Bioconversion of different carbohydrate substrates to ethanol and acetic acid

A

DISSERTATION REPORT

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CERTIFICATE

This is to certify that the thesis entitled "**Bioconversion of different carbohydrate substrates to ethanol and acetic acid**" submitted by Ms. Deepinder Kaur in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under her supervisor. The report has not been submitted for the award of any other degree or certificate in this or any other University or institute.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"Bioconversion of different carbohydrate substrates to ethanol and acetic acid"** in partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from Jan 2012 to June 2012, under the supervision of **Dr. Dinesh Goyal, Professor**, Department of Biotechnology & Environmental Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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Abbreviations

α	Alpha
@	at the rate of
β	Beta
CH_3COOH	Acetic acid
CH_3COONa	Sodium acetate
CO_2	Carbon dioxide
Conc.	Concentration
°C	Degree Celsius
DNS	3,5-dinitrosalicylic acid
et al	and others
g	Gram
GL	Gallons
GYE	Glucose Yeast Extract
Gy	Gray
h	Hour
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
kJ	Kilojoules
L	Litre
µg	Microgram
µl	Microlitre

min	Minute
MJ	Millijoules
mL	Millilitre
mM	Millimolar
M	Molar
mol	moles
N	Normal
NaOH	Sodium Hydroxide
NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulphate
nm	Nanometer
O.D.	Optical Density
O_2	Oxygen
%	Percent
rpm	Rotation per minute
s	Second
v	Volume

ABSTRACT

Bioconversion of different carbohydrate substrates to ethanol and acetic acid was studied in glucose, fructose, jaggery, grape juice, kinnow pulp juice, sugarcane juice and apple juice by inoculating *Saccharomyces cerevisiae* G. Maximum ethanol production of 8.72% was observed with sugarcane juice followed by grape juice which was found to be 7.92%. After wine production, sugarcane wine and grape wine were further used for production of vinegar using *Acetobacter aceti* NRRL 746 with titrable acidity of 4.1 and 4.5, respectively after 25 days of incubation. Treated and untreated mixed agricultural waste was also used as substrate for the production of ethanol using cellulose degrading bacterial strain N11 and *S. cerevisiae* G via simultaneous saccharification and fermentation. Ethanol production was observed over a period of 7 days and the maximum ethanol production (0.3%) was observed in case of acid pretreatment followed by alkali pretreated mixed agricultural waste which was found to be 0.1%. Acid pretreated agricultural waste biomass was found to be promising substrate for ethanol production.

OBJECTIVES

1. Ethanolic fermentation of different carbohydrate substrates.
2. Fermentation of wine to vinegar.
3. Conversion of agricultural waste biomass to ethanol by simultaneous saccharification and fermentation.

INTRODUCTION

The world's present economy is highly dependent on various fossil energy sources such as oil, coal, natural gas etc which are being used for the production of fuel, electricity and other goods (Uihlein *et al*, 2009). With the expansion of human population and increase of industrial prosperity, global energy consumption also has increased gradually. Excessive consumption of fossil fuels, particularly in large urban areas, has resulted in generation of high levels of pollution during the last few decades due to which the level of greenhouse gasses in the earth's atmosphere has drastically increased (Ballesteros *et al*, 2006). Annual global oil production will begin to decline within the near future (Campbell and Laherrere, 1998). Import of transport fuel is affected by limited reserves of fossil fuel. To overcome this crisis scientist moved towards establishing new alternative sources of energy (Zaldivar *et al*, 2001). Between 1980 and 2005, worldwide production of biofuels increased by an order of magnitude from 4.4 to 50.1 billion L (Armbruster and Coyle, 2006). The world bioethanol production in 2001 was 31 billion L (Berg, 2001), which has grown to 39 billion L in 2006 and is expected to reach 100 billion L in 2015 (Taherzadeh and Karimi, 2007).

The energy content of ethanol was higher than the energy required to produce ethanol (Shapouri *et al*, 2002), indicating that ethanol used as a liquid transportation fuel could reduce domestic consumption of fossil fuels, particularly petroleum (Kim and Dale, 2002). However, the cost of bioethanol production is more as compared to fossil fuels (Sarkar *et al*, 2012). Ethanol derived from biomass, one of the modern forms of biomass energy, has the potential to be a sustainable transportation fuel, as well as a fuel oxygenate that can replace gasoline (Wang *et al*, 2000). Brazil and the USA are the two major ethanol producers accounting for 62% of the world production (Kim and Dale, 2004). Biomass used for production of ethanol is mainly corn glucose in U.S (MacDonald *et al*, 2001) and sucrose in Brazil (Rosillo and Cortez, 1998). Globally, bioethanol production from rice straw, wheat straw, corn straw and sugarcane bagasse is now a matter of interest out of which rice straw is the most abundant waste and it can potentially produce 205 billion L bioethanol per year (Sarkar *et al*, 2012). China and India contributed 11% to global ethanol production in 2006 (Nigam and Singh, 2010). Bioethanol production has been found from lignocellulosic waste materials (John *et al*, 2011) such as crop residues (Kim and Dale, 2004), municipal solid waste (Mtui and Nakamura, 2005), forest product industry wastes (Kadar *et al*, 2004), leaf and yard waste (Lissens *et al*, 2004) as well as dairy and cattle manures (Wen *et al*, 2004).

Cellulose, one of the most abundant organic macromolecules in the ecosystem (Ross *et al*, 1991) is a linear unbranched homopolysaccharide consisting of glucose subunit joined together by β 1-4 glycosidic linkages which is totally insoluble in water (Lederberg, 1992). Individual cellulose molecules (polymer) vary widely in length and are usually arranged in bundles or fibrils (Walsh, 2002), can occur in crystalline or paracrystalline (amorphous) structures (Walter, 1998). Ethanol is produced from sugars by fermentation with the help of microorganisms so cellulose must be first hydrolyzed to sugars, generally by mineral acids for conversion to ethanol (Lin *et al*, 2006).

Lignocellulosic materials are renewable, low cost and are abundantly available. Rice straw, wheat straw, corn straw and sugarcane bagasse are the major agricultural wastes in terms of quantity of biomass available (Kim and Dale, 2004). Extensive research has been carried out on ethanol production from lignocellulosics in the past two decades (Binod *et al*, 2010). Hence bioethanol production could be the route to the effective utilization of agricultural wastes. Biomass resources used for ethanol production can be grouped into four categories- Wood residues are by far the largest current source of biomass for energy production. Municipal solid waste is the next largest, followed by agriculture residues and dedicated energy crops. Among these energy crops seem to be the largest, most promising, future resource of biomass. This is because of the reduced average annual costs for establishing and managing energy crops, particularly in comparison to conventional crops (Monique *et al*, 2003). The main components of lignocellulosic materials are cellulose (35-50%), followed by hemicellulose (20-35%) and lignin (10-25%). Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992). The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa (Alexander, 1961).

Industrial ethanol production has been reported using various starchy materials such as corn, wheat, starch and potatoes, cassava root (Lindeman and Rocchiccioli, 1979), corn stover (Kadam and McMillan, 2003), and starch (Maisch *et al*, 1979). Cassava starch, a root crop produced in more than 80 countries is used as a cheap source of fermentation. The fermentable substrate in dried cassava is upto 80% but the waste is toxic and thus its processing is difficult (Sasson, 1990).

The most commonly used microorganism for fermentation is yeast *Saccharomyces cerevisiae* which can produce ethanol up to concentration of 18% of the fermentation broth by the

Embden–Meyerhof (EM) pathway (Matthew *et al*, 2005). Higher concentrations of sugar and the accumulation of ethanol in the cell can inhibit the growth of yeast (Ribereau *et al*, 1985). Currently, approx 80% of total world ethanol production is obtained from the fermentation of simple sugars by yeast (Lin and Tanaka, 2006).

Wine is an alcoholic beverage obtained from grape juice with yeast used as a fermenting organism (Reddy *et al*, 2011). Wine has a rich history dating back thousands of years, with the earliest known production occurring around 8,000 years ago on the territory of modern-day Georgia (Berkowitz, 1996). Vinegar may be defined as a condiment made from various sugary and starchy materials by alcoholic and subsequent acetic fermentation. Vinegar can be produced by different methods and from various raw materials. Wine, fruit musts, malted barley or pure alcohol are used as substrates. Vinegar production ranges from traditional methods employing wood casks and surface culture to submerged fermentation in acetators (Morales *et al*, 2001). Acetic acid is formed in a four-step reaction involving conversion of starch to sugar by amylases, anaerobic conversion of sugars to ethanol by yeast fermentation, conversion of ethanol to hydrated acetaldehyde, and dehydrogenation to acetic acid by aldehyde dehydrogenase (Canning 1985). The last two steps are performed aerobically with the aid of acetic acid forming bacteria.

The current work is on wine and vinegar production using different carbohydrate substrates and ethanol production using mixed agricultural waste biomass via simultaneous saccharification and fermentation.

REVIEW OF LITERATURE

Cellulose

Cellulose, the major chemical component of the fiber wall, is a homopolysaccharide composed entirely of D-glucose linked together by β-1,4-glycosidic bonds with degree of polymerization ranging from 1,000 in bleached kraft pulps to 10,000 in native wood (Schmidt, 2006). It is a linear structure that has a strong tendency to form intra or intermolecular hydrogen bonds resulting in the formation of cellulose microfibrils. The structure of cellulose with its hydrogen bond makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation (Jorgensen, 2003).

Ethanol

With the inevitable depletion of the world's petroleum supply (Kerr, 1998) there has been an increasing worldwide interest in alternative, non-petroleum-based sources of energy. As petroleum supplies 97% of the energy consumed for transportation (Putsche and Sandor, 1996), industry and governments worldwide have been actively identifying, developing and commercializing technology for alternative transportation fuels over the past 20 years (Sheehan, 1994). Feedstock containing significant amounts of sugar, or materials that can be converted into sugars, such as starch or cellulose, can be used in the production of ethanol (Aggarwal *et al*, 2001). Expansion of the market for ethanol to as much as 38-53 billion L per year, or nearly enough for a 10% ethanol blend of gasoline used in the United States, could occur if all available agricultural residues were converted to ethanol (Sheehan, 2001). The total potential bioethanol production from crop residues and wasted crops is 491 GL/year, about 16 times higher than the current world ethanol production. The potential bioethanol production could replace 353 GL of gasoline. Asia is the largest potential producer of bioethanol from crop residues and wasted crops, and could produce up to 291 GL/year of bioethanol (Kim and Dale, 2004). Ethanol can be combined and blended with petrol or burned in its pure form within modified spark-ignition engines. In comparison, a litre of ethanol contains 66% of the energy provided by a litre of petrol but has a higher octane level, and when mixed with petrol for transportation it improves the performance of the petrol and fuel combustion in vehicles, thereby reducing the emission of carbon monoxide, unburned hydrocarbons and carcinogens. (Nigam and Singh, 2010). In comparison to petrol, ethanol contains only a trace amount of sulphur. Therefore, mixing ethanol with petrol helps to reduce the fuel's sulphur content and thereby lowers the emissions of sulphur oxide, a major

component of acid rain, and a carcinogen (Hoekman, 2009). While the reality of global warming continues to be discussed (Sheehan, 1994), the use of fuel ethanol will significantly reduce net carbon dioxide emissions when it replaces fossil fuels, because fermentation derived ethanol is already part of the global carbon cycle (Wyman, 1994).

Raw material

Alcoholic fermentation has been carried out using a number of sugary materials depending upon their availability and suitability in particular geographic situations. Various raw materials like sugarcane juice and molasses, sugar beet, beet molasses (Agrawal *et al*, 1998), sweet sorghum (Bulawayo *et al*, 1996) and starchy materials like sweet potato (Sree *et al*, 2000), corn cobs and hulls (Arni *et al*, 1999), cellulosic materials like cocoa, pineapples and sugarcane waste (Othman *et al*, 1992) and milk/cheese/whey using lactose hydrolyzing fermenting strains (Silva *et al*, 1995) have been reported. Of these, simple sugar bearing materials are the easiest to process, since the yeast ferment these directly while other carbohydrates like starch/cellulose have to be first hydrolyzed to fermentable sugars using current commercial technologies (physio-chemical/enzymatic preparation) before they can be fermented to yield ethanol.

Ethanol production from hydrolysed wheat starch has been successful used for saccharification using *S. cerevisiae* (Dabas *et al*, 1997). In India and other developing countries, ethanol is mainly produced by fermentation of dilute molasses employing *S. cerevisiae* (Bulawayo *et al*, 1996). Cane molasses is a complex mixture that varies in composition according to geographical sources, agricultural practices and sugar mill operations. Common sugar crops used as feedstocks are sugar cane, sugar beet and to a much lesser extent sweet sorghum (Aggarwal *et al*, 2001).

The utilization fraction of wheat straw, rice straw and corn straw is too low (Kim and Dale, 2004). Only a small portion of globally produced rice straw is used as animal feed, the rest is removed from the field by burning, a common practice all over the world, increasing air pollution and affecting human health (Wati *et al*, 2007). Less than 1% of corn straw is collected for industrial processing and about 5% is used as animal feed and bedding. More than 90% of corn straw in United States is left in the fields (Glassner *et al*, 1999). It has been estimated that 442 billion L of bioethanol can be produced from lignocellulosic biomass and that total crop residues and wasted crops can produce 491 billion L of bioethanol per year, about 16 times higher than the actual world bioethanol production (Kim and Dale, 2004).

Table 1. Different raw materials used for production of ethanol

Crop	Residue/crop	Dry matter (%)	Lignin (%)	Carbohydrates (%)	Ethanol yield ratio (%)
Barley	1.2	88.7	2.9	67.1	0.41
Barley straw		81.0	9.00	70.00	0.31
Corn	1	86.2	0.60	73.70	0.46
Corn stover		78.5	18.69	58.29	0.29
Oat	1.3	89.1	4.00	65.60	0.41
Oat straw		90.1	13.75	59.10	0.26
Rice	1.4	88.6		87.50	0.48
Rice straw		88.0	7.13	49.33	0.28
Sorghum	1.3	89.0	1.40	71.60	0.44
Sorghum straw		88.0	15.00	61.00	0.27
Wheat	1.3	89.1		35.85	0.40
Wheat straw		90.1	16.00	54.00	0.29
Sugarcane		26.0		67.00	0.50
Bagasse	0.6	71.0	14.50	67.15	0.28

Source: Kim and Dale (2004).

