Lab-Scale Production of Bioethanol from Paddy Straw and Banana Peel : An Eco-Friendly Approach

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ABSTRACT

Agro-industrial waste serves as a viable substitute material for the manufacturing of biofuels. The objective of current research was to production of bioethanol using agricultural by-products and waste materials under laboratory scale. Pretreatment was conducted on rice straw and banana fruit peels, and the cellulose degrading bacterium was isolated and identified as *Bacillus* sp. The hydrolyzed sample underwent fermentation using adapted yeast (*Saccharomyces cerevisiae*) in a controlled laboratory environment. The fermented sample analyzed by potassium dichromate method, and spectroscopic investigations to quantify bioethanol. The results of the study demonstrated that banana peel, rice straw and their combinations yielded bioethanol *i.e.* banana peel - 4.6 mg/mL, rice straw - 5 mg/mL, and a combination of banana peel and rice straw - 5.04 mg/mL. This yield is lower than industrial-scale production, indicating that the study is at a preliminary laboratory scale and requires further optimization. The outcome of study suggests that the combinations of banana peel and rice straw substrate yielded the maximum quantity of bioethanol compared to other substrates.

Keywords: Bioethanol production, Agricultural biowaste, byproducts, fermentation, Ecofriendly approach

INTRODUCTION

The ongoing growth of industrialization and population has resulted in a persistent increase in the global demand for energy. Currently, over 80% of global energy production relies on the use of fossil fuels. Fossil fuels have played a substantial role as a major energy source worldwide. According to Hamzah et al. (2019), it is certain that both natural gas and petroleum will eventually be depleted. The increasing energy requirements, along with the exhaustion of fossil resources, have prompted a shift towards the utilization of materials for the efficient and economical production. The use of fossil fuel is linked to climate change, global warming, and several energy and security concerns. In addition, fossil fuels are not equitably distributed among nations and are also non-renewable (Danmalikiet al., 2016). The use of fossil fuels for energy has placed a substantial burden on both humans and the environment, resulting in problems such as air and water pollution, as well as global warming. The exponential increase in energy consumption and our heavy dependence on fossil fuels has led to the buildup of greenhouse gases and subsequent alteration of the Earth's climate. Consequently, significant efforts have been made to investigate, assess, and adopt clean and sustainable fuel alternatives (Keller, 2018). Biofuel, specifically bio-ethanol is an alternative energy source that can be used in addition to energy obtained from fossil fuels. The utilization of biofuels for energy generation is significantly more environmentally sustainable and less polluting when compared to processes employing fossil fuels, natural gas, and petroleum. Biofuels are regarded as a feasible substitute for petroleum and natural gas due to the anticipated depletion of these limited resources in the future. Bio-Ethanol manufactured mostly from maize grain and sugar cane, is widely recognized as one of the most prominent renewable fuels now in use. This ethanol, obtained through the process of fermenting carbohydrates, is manufactured from biomass or waste materials. Agricultural wastes that contain a high amount of sugars that may be fermented have been shown to be excellent materials for producing ethanol. This is a promising option for generating energy, especially considering the limited availability of crude oil. It serves as a sustainable substitute that reduces the reliance on crude oil and mitigates environmental degradation. Bioethanol is derived from a diverse range of raw materials such as corn, starch, sugar cane, and lignocellulosic waste materials such biomass from paddy straw and banana (Busic et al., 2018). Currently, bio-ethanol is exclusively manufactured on an industrial scale by converting sugar molasses into ethanol. There is a requirement to enhance the manufacturing of alcohol by utilizing agricultural byproducts and garbage due to its abundant availability (Soma Prabha et al., 2023). Based on the above cited information's, the present study was focused on bioethanol production using agricultural byproduct (paddy straw) and agriculture waste (banana fruit peel) under laboratory condition.

MATERIALS AND METHODS

Sample collection

The agricultural byproduct, paddy straw, was gathered from the agricultural field in the vicinity of Omalur, Salem, Tamil Nadu, India. Additionally, the agricultural waste, banana peel, was collected from nearby fruit shops in Salem District. The rice straw and banana peel were cleansed and dehydrated at room temperature (28 ± 2°C) for duration of 6 days.

Bacterial enumeration and streaking technique

In this procedure, 1 gram of banana peel powder and rice straw powder was separately mixed with 10 milliliters of distilled water, resulting in a stock solution. A 1mL aliquot of the stock sample was diluted with 9mL of distilled water in a set of 10 test tubes. The entire process of serial dilution was conducted in a laminar air flow chamber, ensuring perfect sterility. The samples were diluted in a series, with concentrations of 10^{-6}, 10^{-7}, and 10^{-8}. These diluted samples were then distributed onto nutritional agar plates using the spread plate technique. The plates were then incubated at a temperature of 37°C for duration of 24 hours. To isolate a pure culture from the incubated plates, streaking was conducted. One colony was obtained from the plate that was injected. The colonies obtained from the nutrient agar plate were streaked onto CMC agar in order to isolate bacteria that are capable of digesting cellulose. The CMC agar was formulated using the following components: Carboxymethyl cellulose -10 grams. The composition of the plates includes 2g of Tryptone, 4g of KH_2PO_4, 0.2g of NA_2HPO_4, 0.2g of MgSO4 .$7H_2O$, 0.001g of $CaCl_2$, and 0.001g of $FeSO_4$.$7H_2O$. The plates are then placed in an incubator at a temperature of 37°C for duration of 24 hours.

Cellulose degrading bacterium identification

The bacteria obtained from the sample were subjected to staining in order to determine their gram classification. The sample contained gram-positive bacteria that were rod-shaped. The cellulose degrading capacity of the bacterial isolates was assessed using Congo red staining (1g/L) for duration of 5 minutes, followed by rinsing with a 1M NaCl solution. The degrading capacity was determined based on the diameter of the zone of clearing surrounding the colony. The colony that had the largest zone of clearing, indicating a strong ability to degrade CMC was selected and preserved for future use. Biochemical assays are employed to determine the genus of a bacterium by analyzing the specific biochemical activities displayed by various bacterial species. Multiple biochemical assays (Indole, Methyl red, Voges Proskauer test, Citrate Utilization test, Catalase, TSI-tests) have been used to identify the genus of bacterial isolates.

Pre-treatment of plant materials

Mechanical pretreatment

The pretreatment of lignocellulosic biomass before hydrolysis is essential for bioethanol production as it directly affects the amount of bioethanol that can be generated by fermentation. The objective of pretreatment is to decrease the density, durability, and crystalline structure of cellulose, facilitating the hydrolysis of lignocellulosic biomass into individual sugar molecules. Mechanical pre-treatment involved chopping and grinding to decrease the total particle size of the banana peels and rice straw.

