OPTIMIZING THE PRODUCTION OF BIO-ETHANOL FROM *Prosopis juliflora* LEAVES USING *Saccharomyces cerevisiae*

M.Sc. Thesis

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As thesis research advisors, we hereby certify that we have read and evaluated this thesis prepared under our guidance by **Tadese Kibret** entitled: **Optimizing the Production of Bio-ethanol from *Prosopis juliflora* leaves Using *Saccharomyces cerevisiae.*** We recommended that it be submitted as fulfilling the thesis requirement.

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LIST OF ABBREVIATIONS/ACRONYMS

CDM	Clean Development Mechanism
CRD	Completely Ranidomized Factorial Designs
EDRI	Ethiopian Development Research Institute .
EEPFE	Environmental Economics Policy Forum for Ethiopia
EMD	Electronic Miscellaneous Document
GDP	Gross Domestic Product
GHG	Greenhouse Gas
NRS	National Regional State
UNCTAD	United Nations Conference on Trade and Development
USD	United States Dolar
XDH	Xylitol dehydrogenase
XK	Xylulokinase
XR	Xylose reductase

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Optimizing the Production of Bio-ethanol from *Prosopis juliflora* leaves Using *Saccharomyces cerevisiae*

ABSTRACT

Production of bio-ethanol from Lingocellulosic materials is naturally abundunt renewable resources that can potentially provide a long-term sustainable fuel supply. Prosopis juliflora, an invasive plant is one source of lignocellosic materials to serve as feedstock for bio-ethanol production. With the objective of evaluating its potential for bio-ethanol production, differen concentrations (10g, 20g, 30g, and 40g) of P. juliflora leaves treated with dilute sulfuric acid and untreated were incubated for fermentation over 20 days with 0.5 or 1% yeast (Saccharomyces cerviceae) inoculums. Percent bio-ethanol, cell density and amount or reducing sugars were measured at an interval of 4 days starting from the begining of fermentation. Results showed that ethanol production was observed starting from the 4th day of fermentation, but its amount peaked 29.05% from 40g substration with 1% inoculum on the 12th day of fermentation, and declined afterwards on 16^{th}&20^{th}days of fermentation(19.25% &16.38%) respectively at the same substrate concentration. In agreement with ethanol production, cell density and reduction in reducing sugar were observed in the same pattern. Compared ethanol production between untreated substrates yieled of 25.35%, and acid pre-treated substrates yielded of 29.05% then acid pretreated showed significantlly higher ethanol production than untreated at the same substration concentration of 40gram inoculated with 1% yeast cell. Overall, this study showed that acid pre-treatment, inoculum concentration, fermentation period and substrate concentration affect the amount of bio-ethanol production.

Key word: *Bio ethanol, distillation, fermentation, Prosopis juliflora, Saccharomyces cerevisiae*

1. INTRODUCTION

Production of fuel from renewable resources have deserved a great deal of interest during the past decades mainly due to concerns about fossil fuels depletion and environment pollution. It has advantages over fossil fuel in that less environmental impact and sustainability of resource. Shortage of oil supplies, the growing pressure on reduction in the net emission of CO_2, global warming, and the use of food crops for ethanol production resulted in global food crises have maintained research and development on the ethanol production from non edible renewable raw materials (Himmel, 2007). Bioethanol production from renewable biomass such as lignocellulosic materials is widely investigated nowadays. Lignocellulosic feed stocks are cheap, renewable, abundant and do not compete with the food production. They are rich in holocellulose and lignin which form a recalcitrant lignocellulose complex that resists saccharification processes (Nikolic et al., 2009). Hydrolysis of lignocellulosic materials by dilute sulfuric acid is a promising method till date to obtain fermentable sugars. Despite achieving higher sugar yields with dilute acid hydrolysis method, it is accompanied with formation of toxic inhibitory compounds (Srilekha Yadav et al., 2011).

Prosopis juliflora a popular plant known as mesquite, is native to Central and South America and has spread to North America. In East Africa, P. Juliflora was introduced in the 1970s' through collaborative projects involving local governments and outside agencies (Coppock, 2005). In the Afar region of Ethiopia, where P. juliflora is having dramatic impacts across the landscape, its spread and impacts on resources has been ranked as one of the leading threats to the range lands and biodiversity, ranked as the most problematic plant invader in Ethiopia (Tessema, 2007; EEP, 2006). The potential use of P. juliflorais that it is a fast growing plant, which can grow on any marginalized areas; this may be taken as advantageous to use as a sustainable feedstock for bioethanol production. Hydrolysis of the pod of P. juliflora , into simple sugar with water in the presence of acid shows that the potentila as feed stock for ethanol production (Negusu, 2009). The P. juliflora has recently been suggested to be used as raw material for long-term sustainable production of cellulosic ethanol (Hopkins, 2007). Its nature to tolerate drought, grazing, heavy soil, sand as well as saline dry flats and indigestibility by animals made it a potential feedstock for ethanol production. Among the various types of lignocellulosic substances *Prosopis* can therefore be considered as a potential resource for bioconversion into ethanol. However, no

research has been done in Ethiopia on the production of bio-fuel (Ethanol) from this invasive weed so far. Therefore, this research was design with the focus of producing bio-ethanol from *Prosopis juliflora* with the following objectives.

General Objective

The general objective of this research work was Optimizing the production of bio-ethanol from *Prosopis juliflora* leaves.

Specific objectives

- To identify the optimum substrate concentration for bio ethanol production from *Prosopis juliflora* leaves.
- To determine the effect of inoculum (yeast) concentration on the rate of ethanol production from *Prosopis juliflora* leaves.
- To determine cell density, and reducing sugar concentrations at different fermentation time.

2. LITERATURE REVIEW

2.1. Bio-fuel

Bio-fuel is defined as liquid fuels produced from biomass. Biofuels can be derived directly from plants, or indirectly from agricultural, commercial, domestic, and/or industrial wastes. Renewable biofuels generally involve contemporary carbon fixation, such as those that occur in plants or microalgae through the process of photosynthesis. Other renewable biofuels are made through the use or conversion of biomass. This biomass can be converted to convenient energy containing substances in three different ways: thermal conversion, chemical conversion, and biochemical conversion. This biomass conversion can result in fuel in solid, liquid, or gas form. The two most important biofueles are ethanol and biodiesel (De Oliveira and de Sousa, 2007).

2.2. Ethanol as fuel

The energy crisis of the 1970s renewed interest in ethanol production for fuels and chemicals. Although the interest waned in the following decade due to oil price abatement, the environmental issue of reducing greenhouse gas, rising vehicle fuel demand, and the security of energy supply sustain the development of ethanol production from renewable resources. Ethanol is used in vehicles either as a sole fuel or blended with gasoline. As an oxygenated compound, ethanol provides additional oxygen in combustion, and hence obtains better combustion efficiency. Since the completeness of combustion is increased by the present of oxygenated fuels, the emission of carbon monoxide is reduced by 32.5% while the emission of hydrocarbons is decreased by 14.5% (Rasskazchikova *et al*., 2004).

2.3. Bio-fuels Production in Ethiopia

Currently, there are five potential developers of ethanol in the country of which four are government owned sugar factories and one is private company. The three state owned sugar factories Fincha, Metahara and Wonji have been in operation for long time. Fincha has been producing 8 million litres of ethanol every year since 2009. Metahara Sugar Factory started producing some 12.5 million litres of ethanol per year since mid 2011. The fourth factory whose construction is completed this year is the Tendaho Sugar Factory. This will be the biggest and

most promising factory expected to produce a large amount of ethanol. The company has plans to produce a total of 22.6 million liters of ethanol this year, 2012. Out of this 15 million litres will be used for blending with gasoline while the remaining will be used by households for house cooking stoves (MoWE, 2012).

However, although the government had issued a directive allowing Finchaa to produce and sell fuel alcohol to oil companies, who would in turn blend it with gasoline and distribute it to motorists, it could not sell its fuel alcohol on the market at that time. The major reasons for the refusal of the oil companies appeared to be the need for rehabilitating the existing old fuel stations and lack of interest in investing in a fuel sales operation that gives them little profit. This was also viewed as a lack of understanding and absence of commitment to alleviate one of the major problems of the country. However, the interest in biofuels development has been revitalized with the recent hike in oil prices. Several local and international private and public biofuels companies (developers) have registered in the country since 2006. For example, by 2010 there were more than 82 registered biofuel investors. Most of which were registered for the cultivation of energy crops for biodiesel production. In the case of bioethanol, however, there are only a few developers in the country, most of which are public owned sugar factories that intend to produce bioethanol as a byproduct of sugar production. Reports also indicate that about 1.5 to 2 million hectares of land have already been offered for biofuels investment (ABN, 2007; Lashitew, 2008; Lakew and Shiferaw, 2008; Beyene, 2011).

