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PRODUCTION OF BIOETHANOL FROM AGROWASTE USING CELLULASE ENZYME ISOLATED FROM *TRAMETES SP.*

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ABSTRACT

Bioethanol is one of the most important alternative renewable energy sources that substitute the fossil fuels. Lignocellulosic substances such as agricultural wastes are attractive feedstocks for bioethanol production because agricultural wastes are cost effective and renewable. The main objective of the current study deals with the conversion of lignocellulosic waste materials such as Banana pseudostem, Bengal gram husk, coir pith, cotton pod and wheat bran into liquid based energy fuel. The experimental studies have been carried out to optimize the pretreatment process of ligncellulosic substances and efficient conversion of cellulose to sugars using the enzyme cellulose, isolated from *Trametes sp.* In the pretreatment process, the lignin, hemicellulose, and cellulose are separated to enhance the hydrolysis process. The cellulase was used for the hydrolysis process, which helps in converting the cellulose to sugar which was analysed using Dinitrosalicylic acid. The reducing sugars were fermented to produce bioethanol using the yeast inoculums and the yield was estimated using HPLC. Among the lignocellulosic substances used in the present study, wheat bran found to be a good producer of ethanol.

Keywords: Dinitrosalicylic acid, HPLC, Lignocellulose, Bioethanol, *Trametes* sp., Cellulase.

INTRODUCTION

Biofuels obtained from renewable sources can be classified on the basis of their production technologies, biofuels of first and second generation and biofuels of third and fourth generation. The first-generation fuels refer to biofuels made from plants rich in oil or sugar. The second-generation biofuels (*Biomass to Liquid*) are made from organic materials, such as straw, wood residues, agricultural residues, reclaimed wood, sawdust, and low-value timber. Biofuels of the third and fourth generation are produced from algae by using modern gene and nanotechnologies. Disposal of agrowastes by burning was an accepted practice. This practice is now being challenged due to concern over the health effects of smoke from burning fields (Ballerini*et al.*, 1994). The lignin component acts as a physical barrier and must be removed to make the carbohydrates available for further transformation processes. Therefore, the pretreatment is a necessary process for utilization of lignocellulosic materials to obtain ultimately high degree of fermentable sugars (Patel *et al.*, 2007). Pretreatment is generally conducted using an acid, alkali, steam or organic solvent, etc., To efficiently utilize lignocellulosic products; pretreatment is required to hydrolyse the hemicelluloses to make the celluloses more accessible to the enzymes. The goal of the pretreatment process is to remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by hydrolysis, (2) avoid the degradation or loss of carbohydrate, (3) avoid the formation of byproducts that are inhibitory to the subsequent hydrolysis and fermentation processes, and (4) be cost-effective (Kumar *et al.*, 2009). Bioconversion of cellulosic biomass into fermentable sugar, for production of ethanol using microorganisms, especially cellulose degrading fungi, makes bioethanol production economic, environmental friendly and also renewable (Patel *et al*, 2007). For the conversion of biomass to fuel, the cellulose and hemicellulose must be broken down into their corresponding monomers (sugars), so that microorganisms can utilize them. Three major hydrolysis processes are typically used to produce a variety of sugars suitable for ethanol production: dilute acid, concentrated acid, and enzymatic hydrolysis. Enzymatic activity in lignocellulose hydrolysis gives a good yield and minimum amount of by-products; it has lower energy consumption, milder operating conditions and represents an environmentally friendly processing method (Wingren*et al.*, 2005). Cellulase activity is mainly evaluated using a reducing sugar assay to measure the end products of cellulase hydrolysis activities (Wu *et al.*, 2006). Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular

enzymes: 1, 4- β endoglucanase, 1, 4- β- exoglucanase, and β-glucosidase (β-D-glucosideglucohydrolase or cellobiase). One of the main constraints in obtaining higher rates of ethanol production is the inhibition of yeast metabolism by both high concentration of sugar substrate as well as the end product. Normal fermentation processes typically cease when a beverage has achieved an alcohol content of 10 to 15 percent. Distillation is the process by which ethanol is boiled from the fermented mixture and captured, producing a liquid with a much higher concentration of alcohol.The aim of the study deals with the optimization of different agrowastes with pretreatment process. Specifically, the White rot fungus *Trametes* sp. as a biological pretreatment agent for agrowastes was explored.

