ABE ) were produced from organic wastes material of agriculture and municipal sources by using termite based bacterial isolates (Figures 2-3 ).

**Biological Pretreatment**

Results displayed in table 3 indicates amount of sugar released by different bacteria isolates. It was observed that bacterial isolates 9x (xylanase enzyme )has provided higher amount of sugar (27.84±0.48mM/l ) from wheat straw ( Table 3), which was higher than all other substrates analyzed.

**Table1.Chemical pretreatment of biomass samples with different concentrations (%) of NaOH and $H_2SO_4$,to release of sugars ( %).**

| Substrates | Chemicals | | | | | |
|---|---|---|---|---|---|---|
| | $H_2SO_4$ concentration | | | NaOH concentration | | |
| | 1% | 2% | 3% | 1% | 2% | 3% |
| Wheat straw | 15.38± 1.24 | 19.74±1. 25 | 6.38±0. 86 | 14.71 ±0.46 | 15.95±0.08 | 16.85±0.15 |
| Corn stover | 14.57± 0.18 | 13.73±1. 12 | 4.27±0. 81 | 13.69±0. 46 | 13.65±0.08 | 13.82±0.14 |
| Cotton stalk | 1.53±0 .04 | 1.35±0.0 5 | 0.28±0. 02 | 0.86±0.0 6 | 0.87±0.11 | 0.97 ±0.01 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Rice straw** | 16.85± 0.15 | 15.38±0. 17 | 3.44±0. 15 | 15.07±0. 17 | 14.32±0.28 | 13.39±0.59 |

**Pretreatment of biomass samples**

| **Bacterial Isolates** | **Corn stover** | | | | **Wheat straw** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Acetate** | **Formate** | **Lactate** | **Ethanol** | **Acetate** | **Formate** | **Lactate** | **Ethanol** |
| **Isolate 9x** | 1.15±0.06 | _ | 1.41±0.18 | 5.73±0.28 | 3.04±0.65 | _ | 1.65±0.79 | 3.34±0.41 |
| **Isolate 10** | 1.28±0.14 | _ | 3.44±0.34 | 6.98±0.58 | 1.55±0.28 | 1.24±0.17 | 6.14±0.55 | 5.99±0.26 |
| **Isolate 31** | 1.29±0.34 | 1.98±0.39 | 8.57±0.59 | 9.21±0.54 | 1.72±0.07 | 1.63±0.28 | 3.58 ±0.26 | 6.43±0.49 |

**END PRODUCT ANALYSIS**

**Simultaneous Sccharification and Fermentation**

It was observed that there are variation for growth of different isolates on differentsubstrates that might be due to availability of amount sugars and other similar byproducts

**Table 2. Various fermentation products (mM/l) obtained from biomass samples**

**Various Fermentation products**

**Biomass analysis**

Data in table 6 represents various parameter found in biomass samples. Whereas ligno-cellulosic contents of the samples are given in table 7. It was observed that Cogon grass has higher cellulosic contents as compared to other substrates used for analysis.

**Table 3; Proximate analysis ( %) of biomass samples**

| Substrate | Dry Matter | Moisture | Crude Protein | Crude Ash | Crude Fiber |
|---|---|---|---|---|---|
| Peel(wastes) | 92.41±0.48 | 7.53±0.34 | 7.93 ±0.23 | 5.91± 0.45 | 33.87±0.33 |
| Cogon grass | 93.11±0.27 | 6.89±0.26 | 5.12 ±0.21 | 9.18±0.34 | 35.41±0.42 |

Analysis of organic wastes samples

**Table 4. Chemical analysis (% )of biomass samples**

| Substrate | NDF | ADF | Hemicellulose | Cellulose | Lignin |
|---|---|---|---|---|---|
| Peel(Fruit wastes ) | 79.6±0.51 | 52.1±0.31 | 26.3±0.34 | 29.6±0.67 | 21.5±0.43 |
| Cogon grass | 82.06±0.72 | 48.41±0.42 | 29.6±0.52 | 34.2±0.83 | 15.32±0.25 |

Mean ± standard deviation (n=3) NDF = Neutral Detergent Fiber and ADF=Acid Detergent Fiber

**Table 5; Recovery of solid mass (% ) due to treatment with Acid under various conditions.**

| Pretreatment conditions. | | Total solid recovery (g/100g dry biomass) | |
|---|---|---|---|
| Time (min.) | $H_2SO_4$Concentration | Peel ( wastes) | Cogon grass |
| 15 | 1.0 | 63.52±0.20 | 76.86±0.61 |

| | | | |
|---|---|---|---|
| | 1.5 | 62.02±0.13 | 74.24±0.43 |
| | 2.0 | 57.11±0.34 | 70.33±0.25 |
| 30 | 1.0 | 56.61±0.43 | 68.86±0.48 |
| | 1.5 | 56.07±0.22 | 67.10±0.35 |
| | 2.0 | 52.37±0.20 | 64.76±0.24 |
| 45 | 1.0 | 53.07±0.32 | 64.62±0.42 |
| | 1.5 | 52.11±0.51 | 64.02±0.36 |
| | 2.0 | 51.23±0.44 | 63.50±0.56 |

**Dilute $H_2SO_4$ pretreatment**

The samples of the various biomass were pretreated with dilute acid 1, 1.5 and 2% concentration, an autoclave at temperature of 105, 120 and 135$^oC$ for the period of 15, 30 and 45 minutes. The temperature 120 °C is considered best for both the samples while the retention time of 15 minutes was suitable for peel wastes and

30 minutes for cogon grass at the concentration of 1.5% and 1% respectively, these are the optimized conditions that was used for enzymatic experiment ( Figures 6-7).

