ETHANOL PRODUCTION FROM SORGHUM STRAW WITH ACID PRETREATMENT USING *Saccharomyces cerevisiae*

MSc THESIS

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JULY, 2018

HARAMAYA UNIVERSITY, HARAMAYA

Ethanol Production from Sorghum Straw with Acid Pretreatment Using *Saccharomyces cerevisiae*

A Thesis Submitted to the College of Natural and computational Sciences, School of Biological Science and Biotechnology

In partial Fulfillment of the Requirements for the Degree of MASTER OF SCIENCE IN BIOTECHNOLOGY

Kalid Ahmed Yusuf

July, 2018

HARAMAYA UNIVERSITY, HARAMAYA

APPROVAL SHEET

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As member of the board of examiners of the M.Sc. thesis open defense examination, we certify that we have read and evaluated the thesis prepared by **Kalid Ahmed Yusuf** and examined the candidate. We recommend that the thesis be accepted as fulfilling the thesis requirement for the degree of **Master of Science in Biotechnology.**

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank the almighty God in supplying health, strength patience and persistence to complete this study.

I would like to sincerely thank all the individuals who in one way or another assisted me with the challenge that this work presented to me. Specifically, my great appreciation goes to my advisors, Dr.Meseret Chimdessa and Dr.Manikandan Muthuswamy for intellectual support, encouragement, and enthusiasm throughout my study, and for their ability to continue doing research in correcting both my stylistic and scientific errors.

I would like to acknowledge with sincere gratitude the staff members of the Biology Department of Haramaya University, for properly guiding me in pursuing my M.Sc. studies.

I would like to say special thanks to Botanical Lab Assistants who assisted me in the arrangement of chemical reagents and apparatus during the course of this research work. Successful and timely accomplishment of this study would have been very difficult without their cooperation.

I would like to be thankful to all of my family and my best friends for their constant support and encouragement which have motivated me through my entire endeavor

LIST OF ABBREVIATION / ACRONOMYS

GHG	Greenhouse Gas
LB	Lignocellulose Biomass
LCC	Lignin-Carbohydrate Complex
DP	Degree of Polymerization
ARP	Ammonia Recycle Percolation
VOC	Volatile Organic Compounds
AFEX	Ammonia Fiber Explosion
FAO	Food and Agriculture Organization
HMF	Hydroxyl Methyl furfural
OD	Optical Density
MTBE	Methyl Tertiary Butyl Ether
CRFD	Completely Randomized Factorial Design
LSD	Least Significant Difference
DNSA	Dinitrosalicylic Acid
SPSS	Statistical Package for Social Studies

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Ethanol Production from Sorghum Straw with Acid Pretreatment Using *Saccharomyces cerevisiae*

ABSTRACT

Fermentation is a metabolic process of microorganisms to obtain energy by breaking down organic compound. Sorghum straw was fermented with different concentration substrate (15gm, 30gm , 45g and 60gm) with 0.5% ,1% yeast in different fermentation period (4th day, 8th day, 12th and 16th days) with the aim of ethanol production from Sorghum straw. Among the different substrate concentration 15 gram of substrate mixed with 1% yeast produced high quantity of ethanol on 4th day of fermentation. After the acid pre-treatment and addition of high amount of inoculum concentration (1% yeast), ethanol production was increased as compared with untreated 15 gram substrate mixing with 1% inoculum concentration. The maximum ethanol production for 15g untreated substrate was 0.04%, where as that of acid-pretreated was 0.15%. The reducing sugars contents increased through process as the fermentation period increased. Moreover, on 12th day of fermentation period, the highest reducing sugar concentration (1.36mg/ml) was gained from acid pretreated 30gram Sorghum straw substrate mixed with 1% inoculums concentration, whereas the same substrate concentration showed 1.33mg/ml in untreated substrate. The cell density was higher in acid pre-treated substrate combine with 1 % inoculum (yeast) concentration (2.46mg/ml) than untreated ones (2.30mg/ml). The maximum ethanol production was observed on 4th day of fermentation in acid pre-treated substrate and high inoculums concentration (1% yeast). After 4th day of fermentation period, the rate of ethanol production decreased in treated and untreated substrate. The study revealed that it is possible to produce bio-ethanol from Sorghum straw. Therefore, it is recommended to conduct experimental studies to increase ethanol yield.

Key word*: batch fermentation, cell density, untreated substrate, acid pretreated substrate and Inoculums.*

1. INTRODUCTION

In the 20th century, the world economy has been dominated by technologies that depend on fossil energy, such as petroleum, coal, or natural gas to produce fuels (Paster, 2003). The continuous use of fossil fuels to meet the majority of the world's energy demand is threatened by increasing concentration of CO_2in the atmosphere and concerns over global warming (Demirbas *et al.*, 2004). The combustion of fossil fuel is responsible for 73 % of the CO_2 emission (Wildenborg and Lokhorst, 2005).The heightened awareness of the global warming issue has increased in the development of methods to mitigate greenhouse gases emission (Lombardi, 2003).Much of the efforts to control such emission focus on advancing technologies so as to switch to lower carbon content fuels. Reducing use of fossil fuels would reduce the amount of CO_2 produced and other pollutants (Demirbas, 2006)

The fluctuation of fossil based fuel price and their greenhouse gases (GHG) emissions and environmental concern have got attention to explore cheaper and environmentally friendly bio-fuels as strategy for reducing global warming. Lingo cellulosic biomass (LB) has been considered as one of environmentally friendly renewable energy source. It can be obtained from non-food biomass, and avoids competition between food and fuel (Tan and Lee, 2004). Lignocellulosic biomass are mainly composed of cellulose, hemicelloses, lignin and other minor components (Agues *et al.*, 2012; Berber *et al.*, 2014)

The second generation of bio-ethanol which is obtained from Lignocellulose biomass plays a greater role in the production of biofuels, which is organic in concentration and has the same properties and energy content like petroleum based energy (Lee, 2012). Replacing petroleum with biofuels can reduce air pollution, improving rural economies by creating job opportunities and raising farm incomes, diversify energy portfolios, minimize dependence on foreign oil and improve trade balances in oil-importing nations to reduce the net concentration of GHG to the atmosphere.

Many agricultural raw materials rich in fermentable carbohydrates have been tested worldwide for bio conversion from sugar to ethanol, but the cost of carbohydrates raw materials has become a limiting factor for large scale production by the industries employing fermentation processes. Since the price of feed stock contributes more than 55% to the production cost, in expensive feed stocks such as lingo-cellulosic biomass, agro-food wastes such as sorghum straw are being considered to make bio ethanol competitive in the open market (Campo *et al.*, 2006).The production of ethanol from comparatively cheaper source of raw materials using efficient fermentative microorganisms is the only possible way to meet the great demand for ethanol in the present situation of energy crisis (Pramanik and Rao, 2005).

Biomass is a potential renewable energy source that could replace fossil energy for transportation (Paster, 2003). Biomass is solar produced organic matter such as wood, agricultural byproducts (Mohaghegi*et al*., 1992), energy crops such as fast-growing trees and grasses (Galbe and Zacchi 2002),municipal wastes, paper mill sludges(Lynd et al.,2001),and living cell materials(algae,sewages,*etc*). Biomass is naturally plentiful and renewable. In the United States, about 500 to 600 million tonnes of biomass resources can be produced annually (Paster, 2003). Sorghum straw is also among the potential biomass resources for ethanol production.

Utilization of different types lignocellulose substances is a potential resource available in many tropical regions of the world. Considerable amount of laboratory work is in progress for bioconversion of various Lignocellulose materials in to ethanol using sequential steps of hydrolysis, scarification and fermentation. However, there is very little reported work for bioconversion of sorghum straw in to fuel ethanol production in Ethiopia. Therefore, this research is initiated with the following general and specific objective

General objective of the study:

- To produce ethanol from agricultural waste material (sorghum straw) through batch fermentation by using *Saccharomyces cerevisiae*.

Specific objectives of the study:

- To identify the optimum substrate concentration for bioethanol production from sorghum straw.
- To determine the effect of inoculum (yeast) concentration on the rate of ethanol production from sorghum straw.
- To determine cell density, and reducing sugar concentration of different fermentation time.
- To assess the effect of pre-treatment on the rate of ethanol production from sorghum straws.

