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Research Article

BIOETHANOL PRODUCTION FROM GROUNDNUT SHELL USING MICROORGANISMS DERIVED FROM SUGARCANE ROOT SOIL

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ABSTRACT

Bioethanol is a form of renewable energy that can be produced from fuel or energy crops. Ethanol is produced by the fermentation of sugars present in agricultural feed stocks and crop residues. This study investigates the use of agro wastes like groundnut shells for ethanol production. Initially, the groundnut shells were washed, dried and grinded into powder. Then it was subjected to ethanol production using yeast. After 20 days of incubation the ethanol was estimated using potassium dichromate method. The maximum ethanol yield (1.55%) was obtained when 1% yeast was used. To increase the efficiency of ethanol production, the cellulolytic bacteria was isolated from the cow dung dumped soil site. The 10 bacterial isolates were screened for cellulase enzyme production. Among them one bacterium showed maximum decolourization of congored which was subjected for enzyme production in nutrient broth. The organism showed maximum enzyme activity of 558.12 U/ml. The isolated cellulolytic bacteria were identified as Bacillus anthracis using 16s rDNA sequencing. Ethanol production from the groundnut shells was again carried out with various concentration of crude cellulase enzyme isolated from the bacteria. The estimation result showed 3.8% of ethanol as maximum. Then the ethanol was condensed using a rotary evaporator and when again estimated, showed 7.3% of ethanol. Finally, the presence of ethanol was confirmed by iodoform test. Thus, groundnut shells can efficiently be used for the production of ethanol, which can be used as a high potential fuel source for transportation in the future.

Keywords: Bioethanol, *Sacchraromycess* .sp., *Bacillus.* sp., Groundnut shell.

INTRODUCTION

This study investigated the production of ethanol from groundnut shells. The shells were first powdered, dried, and cleaned. After that, the powder was fermented with yeast to produce ethanol. With 1% yeast, the highest yield (1.55%) was obtained. Cellulolytic bacteria were obtained from cow dung soil in order to increase production. The bacteria that produced the greatest cellulase enzyme were identified as *Bacillus anthracis*. This enzyme raises the ethanol production during fermentation to 3.8%. The ethanol content was increased to 7.3% by further concentration using a rotary evaporator. The presence of ethanol was confirmed by an iodoform test. This demonstrates that groundnut shells may effectively create ethanol for possible fuel use (Dharanipriya *et al.,* 2019). The goal of this study was to identify soil-dwelling bacteria that produce the enzyme cellulase, which degrades cellulose. Out of the seven microorganisms they tested, a few were able to manufacture cellulase like *Staphylococcus aureus, E. coli*. They investigated whether these bacteria could aid in the ethanol production from leaves and paper. To produce ethanol, the materials were fermented with yeast after being treated with acid to eliminate lignin. They used an iodoform test to validate the generation of ethanol. This demonstrates that agricultural waste can be converted into ethanol, which suggests that using this waste could be beneficial (Thomas *et al.,* 2021).

The growing dependence on non-renewable fuels has led to an increase in the quest for renewable energy sources, such as bioethanol. The process of enzymatic hydrolysis, which converts biomass into fermentable sugars, is essential to the synthesis of bioethanol. Enzyme-

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based techniques are being investigated for the efficient manufacture of bioethanol from a variety of sources, including wood, agricultural waste, and algae. One sustainable method for making bioethanol and other chemicals is to use ligno cellulosic biomass, which includes waste from forestry and agriculture. The process of turning biomass into glucose is primarily dependent on enzymatic hydrolysis, which uses less energy and is influenced by pH and temperature. Polyethylene glycol and other non-ionic surfactants can improve this procedure. Biofuels derived from biomass have advantages for the environment, support sustainability, promote rural development, and lessen reliance on oil imports. Pollution can be reduced by using byproducts properly, and biofuels can assist (Katja Vasic *et al.*, 2021).

MATERIALS AND METHODS

Sample collection

Groundnuts were purchased from the local market of Tirupur, Tamil Nadu and manually the shells were removed. Groundnut shells were sun dried, powdered and were used as a raw material for further study, and to collect Sugarcane root, soil and commercial yeast.

