# BIOCONVERSION OF PLANTS BASED BIOMASS INTO ETHANOL BY USING FERMENTATION PROCESS

Iram Batool [1], Faryal Kabir [2], Hania Naeem[2] and, Nazish Manzoor[3]

Corresponding author ; Iram06ali@gmail.com

## ABSTRACT

Cellulosic organic wastes like wheat, rice, cotton straws and corn stoves are being utilized as feeds of animals since past history. However, ample amount of cellulosic materials are being wastes every year and not being utilized for generation of economical resources.

Second generation biomass in Pakistan and some neighbor countries are just putting on fire to clean up land for cultivation of next seasonal crops which is adversely affecting the our environment. .A study was conducted for the production of bioethanol using wheat , cotton and rice straws, corn stove as well as peel wastes . The experimental was conducted by assessing various steps like acidic/ alkali pretreatment, cellulases hydrolysis along with effect of acid proteases on cellulose degradation as well as yeast fermentation processes . Results indicates that higher level of ethanol was produced depending upon on substrates concentration , optimized condition of fermentation. Higher level of glucose (g/L) was obtained from by using acid treatment and higher amount of ethanol was obtained from after during fermentation process . It was observed that use of acid protease enhanced final recovery of ethanol. It is expected that outcome of this study will help to increase production of biofuels to reduce burden of foreigner exchange that is currently being utilize to import fossil fuel from other countries.

**Key words;** Organic wastes , Polysaccharides , Fermentation, Bio fuels

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[1] Department of Biochemistry, University of Narowal, Narowal, Pakistan

[2]UIBB –PMAS Arid Agriculture University Rawalpindi. Pakistan

[3] Department of Microbiology KUST Kohat, Pakistan

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## INTRODUCTION

Fossil fuels, including coal, oil and natural gas, are currently the world's primary energy source. Formed from organic material over the course of millions of years. When fossil fuels are burned,

they release carbon dioxide and other greenhouse gases, which in turn trap heat in our atmosphere, making them the primary contributors to global warming and climate change ( Galbe and Zacchi, 2007).

Biofuel, any fuel that is derived from biomass—that is, plant or algae material or animal waste

Biomass is a vital energy source in Pakistan because of agriculturally 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 ( Ariffin *et al*., 2006; 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%) ( Becerra *et al*., 2015). Various renewable energy resources include different agricultural substances like green leaves, fruit shells, straws, nut shells and fruit seeds (Bergey *et al*.,1994). Most commonly used feed stocks are wheat straw, wheat bran, corn stover, corn steep liquor and apple pomace ( Breznak and Brune,1994). Now a day, agricultural waste is used to produce 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 . 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 (Brune *et al*., 1995).

Exploration of various sources for alternate energy have been increased because of increasing concerns about energy security and climatic changes. 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 (Chaudhry *et al*., 2009). Various alternatives steps to generate sustainable biofuels from biomass has been investigated. Important biological energy resources are like 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 potential fuel for transport sector (Dheeran *et al*., 2012). 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 growing rural economics. The global ethanol production is 51,000 million liters ( Galbe and Zacchi, 2007; Iram *et al*., 2021). 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. While ethanol has greater octane rating properties ( Lehman, 2005).

The simultaneous saccharification and fermentation process combines polysaccharide hydrolysis and fermentation in one step, but still relies on the addition of exogenously produced enzymes. In addition to pretreatment and addition of exogenously enzymes, the major rate limiting step in enzymatic hydrolysis is the protease attack that reduces the enzyme activity, especially cellulases. The simultaneous saccharification and fermentation that occurs in this type of process is an attractive method for keeping monomeric sugars at low enough concentrations to avoid enzyme inhibition, thus reducing costs by decreasing the amount of enzyme needed for the process ( Lynd *et al*., 2008).

Converting lignocellulosic biomass to ethanol involves four stages: pretreatment, hydrolysis, fermentation, and ethanol recovery by distillation. Pretreatment increases biomass digestibility for efficient fermentable sugar production, which reduces the cost of bioethanol production . Various pretreatment methods have been suggested, depending on the purpose of removing hemicellulos. or lignin from the biomass Dilute acid pretreatment is a promising pretreatment capable of high solubilization of hemicellulose This process degrades most of the hydrogen bonds in hemicelluloses and partially degrades cellulose and lignin ( Mahon *et al*., 2011).

