PRODUCTION OF BIOETHANOL FROM AGRO WASTES

Project Reference No.: 41S_B_MSC_019

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KEYWORDS: Sugarcane bagasse, Aspergillus niger, Saccharomyces cerevisiae, Bioethanol, GC-MS Method.

INTRODUCTION: Bioenergy is energy derived from biofuels, biofuels are fuels such as bioethanol and biodiesel produced directly or indirectly from organic materials including plant (Agro wastes) and also animal waste. Bioethanol is one of the most promising alternative biofuel. Ethanol has been frequently used for the blended gasoline in concentration ranges 10-85 % (v/v). Sugarcane bagasse and corn feedstock, are the main source of ethanol. Bioethanol is most important alcohol can be produced by converting the sugar content of any starchy material into alcohol with the evolution of carbon dioxide (CO2) under controlled environmental conditions. Other benefits come from using bioethanol as biofuel: it is totally biodegradable and Sulphur free and the products from its incomplete oxidation (acetic acid and acetaldehyde) are less toxic in comparison to other alcohols. The microorganisms chosen for alcoholic fermentation are usually yeasts, mainly belonging to Saccharomyces genus. The preferred characteristics for industrial bioethanol production are: high ethanol yield; high ethanol tolerance; high ethanol productivity (>5.0 g/L/h); aptitude to grow in simple, inexpensive, and undiluted media; aptitude to grow in presence of inhibitors, at low pH, or high temperature. The temperature is a fundamental parameter of the fermentation process. In India, bioethanol is mostly produced from sugarcane molasses which is a waste by-product obtained after the removal of sucrose from the sugarcane juice for sugar production. Hence the present study was aimed at production of bioethanol using various agro wastes as substrates.

OBJECTIVES:

- Isolation of substrate hydrolyzing enzyme producing microorganisms and selection of substrates.
- Standardization and optimization of media for scale up of bioethanol.
- Determination of bioethanol Production and quantification by GC-MS.

METHODOLOGY:

Preparation and pre-treatment of substrate: Sugarcane bagasse, as agro wastes were collected from different regions of our davangere then washed and dried. Pre-treatment of the sample was carried out by 1% NaOH for a period of 2 hours (Ali et al., 2011).

Microorganisms and maintaining the culture: Soil samples were collected from near davangere university, Tholahunase, Davangere is used (for Saacarification). The soil samples were collected at a depth of 10 -20 cm in the fields in sterile air tight containers and transported to the laboratory. Then that soil was screen for the potent microorganisms to hydrolyze substrate. The predominant fungal culture
will isolate and identify by preparing a wet mount using lacto phenol cotton blue.

**Use of ferment medium and Saccharomyces cerevisiae**: The fermentation media was use 0.2% yeast extract, 0.2% (NH4) NO3, 0.1% MgSO4.7 H2O, 0.2% KH2PO4 and 5 gm. of each substrate are dissolved in 100ml distilled water and then flasks were sterilized by autoclaved at 121°C for 20 minute and then add 5 % (V/V) inoculum of Aspergillus niger and 10% (W/V) Saccharomyces cerevisiae (Baker yeast) and pH was adjusted to 5.5 and fermentation was carried out for 10 days in rotary shaker at 120pm, after 10 days of incubation, the broth was centrifuged at 6000rpm for 10 minute, the supernatant was collected and fed in to a simple distillation column. (Gendy et al., 2013)

**Reducing sugars estimation**: (Somyogi method) Somogyi in 1952 estimated reducing sugars of the sample with glucose as standard. 100mg of sugar bagasse was taken and sugars were extracted with hot 80% ethanol twice (5ml each time) supernatant was collected and evaporated keeping it on a water bath at 80°C and then 10 ml of water was added to dissolve the sugars and followed the procedure as Aliquots of 0.2, 0.4, 0.6, 0.8, 1.0 ml of the working standard solution (1mg/1ml) was pipetted out into a series of test tubes. Volume of standard and sample tubes were made to 2ml with distilled water. One tube set as blank and adds 1ml of alkaline copper tartrate reagent was added in each tube. And then all tubes were placed in boiling water bath for 10 min. Then the tubes were cooled and 1ml of arsenomolybdic acid reagent was added in to each tube. The optical densities of standard and test samples were measured at 660nm and a graph was plotted.

