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Isolation and Identification of Microorganisms from Palm Wine for Ethanol Production

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Abstract
Lignocellulosic feed stock materials are most abundant renewable bio resource material. It is primarily composed of cellulose, hemicellulose and lignin which are strongly associated with each other. Pre-treatment are mainly involved in effective separation of these complex. In the present work Corn Stover degrading microorganisms were isolated from palm wine. Fourteenth microorganisms were isolated, among these microorganisms, twelve were bacterial strains and two were yeast strain. Corn Stover's were treated with physical and chemical methods. The isolated microorganisms were optimized at different pH, Carbon sources, incubation time, incubation temperature for the maximum ethanol production.

Keywords
Lignocellulose, Palm wine, Pretreatment, Corn Stover, Optimization.

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INTRODUCTION
Ethanol is a clear, colourless, flammable, oxygenated hydrocarbon with the chemical formula C_2H_5OH. Ethanol has been made since ancient times by fermenting sugars. All the ethanol used for fuel and alcoholic drinks and most industrial ethanol, is made by this process. Fuel ethanol is also known as Bioethanol. Fuel ethanol is the largest market by far, accounting for 60% of total ethanol production worldwide. The term biofuel is attributed to any alternative fuel that derives from organic material, such as energy crops (corn, wheat, sugar cane, sugar beet, cassava, among others), crop residues (e.g. rice straw, rice husk, corn Stover, corn cobs) [1]. The most common way of Bioethanol production today is fermentation using the yeast *saccharomyces cerevisiae* with high ethanol yields from starch based substrates. In the past decades, thermophilic bacteria have gained more attention because of fast growth rates and their ability to degrade a broad variety of both hexoses and pentose's [2-3]. Industrial ethanol is mainly produced petrochemical through the acid-catalyzed hydration of ethylene. Ethanol for use in alcoholic beverages, and the vast majority of ethanol for use as biofuel, is produced by fermentation where certain species of yeast or bacteria metabolize sugars in oxygen-lean conditions to produce ethanol and carbon dioxide [4]. The effect of various carbon, nitrogen sources and environmental factors were investigated by one-factor-at-a-time method, and then the concentration of medium components was optimized using Taguchi method as a fractional

factorial design. Also, the effect of some low cost materials such as wheat bran, rice bran, whey, potato wastes and barley flour on ethanol production was evaluated in some details [5]. Lignocellulose is an abundant natural carbohydrate formed by biopolymers such as cellulose, hemicellulose and lignin, which can be transformed into substitute renewable energy resource by microbial conversion [6].

Bioethanol is being considered as a potential liquid fuel due to the limited amount of natural resources. Cellulose biomass is also being investigated as a potential substrate for Bioethanol production [7]. The fuel ethanol can be obtained from energy crops and lignocellulosic biomass. The complexity of the production process depends on the feedstock. In this way, the spectrum of designed and implemented technologies goes from the simple conversion of sugars by fermentation, to the multi-stage conversion of lignocellulosic biomass into ethanol. The big diversity of technological alternatives requires the analysis of the global process along with the design and development of each one of the involved operations. Among the new research trends in this field, process integration has the key for reducing costs in ethanol industry and increasing Bioethanol competitiveness related to gasoline [8]. Palm wine is the fermented sap of various palm trees; the sap should be collected from a growing palm. It is collected by tapping the palm this involves making a small incision in the bark about 15cm from the top of the trunk a clean gourd is tied around the tree to collect the sap which runs into it the sap is collected each day and should be consumed with in 5-12 hours of collection fresh palm juice is a sweet, clear, colorless juice containing 10-12% sugar. The sap is an excellent substrate for microbial growth fermentation starts soon after the sap is collected and within an hour (or) two. Becomes reasonably high in alcoholic (upto4%) if allowed to continue to ferment for more than a day, it starts turning into vinegar [9].

MATERIALS AND METHODS

Collection of samples

Fresh palm wine samples were collected from Palakkadu, Calicut, Kochi and Nagercoil.