**Fermentation**

Cogon grass produces 10% of ethanol where as Peel wastes produces 7.4% of ethanol.as shown in Table 9. As the time period increases, glucose concentration was reduced but ethanol concentration was enhanced but up to certain time limit. However, after 72 hours glucose concentration was not sufficient to maintain the ethanol production. Higher cellulosicbut lower lignin contents of cogon grass was compared to Peel wastes and it was found that these contents make cogon grass a better candidate for ethanol production.

**Table 6. Ethanol production from Cogon grass and peel wastes samples**

| Sample | Ethanol Production (% v/w) |
|---|---|
| Cogon Grass | 10.5 |
| Peel wastes | 7.4 |

**Table 7; Sugars and other products (%) obtained from grasses**

| Substrate | Glucose | Xylose | Lignin | Dry matter | Moisture | Ash |
|---|---|---|---|---|---|---|
| Cogon grass | 32.36. ±1.14 | 18.85 ±1.18 | 6.93 ±0.44 | 90.33 ±1.85 | 9.67 ±0.54 | 5.77 ±0.46 |
| Peal wastes | 27.32. ±2.15 | 15.37 ±1.13 | 4.75 ±0.54 | 92.46 ±1.24 | 8.56 ±0.55 | 4.89 ±0.58 |

% age values of various parameters of biomass samples.

**Dilute sulfuric acid pretreatment of Peel wastes**

Glucose content in peel waste was 27.33±2.15% (Table 10). There was increased in the glucose content after treatment (Figures 10 -11) The solid fraction of samples has given larger quantity of glucose when it was treated with dilute acid concentration ( 1.8 % ) for 30 minutes and the temperature of reaction was maintained at 110 °C. It proved that moderate temperature and acid concentration play key role to enhance the glucose contents during pretreatment. Similar finding on acid hydrolysis of orange peel at low temperature has been reported by Talo et al. (2014).

**Percentage decrease in xylose content in solid fraction of peel wastes after $H_2SO_4$Pretreatment**

In peel wastes xylose content was 15.37%±1.13 (Table 10) and after pretreatment decrease in xylose content was observed. An acid concentration of 1.10% and incubation temperature of 110 °C for 30 minutes was found optimum to achieve minimum xylose in the solid fraction (Figure 11).With dilute acid pretreatment 100% removal of hemicellulose is possible (Sunand Cheng, 2005). Wyman et al. (2005) has also reported that maximum xylose solublization occurs at moderate temperature.

**Percentage increase in lignin content in solid fraction after $H_2SO_4$pretreatment**

Contour plot (Figure 10 and 11) also indicates that optimum acid dose of 2.0% for 37 minutes at 125 °C is enough to get minimum lignin in solid fraction. Although the reaction has shown apparently 7.32% increases in lignin content but values in real sense was in decreasing order . The apparent increase in lignin content was due to huge removal of xylose after $H_2SO_4$ pretreatment. If the temperature is kept constant, then the solublization of lignin becomes higher with elevating reaction time to ascertainmaximum value.