According to a survey conducted at national level, regarding water resource and canal development opportunities, it is shown that the country has a potential of more than 500,000 ha of land that is suitable for sugarcane plantation (SC, 2012). In order to raise sugar production, the government is carrying out agricultural expansion projects around existing sugar factories and the Sugar Corporation has plans to build nine new sugar factories within the coming five years (2010-2015). Fincha sugar factory has currently 14, 398 ha of sugar plantation, and the agricultural expansion project carried out there will bring the total sugar plantation close to 21,000 ha. Wonji/Shoa Sugar factory will expand its 8,662 ha of sugarcane plantation to 16,000 ha; Matahara Sugar factory is working towards expanding its current 11,180 ha of sugar plantation to 21,000 ha. In addition, the Tendaho Sugar development project aims to expand its

cultivation to 50,000 ha from its current level of 4,394 ha. The Kessem Sugar Development Project, which is planned to be completed by 2013 with a total of 20,000 ha of land earmarked for sugarcane production, out of which on 943 ha is already covered by cane. Similarly, the Tana-Beles Sugar Development project will have two sugar factories with 25,000 ha of sugarcane plantation each. Moreover, the Kuraz Sugar Development Project will have six factories each having 25,000 ha of sugar cane plantation; and the Welkaiyt Sugar Development Project will have one sugar factory with a 25,000 ha of sugarcane plantation. The Al-Habasha Sugar Mill is the only private company growing sugarcane on 4,000 ha. Totally, upon the completion of all sugar factories, including the private one; the country expected to increase its current annual production of 18 million litres per year to195 million litres per year by 2015.

Table 3.1 Summary of production projection of ethanol from expansion and existing areas of all state owned and private sugar factories

No.	Name of the Sugar Factory/Project	Land under cultivation(ha)2011/2012	Land expansion(ha)2014/2015	Ethanol production capacity(litres annually) in 2015
1	Fincha'a SF	14,398	21,000	20,000,000
2	Wonji/Shoa SF	8,662+(3923 OGs[8])	16,000	
3	Metehara SF	11,180	21,000	25,500,000
4	Tendaho SF	4,394	50,000	55,400,000
5	Kessem SF	943	20,000	20,000,000
6	Tana-Beles Sugar DP	Na	50,000	
7	Kuraz Sugar DP	Na	150,000	
8	Welkaiyt Sugar DP	Na	25,000	
9	Al-Habasha Sugar Mill	4,000	28,000	
	Total	47,500	381,000	120,900,000

Source: (Ethiopian Sugar Corporation, 2012)

2.4. Status of Bio-Ethanol Production in Ethiopia

Ethanol production in Ethiopia is linked with sugar factories. Thus, the total identified irrigable land for sugarcane plantation in the country is about 700, 000 hectares, estimated at a potential to produce one billion liters of ethanol (MoWE, 2013). At present, the main supply line in the domestic market is dominated by five sugar factories in the country of which fours are government owned sugar factories and one is private company. These are Fincha, Metehara, Kessem and Tendaho with the combination of their annual production capacity at around 120 million liters (Ethiopian Sugar Corporation, 2012) . Wonji/Shoa,Tana Beles,Kuraz,Welkaiyt,and Al-Habasha sugare development projects are under constraction to increase the capacity of ethanol production significantly (Ethiopian Sugar Corporation, 2012) . In order to transform this potential into reality, the government developed a strategic plan in 2007 considering jatropha as a principal feedstock for biodiesel production and sugarcane as a principal feedstock for bioethanol production. Among other things the strategy focused on establishing biofuel program, encouraging feedstock development, motivating customer demand, improving environmental sustainability, awareness conception and promotion of biofuels, and renewing energy policy to incorporate bioenergy in detail. As a continuation of this endeavor there have been repeated efforts to initiate using bioethanol for domestic use and particularly, blending 5% of ethanol with gasoline in the year 2008 followed by10% in the year 2011 and to increase the percentage in the years to come was the plan set out. With these of course the plan includes expansion of sugar factories and building new ones though delayed during implementation (MoWE, 2013).

2.5.Composition of Lignocellulosic Biomass

The lignocellulosic materials are the most abundant organic compounds in the biosphere, participating in approximately 50% of the terrestrial biomass. The term lignocellulose structure is related to the part of the plant which forms the cellular wall (half-lamella, primary secondary walls), composed of fibrous structures, basically constituted of polysaccharides cellulose (40-60%) and hemicellulose (20-40%)]. These components are associated to a macromolecular structure containing aromatic substances, denominated lignin (15-25%) (Sun and Cheng, 2002). In a general way, it can be affirmed that those materials possess in their compositions

approximately, 60-70% of polysaccharides (in a dry basis), which contain in their monomeric units valuable glycosides (sugars).

Cellulose is a polysaccharide, polymer of D-glucose, forming chains of β-1,4 bonds, and maintaining a linear and plane structure. Cellobiose, disaccharide 4-0-(β-D-glycopyranosil-D-glucopyranose), is the repeated polymer unit. In natural celluloses, the chains are aligned in a way of forming complex organized fibrils, whether in crystalline or amorphous structures. These fibrils are established amongst them with inter and intra hydrogen bonds, which individually are weak, but collectively, they result in a great binding strength, giving to the cellulose a high resistance to the hydrolysis attack .Ebringerova *et,al.*,(2005) .

Hemicelluloses are closely associated with cellulose in plant tissues and together with cellulose they are the most abundant carbonic material in plants. These macromolecules, contrarily to cellulose, present heteropolysaccharic nature and a considerable degree of ramification, consequently not presenting crystalline regions. They are constituted, in their great majority, of a mixture of polysaccharides with a low molecular mass, as follows: xylans, arabinans, arabinoxylans, mannans and galactomannans. The fundamental units (monomers) are, basically, molecules of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, D-glucuronic acid, D-galacturonic acid, α-D-4-O-methylglucuronic acid and also some oxidation products, as for example, acetates (Girio *et,al.*,2010).

Differently to cellulose, the hemicellulose structure does not present a high crystallinity, therefore, being more susceptible to the chemical hydrolysis under milder conditions. The varieties of bindings and ramifications, just as the presence of different monomeric units, contribute to the hemicellulose structure complexity and its different conformations (Jacobsen, 2000).

Lignin is a natural macromolecule composed by *p*-propylphenolic units with methoxyl substituents on the aromatic ring and, between these units, exist principally ether-type bounds. Lignin presents a highly complex structure, formed by polymerization of three different monomers: coumaric alcohol (I), coniferyl alcohol (II) and synapyl alcohol (III), which differ from one another by possessing different substituents in their aromatic ring. This structure is also responsible for the hardness of the cell wall, constituting in a binding material (“glue-like substance”), which holds the cellulosic fibers. Lignin possesses high molecular mass and

presents about 25% of the photosynthesis biomass produced yearly on earth, retaining 50% more carbon than cellulose (Li,Weng,.2008).

2.6.Fermentation

The most commonly used yeast is *Saccharomyces cerevisiae* (Pretorius, 2000) because it can produce ethanol to a concentration as high as 18% in the fermentation broth. *Saccharomyces* is also generally recognized as safe (GRAS) as a food additive for human consumption (Lin and Tanaka, 2006).

In ideal fermentation, about 95% of sugar is converted to ethanol and carbon dioxide, 1% is converted into cellular matter of the yeast cells, and 4% is converted into other products such as glycerol (Boulton *et al*., 1996). Yeast use accounts for about 10% of the ethanol production cost (Wingren *et al*., 2003). Lignocellulose is often hydrolyzed by acid treatment. The hydrolysate obtained is then used for bioethanol fermentation by microorganisms such as yeast. Because such lignocellulose hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides, microorganisms should be required to efficiently ferment these sugars for the successful industrial production of bio-ethanol (Katahira *et al.*, 2006).

In general, the conversion of lignocellulosic material to sugar and then ethanol is governed by equation (3) below:

$$(C_6H_{10}O_5)n + nH2O \rightarrow nC_6H_{12}O_6 + \text{yeast} \rightarrow 2nC_2H_5OH + 2nCO_2 \qquad (1)$$

According to the reactions, the theoretical maximum yield is 0.51 kg bioethanol and 0.49 kg carbon dioxide per kg of xylose and glucose (Hamelinck *et al.*, 2003, 2005). The overall reaction of this fermentation of hexose sugar (glucose) by yeast has been expressed by Gay-Lussac which forms the basis of calculating fermentation efficiency as:

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2, \qquad (2)$$

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$ (3)

The microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, cellulosic biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to bioethanol. To get an efficient fermentation severe inhibition should be avoided. There are four different strategies to do this. These are modifying the hydrolysis process, detoxification, i*n-situ* detoxification and using less sensitive microorganisms to inhibitors (Taherzadeh, 1999). Microorganisms, presently convert an inadequate portion of the sugars from biomass to bio-ethanol (Demirbas, 2005). There are a number of microorganisms that produce significant quantities of bioethanol (Stewart and Russell, 1987).

Xylose-fermenting microorganisms are found among bacteria, yeast and filamentous fungi (Hahn-Hagerdal *et al.*, 2006). One of the most effective bioethanol producing yeasts, *S. cerevisiae*, has several advantages owing to its high bioethanol production from hexoses and high tolerance to bioethanol and other inhibitory compounds in the acid hydrolysates of lignocellulosic biomass. However, because wild-type strains of this yeast utilize pentoses, such as xylose and arabinose, and celloligosaccharides, bioethanol production from a lignocellulose hydrolysate is inadequate (Katahira *et al.*, 2006). For xylose-using *S. cerevisiae*, high bioethanol yields from xylose also require metabolic engineering strategies to enhance the xylose flux (Hahn-Hagerdal *et al.*, 2006).

