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Production of Biofuel using Waste Papers from *Pseudomonas aeruginosa*

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Abstract: The Present world energy scenario is focused at nonconventional sources. The biomass has emerged as one of the dependable nontraditional feed stocks for the production of biofuel. The present research enlightens various feed stock viz waste papers, sugar beets, sugar cane, corn, wheat, barley etc and fermentation methods for the production of biofuel. The microorganisms *Pseudomonas aeruginosa* has the ability to produce biofuel by optimization. The optimized parameters are incubation period of 72 hours, temperature at 35-37˚C, pH 8, nitrogen source Yeast extract and carbon source cellulose were found. Also the yeast *Saccharomyces cerevisiae* showed excellent co-fermentability of glucose, mannose and galactose in hydrolysate of waste papers. Industrial biofuel producing microorganisms which are capable of fermenting all of the sugar present in feed stock: attracted much attention in recent years with the recent advances in biotechnology.
Keywords: Paper sludge, ethanol, cellulose, *Pseudomonas aeruginosa.*

Introduction

In the modern age of development the increasing quantity of solid waste is one of the growing problems in both developed and developing countries. Due to the rapid growth in industrialization, the most of the rural population have shifted towards the urban area in search of employment. India produces 1,20,000 tonnes of solid wastes every day. The rapid increase in the volume of waste is one aspects of the environmental crisis, accompanying global development. Most common practices of waste processing are uncontrolled dumping which causes mainly water and soil pollution. Although various physical, chemical and microbiological methods of disposal of organic solid wastes are currently in use, these methods are time consuming and expensive[1]. A biofuel is a fuel that is derived from biological materials. Important area of the modern biotechnology is a conversion of non-edible plant biomass to valuable bioproducts, biofuels and biochemicals. World pulp and paper industry produces about 300-350 million tons of various types of paper and board. Smaller part of waste paper materials is recycled, while the most of the used materials is thrown out or burned. Plant-based biomasses including paper materials are regarded as abundant, renewable and inexpensive sources of raw materials that accumulate in the world in huge amounts.

Biofuel is a form of quasi-renewable energy that can be produced from agricultural feedstocks. It can be made from very common crops such as sugarcane, potato, cassava and corn. Concerns about its production and use relate to increased food prices due to the large amount of arable land required for crops, as well as the energy and pollution balance of the whole cycle of fuel production, especially from corn. Recent developments with cellulosic biofuel production and commercialization may allay some of these concerns[2].

The basic steps for large-scale production of ethanol are: microbial (yeast) fermentation of sugars, distillation, and dehydration and denaturing. Prior to fermentation, some crops require saccharification or hydrolysis of carbohydrate such as cellulose and starch into sugars. Saccharification of cellulose is called cellulolysis. Enzymes are used to convert starch into sugar. Biofuel is produced by microbial fermentation of

the sugar. Microbial fermentation currently only works directly with sugars. Two major components of plants, starch and cellulose, are both made of sugars and can, in principle, be converted to sugars for fermentation. Currently, only the sugars (e.g., sugarcane) and starch (e.g., corn) portions can be economically converted. There is much activity in the area of cellulosic ethanol, where the cellulose part of a plant is broken down to sugars and subsequently converted to ethanol. For the ethanol to be usable as a fuel, the majority of the water must be removed. Most of the water is removed by distillation, but the purity is limited to 95-96% due to the formation of a low-boiling water-ethanol azeotrope with maximum (95.6% m/m (96.5% v/v) ethanol and 4.4% m/m (3.5% v/v) water). This mixture is called hydrous ethanol and can be used as a fuel alone, but unlike anhydrous ethanol, hydrous ethanol is not miscible in all ratios with gasoline, so the water fraction is typically removed in further treatment to burn in combination with gasoline in gasoline engines[3].

The cumulative impacts of these concerns have increased the interest in developing biofuels produced from non-food biomass. Feedstocks from ligno-cellulosic materials include cereal straw, bagasse, forest residues, and purpose-grown energy crops such as vegetative grasses and short rotation forests. These second-generation biofuels could avoid many of the concerns facing first-generation biofuels and potentially offer greater cost reduction potential in the longer term [4].