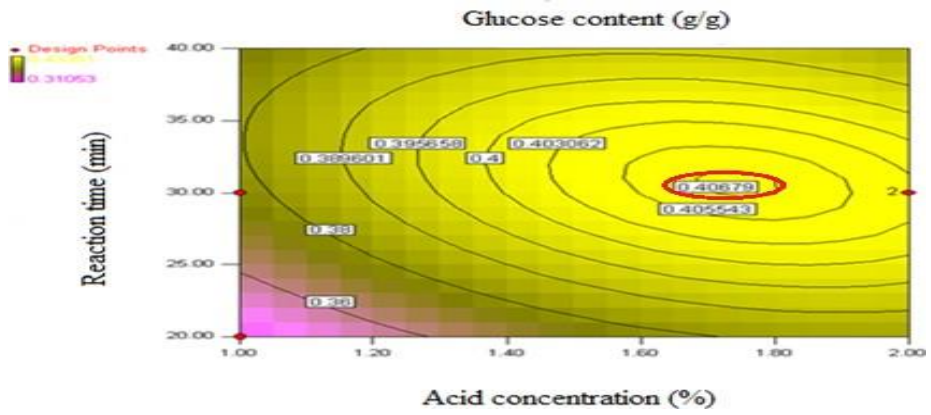


**Figure 10; Contour plot for glucose content in relation to acid concentration and reaction time**

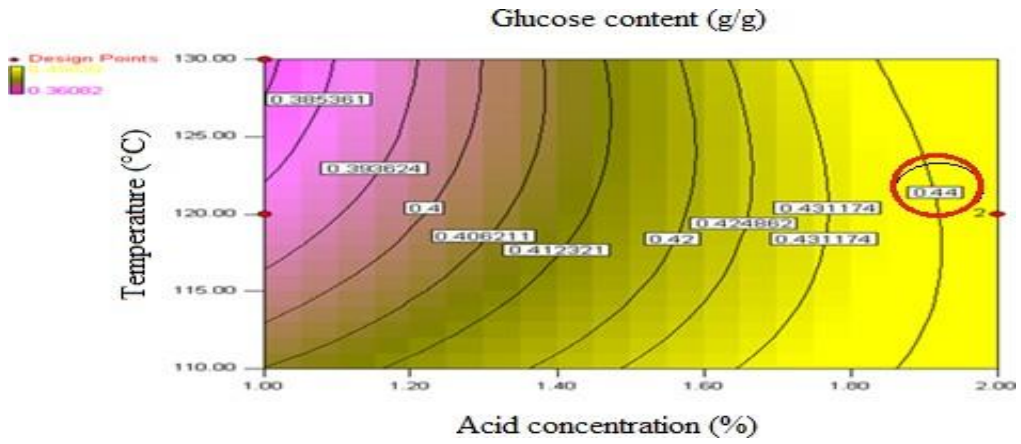


**Figure 11; Contour plot for glucose content in relation to acid concentration and temerature**

**Table 8; Maximum Sugar yields after enzymatic hydrolysis of substrates at pH 4.8, 50 °C, 120 rpm.**

| Substrate | $Glucose_{SF}$ (2.5g/50mL) | $Glucose_{SF}$ (50g/L) | Glucose(g/L) | $Rate_{sac}$ (%) | $Time_{OPT}$(hours) |
|---|---|---|---|---|---|
| Cogon grass | 1.10 | 22.00 | 17.72 | 80.54 | 72 |
| Peal wastes | 0.98 | 18.5 | 14.7 | 78.35 | 72 |

* SF = Solid fraction Y = Yield Sac = saccharification Opt = optimum

The reason behind higher saccharification (80.54%) was achieved as there was no accumulation of sugar like cellobiose occurred although cellobiose was available in reaction mixture. Xue et al. (2012) has also pointed out that the performance of celllases was actually enhanced (due to absence of cellubioses), and the results in higher sugar recovery after enzymatic hydrolysis.

**Table 9 .Chemical analysis of various crops samples**

| Parameters | Cotton stalks | Corn stove |
|---|---|---|

| | | |
|---|---|---|
| Moisture contents | 6.5 | 7.0 |
| Volatile Matter | 77.0 | 75.0 |
| Fixed Carbon | 9.5 | 19.5 |
| Ash contents | 8.7 | 6.0 |
| Crude Fiber | 31.0 | 32.0 |
| Ether extract | 1.8 | 2.5 |
| Crude Protein | 4.2 | 3.8 |
| Cellulose | 34.5 | 33.6 |
| Hemicellulose | 29.5 | 32.5 |
| Lignin | 14.8 | 18.5 |

Various parameters of bioma

**Analysis of Sugar after Pretreatment and Enzymatic Hydrolysis**

In this study three different substrates i.e. corn stover, wheat straw and rice straw were used for sugar production by enzymatic hydrolysis in 500 mL Erlenmyer flask at 50 °C for three days. For sugar production, pretreatment process of lignocellulose is necessary to break down lignin and it increases the accessibility of enzymes and microbes to carbohydrates( Figures 12-13). Two types of pretreatment methods have been applied on these substrates. In physical pretreatment these substrates were first groundto fine powder and passed through 80 mesh size sieve to reduce the size of particles. Then these substrates were subjected to chemical pretreatment. In order to interrupt the structure of lignocellulosic biomass, acidic and basic pretreatment (Chemical pretreatment) conditions were applied ( Table 12).

**Dilute Acid Pretreatment**

Different concentrations of sulphuric acid $H_2SO_4$ were used for pretreatment of agricultural waste samples. The samples were pretreated at a solid loading of 20% (w/v) slurry and heated at a temperature of 100, 110 and 120 $^0$C in an autoclave. The reaction was performed at a retention time of 10, 15 and 20 minutes and at three different concentrations of sulphuric acid i.e. 0.5, 1 and 1.5%. At each temperature, substrate was pretreated with three different concentrations of sulphuric acid. A sample was pretreatedtriplicate at same temperature and

reaction time. Total 9 treatments of three samples were pretreated at a time in 100ml reagent bottles. Total 9 experiments were done (9 × 9 = 81) so, 81 treatments of three samples were performed at 3 different temperatures to check the suitable condition for acidic pretreatment ( Figure 12).