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Acid pretreatment (fig.1)

A 5% solution of sulfuric acid (H_2SO_4) was combined with ground banana peels and rice straw as a second stage in the pre-treatment process. This acid pre-treatment was performed to further modify the lignocellulosic structure of the banana peel and rice straw biomass. The weight of the banana peels and rice straw was approximately 40 g each and they were separately added to 200 mL of 5% H_2SO_4. The combination of banana peels and rice straw, along with a 5% solution of H_2SO_4, was subsequently introduced into an autoclave for sterilization. The sterilization procedure lasted for 15 minutes at a temperature of 120°C. Following a 15 min autoclaving process, the mixture was subsequently cooled to room temperature for duration of 3 hours. Once the combination had reached a lower temperature, the solid residue and the solution were separated using filtration. The solid residues were subsequently rinsed with distilled water and dried using a microwave for 30 min at a temperature of 40°C.

Hydrolysis

The objective of hydrolysis is to further break down the polysaccharides found in the processed lignocellulosic biomass of banana trash into individual subunits of monosaccharides. The monosaccharides that will be generated by hydrolysis will augment the fermentation process by *S. cerevisiae*. The bacteria that were separated were utilized to break down the banana peel and rice straw powder through hydrolysis (Kumar et al, 2019). Three sets of samples were prepared for hydrolysis. The first set consisted of twenty grams (20g) of banana peel powder. The second set consisted of twenty grams (20g) of rice straw powder. The third set was a mixture of banana peel and rice straw, with ten grams (10g) of each. These samples were placed in a 500mL sterile conical flask. To suspend the substrate, 250mLs of distilled water were added to the flask. Next, 5mL of a purified bacterial culture was introduced into the substrate and placed on a rotary shaker at a speed of 200rpm. The mixture was then incubated at room temperature for a period of 7 days. The identical technique was replicated for each of the three sets of samples (fig 2).

Estimation of total reducing sugar

Prior to fermentation, the estimation of total reducing sugar was conducted to determine if sugar was present or absent in the hydrolysis of banana peel and rice straw. The quantification of the reducing sugar in the hydrolysis sample was determined using two different methods.

Benedict's test

The Benedicts test was employed to quantify the quantity of reducing sugars produced by the pretreatment procedures. The underlying premise of the Benedicts test is that reducing sugars cause the blue copper sulfate in the Benedicts reagent to transform into a red copper oxide precipitate. A 1mL aliquot of the hydrolyzed sample was transferred into a test tube, followed by the addition of 2mL of Benedict's reagent. The mixture was then placed in a boiling water bath for 5 minutes and any resulting color changes were noticed.

DNS approach

The spectrophotometry approach was employed to measure the concentration of reducing sugar in the fermenting sample broth, with D-glucose serving as the standard. A 0.05 mL sample that had undergone hydrolysis was combined with 0.35 mL of citrate buffer at a pH of 6.5, as well as 0.6 mL of Dinitro-salicylic acid (DNS). The resulting combination was then heated for duration of 5 minutes in order to promptly halt the reaction. Subsequently, the spectrophotometer was employed to quantify the absorbance at a wavelength of 540 nm. The concentration of reducing sugar in the sample was determined by correlating the absorbance.

Fermentation Process

Inoculum and Fermentation Preparation

Commercial *Saccharomyces cerevisiae* was employed for fermentation as it transforms the monosaccharides and certain disaccharides generated during hydrolysis into ethanol, facilitated by the invertase and zymase enzymes found in *S. cerevisiae*. Preparations of inoculums were made using dehydrated Baker's yeast, specifically *S. cerevisiae*. The broth was produced by combining 10g of peptone, 5g of sodium chloride (NaCl), and 3g of yeast extract in distilled water. Subsequently, the broth was transferred to an incubator shaker and allowed to incubate for duration of 24 hours prior to its utilization in the fermentation process. The yeast strain was adapted by gradually exposing it to increasing substrate concentrations over 3-5 days to improve tolerance and fermentation efficiency. The fermentation process was begun by combining 25mL of yeast inoculum with 100mL of hydrolyzed culture and incubating it at room temperature for a duration of seven days (fig.3). The entire fermentation process was done as per the modified methods of Malik, and Sushil (2019).

Distillation

The extraction of bioethanol was conducted using the distillation technique. The process of distillation was conducted using a soxhlet heating equipment to eliminate the water content from the fermented samples. The heating mantle was set to a temperature of 78^{o}C to heat the round bottomed flask containing the ethanol-water mixture. This specific temperature was chosen since ethanol can only be distilled at 78^{o}C.

Bioethanol estimation

Initial assessment of bioethanol

The generation of bioethanol was analyzed using the potassium dichromate technique. A 2% solution of $K_2Cr_2O_7$, together with 5 mL of concentrated H_2SO_4, was combined with 3 mL of the sample. The process of oxidizing ethanol to acetic acid was achieved by using potassium dichromate in the presence of sulfuric acid, resulting in the formation of a blue-green tint. The transition from a pink hue to a blue-green shade signifies the existence of bioethanol in the fermented sample.

Studies using Fourier Transform Infrared (FT-IR) Spectroscopy

FTIR is an analytical technique used to detect the presence of functional groups in bioethanol. Pellets for FTIR measurement were prepared by combining bioethanol with KBr. The analysis was conducted within the range of 400 to 4000 cm^{-1}, as described by Irfan et al. (2011). The material was subjected to FTIR analysis at the Department of Biotechnology, Periyar University, Salem.

Spectral Analysis using UV spectroscopy

The quantity of ethanol generated by fermentation was determined using the potassium dichromate technique. The experiment involved dissolving 1g of$K_2Cr_2O_7$ in 27mL of 98%H_2SO_4. Five sterile clean test tubes were taken with volumes of 0 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, and 1.00 mL of ethanol with known concentrations and the sixth was set as blank. Next, 3 mL of a prepared chromic acid solution was added to each test tube, and placed in a water bath at 60°C for 10 minutes. Finally, a 1mL of Rochelle salt (40%) was added to ensure color stability. A UV-VIS spectrophotometer was used to measure the absorbance at 600nm. (Nitin Mahendra Chauhan et al., 2021)

RESULTS AND DISCUSSION

Screening of bacterial isolates

Five bacterial colonies (rod-shaped gram-positive bacteria) were obtained from the serial dilution of a sample of banana peel powder ranging from 10^{-5} to 10^{-8} (Fig.4-5). The bacterial isolates were evaluated using biochemical assays and confirm *Bacillus* sp. (Table 1; Fig 6). The cellulose degrading ability of the isolated bacteria was assessed by screening them using Congo red, resulted the formation of a zone on a CMC agar plate treated with Congo red. The diameter of the zone formed was measured to be 19mm (Fig.5B).

