EVALUATION OF SEDGE GRASS (*S. tabernaemotani*)

FOR BIOETHANOL PRODUCTION

MSc. THESIS

By

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HAWASSA UNIVERSITY COLLEGE OF AGRICULTURE

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FREWEYNI HAILU ASFAHA

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ADVISORS'APPROVAL SHEET

(Submission Sheet – 1)

This is to certify that the thesis entitled Evaluation of Sedge Grass (*S. tabernaemotani*) for Bioethanol Production submitted in partial fulfillment of the requirements for the degree of Masters of Sciences with specialization in Bioenergy Science and Technology, Graduate Program of the School of Plant and Horticultural Sciences, College of Agriculture, Hawassa University and is a record of original research carried out by **Freweyni Hailu**, under our supervision, and no part of the Thesis has been submitted for any other degree or diploma. The assistance and help received during the course of this investigation have been duly acknowledged. Therefore, we recommend that it can be accepted as fulfilling the Thesis requirements.

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We, the undersigned, members of the board of Examiners of the final open defense by Freweyni Hailu, have read and evaluated his thesis entitled Evaluation of Sedge Grass (*S. tabernaemotani*) for Bioethanol Production and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree of Masters of Science in Bioenergy Science and Technology.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my own work and that all sources of materials used for the thesis has been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for M.Sc. degree, the graduate program of Hawassa, and I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: Freweyni Hailu Asfaha **Signature**: _____

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Date of Submission: December 3, 2021

DEDICATION

This scientific paper is dedicated to my beloved mother, Letebrhan Gebreselassie, my father Hailu Asfaha, my brother Hailemichael Hailu and all my family for their constant support, love and inspiration.

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LIST OF ABBREVATIONS AND ACRONYMS

AAU	Addis Ababa University
AIR	Acid Insoluble Residue
ANOVA	Analysis of Variance
AS	Absorbance of Sample
AU	Absorbance of Standard
CS	Concentration of Sample
CU	Concentration of Standard
CV	Coefficient of Variance
EY	Ethanol Yield
FTIR	Fourier Transform Infrared Spectroscopy
HMF	5- Hydroxyl Methyl Furfural
LSD	List Significant Difference
NREL	National Renewable Energy Laboratory
ODS	Oven Dried Sample
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
SPORL	Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose
TRSC	Total Reducing Sugar Concentration
TRSY	Total Reducing Sugar Yield

Evaluation of Sedge Grass (*S. tabernaemotani*) for Bioethanol Production

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ABSTRACT

Biofuel production from first generation biomasses, basically human food, might lead to problem of food crisis. Non-edible lignocellulosic biomass which is abundant with low production cost would be considered as an appropriate feedstock for ethanol production. Sedge grass (Schoenoplectus tabernaemontani) is one of environmental friendly non-edible lignocellulosic grasses.However; there are no reports on the use of sedge grass for bioethanol production. Therefore, this study was conducted to investigate the yield of total reducing sugar from sedge grass for ethanol production, with three levels of hydrolysis time (40, 60 and 80 min), three levels of H_2SO_4 concentration (1.5, 2.5 and 3.5%) and three levels of temperature (115, 125 and 135^oC) designed in Complete Randomized Design (CRD) with three replications. The chemical compositions (extractives, cellulose, hemicellulose and lignin) of the plant were also determined by using National Renewable Energy Laboratory (NREL) protocol. The reducing sugar yield was determined by Benedict's solution using spectrophotometer. Simple distillation was carried out to separate ethanol from water and the functional group of the produced ethanol was analyzed using Fourier Transform Infrared (FTIR).To estimate the ethanol yield, potassium dichromate method was used. As the result indicated, the content of hemicellulose, cellulose, lignin and extractives were 42%, 39.87%, 13.07% and 5.06%, respectively. The two-way interaction of treatments exhibited significant differences on total reducing sugar yield. Concerning the three-way interaction, highest total reducing sugar was produced when the feedstock was hydrolyzed at a temperature of 125^0C for 60 min by 2.5% dilute H_2SO_4 solution. However, the lower percent of total reducing sugar yield (38.29%) was recorded from hydrolyzed sample at a temperature of 135^0c for 80min using 3.5% dilute H_2SO_4 solution. Eventually, the highest ethanol yield (51.02%) was recorded from 49.83% of hydrolyzed sample reducing sugar fermented by F. oxysporum for 7 days fermentation. Therefore, sedge grass biomass is recommended as raw materials for bioethanol production, which is a promising alternative energy source against the depleting petroleum.

