UTILIZATION OF PINEAPPLE (*Ananas comosus* (L.) Merr.) BIOMASS FOR BIOFUEL PRODUCTION

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(2009-09-113)

M.Sc. (INTEGRATED) BIOTECHNOLOGY

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by

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"UTILIZATION OF PINEAPPLE (*Ananas Comosus* (L.) Merr.) 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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Certified that this thesis entitled **"UTILIZATION OF PINEAPPLE (*Ananas comosus* (L.) Merr.) BIOMASS FOR BIOFUEL PRODUCTION"** is a record of research work done independently by **Mr. Anoop P. (2009-09-113)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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ANOOP P.

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
ADF	Acid detergent fibre
ANOVA	Analysis of variance
bp	Base pair
C	Cytosine
cDNA	Complementary DNA
CTAB	Cetyl trimethyl ammonium bromide
cm	Centimeter
DNA	Deoxyribo nucleic acid
DNS	Dinitro salicylic acid
dNTPs	Deoxy nucleotide tri phosphates
EDTA	Ethylene diamine tetra acetic acid
EC/TDS	Electrical conductivity/ Total dissolved solids
F	Forward primer
G	Guanine
g	gram
g/l	gram per litre
h	Hour
ha	Hectare
HCl	Hydrochloric acid
kbp	Kilo basepair
kDa	Kilodalton

kg	Kilogram
M	Molar
mg	milligram
min	Minute
ml	Millilitre
N	Normality
nBLAST	Nucleotide basic local alignment search tool
NaCl	Sodium chloride
NCBI	National center for biotechnology information
NDF	Neutral detergent fibre
nm	Nanometre
^{o}C	Degree Celsius
OD	Optical density
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ppm	Parts per million
R	Reverse primer
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal RNA
s	Second
SHF	Separate hydrolysis and fermentation
SSB	Simultaneous saccharification and fermentation
SBB	Single batch bioconversion
sp.	Species
t	Tonne
T	Thymine
TE	Tris-EDTA buffer
Tris HCl	Tris (Hydroxy Methyl) aminomethane hydrochloride

U	Enzyme unit
UV	Ultra violet
V	Volt
v/v	volume/volume
w/v	weight/volume

Introduction

1. INTRODUCTION

The consumption of crude oil has increased exponentially during last few years across the globe. As a result, the oil resources are getting depleted and a fear of run out of oil is a hitch amongst the rapidly growing countries like India. In addition, the unsteadiness of oil prices and concern about climate, demand for substitute fuels have all contributed to explore new avenues of energy production (Raikar, 2012). In this context, bioenergy which is derived from biomass is gaining importance. Biofuels represent an alternative to petroleum-based fuel. Bioethanol is the most widely used biofuel for transportation (Balat, 2010). It is an ecofriendly renewable alternative to fossil fuel which is being depleted at an alarming rate. Green house emissions and resultant climate change also demand a substitute for conventional fuel by plant-based bioethanol.

The major lacuna in bioethanol production is the availability of raw materials. The sugar and starch based feedstock are the raw materials currently in use. However, these raw materials may not be sufficient to meet the demand for biofuel production. Moreover, being food crops, the controversy over use in biofuel production lingers (Greene, 2004). Besides, price of these raw materials is highly unstable and has a big impact on the production costs of bioethanol.

Due to availability and low cost, lignocellulosic biomass is considered a promising feedstock. But an effective conversion protocol has to be developed. The use of residual biomass for bioethanol production has an added advantage of transforming a waste material into a valorized product. Cellulose materials represent the most abundant global source of biomass and have been largely unutilized. The global production of plant biomass, of which over 90 per cent is lignocellulose, amounts to about 200×109 tons per year. About 8 to 20×109 tons of the primary biomass remains potentially accessible (Lin and Tanaka, 2006).

Pineapple waste is rich in sugars and lignocellulosic components. Pineapple (*Ananas comosus*) is one of the most important fruits in the world and is the leading

edible member of the family Bromeliaceae. This fruit juice is the third most preferred worldwide after orange and apple juices (Cabrera *et al*., 2000). The area under pineapple plantation was almost 920,536.05 ha with an estimated production of more than 21,582,237 per ha (FAO, 2011). It is one of the commercially important fruit crops of India with an area of production 89000 ha and the annual output was 1,415,000 per ha. Kerala has great opportunities for pineapple cultivation because it can be grown in all the districts in Kerala, favorable climate to produce pineapple throughout the year and no additional land area is required as it can be grown as an intercrop in rubber, coconut and cashew. At present pineapple is grown in about 12500 ha in Kerala. The production of pineapple is about 315000 tons (RARS Vazhakkulam 2011).

Tropical and subtropical fruits processing have considerably higher ratios of by-products than the temperate fruits (Schieber *et al*., 2001). Pineapple by products consist of basically the residual pulp, peels, stem and leaves. The increasing production of pineapple processed items, results in massive waste generations. Fruit processing for the production of juice, nectar or canned fruit yields a large amount of industrial residues that are often infra-utilized as feed or as fertilizer. During pineapple processing, large amount of unusable waste material are generated, 40-80 per cent of pineapple is discarded as waste (Ban-koffi and Han, 1990; Tanaka *et al*., 1999). Pineapple peel from fruit canned industries is generated in large amount everyday approximately 35 per cent based of raw material (Vimal and Adsule, 1976) or about 10 ton per day from one medium size industry (DIW, 2008). Pineapple waste contains sufficient quantities of simple and complex sugars that may be used for bioethanol production (Nigam, 2000). These wastes are usually prone to microbial spoilage thus limiting further exploitation. Further, the drying, storage and shipment of these wastes is not cost effective and hence efficient, inexpensive and eco-friendly utilization is becoming more and more necessary. Researchers have focused on the utilization of pineapple waste primarily for extraction of bromelain enzyme and secondarily as low-cost raw material for the production of ethanol, phenolic anti-oxidants, organic acids, biogas and fiber production. Interest in the economic conversion of renewable

resources into alcohol using low cost substrate, such as pineapple waste, has been increasing since the last decade (Upadhyay *et al*., 2010).

In the present study, the feasibility of obtaining ethanol from pineapple biomass will be studied with the following objectives

- Production of ethanol from pineapple biomass through saccharification and fermentation processes.
- Isolation and characterization of microorganism causing *in situ* biodegradation of pineapple biomass.
- Enhanced production of bioethanol using mixed cultures of *Saccharomyces cerevisiae/ Zymomonas mobilis* and native organism.

Review of literature

2. REVIEW OF LITERATURE

2.1 Bioethanol as a potential alternate fuel source

The consumption of crude oil has increased exponentially during last few years across the globe. As a result, the oil resources are getting depleted and a fear of *run out of oil* is a hitch amongst the rapidly growing countries like India. In addition, the unsteadiness of oil prices, green house emissions and resultant climate change also demand a substitute for conventional fuel (Raikar, 2012). In this context, bioenergy which are derived from biomass are gaining importance.

Biofuels represent an alternative to petroleum-based fuel. Bioethanol which is an ecofriendly renewable alternative to fossil fuel, is the most widely used biofuel for transportation (Balat, 2010). Bioethanol is obtained by fermenting any biomass high in carbohydrates viz., starches, sugars or celluloses. Its combustion does not increase the greenhouse effect due to zero carbon dioxide emission. It is also well known as an octane number enhancer. Moreover, the addition of bioethanol to conventional fuel leads to decreased emissions of gaseous pollutants, such as carbon monoxide (CO) and carcinogenic volatile organic compounds (Kujawski and Zieliński, 2006).

According to Borah and Mishra (2011), world ethanol production for transport fuel tripled between 2000 and 2007 from 17 billion to more than 52 billion litres. From 2007 to 2008, the share of ethanol in global gasoline type fuel use increased from 3.7 percent to 5.4 percent. In 2009, worldwide ethanol fuel production reached 19.5 billion gallons (73.9 billion litres). Ethanol is widely used in Brazil and in the United States, and together both countries were responsible for 89 percent of the world's ethanol fuel production in 2009. Most cars on the road today in the U.S. can run on blends of up to 10 percent ethanol, and the use of 10 percent ethanol gasoline is mandated in some U.S. states and cities. Since 1976, the Brazilian government has made it mandatory to blend ethanol with

gasoline, and since 2007, the legal blend is around 25 percent ethanol and 75 percent gasoline.

2.2 Bioethanol production from different agricultural feedstock

Bioethanol is a clean fuel for combustion engines made from plant-based feedstocks. The production of bioethanol is from starch crops like corn and wheat and from sugar crops like sugar cane and sugar beet. Conventional crops are unable to meet the global demand due to their primary value as food and feed. Therefore, lignocellulosic materials such as agricultural waste are attractive feed stocks for bioethanol production. They are cost effective, renewable and abundant (Upadhyay *et al.,* 2010).

Lignocelluloses wastes refer to plant biomass wastes are composed of cellulose, hemicellulose, and lignin. They may be grouped into different categories such as wood residues (including sawdust and paper mill discards), grasses, waste paper, agricultural residues (including straw, stover, peelings, cobs, stalks, nutshells, non food seeds, bagasse, domestic wastes (lignocellulose garbage and sewage), food industry residues, municipal solid wastes etc. (Qi *et al*., 2005; Roig *et al*., 2006; Rodríguez *et al*., 2008). The lignocellulosic biomass, which represents the largest renewable reservoir of potentially fermentable carbohydrates on earth (Mtui and Nakamura, 2005) is mostly wasted in the form of preharvest and postharvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great potential in utilizing lignocellulosic waste for the production and recovery of many value-added products including bioethanol (Pandey *et al.,* 2000; Das and Singh, 2004; Foyle *et al*., 2007).

Bioethanol production could be the route to the effective utilization of agricultural wastes. Singh and Jain (1995) studied on the production of ethanol by batch fermentation of cane molasses. Green and Shelef (1989)

used municipal solid waste in ethanol production while agricultural waste was tried by Schugerl (1994). Rice straw, wheat straw, corn straw, and sugarcane bagasse are the major agricultural wastes utilized for ethanol production in terms of quantity of biomass available (Kim and Dale, 2004)

The fruit wastes like pineapple (Muttara and Nirmala, 1982), banana peels (Joshi *et al*., 2001), mangoes (Reddy and Reddy, 2007), papaya (Akin-Osanaiye *et al*., 2008) and grapes (Pramanik and Rao, 2005; Asli, 2010) were used in the production of ethanol.

Lalitha and Sivaraj (2011) demonstrated that fruit biomass peel residue could be used to produce fuel grade ethanol. Santi *et al.* (2012) utilized the orange peel waste, a solid residue of orange juice production for bioethanol production.

Bioethanol from agricultural feedstock could be a promising technology though the process has several challenges and limitations such as biomass transport and handling, and efficient pretreatment methods for total delignification of lignocellulosics. Proper pretreatment methods can increase concentrations of fermentable sugars after enzymatic saccharification, thereby improving the efficiency of the whole process. Conversion of glucose as well as xylose to ethanol needs some new fermentation technologies, to make the whole process cost effective (Sarkar *et al*., 2012).

2.2.1 Characterisation of the feedstock

The characterization of the feedstock is carried out to use a new potential lignocellulosic bioresource that has several attractive agroenergy features for ethanol production. The plant-based feedstocks contain a complex mixture of organic materials, commonly known as carbohydrates, fats, and proteins. The amounts of these organic components vary with the type of feedstock. Rapid and cost effective quantification of lignocellulosic components of agricultural biomass

is essential to determine the various pretreatments on biomass used as feedstock for biofuel industry (Adapa *et al*., 2011).

The characterization of bagasse was carried out to determine the bioethanol production potential. Total carbohydrate (65per cent), lignin (18.4per cent) Glucose (38.1per cent), Xylose (23.3per cent) were estimated in the study (Pandey *et al*., 2000; Kim and Dale, 2004; Georgieva *et al*., 2008).

Corredor *et al*. (2009) evaluated and characterized the forage sorghum as feedstock for fermentable sugar production. Moisture content (8 per cent), total carbohydrate (59-66 per cent), cellulose (24-38 per cent), hemicelluloses (12-22 per cent) and lignin (17-20 per cent) were noticed on chemical composition analysis of the feedstock.

Mohapatra *et al*. (2010) characterised different parts of banana viz., banana fruits, peel, leaves, pseudostem, sheath etc. for their utilisation in different industries including ethanol production.

Compositional analysis of pineapple wastes was 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. The dry peel waste gave a moisture content (92.2 per cent), total solids (7.8 per cent), pH (4.7), cellulose (19.8 per cent) and hemicelluloses (11 per cent).

Arumugam and Manikandan (2011) evaluated the chemical composition of fruit wastes (pulp and peels) of Banana and Mango in order to explore their potential application in bio ethanol production.

The moisture content, total solids, reducing sugars, non-reducing sugars, cellulose and lignin content of fresh and dry peel of mango was estimated in a study on the production of ethanol from mango peel. The moisture content, total

solids, reducing sugars, non-reducing sugars and cellulose and lignin content of fresh peel were estimated as 70 per cent, 25.6 per cent, 7 per cent, 5.9 per cent and 25.2 per cent respectively and those for dry peel were 10 per cent, 70.5 per cent, 30 per cent, 4.3 per cent and 23 per cent respectively (Reddy *et al*., 2011).

Shilpa *et al*. (2013) reported sugar content of peel waste of pineapple (31.53g/l), banana (32.21g/l), orange (37.00 g/l) and pea (31.53g/l) during the assessment of their alcohol production efficiency.

2.2.2 Pretreatment of the feedstock

The most important processing challenge in the production of biofuel is pretreatment of the biomass. Lignocellulosic biomass is composed of three main constituents namely hemicellulose, lignin and cellulose. Pretreatment involves 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 during hydrolysis (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).

Goals of an effective pretreatment process are (i) formation of sugars directly or subsequently by hydrolysis (ii) to avoid loss and/or degradation of sugars formed (iii) to limit formation of inhibitory products (iv) to reduce energy demands and (v) to minimize costs. Physical, chemical, physicochemical and

biological treatments are the four fundamental types of pretreatment techniques employed. In general a combination of these processes is used in the pretreatment step (Sarkar *et al*., 2012)**.**

The chemical and physico- chemical pretreatments are normally preceded by mechanical breakdown to fine pieces, due to the bulkiness of lignocellulosic materials such as agricultural wastes. These can be broken by a combination of chipping, grinding and milling to reduce cellulose crystallinity. Agricultural biomass was broken into small pieces of 2mm length (Jaafaru and Fagade, 2007), 0.5mm (Ojumu *et al.,* 2003) and into powdered form (Vyas *et al.,* 2005) before pre-treatments.

Pretreatment of naturally resistant cellulosic materials is essential to achieve high yields from biological operations; this operation is projected to be the single, most expensive processing step, representing about 20 per cent of the total cost.

Pretreatment has pervasive impacts on all the major operations in the overall conversion scheme from choice of feedstock through to size reduction, hydrolysis, and fermentation, and on to product recovery, residue processing, and co-product potential. Different pretreatments involve biological, chemical, physical, and thermal approaches but those that employ chemicals offer the high yields and low costs vital to economic success. Among the most promising are pretreatments using dilute acid, sulfur dioxide, alkaline hydrogen peroxide, near-neutral pH control, ammonia expansion, aqueous ammonia, and lime, with significant differences among the sugar-release patterns (Yang and Wyman, 2008; Lalitha and Sivaraj, 2011).

2.2.2.1 Acid pre-treatment 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. 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 (Balat *et al*., 2008; Cardona *et al*., 2010).

However, acid pretreatment results 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. Moiser *et al*., (2005) reported higher hydrolysis yield from lignocellulose pretreated with diluted H_2SO_4 compared to other acids. A saccharification yield of 74per cent was obtained from wheat straw when subjected to 0.75 per cent v/v of H_2SO_4 (Saha, 2005). Boopathy (2005) reported an ethanol yield of 11g/l from acid pretreated cotton waste. The optimal acid pre-treatment was found to be 0.2 mol/L of H_2SO_4.

Patle and Lal (2007) reported that acid hydrolysis of fruit and vegetable residue with 0.75per cent (v/v) H_2SO_4 which yielded 49-84 g l^{-1} reducing sugars and 29-32 g l^{-1} ethanol.

Lalitha and Sivaraj (2011) carried out a study for determining the optimal pretreatment conditions for high efficiency ethanol production from the fruit biomass peel residue. The residue was subjected to sulphuric acid pretreatments, which process effectively removed lignin. An ethanol yield 12 g l^{-1} was obtained with acid treatment 0.2 mol l^{-1} H_2SO_4 and fermenting for 15 days.

In a study to determine the influence of different pretreatment methods on sugar conversion and ethanol production of rye straw nitric acid pretreatment gave highest cellulose to glucose conversion rate of 324 g/kg and highest ethanol yield of 96.9 g/kg compared to pre treatments with sulphuric acid and potassium hydroxide (Tutt *et al*., 2012).

Pre-treatment with dilute acids though, significantly improved the cellulose hydrolysis, a neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes. However, this method of biomass pre-treatment has lost popularity due to production of several potent inhibitors such as furfural and hydroxymethyl furfural (Palmqvist and Harn-Hagerdal, 2000).

2.2.2.2 Alkaline pre-treatment 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. Hydroxides of sodium, potassium, calcium and ammonium and alkaline hydrogen peroxide are used in this process.

Oxidative delignification lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of hydrogen peroxide (Azzam, 1989). The susceptibility of cane bagasse to enzymatic hydrolysis was significantly enhanced by pre-treatment with hydrogen peroxide. About 50per cent lignin and most hemicelluloses were solubilized by 2 per cent H_2O_2 at 30°C within 48 hrs, and 95per cent efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45°C for 24 hrs (Azzam, 1989). Bjerre *et al*. (1996) used wet oxidation and alkaline hydrolysis of wheat straw to achieve 85per cent conversion yield of cellulose to glucose.

Sun *et al.* (1995) studied the effectiveness of different alkaline solutions by analyzing the delignification and dissolution of hemicellulose in wheat straw. They found that the optimal process condition was that using 1.5 per cent sodium hydroxide for 144 h at 20°C, releasing 60 per cent and 80 per cent lignin and

hemicellulose respectively. Sodium hydroxide (NaOH) has been reported to increase hardwood digestibility from 14 per cent to 55 per cent by reducing lignin content from 24-55 per cent to 20 per cent. Dilute sodium hydroxide 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) carried out alkaline hydrolysis of fruit and vegetable residue with 4 per cent (v/v) NaOH and yielded 49-84 g l^{-1} reducing sugars and 29-32 g l^{-1} ethanol.

Alkaline pretreatment of oil plam empty fruit bunch fiber was conducted to improve enzymatic saccharification for ethanol production. Alkali pretreatment using 1N NaOH at 30^{o}C and 90 minutes was found to be optimum resulting in a loss of 45.8 per cent and 35.6 per cent, lignin and hemicellulose respectively (Sudiyani *et al*., 2010)

Lalitha (2011) obtained an ethanol yield of 11.5 mgl^{-1} when fruit peel biomass was subjected to pretreatment with 2per cent alkaline hydrogen peroxide at pH 13. After 8 h of treatment, 45 per cent lignin was removed.

Alkali pretreatment is a relatively cheap and preferred method of biomass pretreatment in the lignocelluloses ethanol process since it is not accompanied with production of inhibitors. The effect is however dependent on the lignin content of the material. In general, alkaline pretreatment is more effective on hardwood, herbaceous crops, and agricultural residues with low lignin content than softwood with high lignin content (McMillan, 1994)

2.2.3 Fermentation of the feed stock

The hydrolysed or saccharified biomass is used for fermentation by several microorganisms. 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). An ideal microorganism for a commercially viable ethanol production method, should have broad substrate utilization, high ethanol yield and productivity, ability to withstand high concentrations of ethanol and high temperature, tolerance to inhibitors present in hydrolysate and cellulolytic activity (Sarkar *et al*., 2012)

Hydrolysate obtained after pretreatment need to be subjected for fermentation by microorganisms as it contains glucose and different mono saccharides such as xylose, galactose, mannose, arabinose and oligosaccharides. (Katahira *et al.*, 2006). A number of microorganisms can bring about fermentation resulting in significant production of bioethanol (Steward and Russell, 1987).

Xylose fermenting microorganisms include bacteria, yeasts and filamentous fungi (Hahn-Hagerdal *et al.*, 2006). Microorganisms utilize carbohydrates with 6 carbon atoms, one of the most common being glucose. Cellulosic materials containing high levels of glucose or glucose precursors are most easily converted into bioethanol (Balat *et al.,* 2008).

The yeasts, particularly members of the genera *Saccharomyces, Schizosaccharomyces, Kluyveromyces, Candida* and *Pachysolen* are of primary interest to industrial operations in fermentation of ethanol. Although many microbes are used in ethanol production, the yeast species *Saccharomyces cerevisiae,* which has a high tolerance to ethanol and other inhibitory compounds is primarily used in the industry (Bai *et al*., 2008). It has long been used to produce ethanol from hexoses. It can yield up to 18per cent ethanol; it utilizes both monosaccharide such as glucose and disaccharides including sucrose. (Lin and Tanaka, 2006).

Although *Saccharomyces cerevisae* is the organism of choice as the fermentation process biocatalyst in fuel ethanol industry, others such as *S. uvarum, Schizosaccharomyces pombe, Kluyveromyces fragilis, Kluyveromyces marxianus, Candida utilis* (Vallet *et al*., 1996), *Pachysolen tannophilus* (Sanchez *et al*., 1999) are also known to ferment glucose efficiently to ethanol.