2.MATERIALS AND METHODS

2.1 Fungal Culture

Trametes sp. obtained from Mushroom collection centre, CAS in Botany, University of Madras, Chennai. The culture was maintained through periodic transfer onto PDA medium at 28±2ºC.

2.2 Method of raising the fungus

The test fungi were first raised on PDA. The discs were made with a sterile cork borer of 8 mm diameter from the margin of the 7-day-old dark grown culture. These discs were then transferred to petridishes of 8 cm diameter containing Potato Dextrose Agar medium.

Whenever a liquid medium was used 50mL of basal medium was dispensed in to 250 mL Erlenmeyer flasks and inoculated with 8 mm discs of the mycelium.

2.3 Quantitative Screening for Cellulase activity

The culture was screened for cellulase activity by the method of Mandel*et al.*, 1976 as follows: culture supernatant (0.5 mL) was added to 0.5 mL of 0.05 *M* citrate buffer, pH 4.8 along with 2 % Carboxymethyl cellulose (CMC), at 50° C for 30 min. The enzymatic reaction was terminated by the addition of 3 mL of dinitrosalicylic acid (DNS) reagent. One unit of cellulolytic activity (CMCase) was expressed as milli IU. The best cultures were selected for further studies.

2.4Mass Production of Cellulase enzyme

Cellulase were produced by *Trametes*sp. in Bold Basal medium (Glucose- 10 g/L, Peptone- 1 g/L, Yeast extract- 3 g/ L, KH_2PO_4- 1 g/L, $MgSO_4$. 7 H_2O- 1g/L) incubated at 28 ± 2ºC for 25 days in a Haffin flasks. After 30th day, mycelial mat was harvested and the culture filtrate was taken and used for further studies.

2.5Substrates

Coir pith, Banana pseudostem, Wheat bran, Bengal gram, Cotton pod were used. The wastes was washed with running water, dried and used for study.

2.6Two step pretreatment of Substrates

Substrates were treated with 50 mL of 0.1 N HCl, 0.1N NaOH, 0.1N H_2SO_4 initially followed by enzymatic treatment (the initially pretreated samples were filtered, washed thoroughly with distilled water and the residue was used as the pretreatment processes) and maintained at 100 rpm at room temperature at different time intervals for studying the release of sugar (Saccharification). The released sugars were estimated and tabulated.

2.7Preparation of Yeast inoculum

The yeast inoculum was prepared as described by(Scholar and Benedkite 1999) . One gram of dry baker's yeast was grown on Yeast medium agar plate at 30°C for 24 h. A loopful of the yeast colony was transferred from the agar plate into 100 ml medium and incubated at room temperature on a shaker at 100 rpm.

2.8Submerged fermentation

The neutralized extracts were used for the submerged fermentation. The extracts were mixed with other essential nutrients. The nutrient were be added at a concentration of $(NH_4)_2HPO_4$ - 1.0 g/L, $MgSO_4.7H_2O$ - 0.5 g/L and Yeast extract - 2g/L. The medium were autoclaved and inoculated with yeast inoculums. The inoculated flasks were incubated at 28± 2°C under shaker condition. Aliquots of the sample were taken and assayed for ethanol content by HPLC.

2.9Analysis of Total Sugars (Dubois *et al.*, 1956*)*

To one ml of the sample, 1.0 ml of 5% phenol and 5.0 ml of concentrated sulphuric acid were added and the content was mixed thoroughly, by shaking. Then the solution was allowed to stand for 15min at boiling water bath. After cooling the O.D of the solution was read at 490 nm in a spectrophotometer. The amount of total carbohydrate was calculated using a standard graph prepared from D-glucose. The values are expressed as mg/gram.

2.10Analysis of Reducing Sugars (Miller, 1959)

To one ml of the sample, 3.0 ml of DNS were added, mixed and kept in boiling water bath for 5 mins. After cooling, the absorbance was measured spectrophotometrically at 540 nm. The reducing sugars were calculated using a standard graph prepared for D-Glucose. The values are expressed as mg/gram of paper mill wastes.

2.11Analysis of Biomass production

The yeast multiplication was tested during the fermentation process is necessary to the conversion of glucose to ethanol.

2.12Estimation of Ethanol production by HPLC

High Performance Liquid Chromatography (HPLC) is now a commonly used technique for monitoring the progress of the fermentation in the ethanol production laboratory through the use of highly efficient column materials (the stationary phase), pumping systems that provide a consistent flow of the mobile phase, and detection components designed to accommodate the needs of the analysis.

