

PRODUCTION OF BIOETHANOL FROM DIFFERENT PLANT SOURCES

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ABSTRACT

Bio fuels from various sources such as *Jatropha curcas*, *Pongamia pinnata*, *Azadirachta indica*, *Madhuca indica* etc are produced till now. Ethanol is made biologically from cellulosic biomass, and using substrates such as molasses, fruit pulps and peels etc. are well known. Since the need of bioethanol has been increasing, in this study we used peels of citrus, non-citrus fruits, Pseudo stem of banana as substrates, due to its cheaper, ecofriendly and whole year availability. A chemical pre-treatment process using acid hydrolysis was applied. The amount of carbohydrate and alcohol was determined before and after the pretreatment. Cellulose degrading bacteria isolated from the midgut of the earthworms was inoculated into the pretreated samples, fermenting the substrate for alcohol production. Ethanol production in culture samples was determined by spectroscopic method. Results indicate that ethanol can be made from fruit peel residue and that the acid hydrolysis was effective in removal of lignin, which acts as physical barrier to cellulolytic enzymes and causes the release of 20%-30% more sugar.

KEYWORDS

Ethanol, cellulose, lignin, acid hydrolysis, pretreatment, cellulose degrading bacteria (CDB).

1. INTRODUCTION

Ethanol is one of the most important renewable fuels contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels. The rapid depleting non-renewable resources has already reached pinnacle. Now there has been an urgent need for a renewable, sustainable energy sources. In recent years ethanol had been a promising renewable source. . Ethanol is earning increasing attention as a potentially cleaner, renewable, and domestically produced alternative to fossil fuels for transportation. Ethanol is primarily produced from two major categories, catalytic hydration of ethylene (Synthetic ethanol) and bio-fermentation of agricultural feed stocks fruits, vegetables, and cereals (Balasubramanian.K *et al* 2011). Hence ethanol is produced from different biological sources such as sugar cane, bagasse, miscanthus, sugar beet, sorghum, grain sorghum, switch grass, barley, hemp, kenaf, potatoes, sweet potatoes, cassava, sunflower, fruit, molasses, corn, Stover, grain, wheat, straw, cotton.

Since the price of feedstock contributes more than 55% to the production cost, inexpensive feedstocks such as lignocellulosic biomass and agro-food wastes, are being considered to make bioethanol competitive in the open market (Campo *et al* 2006). The production of ethanol from comparatively cheaper source of raw materials using efficient fermentative microorganism is the only possible way to meet the great demand for ethanol in the present situation of energy crisis (Pramanik, K.and Rao, D.E., 2005). The ripe fruit biomass as raw materials for fermentation, enzymatic hydrolysis using microbial enzymes could be a possible solution to reduce the energy and input costs in ethanol production (Hammond *et al* 1996). The solid wastes generated by fruit processing industries can serve as potential raw materials for the production of secondary metabolites of industrial significance by microorganisms. Peels are the major by-products obtained during the processing of various fruits and these were shown to be a good source of various bioactive compounds which possess various beneficial effects. But, significant quantities of fruit

peels are discarded as waste by the processing industries which cause a real environmental problem (Zhang *et al* 2005). These fruit-processing wastes can be used as potential feedstock for bioethanol production and this could also be an attractive alternate for disposal of the polluting residues (Wyman, Ch.E., 2001). Some few research articles deal with different practical applications of these fruit wastes (banana and mango), e.g., production of microbial enzymes for industrial uses (Essien *et al* 2005), production of alcohol (Hammond *et al* 1996), production of wine, vinegar, production of biogas (Guneseelan, N.V., 2004) and food for livestock (Onwuka *et al* 1997).

The amount of reviews covering ethanol production from other types of feedstock like sucrose-based or starchy materials is more reduced. However, little effort has been made on ethanol production from pretreated enzyme saccharified fruit wastes by simple fermentation techniques.

