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PRODUCTION AND UTILIZATION OF BIOETHANOL AS FUEL TO MINIMIZE ENVIRONMENTAL EFFECT

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ABSTRACT

Bioethanol is a high octane number fuel which is produced from fermentation of different types of plant base biomass

Bioethanol may be used as a fuel in an internal combustion engine and it is more similar to gasoline.. Bioethanol is produced from starch- or sugar-based material such as corn grain , sugar cane or from cellulosic feed stocks

Biofuel can play a great role in Pakistan because country is oil dependent. 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 develop a approach for the management of agriculture as well as other organic wastes utilization for production of alcoholic fuels. Therefore cellulosic materials like wheat and rice straws as well as fruit wastes were used in this study. Samples were analyzed for different parameters. Biological and chemical pretreatments were compared for each substrates. Efficiency of microbial enzymes for saccharification of agricultural substrates was evaluated. It is expected that outcome of this study will help to increase production of biofuels and to reduce burden of imported fossil fuels.

Key words; Fossil fuels, Bio ethanol , Biomass, Green house gases, Climatic changes

INTRODUCTION

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 or biodiesel could reduce environmental impacts and give advantages on social as well as economical levels (Malakar et al., 2020) .

Various alternatives to generate sustainable biofuels are being investigated. Biological energy resources are like bioelectricity, biogases, biodiesel and bio alcohols. 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 (Chenubini , 2010).The production of alcoholic fuels 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 (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 Wong, 2001).

Biomass is a vital energy resource in Pakistan because of agricultural 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 (Malakar et al., 2020). 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%) (Li et al., 2014).Various renewable energy resources include different agricultural substances like green leaves, fruit shells, straws, nut shells and fruit seeds . 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 agro waste 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 grasses can be grown all over the year worldwide, 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.

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 Dale. 2005). *Saccharomyces cerevisiae* (known as baker s years) single celled eukaryotes which is frequently used in fermentation process for production ethanol and other alcoholic products. Therefore current study was undertaken for Chemical and biological analysis of cellulosic biomass for various parameter required for alcohol fuels like bioethanol production

MATERIAL AND METHODS

Collection of Agricultural Substrates

About 1 Kg of biomass samples like wheat straw, rice straw and corn stover 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 . 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 (AOAC, 1990).

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).

Chemical Pretreatment

Acid like H_2SO_4 based pretreatment experiment was performed (1.0, 1.5 and 2% of acid) 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 each bottle and the contents were emptied on filter paper. After filtration, the solid washed away with 300 ml distilled water in order to neutralize the pH. The filter paper was than dried at 105 °C and weighed.

Figure 1. Biomass

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 (Riberio 2013; Iram et al., 2021; Maria et al., 2021).

Saccharomyces cerevisiae

Saccharomyces cerevisiae (also known as "Baker's Yeast" or "Brewer's Yeast") is a unicellular fungus responsible for alcohol production and bread formation.. As a rapidly reproducing eukaryote, *Saccharomyces cerevisiae* is a widely used model organism that has allowed scientists to better understand molecular, cellular, and biochemical processes. The species has been instrumental in brewing for ethanol and other alcohols from different substrates , mostly provide good production at 25^{0}C and pH 5. Therefore in current study about 100 ml enzymatically hydrolysed solution was used for fermentation in reaction bottle for separate hydrolysis and fermentation. These reaction bottles were placed in shaking incubator having 120 rpm at 37^{0}C for 72 hours and pH 5. The ethanol concentration was determined by alcohol meter after 72 hours, and values were expressed as % ethanol obtained from wheat straw, from rice straw and from corn stover. Among these three substrates wheat straw produced higher yield of various alcohols (Tran et al., 2013).

Analysis of sugar and alcohol by using HPLC

All the samples and standard solution of glucose was passed through the 0.22 μm filter prior to analysis. About 20 μl of 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 (Feranndes et al., 2015; Sluiter et al., 2008; Tao et al., 2014; Zhao et al., 2012).

Statistical analysis

Data obtained was statistically analyzed by using ANOVA 1 for means and standard deviation

RESULTS AND DISCUSSION

Results regarding chemical analysis of biomass samples as well as fermentation of sugars into ethanol and other products l are given in the following sections. Therefore in current study acetone- butanol - ethanol (ABE) were produced from organic wastes material of agriculture and municipal sources by using bacterial fermentation.

Biomass analysis

Data in table 1 represents various parameter found in biomass samples. Whereas ligno-cellulosic contents of the samples are given in table 2. It was observed that wheat straw has higher cellulosic contents as compared to other substrates used for analysis.