The microorganism is capable of growing at a pH as low as 5.0 and temperatures as warm as 34.85^0 C. Natural xylose-fermenting yeasts, such as *Pichia stipitis, Candida shehatae*, and *C. parapsilosis*, can metabolize xylose via the action of xylose reductase to convert xylose to xylitol, and of xylitol dehydrogenase (XDH) to convert xylitol to xylulose. Therefore, bioethanol fermentation from xylose can be successfully performed by recombinant *S. cerevisiae* carrying heterologous XR and XDH from *P. stipitis*, and xylulokinase (XK) from *S. cerevisiae* (Katahira *et al.*, 2006). Microorganisms for bio ethanol fermentation can best be described in terms of their performance. Parameters and other requirements such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Demirbas, 2004). All the recombinant strains are mesophilic organisms and

function best between 29.85^0c and 37.85^0c (Hettenhaus, 1998). An organism must maintain fairly constant balance of pH to survive. Most bacteria grow best in a narrow range of pH from 6.5 to 7.5 (Aminifarshidmehr, 1996).

Yeast and fungi tolerate a range of pH 3.5–5.0. The ability to lower pH below 4.0 offers a method for present operators using yeast in less than aseptic equipment to minimize loss due to bacterial contaminants. The majority of organisms cannot tolerate bio ethanol concentrations above 10–15 % (w/v) (Hettenhaus, 1998).

2.7. Distillation

Distillation is one of the steps of the purifications. Distillation is the method used to separate two liquid based on their different boiling points. However, to achieve high purification, several distillations are required. This is because all materials have intermolecular interactions with each other, and two materials will co-distill during distillation. This means that proportion between two materials, in this case ethanol and water can be changed, and still, there are two materials in layers, the liquid and the vapor layers (Onuki, 2005). Whatever method of preparation is used, the ethanol is initially obtain in the mixture the ethanol is then extracted from the solution by fractional distillation .Although the boiling point of ethanol 78.3^0C, is significantly lower than the boiling point of water, 100^0C, these materials cannot be separated completely by distillation. Instead, an azeotrope mixture (i.e. a mixture of 95% ethanol and 5% water) is obtained, and the boiling point of the azeotrope is 78.15^0C. In a distillation, the most volatile material (i.e. the material that has the lowest boiling point) is the first material to distill from the distillation flask, and this material is the azeotrope of 95% ethanol which has the lowest boiling point. If an efficient fractionating column is used, 95% alcohol could be obtained first and then a small intermediate fraction of lower concentration, and then water. But no matter how efficient the fractionating column used, 95% alcohol cannot be further concentrated by distillation because the vapor has exactly the same composition as the liquid; towards distillation, then, 95% alcohol behaves exactly like a pure compound (Jackman, 1987).

2.8. Acid Pretreatment

Acid Pretreatment can employed using either concentrated or diluted acid. Concentrated acid which is widely used are sulfuric acid (H_2SO_4) and hydrochloric acid are used for treatment of lingo cellulosic materials, because they are powerful agents for cellulose hydrolysis (Sun and Cheng, 2002; Wondale, 2012. The main advantage of concentrated acid hydrolysis are flexible with types of feed stock choice, high fermentable sugars yields as well as mild condition of temperature are required. The disadvantages of using concentrated acid technology are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In addition, it must be recovered after hydrolysis to make the process economically feasible.

2.9.Yeasts and Ethanol Production

The yeasts particularly members of the genera *Saccharomyces*, *Schizosaccharomyces*, *Kluyveromyces*, *Candida* and *Pachysolen* are of primary interest to industrial operations in fermentation of ethanol. Yeast, under anaerobic conditions, metabolizes glucose to ethanol primarily by way of the Embden-Meyerhof pathway (Lin and Tanaka, 2006). Baker's yeast (*Saccharomyces cerevisiae*), has long been used to produce ethanol from hexoses. It can yield up to 18% ethanol; it utilizes both monosaccharide such as glucose and disaccharides including sucrose and it is generally recognized as safe (GRAS) for consumption (Kiran *et al*., 2003; Lin and Tanaka, 2006). It is the organism of choice for the production of alcoholic beverages and for leavening bread. In addition, yeast cells are tolerant to high ethanol and inhibitor concentrations and can grow at low pH values which avoid bacterial contaminations.Although *Saccharomyces cerevisae* is the organism of choice as the fermentation process biocatalyst in alcoholic beverages and fuel ethanol industry, others such as *saccharomyces uvarum, Schizosaccharomyces pombe, Kluyveromyces fragilis, Kluyveromyces marxianus, Candid autilis* (Vallet *et al*., 1996), *Pachysolentannophilus* (Sanchez *et al*., 1999) are also known to ferment glucose efficiently to ethanol.

2.10. Invasive Plant Species

Invasive species cause a wide range of environmental, societal and economic impacts. Invasion by introduced species is the second greatest threat to biodiversity after habitat destruction. Invasive species often out-compete native species and can irreversibly alter ecosystem functioning and hydrology. Invasive species may also introduce new pathogens that damage ecosystems and human health. Non-native Species Secretariat, 2014.

This plant was described by De Candolle under the name of Prosopis juliflora. The specific name juliflora, comes from julus meaning whip-like; referring to the long inflorescence, and flora being flower (Havard, 1884). The genus Prosopis was systematically described and organized by Burkart (1976) in to five sections that together contains 44 species and with many varieties (Pasiecznik et al., 2001). Prosopis juliflora belonging to the family Leguminaceae (Fabaceae) and subfamily Mimosoideae, section Algarobia that has six series; specifically it belongs to the series Chilensis that contain eleven species and many varieties. Prosopis juliflora is particularly closely connected to Prosopis pallida. It is a tree or shrub sized woody perennial plant found mainly in the arid and semi arid regions of the world (Geezing et al., 2004). The plant is predominantly xerophilous spiny and sometimes unarmed evergreen tree with height of 3-15 meters depending on genetic difference and other environmental factors, but under favorable environmental conditions some individuals may reach up to 20m (Pasiecznick et al., 2003). Prosopis juliflora landraces often have multi-stemmed, coppiced and prostate shrub forms with long branches and a crown that even touches the ground and have erect, flat topped and decumbent tree forms. Prosopis juliflora produced coppices except those stumped at 10 cm below the ground (Hailu Shiferaw et al., 2004).

Mean monthly maximum temperature above 300^0C linked to the availability of soil moisture between 40-60% favored the weed germination and establishment. With increasing temperature and fluctuating precipitation, weeds may pose threats to the biodiversity. Prosopis juliflora can thrive in a wide range of rainfall. It extends from areas with an annual rainfall of only 50 mm in dry coastal zones to1500 mm mean annual precipitation of high altitude and grows well in areas receiving 250-600 mm annual rainfall (FAO, 2002; Pasiecznik et al., 2001). It can also survive

areas where lowest rainfall recorded in Arabian and Atacama Desert of the world (Ibrahim et al., 1988). If the root system is able to find water during drought, Prosopis juliflora will stay in green leaf throughout the dry season. Altitude does not appear to be limiting factor for the distribution of the plant. It is generally well adapted to different altitudes ranging from 200 meters above sea level up to 1500 meters above sea levels (Pasiecznik et al., 2001).

2.11.*Prosopis juliflora* in Ethiopia

Prosopis juliflora in AmibaraWoreda of Afar NRS (National Regional State) is thought to be the assumed starting point for the spread of *Prosopis juliflora* in Ethiopia. The area represents degraded semi-arid ecosystem in the country to the purposes of its introduction and *Prosopis juliflora* is rapidly invading the traditional agro- and silvo-pastoral land of the Afar and Isa ethnic groups in the Afar National Regional State and has encroached hundreds of kilometers away from the initial plantation area. *Prosopis juliflora* now occupies about over 700,000 hectares of prime grazing land and cultivable land following the Awash River in the Afar Region. It was also pointed out that the species has spread rapidly in eastern Ethiopia, from the Middle Awash Valley in to the Upper Awash Valley and Eastern Hararghe and some localities of Raya Azeboplaines of South Tigray. It is now a common sighting from Awash Arba all the way to Dire Dawa and Harar (Senayit *et al*., 2004; Taye *et al*., 2004).

3. MATERIALS AND METHODS

3.1. Description of the Study area

The experiment was conducted in microbiology laboratory, Department of biology at Haramaya University which is located at latitude of 9º26' N, longitude of 42º03'E and altitude of 1980 m.a.s.1. (FAO,1990). The mean annual temperature is 17ºC with mean minimum and maximum temperature of 3.8 and 25ºC, respectively.

3.2. Substrate preparation

Fresh *Prosopis juliflora* leafves were collected from around Dire Dawa town, Eastern Ethiopia. After collection, leaves were washed with water vigorously, chopped and washed with distilled water and dried in oven at 60 ± 3ºC for 24 h prior to pretreatment. The dried leaves were then ground, sieved and 1200grams of prosopis juliflora leaves powder stored in refrigator for subsequent use (Singh *et al*., 2014).

3.3. Inoculums Preparation

Inoculums were prepared from dried baker's yeast (*Saccharomyces cereviciae*). The yeast was purchased from local market Neway private company in Addis Ababa, Ethiopia. For preparing 0.5% inoculums dissolved 1.5gram of *Saccharomyces cerevisiae* with 300ml of distilled water and to prepare 1% inoculums concentration dissolved 3grams of *Saccharomyces cerevisiae* with 300ml of distilled water . For fermentation of each substrate 10ml of the solutions was used as inoculums concentration for batch fermentation process (Dhopeshwarker *et al.*, 2001).