2. LITERATURE REVIEW

2.1. Ethanol

Ethanol is a clear liquid alcohol that is made by the fermentation of different biological materials. It is a liquid alcohol made of oxygen, hydrogen and carbon is obtained from the fermentation of sugar or converted starch contained in grains and other agricultural or agro-forest feed stocks(Prescott and Dunn,1959).Species of yeast (Saccharomyces cerevisiae) metabolite sugar in the absence of oxygen and produces ethanol and carbon dioxide. Ethanol is particularly useful in industrial applications because of its relatively high affinity for both water and organic compounds. The composition of other alcohols limits their flexibility as compared to ethanol and melts at -114.1^{o}c,boils at 78.5^{o}c and has a density of 0.789 g/ml at 20^{o}c (Kauri and Kocher, 2002).

Ethanol is a clean-burning, high-octane fuel that is produced from renewable sources.. Ethanol is beginning to be used all around the world as a transportation fuel and it has some distinct advantages, fuel that burns too quickly make the engine “knock”, a characteristics rattling sound. The higher the octane rating, the slower the fuel burns, and the less likely the engine will knock. When ethanol is blended with gasoline the octane rating of the petrol goes up by three full points, without using harmful additives. Similar to the case of biodiesel, adding ethanol to gasoline “oxygenates “the fuel. It adds oxygen to the fuel mixture so that it burns more completely and reduces polluting emissions such as carbon monoxide (carvalho *et al.,* 1993).

2.2. The History of Fuel Ethanol

The use of ethanol as an automobile fuel is not a new invention. Already in 1908, Ford’s mode1 T could be adjusted to run on either gasoline or alcohol (DiPardo, 2000). However, after World War II the interest in using ethanol as a fuel declined because cheap gasoline made from petroleum was available. Moreover, ethanol has become used as an additive gasoline. MTBE (methyl tertiary butyl ether) is used as a gasoline additive to increase the oxygen content and the octane number. During the last few years, the use of MTBE has been banned in several states in the USA due to the risk of contamination of water. Many companies have replaced MTBE with ethanol to give the gasoline similar clean burning and octane boosting properties as MTBE blended gasoline (F.O. Lech's World ethanol and biofuels report 2006; Sun and Cheng, 2002).. Brazil has for a long time been the leading ethanol producer of the world. However, during the last years USA has increased its production and today both countries have an annual production of about 16 000 000 m^3 (F.O. Licht's World ethanol and biofuels report, 2006).The Brazilian ethanol is mainly produced from sugarcane. Brazil is the world leader in the use of ethanol as an automobile fuel. In Brazil, the ordinary gasoline, which is used in about 7,000000 cars, contains about 24% ethanol. In addition, 4,000,000 automobiles drive on a blend of 95% ethanol and 5% water (BAFF, 2006).In the USA; ethanol is mainly produced from corn. In Sweden, about 55000m^3 of fuel ethanol is produce per year from wheat and about 18000$m^3$from spent sulfite liquor (Agro etalon AB, 2006; Jordruksverket, 2006). However, initiatives have been taken to increase the future national ethanol production. In 2004 an ethanol from lingo cellulose pilot plant was inaugurated in the city of ornskoldsuik, Agro-ethanol plants to expend it is production of ethanol from grain with 150,000m^3 in 2008.

2.3. Ethanol and Its Application as a Fuel

Ethanol, also known as ethyl alcohol with the chemical formula C_2H_5OH,is a flammable clear, colorless and slightly toxic chemical compound with acceptable odor, it can be produced either from petrochemical feed stocks by the acid-catalyzed hydration of ethane, or from biomass feed stocks through fermentation ,on a global scale, synthetic ethanol accounts for about 5-10% of total production while the rest is produced from fermentation of biomass mainly sugar crops ,e.g. cane beet and grains(mainly corn)(Licht,2006 ethanol as a neat fuel or even in the blended form with gasoline has a long history as automotive fuel. In 1860, German inventor Nicholas Otto used ethanol as a fuel in an early prototype of an internal combust ion engine because it was widely available throughout Europe for use in spirit lamps. Few years Later,Henry ford built his first automobile with an engine that could run on ethanol, in 1908, ford unveiled his mode 1 Engine equipped with carburetors that could be adjusted to use alcohol, gasoline or a mixture of both fuels(Solomon *et al*,2007).

Ethyl alcohol as “the fuel of the future” was presented by him for the first time. In 1925, he told the New York Times “the fuel of the future is going to come from fruits like that sumac out by the road, or from apples, weeds, sorghum straw, sawdust-almost anything. There is fuel in every bit of vegetables matter that can be fermented. There is enough alcohol in one year’s yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years. “However, fossil fuels were predominantly used for automobile transportation throughout the last century, obviously due to their lower production cost. As an automotive fuel, hydrous ethanol can be used as a substitute for gasoline in dedicated engines. Anhydrous ethanol, on the other hand, is effective octane booster when mixed in blends of 5% to 30% with no engine modification requirement (Licht, 2006).

2.4. Environmental Impact of Ethanol

Ethanol is harmless to the environment. The main environmental advantages of fuel ethanol are its sustainability in using a renewable resource as a feedstock, thus promoting independence of fossil fuel, and maintaining the level of greenhouse gas (co_2). Carbon dioxide in the atmosphere is assimilated through photosynthesis and metabolized to a building block of plants. The energy of sunlight is used to make carbohydrates stored in crops and in the whole plant body. While crops are useful as energy sources for human and animals, some crops like starch roil containing crops can be converted to fuels or chemicals. Combustion of these fuels produces co_2 gas which would be assimilated again by plants. In total, almost no net co_2 is produced by using biofuels generated from biomass. How effectively ethanol reduces greenhouse gas emission has been widely discussed. The issues are mainly related to the net energy content in ethanol, and depend on the assumption of ethanol production routes.a number of life-cycle assessments have been studied, and show that a change from fossil fuel to biofuels could reduce co2 emission by factor of 1/2 to 1/5, depending on how significant the use of renewable fuels is at all stages in the process (berenson, 2006; walter -2006blottnitz and curran, 2006; Kim and dale, 2005; Rosillo-calle et al, 2004).in ground water and soil mixtures, ethanol can be rapidly degraded both aerobically (100 ml/l in 7 days) and anaerobically (100 mg/l in3- 25 days) (Armstrong, 1999).

Bioethanol in surface water is also rapidly degraded and thus not harmful to the biotope as long as it is not present in concentrations directly toxic to microorganisms. The half-time of bioethanol in surface water is 6.5 to 26 hours. While bio-ethanol releases volatile organic compounds (VOC) due to its low vapor pressure, degradation of bioethanol in the atmosphere is also predicted to be rapid. Exposure of humans to bio-ethanol is harmless. The exposure may be carried out mostly by inhalation of bioethanol vapor as VOC, and by body contact or, rarely, ingestion from either blended fuel or denatured fuel. The occupation standard for bioethanol in the air is 1000 ppm (1900 mg/m3) on an eight hour basis. Above this standard concentration, ethanol vapor causes eye and upper respiratory tract irritation, fatigue, headache and sleepiness (Massed *et al.*, 1985)

2.5. World Ethanol Production

The main use of ethanol is a motor fuel and fuel additives. Efficient method conversion of biomass in to fuel is ethanol production because ethanol is an economical as well as environmentally friendly fuel. Ethanol has the advantages of being renewable, cleaner burning and produces no GHG (Atlanta's *et al.*, 2002). In 2005, the world ethanol production was approximately 46 billion liters per year, which is expected to reach 76 billion liters per years by 2010(Olfert and Weseen, 2007).

Table 1. World annual ethanol production by country (2005)

Country	Production(millions of liters)	Country	Production(millions of liters)
USA	16,139	South Africa	390
Brazil	15,999	Spain	352
China	3,800	United kingdom	348
France	908	Thailand	299
Russia	749	Ukraine	246
German	431	Canada	231
		Others	1,707

Source: The Saskatchewan institute of public policy, (2007) Global production of bio-ethanol increased substantially during 2005 to 2013 (Figure 1). In 2005 the total amount of bio-ethanol was estimated as 30 million m3 and increased to about 90 million m^3 in 2013. The leading producer of bio-ethanol is the United States followed by Brazil. Whereas corn is the major source of bio-ethanol in the U.S., sugarcane and sugarcane molasses are the major sources in Brazil (Balat and Balat, 2008). Most of the bio-ethanol in the EU is produced from sugar beet molasses. The bio-ethanol sector in EU member states has responded to policy initiatives and has started growing rapidly. The demand for bio-ethanol for transportation was 12.6 billion liters in 2010 (Crop Energies AG, 2014).Bioethanol is appropriates for the mixed fuel in the gasoline engine due to its high octane number and its low octane number and high heat of vaporization impede self-ignition in the diesel engine. So, ignition improver, glow-plug, surface ignition, and pilot injection are applied to promote self-ignition by using diesel-bioethanol blended fuel(Kim et al.,2005).In Brazil, bioethanol for fuel is derived from sugar cane and is used pure or blended with gasoline in a mixture called gasohol(24% bioethanol,76% gasoline)(Oliver *et al.*,2005).In several states of the united states, small amount of bioethanol (10% by volume) is added to gasoline, known as gasohol, blends having higher concentrations of bioethanol in a gasoline are also used,e.g. inflexible – fuel vehicles that can operate on blends of up to 85% bioethanol (Malacca and Freire,2006).