Isolation and identification of microorganisms

0.1mL of palm wine samples were spreaded on to Sabouraud Dextrose Agar (SDA) and Nutrient Agar (NA) agar plate. The nutrient agar plates were incubated at 37ºC for 24 hours and the Sabouraud dextrose agar plates were incubated at 30ºC for 48 hours. Colonies were isolated and selected for the fermentation process. The isolated microorganisms were identified by staining techniques and biochemical characterization.

Effect of different carbon sources

To find a suitable medium and condition for ethanol production by isolates, different carbon sources were examined including glucose, xylose, arabinose, sucrose and fructose were used in the fermentation medium.

Analytical methods

Estimation of sugar concentration by DNSA method

1mL of sample/standard solutions and 1mL of water samples were taken and mixed thoroughly. 3mL of the DNSA reagent was added and were kept tubes in a boiling water bath for 10 min. OD values were taken by UV spectrophotometer at 575nm.

Estimation of ethanol concentration by potassium dichromate method

34g of potassium dichromate was dissolved in 500mL distilled water. And slowly 325mL concentrated sulfuric acid was added in the flask an ice bucket.7.5mL of distillate the 12.5mL potassium dichromate solution was added and the final volume was made up to 25mL.Kept at 60ºC for 30 minutes and read absorbance at 600nm.

Pretreatment of the Corn Stover substrate

Physical pretreatment method

Steam explosion: The corn Stover substrate was autoclaved at 121ºC for 20 min; the contents were cooled and incubated at room temperature [10].

Chemical pretreatment methods

Lignocellulosic Biomass Pretreatment Techniques

The lignocellulosic biomass pretreatment is to separate the biomass components *i.e.* cellulose, hemicellulose and lignin without losing hemicellulose while decreasing the crystallinity of cellulose and increasing the porosity of the biomass material. A number of techniques are available for the pretreatment of biomass; these include Organ solvation and Acid hydrolysis of lignocellulosic biomass [11].

Organ solvation

The biomass was treated with a mixture of organic/aqueous organic solvents were used are solvents are methanol, ethanol and acetone. The process facilitates simultaneous hydrolysis and delignification of lignocellulosic biomass. Lignin can be recovered as a fine precipitate by flash exposure of the liquor to atmospheric pressure, followed by rapid dilution with water. Other products such as sugars and sugar degradation products can be recovered from the water soluble stream.

Acid Hydrolysis of Lignocellulosic Biomass

The biomass was treated with the acid. The treatment process converts the cellulose and hemicellulose in to sugars. Acid hydrolysis is the most common methodology for biomass conversion of fermentable sugars. The substrate was treated with 50% of sulphuric acid solution at room temperature for 15mins.

Determination of glucose and ethanol concentration of the isolated microorganisms by using substrate

Glucose concentration was determined by DNS method. Ethanol was determined using potassium dichromate method.

Optimization of temperature, pH and time for ethanol production

To examine the effects of temperature, initial pH, time of fermentation on ethanol production, isolates were cultivated at a range of temperatures 25, 30, 35, 40ºC; various pH 2, 4, 6, 8; different fermentation time 12, 24, 36, 48 hours.

Distillation

The mixture after fermentation broth was distilled out with the reflux column and reflux condenser for the maximum percentage of Bioethanol.

MALDI-TOF MS identification of the bacteria isolates

The identification of the bacteria by MALDI-TOF MS was performed on a Bruker Micro flex system (Bruker, Germany) instrument equipped with a nitrogen laser with an output wavelength of 337nm used at a repetition rate of 60Hz. All spectra were acquired in the linear positive mode within a range of 2-20 kDa.

RESULTS

Isolation and identification of microorganisms from palm wine

Twelve different bacterial colonies were isolated in nutrient agar plate and two yeast colonies were isolated in Sabouraud Dextrose Agar (SDA) plate.