3.4. Preparation of Nutrient Solution

Nutrient supplements were prepared by adding 0.1 g KH_2PO_4, 0.5 g $CaCl_2$, 0.5 g $MgSO_4$, 0.1 g Na_2SO_4 and 0.1 g $(NH_4)_2SO_4$ per litter by addition of distilled water (Abouzeid and Reddy, 1986).

3.5. Experimental Design

The experimental design was CRD factorial with eight treatment combinations in three replications for each treatment and control. Batch fermentation was carried out on *Prosopis juliflora* leaves as substrate and using baker's yeasts as fermenter. Different amounts (10, 20, 30

and 40g) of acid-treated substrates and inoculums concentrations (0.5% and 1%) were treatments (Table 2). Acid-untreated substrates with inoculum were controls. In all cases, the pH was adjusted to 4.5 with buffer solution. The fermentation process was allowed for 20 days at 30^{o}C. The production of bio-ethanol and other parameters were estimated at 4 days interval starting from the beginning of fermentation (Akin *et al.*, 2005).

Table 2. Treatments for Batch fermentation of *Prosopis juliflora* leaves

Sample No	*Prosopis juliflora* **(gm)**	**Volume of Nutrient Solution(ml)**	**Inoculums Concentration**
A	10	100	0.5%
B	10	100	1%
C	20	100	0.5%
D	20	100	1%
E	30	100	0.5%
F	30	100	1%
G	40	100	0.5%
H	40	100	1%

Note: A=10g +0.5% yeast , B=10g +1% yeast , C=20g+0.5% yeast , D=20g+1% yeast, E=30g +0.5% yeast, F=30g +1% yeast , G=40g +0.5% yeast , H=40g +1% yeast

3.6.1. Estimation of total reducing sugar

The amount of reducing sugar in the fermenting sample broth was estimated spectrophotometrically following the method used by Nelson (1944) and Somogyi (1945) using D-glucose as standard. A fermention broth (0.05ml) was mixed with 0.35ml of citrate buffer (pH=6.5) and 0.6ml of Dinitrosalicylic acid (DNS) and then the mixture was boiled for 5 minutes immediately to stop the reaction. Then the absorbance was measured at 540nm using spectrophotometer (Benn *et al.*, 1971). The amount of reducing sugar in the sample was calculated by relating absorbance of different concentrations of glucose

(250g/ml,125g/ml,62.5g/ml,31.25g/ml) on standard curve (Sadasivam and Manickam, 1996).

3.6.2. Determination of Cell Density (Biomass)

Cell density was measured at the 4^{th}, 8^{th}, 12^{th} 16^{th} and 20^{th} day of fermentation using spectrophotometer (Humas Think HS 3300, Korea) at 600 nm absorbance (Summer *et al*., 2004). Dry weight method of cell measurement was used. The cell in the broth sample was separated by centrifugation and the wet weight of the culture was measured immediately and allowed to dry in oven at 100^{o}C for six hour. The difference in weight was calculated and expressed the dry weight in mg /ml. Then the sample was diluted and measured the absorbance of it with a spectrophotometer at 600 nm. The calibration curve to relate the absorbance with cell dry weight was generated.

3.7. Qualitative Determination of Bio-ethanol

Presence of alcohol in the distillate was checked by functional group classification test (Bordwell and Wellman, 1962). For this, 1 drop of the unknown sample was added to 1 ml of reagent grade acetone in a test tube. Then, a drop of the chromic acid/sulfuric acid reagent was directly added into the solution and the mixture was shaken. A primary or secondary alcohol were reduce the orange-red sulfuric acid reagent to an opaque green or blue suspension of Cr(III) salts.

3.7.1. Quantitative Estimation of Bio-ethanol

One ml of the fermented sample was taken into 500 ml distillation flask containing 30 ml of distilled water and distilled. The distillate was collected in 50 ml flask containing 25 ml of potassium dichromate solution (33.76 g of $K_2Cr_2O_7$ dissolved in 400 ml of distilled water with 325 ml of sulphuric acid and volume raised to 1 liter by addition of distilled water). About 20 ml of distillate was collected in each sample and the flasks were kept in a water bath maintained at 6o°C for 20 minute. The flasks were allowed to cool at room temperature and the volume was made up to 50 ml. Five ml of this solution was diluted with 5 ml of distilled water for measuring the optical density at 600 nm using spectrophotometer (Caputi

et al., 1968). A standard curve was prepared under similar set of conditions by using standard solution of ethanol containing 0 to 20% (v/v) ethanol in distilled water and then ethanol content of each sample was estimated (Yoswathana and Phuriphipat, 2010).

3.8. Data Analysis

The data were analyzed using (SPSS version 17).Duncan's multiple range tests and LSD (least significant difference) tests were used to identify significant differences among treatment means. P values < 0.05 were considered significant in all cases.

4. RESULTS AND DISCUSSION

4.1. Effects of acid pre-treatment, substrate and inoculum concentrations and Fermentation Period on Ethanol Production

Ethanol production was noticed on 4th day of fermentation in both acid pre-treated and untreated substartes. However, the amount of etanol measured(5.54%) on the 4th day was significantly lower than that measured on other 8^{th} 12^{th} 16^{th} days of fermentation and significantly higher than 20^{th} day(4.58%) of fermentation. Low amount of ethanol obtained on the early days of fermentation may be ascribed to less population size of yeasts that need to adapt to the environment and reproduce aggresively to convert the available sugars into ethanol through fermentation. Ethanol production reached its maximum(29.05%) on the 12th days of fermentation and declined on 16^{th} and 20^{th} days of fermentation (Table 3), suggesting there are high population size of yeast to convert sugars into ethanol. The decline in the amount of ethanol after the 12th day may be due to less amount of fermentable substrates and/or accumulation of toxic susbtances that inactivate the activity of yeasts (Aikin-osanaiye *et al*., 2005).

Lignin is one of the factors affecting the enzymatic hydrolysis of biomass. Acid-treated substrates resulted in significantly ($p<0.05$) higher (29.05%) amount of ethanol production than untreated substrates(25.03%) at the same substration concentration of 40g inoculated with 1% yeast cell (Table 3). This might be due to acid pre-treatment of lignocellulose breaks down lignin and increase accessibility of enzymes and microbes to carbohydrates (Demirbas, 2005; Hendricks and Zeeman, 2009). Lloyd and Wyman (2005) and Sharma (2007) also reported that treatment of lignocellolosic substrates by acid prior to fermentation facilitates the availability of fermentable substances to enzymatic reaction. Esteghlalian *et al*. (1997) also reported that dilute sulfuric acid pretreatment can result in high reaction rates and significantly improve cellulose hydrolysis. In both acid treated and untreated substrates, ethanol production found to increase with increasing concentration of inoculums, suggesting more enzymes from yeast are facilitating conversion of more substrates into ethanol. This result agrees with the works of Giampietro *et al*. (1997); Mohd *et al*. (2011); Templer and Murphy (2012) who reported that bio-ethanol in high quantity can be derived from cellulosic biomass through acid or enzymatic hydrolysis followed by fermentation.Ethanol production was significantly affected by susbstrate concentration with

the highest amount (29.05%) of ethanol measurement was observed when 40gm of substrate was inoculated with 1% yeast. mented and declined thereafter 16th,and 20th dars of fermentation(19.25% &16.38%) respectively at the same substrate concentrationof 40gm inoculated with 1% yeast suggesting that this amount of substrate is optimal. For every enzymatic reaction, there will be optimal substrate level to yield maximum product, and increasing the amount of substrate beyond optimal level may be limited by the amount of enzyme produced in the system (Grubb and Mawson, 1993; Reddy, 2006; Hoyer *et al*., 2009).

Table 3- Ethanol production from *Prosopis juliflora* leaves using *S.cerevisiae* (mean ± SD, n=3)