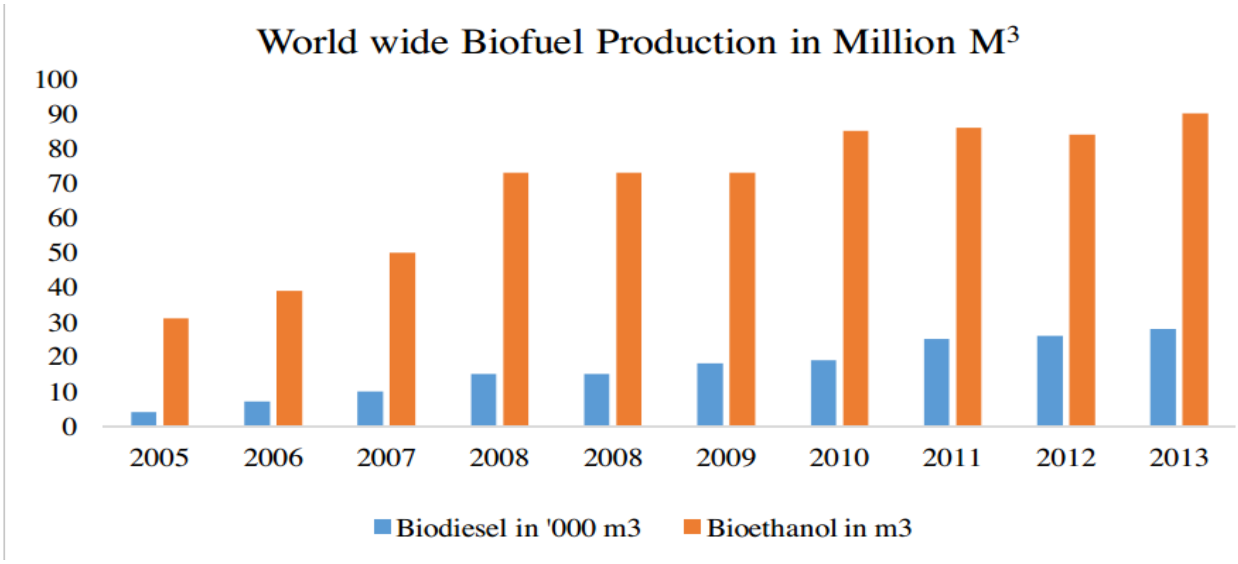

Figure 1 Worldwide liquid bio-fuel production 2005—2013

Source: Crop Energies AG, 2014

2.6. Ethanol Production in Ethiopia

The worldwide recent awareness for the use of ethanol to replace petroleum and generation of power along with sugar mill plants should have led to setting up of number of ethanol plants and co-generations. Ethiopia has several sugar real estate (Fincha, Methara and Wonji Shao) industries which are run and administered by Sugar Development Agency. Among molasses derived products ethanol takes the largest part, but its utilization must attract the attention of the government policy makers in order to utilize as a bioethanol. Bioethanol or biofuel is ethanol based products that can process into liquid fuels for transport purposes (ESDA, 2005)

Table 2.Projected ethanol production by year in million liters

Site of Industry	2009/10	2010/11	2011/12	2012/13	2013/14
Fincha	6.9	13.2	15.7	17.1	21.5
Wonji	-	-	10.6	10.6	10.6
Tendaho	-	15.9	30.1	43.6	55.4
Methara	8.9	12.8	17.5	21.3	21.3
Total	15.8	41.9	73.9	92.6	108.8

Source: Ethiopian sugar Agency, 2009

Table 3.Actual amount of Bio-ethanol produced at Finch and Methara used for blending

Year	Amount produced in (1000m$^{3)}$	Amount used for blending	Amount in millions of USD saved due to blending
2008	5.8	6.7	5.5
2009	7.1	3.4	1.6
2010	7.1	9.8	7
2011	13.8	10.6	9.2
2012	6.5	8.6	7.6
2013	9.8	7.5	7.6
Total	50.8	46.8	38.7

Source: Ethiopian Sugar Corporation, 2012

2.7. Lignocellulose as a Feed Stock for Ethanol Production.

There are three “generations” of bioethanol: first-generation or conventional bioethanol is bioethanol produced from sugar substances, such as sugar cane juice, sugar beets and molasses, as well as starch-based materials such as wheat, corn, sorghum, potatoes or cassava. Sucrose can be fermented directly to ethanol, but starch must be hydrolyzed to glucose before it can be fermented by yeast, generally by saccharomyces cerevisiae (Sameer *et al.,* 2011). Even though the production of ethanol from starch biomass materials represent the most convenient and technically advanced option for bioenergy, such practices would result in severe competition between energy and food supplies. The raw materials account for 40-70% of the total ethanol production costs based on current sugar-or-starch-containing feedstock. Reduction of the production cost would increase the competitiveness of ethanol with fossil fuels. Lignocellulose biomass is believed to be less expensive and more plentiful than either starch or sucrose-containing feed stocks (Hu *et al.,* 2008).

Second generation of bioethanol is bioethanol produced from sustainable feed stock that cannot be used directly for food production. Examples of wastes that could potentially be converted in to bioethanol include perennial grasses, agricultural wastes and forest residues. The conversion of these potential sources of feed stocks for bioethanol offers the double benefits of a reduction in global waste and the generation of valuable transportation fuels (Kayla *et al.,* 2000).

Third generation bioethanol is produced via, direct cellulose fermentation (cellulose production, substrate hydrolysis and fermentation are accomplished in a single process step by cellulolytic microorganisms.

Table 4. Chemical composition of some lignocellulose biomass

Source	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Sorghum straw	32-35	24-27	15-21
Hard wood	40-55	24-40	18-35
Soft wood	45-50	25-35	25-35
Corn Stover	38-40	28	7-21
Corn cobs	45	35	15
Bagasse	32-48	19-24	23-32
Wheat straw	33-38	26-24	23-32
Rice straw	28-36	23-28	12-14
Sorghum stalks	27	25	11
Barley straw	31-35	27-38	14-19
Leaves	15-20	80-85	0

Adapted from (Kumar *et al.*, 2009)

2.8. Pre-treatment of Lignocelluloses Biomass

One of the main problems in application of lingo-cellulosic materials is their resistance against enzymatic de polymerization. Therefore, biomass pretreatment is a crucial step as it breaks down the crystalline structure of cellulose and releases the fermentable sugars, so that the hydrolysis of carbohydrate can be achieved more rapidly and with greater yields (Mosier *et al* .,2005). The carbohydrate polymers in lingo-cellulose are tightly bound to lignin mainly by hydrogen bonds as well as by some covalent bonds which make it a recalcitrant substrate for hydrolysis and ethanol production. Thus, delignification is a crucial step prior to de polymerization and Fermentation steps which can highly increase the rate of subsequent hydrolysis reaction. Delignification liberates cellulose and hemi-cellulose from their complex with lignin (Lin, 2006).