The HPLC system is typically used to profi le the carbohydrate, alcohol, and organic acid content of the fermentation broth.

A high performance liquid chromatographic equipment (Waters 600S) was employed. A small volume of the sample solutions was introduced into the fluid flow using a sample injector. The stainless steel column (3.9 × 300 mm) was packed with

15 µm C18 packing (Lichrosphere, Merck). The flow rate of the mobile phase was varied within a range of 0.6 mL/min to 1.4 mL/min. Acetonitrile/water mixtures ranging in the composition of acetonitrile from 50 to 70 (v/v %) were used as the mobile phase.

Calibration of the HPLC system by use of a standard solution of the components of interest will allow the user to obtain, directly, as weight percent for the analytes of the broth samples. This data can be used to evaluate the progress of the fermentation and what intervention may be necessary at a future time to maximize the production of ethanol and minimize the production of further oxidation products.

2.13Fractional distillation for separation of ethanol

Distillation was done by rotary evaporator (BUCHI Heating Bath B – 490). The rotary evaporator is a device for gently and efficiently evaporating solvents from a mixture. It consists of a heated rotating vessel (usually a large flask) which is maintained under a vacuum though a tube connecting it to a condenser. The fermented broth was dispensed into round bottom flasks fixed to a distillation column. A conical flask was fixed to other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 78°C was used to heat the round bottomed flask containing the fermented broth. The condensed ethanol was analyzed for moisture and percentage of purity.

3.RESULTS

3.1 Effect Of Pretreatment Of Five Substrates By Primary Treatment:

All the agrowastes were subjected to pretreatment with sterile water,0.1 N H_2SO_4 and 0.1 N NaOH. All the samples were kept in the ortibal shaker at 100 rpm for 24h. The filtrates were then used for the detection of fermentable sugars.

3.2 Acid Pretreatment of the samples:

In acid pretreatment wheatbran and cotton pod was found to have 100.2mg/g and 92.08mg/g of sugars respectively. Banana pseudostem and Bengal gram husk showed 75.46 mg/g and 46.16mg/g of sugars respectively. Coir pith was found to be27.44mg/g of sugars which was comparatively lower than the other substrates. (figure). The samples which were pretreated with acid were subjected to alkali treatment. During this treatment,wheatbran and Bengal gram husk were found to possess 410.28mg/g and 410 mg/g of sugar. Coir pith and cotton pod showed395.77mg/g and 275.19mg/g of sugars, respectively, whereas banana pseudostem was found to contain 228.75mg/g of sugar.

3.3 Alkali Pretreatment of the Samples:

In alkali pretreatment wheatbran and banana pseudostem were found to have high content of sugar 289.70mg/g and 284.6 mg/g,respectively. Coir pith showed 199.20mg/g of sugar,which was comparatively lower than that of other substrates.(figure1). In samples pretreated with alkali were subjected to acid treatment. Wheatbran and Bengal gram husk showed 52.24mg/g and 51.71 mg/g of sugar, respectively. Cotton pod showed 51.18mg/g of sugar, whereas coir pith and banana pseudostem showed 29.81mg/g and 28.49mg/g of sugar, respectively.