**Dilute Alkali Pretreatment**

Dilute alkali was used for pretreatment of agrowaste samples. Different concentrations of sodium hydroxide (NaOH) were used at different temperature and different retention time to optimize the condition which may give maximum yield of glucose. The samples were pretreated at a solid loading of 20% w/v slurry and heated at 100, 110 and 120 $^0$C in autoclave for 10, 15 and 20 minutes of reaction time. For pretreating the sample ofdifferent concentrations of sodium hydroxide 0.5%, 1% and 1.5% were used. Total 9 experiments were done (9 × 9 = 81) , therefore total 81 treatments of three samples were performed at 3 different temperatures to check the suitable condition for basic pretreatment.

**Spectrophotometric analysis and Comparison of sugar production in three agrowaste samples**

Better glucose yields were obtained from wheat straw in all 9 experiments after 72 hours of enzymatic hydrolysis. It was observed during experiment that by increasing the concentration of $H_2SO_4$ from 0.5 to 1.5% the amount of sugar was also increased. In all experiments, higher yield of glucose was also recorded at a retention time of 20 minutes rather than 10 and 15 minutes. For acidic pretreatment conditions of wheat straw, the conditions were optimized at 120 $^0$C, 20 minutes of retention time with 1.5% of sulphuric acid (Figure 14). At this concentration, glucose concentration was at peak. During alkali pretreatment conditions, the glucose yield was increased by increasing the temperature and higher yield was recorded at 120 $^0$C. Meanwhile at similar temperature high yield of glucose was recorded when 1.5 % concentration of NaOH was used ( Figure 16 ) . By increasingthe time of enzymatic hydrolysis from 0 to 48hours sugar yield was increased but when thetime is increased further to 72 hours sugar concentration was chopped.The decrease in glucose concentration was probably due to production ofinhibitors by higher acid concentrations. Maximum reducing sugars (7.73 g/L) were obtained at 120$^0$C , when 1% NaOH concentration and reaction time of15 minutes were used .

Rice straw has shown higher glucose yieldin acidic pretreatment conditions at 110 $^0$C, acid concentration (1.5% ) and retention time 10 minutes was used ( Figure 15 ).The optimum condition used for rice straw analysis in case of alkaline pretreatment, temperature (100 $^0$C ), sodium hydroxide concentration (0.5% ) and retention time ( 20 minutes) . Higher yield was obtained after 72hours of enzymatic hydrolysis ( Figure 17).

During acidic pretreatment high yield of glucose was obtained at a temperature( 120$^0$C ), $H_2SO_4$ concentration ( 1.5% )and reaction time of 15 minutes (Figures 13-14). The optimum condition for corn stover at alkaline pretreatment condition was temperature 100 $^0$C, concentration of sodium hydroxide (1.5% ) and retention time applied was 20 minutes (Figure 18).