Lignocellulosic biomass consists mainly of lignin and the polysaccharides cellulose and hemicellulose. Compared with the production of ethanol from first-generation feedstocks, the use of lignocellulosic biomass is more complicated because the polysaccharides are more stable and the pentose sugars are not readily fermentable by *Saccharomyces cerevisiae*. Inorder to convert lignocellulosic biomass to biofuels the polysaccharides must first be hydrolysed, or broken down, into simple sugars using either acid or enzymes. Several biotechnology-based approaches are being used to overcome such problems, including the development of strains of *Saccharomyces cerevisiae* that can ferment pentose sugars, the use of alternative yeast species that naturally ferment pentose sugars, and the engineering of enzymes that are able to break down cellulose and hemicellulose into simple sugars [5].

Lignocellulosic biomass has an advantage over other agriculturally important biofuels feedstocks such as corn starch, soybeans, and sugar cane, because it can be produced quickly and at significantly lower cost than food crops. Lignocellulosic biomass is an important component of the major food crops; it is the non-edible portion of the plant, which is currently underutilized, but could be used for biofuel production. In short, lignocellulosic biomass holds the key to supplying society's basic needs for sustainable production of liquid transportation fuels without impacting the nation's food supply[6] .

Biologically produced alcohols, most commonly ethanol and less commonly propanol and butanol, are produced by the action of microorganisms and enzymes through the fermentation of sugars or starches or cellulose. Biobutanol is often claimed to provide a direct replacement for gasoline, because it can be used directly in a gasoline engines. The ethanol production methods used are enzyme digestion (to release sugar from stored starches), fermentation of the sugars, distillation and drying. The distillation process requires significant energy input for heat (sometime unsustainable natural gas fossil fuel), but cellulosic biomass such as bagasse, the waste left after sugar cane is pressed to extract its juice, is the most common fuel in Brazil, while pellets, wood chips and also waste heat are common in Europe Waste steam fuels ethanol factory, where waste heat from the factories also is used in the district heating grid [7].

A number of chemicals are produced in the ethanol industry and potentially even more in the 2nd generation biofuel industry, serving a wide range of uses in the pharmaceuticals, cosmetics, beverages and medical sectors as well as for industrial uses. The market potential for bioethanol is therefore not just limited to transport fuel or energy production but has potential to supply the existing chemicals industry.

Bioethanol has mostly been used as a biofuel for transport, especially in Brazil. Indeed it was in Brazil where the first bioethanol fuelled cars emerged on am large-scale. Although generally unknown to the average consumer, a large volume of bioethanol is already used in Europe as it is blended with petrol at 5%. It is used as a substitute for lead as an oxygenating additive and has a high octane rating, which improves performance. Although the eventual target is the private consumer, few are aware of bioethanol's potential too, atleast partly replace petrol as a transport fuel in Europe [8].

2. Materials and Methods

2.1 Collection and processing of samples

Waste papers were collected from Karunya University premises, Coimbatore. The materials were collected in polythene bags and kept in the laboratory where the experiments were performed. The cellulose substrate was cut into small pieces using a pair of scissors, while the starch substrate was sun dried and using electric blender the papers were made into small particles.

2.2 Physical and chemical pretreatment

Waste papers were shredded into small pieces, dried in oven at 65˚C for 24 hours and pulverisized in electric blender to form fluffy wool like substrate. 20g of pulverized substrate and 300ml of 1% NaOH solution are mixed in 500ml conical flask and kept it for hydrolysis for 6 hours. After hydrolysis the substrate was washed in running tap water and excess water is removed by squeezing. Again the substrate dried in oven at 65˚C for 24 hours.

2.3 Subculturing of *Pseudomonas aeruginosa*

Weighed 1.3g of Nutrient broth and 2.0g of nutrient agar in 100ml distilled water. The mixture was sterilized for 20 minutes. After sterilizing, the mixture was cooled down and using laminar air flow chamber the microorganism culture of *Pseudomonas aeruginosa* a small quantity was added into the conical flask containing the mixture. The mixture was kept in shaker at 120rpm for 24 hours. After that the sub cultured conical flask was stored in a refrigerator at 4˚C for further use.

2.4 Plating of culture

Weighed 1.3g of nutrient broth and 2.0g of nutrient agar in 100ml distilled water. The mixture and the petriplates were kept for sterilization for 20 minutes. After sterilization, the mixture and plates were kept in laminar air flow chamber and allowed to cool. After it cools for sometime the mixture can be poured into plates and allowed it to solidify. Using Streaking method the culture can be streaked in the solidified plates using loop aseptically and sealed with parafilm and stored in refrigerator.

2.5 Preparation of yeast culture

Measured 0.1g of commercial yeast (*Saccharomyces cerevisiae)* in a weigh balance and dissolve in 10ml of distilled water. Sterilize the conical flask containing 250ml distilled water and 10g of potato dextrose agar and also the petriplates. After sterilization, pour 15-20ml of the agar into the petriplates in a laminar air flow chamber (LAF). When it solidifies, introduce the yeast using streak plate method using loop aseptically. Seal the plates using parafilm maintain in incubator at 26˚C.

