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Synthesis of Bio-Ethanol by *Saccharomyces cerevisiae* Using Lignocellulosic Hydrolyzate from Pretreated Waste Paper Fermentation

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Abstract: The presence of conventional energy sources decreasing day by day. This inevitable situation leads us and increased the searching interest of bio based fuel. The present work illustrates that the synthesis of Bio-Ethanol from waste papers in particular, newspapers and magazine papers since it contains 40-55%, 25-30% of cellulose contents respectively. Several experiments were carried out to describe the Bio-Ethanol synthesis from waste papers by conducting series of chemical and biochemical reactions including acid pre-treatment, Delignification, Distillation and Fermentation. This work also summarizes the systematic study of Bio-Ethanol synthesis by fermentation process and the purity comparison of resultant obtained from various lignocellulosic sources. The Bio-ethanol obtained from the various waste papers was analyzed by Gas Chromatography (GC). The by-product i.e., solid residue obtained from the fermentation tank is used as a Solid Fuel. This study confirms that the production of bio ethanol was effective by using Saccharomyces *cerevisiae* in the pretreated waste paper.
Keywords: Bio ethanol, Fermentation, Lignocellulose.

Introduction

Energy consumption is inevitable for human existence. There are various reasons for the search of an alternative fuel that is technically feasible, environmentally acceptable, economically competitive, and readily available. The first foremost reason is the increasing demand for fossil fuels in all sectors of human life, be it transportation, power generation, industrial processes, and residential consumption [1]. Depletion of world petroleum reserves and the impact of environmental pollution of increasing exhaust emissions have led to the search for suitable alternative fuels for diesel engines [2]. The requirement of fuels for the production of electricity and running of vehicles is increasing day by day. Today every country draws its energy needs from a variety of sources. The sources can be broadly categorized as commercial and noncommercial. The commercial sources include the fossil fuels (coal, oil and natural gas), hydroelectric power and nuclear power, while the non-commercial sources include wood, animal wastes and agricultural wastes. In an industrialized country like, U.S.A., most of the energy requirements are met from commercial sources, while in an industrially less developed country like India, the use of commercial and non-commercial sources are about equal [3]. Fuel ethanol as an alternative fuel replacing the fossil fuels based one has been attracting worldwide interest because of the increasing demand for energy resources. Ethanol (C_2H_5OH) is produced naturally by certain micro-organisms from sugars under acidic conditions at the pH level of 4 to 5. This alcoholic fermentation process is used worldwide to produce alcoholic drinks. The most common micro-organism, the yeast *Saccharomyces cerevisiae*, is poisoned by C_2H_5OH concentrator greater than 10%, and so higher concentrations upto 95% are produced by distilling and fractionating. When distilled, the remaining constant boiling point mixture is 95% ethanol, 5% water. Anhydrous ethanol is produced commercially with azeotropic removal of water by distillation with solvents such as benzene. Only about 0.5% of the energy potential of the sugars required for

