UTILIZATION OF BANANA (*Musa* spp.) BIOMASS FOR BIOFUEL PRODUCTION

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UTILIZATION OF BANANA (*Musa* spp.) BIOMASS FOR BIOFUEL PRODUCTION

by

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(2009-09-116)

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2014

DECLARATION

I, hereby declare that the thesis entitled **"UTILIZATION OF BANANA (*Musa* spp.) BIOMASS FOR BIOFUEL PRODUCTION"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

ADF	Acid Detergent Fiber
CMC	Carboxy Methyl Cellulose
cm	Centimeter
cc	Cubic centimeter
DNA	Deoxyribo Nucleic Acid
^{0}C	Degree celcius
DNS	Dinitro salicylate
EC/TDS	Electrical Conductance/Total Dissolved Solids
et al.	And others
Fig.	Figure
g	Grams
h	Hours
HCl	Hydrochloric acid
H_2SO_4	Sulfuric acid
KCl	Potassium Chloride
KI	Potassium Iodide
M	Molar
mg	Milligrams

min	Minutes
ml	Millilitre
mM	Millimolar
µg	Micrograms
μsm^{-1}	Micro siemens $meter^{-1}$
N	Normal
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NDF	Neutral Detergent Fiber
nm	Nanometer
nBLAST	Nucleotide Basic Local Alignment Search Tool
PCR	Polymerase Chain Reaction
pH	Per hydrogen
ppm	Parts per million
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SBB	Single Batch Bioconversion
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation

INTRODUCTION

1. INTRODUCTION

India has 0.5% of the oil and gas resources of the world but 16% of the world's population with the result that the country depends heavily on oil imports to meet the domestic demand. More than 70% of the needs of the country are met from imports of crude oil and natural gas. World faces the progressive depletion of its energetic resources mainly based on non-renewable fuels. At the same time, energy consumption grows at rising rates. The demand for motor gasoline has been growing at an average annual rate of ~7% during the last decade and it shows an increasing trend. The current consumption of petrol for transportation needs (motor gasoline) is estimated at 15.23 billion liters annually. Conversion of lingo-cellulosic feedstock into biofuels is an important choice for the exploitation of alternative energy sources and reduction of polluting gases. In addition, the utilization of biofuels has important economic and social effects. Ethanol (ethyl alcohol, bioethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer. Ethanol has some advantages when it is used as an oxygenate. Firstly, it has a higher oxygen content that implies a less amount of required additive. The increased percentage of oxygen allows a better oxidation of the gasoline hydrocarbons with the consequent reduction in the emission of CO and aromatic compounds. Compared to other fuels ethanol has greater octane booster properties, it is not toxic, and does not contaminate water sources (Sanchez and Cardona, 2008).

The current share of biofuels in the consumption of transportation fuels is extremely low and is confined mainly to 5% blending of ethanol in gasoline which the government had made mandatory in the states of Andhra Pradesh, Goa, Gujarat, Haryana, Karnataka, Maharashtra, Punjab, Tamil Nadu, Uttar Pradesh and Uttaranchal and in the union territories of Daman and Diu, Dadra and Nagar Haveli and Chandigarh (Sukumaran *et al*. 2010). In India, sugar cane molasses is the main raw material for

ethanol production. But the short supply and increased cost is the main hindrance for its use. Current ethanol production based on corn, starch and sugar substances may not be desirable due to their food and feed value (Taherzadeh and Karimi, 2007). The cellulosic materials are cheaper and available in plenty but their conversion to ethanol involves many steps and is therefore expensive. Under such circumstances a novel approach is essential to use renewable substrates such as fruit waste. Research efforts are focused to design and improve a process, which would produce a sustainable transportation fuel using low cost feed stocks.

Many agricultural raw materials rich in fermentable carbohydrates were tested worldwide for bioconversion from sugar to ethanol, but the cost of carbohydrate raw materials has become a limiting factor for large scale production by the industries employing fermentation processes. Since the price of feedstock contributes more than 55% to the production cost, inexpensive feed stocks such as lingo-cellulosic biomass and agri-food wastes, are being considered to make bioethanol competitive in the open market (Campo *et al*., 2006). The production of ethanol from comparatively cheaper source of raw materials using efficient fermentative microorganism is the only possible way to meet the great demand for ethanol in the present situation of energy crisis.

Banana is one of major constitute the principal food resources in the world and occupy the fourth world rank of the most significant foodstuffs after rice, corn and milk (Arumugam and Manikandan, 2011). India is the largest producer of banana in the world and accounts for nearly 30% of the total world production. In Kerala, the area under cultivation of banana was 61011 hectares and the fruit production was around 515607 tonnes during the year 2012-2013 (Department of economics and statistics, 2014). About 20 - 30% of banana fruit peels are discarded as waste by the processing industries of which a major part is discarded as raw peel. Banana raw peel can be used as a potential feedstock for bioethanol production due to its easily availability and convertible sugar contents. The banana crop also generates large amount of waste after harvest. The fruit processing wastes and plant residue of banana if utilized effectively

for bioethanol production, could also be an attractive alternate for disposal of the polluting residues. However, major hindrance is the identification of suitable micro-organisms and procedures to enhance the conversion rate during alcohol production. The present study is an attempt to produce cost effective and environmentally sustainable ethanol using banana fruit and plant residues, a major agro-waste in Kerala.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Bioethanol as a potential alternate fuel source

The main problems associated with the increasing use of fossil feed stocks as energy source are the related environmental pollution issues as well as the continuous depletion of these reserves. In recent years, the fermentative production of energy from renewable resources has been considered as an alternative to petrochemical processes. More than a dozen alternative fuels are now under production or development for use in alternative fuel vehicles and advanced technology vehicles. Ethyl alcohol or ethanol is considered to be a good alternative to petroleum and is produced by chemical synthesis and by fermentation or biosynthetic processes (Rath *et al*., 2014).

Ethanol (ethyl alcohol, bioethanol) is the most employed liquid biofuel either as a fuel or as a gasoline enhancer (Thomas and Kwong, 2001). Ethanol has some advantages when it is used as an oxygenate. Firstly, it has a higher oxygen content that implies a less amount of required additive. The increased percentage of oxygen allows a better oxidation of the gasoline hydrocarbons with the consequent reduction in the emission of CO and aromatic compounds. Related to fossil fuels, ethanol has greater octane booster properties, it is not toxic, and does not contaminate water sources (Sanchez and Cardona, 2008).

Ethanol production process only uses energy from renewable energy sources; no net CO_2 is added to the atmosphere, making ethanol an environmentally beneficial energy source. In addition, the toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources (Wyman and Hinman, 1990). Ethanol represents closed carbon dioxide cycle because after burning of ethanol, the released CO_2 is recycled back into plant material as the plants use CO_2 to synthesize cellulose during photosynthesis cycle (Chandel*et al*., 2007). Ethanol derived from biomass is the only

liquid transportation fuel that does not contribute to the greenhouse gas effect. According to Ali and Mohammed (2011), ethanol contains 35% oxygen that helps complete combustion of fuel and thus reduces particulate emission that pose health hazard to living beings.

Large scale production of fuel ethanol is mainly from sugarcane and corn Gnansounou and Dauriat (2005). However, ethanol production based on corn, starch and sugar substances is not desirable due to their food and feed value. Hence, the present study is concentrated on utilizing the cost effective feedstock like agricultural wastes and lignocellulosic biomass for the production of bioethanol.

2.2 Bioethanol production from different agricultural feedstocks

Fruit wastes are cheap, easily available, potential source for the production of bioethanol. They also have very good antimicrobial and antioxidant potential (Janani *et al*., 2013).

Osanaiye *et al.* (2005) carried out a study on yeast fermentation of *Carica papaya* (pawpaw) agricultural waste using dried active baker's yeast and brewer's yeast strain (*Saccharomyces cerevisiae*). The fermented pawpaw yielded ethanol contents of 3.83 to 5.19% (v/v). Rotten rambutan fruit wastes were used to produce bioethanol fuel using *Saccharomyces cerevisiae* by Hadeel *et al*. (2011). Lalitha and Rajeshwari (2011) utilized fruit biomass peel residue for the production of fuel ethanol using *Aspergillus niger*. Reddy *et al*. (2011) investigated the suitability of dried mango peel for bioethanol production and obtained 5.3% of ethanol. Different fruit peel wastes of papaya, banana and apple are also reported to be utilised for the production of ethanol by Kandari and Gupta (2012). They reported that the maximum production of alcohol was within 36h of fermentation in papaya peels extract followed by banana and apple peel extract (5.90-4.94%). Raikar (2012) in his study observed 7.6% of ethanol production in grape waste using *Saccharomyces cerevisiae.* Santi *et al.* (2012) studied the production of bioethanol from orange peel waste. The pretreated orange peel

yielded 50.35 w/w ethanol after fermentation. Mishra *et al.* (2012) produced ethanol from fruits of pineapple, orange and sweet lime. They obtained a maximum result of 1.87, 1.46 and 1.32 %v/v of ethanol for pineapple, sweet lime and orange respectively.

Effective production of bioethanol from agricultural wastes involves characterization of the feedstock and pretreatment of the feedstock.

2.2.1 Characterisation of the feedstock

Patle and Lal (2007) have characterized the agricultural wastes for total solids, total sugar, total reducing sugar and moisture content. Total sugars are estimated using the anthrone method and total reducing sugars were estimated by the dinitrosalicylic acid (DNS) method. The dried samples were incinerated at 600^0C for 6 h and the ash content was determined.

Compositional analysis of pineapple wastes have been carried out by Upadhyay *et al*. (2010) for moisture content, pH, cellulose, hemicellulose, total soluble solids, reducing sugar, non-reducing sugar and lignin for ethanol production. Arumugham and Manikandan (2011), estimated the ash content, moisture content, starch content, total lipid content, protein content and the fiber content of the mango and banana fruit samples. The proximate composition of banana fruitpulp was 76.63% moisture, 5.65% protein, 1.37% lipid, 19.75% ash and 0.632% starch. Similarly for mango, the proximate composition of fruit pulp was 81.26% moisture, 7.96% protein, 1.48% lipid, 13.08% ash and 0.507% starch.

Analysis for the determination of moisture, non-reducing sugars, protein, total soluble solids, cellulose and lignin was carried out in mango peel by Reddy *et al.* (2011). The results obtained were 70.5%, 25.6%, 7.0%, 5.9% and 25.2% for moisture content, total soluble solids, reducing sugar, non-reducing sugar, cellulose and lignin content respectively.

Itelima *et al*. (2013) determined the biomass yield, cell dry weight, reducing sugar concentration and the ethanol yield at 24 hours interval. The results of the study showed that after 7 days of fermentation, pineapple peels had the highest biomass yield of 1.89 (OD), followed by banana peels 1.60 (OD), while plantain peels had the least (0.98 OD). The reducing sugar concentrations ranged between 0.27-0.94 mg/cm^3 for pineapple, 0.20 – 0.82 mg/cm^3 for banana and 0.16 – 0.45 mg/cm^3. The optimal ethanol yields were 8.34% v/v, 7.45 % v/v and 3.98 % v/v for pineapple, banana and plantain peels respectively.

Sanches, *et al*. (2014) characterized different fruit wastes like orange bagasse, orange peel, banana peel and mango peel in terms of moisture content, lignin content, cellulose and hemicellulose content for utilizing them as an alternative feedstock for bioethanol production. They have observed 85.30% moisture content, 25.52% hemicellulose content, 11.45% cellulose content and 9.82% lignin in banana peel.

2.2.2 Pretreatment of the feedstock

Lignocellulosic biomass is composed of three main constituents namely hemicellulose, lignin and cellulose. Pretreatment methods refer to the solubilization and separation of one or more of these components of biomass. It makes the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). The lignocellulosic complex is made up of a matrix of cellulose and lignin bound by hemicellulose chains. The pretreatment is done to break the matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack (Sanchez and Cardona, 2008). Pretreatment is undertaken to bring about a change in the macroscopic and microscopic size and structure of biomass as well as submicroscopic structure and chemical composition. It makes the lignocellulosic biomass susceptible to quick hydrolysis with increased yields of monomeric sugars (Mosier *et al.,* 2005).

Commonly used pretreatment methods for the hydrolysis of lignocellulosic biomass include dilute acid pretreatment and alkaline pretreatment.

2.2.2.1 Acid pretreatment of the feedstock

Acid pretreatment is considered as one of the most important techniques and aims for high yields of sugars from lignocellulosics. Sulfuric acid is widely used for acid pretreatment among various types of acid such as hydrochloric acid, nitric acid and phosphoric acid (Aden *et al.* 2002). Acid pretreatment can utilize either dilute or concentrated acids to improve cellulose hydrolysis. The acid medium attacks the polysaccharides, especially hemicelluloses which are easier to hydrolyze than cellulose (Cardona *et al.,* 2009). However, acid pretreatment can result in the production of various inhibitors like acetic acid, furfural and 5 hydroxymethylfurfural. These products are growth inhibitors of microorganisms. Hydrolysates to be used for fermentation therefore need to be detoxified. Mosier *et al*. (2005) reported higher hydrolysis yield from lignocellulose pretreated with diluted H_2SO_4 compared to other acids. A saccharification yield of 74% was obtained from wheat straw when subjected to 0.75% v/v of H_2SO_4 at 121^0C for 1 h (Saha *et al*., 2005).

A neutralization of pH is often necessary for the downstream enzymatic hydrolysis or fermentation processes after acid hydrolysis. Although this method is an old and common method of biomass pre-treatment, it has lost popularity due to production of several potent inhibitors such as furfural and hydroxymethyl furfural (Palmqvist and Harn-Hagerdal, 2000).

Patle and Lal (2007) compared acid, alkaline and enzymatic hydrolysis of agricultural crop wastes for yields of total reducing sugars with the hydrolysates being evaluated for ethanol production using a mixed culture of *Zymomonas mobilis* and *Candida tropicalis*. Acid hydrolysis of fruit and vegetable residues yielded 49– 84 g reducing sugars and 29–32 g ethanol. Sirkar *et al*. (2008) have reported that acid

pretreatment method was found to be optimal for better yield of fermentable sugars from fruit peels.

Arumugam and Manikandan (2011) studied the effect of liquid hot water treatment (LHW), dilute acid pre-treatment and enzymatic saccharification on banana and mango peel wastes. They found that in peels the dilute acid pretreatment significantly increased the sugar release by nearly 20% over the LHW. The study revealed that the fermentation of hydrolysates obtained from the dilute acid pretreatment followed by enzymatic saccharification of mixed fruit pulps (banana and mango) and the banana fruit peels were found to be best for higher ethanol production at optimized conditions.

Lalitha (2011) carried out a study to determine the optimal pretreatment conditions for high efficiency ethanol production from the fruit biomass peel residue. The biomass residue was subjected to sulfuric acid pretreatments, followed by three weeks of fermentation using the *Fusarium solani,* the pretreatment process effectively removed lignin.

2.2.2.2 Alkaline pretreatment of the feedstock

Alkaline pretreatment of lignocellulosics digests the lignin matrix and makes cellulose and hemicellulose available for enzymatic degradation (Pandey *et al.,* 2000). Alkali treatment of lignocellulose disrupts the cell wall by dissolving hemicelluloses, lignin, and silica, by hydrolyzing uronic and acetic esters, and by swelling cellulose. Crystallinity of cellulose is decreased due to swelling. By this process, the substrates can be fractionated into alkali-soluble lignin, hemicelluloses and residue, which makes it easy to utilize them for more valuable products. The end residue (mainly cellulose) can be used to produce either paper or cellulose derivatives (Cardona *et al.,* 2009). Hydroxides of sodium, potassium, calcium and ammonium are used in this process. Alkaline pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier *et al.,* 2005).

