BIOETHANOL PRODUCTION EFFICIENCY OF CELLULASE ISOLATED FROM DIFFFRENT BIOLOGICAL SOURCE

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ABSTRACT:

Bioethanol production can be produced by plant residues that are rich in lignocellulose and cellulose. Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as biofuel, is produced by fermentation, in which certain species ofyeast (e.g., Saccharomyces cerevisiae) or bacteria (e.g., Zymomonasmobilis) metabolize sugars in oxygen-lean conditions to produce ethanol and carbon dioxide. In this study the bioethanol production from husk extract was carried out by endosymbiotic facultative anaerobic bacteria.Out of the four of cellulase-producing bacterial strains isolated from termite gut during this study, the ES1 isolate was found to have cellulase and amylase enzyme production. This isolate was known as *Bacillus cereus*. The effects of different growth parameters, including pH and shaking were investigated to optimize the growth conditions of the bacterium. The maximum cellulase activity was achieved at pH of 7 and an incubation temperature of 35°C. The results showed the liberation of 2.4 g/L of reducing sugar during fermentation. This total reducing sugar produced 17.0 g/L of ethanol after 48 hours when *B.cereus* was used as a fermentation agent. Hence, bioethanol was successfully produced from the cellulose of rice husk residue using the cellulase enzyme from *B. cereus.*

INTRODUCTION:

Ethanol (CH_3CH_2OH) is the most democratic alcoholic bio fuelavailable in the present world market. Henry Ford termed ‘‘fuel of the future’’ for the ethanol. There are several reasonsfor being its use as alternative fuel such as (i) it is produced from the renewable agricultural products like corn, sugar and molasses including other products rather than non-renewable petroleum products. The world total ethanol production during 2009–2010 was almost 100 billion liters .Bio ethanol is a liquid bio fuel. It is produced from several different biomass feed stocksmainly sugarcane in India, Colombia and Brazil while it is predominantly corn in other areas such as the United States, China and European Union (Mustafa *et al.,* 2014).

Bio ethanol production can be produce by the use of plant residues that are rich in lingo cellulose and cellulose. Ethanol is usedfor alcoholic beverages, and the vast majority of ethanol is used for bio fuel. It is produced by fermentation, in which certain species of Bacteria (e, g.*Zymomonas) or* yeast(e.g., *Saccharomyces cerevisia).* The enzymes that are used to hydrolyze cellulose are expensive, the production of second-generation bio ethanol uses cellulose-released sugars(Iqbal*et al.,* 2012). To develop this generation of bio ethanol ,a number of cellulose-containing agricultural by products, such as wood trimmings, husks, straw, bamboo, rapeseedmobilis are used, it is called “first-generation” bio ethanol, and when lingo cellulosic raw materials (sustainable feedstock that cannot be used directly for food production) are used, it is called “second-generation” bio ethanol. Algal bio ethanol, which is called “third-generation” bio ethanol, is still under investigation. Although oil, and sawdust(Qian *et al*., 2014).

DIVERSITY OF CELLULASE IN BACTERIA AND FUNGI

The hydrolysis ofthe cellulose in plants cell wall is the global carbon cycle. It is the most abundant source of carbon on land. Anaerobic rumen bacteria, fungi, or protozoa are used for the cellulose hydrolysis. Cellulolytic microorganishms are used for degrade cellulose although cellulase that are used to degrade cellulose although cellulases. Cellulose is mostlyoccur in plant cell walls, which are very difficult to degrade. Since Bacteria need to secrete their cellulases and most anaerobic bacteria that produce cellulosomes attach them to their outer surface.Because of the recalcitrance of plant secrete up to 50% of highlights the potential utilization of fungal and bacterial speciesfor and their applications in diverse fields and industries(David B Wilson, 2011).

Cellulases are used in textile,medical, food, , laundries, agriculture, textile, enhancement of animal feed digestibility, paper and pulp industry. Cellulase is a synergetic enzyme which is accustomed to split cellulose into glucose and/or different oligosaccharide compounds .Cellulose enzymes may be divided into 3 types: endoglucanase (endo-1, 4-*β*-D-glucanase,)cell biohydrolaseorexoglucanase (exo-1, 4-*β*-D-glucanase, CBH) and *β*-glucosidase (1, 4-*β*-D-glucosidase) studied extensively among these organisms because of their elongated hyphae which produce mechanicalpressure on the cellulose structure,inflicting them to supply massive amounts of Cellulases are inducible enzymes obtained from fungi,

*actinomyce*tes and bacteria during theirgrowth on cellulosic material. Though fungi arebetter cellulase producers than bacteria, there is increasing interest for bacterial cellulases as they are more effective biocatalysts stable under harsh conditions required for various industrial applications. Moreover, bacteria have a higher growth rate, and product recovery is simpler than from fungi. As a source of cellulase producing bacteria, termites were collected from nest or mound *(Odontotermessp*. from the family Termitidae) and woody materials (*Heterotermessp.* from the family Rhinotermitida)(Muhammad *et al*., 2015).