***Key words**: Bioethanol, Fermentation, Fossarium oxysporum, Saccharomyces cerevisiae, Schoenoplectus tabernaemontani .....*

1. INTRODUCTION

1.1. Background of the study

As report of Bauer *et al*., (2016) indicated that, Fossil fuel is the backbone of the world energy demand. Since it is non renewable energy source, the energy from such source is diminishing from time to time and it is more likely to be exhausted in near future as reported by Birhanu and Ayalew, (2017). It is a popular fact that the world's dependency on fossil fuel has caused unfavorable effects, including reducing crude oil reserve, causing air pollution, rising global temperature, contributing to unpredictable weather change, and so on. Thus, biofuel could play a critical role in improving the health of billions of people (Aditiya *et al.,* 2016). Furthermore, due to different factors such as political unrest in the fossil fuel producing countries, the price of the fuels is rising. All the mentioned earlier factors and other challenges lead to search for alternatives sources which are renewable, environmentally friendly and cost effective fuels (Ahorsu *et al*., 2018)

Biofuel is a fuel derived from biological feedstocks that contain appreciable amounts of sugar (Demirbas and Demirbas, 2016). Biofuels are mainly produced from wood, agricultural crops and products, aquatic plants, forestry products, wastes and residues, and animal wastes. They are renewable since they are produced from biomass, organic matter such as plants (Birhanu and Ayalew, 2017). It has been reported that, biofuel can be considered as the most promising source which can be used by blending or alone in the fossil fuel machine without more modification (Hammond *et al*., 2008). They generate about the same amount of carbon dioxide (CO_2) from the tailpipe as fossil fuels, but the plants that are grown to produce the biofuels actually remove carbon dioxide (CO_2) from

the atmosphere. Therefore, the net emission of carbon dioxide is assumed to be close to zero (Hailemichael, 2016).

The two most important biofuels are ethanol (i.e., bioethanol) and biodiesel (Dharma *et al*., 2016). 'Bioethanol' is produced from microbial conversion of biomass materials through fermentation and contains 35% oxygen while 'biodiesel' is produced from bio-oil (Gebreegziabher *et al.,* 2017). Bioethanol is a renewable and sustainable liquid fuel that is believed to have a promising future in tackling today's global energy crisis and the worsening environmental quality (Aditiya *et al.,* 2016). One major problem with bioethanol production, however, is the availability of raw materials for the production. The availability of feed stocks for bioethanol production can vary considerably from season to season, and also depend on geographic locations (Balat *et al.,* 2008). The development of second and third generation bioethanol production may not be as advanced as the first generation bioethanol production. However, looking at the feedstock's availability, second and third generation hold a gigantic potential if implemented nationally (Aditiya *et al.,* 2016).

The production and use of biofules has increased significantly in many countries around the world. Nowadays, the most used liquid biofuel in the world (about 84% of its total world production) is used for transportation. The possible reason could be that fact that compared to the fossil fuels; use of biofuel can reduce greenhouse gas emissions from 30 up to 85% and contribute to lowering the generation of particulate matter in the atmosphere, approximately up to 50% (Di Donato *et al.,* 2019). Some of the major producer and user countries of biofuel are United States of America and Brazil. The bioethanol produced in the USA in 2017 reached 50.6% of the global production, and it is mainly used as a 10% petrol additive. In Brazil, the bioethanol's total production in 2017

was about the 23.8% of the world's total . It is not less true in Ethiopia, which endowed with diverse ecological varieties that have an ample potential for biofuel production (Birhanu and Ayalew, 2017). Though there are many non-edibles naturally occurring biofuel feedstocks in Ethiopia such as Jatropha, castor been, sedge grass, water heyacine, the potential of the country is not yet exploited.

Obviously, the production of ethanol from agricultural wastes can only be applied for the short term purpose since the amount of such waste won't be sufficient in the future (Wongwatanapaiboon *et al*., 2012). Therefore, in order to maintain long-term and successfully sustainable production of the alternative energy, the cultivation of energy crops together with the utilization of agricultural wastes and the search for the potential plants for the production of ethanol should be implemented. As reported by Wongwatanapaiboon *et al*., (2012), grasses are considered as the most suitable energy crops from their longevity, regeneration after the cutoff, and effective capability to withstand the drought.