Impact of acid pre-treatment

The composition of lignocellulosic biomass from banana peel and rice straw was examined both before and after undergoing pretreatment with dilute acid. An estimation was made of the crude fiber content of both untreated and biomass treated with dilute acid. The pretreatment banana peel had a crude fiber value of 18%, whereas the native banana peel had a crude fiber content of 21%. As for rice straw, the pretreated sample contained 2% crude fiber, while the native rice straw had a crude fiber content of 8.5%. This could be attributed to the dissolving of non-crystalline substances derived from the biomass.

Determination of lowering sugar

Benedict Test

The alteration in color resulting from Benedict's reaction provides a partially quantitative or approximate assessment of the concentration of reducing sugars in a given sample. The concentration of decreasing sugar in a sample can be measured by observing a color change. The different colors of the product are categorized based on their sugar content. Blue represents the absence of sugar, Green has 0.5% sugar, Yellow contains 1% sugar, Orange contains 1.5% sugar, Red contains 2% sugar, and Brown has the greatest level of sugar (Fig 7).

DNS Method

The quantity of reducing sugar was measured using DNS after hydrolysis, with D-glucose serving as the reference standard. The sugar content in the banana peel was increased by 3.6 mg/mL following hydrolysis, while the rice straw sample showed increase to 3.8 mg/mL. However, when compared to the standard concentration of 4.3 mg/mL, both samples exhibited lower sugar concentrations (Fig. 8).

Estimation of bioethanol

Initial assessment of bioethanol

The initial assessment of bioethanol was conducted using potassium dichromate and sulfuric acid, in which a redox reaction occurs and the color transition from orange to green signifies the existence of bioethanol in the extract (fig.9).

Fourier Transform Infrared (FT-IR) spectroscopic investigations

Analysis of bioethanol produced from various substrates using FT-IR

The FTIR analysis was employed to identify the different functional groups present in the bioethanol produced from banana peel, paddy straw and both (Fig). The FTIR spectrum of the produced bioethanol reveals the existence of chemical bonds and functional groups. The bands observed several peaks at various intensity for bioethanol produced from all substrates indicate the presence of several compounds, including alcohol, alkenes, and primary amide (Fig. 10; Table 2).

UV spectroscopic analysis of bioethanol

The quantities of unknown ethanol from three different substrates were determined by measuring the absorbance of known ethanol concentrations by UV visible spectroscopy at a wavelength of 600 nm. The ethanol was produced different ranges i.e. banana peel (0.9 mg/mL to 4.6 mg/mL), rice straw (1.09mg/mL to 5mg/mL) and both substrate (0.8mg/mL to 5.04mg/mL) respectively while the positive control yielded ethanol range between 1.5 mg/mL to 6 mg/mL (Fig. 11).

SUMMARY AND CONCLUSION

The primary objective of the current investigation was to generate bioethanol from lignocellulosic biomass. At now, bioethanol is derived by the process of alcoholic fermentation using molasses obtained from crops that include starch or sugar. This study evaluated the comparison of bioethanol production from banana peel and rice straw employing cellulose degrading bacteria *Bacillus* sp. for hydrolysis and fermentation with adapted *Saccharomyces cerevisiae*. The process of bioethanol synthesis using banana peel and rice straw involved mechanical and acid processing. Additional hydrolysis was conducted to break down the polymeric carbohydrate into simple sugars, which will then be fermented into alcohol using *Saccharomyces cerevisiae*. The process of breaking down the substrate was conducted by utilizing the naturally occurring bacteria that were extracted from the banana peel sample. This work involved drawing a calibration curve to accurately quantify the total ethanol concentration in water derived from sugarcane samples. The maximum ethanol yield obtained was 5.04 mg/mL using the combined substrate. The ethanol yield (5.04 mg/mL ≈ 5.04 g/L) is lower than industrial yields (40-100 g/L), indicating a preliminary lab-scale study. Compared to previous studies reporting 20–80 g/L ethanol, the present yield is lower, suggesting optimization is required. This suggests that further optimization is required to improve production efficiency.