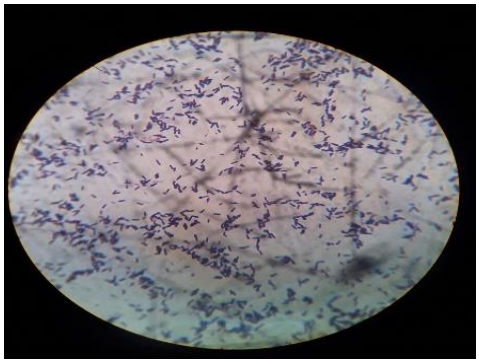


Figure: 1PW1

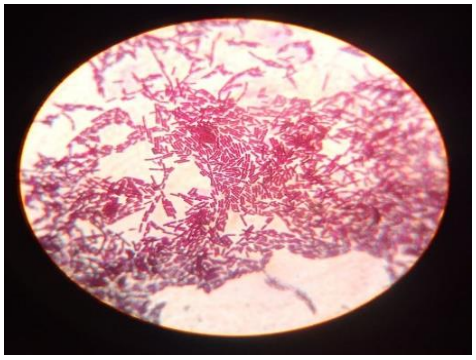
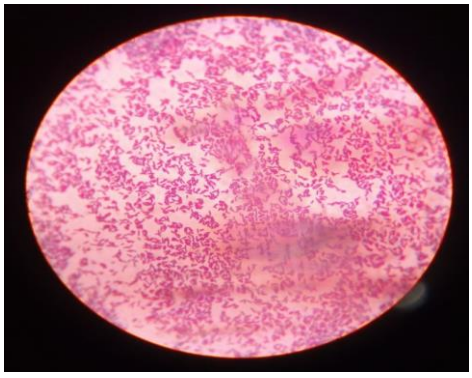


Figure:2 PW2



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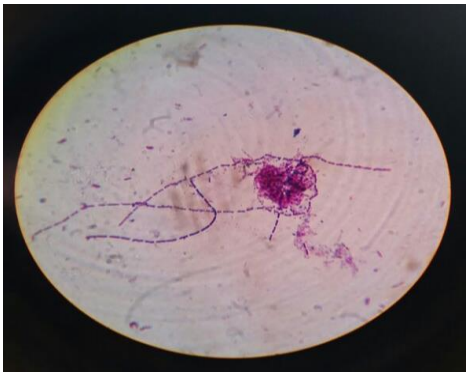

Figure: 4 PW4

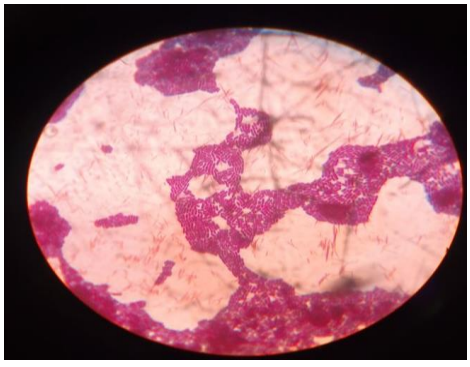

Figure:5 PW5

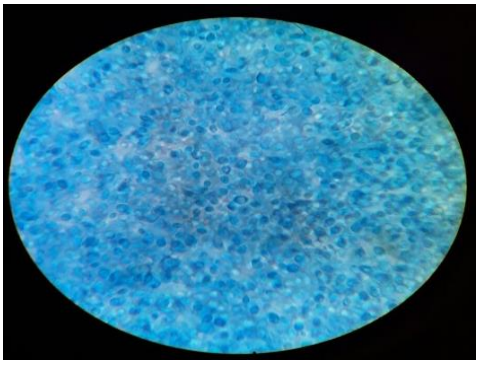

Figure:6 Y1

Biochemical Characterization

S.No	Sample	Indole	MR	VP	Citrate	TSI	Catalase	Oxidase	Starch hydrolysis	Gram staining
1	Pw1	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve

Note: +ve (positive), -ve (negative), TSI (triple sugar iron test), MR (methyl red), VP (vogues proskauer test).