Saccharomyces cerevisiae was utilized for ethanol production from *Washingtonia robusta* fruits by Mazmanci (2011). Raikar (2012) reported the production of ethanol from grape waste using *Saccharomyces cerevisiae* (baker's yeast). The production of ethanol was increased with the progress of fermentation and reached the maximum value at 48 h of fermentation, which was confirmed with the continuous decrease in the value of specific gravity of fermented sample.

Ethanologenic bacteria which are used for bioethanol production include *Escherichia coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* (Dien *et al.*, 2003). *Zymomonas mobilis* serves as a biocatalyst for ethanol production. It has a higher ethanol yield (5-10 percent more ethanol per fermented glucose) and has 2.5 times higher specific ethanol productivity than *Saccharomyces* sp. (Sprenger 1996). Lin and Tanaka (2006) opined that the high ethanol yield and productivity observed for *Zymomonas* are a consequence of its unique physiology wherein it metabolizes glucose anaerobically using the ED pathway. Hahn-Hagerdal *et al.*, (2006) opined that *Zymomonas mobilis* can efficiently produce ethanol from hexose, but not from pentose.

2.3 Enhancement of alcohol production

2.3.1 Isolation of native microorganism for cellulase activity and its characteristaion

Cellulose is a linear polysaccharide of glucose residues with β-1, 4-glycosidic linkages. Abundant availability of cellulose makes it an attractive raw material for producing bioethanol. With the help of cellulolytic system, cellulose can be converted to glucose. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system. Source for cellulase system is from microbial system thriving on cellulosic biomasses as their major feed (Gupta *et al*., 2012).

Cellulases are secreted by a number of fungi, bacteria and protozoa. The enzymes from fungi and bacteria are preferred due to the ease of cultivating the organisms *in vitro*. Bacteria have a great potential in the cellulase enzyme industry because they grow rapidly and their cellulases are often more complex providing increased function and synergy (Maki *et al*., 2009).

The cellulolytic enzymes of *Bacillus sp*. have potential use in the conversion of agricultural wastes into useful products (Ozaki *et al*., 1990). Chundakkadu (1999) reported efficient cellulase activity of *Bacillus subtilis CBTK 106* on banana waste. Muhammad Salem Akhtarif *et al*. (2001) confirmed the saccharification potential of *B. subtilis* in wheat straw, rice straw and bagasse. Production of cellulase using carboxy methyl cellulose as substrate by *B.pumilus EB3* was studied by Ariffin *et al*. (2006).

Cellulase activity is mainly evaluated using a reducing sugar assay to measure the end products of cellulase hydrolysis activities. Thus, the results of such an assay are typically expressed as the hydrolysis capacity of the enzymes (Dashtaban *et al*., 2009).

Microorganisms were identified conventionally using morphological and biochemical characterization. Later, full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant microorganisms.

Basavaraj *et al.,* (2014) isolated and characterized 57 microorganisms from soil based on morphology and biochemical characterization such as Methyl red test, Voges Proskauer test, Citrate utilization test, Starch hydrolysis test, Gelatin hydrolysis test, Nitrate reduction test, Catalase test, Oxidase test, Glucose fermentation test, Lactose fermentation test and Indole test, Urea hydrolysis test, H2 S production test. Among all isolated strains, maximum cellulase activity were showed by *Bacillus cereus* (0.440 IU/ml/min and 0.410 IU/ml/min), followed by *Bacillus subtilis* (0.357 IU/ml/min) and *Bacillus thuringiensis* (0.334 IU/ml/min).

Later, full and partial 16S rRNA gene sequencing methods have emerged as useful tools for identifying phenotypically aberrant microorganisms. The analysis of the nucleotide sequences of the 16S ribosomal RNA gene (16S rRNA), which has emerged as the single best method to identify bacteria (Kolbert and Persing, 1999; Drancourt *et al*., 2000) The reasons for using 16S rRNA gene sequences to study bacterial phylogeny and taxonomy 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 (Patel, 2001).

16S rRNA sequencing has been used for characterization of bacterial species *Aeromonas veronii* and *Pseudomonas putida* by Singh *et al*. (2011) and Kumari *et al.* (2012). Yadav *et al*. (2012) carried out molecular characterization of cellulose degrading bacterial flora from wood sample by analysis of 16S rRNA sequence.

2.3.2 Fermentation of the feedstock using mixed cultures of microorganisms

Microorganisms usually applied for bioethanol production cannot utilize all the sugar sources derived from hydrolysis. For example, the wild-type strain of *S. cerevisiae* is unable to use pentose, and this represents a waste of biomass and reduces the bioethanol yield. A microbial consortium or cellulosic enzyme cocktails during fermentation could convert a wide range of both hexoses and pentoses.

Ban-koffi and Han (1990) reported that pineapple waste when fermented using *Zymomonas mobilis* and *Saccharomyces cerevisiae* gave 8 percent ethanol in 48h.

Zayed and Meyer (1996) obtained an ethanol yield of 16.9per cent from wheat straw employing the fungus *Trichoderma viride* and the yeast *Pachysolen tannophylus* by single batch bioconversion (SBB) process. In SSB process, fermentation is allowed only after the termination of enzyme production along with enzymatic hydrolysis of pretreated biomass in the same culture broth. The enzymatic hydrolysis is terminated by heat-inactivation.

Krishna *et al*. (1998) carried out ethanol production by simultaneous saccharification and fermentation (SSF) of sugarcane leaves using *T. reesei* and S. cerevisiae. The SSF processes, firstly described by Takagi *et al*. (1977) combines enzymatic hydrolysis of cellulose with simultaneous fermentation of its main derived sugar (glucose) to ethanol. 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. In addition, the presence of ethanol causes the medium to be less vulnerable to invasion by undesired microorganisms (Ballesteros *et al*., 2004; Olofsson *et al*., 2008).

Patle and Lal (2007) reported ethanol production from hydrolysed agricultural wastes using mixed culture of *Zymomonas mobilis* and *Candida tropicalis.*

Mishima *et al*. (2008) reported an ethanol yield of 0.14g/g dry substrtae through simultaneous saccharification and fermentation (SSF) of pretreated water hyacinth using commercial cellulase and *S. cerevisiae.*

Gupta *et al*. (2009) resorted to separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *S. cerevisiae* and *Pichia stipitis*. The fermentation of both acid and enzymatic hydrolysates, containing 18.24 g/L and 37.47 g/L sugars, with Pichia stipitis and Saccharomyces cerevisiae produced 7.13 g/L and 18.52 g/L of ethanol with corresponding yield of 0.39 g/g and 0.49 g/g, respectively.

According to Tomas-Pejo *et al*., (2008), in SHF process, enzymatic hydrolysis is performed separately from the fermentation step. Since hydrolysis and fermentation occur separately, each step can be performed at optimum conditions. It enables enzymes to operate at optimum activities to produce more substrates for yeast fermentation. The optimum temperature for cellulase is usually between 45 and 50 °C, depending on the cellulose-producing microorganism whereas; the optimum temperature for most of the ethanol producing microorganisms is between 30 and 37°C. (Saha *et al*., 2005; Olsson *et al*., 2006).

Mukhopadhayay and Chatterjee (2010) used *Saccahromyces cerevisiae* and *Pachysolen tannophilus* for bioconversion of water hyacinth hydrolysate yielding 0.21 g/g of ethanol. *S. cerevisiae* was employed for fermentation of hexose sugars in the hydrolysate. Since xylose was also present as second predominant sugar *P. tannophilus* was used in addition to make biomass to ethanol process more economical.

Mishra (2012) investigated the ethanol production from various agro fruit residues by using *Saccharomyces cerevisiae and Candida albicans* during fermentation.

Itelima *et al*., (2013) reported bioethanol production from banana, plantain and pineapple peels by SSF Process using a mixture of starch digesting fungus A. niger and non starch digesting sugar fermenter (*S. cerevisiae*). Ethanol yield of three substrates were 8.34per cent (v/v) for pineapple peel, 7.45per cent (v/v) for banana peel and 3.98per cent (v/v) for plantain peel.

2.4 Pineapple as a feedstock for bioethanol production

One of major challenges faced by fruits and vegetable processing industries is waste disposal or rather its utilisation. Waste from pineapple processing units could be utilized for industrial purposes *viz*. fermentation, extraction of bioactive components, extraction of functional ingredients etc. It is utilised as a low-cost raw material for the production of ethanol, phenolic anti-oxidants, organic acids, biogas and fiber production (Upadhyay *et al.* 2010).. Pertinent scientific and technological implications would produce better and more profitable markets for pineapple wastes.

Pineapple waste contains many reusable substances of high value. The wastes from canneries have high exploitation potential with encouraging future. New and emerging technologies, such as green technology for biogas or bioethanol production is highly likely with pineapple residues (Upadhyay *et al.,* 2010).

Golden sweet pineapples were used in the study by De pradose *et al.* (2010). In their study pineapple waste (core and peel) was separated, blended and characterized in terms of total soluble solids (TSS) by refractometry, fermentable sugars by ionic chromatography (sucrose, glucose and fructose), pH and water content. The pineapple waste was pressed in pilot plant scale pneumatic equipment, which yielded a liquid phase and a press cake. Both streams (core and peel) were also characterized in terms of TSS, pH and sugar content and moisture content.

Ban-koffi and Han, (1990) reported that pineapple waste consist of 19 per cent cellulose, 22 per cent hemicelluloses, 5 per cent lignin and 53 per cent cell soluble matters but However, relatively low levels of fermentable sugars (11.7 per cent total sugar) and high amounts of fiber concentration necessitated the pre treatment of the substrate which increased the sugar content. Initial sugar concentration of more than 20 per cent is the necessary minimum for economic alcohol fermentation.

In a study, De Prados *et al.* (2010) obtained 12-15per cent ethanol from pineapple waste by simultaneous saccharification and fermentation (SSF) and concluded that industrial pineapple waste as a feasible source to produce bioethanol which would contribute to valorisation of pineapple industrial residues.

Hossain and Fazliny (2010) used *Saccharomyces cerevisiae* for ethanol production from rotten pineapples waste through fermentation. Nadya *et al*. (2012) could obtain 8.637 per cent ethanol from pineapple peel extract using *Saccharomyces ellipsoideus.* Shilpa *et al.* (2013) reported that pineapple peel yielded 8.34per cent of alcohol using *Saccharomyces cerevisiae* and *Aspergillus niger.*

2.5 Statitical analysis

Saliu (2012) used Duncan Multiple Range Test for analysis of data obtained and the data were subjected to analysis of variance and the sample means tested for significant differences. This was carried out with the statistical package, SPSS 15.0. and graphs were plotted with Microsoft Excel. Wong and Sanggari (2014) used one way analysis of variance used one way analysis in anova for optimizing the parameters of fermentation**.**

Materials and methods

3. MATERIALS AND METHODS

The present study entitled "Utilization of pineapple (*Ananas comosus* (L.) Merr.) biomass for biofuel production" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period from 2013-14. The investigation was carried out in two phase *viz*., bioethanol production from pineapple biomass and enhancement of bioethanol production. The details of experimental materials and methodology adopted for the study are presented in this chapter.

3.1 BIOETHANOL PRODUCTION FROM PINEAPPLE BIOMASS

3.1.1 Characterisation of the feedstock for alcohol production

Sample collection and preparation

Ripe pineapple samples (*Ananas comosus* (L.) Merr.) cv. Mauritius (40 numbers) were collected from a pineapple plantation located at Cheriyakolla, Thiruvananthapuram. The three types of feed stocks viz., pineapple peel waste, pineapple fruit waste and pineapple plant residue were used for the study.

Pineapple fruits weighing 1300 g-1600 g was thoroughly washed in tap water. The fruit were peeled to an approximate thickness of 2 cm using a clean knife to prepare the peel waste. The peeled fruit samples were chopped in to small pieces and ground thoroughly in an electric grinder. The fruit pulp thus obtained was sieved through a clean plastic mesh of pore size 1 mm to obtain the fruit waste. The fresh leaves were collected and chopped to a size of 1 cm^2 to prepare the plant residue.

The peel waste, fruit waste and plant residue thus obtained were packed separately in paper covers and oven dried at a temperature of 60^0C till constant drying percent was obtained. The oven dried samples were finely ground and stored in air tight containers for further analysis and characterization of feedstock.

3.1.1.1 Moisture content

The moisture content of the samples were estimated using gravimetric method (AOAC, 2000). Ten gram of the wet sample was taken in a petriplate and left open in the oven at a temperature of 60°C until constant moisture per cent was obtained. Five replications were performed.

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 solution and an Ec. value was noted from the display unit.

3.1.1.3 Total carbohydrates

Total carbohydrate is estimated by Anthrone method (Hedge and Hofreiter, 1962). The oven dried sample, 100 mg was taken in a boiling tube, 5 ml of 2.5N HCl was added and kept in a boiling water bath for 3 hrs for hydrolysis. It was then neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml, transferred to 15 ml centrifuge tubes and centrifuged at 4500 rpm in refrigerated centrifuge for 5 minutes. The supernatant was collected and 1 ml aliquot was taken for analysis. The standard solutions were prepared using 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard, 0 ml was kept as a blank and the volume was made up to 1 ml using distilled water in all the tubes. Then 4 ml of ice cold anthrone reagent (Appendix-I d) was added to the tubes which were prior cooled on ice. These were then heated for 8 min in a boiling water bath. The samples were cooled and absorbance at 630nm was recorded using UV-visible spectrophotometer. The standard graph was prepared by plotting concentration of the standard on X-axis and absorbance on Y-axis. The amount of carbohydrate present in the sample was calculated using the following formula from the graph.

Calculation

Amount of carbohydrate present in 100 mg of the sample =

$$\frac{\text{mg of glucose as read from std. graph X100}}{\text{Volume of test sample}}$$

3.1.1.4 Total dissolved solids

Total dissolved solids 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 solution and Ec. 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 per cent alcohol twice by using 5 ml every time. The supernatant was collected and evaporated on water bath. 10 ml of distilled 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 hydrolysed by heating at 49 ºC 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 esimated by DNS method. Total non-reducing sugar of the raw, acid treated and alkali treated feed stocks was estimated by this method.

Calculation

Non-reducing sugars

$$= \frac{\text{Sugar value from graph (µg) x Total vol. of extract (10 mL)}}{\text{Aliquot sample (1 mL) x Wt. of sample (100 mg) x1000}}$$

3.1.1.6 Sucrose

Sucrose content was estimated by redox titration method (KAU, 2012) by using Fehling's solution A and B (Appendix-I a).

To 2 g 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 conc. 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. About 5 ml of Fehling`s solutions A and B were pipetted out into a clean conical flask and diluted with 10 ml 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 1 ml at a time. After each dilution of sample solution a few seconds were allowed for the reduction to take place. When the blue colour 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 colour appeared.

Calculation

Let the volume of the made up solution required to react with 10 ml of Fehling’s solution be V ml.

V ml of this solution contains enough glucose to react with 10 ml of Fehling’s solution.

10 ml of Fehling’s solution = 0.05 gm of monosaccharides

Wt. of monosaccharides in 250 ml of

the made up solution $= \frac{250 \times 0.05 \text{ gm}}{V}$

Weight of glucose and fructose in

100 ml of the original sucrose solutions $= \frac{250 \times 0.05 \times 100 \text{ gm}}{V \times 20}$

1 gm of the monosaccharides is equivalent to 0.95 gm of sucrose.

Weight of 100 ml of the given solution $= \frac{250 \times 0.05 \times 5 \times 0.95 \text{gm} \times Df}{V}$

Df - Dilution factor

3.1.1.7 Estimation of total reducing sugar

Total reducing sugar was estimated by using DNS method (Miller, 1972; sadasivam and Manickam, 1992). About 100 mg of sample was weighed and the sugars were extracted with hot 80 per cent ethanol twice by using 5 ml every time. The supernatant was collected and evaporated it by keeping it on a water bath at 80°C. 10 ml distilled water was added to the above reaction mixture to dissolve the sugars. 2 ml of extract was pipetted out in 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 f) was added. 1 ml of 40 percent Rochelle salt solution was also added to the warm tubes containing the reaction mixture. 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 dark red colour was read at 510 nm in a UV-visible spectrophotometer (spectronic gene sys 5). The amount of reducing sugars present in the sample was calculated using standard graph. Total reducing sugar of the raw, acid pretreated and alkali pretreated feed stocks was estimated by this method.

3.1.1.8 Glucose and Xylose

The glucose content of the feed stocks were estimated by redox titration method (KAU, 2012) using Fehling's solution A and B (Appendix-I a). To 2 g 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 untill 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 1 ml at a time. After each dilution of sample solution few seconds were allowed for the reduction to take place. When the blue colour of the Fehling's solution nearly faded, 3 drops of 1 percent 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 the blue colour of indicator and the appearance of bright red colour 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

Let the volume of glucose reacting with 10 ml of Fehling's solutions be V ml.

V ml of the solution contains enough glucose to react with 10 ml Fehling's solutions.

10 ml of Fehling's solution = 0.05 gm of glucose

That is V ml of the given solution contains 0.05 gm glucose

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

3.1.1.9 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 b) was added. Then 7 ml of diluted HCl was added. 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard was pipette 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^{o}C for exactly 10 min. The tubes were removed and cooled by immersing in tap water for 5 min. 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.

3.1.1.10 Cellulose

Cellulose was estimated by spectrophotometric method (Updegroff, 1969). Initially 0.5g of the sample taken in a test tube and 3ml 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 (Hermle Z383 K) 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. 1 ml of the above solution was diluted to 100 ml. To 1 ml of this diluted solution, 10 ml of anthrone reagent was added with proper mixing. The tubes were heated in a boiling water-bath for 10 min. The intensity of the colour was measured at 630 nm in a UV-visible spectrophotometer (Spectronic Gene Sys 5). 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.

3.1.1.11 Hemicellulose

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

Estimation of Neutral Detergent Fibre

One gram of the powdered sample taken in a refluxing flask 10 ml of cold neutral detergent solution (Appendix-I c), 2 ml of decahydro naphthalene and 0.5 g sodium sulphite was added. 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° C for 8 hrs. 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

One gram of powdered sample was taken in a round bottom flask containing 100 ml of acid detergent solution (Appendix-I c). Then it was heated to boil in 5-10 min. When the mixture started to boil, heat was reduced to avoid foaming. It was 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 100ºC for overnight. The contents 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 percentage = Neutral detergent fiber (NDF) percentage - Acid detergent fiber (ADF) percentage

3.1.1.12 Lignin

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

Acid Detergent Fiber (ADF)

One gram of powdered sample was taken in a round bottom flask and 100 ml of acid detergent solution (Appendix-I c) was added. Then it was heated to boil in 5-10 min. The heat was reduced to avoid foaming when it started to boil. Then it was refluxed for 1h 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 become colorless. The contents were dried at 100^0C 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 Fiber (ADF) 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 3h 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 2h. 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 3h. The crucible was cooled in a desiccator and weighed. The ash content was calculated. For blank taken 1 g asbestos, and 72 percent H_2SO_4 was added and the same steps for sample were followed.

Calculation

$$\text{Percentage of ADL} = \frac{(\text{Weight 72 per cent } H_2SO_4 \text{ washed fiber} - \text{Ash}) \times 100}{\text{Weight of sample}}$$

3.1.2 Pretreatment 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

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

3.1.2.2 Alkaline hydrolysis

Five gram of the sample was weighed in a culture bottle. Approximately 40 ml of 2 per cent 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 by following the procedures 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* and *Zymomonas mobilis.* 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 pineapple peel, pineapple fruit waste and pineapple plant residue was taken in a 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 (biotech system yorco). Under

sterile conditions in a laminar air flow cabinet (Clean air systems CAH-900) *Saccharomyces cerevisiae* and *Zymomonas mobilis* were inoculated to the samples for fermentation in separate flasks, five replications were performed. This was incubated for 6 days at 37°C by continuous shaking in a shaking incubator (pelican equipments) with 60 rpm. After incubation the alcohol content was estimated. pH value pH/Conductivity meter (Eutech PC 510), 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. About 20ml 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 minutes. The flask removed from the water bath and 10ml of 0.5M KI was added. the contents were titrated with 0.1M sodium thiosulphate solution. When the brown color of the solution gets a green tinge, a few drops of 1% starch indicator was added which was prepared in boiling water. The addition of sodium thiosulphate solution was continued until the solution gets a clear, green-blue color which was the endpoint of titration.

Calculation

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

Where V was the burette reading

Extra moles of dichromate spent by thiosulphate = $n/6$

No. of moles of dichromate reacted to oxidize alcohol (n_1) = no. of moles added - moles spent by thiosulphate

No. of moles of alcohol = $3 \times n_1$

Volume of alcohol in the sample = $[3n_1 \times 58.6]$

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

Volume of in 100 ml diluted sample= $[(3n_1 \times 58.6) \times 50]$

Percentage of alcohol present in 10 ml of original sample =

$$[(3\ n_1 \times 58.6) \times 50 \times 10]$$

3.2 Enhancement of alcohol production

3.2.1 Isolation of native microorganism and its characterisation

Cellulolytic microorganisms were isolated from degraded samples of pineapple fruit biomass. Pineapple feed stocks collected from different sources were kept in different containers for one month. Microorganisms were isolated from degraded biomass using carboxy methyl cellulose (CMC) agar medium (Appendix-II c). Five replications were maintained for each sample. The plates were incubated for 48hrs. Single colonies were identified and transferred to slants. Cellulolytic microbial colonies were identified by confirmation test. Single colonies were flooded with Congo red (0.1 per cent) solution followed by addition of 1M NaCl after 15 minutes. The colonies showing clear zones were isolated and maintained the pure cultures. Morphological, biochemical and molecular characterization of the isolated native microorganism was done.