In addition, acid pretreatment permits hemicellulose hydrolysis of pentoses and hexoses, removes some of the lignin, and makes the cellulose structure more accessible, so that a fraction can be converted to glucose enzymatically.. The choice of pretreatment technology for a particular raw material depends on several factors, some of them directly related to the enzymatic hydrolysis step such as sugar-release patterns and enzymes employed. Thus, the combination of the composition of the substrate in addition to the pretreatment conditions has a great influence on biomass digestibility ( Reiner, 2010 )

A yeast is a unicellular fungus which reproduces asexually by budding or division, especially the genus *Saccharomyces* which is important in food fermentations ( Rogers, 2008). Most yeasts are larger than most bacteria and their participation are importance in the food industry.

*Saccharomyces cerevisiae* (commonly known as baker's yeast) is a single-celled eukaryote that is frequently used in scientific research. *S. cerevisiae* is an attractive model organism due to the fact that its genome has been sequenced, its genetics are easily manipulated, and it is very easy to maintain in the lab. Most yeasts require an abundance of oxygen for growth, therefore by controlling the supply of oxygen, their growth can be checked. In addition to oxygen, they require a basic substrate such as sugar. Some yeasts can ferment sugars to alcohol and carbon dioxide in the absence of air but require oxygen for growth. They produce ethyl alcohol and carbon dioxide from simple sugars such as glucose and fructose.Yeasts are active in a very broad temperature range - from 0 to 50° C, with an optimum temperature range of 20° to 30° C.

The optimum pH for most micro-organisms is near the neutral point (pH 7.0) and are usually acid tolerant. Yeasts can grow in a pH range of 4 to 4.5 (Scharf and Boucias, 2010)

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Although C. thermocellum is a proven industrial ethanol producer in traditional starch-based processes, it will be no easy task to provide this microorganism with the ability to convert lignocellulosic biomass to ethanol. The carbohydrate components of lignocellulose (cellulose and hemicellulose) are tightly bound to lignin, making the sugars largely inaccessible to enzymes. Before enzymatic hydrolysis, pretreatment with acid or alkali and increase activity of

cellualses by action of protease inhibitors that reduce the activity of proteases is generally needed to fully maximize the release of sugars from any lignocellulosic biomass

Keeping in view importance of biofuels study was conducted with aims and objective like (i) Treatment of lignocellulosic biomass to break down into cellulose, hemicellulose and lignin by using acid/ alkali treatment .(ii) Optimization of various condition for yeast and bacterial fermentation process (iii) Production of higher yields ethanol by using cellulase and acid protease.

## MATERIAL AND METHODS

### Collection of Agricultural Substrates

Various samples (wheat, rice, and cotton straws as well and corn stover wastes) were collected from different areas of Punjab. The samples were dried, grinded passed through 40 mesh standard size sieve.

### Analysis of Biomass Samples

All samples were analyzed for moister, ash, dry matter, crude protein, crude fiber, crude fat as well as dry weight contents were determined (AOAC, 1990). Cellulose, hemicellulose and lignin contents were quantified by using standard method described by many authors including Scharf and Tartar (2008).

### Acidic and Alkaline Pretreatment of biomass samples

Pretreatment process 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 samples in each bottle and the contents were emptied on filter paper. After filtration, the solid was wash away with 300 ml distilled water to neutralize the pH and filter paper was than dried at 105 °C and weighed.

**Enzymatic Hydrolysis**

The biomass samples after pretreatment 5% (w/v) was hydrolyzed with cellulase 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. Chloromphenicol (100 µg/ml) and ampicilin (100 µg/ml) were also added during reaction to inhibit microbial growth. Cellulases from *T. reesei,*cellobiase from *Aspergillusniger* and Novozyme 188 was delivered by Novozyme A/S, Bagsvaerd, Denmark having activity of (30FPU g-1). The samples were withdrawn from reagent bottle after every 12 hours to determine the concentration of sugar ( Shields and Cathcart, 2010).