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APPENDIX

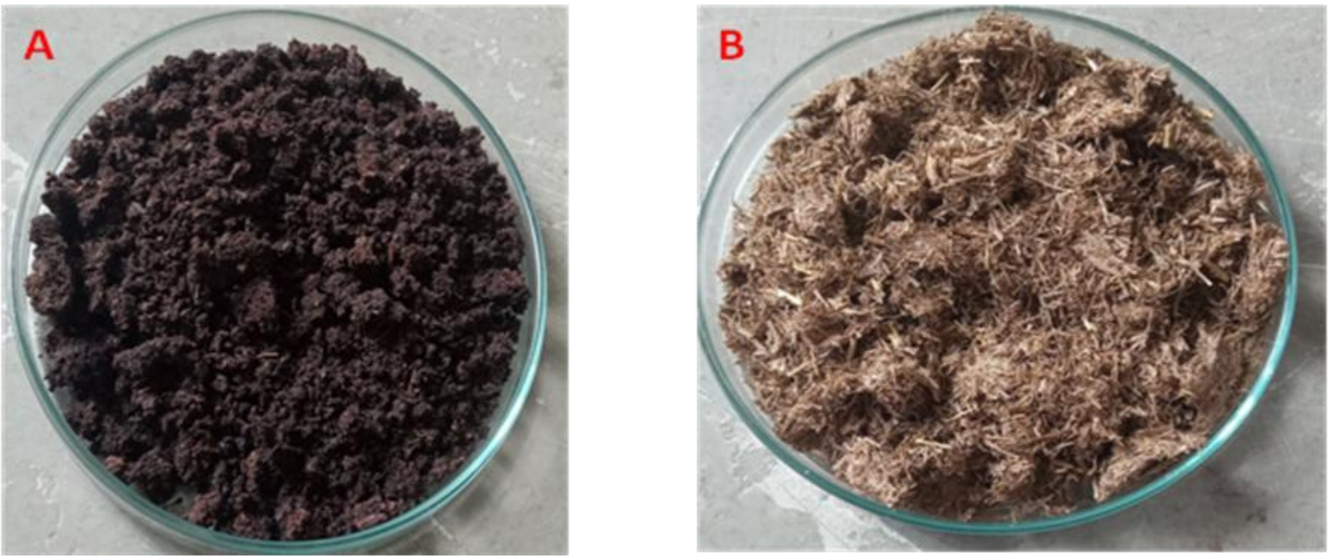

Fig 1: Acid pretreated samples (A) Banana peel (B) Rice straw

Fig 2: Hydrolysis of samples

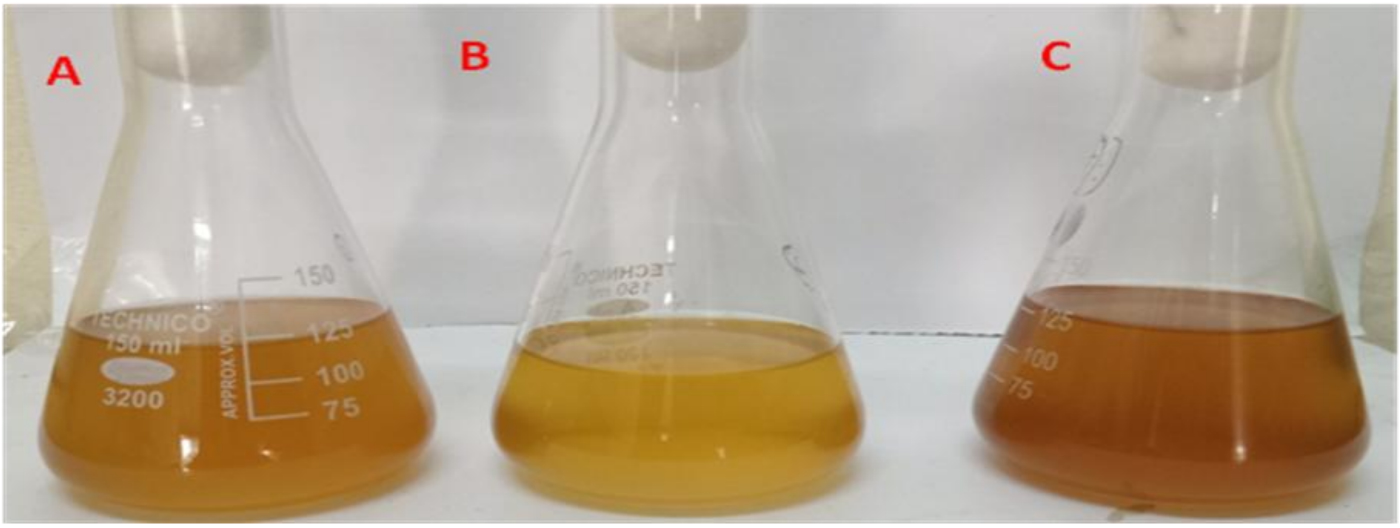

Fig 3: Fermented samples (A) Banana peel, (B) Rice straw (C) Banana peel and rice straw

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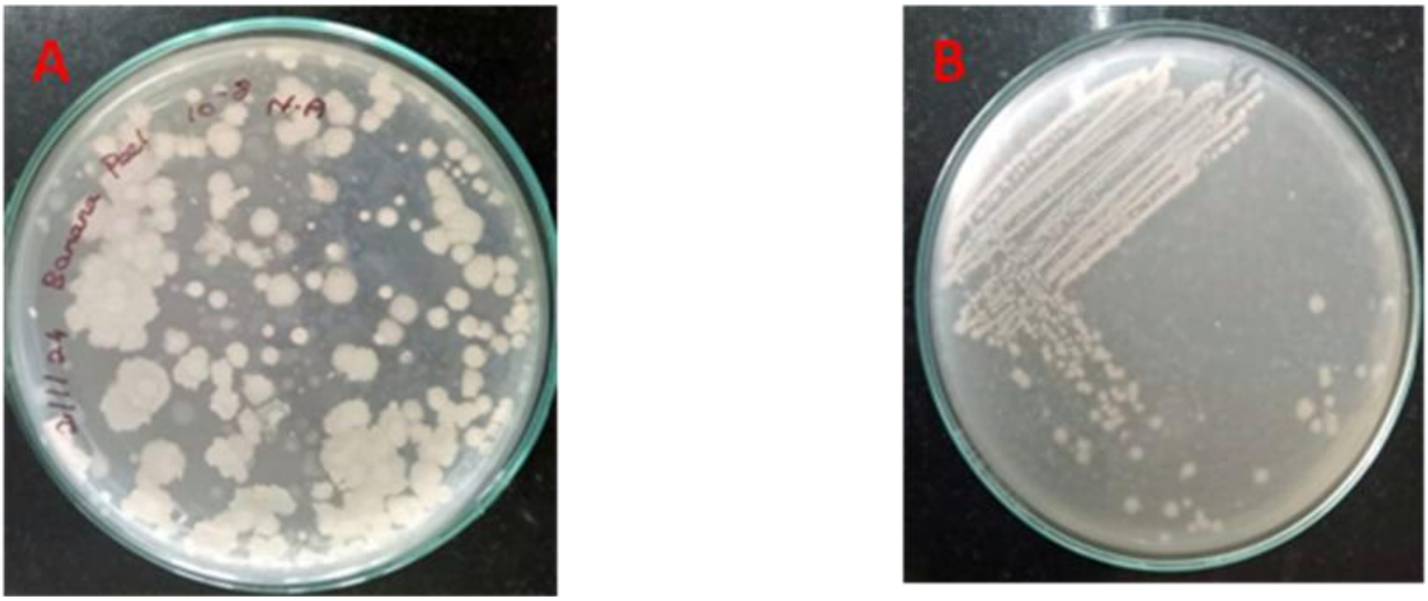


Fig 4: (A) Spread plate technique-dilution 10^{-8} (B) Streak plate technique - dilution 10^{-8}