Azzam (1989) observed that the susceptibility of cane bagasse to enzymatic hydrolysis was significantly enhanced by pre-treatment with hydrogen peroxide. About 50% lignin and most hemicelluloses were solubilized by 2% H_2O_2 at 30°C within 8h, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45°C for 24 h.

Alkali pre-treatment is a relatively cheap and preferred method of biomass pre-treatment in the lignocelluloses ethanol process since it's not accompanied with production of inhibitors. Its effect is however dependent on the lignin content of the materials (McMillan, 1994). Bjerre *et al*. (1996) used wet oxidation and alkaline hydrolysis of wheat straw and achieved 85% conversion yield of cellulose to glucose.

Dilute sodium hydroxide (NaOH) treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Sun and Cheng, 2002).

Patle and Lal (2007) compared acid, alkaline and enzymatic hydrolysis of agricultural crop wastes for yields of total reducing sugars with the hydrolysates being evaluated for ethanol production. Alkaline hydrolysis did not give significant amount of reducing sugars.

In her study, Lalitha (2011) investigated the optimal pretreatment conditions for high efficiency ethanol production from the fruit biomass peel residue. The residue was subjected to alkaline hydrogen peroxide pretreatments and sulfuric acid pretreatments, followed by three weeks of fermentation using the *Fusarium solani,* the pretreatment process effectively removed lignin. Alkaline treatment using 2% H_2O_2 at pH 13 soaked for 8 h removed 45% lignin and ethanol produced was found to be 115 mg/L.

2.2.3 Fermentation of the feedstock

The industrial utilization of lignocelluloses for bioethanol production is hindered by the lack of ideal microorganisms which can efficiently ferment both pentose and hexose sugars (Talebnia *et al.,* 2010). For a commercially viable ethanol production method, an ideal microorganism should have broad substrate utilization, high ethanol yield and productivity, should have the ability to withstand high concentrations of ethanol and high temperature, should be tolerant to inhibitors present in hydrolysate and have cellulolytic activity (Sarkar *et al.,* 2012).

Some native or wild type microorganisms used in the fermentation are *Saccharomyces cerevisiae, Escherichia coli, Zymomonas mobilis, Pachysolentannophilus, Candida brassicae* and *Mucor indicus* (Bjerre *et al.,* 1996; Balat *et al.,* 2008; Sanchez and Cardona, 2008; Talebnia *et al.*, 2010; Girio *et al.,* 2010). *Saccharomyces cerevisiae* and *Zymomonas mobilis* are the best known yeast and bacteria employed in ethanol production from hexoses (Talebnia *et al.,* 2010).

In a study by Osanaiye *et al.* (2005) on yeast fermentation of C*arica papaya* (pawpaw) agricultural waste using brewer's yeast strain *Sacchromyces cerevisiae,* the fermented pawpaw yielded ethanol contents of 3.83 to 5.19% (v/v).

Production of bioethanol from mango peel extract was done using *Saccharomyces cerevisiae* CFTRI101 by Reddy *et al.* (2011). Direct fermentation of mango peel extract gave 5.13% (w/v) of ethanol. Oyeleke *et al.* (2012) studied the production of bioethanol from cassava and sweet potato peels using *Zymomonas mobilis* and *Saccharomyces cerevisiae*. When only *Zymomonas mobilis* was used for fermentation, the mass of bioethanol produced from cassava peels and sweet potato peels were 10.6 g/cm3 (23%) and 5.9 g/cm3(12%) respectively and when only *Saccharomyces cerevisiae* was used for fermentation, the mass of bioethanol produced from cassava peels and sweet potato peels were 10.36 g/cm3 (22%) and 5.68 g/cm3 (12%) respectively.

In his study on production of ethanol from grape waste, Raikar (2012) states that when *Saccharomyces cerevisiae* was used for fermenting the fruit waste. The best ethanol production rate was observed at optimum pH value of 5 with 16 % sugar concentration at 35°C.

In by Janani *et al.* (2013), a comparison of different fruit wastes like pawpaw, grape, banana and apple as raw material for the production of bioethanol using *Saccharomyces cerevisiae* was carried out. The results showed that the rate of ethanol production by fermentation using *Saccharomyces cerevisiae* (baker's yeast**)** yields is highest (6.21%) for grape fruit waste. It was maximum at pH 5.4, temperature 30°C and specific gravity 0.872.

Fermentation of unhydrolyzed potato waste to ethanol by cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae* was investigated by Rath e*t al.* (2014). Fermentation was done for 7 days for potato waste and the ethanol content was measured every 24 hours. The maximum ethanol yield from waste potatoes was 12.124%.

2.3 Enhancement of alcohol production by mixed culture of microorganisms

Enhancement of alcohol production is usually done by hydrolysis of the lignocellulosic feedstock or using different combinations of microorganisms.

Microorganisms usually applied for bioethanol production cannot utilize all the sugar sources derived from hydrolysis. For example, the wild-type strain of *Saccharomyces cerevisiae* is unable to use pentose, and this represents a waste of biomass and reduces the bioethanol yield. To overcome this problem, recombinant yeast or cellulosic enzyme cocktails are introduced during fermentation to convert a wide range of both hexoses and pentoses (Wyman, 1996).

Commonly used methods for enhancing ethanol production using mixed cultures of microorganisms include separate hydrolysis and fermentation (SHF),

simultaneous saccharification and fermentation (SSF) and single batch bioconversion (SBB). The major advantage of separate hydrolysis and fermentation method is that it is possible to carry out the cellulose hydrolysis and fermentation at their own optimum conditions. The optimum temperature for cellulase is usually between 45 and 50°C, depending on the cellulose-producing microorganism (Soderstrom *et al*., 2003; Wingren *et al*., 2003; Saha *et al*., 2005; Olsson *et al*., 2006). However, the optimum temperature for most of the ethanol-producing microorganisms is between 30 and 37°C.

Itelima *et al*. (2013) reported bioethanol production from banana, plantain and pineapple peels by simultaneous saccharification and fermentation process. Ethanol yield of the three substrates were found to increase gradually from the first day to the seventh day with the pineapple peel having the highest yield of 8.34% (v/v), followed by banana peel 7.45% (v/v), while the least was obtained from plantain peel 3.98% (v/v). The study shown that simultaneous saccharification and fermentation of waste materials from banana, plantain and pineapple to ethanol by a mixture of starch digesting fungus *Aspergillus niger* and non-starch digesting sugar fermenter like *Saccharomyces cerevisiae* is feasible. Simultaneous saccharification and fermentation has been found to effectively remove glucose, which is an inhibitor to cellulase activity, thus increasing the yield and rate of cellulose hydrolysis.

Fermentation of unhydrolyzed potato waste to ethanol by cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae* was investigated at different temperatures (20°C to 50°C) and at different pH (4 to 7) by Rath *et al.*(2014). The potatoes were fermented for 7 days and the ethanol content was measured every 24 hours. The maximum ethanol yield from waste potatoes was 12.124%.

2.4 Banana biomass as a feedstock for bioethanol production

Being one of the largest producing crops in the world, banana has great potential to be utilized for bioethanol production.

Brooks (2008) has reported the ability of different yeast strains isolated from ripe banana peels to produce ethanol. *Saccharomyces cerevisiae* R-8 exhibited the best attributes for ethanol production by being highly flocculent, tolerant to 6 - 12% (v/v) ethanol, fermentatively active at 37 - 42^0C and fermented 40% (v/v) glucose. *Saccharomyces cerevisiae* T-7 and *Saccharomyces cerevisiae* R-2 showed rapid fermentative activity on maltose by liberating 150 and 120 ml of CO_2 in 6 h, respectively. *Debaryomyces hansenii* B-2 and *Saccharomyces kluvveri* K-6 each fermented 40% (v/v) glucose at 30^oC to yield 3.6% and 5.8% ethanol, respectively.

Kinetics study on ethanol production from banana peel waste by mutant strains of *Saccharomyces cerevisiae* was conducted by Manikandan *et al*. (2008). Five different strains of *Saccharomyces cerevisiae* were used for the study. The effect of temperature, pH and initial substrate concentration were studied and optimized. A temperature of 33^0C, pH of 4.5 and an initial substrate concentration of 10% were found to be optimum.

Dhabekar and Chandak (2010) have used *Saccharomyces cerevisiae* for production of ethanol from banana peel waste. Maximum production of alcohol was shown by media containing dextrose *i.e.* 3.07% on 12th day, while the maximum alcohol production from beet waste and banana peels was 2.15% and 1.90% at 4th day. Alcohol produced from dextrose within 4th day was found to be 2.05% and was equivalent to the ethanol produced from waste within less time period.

In a study by Hossain *et al*. (2011), fermentation of banana waste was conducted using *Saccharomyces cerevisiae*, Type II under anaerobic condition. The fermented banana fruit waste produced 4.1 to 7.1% bioethanol. The bioethanol yield from mixture of rotten banana fruit increased with increase in fermentation period. It is also increased with yeast concentration, using 35% of water at 35°C. The optimum shaking hours for fermentation was 6 h at pH 5.8.

Kumar *et al.* (2011) reported utilization of dried and ground peel biomass, ripe waste banana and hydrolyzed peels of green and red banana for bioethanol production using *Saccharomyces cerevisiae*. The substrate was given with different concentration as 1%, 2.5%, 5%, 7.5% and 10% (w/v) along with 1% inoculum. *Saccharomyces cerevisiae* exhibited maximum yield of ethanol in ripened red banana and their hydrolyzed peels about 1.3% and 0.27% (v/v) in 10% substrate concentration.

Filho *et al.* (2013) evaluated the possibility of using *Musa cavendischii* banana tree pseudostem as a substrate for alcoholic fermentation. The ethanol yield obtained after fermentation was 0.90 g ethanol $L^{-1}h^{-1}$.

Simultaneous Saccharification and Fermentation (SSF) of banana peels to ethanol by cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae* was investigated at different temperatures (20°C to 50°C) and at different pH (4 to 7). Fermentation was done for seven days for banana peels and the ethanol content was measured every 24 h. The optimum pH and temperature for the fermentation of banana peels was found to be 6 and 30°C respectively. Using a 12%, 9%, 6%, 3% yeast inoculum, maximum ethanol production was completely achieved in 2, 3, 5, 7 days respectively (Singh *et al*., 2014).

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Utilization of banana (*Musa* spp.) biomass for biofuel production" was conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period 2013 - '14. The investigation was carried out in two phases *viz.,* bioethanol production and enhancement of bioethanol production. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 BIOETHANOL PRODUCTION FROM BANANA BIOMASS

3.1.1 Characterisation of the feedstock for alcohol production

Sample collection and preparation

Banana variety Nendran (*Musa* AAB) biomass (banana raw peel, banana ripe peel and banana pseudo stem) were utilized for the study. Samples were collected from Instructional Farm, College of Agriculture, Vellayani.

Banana fruits (raw and ripe) were washed thoroughly under tap water. Peels were separated from banana fruit and chopped into small pieces (~ 2-3 cm). Banana pseudo stem was also washed with tap water and chopped into small pieces of approximately 3-4 cm using clean knife. The samples were oven dried separately at a temperature 60^0C for 48 h in paper covers. The oven dried samples were finely ground and stored in air tight containers for further analysis (Plate-1).

The three types of feed stocks such as banana raw peel, banana ripe peel and banana pseudo stem were separately blended and characterized for alcohol production.

3.1.1.1 Moisture content

The moisture content of the feed stocks banana raw peel, banana ripe peel and banana pseudostem was estimated by following the standard methods prescribed by Association of Official Analytical Chemists (AOAC, 2000). It was estimated by

Plate 1. Powdered samples of banana

gravimetric measurement of weight loss of 10 g of sample. The sample after drying in an oven at 60°C until a constant weight was obtained.

3.1.1.2 Total soluble salt

Total soluble salt content of the samples were estimated by using the EC/TDS meter. 2 g of the sample was digested with diacid (Nitric acid and Perchloric acid taken in 8:1 ratio) and the volume was made up to 50 ml. The electrode was then introduced into this solutions and EC values were noted from the display unit and was expressed as micro siemens meter^{-1} (µsm^{-1}).

3.1.1.3 Total carbohydrates

Total carbohydrate was estimated by Anthrone method (Hedge and Hofreiter, 1962). 100 mg of the sample was taken into a boiling tube. The sample was hydrolyzed by keeping it in a boiling water bath for three hours by adding 5 ml of 2.5N HCl and cooled to room temperature. Then it was neutralized with solid sodium carbonate until the effervescence ceases. The volume was made to 100 ml and was centrifuged in a refrigerated centrifuge. The supernatant was collected and 0.5 and 1ml aliquots were taken for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard. Zero ml was kept as a blank. The volume was made upto 1 ml in all the tubes by adding distilled water. The contents of all the tubes were cooled on ice before adding ice-cold anthrone reagent. Then 4 ml of anthrone reagent (Appendix I a) was added and was heated for eight minutes in a boiling water bath. The contents were cooled rapidly and the green to dark green color was read at 630 nm in a UV-visible spectrophotometer. The standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on Y-axis. From the graph the amount of carbohydrate present in the sample was calculated using the following formula.

Calculation

Amount of carbohydrate present in 100 mg of the sample = mg of glucose (as read from the standard graph) x 100 / Volume of test sample.

3.1.1.4 Total Dissolved Solids

Total dissolved solids was estimated by using EC/TDS meter. 2 g of the sample was digested with diacid (Nitric acid and Perchloric acid taken in 8:1 ratio) and the volume was made up to 50 ml. The electrode was then introduced into this solutions and TDS values were noted from the display unit.

3.1.1.5 Total non-reducing sugar

Total non-reducing sugar is estimated by using DNS method (Malhothra and Sarkar, 1979). 100 mg of the sample was weighed and the sugars were extracted with hot 80% alcohol twice by using 5 ml every time. The supernatant was collected and evaporated on water bath. 10 ml of water was added and the sugars were dissolved. 1 ml of extract was pipetted out and 1 ml of 1N H_2SO_4 was added. The mixture was hydolysed by heating at 49^0C for 30 min. The tubes were cooled and 2 drops of methyl red indicator was added. The contents were neutralized by adding 1N NaOH drop wise from a pipette. An appropriate reagent blank was maintained. Then the total reducing sugars were estimated by DNS method. Total non-reducing sugar of the raw, acid treated and alkali treated feed stocks was estimated by this method.

Calculation

$$\text{Non-reducing sugars (\% mg)} = \frac{\text{Sugar value from graph } (\mu g) \times \text{Total vol. of extract (10 ml)}}{\text{Aliquot sample (1 ml)} \times \text{Wt. of sample (100 mg)} \times 1000}$$

3.1.1.5.1 Sucrose

Sucrose content was estimated by titration method by using Fehling's solution A and B (Appendix-I b).

To 2g of oven dried finely ground sample absolute alcohol was added. The above solution was kept in 60°C water bath for 15 minutes. It was left open till the alcohol was completely volatilized. It was made up to 100 ml in a volumetric flask using distilled water. The sample was then filtered and used for the estimation.

The above prepared sample was taken into a 250 ml beaker and diluted with about 20 ml of water. About 5 ml of concentrated hydrochloric acid was added with constant stirring. The above solution was maintained at 67°C-70°C for 10 minutes. Then it was neutralized with the addition of 1N sodium hydroxide solution. The neutralization was completed by the addition of solid sodium carbonate. The above solution was filtered into a 250 ml measuring flask and the volume was made up with distilled water. 5 ml of Fehling`s solutions A and B were pipetted out into a clean conical flask and diluted with 10ml of distilled water. A few glass beads were added and heated to boiling on a wire gauze. When the solution started to boil, the sample solution was added from a burette about 1ml at a time. After each dilution of sample solution a few seconds were allowed for the reduction to take place. When the blue color of the Fehlings solution nearly faded, three drops of methylene blue indicator was added and the titration was continued by addition of sample solution in drops till a brick red color red appeared.