Production of ethanol from different carbon source: The effect of the carbon source for the production of ethanol were studied in the fermentation broth was supplemented with different carbon source such as glucose, xylose, arabinose, sucrose and fructose then incubated static condition at 35ºc. The results in table-5.1 showed that the best carbon source for the isolates was arabinose and xylose. The results revealed that the isolates were able to produce high ethanol when consumed five different carbon sources.

Ethanol estimation by potassium dichromate method

S. no	Sample	Starch	xylose	sucrose	arabinose	Cellulose
1	PW1	0.067	1.937	1.998	2.011	0.058
2	Y1	0.068	2.208	1.832	1.996	0.061
3	MV1	0.083	1.943	1.612	1.785	0.067
4	MV2	0.062	1.926	1.068	2.136	0.058

Table .1 Production of ethanol from different carbon source

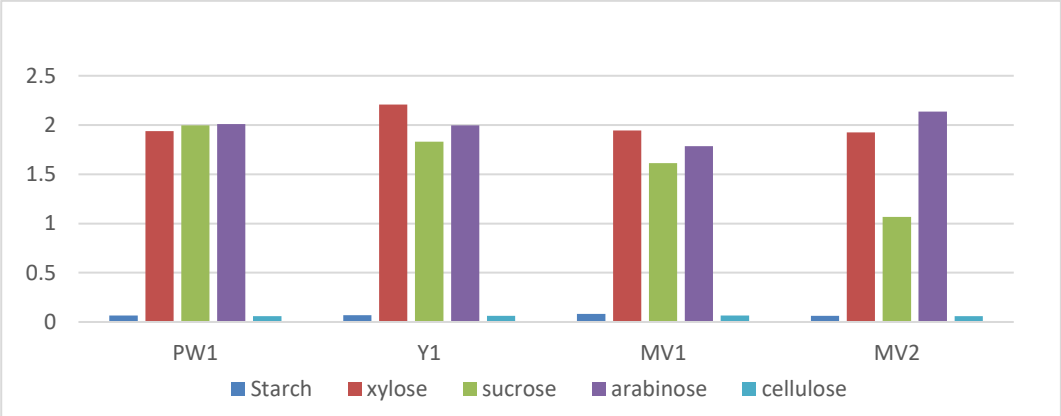

Chart: 1 Estimation of ethanol

Sugar estimation by DNSA method

S. no	Sample	Starch	xylose	Sucrose	arabinose	Cellulose
1	PW1	0.024	2.110	2.190	1.197	0.004
2	Y1	0.027	1.955	1.500	1.939	0.002
3	MV1	0.014	2.246	1.049	1.344	0.013
4	MV2	0.01	2.853	1.303	1.741	0.007