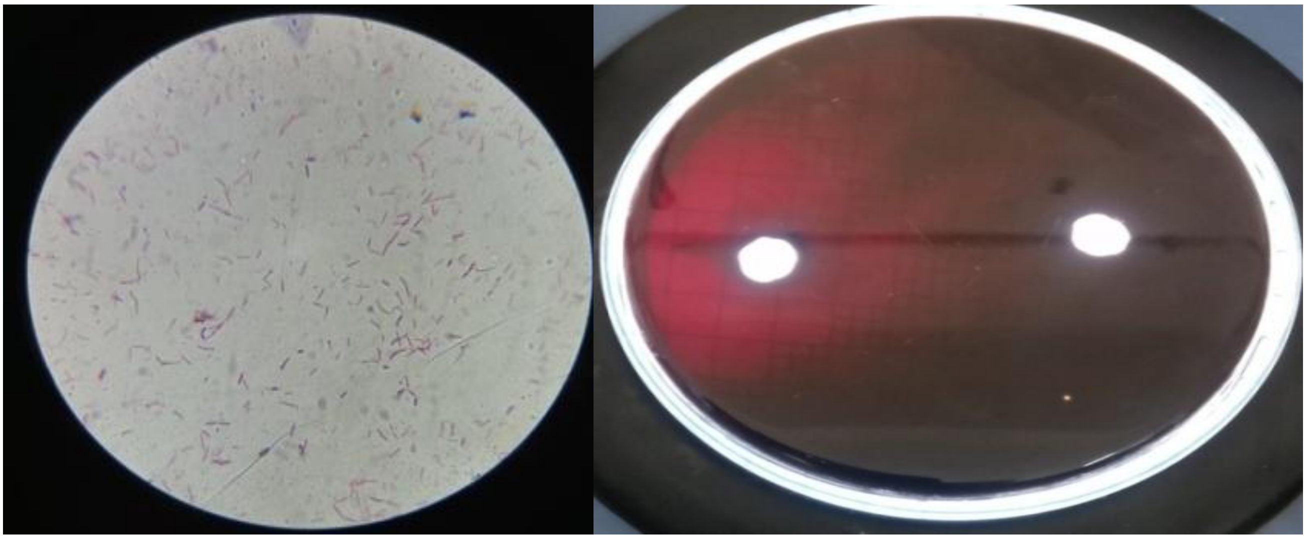


Fig 5: Gram staining (A) and Cellulose degrading ability of bacterial isolate (B)

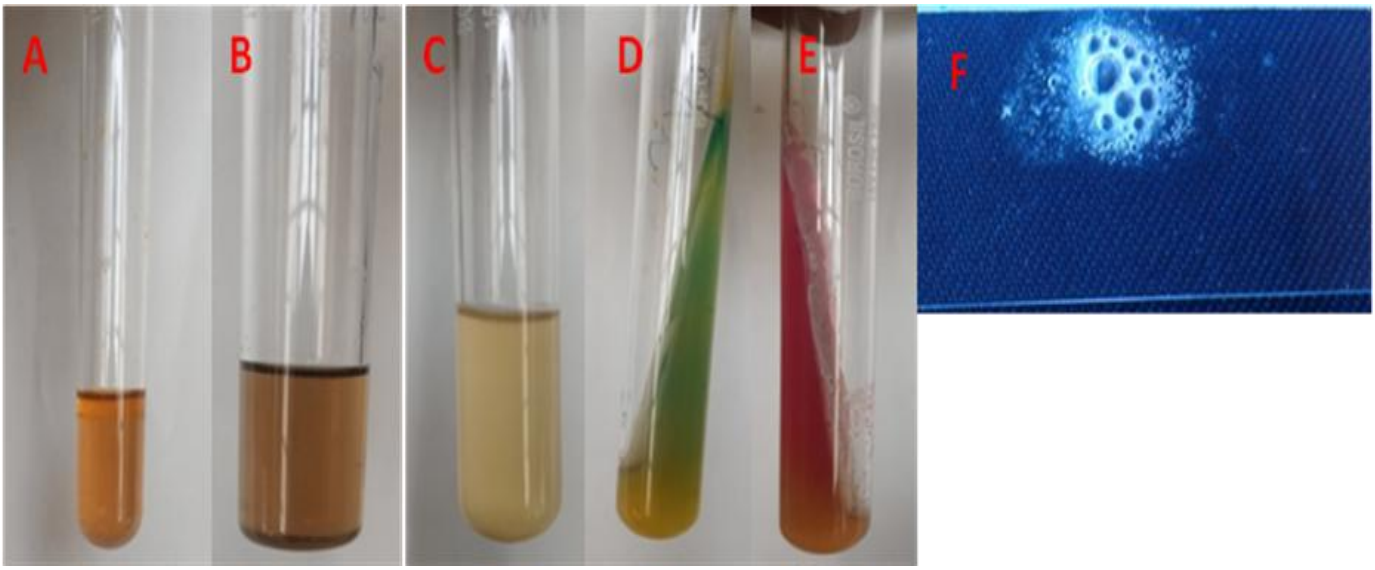


Fig 6: Biochemical assays of bacterial isolate (A): Indole test,(B): Methyl red,(C): Vogues Proskauer, (D) Simmon citrate, (E): triple sugar iron, (F): Catalase.

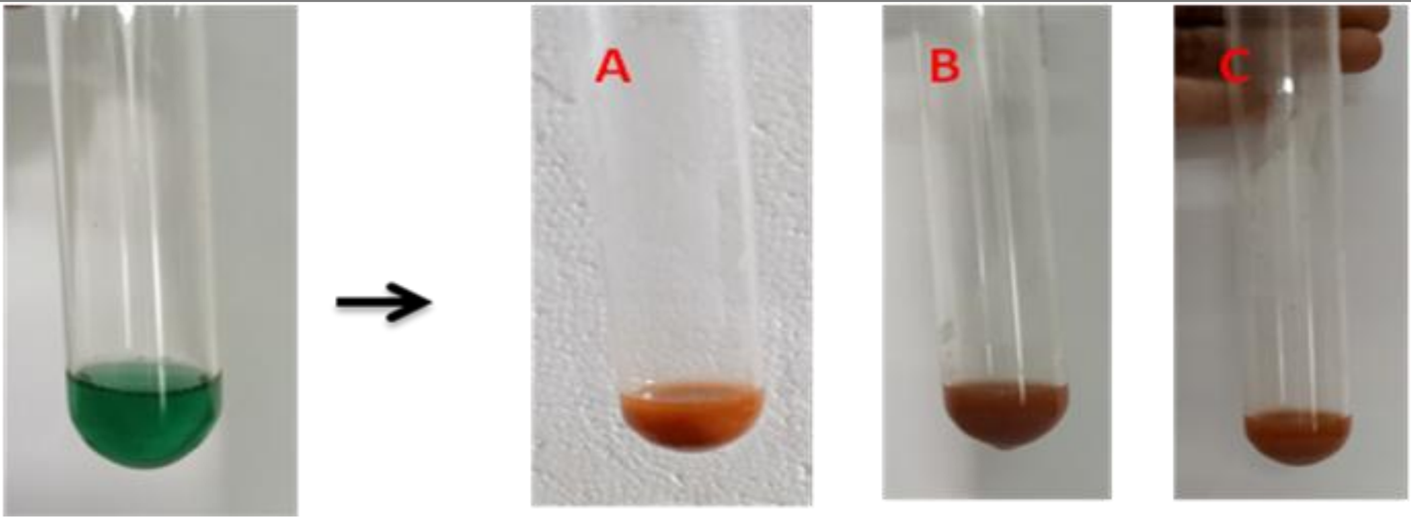


Fig 7: Show color changes from green to red indicate the presence of reducing sugar in the hydrolyzed sample (A) Banana peel, (B) Rice straw, (C) Banana peel and Rice straw