Ethanol producing microorganisms

Out of the various ethanol producing micro-organisms used in fermentation, yeast belonging to *S. cerevisiae* isolated from spoiled high sugar foods (Ok and Hashinanga, 1997) have been used most commonly. Yeast metabolizes most sugars through the glycolytic pathway to produce energy and necessary growth intermediates. To limit high concentrations of acidic end products synthesized in glycolysis, such as pyruvic acid and acetic acid, yeast convert these acids to ethanol and carbon dioxide. Although yeast are not very tolerant of acidic products, they are very tolerant of ethanol. *S. cerevisiae* are able to produce up to 51.1% weight ethanol/weight glucose from glucose derived from starch (Ingledew, 1999). Alternative organisms are often studied; however, these species rarely produce ethanol as efficiently and at the high concentrations of *Saccharomyces*. *Pichia stipitis* produce ethanol

only upto 61 g/L ethanol in 350 h from 154 g/L xylose (Slininger *et al*, 2006). *Zymomonas* were compared for ethanol production, with some strains being more tolerant of high sugar or ethanol concentration and high incubation temperature than others. One of the most promising ethanol producing organism is the bacterium *Zymomonas mobilis* which is used to make palm wines. *Zymomonas* is the only microorganism that metabolizes glucose anaerobically using the ED pathway as opposed to the EM or glycolytic pathway (Matthew *et al*, 2005) but the disadvantage of this process is the production of various by-products, primarily acetic and lactic acid and moreover it can't utilize all substrates (Wu *et al*, 1986). This bacterium can produce up to 1.9 mol of ethanol from each mole of glucose fermented (Skotnicki *et al*, 1981). In a comparative study on ethanol production from molasses using *S. cerevisiae* and *Z. mobilis*, yeast was found to be more ethanol tolerant and produced more ethanol at sugar concentration above 15% (v/v) (Bansal and Singh, 2003).

S. cerevisiae from palm wine, produced increased amounts of ethanol in yeast extract peptone dextrose medium (Uma and Polasa 1990). Bertolini *et al* (1991) also isolated new strains of *S. cerevisiae* on basal medium containing 48% sucrose from fermenting sample collected from Brazilian alcohol factories.

Table 2. Potential of different microorganisms studied for ethanol production using different substrates.

Microorganisms	**Temp (°C)**	**Sugar (g/L)**	**Ethanol(g/L)**	**Reference**
Candida tropicalis 1369	37	Xylose (100)	5.4	Jeffries, 1981
Clavispora species 83-877-1	25	Xylose (60)	10.9	Nigam *et al*,1985
Brettanomyces clausenii	37	Xylose (20)	21.4	Parekh *et al*.,1988
Bakers' yeast- *S. cerevisiae*	28	Sucrose (220)	96.71	Caylak and Vardar, 1996
S. cerevisiae 24860	30	Molasses (1.6–5.0)	5-18.4	Ergun and Mutlu, 2000
Kluyveromyces fragilis 27774	30	Glucose (20–120)	48.96	Vallet *et al*, 1996
Kluyveromyces marxianus 30016	30	Glucose (100)	44.4	Vallet *et al*, 1996
Candida utilis 30091	30	Glucose (100)	44.4	Vallet *et al*, 1996
Pachysolen tannophilus 32691	30	Glucose (0-25)	7.8	Sanchez *et al*, 1999

Source: Banat *et al*, 1998; Lin and Tanaka, 2006.

Simultaneous saccharification and fermentation (SSF)

The process for producing ethanol from lignocellulosic material includes three main steps: steam pretreatment, enzymatic hydrolysis and fermentation. The subsequent enzymatic hydrolysis of the cellulose and hemicellulose can be performed simultaneously with fermentation of the produced monomeric glucose and is then denoted SSF (Linde *et al*, 2007). The SSF process increases the yields of ethanol by minimizing product inhibition as well as eliminates the need for separate reactors for saccharification and fermentation (Deshpande *et al*, 1983). The SSF process was also shown to be superior to saccharification and subsequent fermentation (Krishna *et al*, 1999) due to the rapid assimilation of sugars by yeast during SSF.

Strategy of pretreatment

Steam explosion (autohydrolysis)

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials (McMillan, 1994). In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Ninety percent efficiency of enzymatic hydrolysis has been achieved in 24 h for poplar chips pretreated by steam explosion, compared to only 15% hydrolysis of untreated chips (Grous *et al*, 1986). The advantages of steam explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The conventional mechanical methods require 70% more energy than steam explosion to achieve the same size reduction (Holtzapple *et al*, 1990). Steam explosion is recognized as one of the most cost effective pretreatment processes for hardwoods and agricultural residues, but it is less effective for softwoods (Clark and Mackie, 1987). Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes (Mackie *et al*, 1985).

Acid pretreatment

Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic,

corrosive and hazardous and require reactors that are resistant to corrosion. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995). Dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian *et al*, 1997). At moderate temperature, direct saccharification suffered from low yields because of sugar decomposition. High temperature in dilute acid treatment is favorable for cellulose hydrolysis (McMillan, 1994). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or ammonia fiber expansion technique (AFEX). A neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes.

Alkaline pretreatment

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (McMillan, 1994). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969). Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity and disruption of the lignin structure (Fan *et al*, 1987). The digestibility of NaOH-treated hardwood increased from 14% to 55% with the decrease of lignin content from 24–55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet *et al*, 1976). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10–18% (Bjerre *et al*, 1996). Chosdu *et al* (1993) used the combination of irradiation and 2% NaOH for pretreatment of corn stalk, cassava bark and peanut husk. The glucose yield of corn stalk was 20% in untreated samples compared to 43% after treatment with electron beam irradiation at the dose of 500 kGy and 2% NaOH, but the glucose yields of cassava bark and peanut husk were only 3.5% and 2.5%, respectively. Ammonia was also used for the pretreatment to remove lignin.

Ethanol represents closed carbon dioxide cycle because after burning of ethanol, the released CO_2 is recycled back into plant material because plants use CO_2 to synthesize cellulose during photosynthesis cycle (Chandel *et al*, 2007). Ethanol production process only uses energy from renewable energy sources; no net CO_2 is added to the atmosphere, making ethanol an environmentally beneficial energy source. In addition, the toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources (Wyman and Hinman, 1990). Ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the green house gas effect (Foody, 1988).

MATERIAL AND METHODS

Different substrates such as glucose, fructose, jaggery, grape juice, kinnow pulp juice, sugarcane juice and apple juice were used for production of wine using *Saccharomyces cerevisiae* G and further to vinegar using *Acetobacter aceti* NRRL 746. The mixed agricultural waste biomass was degraded using different pretreatments and then subjected to SSF which led to production of bioethanol.

Strains

Saccharomyces cerevisiae G and *Acetobacter aceti* NRRL 746 were procured from Departmant of Microbiology, Punjab Agriculture University (PAU) Ludhiana, Punjab (India).

Maintenance of cultures

1. *S. cerevisiae* G was maintained on Glucose Yeast Extract (GYE) agar slants at 4°C.
2. *A. aceti* NRRL 746 was maintained on Tryptone agar slants at 4°C.
3. Cellulose degrading bacterial strain N11 (Procured from Science and Technology Entrepreneur's Park, Thapar University Patiala, Punjab) was maintained on Nutrient Agar (NA) slant.

Microscopy of the strains

Lactophenol cotton blue staining of *S. cerevisiae* G

Lactophenol cotton blue stain was used for the microscopic examination of *S. cerevisiae* G strain (Jordan and Pattison, 1996). The lactophenol cotton blue wet mount preparation is the most widely used method of staining and observing fungi and is simple to prepare. The preparation has three components: phenol, which will kill any live organisms, lactic acid which preserves fungal structures and cotton blue which stains the chitin in the fungal cell walls.

Materials

a. Lactophenol dye
b. Alcohol (70%)

Method

1. Placed a drop of alcohol (70 %) on a clean grease free microscope slide.

2. Immersed the actively grown culture of *S. cerevisiae* G in a drop of alcohol.
3. Two drops of the lactophenol cotton blue were added before the alcohol dries out.
4. Folding the cover slip between forefinger and thumb, touch one edge and mountain avoiding air bubbles. The preparation was ready for examination.
5. Visualized under phase contrast microscope (Nikon Eclipse 90i, Japan) at different magnifications.

Phase contrast microscopy (Nikon Eclipse 90i, Japan) of actively grown culture of *S. cerevisiae* G was also done under different magnification.

Gram Staining of *A. aceti* NRRL 746

Gram staining is a method of differentiating bacterial species into two large groups namely Gram-positive and Gram-negative. Gram Staining was done as per Christian Gram, 1884.

Materials

a. Crystal violet (0.3%)
b. Grams iodine (1% iodine, 2% potassium iodide in water)
c. Decolorizer (95% ethanol)
d. Safranine (0.25%)

Method

1. Bacterial smear from actively growing cells of culture *A. aceti* NRRL 746 were spread on a clean grease free slide and was heat fixed.
2. Crystal violet was flooded for 1 min on the smear.
3. Washed with tap water to remove excess of crystal violet.
4. Grams iodine was flooded for 1 min and washed briefly with water.
5. Decolorized with acetone and washed with tap water.
6. Safranine was used to counter stain for 30 s.
7. Washed with tap water to remove excessive stain.
8. Visualized under phase contrast microscope (Nikon Eclipse 90i, Japan) at different magnifications. A gram positive *Bacillus* sp. was taken as reference.

Production of wine

Different carbohydrate substrates were used for the production of wine over a period of 7 days using *S. cerevisiae* G.

Materials

a. Glucose
b. Fructose
c. Jaggery
d. Grape juice
e. Kinnow pulp juice
f. Sugarcane juice
g. Apple juice

Jaggery, grape juice, kinnow pulp, sugarcane juice and apple juice were procured from local market of Patiala, Punjab (India). Kinnow pulp was boiled in distilled water and then sieved to obtain juice which was then used for the production of wine.

Media

a. GYE media (Appendix I)

Method

1. *S. cerevisiae* G was activated by adding inoculum (5%) in autoclaved jaggery solution (25g/L).
2. Different substrates were pasteurized and allowed to settle for 24 h.
3. Sugar concentrations were maintained to 18 °Brix (18 g/100 mL) using brixometer (Erma Hand Refractometer, Japan).
4. The autoclaved substrates were inoculated (5%) with overnight grown culture of *S. cerevisiae* G (10^6 cell mL^{-1})
5. Fermentation was carried out for 7 days at 22 ± 2°C.
6. Sampling was done at regular interval for determination of pH, sugar and ethanol.

Ethanol estimation by Gas chromatography (GC)

Gas chromatography is used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. In gas chromatography, the mobile phase is a carrier gas, usually an inert gas such as helium or nitrogen.

The stationary phase is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column. The instrument used to perform gas chromatography is called a gas chromatograph.

Method

1. Different concentrations of ethanol (2 to 10%) were prepared, samples (1 µL) were injected and chromatogram was obtained using flame ionization detector with GC (NUCON SERIES 5700) under following conditions:

- Oven temperature: 155°C (isothermal).
- Inlet temperature: 175°C.
- Detector temperature: 250°C.
- Run time: 5.5 min.
- Column: Porepar Q.
- Carrier gas (N_2) flow rate: 30 mL/min.
- Hydrogen and air flow rate: 30 mL/min and 300 mL/min, respectively.

2. Response factor was calculated

$$\text{Response factor} = \frac{\text{concentration}}{\text{peak area}}$$

3. Samples were prepared by centrifuging at 8000 rpm for 15 min and then filtered through membrane using a membrane filter of 0.45 µm. The samples were kept at 4°C prior to analysis. The samples should not be preserved for more than 30 days.
4. Ethanol concentration of unknown sample was calculated from standard curve.

Calculation

$$\text{Ethanol concentration (\%) of unknown sample} = \text{concentration factor} \times \text{area of unknown sample}$$

Ethanol estimation by potassium dichromate ($K_2Cr_2O_7$) method

Ethanol estimation was done as per the method given by Seo *et al*, 2009.

It involves solvent extraction of ethanol from a culture broth followed by measurements of the ethanol concentration using the dichromate oxidation method. When ethanol is present in an aqueous solution, chromium ions oxidize ethanol, and these ions are reduced from the +6 oxidation state to +3, changing the color from orange to green.

Material

a. Ethanol
b. Potassium dichromate: It was prepared by dissolving 10 g potassium dichromate in 100 ml of 5M sulphuric acid solution.
c. Di-n-butyl phthalate (DBP)

Method

Different concentrations of ethanol were prepared ranging from 2 to 8% and volume was raised to 2 ml.

1. DBP (2 mL) was added to the above tubes.
2. The mixture was shaked vigorously for 30 min on shaker at 250 rpm.
3. The lower phase (1.5 mL) was transferred to a new microtube.
4. Then 1.5 mL of potassium dichromate was added to the microtube.
5. The mixture was shaked vigorously for 30 min on shaker at 250 rpm.
6. Then O.D of the lower phase was taken at 595 nm.
7. Standard curve was prepared and ethanol concentration was estimated from unknown samples.

Table 3. Standard curve of ethanol using potassium dichromate ($K_2Cr_2O_7$) method

Ethanol (µL)	Distilled water (mL)	Ethanol (%)	DBP (mL)	Shaked for 30 min and 1.5 mL of lower layer was taken.	Potassium dichromate (mL)	Shaked for 20 min and lower layer was taken.	O.D taken at 595 nm
0	2	0	2		1.5		
40	1.96	2	2		1.5		
80	1.92	4	2		1.5		
120	1.88	6	2		1.5		
160	1.84	8	2		1.5		

Production of vinegar

Vinegar can be produced by different methods and from various raw materials like wine and any kind alcoholic solution (Morales *et al*, 2001).

Material

a. NH_4NO_3 (0.01%)
b. $(NH_4)_2SO_4$(0.01%)
c. $ZnSO_4$ (0.01%)
d. Yeast extract (0.01%)
e. Mother vinegar (10%)

Media

a. Tryptone media (Appendix II).