Sedge grass is one of the grasses that can be used as the feedstock of bioethanol production. It is one of the grass species from *Cyperaceous* family spread in water bodies all over the world. Like other grass types, its chemical composition is lignocellulose and it is a macrophyte plant (Brown *et al*., 2020). This plant is grown in many lakes of the world including Ethiopia even in Lake Hawassa. It has advantage as carbon sequester, feeding for marine living organisms and prevents sediment. But no information about lignocellulosic composition and Ethanol production ability of sedge grass. So in order to assess the suitability of sedge biomass as a fuel, studies on its lignocellulosic composition, reducing sugar and produced ethanol yield are necessary.

1.2. Statement of the problem

Biomasses that contain starch, sugar and lignocellulose materials have been reported to be alternative bioenergy (bioethanol) sources (Balat *et al*. 2008). However, the use of starch and sugar from cassava, corn, and sugarcane which are basically human food might possibly lead to the problem of food crisis. So, utilization of other non-edible lignocellulosic biomass such as Sedge grass can reduce problem of food crisis, environmental pollution, and dependency on fossil fuel and reduce the cost of importing petroleum products. Sun and Cheng (2002), stated about grass biomass with chemical composition of 25 - 40 % cellulose, 35 - 50% hemicellulose, 10 - 30% lignin and 1 - 5% extractive are preferable for bioethanol production. Sedge biomass (*S. tabernaemontani)* is one of environmental friendly non-edible lignocellulosic grass. But no previous report on the investigation of lignocellulosic composition and bioethanol production from Sedge grass is found in the literature. Reports on other lignocellulosic biomass indicated that, ethanol produced using *F.Oysporum* fermenter gave higher yield than ethanol produced from *S.Cereviciae* fermenter. This is due to inability to metabolize pentoses such as xylose of microbe *S.cereviciae* in fermentation time (Anasontzis and Christakopoulos, 2014). Thus, this study was initiated to investigate the potential of sedge grass as source of bioethanol. The produced bioethanol was analyzed; and the ethanol yields produced from sedge grass by two fermentation aided by microorganisms (*F. oxysporum* and *S.cereviciae*) were compared.

1.3. Objectives

1.3.1. General objective

The overall aim of the current study was to analyze the total reducing sugar content of sedge grass (*S. tabernaemontani*) and production of bioethanol using two fungi species at different fermentation conditions.

1.3.2. Specific objective

The specific objectives of this study were:

- To characterize the chemical composition (cellulose, hemicellulose, lignin and extractives) of sedge grass biomass.
- To investigate the effects of process variables (H_2SO_4 concentration, hydrolysis time and temperature) on total reducing sugar yield of sedge grass.
- To analyze functional group of produced ethanol through FTIR technique and to investigate the effects of fermentation microbes (*S.cereviciae* and *F.oxysporum*) and fermentation days on ethanol yield of sedge grass.

1.4. Significance of the Study

This study will help to get information about potential of sedge grass biomass for bioethanol production and to know the effect of different fermenting microbes on bioethanol yield. Investigation of the lignocellulosic composition of this biomass could help to assess its suitability for bioethanol production with minimize processing cost, shorter time and no competing economic value. This study would serve as a baseline for other researchers who are working on similar biomass to produce bioethanol.

1.5. Scope of the Study

The study generally encompass: pretreating sedge grass sample, total reducing sugar determination and investigation of the effects of hydrolysis variables (temperature, hydrolysis time and H_2SO_4 concentration) on total reducing sugar yield. The effects of fermentation variables (fermenting microbes and fermentation times) on ethanol yield have been investigated, and the produced ethanol was analyzed using FTIR spectroscopy.

2. LITERATURE REVIEW

2.1. Renewable Energy

Renewable energy can replace conventional fuels in four distinct areas: electricity generation, hot water/space heating, motor fuels, and rural energy services. About 16% of global final energy consumption comes from renewable resources, with 10% of all the energy from traditional biomass, mainly used for heating, and 3.4% from hydroelectricity. New renewables (small hydro, modern biomass, wind, solar, geothermal, and biofuels) account for another 3% and are growing rapidly (Ellabban *et al*., 2014). at the international level, at least 30 nations around the world already have renewable energy contributing more than 20% of energy supply. Wind power, for example, is growing at the rate of 30% annually, with a worldwide installed capacity of 282.5 (GW) at the end of 2015 (Araújo *et al*. 2017).