Table 2 DNSA method

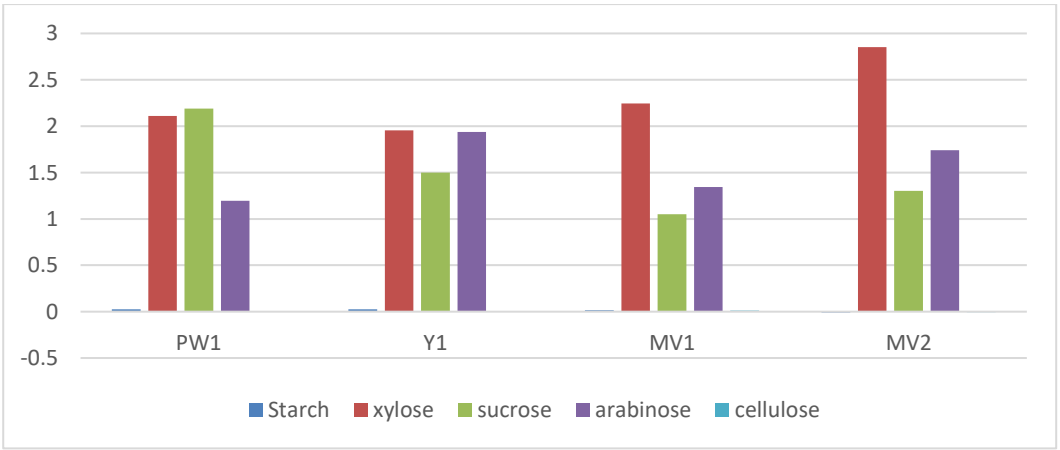

Chart :2 DNSA method

Fermentation using substrate as a corn Stover

Figure 7 fermentation using corn stover as a substrate

Determination of glucose and ethanol concentration

Pretreatment of corn stovers

The corn stovers were treated with physical and chemical method for effective separation of the Cellulose, hemicellulose and lignin complex. After the pretreatment the substrates were used for the ethanol production. Ethanol was estimated by potassium dichromate method.

S. No	Sample	O.D value
1	PW1	2.016
2	Y1	2.315
3	MV2	2.228

Table .6 Estimation of ethanol after the pretreatment of substrate

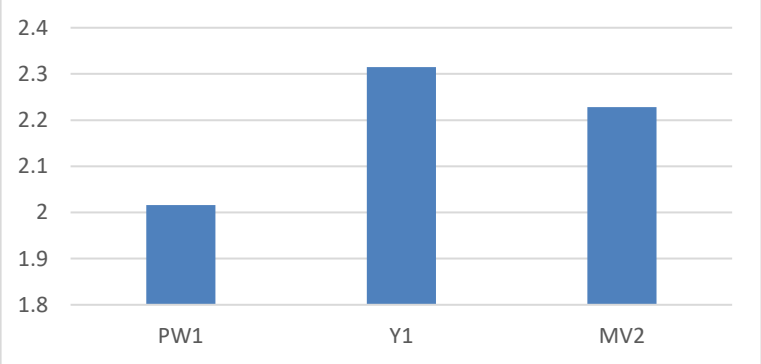

Chart5: Estimation of ethanol after the pretreatment of substrate

Optimization of temperature, pH and time

To determine the effect of temperature on ethanol production, the isolates were cultured at different temperature. The optimum growth temperature for maximum ethanol production was 30ºc. Among the other isolates, Y1 was exhibited the highest ethanol yield.

To determine the effect of pH on ability of isolate PW1 and Y1 to produce ethanol, pH of fermentation broth was adjusted from 2 to 8 then incubated at 35ºC in static conditions for 72 hours. The results indicated that maximum ethanol was produced at pH 6-8. Among the other isolates, Y1 was exhibited highest ethanol yield 2.482 at pH 6.

To study the effect of time on ethanol production, fermentation broth was inoculated with active culture the culture of PW1 and Y1 then incubated static condition at 35ºC for 12, 24, 36 and 48 hours. The results revealed that the ethanol production was increased over time. Ethanol production by Y1 was raised due to increasing fermentation time from 36 to 48 hours. The maximum ethanol was produced by Y1 after 48 hours incubation with 2.824 ethanol, respectively.

S. No	Sample	Temperature		pH		Time	
		Temperature	OD value	pH	OD value	Hours	OD value
1	PW1	25	0.028	2	0.024	12	0.128
		30	0.934	4	0.422	24	0.242
		35	1.824	6	2.104	36	1.326
		40	2.012	8	1.722	48	2.614
2	Y1	25	0.062	2	0.123	12	0.262
		30	2.214	4	0.192	24	0.431
		35	1.642	6	2.482	36	1.528
		40	1.212	8	1.832	48	2.824
3	MV2	25	0.098	2	0.268	12	0.332
		30	2.468	4	0.462	24	0.621
		35	1.926	6	2.628	36	1.652
		40	1.421	8	1.946	48	2.989