Method

1. Ethanol content was diluted to 8%.
2. Inoculation (7.5%) was done with *A. aceti* NRRL 746.
3. 10% of mother vinegar (vinegar having acidity of 4%) was added.
4. Stock (0.01%) of NH_4NO_3, $(NH_4)_2SO_4$, $ZnSO_4$ and yeast extract was prepared and above stock was added to the culture @ 0.1%.
5. Fermentation was carried out at 28 ± 2ºC for 36-48 h at 75 rpm.
6. After regular interval of 2 days shaking was done and fermentation was continued for 25 days.

Estimation of titrable acidity

Titrable acidity was done as per the method given by Ough and Amerine, 1987.

Materials

a. 0.1M Sodium hydroxide (NaOH)
b. Phenolphthalein indicator
c. Vinegar samples

Method

1. Distilled water (200 mL) was taken in a flask.
2. Then 5 mL of sample was added to the above flask.

3. Two drops of indicator phenolphthalein was then added.
4. Burette was filled with 0.1 N NaOH solutions.
5. NaOH was added to the flask drop wise till the pink colour appears.
6. Volume of NaOH used was then noted.
7. Titrable acidity was calculated by the formula.

Calculation:

$$\text{Titrable acidity} = \frac{V1 \times N1 \times 7.5}{V2}$$

Where,

V_1 = Volume of NaOH used

N_1 = Normality of NaOH used

V_2 = Volume of sample added

7.5 is the tartaric acid factor.

Estimation of reducing sugars

Reducing sugars were estimated by using DNS (3,5-dinitrosalicyclic acid) method given by Miller, 1959.

Materials

a. Stock: 2 mg/mL glucose.
b. DNS reagent (Appendix III).

Method

1. Different concentrations of glucose were prepared ranging from 0.2 - 2 mg/mL and volume was made to 1 mL.
2. Then 3 mL of DNS was added and placed in boiling water bath for 10 min.
3. OD was taken at 540 nm.
4. A standard graph was prepared using stock solution.
5. For sample analysis, cell free supernatant (0.5 mL) was taken, volume was made to 1 mL and the same procedure was followed.

Table 4. Standard curve of Glucose using DNS method

Stock (mL)	Distilled water (mL)	Glucose conc. (mg/mL)	DNS (mL)	Boiled for 5 minutes	O.D taken at 540 nm
0	1.0	0	3.0		
0.1	0.9	0.2	3.0		
0.2	0.8	0.4	3.0		
0.3	0.7	0.6	3.0		
0.4	0.6	0.8	3.0		
0.5	0.5	1.0	3.0		
0.6	0.4	1.2	3.0		
0.7	0.3	1.4	3.0		
0.8	0.2	1.6	3.0		
0.9	0.1	1.8	3.0		
1.0	0	2.0	3.0		

Estimation of cellulose

Estimation of cellulose was done by Anthrone assay given by Updegraff, 1969

Cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cellodextrins which get dissolved and hydrolyzed to form glucose molecules on treatment with 67% H_2SO_4. This glucose molecule is dehydrated to form hydroxymethyl furfural which forms green coloured product with anthrone and the colour intensity is measured at 630 nm.

Materials

- Acetic/Nitric reagent: Mix 150 mL of 80% acetic acid and 15 mL of concentrated nitric acid.
- Anthrone reagent: Dissolve 200 mg anthrone in 100 mL of concentrated sulphuric acid. Prepare fresh and chill for 2 h before use.
- 67% sulphuric acid.
- Stock solution of cellulose: 10 mg/mL

Method

1. Acetic/nitric reagent (5 mL) was added to 0.1 g of the sample in a test tube and it was mixed in a vortex mixer.

2. The tubes were placed in a water-bath at 100°C for 30 min.

3. The contents were then cooled and centrifuged at 10000 rpm for 15 min.

4. The supernatant were discarded and residue was washed with distilled water.

5. 67% H_2SO_4 (10 mL) was added to it and it was allowed to stand for 1 h.

7. The above solution was then diluted 100 times.

8. To 0.2 mL of this diluted solution, 2 mL of anthrone reagent was added.

9. The tubes were then heated in a boiling water-bath for 10 min.

10. The contents were cooled and colour was measured at 630 nm.

12. The standard was prepared by taking different concentrations of cellulose and cellulose in unknown sample was then calculated.

Table 5. Standard curve of cellulose using Anthrone assay.

Stock (mL)	Distilled water (mL)	Cellulose (mg/mL)	67% H_2SO_4 (mL)	Diluted to 100 mL and 0.2 mL of diluted sample was taken.	Anthrone (mL)	Boiled for 10 minutes	O.D taken at 630 nm
0	1.0	0	10		2		
0.2	0.8	2	10		2		
0.4	0.6	4	10		2		
0.6	0.4	6	10		2		
0.8	0.2	8	10		2		
1.0	0	10	10		2		

Sample collection and processing

Mixed agricultural waste composed of leaves of different plants viz. Ashoka (*Saraca asoca*), Mango (*Mangifera indica*), Eucalyptus (*Eucalyptus globulus*), Jamun (*Syzygium cumini*), Poplar (*Populus tremula)*, Bamboo (*Bambusa arundinacea*), Rice straw *(Oryza sativa*) and Wheat husk *(Triticum aestivum*) were collected from different places of Thapar University and nearby villages of Patiala, Punjab (India) located at 30º20´N, 76º24´E and 310 m above the sea level. Equal amount (by weight) of agricultural waste from different tree species were taken, washed to remove adhering debris and were air dried and milled by mechanical blender and sieved to 2 mm for further treatment process. The biomass was also pre-treated with different methods in order to obtain maximum ethanol.

Pretreatment methods used for mixed agricultural waste biomass

Acid pretreatment

1. Processed mixed agricultural waste (1 gm) was taken in an Erlenmeyer flask.
2. One percent H_2SO_4 (10 mL) was added to it.
3. The mixed agricultural waste was then autoclaved at 121°C for 15 min.
4. It was cooled and supernatant was discarded.
5. Residue was washed with NaOH (1%) followed by distilled water to neutralize pH.
6. It was then filtered by Whatman filter paper no. 42 and then dried in oven at 70°C for 24 h.

Alkali pretreatment

1. Processed mixed agricultural waste (1 gm) was taken in an Erlenmeyer flask.
2. 1 N NaOH (10 mL) was added to it.
3. The mixed agricultural waste was then autoclaved at 121°C for 15 min.
4. It was cooled and supernatant was discarded.
5. Residue was washed with 0.02 M acetate buffer (Appendix IV) followed by distilled water to neutralize pH.
6. It was then filtered using Whatman filter paper no. 42 and then dried in oven at 70°C for 24 h.

Steam pretreatment

1. Processed mixed agricultural waste (1 gm) was taken in an Erlenmeyer flask.
2. Distilled water (20 mL) was added to the flask.
3. It was given sudden steam explosion in an autoclave at 121°C for 15 min by sudden depressurization by opening steam exhaust valve.
4. The substrate was cooled and filtered by Whatman filter paper no. 42 and then dried in oven at 70°C for 24 h.

Simultaneous saccharification and fermentation (SSF) using treated and untreated mixed agricultural waste biomass

Media composition for SSF

The fermentation media (Table 6) were prepared in 100 ml Erlenmeyer flask for SSF of mixed agricultural waste biomass using cellulose degrading strain N11 and *S. cerevisiae* G.

Table 6. SSF media ingredient for ethanol production

Ingredients	**Amount (g/L)**
Substrate	10
Yeast extract	1
Peptone	1

Experimental Procedure:

1. Media (50 ml) supplemented with mixed agricultural waste biomass (1%) and one blank for the reference were taken in 100 ml Erlenmeyer flask and pH of media was adjusted to 4.5.
2. Media were autoclaved for 15 min at 121˚C and 15 lb/psi and cooled at room temperature.
3. Two percent of overnight grown cellulose degrading bacterial strain (N11) was inoculated in flasks containing fermentation media and incubated at 30˚C for 7 days under shaking condition at 120 rpm.
4. Twenty four hour grown *S. cerevisiae* G (5%) was added to the flask after 24 h of incubation and placed in incubator at 30˚C under shaking condition at 120 rpm.
5. Samples were withdrawn at a regular interval of 48 h and analyzed for reducing sugar and ethanol using DNS method and GC, respectively.

RESULTS AND DISCUSSION

To check the purity of the cultures morphological study of the strain *S. cerevisiae* G and *A. aceti* NRRL 746 was done using phase contrast microscope and these strains were further used for the production of wine and vinegar using different carbohydrate substrates, respectively.

Microscopy of the strains

Lactophenol cotton blue staining of *S. cerevisiae* G

The strain *S.cerevisiae* was stained with lactophenol cotton blue dye. Both stained and unstained cells were then visualized under microscope at different magnification (Figure 1).

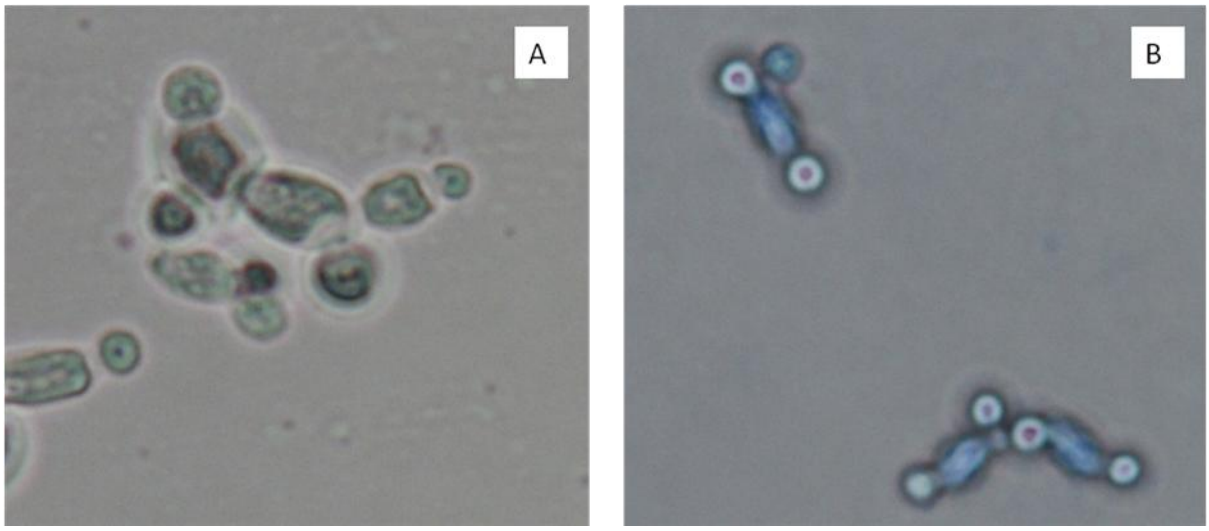

Figure 1. Micrograph of *S. cerevisiae* G. A) Unstained cells B) Stained with lactophenol cotton blue dye under 100X (Nikon eclipse 90i, Japan).

S. cerevisiae is a suitable candidate to produce ethanol (Gunasekaran and Raj, 2001) and was found to be more ethanol tolerant and produced more ethanol at sugar concentration above 15% (v/v) (Bansal and Singh, 2003). So in the present study strain *S.cerevisiae* G has been used for production of ethanol using different carbohydrate substrates and further to acetic acid using *A.aceti* NRRL 746 for its conversion to vinegar.

Gram staining

The strain *A. aceti* NRRL 746 was visualized under phase contrast microscope at different magnification and was found to be Gram negative rod shaped bacteria (Figure 2); *Bacillus* sp. was taken as reference.

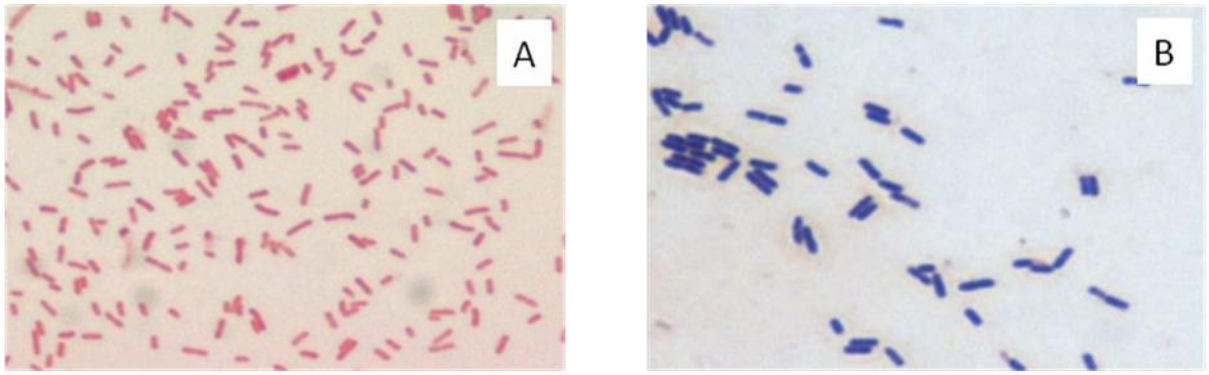

Figure 2. Gram staining of bacterial strain A) Gram negative *A. aceti*; B) Gram positive *Bacillus* sp. at 100X (Nikon Eclipse 90i, Japan).

Production of wine

Wine production was observed over a period of 7 days using different carbohydrate substrate. Different parameters such as pH, reducing sugar and ethanol were studied at regular intervals to monitor the process of wine production.

pH varied from 3.2 to 4.93 among all fermentation with maximum decrease in the pH was observed with glucose as substrate (Table 7).

The °Brix (sugar in g/100 mL) using Brixometer was observed and a significant decrease in sugar concentration was found in all the carbohydrate substrates as the substrate is being utilized by *S. cerevisiae* G for production of ethanol. Maximum decrease in sugar was found within three days of inoculation and bubbling of CO_2 was seen (Figure 3). Highest sugar consumption was found using grape juice and sugarcane juice which was 72.2% and 80%, respectively (Table 8).

Wine is a fermented beverage of cereals, fresh fruits etc. Wine from rice is produced after saccharification of starch by microbes and enzymes (especially, commercial amylase). The wine is a complex mixture of organic and inorganic substances like carbohydrates, proteins, amino acids, ethyl alcohol, organic acids, inorganic acids and micronutrients etc.