the concentration and separation processes. This process heat may be provided by the combustion or gasification of otherwise waste biomass. The improved production of ethanol has been viewed with particular interest since it can be used in existing motor vehicles as a gasoline extender (gasohol) or octane booster. Alcohol-gasoline blends, although they require strict elimination of water from the alcohol, have continued to gain importance. The wide boiling range of gasoline offers a special advantage; the lower boiling components are sufficiently volatile to allow engine start-up at low temperature. Unmodified engines powered by pure methanol or ethanol will not start below 10-15^{0}C. The presence of oxygen in the alcohols gives them compatibility with water, gasoline or diesel fuels and this means that less air is required for combustion. The alcohols have an affinity for water sufficient to draw it from the air, a characteristic that can cause problems in their unprotected use and storage. Ethanol has greater potential for use as an industrial solvent and chemical than as a liquid fuel. For example, about half the ethanol sold in the U.S.A is used as a solvent. Printing inks, shellacs, varnishes, nitrogen cellulose coating, cosmetics, pharmaceuticals and some foods, all use ethanol in varying amounts. Fermentation ethanol may have the best future as a substrate for single-cell protein production. Its advantage in this use include: purity, acceptability; ease of storage and handling; non-toxicity; as a substrate or CO substrate; miscibility with water; relatively low oxygen demand; relatively low temperature fermentation; and high cell yields [4]. Bioethanol is produced by the biochemical reaction called fermentation. Generally, fermentation is the breakdown of complex molecules in organic compound under the influence of ferment such as yeast, bacteria, enzymes etc. Fermentation is a well-established and widely used technology for the conversion of grains and sugar crops into ethanol. About 500million gal ethanol per year by 1985, were produced in the limited states by the use of surplus gain. It is intended for mixing with gasoline to produce gasohol (90 percent gasoline, 10 percent ethanol). This process requires high cost and high energy. One scheme considered for reducing costs of ethanol production by fermentation is in finding less expensive grains or sugars and a process that requires less energy. Glucose produced by hydrolysis of an abundant carbohydrate polymer called lignocellulose is being considered for the former. There various studies have done in the production of bio ethanol from the waste paper. The bio ethanol content depends upon the various compositions in the waste paper. The presence of 70.12 ±4.88% of carbohydrates (holocellulose) makes waste paper a prospective and renewable biomass for bio ethanol production [5]. Glucose production was enhanced by using diluted sulphuric acid during pretreatment. Different incubation periods were tested for saccharification and subsequent Bio ethanol fermentation. The highest yield of glucose (41.90 mg/ml) was shown to increase with 27.20% and 25.90% respectively by increasing the reaction period by 30 min and by increasing the acid concentration by 0.5% [6]. The effects of mixing and temperature on ionic liquid-based catalytic conversion of cellulose to fuel products and suggests the optimal mixing speed and temperature for maximizing the production of cellulosic fuels. At 120^{0}C, maximum amount of production of cellulosic fuels has obtained [7]. The growth and fermentation tests of yeasts for various monosaccharides with 5ml of acid media were carried out by shaking at 120 rpm in a rotary shaker at 30^{0}C and 37^{0}C, respectively [8]. Waste papers (newspaper, office paper, magazines and cardboard in this study) with 50–73% (w/w oven dry weight) carbohydrate contents have considerable potential as raw materials for bio ethanol production [9]. Hydrolysis of cellulose with higher yield, lower cost, less resource consumption and more eco-friendly aspects can be achieved by chemical modification, which has a significant expectation of potential application [10]. Lignocellulosic biomass as an available and cheap source is gaining popularity as a source of fermentable sugars for liquid fuel production [11]. An effective way of utilizing acid lignin with a highly condensed structure may economically develop acid saccharification industries [12].The comparative study has done for sugar, reducing sugar and cellulose in the various sources like rice husk, groundnut hull and newspaper [13]. In the present study, waste news paper and magazine papers were used as a lignocellulosic source.

Materials and Methods

Raw material

The raw materials of waste papers of various sorts namely, office waste papers, newspaper and magazine waste papers are collected in our college campus. The collected waste papers separated individually depending upon their physical properties. Here we separate the paper wastes and they classified into waste newspaper, waste used A4 sheets and waste reading magazines. The collected waste papers are taken for size reduction. Then the tore waste paper is weighed about 1 kg and soaking into the water in the ratio of 1:20 individually i.e., one kg of feed requires 20 litres of water. And it stands for 24 hours. During this period, the fibres of the paper are loosening and it make easier to separate the cellulose, hemicelluloses and lignin components from the fibre of the papers. After completion of one day, the water was filtered the paper was crushed mechanically in mixing and made as pulp.

Deinking Process

In the Deinking process ink pigments has been removed from the slurry because this ink pigments may affect the yield of Ethanol. The ink used for printing in waste papers are mostly carbon based one. It can be easily eliminated by adding concentrated sulphuric acid usually concentration of 10%. In this process, 10% concentrated sulphuric acid is added followed by 5% concentrated sulphuric acid addition. The stirring operation may be adapted in this deinking process.

Delignification

Delignification is the process of removal of the structural polymer lignin from plant tissue, so that it can be used for applications like making paper. This can also be done mechanically. Lignin is a mixture of phenolic compounds that is intermeshed in plant secondary walls, cross-linking the cellulose carbohydrates that can be used to form paper fibers. This complex forms a hydrophobic matrix, meaning it repels water, allowing the plant to transport water up through its system. The removal of lignin from the wood has traditionally taken place by a method called the Kraft process. The mass of fibers remaining after the lignin is removed is known as pulp. The Kraft process produces stronger pulp than the methods used previously, and removes 95% of the lignin from the wood. But in this process, we separate the lignin from the waste paper because the lignin content in the waste paper is less than 5%. This stage is done by adding 0.01 M of NaOH and then doing reflux to remove the lignin on paper. Delignification carries out for 6 hours.

