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Production of Bioethanol from Lignocellulosic Biomass

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ABSTRACT

This study deals with the use of renewable lignocellulosic biomass to synthesis ethanol as an alternative energy source to crude oil using a low-cost feasible process. Lignocellulosic biomass usually contains hemicellulose and cellulose which could be converted to fermentable sugar like glucose. Renewable agricultural byproducts like sugarcane bagasse, wheat straw, water hyacinth, ragi, and rice straw were selected for producing ethanol. They were pretreated with mild, dilute acid using 2% H2SO4 (v/v), followed by enzymatic saccharification using locally isolated fungal species Aspergillus niger to convert complex polysaccharide into simple sugar. Optimization of reaction condition was carried out with the help of chemically predefined media at temperature of 38°C and pH 4.8. The reduced sugars were estimated using the dinitrosalicylic acid method. Fermentation was carried using yeast Saccharomyces cerevisiae by stationary fermentation method at temperature of 35°C under anaerobic conditions. Produced ethanol was estimated using NaOH-Iodine precipitation method. Maximum ethanol yield was obtained with sugarcane bagasse around 11.90 g/lit followed by wheat straw 9.56 g/lit, rice straw 8.84 g/lit, ragi straw 7.01 g/lit, and water hyacinth 6.19 g/lit of ethanol in fermentation.

Key words: *Cellulase, Ethanol, Saccharification, Fermentation, Lignocellulosic biomass, Pretreatment.*

1. INTRODUCTION

Consumption of energy has increased steadily over the last few decades as the population of the world has grown, and more countries have become industrialized. Crude oils have been the major natural resource to meet the growing demand for energy. Along with this, the usage of the fuels direct to global warming, environmental pollution, and other related hazards. If the current trend of crude oil utilization continues then, it is predicted that the annual global oil production would decline from the current 30 billion barrels per annum to approximately 5 billion barrels in 2050 [1]. Therefore, at present, there is a growing demand for alternative energy sources worldwide. One such source for substitution to transportation fuel is the renewable energy which is obtained from biomass. Production of liquid biofuels from lignocellulosic biomass will significantly reduce the dependence on petroleum-based fuels and therefore it has become a research area of great interest to many research scientists and government agencies [2]. Due to the recent advancement in agriculture and biotechnology, it is possible to propose newer and cheaper techniques of producing biofuels like bioethanol from lignocellulosic biomass [3].

Bioethanol has a privileged octane number, broader flammability restrictions, higher flame speeds, and high heats of vaporization than compared to gasoline. These characteristics allow for greater compression ratio and shorter burn time, leaner burn engine, which leads to theoretical efficiency benefits over gasoline in an internal combustion engine. Ethanol is also a safer alternative to methyl tertiary butyl ether, which is common additive to gasoline used to provide cleaner combustion [4]. Ethanol also represents a closed carbon dioxide cycle since after burning of ethanol, released CO_2 gasses are recycled back to plants materials as they use CO_2 to produce cellulose during photosynthesis. Ethanol derived from the biomass is only liquid transportation fuel which does not contribute to greenhouse gas effect [5,6].

Potential source intended for low-cost ethanol production is the utilization of lignocellulosic biomass such as grasses, agricultural residues, wood chips, and

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sawdust. Extensive research is being conducted from last few decades on the conversion of lignocellulosic biomass to ethanol [7]. This conversion involves a two-step process: Hydrolysis of hemicellulose and cellulose in the lignocellulosic biomass to fermentable sugar and the fermentation of sugar into ethanol. The hydrolysis is catalyzed by a cellulase enzyme or by acid hydrolysis, and the fermentation is carried out using yeasts or bacteria. The presence of hemicellulose and lignin make access of cellulase enzymes to cellulose difficult and hence pretreatment steps was carried out to reduce hemicellulose and lignin content [8].

Bioethanol can be used to decrease a nation's dependency on volatile imported fossil fuels and bring about a socio-economic development with a reduced impact on the environment which helps keep a check on climate change while also addressing the inherent challenges of biomass disposal [9,10]. Hence, the present study was undertaken to utilize renewable lignocellulosic biomass to produce bioethanol. The objective of the study deal with the comparison of ethanol production using different biomass likely bagasse, ragi straw, wheat straw, rice straw, and water hyacinth. The production of ethanol is carried by locally isolated fungal strain *Aspergillus niger* which enzymatically saccharifying the extracted hemicellulose and cellulose into simple monosaccharides and the fermentation was carried by *Saccharomyces cerevisiae*.