Substrate(gm)	Treatment	Ethanol produced (%) at different fermentation period				
		4th day	8th day	12th day	16th day	20th day
A	treated	5.54 ± 0.03^{dK}	9.16 ± 0.02^{bK}	10.57 ± 0.01^{aK}	7.61 ± 0.02^{cJ}	4.85 ± 0.008^{eJ}
	Untreated	5.09 ± 0.03^{dK}	7.87 ± 0.08^{cL}	9.23 ± 0.05^{aL}	8.24 ± 0.02^{bI}	5.34 ± 0.027^{dI}
B	treated	7.13 ± 0.07^{cI}	10.14 ± 0.04^{bJ}	11.10 ± 0.03^{aJ}	10.04 ± 0.01^{bH}	7.21 ± 0.041^{cG}
	Untreated	6.63 ± 0.02^{cJ}	9.02 ± 0.09^{aK}	9.03 ± 0.08^{aL}	8.58 ± 0.07^{bI}	5.77 ± 0.031^{dH}
C	treated	8.89 ± 0.01^{dG}	12.44 ± 0.07^{cH}	17.46 ± 0.03^{aH}	15.62 ± 0.03^{bE}	12.76 ± 0.071^{cE}
	Untreated	7.51 ± 0.06^{eI}	11.77 ± 0.04^{dI}	16.04 ± 0.07^{aI}	15.80 ± 0.04^{bE}	12.94 ± 0.031^{cE}
D	treated	9.37 ± 0.01^{eF}	13.56 ± 0.06^{cG}	18.08 ± 0.03^{aG}	14.93 ± 0.03^{bG}	12.76 ± 0.071^{dE}
	Untreated	8.07 ± 0.02^{eH}	12.77 ± 0.04^{cH}	18.00 ± 0.02^{aG}	5.10 ± 0.01^{bF}	12.26 ± 0.062^{dF}
E	treated	12.09 ± 0.02^{dD}	16.1 ± 0.02^{bE}	21.06 ± 0.06^{aE}	15.02 ± 0.04^{cF}	12.17 ± 0.040^{dF}
	Untreated	11.04 ± 0.07^{eD}	14.96 ± 0.05^{cF}	18.66 ± 0.05^{aF}	17.15 ± 0.02^{bD}	14.30 ± 0.028^{dD}
F	treated	14.9 ± 0.01^{dB}	20.64 ± 0.05^{bC}	24.95 ± 0.07^{aD}	15.54 ± 0.07^{cE}	12.66 ± 0.041^{eE}
	Untreated	12.49 ± 0.01^{eC}	19.04 ± 0.06^{cD}	21.50 ± 0.06^{aE}	20.08 ± 0.04^{bB}	17.23 ± 0.017^{dB}
G	treated	14.27 ± 0.05^{eB}	21.25 ± 0.07^{cB}	28.07 ± 0.04^{aB}	22.24 ± 0.04^{bA}	19.37 ± 0.035^{dA}
	Untreated	11.72 ± 0.09^{eE}	19.01 ± 0.06^{cD}	23.61 ± 0.05^{aE}	20.18 ± 0.02^{bB}	17.23 ± 0.017^{dB}
H	treated	16.72 ± 0.02^{dA}	22.74 ± 0.03^{bA}	29.05 ± 0.02^{aA}	19.25 ± 0.06^{cC}	16.38 ± 0.058^{dC}
	Untreated	14.04 ± 0.02^{eB}	19.37 ± 0.01^{cD}	25.31 ± 0.03^{aC}	20.26 ± 0.02^{bB}	17.46 ± 0.090^{dB}

Note: Means followed by different small letters in row are significant at 0.05 probability levels. Means followed by different capital letter in column are significantly different at 5% level of significance . A= 10g + 0.5% yeast, B=10g + 1% yeast, C= 20g + 0.5% yeast, D= 20g + 1% yeast, E= 30g + 0.5% yeast, F= 30g + 1% yeast, G= 40g + 0.5% yeast and H= 40g + 1% yeas.

4.1.1. Qualitative Determination of Bio-ethanol

The presence of alcohol in the distilate was checked by the functional group classification test of chromic acid or sulfuric acid reagent grade color change test to do that prepared experimental group and controled group. In the experimental test tube added a dropped of sulfuric acid reagents and a dropped of fermented sample then added one milliliter of aceton reagent grade the primary or secondar alcohl oxidized and disappeared of the orange-red chromic acid/sulfuric acid reagent to blue colors this posite result indicated that the presence of alcohol in the disitilate and quality of ethanol were checked by color test in the prosopis juliflora leaves.

4.2. **Effects of acid pre-treatment, substrate and inoculum concentrations and Fermentation Period on reducing sugar yield**

Concentration of reducing sugar measured on the 4th day of fermentation was found to be significantly higher in both acid pretreated and untreated substrates, but found to decline with increasing days of fermentation (Table 4). A maximum of 11.75±0.05mg reducing sugar was obtained from the acid pretreated *Prosopis juliflora* leaves at 40gram inoculated with 1% yeast cell biomass. However, with increasing fermentation period, the amount of reducing sugar was declined, and this may be due to the fact that some sugars contained in *Prosopis juliflora* leaves were consumed by yeasts as a substrate to grow on and converted into ethanol.

The comparison of reducing sugar concentration between acid pre-treated and untreated substrates showed that the amount of reducing sugar was significantly higher when substrates were pretreated with diluted sulphuric acid (Table 4). This may be the facilitation of hydrolysis of lignocellulosic material by the acid for conversion into sugar. In line with this, Demirbas (2005) and Hendriks and Zeeman, (2009) reported that acid pretreatment of lignocellulose is important to break down lignin and increase the availability of sugar for microbes to grow on and convert it to ethanol. Arumugam and Manikandan (2011), reported that an initial acid pretreatment of fibrous peel residues breakdown its structure to make it more susceptible to enzymatic reactions. This can be the reason for the valuable amount of reducing sugars librated from the hydrolysates. In all the substrates concentration increasing yeast concentration from 0.5 to 1% resulted in significant increase in ethanol concentration and decrease in sugar concentration. Rate of decrement of reducing sugar agrees with the amount of ethanol

production, suggesting that the more is the reduction in reducing sugar means the more it is converted to ethanol by yeast

Table 4- Reducing sugar concentration (mg/ml) measured at 540nm *from Prosopis juliflora* leaves (values are Mean ±SD, n=3).

Substrate (gm)	Treatment	Reducing sugar concentration(gm/m) at different fermentation period				
		4th day,	8th day,	12th day,	16th day ,	20th day
A	treated	6.93±0.035^{aG}	5.72±0.058^{bH}	4.2±0.023^{cF}	2.67±0.064^{dG}	0.53±0.023^{eG}
	untreated	6.25±0.077^{aH}	5.53±0.077^{bH}	4.41±0.031^{cE}	3.31±0.040^{dE}	1.01±0.045^{eF}
B	treated	6.07±0.023^{aH}	4.44±0.018^{bG}	3.18±0.062^{cG}	2.18±0.058^{dH}	0.47±0.045^{eG}
	untreated	5.24±0.031^{aI}	4.27±0.031^{bG}	3.20±0.049^{cG}	2.21±0.017^{dH}	0.23±0.035^{eH}
C	treated	8.96±0.045^{aE}	8.30±0.046^{bD}	5.92±0.042^{cD}	5.24±0.072^{dC}	0.96±0.067^{eF}
	untreated	8.81±0.036^{aE}	8.18±0.027^{bD}	6.0±0.062^{cC}	5.31±0.071^{dC}	1.20±0.046^{eE}
D	treated	8.30±0.013^{aF}	7.95±0.044^{bE}	6.28±0.085^{cC}	3.68±0.031^{dE}	1.26±0.064^{eE}
	untreated	8.19±0.017^{aF}	7.31±0.045^{bF}	6.46±0.055^{cC}	5.39±0.064^{dC}	2.07±0.04^{eC}
E	treated	10.32±0.036^{aC}	8.61±0.017^{bC}	7.06±0.072^{cB}	6.19±0.020^{dB}	2.34±0.032^{eB}
	untreated	10.19±0.010^{aC}	8.26±0.026^{dD}	7.15±0.031^{cB}	6.25±0.067^{dB}	3.22±0.013^{eA}
F	treated	8.95±0.010^{aE}	7.30±0.013^{bF}	5.80±0.010^{cD}	4.31±0.015^{dD}	1.77±0.023^{eD}
	untreated	8.0±0.023^{aF}	7.21±0.055^{bF}	5.92±0.053^{cD}	4.59±0.059^{dD}	2.36±0.023^{eB}
G	treated	11.53±0.040^{aB}	9.4±0.049^{bA}	7.16±0.071^{cB}	6.69±0.020^{dA}	2.48±0.028^{eB}
	untreated	9.69±0.049^{aC}	9.01±0.040^{bB}	7.47±0.018^{cA}	6.68±0.022^{dA}	3.25±0.045^{eA}
H	treated	11.75±0.058^{aA}	7.93±0.017^{bE}	4.44±0.060^{cE}	3.02±0.013^{dF}	0.85±0.054^{eF}
	untreated	9.55±0.070^{aD}	7.18±0.053^{bF}	4.5±0.032^{cE}	3.03±0.022^{dF}	1.34±0.032^{eE}

Note: Means followed by different small letters in row are significant at 0.05 probability levels. Means followed by different capital letter in column are significantly different at 5% level of significance . A= 10g + 0.5% yeast, B=10g + 1% yeast, C= 20g + 0.5% yeast, D= 20g + 1% yeast, E= 30g + 0.5% yeast, F= 30g + 1% yeast, G= 40g + 0.5% yeast and H= 40g + 1% yeast

4.3 **Effects of acid pre-treatment, substrate and inoculum concentrations and Fermentation Period on Cell Density**

The result showed that cell density increased from 3.79±0.013 to 5.03±0.010 with increasing of fermentation period from 4th to 12th day.However, after 12th day cell biomass found to decline(2.35±0.032) 0n 20th days of fermentation. This may be due to cyto-toxicity of ethanol and limitation of available nutrient resources. In all the substrate concentrations, pre-treated substrate showed higher cell density as compared to untreated substrates. Moreover, a maximum

of 5.03 (g/ml) cell density was observed when 40 gram of substrate was inoculated with 1% of yeast. At this same substrate concentration ethanol production was also highest. In line with this, Akin-Osanaiye *et al.* (2005) reported that increasing ethanol production with increasing cell biomass indicated that the amount of yeast influenced ethanol production in agro waste

Table 5 : Effects of acid pre-treatment, substrate and inoculum concentrations and Fermentation Period on Cell Density. (values are Mean ±SD, n=3)