Acid protease .A protein-digesting enzyme that exhibits maximum activity and stability in acid conditions (pH 2.0–5.0) and is inactivated at pH values above 6.0. Acid protease are helpful in food and wine industries for extraction of higher yields of ethanol. Therefore in current study addition of cellulase was supplemented with acid proteases to increase yield of ethanol

After enzymatic hydrolysis, $H_2SO_4$ (µl) or NaOH was added. Un-hydrolyzed sample was separated by centrifuging for 10 minutes at 13,500 g. Supernatant was collected by means of syringe filters.The amount of sugar was determined by p-hydroxybenzoic acid hydrazide (PAHBAH) method against standard curve(1Mm-25mM of xylose.The best pretreatment condition was selected after enzymatic hydrolysis process. The sample containing higher amount

of released sugar was further selected for fermentation process and solid biomass was stored at 4 °C.

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**Saccharification**

The agro and municipal waste samples ( wheat, cotton , rice straws and corn stover wastes) were taken as a solid loading of 5% (w/v) and then autoclaved. The crude enzymes from bacterial species were added and the ratio of substrate to enzyme was adjusted to 1:1 and placed for 72 hours at 50$^{o}$C. Both of the enzymes were added in separate reaction mixture in order to check the individual enzymatic activity. Also both enzymes were mixed at a ratio of 1:1 to check the combine effect of enzymes and after scarification, the sugar contents were determined ( Tokud and Watanabe, 2007).

**Culture conditions for growth**

*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 cells was carried out in a 5-mL tube of YPD medium containing NaCl 0.9% (w/v) at 30°C for 16 h on a rotary shaker (100 r.p.m.) according to Alfenore *et al*. ( 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 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 ( Watanabe *et al*., 1998).

**HPLC Analysis of Enzymatic Hydrolysate**

The fermentation products like monomer sugars (hexoses and pentoses) acetone- butanoland ethanol as well as others bio products were determined by using method reported by Wenzel *et al*. (2002).

The enzymatically hydrolyzed samples of acidic and alkaline pretreatment of wheat and rice straws as well as corn stover were further analyzed by HPLC. For this purpose, the samples those have shown higher amount of glucose at optimized conditions were used for analysis. The samples those were withdrawn at different time periods during enzymatic hydrolysis, then these were centrifuged at 14,000 rpm, at 4 $^o$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 20 μl 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).

**Statistical analysis**

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

## RESULTS AND DISCUSSION

Results regarding Physical and chemical analysis of biomass samples, pretreatment, enzymatic as well as quantification of end products by using High performance chromatography (HPLC ) technique..

### Proximate Analysis of various Biomass samples

Various samples of biomass were analyzed to get concentration level of dry matter, moisture, crude protein, lipid ash and fiber contents (Table 1). These parameters play important role to maintain quality of feed stock uses for different purposes.

**Table 1**. **Proximate analysis (%) of biomass samples**

| **Parameters** | **Cotton stalks** | **Corn stover** | **Wheat straw** | **Rice straw** |
|---|---|---|---|---|
| **Dry Matter** | 92.5 ± 2.6 | 89.8± 3.1 | 91.4± 1. 5 | 90.5± 0.5 |
| Moisture contents | 6.7± 0.8 | 7.5 ±0.9 | 7.8± 0.6 | 5.7± 0.6 |
| Volatile Matter | 77.6 ± 1.2 | 75.8± 2.6 | 89.3±2.5 | 90.6± 1.5 |
| Fixed Carbon Content | 17.5±1.2 | 19.5± 2.3 | 18.7± 1.3 | 17.5± 1.6 |
| Ash Contents | 8.7± 0.5 | 6.2± 0.4 | 5.1± 0.7 | 3.4± 0.6 |
| Crude Fat Content | 3.5± 0.2 | 3.7 ± 0.8 | 3.6±0.3 | 2.8± 0.4 |
| Crude Protein Content | 4.2 ± 0.6 | 6.8 ± 0.9 | 9.6± 0.4 | 4.7± 0.5 |
| Cellulose Content | 37.5±1.5 | 33.6±2.1 | 38.5± 2.7 | 34.8± 1.7 |
| Hemicellulose Content | 28.5± 2.5 | 26.5± 2.8 | 27.8 ± 3.1 | 26.7± 2.8 |

| | | | | |
|---|---|---|---|---|
| Lignin Content | 14.8± 2.6 | 19.5± 1.5 | 13.7± 2.4 | 15.8± 1.9 |