Biomass is the fourth largest source of renewable energy in the world (Drożyner *et al*., 2013). The two main sources of biomass are the purpose-grown energy crops and wastes materials. Waste cooking oils, animal fats and other biomass residues are an examples for utilizing waste sources for biofuel (Shalaby, 2015). bioethanol which is one of the renewable bioenergy, is known to be a potential alternative to petroleum-derived fuels and has also a potential to meet the increasing demand of energy for industrial processes, heating and transportation (Izmirlioglu and Demirci, 2012). Increase in prices of molasses and its limited availability is another reason to divert the attention toward alternate resources (Anwar and Gulfraz, 2012).

As reported by Yusoff *et al*., (2021), at present, the transportation sector is almost entirely dependent on petroleum-based fuel. It is being responsible for around 60% of the world oil consumption. Nowadays, industrial bioethanol production is mainly focused on corn, wheat and sugarcane, as well as on highly abundant agricultural wastes. The use of residual biomass for bioethanol productions has the added advantage of transforming a waste material into a valorized product. The increase in the prices of fuel and possibility of shortfalls has led to an extensive evaluation of alternative sources of renenable energy to meet the global energy demand (Carrillo-Nieves *et al*., 2019).

Alcohol – based fuel has been reported to have several advantages over gasoline. For instance, alcohol combustion does not cause an increase in atmospheric CO_2 concentration, and thus, it is less polluting than gasoline. This is because alcohol produces less toxic substances and less gaseous emissions. It is also a more secure energy source because it is renewable and can be produced any where in the world from biomass (De Bhowmick *et al*., 2018). Ethanol can be blended up to 20% with diesel or petrol (Hansen *et al*., 2005). currently, is produced from molasses, which is byproduct of sugar industries. The cost of production increases as the demand for molasses has increased (Farooq *et al*., 2020). Hence, it is absolutely necessary to search for an alternate source for ethanol production. The agricultural crop residues such as paddy straw, wheat straw grasses and bagasse are abundantly available having rich source of sugars (Bundhoo, 2019). Ethiopia is currently using 75% of foreign currency earnings from export sector to buy and import oil. the majority of the fossil fuel is used by the transport sector. Currently, importing Biofuel products to Ethiopia can be a substitute for fossil fuel, is given a little emphasis. There is a little effort in the country to produce biofuel energy. This little effort should be organized

and converted to a large scale bio-fuel production level in order to minimize the huge energy gap in the country (Consumption and Resources, 2016).

2.2. Bioethanol

Bioethanol or fuel alcohol refers to ethyl alcohol produced by microbial fermentation that is used as a transportation biofuel. It is a liquid biofuel which can be produced from several/different biomass feed stocks and conversion technologies (Vohra *et al*., 2014). It is an attractive alternative fuel because it is a renewable bio-based resource and it is oxygenated thereby provides the potential to reduce particulate emissions in compression ignition engines (Hailemichael, 2016)

Bioethanol has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline (Bailey,2018). These properties allow for a higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (Balat, 2007). There are also some disadvantages of bioethanol that include its lower energy density than gasoline, its corrosiveness, low flame luminosity, lower vapor pressure (making cold starts difficult), miscibility with water, and toxicity to ecosystems (Demirbas *et al*., 2015). Some characteristics of ethanol fuels are summarized below (Table1).

Table 1: Some characteristics of ethanol fuels (Hailemichael, 2016)

Parameter	**Characteristic properties**
Molecular formula	C_2H_5OH
Molecular mass	46.07 g/mol
Appearance	Colorless liquid
Water solubility	(Between –117°C and 78°C)
Density	0.789 kg/l
Boiling temperature	78.5°C (173°F)
Freezing point	–117°C
Flash point	12.8°C (Lowest temperature of ignition)
Ignition temperature	425°C
Explosion limits	The lowest 3.5% (v/v) Upper 19% (v/v)
Vapor pressure at 38°C	50 mm Hg
Higher heating value (at 20°C)	29,800 KJ/kg
Lower heating value (at 20°C)	21,090 KJ/kg
Specific heat	Kcal/Kg 60°C
Acidity	(p*K*a) 15.9
Viscosity	1.200 maps. s (20°C)
Octane number	99

2.3. Status and Future Prospects of Bioethanol Production

Ethanol has been used as liquor since ancient times and later in the twentieth century as fuel has sparked a widespread interest in its production. This is because it shows great potential in areas including good biodegradability, and reduction in carbon dioxide (10–100%), carbon monoxide (25-30%) and particulate matter emission, as well as high-octane value (Niphadkar *et al*.,2018). The production of bioethanol from renewable resources and the combustion advantages for greener alternatives have led scientists around the world to develop cutting-edge technologies to achieve higher biomass conversion and,

consequently, industrial-level yield and purity. Thus, production of bioethanol can also reduce the consumption of crude oil (Niphadkar *et al*., 2018).