Table 7: Effect of temperature, pH and time on Ethanol production

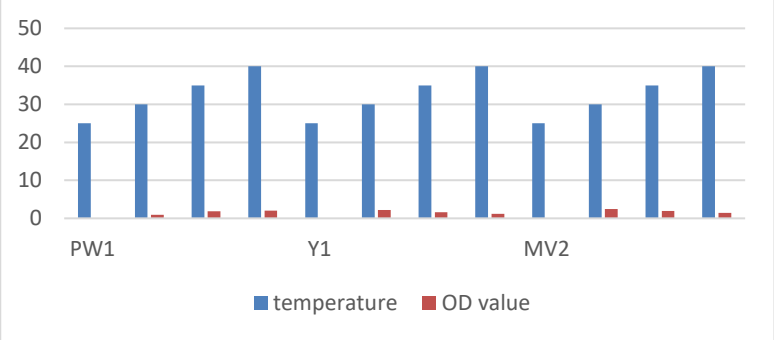

Chart 7: Effect of Temperature

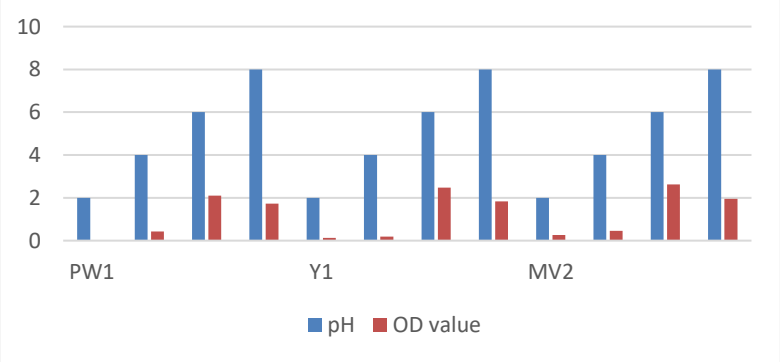

Chart8: Effect of pH

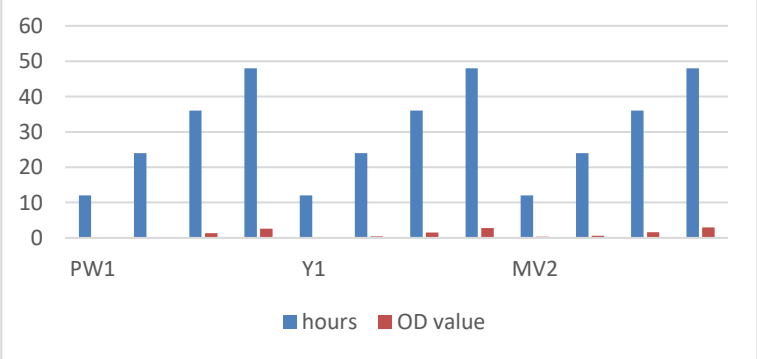

Chart9: Effect of time

MALDI-TOF

The maximum ethanol producing isolate (Y1) was given to MALDI-TOF analysis for the identification. The identified bacteria were *Pseudomonas stutzeri*.

