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Original Research Article

Bioethanol from sawdust using cellulase hydrolysis of *Aspergillus ochraceus* and fermentation by *Saccharomyces cerevisiae*

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A B S T R A C T

The present study aimed with utilizes the sawdust for the production of bioethanol using fungus and yeast isolated from the soil sample of Vellar estuary, Parangipettai. Among the isolated strains, cellulase enzyme producing potent fungus of *Aspergillus ochraceus* and ethanol producing *Saccharomyces cerevisiae* were employed for saccharification and fermentation respectively. To optimize bioethanol production, the substrates was taken in three different variations (5g substrate+ 100 ml Distilled water; 5g substrate+ 100 ml Distilled water + 0.5% Glucose; 5g substrate+ chemically defined medium) and then it was enzymatically saccharified using *A. ochraceus* and fermented by *S. cerevisiae* in stationary and shaking methods. High yield of ethanol was obtained from sawdust amended with chemically defined medium in shaking fermentation than the stationary fermentation. The results provided evidence that the sawdust might be indeed potential substrate for the production of bioethanol.

Keywords

Sawdust, *A. ochraceus*, cellulase, fermentation, bioethanol

Introduction

Biofuel refers to liquid or gaseous fuel mainly for the transport sector that are predominantly produced from various biomass resources such as wheat, sugar beet, corn, paddy straw, wood, municipal wastes and forestry by-products etc. Generally, biofuel offer many benefits that include sustainability, reduction of greenhouse gas emissions, engine compression ratio and also reduce the environmental pollution (Reijnders, 2006). A variety of liquid fuels such as bioethanol, methanol and biodiesel can be used as an alternative fuel for transport, besides other alternatives of gaseous fuels such as Liquid Natural Gas (LNG), Compressed Natural Gas (CNG), Liquefied Petroleum Gas (LPG) and hydrogen (Semin *et al*., 2009).

Combustion of petroleum-based fuels increases net emission of carbon dioxide, different toxic and volatile compounds that are responsible for the health hazards and pollutions such as; benzene, toluene and xylenes (Tillman and San Diego 2001). The combustion of fossil fuels is also responsible for 73% of the CO_2 production which leads to global warming (Wildenborg and

Lokhorst, 2005). One of the solutions is to make use of the biofuels that are made from renewable materials such as lignucellulosic materials instead of fossil fuels. Bioethanol is a best alternative fuel to replace the current fossil fuel, which improves fuel combustion and decrease emissions of CO_2, NOX and hydrocarbons (Rasskazchikova *et al.,* 2004).

Bioethanol (ethyl alcohol, grain alcohol, CH_3-CH_2-OH or ETOH) is a liquid biofuel and it can be blended with petrol (E_5, E_{10}, E_{85}) or used as neat alcohol in engines. When compared to gasoline, higher octane number and decreased rate of evaporation made it as an excellent fuel for future advanced flexi-fuel hybrid vehicles (Chum and Overend, 2001; Kim and Dale, 2005). Since ethanol is less carbon-rich than gasoline, internal combustion engines operating on ethanol results in emission of fewer Green House Gas (GHG).

In biofuel production two categories of substrate has been used that is crops and grains like corn, wheat, sugarcane, soybeans, etc. and the second category contains waste biomass such as straw, corn stover and waste wood. The second category is much more inexpensive because it is a waste material, it is more ethical to use for bioethanol production as compared to the first category. Fermentation of sugars derived from inexpensive wood sawdust is an economical and efficient method for bioethanol production. The sawdust containing cellulose is the most abundant form of organic carbon, synthesized by plants and it is a linear polymer, composed of glucose sub-units held together by β-1,4-glucosidic bonds. Cellulosic materials are renewable natural biological resources which can be used for the production of biofuels (Zhang *et al*., 2007). Various industries utilize huge volumes of cellulosic wastes which provide a low-cost and sustainable resource for production of ethanol (Das and Singh, 2004).