Calculation

10 cc of Fehling's solution = 0.05 g of monosaccharides

Wt. of monosaccharides in 250 cc of the made up solution

$$= \frac{250}{V} \times 0.05 \text{ g}$$

Wt. of glucose and fructose in 100 cc of the original sucrose solutions

$$= \frac{250}{V} \times 0.05 \times \frac{100}{20} \text{ g}$$

1 g of the monosaccharides is equivalent to 0.95 g of sucrose

Percentage of sucrose in the sample $= \frac{250 \times 0.25 \times 0.95 \times \text{dilution factor}}{V \times 100}$

V – Burette reading

3.1.1.6 Estimation of total reducing sugar

Total reducing sugar was estimated by using DNS method (Miller, 1972; Sadasivam and Manickam, 1992). 100 mg of the sample was weighed and the sugars were extracted with hot 80% alcohol twice by using 5 ml every time. The supernatant was collected and evaporated on water bath. 10 ml of water was added and the sugars were dissolved. 2 ml of extract was pipetted out to the test tubes and the volume was equalized to 3 ml with distilled water in all the tubes. Then 3 ml of DNS reagent (Appendix-I c) was added. 1 ml of 40 percent Rochelle salt solution was also added to the warm tubes containing the reaction mixture. The intensity of dark red color was read at 510 nm in a UV-visible spectrophotometer. Working standards were prepared by dissolving 100 mg of glucose in 100 ml distilled water and 50 ml was pipetted out and made to 100 ml. From this, 0.2 to 1 ml (100 to 500 μg) of solution were taken in different tubes and made up to 3 ml with distilled water, as working standards. 1 ml of distilled water was used as blank. The intensity of the color developed was read in a spectrophotometer at 530 nm. The amount of reducing sugars present in the sample was calculated using standard graph.

3.1.1.6.1 Glucose and Xylose content

The glucose content of the feed stocks were estimated by redox titration method using Fehling's solution A and B. Absolute alcohol was added to 2 g of oven dried finely ground sample. The above solution was kept in 60°C water bath for 15 minutes. It was left open until the alcohol was completely volatilized. It was made up to 100 ml in a volumetric flask using distilled water. The sample was then filtered and used for the estimation.

A few glass beads were added and heated to boiling on wire gauze. When the solution started to boil, the sample solution was added from a burette about 1ml at a time. After each dilution of sample solution a few seconds were allowed for the reduction to take place. When the blue color of the Fehling's solution nearly faded, 3 drops of 1 % methylene blue indicator was added. The titration was continued by the

addition of sample solution in drops. The end point was indicated by the disappearance of blue color of the indicator and the appearance of bright red color of cuprous oxide.

Estimation of xylose was carried out by using conversion factor. The amount of xylose present in the sample was one by fourth of that of the amount of glucose.

Calculations

$$\text{Percentage of glucose in the sample} = \frac{100 \times 0.05}{V} \times \text{dilution factor}$$

V – Volume of sample solution reacting with 10 ml of Fehling`s solution (which contains 0.05 g of glucose).

3.1.1.6.2 Fructose

Fructose content was estimated by Spectrophotometric method using Resorcinol reagent (Ashwell, G. 1957). To 2 ml of the sample solution taken in a test tube 1 ml of resorcinol reagent (Appendix-I d) was added. Then 7 ml of diluted HCl was added. 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard were pipetted out and the volume was made up to 2 ml with distilled water. To this 1 ml of resorcinol reagent was added and 7 ml of dilute HCl as above. A blank was maintained along with the working standard. All the contents of the tubes were heated in a water-bath at 80°C for exactly 10 min. The tubes were removed and cooled by immersing in tap water for 5 m. The color was read at 520 nm within 30 min in a UV-visible spectrophotometer.

The standard graph was drawn and the amount of fructose present in the sample was calculated using the standard graph.

3.1.1.7 Cellulose

Cellulose was estimated by spectrophotometric method (Updegroff, 1969). Initially 0.5 g of the sample taken in a test tube and 3 ml of acetic/nitric reagent (Appendix-I e) was added and mixed in a vortex mixer. The tubes were kept in a water-bath at 100^{0}C for 30 min. The tubes were centrifuged in a refrigerated centrifuge for 15-20 min. The supernatant was discarded and the residue was washed with distilled

water. 10 ml of 67 percent sulphuric acid was added and allowed to stand for 1 h. One ml of the above solution was diluted to 100 ml. 10 ml of anthrone reagent was added to 1 ml of this diluted solution, with proper mixing. The tubes were heated in a boiling water-bath for 10 min. The intensity of the color was measured at 630 nm in a UV-visible spectrophotometer. A blank was set up with anthrone reagent and distilled water. 100 mg cellulose was taken in a test tube and the same procedure was followed for sample. Instead of just taking 1 ml of the diluted solution a series of volumes 0.4-2 ml were taken corresponding to 40-200 µg of cellulose and the color was developed. The standard graph was drawn and the amount of cellulose present in the sample was calculated.

3.1.1.8 Hemicellulose

Hemicellulose was estimated by Gravimetric method (Georing and Vansoest, 1975)

Estimation of Neutral Detergent Fibre

10 ml of cold neutral detergent solution (Appendix-I f), 2ml of decahydro naphthalene and 0.5 g sodium sulphite were added to 1 g of the powdered sample taken in a refluxing flask. The mixture was boiled and refluxed for 60 min. The contents were filtered through sintered glass crucible (G-2) by suction and washed with hot water. Finally two washings were performed with acetone. The residue was transferred to a crucible, and dried at 100^0 C for 8 h. The crucible was cooled in a desiccator and weighed. NDF content was expressed in percentage *i.e.*, W/Sx100 where W is the weight of the fibre and S is the Weight of the sample.

Estimation of Acid Detergent Fibre

1 g of powdered sample was taken in a round bottom flask containing 100 ml of acid detergent solution. Then it was heated to boil for 5-10 min. When the mixture started to boil, the heat was reduced to avoid foaming. It was then refluxed for 1 h after the onset of boiling. While refluxing the boiling was adjusted to slow, even level. The

container was removed, swirled and filtered the contents through a preweighed sintered glass crucible (G-2) by suction and washed with hot water twice. Then a wash was performed with acetone and the lumps were broken. The acetone washing was repeated until the filtrate is colorless. It was then dried at 100°C for overnight. The content were weighed after cooling in a desiccator. ADF content was expressed in percentage *i.e.*, W/Sx100 where W is the weight of the fiber and S is the weight of the sample. The hemicellulose was calculated using the formula

Hemicellulose (%) = Neutral detergent fiber (NDF) (%) - Acid detergent fiber (ADF) (%)

3.1.1.9 Lignin

Lignin was estimated by Gravimetric method (Georing and Vansoest, 1975).

Acid Detergent Fiber (ADF)

1g of powdered sample was taken in a round bottom flask and 100 ml of acid detergent solution was added. Then it was heated to boil for 5-10 min. The heat was reduced to avoid foaming when it started to boil. Then it was refluxed for 1 h after the onset of boiling. The boiling was adjusted to slow, even level. Then the contents were filtered through preweighed sintered glass crucible (G-2) by suction and washed with hot water twice. Then washed with acetone and the lumps were broken. The acetone wash was repeated until the filtrate became colorless. The contents were dried at 100^{0}C overnight. After the overnight incubation the contents were cooled in a desiccator and the weight was estimated. ADF content was expressed in percentage i.*e*., W/S x 100, where W is the weight of the fiber and S is the weight of the sample.

Determination of Acid Detergent Lignin (ADL)

Acid detergent fibre was transferred to a 100 ml beaker with 25-50 ml of 72 percent sulphuric acid. 1 g asbestos was added and allowed it to stand for 3 h with intermittent stirring with glass rod. The acid was diluted with distilled water and filtered with preweighed Whatman no.1 filter paper (The filter paper was wetted in hot water,

dried in oven at 102°C for 2 h. Then cooled in a desiccator and weighed in a covered dish). The glass rod and the residue were washed several times to get rid of the acid by filtration. The filter paper was dried at 100^0C and weighed after cooling in a desiccator. The filter paper was transferred to a preweighed silica crucible and ashed with the content in a muffle furnace at 55°C for about 3 h. The crucible was cooled in a desiccator and weighed. The ash content was calculated. One gram asbestos was taken and 72 percent H_2SO_4 was added and the same steps for sample were followed.

Calculation

$$\%\text{Lignin} = \frac{\text{Weight of 72\% } H_2SO_4 \text{ washed fiber - Ash (Test - Asbestos blank) x 100}}{\text{Weight of sample}}$$

3.1.2 Pre–treatment of the feedstock

The purpose of the pretreatment was delignification. The removal of lignin is necessary for cellulose to become readily available for the enzymes, which permit the microorganism to convert the glucose into ethanol.

3.1.2.1 Acid hydrolysis

5 g of the sample was weighed in a culture bottle. Approximately 40 ml of 0.8M H_2SO_4 was added. The bottles were closed and incubated at room temperature for 24 h.

3.1.2.2 Alkaline hydrolysis

5g of the sample was weighed in a culture bottle. Approximately 40 ml of 2 percent H_2O_2 (pH-13) was added. The bottles were closed and incubated at room temperature for 48 h.

The parameters *viz*., total non-reducing sugar, reducing sugar and lignin content were estimated after incubation period following the procedure described in 3.1.1.5, 3.1.1.6 and 3.1.1.9 respectively.

3.1.3 Fermentation of the feed stock

Fermentation of the feedstock (raw and pre-treated) was carried out using *Saccharomyces cerevisiae* (Plate-2) and *Zymomonas mobilis* (Plate-3). The organisms were procured from the Institute of Microbial Technology, Chandigarh, India. The stock cultures were maintained on malt-yeast-agar (Appendix-II b) and yeast extract-glucose- salt- agar slants (Appendix-II a) stored at 4°C were used for the study. 2 g of oven dried and powdered banana raw peel, banana ripe peel and banana pseudostem samples were taken in separate 150 ml screwed conical flasks. 50 ml of distilled water was added and the pH was adjusted to 7 and the samples were sterilized by autoclaving in a horizontal autoclave. Under sterile conditions in a laminar air flow cabinet *Saccharomyces cerevisiae* and *Zymomonas mobilis* were inoculated to the samples for fermentation in separate flasks. This was incubated for 6 days at 37°C by continuous shaking in a shaking incubator with 60 rpm. After incubation the alcohol content was estimated. pH value, total reducing sugars, total non-reducing sugars and total soluble sugars were determined before and after fermentation. The end point of fermentation was determined by estimating alcohol content in 48 h intervals until the reading was constant. The feedstock and fermenting micro-organism yielding more alcohol was selected for enhancement process.

3.1.3.1 Estimation of alcohol content

Alcohol content was estimated by titration method using potassium dichromate and sodium thiosulphate. 4 ml of the fermented sample was pipette out into a 100 ml volumetric flask and made up with distilled water. Then 5 ml of the diluted sample was transferred in a screwed conical flask and 10 ml of 0.05M potassium dichromate was added. 20 ml of 50 percent sulfuric acid solution was added slowly to each flask. Each flask was capped loosely and heated in a water bath at 50°C for 60 min. The flask was removed from the water bath and 10 ml of 0.5M KI was added. The contents were titrated with 0.1M sodium thiosulphate solution. When the brown color of the solution got a green tinge, a few drops of 1% starch indicator was added which was prepared in

Plate 2. ***Saccharomyces cerevisiae***

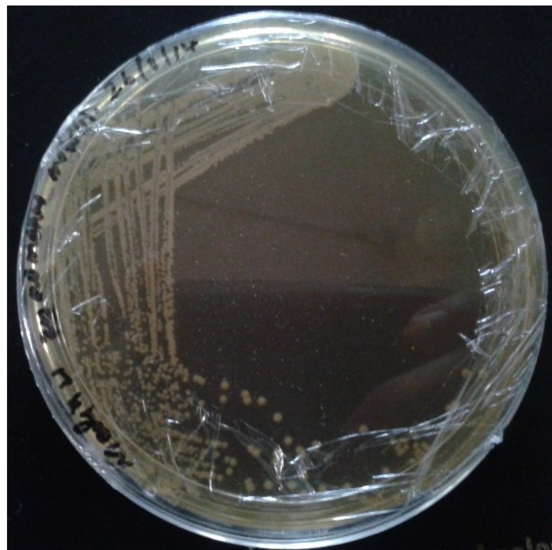


Plate 3. ***Zymomonas mobilis***

boiling water. The addition of sodium thiosulphate solution was continued until the solution got a clear, green-blue color which was the endpoint of titration (Plate-4).

Calculation

Number of moles in X ml 0.1 M sodium thiosulphate = $\frac{24.818 \times X}{1000}$

Where X was burette reading

= Y moles

Extra moles of dichromate spent by thiosulphate = Y/6

No. of moles of dichromate reacted to oxidize alcohol (Z) = No. of moles added **-** moles spent by thiosulphate

No. of moles of alcohol = 3 x Z

Volume of alcohol in the sample = 3Z x 58.6

This was the volume of alcohol present in 5 ml of the diluted sample

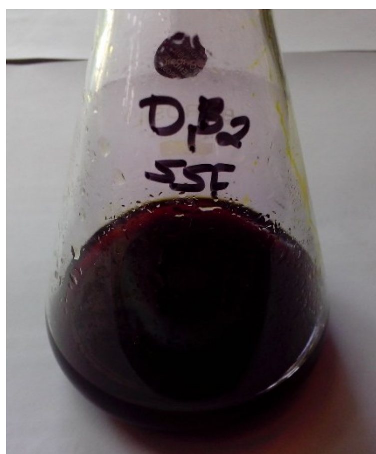
Volume of in 100 ml dilued sample = (3Z x 58.6) x 50

Percentage of alcohol present in 10ml of original sample = (3Z x 58.6) x 50 x 10

3.2 ENHANCEMENT OF ALCOHOL PRODUCTION

3.2.1 Isolation of native micro-organism and its characterization

Cellulolytic microorganisms were isolated from degraded samples of banana raw peel biomass by serial dilution using Carboxy methyl cellulose (CMC) agar medium (Appendix- II c). Five replications were maintained. Cellulolytic microbial colonies were identified by confirmation test using Congo red solution (0.1%) and 1 M NaCl. The colonies showing clear zones were isolated and maintained pure cultures. Morphological, biochemical and molecular characterization of the micro-organism were done.



a) Before titration **b) End point of titration**

Plate 4. Colour change during titration for alcohol estimation

3.2.1.1 Morphological characterization

3.2.1.1.1 Gram staining

A single colony was selected from the microbial pure culture. One drop of sterile water was taken on a grease free glass slide and the selected single colony was transferred to the slide. The culture was thoroughly spread to get a clear smear using sterile loop and the smear was heat fixed. The smear was flooded with crystal violet for one minute and then washed gently in tap water. In the second step, smear was exposed to Gram's iodine for one minute, and then washed with tap water. Then the slide was exposed to decolorizer and washed immediately with tap water. Finally, counter stain Saffranin was added and washed after 30 seconds. After drying, stained slide was examined under oil immersion (100X) to note Gram reaction, morphology and arrangement.