3.2.1.1 Morphological characterization

3.2.1.1.1 *Gram staining and Microscopic observation*

Single colony was selected from the pure culture maintained on CMC agar medium. Sterile water droplet was taken on a grease free glass slide and the above selected colony was transferred to the slide. The culture was thoroughly spread to

get a clear smear using sterile loop and heat fixed the smear. A drop of crystal violet was added to the smear and allowed to completely flooded over it. After one minute the crystal violet was washed out using tap water. Then a drop of Gram's iodine was added and kept for another one minute. The slide was again washed with tap water and flooded the slide with decolorizer for five seconds. The decolouriser was rinsed off with tap water. Then the slide was flooded with safranin and allowed to remain for thirty seconds and rinsed the slide with tap water. The slide was dried and placed in upright position and it was microscopically examined under oil immersion (100X) objective.

3.2.1.2 Biochemical characterization

3.2.1.2.1 Catalase test

The native micro organism 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 48 h the slant was scrapped with sterile non metallic instrument and was suspended in a drop of 3 per cent 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-IIe to IIi) containing the specific carbohydrates glucose, sucrose, lactose, mannitol and maltose respectively were prepared separately. Then a Durham tube was inserted to each tube in inverted position. The broth was sterilized by autoclaving in a horizontal type autoclave (biotech system yorco). 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 (biotech system yorco). Under sterile conditions it was inoculated with native microorganism from the pure culture maintained. An uninoculated tube was kept as control. All the tubes were incubated for 37 °C for 48h. 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***

The native organism was inoculated using sterile technique to MR-VP broth (Appendix-II m). An uninoculated tube was kept as control. All the tubes were incubated at 37 °C for 24h. After incubation 1 ml of methyl red indicator was added to all the tubes and observed the change in color of the broth.

3.2.1.2.3.3 ***Voges Proskauer test***

MR-VP broth (Appendix-II m) was prepared and sterilized by autoclaving in a horizontal type autoclave (biotech system yorco). Native organism was inoculated under sterile conditions in a laminar air flow cabinet (Clean air systems CAH-900). Control was maintained without inoculation. All the tubes were incubated at 37 °C for 24 h. After incubation 12 drops of 1percent alcoholic α-naphthol reagent and 6 drops of 40 percent potassium hydroxide were added in each tube and gently mixed. 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 *l*) slant tubes were prepared and autoclaved for sterilization. Under sterile conditions, the native organism was inoculated by the streak plate method. A slant was maintained as control without

inoculation. All the tubes were incubated for 48h 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) 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 hrs. Following 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 j) was prepared and sterilized by autoclaving in a horizontal type autoclave (biotech system yorco). It was cooled to 55 °C. 20 per cent 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 48h. After incubation the tubes were observed for pink colour 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 solubilised in 0.05 M phosphate buffer at pH 8 was estimated. The isolated native organism was inoculated on sterile pineapple fruit 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 (Eppendorf 5427 R). Clear supernatant was pipetted out which served as a crude enzyme source. This was added to 0.05 ml of 1percent CMC in 0.05 M phosphate buffer and incubated at 50 °C for 30 minutes taken in a test tube. The Test tube

without 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 1.5 ml DNS reagent followed by boiling at 100 °C in water bath (Labline SWB1) for 10 min. Sugar liberated was determined by measuring absorbance at 540 nm in a UV-visible spectrophotometer (spectronic gene sys 5). 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 (Qiagen).

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 (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

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 (Table1) and the template DNA.

Table 1. Primers used for PCR amplification

Target	**Primer Name**	**Direction**	**Sequence(5' → 3')**
16S rRNA	16S-UP-F	Forward	GAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG
		Reverse	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT
23S rRNA	16S-UP-R	Forward	GATGTGGAGTTGCTTAGACA
		Reverse	CTTTTATCCGTTGAGCGATG

The PCR amplification was carried out in a PCR thermal cycler (Bio Rad PDS 1000/HE system).

PCR amplification profile

16S rRNA/23S rRNA

95 ºC	-	5.00 min	
95 ºC	-	0.30 min	35 cycles
54 ºC	-	0.40 min	
72 ºC	-	1.00 min	
72 ºC	-	7.00 min	
4 ºC	-	∞	

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 (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).The PCR products were stored in -20 refrigerator (Samsung RS21HUTP1). This was sequenced from Regional DNA Finger printing laboratory Rajiv Gandhi Centre for Biotechnoloy, Thiruvananthapuram (Appendix III).

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

Three different modes of saccharification and fermentation processes tried to optimize the process for enhanced production of ethanol from pineapple

biomass. The microbes used in enhancement process were subjected to compatibility check.

3.2.2.1 Separate Hydrolysis and Fermentation (SHF)

Oven dried pineapple fruit waste was taken in 150ml screw capped conical flasks. 50 ml of distilled water was added; pH was adjusted to 7 and sterilized the feed stock by autoclaving in a horizontal type autoclave (biotech system yorco). Under sterile conditions in a laminar air flow cabinet (Clean air systems CAH-900) the isolated native microorganism was inoculated to the sample for saccharification and five replications were kept. The inoculated sample was incubated at 37 °C by continuous shaking in a shaking incubator (Pelican equipments) at 60 rpm for 6 days. After incubation 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 (Pelican equipments) at 60 rpm. After incubation the alcohol content was estimated.

3.2.2.2. Simultaneous Saccharification and Fermentation (SSF)

Oven dried pineapple fruit waste was taken in 150 ml srew capped conical flasks and 50 ml of distilled water was added. The samples were sterilized by autoclaving in a horizontal type autoclave (Biotech system yorco). Under sterile conditions the isolated native microorganism and *Saccharomyces cerevisiae* were inoculated to the sample for Simultaneous Saccharification and Fermentation in a laminar air flow cabinet (Clean air systems CAH-900) and kept five replications. The inoculated sample was incubated at 37 °C by continuous shaking in a shaking incubator (Pelican equipments) at 60 rpm for 6 days. After incubation the alcohol content was estimated.

3.2.2.3 Single Batch Bioconversion (SBB)

Oven dried pineapple fruit waste was taken in 150 ml screw capped conical flasks. 50 ml of distilled water is added and pH (pH/Conductivity meter (Eutech PC 510)) was adjusted to 7. The sample was sterilized by autoclaving in a

horizontal type autoclave (biotech system yorco). The isolated native microorganism was inoculated to the sample under sterile conditions for saccharification with five replications each. The inoculated sample was incubated at 37 °C by continuous shaking in a shaking incubator (Pelican equipments) for 6 days at 60 rpm. After incubation where the saccharification process was completed 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.

3.3 Statistical analysis

The experimental design followed in the study was completely randomized design, with five replicates. All data were expressed as mean values ± SE. The comparison between the mean values were tested using Duncan's multiple range test and the ANOVA was performed to find out the LSD ($p<0.05$).

Results

4. RESULTS

The present study entitled "Utilization of pineapple (*Ananas comosus* (L.) Merr.) biomass for biofuel production" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period from 2013-14. The investigation was carried out in two phase *viz*., bioethanol production from pineapple biomass and enhancement of bioethanol production. The results of the experiments are presented in this chapter.

4.1 BIOETHANOL PRODUCTION FROM PINEAPPLE BIOMASS

4.1.1 Collection and preparation of the feed stock

Ripened pineapple (*Ananas comosus* (L.) Merr.) cv. Mauritius (approximately 40 numbers) were collected from pineapple plantation located at Cheriyakolla, Thiruvananthapuram (Plate 1). The feed stocks pineapple peel, pineapple fruit waste and pineapple plant residue were separated (plate 2), oven dried and finely ground samples were stored in air tight containers for analysis (Plate 3).

4.1.2 Characterisation of the feedstock for alcohol production

Three types of feed stocks were separately blended and characterized for alcohol production and the results of the biochemical analysis are presented in tables 2 to 5.

4.1.2.1 Moisture content

The moisture content of the feed stocks pineapple peel, pineapple fruit waste and pineapple plant residues were estimated by standard methods prescribed by Association of Official Analytical Chemists (AOAC, 2000). Significant variation was found among the three feed stocks with respect to moisture content (Figure 1). The pineapple plant residue recorded maximum moisture content (84.54 per cent), followed by pineapple fruit waste

Plate 1: Collection of pineapples from farms

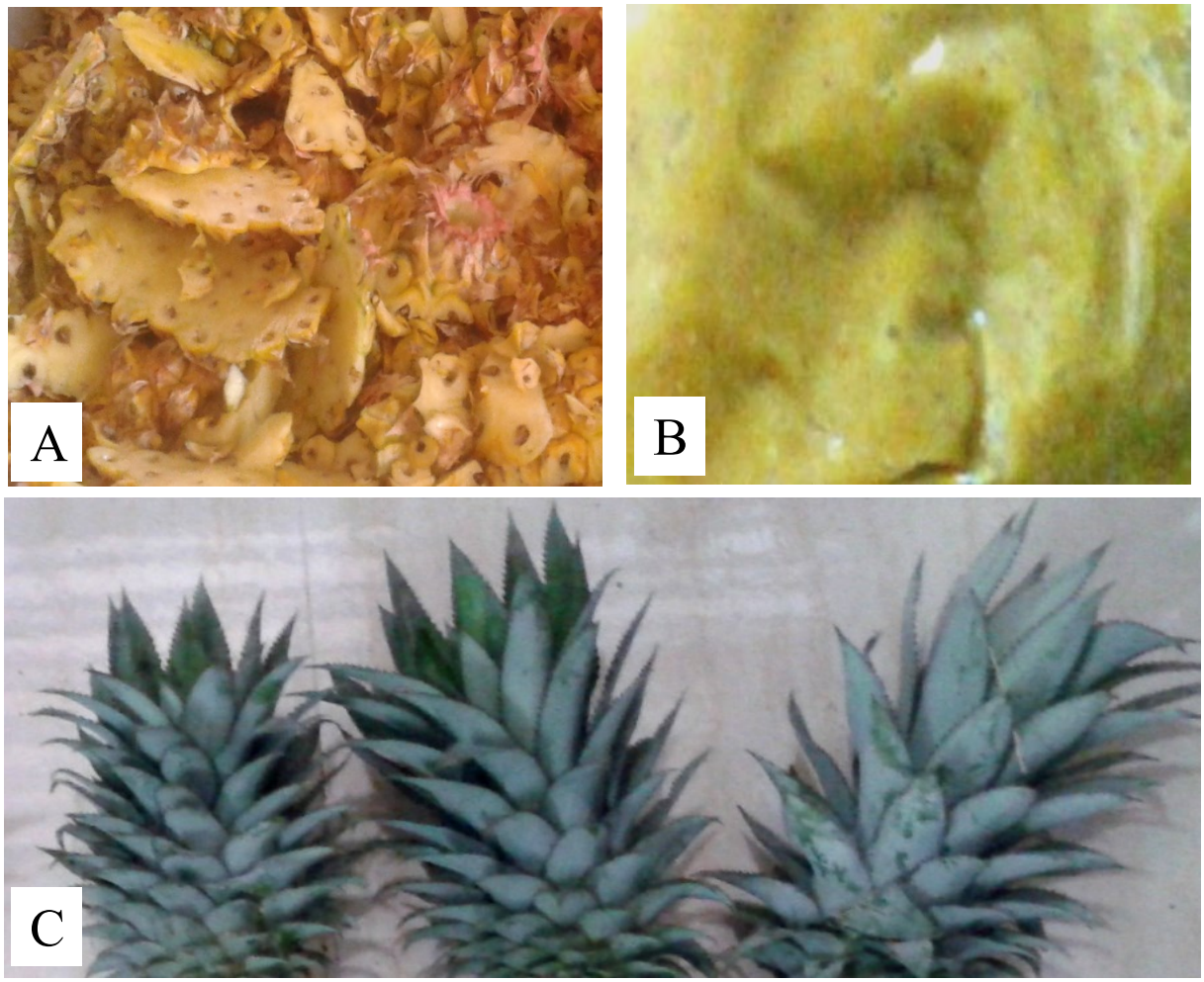

Plate 2: Pineapple feed stocks for the study (A) Pine apple peel (B) Pineapple fruit waste

(C) Pineapple plant residue

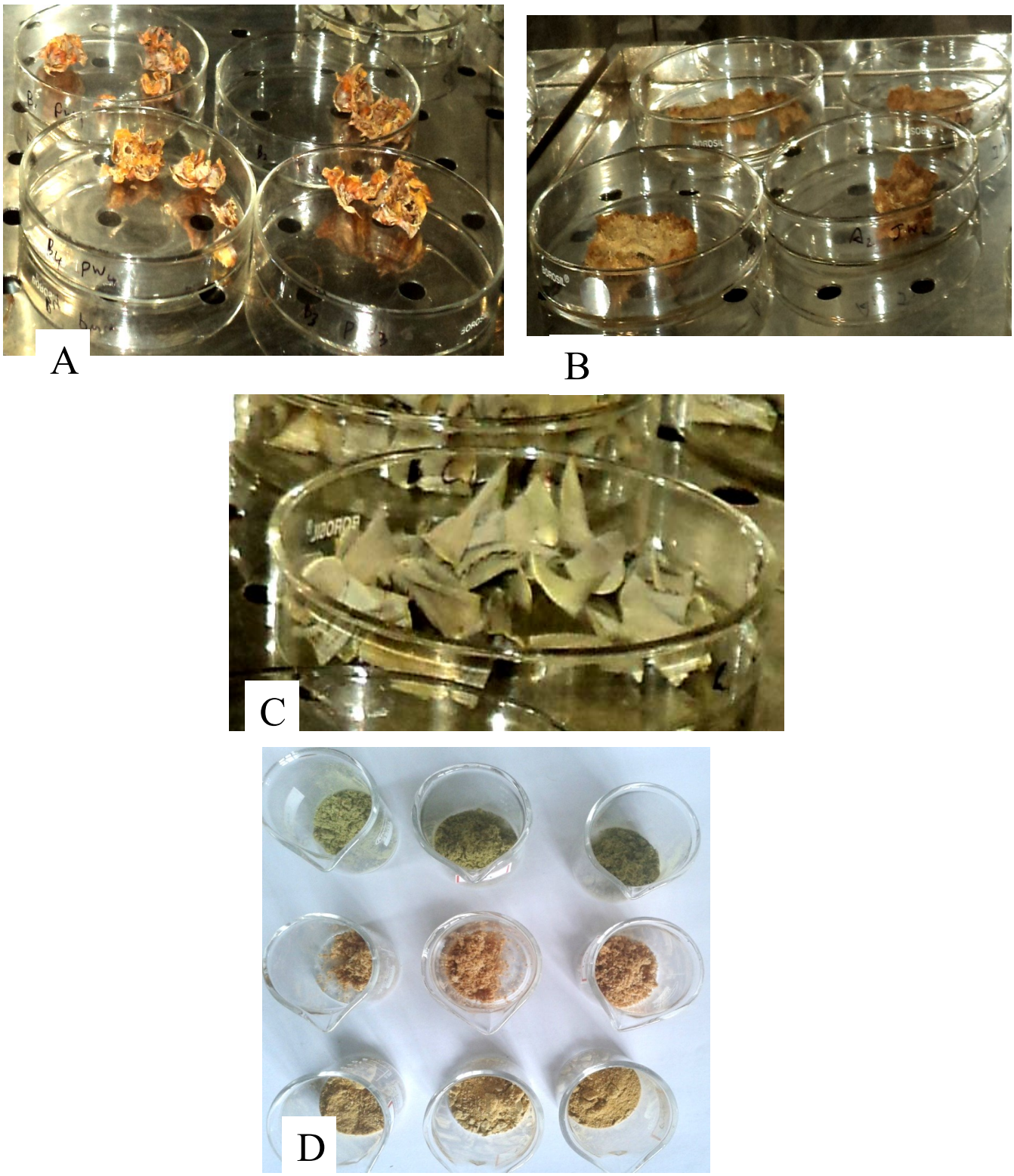

Plate 3: Sample preparation for analysis (A) Oven dried pineapple peel waste (B) Ovendried pineapple fruit waste (C) Oven dried pineapple plant residue (D) Ground samples for biochemical characterisation

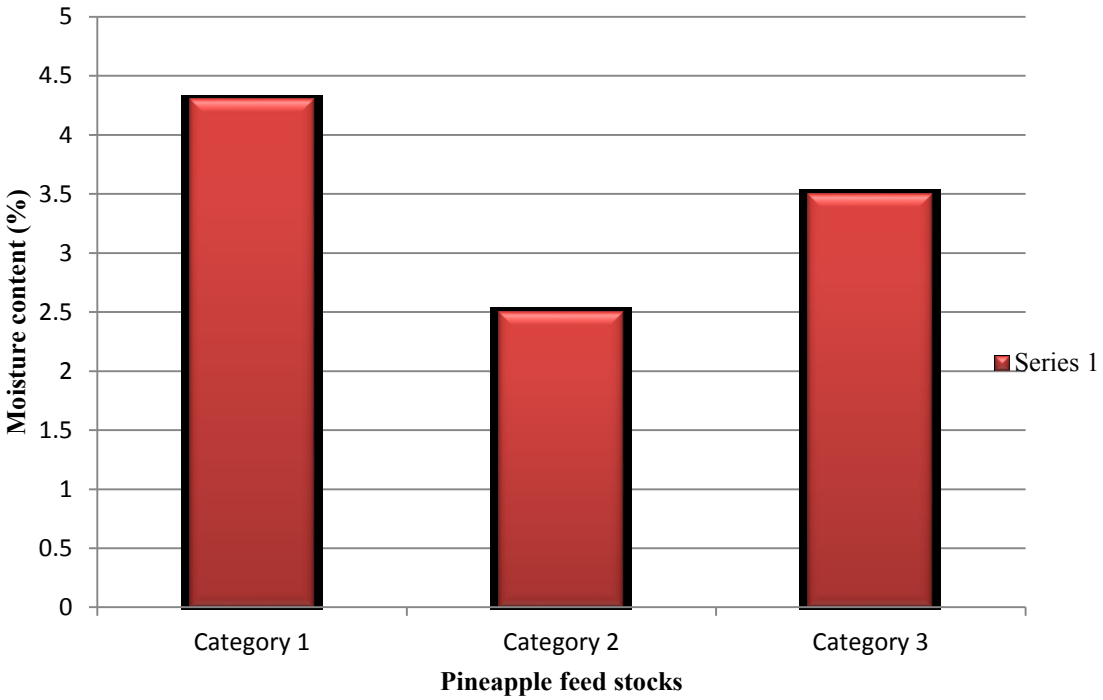

Figure 1: Moisture content of different feedstocks of pineapple.

(81.42 per cent). Among the three feed stocks, pineapple peel recorded the lowest moisture content (75.60 per cent).

4.1.2.2 Total soluble salts

Total soluble salt content of the samples were estimated by using the EC/ TDS meter. Significant variation was observed with respect to total soluble salt (TSS) content among the three feed stocks tried (Table 2). Pineapple peel recorded maximum TSS content (0.206 ppm). This was found to be on par with the TSS content (0.204 ppm) of pineapple fruit waste. Pineapple plant residue showed significantly lower TSS content (0.115 ppm).

4.1.2.3 Total carbohydrates

Total carbohydrate was estimated by Anthrone method (Hedge and Hofreiter, 1962). The three feed stocks used in the experiment showed significant variation with respect to total carbohydrates (Table 3). Pineapple fruit waste reported the maximum total carbohydrate content (70.68 percent). Which was followed by pineapple peel waste (60.43 percent) and pineapple plant residue which recorded the lowest value (25.63 per cent).

4.1.2.4 Total dissolved solids

Total dissolved solids was estimated by using EC /TDS meter apparatus. No significant variation was found with regard to total dissolved solids among the three feed stocks tried (Table 2). However the highest value was noticed in pineapple fruit waste (28.12 ppm). This was found to be on par with that of plant residue (27.44 ppm) and pineapple peel waste (27.24 ppm).

4.1.2.5 Total non reducing sugar

Total non reducing sugar was estimated by using DNS method (Malhothra and Sarkar, 1979). Significant variation was found among the three feed stocks with respect to the total non reducing sugar content (Table 3). The pineapple fruit waste recorded the maximum total reducing sugar content (18.2 per cent). This

was followed by pineapple peel waste (12.99 per cent). The pineapple plant residue recorded the lowest total non reducing sugar content of 5.21per cent.

4.1.2.6 Sucrose

Sucrose content was estimated by redox titration method by using Fehling's solution A and B (Appendix-I a). Sucrose content of the three feed stocks significantly varied (Table 4). The pineapple fruit waste showed maximum sucrose content (8.37 per cent). Which was followed by pineapple peel waste (2.96 per cent), while the lowest value of 0.21 per cent was recorded in pineapple plant residue.

4.1.2.7 Total reducing sugar

Total reducing sugar is estimated by using DNS method (Miller, 1972; Sadasivam and Manickam, 1992). Significant variation was found among the three feed stocks with respect to total reducing sugar content (Table 3). Maximum value for total reducing sugar content (27.2 per cent) was recorded in pineapple fruit waste, which was followed by pineapple peel waste (25.56 percent) while pineapple plant residue recorded the lowest value (11.72 percent).