MATERIALS AND METHOD

Termites collection

Termites were collected from Perambalur, Tamilnadu. They live in woody materials (*Cryptotermes* sp., Kalotermitidae). These termites were transfer migrated into the container.

Isolation of cultivable Bacteria from the Termite gut

Twenty worker termites were surface sterilized by using 70% ethanol and degutted using sterile forceps according to Long *et al*. (2010). The guts were homogenized, and dilution series (up to a dilution of 10^{-12}) were spread-plated on Nutrient agar to isolate bacteria followed by 24 hours incubation at 37° C. After incubation, the number of colonies in each plate was counted using a colony counter(Gincy *et al*., 2011). Average of the count obtained was calculated and total bacteria present per gram of termite was calculated as

No. of colonies x Dilution factor No. of bacteria/g / Volume of the sample

Isolation of Cellulose Degrading Bacteria

The sesame processed samples were inoculated in a basal salt medium (KH_2PO_4- 5 g, $KHPO_4$- 10 g, $(NH_4)2SO_4$- 0.5 g, NaCl - 0.2 g, $MgSO_4$-0.1 g, yeast extract - 10 g, Carboxy-Methyl Cellulose (CMC) - 10 g in a liter) with a pH 7for the isolation of cellulolytic bacteria. The cultures were incubated for 2 days in a shaker incubator at 30

°C and 120 rpm. In order to isolate the bacterial colonies have the capable of utilizing cellulose as sole source of carbon.The colonies were grown on cellulose rich starch agar media. To confirm the cellulose-degrading ability of bacterial isolates, the isolates were streaked on the cellulose Congo red agar medium. Congo red is regarded as an indicator for cellulose degradation in an agar medium; colonies showing discoloration of Congo red were taken as positive cellulose-degrading bacterial colonies (Gupta *et al.*,2012).

Primary screening of amylase

All the selected isolates were individually examined for primary screening to determine their amylase production capability using starch agar medium. Briefly, each bacterial isolate was streaked on to 27 starch agar plates and incubated for 24hours at 37°C. After incubation, the plates were spreaded with iodine solution (0.3% iodine and 1% KI). Amylase positive bacterial strains were identified and recorded based on the clear zone formation around the bacterial growth(Vishwanatha et al., 2020).

Biochemical characterization:

Gram's staining

Thin smear of isolated pathogens were prepared on a clean glass slide and air dried. The smear was covered by crystal violet (CV) for 1 min. and then washed. The slide was treated with grams iodine (I) for 1 min. The CVI complex destained by 70 percent ethanol was added over the slide. After 30 sec., the slides were washed and counter stained by Saffranin for 1 min. and then washed. Stained slide was air dried and observed under microscope followed by Ann C. Smith and Marise A. Hussey(2005) protocol.

Indole test

Sterile Indole medium were prepared and sterilized by autoclaving at 15 lbs (121° C) pressure for 15 min. The isolated *Rhodopseudomonas* sp. was inoculated on indole broth and incubated at 35°C for 24 h. After that, few drops of Kovacs reagent were added to the test tubes and the results were recorded(Charles*et al.*, 2015).

Methyl red test

MR-VP was prepared and sterilized by autoclaving at 15 lbs (121° C) pressure for 15 min. Isolated *Rhodopseudomona*s was inoculated and labeled respectively in the MR-VP broth and incubated at 35° C for 24 hours (Lekh *et al.*, 2012). After incubation 5 drops of 0.4 per cent methyl red reagent were added to each tubes and the color change were observed.

Voges-Proskauer test

Isolated *Rhodopseudomonas* was inoculated and labeled respectively in the MR-VP broth and incubated at 35° C for 24 hours(Priyanka*et al.*, 2017). After incubation 1-2 drops of Barritt's Reagent A (α-napthol) and 2-3 drops of Barritt's B (40 % KOH) were added and color change were recorded.