While ethanol can be produced from agricultural products, which are abundant renewable resources found worldwide (Krishna S H and Chowdary G V., 2000) but the close physical and chemical associations between lignin and plant cell wall polysaccharides, together with cellulose crystallinity limits the ethanol production (Gould J M and Freer S N., 1984). Lignin forms a protective shield around cellulose and hemicellulose, protecting the polysaccharides from enzymatic degradation. To convert the biomass into ethanol, the cellulose must be readily available for cellulose enzymes. Thus, by removing the lignin, the cellulose becomes vulnerable to enzymes and allows the cellulose degrading bacteria to convert the glucose into ethanol during fermentation. Therefore, a pretreatment must be applied to degrade the lignin in fruit residue, decrease cellulose crystallinity, and increase the surface area for enzymatic activity.

The peel contains various carbohydrate polymers, which make it an interesting choice for production of metabolites such as ethanol by appropriate microorganisms. An individual or combination of mechanical, chemical, and biological pretreatments, however, is required to break down cellulose, hemicellulose and pectin polymers present in the cell walls of peels and convert them into their sugar monomers which can further be fermented to ethanol (Grohmann *et al.*, 1995).

Cellulose degrading bacteria have been isolated for obtaining more effective cellulases from variety of sources such as soil, decayed plant materials, organic matters, faeces of ruminants and composts. The conversion of cellulosic mass to fermentable sugars from cellulolytic microorganisms has been suggested as a possible process that possesses potential to reduce the use of fossil fuels and reduce environmental pollution. The gut of earthworms is the residence for the production of the beneficial microorganisms and one among them is the cellulose degrading bacteria.

Hence, the present study was to examine the production of bioethanol using the peels of citrus and non-citrus fruits and Pseudo stem of banana as raw material by subjecting them to acid pretreatment. By further subjecting them to the carboxy methyl cellulase producing potential of the earthworm gut bacterial isolates will ferment the substrate for ethanol production.

2. MATERIALS AND METHODS

2.1. RAW MATERIAL (SUBSTRATE)

Fruit biomass peels of orange, papaya and pseudo stem of banana were collected and were chopped into small pieces and dried under the sunlight. The dried substrate was powdered with an electric grinder packed in polyethylene bags and stored at room temperature for further analysis.

2.2. PRE-TREATMENT OF RAW MATERIALS

The purpose of acid hydrolysis was to remove lignin from the fruit biomass peel residue, which hinders enzymatic hydrolysis of cellulose for ethanol fermentation. Dilute sulfuric acid (H_2SO_4) concentration of 0.8M used in this pretreatment.

For the acid hydrolysis pretreatment, approximately 5 g of dry fruit biomass peel residue was placed into anaerobic bottles containing 100mL of DI water and 0.2M H_2SO_4 and allowed to soak for 24 h. The bottles were subsequently autoclaved and allowed to cool. (G.Lalitha and RajeshwariSivraj., 2011)

2.3. DETERMINATION OF CARBOHYDRATES

Estimation of total sugar by anthrone method was carried out. For this 100mg of the powdered sample was taken and subjected to hydrolysis by keeping it in boiling water bath for 3 hrs with 5ml of 2.5N HCL. Cool the contents to room temperature and neutralize it with solid sodium carbonate until the effervescence ceases. Make up the volume to 100ml and centrifuge, collect the supernatant, and add the anthrone reagent. Read the absorbance at 630nm. Standard glucose is prepared and used to obtain the standard graph. (Dubio *et al* 1951)

2.4. ISOLATION OF CELLULOSE - DEGRADING BACTERIA

2.4.1. Collection of earthworms and processing

Earthworms were collected from the vermi compost and were washed with sterile water. They were then placed in a petriplate containing moistened filter paper for 24hrs. Clean them externally with 70% ethanol and dissect them to obtain the mid gut portion.

2.4.2. Isolation of bacteria from the mid gut of the earthworm

Isolation was carried out by plate dilution method of Cappuccino and Sherman 2008. Midgut was collected in 10 ml of 85% NaCl and homogenized in a vortex mixture for 5 minutes. The sample was then serially diluted (10^{-1} to 10^{-7}) triplicates of each dilution was plated on to nutrient agar plates and were incubated at 30° C for 24 hours.