4.1.2.8 Glucose and Xylose

The glucose content of the feed stocks was estimated by redox titration method using Fehling’s solution A and B (Appendix-I a). Glucose content showed significant variation among the three feed stocks used in the experiment (Table 4). The pineapple fruit waste showed the maximum glucose content (3.06 per cent), followed by pineapple peel waste (2.23 per cent). The pineapple plant residue was observed to record the lowest glucose content (0.76 per cent) among the three feedstocks.

Among the three feed stocks, significant variation was observed with respect to xylose content (Table 4). It was observed that the pineapple fruit waste recorded maximum xylose content (0.75 per cent), which was followed by

Table 2. Total soluble salts and total dissolved solids in different feed stocks of pineapple

Sl. No.	Feed stocks	Total soluble salts (ppm)	Total dissolved solids (ppm)
1	Pineapple fruit peel	0.204 ± 0.004	27.24 ± 0.23
2	Pineapple fruit waste	0.206 ± 0.004	28.12 ± 0.45
3	Pineapple plant residue	0.115 ± 0.002	27.44 ± 0.35
C.D. (0.05%)		0.004	0.53

Data represents the mean of five replications

Table 3. Total carbohydrate, total non reducing sugar and total reducing sugar content in different feed stocks of pineapple

Sl. No.	Feed stocks	Total carbohydrate (%)	Total non reducing sugar (%)	Total reducing sugar (%)
1.	Pineapple fruit peel	60.43 ± 0.81	12.99 ± 0.24	25.56 ± 0.54
2.	Pineapple fruit waste	70.68 ± 0.16	18.20 ± 0.17	27.20 ± 0.72
3.	Pineapple plant residue	25.63 ± 0.13	5.21 ± 0.07	11.72 ± 0.40
C.D.(0.05%)		0.59	0.23	0.42

Data represents the mean of five replications

pineapple peel waste (0.56 per cent). The pineapple plant residue was observed to record the lowest xylose content (0.14 per cent).

4.1.2.9 Fructose

Fructose content was estimated by spectrophotometric method by using resorcinol reagent (Ashwell, 1957). Significant variation was observed among the three feed stocks with respect to fructose content (Table 4). The maximum fructose content (2.05 per cent) was observed in the pineapple fruit waste, followed by pineapple peel waste (1.86 per cent), while pineapple plant residue recorded the lowest fructose content (0.78 per cent).

4.1.2.10 Cellulose

Cellulose was estimated by spectrophotometric method (Updegroff, 1969). Significant variation was found among the three feed stocks with respect to cellulose content (Table 5). The cellulose content was maximum in pineapple plant residue (69.20 per cent). This was followed by pineapple fruit waste (41.74 per cent), while the lowest value for cellulose content was observed in pineapple peel waste (18.83 per cent).

4.1.2.11 Hemicellulose

Hemicellulose was estimated by gravimetric method (Georing and Vansoest, 1975). Hemicellulose content showed significant variation among the three feed stocks (Table 5). The pineapple peel waste was observed to report maximum hemicellulose content (23.08 per cent) followed by pineapple plant residue (21.85 per cent). Pineapple fruit waste recorded the lowest hemicellulose content (20.47 per cent).

4.1.2.12 Lignin

Lignin was estimated by gravimetric method (Georing and Vansoest, 1975). Significant variation was observed with respect to lignin content of the different feed stocks used in the study as inferred from the Table 5. The highest

Table 4. Glucose, fructose, xylose and sucrose content in different feed stocks of pineapple

Sl. No.	Feed stocks	Glucose (%)	Fructose (%)	Xylose (%)	Sucrose (%)
1	Pineapple fruit peel	2.23 ± 0.71	1.86 ± 0.08	0.56 ± 0.02	2.96 ± 0.27
2	Pineapple fruit waste	3.06 ± 0.71	2.05 ± 0.06	0.75 ± 0.02	8.37 ± 0.07
3	Pineapple plant residue	0.76 ± 0.22	0.78 ± 0.02	0.14 ± 0.06	0.21 ± 0.02
C.D. (0.05%)		0.60	0.08	0.06	0.24

Data represents the mean of five replications

Table 5. Cellulose, hemicellulose and lignin content in different feed stocks of pineapple.

Sl. No.	Feed stocks	Cellulose (%)	Hemicellulose (%)	Lignin (%)
1	Pineapple fruit peel	18.83 ± 0.43	23.08 ± 0.11	6.17 ± 0.11
2	Pineapple fruit waste	41.74 ± 0.95	20.47 ± 0.12	3.75 ± 0.19
3	Pineapple plant residue	69.20 ± 0.22	21.85 ± 0.42	11.16 ± 0.41
C.D. (0.05%)		0.99	0.41	0.34

Data represents the mean of five replications

lignin content (11.16 per cent) was recorded in the pineapple plant residue. This was followed by pineapple peel waste (6.17 per cent). While the lowest value (3.75 per cent) was recorded in pineapple fruit waste.

4.1.3 Pretreatment of the feedstock

Pretreatments of the three feed stocks were carried out by treating with dilute acid (0.8 M sulphuric acid) and 2 per cent alkaline hydrogen peroxide (pH 13) for 48 h.

4.1.3.1 Characterisation of pretreated feedstock

4.1.3.1.1 *Total non reducing sugar*

Alkaline and acid pretreatment of feed stocks indicated an increase in total non reducing sugar content (Table 6), (Figure 2). Significant variation in the total non reducing sugar content was obtained for different feed stocks. In the untreated sample, highest value (18.20 per cent) was obtained in pineapple fruit waste followed by pineapple fruit peel (12.99 per cent). The lowest total non reducing sugar (5.21 per cent) was found in plant residue. Similar trend was noticed in alkaline and acid pretreated samples. Among the alkaline treated feedstock, fruit waste recorded 18.55 per cent total non reducing sugar and lowest was recorded in pine apple plant residue (5.75 per cent). On acid pretreatment, fruit waste recorded maximum total nonreducing sugar (18.92 per cent), among the feed stocks tried and the lowest value in plant residue (5.89 per cent).

Significant variation was observed with respect to percent saccharification to non reducing sugars among different pretreatents. Acid pretreatment gave more sacharification compared to alkali pretreatments. Among the feedstocks pineapple plant residue gave maximum saccharification followed by pineapple fruit peel and the lowest was recorded in fruit waste. Among the six treatments tried, maximum sachharification was found in acid treated pineapple plant residue (11.38 per cent) and the lowest saccharification (1.84 per cent) was recorded in alkali treated fruit waste.

4.1.3.1.2 *Total reducing sugar*

Total reducing sugar is estimated by using DNS method (Miller, 1972; Sadasivam and Manickam, 1992). An increase in total reducing sugar content was observed in acid as well as alkali pretreated feed stocks (Table 7), (Figure 3). Pineapple fruit waste recorded the highest reducing sugar content (27.20 per cent) followed by fruit peel (25.56 per cent) and the lowest value (11.72 per cent) in plant residue, among the untreated feedstock. Similar trend was found with respect to alkali and acid pretreated samples. In the alkali treated and acid samples, maximum value (28.11 per cent and 27.41 per cent respectively) was obtained in fruit waste and lowest (15.24 per cent and 15.58 per cent respectively) in plant residue.

Saccharification also showed significant variation among different pretreatments. With respect to plant residue, acid pretreatment 24.78 per cent gave saccharification, which was significantly higher among the six treatments. Alkali treatment gave higher saccharification in fruit waste and fruit peel. The lowest saccharification (0.03 per cent) was obtained in acid treated fruit peel.

4.1.3.1.3 *Lignin content*

Gravimetric method (Georing and Vansoest, 1975) was used for the estimation of lignin in the pretreated feed stocks. A decrease in lignin content was observed in alkaline as well as acid pretreated feed stocks (Table 8), (Figure 4). Significant variation was observed in lignin content among the differently pretreated feed stocks used. Among the untreated feedstock, the highest lignin content (11.16 per cent) was observed in plant residue, followed by fruit peel (6.17 per cent) and the lowest (3.75 per cent) in fruit waste. Same trend was noticed with respect to different pretreatments. However, lignin content of the alkaline pretreated feed stocks were comparatively lower than the acid treated feed stocks. In case of alkali treated feedstock, maximum lignin content (6.32 per cent) was found in plant residue and the lowest (2.41 per cent) in fruit waste. Plant residue recorded 7.49 percent lignin content, which was the highest among the

Table 6. Total non reducing sugar content and percentage saccharification of pretreated feedstocks of pineapple

Sl. No.	Feed stocks	Untreated sample (%)	Alkali pretreated sample (%)	Acid pretreated sample (%)	Percent saccharification to non reducing sugar*	
					Alkali pretreated sample	Acid pretreated sample
1	Pineapple fruit peel	12.99 ± 0.24	13.64 ± 0.41	13.61 ± 0.45	4.67 ± 0.52	4.5 ± 0.66
2	Pineapple fruit waste	18.20 ± 0.17	18.55 ± 0.37	18.92 ± 0.20	1.84 ± 0.68	3.78 ± 0.77
3	Pineapple plant residue	5.21 ± 0.07	5.75 ± 0.26	5.89 ± 0.30	9.33 ± 0.34	11.38 ± 0.86
C.D. (0.05%)		0.23	0.14	0.13	0.71	

Data represents the mean of five replications

Table 7. Total reducing sugar content and percentage saccharification of pretreated feedstocks of pineapple

Sl. No.	Feed stocks	Untreated sample (%)	Alkali pre treated sample (%)	Acid pre treated sample (%)	Percentage saccharification to reducing sugar	
					Alkali pretreated sample	Acid pretreated sample
1	Pineapple fruit peel	25.56 ± 0.54	26.38 ± 0.67	25.57 ± 0.52	3.06 ± 0.54	0.03 ± 0.01
2	Pineapple fruit waste	27.20 ± 0.72	28.11 ± 0.98	27.41 ± 0.67	3.18 ± 0.30	0.69 ± 0.15
3	Pineapple plant residue	11.72 ± 0.40	15.24 ± 0.09	15.58 ± 0.39	23.12 ± 0.33	24.78 ± 2.61
C.D. (0.05%)		0.42	0.59	0.57	0.15	

Data represents the mean of five replications

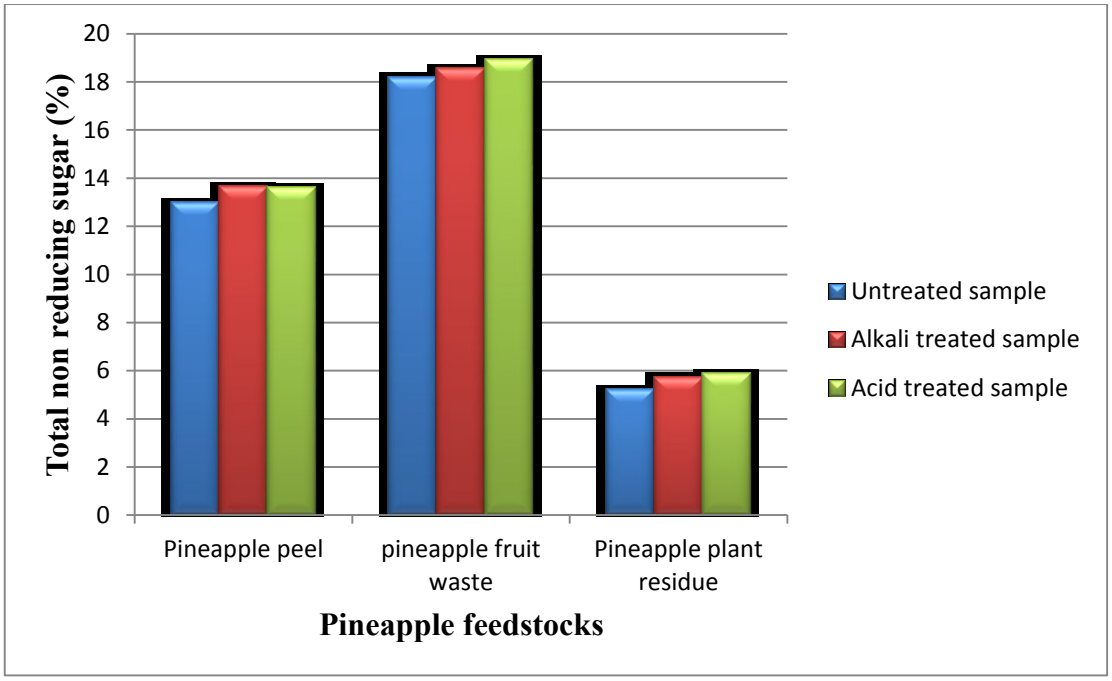

Figure 2: Total non reducing sugar content of untreated and pretreated feedstocks of pineapple

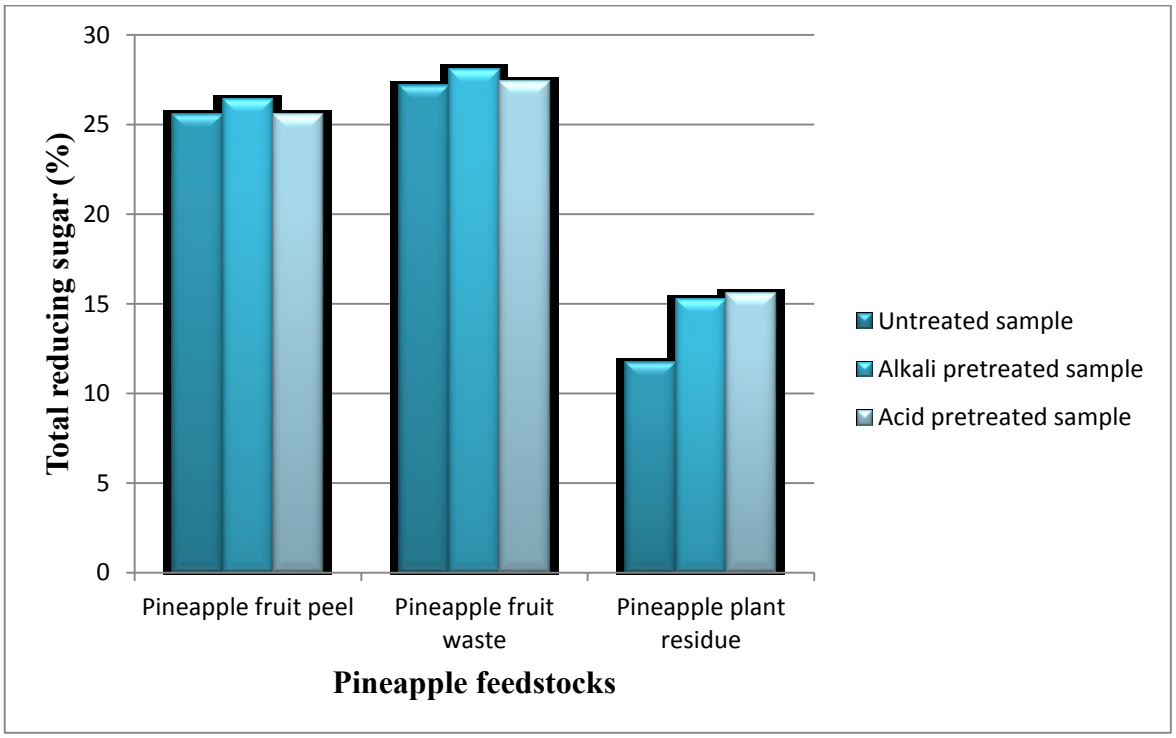

Figure3. Total reducing sugar content of untreated and pretreated feedstocks of pineapple

Table 8. Lignin content and percentage removal of lignin in pretreated feed stocks of pineapple

Sl. No.	Feed stocks	Untreated sample (%)	Alkali pre treated sample (%)	Acid pre treated sample (%)	Percentage removal of lignin*	
					Alkali pre treated sample (%)	Acid pre treated sample (%)
1	Pineapple fruit peel	6.17 ± 0.11	3.78 ± 0.48	5.06 ± 0.05	38.85 ± 6.88	17.94 ± 1.11
2	Pineapple fruit waste	3.75 ± 0.19	2.41 ± 0.33	3.26 ± 0.18	35.63 ± 8.3	12.8 ± 0.61
3	Pineapple plant residue	11.16 ± 0.41	6.32 ± 0.30	7.49 ± 0.33	43.34 ± 2.67	32.82 ± 3.29
C.D. (0.05%)		0.34	0.30	0.61	0.31	

Data represents the mean of five replications

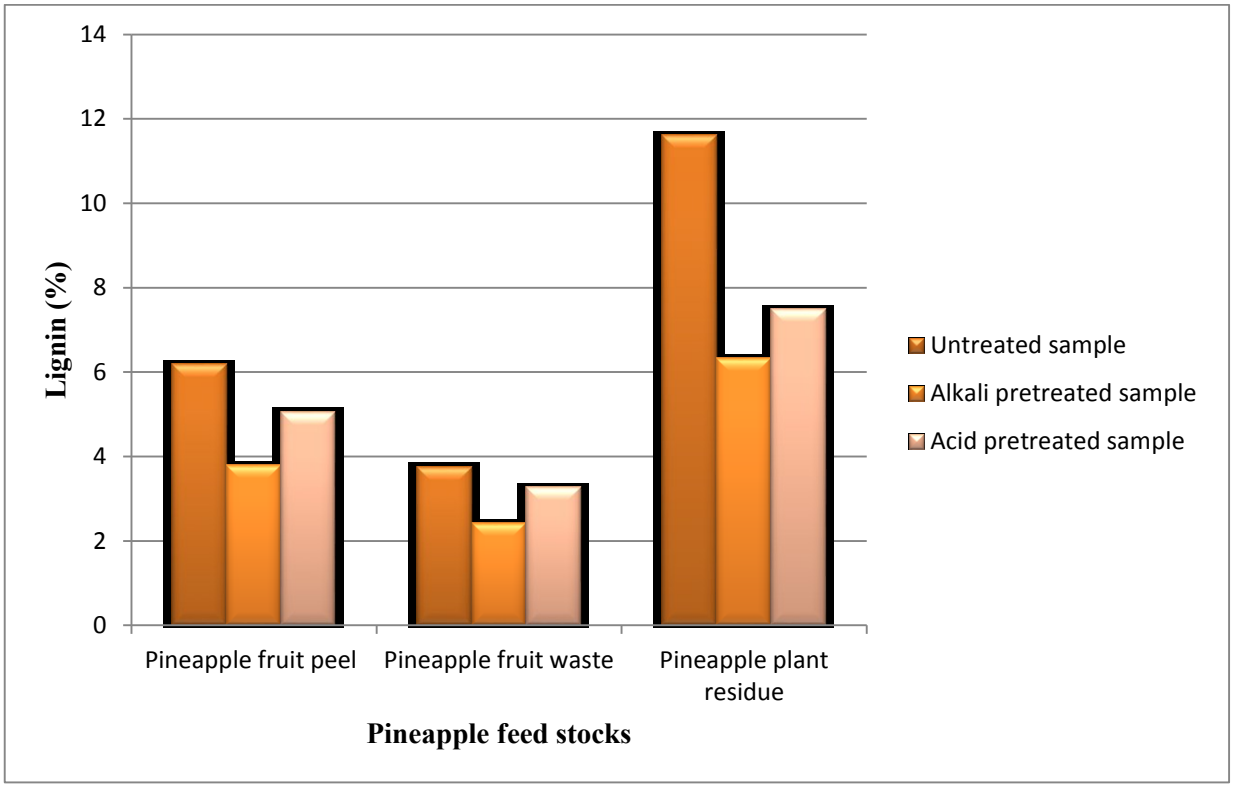

Figure 4: Lignin content of untreated and pretreated feedstocks of pineapple

feedstocks and the lowest lignin content (3.26 per cent) was recorded in fruit waste in acid pretreatment.

Percentage removal of lignin showed significant variation among the six treatments treatments. Alkali pretretament was found to remove more lignin compared to acid pretreatment. The highest lignin removal (43.34 per cent) was observed in alkali treated plant residue and the lowest value (12.8 per cent) was observed with acid treated fruit waste.

4.1.4 Fermentation of the feed stock

Fermentation of the three feed stocks was done by using *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Plate 4). Both raw and pretreated feed stocks were used for fermentation and the results are presented in the Table 8.

4.1.4.1 Estimation of alcohol content

Alcohol content was estimated by redox titration method by using potassium dichromate. Significant variation was found with respect to alcohol yield among the various treatments tried (Table 9). Maximum alcohol yield (8.34 per cent) was obtained in on fermenting untreated pineapple fruit waste using *Saccharomyces cerevisiae* (Figure 6). The lowest alcohol yield (0.48 per cent) among the 18 treatments was obtained on fermentation of untreated plant residue with *Zymomonas mobilis* (Figure 5).

The lowest yield (1.47 per cent) using *Saccharomyces cerevisiae* was obtained in alkali pretreated plant residue. Untreated feedstocks gave higher yield compared to pretreated ones on fermentation using *S. cerevisiae*. Among the different pretreatments, fruit waste gave maximum yield (8.34 per cent in untreated, 5.83 per cent in alkali treated and 2.74 per cent in acid treated).