Figure 1

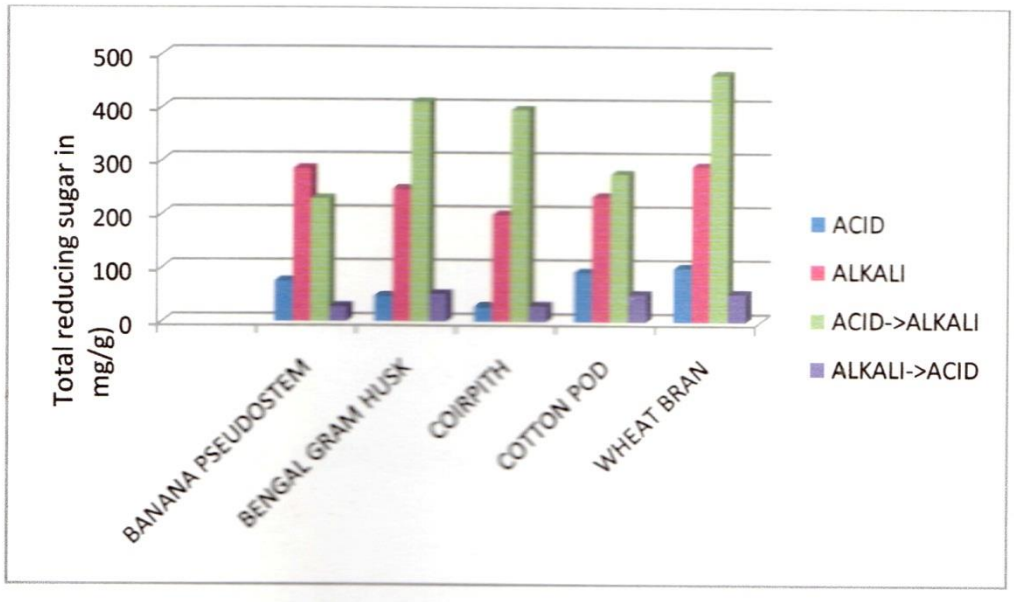

Figure 1:Pretreatment of the substrates

3.4 Effect Of Pretreatment Of Five Substrates By Secondary Treatment:

Fresh and pretreated samples of banana pseudostem, Bengal gram husk,cotton pod, coir pith and wheatbran residue were treated with cellalase enzyme. The candidate fungus, *Tramatussp.*was allowed to grow in Erlen Meyer flasks having Potato Dextrose broth and was incubated at room temperature for 7 days. The culture filtrate was then used to hydrolyse cellulose in the substrates as a sources of cellulose enzyme. The samples obtained after the pretreatment process were neutralized using 1N NaOH. These samples were checked for the presence of reducing sugar through Dinitrosalicyclic Acid method.

3.5 Estimation Of Reducing Sugar Through DinitroSalicyclic Acid Method

Samples were then subjected to sterile water pretreatment wheat bran,cotton pod, Bengal gram husk and coir pith were found to possess 21.89mg/g, 20.05mg/g and 18.46mg/g of reducing sugar, respectively. Wheatbran and banana pseudostem showed 17.94mg/g and 17.41mg/g of reducing sugar,respectively.(Figure 2). Fresh samples were treated with concentrated sulphuric acid and then treated with cellulase enzyme. The acid treated samples wheatbran, bengalgram husk and cotton pod were found to have 11.08mg/g,7.38mg/g, respectively, whereas banana pseudostem and coirpith showed 5.80mg/g and 4.48mg/g of reducing sugar,respectively . Fresh samples were treated with an alkali(NaOH) and then treated with cellulose enzyme. In alkali pretreatment wheat bran and cotton pod showed 45.38mg/g and 25.85mg/g of reducing sugar, respectively, whereas coir pith and Bengal gram husk showed 18.73 mg/g and 12.40 mg/g of reducing sugar, respectively. Banana pseudostem showed 8.44 mg/g of reducing sugar. Fresh samples were treated with acid followed by alkali pretreatment, which were then treated with cellulose enzyme. The acid-alkali pretreatment of the samples showed the presence of 45.90 mg/g and 27.96 mg/g of reducing sugar in wheat bran and coir pith, respectively. Cotton pod and banana pseudostem showed 23.21 mg /g and 14.24 mg/g of reducing sugar, respectively, whereas Bengal gram husk showed 10.55mg/g of reducing sugar. In alkali followed by acid pretreatment wheatbran and Bengal gram husk were found to have 21.89 mg/g and 16.35 mg/g of reducing sugar, whereas banana pseudostem and coir pith showed 15.56 mg/g and 13.45 mg/g of reducing sugar. Cotton pod showed 12.66 mg/g of reducing sugar.

Figure 2:

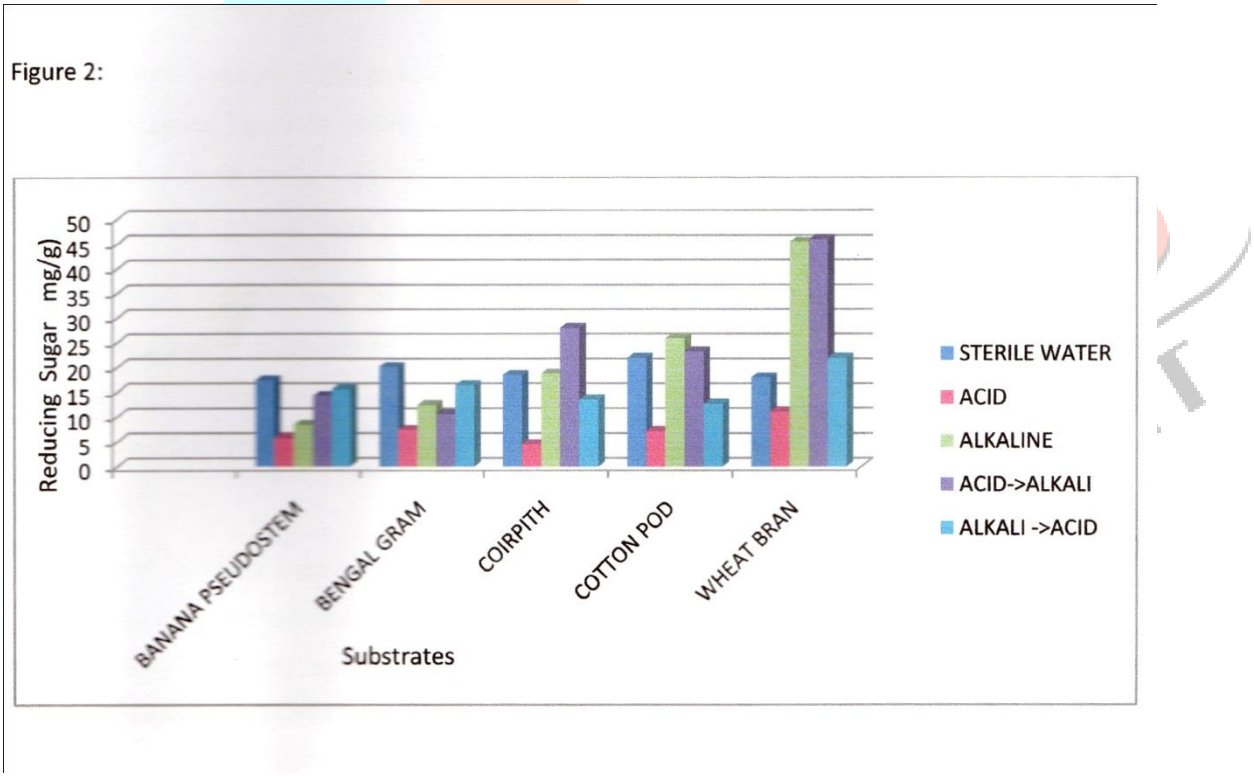

Figure 2:Efffect of reducing sugars of the substrates

3.6 Preparation of yeast

The baker's yeast was used in this study. The yeast was cultured in Luria Broth (LB) medium maintained at pH 4.8 and incubated in 30°C at 200rpm. After 24h the yeast cells were harvested by centrifugation at 5000 rpm for 10 min. The supernatant was discarded and the cells were washed with 1% of phosphoric acid for three times to remove residual sugars in the medium. This purified yeast biomass was used for fermentation process. The yeast inoculum was added on the neutralized extract and allowed to incubate at 28°C temperature for 120h. The samples were kept in orbital shaker at 100rpm. An aliquot of 0.5ml was withdrawn on alternative days and analyzed for fermentable sugar.

3.7 Estimation Of Total Sugar:

After complete pretreatment process,the substrates were extracted with 0.1N H2SO4, 0.1N NaOH followed by enzymatic pretreatment. However, pretreatment with enzyme showed very little quantity sugars extractable with NaOH . The wheat bran treated with *Trametes*sp has the maximum total sugar yield for primary treatment, followed by secondary pretreatment. In Sterile water pretreatment, cotton pod and wheatbran showed 543.53 mg/g and 438.25 mg/g of fermentable sugar, respectively, wheras Bengal gram husk and coir pith were found to have 203.16 mg/g and 146.17 mg/g, respectively. Banana pseudostem showed 62.79 mg/g of fermentable sugar. In acid pretreated cotton pod and wheatbran were found to have 247.22 mg/g and 218.99 mg/g of fermentable sugar. Banana pseudostem and coir pith showed 201.31 mg/g and 148.01 mg/g of fermentable sugar whereas Bengal gram husk showed 148.01 mg/g of fermentable sugar. In alkali pretreatment wheat bran and cotton pod showed 670.97 mg/g and 558.30 mg/g of fermentable sugar whereas coir pith and banana pseudostem were found to be 531.65 mg/g and 492.08 mg/g of fermentable sugar. Bengal gram husk showed 148.01 mg/g of fermentable sugar.In acid followed by alkali pretreatment wheat bran and coir pith were found to have 707.38 mg/g and 368.07 mg/g of fermentable sugar whereas banana pseudostem and cotton pod showed 262.53 mg/g and 182.84 mg/g of fermentable sugar. Bengal gram husk showed 70.71 mg/g of fermentable sugar (figure 3).