Table 7. pH of different substrates over a period of 7 days.

S.No.	**Substrates**	**pH**				
		0 day	1 day	3 days	5 days	7 days
1	Glucose	5.50	4.98	4.32	3.72	**3.20**
2	Fructose	5.50	4.70	4.24	3.98	3.42
3	Sugarcane juice	5.50	5.25	5.10	4.98	**4.93**
4	Jaggery	5.50	5.20	4.95	4.60	4.23
6	Kinow pulp	5.50	5.16	4.82	4.65	4.50
7	Apple juice	5.50	4.94	4.32	4.16	3.98
8	Grape juice	5.50	4.89	4.28	4.07	3.96

Table 8. °Brix (g/100mL) of different substrates over a period of 7 days.

S.No.	**Substrates**	**°Brix (g/100mL)**				
		0 day	1 day	3 days	5 days	7 days
1	Glucose	18.0	16.5	16.5	16.0	15.0
2	Fructose	18.0	16.8	16.1	15.8	15.0
3	Sugarcane juice	18.0	14.0	7.2	5.4	**3.6**
4	Jaggery	18.0	13.1	6.5	5.8	5.4
6	Kinow pulp	5.0	4.0	3.0	2.6	2.2
7	Apple juice	9.5	7.0	5.0	4.9	3.7
8	Grape juice	14.0	9.5	5.6	4.2	**3.9**

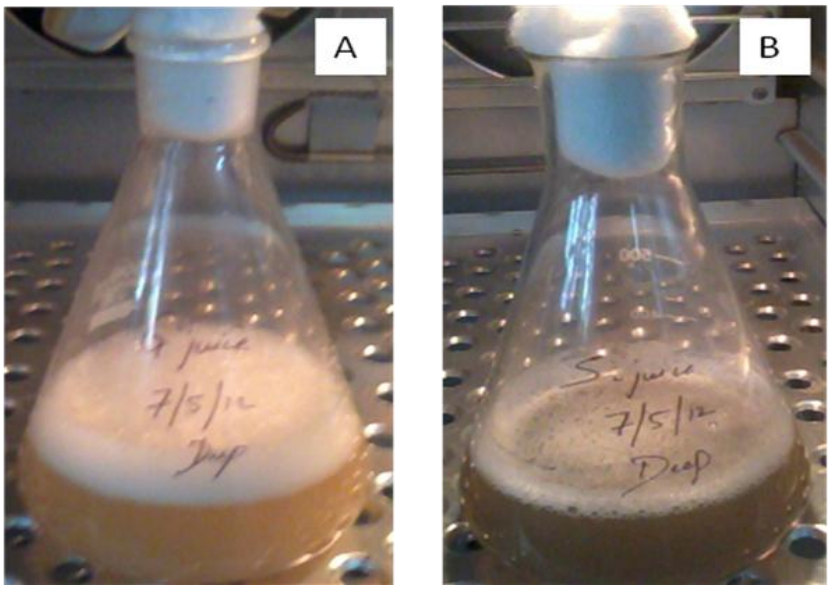

Figure 3. Fermentation in grape juice and sugarcane juice after 24 h of inoculation.

The amount of ethanol produced was monitored over a period of 7 days by GC and potassium dichromate method (Seo *et al*, 2009).

Table 9. Standard curve for ethanol using potassium dichromate method

Ethanol (%)	O.D at 595 nm
2	0.144
4	0.498
6	1.008
8	1.299

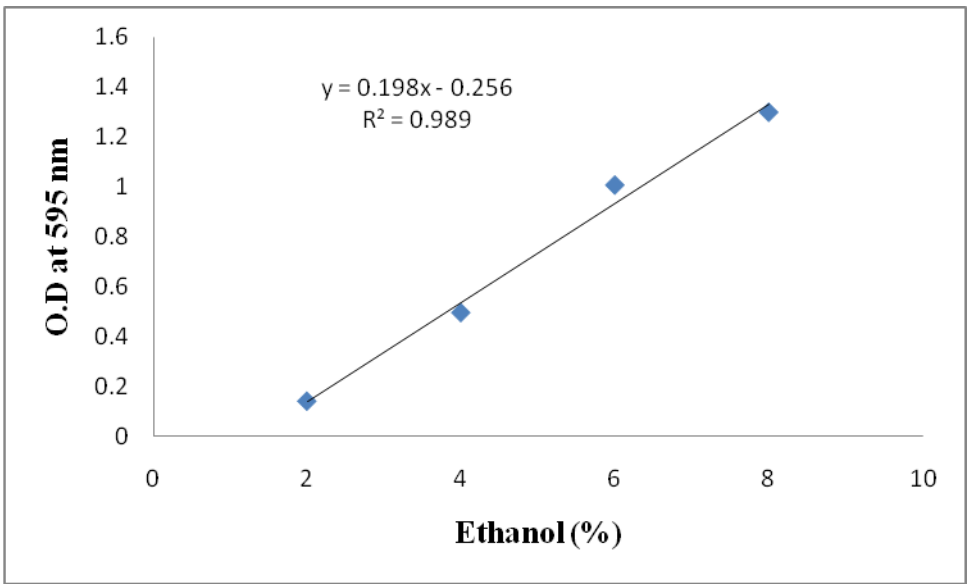

Figure 4. Standard curve for ethanol using potassium dichromate method (y = 0.198x-0.256 and R^2 = 0.989)

Kocher *et al* (2003) reported 8.72% (v/v) ethanol and 80% reduction in the sugar content of the juice during the fermentation of the sugarcane juice using *S. cerevisiae* G. The ethanol produced by fermentation of grape juice was 7.92% which was found to be similar by Heard and Fleet (2008) using grape juice.

Ethanol production using different substrate with *S. cerevisiae* G was observed over a period of 7 days (Figure 6, 7, 8, 9, 10, 11, 12). Maximum ethanol production was observed with sugarcane juice (8.72%) and minimum with apple juice (0.39%) (Table11).

Table 10. Calibration curve of ethanol using GC

Ethanol (%)	Retention time (min)	Area	Concentration factor
2	2.76	2789510	0.00000072
4	2.74	5714285	0.00000070
6	2.71	7730443	0.00000078
8	2.71	9907156	0.00000081
10	2.68	13589253	0.00000074

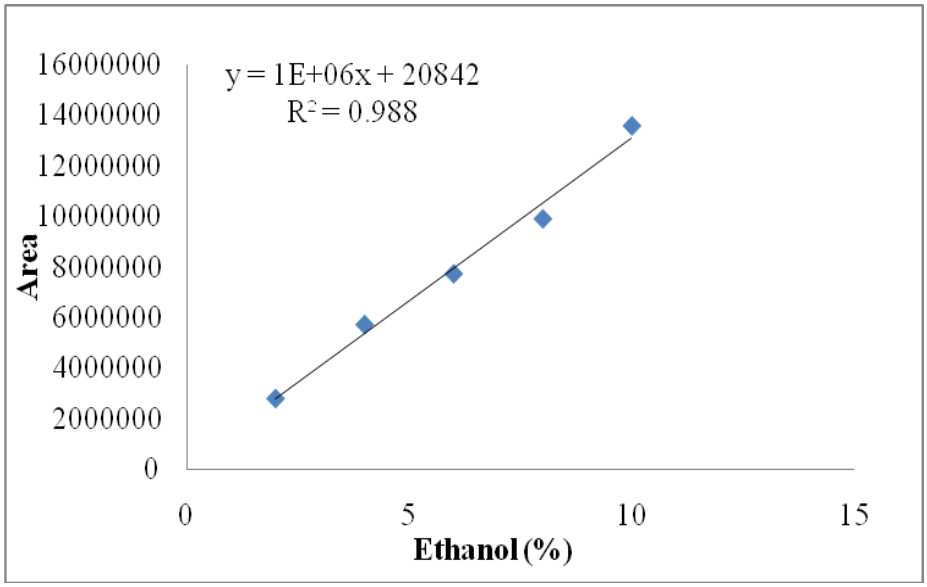

Figure 5. Calibration curve for ethanol using GC.

Table 11. Ethanol production after 7 days of fermentation process

S.No.	Substrates	Retention time (min)	Ethanol (%) by GC	Ethanol (%) by potassium dichromate method
1	Glucose	2.73	2.37	1.97
2	Fructose	2.68	1.70	1.45
3	Sugarcane juice	2.68	**8.72**	6.83
4	Jaggery	2.69	5.67	5.006
5	Kinu pulp	2.73	2.17	3.63
6	Apple juice	2.68	0.39	6.26
7	Grape juice	2.71	**7.92**	4.37

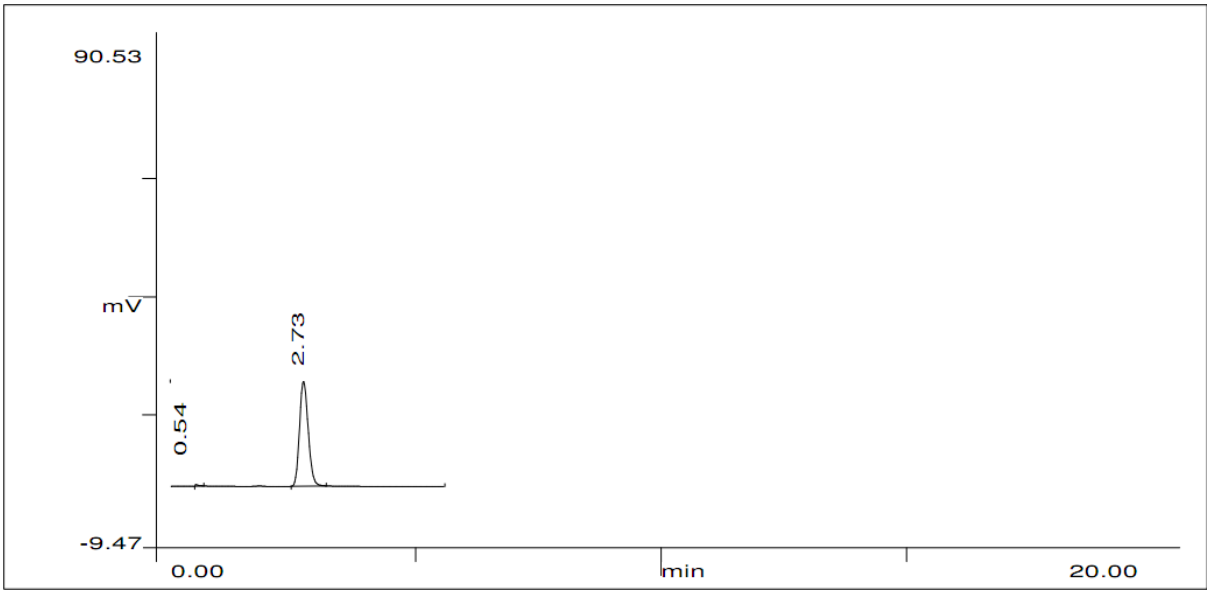

Figure 6. Gas chromatogram of ethanol produced after 7 days of fermentation using glucose as substrate.

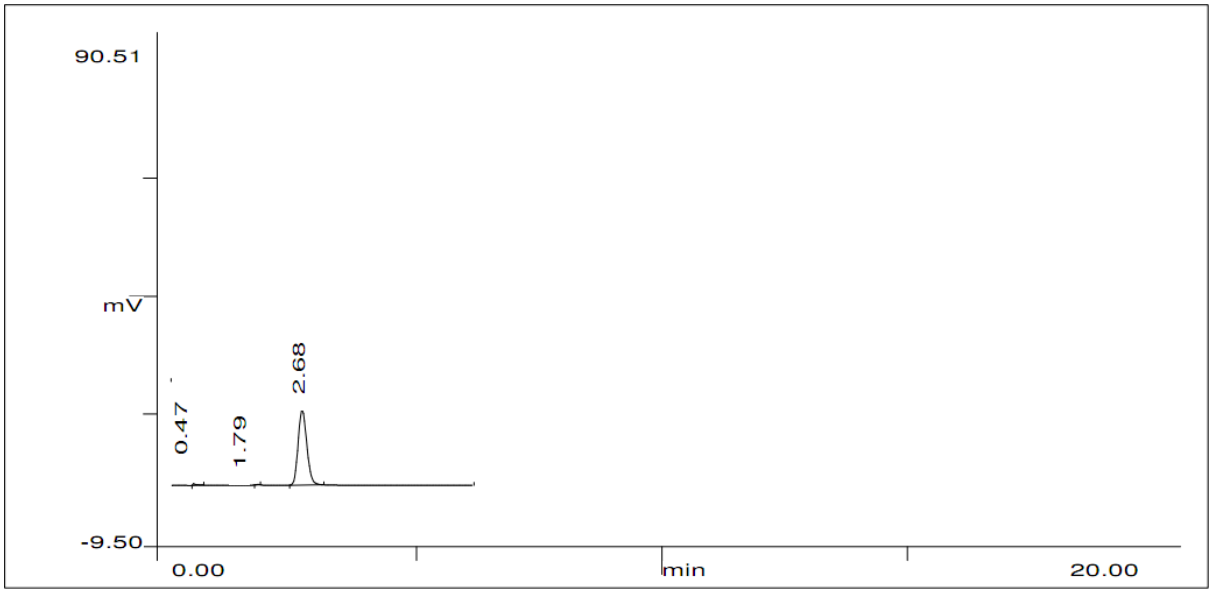

Figure 7. Gas chromatogram of ethanol produced after 7 days of fermentation using fructose as substrate.