The bioethanol production through microbial fermentation provides an economically competitive source of energy (Mcaloon *et al.*, 2000; Yasuyuki *et al*., 2011). There are a variety of biotechnological processes and microbial mechanisms for biological fuel (ethanol) production from lignocellulosic and cellulosic materials. Microorganisms are required to produce ethanol from lignocellulosic hydrolysates with a high yield from all sugars present using an economically feasible process. Different fermentation organisms among bacteria, yeast, and fungi (natural as well as recombinant) have been reviewed (Park *et al.,* 2010; Talebnia *et al.,* 2010) with emphasis on their performance over lignocellulosic hydrolysates. The simultaneous saccharification and fermentation (SSF) (Hari Krishna *et al.,* 1998; Yamane *et al.,* 2002) process is a favored option for conversion of the lignocellulosic biomass into ethanol using different fungus because it provides enhanced rates, yields and concentrations of ethanol. There are mainly two processes involved in the conversion (i) hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars (hexoses as well as pectoses) and (ii) fermentation of the reducing sugars to ethanol.

Hence, to reduce the several impacts of fossil energy, renewable sources of energy have been proposed as alternatives. These are sustainable in that they do not pose serious threat to the environment when properly managed. Also they can be replaced rapidly by natural on-going processes. The objective of the present study was to produce ethanol as a fuel from

renewable sawdust cellulosic wastes through microbial extracellular enzymatic hydrolysis and fermentation. The process was carried out in two steps saccharification and fermentation, with saccharification at 30^{o} C by *Aspergillus ochraceus* and fermentation by *Saccharomyces cerevisiae* at 30^{o} C. For comparative studies stationary and shaking methods of fermentation were adopted.

The main of this study includes to collect the mangrove rhizospheric soil from Vellar estuary, Parangipettai, India. Then, isolate and identify the fungus and yeast (*Saccharomyces cerevisiae*) by standard protocols. And also to determine cellulase activity and to find out the potent fungal isolates on cellulose supplemented medium. Then, prepare the substrate of sawdust for bio ethanol production. To screen the substrate for the saccharification process using potent cellulase enzyme producing fungus and fermentation by *Saccharomyces cerevisiae* in stationary and shaking fermentation method.

Materials and methods

Isolation of fungi

In the present study, the rhizospheric soil sample was collected from Vellar estuary for the isolation of fungus. The fungal strains from sediment sample were isolated using serially diluted pour plating technique. One gram of collected soil sample was diluted in 99 ml blank and from the dilution 1 ml was serially diluted to the test tubes containing 9ml of sterile distilled water and dilutions were made up to 10^{-4} . From the each dilution, 1 ml of dilution suspension was pipette out and plated on sterile Potato Dextrose Agar (PDA). Bacterial contamination was inhibited by adding 0.05% of chloramphenicol in PDA. All plates were incubated at 25˚C for 5-7 days.

Isolation of Yeast

For isolation of yeast same serial dilution method was followed and the samples were inoculated on selective medium of sterile Yeast-extract, Peptone and Dextrose (YEPD) plates. The plates were incubated at 30˚ C for 5-7 days.

Screening of cellulase enzyme activity

The agar diffusion method of Hankin and Anagnostakis (1977) was employed to screen cellulolytic activities of the isolated fungi. The medium comprised of 1% carboxyl methyl cellulose (CMC) incorporated into Mandels mineral salt medium (Mandels *et al*., 1974) and solidified with agar (15 g/l). Medium was autoclaved and poured into petri dishes. Each plate was inoculated by streaking once across the middle of the plate and incubated at 28 ± 2 °C. Cellulolytic activity was detected after growth, by flooding the plates with 1% congo red solution for 15 minutes. The dye was drained and plates were flooded with 1N sodium chloride solution for another 15 minutes. Clearance around growth of isolate represents cellulase production. The diameter zone of clearance was measured at five different locations and the mean was used to represent cellulase activity of the organism.

Preparation of substrate

The saw dust obtained from a wood mill was processed mechanically to reduce the length of fibers, prior to pretreatment. After removing the minor impurities by means of washing, they were oven-dried at 60º C for overnight.

Pre-treatment of substrates

The substrates were treated chemically with

1%NaOH for 2 hrs (Solomon *et al*., 1999). The material recovered by filtration was washed with distilled water and dried at 65^{o} C to a constant weight.