2.4.3. Screening of cellulose degrading bacteria

The isolated colonies obtained on the nutrient agar plates were inoculated on to CMC agar plates and incubated at 30° C for 24hrs. The colonies obtained on this were streaked on CMC slants. The CMC agar plates were flooded with 1% congo red, a clear zone of lysis around the colonies on washing the plates with 1M NaCl indicates the presence of cellulose degrading bacteria. (Krupa Mary Jacob *et al.* 2014).

2.5. INNOCULUM USED:

The pretreated samples with 0.8M H_2SO_4 were inoculated with the cellulose degrading bacteria strain 1 and strain 2 obtained from the mid gut of the earthworms.

2.6. ESTIMATION OF REDUCING SUGAR BY DNS METHOD

The inoculated samples of 0.5ml were removed periodically at intervals of 2,5,7,9 and 12 days and the amount of reducing sugar present were estimated. The samples were made up to 1ml with distilled water followed by the addition of 1ml of DNS reagent and kept in boiling water bath for 10 minutes. Readings were taken at optical density of 540nm. (Miller, 1959)

2.7. ESTIMATION OF ALCOHOL BY MODIFIED CERRIC AMMONIUM NITRATE METHOD

Standard alcohol samples from 0.1-1.0ml were pipette out in series. The samples of 0.1ml were taken at intervals of 2,5,7,9 and 12 days and the amount of alcohol present was determined.All the test tubes were made up to 1ml with distilled water and followed by the addition of 2ml of 10% cerric ammonium nitrate to all the tubes just before taking the readings. Readings were taken at optical density of 486nm. (Reid and Truelove 1952)

3. RESULTS

3.1 Before pretreatment

Plate 1. Amount of carbohydrates in the fruit peel biomass before pretreatment.

Trials	1st	2nd	3rd	4th
orange	5	15	4.4	2.6
papaya	4.6	3	0.4	1.4
pseudostem	2.4	12	1.6	2

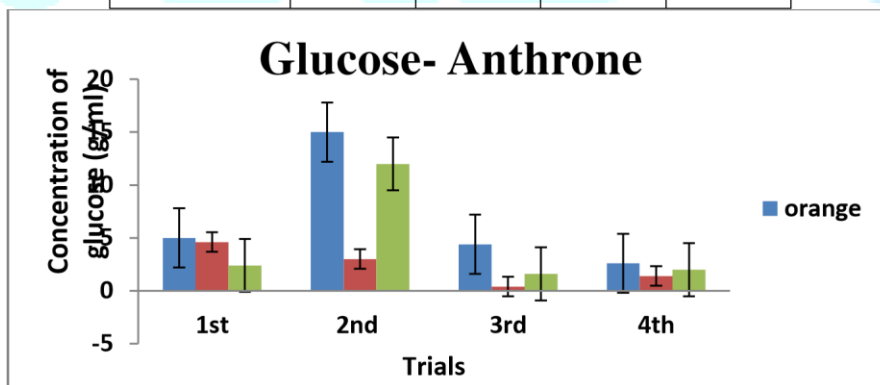


Plate 1. Amount of carbohydrates in the fruit peel biomass before pretreatment.

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3.2. After pretreatment

3.2.1. Determination of carbohydrates – DNS method

Trials	1st	2nd	3rd	4th
Orange	45	19	11	11
Papaya	28	16	0.5	0.3
Pseudostem	20	34	0.8	0.9