2.8.1. Physical pretreatment

Physical pretreatment includes mechanical comminuting, steam explosion and microwave, Radiation. Mechanical comminuting can be a combination of chipping, milling and grinding. It aims to reduce the particle size of the biomass to attain a larger surface area for enzyme access. The desired final particle size determines the appropriate technique to apply. For example, chipping is used when 10-30mm particle size is required whilst milling and grinding are for more fine particles (0.2-2mm). The higher energy cost of mechanical comminuting especially for large-scale applications makes it an unattractive approach for pretreatment (Evander, 2005). It can result in significant changes in the physical characteristics of biomass, including smaller Size, as well as a lesser degree of both crystallinity and polymerization. It has been widely applied in the corn-to-ethanol process, making corn kernels more open to enzymatic hydrolysis. Lignocelluloses biomass, however, requires much more mechanical energy input than corns, thus making comminuting a less economically competitive approach for ethanol production. Although power in put for this process greatly depends on the final size requirement and the physical characteristic of the selected feedstock (Sun and Cheng, 2002).Steam explosion microwave provides swift thermal expansion which opens up the structure of the target biomass. It is recognized as a suitable pretreatment method for hardwoods and agricultural residues, but less effective for softwoods. This kind of pretreatment is typically initiated at a temperature of 160-260 °C and the residence time varies from seconds to a few minutes (Sun and Cheng, 2002). The main factors affecting the results of steam explosion are residence time, temperature, particle size as well as moisture content (Duff and Murray, 1996).

2.8.2. Acid pretreatment

The common pretreatment practice in the last two decades has been to incubate lignocellulose materials in the medium of dilute sulfuric acid (0.5 to 1.5% $H2SO4$) at 100 to 150°C (Wingren *et al*., 2003). In this process the hemicellulose is hydrolyzed into sugar monomers which are recovered in the liquid fraction after filtration. The residual solids will contain cellulose and lignin which will be further subjected to hydrolysis by cellulases (Taherzadeh and Karimi, 2008). This process has been shown to result in by products such as furfural (pentose sugars byproduct), and hydroxyl methyl furfural (HMF; hexose sugar byproduct), phenolic acid (lignin byproduct) and acetate (diacetylation product of hemicellulose). Each of these by products at concentration 5 mm or above have been shown to have significant inhibitory effect on the fermentation process (Martinez *et al.,* 2000; Palmqvist *et al.,* 2000b; Klinke *et al.,* 2004). Use of higher concentrations of acid and higher temperatures yield higher reducing sugar concentrations, however very under such conditions reducing sugars are degraded at a much higher level (Zhu *et al.,* 2009). Use of concentrated acid alone has been shown to (i) yield very high levels of sugar (90%), (ii) can handle diverse feedstock, (iii) is relatively rapid (10 to 12 hours), and (iv) causes less degradation. The latter requires more expensive corrosion resistant equipment (Hamelinck *et al.,* 2005) and the hydro lysate have to be neutralized by the addition of lime and contaminants removed by treatment with activated charcoal (Gupta *et al.,* 2009; Shleser, 1994). In the later process, over-liming has been shown to catalyze condensation of lignin-derived compounds. The advantage of these reactions is that they detoxify the hydro lysate and these reactions occur at much higher rate at higher PH. On the other hand, at pH 11 as much as 30% of glucose is converted to HMF. Additionally, over-liming the hydrolyses at an industrial scale and accurately controlling pH in fermenters pose a major challenge. Steam treatment of H_2SO_4 impregnated lignocellulose is scarified at a much higher rate. The most favorable conditions for this process require that the samples be treated with 0.5%$H_2SO_4$and hold the sample at 200°C for 4 to 8 min (Sassner*et al.,* 2008). Yet another pretreatment method used a mixture of H2SO4 and acetic acid with an efficiency of 90% scarification (Rach et al., 2010).

2.8.4. Acid hydrolysis

There are two types of acid hydrolysis; dilute and concentrated acid hydrolysis. Dilute acid is conducted at high temperatures and pressure and has short reaction time. The hemicellulose component is depolymerized at lower temperatures than the cellulosic fraction (Chandel *et al.,* 2007). The biomass is mixed with dilute sulphric acid and held at temperatures ranging from 120-220**°c** for short period of time to hydrolyses the hemicellulose to xylose and other .DE polymerization of the hemicellulose fraction of the cell wall enhances Cellulose digestibility in the residual solids (Dien *et al.,* 2006; Saha *et* *al.,* 2005).The disadvantage with dilute acid hydrolysis is that at high temperature or long residence time the monosaccharaides produced degrades and gives rise to fermentation inhibitors like furan compounds, weak carboxylic acids and phenolic compounds (Klinke *et al.,* 2004; Larsson *et al.,* 1999). These inhibitors affect the performance of the ethanol producing microorganism (Chandel *et al.,* 2007). To remove these inhibitors and increase the hydro lysate ferment ability, several chemicals and biological methods have been used. These methods include over liming (Martinez *et al.,* 2000), charcoal adsorption (Chandel *et al.,* 2007), ion exchange (Nilvebrant *et al.,* 2001), detoxification with lactase (Martin *et al.,* 2002; Chandel *et al.,* 2007), and biological detoxification (Lopez *et al.,* 2004). The cost is however higher than the benefits achieved (Palmqvist and Hahn Hagerdal, 2000).

Concentrated acid hydrolysis uses concentrated acid followed by dilution with water to dissolve the hydrolyzed sugar. The process allows for complete and rapid conversion of cellulose to glucose and hemicelluloses with a little degradation (Chandel *et al.,* 2007). The concentrated acid process uses 70% sulphuric acid at between 40 –50^{o}C for 2 to 4hrs. The low temperature and pressure leads to minimize the sugar degradation.

2.8.5. Enzymatic hydrolysis

Lignocellulose materials can be scarified enzymatically to get fermentable sugars (Itoh *et al.,* 2003; Tucker *et al.,* 2003). Bacteria and fungi are a good source of cellulases and hemicellulase that could be used for the hydrolysis of pretreated lignocelluloses. This usually is enzymatic cocktail consisting of different hydrolytic enzymes such as cellulases, xylanases and mannanases (Chandel *et al.,* 2007). Large numbers of microorganisms are capable of degrading cellulose (Miyamoto, 1997). For fungi, members of the genera that have received considerable attention under aerobic conditions are *Chaetomium,* and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum and Serpula* (Basidiomycetes); *Aspergillums, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium, and Trichoderma* (Deuteromycetes) and *Mucor* (Zygomycetes). The genera with prominent cellulolytic activity within anaerobic fungal division Chytridio mycetes are Neocallimastix, Piromyces, Caecomyces, Orpimomyces and Anaeromyces (Lynd *et al.,* 2002). However, only a few of these microorganisms are known to produce significant quantities of cell-free enzymes capable of completely hydrolyzing crystalline cellulose in vitro.Fungi of the genera *Trichoderma* and *Aspergillus* are thought to be prominent cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use (Rajesh *et al.*, 2008).

2.9. Fermentation

Microorganisms play a significant role in production of ethanol from renewable resources and thus, selection of suitable strain is essential for the industrial process. One of the most successful microorganisms for bio-ethanol production is Saccharomyces cerevisiae. Although the wild-type strain has high bio-ethanol productivity and very tolerant to high ethanol concentrations and inhibitory compounds, it is unable to ferment pentose (hemicelluloses) (Hahn-Hagerdal *et al.,* 2007). Pichiastipitis, Candida shehatae and Pachysolantannophilusare promising microbes that are capable of fermenting both hexoses and pentose's (Lin and Tanaka, 2006). However, S. cerevisiae is still the most commercialized and dominated strains for bio-ethanol production (Lin and Tanaka, 2006).The disadvantage of *S. cerevisiae* can be overcome by introducing geneticinformation of Xylose reductase and xylitol dehydrogenase. Although the fermentation can be performed as a batch, fed batch or continuous process, most ethanol production industries use the batch mode (Tomas *et al.,* 2008)

Ethanol production is much more challenging and difficult when lingo-cellulosic and/or cellulosic materials are to be used as raw materials. Unlike the starch-based materials, pretreatment and hydrolysis of lingo-cellulosic materials produce a mixture of pentose and hexoses along with other inhibiting compounds, causing many problems in the fermentation step. Therefore, capability of consuming both pentose and hexose sugars, high tolerance against substrate, ethanol as well as inhibiting compounds, high ethanol yield and minimum nutrient requirements are the essential features of an ideal microorganism. Although no microorganism has been found yet to meet all these requirements, development of a desirable strain is focus of many studies. Thus far, wide varieties of microorganisms including yeasts, bacteria and fungi have been exploited offering different advantages and disadvantages (Olsson and Hahn-Hagerdal, 1993).

