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Production of Bioethanol from Lignocellulosic Biomass by Simultaneous Saccharification and Fermentation

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Abstract: ***Cellulose degrading microbes such as Bacillus subtilis, Aspergillus niger, and Trichoderma viride were isolated. Lignocellulosic biomasses selected for the production of ethanol by Simultaneous Saccharification and Fermentation were sugarcane bagasse, saw dust, rice chaff. These lignocellulosic biomasses were pretreated by alkaline pre treatment method. Enzymatic Hydrolysis was done by cellulose degrading microbes on the lignocellulosic biomass. Two fermentation microbes were selected which were Saccharomyces cerevisiae and Zymomonas mobilis (MTCC 2428) which was obtained from IMTech, Chandigarh. The distillate obtained from the fermentation broth was analyzed using High Performance Liquid Chromatography.***

Keywords: *Bacillus subtilis, Trichoderma viride, Aspergillus niger,* Lignocellulosic biomass, Simultaneous Saccharification and Fermentation.

1. Introduction

The production of bio ethanol has been tried with the food crops and also with the lignocellulosic biomass. The ethanol produced from the food crops were called first generation bio ethanol, where as the ethanol produced from lignocellulosic biomass were called as second generation biomass. But there were some concerns related to the first generation bio ethanol. Some of them were food security in which there was an issue that the use of food crops will increase the food prices. Large land acquisition will be required for the production of bio ethanol. Green house gases balance also seems to shift as the production of bio ethanol involves more and more food crops. And some environmentalist concern that bio ethanol production from crops might affect the biodiversity and water preservation[1].Fearing that the first generation of bio ethanol production might not be a suitable substrate for production of bio ethanol was research was switched over to lignocellulosic biomass, for bio ethanol production. Biomasses are biological materials that are taken from living organisms [2].These are the sources from which the energy can be derived. Lignocellulosic biomass refers to plants organic matter. The plant matter is made of lignocelluloses content which is the composition of three parts cellulose, hemicelluloses and lignin.

Cellulose is a linear and crystalline organic polymer. It consists of repeating sugar units of glucose that are linked together by β-1,4 glycosidic bonds[3]. . Hemicellulose which is made up of many heteropolymers, present along with cellulose in almost all plant cell walls. While cellulose is strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. Lignin is a complex of chemical compound commonly obtained from wood, and form an important part of the secondary cell walls of plants. Various pre-treatment methods are now available to fractionate, solubilise, and separate each component from lignocellulosic biomass. These include acid pre treatment such as concentrated sulphuric acid or hydrochloric acid, alkaline pre treatment such as sodium hydroxide, lime treatment, ammonia fibre expansion (AFEX) and many others such as sulphur dioxide (SO2), hydrogen peroxide (H2O2), steam explosion (auto hydrolysis), , wet oxidation, liquid hot water, carbon dioxide (CO2) explosion and organic solvent treatments [4]. There are five major steps which describes the process of conversion of lignocellulosic biomass to bio ethanol: (i) pre treatment of biomass to free the cellulose and hemicellulose from lignocellulosic biomass, (ii) break down of cellulose and hemicellulose to their simpler monomer units. From cellulose C6 sugars (hexoses) and from hemicellulose C5 sugars (pentose), (iii) Applying fermentation process and converting these sugars to produce ethanol, (iv) ethanol recovery by distillation process and (v) effluent treatment to remove any harmful chemicals before letting out the effluent in the environment [5].But to assume how effective is any given pre treatment it should meet the following criteria: (i) it should increase the yield fermentable sugar yields, (ii) to avoid, degradation of carbohydrates, (iii) minimize the formation of microbial growth inhibiting by-products, and (iv) minimize energy consumed, capital and operating costs. It is know that many of this pre treatment are utilize but their concentration and effectiveness differs with respect to any given substrate [6].

The current study is based on the isolation of cellulose degrading microbes and utilizing their ability for enzymatic degradation of the lignocellulosic biomass and production of

bio ethanol by using fermentation microbes and employing simultaneous saccharification and fermentation

2. Material and Methods

2.1 Isolation and Identification of microbes

Cellulose degrading microbes were isolated from soil and decaying wood. Bacteria were identified by Bergey's manual of determinative bacteriology[7], and fungi were identified by lactophenol cotton blue staining. *Saccharomyces cerevisiae* was obtained from the lab and *Zymomonas mobilis* (MTCC 2428) was procured from IMTech, Chandigarh.

