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# Production of Lignocellulosic Biofuel by Agro-Industrial Waste

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**ABSTRACT:** *World energy consumption is increasing annually due to use of high energy consuming products and industrial revolution. World is facing dilemma of energy shortage, which signifies the need to shift from traditional energy generating procedures to modern cost effective and durable energy generating resources. Biofuels presents an excellent alternative to traditional energy resources by its use an environment friendly fuel. The present study aims to generate bioethanol using different lignocellulosic substances i.e. sugarcane bagasse, leaves of neem plants and different grasses respectively collected from local sources. Fermentation was carried out using different bacterial isolates also obtained from local environment. Each substrate was subjected to pretreatment by 5% and 2% sulphuric acid and stream heat treatment before initializing bioethanol production. pH and reducing sugar content of each sample was determined. Bacterial isolates were isolated from agricultural sites. Bacillus thuringiensis was isolated from the soil of agricultural field and E.coli was isolated from locally collected waste water. Bioethanol was separated using the process of freeze drying. Total content of bioethanol obtained from E.coli was 0.15% and 0.09% from Bacillus thuringiensis.*

**Keywords**: Bioethanol, Bagasse, Lignocelluloses, Bacillus, hydrolysis.

## INTRODUCTION

Energy generation using renewable energy sources has always been a topic of interest in scientific community. Excessive use of fossil fuels has resulted in the global warming and climate change. Therefore, there is a thrust towards replacing fossil fuels with cleaner and renewable fuels such as bioethanol and biodiesel. Moreover, there is a rapid increase in the consumption of conventional fossil fuels and because of the unpredictable rise in their prices; there is an urgent need to develop an alternative renewable source of energy for taking care of the national energy securities. Lignocellulosic raw materials which include fruit and

vegetable waste, forestry waste, agro-residues, MSW etc. Biofuels are sustainable, safe and economical alternative for fossil fuels and could lead to minimize the emission of carbon dioxide. Biofuels could cut down the world's dependence on fossil fuel (deoliveira *et al*, 2005). Bioethanol and biodiesel are two commonly produced and used biofuel. Bioethanol is produced by the fermenting the sugar and starch component of plant. It is also produced by the products and agricultural waste (Booddey, 1993).Brazil produced bioethanol for the very first time from sugarcane. USA produced 6.49 billions of gallons of ethanol in 2007. Similarly Brazil produced 5.02 billions of gallons, china produced 0.49 and Canada produced 0.21 billions of gallons of ethanol in a same year (USDE, 2013*).*

Bioethanol is a pure form of ethanol and it must be blended with gasoline before use for vehicles as fuel. It has successfully substituted traditional fossil fuel. Lignocellulosic components present an excellent substrate for generation of bioethanol. These substrates include sugarcane bagasse, fruit and vegetable waste and various agricultural wastes. They are rich in cellulose and hemicelluloses which are the utilized by different microbes to generate fermentable sugars which eventually helps in generation of bioethanol. Different approaches can be used for extraction of ethanol. Mostly enzymatic hydrolysis and chemical hydrolysis is utilized for breakdown of lignocellulosic component of different substrates. The sugar and starchy biomass is converted into fermentable sugars through enzymatic process (IEA, 2007). Acid hydrolysis and steam heat pretreatment techniques are thought to be effective because of contaminant free process (Sun *et al.*, 2004). Both methods are also cost effective.

Various microorganisms are currently being used for ethanol production by the process of fermentation. Microorganisms such as yeast, bacteria and fungi have great potential to convert plant biomass derived sugars into biofuel. *Saccharomyces cerevisiae*is a major biocatalyst that is currently used to produce Bioethanol at industrial scale (Sasser *et al.,* 2008). The selection of organism for bioethanol production is dependent on substrate and pH and temperature tolerant biocatalyst. The high yield of bioethanol is dependent on high tolerant biocatalyst and commercial yeast is known as tolerant microorganism (Pasha *et al.,* 2007). In the present study our main goal was to generate bioethanol utilizing bacterial species of *E. coli*and *Bacillus thuringiensis* isolated from different sources by utilization of lignocellulosic substrates i.e. neem leaves and sugarcane bagasse and local grasses through fermentation process.