3.2.1.2 Biochemical characterization

3.2.1.2.1 Catalase test

The native microorganism was inoculated on a trypticase soya agar (Appendix-II d) slants and incubated for 48 h at 37°C. An uninoculated slant was kept as control. After 48h the slant was scrapped with sterile non-metallic instrument and was suspended in a drop of 3 percent hydrogen peroxide on a grease free slide. The slide was examined immediately for bubble formation.

3.2.1.2.2 Carbohydrate fermentation test

Fermentation broth (Appendix-II e to i) containing the specific carbohydrates glucose, sucrose, lactose, mannitol and maltose respectively were prepared. Then a Durham tube was inserted to each tube in inverted position. The broth was sterilized by autoclaving in a horizontal type autoclave. All the tubes were inoculated with the native organism maintained as pure culture using sterile technique. One uninoculated tube was kept as control for each fermentation broth. All the tubes were incubated for 72 h. After 72 h incubation the broth was examined for bubble formation.

3.2.1.2.3 Indole Methyl Red Voges Proskauer Citrate Utilization (IMVIC) test.

3.2.1.2.3.1 Indole production test

Trypticase broth (Appendix-II d) tubes were prepared and sterilized by autoclaving in a horizontal type autoclave. It was inoculated with native microorganism under sterile conditions. An uninoculated tube was kept as control. All the tubes were incubated for 37°C for 48 h. After incubation Kovac's reagent was added into all the tubes and gently mixed. The reagent was allowed to come to the top. The tube was examined for the development of cherry red color in the top reagent layer.

3.2.1.2.3.2 Methyl red test

Native organism was inoculated using sterile technique to MR-VP broth (Appendix-II j). An uninoculated tube was kept as control. All the tubes were incubated at 37°C for 24 h. One milli liter of methyl red indicator was added to all the tubes after incubation and observed for change in color.

3.2.1.2.3.3 Voges Proskauer test

MR-VP broth was prepared and sterilized by autoclaving in a horizontal type autoclave. Native organism was inoculated under sterile conditions in a laminar air flow cabinet. Maintained a control without inoculation. All the tubes were incubated at 37°C for 24 h. After incubation 12 drops of 1% alcoholic α-naphthol reagent and 6 drops of 40% potassium hydroxide were added in each tube and gently mixed and observed for the development of crimson ruby pink color of the medium.

3.2.1.2.3.4 Citrate utilization test

Simmon's citrate agar (Appendix-II k) slant tubes were prepared and autoclaved for sterilization. Under sterile conditions, the native organism was inoculated by streak plate method. A slant without inoculation was maintained as control. All the tubes were incubated for 48 h at 37°C. After incubation the slants were observed for the growth of the organism and development of deep prussian blue color.

3.2.1.2.4 Gelatin hydrolysis test

Nutrient gelatin medium (Appendix-II l) was prepared and sterilized. It was dispensed into sterile test tubes and allowed to solidify. Media was inoculated with large quantity of inoculum by stabbing all the way to the bottom of the tubes. Uninoculated tubes were kept as control. The tubes were incubated at 37°C for 48 h. After incubation, the tubes were placed in the refrigerator at 4°C for 30 min. Then the tubes were kept in room temperature for 30 min and observed change in consistency of the medium.

3.2.1.2.5 Urease test

Basal media urease test broth (Appendix-II m) was prepared and sterilized by autoclaving in a horizontal type autoclave. It was cooled to 55°C. Twenty percentage urea was added to the medium and poured into sterile test tubes. The tubes were inoculated with isolated native micro-organism under sterile conditions and incubated at 37°C for 48 h. After incubation the tubes were observed for pink color formation in the media.

3.2.1.2.6 Cellulolytic activity

Cellulolytic activity was assayed using DNS method. The reducing sugar released from CMC that are solubilized in 0.05M phosphate buffer at pH 8 was estimated. The isolated native organism was inoculated on sterile banana raw peel waste taken in sterile test tubes. This is incubated at room temperature by continuous shaking with 60 rpm. The cellulase assay was done in 48 h interval until endpoint of cellulase activity was obtained. After incubation the sample was centrifuged at 14000 rpm for 10 min at 4°C in a refrigerated micro centrifuge. Clear supernatant was pipetted out which served as a crude enzyme source. This was added to 0.05 ml of 1 % CMC in 0.05M phosphate buffer and incubated at 50°C for 30 min. in a test tube. The test tube without adding CMC served as blank and non-fermented sample kept in another test tube served as control. After incubation reaction was stopped by the addition of 1.5ml DNS reagent followed by boiling at 100°C in water bath for 10 min. Sugar liberated

was determined by measuring absorbance at 540 nm in a UV-visible spectrophotometer. Cellulase production was estimated by glucose calibration curve. One unit of enzyme activity was expressed as the quantity of the enzyme which is required to reduce 1µmol of glucose per minute under standard assay conditions (Muhammad *et al.* 2012).

3.2.1.3 Molecular studies-16S and 23S ribosomal RNA sequence

3.2.1.3.1 Genomic DNA isolation

Genomic DNA was isolated from the culture using DNeasy® Blood and Tissue Kit.

3.2.1.3.2 Agarose gel electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8 percent agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator and the image was captured under UV light using Gel documentation system.

3.2.1.3.3 PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and the template DNA. The PCR amplification was carried out in a PCR thermal cycler.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-UP-F	Forward	CGAATTCGTCGACAACAGAGTTTGATC CTGGCTCAG
	16S-UP-R	Reverse	CCCGGGATCCAAGCTTACGGCTACCTT GTTACGACT
23S rRNA	F	Forward	GATGTGGAGTTGCTTAGACA
	R	Reverse	CTTTTATCCGTTGAGCGATG

The PCR amplification was carried out in a PCR thermal cycler.

PCR amplification profile

16S rRNA/23S rRNA

95 ^{o}C - 5.00 min

95 ^{o}C - 0.30 min }

54 ^{o}C - 0.40 min } 35 cycles

72 ^{o}C - 1.00 min }

72 ^{o}C - 7.00 min

4 ^{o}C - ∞

3.2.1.3.4 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2 percent agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV-transilluminator and the image was captured under UV light using Gel documentation system (Bio-Rad). The PCR products were stored in -20^{0}C refrigerator. This was

sequenced from Regional DNA Finger printing laboratory Rajiv Gandhi Centre for Biotechnoloy, Thiruvananthapuram.

3.2.2 Bioethanol production using mixed cultures of *Saccharomyces cerevisiae* and native organism

Three different modes of saccharification and fermentation processes were tried to optimize the process for enhanced production of ethanol from banana raw peel biomass.

3.2.2.1 Separate Hydrolysis and Fermentation (SHF)

Oven dried banana raw peel powder was taken in 150 ml conical flasks with screw cap. 50 ml of distilled water was added; pH was adjusted to 7 and sterilized the feed stock by autoclaving in a horizontal type autoclave. The isolated native microorganism was inoculated to the sample for saccharification under sterile conditions. The inoculated sample was incubated at 37°C by continuous shaking in a shaking incubator at 60 rpm for 8 days. After incubation *Saccharomyces cerevisiae* was inoculated to the sample for fermentation. This was again incubated for 6 days at 37°C by continuous shaking in a shaking incubator at 60 rpm. After incubation the alcohol content was estimated.

3.2.2.2. Simultaneous Saccharification and Fermentation (SSF)

Oven dried banana raw peel powder was taken in 150 ml conical flasks with screw cap and 50 ml of distilled water was added. Sterilized the sample by autoclaving in a horizontal type autoclave. Isolated native microorganism and *Saccharomyces cerevisiae* were inoculated to the sample under sterile conditions. The inoculated sample was incubated at 37°C by continuous shaking in a shaking incubator with 60 rpm for 6 days. After incubation the alcohol content was estimated.

3.2.2.3 Single Batch Bioconversion (SBB)

Oven dried banana raw peel was taken in 150 ml conical flasks with cap. 50ml of distilled water is added. pH was adjusted to 7. Sterilized the sample by autoclaving in a horizontal type autoclave. The isolated native microorganism was inoculated to the sample for saccharification under sterile conditions and kept three replications. The inoculated sample was incubated at 37°C by continuous shaking in a shaking incubator for 8 days with 60 rpm. After incubation, the sample was autoclaved and completely killed the native organism for single batch bio conversion. Then under sterile conditions *Saccharomyces cerevisiae* was inoculated to the sample for fermentation. This was again incubated for 6 days at 37°C by continuous shaking in a shaking incubator with 60 rpm. After incubation the alcohol content was estimated.

RESULTS

4. RESULTS

4.1 BIOETHANOL PRODUCTION FROM BANANA BIOMASS

4.1.1 Characterisation of the feedstock

4.1.1.1 Moisture content

The moisture content of the banana feed stocks were determined by gravimetric measurement of weight loss of 10 g of sample drying the samples at 60^0C for 48 h. The results showed significant variation. Maximum moisture content was obtained for banana pseudostem (95.32%) and the lowest value was obtained for banana raw peel (86.14%) (Fig. 1).

4.1.1.2 Total soluble salts (TSS)

The result of the TSS of the sample are presented in Table 1. From the table it was inferred that significant variation was observed among the feed stocks. The highest value was obtained for banana ripe peel (0.082 μsm^{-1}) and the lowest value was obtained for banana pseudostem (0.007 μsm^{-1}).

4.1.1.3 Total dissolved solids (TDS)

Significant variation was observed among the feed stocks as inferred from Table 1. The highest total dissolved solids value was observed for banana pseudostem with a value of 21.90 ppm and the lowest value was observed for banana ripe peel (19.47 ppm).

4.1.1.4 Total carbohydrates

From the data presented in Table 2, the results were found to vary significantly. Maximum carbohydrate content was obtained for banana ripe peel with a value of 42.63%. The lowest carbohydrate content was observed in banana pseudostem with 15.33%.

Fig. 1 Representation of moisture content of different feed stocks of banana

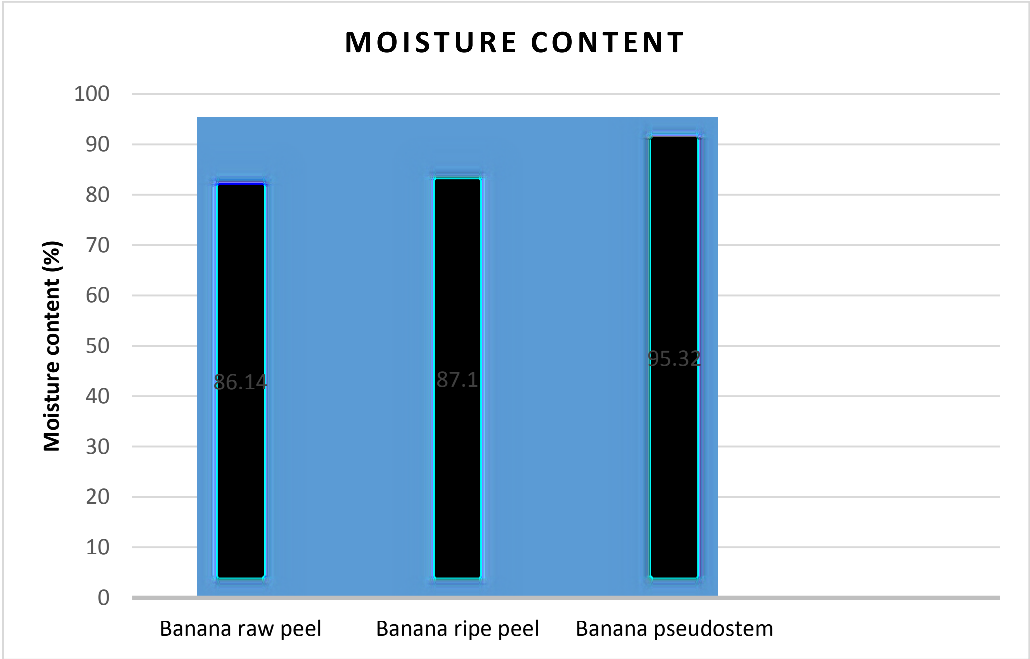


Table 1. Total soluble salts and Total dissolved solids contents in different feed stocks of banana

Feedstock	**Total soluble salts (µsm^{-1})**	**Total Dissolved solids (ppm)**
Banana raw peel	0.062±0.004	20.27±0.13
Banana ripe peel	0.082±0.002	19.47±0.09
Banana pseudostem	0.0070±0.001	21.9±0.13
CD	0.004334	0.204182

No. of replications = 5

Table 2. Total carbohydrate content, Total reducing sugar content and Total non-reducing sugar content in different feed stocks of banana

Feedstock	**Total carbohydrate content (%)**	**Total reducing sugar content (%)**	**Total non-reducing sugar (%)**
Banana raw peel	39.28±0.92	18.12±0.79	9.85±0.25
Banana ripe peel	42.63±0.88	21.98±1.43	12.05±0.36
Banana pseudostem	15.33±0.94	6.75±0.40	3.14±0.12
CD	1.446384	1.229962	0.492344

No. of replications = 5

4.1.1.5 Total reducing sugar content

Total reducing sugar content was determined by DNS method. As per the values of Table 2, significant variation was observed among the results. The result showed that banana ripe peel is having the highest reducing sugar content with a value of 21.98% while the banana pseudostem showed the lowest value of 6.75%.

4.1.1.6 Total non-reducing sugars

From Table 2, it was inferred that the values for total non-reducing sugar varied significantly. Maximum value was observed in banana ripe peel (12.05%). Total non-reducing sugar was observed to be the lowest in banana pseudostem with a value of 3.14%.

4.1.1.7 Glucose content

From Table 3, it was observed that different feed stocks showed significant variation. Maximum value for glucose content was observed in banana ripe peel (10.89%). The value was observed to be lowest in banana pseudostem (3.01%).

4.1.1.8 Fructose content

Results obtained varied significantly as per Table 3. The value of fructose was found maximum in banana ripe peel (7.44%). The lowest value of fructose was found in banana pseudostem (2.70%).

4.1.1.9 Xylose content

The analytical values for xylose content in banana feed stocks showed significant variation (Table 3). Xylose percentage was found to be maximum in banana ripe peel (2.72%). The lowest value of xylose was observed in banana pseudostem (0.76%).

4.1.1.10 Sucrose content

Sucrose is a non-reducing sugar which is found to be present in banana feedstock was determined by titration method using Fehling's solutions. The results analysis obtained varied significantly (Table 3). The value of sucrose was observed

Table 3. Glucose, Fructose, Xylose and Sucrose contents in different feed stocks of banana

Feedstock	Glucose content (%)	Fructose content (%)	Xylose content (%)	Sucrose content (%)
Banana raw peel	6.30±0.71	6.21±0.30	1.57±0.01	2.46±0.13
Banana ripe peel	10.89±0.26	7.44±0.11	2.72±0.06	3.38±0.04
Banana pseudostem	3.01±0.11	2.70±0.08	0.76±0.03	0.48±0.05
CD	0.695803	0.116615	0.061532	0.103402

No. of replications = 5

maximum in banana ripe peel (3.38%) and banana pseudostem was observed to be having the lowest value for sucrose content with a value of 0.48%.

4.1.1.11 Cellulose content

The percentage of cellulose present in the feedstock was determined by spectrophotometric method. From the Table 4, it was observed that the results varied significantly. Maximum cellulose percentage was observed in banana pseudostem with a value of 27.57% while the lowest cellulose percentage was found in banana ripe peel (9.97%).

4.1.1.12 Hemicellulose content

Hemicellulose was estimated by Gravimetric method. Significant variation was observed among the feed stocks as per the values of Table 4. Hemicellulose content was found to be highest in banana raw peel (27.14%). The lowest value for hemicellulose was observed in banana ripe peel (15.29%).