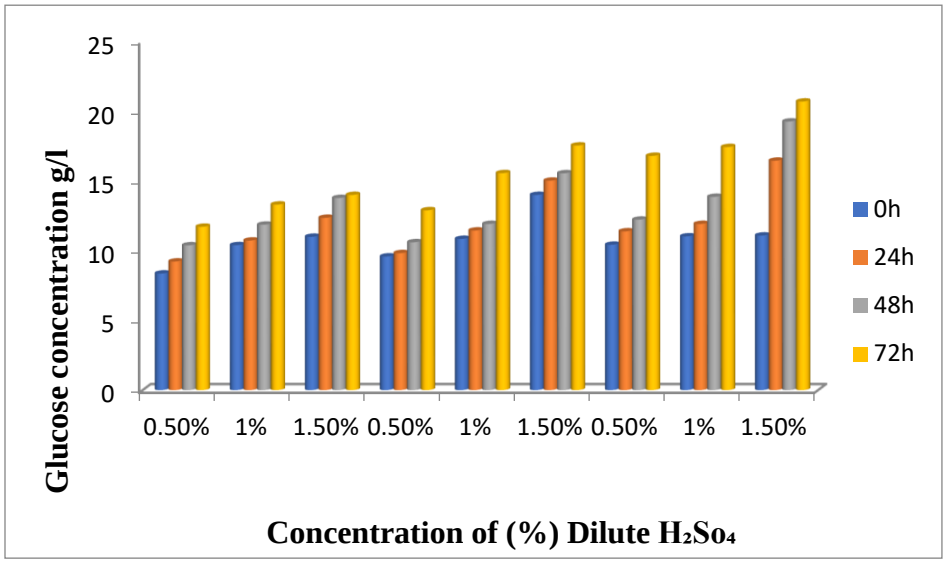


**Figure 14. Glucose yield obtained from Wheat straw at $H_2SO_4$ pretreatment conditions at 120$^0$C**

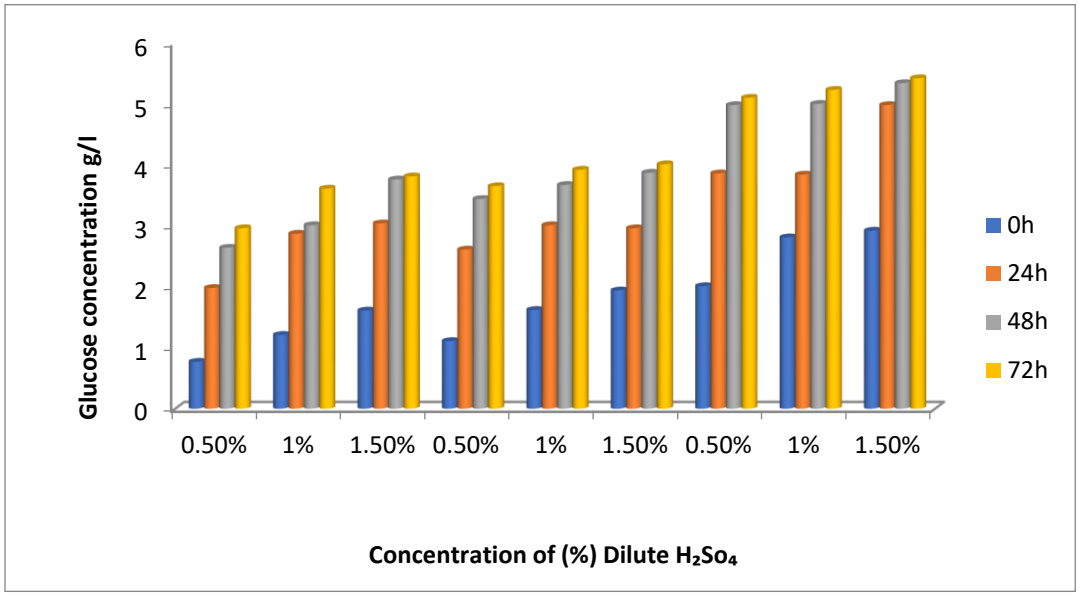


**Figure 15. High glucose yield obtained from Rice straw at $H_2SO_4$ pretreatment conditions at 110 $^0$C**

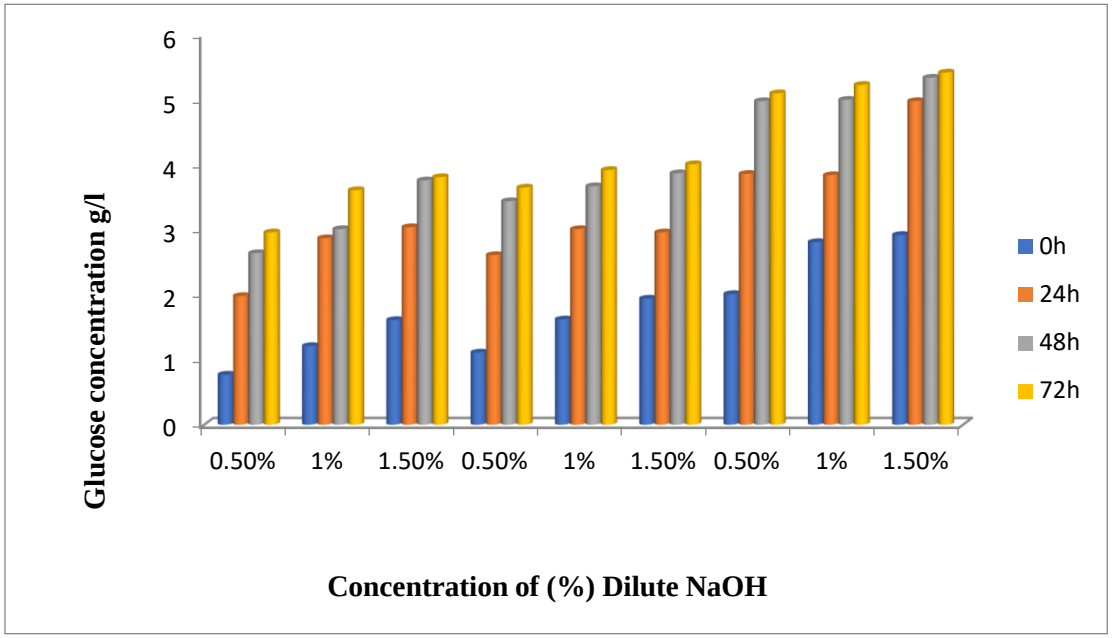


**Figure 16. High glucose yield obtained from Wheat straw byNaOH pretreatment conditions at 120 $^0$C**

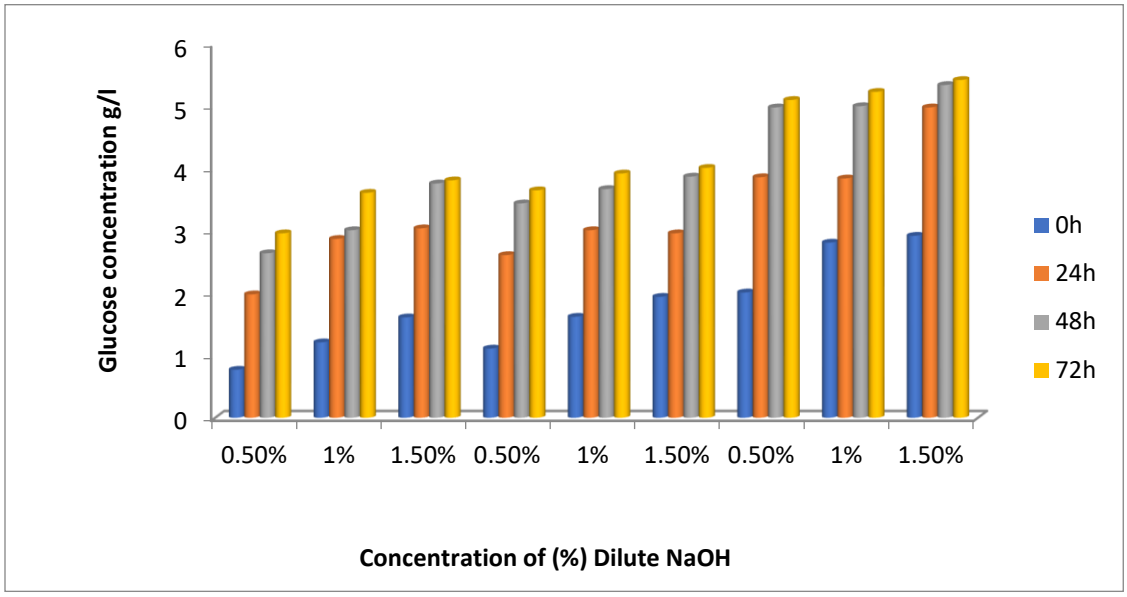


**Figure 17. Glucose yield obtained from Rice straw at NaOH pretreatment conditions at 100 $^{0}$C**

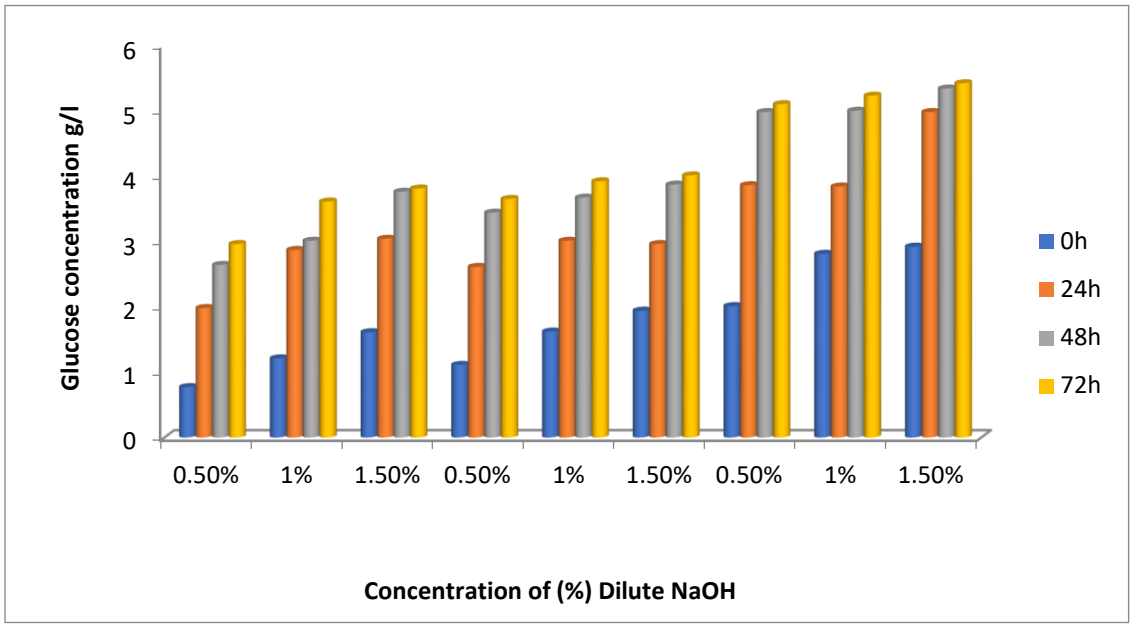


**Figure 18. Glucose yield obtained from Corn stover byNaOH pretreatment conditions at 100 $^{0}$C**

**HPLC Analysis of Enzymatic Hydrolysate**

The enzymatically hydrolyzed samples of acidic and alkaline pretreatment of wheat and rice straws as well as corn stoverwere further analyzed by HPLC. For this purpose, the samples those have shown higher amount of glucose at optimized conditions were used for analysis. Thesamples those were withdrawn at different time periods during enzymatic hydrolysis, then these were centrifuged at 14,000 rpm, at 4 $^{0}$C for 15 minutes.Supernatant was separated and then filtered by using 0.22 μm syringe filter. An aliquot of the sample (500 μl) was diluted with 1ml methanol to bring the concentrations of the samples within the range of calibration curve. Methanol was used due to the solubility of the sugars. All the samples and standard solution of glucose was passed through the 0.22 μm filter prior to analysis. About 20μl of agrowaste sample was injected through injection loop into HPLC system. In order to analyze the glucose, enzymatically hydrolyzed samples were run in the gradient mode for 10 minutes ( Shields and Cathcart.2010).