Estimation of Cellulose Content

After the completion of pre-treatment, it is essential to determine the cellulose content in the sample because it gives an idea about the appropriate volume of bio-ethanol is obtained from the sources i.e., our waste papers. Using Carboxyl Methyl Cellulose is used as a standard component to determine the cellulose content in the raw material. Cellulose undergoes acetolysis with acetic/nitric acid reagent forming acetylated cellodextrins which gets dissolved and hydrolyzed to form glucose molecules on treatment with 67% H_2SO_4. This glucose molecule is dehydrated to form hydroxyl methyl furfural which forms green colour product with a throne and the colour intensity is measured at 630nm.

Acid Hydrolysis

The cellulose molecules are composed of long chains of sugar molecules. In the hydrolysis process, these chains are broken down to free the sugar before it is fermented for alcohol production. There are two major cellulose hydrolysis processes a chemical reaction using acids, or an enzymatic reaction. In Acid hydrolysis method they are further classified into two types. Dilute Acid Method, Concentrated Acid Method. In Dilute acid method may be used under high heat and high pressure, or more concentrated acid can be used at lower temperatures and atmospheric pressure. A decrystalized cellulosic mixture of acid and sugars reacts in the presence of water to complete individual sugar molecules (hydrolysis). Here we add 20% concentrated Sulphuric acid to the slurry and heated at 100^{o}C for 10 minutes. During this process the cellulose will be converted into sugar components.

Estimation of Glucose Content

3, 5-Dinitrosalicylic acid (DNSA) is used extensively in biochemistry for the estimation of reducing sugars. It detects the presence of free carbonyl group (C=O) of reducing sugars. This involves the oxidation of the aldehyde functional group (in glucose) and the ketone functional group (in fructose). During this reaction DNSA is reduced to 3- amino- 5-nitrosalicylic acid (ANSA) which under alkaline conditions is converted to a reddish brown colored complex which has an absorbance maximum of 540 nm. To make dinitrosalicylic acid reagent 1g dinitrosalicylic acid was dissolved by stirring with 200mg crystalline phenol an 50mg sodium sulphite in 100ml 1% NaOH.

Store at 4^{o}C (since the reagents deteriorates due to sodium sulfite if long storage is required sodium sulfite may be added at the time of use 40% Rochelle salt solution (potassium sodium tartrate) and cooled then read the intensity of dark red color at 540 nm using UV-Vis spectrophotometer. The reaction involved in this process is followed by, 3,5,dinitrosalycylic acid is converted into 3- amino 5-dinitrosalycylic acid.

Neutralization

After the completion of determining the glucose content in the various samples, the pH level of the samples must be checked. It is inevitable to confirm the pH to be neutral in order to make sure the growth of yeast in fermentation step. Because during anaerobic stage, the yeast of Sacchromyces *cerevisiae* will grow better conditions at pH level of 4-7. The pH level of various samples might be less than 2 which show the need of neutralization step because of the hydrolysis process. In order to neutralize or raise the pH range we add one litre of 25% Calcium Carbonate solution to the slurries. During this process the evolution of Carbon dioxide must neutralize the acid level. After adding Calcium Carbonate Solution checking of the pH level is being carried out. If the pH level of the slurry is satisfied about 4-7 then the process is stopped. Otherwise continuously added the Calcium Carbonate solution and repeat the process.

Fermentation

Fermentation can be performed as a batch, fed-batch or continuous process. The choice of most suitable process will depend upon the kinetic properties of microorganisms and type of Lignocellulosic hydrolysate in addition to process economic aspects. After check the pH level the slurry must be weighed and poured into the fermentation tank. Then 2.5g/litre slurry of *Saccharomyces cerevisiae* added into the fermentation tank and mix well. *Saccharomyces cerevisiae* is in the fungi kingdom. The reasons for this classification are because it has a cell wall made of chitin, it has no peptiodglycan in its cell walls, and its lipids are ester linked. It also uses DNA template for protein synthesis and it has larger ribosomes. It is then consider yeast because it is a unicellular organism so it cannot form a fruiting body; like other fungi. The common name of *Saccharomyces cerevisiae is* Brewer's yeast/ Baker's yeast. Here the Fermentation Process is an anaerobic batch type. Then the fermentation tank is kept without air and maintained in dark room for four days. Here the Fermentation Process is a batch type.