With respect to *Zymomonas mobilis* highest alcohol yield (6.57 per cent) was obtained in untreated pineapple fruit waste. Among the different pretreatments, fruit waste recorded higher alcohol yield (6.57 per cent in

untreated, 5.53 per cent in alkali treated and 2.76 per cent in acid treated) compared to other feed stocks.

Saccharomyces cerevisiae is found to be more efficient than the *Zymomonas mobilis* in fermenting sugar to ethanol.

As the pretreatments did not improve the alcohol yield, percent conversion of sugar to alcohol was calculated on fermenting the untreated feed stocks only (Table 10). It was observed that the pineapple fruit waste recorded highest conversion rate of reducing sugar to alcohol (30.22 per cent). The lowest conversion rate (20.42 per cent) was found in pineapple peel waste, which was on par with that of pieapple plant residue (20.71 per cent). Whereas, the percent conversion of non reducing sugar is found to be maximum (28.81per cent) with pineapple peel waste followed by pineapple fruit waste (17.76 per cent) and the lowest value was recorded in the pineapple plant residue (10.85 per cent).

Change in pH of the fermented feed stocks were examined before and after fermentation (Table 11) and the results showed that decrease in pH after fermentation. The untreated feed stocks give an acidic pH before fermentation ranging from 3.9 for pineapple fruit waste 4.17 for pineapple fruit peel and 3.94 for pineapple plant residue. The pH was adjusted to near neutral in prior to fermentation in all feed stocks. After fermentation the pH was fall in to an acidic range, 3.55 to 4.18 in all feed stocks untreated samples and 3.66 to 6.95 in acid and alkali pretreated samples.

4.2 Enhancement of alcohol production

4.2.1 *Isolation of native microorganism and its characterisation*

Cellulolytic bacteria (Plate 4C) was isolated from degraded pineapple peel waste. The cellulolytic activity was confirmed by culturing on CMC agar followed by congo red test.

9. Alcohol yield from untreated and pretreated feed stocks of pineapple after fermentation

Feed stocks	Fermentation using *Zymomonas mobilis*			Fermentation using *Saccharomyces cereviceae*			C.D.# (0.05%)
	Untreated sample (%)	Alkali pre treated sample (%)	Acid pre treated sample (%)	Untreated sample (%)	Alkali pre treated sample (%)	Acid pre treated sample (%)	
Pineapple fruit peel	2.38 ± 0.03	2.24 ± 0.08	2.38 ± 0.15	6.56 ± 0.15	2.18 ± 0.02	2.43 ± 0.06	0.09
Pineapple fruit waste	6.57 ± 0.02	5.53 ± 0.20	2.76 ± 0.02	8.34 ± 0.17	5.83 ± 0.03	2.74 ± 0.03	
Pineapple plant residue	0.48 ± 0.03	1.47 ± 0.04	1.80 ± 0.04	2.05 ± 0.03	1.47 ± 0.03	1.77 ± 0.06	
C.D. (0.05%)	0.08	0.37	0.26	0.39	0.08	0.15	-

#Represents C.D. (0.05%) value of 18 treatments.

10. Non fermentable sugar content of different feed stocks of pineapple after fermentation using *Saccharomyces cerevisiae*

Feed stocks	Total reducing sugar			Total non reducing sugar		
	Before (%)	After (%)	conversion to alcohol (%)	Before (%)	After (%)	conversion to alcohol (%)
Pineapple fruit peel	25.56 ± 0.54	20.34 ± 0.55	20.42 ± 1.62	12.99 ± 0.24	9.22 ± 0.68	28.81 ± 6.55
Pineapple fruit waste	27.20 ± 0.72	18.95 ± 0.58	30.36 ± 3.59	18.20 ± 0.17	14.97 ± 0.75	17.76 ± 3.41
Pineapple plant residue	11.72 ± 0.40	9.28 ± 0.22	20.71 ± 4.08	5.21 ± 0.07	4.64 ± 0.19	10.85 ± 4.71
C.D. (0.05%)	0.42	-	1.99	0.23	-	6.03

#Represents the mean of five replications

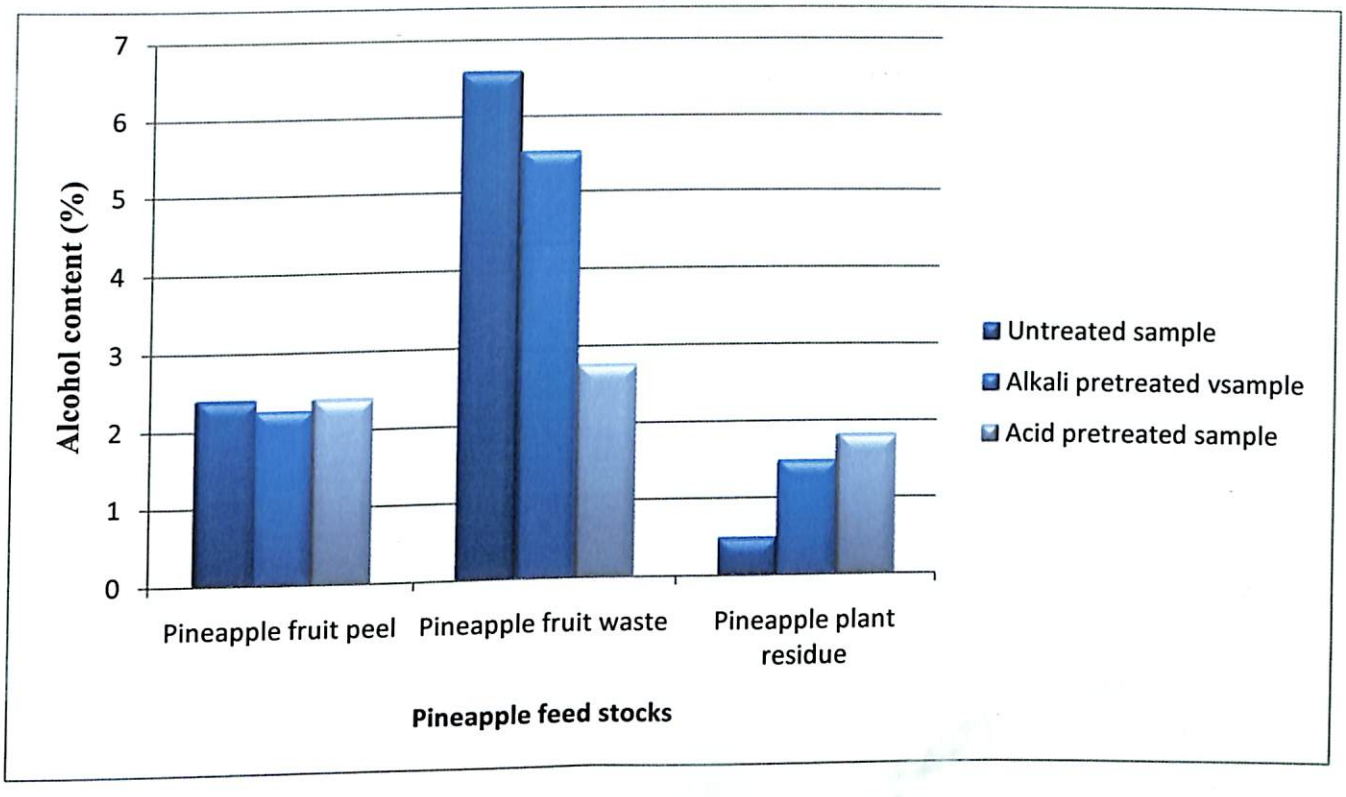

Figure 5. Alcohol yield from untreated and pretreated feed stocks of pineapple after fermentation with *Z.mobilis*

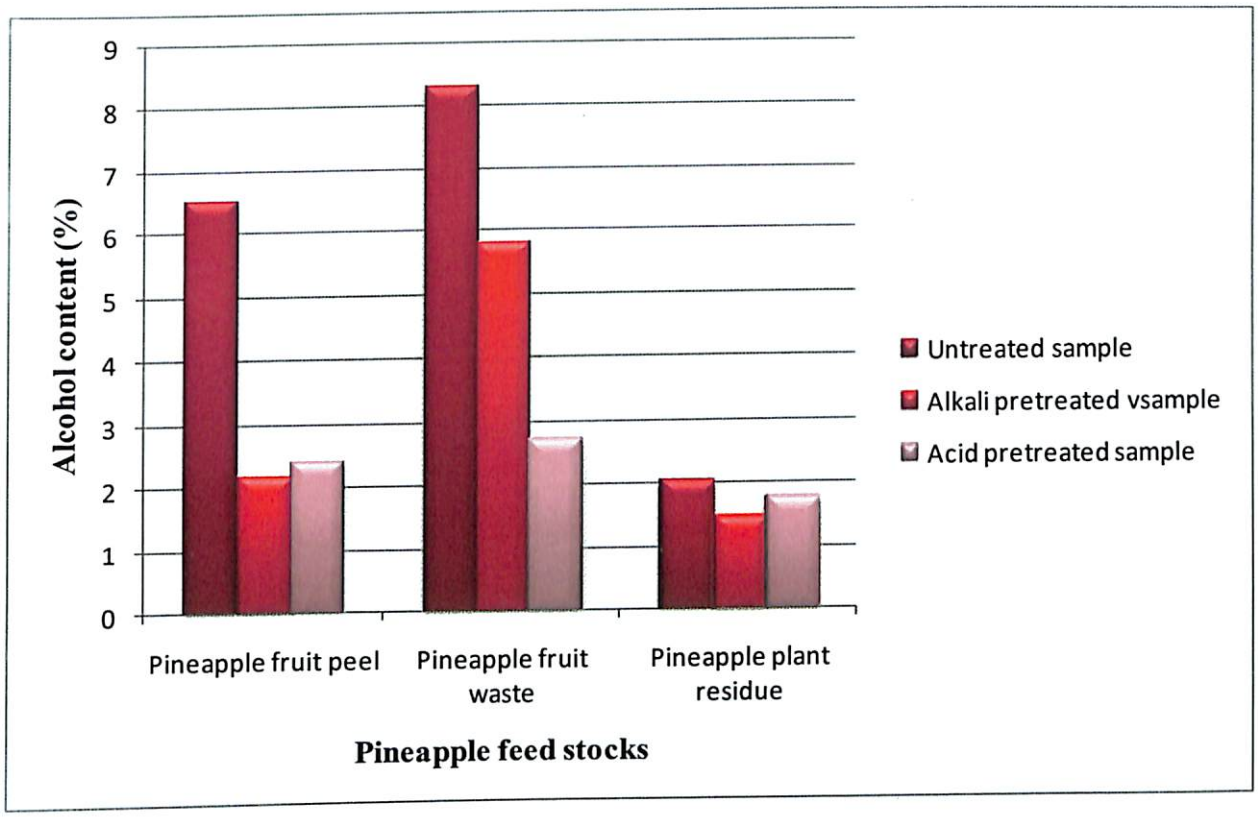

Figure 6¨Alcohol yield from untreated and pretreated feed stocks of pineapple after fermentation with *S.cereviceae*

Table 11. Change in pH during fermentation

Sl. No	Feed stocks	pH of the untreated sample	Untreated sample				Fermentation with *Zymomonas mobilis*				Fermentation with *Saccharomyces cerevisiae*			
			Fermentation with *Zymomonas mobilis*		Fermentation with *Saccharomyces cerevisiae*		Alkali treated		Acid treated		Alkali treated		Acid treated	
			Initial**	final	Initial	Final	Initial	final	Initial	final	Initial	final	Initial	Final
1	Pineapple fruit peel	4.17	7.00	3.80	7.00	3.60	7.00	5.67	7.00	4.21	7.0	5.35	7.0	5.03
2	Pineapple fruit waste	3.93	7.00	3.55	7.00	3.58	7.00	4.32	7.00	3.66	7.0	3.95	7.0	5.46
3	Pineapple plant residue	4.94	7.00	4.18	7.00	4.17	7.00	6.95	7.00	6.09	7.0	5.95	7.0	5.7 8

** pH was adjusted to 7 before fermentat

4.2.1.1 *Morphological characterization*

4.2.1.1.1 Gram staining and Microscopic observation

The bacteria obtained from the degraded samples of pineapple peel waste was subjected to gram staining. They were gram negative, rod shaped cell (Plate 5). The isolated colonies were slimy, white, flat and circular with undulate margin.

4.2.1.2 Biochemical characterization

4.2.1.2.1 *Catalase test*

The native microorganism which was isolated from degraded samples of pineapple peel waste was subjected to catalase test. It was observed that the bacteria was catalase positive (Plate 6).

4.2.1.2.2 *Carbohydrate fermentation test*

The isolated organism was tested for its carbohydrate fermentation activity. Different carbohydrates tested were glucose, fructose, sucrose, mannitol, maltose and lactose. The bacteria was inoculated to the broth containing each carbohydrate and incubated for 72 h. Bubble formation was examined inside the durham's tube inserted inside the broth. There was no bubble formation inside the tube, which confirmed that the bacteria was negative to carbohydrate fermentation test (Plate 7).

4.2.1.2.3 IMVIC test

4.2.1.2.3.1 *Indole production test*

The bacteria was inoculated to trypticase broth (Appendix-II d) for indole production test. After 48 h of incubation the broth was treated with kovac's reagent and observed for development of cherry red colour. There was no colour development which indicated that the organism was negative to the indole production test (Plate 8A).

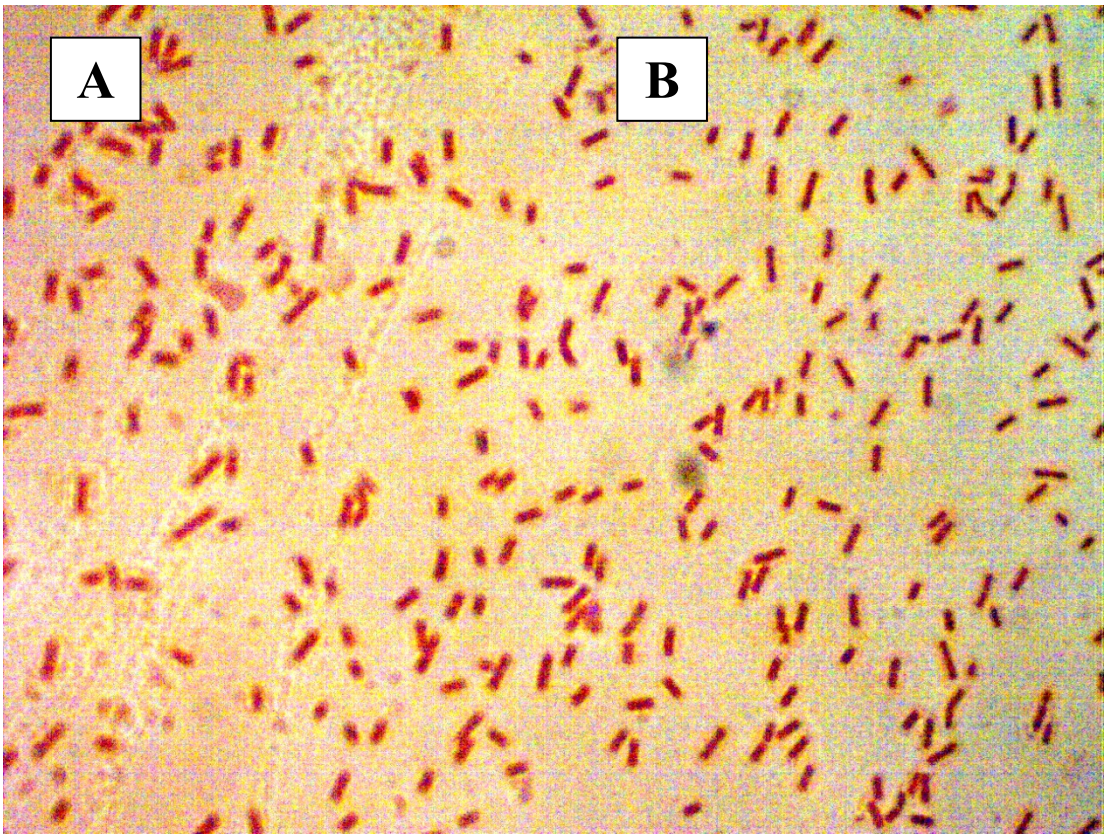

Plate 5: Gram staining of isolated microorganism (-ve)

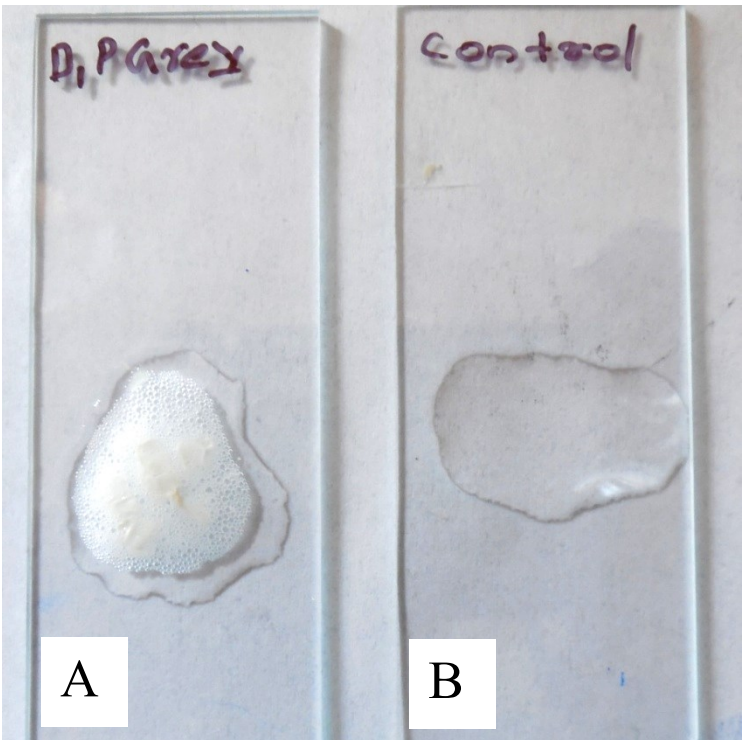

Plate 6: Catalase test (A) Isolated microorganism (+ve) (B) Control

4.2.1.2.3.2 *Methyl red test*

The micro organism was inoculated in the MR-VP broth for methyl red test. Methyl red indicator was added and observed for colour development. Colour change in the broth indicated that the microbe was positive to methyl red test (Plate 8B).

4.2.1.2.3.3 *Voges Proskauer test*

The microbe was inoculated in the MR-VP broth (Appendix-II m). After incubation at 37°C for 24 h, 12 drops of 1 per cent alcoholic α-naphthol reagent and 6 drops of 40 percent potassium hydroxide were added in each tube and gently mixed. Development of crimson ruby pink color in medium indicated that organism was Voges Proskauer test positive (Plate 8C).

4.2.1.2.3.4 *Citrate utilization test*

The isolated microorganism was inoculated in the Simmon's citrate agar (Appendix-II l) slant and incubated at 37°C for 48h. After incubation, the slants were observed for the growth of the organism and development of deep prussian blue colour. Growth of the organism was observed in the slant and the development of deep prussian blue colour. It showed that the microbe utilizes citrate and it is positive for citrate utilization test (Plate 8D).

4.2.1.2.4 *Gelatin hydrolysis test*

Nutrient gelatin medium (Appendix-II) was prepared and microbe was inoculated in to the media. After 48 h of incubation at 37°C, the tubes were kept in refrigerator for 30 min followed by 30 min incubation at room temperature.The inoculated media failed to solidify indicating that the organism was gelatin hydrolysis positive (Plate 9).