Some species of bacteria such as Zymomonas mobilis and the genetically engineered Escherichia colican produce ethanol at higher yields, but they are less resistant to the end product (ethanol) and other compounds present in the hydrolyzates when compared to the yeast. During the evolution of yeast S. cerevisiae, the ability to override the glucose repression circuit that suppresses respiration of glucose and other hexose sugars above 20-40Mm threshold concentration in the presence of oxygen was developed. This phenomenon, known as the „Crabtree effect‟, provided the ancestor of S. cerevisiae with an advantage over its competitors because high ethanol levels (>4% v/v) are toxic to most other microorganisms. In contrast with many advantages offered by using yeast in ethanol production, it lacks the mechanism to take up pentose sugars as substrate. Attempts to add this ability by genetic manipulation are still at the laboratory stage (Jeffries, 2006).

2.10. Factors Affecting Fermentation

Microorganisms for 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, pH, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Demirbas, 2005).

2.10.1. Effect of sugar concentration

The concentration of sugar can affect the microbial ethanol fermentation in various ways. Use of concentrated sugar substrate is one of the ways to obtain high ethanol yield during fermentation. The amount of ethanol produced is proportional to the amount of sugar added; thus, high sugar concentrations are desired. However, too high sugar concentrations can inhibit metabolism due to increased osmotic pressure. Very low levels of sugar may limit the rate of ethanol production (Jones *et al.*, 1981).Hence; each fermentation process will have an optimal glucose or equivalent sugar concentration (Sofer and Zaborsky, 1981).

Sugar concentration of 10-18% is usually satisfactory, although other concentrations are used (Dunn, 1959). Borzani *et al.* (1993) studied fermentation with various initial concentrations of sugar. They also demonstrated the logarithmic relationship between time of fermentation and initial concentrations of sugar. Bertolini *et al.* (1991) isolated yeast strains from sample collected from Brazilian alcohol factories. These strains were capable of fermenting up to 30% of sucrose efficiently. The efficiency of selected strains varied from 89% to 92% depending upon the utilization of total sugar available in the medium. A maximum amount of 19.7% (v/v) ethanol accumulated from fermentation of 30% sugar as compared to two reference strains, which produced 18.0 (v/v) and 15.6 (v/v). A repeated batch fermentation system was used to produce ethanol using an osmotolerant S. cerevisiae immobilized on calcium alginate (Sree *et al.,* 2000). Fermentation was carried out with initial concentration of 150, 200, 250 g glucose per liter at 30 ºC. The maximum amount of ethanol produced by immobilization osmotolerant S. cerevisiae cells using 150, 200 and 250 g/L glucose was 72.5, 93 and 83 g ethanol per liter at 30 ºC after 48 hours. Maximum yield was obtained at initial sugar of 20% with fermentation efficiency of 90%.

2.10.2. Effect of temperature

Temperature plays a major role in the production of ethanol, since the rate of alcoholic fermentation increases with the increase in temperature. The optimum temperature of ethanol ranges between 25°C to 40°C which depends on room temperature. When temperature goes below the optimal range, their ability to catalyze the intended reaction slows down. On the other hand, when the temperature increases, enzymes begin to denature or unfold and thus become inactive. Each enzyme will have a different temperature range where it becomes inactive. Even if one essential enzyme stops working, the organism fails to grow. Hence, the first essential enzyme that gets deactivated defines the maximal temperature at which that organism can grow. At the lower end, it gets more complicated. Usually, the enzymes are not inactivated but rather just slow down (Sanchez, 2007).

2.10.3. Effect of pH

PH value has significant influence on Bio-ethanol fermentation. PH of bio-ethanol produced from different fruit wastes was determined. The pH values of ethanol produced by the process of fermentation range from 4 to 6. Yeast survives in a slightly acidic environment that is with pH of between 4 and 6. Ethanol with high content of alcohol produced from different agro waste materials exhibited different pH value. (Asli, 2010).

2.10.4. Effect of specific gravity

Specific gravity is used to measure the sugar content. As the fermentation progressed, the specific gravity considerably decreased. The decrease in specific gravity is clear indication of yeast fermenting the sugar resulting in ethanol production. The specific gravity reaching a constant value after incubation period is the indication of end of fermentation (Asli, 2010).

2.10.5. Effect of Ethanol concentration

The concentration of ethanol in the fermentation broth can directly affect the growth rate of the culture and its ability to convert sugar to ethanol. Inhibitory and toxicity level of ethanol vary from culture to culture. Higher temperature lowers the tolerance of the organism. At temperatures above 35 °C, strains lose viability at ethanol concentrations of 10 % (w/v) (Hettenhaus, 1998). With the increase in initial sugar concentration, the ethanol production increased significantly. Concentration is the measure of ethanol content present in the distillate. Concentration of ethanol was expressed in terms of percentage (%) (Sanchez, 2007).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The experiment was conducted in Botanical Science laboratory, School of Biological Sciences and Biotechnology 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).

3.2. Substrate Preparation.

Agricultural crops residues such as sorghum straws were collected from Haramaya University, Eastern Ethiopia and it was cleaned with water vigorously prior to bring to the laboratory. The collected crop residues were chopped, washed with distilled water and dried at 60 ± $3c^0$ for 24 h prior to pretreatment. Sorghum straws were subjected to acid pretreatment with 10 mL of 0.5 % (v/v) H_2SO_4, at 11-12 bars for approximately 45 minutes, 180^ocautoclaving (Karimi*et al*., 2006) and the solid residue will be sieved and stored in polypropylene for subsequent use (Singh *et al*., 2014). The low acid consumption is a major advantage in terms of cost and process severity (Galbe, and Zacchi, 2002). Moreover, low acid concentrations (<1% w/v sulphuric) release essential nutrients (S and P) that enhance downstream fermentation (Zhang and Wu, 2012).

3.3. Inoculums Preparation

Inoculums were prepared from dried baker's yeast, *Saccharomyces cerevisiae*. For preparing 0.5 and 1% inoculums concentration, 0.5 and 1 g of yeast were dissolved in 99.5 and 99ml of distilled water, respectively. For fermentation of each substrate, 10ml of the solutions was taken and used as inoculums for batch fermentation process (Dhopeshwarker *et al.*, 2001).

3.4. Preparation of Nutrient Solution

Nutrient supplements were prepared by dissolving 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 of $(NH_4)_2SO_4$ in 1000ml of distilled water (Abouzeid and Reddy, 1986).

3.5. Experimental Design and fermentation

The experimental design was based on CRD factorial and eight treatment combination with three replications for each treatment and control. Batch fermentation was carried out by using sorghum straw (substarte) by using baker's yeasts. The different proportion of substrates (15g, 30g, 45g and 60g) and inoculums (0.5% and 1%) were prescribed in table-5. 500 ml Erlenmeyer flask were used for batch fermentation. The fermentation medium contain the substrates, inoculums (0.5% and 1%), 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 $(NH_4)_2SO_4$. The pH was adjusted to 4.5 with buffer solution. The fermentation process was allowed for 16 days at 30°C. The production of bio-ethanol and other parameters were estimated at the interval of 4th, 8th, 12th, and 16th days starting from the beginning of fermentation (Akin *et al.*, 2005).

Table5.Batch fermentation of Sorghum straw with different combination of yeast inoculums (All treatments with three replicates).

Samples	**Sorghum straw (gm)**	**Volume of Nutrient Solution(ml)**	**Inoculums Concentration**
A	15	100	0.5%
B	15	100	1%
C	30	100	0.5%
D	30	100	1%
E	45	100	0.5%
F	45	100	1%
G	60	100	0.5%
H	60	100	1%

A=15g +0.5% yeast, B=15g +1% yeas, C=30g+0.5% yeast, D=30g+1% yeast, E=45g +0.5% yeast, F=45g +1% yeast, G=60g +0.5% yeast, H=60g +1% yeast

3.5.1. Determination of cell density

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

3.5.2. Estimation of total reducing sugar

The reducing sugar content of the samples of fermentation were estimated by the method of Nelson (1944) and Somogyi (1945); Caputi *et al.* (1968), using D-glucose as standard. Analysis of reducing sugar in untreated and pretreated samples were determined by using 3, 5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). A sample (0.05ml) was taken from filtrate, (0.35ml) citrate buffer (pH=6.5) and (0.6ml) of Dinitro salicylic acid (DNS) was added and then the sample was boiled for 5 minutes immediately to stop the reaction. The absorbance was measured for reducing sugar at 540nm using spectrophotometer (Bennet, 1971). The amount of reducing sugar in the sample was calculated using standard graph prepared from working standard Glucose (Caputi *et al.*, 1968).