**Protein determination**: The Bradford protein assay which was described by the Bradford and this technique is faster and sensitive than any other protein assay method (Bradford s, 1976). The protein content of all the substrates was determined with bovine serum albumin as standard. It is a simple procedure for the determination of protein concentration in the given sugar bagasse sample solution. The Bradford protein assay which was described by the Bradford and this technique is faster and sensitive than any other protein assay method (Bradford s, 1976). Aliquots of 0.2, 0.4, 0.6, 0.8, 1.0 ml of the working standard solution (1mg/1ml) was pipetted out into a series of test tubes. Volumes of standard & sample tubes were made up to 3ml with phosphate buffer. Add 5ml of Bradford reagent to each tube and then kept all the tubes for incubation at room temperature for 30min. Then the tubes were cooled and then optical densities of the standard and test samples were measured at 595nm.

**Optimization of fermenting media such as different pH and different incubation time for bioethanol production**: The chemically pre- treated substrates were used for all experiments. In order to optimize bioethanol production the "Substrates were taken in different variations,(5g, 6g, 7g, 8g, 10g) and different incubation time ( 2 days, 4 days, 6days, 8days, 10 days ) and different pH ( 3.5, 4.5, 5.5, and 6.5, 7.5) (Duhan et al., 2013).

**Separation of ethanol from water mixtures by distillation method**: To separate a mixture of alcohol (ethanol) and water mixture, we can use a process known as fractional distillation. This technique relies on the fact that the compounds in the mixture have different boiling points, since ethanol boils at a lower temperature (78.5°C) than water; the alcohol vaporizes while most of the water remains liquid. At this point, we have competed distillation.

**Determination of Ethanol**: Ethanol was estimated by Potassium dichromate method. (Caputi et al., 1968). According to the potassium dichromate method, ethanol was estimated by taking different concentrations of ethanol (2- 10%) was prepared using deionized water, 2ml of each ethanol solution was taken and the volume was adjusted to 50ml using deionized water in a volumetric flask. Further, this solution was transferred to a round bottom distillation flask. Distillates was carried out at 55°C using a
small distillation unit until 15ml of the distillate was taken. 1.85 ml of distillate was taken in a separate set of test tubes. 3.12 ml of freshly prepared chromic acid was added and 1.25ml of distilled water was added. The test tubes were incubated at 50°C for 30 min. the test tubes were cooled to room temperature and the color developed was observed in a UV -Visible spectrophotometer at 660nm against a reagent blank. A standard graph was plotted against concentration of ethanol v/s optical density. Alcohol concentration in sugar bagasse was estimated by following the same protocol and using the standard graph of ethanol. (Caputi et al., 1968).

Quantification by GC-MS Method: Gas chromatography (GC) is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. The comparison of retention times is what gives GC its analytical usefulness. Mass spectroscopy is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The basic principle involved in the mass spectrometer generates multiple ions from the sample under investigation, it then separates them according to their specific mass to charge ratio (m/z), and then records the relative abundance of each ion type.

RESULTS AND DISCUSSION:

The sugar released, from sugarcane bagasse as substrate, maximum amount of sugars were released from sugar bagasse is 1150µg/ml before the substrate is undergoing for fermentation for production of bioethanol but after 10 days of fermentation, it is almost of about 600µg/ml of sugar released by sugar cane bagasse, which is estimated by somyogi method, this showed that the sugar is being fermented by the help of the microorganism yeast for the production of bioethanol.

The utilization of the protein from sugar bagasse can be of immense importance for bioethanol production. Protein content of sugar bagasse obtained before undergoing for fermentation was found to be 600µg/ml respectively without addition of microorganism. Then the protein content after fermentation with the addition of A. niger and yeast result in gradually increased variation among protein content was found to be 1090µg/ml of sugar bagasse which enhanced the biomass production for bioethanol production. And the concentrations were mentioned in graphical representation. A niger produce cellulolytic and lignin lytic enzymes at the initial stage of composting process and making the substrate available for biodegradation.

The unknown concentration of bioethanol produced by sugar bagasse, by optimized all conditions of ferment broth was found to be 6.81 %( v/v) in 50ml ferment broth after 10 days of incubation.

The unknown concentration of bioethanol produced by sugar bagasse, by optimized at different substrate concentration like 2.5g, 3.0g, 3.5g, 4.0g, 5.0g/ 50 ml of ferment broth was found to be 2.5g/50ml showed the highest amount of bioethanol produced of about 5.9%(V/V) because this substrate concentration favours Saccharomyces cerevisiae which converts the sugar present in the medium to ethanol. With the increase in the concentration of Substrate, the time required for the completion of fermentation decreased dramatically.