2. MATERIALS AND METHODS

2.1. Raw Materials

Renewable lignocellulosic biomass such as sugarcane bagasse, wheat straw, rice straw, ragi straw, and water hyacinth were collected from various locations around Bengaluru to be utilized as substrates. These substrates were first washed with water for removing impurities present on them then they were subjected to sun-drying to remove moisture from the substrates. To convert the biomass into extremely fine powdered particles, the biomass was first treated in a mixer-grinder to reduce the size of particles; the size was further reduced by putting the biomass in a ball mill. Finally, the grounded material was sieved to get a uniform size. The particle sizes of substrates considered were 0.300 mm (wheat straw), 0.600 mm (bagasse), 0.425 mm (rice straw), 0.600 mm (water hyacinth), and 0.850 mm (ragi straw).

2.2. Pretreatment of Biomass

Pretreatment of biomass was carried by dilute acid hydrolysis method. The biomass was soaked in H_2SO_4 (2% V/V) and the slurry was subjected to high-pressure steam at 121°C in a vertical autoclave for 2 h, after which the steam of the autoclave was released, and the solids were separated from the liquid by filtration using a muslin cloth [11,12].

2.3. Chemical Analysis of Biomass

Biomass was subjected to estimation of total sugars, reducing sugars, non-reducing sugars (cellulose) and protein estimation [13-15].

2.4. Microorganisms Used

A. *niger* was isolated from soil samples taken from 3 different locations of Bengaluru such as Koramangala, Lalbagh and BMS College. The soil samples were mixed with sterile distilled water (DW) to prepare a stock solution, and a series of dilutions were made using sterile test tubes up to 10-7 dilution factor. The fungus was cultured and maintained on potato dextrose agar plates at 32°C; sub cultured until a pure isolate was obtained [16]. The grown isolates were identified as A. *niger* with the help of microscopic characterization [17] and MALDI-TOF sequencing method.

2.4.1. Inoculum development

For the preparation of inoculum 300 ml of DW was mixed with potato dextrose broth in an erlenmeyer flask, autoclaved, and allowed to cool. Inoculum was developed by transferring the culture of A. *niger* grown on potato dextrose Agar plates under sterile conditions of the laminar air flow chamber, the flasks were kept in a biological oxygen demand (BOD) incubator at 32°C for 7 days for the formation of mycelial mat which is used as inoculum in saccharification process [18].

S. *cerevisiae* (MTCC No: 3976) was obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh India. The organism was used to carry out fermentation process; it was sub-cultured in a YEPDA growth medium [19]. The plates were incubated at 30°C for 48 h.

2.5. Production of Bioethanol

The production of bioethanol is carried out using two important processes, namely saccharification and fermentation.

2.5.1. Saccharification

The substrates which had undergone pretreatment are used for saccharification process. Locally, isolated fungal species A. *niger* was used as inoculum. The substrates were autoclaved and inoculated with sporulating mycelial mat of A. *niger*. To optimize bioethanol production; the substrates were taken in three dissimilar variations as shown in Table 1.

Chemically defined medium is prepared as per the composition given in Table 2 for increasing the yield of reducing sugars necessary for fermentation [20].

Table 1: Design of saccharification experiment.

Experement no	1	2	3
Bagasse	100 g+200 ml DW	100 g+200 ml CMC	100 g+200 ml CDM
Rice straw	100 g+150 ml DW	100 g+150 ml CMC	100 g+150 ml CDM
Ragi straw	100 g+160 ml DW	100 g+160 ml CMC	100 g+160 ml CDM
Wheat straw	100 g+180 ml DW	100 g+180 ml CMC	100 g+180 ml CDM
Water Hyacinth	100 g+250 ml DW	100 g+250 ml CMC	100 g+250 ml CDM

DW=Distilled water, CMC=Carboxy methyl cellulose, CDM=Chemically defined media

Table 2: Composition of chemically defined media.

Constituents	g/L	Constituents	g/L
L-glutamic acid	0.3	Tween 20	3.3
NH_4NO_3	1.4	$MnSO_4$	1.6
KH_2PO_4	2.0	$ZnSO_4$	1.4
$CaCl_2$	0.3	$FeSO_4$	5
Peptone	7.5		

The stationary method of saccharification was employed, and the fermenter trays with substrates were kept in a BOD incubator at temperature of 34°C and pH 4.8 was maintained, the total sugars released was monitored on a daily basis for 7 days by dinitrosalicylic acid (DNS) method.