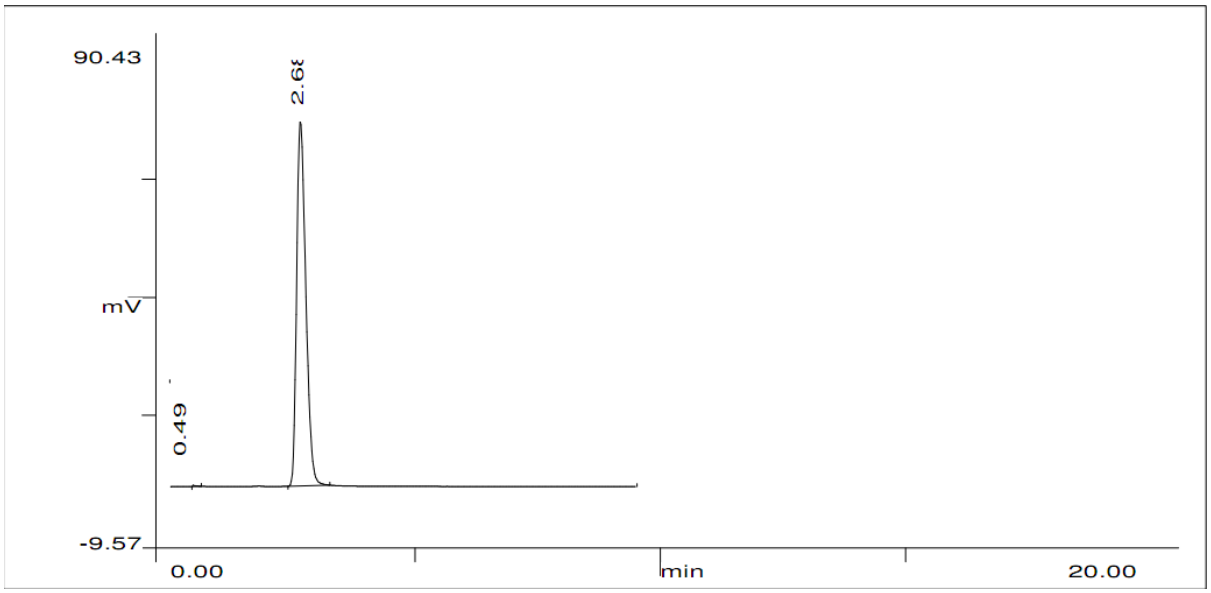

Figure 8. Gas chromatogram of ethanol produced after 7 days of fermentation using sugarcane juice as substrate.

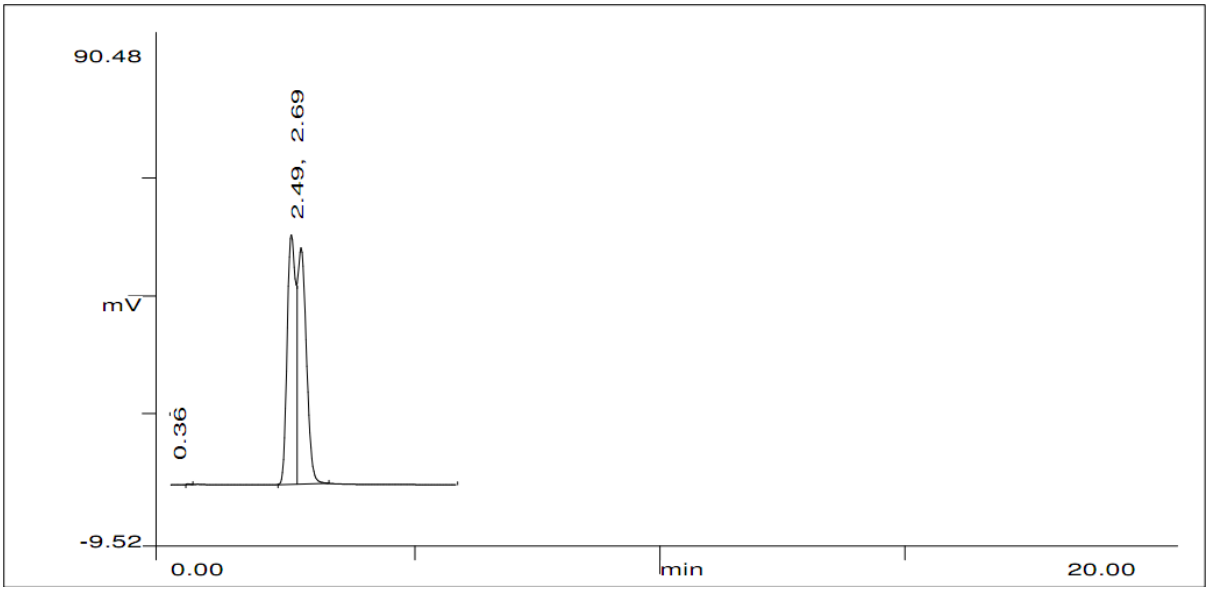

Figure 9. Gas chromatogram of ethanol produced after 7 days of fermentation using jaggery as substrate.

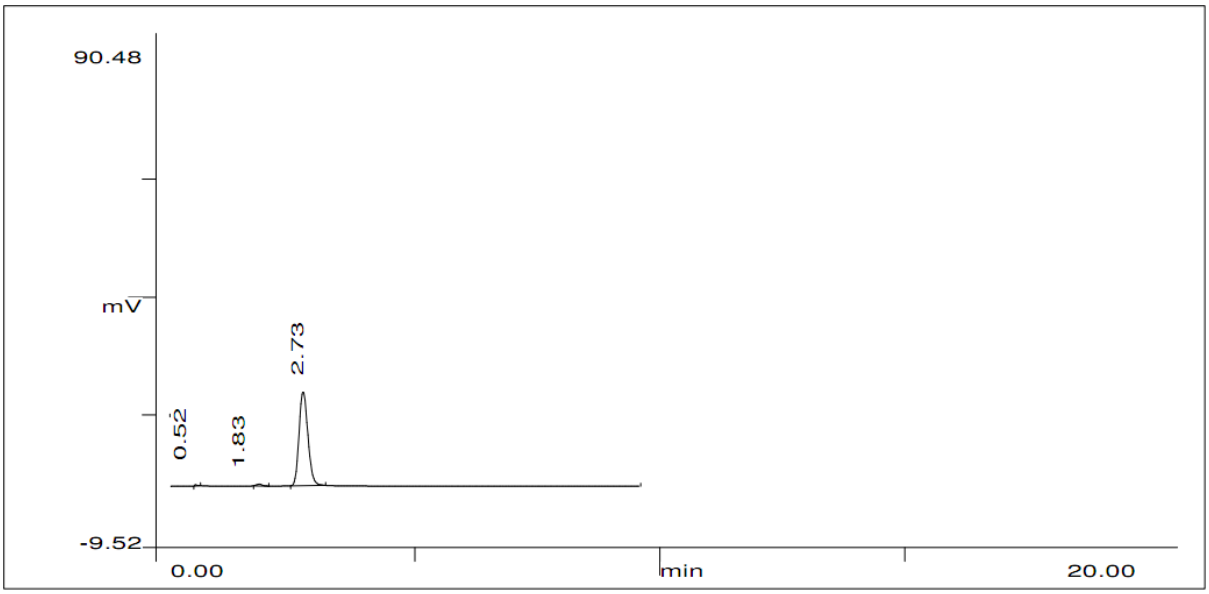

Figure 10. Gas chromatogram of ethanol produced after 7 days of fermentation using kinnow pulp juice as substrate.

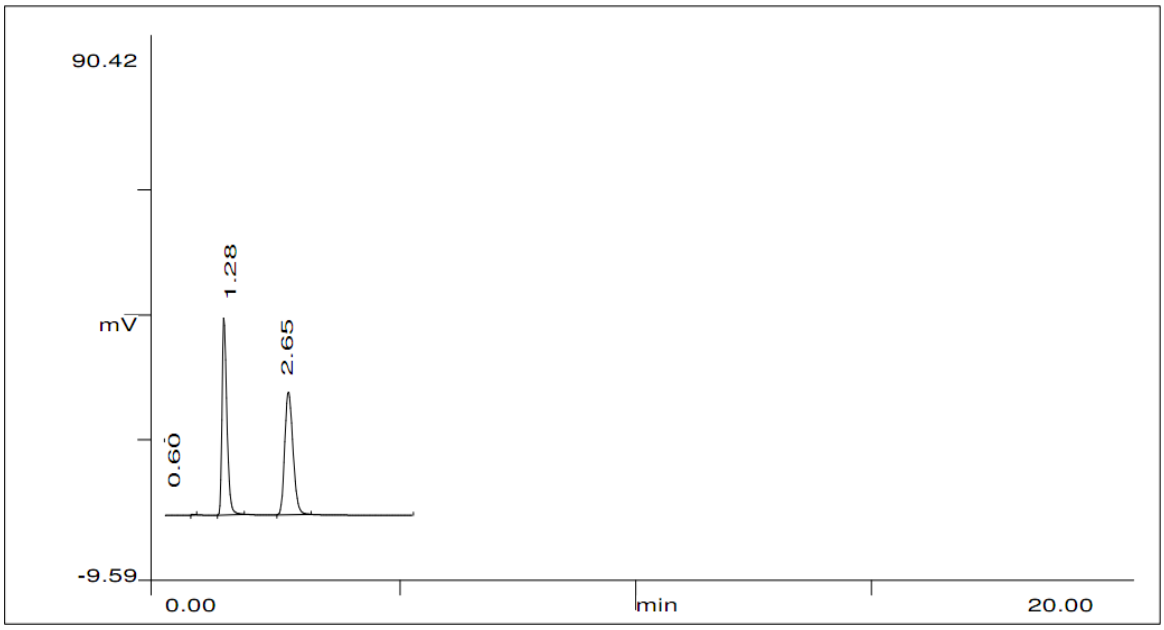

Figure 11. Gas chromatogram of ethanol produced after 7 days of fermentation using apple juice as substrate.

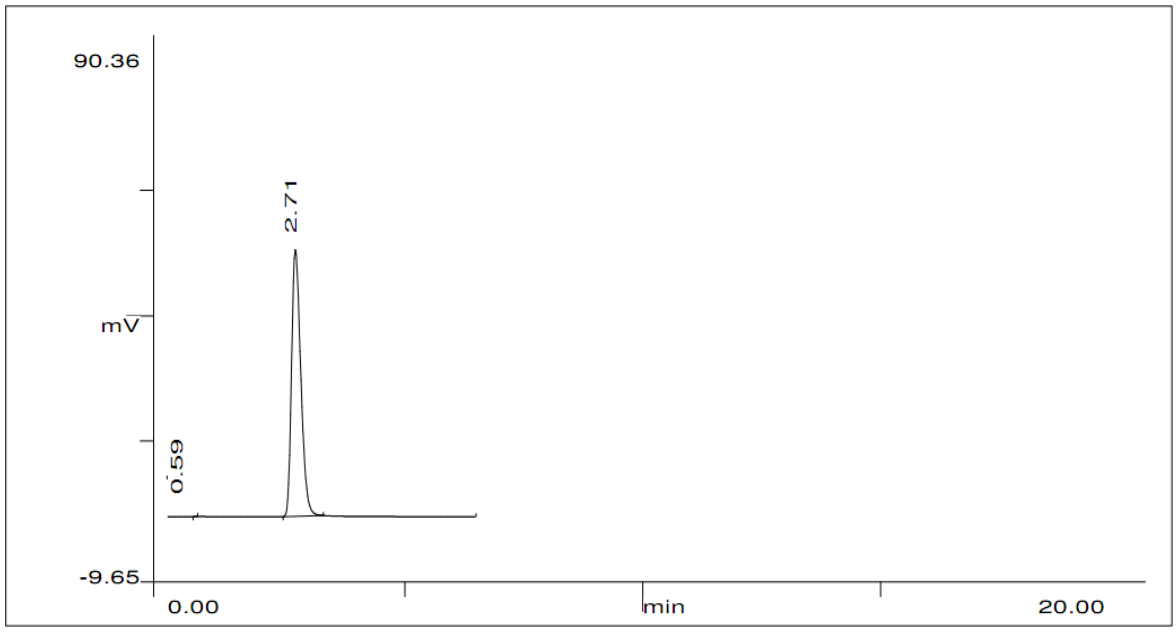

Figure 12. Gas chromatogram of ethanol produced after 7 days of fermentation using grape juice as substrate.

Production of vinegar

The grape wine and sugarcane wine having ethanol concentration of 7.92% and 8.72%, respectively was further used for vinegar production using *A. aceti* NRRL 746. Titrable acidity and pH of the samples were recorded at regular intervals of 5 days over a period of 25 days.

Table 12. pH and titrable acidity of vinegar at different time intervals

S.No	Substrate	5 days		10 days		15 days		20 days		25 days	
		pH	#Acidity (%)	pH	Acidity (%)	pH	Acidity (%)	pH	Acidity (%)	pH	Acidity (%)
1	Sugarcane juice	4	0.85	3.6	1.64	3.1	2.98	2.5	3.63	2.1	4.1
2	Grape juice	3.9	0.95	3.3	1.8	2.7	3.07	2.2	3.98	1.9	4.5

Titrable acidity

Titrable acidity in grape vinegar was found to be 4.5% whereas 4.1% acidity was observed with sugarcane vinegar after 25 days (Table 12). The produced vinegar was diluted to 4% acidity using sterile water and then bottled aseptically.