Based on the report of Niphadkar *et al*., (2018) the annual global ethanol production has increased to almost 41,000 million gallons, if recent market reviews are to be believed. Recent advances in technologies such as the use of agricultural wastes containing polysaccharides, or algal polysaccharides, and genetic manipulation to develop crops containing high carbon content, or to contain cellulose in their leaves, have opened a new horizon in bioethanol production. Bioethanol production is evolved from first generation production to fourth generation bioethanol production. However there are various constraints and challenges involved (Ayodele *et al.*,2020).

As Di Donato *et al.*, (2019) reported that, bioethanol is nowadays the most used liquid biofuel in the world (about 84% of its total world production is used for transportation) since, compared to the fossil fuels, it can reduce greenhouse gas emissions from 30 up to 85% and contribute to lowering the generation of particulate matter in the atmosphere, approximately up to 50%. The main countries producing and using bioethanol as a fuel are United States of America and Brazil. As seen below (Figure 1) (Di Donato *et al*., 2019), the bioethanol produced in the USA in 2017 reached 50.6% of the global production, and it is mainly used as a 10% petrol additive. In Brazil, the bioethanol's total production in 2017 was about 23.8% of the world's total (Di Donato *et al.*, 2019)

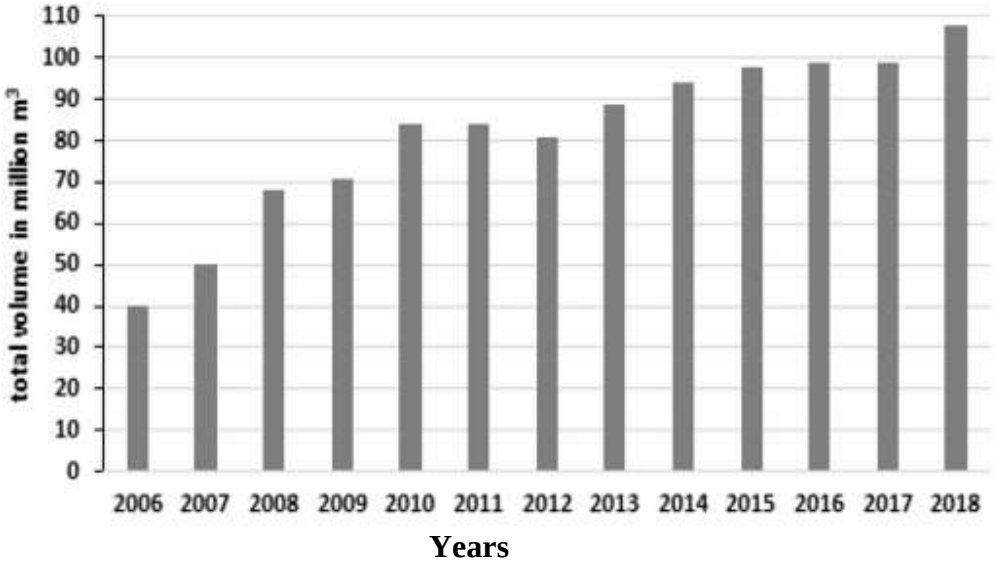

Figure 1: World bioethanol production

Based on the report of Birhanu and Ayalew, (2017), about 95% of the total energy consumption is composed of traditional biomass fuels with only 5% coming from modern energy sources in Ethiopia. The biomass is mainly obtained from firewood, charcoal, dung and crop residues that mainly depend on the surrounding forest resources and agricultural residues (Surendra *et al*., 2013). As a result, the country's forest and land resources are being disappeared and degraded with an alarming rate, resulting in desertification, reduction in agricultural production and recurrent drought (Birhanu and Ayalew, 2017). However, currently ethanol is being produced in Ethiopia as modern energy source (Gabisa and Gheewala, 2020).

Ethanol production in Ethiopia is linked with sugar factories. 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 (Ministry of Water & Energy, 2010). At present, the main supply line in the domestic market is dominated by two sugar factories (Fincha and Metehara) with the combination of their annual production capacity at around 11.1 million liters. 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 (Tekalign, 2018).