4.1.1.13 Lignin content

Lignin content of the feed stocks were estimated by Gravimetric method. Results revealed that banana pseudostem varied significantly from other feed stocks. The percentage of lignin was found maximum in banana pseudostem (15.44%). The lowest value of lignin was obtained in banana raw peel (8.42%) which was on par with the value of banana ripe peel (8.86%) (Table 4).

4.1.2 Pre-treatment of the feedstock

4.1.2.1 Total reducing sugar content after pre-treatment

The results of total reducing sugar percent of the feed stocks are presented in Table 5. The values showed that acid pre-treated feed stocks were having significant variation. Maximum value was obtained for acid treated banana ripe peel (22.85%). The lowest value was observed for banana pseudostem with 7.19%.

The values of alkali pre-treated samples varied significantly (Table 5). The highest total reducing sugar content in alkali treated banana feed stocks was noticed in

Table 4. Cellulose, Hemicellulose and Lignin contents in different feed stocks of banana

Feedstock	**Cellulose content (%)**	**Hemicellulose content (%)**	**Lignin content (%)**
Banana raw peel	15.14±0.54	27.14±0.86	8.42±0.51
Banana ripe peel	9.97±0.28	15.29±0.39	8.86±0.30
Banana pseudostem	27.57±0.55	22.17±0.40	15.44±0.42
CD	0.818263	0.85442	0.54449

No. of replications = 5

Table 5. Total reducing sugar content, Total non-reducing sugar content and lignin content of the banana samples after the pre-treatments

	Feedstock	**Acid treated samples**	**Alkali treated samples**
Total reducing sugar content (%)	Banana raw peel	20.60±1.01	18.81±1.21
	Banana ripe peel	22.85±1.57	22.41±1.59
	Banana pseudostem	7.19±0.41	6.88±0.41
	CD	1.42206	1.396811
Total non-reducing sugar content (%)	Banana raw peel	10.22±0.40	9.97±0.21
	Banana ripe peel	12.41±0.47	12.16±0.38
	Banana pseudostem	3.27±0.21	3.21±0.21
	CD	0.590435	0.517244
Lignin content (%)	Banana raw peel	6.27±0.50	7.82±0.58
	Banana ripe peel	7.22±0.46	8.53±0.39
	Banana pseudostem	11.44±0.26	13.45±0.56
	CD	0.604269	0.659433

No. of replications = 5

banana ripe peel (22.41%) while the lowest value was obtained for alkali treated banana pseudostem (6.88%).

4.1.2.2 Total non-reducing sugar content after pre-treatment

The results of analysis of total non-reducing sugar showed that the values of acid treated samples varied significantly (Table 5). The highest value was obtained for banana ripe peel (12.41%) whereas the lowest value was noticed in banana pseudostem with a value of 3.27%.

From the data presented in Table 5, the results of alkali treated samples varied significantly. In the case of alkali treated samples, maximum non-reducing sugar content was observed as 12.16% in banana ripe peel. The lowest value was found to be 3.21% of that of banana pseudostem.

4.1.2.3 Lignin content after pre-treatment

The results showed that lignin content of the acid treated samples varied significantly. From the data given in Table 5, the highest value of lignin was observed in banana pseudostem (11.44%). The value was found to be lowest in banana raw peel with a value of 6.27%.

The values of alkali treated samples were also varied significantly as per Table 5. Maximum lignin percentage was noticed in banana pseudostem with 13.45% while the lowest value was observed in banana raw peel (7.82%).

4.1.2.4 Percent saccharification

The analytical values given in Table 6 showed that the results of percent increase in total reducing sugar varied significantly. Maximum increase in total reducing sugar was observed in acid treated banana raw peel (12.00%). The lowest value was observed in alkali treated banana ripe peel (1.86%) which was on par with that of alkali treated banana pseudostem (1.97%).

From the Table 6, it was inferred that the results of percent increase in total non-reducing sugar varied significantly. The percent increase was found to be highest

Table 6. Percent saccharification and percent removal of lignin

	Feedstock	**Percent increase in total reducing sugar**	**Percent increase in total non-reducing sugar**	**Percent removal of lignin**
Acid treated samples	Banana raw peel	12.00±1.21	3.51±1.93	25.58±2.31
	Banana ripe peel	3.79±0.55	2.83±1.03	18.54±3.08
	Banana pseudostem	6.09±0.92	4.11±1.68	25.92±0.48
Alkali treated samples	Banana raw peel	3.55±1.34	1.17±0.72	7.13±1.89
	Banana ripe peel	1.86±1.06	0.89±0.37	3.76±1.20
	Banana pseudostem	1.97±0.19	2.07±0.84	12.94±1.35
	CD	1.600175	1.408684	2.651361

No. of replications = 5

in acid treated banana pseudostem (4.11%) whereas the value was noticed lowest in alkali treated banana ripe peel (0.89%) which was on par with alkali treated banana raw peel (1.17%).

4.1.2.5 Percent removal of lignin

The results observed to be varied significantly according to the Table 6. The lignin removal percentage was observed to be highest in acid treated banana pseudostem (25.92%) which was on par with the value of acid treated banana raw peel (25.58%). The lowest lignin removal percentage was noticed in alkali treated banana ripe peel with 3.76%.

The above results revealed that the percent increase in total sugars and percent removal of lignin were found to be maximum in acid treated samples of banana. The values were significantly higher than that of alkali treated samples. So it was inferred that acid treatment was the best pre-treatment method for banana biomass.

4.1.3 Fermentation of the feedstock

4.1.3.1 Ethanol production using Saccharomyces cerevisiae and Zymomonas mobilis

Percentage of ethanol produced after fermenting the banana samples using *Saccharomyces cerevisiae* are given in Table 7. The values of ethanol percentage obtained after fermentation showed that the samples without pre-treatment varied significantly from the acid treated and alkali treated samples. Maximum ethanol percentage was obtained for the untreated banana raw peel sample (6.63%) and the lowest value was observed for alkali treated banana pseudostem sample (0.82%).

Zymomonas mobilis was used for the fermentation of raw as well as pre-treated feed stocks of banana. The values of ethanol produced by this microorganism are given in Table 7. As per the table, the results varied significantly. The highest ethanol percentage was observed in acid treated banana raw peel (3.11%). It was observed that this value is on par with the values of alkali treated banana raw peel (3.03%) and untreated banana raw peel (3.00%). The lowest value was noticed for alkali treated

Table 7. Ethanol production from banana feed stocks using *Saccharomyces cerevisiae* and *Zymomonas mobilis*

	Feedstock	Ethanol percentage (Untreated samples)	Ethanol percentage (Acid pre-treated samples)	Ethanol percentage (Alkali pre-treated samples)
Fermentation using *Saccharomyces cerevisiae*	Banana raw peel	6.63±0.18	5.22±0.39	4.60±0.81
	Banana ripe peel	4.26±0.19	3.50±0.23	3.16±0.24
	Banana pseudostem	1.65±0.21	1.29±0.44	0.82±0.18
Fermentation using *Zymomonas mobilis*	Banana raw peel	3.00±0.05	3.11±0.06	3.03±0.04
	Banana ripe peel	2.62±0.20	2.67±0.05	2.25±0.18
	Banana pseudostem	0.38±0.33	0.38±0.04	0.26±0.03

No. of replications = 5

CD = 0.270511

banana pseudostem with a value of 0.26% which was on par with untreated banana pseudostem (0.38%) and acid treated banana pseudostem (0.38%).

As per the above results *Saccharomyces cerevisiae* is the better microorganism for the fermentation of banana biomass. The ethanol percentage obtained for the banana biomass fermented with *Saccharomyces cerevisiae* were found to be high and varied significantly from the ethanol percentage of banana samples fermented with *Zymomonas mobilis.*

The results also showed that the pre-treatments carried out in this study were insignificant as the ethanol percentage was lower than the values of untreated feed stocks.

4.1.3.2 End point of fermentation

From the Figure 2, it was inferred that end point of fermentation was observed to be at the 6th day after the inoculation for *Saccharomyces cerevisiae* and at the 8th day for *Zymomonas mobilis.* At the 6th day of incubation, the ethanol percentage was found to reach maximum (6.63%) for *Saccharomyces cerevisiae*. At the 8th day of incubation, the ethanol percentage was observed to reach the maximum value of 3.11% for *Zymomonas mobilis.* After that the ethanol percentage tend to be steady.

4.1.3.3 Change in pH

Change in pH of the different feed stocks were determined after fermentation. The results revealed that the pH values of the samples reduces significantly after the production of ethanol. Banana raw peel without pre-treatment showed the most significant fall in the pH value after fermentation using *Saccharomyces cerevisiae* with a value 4.57 where the initial pH was 7.00. The pH values of pre-treated banana samples did not show significant variation after fermentation from the initial pH value (Table 8).

Fig. 2 Representation of end point of fermentation

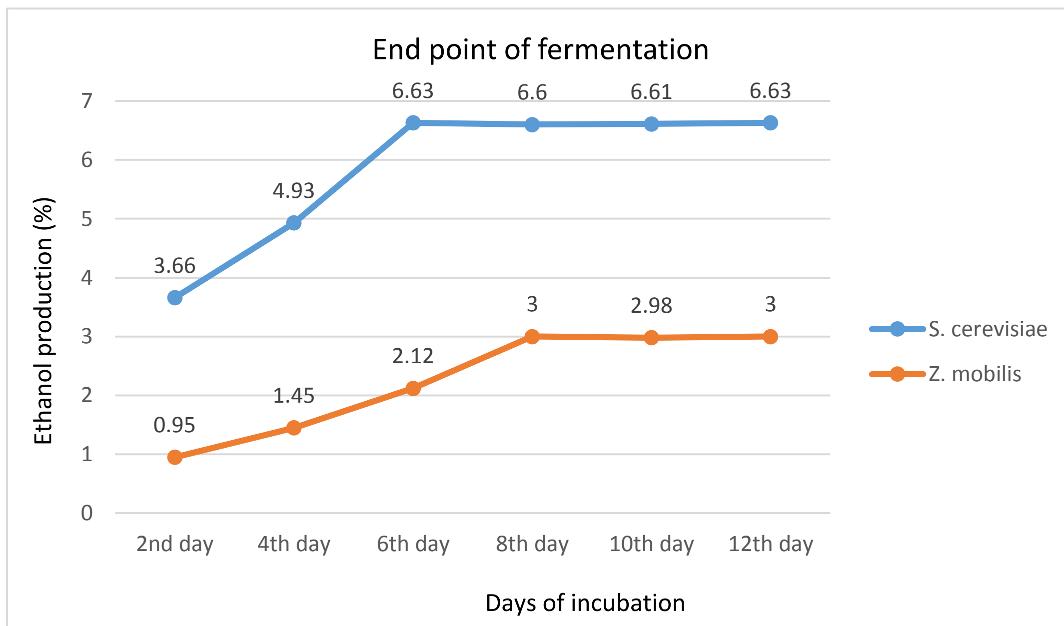


Table 8. Change in pH of the banana feed stocks after fermentation

	Feed stocks	**pH before fermentation**	**After fermentation using *S. cerevisiae***	**After fermentation using *Z. mobilis***
Untreated samples	Banana raw peel	7.00	4.57	5.31
	Banana ripe peel	7.00	5.06	5.64
	Banana pseudostem	7.00	5.48	6.15
Acid treated samples	Banana raw peel	7.00	6.04	6.79
	Banana ripe peel	7.00	6.25	6.66
	Banana pseudostem	7.00	6.90	6.94
Alkali treated samples	Banana raw peel	7.00	6.71	6.78
	Banana ripe peel	7.00	6.75	6.78
	Banana pseudostem	7.00	6.92	6.99

4.1.3.4 Initial sugar content and non-fermentable sugar content

Initial sugar content and non-fermentable sugar content of the untreated banana feed stocks after fermentation using *Saccharomyces cerevisiae* were determined. The results revealed that the values showed significant variation (Table 9). The highest value for initial total reducing sugar content was observed for banana ripe peel (21.98%). The lowest value of total reducing sugar was obtained for banana pseudostem (6.75%).

The values of non-fermentable total reducing sugar varied significantly. The highest value was noticed in banana ripe peel (8.78%) and the lowest was observed in banana raw peel (2.19%).

Initial total non-reducing sugar content was found to be maximum in banana ripe peel (12.05%). The lowest value was noticed for banana pseudostem (3.14%).

Highest value for non-fermentable total non-reducing sugar was observed in banana ripe peel (6.73%) and the lowest value was observed in banana pseudostem (2.61%).

4.1.3.5 Percent conversion of sugars to alcohol

Percent conversion of total reducing sugar and total non-reducing sugars to ethanol were determined and the values are given in Table 9. From the table, it was inferred that the results varied significantly. The highest value for percent conversion to alcohol was observed in banana raw peel as 87.87% for total reducing sugar. The lowest value was observed in banana pseudostem as 47.12%.

The maximum percent conversion of total non-reducing sugar to alcohol was noticed in banana raw peel (45.90%) which was on par with banana ripe peel (44.12%). The lowest value was observed in banana pseudostem (16.88%).

Table 9. Non-fermentable sugar content and percent conversion of sugar to alcohol in the untreated samples of banana fermented using *Saccharomyces cerevisiae.*

	Feedstock	Initial sugar content (%)	Non-fermentable sugar content (%)	Percent conversion to alcohol
Total reducing sugar	Banana raw peel	18.12±0.79	2.19±0.19	87.87±1.39
	Banana ripe peel	21.98±1.43	8.78±0.69	59.85±4.86
	Banana pseudostem	6.75±0.40	3.57±0.37	47.12±3.69
	CD	1.229962	0.677336	5.898901
Total non-reducing sugar	Banana raw peel	9.85±0.25	5.33±0.27	45.90±2.91
	Banana ripe peel	12.05±0.36	6.73±0.51	44.12±4.04
	Banana pseudostem	3.14±0.12	2.61±0.18	16.88±0.93
	CD	0.492344	0.406336	2.614786

No. of replications = 5

4.2 ENHANCEMENT OF ALCOHOL PRODUCTION

4.2.1 Isolation of native microorganism and its characterization

4.2.1.1 Morphological characterisation

Yellow colored circular bacterial colonies were observed (Plate-5). Morphological characteristic of isolated native microorganism was studied by Gram's staining method. The bacteria was observed as pink colored cocci shaped cells after staining (Plate-6). Hence the organism was inferred as Gram negative.

4.2.1.2 Biochemical characterization

4.2.1.2.1 Catalase test

Bacterial isolate was studied for catalase production. The microbial colonies produced bubbles when suspended with hydrogen peroxide solution. So the microorganism was inferred as catalase positive (Plate-7).

4.2.1.2.2 Carbohydrate fermentation test

Bacterial isolate were studied for the fermentation of carbohydrates. The formation of bubbles were not observed. So the bacteria was inferred to have negative result for carbohydrate fermentation (Plates 8-12).

4.2.1.2.3 Indole production test

After testing the bacterial isolate for indole production, it was observed that the tubes did not showed any change in color. So the organism was inferred to have negative result for indole production (Plate-13).

4.2.1.2.4 Methyl red test

Methyl red test of the bacteria showed negative results since the tubes did not showed any change in color (Plate-14).