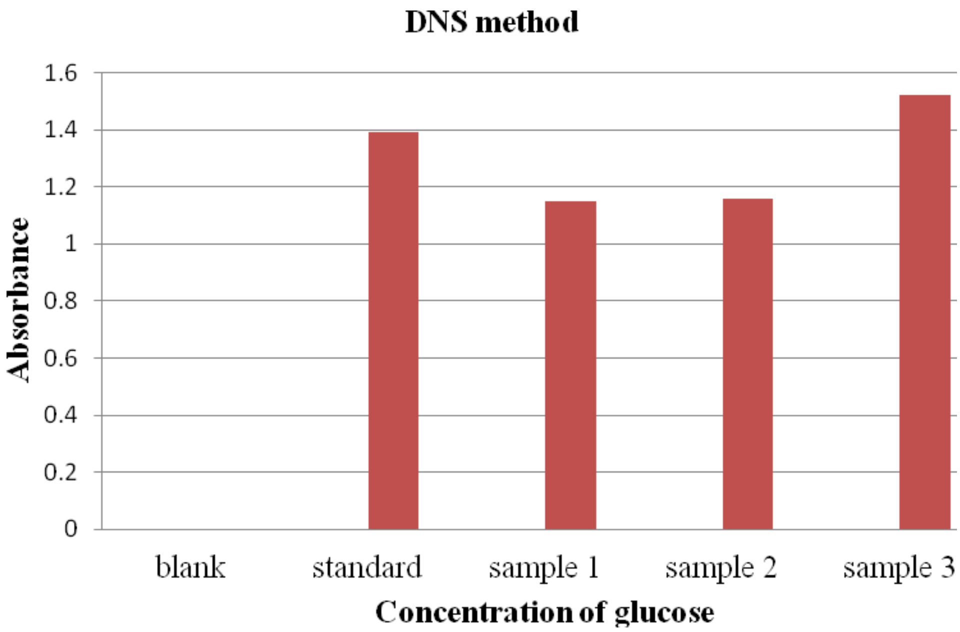


Fig 8: Estimation of reducing sugar by DNS method

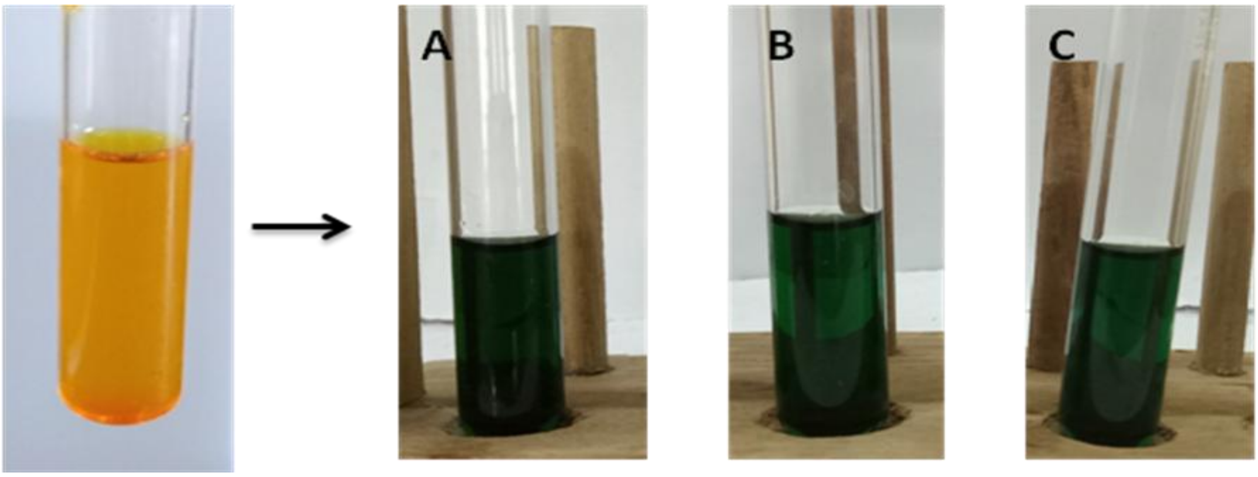
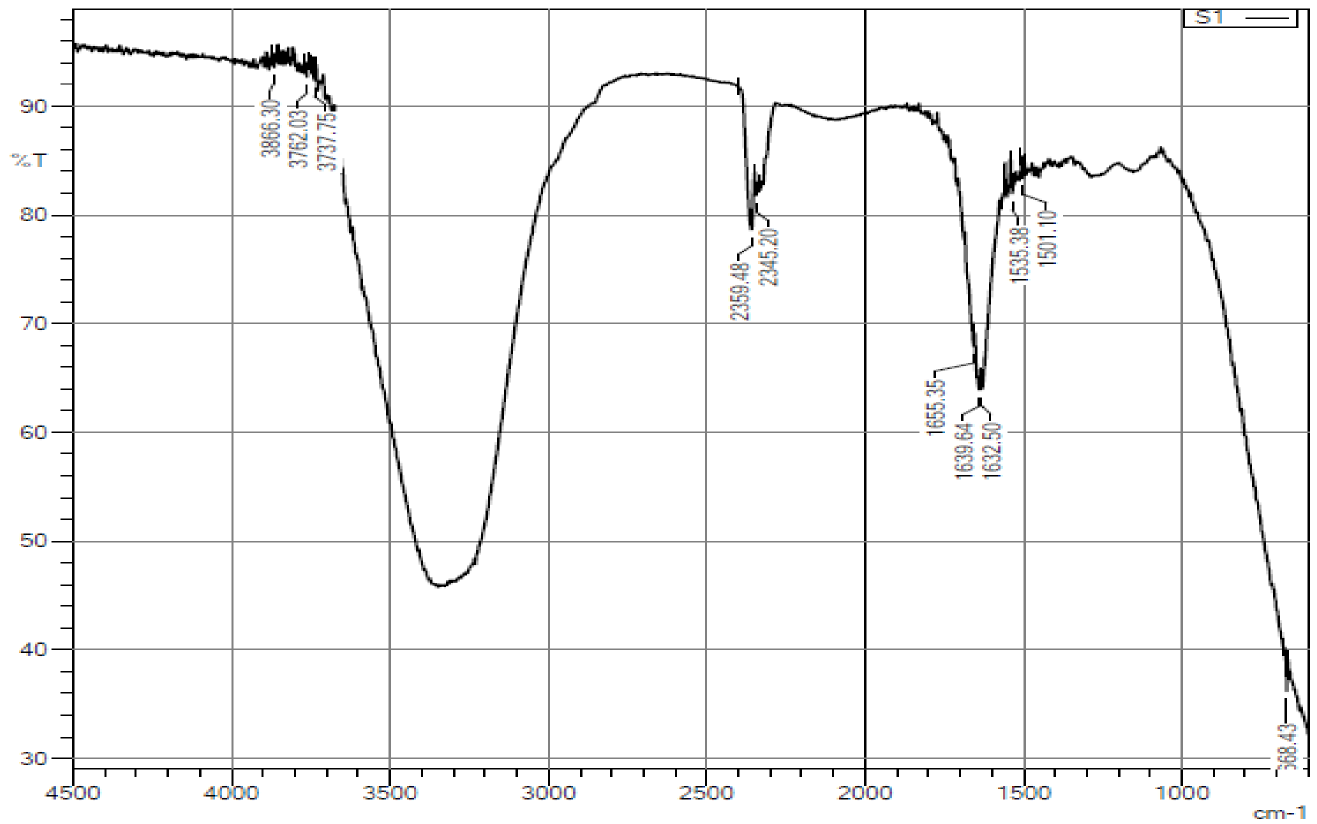