Citrate utilization test

*Rhodopseudomonas*isolated colonies werecollected from a straight wire and inoculated into Simmon's citrate slants and incubated overnight at 37° C. If the organism has the capable to utilize citrate, the mediumcolor changes green to blue.

Catalase test

A loop full *Rhodopseudomonas* culture was taken on the clean glass slide.3 % H_2O_2 were added on the slide and allowed to react for 30 sec. The presence of the effervescence was recorded as catalase positive and absence were catalase negative.

Oxidase test

Plates were prepared with isolated *Rhodopseudomona*s sp. and sterile oxidase disc were placed over the surface of colonies. The change of color on disc was noted. If the inoculated disc area turns dark blue to maroon to almost black, then the result is positive. If a color change does not shows within 3 minutes then the results are negative.

Anaerobic test

The medium 1 for OF test contained 2.0 g L^{-1} peptone, 5 g L^{-1}NaCl, 0.3 g L^{-1} KH_2 PO_4, 3 g Agar and 3 mL 1% Bromothymol blue. All materials were dissolved together and the pH was adjusted to 7.1. 5 mL of medium 1 was added to each test tube (13 cm diameter) and kept for sterilization at 121°C for 20 min. After 0.5 mL filter was

sterilized glucose was added to each test tube. Bacterial isolates were inoculated to each two test tubes and one test tube was covered with pure Vaseline. The test tubes were incubated at 30°C for 18-24 hours (Abada *et al.*, 2018).

Cellulase Production

The cellulose degrading bacteria were cultured on production media. The media composed of ($MgSO_4$- 0.25 g, KH_2PO_4- 0.5 g, CMC - 2.0 g, Gelatin - 2 g, Agar - 15 g and 1 L distilled water). The cultures were grown at optimum temperature at different pH, and 120 rpm. After 24 hours of incubation period, the cultures were centrifuged for 10 min at 8000 rpm and at 4°C. The supernatant were collected and stored as crude enzyme at -20 °C for further investigations (Tailliez *et al.,* 1989).

Cellulase Enzyme Activity Assay

The measuring the amount of reducing sugar method is used for determining the cellulose activity. The enzyme activity was determined by the International Union of Pure and Applied Chemistry (IUPAC) commission on biotechnology (T.K.Ghose *et al.,* 1987). 0.5 mL of supernatant was taken to 0.5 mL of 2% CMC in 0.05 mL sodium acetate buffer solution (pH 4.8). The mixed solution was kept at optimum temperature for 1 hour. The reaction was completed by adding 3 mL of 3,5- dinitrosalicylic acid (DNS) reagent to 1 mL of reaction mixture (Miller 1959). The mixed solution were boiled in water bath then allowed to cool by using ice. The colour intensity was studied at OD 540 nm.3,5-dinitrosalicylic acid is used for determining the reducing sugars spectrophotometrically by usingpreviously prepared glucose standard curve. The enzymatic activity of cellulase termed in international units (IU).The amount of enzyme that releases 1 µmol reducing sugars (measured as glucose) per mL per minute is known as 1 unit of enzymatic activity.

Production and estimation of bioethanol

To initiate the saccharification process, the cellulose degrading bacteria were grown in basal medium prepare in 1% of husk extract at optimum fermentation conditions with shaking at 120 rpm for 48 h. The culture was centrifuged; the supernatant was resterilized by 0.2 µm filter for the growth of *B.cereus* at 37°C for 48 hours. Before and after the fermentation process , the total reducing sugar was

estimated by using DNS method (Devarapalli*et al.*, 2015).The fermentation broth samples were centrifuged at 8000 rpm at 4 °C for 3 min to separate suspended particles, and the clear liquid was analyzed for the presence of fermentation products. Then, the clear samples were filtered. Ethanol concentration was measured according to Ire et al. (2016) using Gas Chromatography at CECRI (Shimadzu-2014, Tokyo, Japan) with a packed column (Gaskuropack5460/80;GC-2014Glass ID:3:2φ X 2:1 m, GL Science Co. Ltd., Tokyo, Japan), with the following operational conditions: temperature of column and detector were 110 and 250 °C, respectively, nitrogen gas flow rate 60 mL/min and the injected sample volume 2 μL.