Isolation of bacteria & yeast from soil

To isolate the Saccharomyces from Sugarcane roots and soil. In order to isolate cellulolytic bacteria, a soil sample was taken from the location where cow dung was disposed of. First, 100 milliliters of distilled water were used to dissolve 1 gram of soil sample. One milliliter was extracted from this and put into the test tube along with 9 ml of distilled water. It was regarded as a dilution of 10^{-2}. By moving 1 ml from tube to tube, tenfold dilution was also made from tube 1 to 7. Using a L rod, 0.1 ml of each dilution was individually added to the nutrient agar plates and dispersed around the agar surface. Following that, the plates were incubated for 24 hours at 37°C. Following incubation, the chosen bacterial isolates were pure-cultured and employed in an additional screening procedure.

Screening of cellulase producing bacteria and yeast

Using cellulose congo red agar plates, the bacterial isolates were tested for cellulose breakdown. The cellulose congo red agar plate was covered with the bacterial isolates, and it was cultured for 48 hours at 37°C. Following incubation, the bacterial isolates were examined for congo red decolorization, a sign of the bacteria's capacity to break down cellulose Morphological and Molecular analysis (MALDI-DOF) was done for confirmation.

Morphological identification of cellulaseproducing bacteria & yeast gram's staining

The isolated colony was thinly sliced, allowed to air dry, and then heated. After applying crystal violet to the smear for 60 seconds, distilled water was used to remove the discoloration. The extra water was drained, Gram's iodine solution was applied to the smear, and it was left for 60 seconds. The slide was rinsed with distilled water. The slide was submerged in 95%ethanol for 30seconds and cleaned with distilled water. The counter stain safranin was added and left for 60 seconds. Again, the stain was eliminated by giving the area a brief rinse with distilled water. After air drying, the slide was inspected under a light microscope.

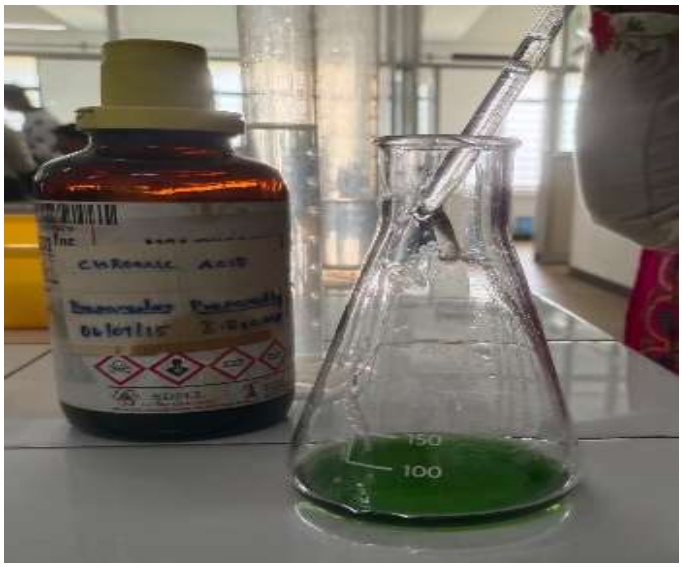

Figure 1. Ethanol estimation using potassium dichromate method.

Cellulase activity assay

A loopful of bacterial culture was inoculated into 100 ml nutrient broth. To broth was incubating for 24hrs after incubation the overnight grown culture was taken and centrifuged at 10,000rpm for 15mins. The pellet was discarded and the supernent was transferd to fresh tube. This supernent was taken as crude enzyme source and tested for the enzyme production. The carboxy methyl cellulase activity of crude enzyme was assayed using a method described by miller (1959) with some modification. Varying concentration of culture supernent such as 1 ml to 5ml of 1% CMC prepared in 50nm sodium citrate buffer (pH 4.8) was added and incubated 60°C for 30mins. The reaction was terminated by adding 3ml of DNSA reagent and sequent placing the tube in boiling water bath at 100°C For 15mins. Finally, the absorbence was measured at 575nm using spectrophotometer.