Total sugar estimation

The content of total sugar was also measured in the fermentation medium before and after fermentation process as described by Dubios *et al*., (1956). Briefly, 1ml of prepared substrate sample was taken in test tube. To this 1 ml of 5% phenol and 5 ml of concentrated sulphuric acid was added. This mixture was incubated at 29° C for 15 minutes to develop the color. The optical density was measured at 490 nm. The sugar content was determined by using D-Glucose as a standard.

$$\text{Total sugar (mg.g-1)} = \frac{\text{Std. OD value x OD value of sample}}{\text{Total volume x Weight of the sample x Volume of sample taken for estimation.}}$$

Fermentation

The fermentative production of bioethanol was carried out in two steps of saccharification and fermentation by stationary and shaking conditions. The chemically pre-treated substrates were used for all the experiments. In order to optimize bioethanol production the substrates were taken in three different variations in the following manner (Table-1).

Chemically defined medium (Table-2) was used in experiment-3. All the flasks were autoclaved at 15 lbs for 15 minutes. Saccharification and fermentation studies were performed in 250 ml Erlenmeyer flasks in which 5 grams of substrate was taken in each flask (as presented in Table-1) and fermentation experiments were carried out.

Saccharification of substrates by *Aspergillus ochraceus*

For saccharification of substrates isolated fungal culture of potent cellulase enzyme producing *A. ochraceus* was employed. The chemically treated substrates were autoclaved and inoculated with sporulating mycelial mat of *A. ochraceus*. Saccharification was carried out in stationary and shaking methods for a period of six days at 30^{o} C and total sugars was monitored before and after process. For the shaking method an orbital shaking incubator was employed and shaking was performed at 100 rpm at 30^{o} C. The *A. ochraceus* was selected for saccharification as it is cellulolytic in nature and can hydrolyze cellulose present in the substrates to simple sugars which can be fermented. Fermentation was carried out using *Saccharomycese cerevisiae.*

Estimation of Ethanol (Caputi et al. 1968)

The ethanol content in the fermentation medium was estimated after appropriate incubation time using chromic acid method adopted by Caputi et al. (1968). 10 ml of fermented medium was taken and underwent distillation process. Approximately 5ml of distillate was collected and combined with 25ml of chromic acid and content was made up to 51ml with sterile distilled water and kept in water bath at 80ºC for 15 minutes, after cooling read at 600nm. Thus the obtained values plotted with standard values.

Result and Discussion

Isolation and Identification of fungi

Degradation of the lignocellulosic complex to liberate cellulase can be brought about with the help of microorganisms especially fungi like brown rot, white rot and soft rot

fungi. Lignocellulosic materials are renewable, largely unused, and abundantly available sources of raw materials for the production of ethanol. It's a potential source for future low-cost ethanol production is to utilize lignocellulosic materials such as sawdust, crop residues, grasses and trunks (Millati et al., 2002).

In the present study, totally 6 fungi viz., *Aspergillus amastelodami, Aspergillus* sp., *Aspergillus terreus, Aspergillus* sp., *A. ochraceus, Penicillium* sp., were isolated and identified by lactophenol cotton blue staining method. The potent cellulase enzyme producing *A. ochraceus* was selected for sawdust saccharification process. The wide diversity of fungal taxa found on mangrove soil samples and the predominance of *Aspergillus* and *Penicillium* genera were encountered. The saprophytic condition of the soil samples isolates reinforces their ability to use lignocelluloses as sole carbon source, however not all of the isolated fungi were able to secrete significant amounts of cellulase activities for biotechnological use. Most of the authors reported that the cellulosic degradation of sawdust using fungi for ethanol production (Ojumu et al., 2003; Ali et al., 2011; Kathiresan et al., 2011; Nwakaire et al., 2013).

Isolation and Identification of yeast

Yeast was isolated from soil sample using selective medium of Yeast extract, Peptone and Dextrose (YEPD). The grown yeast isolates were identified as *Saccharomyces cerevisiae* by studying some of the morphological characteristics (Kregervan Rij, 1984).

Cellulase enzyme activity

All the isolated fungi were screened for cellulase enzyme activity. Among them the *A. ochraceus* was showed maximum (8 mm) zone of enzyme activity and it was selected for further saccharification process.