2.2 Collection of sample

Three lignocellulosic biomass were chosen that were, saw dust which was collected from the local saw mill, sugarcane bagasse collected from juice shop, and rice chaff was collected from rice mill at Shimoga, Karnataka.

2.3 Pre-treatment

The biomass was alkaline pre-treatment with ammonia. For 1g of substrate was taken and 10ml of ammonia was added and was steamed at 100°C in pressure cooker for 30-45 min. After pre-treatment the biomass was neutralized by washing with water and was dried in sun before being used as substrate. Amount of reducing sugars were measured before and after the pre treatment.

2.4 Evaluating the enzyme assay of lignocellulosic biomass

The lignocellulosic biomass were taken as substrate in Minimal Salt media, and inoculated with respective organism. Cellulase enzyme activity was found according to CMC assay. The substrate and the microorganism showing the highest enzyme activity was taken for the production of bio ethanol by simultaneous saccharification and fermentation.

2.5 Fermentation

The fermentation was done based on Simultaneous Saccharification and Fermentation (SSF). Separate fermentation media was prepared for Bacteria and fungi. For bacteria the fermentation media was minimal salt mineral media containing $NaNO_3$ 0.2 g, KH_2PO_4 1g, $MgSO_4.7H_2O$ 2 g, $FeSO_4.7H_2$ O 0.05 g, KCL 2 g, yeast extract 5 g, tryptone 10g, carbon source 10 g, distilled water-1000ml pH-7.0[8]. For fungi the fermentation media composition were $NaNO_3$ 3.0g, KCI 0.5g, MgS04.7H2O 0.5g, KH2PO4 1.0g, FeSO4. 7H2O, 0.01 g, 10g of carbon source distilled water 1000ml at pH 5.6 [9]

2.6 Distillation

After the fermentation process was completed, the fermentation broth was taken for the process of distillation. The broth was heated up to 80°C and the distillate was collected in a conical flask. The distillate was measured with the help of measuring flask, and then transferred into glass bottle and wrapped with parafilm.

2.7 Analysis of distillate by HPLC

The process of estimation of ethanol was done by High Performance Liquid Chromatography (HPLC).Column used was C16, and the mobile phase prepared was water: methanol (70:30). Flow rate of the mobile phase was maintained at 1ml/min. Absorbance was measured at 210nm [10].

3. Result

3.1Isolation and identification of Cellulose degrading microbes

Cellulose degrading microbes such *as Bacillus subtilis*, *Aspergillus niger*, *Trichoderma viride*, were isolated and identified.

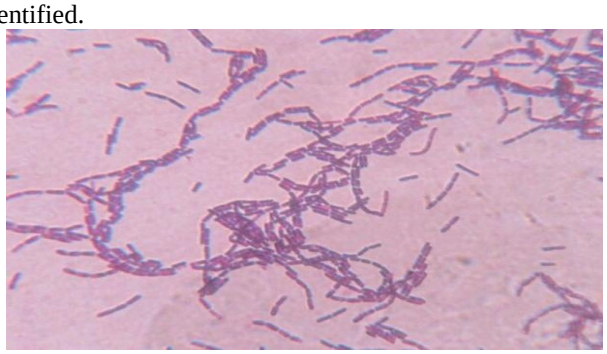

Figure 1: Gram positive rods *Bacillus subtilis*

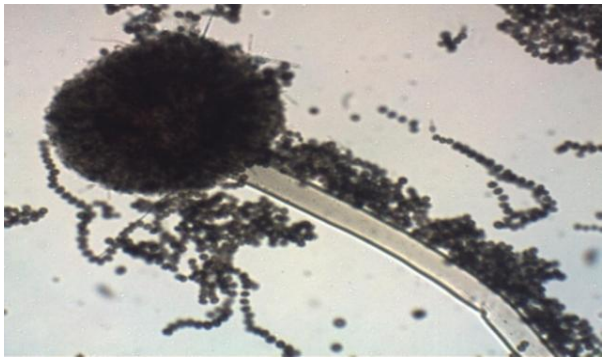

Figure 2: Lactophenol cotton blue staining of *Aspergillus niger*

3.2 Pre-treatment of Lignocellulosic Biomass

Three lignocellulosic material viz., sugarcane bagasse, saw dust, rice chaff, and were subjected to alkaline pre-treatment. The amount of reducing sugar before and after the pre-treatment was done by Dinitrosalicylic acid method. The highest content of reducing sugar was found in sugarcane bagasse, followed by saw dust and the least was found in rice chaff.