Figure 2

Figure 3

Figure 2 and 3 Groundnut shells raw material and Groundnut shells powdered

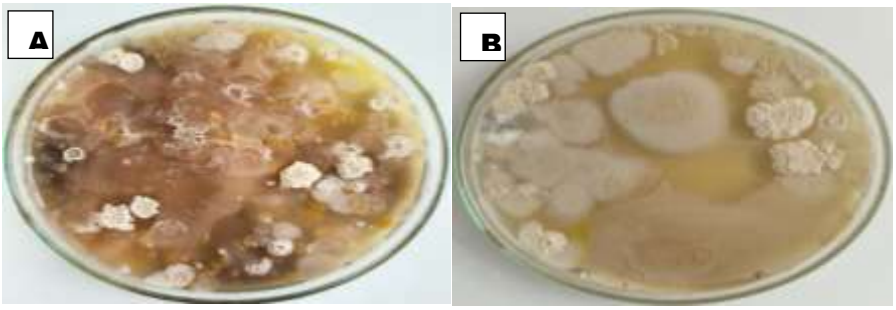

Figure 4. Mother plate isolates Sugarcane roots and soil; A- Bacterial isolates and B- yeast.

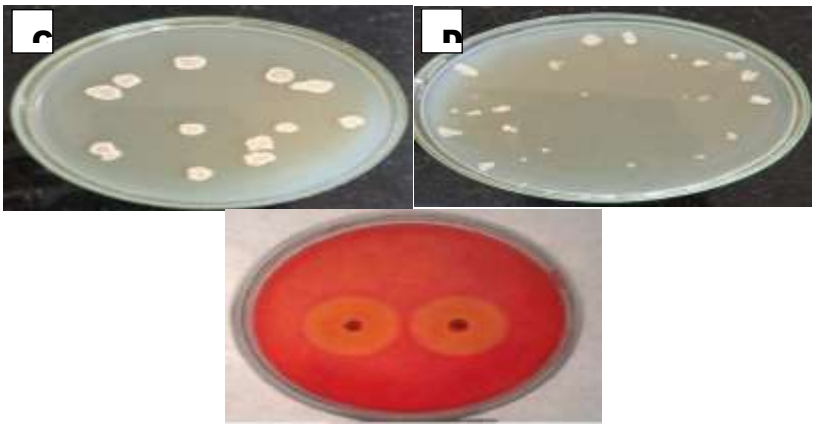

Figure 5. Two Microbial isolate C and D chosen based on the appearance and cellulolytic activity, E shows the confirmation of cellulose degradation (Decolorisation of Congo Red).

Production of ethanol from groundnut shell using yeast

10g groundnut shell powder was physically pretreated and added to 100ml of distilled water. It was then autoclaved at 121°C. To this, varying concentration of yeast (0.25% / 1%) was added and keeps it for incubation days at room temperature for 20days.

Ethanol estimation using potassium dichromate method

Following incubation, 10 milliliters of the fermented broth were put into a centrifuge tube and centrifuged at 10,000rpm for 15 minutes. We collected the supernatant and utilized it to estimate the amount of ethanol. 12.5 milliliters of chromic acid were added to 5 milliliters of supernatant from each concentration, which was then placed in a conical flask. Next, distilled water was used to fill each flask to the brim with 25 ml, and they were all placed in a boiling waterbath at 80 °C for 15 minutes. Following incubation, the tubes were allowed to cool, and a spectrophotometer set to 600 nm was used to measure the absorbance.

Enzymatic saccharification and fermentation

Crude cellulase enzyme was given to tubes containing 1% yeast at varying concentrations to boost the generation of ethanol. In 100 milliliters of distilled water, 10 grams of groundnut shell powder were used for this. The physical preparation was done by autoclaving for 15 minutes at 120 °C. Next, for the goal of saccharification, different concentrations of crude enzyme (ranging from 1 to 5%) were introduced to each flask. The flasks were incubated for 20 days at room temperature after the pH was measured. Using the potassium dichromate technique, the samples were examined for the formation of ethanol after incubation.

GC- MS analysis for the presence of ethanol

Gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC–MS) analyses were performed using GC Agilent Technologies 7890B GC-System coupled with MS 5975C GC/MSD. The mobile phase was helium and with a stationary phase (0.25 µm HP-5 5% cross-linked with phenylmethyl polysiloxane) with an internal diameter of 0.320 nm and length 30 m The volume injected was 1 µl per run.