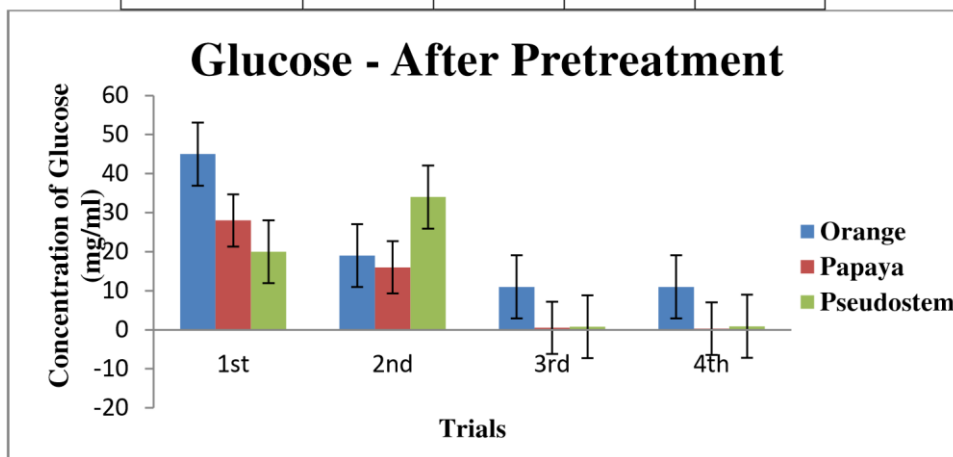
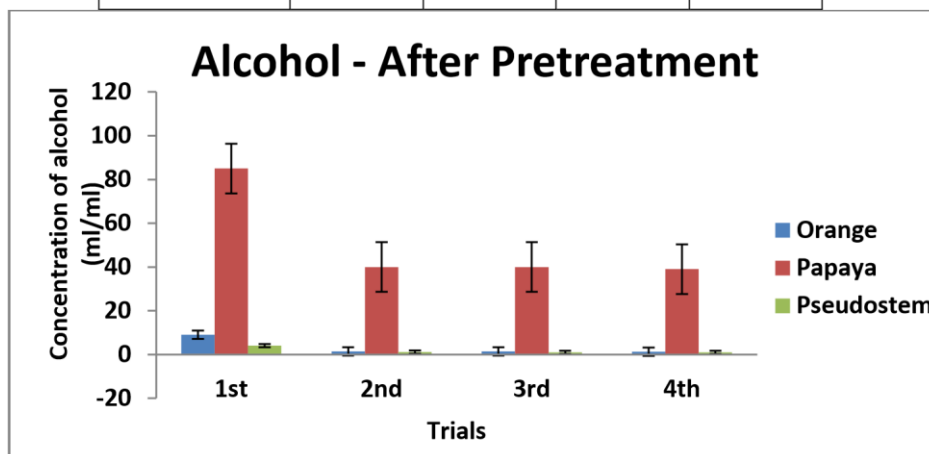


Plate 2. Amount of reducing sugars in the fruit peel biomass after pretreatment.

3.2.2 Alcohol - After pretreatment Estimation of alcohol

Trials	1st	2nd	3rd	4th
Orange	9	1.4	1.4	1.3
Papaya	85	40	40	39
Pseudostem	4	1.2	1	1



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Plate 3. Amount of alcohol in the fruit peel biomass after pretreatment.