Stationary fermentation

In stationary fermenation *A. ochraceus* was used for the sawdust saccharificaation, the highest amount (51%) of total sugar was obtained in 5g substrate added with chemically defined medium, followed by 5g substrate + .05% glucose added medium (48%) and the lowest amount was recorded in 5g substrate with distilled water (41%).

In stationary fermentation ethanol production was observed after 6^{th} day. Among three fermentation designs highest (6g/100g) ethanol production was observed in 5g substrate added with chemically defined medium, followed by 5g substrate + .05% glucose added medium (4.8g/100g) and the lowest amount was recorded in 5g substrate with distilled water (3.5g/100g). These indicating that the efficiency of saccharifying and fermenting enzymes on substrates shows variations in performance.

Shaking fermentation

In shaking fermentation *A. ochraceus* was used for the sawdust saccharification, the highest amount (59%) of total sugar was obtained in 5g substrate added with chemically defined medium, followed by 5g substrate + .05% glucose added medium (54%) and the lowest amount was recorded in 5g substrate with distilled water (42%).

In shaking fermentation comparatively high yield of ethanol was obtained than the stationary method. The total sugar released was similar to stationary fermentation with a minor fluctuation.

Table.1 Design of fermentation experiments

1.	5 g substrate + 100 ml distilled water
2.	5 g substrate + 100 ml distilled water + 0.5% glucose
3.	5 g substrate + 100 ml chemically defined Media

Table.2 Composition of chemically defined medium (%)

L-Glutamic acid	0.03
NH_4No_3	0.14
KH_2PO_4	0.2
$CaCl_2$	0.03
$MgSO_4$	0.03
Proteose peptone	0.75
$FeSO_4$	0.5
$MnSO_4$	0.16
$ZnSO_4$	0.14
Tween 80	2%