**Pretreatment of Agricultural substrates**

Samples of wheat, cotton, rice (straws) and corn stover wastes were used for pretreatment process. Maximum amount of sugar (17.5 ±1.6mM/l ) was found in wheat straw, when sample was treated with $H_2SO_4$ . At a concentration of 3 % very less amount of sugar was detected 6.38±0.86 mM/l (Table 2). Probably amount of released sugar may be converted into inhibitors such as hydroxymethyl furfural and similar others products (Garcia et al., 2011). During alkali pretreatment higher amount of sugar (16.5 ±0.1mM/l) was released when sample was treated with NaOH (3%)( Table 3) . In cotton stalk, higher amount of sugar (14.2 ±0.03mM/l) was obtained when 1 % $H_2SO_4$ was used.When sample was treated with 3 % NaOH the sugar concentration and acidic pretreatment of rice straw higher amount of released sugar all values are mentioned in table 2. It was observed that values of sugar obtained by two different treatments are according to results reported by other authors.

**Table2. Chemical pretreatment of biomass samples with different concentrations (%) of $H_2SO_4$ release of sugars ( %), after 72 h duration**

| $H_2SO_4$ (%) | Temp (C) | Time ( min) | Wheat Straw | Rice Straw | Cotton stalk | Corn stover |
|---|---|---|---|---|---|---|
| 1 | 105 | 10 | 11.9± 0.3 | 7.8 ± 0.5 | 6.7 ± 0.8 | 14.9 ± 0.4 |
| | | 15 | 11.8± 0.4 | 9.8 ± 0.4 | 9.2 ± 0.5 | 15.2 ± 0.7 |
| | | 20 | 11.5± 0.8 | 8.5 ± 1.5 | 9.8 ± 0.6 | 15.1 ± 0.7 |
| | 115 | 10 | 8.6± 0.5 | 11.5 ± 0.4 | 10.3 ± 0.5 | 13.8 ± 0.5 |
| | | 15 | 7.9± 0.2 | 12.8 ± 0.3 | 11.7 ± 0.3 | 13.9 ± 0.4 |
| | | 20 | 8.1± 0,7 | 9.5 ± 0.1 | 9.8 ± 0.1 | 10.8 ± 0.7 |
| | 125 | 10 | 12.1± 0.7 | 13.8 ± 0.2 | 10.8 ± 0.2 | 14.9 ± 0.1 |
| | | 15 | 12.7± 0.8 | 14.2 ± 0.4 | 12.90 ± 0.1 | 14.8 ± 0.1 |
| | | 20 | 15.6± 0.7 | 9.1 ± 0.7 | 15.01 ± 0.5 | 15.1 ± 0.3 |
| 1.5 | 105 | 10 | 11.2± 0.7 | 15.3 ± 0.6 | 12.6 ± 0.1 | 14.9 ± 0.1 |
| | | 15 | 11.9± 0.5 | 13.5 ± 0.6 | 13.1 ± 0.4 | 14.2 ± 0.4 |
| | | 20 | 9.5± 0.6 | 12.0 ± 0.8 | 13.5 ± 0.7 | 15.1 ± 0.3 |
| | 115 | 10 | 11.6 ± 0.8 | 12.8 ± 0.6 | 13.0 ± 0.2 | 13.2 ± 0.0 |
| | | 15 | 9.5±0.3 | 16.2 ± 0.5 | 9.1 ± 0.5 | 9.2 ± 0.1 |
| | | 20 | 12.5 ±0.7 | 14.6 ± 0.1 | 10.6 ± 0.2 | 16.6 ± 0.7 |
| | 125 | 10 | 14.6±0.8 | 11.2 ± 0.6 | 14.1 ± 0.6 | 14.8 ± 0.5 |
| | | 15 | 16.5±0.9 | 12.8 ± 0.7 | 9.1 ± 0.2 | 15.9 ± 0.2 |
| | | 20 | 15.6±0.6 | 11.6 ± 0.2 | 10.5 ± 1.0 | 16.1 ± 0.9 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2.0 | 105 | 10 | 13.2±0.5 | 13.2 ± 0.6 | 14.2 ± 0.4 | 15.1 ± 0.6 |
| | | 15 | 14.3±0.7 | 11.9 ± 0.5 | 12.2 ± 0.6 | 14.0 ± 0.7 |
| | | 20 | 12.6±0.9 | 11.3 ± 0.6 | **16.4 ± 1.0** | **17.1 ± 0.9** |
| | 115 | 10 | 12.3±0.8 | 16.1 ± 0.2 | 12.2 ± 0.4 | 15.1 ± 0.1 |
| | | 15 | 13.7±0.5 | 12.2 ± 0.7 | 12.6 ± 0.5 | 15.2 ± 0.6 |
| | | 20 | 14.5±0.6 | 16.4 ± 1.0 | 13.1 ± 0.8 | 14.9 ± 0.5 |
| | 125 | 10 | 14.6±0.3 | 14.0 ± 0.3 | 12.8 ± 0.7 | 15.1 ± 0.2 |
| | | 15 | 16.7±0.8 | 15.1 ± 0.1 | 14.2 ± 0.5 | 14.8 ± 0.1 |
| | | 20 | **17.5±1.6** | **16.5 ± 0.1** | 14.2±0.3 | 15.6 ± 0.1 |