Fig 9: The color changes from orange to green indicate the presence of bioethanol(A): Banana peel, (B): Rice straw sample, (C): Banana peel and rice straw sample.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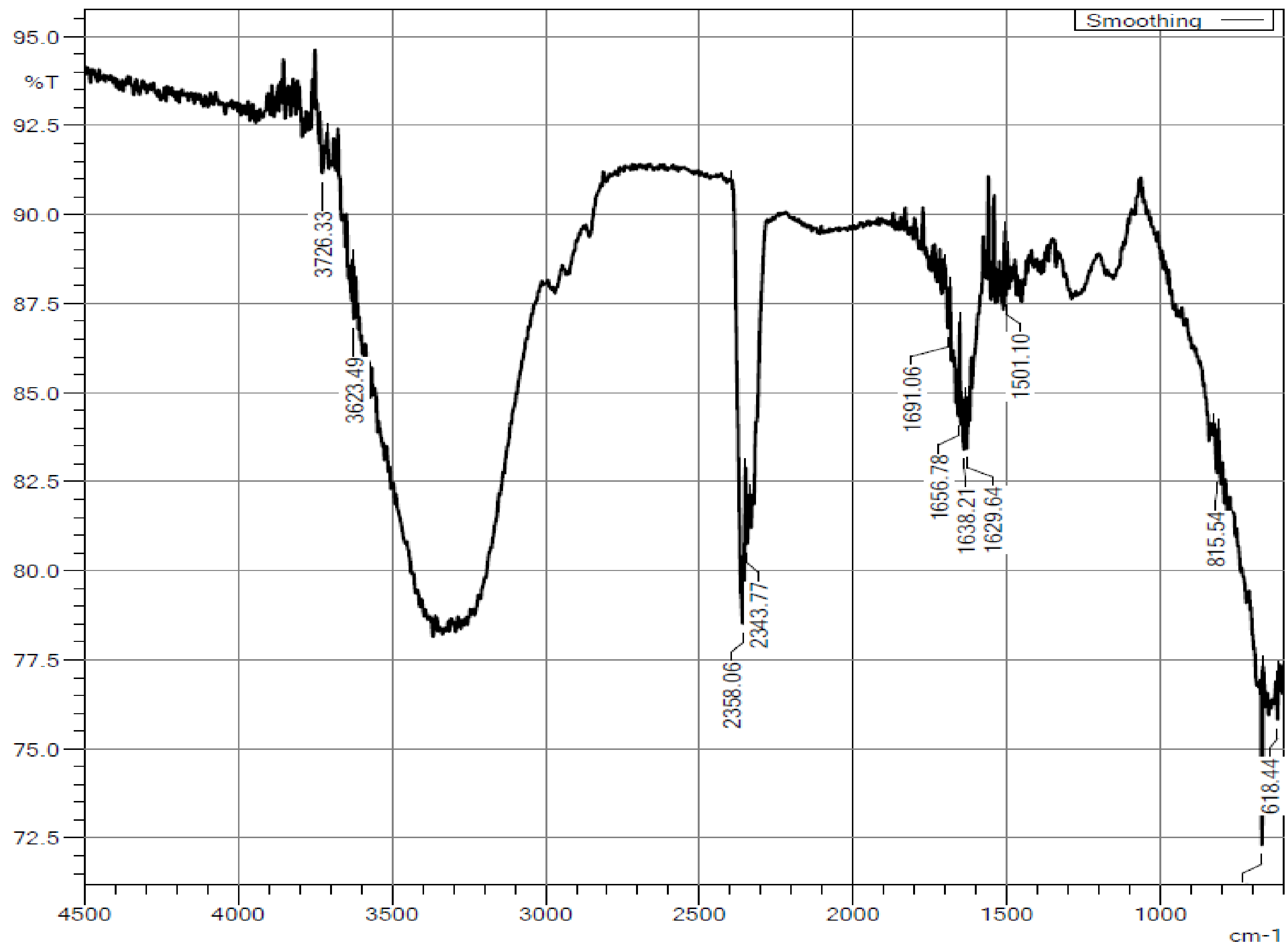
(A)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3726.33
3623.49
2358.06
2343.77
1691.06
1656.78
1638.21
1629.64
1501.10
815.54
618.44

(B)