The identification of peak as based on the retention time $t_R$.Identification of glucose in three samples i.e. wheat straw, rice straw and corn stover were confirmed by the known standard injected through HPLC and its only one prominent peak was observed at a retention time of 3.255 minutes ( Table 13 and Figures 19-20).

**Table 10; Analysis of wheat and rice straws samples for sugars with HPLC**

| **Components** | **Retention time ( min)** | **Concentration (mg/ml) Rice straw** | **Concentration (mg/ml) Wheat straw** |
|---|---|---|---|
| Glucose | 8.6 | 22.52 | 28.3 |
| Cellobiose | 7.1 | 1.02 | 1.05 |
| Xylose | 11.6 | 4.3 | 5.6 |
| Arabinose | 12.0 | 1.4 | 1.8 |
| Mannose | 13.2 | 1.5 | 2.8 |

| | | | |
|---|---|---|---|
| Galactose | 15.5 | 1.2 | 1.5 |
| Furfural | 42.5 | 1.4 | 2.65 |
| HMF | 28 | 1.2 | 2.84 |

Analysis of sugar with HPLC

**Fermentation with*Clostridium acetobutylicum***

The major product of this type of fermentation is known as ABE (acetone, butanol and ethanol) fermentation. The ratio of the acetone, butanol and ethanol in the fermentation process ismostly 3:6:1 as reported earlier by many authors. It was estimated that *Clostridium acetobutylicum* yields higherbutanol quantity at acidic pretreatment conditions as compared to alkaline pretreatment conditions. Although alkaline pretreatment conditions are best for butanol production because the chances for the production of fermentation inhibitors are very low. But in this experiment the reason for low butanol production might be due to low quantity of glucose obtained at alkaline conditions.Among three substrates the yield of butanol was high in wheat straw because of number of factors i.e. the amount of carbohydrate was high in wheat straw as compared to rice straw and corn stover. Low lignin content in wheat straw is responsible for high glucose yield as well as high yield of butanol than other two substrates. Wheat straw contains low lignin contents as compared to rice straw and corn stover. The higher butanol production from wheat straw may be due to its low lignin contents. Among three substrates wheat straw yields highest quantity of butanol at acidic pretreatment conditions. It was estimated from previous studies that wheat straw hydrolysate contain furfural and hydoxymethyl furfural that supported the production of biobutanol by fermentation.. It is concluded that wheat straw is a superior fermentation substrate probably fermentation stimulatory chemicals are present in wheat straw.

**Table 11; Proximate analysis of straws samples**

| Substrates | Dry matter% | Moisture% | Crude protein% | Crude fat% | Crude fiber% | Ash% |
|---|---|---|---|---|---|---|
| Corn stover | 91 | 5.32 | 7 | 2.9 | 2.5 | 3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Wheat straw | 92.8 | 7.2 | 17.5 | 3.6 | 15 | 23.5 |
| Rice straw | 90.8 | 5.40 | 4.37 | 1.9 | 11 | 24 |

Analysis of biomass samples

**Table 12;Chemical analysis of straws samples**

| Samples | Cellulose % | Hemicellulose% | Lignin% |
|---|---|---|---|
| Corn stover | 30 | 21 | 7 |
| Wheat straw | 40 | 25 | 13 |
| Rice straw | 35 | 22 | 20 |

**Chemical analysis of biomass samples**

***Clostridium acetobutylicum*function for butanol**

The clostridium specie *Clostridium acetobutylicum*was maintained at at -20$^0$C. Enzymatically hydrolysed sample was then used for fermentation. The pH was maintained at 6.5 with NaOH. 1ml *C. acetobutylicum*spores were added in 100ml enzymatically hydrolysed solution inreaction bottle for separate hydrolysis and fermentation. These reaction bottles were placed in shaking incubator having 120rpm at 37$^0$C for 72 hours. The butanol concentration was determined by alcohol meter after 72 hours, and values were expressed as % butanol obtained from wheat straw, from rice straw and from corn stover. Among these three substrates wheat straw produced highyield of butanol ( Tables 16 -18).