3.3. After Inoculation of cellulose degrading bacteria strain 1 and 2

3.3.1. Determination of carbohydrates – DNS method

Strain 1

e/days				
e				
a				
ostem				

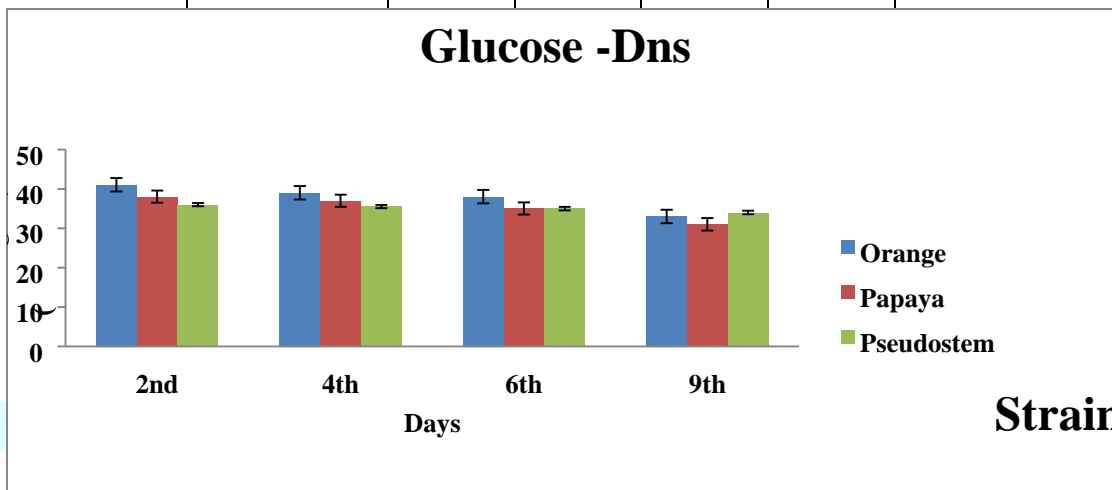


Plate 4. Amount of reducing sugars in the fruit peel biomass after adding cellulose degrading bacteria strain 1.

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Strain 2

Sample/days	2nd	4th	6th	9th
Orange	40	39	34	31
Papaya	38	36	35	30
Pseudostem	35.5	35	34	33

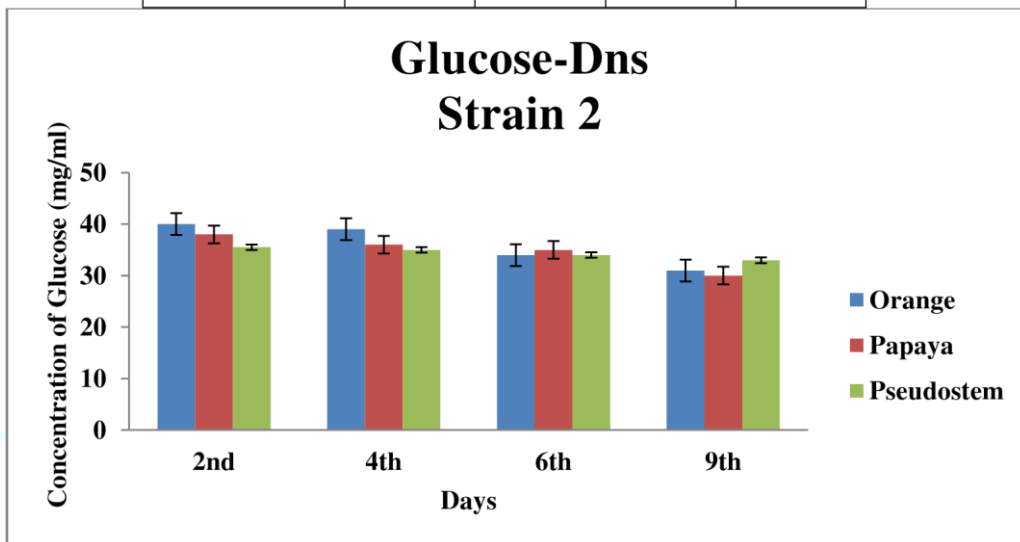


Plate 5. Amount of reducing sugars in the fruit peel biomass after adding cellulose degrading bacteria strain 2.

3.3.2. Estimation of Alcohol

Strain 1

Sample/days	2nd	4th	6th	9th
Orange	8	9	10	17
Papaya	53	54	57	61
Pseudostem	3	4	5	5

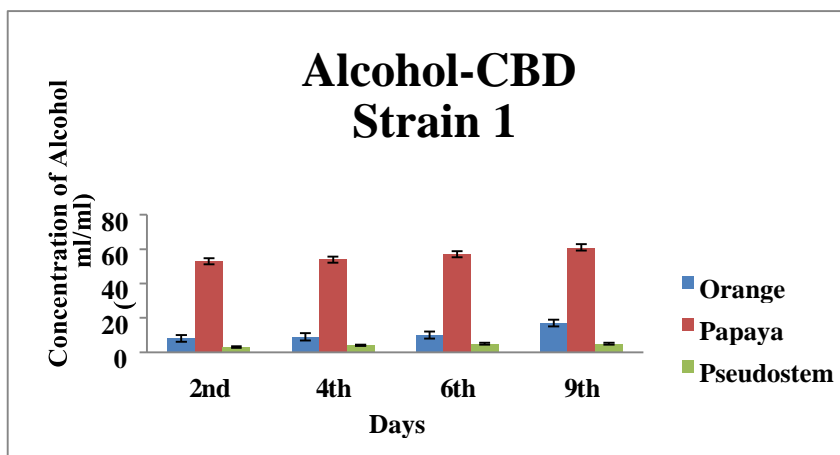


Plate 6. Amount of alcohol in the fruit peel biomass after adding cellulose degrading bacteria strain 1.