RESULTS AND DISCUSSION

Isolation and Identification

Figure 1(a and b) shows the isolated bacterial colonies on agar plates. The colony forming unit was 23X $10^{7.}$ coloniesare opaque, transluscent, circular and irregular rhizoidal in nature. It can be seen in Table 1. Morphologically four different genera were isolated. Out of four, oneis Gram positive rod and another one is gram positive cocci. Out of four, two were gram negative (rod and cocci). Results of biochemical tests (Table 2) showed that out of four, 3 isolates were facultative anaerobes and catalase-positive and oxidase positive. Based on biochemical characters isolates were identified as *B.cereus Enterococcus* sp, *Lactococcus* sp and *Enterobacter* sp respectively designated as endosymbiont ES1 to ES4. Table 2 shows the characteristic of Isolates. Out of 4, 3 were gram positive and one is gram negative(Figure 2) Isolate ES1 Gram positive rod With endospore was identified *asB.cereus*. Isolate ES2 another gram positive rod was found to be Lactobacillus sp. Isolated strain ES3 and ES 4 was belongs to *Enterococcus sp* and *Enterobacter sp.*. The lingo cellulolytic activity of*Enterobacters*was investigated by Borji*et al.* (2003). Deschamps*et al.* (1980) has demonstrated that the*Enterobacter*is able to assimilate differentphenolic compounds.

The digesting lignocellulosic materials in the gut gives termites an important role in the carbon cycle in the symbiotic relationship with the microorganisms (Brune, 2014). The present study showed that bacteria found in the termite gut contain 4 genera can degrade different types of organic materials. This implies that bacteria have an important role in lignindegradation in termites. Several studies have demonstrated

degradation of lignin and lingo cellulosic materials by bacteriafrom the termite gut, but information about their effect on the chemical composition of agricultural by-products as feed for ruminants is scarce. Consistent with the results, several experiments have isolated *Bacillus and Lactobacillus* from the gut of termites *Reticulitermessantonensis* in liquid and solid media containing aromatic compounds as a carbon source (Kuhnigk and Konig, 1997).

Table 1: Colony and cell morphology

Strain code	Colony morphology	Gram stain
ES1	Curled white irregular colony.	Gram positive rod
ES2	circular flat opaque	Gram positive rod
ES3	opaque irregular Rhizoidal	Gram positive cocci
ES4	Small circular translucent	Gram negative rod

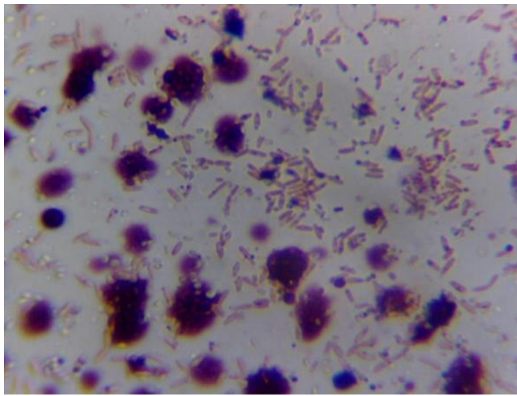
Table 2: Biochemical characters

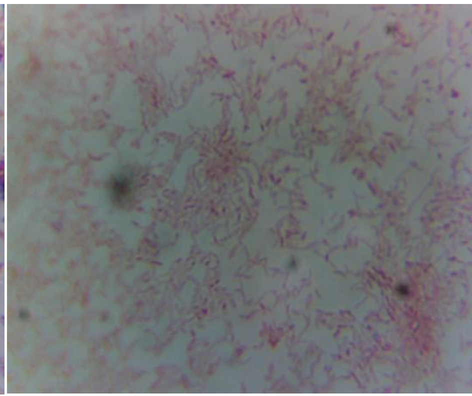
Characteristics	ESI	ES2	ES3	ES4
Indole	Negative	positive	negative	positive
MR	Negative	positive	positive	positive
VP	Positive	Negative	Negative	Negative
Citrate	negative	positive	Negative	positive
Catalase	Positive	positive	positive	Negative
Oxidase	Positive	positive	positive	Negative
GENERA	*B.cereus*	*Lactococcus*sp	*Enterococcus sp*	*Enterobactersp*