RESULT AND DISUCSSION

3kg of groundnut shells were obtained from 7kg of groundnuts (Plate 3.1.1)**.** From 2kg of groundnut shells, 600g of powder was obtained and was used for the study (Plate 3.1.2). After incubation, based on the different colony morphology on the nutrient agar, 5 bacterial isolates were selected and then the pure cultures of the colonies were isolated. A total of 13 microbial cultures (A- mother plate of bacteria and B-mother plate of yeast) were isolated based on morphology and biochemical characterization from the soil samples. Among the 5 bacterial and yeast isolates, two bacterial isolates (C and D) showed the maximum decolorization of Congo red after 48 hours of incubation, which indicated the cellulose degrading ability of the bacteria. Cellulolytic bacteria isolated from agricultural field showed maximal cellulase production after 48 hours of incubation at 45^0C in medium containing 1.5% carboxymethyl cellulose (CMC) as substrate. Among 13 isolated strains, two cellulolytic bacterial strains showed maximum enzyme activity. In most of the studies, the cellulose degrading organisms showed decolourisation of Congo red after 48hours of incubation. The colony morphology of the bacterial isolate was flat or slightly convex with irregular borders and a ground-glass appearance. The isolated bacterium was identified as gram-positive, rod-shaped bacterium Gram's staining. The cellulase producing capacity of the bacterial isolates was estimated using the DNSA method and it showed 558.12 U/ml of enzyme activity. A reducing sugar test is the primary method used to measure the final products of cellulase hydrolysis activities in order to assess cellulase activity. As a result, the assay's results are usually expressed as the enzymes' hydrolysis capability. It is difficult to quantify, has no theoretical foundation, and ignores important variables like the ratio of crystalline to amorphous cellulose, the hydrolysis time, and the concentration of cellulose and cellulase. Enhancing previously commonly used techniques for measuring cellulase activity has been the primary focus of research. For this reason, a precise and repeatable experiment is still needed to determine the rate of cellulase hydrolysis.

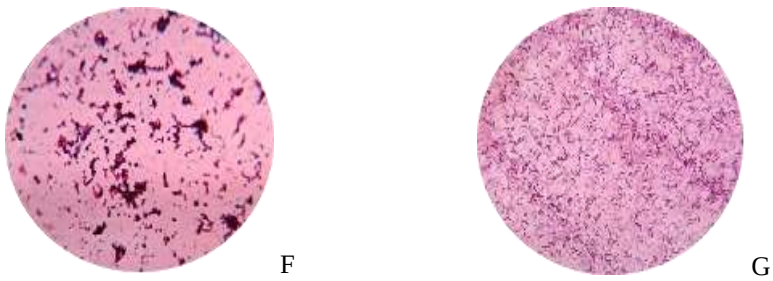

Figure 6. Gram's staining microscopic image of two selected isolates F and G.

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Patient Name	Ms. SREE NITHI B (DR NGP COLLEGE)	Lab No	2311233
UHID/IP No	MICROBIOLOGY	Sample Date	02/02/2024 6:18PM
Age/Gender	315158	Receiving Date	02/02/2024 6:37PM
Bed No/Ward	1 Day/Female	Report Date	07/02/2024 10:04AM
Referred By	OPD	Report Status	Final
	Dr. EMD		

BACTERIOLOGY-INVESTIGATION

Test Name	Result	Unit	Biological Ref. Range	Method
Specimen type	Pure culture from Soil			
Investigation	Bacterial identification			
Smear	Gram positive spherical or oval seen			
Identification	Saccharomyces sp			
Probability	99.8%			
Method	VITEK MS PRIME			
Notes	The organism identified shows 99.8 % Probability in VITEK MS PRIME, which is a mass spectrometry system using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) for the identification of microorganisms cultured from given specimens. The bacterial identification is given for the pure culture given by Ms. SREE NITHI B (II MSc Microbiology - Dr.N.G.P Arts and science college).			

--End Of Report--

Dr. CHINNASWAMY P
Chief Of Laboratory Medicine
Selects Blood Bank

Dr. GANDHIRAJ D
Chief Microbiologist

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Figure 7. Laboratory report proof for finding the unknown isolate as *Saccharomyces sp.*

Duplicate Copy

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Patient Name	Ms. SREE NITHI B (DR NGP COLLEGE)	Lab No	2311233
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BACTERIOLOGY-INVESTIGATION

Test Name	Result	Unit	Biological Ref. Range	Method
Specimen type	Pure culture from Soil			
Investigation	Bacterial identification			
Smear	Gram positive rod shaped seen			
Identification	*Bacillus sp*			
Probability	99.9%			
Method	VITEK MS PRIME			
Notes	The organism identified shows 99.9 % Probability in VITEK MS PRIME, which is a mass spectrometry system using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) for the identification of microorganisms cultured from given specimens. The bacterial identification is given for the pure culture given by Ms. SREE NITHI B (II MSc Microbiology - Dr.N.G.P Arts and science college).			