## MATERIALS AND METHODS

### Collection of samples

To produce lignocellulosic biofuel, Sugarcane bagasse was collected from a local juice shop of Lahore, Neem leaves (*Azadirachta indica*) were collected from a local garden and grass samples from the premises of Lahore Garrison University in the year 2018.

### Pretreatment of Biomass

Each collected sample was air dried and individually grinded. The grinded samples were then stored in polythene bags till further analysis. Two treatment groups were formed for pretreatment of biomass. In one group 40g

of each sample was taken in different flasks and in each flask 15ml of 2% Sulphuric acid was added. In second group 40g of respective samples were taken in three different flasks and in each flask 15ml of 5% Sulphuric acid was added for the delignification to the lignocelluloses into the cellulose and hemicelluloses and for de-polymerization of carbohydrates into reducing sugars. This process is also known as acid hydrolysis assay (Dein, 2006).

**Steam heat treatment**

For effective solubilization of lignin, samples were further subjected to steam heat treatment after acid hydrolysis. For steam heat treatment, the contents of flasks obtained after acid hydrolysis were autoclaved at 121°C for 15 minutes and allowed to cool. Contents of flasks were filtered before inoculation step.

**Determination of reducing sugar**

To determine the reducing sugars, filtrate biomass was treated with DNS (dinitrosalicylic acid). A graph was plotted by taking OD at 540nm using spectrophotometer. Concentration of reducing sugars in different samples was determined. For color change detection, 30% of sodium potassium titrate was added into mixture containing test tubes and were placed in water bath for 10 minutes.

**PH Adjustment of Biomass**

The pH values of pretreated biomass were adjusted per biocatalyst being inoculated into it. pH values of substrates for both bacterial isolates were adjusted at 4.

**Isolation of Bacteria**

The microorganisms that were used as biocatalysts to derive the fermentation process were isolated from different medium sunder the required and appropriate conditions at the biological lab of Lahore Garrison University. The appropriate and favorable ingredients and different media were selected for the growth of these microorganisms.

***Isolation of Bacillus thuringiensis***

Agricultural soil sample collected from Central Park Lahore was screened for *Bacillus thuringiensis* presence. Two-fold serial dilution of soil sample was made. 1g of soil was added into 10ml of distilled water and seven dilutions were prepared accordingly. 0.02ml of dilution $10^3$ was taken and spread over Nutrient-agar. Same procedure was repeated for $10^5$ and $10^7$ dilutions. The Petri plates were incubated at 37°C for 24 hours. For the selective isolation of isolates of *Bacillus thuringiensis*, minimal selective media was prepared as per manufacturer instructions with the addition of L-serine and M9-salt (Andrezjczak *et al*., 2008). Then selected colonies from N-agar were streaked over selective media and incubated at 37°C for 48 hours. Different morphological tests were performed to confirm its morphological characteristics.

***Isolation of Escherichia Coli***

Waste water was collected for the isolation of *Escherichia coli*. 2-fold serial dilutions were made by addition of 1ml waste water into 10 ml distilled water. Dilutions were made accordingly e.g. $10^1$, $10^2$,$10^3$ till $10^{10.}$ and cultured on Eosin Methylene Blue (EMB) agar. 0.02ml of inoculum was spread over media plates and incubated at 37°Cfor 24 hours.

**Identification of bacterial isolates**

Bacterial isolates were pure cultured through repeated streaking on their respective growth media. Grams staining, motility test and endospore staining of the isolates were performed for determination of bacterial characteristics. Gram negative bacterial isolate was identified on biochemical basis using API 20E strips (bioMerieux, Inc.) as described in Bergey's manual (Bergey, D. *et al.*, 1994).

**Fermentation**

The isolated organisms (Biocatalysts) were inoculated into biomasses under the appropriate conditions. *Escherichia coli and Bacillus thuringiensis* were used as fermenting bacteria. Each bacterial isolate was inoculated into 10ml of each pretreated biomass. Each treatment had two replicas and one control test tubes. All the inoculated test tubes were incubated into shaking incubator at 150rpm at 37°C for 7days for completion of fermentation.