3.5. 3. 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 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 was reduced the orange-red chromic acid/sulfuric acid reagent to an opaque green or blue suspension of Cr (III) salts.

3.5.4. Quantitative Estimation of Bio-ethanol

Two ml. of the fermented sample was taken into 500 ml Pyrex distillation flask containing 30 ml of distilled water and was 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 sulfuric acid and volume raised to 1 liter). About 20 ml of distillate was then collected in each sample and the flasks were kept in a water bath maintained at 60°C for 20 minute. The flasks were cooled to room temperature and the volume were raised to 50 ml. Five ml. of this was diluted with 5 ml of distilled water for measuring the OD 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 summarized into tables and graphs by using Microsoft office excel spreadsheet and analyzed using Statistical Package for Social Studies (SPSS version 20). Least significant difference (LSD) test was used to identify significant differences among treatment means. P values ≤ 0.05 was considered as statistical significance at 95% of confidence interval.

4. RESULTS AND DISCUSSION

4.1. Effect of Pretreatment on Ethanol Production

In both acid treated and untreated substrates (Table-6), the amount of bio-ethanol was found to decrease subsequently with increasing days of fermentation. This may be due to the fewer amounts of cell biomass and total reducing sugars observed with increasing time of fermentation. Compared to untreated substrate, acid treated substrates showed higher amount of bio-ethanol. This shows that acid treatment of substrates facilitates the breakdown of the complex lignocellulosic materials into sugars that yeasts can consume and convert them into bio-ethanol. The result showed that acid pretreatment significantly increased bio-ethanol production through fermentation period, this may due to the fact that acid pretreatment facilitates degradation of cellulosic material into simple sugar then converted into bio-ethanol (Hendricks and Zeeman, 2009).

Table 6. Ethanol production from Sorghum straw using *S. cerevisiae* at different fermentation period.

Substrate	treatment	Ethanol concentration in (%) produced at different fermentation period			
		4th day	8th day	12th day	16th day
A	Treated	0.03±0.004Aa	0.03±0.002Bb	0.04 ± 0.008Cc	0.02±0.003Dd
	Untreated	0.03±0.012Ea	0.04±0.004Fb	0.03 ± 0.005Gc	0.03±0.004Hd
B	Treated	0.15±0.19Aa	0.03±0.002Bb	0.03 ± 0.006Cc	0.03±0.002Dd
	Untreated	0.04±0.009Ea	0.03±0.001Fb	0.03 ± 0.006Gc	0.04±0.002Hd
C	Treated	0.03±0.011Aa	0.02±0.004Bb	0.04 ± 0.006Cc	0.03±0.004Dd
	Untreated	0.04±0.002Ea	0.03±0.005Fb	0.04 ± 0.005Gc	0.03 ±0.002Hd
D	Treated	0.03±0.004Aa	0.03±0.005Bb	0.04 ± 0.003Cc	0.03±0.003Dd
	Untreated	0.04±0.005Ea	0.03±0.005Fb	0.03 ± 0.006Gc	0.04±0.001Hd
E	Treated	0.04±0.002Aa	0.04±0.004Bb	0.03± 0.003Cc	0.04±0.002Dd
	Untreated	0.04±0.003Ea	0.04±0.001Fb	0.03± 0.002Gc	0.03±0.001Hd
F	Treated	0.04±0.004Aa	0.03±0.004Bb	0.04 ± 0.001Cc	0.03±0.003Dd
	Untreated	0.04±0.001Ea	0.03±0.004Fb	0.04 ± 0.004Gc	0.04±0.002Hd
G	Treated	0.02±0.003Aa	0.03±0.006Bb	0.04 ± 0.003Cc	0.03±0.002Dd
	Untreated	0.03±0.004Ea	0.02±0.005Fb	0.04 ± 0.007Gc	0.02±0.002Hd
H	Treated	0.02±0.002Aa	0.04±0.003Bb	0.04 ± 0.008Cc	0.03±0.003Dd
	Untreated	0.03±0.003Ea	0.03±0.004Fb	0.04 ± 0.003Gc	0.03±0.004Hd

A=15g +0.5% yeast , B=15g +1% yeast , C=30g+0.5% yeast , D=30g+1% yeast ,E=45g +0.5% yeast, F=45g +1% yeast , G=60g +0.5% yeast , H=60g +1% yeast.

Means with the same letter (lower case) in the same row are not significantly different at $p<0.05$; means with the same letter (upper case) in the same column are not significantly different at $p<0.05$.Esteghlalian *et al.* (1997) also reported that dilute sulfuric acid pretreatment can result in high reaction rates and significantly improve cellulose hydrolysis. Moreover, highest ethanol productions (0.15±0.19) were observed at 4th day of fermentation with 15 gram substrate in combination with 1% yeast inoculums but found to be decline thereafter (Table 6). This could be due to depletion of substrates by yeast to be converted to ethanol. Moreover, this mix ration is found to be optimum compared to the rest of substrate inoculums concentrations used in this experiment.

The decrease in ethanol production at high substrate concentration beyond the optimum might be attributed to various factors including increments of viscosity in fermentation medium, osmotic pressure, and production of other compounds such as glycerol or acetic acid that have inhibitory effect on yeast growth.. In line with this Reddy (2006) reported that ethanol production increases with substrate concentration up to certain level and decreases thereafter. This finding is in agreement with those of Hoyer *et al*. (2009) and Grubb and Mawson (1993).

4.2. Qualitative Determination of Bioethanol

The presence of alcohol in the distillate was checked by functional group classification test by using chromic acid in the presence of sulfuric acid. Upon treatment with chromic acid, alcohol functional group is expected to be oxidized while Cr (VI) is being reduced to the blue-green Cr(III) and change its color into blue-green color. In this experiment the same color change occurred, suggesting that ethanol was really produced in the course of incubation (Caputi *et al*., 1968).

4.3. Effect of Pre-treatments on reducing sugar yield

Concentration of reducing sugar measured on the 12th 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 7). Moreover, highest reducing sugar concentration (1.36mg/ml) was observed in 30g Sorghum straw mixed with 1% yeast. The reduction in sugar concentration indicated that some sugar contained in Sorghum straw has been consumed by yeast as a substrate to grow and converted into ethanol. Comparison of reducing sugar concentration between acid pretreated and untreated substrates showed that the amount of reducing sugar was significantly higher when substrates were pretreated with acid, suggesting the facilitation of hydrolysis of lignocellulosic material by the acid for conversion into sugar. In line with this, Hendricks 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. 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 7. Reducing sugar concentration (mg/ml) measured at 540nm from Sorghum straw at different fermentation period (values are Mean ±SD, n=3).

Substrate	Treatment	Reducing sugar concentration(gm/ml) at different fermentation period			
		4th day	8th day	12th day	16th day
A	Treated	0.73±0.049Aa	0.70±0.112Bb	1.23±0.030Cc	0.88±0.030Dd
	untreated	0.88±0.071Ea	1.21±0.239Fb	1.14±0.112Gc	1.08±0.142Hd
B	Treated	0.73±0.035Aa	0.93±0.037Bb	1.05±0.120Cc	0.90±0.033Dd
	Untreated	0.84±0.008Ea	1.05±0.119Fb	0.88±0.161Gc	0.92±0.012Hd
C	Treated	0.90±0.082Aa	1.21±0.269Bb	1.36±0.064Cc	1.16±0.041Dd
	Untreated	0.64±0.021Ea	0.99±0.161Fb	1.33±0.153Gc	0.91±0.097Hd
D	Treated	0.84±0.083Aa	0.73±0.037Bb	1.14±0.090Cc	0.90±0.011Dd
	Untreated	0.78±0.172Ea	0.85±0.038Fb	0.90±0.105Gc	0.84±0.011Hd
E	Treated	0.84±0.093Aa	0.75±0.355Bb	0.79±0.108Cc	0.79±0.052Dd
	Untreated	0.78±0.149Ea	0.63±0.094Fb	0.72±0.038Gc	0.71±0.031Hd
F	Treated	0.73±0.089Aa	0.45±0.034Bb	0.99±0.023Cc	0.71±0.015Dd
	Untreated	0.74±0.116Ea	0.50±0.067Fb	0.68±0.146Gc	0.64±0.030Hd
G	Treated	1.03±0.376Aa	0.46±0.015Bb	0.58±0.204Cc	0.61±0.078Dd
	Untreated	0.95±0.296Ea	0.36±0.034Fb	0.87±0.165Gc	0.64±0.011Hd
H	Treated	1.16±0.343Aa	0.64±0.215Bb	0.83±0.056Cc	0.71±0.127Dd
	Untreated	1.10±0.079Ea	1.30±0.164Fb	0.99±0.399Gc	0.88±0.413Hd

A=15g +0.5% yeast , B=15g +1% yeast , C=30g+0.5% yeast , D=30g+1% yeast ,E=45g +0.5% yeast, F=45g +1% yeast , G=60g +0.5% yeast , H=60g +1% yeast.