Table 1. Proximate analysis (%) of biomass samples

Substrate	**Dry Matter**	**Moisture**	**Crude Protein**	**Crude Ash**	**Crude Fiber**	**Ash**
Wheat straw	92.13±0.48	8.73±0.24	8.25 ±0.23	8.19±0.36	35.46±0.44	6.38±0.6
Rice straw	90.16±0.26	7.16±0.25	7.26 ±0.22	8.15±0.36	36.45±0.45	6.75±0.3
Corn stover	93.46±0.45	8.86±0.34	6.65 ±0.26	6.82± 0.45	37.96±0.37	5.76±0.5

Analysis of organic wastes samples

Table 2. Chemical analysis (%)of biomass samples

Substrate	NDF	ADF	Hemicellulose	Cellulose	Lignin
Wheat straw	84.5±0.56	56.75±0.27	29.13 ±0.26	31.17±0.38	25.47±0.48
Rice straw	83.14±0.25	55.16±0.28	27.16 ±0.24	29.18±0.46	26.43±0.47
Corn stover	78.5±0.57	53.5±0.34	25.6±0.36	27.5±0.68	24.5±0.45

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

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^{o}C 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 (Figs 2-5).

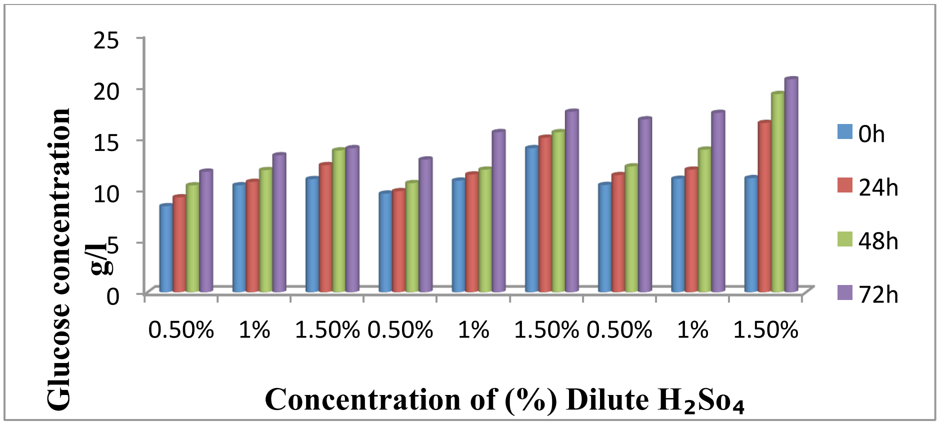

Figure 2. Glucose yield obtained from Wheat straw at H_2SO_4 pretreatment conditions at 120^0C

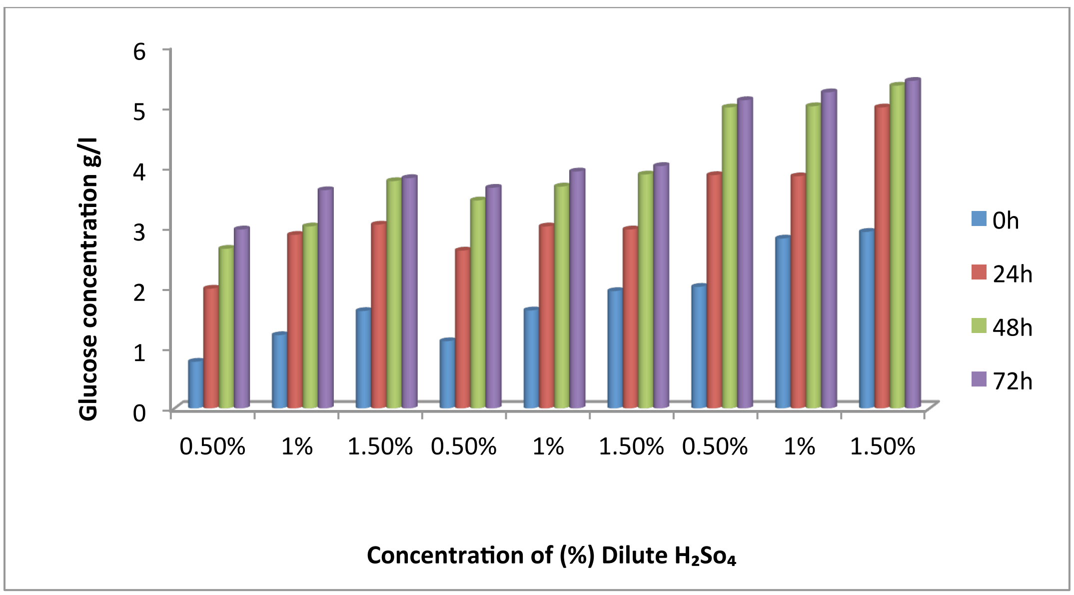

Figure 3. High glucose yield obtained from Rice straw at H_2SO_4 pretreatment conditions at 110 ^oC.