The unknown concentration of bioethanol produced by sugar bagasse, by optimized at different pH like 3.5, 4.5, 5.5, 6.5, 7.5 was found to be pH 5.5 showed the highest amount of bioethanol produced of about 6.3%(V/V) because this pH favours Saccharomyces cerevisiae which converts the sugar present in the medium to ethanol and also provides acidic condition which prevents the bacterial contamination during fermentation. As the pH decreases, the fermenting broth became more acidic, thus changing the metabolic activities of the yeast for increased ethanol production.
The unknown concentration of bioethanol produced by sugar bagasse, by optimized at different incubation time like 2days, 4days, 6days, 8days, 10 days of ferment broth was found to be **10 days** showed the highest amount of bioethanol produced of about 6.91%(V/V). Because these 10 days of incubation time, the depletion of sugar was very rapid, this phase was believed to be the exponential phase which was the period of rapid cell multiplication indicated by active fermentation. Increases with the increasing number of days while sugar concentration decreases. This shows the sugar is being fermented by the help of the microorganism yeast for the production of ethanol.

**Quantification of bioethanol by GC-MS Method.**

A Gas chromatography was used in the quantitative analysis of ethanol in the all fermentation media. Bioethanol obtained from sugarcane bagasse by potassium dichromate method was found to be 6.81 %(v/v) and confirmed by Gas chromatography which yielded 9.99 % (v/v) the Retention time (min) was 20.08 as shown in below chart the peak 207 shows highest ethanol concentration.

Chart for the estimation of glucose by Somoyogi method:

<table>
<thead>
<tr>
<th>Before fermentation</th>
<th>After fermentation</th>
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Chart for the estimation of protein by Bradford’s method:

<table>
<thead>
<tr>
<th>Before fermentation</th>
<th>After fermentation</th>
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</table>

![Chart for glucose estimation](image)

![Chart for protein estimation](image)
The chart for the general ethanol production

The chart for the different Substrate Concentration

<table>
<thead>
<tr>
<th>Substrate concentration in g</th>
<th>Ethanol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5.8%</td>
</tr>
<tr>
<td>1</td>
<td>3.7%</td>
</tr>
<tr>
<td>1.5</td>
<td>2.8%</td>
</tr>
<tr>
<td>4</td>
<td>1.8%</td>
</tr>
<tr>
<td>5</td>
<td>4.5%</td>
</tr>
</tbody>
</table>

The chart for the different pH

<table>
<thead>
<tr>
<th>Different pH</th>
<th>Ethanol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>1.9%</td>
</tr>
<tr>
<td>4.5</td>
<td>2.7%</td>
</tr>
<tr>
<td>5</td>
<td>6.3%</td>
</tr>
<tr>
<td>6</td>
<td>5.6%</td>
</tr>
<tr>
<td>7</td>
<td>4.6%</td>
</tr>
</tbody>
</table>

The chart for the Quantification of bioethanol by GC-MS Method

CONCLUSION: The result of this study shows that agricultural waste namely sugarcane bagasse, known to contain sugar is good substrates for ethanol production. Therefore the findings of this work suggest that ethanol can be produced from agricultural wastes rather than allowing it to contribute a nuisance to the environment. Therefore, there should be the development of an environmentally friendly, pre-treatment procedure highly effective enzyme system for conversion of pre-treated waste to fermentable sugars, Effective microorganism to convert multiple sugars to ethanol. The use of alternate sources for the production of ethanol has been found to be economical and effective. This process of utilizing the solid waste those are very rich in cellulose, hemicellulose and lignin, gives rise to zero waste generation techniques. The maximum ethanol obtained from sugar cane bagasse was 6.8 % (v/v) at 10th day. And the fermentation extract confirms the presence of ethanol which is quantified by potassium dichromate method. According to the policy statement, ethanol production for blending with fuel in 2017-18 will be around 150 crore liters, resulting in foreign exchange savings up to Rs 4,000 crore. The production of biofuels is expected to control the carbon dioxide emissions by 20,000 tons.
SCOPE FOR FUTURE WORK: Bioethanol offers great benefits for safeguarding the environment, boosting the rural economy and ensuring fuel security. Bioethanol production process successful at industrial scale with reduction in capital and operation cost.

PHOTOGRAPHS OF BIOETHANOL