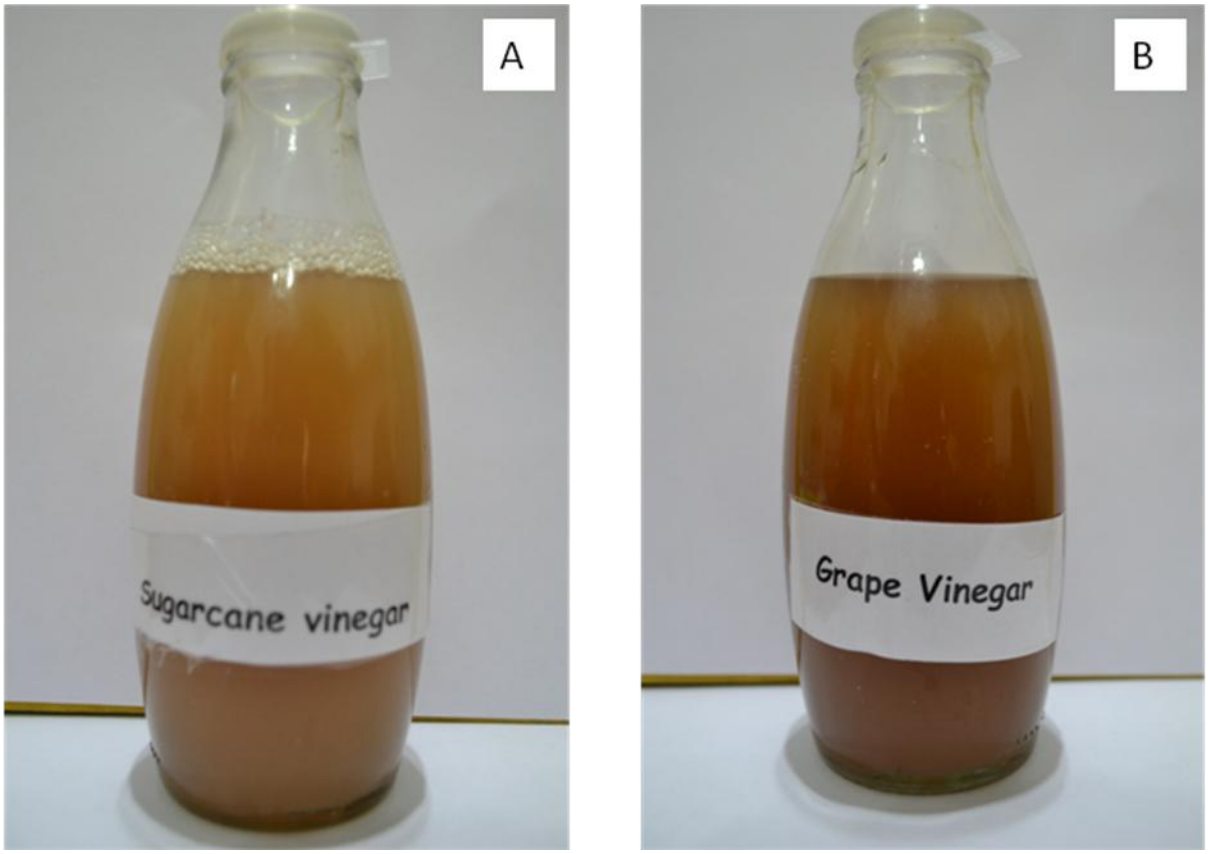

Figure 13. Bottled vinegar A) Sugarcane vinegar B) Grape vinegar

Simultaneous saccharification and fermentation (SSF)

Different pretreatments of mixed agricultural waste were done to hydrolyze cellulosic component of biomass to increase reducing sugars and ethanol yield.

Estimation of cellulose

The amount of cellulose can be estimated by using standard curve of cellulose having

$y = 0.070x + 0.520$ and $R^2 = 0.988$

Table 13. Standard curve for cellulose

Cellulose (mg/mL)	O.D at 630 nm
2	0.635
4	0.818
6	0.974
8	1.071
10	1.21

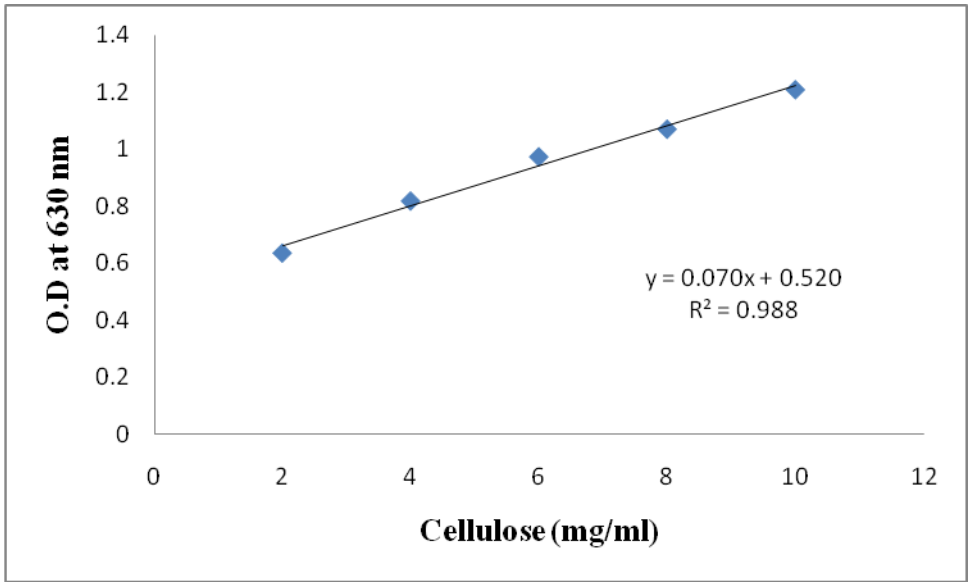

Figure 14. Standard curve for cellulose ($y = 0.070x + 0.520$ and $R^2 = 0.988$)

Table 14. Standard curve of glucose

Glucose (mg/mL)	O.D at 540 nm
0.2	0.221
0.4	0.518
0.6	0.914
0.8	1.237
1	1.565
1.2	1.889
1.4	2.178
1.6	2.554
1.8	2.855
2	3.156

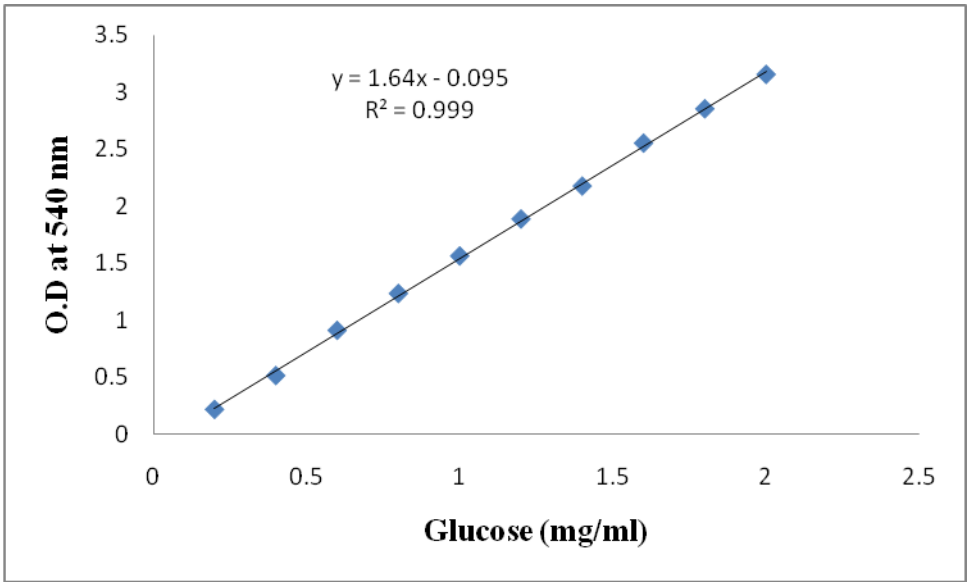

Figure 15. Standard curve for glucose (y = 1.64x – 0.095 and R^2 = 0.999)

Table 15. Cellulose and residual sugar content of treated and untreated mixed agricultural waste biomass.

Analytical parameter	**Untreated biomass**	**Treated biomass**		
		Acid	Alkali	Steam
Cellulose (mg/g)	174.35 ± 1.43	154.71 ± 1.24	163.76 ± 1.31	169.75 ± 1.49
Reducing sugars (mg/g)	2.63 ± 0.13	3.41 ± 0.05	3.03 ± 0.11	2.93 ± 0.12

Values are means of triplicate value with SE

Table 16. Reducing sugar over a period of 7 days in SSF

S.No.	**Biomass**	**Reducing sugar (mg/mL)**				
		0 day	1 day	3 days	5 days	7 days
1	Untreated	0.05 ± 0.002	0.06 ± 0.001	0.05 ± 0.002	0.03 ± 0.0011	0.02 ± 0.0012
2	Alkali pretreated	0.06 ± 0.003	0.07 ± 0.005	0.05 ± 0.002	0.03 ± 0.002	0.01 ± 0.001
3	Steam pretreated	0.06 ± 0.004	0.06 ± 0.003	0.05 ± 0.003	0.04 ± 0.002	0.02 ± 0.0012
4	Acid pretreated	0.07 ± 0.005	0.09 ± 0.004	0.06 ± 0.003	0.02 ± 0.0015	0.01 ± 0.001

Values are means of triplicate value with SE

Estimation of ethanol

Ethanol produced using mixed agricultural waste as substrate in SSF media was observed over a period of 7 days and samples were withdrawn at an interval of 2 days and ethanol estimation was done using GC.

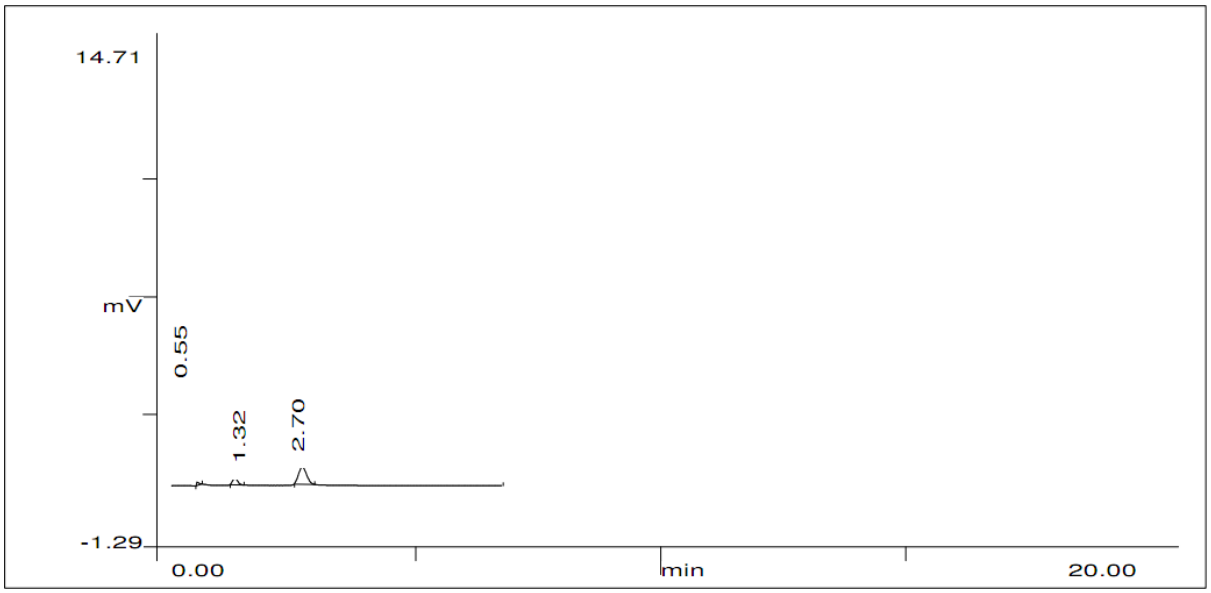

Figure 16: Gas chromatogram of ethanol produced by SSF using alkali pretreated biomass as substrate after 3 days of fermentation.

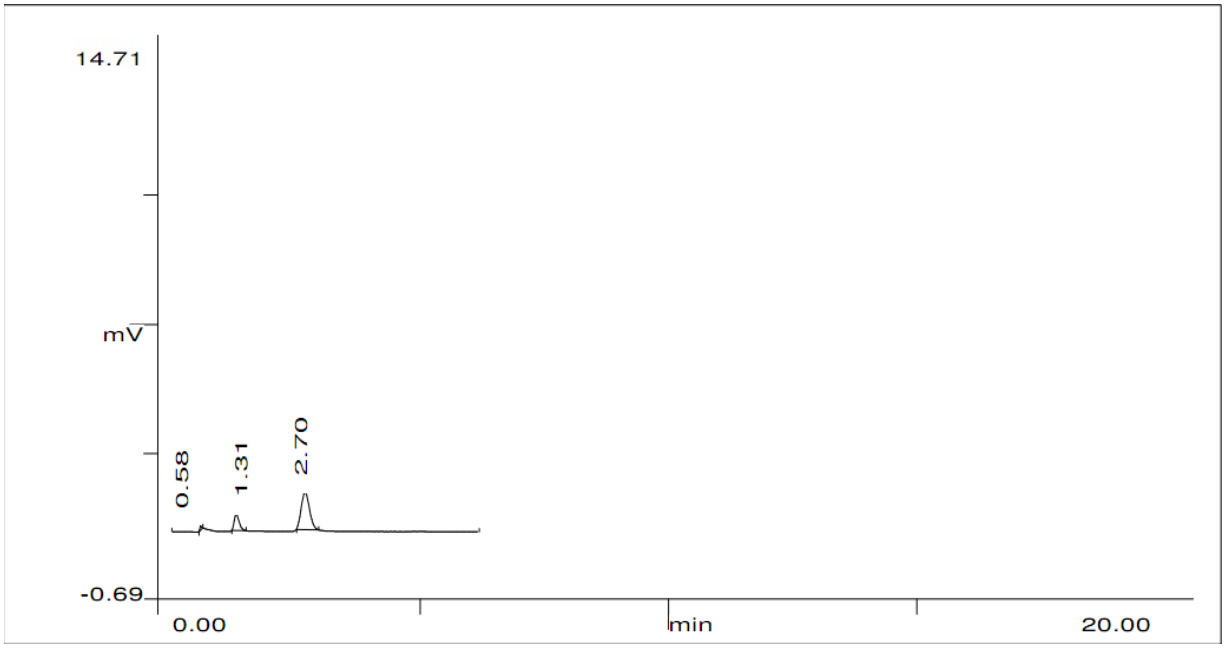

Figure 17: Gas chromatogram of ethanol produced by SSF using alkali pretreated biomass as substrate after 5 days of fermentation.