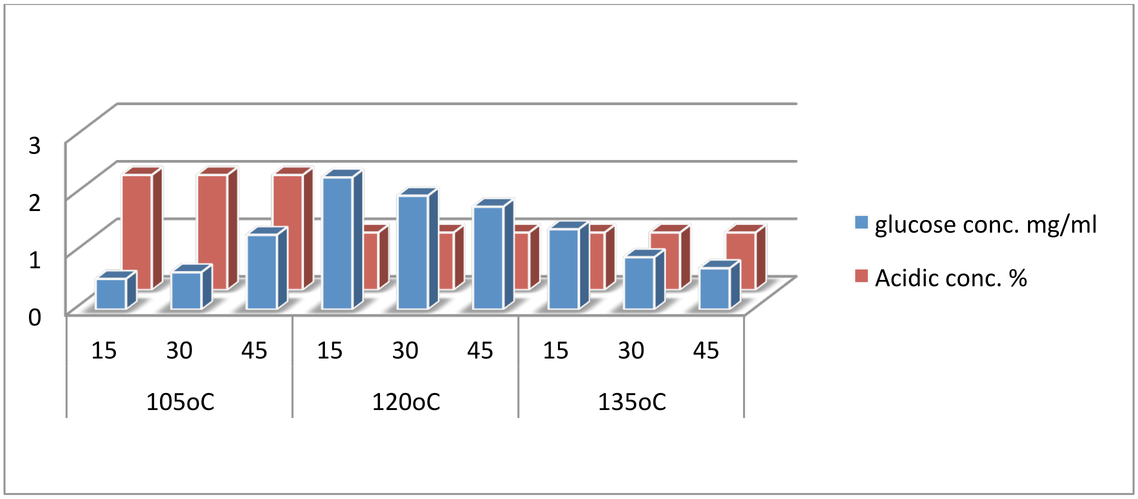

Figure 4. Comparison of glucose concentration at various temperatures after dilute acid pretreatment.

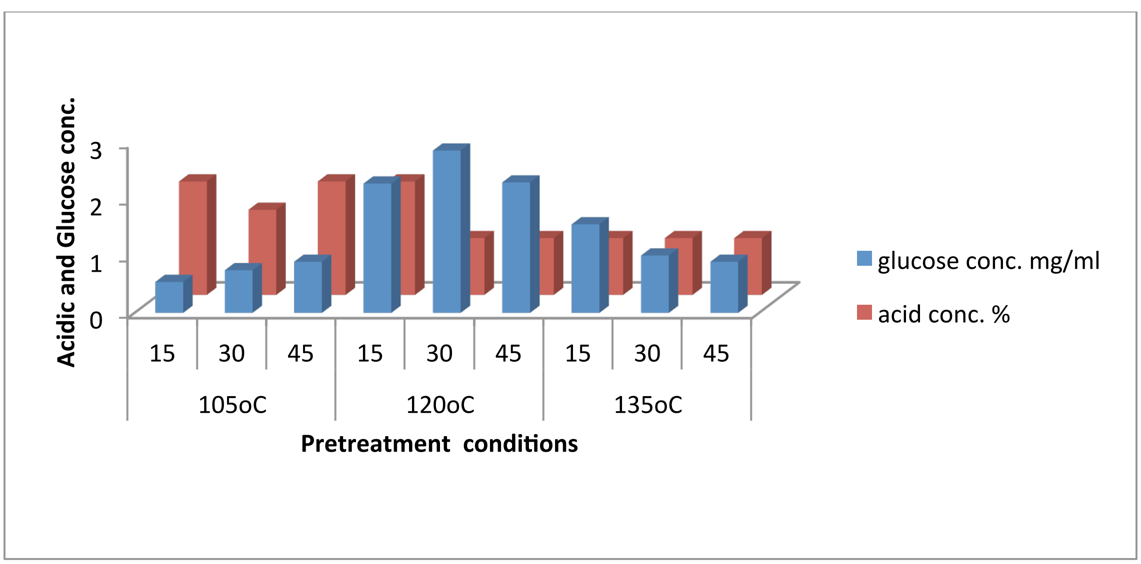

Figure 5. Comparison of glucose concentration at various temperature

Saccharification of biomass samples with enzymes

Higher maximum amount of glucose was released from agro waste up to 36 hours so there was no need to run experiment for more hours (Fig. 6). This released sugar, can than further be used for fermentation experiments (Huang et al., 2015; Dheeran et al., 2012; Garcia et al., 2011). Glucose (11.55 mg/ml) released during the first 48 hours after the addition of enzyme and after that sugar released and became constant as observed from the straight line (Fig.6).