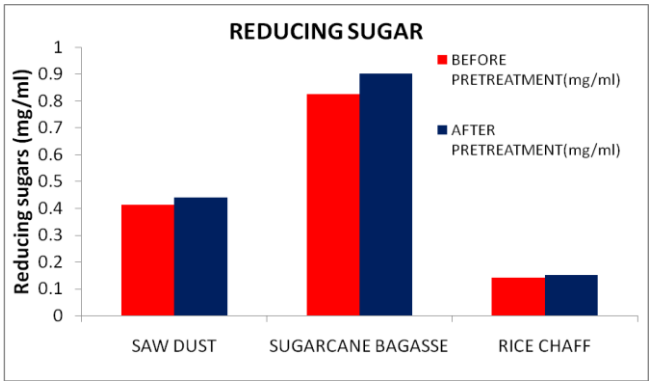

Figure 3: Estimation of reducing sugar in different substrate

Table 1: Estimation of reducing sugar content

Substrate	*Before pre-treatment(mg/ml)*	*After pre-treatment(mg/ml)*
Saw dust	0.412	0.44
Sugarcane bagasse	0.8241	0.9
Rice chaff	0.1413	0.15

3.3 Enzyme assay of Lignocellulosic biomass

1g of each pre treated lignocellulosic biomass was taken and added as a carbon source in 100ml of MSM media. The microbes were inoculated with each respective biomass at a constant pH of 5.6 for fungi and 7.0 for bacteria. Every 48 hr 10ml of cultural broth was taken and was checked for enzyme activity by CMC assay. It was found that *Aspergillus niger* showed the highest enzyme activity of 600.52IU/ml in sugarcane bagasse. *Trichoderma viride* showed the highest enzyme activity of 480.42 IU/ml in rice chaff where as *Bacillus subtilis* showed the highest enzyme activity of 390.34IU/ml in sugarcane bagasse. These biomass were selected as substrates for the production of bioethanol.

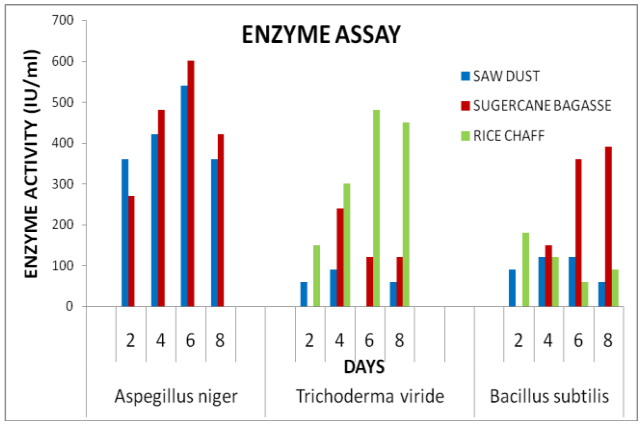

Figure 4: Enzyme activity of Microbes on different substrate

3.4 Fermentation

The microbes that showed the highest activity with respect to the given substrate were chosen. *Aspergillus niger* showed the highest enzyme activity on sugarcane bagasse. *Trichoderma viride* showed the highest enzyme activity on rice chaff. *Bacillus subtilis* showed the highest activity on sugarcane bagasse. 10g substrate was taken as a carbon source in MSM and the fermentation media was made up to 1liter.For fermentation by bacteria the pH was set to 7.0 and for both the fungi the pH was set to 5.6. After 5 days *Saccharomyces cerevisiae* was inoculated in the fermentation broth and after 10 days the fermentation broth was taken out for the process of distillation. Another set of batch was prepared and the above process was repeated with *Zymomonas mobilis* (MTCC 2428). The batch was then inoculated with *Zymomonas mobilis* (MTCC 2428) and after 10 days fermented broth was taken out for distillation. Simultaneous Saccharification and Fermentation process (SSF) was performed.