4.2.1.2.5 *Urease test*

The native micro organism was inoculated in the basal media; urease test broth (Appendix-II j). After incubation at 37°C for 48 h, the broth was examined

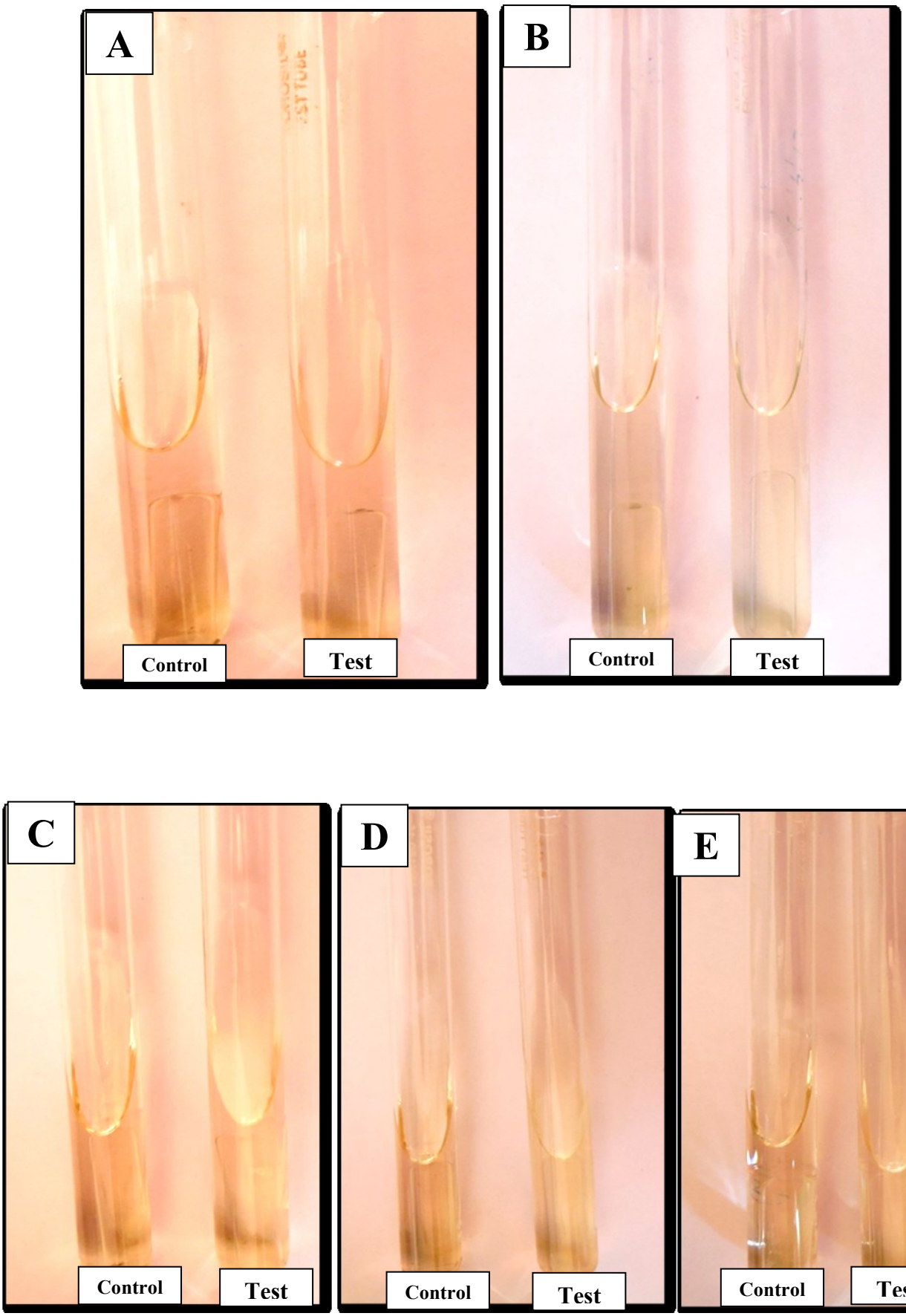

Plate 7: Carbohydrate fermentation test (-ve) (A) Glucose (B) Sucrose (C) Lactose (D) Maltose (E) Mannitol

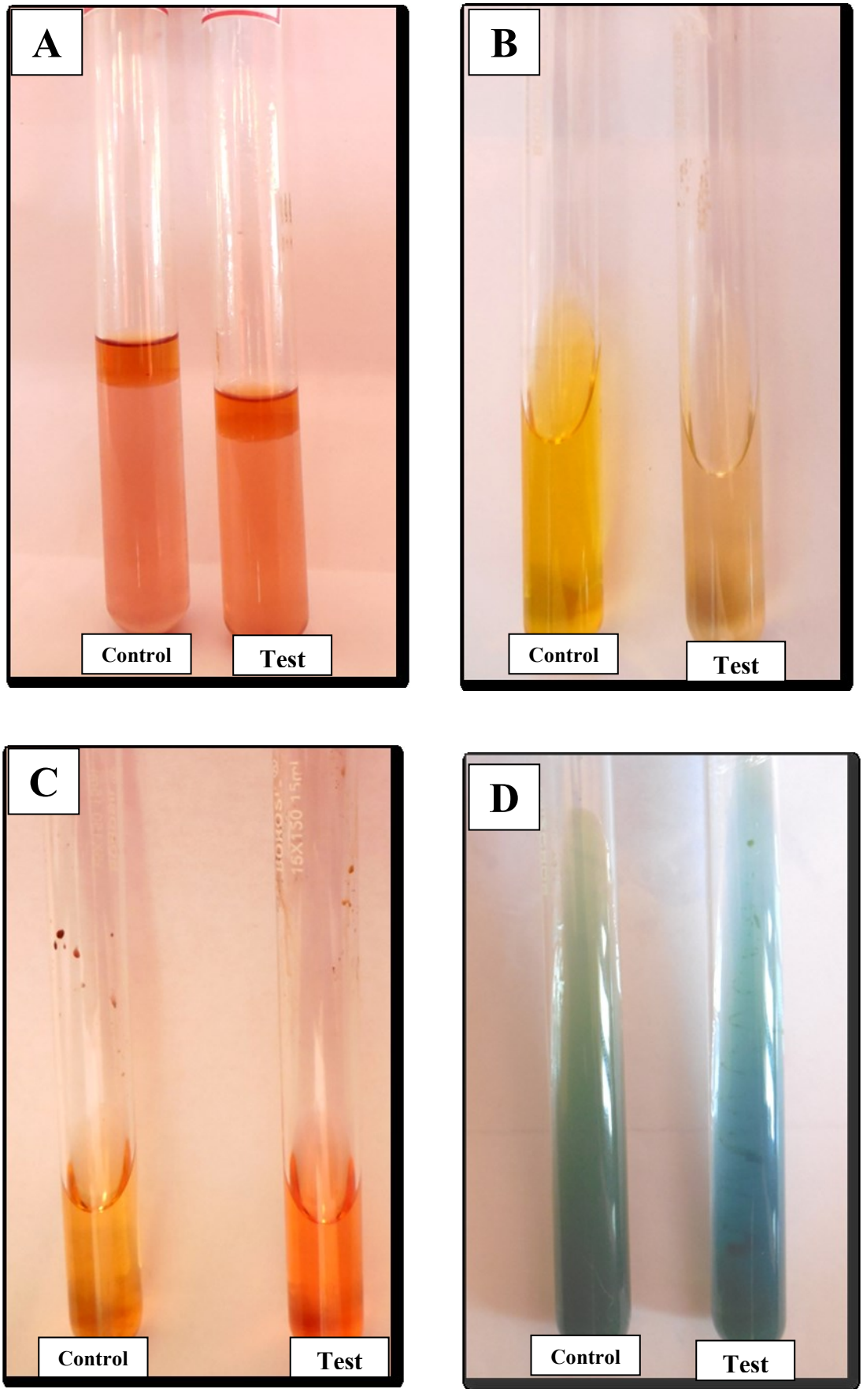

Plate 8: IMVIC test (A) Indole test (-ve) (B) Methyl red test (+ve) (C) Vogues proskuer test(+ve) (D) Citrate utilisation test (+ve)

for the development of pink color formation. There was no color formation after incubation, which indicated that the organism was urease negative (Plate 10).

4.2.1.2.6 *Cellulolytic activity*

Cellulolytic activity of the bacteria was tested by cellulase assay using carboxymethyl cellulose and maximum cellulase activity was observed on sixth day of incubation and then the activity was reduced (Figure 7).

4.2.1.3 Molecular characterisation of isolated microorganism

4.2.1.3.1 *Genomic DNA Isolation*

Genomic DNA was extracted from the native organism. The agarose gel electrophoresis (0.8 per cent) of the extracted genomic DNA showed the presence of good quality unsheared DNA bands (Plate 11). Further absorbance reading of the extracted genomic DNA by using spectrophotometric method revealed good quality and quantity of DNA (Table 12).

4.2.1.3.2 *PCR amplification of plasmid DNA*

The DNA was amplified using the primers and checked using agarose gel electrophoresis (1.2 percent). The PCR amplicons showed the presence of good quality bands on the gel (Plate 12). The PCR products were sent for sequencing to Rajive Gandhi Centre for Biotechnology.

The sequences obtained (Appendix III) showed similarity to 16s and 23s ribosomal RNA sequences. Nucleotide BLAST was used for sequence similarity search. The sequenced data showed high similarity to *Bacillus subtilis* (Figure 7 and 8).

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

The results of characterization of feed stocks and fermentation indicate that the pineapple fruit waste is the best feed stock and *Saccharomyces cerevisiae*

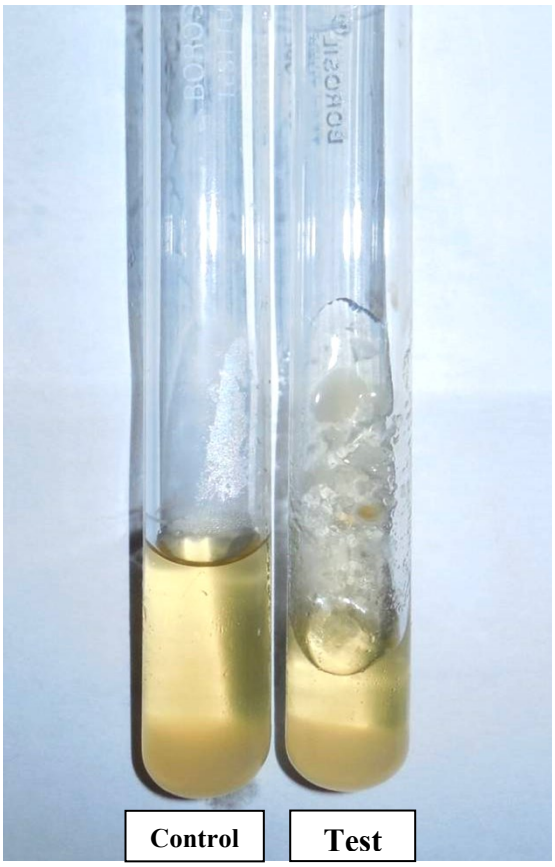

Plate 9: Gelatin hydrolysis test (+ve)

Plate 10: Urease test (-ve)

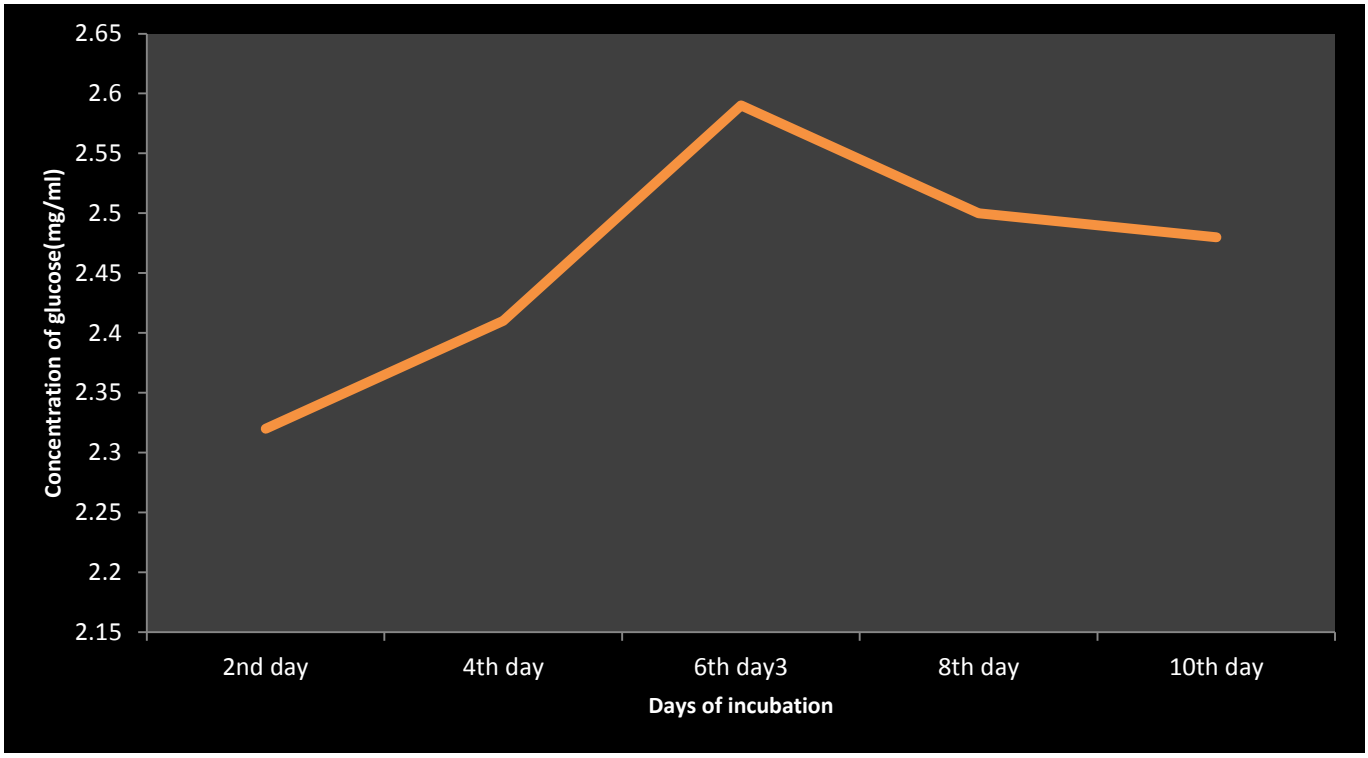

Figure 7: Cellulolytic activity of *Bacillus* sp.

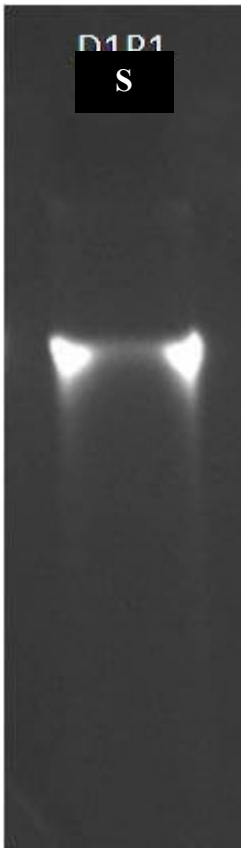

Plate 11 :. DNA isolated from native microorganism

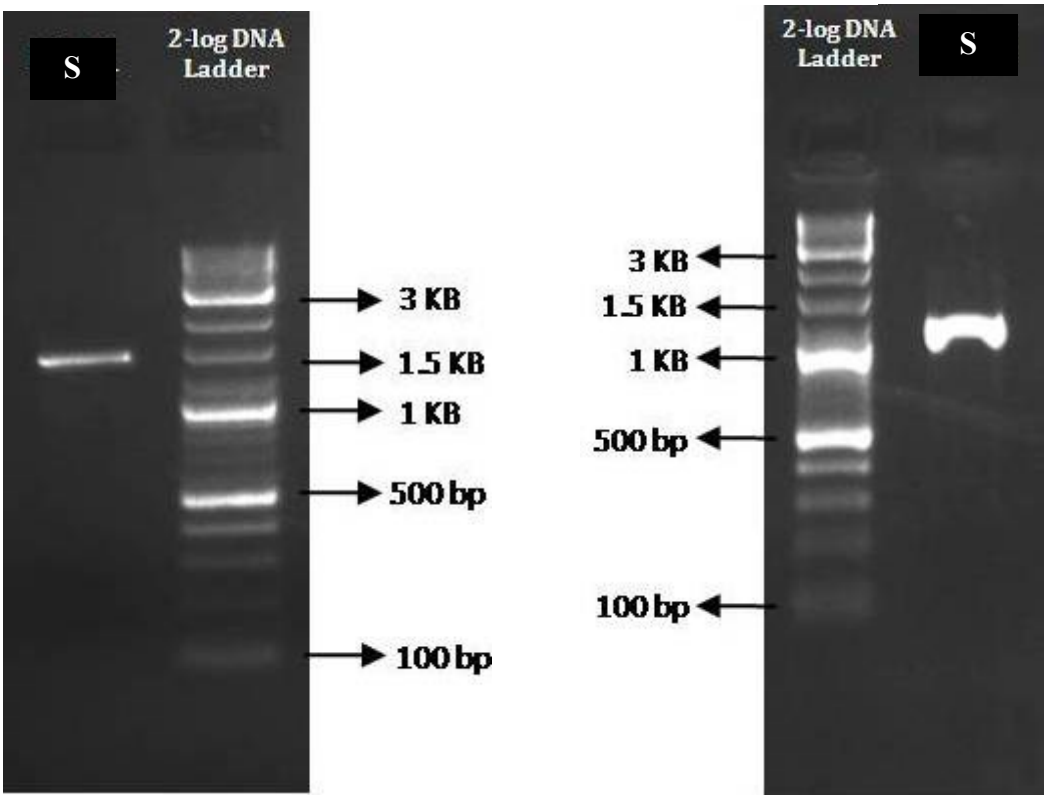

Plate 12: PCR amplification of 16S rRNA and 23S rRNA (S- Sample)

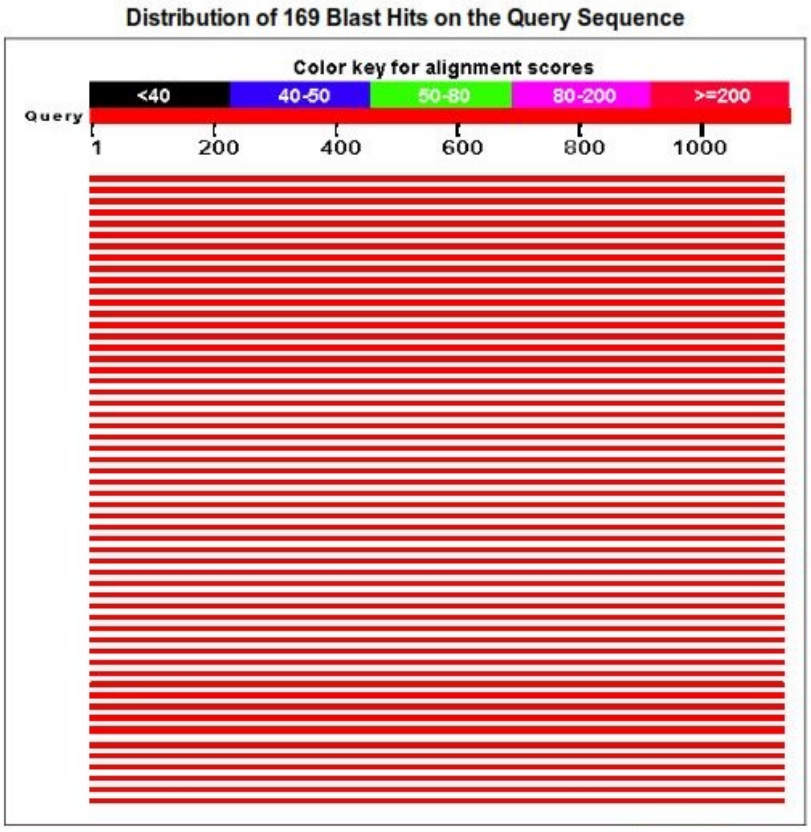

Figure 8: Graphical representation of 16S rRNA sequence alignment

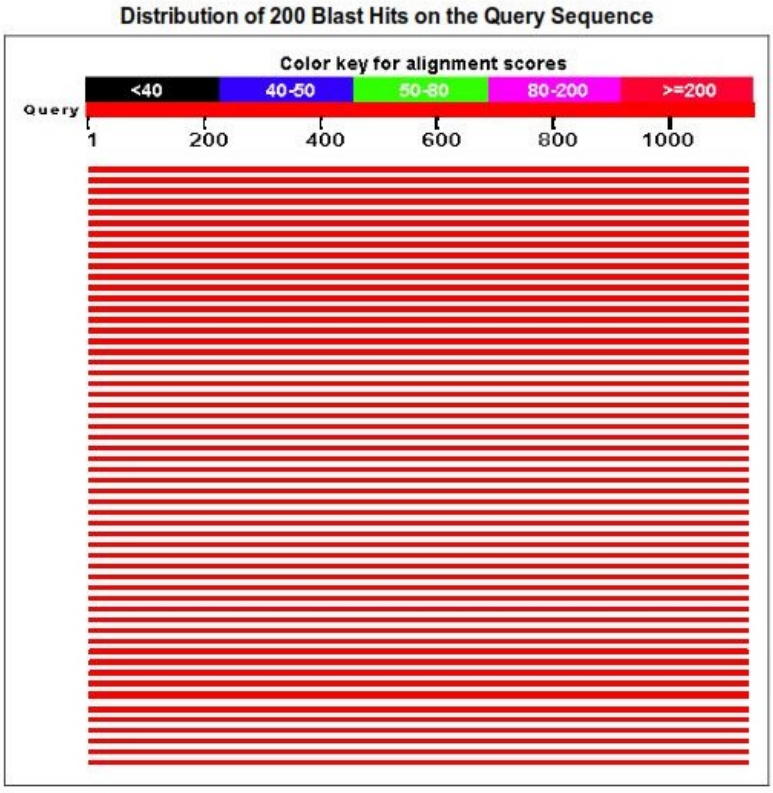

Figure 9: Graphical representation of 23S rRNA sequence alignment

is the most amenable among the organisms tried for alcohol production. *Bacillus subtilis* isolated was used in combination with *Saccharomyces cerevisiae* to enhance the alcohol yield. The compatibility check shows slight negative interaction towards *Saccharomyces cerevisiae.*

Three modes of fermentation viz., separate hydrolysis and fermentation, simultaneous saccharification and fermentation and single batch bioconversion were performed. The end point of the fermentation was estimated by determining alcohol per cent at two days interval. The alcohol percentage found to become steady at 6^{th} day of fermentation (Figure 10). Hence alcohol yield on the 6^{th} day is taken as the final value.

Significant variation was observed among different modes of fermentation in with respect to alcohol content. On the 6^{th} day of incubation, Single batch bioconversion (SBB) indicated maximum (Table 13) alcohol yield (11.09 per cent). Simultaneous saccharification fermentation (SSF) yielded (8.76 per cent) alcohol, which was the lowest. This was found to be on par with the alcohol yield of the pineappale fruit waste using *S. cerevisiae* alone. Separate hydrolysis and fermentation (SHF) yielded 9.50 per cent alcohol (Figure 11).