Distillation

After the fermentation stage is completed the clear liquid in the upper layer should be collected. The Ethanol and other constituents present in the fermentation tank should be separated by the filtrate and the solid should be kept in the tray for further use. The fermented liquor should pour into the distillation column. The temperature range between 78-80°C is maintained in distillation column setup. At this temperature, ethanol has evaporated. At the first time the ethanol should come along with some of the water vapor. Then, the fermented liquid from the fermented tank must be in large amount and it contains many other components rather than ethanol and water. It is impossible to measure correctly the volume of ethanol present in the solution. So we obtained ethanol only by reflux method i.e., the top product from the distillation column is do again distillates to get only ethanol. In order to get purified ethanol as the final product we must add Calcium Oxalate to the top product of the final distillation column. While adding Calcium Oxalate the water molecules absorbed by calcium molecule and forms Calcium Hydroxide and the 99% pure ethanol has collected from the top of the distillation column. The bottom product from the fermentation tank i.e., solid residue should be drained in sunlight for 4-5 days to remove the moisture content in the solid residue and used as a solid fuel.

Results and Discussion

The raw materials of various types of papers are taken and these are containing the glucose, xylose, other sugars, and lignin and ash contents. 46-53% glucose, 5-10% xylose, 2-8% other sugars, 17-25% lignin, 1-25% ash are the basic constituents of waste papers. By using anthrone method the cellulose content in various papers are estimated by plotting the standard graph initially. The result obtained for the various concentrations of sample from UV-Visible spectrophotometer are shown in Figure 2.

From the absorbance values of prepared Concentration of cellulose have been calculated. The Slope obtained from the plot is 0.002.

For first sample of office papers the absorbance value obtained from UV-visible spectrophotometer is 0.241. So, the cellulose concentration in the sample of 0.5gram of office waste paper is calculated as 120.5 mg/l.