3.5 Distillation

The distillate obtained from the fermentated broth was collected and was measured. The highest distillate obtained was from the substrate sugarcane bagasse that was simultaneously saccharified and fermented by *Aspergillus niger* and *Saccharomyces cerevisiae* which was found to be 35ml. With same substrate *Bacillus subtilis* and *Saccharomyces cerevisiae* distillate was found to be 18ml. With *Aspergillus niger* and *Zymomonas mobilis(MTCC 2428)* the distillate was found to be 15ml. Rice chaff that was simultaneously saccharified and fermented by *Trichoderma viride* and *Saccharomyces cerevisea* the distillate obtained was 25ml.

Table 2: Amount of distillate obtained from the biomass and type of microorganism used

Substrate	**Microorganism**	**Distillate(ml)**
Sugarcane bagasse	*Aspergillus niger* and *Saccharomyces cerevisiae*	35
Sugarcane bagasse	*Bacillus subtilis* and *Saccharomyces cerevisiae*	18
Rice chaff	*Trichoderma viride and Saccharomyces cerevisiae*	25
Sugarcane bagasse	*Aspergillus niger and Zymomonas mobilis MTCC 2428*	15

3.6 Analysis of distillate by HPLC

After the distillates were obtained they were filtered and sonicated. The samples prepared were injected into the HPLC machine and the chromatogram was observed.

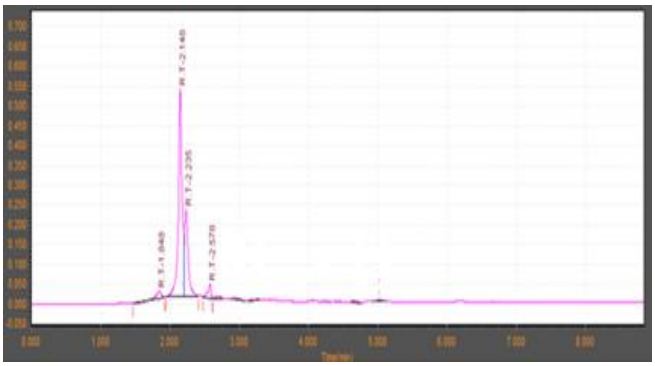

Figure 5: Chromatogram showing peak of 5% ethanol run in HPLC as standard

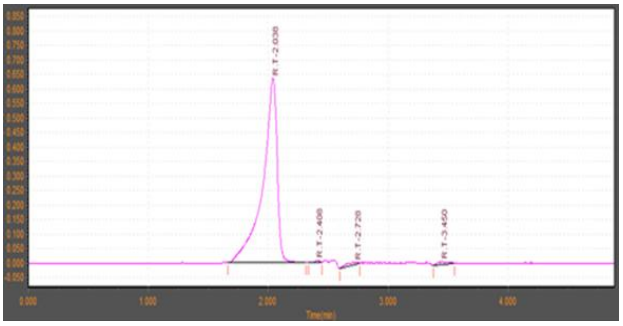

Figure 6: Chromatogram showing distillate sample obtained from sugarcane bagasse which was simultaneous saccharified and fermented by *Bacillus subtilis* and *Saccharomyces cerevisiae.*

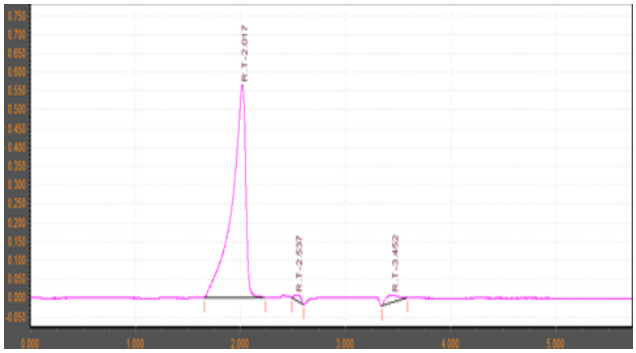

Figure 7: Chromatogram showing distillate sample obtained from sugarcane bagasse which was simultaneous saccharified and fermented by *Aspergillus niger* and *Saccharomyces cerevisiae.*