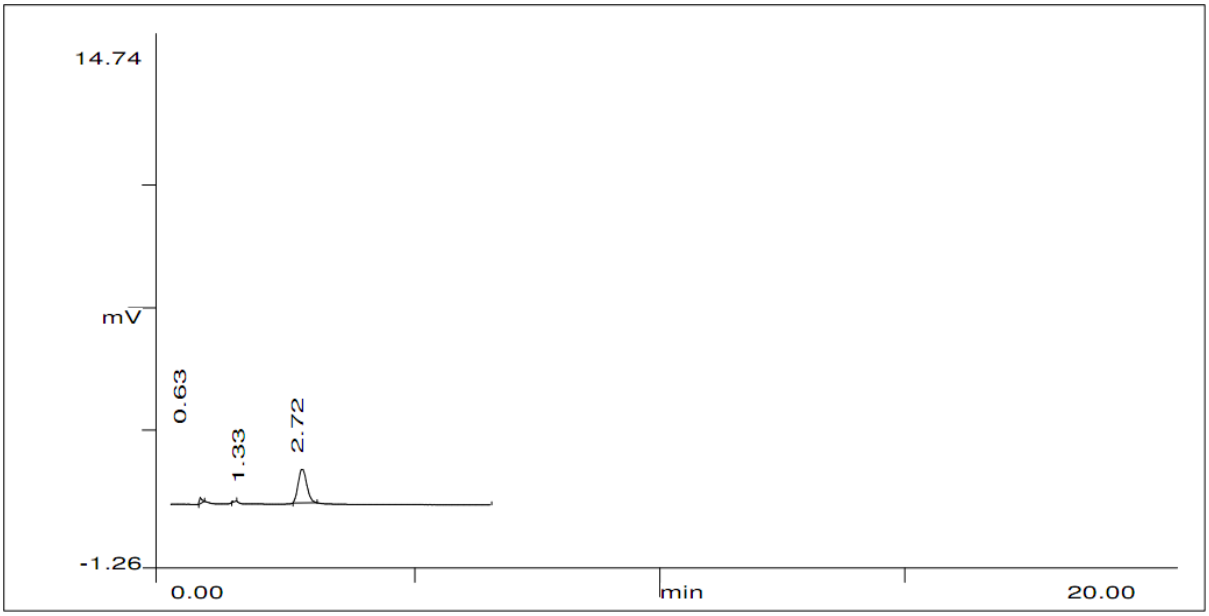

Figure 18: Gas chromatogram of ethanol produced by SSF using alkali pretreated biomass as substrate after 7 days of fermentation.

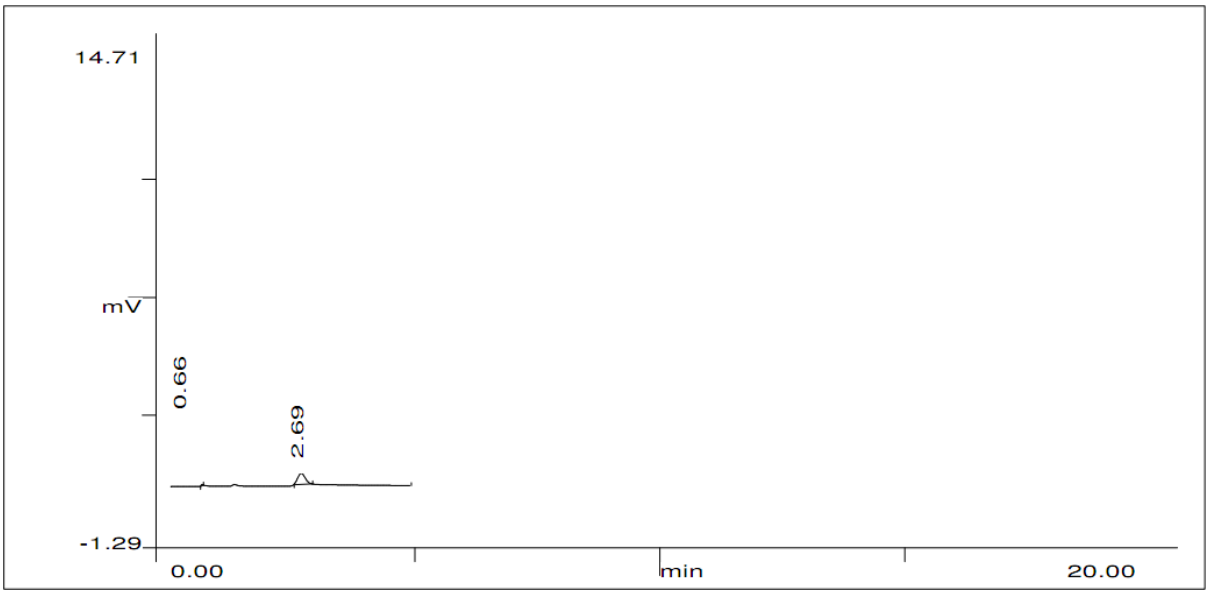

Figure 19: Gas chromatogram produced by SSF using steam pretreated biomass as substrate after 3 days of fermentation.

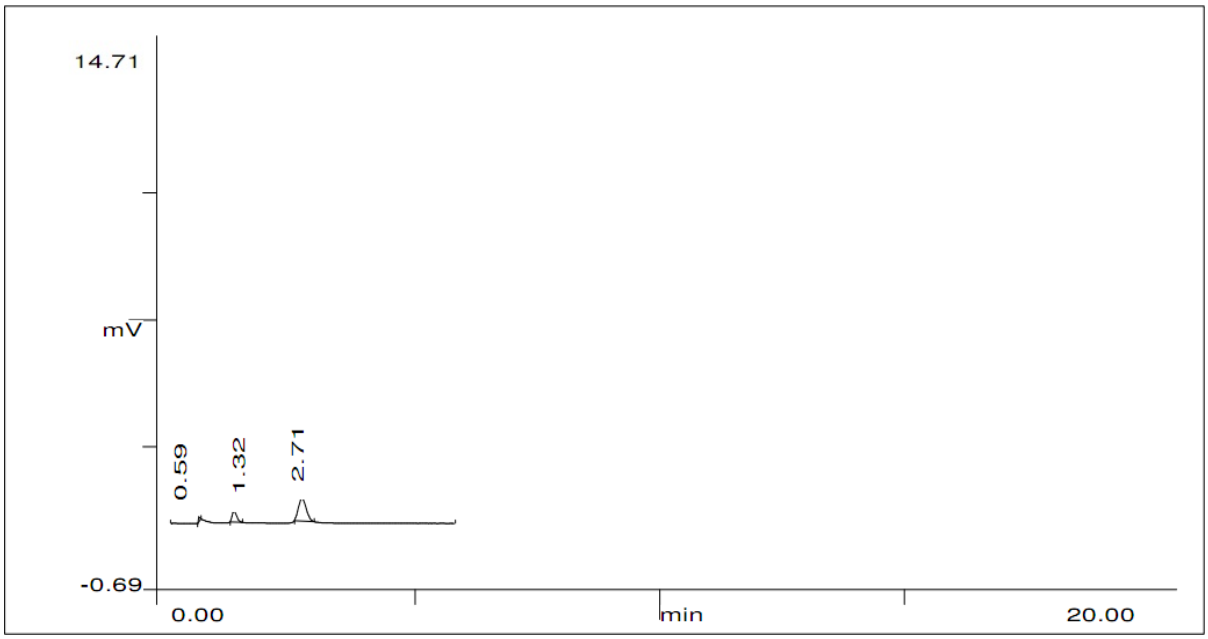

Figure 20: Gas chromatogram produced by SSF using steam pretreated biomass as substrate after 5 days of fermentation.

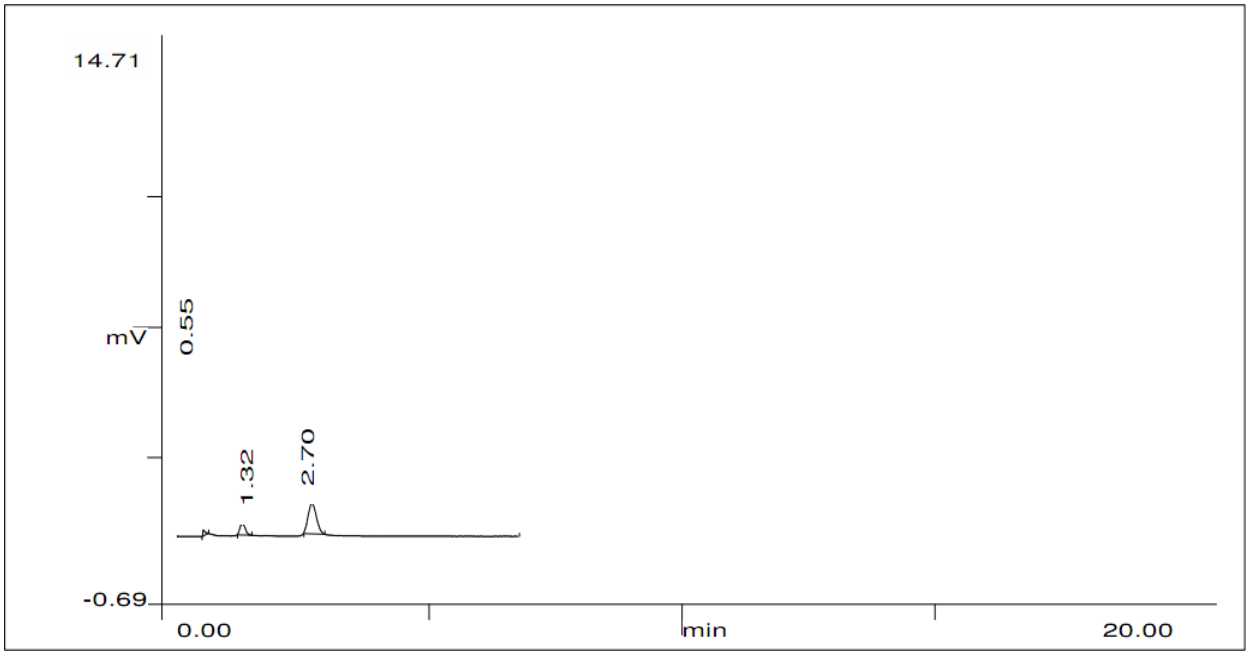

Figure 21: Gas chromatogram produced by SSF using steam pretreated biomass as substrate after 7 days of fermentation.

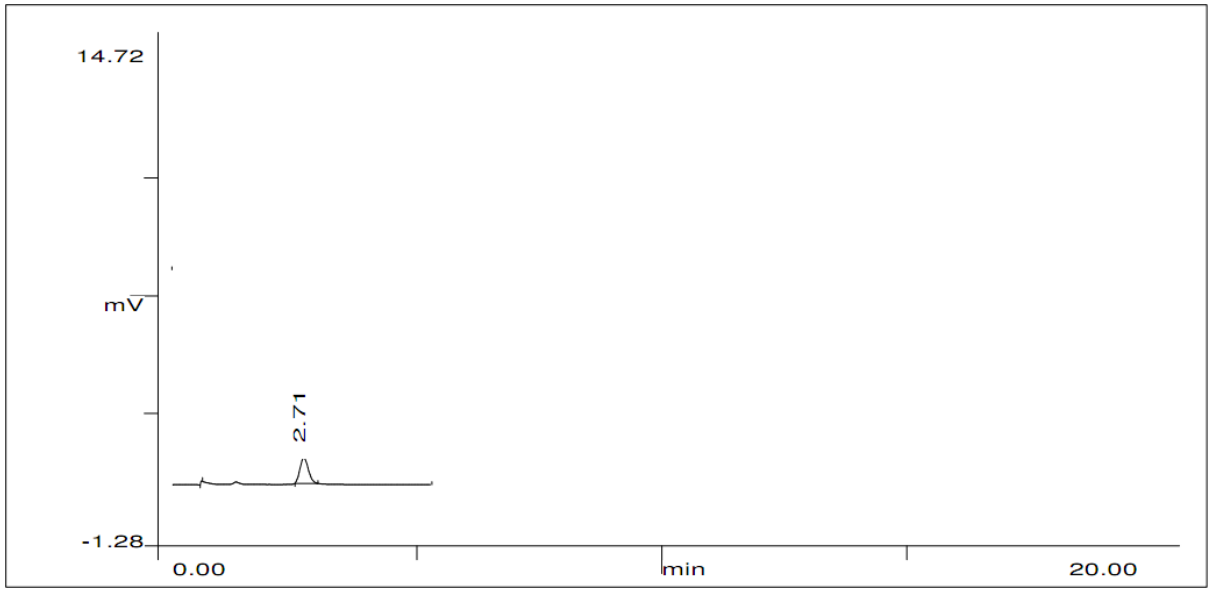

Figure 22: Gas chromatogram produced by SSF using acid pretreated biomass as substrate after 3 days of fermentation.

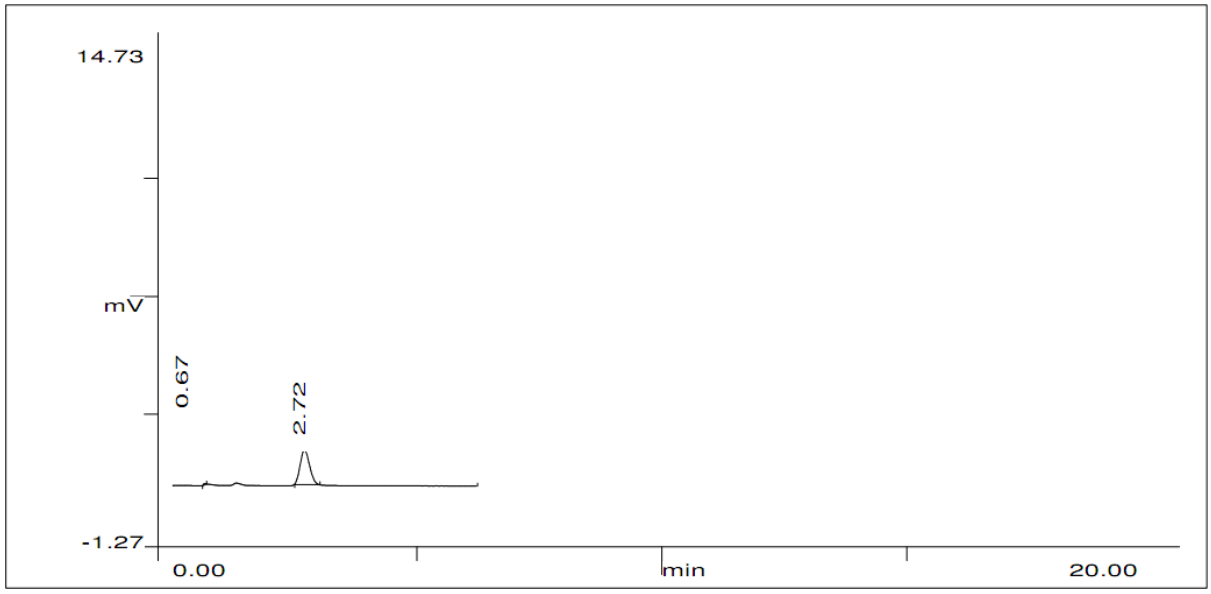

Figure 23: Gas chromatogram produced by SSF using acid pretreated biomass as substrate after 5 days of fermentation.