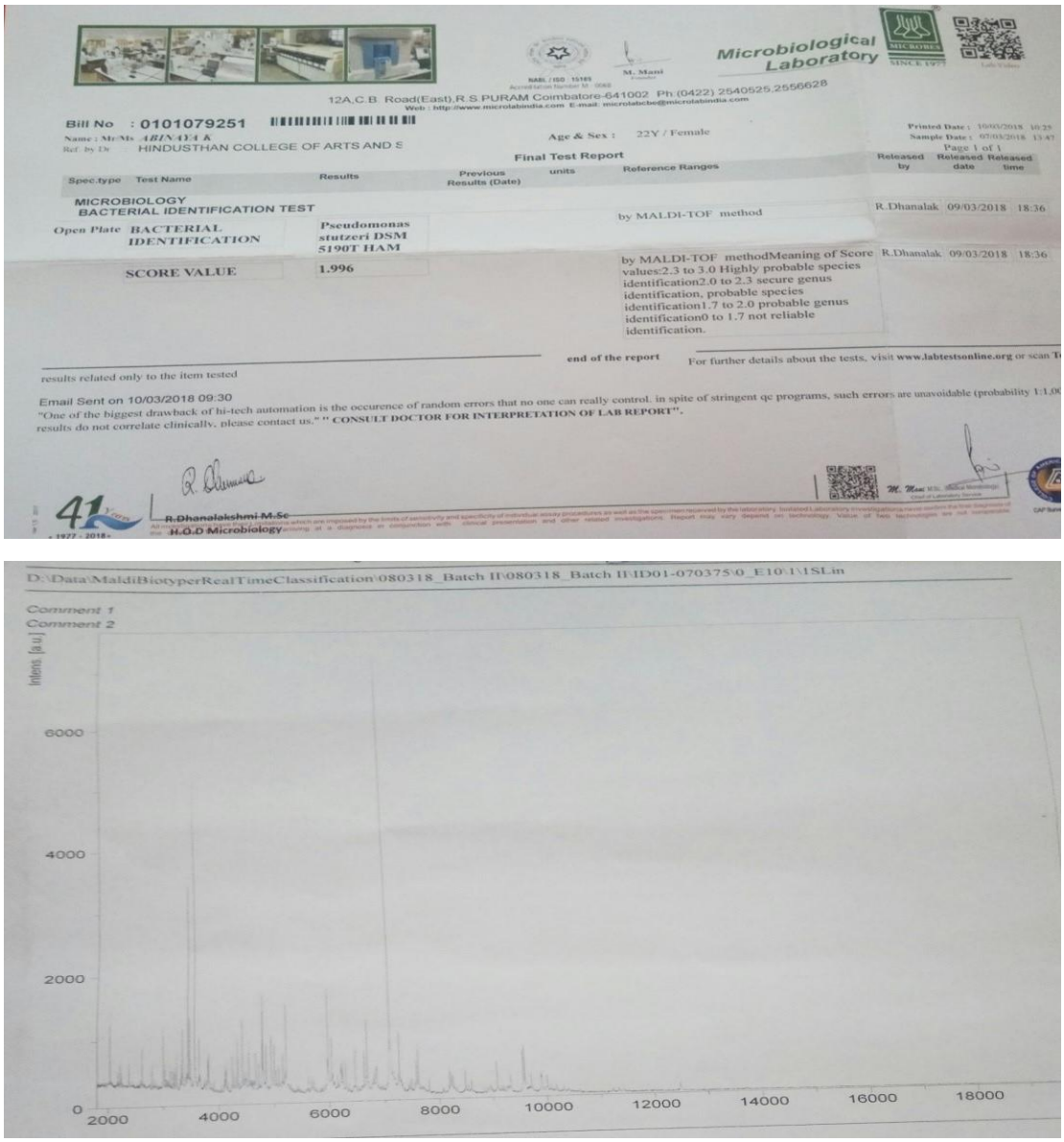

Figure 8 MALDI-TOF

Distillation

The sample was given for the extraction and distillation of the ethanol in South Indian Textile Research Association (SITRA)Coimbatore.12ml of ethanol was eluted from the fermentation broth.

DISCUSSION

Bioethanol is a liquid energy for automobiles and industry. Ethanol is used as a universal solvent and also fuel. Ethanol is used as an alternative to petroleum. Bioethanol offer several advantage over gasoline, higher flame speed broader flammability and increase the heat of vaporization. The results obtained in this study are useful in scaling up the fermentation processes for ethanol production using corn stover.

The microorganisms are isolated from palm wine for the Bioethanol production. Twelve different bacterial colonies were isolated on nutrient agar plate and two yeast colonies were isolated on Sabouraud Dextrose Agar (SDA) plate. The substrate was treated with physical and chemical methods for the effective separation of cellulose, hemicellulose and lignin complex. After the pretreatment corn stover yield maximum ethanol and the isolates were optimized at different incubation time, temperature, different carbon sources and pH for the maximum ethanol

production. Among the other isolates, *Psudomonasstutzeri* was identified as the maximum ethanol producing organism.

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