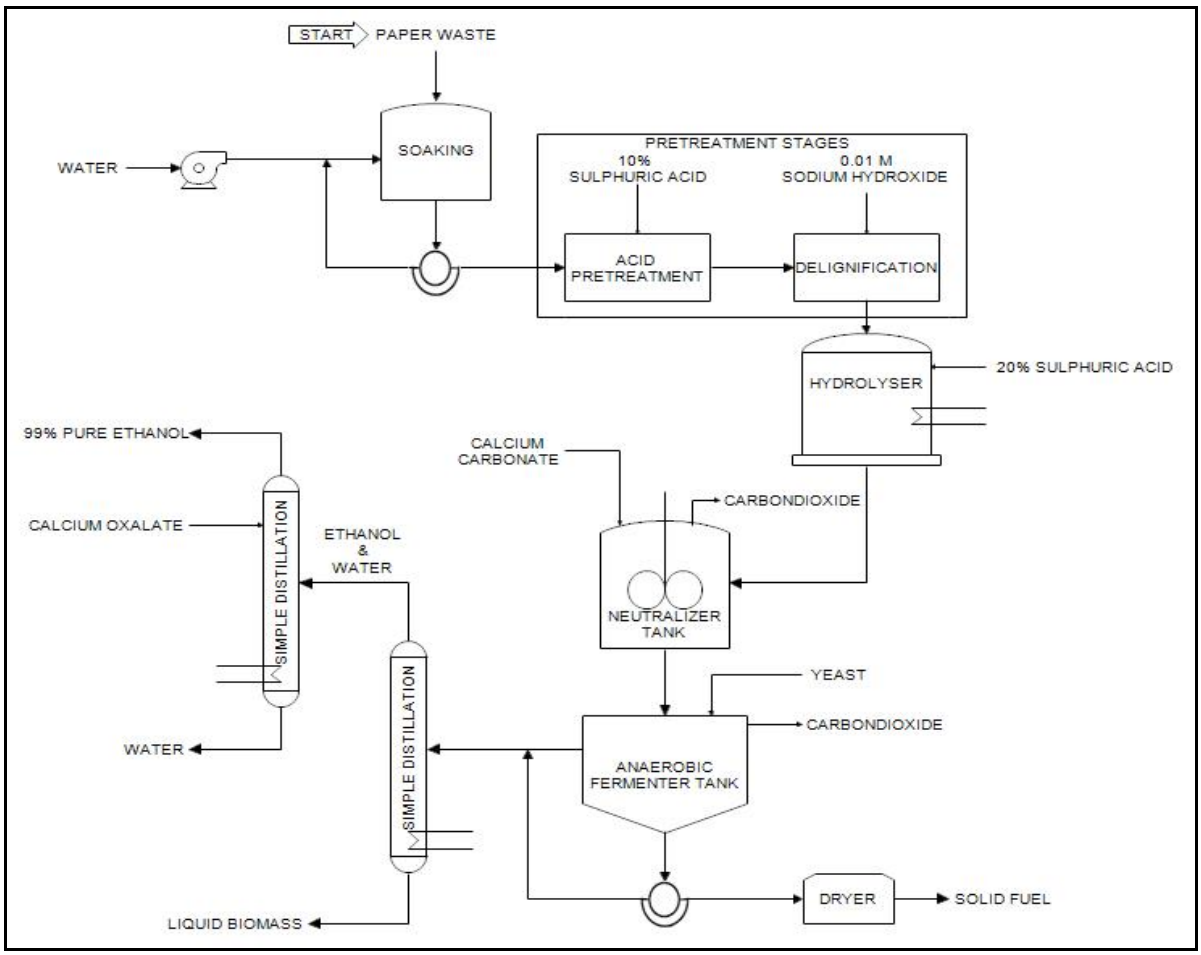


Figure 1 Flow sheet for Synthesis of Bio ethanol from the Waste Papers

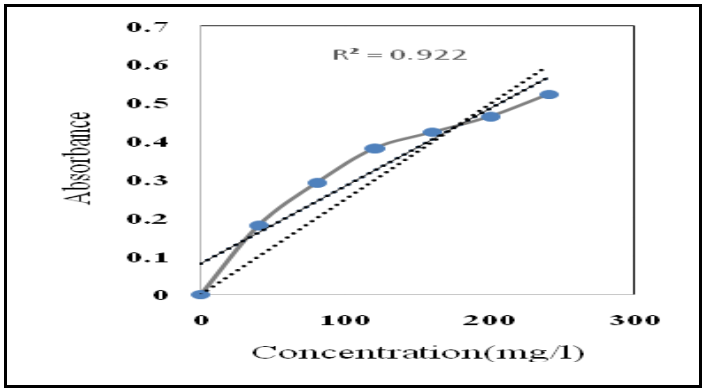


Figure 2 Plot for various Concentration of CMC vs. Absorbance Value obtained from UV-visible Spectrophotomete

The percentage of cellulose present in the sample of office waste paper of 0.5gram is given as 24.1%. For second sample of newspaper the absorbance value obtained from UV-visible spectrophotometer is 0.232. So the cellulose concentration in the sample of 0.5gram of newspaper is calculated as 116 mg/l. The percentage of cellulose present in the sample of newspaper of 0.5gram is given by 23.2%. For third sample of magazines paper the absorbance value obtained from UV-visible spectrophotometer is 0.239. So, the cellulose concentration in the sample of 0.5gram of magazine paper is calculated as 119.5 mg/l. The percentage of cellulose present in the sample of magazines paper of 0.5gram is 23.9%.

Comparison of Glucose Content in Various Papers

By using DNSA method the glucose content in various papers are estimated by plotting the standard graph initially. The result obtained for the various concentrations of sample from UV-Visible spectrophotometer were shown in Figure 3.

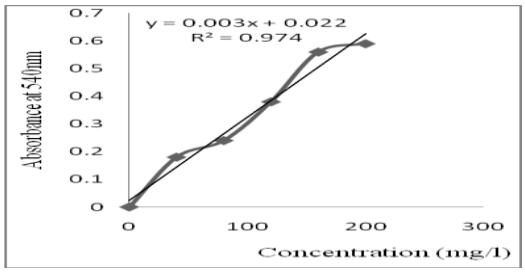


Figure 3 Plot for Concentration of Glucose Sample Vs. Absorbance value obtained from UV-visible Spectrophotometer