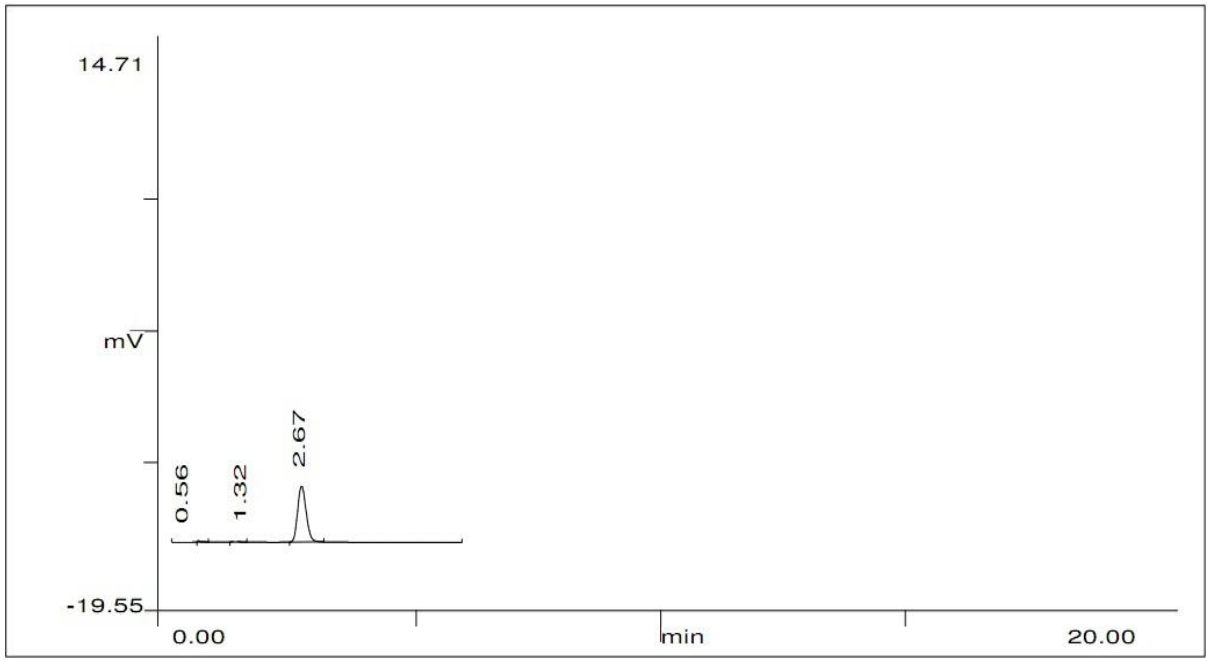

Figure 24: Gas chromatogram produced by SSF using acid pretreated biomass as substrate after 7 days of fermentation.

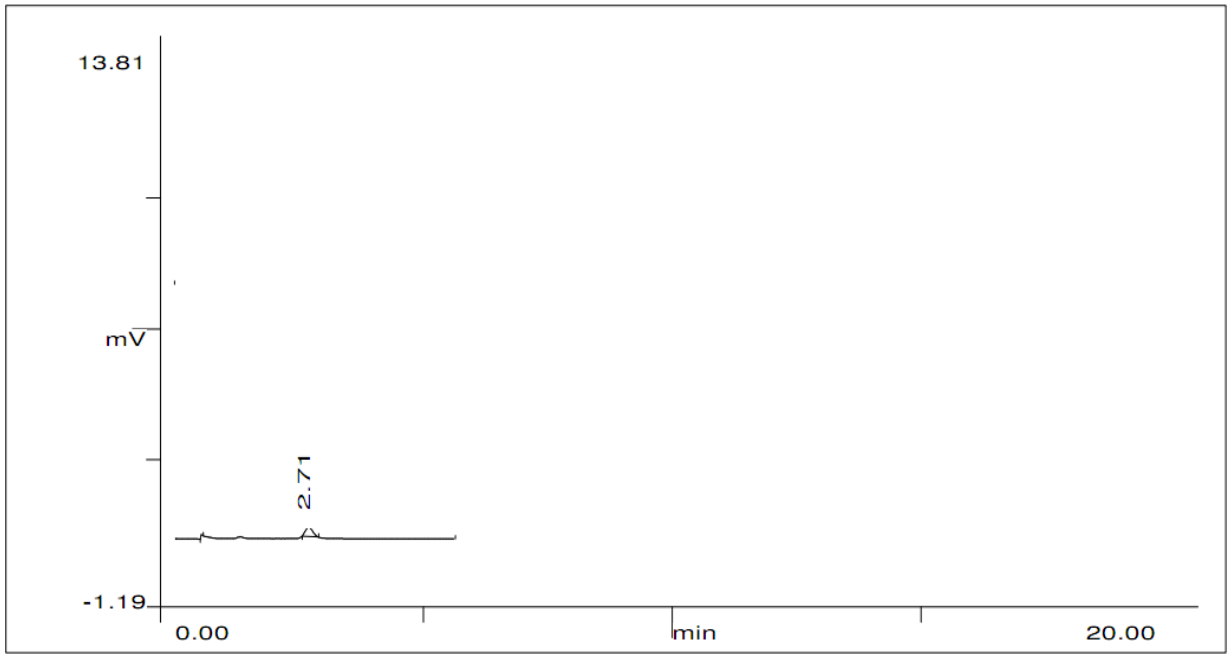

Figure 25: Gas chromatogram produced by SSF using untreated biomass as substrate after 3 days of fermentation.

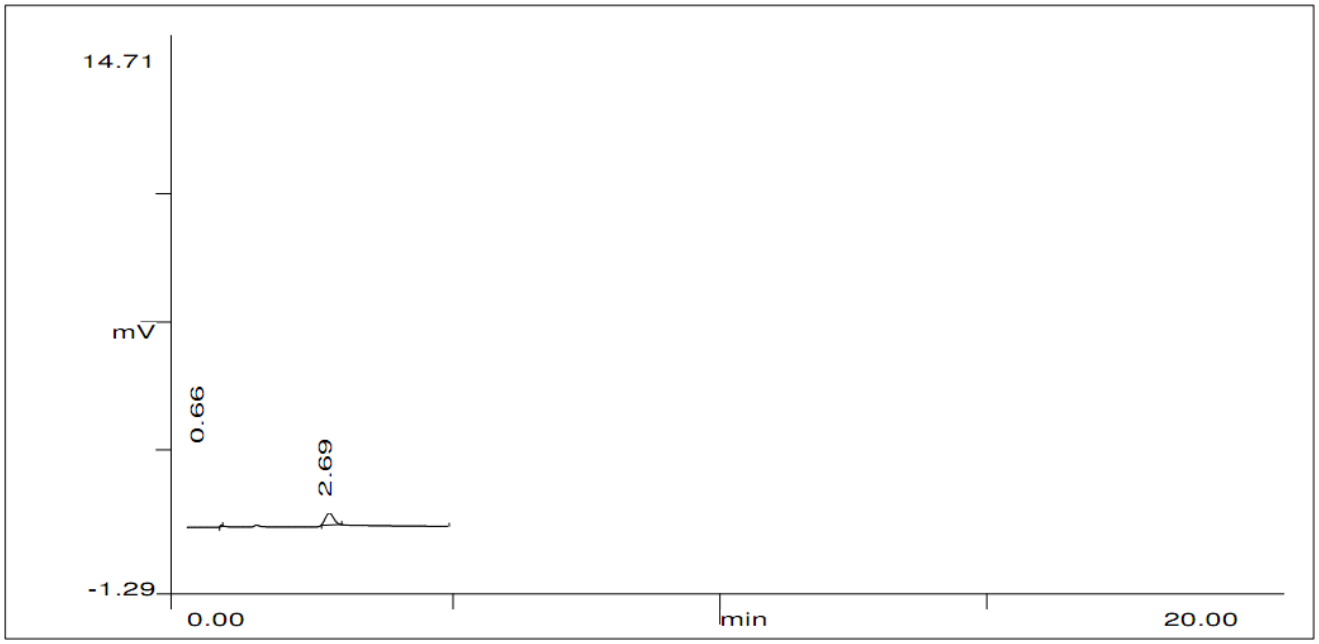

Figure 26: Gas chromatogram produced by SSF using untreated biomass as substrate after 5 days of fermentation.

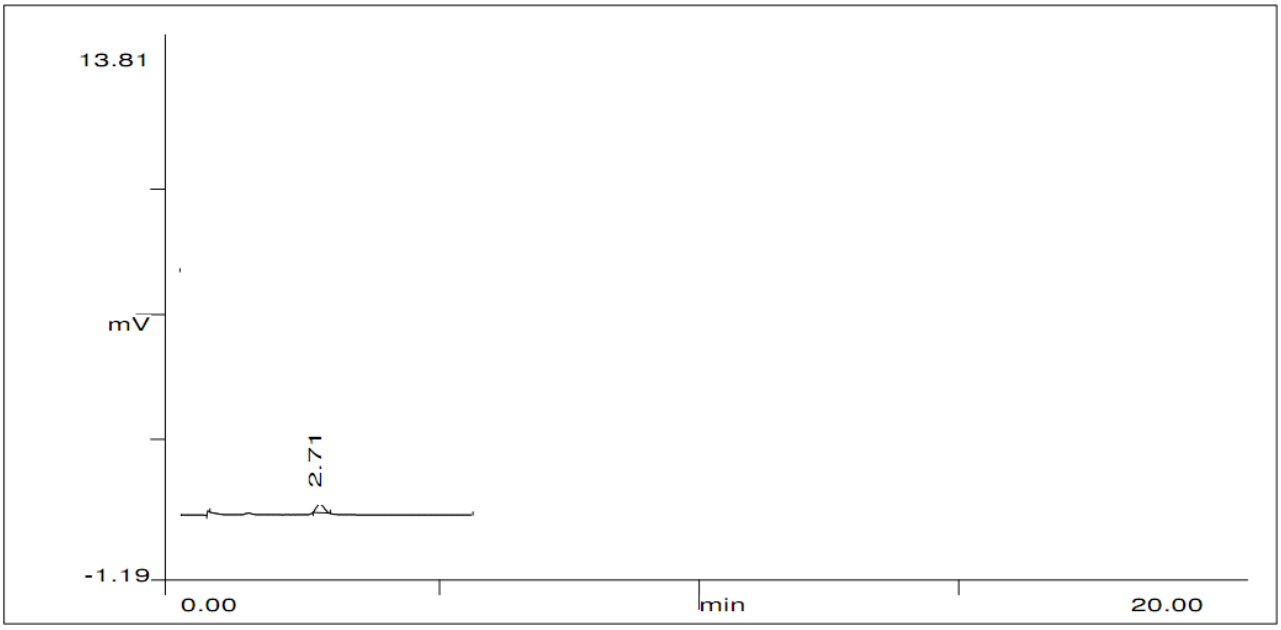

Figure 27: Gas chromatogram produced by SSF using untreated biomass as substrate after 7 days of fermentation.

Table 17: Ethanol estimation at regular intervals during SSF using GC

Biomass	Ethanol (%)		
	3 days	5 days	7 days
Untreated	0.010	0.02	0.030
Alkali pretreated	0.051	0.08	0.104
Steam pretreated	0.025	0.04	0.065
Acid pretreated	0.065	0.12	0.280

Depending on the amount of ethanol produced, acid pretreatment was found to be the best of the three pretreatment methods carried out. Recent studies have shown that when acids are combined with alkali, they play more effective role in lignocellulosic waste pretreatment than acids or alkalis alone (Damisa *et al*, 2008). Using steam explosion as pretreatment, 0.065% of ethanol was produced at end of 7 days (Table 17). Using alkali pretreatment method maximum ethanol was found to be 0.104% (Table 17). Using acid method as a pretreatment strategy, maximum ethanol (0.28%) was obtained (Table 17). Acid pretreatment gave best results of the three different pretreatments carried out, as in case of acid pretreatment both acid and alkali are used, which resulted in higher delignification of the substrates. By acid pretreatment, the structure of substrate becomes less complex, and it becomes more accessible to the enzyme, and hence, more reducing sugars are released. These sugars are then converted to ethanol by yeast, the fermenting organism (Mutreja *et al*, 2011)

CONCLUSION

1. Different carbohydrate substrates such as glucose, fructose, jaggery, grape juice, kinnow pulp juice, sugarcane juice and apple juice were used to produce ethanol and maximum ethanol concentration (8.72%) was found in sugarcane juice followed by grape juice (7.92%).

2. Sugarcane wine and grape wine were further used for production of acetic acid and the acidity was found to be 4.1% and 4.5%, respectively after 25 days of incubation.

3. Mixed agricultural waste was used for production of ethanol via using SSF and maximum ethanol was observed by acid pretreated biomass (0.28%) followed by alkali pretreated biomass (0.104%)

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Appendix I

GYE media

Composition (g/l)

Glucose	10
Peptone	5
Yeast extract	5

Maintain pH at 5.5

Appendix II

Tryptone media

Composition (g/l)

Glucose	5
Tryptone	20
Yeast extract	7.5

Maintain pH at 7.0

Appendix III

DNS reagent

Mix:	Distilled Water	1416 mL
	DNS	10.6 g
	NaOH	19.8 g

Dissolve the above and then add:

	Rochelle salts (Na-K tartatate)	306 g
	Phenol (melt at 50°)	7.6 mL
	Sodium metabisulphite	8.3 g

Appendix IV

0.02 M acetate buffer (pH = 4.4)

Make

0.2 M CH_3COONa solution by adding 2.722 g in 100 mL distilled water

0.2 M CH_3COOH by adding 1.15 mL in 98.85 mL distilled water

Mix 37 mL of 0.2 M CH_3COONa and 63 mL of 0.2 M CH_3COONa and then dilute it 10 times by adding distilled water.