Means with the same letter (lower case) in the same row are not significantly different at $p<0.05$; means with the same letter (upper case) in the same column are not significantly different at $p<0.05$.

In line with this (Aden *et al.*,2002) pointed out that the main advantage of dilute acid pretreatment related to other pretreatment methods is the higher recovery of sugars derived from hemi-cellulose. The dilute acid pretreatment has the advantage of not only solubilizing hemicelluloses but also converting solubilized hemicelluloses to fermentable

sugars (Saha *et al.*, 2005). In addition to this Sirkar *et al.* (2008) reported that acid pretreatment method was found to be optimal for better yield of fermentable sugars from lignocellulosic material.

4.4. Effects of acid pretreatment and inoculums concentration on cell density

The cell densities were observed and indicated in the table 8, the cell density were high in the 4th day of fermentation. However, after 4th day of fermentation the cell biomass concentration found to be decline. This may be due to cyto-toxicity of ethanol and limitation of available nutrient resources. Moreover, 4th day showed highest percentage of ethanol production with high concentration of cell density. The increasing ethanol production with increasing cell biomass indicated that the amount of yeast influenced ethanol production (Akin-Osanaiye *et al.*, 2005). The substrate concentrations increase with increase the cell density up to 30gram substrate after that it decline. This may be due to high alcohol content and decrement in fermentable sugar. In both acids pretreated and untreated substrates. The highest cell biomass (2.46) was observed with 30gram Sorghum straw substrate with 1% yeast.

Table 8.Cell density (mg/ml) measured at 540nm from Sorghum straw at different fermentation period (Mean ±SD, n=3)

Substrate	**Treatment**	**Treatment Cell density (mg/ml) at different fermentation period**			
		4th day	**8th day**	**12th day**	**16th day**
A	Treated	0.42±0.099Aa	0.83±0.299Bb	1.90±0.565Cc	0.32±0.165Dd
	Untreated	1.46±0.386Ea	2.02±1.521Fb	1.72±0.638Gc	1.32±0.606Hd
B	Treated	0.56±0.076Aa	1.22±0.114Bb	1.31±0.450Cc	0.99±0.809Dd
	Untreated	1.17±0.529Ea	1.56±0.378Fb	1.67±0.349Gc	1.44±0.273Hd
C	Treated	1.27±0.348Aa	2.04±0.817Bb	1.41±0.469Cc	1.06±0.686Dd
	Untreated	1.16±0.584Ea	1.14±0.453Fb	1.74±0.840Gc	1.26±0.650Hd
D	Treated	2.46±0.622Aa	1.41±0.593Bb	1.57±0.692Cc	1.65±0.243Dd
	Untreated	2.30±0.333Ea	1.08±0.443Fb	1.14±0.613Gc	1.51± 0.099Hd
E	Treated	1.29±0.280Aa	1.09±0.734Bb	1.18±0.632Cc	1.69±0.038Dd
	Untreated	1.40±0.428Ea	1.39±0.663Fb	1.01±0.522Gc	1.29±0.689Hd
F	Treated	1.18±0.373Aa	0.83±0.242Bb	1.07±0.338Cc	0.68±0.076Dd
	Untreated	0.90±0.401Ea	1.36±0.417Fb	1.04±0.611Gc	1.44±0.776Hd
G	Treated	0.68±0.136Aa	0.79±0.288Bb	0.39±0.462Cc	0.98±0.818Dd
	Untreated	1.43±0.831Ea	0.96±0.550Fb	0.85±0.305Gc	1.42±0.824Hd
H	Treated	1.15±0.689Aa	1.03±0.643Bb	1.41±0.523Cc	0.51±0.015Dd
	Untreated	1.46±0.447Ea	1.13±0.401Fb	1.14±0.483Gc	1.58±0.147Hd

A=15g +0.5% yeast , B=15g +1% yeast , C=30g+0.5% yeast , D=30g+1% yeast ,E=45g +0.5% yeast, F=45g +1% yeast , G=60g +0.5% yeast , H=60g +1% yeast.

Means with the same letter (lower case) in the same row are not significantly different at $p<0.05$; means with the same letter (upper case) in the same column are not significantly different at $p<0.05$.

5. SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary

With the aim of ethanol production from Sorghum straw, a series of experiments were carried out with different substrate concentration (15gm, 30gm, 45g and 60gm) using batch digester up to 16th day. The fermentation was carried out to produce ethanol, to determine optimum substrate concentration for maximum ethanol production, to determine reducing sugar and cell density. Though all substrate concentrations resulted in ethanol production starting from the fourth day of fermentation, 15g substrate was found to yield high amount of ethanol throughout the incubation period. Therefore it can be considered as the optimum concentration. Moreover, it was observed that after 4th day of fermentation ethanol production decreased as time for fermentation increased .The reducing sugar concentrations were analyzed for acid pretreated and untreated substrate. The reducing sugars decreased gradually as the fermentation period increased. Moreover, on 12th day of fermentation period, the highest reducing sugar concentration (1.36mg/ml) was obtained from acid pretreated 30gram substrate mixed with 1% of yeast. The cell density was also observed and it was indicated that yeast biomass was higher at 4th day but go down there after.

5.2. Conclusion

As proved from the experiment, that the bio-ethanol could be produced from Sorghum straw. Bio-ethanol production from treated and untreated substrates was statistically significant at $p<0.05$. Among the different substrate concentration 15 gram substrate combination with high yeast concentration (1%) showed the highest percentage of ethanol production.

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 4th day of fermentation. This may be due to substrate limitation and decrease in cell biomass (yeast).

5.3. Recommendations

- ✓ The study revealed that it is possible to produce bio-ethanol from Sorghum straw. Therefore, it is recommended to conduct experimental studies to increase ethanol production from lignocellulosic materials
- ✓ More work should be undertaken to optimize the production of bio-ethanol using Sorghum straw at appropriate condition.
- ✓ Further investigation should be done to analyze the potential of bio-ethanol production from Sorghum straw using combination of cellulosic enzyme.
- ✓ Further investigation could done on economic feasibility analysis of the overall conversion process from Sorghum straw to ethanol is necessary for the purpose of commercialization.
- ✓ Additional work is encouraged to see the effect of inhibitor on bio-ethanol production as a result of pretreatment.
- ✓ It is also recommended to check the bio-ethanol quality of Sorghum straw by Gas chromatography too.
- ✓ Further study is very important to describe how absolute bio-ethanol can be produced from Sorghum straw 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 (78^0c) and distillation by rotary evaporator produced only 95% pure alcohol.

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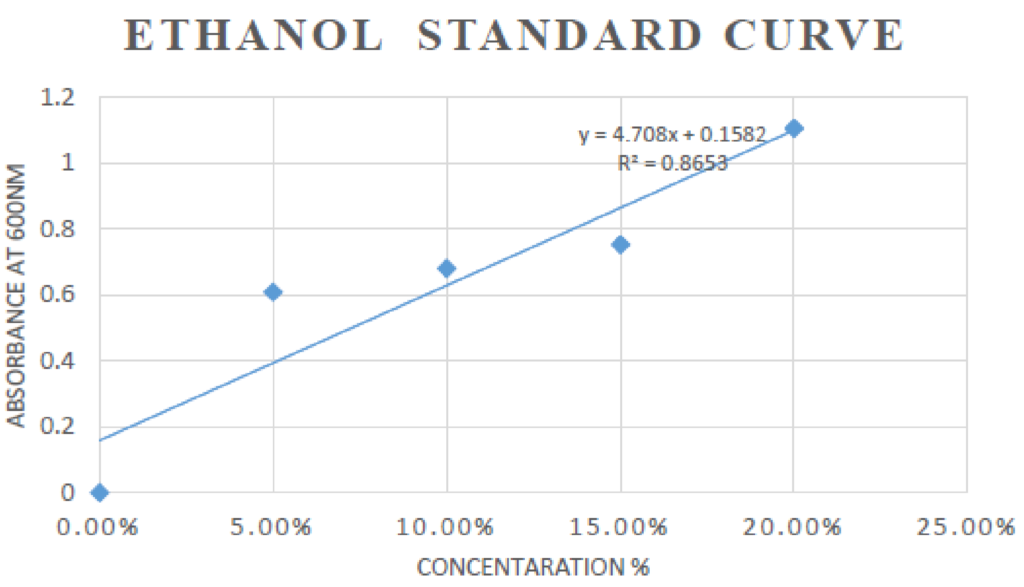
7. APPENDICES