Figure 1: Isolation of Bacteria from Termite gut

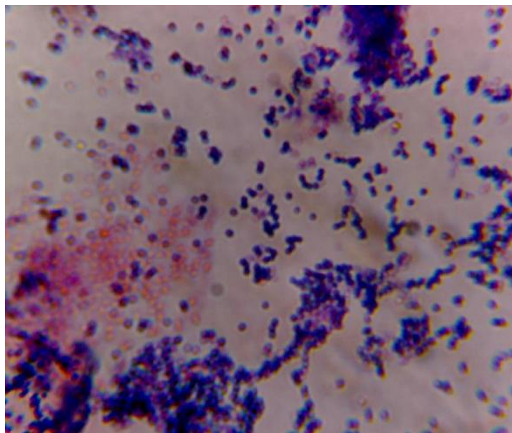
a)Termite b)isolated bacteria

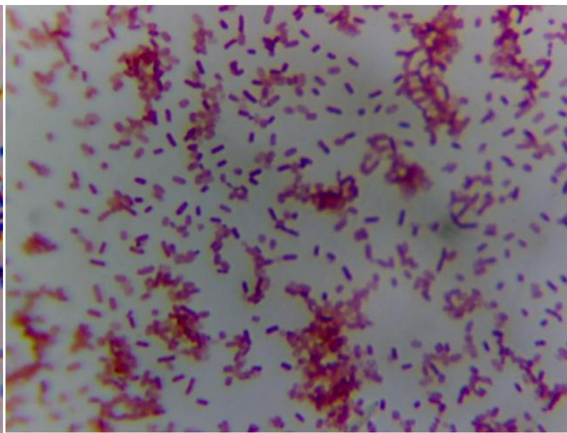
Figure 2: Gram staining morphology of isolates





***ES1 B.cereus*(G$^+$)** **ES2 *Lactococcus*sp(G$^+$)**





ES3 *Enterococcus*sp(G$^+$) **ES4 *Enterobacter*sp (G$^-$)**

Cellulase and amylase producer

The four isolates were subjected to qualitative analysis of cellulase and amylase and results were given in table 3. In this study all four bacterial isolates (75%) were able to grow on CMC media indicating their cellulolytic capability but only on ES1 *B.subtils* found to be amylase producer (Figure 3). Wenzel*et al*. (2002) had detected some cellulolytic bacteria from the gut of termite they have used CMC medium for their

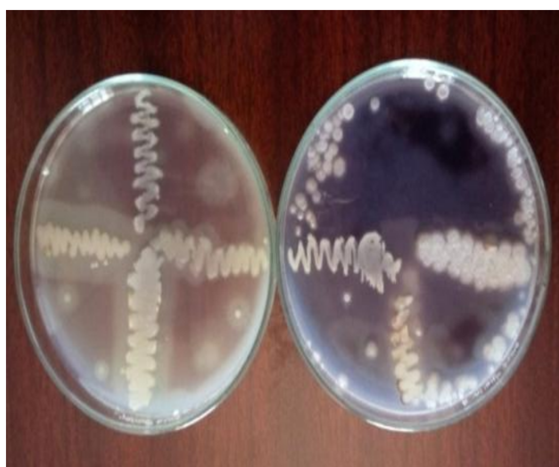
identification.*Enterobacteraerogenes*and*Enterobacter cloacae*has been investigated and the capability of these bacteria is clear to perform a series of anaerobic reactions such as O-demethylation, decarboxylation (Kuhnigk*et al.*, 1994). In this studydescribed the facultative bacteria with the ability of degrading lignocellulose compounds are found in terminte gut on the periphery while the strict anaerobes are in the center of the gut (M.Ramin *etal.*, 2008).

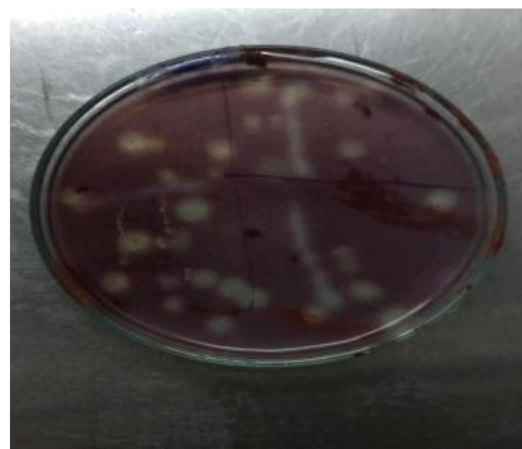
Table 3: Qualitative Enzyme Screening Plate

Strain code	Cellulase	Amylase
ES1	Positive	Positive
ES2	Positive	Negative
ES3	Positive	negative
ES4	Positive	negative