Strain 2

Sample/days	2nd	4th	6th	9th
Orange	11	12	15	18
Papaya	68	69	70	74
Pseudostem	3	4.5	4	5

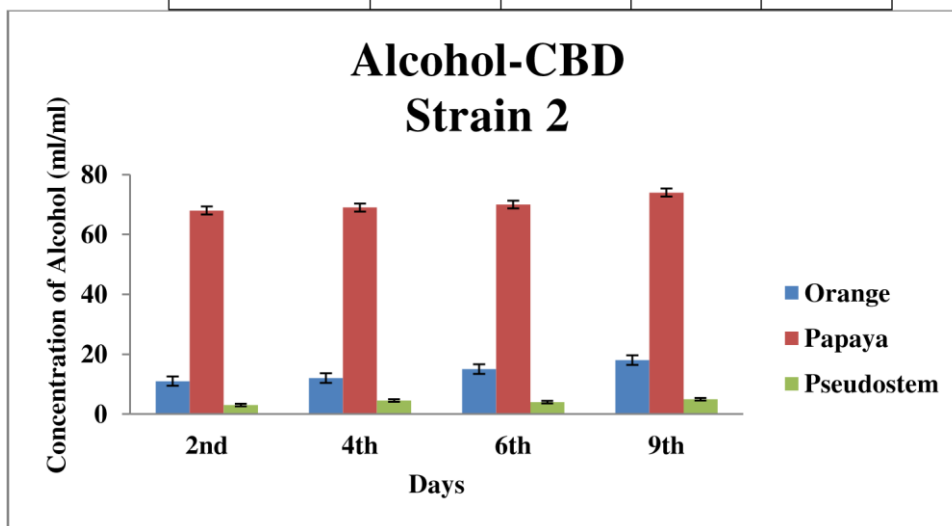


Plate 7. Amount of alcohol in the fruit peel biomass after adding cellulose degrading bacteria strain 2.

4. DISCUSSION

In the study conducted, biofuels was produced by using wastes such as peels of citrus and non-citrus fruits and pseudo stem of banana. The amount of carbohydrates in the fruit peels is shown in plate 1. This indicates that the orange showed higher carbohydrates levels than papaya and pseudostem. Effect of pretreatment on fruit peels

The reducing sugar yields from the fruit peel biomass after subjecting them to acid hydrolysis pretreatment are shown in plate 2. It was analyzed that the dilute acid pretreatment significantly increased the sugar release by nearly 20%-30%.

Sirkar et al. have reported that acid pretreatment method was found to be optimal for better yield of fermentable sugars from fruit peels. An initial pretreatment stage, in case of fibrous peel residues, is needed to breakdown its structure to make it more susceptible to an enzymatic attack. Aden et al. pointed out that the main advantage of dilute acid pretreatment related to other pretreatment methods is the higher recovery of sugars derived from hemicellulose. The dilute acid pretreatment has the advantage of not only solubilizing hemicelluloses but also

converting solubilized hemicelluloses to fermentable sugars in wheat straw and it thus, eliminates or reduces the need for use of hemicellulase enzymes mixtures. In a similar fermentation experiment, Nigam has reported more than 60% sugar yield in the hydrolysates obtained from water hyacinth by dilute acid treatment. Acid, alkaline pretreatment of biomass has been extensively studied and in an experiment using agricultural wastes, Patle and Lal has observed reducing sugar yield of 49 84 g L and ethanol production of 23 32 g L in the acid pretreated fruits and vegetable residues and the enzymatic hydrolysates yielded 36 123 g L of reducing sugars and 11 54 g L of ethanol.