The slope obtained from the graph is 0.003. From the absorbance values of prepared samples the concentration of glucose has been calculated. For first sample of office papers the absorbance value obtained from UV-visible spectrophotometer is 0.421. So, the glucose concentration in the sample of 0.5gram of office waste paper is calculated as 140.33 mg/l. The percentage of glucose present in the sample of office waste paper of 0.5gram is given by 28.066 %. For second sample of newspaper the absorbance value obtained from UV-visible spectrophotometer is 0.374. So, the glucose concentration in the sample of 0.5gram of newspaper is calculated as 124.67 mg/l.The percentage of glucose present in the sample of newspaper of 0.5gram is given by 24.93 %. For third sample of magazines paper the absorbance value obtained from the UV-visible spectrophotometer is 0.351. So, the glucose concentration in the sample of 0.5gram of magazine paper is calculated as 117 mg/l .The percentage of glucose present in the sample of magazines paper of 0.5gram is given by 23.4%

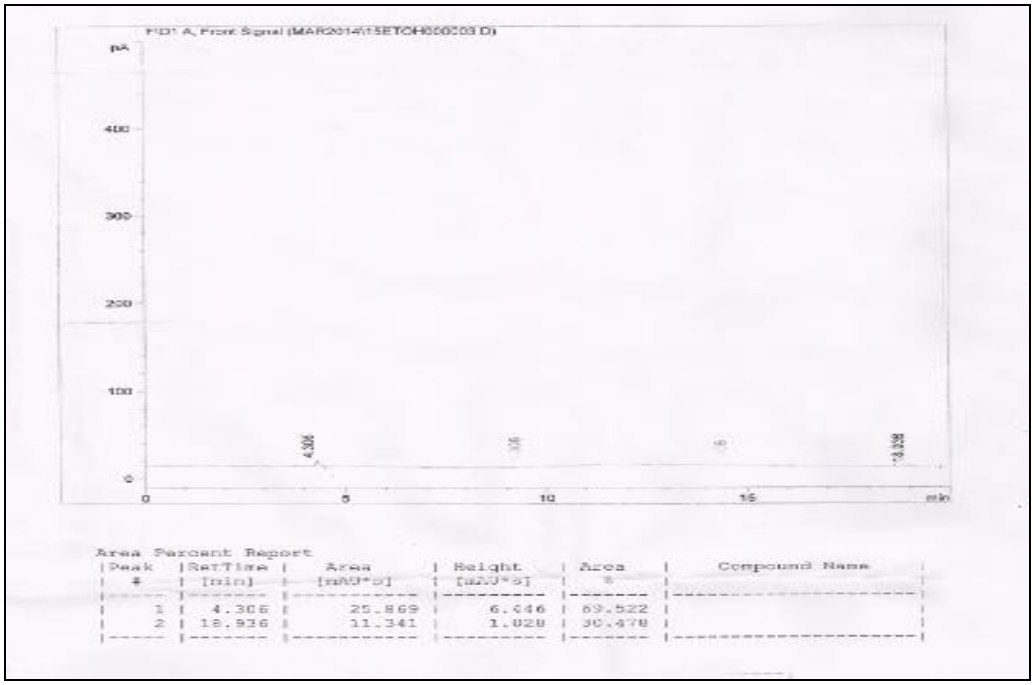
Chromatogram of Standard Sample (Ethanol)

Bio ethanol production from the waste paper has been analysed using the Gas Chromatography. Before doing the test the system has been calibrated using the standard solution. The standard solution result has present in the Figure 4. The purity of the ethanol is 99.737%.

Figure 4 GC Result for Standard Sample (Ethanol)

Chromatogram of Bio ethanol obtained from waste News papers

After the various pre treatment, Hydrolysis and neutralises of waste news paper has send to the fermentation tank. Product from the fermentation tank has been send to the simple distillation column. The product from the distillation column has been tested using the GC with reference to the ethanol. The result obtained from the GC has conform that, it contain 69.52% of bio ethanol. It is shown in the figure 5.