The present study concluded that fruit waste is the best candidate for bioethanol production than other pineapple feed stocks tried. Single batch bioconversion using the cellulolytic organism, *Bacillus* sp. and fermenting organism, *S. cerevisiae* could bring about a substantial enhancement in alcohol yield.

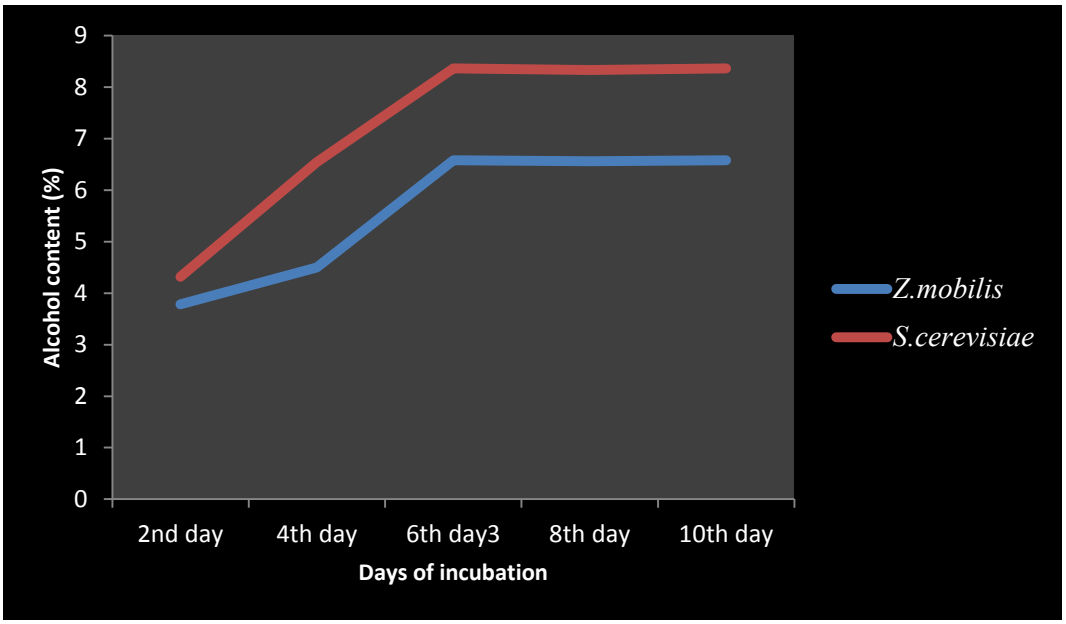

Figure 10: Endpoinnt of fermentation for *Z.mobilis* and *S.cerevisiae*

Table 12. Absorbance reading of the extracted genomic DNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield ($ng\mu l^{-1}$)
1.	Sample 1	0.012	0.007	1.71	360

Table 13. Alcohol yield from pineapple fruit waste via different modes of saccharification and fermentation using mixed cultures of *Bacillus sp*. and *S.cerevisiae*

Sl. No.	Modes of Saccharification and fermentation	Alcohol (%)
1	Separate Hydrolysis and Fermentation (SHF)	9.50 ± 0.100
2	Simultaneous Saccharification and Fermentation (SSF)	8.76 ± 0.66
3	Single Batch Bioconversion (SBB)	11.09 ± 0.045
4	Using single organism, S. cerevisiae (Control)	8.34 ± 0.17
C.D. (0.05%)		0.49

Data represents the mean of five replications

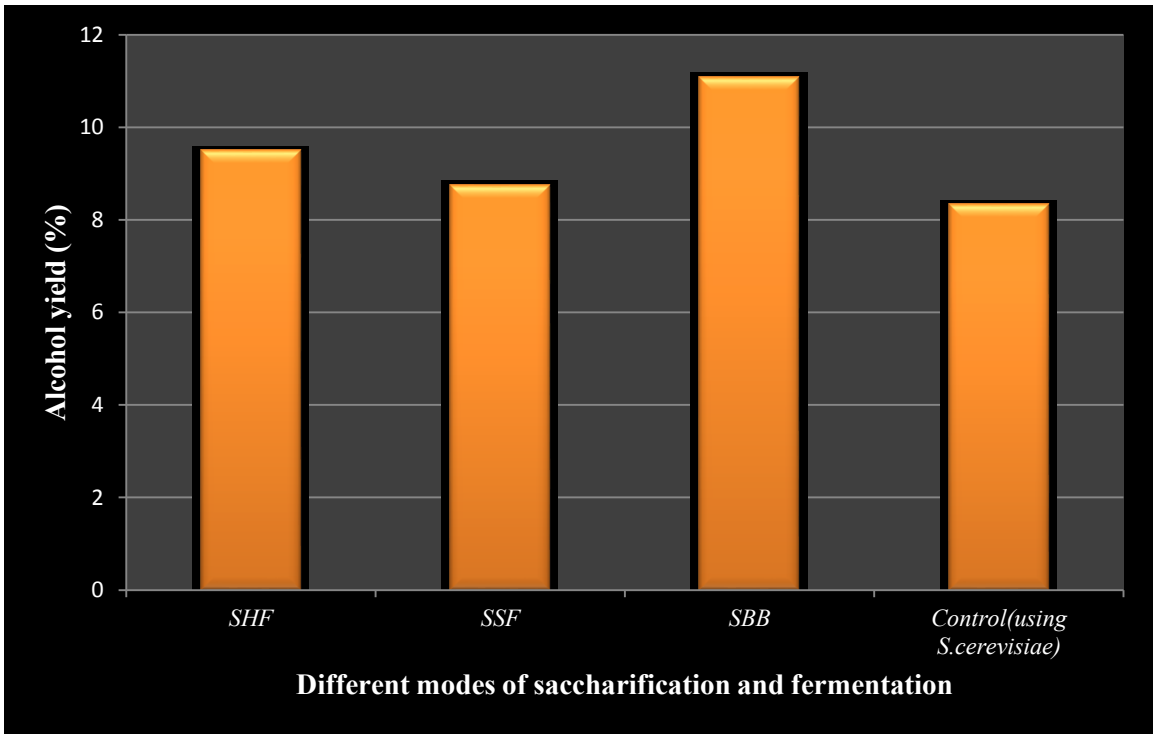

Figure 11: Alcohol yield from pineapple fruit waste via different modes of saccharification and fermentation using mixed cultures of *Bacillus* sp. and *S.cerevisiae*

Discussion

5. DISCUSSION

During the most recent decades, there is an increased interest in fuel from biomass worldwide. Rising concern over depleting fossil fuel and greenhouse gas resulted in a high level of interest in non-conventional fuel originating from bio-renewable sources including sugars, starches and lignocellulosic materials. Bioethanol market is expected to reach 100 x 109 liters in 2015 (Bhatia, 2012). Corn-based and sugar based-ethanol have become the primary feedstocks for bioethanol production for the past several years. But they are not sufficient to replace a considerable portion of the one trillion gallons of fossil fuel presently consumed worldwide each year. Furthermore, the ethical concerns about the use of food as fuel raw materials have encouraged low-cost large-scale processes for lignocellulosic feedstocks originating mainly from agricultural and forest residues along with herbaceous materials and municipal wastes. Although lignocellulose derived biofuel is a promising technology, there are some obstacles that interfere ith bioconversion processes reaching optimal performance associated with minimal capital investment. Lignocellulosic biomass materials constitute a substantial renewable substrate for bioethanol production that do not compete with food production and animal feed. Moreover, the utilization of these materials also contribute to environmental sustainability.

During pineapple processing, the crown and stem are cut off before peeling. The core is then removed for further processing. These wastes (peel, core, stem, crown, and leaves) generally account for 50 percent (w/w) of total pineapple weight Ketnawa, *et al*., (2012). Therefore, with increasing pineapple production, pineapple wastes are also proportionally increasing. So, pineapple waste biomass is a good choice for bioethanol production.

Pineapple waste contains sufficient quantities of simple and complex sugars that may be used for bioethanol production (Nigam, 2000). In addition, pineapple waste has relatively lower lignin content compared to other waste materials such as banana stem, coconut waste, and oil palm empty fruit bunches, which suggests that it can undergo hydrolysis step more easily with the utilization

of lesser amount of chemicals. Reports on the use of pineapple waste for the production of industrially relevant metabolites such as cellulase through fermentation processes are limited. Considering these factors, the present study on utilization of pineapple (*Ananas comosus* (L.) Merr.) biomass for biofuel production was undertaken at the Department of Plant Biotechnology, College of Agriculture, Vellayani. The results of the study are discussed in this chapter.

The feed stocks were prepared by drying and grinding of pineapple peel, pineapple fruit waste and pineapple plant residue separately. This is a method of physical pretreatment, alter the structures of cellulosic biomass to increase the digestibility of enzyme to convert the carbohydrate polymers in to fermentable sugars. Sun and Cheng (2002) reported that the first step for ethanol production from agricultural solid wastes is comminution through milling, grinding or chipping to reduce cellulose crystallinity.

Moisture content of the feed stocks pineapple peel, pineapple fruit waste and pineapple plant residues was estimated and the results of the study showed that maximum percent of moisture content was present in pineapple plant residue followed by pineapple fruit waste and pineapple peel waste. Lonsane *et al*. (1985) observed that moisture content has significant effect on microbial growth and metabolite production and in turn it affects the efficiency of fermentation.

The present investigation of total sugar content of feed stocks showed that pineapple fruit waste had recorded the highest values for glucose, fructose, xylose and sucrose compared to pineapple peel waste and pineapple plant residue. Besides, it was found that sucrose content was significantly higher in pineapple fruit waste (8.37 per cent). Xylose content was found to be low compared to other sugars in all the feedstocks analysed. From the results obtained, it can be confirmed that sucrose is the major sugar present in the pineapple fruit waste. Same trend was reported by Roha *et al.* (2013) in pineapple biomass. The fructose content was 2.24 per cent in core followed by peel (2.04 per cent) and crown (0.87 per cent). Glucose content was highest in the core (2.56 per cent) followed by the peel (2.18 per cent) and the crown (0.53 per cent). The sucrose content of

9. Alcohol yield from untreated and pretreated feed stocks of pineapple after fermentation

Feed stocks	Fermentation using *Zymomonas mobilis*			Fermentation using *Saccharomyces cereviceae*			C.D.# (0.05%)
	Untreated sample (%)	Alkali pre treated sample (%)	Acid pre treated sample (%)	Untreated sample (%)	Alkali pre treated sample (%)	Acid pre treated sample (%)	
Pineapple fruit peel	2.38 ± 0.03	2.24 ± 0.08	2.38 ± 0.15	6.56 ± 0.15	2.18 ± 0.02	2.43 ± 0.06	0.09
Pineapple fruit waste	6.57 ± 0.02	5.53 ± 0.20	2.76 ± 0.02	8.34 ± 0.17	5.83 ± 0.03	2.74 ± 0.03	
Pineapple plant residue	0.48 ± 0.03	1.47 ± 0.04	1.80 ± 0.04	2.05 ± 0.03	1.47 ± 0.03	1.77 ± 0.06	
C.D. (0.05%)	0.08	0.37	0.26	0.39	0.08	0.15	-

#Represents C.D. (0.05%) value of 18 treatments.

10. Non fermentable sugar content of different feed stocks of pineapple after fermentation using *Saccharomyces cerevisiae*

Feed stocks	Total reducing sugar			Total non reducing sugar		
	Before (%)	After (%)	conversion to alcohol (%)	Before (%)	After (%)	conversion to alcohol (%)
Pineapple fruit peel	25.56 ± 0.54	20.34 ± 0.55	20.42 ± 1.62	12.99 ± 0.24	9.22 ± 0.68	28.81 ± 6.55
Pineapple fruit waste	27.20 ± 0.72	18.95 ± 0.58	30.36 ± 3.59	18.20 ± 0.17	14.97 ± 0.75	17.76 ± 3.41
Pineapple plant residue	11.72 ± 0.40	9.28 ± 0.22	20.71 ± 4.08	5.21 ± 0.07	4.64 ± 0.19	10.85 ± 4.71
C.D. (0.05%)	0.42	-	1.99	0.23	-	6.03

#Represents the mean of five replications

pineapple core and peel extract was observed to be 8.92 per cent and 3.87 per cent, respectively. However, sucrose was not detected in pineapple crown. This is in confirmatory with our finding that pineapple fruit waste extract had the highest values of fructose, glucose and sucrose compared to the other pineapple feed stocks.

Bartolome *et al.* (1995) also observed that in pineapple, the sucrose content was approximately two-third of the total sugar. Masniza *et al*. (2000) also reported that pineapple contains 12-15 per cent soluble sugars, of which two-third is in the form of sucrose and the rest were glucose and fructose.

The total non reducing sugar and reducing sugars of pineapple feed stocks were estimated and highest percentage (18.20 percent and 27.2 percent, respectively) was noticed in pineapple fruit waste followed by pineapple peel waste. The lowest value 5.52 percent and 11.72 per cent respectively was recorded in pineapple plant residue. Rani *et al*. (2004) reported 4.9 per cent of non reducing sugar and 27.8 per cent reducing sugars in dried pineapple waste.

Total dissolved solids of pineapple feed stocks obtained as 27.24 ppm, 28.12 and 27.44 respectively for pineapple peel waste, pineapple fruit waste and pineapple plant residue. Pineapple feed stocks recorded the highest value (0.206 ppm) of total soluble salts in pineapple fruit waste followed by fruit peel (0.204 ppm). The lowest value (0.115 ppm) was obtained in pineapple plant residue.

Total carbohydrate was found to be maximum (70.68 per cent) in pineapple fruit waste followed by pineapple peel waste (60.43 per cent) and the lowest (25.63 per cent) in pineapple plant residue. Rosma *et al*. (2005) reported that pineapple waste consisting of peel, core and plant residues accounts for 60.14 per cent of total carbohydrates.

Lignin interferes with cellulose hydrolysis in lignocellulosic substrate because it acts as a physical barrier that prevents the contact of cellulase to cellulose (Umamaheswari *et al.,* 2010). This special and complicated structure makes cellulose resistant to both biological and chemical treatments. (Morohoshi, 1991; Delmer and Amor, 1995; Ha *et al*. 1998). Hemicellulose is less complex,

and its concentration in lignocellulosic biomass is 25 to 35 percent and it is easily hydrolysable to fermentable sugars (Saha *et al*., 2007). The results obtained showed that pine apple fruit waste have 20.47 per cent hemicellulose and a low lignin content of 3.75 per cent.

Estimation of cellulose, hemicelluloses and lignin content of the feed stocks revealed that pineapple plant residue have maximum cellulose content (69.20 per cent) followed by pineapple fruit waste (41.74 per cent) and pineapple peel waste (18.83 per cent). Pineapple peel waste had reported a maximum hemicellulose content (23.08 per cent) followed by pineapple plant residue (21.85 per cent) and pineapple fruit waste (20.47 per cent). Lignin content was found to be maximum (11.16 per cent) in plant residue followed by pineapple peel waste (6.17 per cent) and pineapple fruit waste (3.75 per cent). Upadhyay *et al.* (2010) reported 19.4 per cent cellulose, 22.4 per cent hemicellulose and 11 per cent lignin in dried pineapple waste.

Relatively low levels of fermentable sugars and high amount of fibre necessitated a pretreatment of the pineapple waste to increase the sugar content prior to fermentation. To obtain an efficient conversion, pre treatment was performed on three feed stocks with acid and alkali which reduced the lignin content and made the sugar molecules accessible for fermentation.

Total reducing sugar and non reducing sugar content indicated an increase on alkaline and acid pretreatment of the feed stocks. The increase in sugar concentration in pretreated feed stocks might be due to the hydrolysis of cellulose and hemicellulose in to sugars. The use of an alkali causes the degradation of ester and glycosidic side chains resulting in structural alteration of lignin, cellulose swelling, partial decrystallization of cellulose (Cheng *et al*., 2010; McIntosh *et al*., 2010; Ibrahim *et al*., 2011) and partial solvation of hemicelluloses (McIntosh *et al*., 2010; Sills *et al*., 2011). During acid pre-treatment strong acid allows complete breakdown of the components in the biomass to sugars (Goldstein and Easter, 1992). The results of the present study corresponds to the findings by

Dhabhai *et al*., (2012) that the total reducing and non reducing sugar content increased with pre treatments.

In the present study, a decrease in lignin content was observed for alkaline as well as acid pretreated feed stocks. However, higher percentage of removal of lignin was observed with alkaline pretreated pineapple feed stocks. The removal of lignin is necessary for cellulose to become readily available for the enzymes to act, which permit the organism to convert the glucose into ethanol. Wyman (1996) reported that lignin was removed on chemical pretreatment which was indicated by weight loss of the residue. Krishna and Chowdary (2000) concluded that alkaline peroxide pretreatments were effective in the fractionation of the hemicellulose and lignin components and resulted in efficient hydrolysis in linn leaves. Gould and Freer (1984) observed that wheat straw when treated for several hours at room temperature with 1per cent H_2O_2 at a pH of 1.5 released slightly more than one half of its lignin as water-soluble degradation products. They found that increase in concentrations of H_2O_2, more alkaline pH, or repeated H_2O_2 pretreatments did not alter the total amount of lignin solubilised.

In the present study, an increased alcohol yield was observed with fermentation of untreated feedstock than pretreated feed stocks. The results of the study are contradictory to the results obtained by Lalitha (2011) who reported that pre-treatment of feed stocks removed lignin from the fruit biomass peel residue, which resulted in higher production of ethanol. In the study it was observed that with pretreatments, the lignin content was reduced. But this did not produce any increase in alcohol yield. This may be due to the action of various inhibitors like acetic acid, furfural and 5- hydroxymethylfurfural during the pretreatment. These products were found to inhibit the growth of fermenting microorganisms. Moreover, the addition of NaOH and HCl to acid and alkali pretreated feed stocks, respectively for adjusting the pH to near neutral increased the salt concentration which in turn might have indused a toxic effect and inhibited the growth of inoculated microorganism. Casey *et al.* (2013) reported that salts of chlorides, sulphates and sodium in higher concentrations can be significantly inhibit the growth of *Saccharomyces cerevisiae*.

In the study, the alkali and acid pretreated feed stocks and untreated feed stocks were fermented using *Saccharomyces cerevisiae* and *Zymomonas mobilis,* separately. Fermentation using *Saccharomyces cerevisiae* in untreated pineapple fruit waste yielded maximum alcohol percentage (8.34 per cent) compared to other feed stocks. This could be attributed to the increased level of fructose, glucose and sucrose compared to the other feed sttocks. Shilpa (2013) also reported an alcohol content of 8 per cent in pineapple peel with *S.cerevisiae*. In the study, untreated pineappale peel recorded an alcohol yield of 6.56 per cent.

Fermentation using *Zymomonas mobilis* also give an higher alcohol percentage (6.57 per cent) in untreated pineapple fruit waste. But this alcohol yield is significantly lower than alcohol obtained with *Saccharomyces cerevisiae.* Hence, *Saccharomyces cerevisiae* was found to be more efficient than *Zymomonas mobilis* for fermenting the pineapple waste biomass. These results found to contradict to the results obtained by Raman and Pothiraj, (2008) that ethanol production after 36 h of fermentation at pH 6.0 was highest with *Z.mobilis* than the ethanol production rate of *S.cerevisiae*.

Ban-Koffi and Han (1990) reported that pretreatment of pineapple waste with cellulase and hemi-cellulase and then fermentation with *S. cerevisiae* or *Z. mobilis* produced about 8 percent ethanol from pineapple waste in 48 hrs.

The results of percent conversion rate of reducing sugar after fermentation indicate that pineapple fruit waste have higher conversion rate (30.35) than other feed stocks where as the percent conversion of non reducing sugar was found to be maximum with pineapple peel waste (28.81). Hong *et al*., (2013) observed the conversion rate of 34.2 percent *with S. cerevisiae* from reducing sugar to ethanol.

In the study, it was observed that pH shifts towards acidic range (3.5-5) as the fermentation progressed. The decrease in the pH may be due to the release of microbial metabolism by products into the fermentation medium.

For the enhancement of fermentation a native microorganism having cellulolytic activity was isolated from pineapple waste. The colonies were white, flat, slimy and circular with undulate margin and rod shaped gram negative cells.

The bacteria was catalase positive, negative to carbohydrate fermentation test, indole production test, urease test and positive to methyl red test, gelatin hydrolysis, Voges Proskauer test and citrate utilization test. Similar characterisation of cellulolytic microorganism was conducted by Basavaraj *et al*. (2014).

The biochemical and molecular characterisation indicted the similarity of the isolated organism to *Bacillus subtilis*. Which are gram positive bacteria but the isolated bacteria was found to be gram negative on staining. This may be due to the fact that gram positive organisms at sporulation stage might give gram negative response on staining. Barenfanger and Drake (2001) discussed that *Bacillus sp.* and *Clostridium sp.* in sporulation stage gave gram negative staining.

Cellulolytic activity of the isolated microorganism was tested by cellulase assay using carboxymethyl cellulose and maximum cellulase activity was observed on the sixth day of incubation; thereafter cellulolysis was found to decrease. Hence, period of incubation for maximum saccharification was determined as six. Robson and Chambliss (1984) reported that the cellulolytic activity was not produced until the stationary phase of growth was achieved. From this point, the level of cellulolytic activity was found to increase followed by a gradual decrease in the level of cellulolytic activity was observed due to cell lysis.