**Fractional Freezing**

After incubation, the Fermented broths were filtered through Gauze swabs or Sponges-B.P by (*SURGITEX).* This step was performed to increase concentration of ethanol. Fermented samples were treated for two consecutive days to minimize crystal formation time (Haizum *et al.*, 2015).

**Oxidation of Ethanol**

To confirm the ethanol production into fermented solution, the solution was treated with the acidified potassium dichromate.10 mL of each fermented solution was taken into test tube. 1M of potassium dichromate was added into it along with few drops of sulphuric acid and heat treatment was given in water bath which was setup at 70°C - 80°C for 30-60minutes and observed for color change.

**Extraction of Ethanol**

Ethanol solution in reagent bottle was placed at rest position for one day and ethanol layer appeared over the surface that was separated into separate reagent bottle. Eff is the concentration efficiency in % and Co is initial concentration of fermented solution that was 200mL where Cl is the concentration of Bioethanol after fractional freezing. Efficiency was measured by putting the values in formula given below:

Efficiency %= Co-Cl/Cl*100

## RESULTS

Production of biofuel was carried out using different kind of lignocellulosic substances. Each substrate was selected for maximum production of bioethanol. They have high content of cellulose, hemicellulose and lignin in different ratios. First different substrates were subjected to pretreatment to recover sugars then treated samples were subjected to fermentation to initiate the production of bioethanol.

Sugarcane bagasse, neem leaves and grasses were treated initially with 2% and 5% Sulphuric acid to release free sugars. Additional step of steam treatment was added to amplify the recovery process. Each pretreated sample reducing sugar was measured using DNS method and pH of samples were adjusted accordingly.

**Isolation of Biocatalysts**

***Bacillus thuringiensis*:**

Three of gram-positive and spore forming bacilli were isolated from soil. Morphological and physiological characters of isolates are shown in (Table 1). Isolates showing required characters were transferred to M9 minimal media. After incubation, mucoid, smooth, flat colonies of Bacillus were obtained. Microscopic examination showed they were gram positive with typical rods.

***Escherichia Coli*:**

*Escherichia coli* was used a fermentative microbe and it was isolated from waste water collected from local area and it was spread plated over EMB media and morphological analysis of *E. coli* colonies was done to check its colony characters. Small pink colonies with green metallic sheen were obtained over EMB agar. Microscopic examination was done and isolated colonies were observed as rod shaped and gram negative. Motility test confirmed bacterial isolates to be non-motile. Endospore staining confirmed that test subject does not contain spores inside cell. Morphological analysis of *E coli* is given in the table (Table 2). To further confirm the identity of isolated bacteria API strip was used. It confirmed the presence of *E coli* by giving characteristics results associated with *E. coli* (Fig 1).

**Physiological Characterization**

***Biocatalysts***

Temperature for bacterial biocatalysts (*Escherichia coli* and *Bacillus thuringiensis)* was adjusted at 37˚Cas optimal temperature. The incubation time for *Escherichia coli* was 24 hours. *Bacillus thuringiensis* grew slowly within 24 hours but showed well growth after 48 hours. pH for bacterial biocatalysts (*Escherichia coli* and *Bacillus thuringiensis*) was adjusted at 4 (Table 3)

***Fermentation***

Each pretreated Feedstock used as a substrate for second generation biofuel production, were obtained in dried fine powder and were pretreated with 2% and 5% sulphuric acid and gave thick solution and appeared in dark color after steam heat treatment that was the indication of delignification and depolymerization of carbohydrates or reducing sugar For the confirmation of delignification and depolymerization of carbohydrates, all the pretreated solutions e.g. Sugarcane bagasse, Neem leaves and grasses were individually treated with DNS reagent that gave the positive results as brown color was observed on boiling the pretreated solutions with DNS reagent and sodium potassium titrate .

***Oxidation test of Bioethanol***

The positive results were obtained within 30-45 minutes of treatment and fermented solution turned into green color that was the indication of oxidation of Bioethanol. Results varied at different conditions.