Figure 3.Screening of enzyme producing bacteria

A). isolated colonies B) amylase C)cellulose





Effect Of pHand agitation on growth and enzyme production

The cellulase activity of the *B. cereus* isolated from termite gut was taken and impact of hydrogen ion concentration evaluated by measuring growth different pH ranges (5,7,9,11) . The maximum growth rate was obtained at pH7 with optical absorption 1.2 at 600 nm followed by 0.6, 0.8, and 0.07 at pH of 5,9 and 11 under 100 rpm. Growth at static condition was found to be moderate and comparatively less than shaking. The growth of the bacteria is affected by the growth phase at which it grows under static. The results agreed with those for *B. amyloliquefaciens* and *B. alcalophilus*,

which also possed a maximum cellulase activity at a 3% inoculum concentration (Abou-Taleb et al. 2009). Cellulase activity was measured by DNS method achieved at a pH of 7.0 and was 25 IU/mL followed by 22, 20 and 15 U/Ml was recorded at pH 5,9 and 11 (Fig. 4). The threedimensional shape of the active site of enzyme is maintained by an optimum pH.Due to alteration in the ionic bonding of enzyme is leads to the loss of functional shape of enzyme. The obtained bacterial isolate had an optimum pH at 7 above or below this pH, the bacterial growth is affected and in turn reduce cellulase activity.

Bioethanol production

Fermentation was begins with initial reducing sugar 0.2 g/L of husk extract minimal medium and fermentation was carried upto 48hours. The results were showed that there was a high concentration of ethanol when *B. cereus* was used to degrade the husk cellulose residues (Fig. 6) which suggested there was a higher conversion of the substrate to reducing sugars.the increasing concentration of reducing sugar was estimated as from to 2.4g/L at the end of 48h incubation(Abada*et al.*, (2018). The reducing sugar content was 0.28 g/L for the fermentation medium before the saccharification process. After the growth of the *Bacillus cereus* for 48 h under optimum fermentation conditions, the reducing sugar content was elevated as measured by the DNS method (Fig. 7). Banerjee et al. (2010) explained that enzymatic hydrolysis is done by the highly substrate specificcellulase enzyme. The analysis of the fermentation medium followed by distillation (Fig. 8) by GC-MS showed an ethanol concentration of 17.0 g/L(Fig 5). The obtained yield can be compared with the yield obtained from other *B.subtils*. The bioethanol yield obtained in this study was 8 higher than the yield (7.5 g/L) obtained from the fermentation of sugarcane bagasse hydrolysate using *Pichiastipitis* DSM 3651, as reported by Ira et al. (2016).

Figure 4 .Growth of *B.subtilis* and cellulase enzyme assay

Figure 5. GCMS analysis of bioethanol

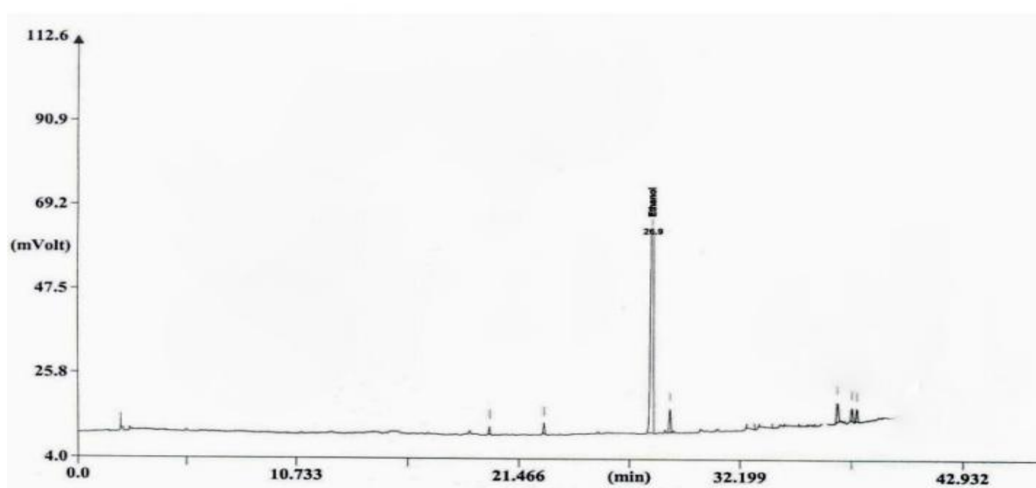


Figure 6: Husk substrate and extract

Figure 7: Sugar estimation of DNS

Figure 8: Distillation of fermented broth

SUMMARY AND CONCLUSION

Bio-ethanol is regarded as an important renewable fuel to partly replace fossil-derived fuels. In this study, bioethanol production, which includes cellulase production, saccharification of the cellulose content of husk residue, and ethanol production, was investigated (Bharat et al., 2008).Out of the four of cellulase-producing bacterial strains isolated from termite gut during this study, the ES1 isolate was found to have cellulase and amylase enzyme production. This isolate was known as *Bacillus cereus*. The effects of different growth parameters, including pH and shaking were investigated to optimize the growth conditions of the bacterium.