In alkali followed by acid pretreatment wheatbran and coir pith were found to have 222.16 mg/g and 173.61 mg/g of fermentable sugar wheras Bengal gram husk and cotton pod showed 147.22 mg/g and 94.45 of fermentable sugar. Banana pseudostem showed 79.41 mg/g of fermentable sugar.

Figure 3.

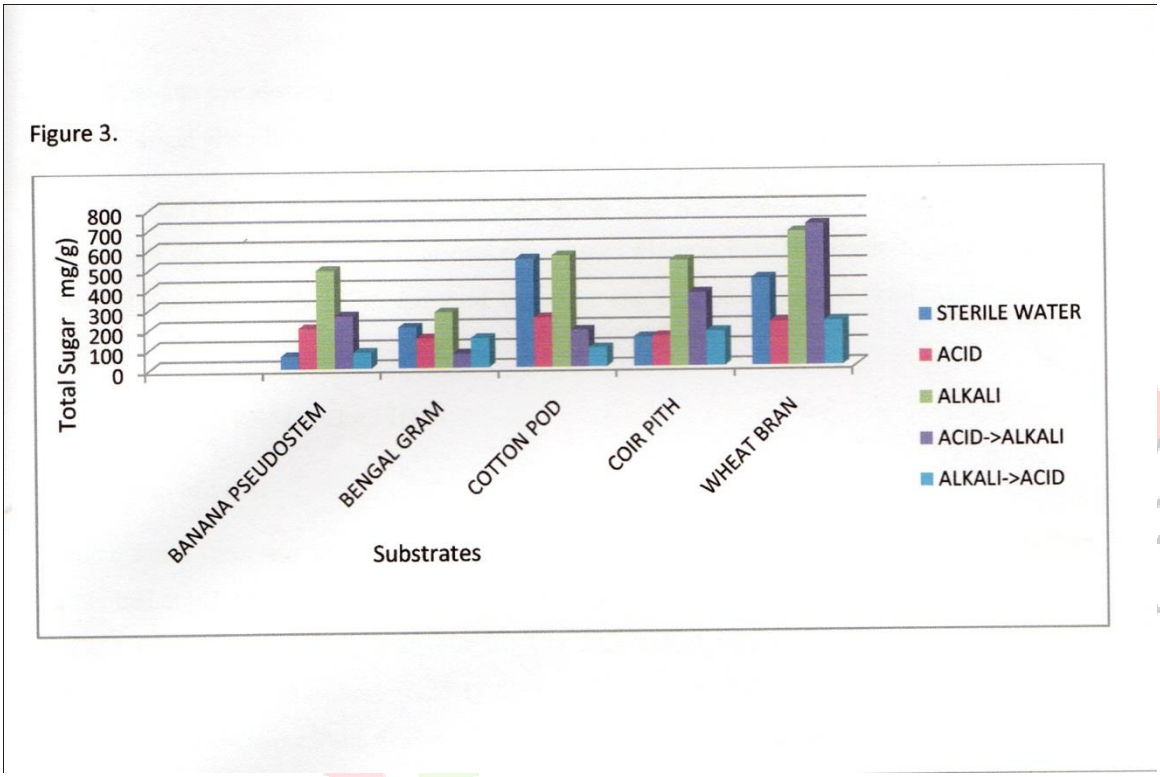

Figure 3:Effect of total sugar of the substrates

3.8Estimation Of Ethanol

Samples pretreated with acid , alkali, acid-alkali, alkali-acid were treated with enzymes and yeast inoculation were used to estimate ethanol production . In sterile water, wheatbran and coir pith showed 1.2 ml and 2.5 ml of etanol. Cotton pod and Bengal gram husk were found to be 1.5ml and 0.9 ml respectively, of ethanol. Banana pseudostem showed 0.7 ml of ethanol (figure 4). In acid wheatbran and cotton pod were found to be 1.6 ml and 0.95 ml of ethanol. Whereas coir pith and banana pseudostem showed 0.92 ml and 0.9 ml of respectively ethanol. Bengal gram husk showed 1.6 ml of ethanol. In alkali wheatbran and coir pith were found to be 3 ml and 2.5 ml respectively of ethanol, whereas Bengal gram husk and banana pseudostem showed 2.9 ml and 0.8 ml of ethanol. Cotton pod showed 0.7 ml of ethanol. In acid followed by alkali wheatbran and coir pith were found to be 1.9 ml and 1.5 ml respectively of