***Efficiency of bioethanol***

After extraction and purification of ethanol utilizing different methods. Efficiency of ethanol was measured. 5% Sulphuric acid treated biomass showed more efficiency using *E coli* as a biocatalyst. 5% treated bagasse showed most efficiency of 0.15% bioethanol followed by grass and neem leaves. By using *B. thuringiensis* as biocatalyst 5% acid treated bagasse has efficiency of 0.07% (Figs 2-3).

**Table 1: Morphological characterization of *Bacillus thuringiensis***

| Characteristics | Observation |
|---|---|
| Gram nature and cell arrangement | Gram positive rods |
| Color of colony | Off white |
| Size of colony | Small to medium |
| Elevation | Flat |
| Nature | Mucoid |
| Margins | Smooth |
| Shape | Circular |
| Margins | Smooth |
| Shape | Circular |

**Table 2: Morphological Characterization of *Escherichia coli:***

| Serial no. | Characteristics | Observation |
|---|---|---|
| 1 | Shape | Rods |
| 2 | Capsule | Variable |
| 3 | Spore | Non-spore forming |
| 4 | Flagella | Flagellated |
| 5 | Motility | Non-motile |


**Fig 1: API strip showing different result after incubation confirming presence of *E coli*.**

**Table 3: Characteristics of Biocatalyst for carrying out the fermentation process.**

| Characteristics of Biocatalysts | | | |
|---|---|---|---|
| *E. coli* | | *B. thuringiensis* | |
| pH | 4 | pH | 4 |
| Temperature | 37°C | Temperature | 37°C |
| Incubation time | 24 hours | Incubation time | 48 hours |