Substrate + Yeas	Treatment	Cell density observed from fermented p. juliflora leaflet at 600nm(g/ml)				
		4th day,	8th day,	12th day,	16th day ,	20th day
A	treated	2.87 ± 0.032^{cD}	3.21 ± 0.040^{bF}	3.41 ± 0.018^{aC}	2.55 ± 0.017^{cF}	0.96 ± 0.027^{dF}
	Untreated	2.79 ± 0.080^{cE}	3.02 ± 0.083^{bF}	3.28 ± 0.054^{aD}	2.64 ± 0.050^{cF}	1.04 ± 0.038^{dE}
B	treated	3.45 ± 0.049^{cC}	3.81 ± 0.073^{bD}	4.44 ± 0.027^{aC}	3.72 ± 0.054^{bD}	0.49 ± 0.010^{dH}
	Untreated	3.38 ± 0.022^{cC}	3.79 ± 0.013^{bD}	4.34 ± 0.032^{aC}	3.79 ± 0.015^{bD}	1.05 ± 0.027^{dE}
C	treated	2.91 ± 0.022^{cD}	3.45 ± 0.027^{bE}	4.37 ± 0.018^{aC}	4.11 ± 0.044^{aC}	1.46 ± 0.038^{dD}
	Untreated	2.88 ± 0.040^{cD}	3.35 ± 0.027^{bE}	4.32 ± 0.058^{aC}	4.12 ± 0.025^{aC}	2.17 ± 0.040^{dB}
D	treated	3.69 ± 0.074^{cB}	4.15 ± 0.027^{bC}	4.73 ± 0.015^{aB}	3.86 ± 0.023^{cD}	0.98 ± 0.027^{dF}
	Untreated	3.67 ± 0.047^{cB}	4.09 ± 0.018^{bC}	4.67 ± 0.013^{aB}	3.99 ± 0.067^{bD}	0.87 ± 0.094^{dG}
E	treated	2.85 ± 0.037^{cD}	4.87 ± 0.018^{aA}	4.46 ± 0.017^{bC}	4.37 ± 0.089^{bB}	1.90 ± 0.070^{dC}
	Untreated	2.80 ± 0.085^{cD}	4.85 ± 0.059^{aA}	4.39 ± 0.064^{bC}	4.37 ± 0.013^{bB}	2.30 ± 0.036^{dA}
F	treated	3.79 ± 0.013^{cA}	4.50 ± 0.037^{bB}	4.98 ± 0.031^{aA}	3.52 ± 0.031^{cE}	1.57 ± 0.015^{dD}
	Untreated	3.74 ± 0.044^{cA}	4.39 ± 0.064^{bB}	4.92 ± 0.058^{aA}	3.38 ± 0.015^{cE}	1.63 ± 0.017^{dD}
G	treated	2.87 ± 0.033^{dD}	3.82 ± 0.015^{cD}	4.80 ± 0.050^{aB}	4.38 ± 0.010^{bB}	1.33 ± 0.042^{eE}
	Untreated	2.85 ± 0.038^{dD}	3.76 ± 0.013^{cD}	4.73 ± 0.030^{aB}	4.39 ± 0.017^{bB}	2.26 ± 0.023^{eAa}
H	treated	3.79 ± 0.013^{cA}	4.57 ± 0.023^{bB}	5.03 ± 0.010^{aA}	4.75 ± 0.028^{bA}	2.35 ± 0.032^{dA}
	Untreated	3.75 ± 0.040^{cA}	4.92 ± 0.030^{aA}	4.97 ± 0.049^{aA}	4.73 ± 0.022^{bA}	2.25 ± 0.027^{dA}

Note: Means followed by different small letters in row are significant at 0.05 probability levels. Means followed by different capital letter in column are significantly different at 5% level of significance . A= 10g + 0.5% yeast, B=10g + 1% yeast, C= 20g + 0.5% yeast, D= 20g + 1% yeast, E= 30g + 0.5% yeast, F= 30g + 1% yeast, G= 40g + 0.5% yeast and H= 40g + 1% yeast

5. SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary

The potential of producing bioethanol through conversion of lignocellulosic biomass can be a viable and important perspective as regards both the synthesis of a renewable fuel as well as by solving the important and burning problem of environment. Ethanol from lignocellulosic biomass holds great potential due to the widespread availability, abundance, and relatively low cost of cellulosic materials. *Prosopis juliflora* can be considered a promising alternative to synthesis of ethanol by using *Saccharomyces cerevisiae* .

The present study indicated that the maximum ethanol production was recorded (29.05%) from the 12th daypre-treated *Prosopis juliflora* leaflets of the highest substration concentration of 40g with 1% yeast combination and the minimum ethanol production was recorded (5.09%) from 10g untreated substrated with 0.5% inoculums and the reducing sugar decrease as fermentation period increases and the maximum reducing sugar was recorded (11.75±0.05) was obtained from pre-treated *Prosopis juliflora* leaflets of 40g with 1% inoculums and the minimum was obtained (0.23±0.035mg/ml) from 10g untreated *Prosopis juliflora* leaflets and the maximum cell density recorded (5.03±0.049mg/ml) was obtained from 40g with 1% inoculums untreated leaflets and the minimum was (0.49±0.01mg/ml) was obtained from 10g with 1% yeast cells.

5.2 Conclusion

The finding of present study revealed that *Prosopis juliflora* leaflets exhibited significant result for the production of bioethanol. The amount of bio-ethanol produced from those substrates concentration was different and statistically significant at $p<0.05$.Among the different substrate concentration, 40 gram of treated substrate showed the highest percentage of ethanol production with high yeast concentration. Therefore, substrate concentration and inoculums concentration are directly proportional until it reached the optimum level for ethanol production. Comparatively the reducing sugar utilization was more in pretreated substrates than untreated ones. Bio-ethanol production increased slightly when initial substrate concentration increased. But, it was decreased after 12th day of fermentation. This may be due to substrate limitation and decrease in cell biomass (yeast).

5.3 Recommendation

Based on the findings of the experiments, the following recommendations were suggested

- To check the bio-fuel quality of *Prosopis juliflora* leaflets by Gas chromatography too.
- Further study is very important to describe how absolute bio-ethanol can be produced from *Prosopis juliflora* leaflets by using rotary evaporator, because it is difficult to make pure ethanol since there are other chemicals that can evaporate below the boiling point of ethanol ($78^{o}C$).
- It recommended that government or other investor's to initate piolet study on *Prosopis juliflora* leaflets for production of bio-fuel.

6. **REFERENCES**

ABN (African Biodiversity Network). 2007. “Agrofuels in Africa: The Impacts on Land, Food and Forests. http://www.africanbiodiversity.org/sites/default/files/PDFs/Agrofuels%20in%20Africa Impacts%20on%20land%2C%20foods%20%26%20forests.pdf (retrieved May 30, 2011. agricultural waste to ethanol by SSF using recombinant cellulase from *Clostridium.*

Akin-osanaiye, B., Nzelibe, C. and Agbaji, A. S. 2005. Ethanol production from carica papaya (pawpaw) fruit wastes.Asian, J. Bioche., 3: 188-193.

Amadi, E., Agomuo, N. and Ibegbuan, C.O. 2009. Research methods in Biochemistry supreme publishers,owerri,Nigeria; 93-99.

Aminifarshidmehr, N.1996. The management of chronic suppurativeotitis media with acid media ancient art of winemaking. Yeast 16:675-729.

Arumugam, R. and Manikandan, M. 2011. Fermentation of pretreated hydrolyzates of banana assimilating yeast strain. *Appllied. Microbial. Biotechnology*. 72: 1136-1143.

Bayissa, B. 2002. The Prevailing Fuel Crisis and Mitigating Measures. In: Energy in Ethiopia: Status, Challenges, and Prospects. Proceedings of Energy Conference.

Beyene, A. 2011. Biofuels Production and Their Implication on Livelihoods: Ethiopia. Papre presented at ECAS -4th European Conference on African Studies,African Engagements: On Whose Terms? Uppsala 15-18 .

Bordwell, F. G.and Wellman, K. M. J. 1962. Chemistry Education volume 39: 308–310.

Boulton, B., Singleton, V.L., Bisson, L.F. and Kunkee. R.E. 1996. Yeast and biochemistry of industrial yeast strains. *Pure Applied Chemistry*. 59: 1493–500.

Caputi, A. Jr., Ueda, M. and Brown, T. 1968. Spectrophotometric determination of ethanol in wine. Amercan . Journal. Enology and Viticulture., 19: 160-165.

Coppock, D. A. 2005. Agro-Pastoralists Wrath for the Prosopis Tree: the Case of the Il Chamus of Baringo District, Kenya. Utah: ENVS Faculty publication.

De Oliviera, F.B. and de Sousa, S.S.A. 2007. Biomas-To-Liquids: A Potential Path in Brazil. Energy Tribune.

Demirbas, A.2004. Ethanol from cellulosic biomass resources. *International Journal Green Enererprise*. 1: 79–87.

Demirbas, A .2005. Bioethanol from Cellulosic Materials: A Renewable Motor Fuel from Biomass. *Energy Sources* 27: 327-33.

Dhopesh warker,U., Jadav, R., Jadav, L.D.,` souza, and Jayaprakash, D. 2001.