However, main end products of acidic /alkaline pretreatments and enzymatic hydrolysis are glucose and xylose which can be metabolized by clostridium speciesduring growth and acetone – butanol- ethanol( ABE) fermentation ( Qureshi and Blaschek,2000; Moretti and Thorson, 2008 ).*C. acetobutylicum* and *C. beijerinckii* are two major microbes normally used in ABE fermentation. However, in recent years research worker from different countries have investigated various strain (s) their parent micorbes, cultivation conditions and

growth media for various biomass samples used in batch, fed batch and continuous fermenters to produce ABE ( Tables16-18)

**Table 13; Acetone, Butanol and Ethanol production ( %) from various agrowaste by *Clostridium acetobutylicum***

| Samples | Acetone% | Butanol% | Ethanol% |
|---|---|---|---|
| Wheat straw | 1.5 | 6.3 | 1.8 |
| Rice straw | 1.2 | 6.2 | 1.3 |
| Corn stover | 1.0 | 5.2 | 1.1 |

**ABE production from Biomass samples**

**Table 14; Acetone, Butanol and Ethanol production from agrowaste by *Clostridium acetobutylicum*at $H_2SO_4$ pretreated samples**

| Samples | Acetone % | Butanol% | Ethanol% |
|---|---|---|---|
| Wheat straw | 2.5 | 7.2 | 2.1 |
| Rice straw | 1.7 | 4.9 | 2.2 |
| Corn stover | 1.1 | 4.5 | 2.3 |

**ABE production from Biomass samples**

**Table 15; Acetone, Butanol and Ethanol production from agrowaste by *Clostridium acetobutylicum*at NaOH pretreated samples**

| Samples | Acetone % | Butanol% | Ethanol% |
|---|---|---|---|
| Wheat straw | 1.9 | 4.9 | 2.8 |
| Rice straw | 1.5 | 4.6 | 2.4 |
| Corn stover | 1.3 | 4.2 | 2.2 |

**ABE production from Biomass samples**

## DISCUSSION

For the production of alcoholic fuels ( Butanol and Ethanol ) from lignocellulosic feedstock required various technological steps like acid or alkali pretreatment, saccharification and fermentation. To accomplish an cost effective production of biofuels,proper adjusting of all units of system is of great important. In the past different countries significantly improved alcoholic fuels production by refining different process like pretreatment, enzymatic hydrolysis, fermentation, and higher level of ethanol recovery ( Zhao, 2012). The popular cases of biomass based fuels production in developed countriesmay be good references for the developing countries . In addition many novel ideas, such as biorefinery and the concept of oriented conversion of classified composition have been investigated for ethanol production. Similar technology are also applicable for butanol production fromlignocellulosic biomass (García et al., 2011; Demirbas, 2009 ). The cost of fuels may further decreases when it will produce at industrial scale and efficient combination of these processes will result in competitive biofuel production from plant biomass, which is currently not being utilized effectively ( Talo et al., 2014).

Fermentation of available sugars in cellulosic biomass have potential to provides important products like acetone, butanol, ethanol and similar other alcohols, thatcould be used as liquid fuels. Mostly available source of biomass containing carbohydrates are wood wastes , agriculture crops like wheat, rice and cotton straws ,

corn covers, sorghum straws, fruit and vegetable wastes and similar other substrates. Cellulose is considered as major sugar for alcohol (fuel) production and cellulose is complex sugar present in plants materials. This complexcellulosic material is break down into smaller units with help of acid treatment and enzymatic hydrolysis as well as bacterial/ fungal fermentation. These forms of alcohols is important because that may use as fuels. Therefore biofuels may provide solution of (1) combating climate change, as it help to reduce level of carbon emission release from traffic etc. (2) . Biofuel is able to respond growing demand of fossil fuel and energy (3) Biofuels securing energy supply as it provides security to challenges rising for fuels globally (4).Reducing amount of waste and utilizing natural resources, therefore biofuels is excellent example to provide answer of circular economy . In current study various cellulosic materials was used to produce bioethanol and biobutanol. Therefore various order of alcoholic fuels production from cellulosic substrates was obtained . Among all substrates of biomass used straws has provided better yields of alcoholic fuels as compared to others material used.However, amount of acetone, butanol and ethanol produced depends on nature of cellulosic biomass used as well as various distillation process conducted after fermentation for purification of these type of alcohols.

## CONCLUSION

The country ‘s energy demand is expected to increase three fold by 2050, but supply position is not inspiring. Due to similar situation renewable and sustainable energy resources are the best alternative of conventional fuels and energy sources . Bioconversion of lignocellulosic biomass into alcoholic fuels (butanol and ethanol ) provides a sustainable and economical pathway . While, a deep understanding of fundamentals of various pretreatment processes and development of more efficient and economical fermentation processes needs continuing efforts. Moreover, the development of cost-effective detoxification, more efficient microbial strains are required. The processof integration and optimization to reducing energy consumption as well as to increase yields replace currently available fossil fuels those are already in process of depletion. Therefore scientists all over the world are observing different cost effective methods for alternative sources of energy especially by using cellulosic biomass. It is expected that these types of research work could be an important phenomena for the

development of country by using indigenous resources in future.

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