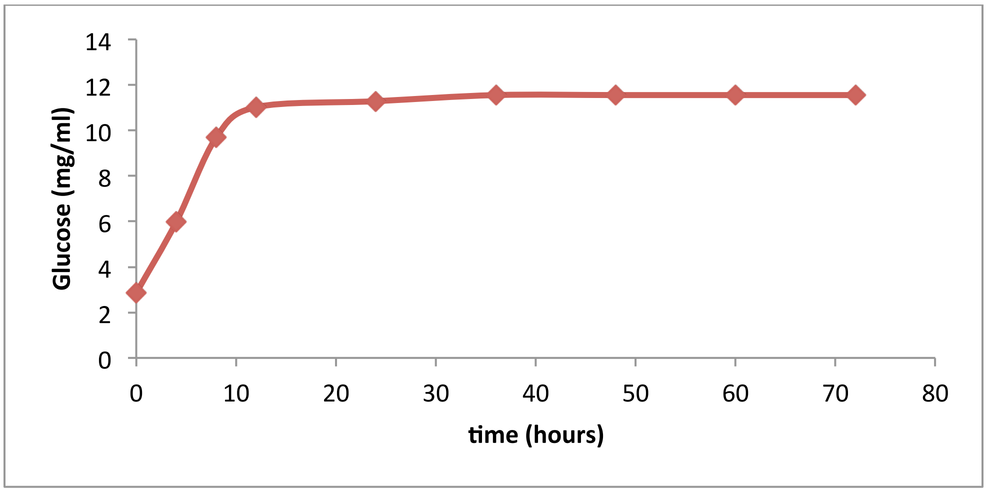

Figure 6. Enzymatic Saacharification of sugar from biomass

Fermentation

Significantly higher concentration of butanol was obtained from different substrates as shown in Tables 3 to 5. 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 cellulosic but lower lignin contents were found and these contents make agrowaste a better candidate for alcohol fuel production (Gregg and saddler, 1996; ; Hanifeng et al., 2015; Jiang et al., 2015).

Fermentation with *Saccharomyces cerevisiae*

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 higher butanol quantity at acidic pretreatment 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 peel waste . 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. 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 (Kathleen et al., 2015; Moretti and Thorson, 2008; Quershi and Blaschek, 2000).

Table 3. Acetone, Butanol and Ethanol production (%) from various biomass samples during fermentation by using *Saccharomyces cerevisiae*

Samples	Acetone%	Butanol%	Ethanol%
Wheat straw	1.7	7.5	2.5
Rice straw	1.5	6.8	1.8
Corn stover	1.6	6.5	2.1

ABE production from Biomass samples

Table 4. Ethanol production along with other products from biomass by H_2SO_4 pretreated samples and *Saccharomyces cerevisiae* fermentation

Samples	Acetone %	Butanol%	Ethanol%
Wheat straw	2.7	5.6	7.5
Rice straw	2.5	5.6	6.2
Corn stover	2.1	5.4	6.3

ABE production from Biomass sample

HPLC Analysis of reaction mixtures

The enzymatically hydrolyzed samples of acidic pretreatment of wheat and rice straws etc., 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 with drawn 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. 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 6).

Table 6. 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	8.5	23.55	26.4
Cellobiose	7.4	2.12	1.15
Xylose	11.5	5.6	5.8
Arabinose	11.0	2.5	1.7
Mannose	12.3	1.6	2.6
Galactose	15.5	1.7	1.8
Furfural	42.5	1.6	2.62
HMF	28	1.3	2.85

Analysis of sugar with HPLC

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 a great importance. 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 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 ethanol production fromlignocellulosic biomass (García et al., 2011; Riberio, 2013). 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, 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 (Iram et al., 2021) . 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 combating climate change, as it help to reduce level of carbon emission release from traffic etc. 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 (Maria et al., 2021).

CONCLUSION

Renewable and sustainable energy resources are the best alternative of conventional fuels and energy sources . Bioconversion of lignocellulosic biomass into alcoholic fuels (ethanol etc) 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 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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