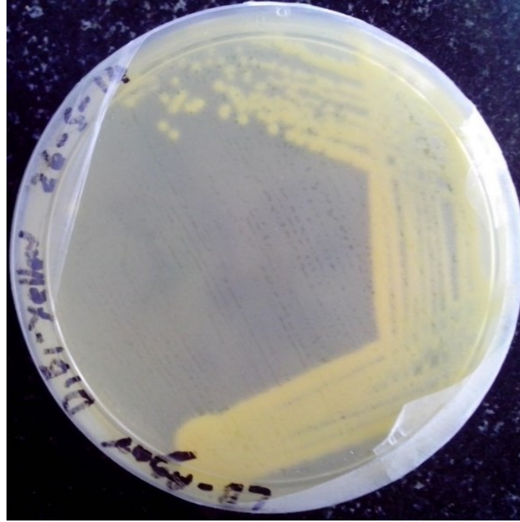


Plate 5. Native microorganism (*Nesterenkonia* sp.)

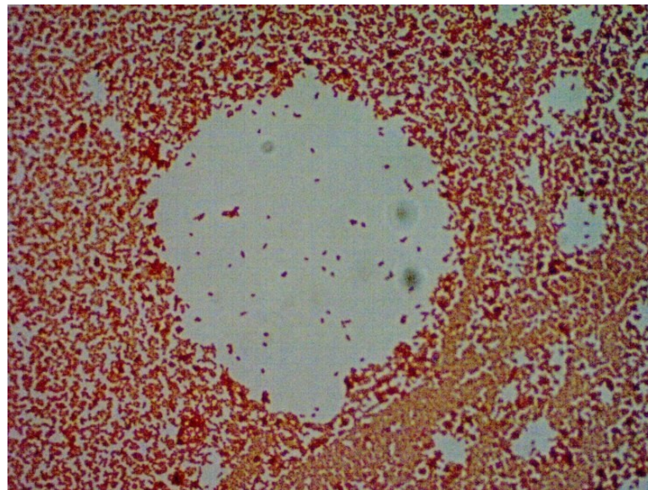


Plate 6. Microscopic view of isolated bacteria after Gram’s staining

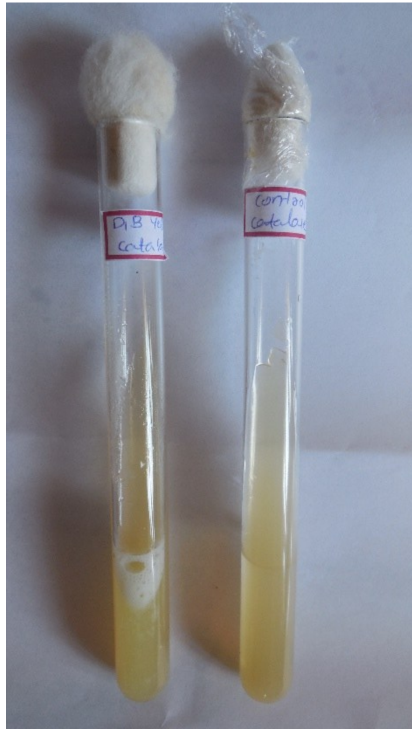


Plate 7. Catalase test

Plate 8. Glucose fermentation

Plate 9. Sucrose fermentation

Plate 10. Lactose fermentation

Plate 11. Maltose fermentation

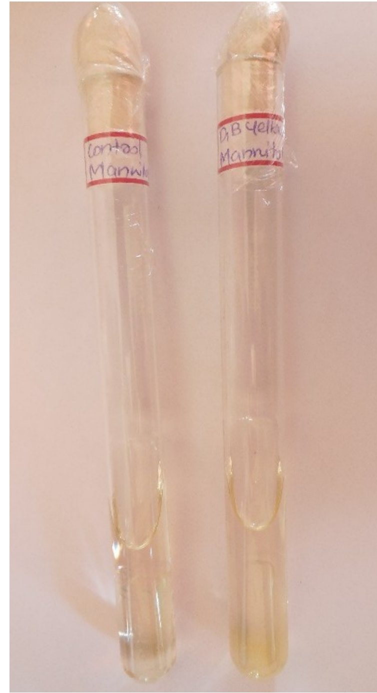


Plate 12. Mannitol utilization test

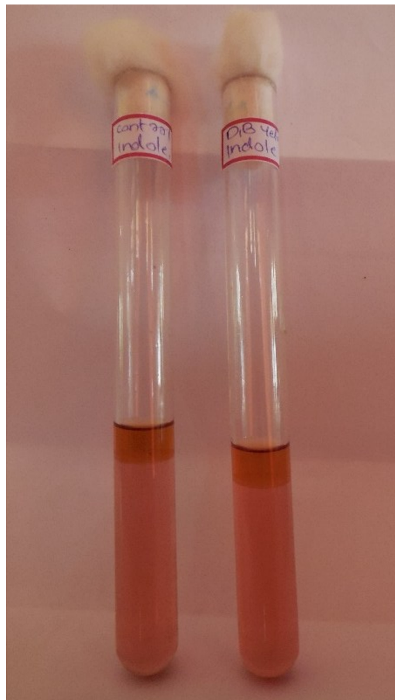


Plate 13. Indole production test

Plate 14. Methyl red test

4.2.1.2.5 Voges Proskauer test (VP test)

The tubes containing the bacterial isolate was not observed to have crimson ruby pink color after VP test. So the organism is inferred to be negative for VP test (Plate-15).

4.2.1.2.6 Citrate utilization test

The bacterial isolates was observed to change the color of the medium to Prussian blue color. So the organism was inferred to be positive for citrate utilization test (Plate-16).

4.2.1.2.7 Gelatin hydrolysis test

The bacterial isolate was found to change the consistency of the medium after incubation. So the microorganism was inferred to be positive for gelatin hydrolysis test (Plate-17).

4.2.1.2.8 Urease test

The color of the medium was not changed to pink after incubating with the bacteria. So the microorganism was inferred to be negative for urease test (Plate-18).

4.2.1.2.9 Cellulolytic activity

Cellulolytic activity of the bacterial isolate was studied. The result of the study showed that the microorganism shows slow rate of cellulolysis and the activity was found to be maximum at the 8th day after inoculation (Fig. 3).

4.2.1.3 Molecular studies – 16S/23S ribosomal RNA sequence

4.2.1.3.1 Genomic DNA isolation

Genomic DNA of the bacteria was isolated. The agarose gel electrophoresis (0.8%) of the extracted genomic DNA showed the presence of good quality unsheared DNA bands on the gel (Plate-19). Further absorbance reading of the extracted genomic

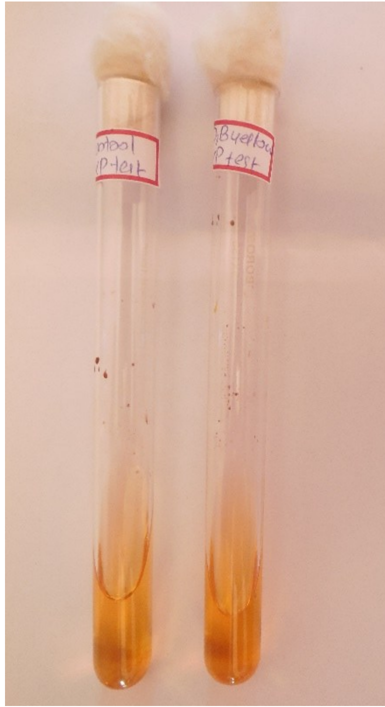


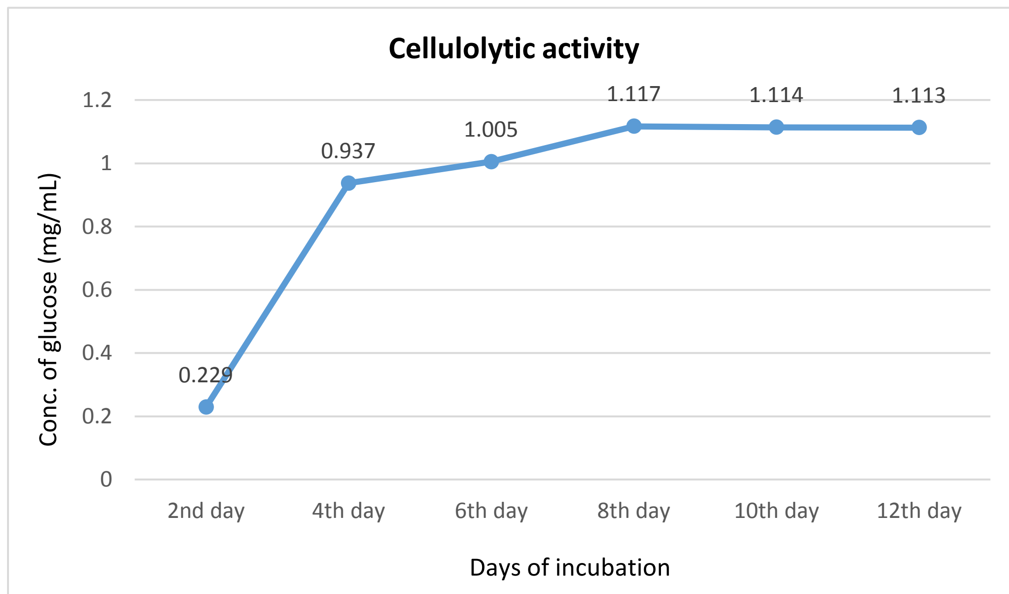
Plate 15. VP test

Plate 16. Citrate utilization test

Plate 17. Gelatin hydrolysis

Plate 18. Urease test

Fig. 3 Representation of cellulolytic activity of *Nesterenkonia* sp.



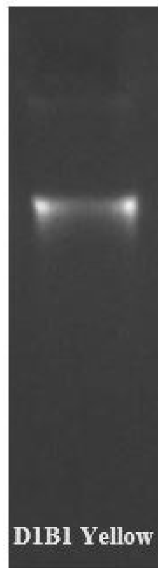


Plate 19. Microbial DNA band

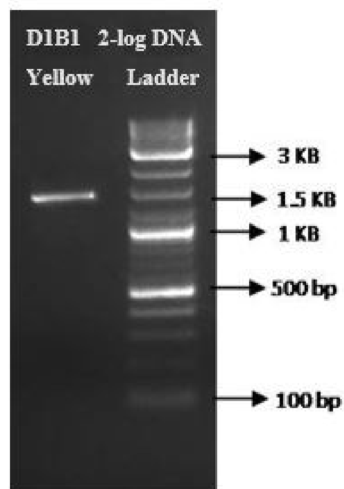


Plate 20. Amplification profile of the isolated bacterial DNA using 16S rRNA primer

DNA by using spectrophotometric method revealed good quality and quantity of DNA (Table 10).

4.2.1.3.2 PCR analysis of genomic DNA with 16S rRNA and 23S rRNA primers

The amplified products were obtained after PCR using 16S rRNA and 23S rRNA primers. The agarose gel electrophoresis (1.2%) of the amplified products showed the presence bands on the gel (Plate-20).

4.2.1.3.3 Sequence analysis using nBLAST

Molecular studies of the bacteria was carried out by sequencing the 16S ribosomal RNA. The sequence obtained (Appendix III) was analysed using nBLAST (Plate-21). The result revealed that the bacteria showed maximum sequence similarity (99%) to the bacterial species *Nesterenkonia* sp. EGI 80099.

From the above the results, the isolated microorganism was found to be cellulolytic bacteria of the species *Nesterenkonia* sp.

4.2.2 Bioethanol production from banana raw peel using mixed cultures of *Saccharomyces cerevisiae* and native organism.

The values of ethanol percentage produced by mixed cultures of *Saccharomyces cerevisiae* and *Nesternkonia* sp. are presented in Table 11. The results showed significant variation. The highest value was observed for single batch bioconversion (6.88%) and the lowest value was obtained for simultaneous saccharification and fermentation (5.88%).

As per the above results, it was inferred that there was not a significant increase in the production of alcohol when *Saccharomyces cerevisiae* and cellulolytic microorganism *Nesterenkonia* sp. were used in mixed cultures for fermentation.

Table 10. Quality and quantity of isolated genomic DNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260} /A_{280}	DNA Yield (ngµl^{-1})
1	Bacterial DNA	0.012	0.007	1.71	360

Table 11. Enhancement of alcohol production from banana raw peel using mixed cultures (*Nesterenkonia* sp. and *Saccharomyces cerevisiae*)

Fermentation method	Ethanol percentage
Separate Hydrolysis and Fermentation (SHF)	6.43±0.14
Simultaneous Saccharification and Fermentation (SSF)	5.88±0.19
Single Batch Bioconversion (SBB)	6.88±0.06
CD	0.19331