Ebringerova, A. Z., Hromadkova, T.and Heinze. 2005. Hemicellulose Advance Polymer Science, the major hemicellulose polymers in cereals are heteroxylans, pp.(186), 1- 67

EEP. 2006. Proceeding of the Workshop on Afar Pastoralist Prosopis Project Immerging issues. Addis Ababa.

Esteghlalian, A., Hahimoto, A .G., Fenske, JJ. And Penner, M. H .1997. Modeling and optimization of fuel from Groundnuts shell waste. *American Journal of engineering.*

Girio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S R.and Bogel-Lukasic.2010. Hemicellulose Bioresour Technology, 101 , pp. 4775-4800.

Gebremeskel, L. and Tesfaye, M. 2008. A preliminary assessment of socioeconomic and environmental issues pertaining to liquid biofuel development in Ethiopia.

Gray, K. and Emptage, M. (2006). Bioethanol. Current opinion in chemistry Biology 10, 141-146.

Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M., Liden, G. and Zacchi, G. 2006. Bio-ethanol the fuel of tomorrow from the residues of today. Trends Biotechnology. 24, 549-556 .

Hamelinck, C.N., Van Hooijdonk, G. and Faaij, A.P.C. 2005. Ethanol from lignocellulosi biomass: technology economic performance in short-, middle- and long-term. Biomass Bioenergy.

Hendriks, ATWM. and Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology 100:10-18.

Hettenhaus, J. 1998. Ethanol fermentation strains: present and future requirements for biomass to ethanol commercialization. USA, United States Department of Energy and National renewable Enery Laboratory, cited; available from: /www.p2pays.org/ ref/38/37753.

Himmel, M. E. 2007. Biomass recalcitrance: Engineering plants and enzymes for biofuels production.

Hopkins, M. 2007. Cooking up a smoky solution. Nature News, doi: 10. 1038/news 070813-l.

Hoyer, K., Galbe, M. and Zacchi, G. 2010. Research Effects of enzyme feeding strategy on ethanol yiedl in fed-batch simultaneous saccharification and fermentation of

spruce at high dry matter. *Biotechnol Biofuels*;3: 14. pmid:20579340.

Jackman, E.A. 1987 . Industrial alcohol. In: Basic Biotechnology London, Academic Press .pp. 39 -336.

Jacobsen, S.E. and Wyman, C.E. 2000. Cellulose and Hemicellulose Hydrolysis Modesl for Application to Current and Novel Pretreatment Processes. *Applied Biochemistry and Biotechnology*, 84 (1-9): 81-96.

Jeffries, T. and Jin,Y. 2000. Ethanol and thermo tolerance in the bioconversion of xylose by yeasts. *Advanced.Applied.Microbial Biology*. 47:221–268 .

Jing-Ke Weng, X. Li.and Chapple, C. 2008. Improvement of biomass through lignin Modification Plant Journal, 54 . pp. 569-581.

Katahira, S., Mizuike, A., Fukuda, H. and Kondo, A. 2006. Ethanol Fermentation from lignocellulosic hydrolysate by a recombinant xylose and cellooligosaccharide assimilating yeast strain. *Appllied. Microbial. Biotechnology*. 72: 1136-1143.

Kiran, S., Sikander, A.and Lkram-ul-Haq .2003. Time course study for yeast invertase Production by submerged fermentation. J BiolSci 3(11):984–988.

Lakew, H. and Shiferaw. Y. 2008.“ Rapid Assessment of Biofuels Development Status in Ethiopia.”In T.Anderson and M. Belay (eds.), Rapid Assessment of Biofuels Development Status in Ethiopia and Proceedings of National Workshop on Environmental Impact Assessment and Biofuels. MELCA Mahiber, Addis Ababa.

Lin and *Tanaka .2006.* Ethanol fermentation from biomass resources: current state and prospects.

Lin,Y. and Tanaka, S. 2006. Ethanol fermentation from biomass resources current state and prospects. *Applied MicrobialBiotechnology*, 69: 627-642.

Lloyd, T.A. and Wyman,. C.E. 2005. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover .

Mohd, A.K., Loh, SK.,Nasrin, A., Astimar, A.and Rosnah, MS. 2011. Bioethanol production From empty fruit bunches hydrolysate using Saccharomyces cerevisiae. *Research Journal* of *Environmental Sciences*.

MoME (Ministry of Mines and Energy). 2007. The Biofuel Development and Utilization Strategy of Ethiopia. MoME, Addis Ababa.

MoWE (Ministry of Mines and Energy), (2012) The Biofuel Development and Utilization Strategy of Ethiopia. MoME, Addis Ababa.

MoWE. 2013. Biofuels Development Coordination Directorate. Head Office, Addis Ababa : Personal Communication, April 23, 2013.

Negusu. 2009. Potential biofuel production from prosopis julifora. Addis Ababa: Addis Ababa Universty.

Nikolic *et al.*, 2009. Nedovic Effect of different fermentation parameters on bioethanol production from corn meal hydrolysates by free and immobilized cells of *Saccharomyces cerevisiae.* var. *ellipsoideus Journal of Chemical Technology* and *Biotechnology* 84 , pp. 497-503.

Non-native Species Secretariat, accessed August 31, 2014.

Onuki, S. 2005. Bioethanol, Industrial production process and recent studies. USA Perry., chemical engineering hand book.

Pretorius, I.S.2000. Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. Yeast, 16:675-729.

Puhan, S., Vedaraman, N., Rambrahaman, B.V.and Nagarajan, G. 2005. Mahua (Madhuca indica) seed oil: a source of renewable energy in India. J Sci Ind Res 64:890−896.

Rasskazchikova, T. V., *et al*., 2004. Ethanol as high-octane additive to automotive gasolines: production and use in Russia and abroad. *Chemistry and Technology of Fuel and Oils*, 40(4):203-210.

Reddy,L.V.,Reddy,O.V.S. and Wee,Y.J. 2011. Production of ethanol from mango(Mangifera india L.) Pee by saccharomyces cerevisiae CFTRI 101. *African Journal of Biotechnology*,10(20): 4183-4189.

Sadasivam, *S.*and Manickam,A. 1996. Biochemical methods. 2nd edition, New Age International p Ltd. Publisher, New Delhi. Pp. 179-186.

Sanchez, P. A., B. Jama and A. I. Niang. 1999. Soil fertility, small-farm intensification and the environment in Africa. In C. Barret and D. Lee (eds.) Agricultural intensification and the environment. Cornell University (in press).

Senayit, R., Agajie, T., Taye, T., Adefires, W. and Getu, E. 2004. Invasive Alien Plant control and Prevention in Ethiopia, Pilot Surveys and Control Baseline Conditions. Report submitted to EARO, Ethiopia and CABI under the PDF-B

phase of the UNEP/GEF Project, removing Barriers to Invasive Plant Management in Africa. EARO, Addis Ababa.

Sharma-shivappa, R.R, R.R, R.R., Boyette, M.D., and Osborne, J. 2007. A comparision of chemical pretreatment methods for improving Saccharification of cotton stalks. *Bioresource, Technol*., 98:300-11.

Srilekha Yadav et al., 2011. Venkateswar RaoBioethanol fermentation of concentrated rice straw hydrolysates using co-culture of *Saccharomyces cerevisiae* and *Pichia stiptis* Bioresour. Technol., 102 (2011), pp. 6473-6478.

Sugar Corporation. 2013. www.etsugar.gov.et. *www.etsugar.gov.et.* Online Sugar Corporation, Ethiopian Sugar Industry Profile.

Stewart, G. and Russell, I. 1987. Biochemistry and genetics of carbohydrate utilization by industrial yeast strains. *Pure Applied Chemistry*. 59: 1493–500.

Summer,J., Georgierva,B. and Ahring, K .2004. Bioconversion of banana wastes to ethyl alcohol. *Biochemistry Translation,* :32-283.

Sun, Y.and Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresource Technology, 83*: 1–11.

Taherzadeh, M. 1999. Ethanol from lignocelluloses*: physiological effects of inhibitors and fermentation strategies*.

Templer, R.and Murphy, RJ. 2012. Environmental sustainability of bioethanol production from waste paper: sensitivity to the system boundary. Energy and *Environmental Science,*. 2: 8281-8293.

Tessema, T. 2007. The Prospects of Biological Controls of Weeds in Ethiopia. Ethiopian Journal of weed Management 1, 63-67.

Wingren, A.M., Galbe, F. and Zacchiu. 2003. Techno-Economic Evaluation of Producing Ethanol from Softwood: Comparison of SSF and SHF and Identification of Bottlenecks. Biotechnology. Program. 19:1109-1117.

Yoswathana, N.and Phuriphipat, P. 2010. Bioethanol Production from Rice Straw. *Energy Research Journal.;*1:26- 31.