ethanol. Banana pseudostem and cotton pod showed 1.0 ml and 0.9 ml respectively of ethanol. Bengal gram husk showed 0.3 ml of ethanol. In alkali followed by acid wheatbran and cotton pod were found to be 1.3 ml and 1.1 ml of ethanol, whereas coir pith and banana pseudostem showed .95 ml and 0.9 ml respectively of ethanol. Bengal husk showed 0.6 ml of ethanol.

Figure 4

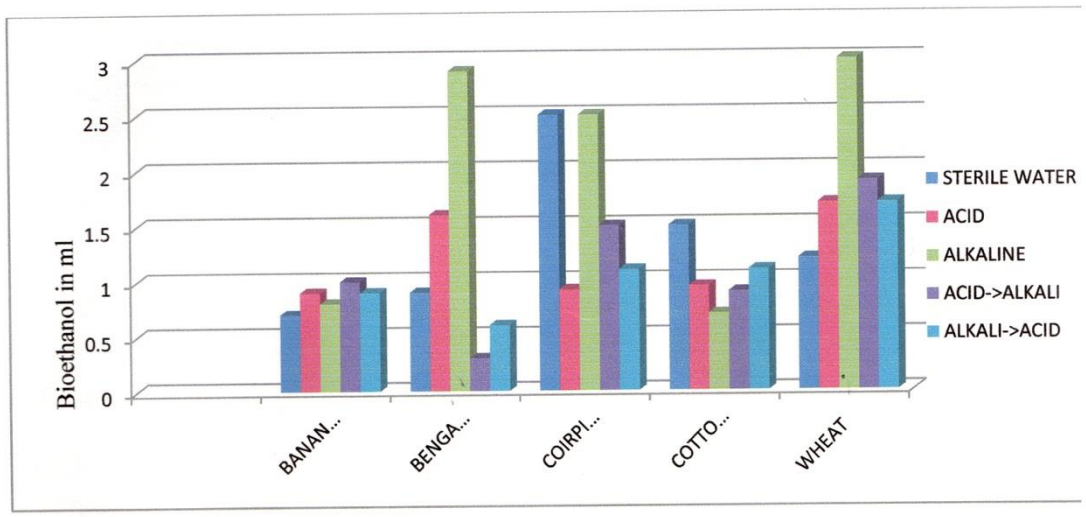

Figure 4:Ethanol production from the different substrates in ml

3.9 Fractional distillation for seperation of ethanol

Seperation of ethanol from other impurities was carried out by distillation. The rotary evaporator was proficiently evaporating solvents from a fermented broth. The temperature was adjusted to 80°C.

3.10 Determination Of Ethanol Production Using Hplc:

The samples after distillation were analyzed using HPLC technique. In this study fermentation was conducted under similar conditions produced 17 g of ethanol/gram of wheatbran on 5^{th} day. No other impurities were observed. Furthermore, Bengal gram husk was found to have 9 gm of ethanol/ gram of substrate on the 5^{th} day. Similarly 5 gm/g, 2 gm/g of ethanol was observed in banana pseudostem and coir pith. Out of five substrates tested wheatbran found to be a good producer of ethanol when compared to all the other substrates (figure 5)