2.6 Conversion process

One liter of water was added to 20g of the starch substrate. The medium composition are 6.60g of K_2SO_4, 3.0g of KH_2PO_4, 0.50g of $MgSO_4$, 1.0g of $CaCl_2.2H_20$ and 5.0g of Peptone was added into the starch substrate. The mixture of substrate was autoclaved at 121˚C for 15 minutes, and then the medium was cooled to room temperature. After it cools down the microorganism (*Pseudomonas aeruginosa*) were added into the substrate in laminar air flow chamber, finally the substrate was kept in Orbital shaker at 35˚C for 140rpm for 48 hours.

2.7 Sugar Analysis by DNS Method

Procedure

1. The above reagents were first prepared.
2. 3 ml of reagent was added to 3 ml of glucose solution in a lightly capped test tube.
3. The mixture was heated at 90°C for 5-15 minutes till red-brown coloration is developed.
4. 1 ml of 40% Na-K tartarate solution was added to stabilize the color.
5. After the solution was cooled to room temperature in a water bath, the absorbance of the solution was recorded with the help of UV spectrophotometer at 575 nm.
6. The above procedure was repeated for each of the filtered samples and graph between optical density Vs concentration was plotted.

2.8 Fermentation and centrifugation

The cellulose were filtered, mixed and diluted with water to adjust the initial concentration. After 48 hours of substrate, it is taken from shaker and kept in a laminar air flow chamber and take yeast cultured plates and dilute the cultures with distilled water as the ratio 10ml per conical flask. Finally introduce 10ml of yeast dilution in 250ml conical flask and keep in incubator for about 72 hours. After fermentation the sugar molecules that can be converted into biofuel by the action of yeast (*Saccharomyces cerevisiae).*The yeast containing substrate that can be centrifuged at 5000 rpm for 10 minutes.

2.9 Distillation and Analysis

During centrifugation the yeast containing substrate that can be separated into pellet and supernatant. For distillation purpose, the supernatant that is poured in the round bottom flasks by setting at 70rpm until the supernatant of sample that can be filtered and converted into biofuels. After the conversion of biofuel that can be analyzed by UV-Spectrometer at 575nm.

3. Results and Discussion

3.1 Collection of papers

Fig 1: Waste paper

Fig 2: Grinded paper

3.2 Pretreatment and subculture

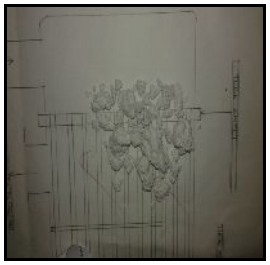


Fig 3: Hydrolyzed paper

Fig 4: Subculture of *Pseudomonas aeruginosa*

3.3 Preparation of yeast culture

Figure 5: Preparation of *Saccharomyces cerevisiae*

3.4 Fermentation

Figure 6. Before fermentation

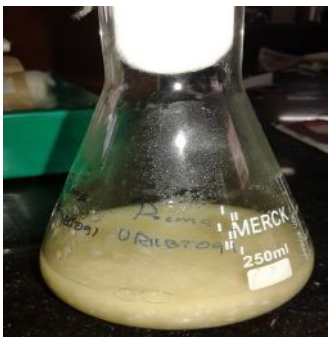


Figure 7: After fermentation

3.5 Centrifugation

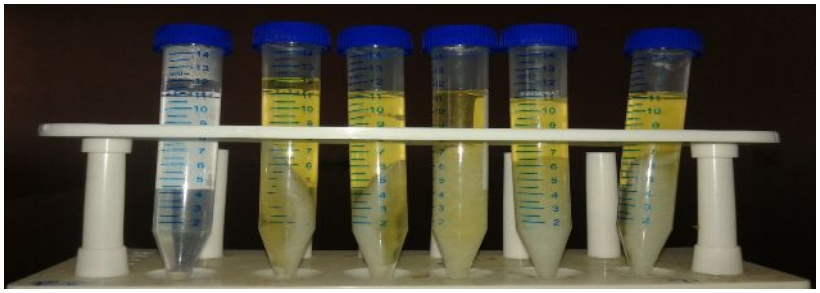


Figure 8. After fermentation, the sugar molecules converted into biofuel by the action of yeast *Saccharomyces cerevisiae.*