7. APPENDICES

7.1. Appendix Tables

Table1. Standard curve for the determination of Ethanol production was used ethanol water solution by range of 0-20%(v/v)

Test tube	cell density	absorbance
1	0	0
2	5%	0.608
3	10%	0.68
4	15%	0.752
5	20%	1.105

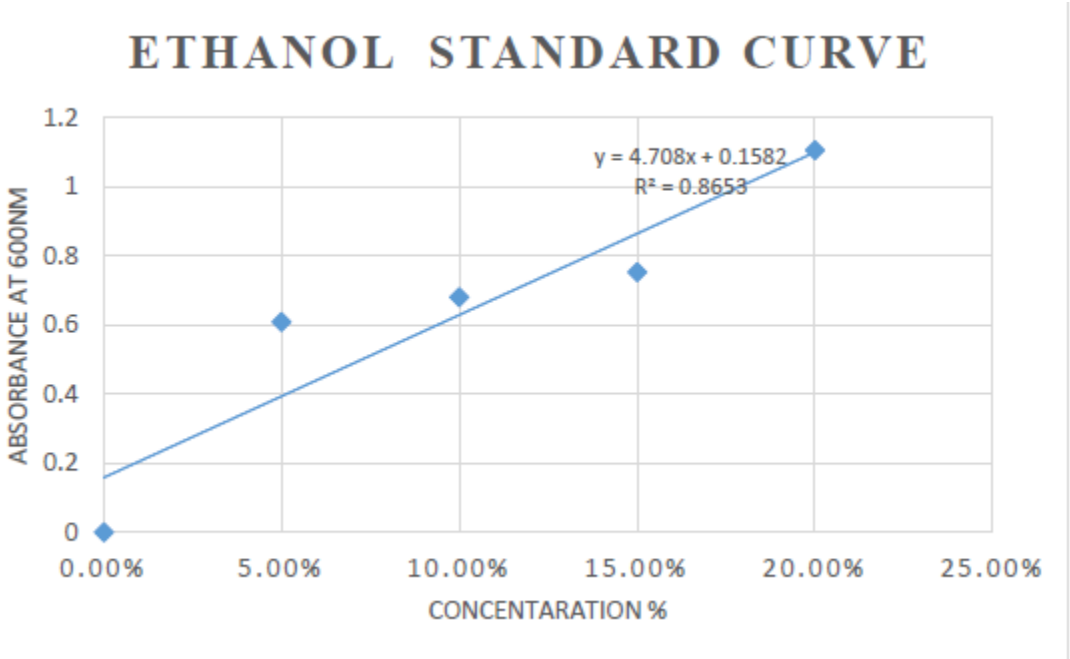


Figure 1. Standard curve for estimation of ethanol content by using D- glucose concentration prepared by taking 100 mg/ml already prepared standard D- glucose.

Table2.Standard curve for the determination of reducing suger by using D- glucose concentration prepared by taking 100 mg/ml already prepared standard D- glucose.

Test tube	cell density	absorbance
1	0	0
2	0.2	0.122
3	0.4	0.231
4	0.6	0.389
5	0.8	0.483
6	1	0.676

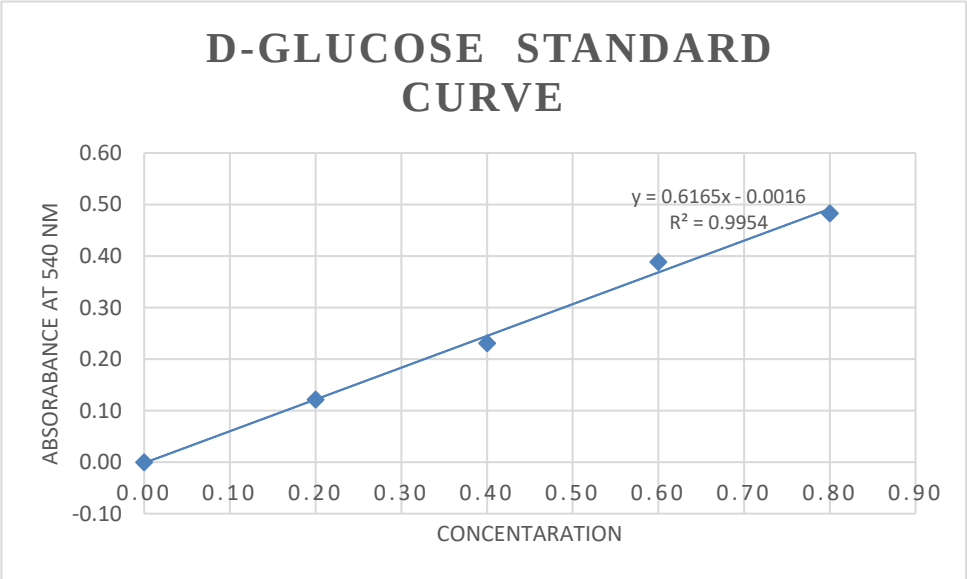


Figure 2. Standard curve for the determination of reducing suger by using D- glucose concentration prepared by taking 100 mg/ml already prepared standard D- glucose

Table 3. Standard curve for determination of cell density prepared by taking 1g baker yeast and dissolve by 10ml distilled water with appropriate serial dilution.

Test tube	cell density	absorbance
1	5	0.5
2	2.5	0.23
3	1.25	0.1

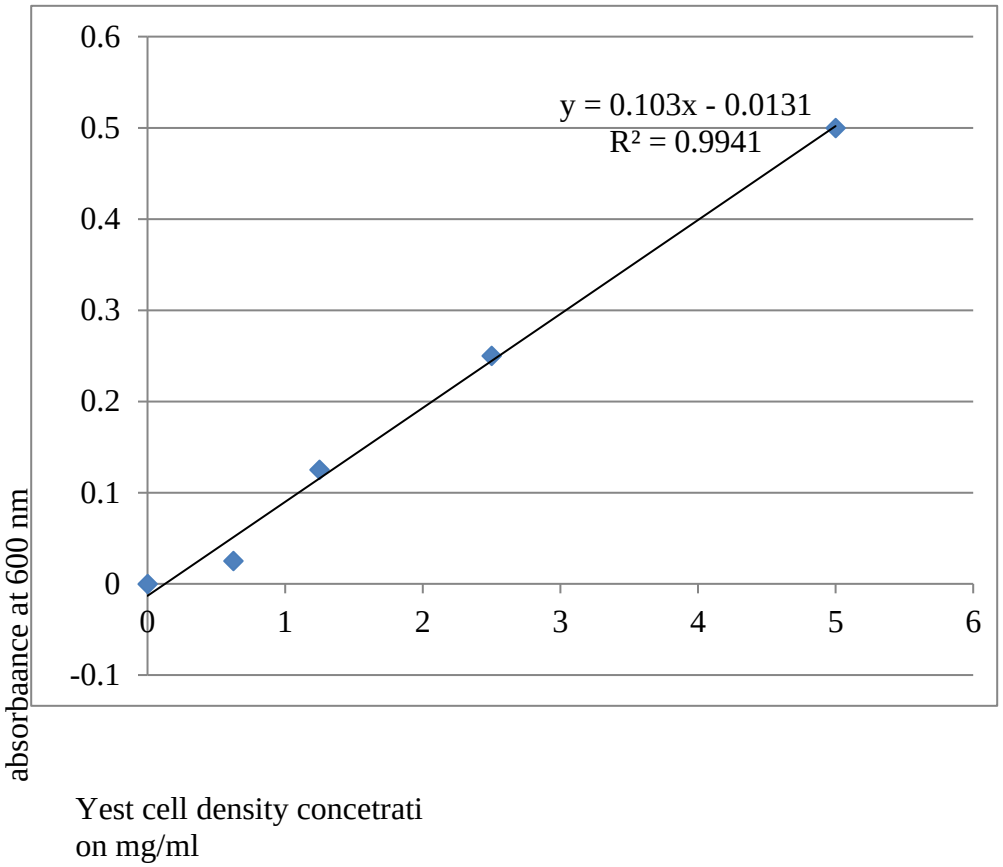
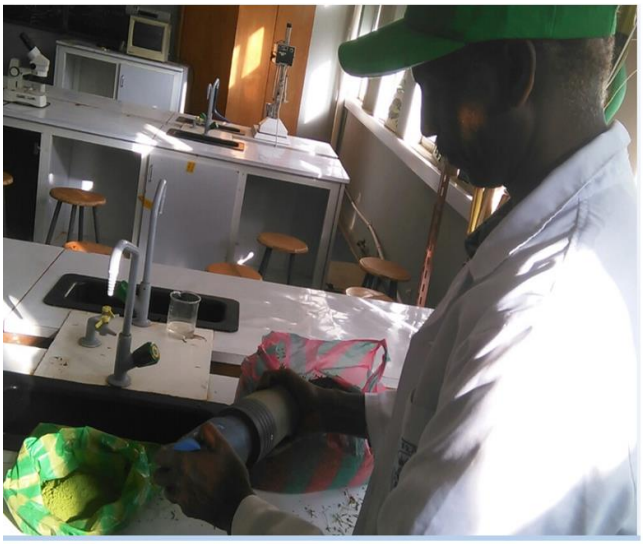


Figure 3. Standard curve for determination of cell density prepared by taking 1g baker yeast and dissolve by 10ml distilled water with appropriate serial dilution.

Collected *prosopis juliflora* plant leaflets were washed and dried in Microbiology labratory



Prosopis juliflora leaflets were grained wih mortar to make it a powder

Grainded Powder of *p.juliflora* leaflets

Prosopis juliflora leaflets substration with its total three replication

p.juliflora leaflets substration were autoclaving at 121c^{o} for 30 minutes and Total fermentater of *p.juliflora* leaflets substrates

Sample were taken at each intervals of 4th days of fermentation for distillation by Rotary evaporater ,to measure reducing sugar and cell density.