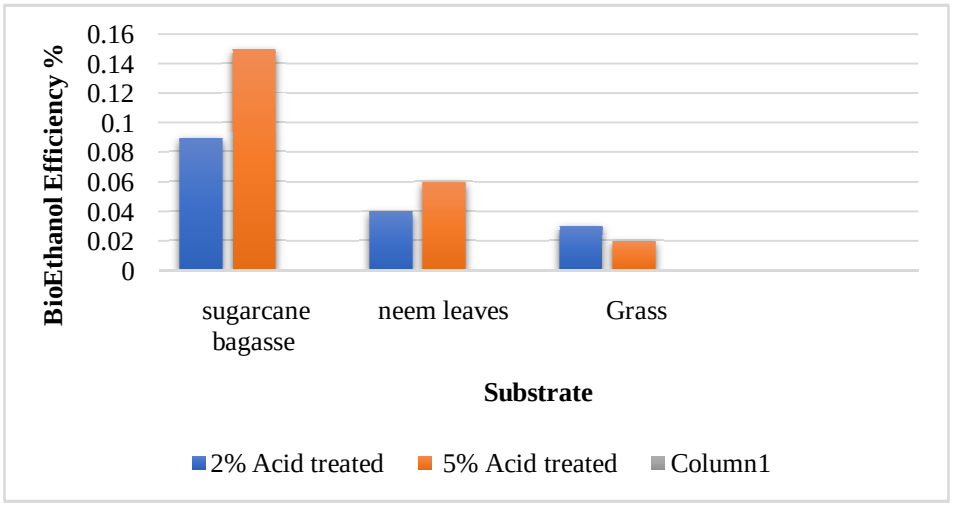


**Fig 2: Efficiency of bioethanol produced using *E.coli* as a biocatalyst.**

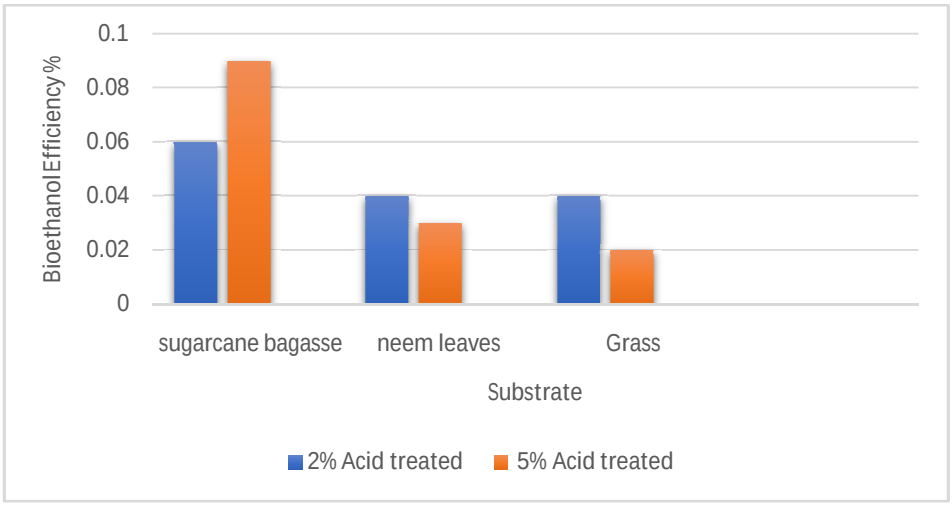


**Fig 3: Efficiency of Bioethanol produced using *Bacillus thuringiensis* as a biocatalyst.**

## DISCUSSION

The present study was carried to analyze production of bioethanol using agricultural substrates and utilizing bacteria as fermentative microbes. Two bacterial species *Bacillus thuringiensis* and *E coli* were isolated from the locally collected soil and waste water samples respectively. Each test isolate was subjected to various morphological, physiological and biochemical tests. The results showed that the test isolates from the soil sample were Gram-positive with rod structure (Table 1). They produced off white color colonies on minimal media, their optimum growth temperatures were 28–37°C. Whereas test isolate from waste water were found gram negative, rods with green color colonies with metallic sheen on EMB media (Table 2). Similar procedure was used by the (Solaimani S *et al.*, 2017) for isolation of desired bacteria. Gram negative bacteria were analyzed using the API 50 CHL (bioMérieux, France) test system according to the protocol set by the manufacturer, and isolate was identified as *E coli.*

The production of bioethanol from agro-waste derived from different agricultural substrates namely sugarcane bagasse (*Saccharum officinarum*), neem leaves (*Azadirachta indica*) and grass samples respectively was carried out. All samples were subjected to acid hydrolysis pretreatment prior to fermentation which is in accordance with work of (Cutzu *et al.*, 2017 and Braide *et al*., 2016). 2 % and 5 % diluted sulphuricacid was added to biomass and steam heat treatment was applied to each substrate (Sun *et al.*, 2004). Pretreatment ensures conversion of complex plant material to simple sugars for utilization by bacterial isolates ((Lal, 2005).)The raw materials were assessed before fermentation to determine if they are good substrates for fermentation, and were found to be suitable based on their color and pH conditions. Each pretreated sample, reducing sugar content was determined using DNS method. Similar method as described previously by (Cutzu *et al.*, 2017) pH was adjusted to 4.0 for bacterial isolates proper functioning.

Pretreated samples from each substrate were subjected to the process of fermentation for the period of 7 days at 37$^{o}$C after inoculation with both bacterial species. After fermentation process of fractional freezing was carried out to enhance the concentration of ethanol (Haizum *et al.*, 2015). To confirm the ethanol production, the solution was treated with the acidified potassium dichromate for oxidation of ethanol. After this process ethanol content of each treated sample was determined. 5% Sulphuricacid treated biomass showed more efficiency using *E coli* as a biocatalyst. 5% treated bagasse showed most efficiency of 0.15% bioethanol followed by grass and neem leaves. By using *B. thuringiensis* as biocatalyst 5% acid treated bagasse showed maximum ethanol efficiency of 0.07%. Similar results were observed by Jahid M., 2018. Acid hydrolysis proved to be effective treatment for obtaining bioethanol prior to fermentation.

## CONCLUSION

The result of this study shows that agricultural substrates namely sugarcane bagasse, neem leaves and grasses which contain fermentable sugars can be used as a substrate for bioethanol production. Therefore the findings of this work suggest that ethanol can be produced from different agricultural substrates rather than allowing it to contribute a nuisance to the

environment. Therefore, there should be effective microorganism to convert multiple sugars to ethanol. This will help to generate environmental friendly fuel for coming generations.

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