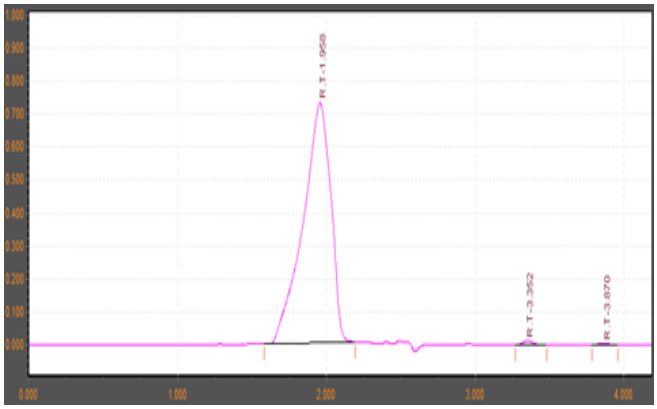

Figure 8: Chromatogram showing distillate obtained from Rice chaff which was simultaneous saccharified and fermented by *Trichoderma viride* and *Saccharomyces cerevisiae.*

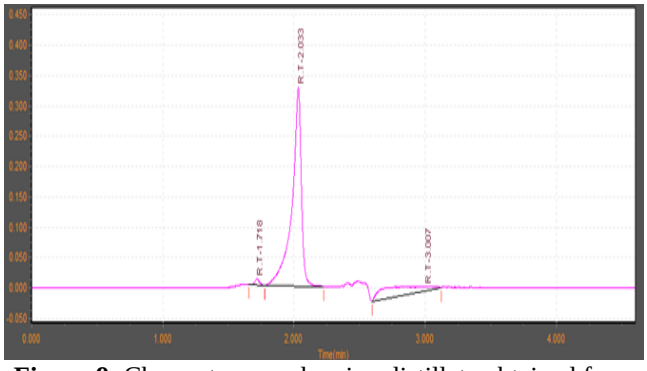

Figure 9: Chromatogram showing distillate obtained from sugarcane bagasse which was simultaneous saccharified and fermented by *Aspergillus niger* and *Zymomonas mobilis(* MTCC 2428).

4. Discussion

The present work carried out with the aim to produce bioethanol from lignocellulosic biomass by simultaneous saccharification and fermentation. Cellulose degrading microbes were isolated and identified. From the above study it was found out that for the production of bio ethanol from lignocellulosic material, firstly the cellulase enzyme activity of each microorganism was checked with each substrate. Sugarcane bagasse and rice chaff were chosen on which the highest cellulase activity of microorganism were recorded. In a study conducted it was found that the substrates such as Banana peel and sugarcane waste had more capability to produce ethanol as compared to waste paper by using cellulase enzyme [11]. Simultaneous Saccharification and Fermentation process was performed. After the saccharification process was over the fermentation microbes such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* (MTCC 2428) were incorporated for the conversion of soluble sugars into ethanol. After the Fermentation process the broth was taken was distillation. The distillate obtained was measured and they were analysed for ethanol content. The reference was made by 5% ethanol and it was runned in HPLC. In the same way the distillate were also runned in HPLC. The peak of the reference and the sample were compared based on their retention time. In a similar study conducted for the analysis of the contents of ethanol were determined using Gas Chromatography (GC), propenol was used as standard [12]. In the comparison of these two fermenting microbes it was found that *Saccharomyces cerevisiae* was able to ferment the soluble sugars easily but *Zymomonas mobilis (MTCC 2428)* showed results only with sugarcane bagasse that was saccharified by *Aspergillus niger*.

5. Conclusion

From the present study conducted it was found that *Aspergillus niger* and *Bacillus subtilis* showed the highest cellulase activity on sugarcane bagasse indicating it as a good substrate for the production of cellulase enzyme and also a very low cost, readily available lignocellulosic biomass for the production of bioethanol.

6. Future Scope

The isolated microorganism can be further studied and used to optimize the fermentation process for the production of Bioethanol.

7. Acknowledgement

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Samuel Xavier Christopher, final year M. Tech student at Department of Biotechnology, Acharya Institute of Technology, Bangalore. For the partial fulfillment of M.tech the project was carried out under the guidance of Dr. S.M.Gopinath, Prof and HOD, under the mentorship of Prof. Ismail Shareef. M and Manasa Satheesh, Director Genewin Biotech in studies of production of bioethanol from lignocellulosic biomass.