Chemical treatment of biomass samples for sugar . Mean ± ST

**Table3. Chemical pretreatment of biomass samples with different concentrations (%) of NaOH to release of sugars ( %).( g/L ) after 72 h duration.**

| NaOH (%) | Temp (C) | Time ( min) | Wheat Straw | Rice Straw | Cotton stalk | Corn stover |
|---|---|---|---|---|---|---|
| 1 | 105 | 10 | 12.5± 0.4 | 5.2 ± 0.7 | 3.7 ± 0.6 | 4.9 ± 0.4 |
| | | 15 | 12.8± 0.3 | 9.01 ± 0.1 | 5.2 ± 0.5 | 5.6 ± 0.4 |
| | | 20 | 13.6± 0.8 | 11.5 ± 1.3 | 10.5 ± 0.2 | 11.1 ± 0.1 |
| | 115 | 10 | 8.7± 0.4 | 4.5 ± 0.9 | 13.1 ± 0.1 | 12.1 ± 0.3 |
| | | 15 | 7.9± 0.2 | 5.8 ± 0.3 | 13.7 ± 0.3 | 12.9 ± 0.4 |
| | | 20 | 8.1± 0,6 | 9.5 ± 0.1 | 9.8 ± 0.1 | 9.8 ± 0.7 |
| | 125 | 10 | 10.6± 0.7 | 13.2 ± 0.2 | 12.8 ± 0.2 | 13.1 ± 0.1 |
| | | 15 | 12.6± 0.8 | 14.2 ± 0.4 | 13.90 ± 0.1 | 14.8 ± 0.1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 20 | 16.3± 0.5 | 9.1 ± 0.7 | 15.01 ± 0.5 | 12.1 ± 0.3 |
| 1.5 | 105 | 10 | 13.6 ± 0.7 | 12.8 ± 0.1 | 13.6 ± 0.1 | 12.9 ± 0.1 |
| | | 15 | 15.5± 0.5 | 14.5 ± 0.1 | 14.01 ± 0.4 | 13.2 ± 0.4 |
| | | 20 | 16.8± 0.9 | 15.0 ± 0.3 | 15.5 ± 0.4 | 15.1 ± 0.3 |
| | 115 | 10 | 11.6 ± 0.8 | 12.5 ± 0.1 | 13.0 ± 0.2 | 13.2 ± 0.0 |
| | | 15 | 15.5±0.3 | 13.2 ± 0.5 | 9.1 ± 0.5 | 9.2 ± 0.1 |
| | | 20 | 17.5 ±0.7 | 14.6 ± 0.1 | 10.6 ± 0.2 | 10.6 ± 0.2 |
| | 125 | 10 | 13.6±0.8 | 11.2 ± 0.0 | 14.1 ± 0.6 | 13.8 ± 0.5 |
| | | 15 | 14.5±0.9 | 9.8 ± 0.1 | 9.1 ± 0.2 | 8.9 ± 0.2 |
| | | 20 | 17.6±0.6 | 11.6 ± 0.2 | 10.5 ± 1.0 | 10.1 ± 0.9 |
| 2.0 | 105 | 10 | 13.5±0.8 | 14.2 ± 0.5 | 14.2 ± 0.4 | 13.9 ± 0.5 |
| | | 15 | 14.6±0.4 | 8.9 ± 0.1 | 12.2 ± 0.6 | 11.0 ± 0.8 |
| | | 20 | 15.3±0.5 | 10.3 ± 1.0 | **16.4 ± 1.0** | **15.1 ± 0.9** |
| | 115 | 10 | 14.2±0.6 | 14.1 ± 0.2 | 13.2 ± 0.3 | 12.1 ± 0.1 |
| | | 15 | 15.8±0.9 | 12.2 ± 0.7 | 13.6 ± 0.1 | 12.2 ± 0.1 |
| | | 20 | 16.2±0.7 | **15.4 ± 1.0** | 12.1 ± 0.2 | 11.9 ± 0.1 |
| | 125 | 10 | 16.6±0.5 | 13.0 ± 0.3 | 10.8 ± 0.3 | 10.1 ± 0.2 |
| | | 15 | 18.7±0.9 | 13.1 ± 0.1 | 13.2 ± 0.2 | 12.8 ± 0.1 |
| | | 20 | **21.5±1.2** | 12.1 ± 0.1 | 12.3+0.3 | 11.01 ± 0.1 |