No. of replications = 5

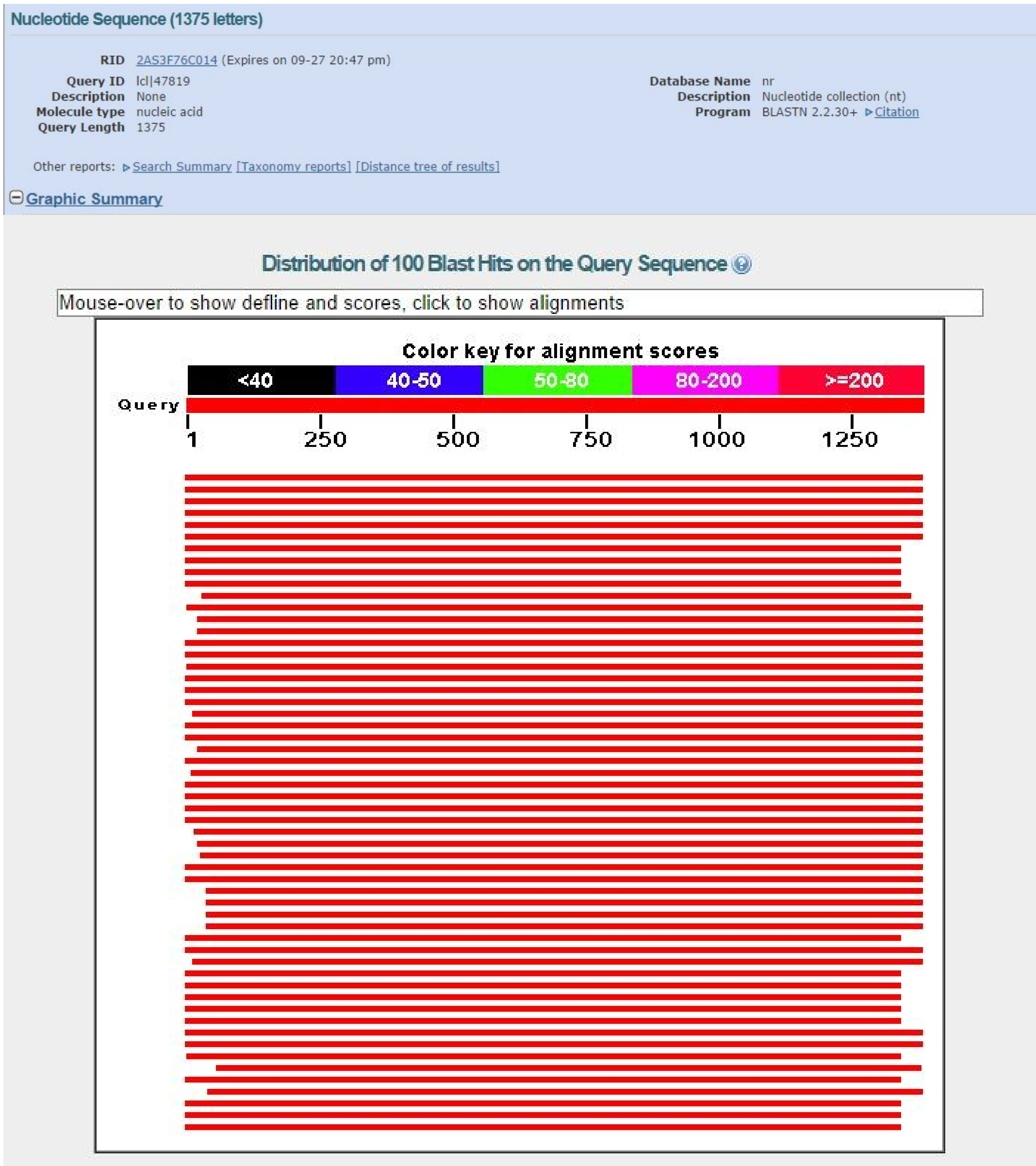


Plate 21a. Screen shot showing the result of nBLAST

Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
	Nesterenkonia sp. EGI 80099 16S ribosomal RNA gene, partial sequence	2527	2527	100%	0.0	99%	KF040423.1
	Nesterenkonia sp. NP1 16S ribosomal RNA gene, partial sequence	2488	2488	100%	0.0	99%	JX424770.1
	Nesterenkonia sp. DY01 16S ribosomal RNA gene, partial sequence	2483	2483	100%	0.0	99%	KF434120.1
	Uncultured bacterium clone AKIW983 16S ribosomal RNA gene, partial sequence	2471	2471	100%	0.0	99%	DQ129577.1
	Nesterenkonia sp. A-10 16S ribosomal RNA gene, partial sequence	2471	2471	100%	0.0	99%	AY914064.1
	Nesterenkonia sp. YB250 16S ribosomal RNA gene, partial sequence	2466	2466	100%	0.0	99%	KF914156.1
	Uncultured bacterium clone nbw630a10c1 16S ribosomal RNA gene, partial sequence	2444	2444	97%	0.0	99%	GQ112405.1
	Uncultured bacterium clone ncd1384f11c1 16S ribosomal RNA gene, partial sequence	2416	2416	97%	0.0	99%	JF120763.1
	Uncultured bacterium clone ncd1403g03c1 16S ribosomal RNA gene, partial sequence	2416	2416	97%	0.0	99%	JF117445.1
	Uncultured bacterium clone ncd1349e07c1 16S ribosomal RNA gene, partial sequence	2405	2405	97%	0.0	99%	JF116251.1
	Uncultured Actinomycetales bacterium clone YNP_ObP_B34 16S ribosomal RNA gene, partial sequence	2385	2385	96%	0.0	99%	DQ243765.1
	Nesterenkonia suensis strain MTA-34-2 16S ribosomal RNA gene, partial sequence	2340	2340	99%	0.0	98%	KJ578740.1
	Nesterenkonia flava strain 42 16S ribosomal RNA gene, partial sequence	2338	2338	98%	0.0	98%	KC843407.1

Plate 21b. Screen shot showing the result of nBLAST

DISCUSSION

5. DISCUSSION

5.1 BIOETHANOL PRODUCTION FROM BANANA BIOMASS

5.1.1 Characterisation of the feedstock

Raw peels, ripe peels and pseudostem of the banana variety AAB were utilized in the present study. Moisture content of the banana peel was determined. The result of the study revealed that the banana peel has showed a moisture percentage of 86-88%. Lesser values of moisture content was reported by Mohapatra *et al.* (2010) in the peels of banana variety AAB with 83.5%. However Sanchez *et al.* (2014) described that banana peel has a moisture percentage of 86.30 which was closer to the result of the present study.

According to the results given in Table 3, the maximum values for glucose, sucrose and fructose in banana peel was observed as 10.89%, 3.38% and 7.44% respectively. Comparatively lesser values were reported by Mohapatra *et al.* (2010). In their study, they observed the contents of glucose, sucrose and fructose in banana peel as 2.4%, 2.6% and 6.2% respectively.

The results of cellulose content in different feed stocks were observed as 9.97% and 27.57% respectively for banana ripe peel and banana pseudostem as per Table 4. Lesser amount of cellulose was observed in banana ripe peel (8.40%) by Mohapatra *et al.* (2010). But they have noticed a higher amount of cellulose in banana pseudostem (34-40%). 13.20% of cellulose content was observed in banana waste by John *et al.* (2006). Sanchez *et al.* (2014) have also studied the cellulose content in banana peel feedstock. They have reported a value of 11.45% cellulose in banana peel.

From the result of this study, it was inferred that the banana peel has a maximum hemicellulose content of 27.14% and 8.86% of lignin content. John *et al.* (2006) reported that banana waste contains 14.00% lignin, 14.8% hemicellulose. However

Sanchez *et al.* (2014) reported 25.52% hemicellulose and 9.82% lignin in banana peel feedstock. These values were closer to the results of the present study.

5.1.2 Pre-treatment of the feedstock

In this study, acid and alkali pre-treatments were carried out for the saccharification of the banana feed stocks. Dilute sulfuric acid with 0.8M concentration and hydrogen peroxide with 2% concentration were used for the treatment (Lalitha, 2011). The reducing sugar content was increased after treating the samples with dilute sulfuric acid. This may be due to the recovery of sugars from cellulose and hemicellulose as reported by Aden *et al.* (2002). Palmqvist and Harn-Hagerdal (2000) have also reported that the pre-treatment of the lignocellulosics with dilute acids can significantly improve the cellulose hydrolysis. Saha *et al.* (2005) stated that the dilute acid pretreatment has the advantage of not only solubilizing hemicelluloses but also converting solubilized hemicelluloses to fermentable sugars.

Alkali treated samples did not showed any significant increase in sugar content as per the results. Similar observation was made Patle and Lal (2007). They have compared acid, alkaline and enzymatic hydrolysis of agricultural crop wastes for yields of total reducing sugars and noticed that acid hydrolysis of fruit and vegetable residues gave a fare percentage of sugar yield. Alkaline hydrolysis did not gave significant amount of reducing sugars. Sirkar *et al*. (2008) have also reported that acid pretreatment method was found to be optimal for better yield of fermentable sugars from fruit peels.

In the present study, the percent release of total reducing sugar after acid treatment was observed as 12.00% and non-reducing sugar release was noticed as 3.51%. Arumugam and Manikandan (2011) observed that dilute acid treatment significantly increased the sugar release nearly by 20%.

Percent removal of lignin from banana feed stocks revealed that maximum removal of lignin was observed in acid treated banana pseudostem (25.92%). Alkali

treated samples showed comparatively lesser removal of lignin. From the results it was inferred that the acid treatment was the best pre-treatment for banana feed stocks compared to alkali treatment. Krishna and Chowdari (2010) stated that an ideal pretreatment would reduce the lignin content and crystallinity of the cellulose. The purpose of pre-treatment was to remove lignin from the fruit biomass peel residue, which hinders enzymatic hydrolysis of cellulose for ethanol fermentation as reported by Lalitha (2011). She also reported that acid treatment removed significant amount of lignin from fruit peel biomass.

5.1.3 Fermentation of the feedstock

Banana feed stocks were fermented using *Saccharomyces cerevisiae* and *Zymomonas mobilis.* From the results obtained, it was inferred that banana raw peel fermented using *Saccharomyces cerevisiae* showed the highest percentage of alcohol production (6.63%). Dhabekar and Chandak (2010) have also used *Saccharomyces cerevisiae* for production of ethanol from banana peel waste. They finally got result of 1.90% alcohol production from banana peel waste. Hossain *et al.* (2011) have reported that the banana peel fermented using *Saccharomyces cerevisiae* produces 4.1 to 7.1% of bioethanol. *Saccharomyces cerevisiae* was reported to be the suitable microorganism for bioethanol production from fruit peels by Reddy *et al.* (2011) and Janani *et al.* (2013).

Zymomonas mobilis was found to be an inefficient microorganism for bioethanol production from banana feed stocks. *Zymomonas mobilis* only managed to produce 3.00% of bioethanol from banana raw peel in the present study. However, some contradictory results were reported by Senthilkumar and Gunasekaran (2005). They have stated that ethanol producing bacteria *Zymomonas mobilis* have attracted more attention than *Saccharomyces cerevisiae* due to its higher growth rate and ethanol production capability. Oyeleke *et al.* (2012) have also reported that *Zymomonas*

mobilis was more effective than *Saccharomyces cerevisiae* for production of ethanol from cassava peels.

The end point of fermentation was determined for both *Saccharomyces cerevisiae* and *Zymomonas mobilis.* The ethanol percentage was determined at two days intervals. The values were observed to reach maximum at 6th day and 8th day for *Saccharomyces cerevisiae* and *Zymomonas mobilis* respectively. After that the ethanol percentage was observed to be unchanged or reduced. So the end point of fermentation was inferred to be 6th day for *Saccharomyces cerevisiae* and 8th day for *Zymomonas mobilis.* Dhabekar and Chandak (2010) reported the production of ethanol from banana peel waste using *Saccharomyces cerevisiae*. The maximum ethanol production was observed as 1.90% at the 4th day of incubation. Another report showed that the ethanol production from pineapple peel, banana peel and plantain using *Saccharomyces cerevisiae* was optimum at the 7th day of incubation (Itelima *et al.,* 2013).

Even though the acid pre-treatment of the banana feed stocks improved the sugar content of the samples, the ethanol production was observed to be lesser compared to untreated samples. This may be because of some inhibitors produced as a result of acid treatment as reported by Cardona *et al.* (2009). They have reported that acid pretreatment results in the production of various inhibitors like acetic acid, furfural and 5 hydroxy methyl furfural. These products are growth inhibitors of microorganisms.

Initially the pH values of the acid treated samples were very low. In order to adjust the pH of the medium to optimum pH for microorganism (pH-7), 1N NaOH solution was added to it. It may have increased the salt concentration of the medium which lead to inhibit the growth of fermenting microorganism.

From the values of change in pH, it was inferred that the pH tend to reduce with the production of ethanol. The lowest pH value was observed in untreated sample of banana raw peel fermented using *Saccharomyces cerevisiae.* This may be due to the

higher production of ethanol from untreated banana raw peel. Hossain *et al.* (2011) reported that the pH value of the banana sample decreased with the production of ethanol. Hadeel *et al.* (2011) have also observed reduce in pH value after fermenting rambutan fruit waste using *Saccharomyces cerevisiae.*

The pre-treatments were observed to be inefficient in the present study. So the untreated banana feed stocks were only studied for non-fermentable sugar content. The results showed that banana peel has a non-fermentable sugar content of 8.78% for reducing sugar and 6.73% for non-reducing sugar. The maximum conversion of sugar to ethanol was noticed as 87.87%. Kandari and Gupta (2012) reported slightly higher values than this result. They have observed 10.40% total non-fermentable sugars and 95.20% conversion to alcohol in banana peel waste.

The results of the present study revealed that untreated banana raw peel sample was the best feedstock among the three feed stocks utilized in this study. Banana raw peel yielded 6.63% of ethanol after fermentation which was significantly higher than the values of banana ripe peel and pseudostem. Pre-treatments carried out in the study were found to be insignificant since the pre-treated samples produced less amount of alcohol compared to untreated samples. Also from the study it was revealed that *Saccharomyces cerevisiae* was the better organism to produce ethanol from banana feed stocks compare to *Zymomonas mobilis.*

5.2 ENHANCEMENT OF ETHANOL PRODUCTION

5.2.1 Isolation of native microorganism and its characterization

The first phase of the study revealed that banana raw peel was the best feedstock for ethanol production. So the cellulolytic native microorganism was isolated from banana raw peel sample. The organism which showed cellulolytic activity when grown in CMC agar plates were isolated and pure cultures were maintained. The organism was found as a member of the bacterial species *Nesterenkonia* sp. when the 16S rRNA was sequenced. The organism showed maximum sequence similarity to the bacterial

species *Nesterenkonia* sp. As reported by Patel (2001), the traditional methods for microbial identification require the recognition of differences in morphology, growth, enzymatic activity, and metabolism to define genera and species. Full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant microorganisms. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes.

The morphological and biochemical characters of the bacterial was also studied. The organism was observed as pale orange colored circular colonies. Gram's staining of the colonies revealed that the organism was gram negative and observed as cocci shaped cells. Biochemical characters of the microorganism showed positive results for Catalase test, Citrate utilization test and Gelatin hydrolysis test. Even though the result of gelatin hydrolysis was positive, the microorganism showed less activity on gelatin. The organism was not a commonly used one for cellulolysis due to its slow activity on cellulose. The results of the present study revealed that the organism showed slow rate of cellulolysis. A similar microorganism was reported to be isolated from soil by Shi *et al.* (2011). The organism was observed as Gram negative, rod shaped orange colored colonies. The organism showed positive results for catalase test and negative for oxidase test and starch hydrolysis. They have sequenced the 16S rRNA of the strain and observed that that the strain formed a separated line within a clade containing the genus *Nesterenkonia* in the phylum Actinobacteria. Collins *et al.* (2002) have also reported a cocci shaped bacterium with occasional branching isolated from saline conditions. The 16s rRNA sequence showed similarity to the species *Nesterenkonia halobia.*

5.2.2 Ethanol production using mixed cultures of *Saccharomyces cerevisiae* and native microorganism.

Banana raw peel fermented with *Saccharomyces cerevisiae* was selected for the enhancement of alcohol production. A cellulolytic bacteria (*Nesterenkonia* sp.) isolated from banana raw peel was utilized in mixed cultures with *Saccharomyces cerevisiae* to try an enhancement in alcohol production. Separate Hydrolysis and Fermentation (SHF), Simultaneous Saccharification and Fermentation (SSF) and Single Batch Bioconversion (SBB) were the types of fermentation systems used. In this process, the stages are virtually same and both are performed in the same bioreactor. Thus, the presence of yeast together with the cellulolytic enzyme complex reduces the accumulation of the inhibiting sugars within the reactor, thereby increasing the yield and the saccharification rates as reported by Ballesteros *et al*. (2004). Another advantage of this approach is that a single bioreactor is used for the entire process, therefore reducing the investment costs. In addition, the presence of ethanol causes the medium to be less vulnerable to invasion by undesired microorganisms (Olofsson *et al*., 2008).

In the present study, among the three fermentation methods namely SHF, SSF and SBB, Single Batch Bioconversion (SBB) produced the highest percent of alcohol (6.88%) and the lowest alcohol percentage was observed in SSF (5.88%). This might have happened because *Nesterenkonia* sp. inhibited the growth of *Saccharomyces cerevisiae* when cultured together in SSF. But Söderström *et al*. (2003); Wingren *et al*. (2003); Saha *et al*. (2005) and Olsson *et al*. (2006) have reported that Separate Hydrolysis and Fermentatio (SHF) was more effective. They observed that the major advantage of separate hydrolysis and fermentation method is that it is possible to carry out the cellulose hydrolysis and fermentation at their own optimum conditions. However Philippidis *et al.* (1993) stated that inhibition of cellulase activity by the released sugars, mainly cellobiose and glucose, is the main drawback of SHF. Another possible problem in SHF is that of contaminations. The hydrolysis process is rather

long, and a dilute solution of sugar always has a risk of microbial contaminations, even at rather high temperature such as 45-50 °C. A possible source of contamination could be the enzymes.

The highest percentage of ethanol from banana raw peel was observed when it was fermented using the co-cultures of *Nesterenkonia* sp. and *Saccharomyces cerevisiae* (6.88%). Itelima *et al*. (2013) have also reported that the bioethanol production from banana peel was increased when mixed cultures of cellulolytic microorganism and fermenting microorganism were used. They have observed a result of 7.45% ethanol production from banana peels in mixed cultures. Singh *et al.* (2014) have reported an ethanol production of 6.29% from banana peel using co-cultures of *Aspergillus niger* and *Saccharomyces cerevisiae.*

An attempt was made to enhance the production of ethanol from banana raw peel waste by using mixed cultures of *Nesterenkonia* sp. and *Saccharomyces cerevisiae* as fermenting organism. But the result of the study revealed that due to the slow cellulolytic activity of the banteria, and antagonistic effect to *Saccharomyces cerevisiae*, there was no significant increase in ethanol production.