--End Of Report--

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Figure 8. Laboratory report proof for finding the unknown isolate as *Bacillus sp.*

Figure 9.1. *Saccharomyces sp* low crystalline formation.

Figure 9.2.*Bacillus sp* high crystalline formation.

Figure 10. Ferment using Cellulose enzyme.

Figure 11. *Saccharomyes sp* from sugarcane root & Soil.

The ethanol produced after 20 days of incubation with different enzyme concentration was measured using potassium dichromate method. Ethanol generation increased gradually during the saccharification of groundnut shell powder as crude cellulase enzyme concentration increased. The highest yield of 3.8% ethanol was achieved with the use of 5% crude cellulase enzyme. Groundnut shell is fermented by *Saccharomyces cerevisiae.* The ethanol yields obtained by using *Saccharomyces cerevisiae alone* and in combination were compared in this study. The combination of *Saccharomyces cerevisiae* produced the highest output of ethanol, 0.955%.

Table 1. Estimation of ethanol in Saccharomyces *cerevisiae*.

Concentration of Yeast (%)	Abs @ 600nm
0.25%	0.745
0.5%	0.859
0.75%	0.915
1%	1.02

Table 2. Estimation of ethanol in *Bacillus sp* (Cellulase enzyme).

Concentration of Enzyme (%)	Ethanol Concentration (%)
1%	3.28%
2%	3.37%
3%	3.46%
4%	3.55%

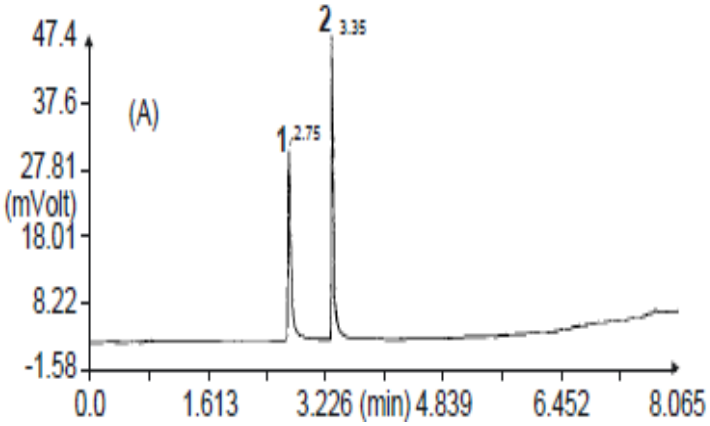

Figure 12. GC-MS analysis of the bioethanol from the groundnut shell.

Saccharification of groundnut shell powder with the increasing concentration of crude cellulase enzyme showed gradual increase in the ethanol production. Maximum production of 3.85% ethanol was obtained when 4% crude cellulase enzyme was used Rabah *et al*., (2011) used *Saccharomyces cerevisiae* and *Bacillus.sp* for the hydrolysis and fermentation of groundnut shell and rice husk. The study compared the ethanol yield when *Saccharomyces cerevisiae* and *Bacillus.sp* used alone and also compared when combination of these two microorganisms used. Maximum ethanol yield of 1.02% was obtained when mixture of *Saccharomyces cerevisiae* and *Bacillus.sp* used, while the lowest concentration of ethanol 3.28% was obtained when *Bacillus.sp* was used alone. GC-MS analysis of bioethanol from groundnut shells provides a comprehensive understanding of its composition, purity, and potential applications. This analytical technique is crucial for ensuring quality control and optimizing bioethanol production processes, contributing to the sustainability of renewable energy sources and industrial applications.

CONCLUSION

There are numerous uses for bioethanol in the chemical, medicinal, and fuel industries. This lessens the need for foreign oil and the amount of CO_2 released into the sky. The goal of the current work was to use yeast (*Saccharomyes sp*) and *Bacillus sp* to produce ethanol from groundnut shells through fermentation. This study shown that groundnut shells are an effective and practical source of ethanol that can be produced on a big scale. It can also be utilized as biofuel in the future.

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