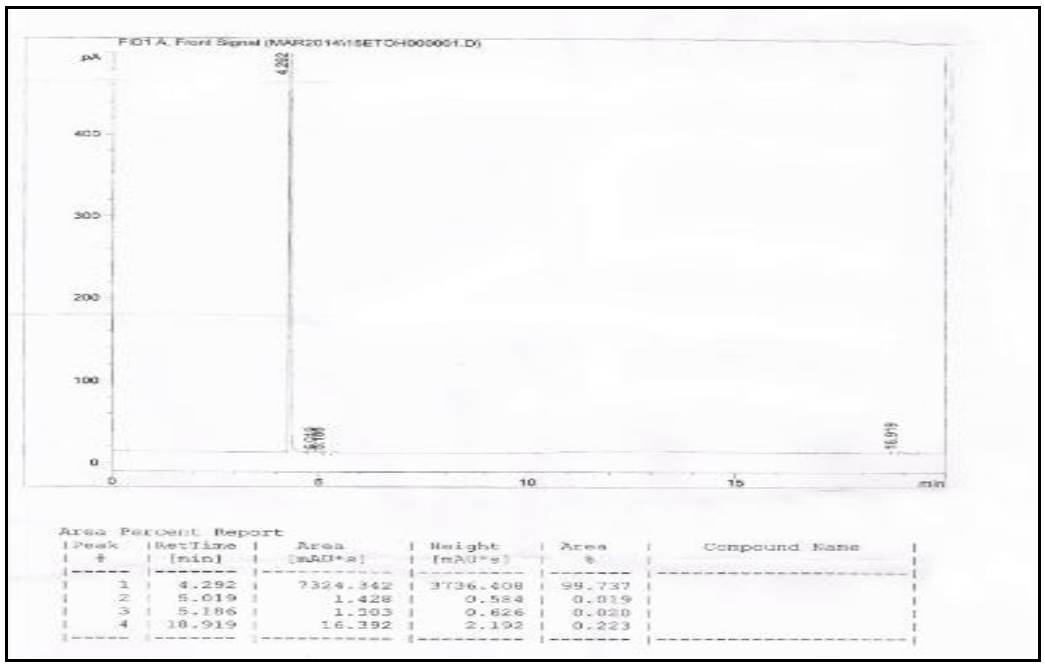
Area Percent Report

Peak #	RetTime [min]	Area [mAU*s]	Height [mAU*s]	Area %	Compound Name
1	4.306	25.869	6.646	69.522	
2	18.936	11.341	1.028	30.478	

Figure 5 GC Result of Bioethanol Obtained from the Waste Newspaper

Chromatogram Result after the Addition of CaO

In addition, the bioethanol has treated with CaO for removing the other components present in the mixture. After treating mixture of bio ethanol in to the CaO, the product has tested with GC the result shows (Figure 6) that 99.72% of ethanol content. It is nearly equal to the pure ethanol.



Area Percent Report

Peak #	RetTime [min]	Area [mAU*s]	Height [mAU*s]	Area %	Compound Name
1	4.292	7324.342	3736.408	98.737	
2	5.019	1.428	0.584	0.019	
3	5.186	1.303	0.626	0.020	
4	18.919	16.392	2.192	0.223	

Figure 6 GC Result of Bioethanol obtained from the used Newspaper after the addition of CaO

Chromatogram of Bioethanol obtained from waste Magazine papers

When using the magazine waste paper, the production of bio ethanol purity is 37.743%. The purity of ethanol can be raised by the addition of Calcium oxide and doing second distillation.

Conclusions

Experimentation on Bioethanol production has been done using various Lignocellulosic sources of newspaper and magazine. The present investigation has proved the successfulness of implementing the Bioethanol production from waste papers of various cellulosic contents as raw materials using *Saccharomyces cerevisiae*. The reaction period for pre-treatment, hydrolysis, fermentation optimized to get the maximum conversion of lignocellulosic materials into ethyl alcohol. There is a 125ml of ethanol has obtained from fermentation liquor of 300ml by distillation. The purity of ethanol has obtained from waste news paper is 69.52% and by second distillation the purity has enhanced to 99.73% after the addition of calcium oxide. Similarly, the purity of Ethanol obtained by magazine papers is 37.74% and it can be raised by doing second distillation. The present work gave us the opportunity for preventing the noxious emissions during the chemical method of ethanol synthesis. The utilization of waste paper as thereby production of biomass based fuels both enhances the economic potential. The ethanol synthesis from waste papers may replenish the fuel availability and it may lead to the sustained development.

Figure 5.7GC result of Bioethanol obtained from Waste Magazine Paper

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