2.5.1.1. Saccharification using *A. niger*

The fungal species was selected due to its cellulolytic nature and can hydrolyze cellulose present in the substrates into simple sugars. This process can be also carried out using strong acids or by commercially available enzymes such as cellulase, pectinase, xylanase, which can be purchased from pharmaceutical companies, the only drawback is that it would be highly expensive. Hence, to carry out a cost-effective process locally isolated *A.niger* was employed to convert complex polysaccharides into simple fermentable sugars [21].

2.5.2. Fermentation

For the production of bioethanol, *S. cerevisiae* was procured from MTCC Chandigarh and employed. Stationary method of fermentation was used wherein the yeast inoculum was added to 500 ml of glucose syrup solution obtained after saccharification process, the reagent bottles containing the glucose solutions were sealed to maintain anaerobic conditions [22]. The bottles were kept in BOD incubator at 35°C, during fermentation process samples were analyzed every 24 h to estimate the amount of ethanol produced using NaOH-iodine precipitation method; the entire process was carried out for 8 days.

Table 3: Chemical composition of pretreated biomass % (w/w)

Biomass	Total sugars	Reducing sugars	Cellulose
Bagasse	22.20	7.9	42
Rice straw	16.32	3.6	39.20
Ragi straw	14.58	4.5	32.52
Wheat straw	19.53	5.4	32.92
Water hyacinth	13.82	2.8	19

3. RESULTS AND DISCUSSION

Bioethanol was produced by saccharification and fermentation of lignocellulosic biomass such as sugarcane bagasse, wheat straw, rice straw, ragi straw, and water hyacinth. Table 3 shows the estimation of total sugars, reducing sugars, and cellulose content of pretreated materials.

The differences in the results obtained when compared to other research works may be attributed to the growing locations, seasons, stage of harvest, harvesting methods, and analytical procedures used.

3.1. Saccharification

During this process, the total reducing sugars (glucose) released was monitored using DNS method, which indicated that the amount of sugars released increased from day 1 to 6. The highest amount was released on the 6^{th} day of saccharification, after which the concentration of sugar released starts to decline slightly. Results of substrates treated with different variations are given in Table 4.

Among the three variations used, it is clear that chemically defined media (CDM) has resulted in producing large quantities of reducing sugars when compared to the other variations. CDM supports the growth of A. *niger* by providing nutrients necessary for the growth and survival of the organism. CDM provides the necessary environmental conditions for the organism to produce cellulolytic enzymes such as cellulase, pectinase, and xylanse which effectively

Table 4: Glucose concentration (µg) during saccharification.

Substrate	Variation	Day 1	Day 3	Day 5	Day 7
Bagasse	DW	1660	2890	3880	4250
	CMC	2110	3450	4760	5220
	CDM	2920	5570	7780	8540
Wheat straw	DW	1400	2180	3120	3380
	CMC	1960	2970	3950	4430
	CDM	2350	4260	5800	6680
Rice straw	DW	1180	1850	2420	2750
	CMC	1680	2450	3270	3490
	CDM	1960	3350	4540	4880
Ragi straw	DW	1240	1960	2660	2990
	CMC	1740	2580	3440	3860
	CDM	2120	3560	4820	5480
Water hyacinth	DW	720	1120	1190	2220
	CMC	940	1880	2500	2790
	CDM	1060	2100	3200	3720

DW=Distilled water, CMC=Carboxy methyl cellulose, CDM=Chemically defined media

break down complex polysaccharides into simple sugars. Bagasse produces the highest amount of reducing sugars around 8600 µg followed by wheat straw at 6700 µg, ragi straw at 5520 µg; rice straw produced 4920 µg and water hyacinth was the least with 3770 µg of sugars produced. The order of reducing sugars produced by substrates is represented as follows:

Bagasse > Wheat straw > Ragi straw > Rice straw > Water hyacinth

3.2. Estimation of Bioethanol

The estimation of ethanol was carried out by NaOH-iodine precipitation method, in which samples were collected from the fermentation media and observed for amount of ethanol produced from day 1 to 8 from the different variations of substrates. The results of ethanol produced are presented in Figures 1-3.