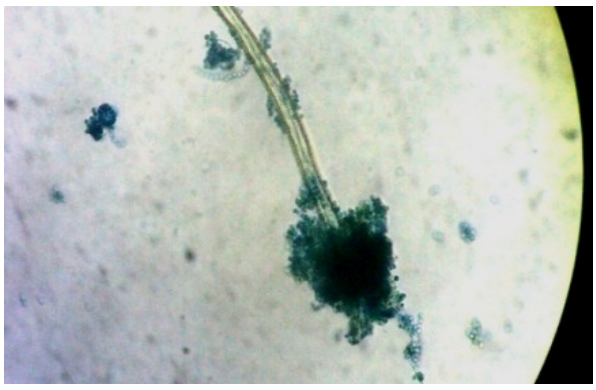

Fig.1 Microscopic view of *Aspergillus ochraceus*

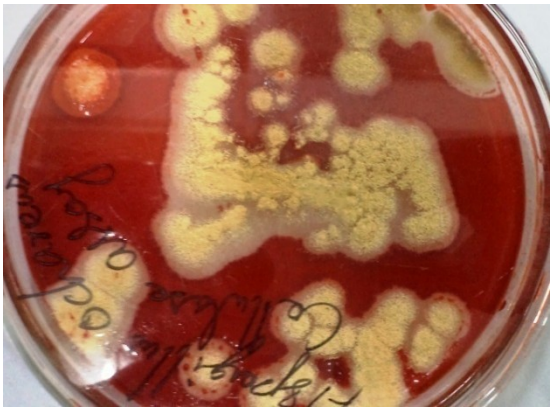

Fig.2 Cellulase enzyme activity

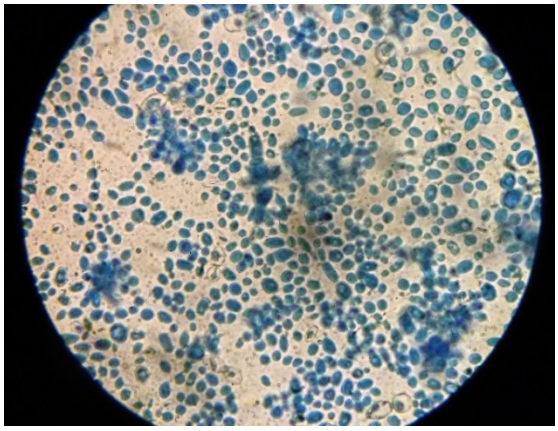

Fig.3 *Saccharomyces cerevisiae*

Fig.4 Experimental setup of ethanol production

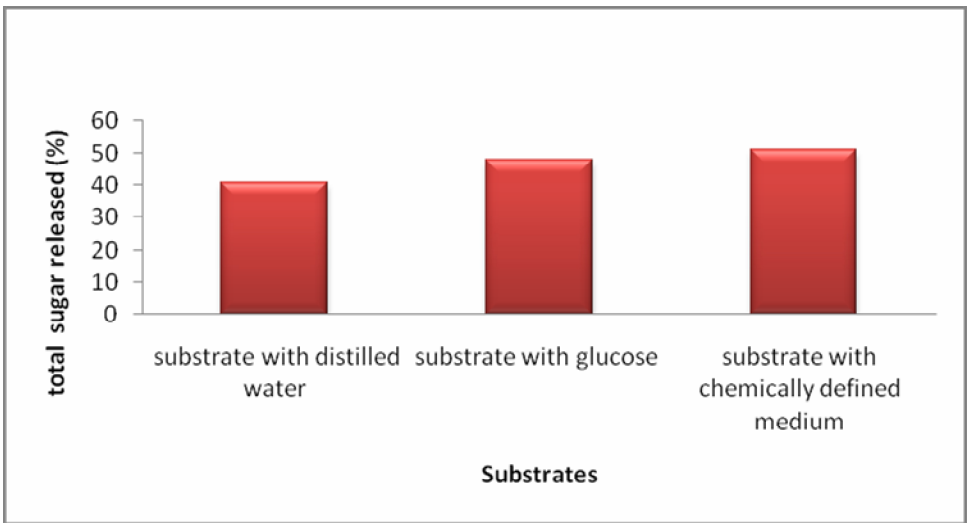

Fig.5 Total sugar estimation by stationary method using *A. ochraceus*

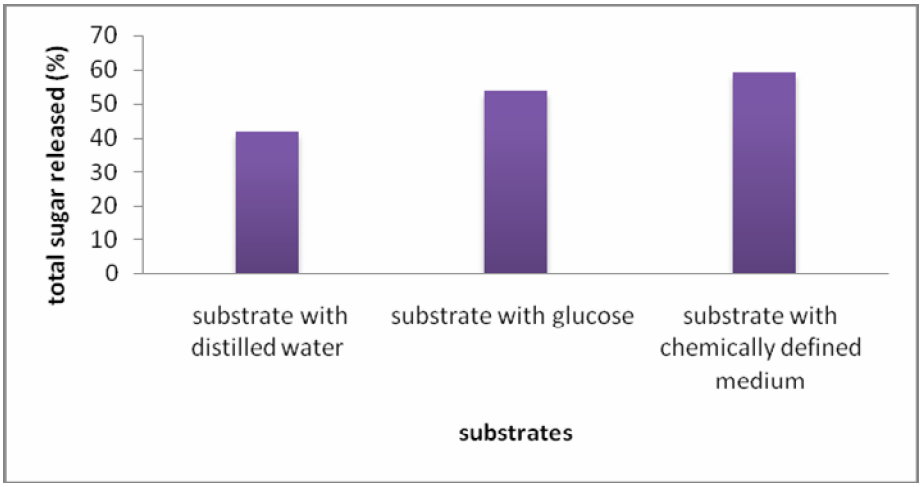

Fig.6 Total sugar estimation by shaking method using *A. ochraceus*

With increase in time of fermentation, ethanol production increased (7.2g/100g) in 5g substrate added with chemically defined medium followed by 5g substrate + .05% glucose added medium (6.5g/100g) and less yield from 5g substrate with distilled water (4.3g/100g).

Sawdust was affirmed as a good substrate for energy production particularly in the production of bioethanol. While A. *ochraceus* demonstrates a high cellulase activity and the supremacy of the cellulases used to hydrolysing the biomass wastes to fermentable sugars finally *S. cereviciae* convert sugar to bioethanol was established. Overall, sawdust hydrolyzed by cellulases of *A. ochraceus* was most productive in terms of ethanol yield and can therefore be harnessed in biofuel production.

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