SUMMARY

6. SUMMARY

A study on "Utilization of banana (*Musa* spp.) biomass for biofuel production" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during the year 2013-14. The salient features of the study are summarized below.

With the ever increasing demand for energy and the fast depleting petroleum resources, globally there is an increased interest in alternative fuels. Bioethanol is one of the important alternatives being considered. Many agricultural raw materials rich in fermentable carbohydrates are being tested worldwide for bioconversion from sugar to ethanol, but the cost of the carbohydrate raw materials has become a limiting factor for large scale production by the industries employing fermentation processes. Also using food and feed crops for bioethanol production is almost unethical in the current world scenario. The production of bioethanol from comparatively cheaper and non-food sources like agricultural wastes or fruit wastes using efficient fermentative microorganism is the only possible way to meet the great demand for ethanol in the present situation of energy crisis. The present study is an attempt to produce cost effective and environmentally sustainable ethanol using banana fruit and plant residues, a major agro-waste in Kerala.

Banana raw peel, banana ripe peel and banana pseudostem feed stocks were utilized for bioethanol production. One of the main criteria for bioethanol production is to characterize the feed stocks.

Different characters of the banana biomass were studied. The results showed that banana pseudostem has the maximum moisture content (95.32%) among the three feed stocks.

Banana ripe peel was observed to have the maximum sugar contents. It showed 21.98% total reducing sugar, 12.05% non-reducing sugars, 10.89% glucose content,

7.44% fructose content, 2.72% xylose and 3.38% sucrose contents. The sugar content was found to be minimum in banana pseudostem.

Total soluble salt content was also found maximum in banana ripe peel (0.082 μsm^{-1}). But the total dissolved solids content was observed maximum in banana pseudostem (21.90 ppm).

Banana pseudostem exhibited a high cellulose content of 25.57% and lignin content of 15.44% whereas the hemicellulose content was found to be maximum in banana raw peel with 27.14%.

In order to remove the lignin content from the feedstock as well as to release the fermentable sugars from cellulose and hemicellulose, different pre-treatments were carried out in this study. Among the acid and alkali treatments, acid treatment was observed to release more sugars with the values of 22.85% reducing sugars and 12.41% non-reducing sugars from banana ripe peel.

The highest value of lignin was observed in banana pseudostem (11.44%) and the value was found to be lowest in banana raw peel with a value of 6.27% when acid treatment was done in the feed stocks. Alkali treatment was inferred to be ineffective for the removal of lignin.

Percent saccharification result also indicated that the acid treatment was the best pre-treatment for banana feed stocks. Maximum increase in total reducing sugar was observed in acid treated banana raw peel (12.00%). The percent increase in non-reducing sugar was found to be highest in acid treated banana pseudostem (4.11%).

The percent removal of lignin from banana feed stocks showed that the highest lignin removal was found in acid treated banana pseudostem (25.92%). The lowest lignin removal percentage was noticed in alkali treated banana ripe peel with 3.76%.

The results of the pre-treated banana samples revealed that acid treatment was the effective pre-treatment method for banana biomass.

The values of ethanol percentage obtained after fermentation showed that maximum ethanol percentage was noticed for the untreated banana raw peel sample (6.63%) and the lowest value was observed for alkali treated banana pseudostem sample (0.82%) when *Saccharomyces cerevisiae* was used for fermentation.

Zymomonas mobilis was found to have produced lesser amount of bioethanol from banana feedstock when compared to *Saccharomyces cerevisiae.* The highest ethanol percentage was observed in acid treated banana raw peel (3.11%). It was observed that this value is on par with the values of alkali treated banana raw peel (3.03%) and untreated banana raw peel (3.00%).

The ethanol percentage obtained for the banana biomass fermented with *Saccharomyces cerevisiae* were found to be high and varied significantly from the ethanol percentage of banana samples fermented with *Zymomonas mobilis.*

The results also showed that the pre-treatments carried out in this study were insignificant as the ethanol percentage was lower than the values of untreated feed stocks.

From the results, it was inferred that end point of fermentation was observed to be at the 6th day after the inoculation for *Saccharomyces cerevisiae* and at the 8th day for *Zymomonas mobilis.* At the 6th day of incubation, the ethanol percentage was found to reach maximum (6.63%) for *Saccharomyces cerevisiae*. At the 8th day of incubation, the ethanol percentage was observed to reach the maximum value of 3.11% for *Zymomonas mobilis.* After that the ethanol percentage tend to be steady.

The results revealed that the pH values of the samples reduces significantly after the production of ethanol. Banana raw peel without pre-treatment showed the most significant fall in the pH value after fermentation using *Saccharomyces cerevisiae* with a value 4.57 where the initial pH was 7.00.

The non-fermentable sugar contents were determined after fermentation. The highest value for non-fermentable total reducing sugar was noticed in banana ripe peel (8.78%). The lowest value was noticed for banana pseudostem (3.14%).

Highest value for non-fermentable total non-reducing sugar was observed in banana ripe peel (6.73%) and the lowest value was observed in banana pseudostem (2.61%).

The highest value for percent conversion to alcohol was observed in banana raw peel as 87.87% for total reducing sugar. The lowest value was observed in banana pseudostem as 47.12%.

The maximum percent conversion of total non-reducing sugar to alcohol was noticed in banana raw peel (45.90%) which was on par with banana ripe peel (44.12%). The lowest value was observed in banana pseudostem (16.88%).

From the results obtained after fermentation, it was inferred that the banana raw peel was the best feedstock among the three feed stocks used in this study. Also it was revealed that *Saccharomyces cerevisiae* was the better fermenting microorganism.

An attempt was made to increase the production of bioethanol from banana feedstock. For that a cellulolytic microorganism was isolated from the degraded samples of banana raw peel. The organism was characterized morphologically, biochemically and molecularly. The results revealed that the microorganism was closely related to the bacterial species *Nesterenkonia* sp. EGI 80099.

Mixed cultures of the cellulolytic microorganism and *Saccharomyces cerevisae* were used in three different methods of fermentation. Among the fermentations, the highest value was observed for single batch bioconversion (6.88%) and the lowest value was obtained for simultaneous saccharification and fermentation (5.88%).

Many attempts were made to produce bioethanol from banana biomass. But this is the first time banana raw peel, banana ripe peel and banana pseudostem are

compared for alcohol production using different fermenting microorganisms. The present study revealed that the banana raw peel is a potential feedstock for bioethanol production than banana ripe peel. Even though it was not statistically significant, a slight increase in the production of ethanol was observed in the banana raw peel when the mixed cultures of *Saccharomyces cerevisiae* and *Nesterenkonia* sp. were used for fermentation. By improving the culture conditions and optimizing pre-treatment methods, it is possible to increase the ethanol production from banana peel wastes.

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APPENDICES

APPENDIX – I

Reagents required for the characterization of the feed stock

a) Anthrone reagent

Anthrone	-	200 mg
95% ice-cold sulfuric acid	-	100 ml

Reagent was prepared fresh before use

b) Fehling's solution A and B

i) Fehling's solution A

Copper sulphate ($CuSO_4$)	-	34.64 g

The components were dissolved in distilled water and made up the volume to 500 ml in a volumetric flask.

ii) Fehling's solution B

Rochelle salt	-	173 g
(Potassium sodium tartarate $KNaC_4H_4O_6 \cdot 4H_2O$)		
Sodium hydroxide NaOH	-	50 g

The components were dissolved in distilled water and made up the volume to 500 ml

Diluted 5ml of this stock to 50ml for working standard.

c) DNS reagent

DNS	-	1 g
10% (w/v) Sodium Hydroxide Solution	-	16 ml
Rochelle salt (Sodium Potassium Tartarate)	-	30 g

Make up to 100 ml

d) Resorcinol reagent

Resorcinol	-	1 g
Thiourea	-	0.25 g
Glacial acetic acid	-	100 ml

e) Acetic/nitric reagent

80% Acetic acid	-	450 ml
Concentrated Nitric acid	-	45 ml

f) Neutral detergent solution and acid detergent solution

i) Neutral Detergent solution

Disodium ethylenediamine tetra acetate	-	18.6 g
Sodium borate decahydrate	-	6.81 g

Dissolve in about 200 ml of distilled water by heating to this added a 200 ml solution of the following in distilled water

Sodium lauryl sulphate	-	30 g
2-ethoxy ethanol	-	10 ml

To the above solution 100ml of the following solution in distilled water was added

Disodium hydrogen phosphate	-	4.5 g

Made up the volume to one liter and adjust the pH to7.0

ii) Acid Dtergent solution

Cetyl trimethyl ammonium bromide	-	20 g

Dissolve the above component in one liter of 1N sulphuric acid.

APPENDIX – II

Media compositions for isolation and characterization of native microorganism

a) Media No. 21 (Yeast Extract Glucose Salt Agar)

Yeast extract	-	10.00 g
Agar	-	15.00 g
Distilled water	-	0.9 L
Glucose	-	20.0 g
Magnesium chloride [$MgCl_2$]	-	10.00 g
Ammonium sulphate [$(NH4)_2SO_4$]	-	10.00 g
Potassium dihydrogen phosphate [KH_2PO_4]	-	10.00 g

b) Media No. 6 (Malt Yeast Agar)

Malt extract	-	3.00 g
Yeast extract	-	3.00 g
Peptone	-	5.00 g
Glucose	-	10.00 g
Agar	-	20.00 g
Distilled water	-	1.00 L
pH	-	7.0

c) Carboxy Methyl Cellulose Agar (1 L)

Peptone	-	10 g
Carboxy methyl cellulose	-	10 g
Dipotassium phosphate	-	2 g
Agar	-	10 g
Magnesium sulphate	-	0.3 g
Ammonium sulphate	-	2.5 g
Gelatin	-	1 g
Distilled water	-	1 L

d) Trypticase soya agar

Tryptone	-	17 g
Soytone	-	3 g
Dextrose	-	2.5 g
NaCl	-	5.0 g
K_2HPO_4	-	2.5 g
Agar	-	15 g
Distilled water	-	1.00L
Final pH	-	7.3 +/- 0.2

Omit agar for broth medium

e) Glucose fermentation broth

Peptone	-	5.00 g
Beef extract	-	3.00 g
Glucose	-	5.00 g
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

f) Sucrose fermentation broth

Peptone	-	5.00 g
Beef extract	-	3.00 g
Sucrose	-	5.00 g
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

g) Lactose fermentation broth

Peptic digest of animal tissue	-	5.00 g
Beef extract	-	3.00 g
Lactose	-	5.00 g
Distilled water	-	1.00 L
Final pH	-	6.9±0.2 (at 25°C)

h) Mannitol fermentation broth

Peptone	-	5.00 g
Beef extract	-	3.00 g

Mannitol	-	5.00 g
Distilled water	-	1.00 L
Final pH	-	6.9±0.2 (at 25°C)

i) Maltose fermentation broth

Peptone	-	5.00 g
Beef extract	-	3.00 g
Maltose	-	5.00 g
Distilled water	-	1.00 L
Final pH	-	6.9±0.2 (at 25°C)

j) MR-VP broth

Peptone	-	7 g
Dextrose	-	5 g
Dipotassium phosphate	-	5 g
Distilled water	-	1 L
pH	-	6.9±0.2 (at 25°C)

k) Simmon's citrate agar

Magnesium Sulphate ($MgSO_4$)	-	0.2 g
Ammonium dihydrogen phosphate	-	1 g
Dipotassium phosphate	-	1 g
Sodium citrate	-	2 g
Sodium chloride	-	5 g

Bromothymol blue	-	0.08 g
Agar	-	15 g
Distilled water	-	1 L
pH	-	6.8±0.2 (at 25°C)

l) Nutrient gelatin medium

Peptone	-	5 g
Beef extract	-	3 g
Gelatin	-	120 g
Distilled water	-	1 L
pH	-	6.8±0.2 (at 25°C)

m) Urease test broth

Yeast extract	-	0.1 g
Urea	-	20 g
Monopotassium phosphate	-	0.091 g
Disodium phosphate	-	0.095 g
Phenol red	-	0.010 g
Distilled water	-	1 L
pH	-	6.8±0.2 (at 25°C)

APPENDIX – III

Nucleotide sequence of the isolated cellulolytic bacteria (*Nesterenkonia* sp.)

16S rRNA sequence

GCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCTTCCTGCTTGCAGGA
AGTGGATTAGTGGCGAACGGGTGAGTATCACGTGAGCAACCTGCCCTTGACTCTGGG
ATAAGCCTGGGAAACTGGGTCTAATACCGGATATAACCTCTTACCGCATGGTGAGTG
GTGGAAAGCTTTTGCGGTCTTGGATGGGCTCGCGGCCTATCAGCTTGACGGTGAGGT
AGTGGCTCACCGTGGCGATGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
GGGCGAAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGGCCTTCGGGTTGTAAA
CCGCTTTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTATCCGGAATTATTG
GGCGTAAAGAGCTCGTAGGCGGTTTGCCGCGTCTGCTGTGAAAGCCCGGGGCTTAAC
CCCGGGTGTGCAGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCC
TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTC
TCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTT
TCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCG
ATGCAACGCGAAGAACCTTACCAAGGCTTGACATGGACCGGATCGCCCTAGAGATAG
GGTTTCCCTTCGGGGCTGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTATGTTGCCAGCACG
TAAAGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGA
CGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTA
CAGTGGGTTGCGATACTGTGAGGTGGAGCTAATCCCTAAAAGCCGGTCTCAGTTCGG
ATCGAAGTCTGCAACTCGACTTCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGC
AACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACTCGCCCGTCAAGTCACGAA
AGTTGGTAACA

ABSTRACT

UTILIZATION OF BANANA (*Musa* spp.) BIOMASS FOR BIOFUEL PRODUCTION

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Abstract of the
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ABSTRACT

A study on "Utilization of banana (*Musa* spp.) biomass for biofuel production" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani during the year 2013-14.

Banana raw peel, banana ripe peel and banana pseudostem feed stocks were utilized for bioethanol production. The results of characterization of feed stocks showed maximum values of sugars in banana ripe peel. It showed 21.98% total reducing sugar, 12.05% non-reducing sugars, 10.89% glucose content, 7.44% fructose content, 2.72% xylose and 3.38% sucrose contents.

Banana pseudostem exhibited a high cellulose content of 25.57% and lignin content of 15.44% whereas the hemicellulose content was found to be maximum in banana raw peel with 27.14%.

Among the pre-treatments carried out, acid treatment was observed as the best treatment compared to alkali treatment. Acid treatment was noticed to have maximum increase in total reducing sugar content of banana raw peel (12.00%). The percent increase in non-reducing sugar was found to be highest in acid treated banana pseudostem (4.11%). Also the lignin removal percentage was observed to be high in acid treated banana pseudostem (25.92%).

Saccharomyces cerevisiae produced 6.63% of ethanol from untreated samples of banana raw peel. However *Zymomonas mobilis* was able to produce only 3.11% of ethanol from acid treated samples of banana ripe peel. From the results, it was inferred that the banana raw peel has the maximum potential to be used as feedstock for ethanol production. *Saccharomyces cerevisiae* was noticed to be the best microorganism.

A cellulolytic microorganism was isolated from the degraded samples of banana. The organism was characterised and it was found to be closely related to

bacterial species *Nesterenkonia* sp. EGI 80099. Mixed cultures of this microorganism and *Saccharomyces cerevisiae* produced a maximum percentage of 6.88% ethanol from banana raw peel in single batch bioconversion.