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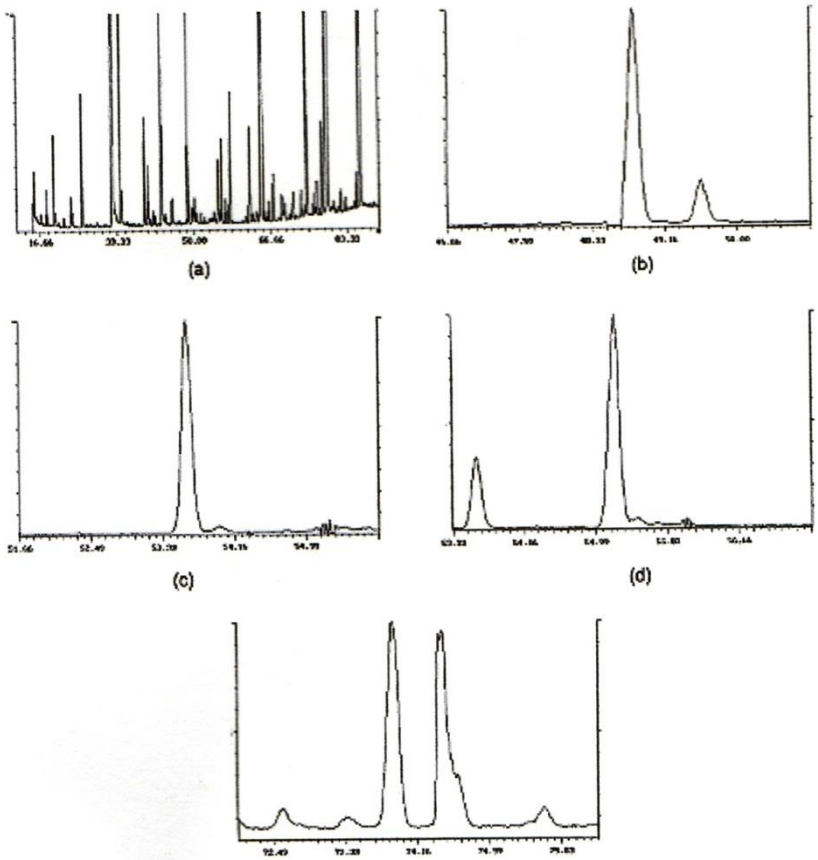

Figure 5: Determination of ethanol production using HPLC a)Coir pith b) Banana pseudostem c) Wheat bran d) Cotton pod e) Bengal gram husk

4.DISCUSSION

India is a larger consumer of petroleum products. The increasing demand for ethanol for various industrial purposes such as alternative source of energy, industrial solvents, cleansing agents and preservatives, has necessitated increased production of ethyl alcohol. Currently the cellulase isolated from*Trametes sp.* under submerged fermentation was used for the pretreatment processes. Pretreatment has been viewed as one of the most expensive processing step within the adaptation of biomass to fermentable sugar. The pretreatment implies the acid,alkaline and enzymatic treatment.Chemical pretreatment was originally developed and have been extensively used in the paper industry for delignification of cellulosic materials to produce high quality paper products(Yi Zheng et al., 2009). In 2007,Gurav and Geetha stated the crop residues were initially subjected to microbiological pretreatment for obtaining reducing sugars. Such treated substrates with and without obtaining the filtrate were further subjected to fermentation. The amount of total fermentable sugar released during chemical pretreatment using H2SO4 and NaoH was found to be greatest extent in wheat bran 100.2mg/g and 298mg/g likewise enzyme treated samples were found out 218.99mg/g and 670.97mg/g. Similarly, reducing sugars during chemical pretreatment exhibited 11.08 mg/g and 45.38 mg/g for H2SO4 and NaOH. The enzymatic pretreatment after chemical pretreatment the yield of sugars found to be high for wheat bran 218.99 mg/g and 670.97 mg/g respectively. (Begum *et al***.** 2009**)** stated that neutral sugar release from T.*viride.* was reported to be 6.3 -15.1%

which indicates that the present pretreatment contained comparatively more amount of fermentable sugar. Similar pretreatment studies in paddy straw showed maximum release of reducing sugar and total sugar were 0.44 mg/g and 0.96 mg/g respectively (Gurav and Geetha, 2007). Fermentation technology produces nearly 80% ethanol as clean fuel and for which *Saccharomyces cerevisiae*is considered to be of most important yeast strain because of its biotechnological application in the field of fermentation technology(Sreenath and Jeffries,1996).

5. CONCLUSION

This study provides the possibility of converting cellulose based waste materials to ethanol via fungal pretreatment and fermentation. Test fungus *Trametes sp*. shown to have better biotechnological potential and can be used to convert wheat bran, banana pseudostem, cotton pod,Bengal gram husk and coirpith waste to fermentable sugars and finally to ethanol.

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