The maximum cellulase activity was achieved at pH of 7 and an incubation temperature of 35°C. The results showed the liberation of 2.4 g/L of reducing sugar during fermentation. This total reducing sugar produced 17.0 g/L of ethanol after 48 hours when *B.cereus* was used as a fermentation agent. Hence, bioethanol was successfully produced from the cellulose of rice husk residue using the cellulase enzyme from *B. cereus.*

REFERENCE

1. Abada, E. (2014). “Production and purification of lipase from Pseudomonas sp. AB2 with potential application in biodiesel production,” J. Pure Appl. Microbio. 8, 133- 142.
2. Abada, E. A., Masrahi, Y. S., Al-Abboud, M., Alnashiri, H. M., and El-Gayar, K. E. (2018). "Bioethanol production with cellulase enzyme from Bacillus cereus isolated from sesame seed residue from the Jazan region," BioRes. 13(2), 3832-3845.
3. Abou-Taleb, K. A. A., Mashhoor, W. A., Nasr, S. A., Sharaf, M. S., and Abdel-Azeem, H. H. M. (2009). “Nutritional and environmental factors affecting cellulase production by two strains of cellulolytic Bacilli,” Australian Journal of Basic and Applied Sciences 3(3), 2429-2436.
4. Amore A., Olimbia P., Veliriaventoniro, Albertie A., vinzenza. (2016) Cellulolytic Bacillus strains from natural habitats. *Journals of peer reviewed* 31(2):49-52.
5. Ann C. Smith, Marise A. HusseyGram Stain Protocols, Americans society for microbiology,30 September 2005.
6. Bharat P., Binod B., Rubin TM. (2014) Production Purification and Characterization of Cellulase from Bacillus subtilis Isolated from Soil. *European journal of bio technology and bioscience* 2 (5):31-37.
7. Borji, M., S. Rahimi, G. Ghorbani, J. VandYoosefi and H. Fazaeli, 2003.Isolation and identification of some bacteria from termites gut capable in degrading straw lignin and polysaccharides. J. Fac. Vet. Med. Univ. Tehran, 58: 249-256.
8. Brune, A., 1998. Termite guts: The world's smallest bioreactors. Trends Biotechnol., 16: 16-21.

9. Charles Darkoh, Cynthia Chappell, Christopher Gonzales, and Pablo Okhuysena, A Rapid and Specific Method for the Detection of Indole in Complex Biological Samples,Applied Environmental Microbiology,2015 Dec; 81(23): 8093–8097.
10. David B Wilson, Microbial diversity of cellulose hydrolysis, Current Opinion in Microbiology 2011, 14:1–5.
11. Deschamps, A.M., G. Mahoudeau and J.M. Lebeault, 1980. Fast degradation of kraft lignin by bacteria. Eur. J. Applied Microbiol. Biotechnol., 9: 45-51.
12. Devarapalli M., Hasan K., Atiyeh. (2015) A review of conversion processes for bioethanol production with a focus on syngas fermentation. *Biofuel Research Journal 7 ,268-280.*
13. Geetashirnalli, Ashwin.M., and Dhanashreegachhi.(2018) Isolation and evaluation of cellulolytic yeasts for production of ethanol from wheat straw.*International current microbial and applied science 7(12):2215-2221.*
14. Gincy Marina Mathew, Yu-Ming Ju, Chi-Yung Lai, Dony Chacko Mathew & Chieh Chen Huang, Microbial community analysis in the termite gut and fungus comb of Odontotermes formosanus: the implication of Bacillus as mutualists,FEMS Microbiol Ecol 79 (2012) 504–517.
15. Gupta, P., Samant, K., and Sahu, A. (2012). “Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential,” International Journal of Microbiology.45:67-71
16. Iqbal MS., Ahmed MS., Ogras TS., Chun L.,MuhammadArshad., Khan Khattak JZ., Javed H A., Saleem U., Bashir R.(2012) Ethanol production from waste material*s. Journal of biochemtech* 4(1):285-288.
17. Ira, F. S., Ezebuiro, V., and Ogugbue, C. J. (2016). “Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse,” Bioresources and Bioprocessing 3(26), 1-12.
18. Kuhnigk T, König H (1997) Degradation of dimeric lignin model compounds by aerobic bacteria isolated from the hindgut of xylophagous termites. J Basic Microbiology, 37:205–211.
19. Lekh R., Kuldeep K., Sandeep S. (2014) Screening Isolation and Characterization of CellulaseProducingMicro-Organisms from Soil. *International Journal of Pharmaceutical Science Invention* 2 12-18.