Chemical treatment of biomass samples for sugar . Mean ± ST

**Comparative study of treatments by using various substrates**

It was observed that higher amount of sugar was produced when wheat straw was treated with Enzymescellulase and acid protease . It was not noted that amount of sugar released in both chemical treatments depends on natural of substrates used for analysis . All agro and municipal wastes substrates used in study contain reliable amount of sugars **(** Tables 2-3**).** Which is good indicator for production of ethanol on commercial scales and similar results are also reported by

Zhao et al. (2012)

The solid fraction of samples has given larger quantity of glucose when it was treated with dilute acid concentration 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).

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 ( Williams , 2009 )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 ( Yoon *et al*., 2007).

**Saccharification of biomass samples with enzymes**

Saccharification process of various biomass samples was carried out after acid/ alkali treatment with cellulase and acid proteases . Results indicates that wheat straw has released glucose followed by rice straw, corn , cotton straws and peel wastes ( table 2-3 ). This released sugar, can than further be used for fermentation experiments.

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

As the timeperiod 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 theethanol production. Higher cellulosic but lower lignin contents of

cogon grass was compared to Peel wastes and it was found that these contents make cogongrass a better candidate for ethanol production.

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

Better glucose yields were obtained from wheat straw in all experiments after 72 hours of enzymatic hydrolysis ( Table 4). 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 .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 . By increasing the time of enzymatic hydrolysis from 0 to 48 hours sugar yield was increased but when the time is increased further to 72 hours sugar concentration was chopped.The decrease in glucose concentration was probably due to production of inhibitors by higher acid concentrations. Maximum reducing sugars (7.73 g/L) were obtained at 120 $^o$C , when 1% NaOH concentration and reaction time of 15 minutes were used .Rice straw has shown higher glucose yield in acidic pretreatment conditions at 110 $^o$C, acid concentration (1.5% ) and retention time 10 minutes was used .The optimum condition used for rice straw analysis in case of alkaline pretreatment, temperature (100 $^o$C ), sodium

hydroxide concentration (0.5% ) and retention time ( 20 minutes) . Higher yield was obtained after 72hours of enzymatic hydrolysis. During acidic pretreatment high yield of glucose was obtained at a temperature ( 120°C ), $H_2SO_4$ concentration