The molecular characterisation of the microorganism by the analysis of the sequences obtained in PCR amplification using specific primers indicted that, it showed sequence similarity to *Bacillus sp.*

The 16S rRNA sequence is used to study the bacterial phylogeny and taxonomy. This is due to the fact that it is present in almost all bacteria. It often exists as multigenic family or operons. The function of the 16Sr RNA gene over the time has not changed, suggesting that random sequence changes are a more accurate measure of evolution. The 16S rRNA gene (1500) is large enough for informatics purpose. The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16S rRNA gene sequencing studies as

opposed to the more cumbersome manipulations involving DNA-DNA hybridization *(*Janda and Abbott, 2007).

Yadav *et al*. (2012) carried out molecular characterization of cellulose degrading bacteria on the basis of 16s rRNA. For sequence analysis, direct sequencing of PCR product was done. The sequence of this 16s rRNA was analysed by various bioinformatics tools like nucleotide BLAST, ClustalW, Drawtree etc.

In the study, enhancement in alcohol production was tried using mixed cultures of cellulolytic isolated bacteria and fermenting organism, *Saccaharomyces cerevisiae*. Three different modes of saccharification and fermentation (SHF, SSF, SBB) was attempted to optimise the process for enhanced production of ethanol from the best identified feed stock, pineapple fruit wate. It was observed that single batch bioconversion (SBB) mode gave maximum alcohol yield of 11.09 per cent. In SSB process, fermentation is allowed only after the termination of enzyme production by heat-inactivation of cellulolytic organism, in the same culture broth. The increased alcohol yield of SBB may be due to the heat inactivation of isolated bacteria before fermentation. This limits the chance of interaction of isolated bacteria with S*accharomyces cereviciae.* During the compatibility test for the isolated bacteria and *S. cereviciae,* a slight incombatility was observed indicated by a very thin clear zone.

In simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF) both the organisms are inoculated into same culture medium and hence, the interaction between the two is more. The decreased level of ethanol in these methods may be due to this interaction and subsequent inhibition of *Saccharomyces cereviciae* resulting low sugar conversion to alcohol. Till date, there is no report on the fermentation of agriculture residues with mixed cultures of *Bacillus sp.* with S*accharomyces cereviciae.*

The end point of fermentation was determined by investigating the alcohol percentage at 2 days interval and it was found that alcohol percentage will be constant from sixth day. Shilpa *et al.* (2013) reported that pineapple peel gave the

highest bioethanol yield after 7 days of fermentation. In the study, saccharification was completed on 6th day of inoculation. Hence, all the three modes, SBB, SSF and SHF required 12 days of incubation for complete saccharification and fermentation.

Krishna *et al*. (1998) carried out ethanol production by simultaneous saccharification and fermentation (SSF) of sugarcane leaves using *T. reesei* and S. cerevisiae. Mishima *et al*. (2008) reported an ethanol yield of 0.14g/g dry substrtae through simultaneous saccharification and fermentation (SSF) of pretreated water hyacinth using commercial cellulase and *S. cerevisiae.* Gupta *et al*. (2009) resorted to separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *S. cerevisiae* and *Pichia stipitis*.

From the study, it was observed that the mixed culture of Bacillus sp.and S. cerevisiae gave a higher alcohol yield compared to the use of single organism, ie S. cerevisiae alone. SHF and SSF yielded 9.5 per cent and 8.76 per cent alcohol, respectively whereas the using single organism it was 8.34 per cent.

Future lines of work

Optimization of different modes of fermentation and suitable feedstock for fermentation is the preliminary requirement for bioethanol production. From the study, the pineapple fruit waste is identified as a potential feedstock for bioethanol production. Hence further studies should be focussed on increasing the yield of alcohol by optimizing the parameters that influence fermentation process *viz*., pH, temperature, inoculums size etc.

Studies should be conducted to identify efficient microorganisms from natural flora or genetically modify the strain of identified organism to improve fermentation efficiency. Instead of using a single microorganism for fermentation, different consortia of microorganisms could be tried to enhance the alcohol yield.

Different combinations of feed stocks (lignocellulosic materials) could be tried for improving the bioethanol production.

Summary

6. SUMMARY

A study on utilization of pineapple (*Ananas comosus* (L.) Merr.) biomass for biofuel production was conducted at College of Agriculture, Vellayani, Thiruvananthapuram during 2013- 2014. The important findings of the above studies are summarised in this chapter.

Pineapple waste is a best candidate for alcohol production due its abundance and ease of availability. It is a cheap substrate for biofuel production due to low lignin content and can undergo hydrolysis step more easily. The feed stocks were prepared by drying and grinding of pineapple peel, pineapple fruit waste and pineapple plant residue separately. This is a method of physical pretreatment; alter the structures of cellulosic biomass to increase the digestibility of enzyme to convert the carbohydrate polymers in to fermentable sugars.

The study on moisture content of the feedstocks showed that pineapple plant residue has higher moisture content followed by pineapple fruit waste and pineapple peel waste. The estimation of total sugar content of feed stocks revealed that, pineapple fruit waste have highest values of glucose, fructose, xylose and sucrose(8.37 per cent) compared to the other feed stocks and the higher levels of sugar content resulted in increased ethanol production during fermentation.

The total non reducing sugar and reducing sugars of pineapple feed stocks were estimated and observed a higher percentage in pineapple fruit waste followed by pineapple peel waste (18.20 percent and 27.2 percent, respectively). While the lowest value was recorded in pineapple plant residue.

Total dissolved solids were found to be maximum in pineapple fruit waste (28.12 per cent). Total carbohydrate was recorded maximum in pineapple fruit waste (70.68 per cent) followed by pineapple peel waste and lowest value was observed in pineapple plant residue. Estimation of cellulose, hemicelluloses and lignin content of the feed stocks revealed that pineapple plant residue have maximum cellulose content (69.20 per cent) followed by pineapple fruit waste (41.74 per cent) and pineapple peel waste (18.83 per cent). Whereas pineapple

peel waste has maximum hemicellulose content. Lignin content was found to be maximum (11.16 per cent) in plant residue followed by pineapple peel waste (6.17 per cent) and pineapple fruit waste (3.75 per cent)

Relatively low levels of fermentable sugars and high amount of fibre necessitated a pretreatment of the pineapple waste to increase the sugar content prior to fermentation. Pre treatment was performed for three feed stocks with acid and alkali which reduce the lignin content and made the sugar molecules accessible for fermentation.

Acid and alkali pretreatment of the pineapple feed stocks resulted an increase in total reducing sugar and total non reducing sugar concentrations. The increase in sugar concentration in pretreated feedstocks is due to the hydrolysis of cellulose and hemicellulose in to sugars. The removal of lignin is necessary for cellulose to become readily available for the enzymes. The acid and alkali pretreatment decreased the lignin content, but a higher percentage removal of lignin was observed with alkaline pretreated pine apple feed stocks.

Fermentation of untreated feedstocks gave higher alcohol percent than pre-treated feed stocks. In this study it was observed that, on pretreatments the lignin content was reduced. But this did not produce any increase in alcohol yield. This may be due to the production of various inhibitors like acetic acid, furfural and 5 hydroxymethylfurfural during the pretreatment. These products inhibit the growth of fermenting microorganisms. Moreover, the addition of NaOH and HCl to acid and alkali pretreated feed stocks, respectively for adjusting the pH to near neutral increased the salt concentration which in turn might have created a toxic effect and inhibited the growth of inoculated microorganism.

In the study, the alkali and acid pretreated feed stocks and untreated feed stocks were fermented using *Saccharomyces cerevisiae* and *Zymomonas mobilis,* separately. Fermentation using *Saccharomyces cerevisiae* in untreated pineapple fruit waste yielded maximum alcohol percentage (8.34 per cent) compared to

other feed stocks. This could be attributed to the increased level of fructose, glucose and sucrose compared to the other feed stocks.

Fermentation using *Zymomonas mobilis* also give a higher alcohol percentage (6.57 per cent) in untreated pineapple fruit waste. But this alcohol yield is significantly lower than alcohol obtained with *Saccharomyces cerevisiae.* Hence, *Saccharomyces cerevisiae* was found to be more efficient than *Zymomonas mobilis* for fermenting the pineapple waste biomass.

The results of percent conversion rate of reducing sugar after fermentation indicated that pineapple fruit waste have higher conversion rate than other feed stocks where as the percent conversion of non reducing sugar is found to be maximum with pineapple peel waste (28.81 percent). pH of the fermentation was tend to become acidic as the fermentation progress. This decrease in pH may be due to the release of yeast metabolism by products into the fermentation medium.

For the enhancement of fermentation a native microorganism having cellulolytic activity was isolated from pineapple waste. The isolated organism have the colony morphology as white, flat, slimy and circular with undulate margin and showed gram negative staining. The microorganism was catalase positive, negative to carbohydrate fermentation test, indole production test, urease test and positive to methyl red test, gelatin hydrolysis, Voges Proskauer test and citrate utilization test. The isolated microorganism was found to show similarity with *Bacillus sp.* Cellulolytic activity of the microorganism was tested by cellulase assay and maximum cellulase activity was observed on the sixth day of incubation.

The biochemical and molecular characterisation indicted the similarity of the isolated organism to *Bacillus sp*. Bacillus is a gram positive organism but the isolated microorganism was found to be gram negative on staining. This may be due to the fact that gram positive organisms at sporulation stage might give gram negative response on staining.

The molecular characterisation of the microorganism by the analysis of the sequences obtained in PCR amplification using specific primers indicted that, it showed sequence similarity to *Bacillus sp.*

In the study, enhancement in alcohol production was tried using mixed cultures of cellulolytic isolated bacteria and fermenting organism, *Saccharomyces cerevisiae*. Three different modes of saccharification and fermentation (SHF, SSF, SBB) was attempted to optimise the process for enhanced production of ethanol from the best identified feed stock, pineapple fruit waste. It was observed that single batch bioconversion (SBB) mode gave maximum alcohol yield of 11.09 per cent. In SSB process, fermentation is allowed only after the termination of enzyme production by heat-inactivation of cellulolytic organism, in the same culture broth. The increased alcohol yield of SBB may be due to the heat inactivation of isolated bacteria before fermentation. This limits the chance of interaction of isolated bacteria with S*accharomyces cerevisiae.* During the compatibility test for the isolated bacteria and *S. cerevisiae,* a slight incompatibility was observed indicated by a very thin clear zone.

In simultaneous saccharification and fermentation (SSF), separate hydrolysis and fermentation (SHF) both the organisms are inoculated into same culture medium and hence, the interaction between the two is more. The decreased level of ethanol in these methods may be due to this interaction and subsequent inhibition of *Saccharomyces cerevisiae* resulting low sugar conversion to alcohol. Till date, there is no report on the fermentation of agriculture residues with mixed cultures of *Bacillus sp.* with *Saccharomyces cerevisiae.*

The end point of fermentation was determined by investigating the alcohol percentage at 2 days interval and it was found that alcohol percentage will be constant from sixth day. In this study, pineapple fruit waste was proved as one of the novel and potential raw material biofuel production. However, optimization of

substrate concentration and environmental conditions will require for an industrial application.

From the study, it was observed that the mixed culture of *Bacillus sp*.and *S. cerevisiae* gave a higher alcohol yield compared to the use of single organism, ie *S. cerevisiae* alone.

The present study concluded that fruit waste is the best candidate for bioethanol production than other pineapple feed stocks tried. Single batch bioconversion using the cellulolytic organism, *Bacillus* sp. and fermenting organism, *S. cerevisiae* could bring about a substantial enhancement in alcohol yield.

Optimization of mode of fermentation and suitable feedstock for fermentation is the preliminary requirement for bioethanol production. From the study, the pineapple fruit waste is identified as a potential feedstock for bioethanol production. Hence further studies should be focussed to increase the yield of fermentation by optimizing the parameters such as pH, temperature, inoculums size etc.

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Appendice

APPENDIX – I

Reagents required for the characterization of the feed stock

a)Estimation of glucose

i)Fehling's solution A

Copper sulphate ($CuSO_4$) - 34.64g

Dissolved the component with distilled water and made up the volume to 500ml in a volumetric flask.

ii)Fehling's solution B

Rochelle salt - 173g

(potassium sodium tartarate $KNaC_4H_4O_6 \cdot 4H_2O$)

Sodium hydroxide NaOH - 50g

Dissolved the components with distilled water and made up the volume to 500ml in a volumetric flask.

b) Estimation of fructose

ii)Resorcinol reagent

Resorcinol - 1g

Thiourea - 0.25g

Glacial acetic acid - 100ml

This solution is indefinitely stable in dark.

ii)Diluted HCl was prepared by mixing five parts of conc. HCl with one part of distilled water.

iii)Standard fructose solution

Fructose - 50mg

Distilled water - 50ml.

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

c) Estimation of hemicellulose

i)Neutral Detergent solution

Disodium ethylenediamine tetra acetate - 18.6g

Sodium borate decahydrate - 6.81g

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

Sodium lauryl sulphate - 30g

2-ethoxy ethanol - 10ml

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

Disodium hydrogen phosphate - 4.5g

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

ii)Acid Dtergent solution

Cetyl trimethyl ammonium bromide - 20g

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

d) Estimation of total carbohydrate

i)2.5N HCl solution is prepared by adding 25.7ml HCl(35.4%,9.7n) in 100ml distilled water.

ii)Anthrone reagent

Anthrone - 200mg

95% ice-cold sulfuric acid - 100ml

This was prepared fresh before use

iii)Standard glucose

Glucose - 100mg

Distilled water - 100ml

10ml of stock diluted to 100ml with distilled water was used as working standard.

e) Estimation of cellulose

i) Acetic/nitric reagent

80% acetic acid - 150ml

Concentrated nitric acid - 15ml

ii)Anthrone reagent

Anthrone	-	200mg
Concentrated sulphuric acid	-	100ml

Prepared fresh and chilled for 2hr before use

67% sulphuric acid was also prepared.

Acid detergent solution 20g of cetyl trimethyl ammonium bromide in one litter of 1N sulphuric acid 72% H_2SO_4 (W/V).

f) Estimation of total reducing sugar

DNS (Dinitro Salicylic Acid) reagent

Dinitro salicylic acid	-	1g
Crystalline phenol	-	200mg
Sodium sulphite	-	50mg
1% NaOH	-	100 mL.

Dissolved by stirring and stored at 4^0C. Since the reagent deteriorates due to Sodium sulphite, for long storage, sodium sulphite was added at the time of use.

APPENDIX – II

Composition of different media

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

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

b)Media No.6 (Malt Yeast Agar)

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

c)Carboxy Methyl Cellulose Agar (1 L)

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

d)Trypticase soya agar

Tryptone	-	17 g
Soytone	-	3 g
Dextrose	-	2.5 g
NaCl	-	5.0 g
K2HPO4	-	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	-	5.000g
Beef extract	-	3.000g
Glucose	-	5.000g
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

f)Sucrose fermentation broth

Peptone	-	5.000g
Beef extract	-	3.000g
sucrose	-	5.000g
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

g)Lactose fermentation broth

Peptic digest of animal tissue	-	5.000g
Beef extract	-	3.000g
Lactose	-	5.000
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

h)Mannitol fermentation broth

Peptone	-	5.000g

Beef extract	-	3.000g
Mannitol	-	5.000g
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

i)Maltose fermentation broth

Peptone	-	5.000g
Beef extract	-	3.000g
Glucose	-	5.000g
Distilled water	-	1.00L
Final pH	-	6.9±0.2 (at 25°C)

j)Urease Test Broth

Yeast extract	-	0.100g
Urea	-	20.000g
Monopotassium phosphate	-	0.091g
Disodium phosphate	-	0.095g
Phenol red	-	0.010g
Final pH	-	6.8±0.2 (at 25°C)

k)Nutrient Gelatin

Peptone	-	5.000g
Beef extract	-	3.000g
Gelatin	-	120.000g
Final pH	-	6.8±0.2 (at 25°C)

***l*)Simmons Citrate Agar**

Magnesium sulphate	-	0.200g
Ammonium dihydrogen phosphate	-	1.000g
Dipotassium phosphate	-	1.000g
Sodium citrate	-	2.000g
Sodium chloride	-	5.000g
Bromothymol blue	-	0.080g
Agar	-	15.000g

Final pH	-	6.8±0.2 (at 25°C)

m)MR-VP broth (Glucose Phosphate Broth)

Buffered peptone	-	7.000g
Dextrose	-	5.000g
Dipotassium phosphate	-	5.000g
Final pH	-	6.9±0.2 (at 25°C)

APPENDIX – III

DNA sequences of the isolated native micro organism

16S Sequences

GCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGC
TCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAA
GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCG
CATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCA
ACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAA
GAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGC
CACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGT
CCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGA
AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCA
GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA
GGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCG
AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCT
TCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTC
AGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
ACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGT
TCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
GCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTG

ATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGG
GATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGT
TCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCAT
TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTG
AGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCC
GCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACA
AGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA
ATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCC
CCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAG
AGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA
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Abstract

UTILIZATION OF PINEAPPLE (*Ananas comosus* (L.) Merr.) BIOMASS FOR BIOFUEL PRODUCTION

by

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8. ABSTRACT

A study on utilization of pineapple (*Ananas comosus* (L.) Merr.) biomass for biofuel production was conducted at College of Agriculture, Vellayani, Thiruvananthapuram during the period of 2013-14.

Rising concern over depleting fossil fuel and greenhouse gas resulted in a high level of interest in nonconventional fuel originating from biorenewable sources including sugars, starches and lignocellulosic materials. Lignocellulosic materials constitute a substantial renewable substrate for bioethanol production that do not compete with food production and animal feed.

Pineapple waste is a promising feed stock for alcohol production due to its abundance and ease of availability. Also it is a cheap substrate for biofuel production due to low lignin content and can undergo hydrolysis steps more easily.

The feed stocks were prepared by drying and grinding of pineapple peel, pineapple fruit waste and pineapple plant residue separately. This is a method of physical pretreatment used for degradation of lignocelluloses and for reduction of cellulose crystallinity.

The study on moisture content of the feedstocks using gravimetric method showed that pineapple plant residue has higher moisture content followed by pineapple fruit waste and pineapple peel waste. The estimation of sugar content of different feed stocks revealed that, pineapple fruit waste have highest values of glucose, fructose, xylose and sucrose compared to the other feed stocks and this higher levels of sugar content resulted in higher ethanol production during fermentation.

Total dissolved solids was found to be maximum in pineapple fruit waste. Similarly total carbohydrate was recorded maximum in pineapple fruit waste followed by pineapple peel waste and lowest value was observed in pineapple plant residue. Estimation of cellulose, hemicelluloses and lignin content of the

feed stocks revealed that pineapple plant residue have maximum cellulose content followed by pineapple fruit waste and pineapple peel waste. Whereas pineapple peel waste recorded maximum hemicellulose content. Lignin content was found maximum in pineapple fruit waste.

To obtain a highly efficient conversion, pre treatment was performed for three feed stocks with acid and alkali which reduce the lignin content and make the sugar molecules accessible for fermentation. Acid and alkali pretreatment of the pineapple feed stocks resulted an increase in total reducing sugar and total non reducing sugar concentrations. The increase in sugar concentration in pretreated feedstocks is due to the hydrolysis of cellulose and hemicellulose in to sugars.

The acid and alkali pretreatment decreased the lignin content, but a higher percentage removal of lignin was observed with alkaline pretreated pineapple feed stocks.

The biochemical characterisation of the feed stocks revealed the sugar content and fermentation potential. To find out the effect of pretreatment fermentation was carried out in untreated and pretreated feed stocks with *Saccharomyces cerevisiae* and *Zymomonas mobilis.* Fermentation of untreated feedstocks gave higher alcohol percent than pre-treated feed stocks inspite of the fact that pretreatments resulted in an increase in total reducing and non reducing sugars and a decrease in the lignin content . This may be due to the production of various inhibitors or due to high salt formation during pH adjustments of the pretreated feedstocks. The results of percent conversion rate of reducing sugar to alcohol indicated that pineapple fruit waste have higher conversion rate than other feed stocks where as the percent conversion of non reducing sugar is found to be maximum with pineapple peel waste. pH of the fermenting medium also tend to become acidic.

Characterisation of feedstocks and alcohol yield after fermentation showed that pineapple fruit waste is the most amenable feedstock for alcohol production than other two. The alcohol yield (8.34 per cent) obtained with untreated fruit

waste using *S.cerevisiae* was found to be significantly higher than all other combinations tried.

For the enhancement of fermentation and subsequent alcohol yield, cellulolytic microorganism was isolated from degraded pineapple waste. It was identified as *Bacillus* sp. by biochemical and molecular characterisation. Three modes of enhancement of fermentation were performed with pineapple fruit waste; Single batch bioconversion, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) using *Saccharomyces cerevisiae* and isolated native microorganism. Single batch bioconversion was found to be the best enhancement method yielding 11.09 per cent alcohol. The decreased level of ethanol in other enhancement methods may be due to the negative interaction of *Bacillus sp.* with *Saccharomyces cerevisiae*.

The present study concluded that fruit waste is the best candidate for bioethanol production than other pineapple feed stocks tried. Single batch bioconversion using the cellulolytic organism, *Bacillus* sp. and fermenting organism, *S. cerevisiae* could bring about a substantial enhancement in alcohol yield.