From the results obtained during fermentation, there seems to be an increasing trend in production of ethanol from the 1st to 6th day, after which there is no drastic increase in the amount of ethanol produced. The highest amount of ethanol was obtained from bagasse around 11.90 g/lit on the 6th day which is also in accordance with release of reducing sugars during saccharification followed by wheat straw 9.56 g/lit, rice straw 8.84 g/lit, and ragi straw with 7.01 g/lit. The least amount of ethanol was produced from water hyacinth about 6.19 g/lit. Among the three variations, CDM has resulted in producing larger quantities of

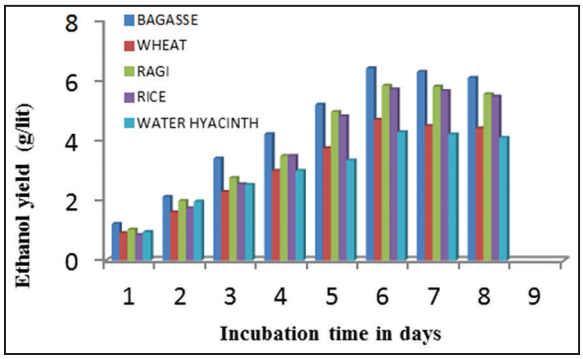

Figure 1: Estimation of ethanol variation.

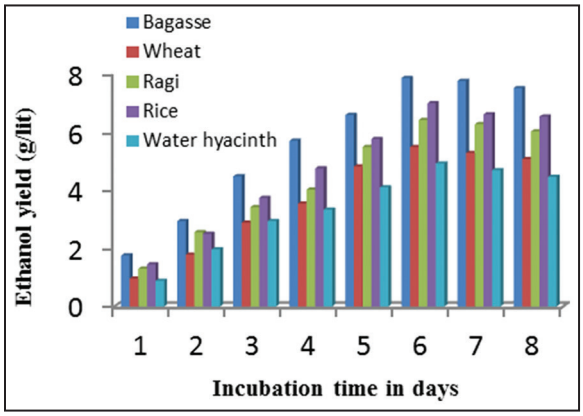

Figure 2: Estimation of ethanol variation: Carboxy.

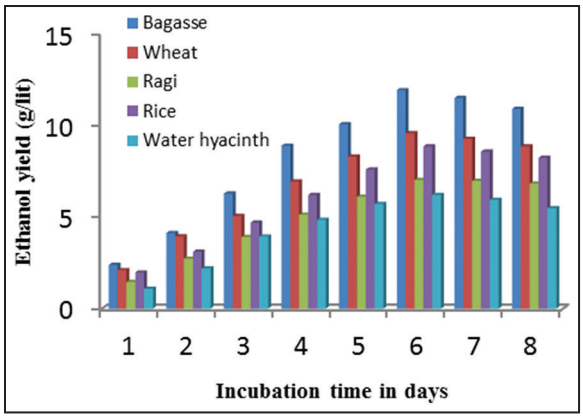

Figure 3: Estimation of ethanol variation: Chemically

ethanol. Based on the obtained results, the increasing order of ethanol production is:

Bagasse > Wheat straw > Rice straw >Ragi straw > Water hyacinth

By comparing the overall results obtained, it shows that cellulose utilization and cellulolytic enzyme activity and bioethanol yield are lower in the reagent bottles that contain glucose syrup from substrates treated with only distill water. Whereas in the bottles that contains glucose syrup from substrates treated with carboxymethyl cellulose and CDM have shown

Table 5: Amount of ethanol produced when treated with CDM.

Substrates	Ethanol (g/lit)	Substrates	Ethanol (g/l)
Bagasse	11.90	Ragi straw	7.01
Wheat straw	9.56	Water hyacinth	6.19
Rice straw	8.84		

CDM=Chemically defined media

increased cellulose utilization, cellulolytic enzyme activity, and bioethanol production. However, maximum amount of ethanol production was seen in substrates treated with chemically defined medium. Results show that CDM has given a higher ethanol yield when compared to other combinations as shown in the following graphs of Figures 1-3.

4. CONCLUSION

Finally, we can conclude that renewable lignocellulosic biomass such as bagasse, wheat straw, water hyacinth, rice straw, and ragi straw can be used as a potential source of raw material for the production of bioethanol, this study focused on achieving a cost-effective process for the production of ethanol and reduction in cost was achieved using freely available raw materials and also not opting for commercially available enzymes which are highly expensive for saccharification process. From the results obtained it can be concluded that pretreated lignocellulosic biomass can be treated with intact pure microorganism like A. *niger* for saccharification in which complex polysaccharides are converted into simple sugars. Furthermore, the use of CDM during saccharification has enhanced the overall cellulolytic activity, increasing cellulose utilization and in turn increases the total yield of reducing sugars needed for fermentation. Fermentation using *S. cerevisiae* in the present work resulted in the production of ethanol under anaerobic conditions at 35°C, the highest yield of ethanol was found in bagasse and wheat straw followed by rice, ragi straw, and water hyacinth which were treated with CDM is as per the Table 5.

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