3.6 Distillation

Figure 9: Purification of biofuel

3.7 Analysis

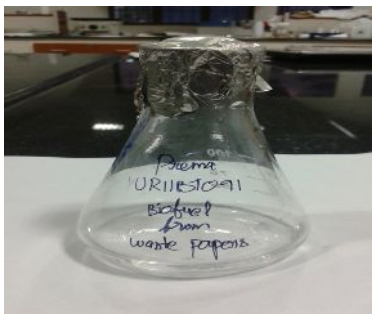


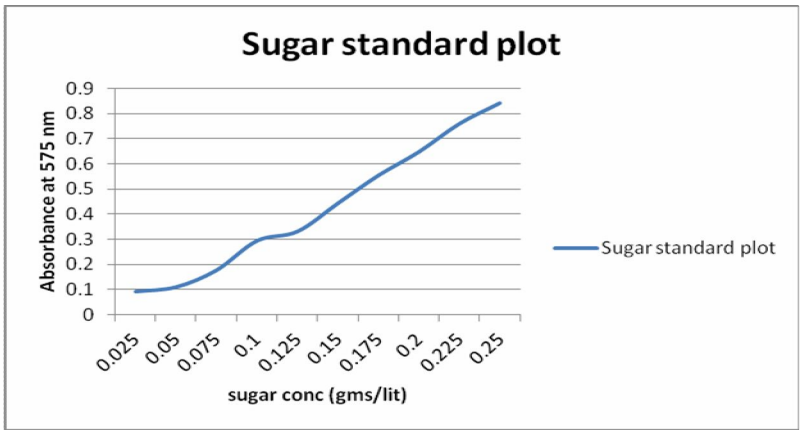
Figure 10: Biofuel yield

4. Analysis of sugar with increasing number of days

Sugar was analyzed by DNS method. The method is explained previously. Initial amount of sugar was 25 mg in 100 ml of water. The standard plot of sugar is shown as follows:

S.No	Amount of Glucose (in ml)	Amount of Water (in ml)	Conc. of Glucose (g/l)	Absorbance At 575 nm
1	0	3.0	0	0
2	0.3	2.7	0.025	0.0928
3	0.6	2.4	0.05	0.111
4	0.9	2.1	0.075	0.178
5	1.2	1.8	0.1	0.295
6	1.5	1.5	0.125	0.332
7	1.8	1.2	0.15	0.444
8	2.1	0.9	0.175	0.555
9	2.4	0.6	0.2	0.6483
10	2.7	0.3	0.225	0.7603
11	3.0	0	0.25	0.8425

4.1 Sugar standard plot



The concentration of sugar before fermentation and after fermentation with increasing number of days is given in the tabular and graphical form:

4.2 Before fermentation (Table no.2)

S.No	Sample	ABS AT 575 nm	Sugar Conc (g/l)
1	*P.aeruginosa*	0.5342	0.2110
2	*E.coli*	0.7532	0.2604

4.3 After fermentation

4.3.1 Day 1 of fermentation (Table no.3)

S.No	Sample	ABS AT 575 nm	Sugar Conc (g/l)
1	*P.aeruginosa*	0.5527	0.2435
2	*E.coli*	0.6223	0.2776

4.3.2 Day 2 of fermentation (Table no.4)

S.No	Sample	ABS AT 575 nm	Sugar Conc (g/l)
1	*P.aeruginosa*	0.5223	0.1356
2	*E.coli*	0.4657	0.1534

4.3.3 Day 3of fermentation (Table no.5)

S.No	Sample	ABS AT 575 nm	Sugar Conc (g/l)
1	*P.aeruginosa*	0.4013	0.1562
2	*E.coli*	0.2875	0.1134

Conclusion

The conversion process employed for waste papers (which are basically made up of cellulose) was achieved by hydrolysis, where the complex cellulose structure was broken down into simple fermentable sugar. The amount of fermentable sugar that was obtained from the hydrolysis of the wastepaper was obtained to be 40%, with only slight variation from the standard. The waste paper substrate (basically made up of starch), was converted into simple fermentable sugar by microbial process i.e., *Pseudomonas aeruginosa.* The microbial hydrolysis of waste paper substrate yielded was 45%. The production of ethanol fuel from organic and food waste yielded 0.86 litres of 95% ethanol from 20g of waste papers and maize substrate, which were respectively converted to 55% and 63% fermentable sugar. Apart from the fact of relieving the environment of pollution which may be attributed to wastepaper, to have economic production of ethanol fuel, it is best to use waste papers which give a higher percentage of yields.

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