7.1 Appendix Tables

Appendix table 1. Standard curve for the determination of Ethanol production was used ethanol water solution by range of 0-20% (v/v)

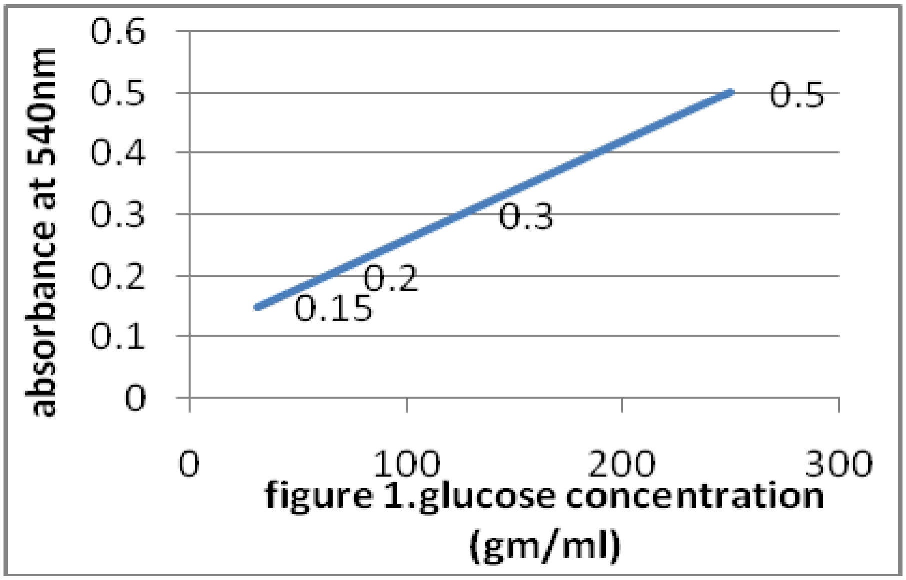
Test tube	cell density	absorbance.
1	0	0
2	5%	0.608
3	10%	0.68
4	15%	0.752
5	20%	1.105



Appendix 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

Appendix table 2. Standard curve for determination of glucose concentration prepared by taking 500 mg/ml already prepared standard D- glucose

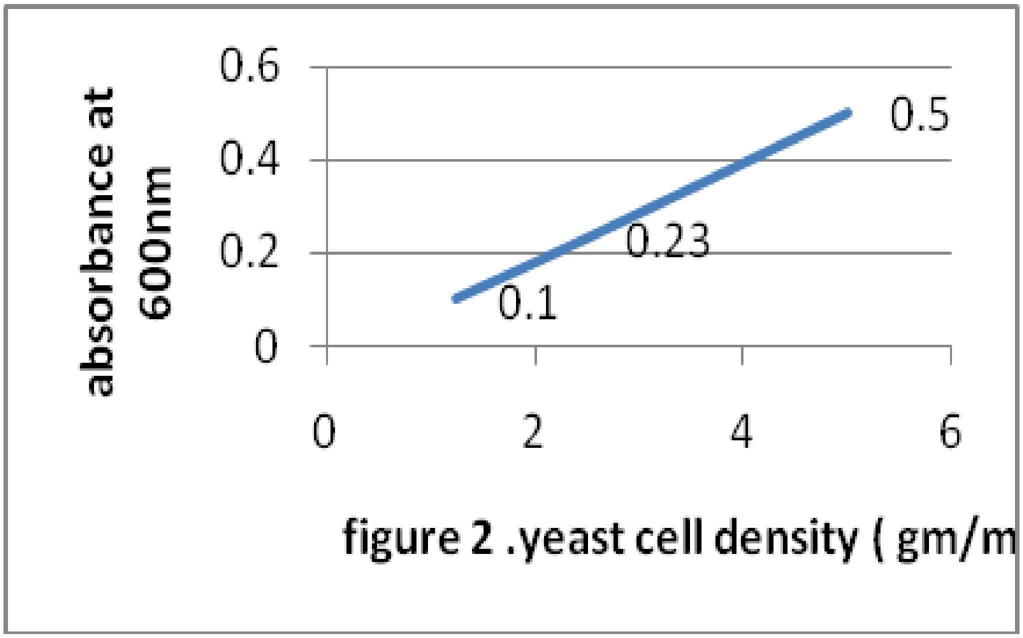
Test tube	Concentration	Absorbance
1	250	0.5
2	125	0.3
3	62.5	0.2
4.	31.25	0.15



Appendix figure 2. Standard curve for determination of glucose concentration prepared by taking 500 mg/ml already prepared standard D- glucose

Appendix 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



Appendix 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

7.2. Appendix figures.

Figures 4.samples ready for fermentation (A.cutted sorghum straw B.chopped sorghum straw C. powdered sorghum straw).

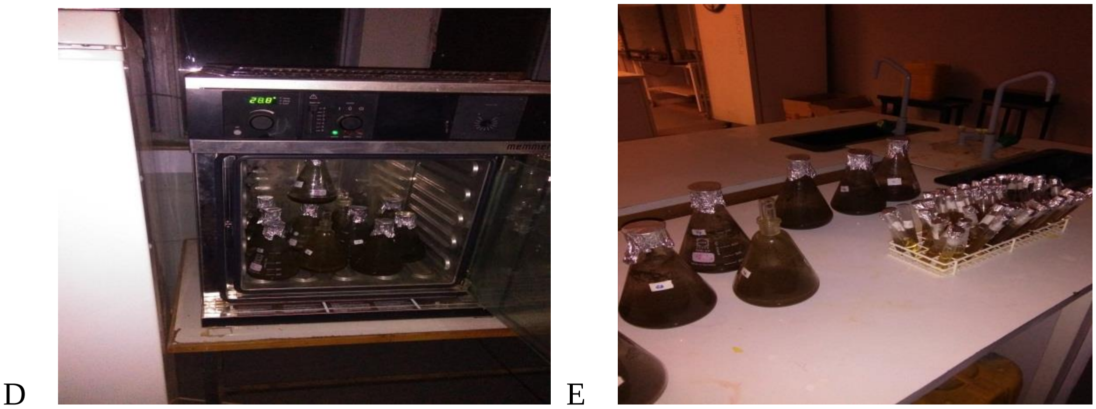


Figure 5.Fermenting process in the incubator and sample taken to measure reducing sugar ,cell density .

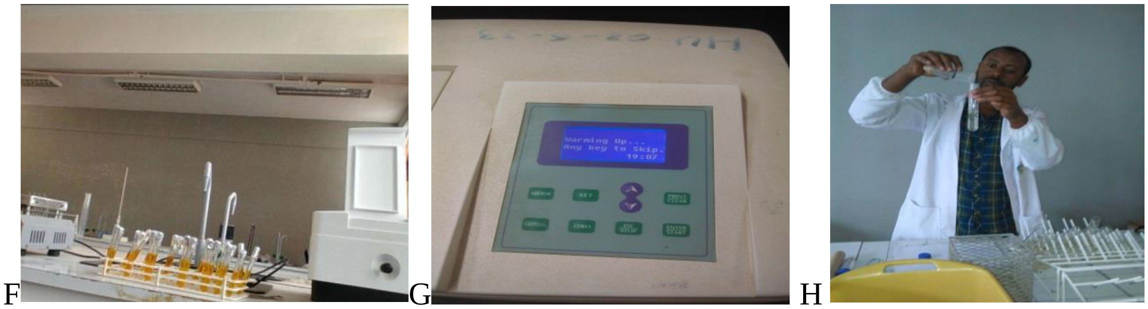


Figure 6.sample ready to read reducing sugar at 540nm and cell density at 540nm by spectrophometre.

Figure 7.distillation process by rotary evaporator and measuring and recording the result