The amount of alcohol after pretreatment varied between the fruit peels, with papaya having the high content of alcohol compared to other samples. The sugars released by the breakdown of lignin brought about the conversion of glucose to ethanol.

The work presented by Amit rajan Prasad singh also supports the above results as the optimal temperature, pH and incubation time for fermentation may enhance ethanol yield and minimize the cost of production could be obtained from banana and orange peels. Addition of cellulose degrading bacterial strain 1 and 2 from the midgut of earthworm to the pretreated samples did not affect the alcohol production but instead showed the same amount of alcohol produced during the pretreatment (plate 4,5,6,7). Therefore this result is in good agreement with the data gathered by T.Shankar *et al.* which provides evidence for cellulase producing ability of the earthworm gut bacterial isolates which inturn causes the conversion of glucose released by pretreatment process to ethanol. The production of cellulase and cellulase-lignocellulosic substrate interactions of bacterial strains in the earthworm gut was also evident. This study gives us a hint that the microbial wealth of cellulase producing bacteria isolated from the earthworm gut can be harnessed for biotechnological processes such as for production of bioethanol. The study from Krupa mary Jacob also indicates the ability of certain bacterial isolates present in the mid gut of composting earthworms to degrade cellulose. The ability of these bacterial isolates to form reducing sugars, which can be used as substrates for the production organic acids like ethanol, has the potential to be an environmentally sustainable alternative.

At the end of the incubation time it was found that the glucose in the fermentation medium over the time reduced and the amount of alcohol increased. Amount of glucose after 6th day in orange and papaya showed a gradual reduction and this might be probably due to reduced substrate concentration or due to the increase in the number of bacterial cells. A rapid decline in the ethanol productivity at the end of incubation time (9th day) was observed, this might be due to decrease in the number of bacterial cells or because of the denaturation of enzyme by the ethanol produced during fermentation or due to reduced substrate concentration. The reduction in the alcohol yield in the fruit peels might be due to the inhibitory effect of high polyphenol content and or less availability of fermentable sugar.

Hence from the above results we can infer that dilute acid pretreatment is more effective in removing the lignin content from the samples, and thereby allowing the conversion of glucose to alcohol. The addition of cellulose degrading bacterial strain 1 and 2 showed more or less the same amount of alcohol productivity with that of pretreatment activity. Therefore among the samples used papaya showed effective production of alcohol and then followed by orange and the least being the pseudo stem of banana.

5. CONCLUSION

Due to rapid increase in population size and exponential growth in industrialization, load is increasing on the fossil fuel resources and thus these resources are being depleted very fast. Production of Bioethanol, a high octane fuel, therefore may be a good replacement. Another benefit over fossil fuels is the greenhouse gas emissions. By encouraging bioethanol's use, the rural economy would also receive a boost from growing the necessary crops. The current research tendencies for improving fuel ethanol production are linked to the nature of employed raw materials, processing steps, and related process engineering issues. Agro-processing techno-economic activities are

to be generated for conservation and handling of agricultural produce and make it usable as food, feed, fibre, fuel or industrial raw material.

In this present study, efforts were made to identify the fruit wastes as potential raw material for bioethanol production. A higher concentration of alcohol using fruit peel wastes (orange, papaya, pseudostem of banana) by fermenting them with the help of cellulose degrading bacteria and also with effective pretreatment method was achieved. It can be said that papaya yielded high amount of alcohol when compared to orange and pseudostem. From this it was concluded that papaya contains maximum of fermentable sugars so produced maximum of fermentable sugar so produced maximum of alcohol at the end of incubation.

As this process is cost effective and do not yield any toxic residues so a common man can develop this technique and can produce it in an industrial level. Finally, it can be concluded that the produced fruit peel- Bioethanol is economically and environmentally viable. And can be a good substitute of Petrol. Although some more research works in different states and different environments should be done to find out any better result. Productivity and economical viability are also some fields to be taken care of to have a wonderful energy source.

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