20. Long YH., Xie L., Liu N., Yan X.,LiMH.,FanMZ.,Wang Q.,(2010) Comparison of gut-associated and nest associated microbial communities of a fungus-growing termite (*Odontotermesyunnanensis*). Insect Sci 17: 265–276
21. M. Ramin, A.R. Alimon , N. Abdullah, J.M. Panandam and K. Sijam, 2008. Isolation and Identification of Three Species of Bacteria from the Termite Coptotermes curvignathus (Holmgren) Present in the Vicinity of University Putra Malaysia. Research Journal of Microbiology, 3: 288-292.
22. Maryam B., Qadir A., Zameer M., Sajid RA., Rubina N., Jamil N., Sameen A., Afzaal R. (2018) Production of Cellulases by Bacillus cellulosilyticus Using Lignocellulosic. *Material Pol Environ Stud 27(6).*
23. Miller, G. L. (1959). “Use of dinitrosalicylic acid reagent for determination of reducing sugar,” Anal. Chem. 31(3), 426-428.
24. Muhammad Imran, Zahid Anwar, Muhammad Irshad, Muhammad Javaid Asad, Hassan Ashfaq, Cellulase Production from Species of Fungi and Bacteria from Agricultural Wastes and Its Utilization in Industry: A Review, Advances in Enzyme Research, Vol.04 No.02(2016).
25. Mustafa Vohraa, Jagdish Manwarb,*, Rahul Manmodec, Satish Padgilwarb, Sanjay Patil, Bioethanol production: Feedstock and current technologies, Journal of Environmental Chemical Engineering 2 (2014) 573–584.
26. Priyanka P., Yuvaraj C., Fahra S., and Aranganathan V. (2017) Isolation of cellulose degrading fungi from soil and optimization for cellulose production using carboxy methyl cellulose. *international journal of life science &pharma research(7)(1) 2250-048.*
27. Qian Kang,Lise Appels,Tianwei Tan and Raf Dewil, Bioethanol from Lignocellulosic Biomass: Current Findings Determine Research Priorities, Review Article, The Scientific World Journal / 2014, volume 2014 |Article ID 298153.
28. Rahman SS.,MahboobHossain MD., Choudhury N. (2018) Bioethanol fermentation from kitchen waste using saccharomyces cerevisiae. *Journal of F1000Research* 7:512.
29. Tailliez, P., Girard, H., Millet, J., and Beguin, P. (1989). “Enhanced cellulose fermentation by an asprogenous and ethanol tolerant mutant of Clostridium thermocellum,” Applied Environmental Microbiology 55, 207-211.

30. Tiwari.S.,JadhavS.,Tiwari KL.(2015) Bioethanol production from rice bran with optimization of parameters by Bacillus cereus strain. *IntJ EnvironSciTechnol 12(12):125.*

31. T. K. Ghose, *“Mesurnment of cellulase activity,” Pure and Applied Chemistry,* vol. 59, pp. 257–268, 1987

32. Vishwanatha T, Keshavamurthy M, Naganagouda V. Kote and Manjula A. C,Molecular Identification and Response Surface Methodological (RSM) Approach for Optimized Production of Amylase from *Bacillus altitudinis* GVK38, *BioscienceBiotechnologyResearch communications,*volume-13-1-jan-mar-2020.

33. Vohra M.,Manwar J.,Manmode R.,Padgilwar S.,Patil S.(2014) Bioethanol production :feedstock and current technologies. *Journal of environmental chemical engineering* 2: 573-584.

34. Wenzel M, Schönig I, Berchtold M, Kämpfer P, König H (2002) Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis.* J Appl Microbiol 92:32–40.