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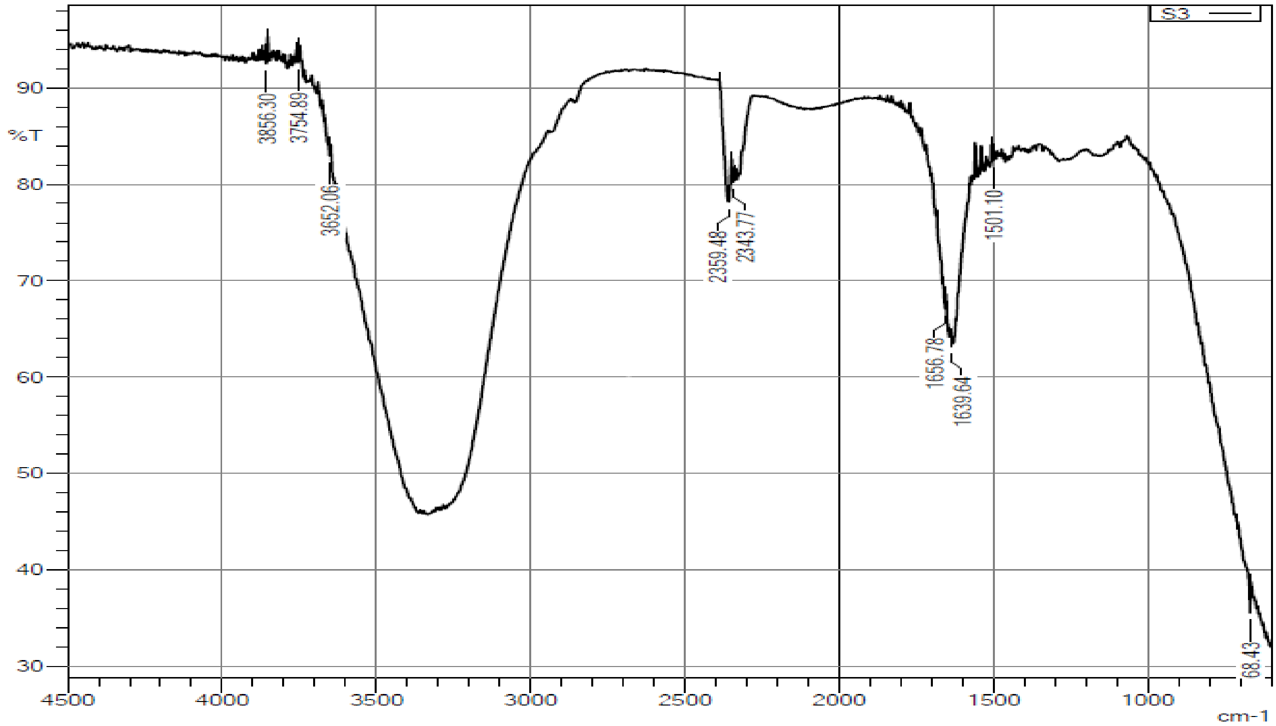
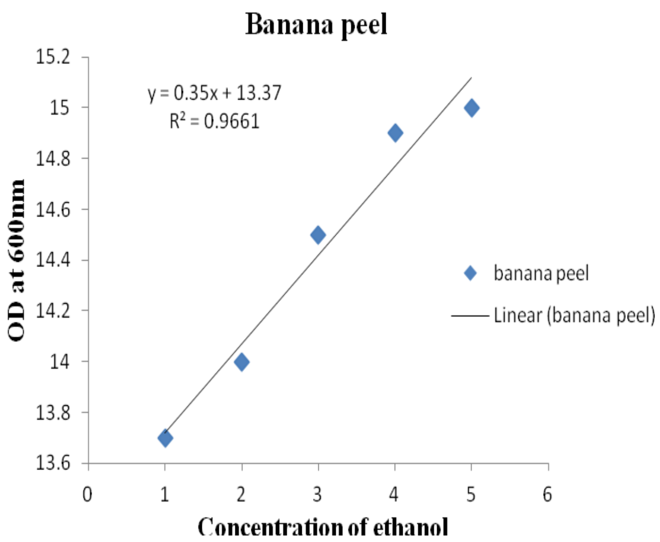
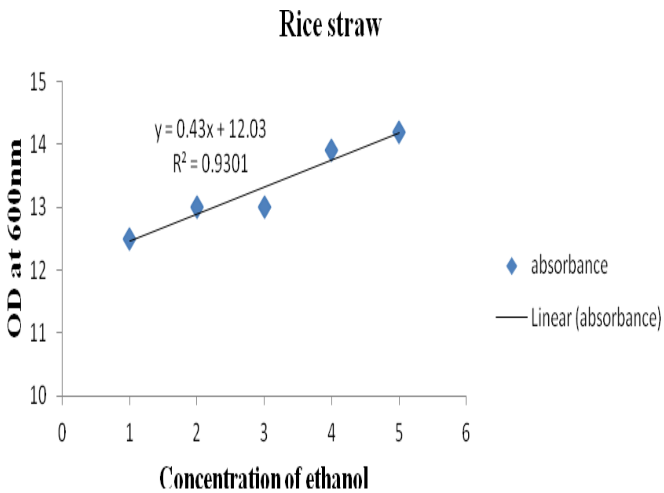


(C)

Fig 10: FT-IR spectrum of bioethanol from different substrates A) banana peel b) Rice straw c) Both A &B





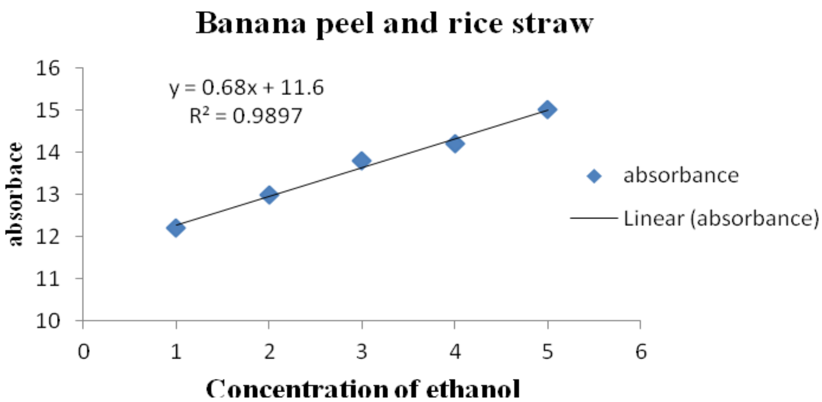


Fig 11: UV visible spectroscopy analysis of bioethanol from different substrate

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a) banana peel b) rice straw c) both a &b

Table 1: Biochemical characterization of isolated bacterium

S.NO	TEST	Inference
1	Indole	+
2	Methyl red	-
3	Vogues Proskauer	-
4	Catalase	+
5	Simmon citrate	+
6	Triple iron sugar	+

Positive (+), Negative (-)

Table 2: FTIR analysis of bioethanol produced from different substrates (banana peel, paddy straw and its combination)

S. No.	Functional group	Stretch/ bonding	Visible intensity		
			Banana peel	Rice straw	Banana peel + Rice straw
1	Alcohol	C – O – H bending	Large (668)	Large (618)	Large (684.3)
2	Alkenes	C-C stretch	Medium (1632)	Medium (1638.21)	Medium (1639.64)
3	Alkenes	C-C stretch	Medium (1639)	Medium (1656.78)	Medium (1656.78)
4	Primary amide	C=O stretch doubled	Medium (1655)	Large (3360)	Large (3370)
5	Alcohol	OH stretch	Large (3400)	Large (3623.49)	Large (3652.06)

* - figures in the parenthesis show the observed frequency value (cm -1)