**Table 4. Products obtained after fermentation process**

| Substrate | Total concentration (g/L) | Total theoretical yield of ethanol (g/L) | Actual yield of ethanol (g/L) | Fermentation Efficiency (%) |
|---|---|---|---|---|
| Wheat straw | 21.7 | 11.7 | 11.3 | 92.3 |
| Rice straw | 17.8 | 9.6 | 10.6 | 82.8 |
| Corn stover | 16.5 | 7.8 | 9.5 | 91.5 |
| Cotton stalk | 18.6 | 10.8 | 9.7 | 90.5 |

Ethanol production from biomass samples Mean ± standard deviation

( 1.5% ) and reaction time of 15 minutes. The optimum condition for corn stover at alkaline pretreatment condition was temperature 100 °C, concentration of sodium hydroxide (1.5% ) and retention time applied was 20 minutes .

**Ethanol recovery**

In the conventional process of producing ethanol biofuel from corn starch, the recovery of ethanol from the fermentation broth is accomplished using a multicolumn distillation system

which yields an ethanol-rich stream near the ethanol–water azeotrope of 95 weight % ethanol could be possible depending variety of biomass used

The identification of peak as based on the retention time $t_R$ .Identification of glucose in five samples i.e. wheat straw, rice straw corn stover, cotton stalk and peel wastes 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 5).

**Table 5. Analysis of sugar by using optimized condition by using HPLC**

| **Components** | **Retention time** | **Rice straw** | **Wheat straw** | **Cotton stalk** | **Corn stover** | **Peel wastes** |
|---|---|---|---|---|---|---|
| Glucose | 8.6 | 22.52 | 28.3 | 17.5 | 16.7 | 12.4 |
| Cellobiose | 7.1 | 1.02 | 1.05 | 1.3 | 1.4 | 1.5 |
| Xylose | 11.6 | 4.3 | 5.6 | 4.7 | 4.5 | 3.8 |
| Arabinose | 12.0 | 1.4 | 1.8 | 1.4 | 1.6 | 1.2 |
| Mannose | 13.2 | 1.5 | 2.8 | 2.1 | 2,5 | 1.8 |
| Galactose | 15.5 | 1.2 | 1.5 | 1.3 | 1.4 | 1.1 |
| Furfural | 42.5 | 1.4 | 2.65 | 1.3 | 1.5 | 1.2 |
| HMF | 28 | 1.2 | 2.84 | 1.6 | 1.7 | 1.4 |

**Analysis of sugar with HPLC**

Protease: Enzyme that hydrolyzes proteins to peptides and/or amino acids. The use of certain proteases in ethanol fermentation has been proven to improve fermentation in the following

ways: • Faster Fermentation Time • Higher Ethanol Yields • Enhanced Yeast Growth • More Efficient Filtration and Evaporation in downstream process steps • More consistent fermentation • More carbohydrate fermented • Reduced carbohydrate in thin stillage

Yeast require certain nutrients to grow and maintain their population in order to convert glucose into ethanol. These may include the following: • Free Amino Nitrogen • Peptides and amino acids • Vitamins and Minerals (Inositols, Zinc, etc.) If yeast nutrition is not maintained, then the fermentation will suffer and result in lower rates and yield of ethanol formation. Nitrogen sources such as Urea, Ammonia, etc. can be added. However, this tends to give only Free Amino Nitrogen.

## 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 (Yoon *et al*., 2007). The popular cases of biomass based fuels production in developed countries may 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 butanolproduction fromlignocellulosic biomass (Zhang *et al*., 2004). 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.

Fermentation of available sugars in cellulosic biomass have potential to provides important products like acetone, butanol, ethanol and similar other alcohols, that could 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 complex cellulosic 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 lignocellulosicbiomass 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 process of integration and optimization to reducing energy consumption as well as to increase yields of alcoholic fuels from raw materials could decrease its cost of production and make it more economically competitive

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