2.4. Raw Materials for Bioethanol Production

Different feedstock generations are available for production of bio-ethanol as it can be derived from any biological raw-materials that can be converted into sugar from starch or cellulose (Balat and Balat, 2009). The availability of feedstocks for bioethanol production can vary considerably from season to season and depends on geographic locations. They can be conveniently classified into three major types such as first generation (sugar and starchy plant biomasses), second generation (non-edible lignocellulose biomasses) and third generation feed stocks (Balat *et al.,* 2008).

2.4.1. Sucrose containing feedstocks

Bioethanol feed stocks such as corn and sugar beet are normally used as fertilizers or animal feed, and thus, external energy are required for those coproducts conversion usually at the expenses of fossil fuels burning. Energy consumption in the industrial phase of corn ethanol production is even higher when compared to sucrose-based ethanol, since additional steps to convert starch to glucose are required (Manochio *et al.,* 2017). Two-third of world sugar production is from sugar cane and one-third is from sugar beet (Linoj *et al.,* 2006). These two biomasses are produced in geographically distinct regions. Sugar cane grows in tropical and subtropical countries, while sugar beet grow only in temperate-climate countries. Generally, sugar beet, sweet sorghum and sugar cane are included under sucrose containing feed stocks in bioethanol production (Muktham *et al*., 2016).

2.4.2. Starchy materials

Starch is a biopolymer that can be defined as a homopolymer consisting only one monomer, D-glucose (Pongsawatmanit *et al.*, 2007). It consists of long chains of glucose molecules and can also be converted to fermentable sugars to produce bioethanol. However, it is necessary to break down the chains of this carbohydrate to obtain glucose syrup, which can be converted into bioethanol by yeasts. This type of feedstock is the most utilized for bioethanol production in North America and Europe (Hafid *et al*., 2017) from these starchy materials (wheat, corn, and barley) are mainly employed as biomasses for bioethanol production purposes (Balat *et al.*, 2008).

2.4.3. Lignocellulosic biomass

Lignocellulosic biomass is composed primarily of cellulose, hemicellulose, and lignin (Yu *et al*., 2017). Cellulose is a homogenous polymer composed of glucose (six carbon sugars), however, hemicellulose is a heteropolymer predominantly composed of five carbon sugar sub-units such as xylose, mannose, and arabinose. However, the composition of different compounds in the hemicellulose varies for the type of biomass. The hemicellulose makes side chain connections between the cellulose and lignin portions. Lignin is a complex aromatic polymer that covers the sugar polymer matrix to serve as a protective barrier from physical and chemical attacks (Taherzadeh, 2008).

Balat *et al.* (2008) reported that, lignocellulosic biomass such as agricultural residues (straw), wood, grasses and energy crops are attractive materials for bioethanol fuel production. It's attractive and sustainable. This is because lignocellulosic biomass is renewable and non-competitive with food crops. Furthermore, lignocellulosic biomass is almost equally distributed on the earth (Nomanbhay *et al.,* 2017). Approximately 75% of lignocellulose is comprised of polysaccharides, which can potentially be converted into

monosaccharides for fermentation. The main constituents of plant secondary cell walls are cellulose, hemicellulose and lignin, and these are present in varying proportions in different feedstocks (Table 2)(Marriot *et al*., 2016). Secondary cell walls of both dicots and grasses also contain.

Table 2 : Typical percentage composition of the main components of cell walls in different sources of lignocellulosic biomass (Marriot et al., 2016)

Lignocellulosic components	Dicots	Grasses	Soft woods	Hard woods
Cellulose	45-50	35-45	25-50	40-55
Hemicellulose	30-30	40-50	20-30	20-35
Lignin	7-10	20	25-35	18-25

Cellulose

Cellulose is the main structural constituent of plant cell walls. It is a crystalline, very fibrous, and rigid due to the hydrogen bond links between cellulose molecules (Pereira *et al*.,2003). It is chemically stable and mechanically robust, making it water insoluble and more resistant to depolymerization. Usually, it is covered by hemicellulose forming a cellulosehemi cellulose complex that inhibits the access of enzymes, influencing the hydrolysis rates, and therefore, the production of fermentable sugars and the digestibility of lignocellulosic biomass (Zabed *et al*., 2016). High cellulose content in lignocellulosic material is a promising condition for biofuel production (Raud *et al*., 2014).

Hemicellulose

Hemicellulose is the second most abundant polymer of plant cell walls and is mainly composed of xylan and mannan (Rocha-Meneses *et al*., 2017). Compared with cellulose, hemicellulose has a lower degree of polymerization. It is chemically heterogeneous, and also has a random and amorphous structure (Yu *et al*., 2017). As hemicellulose is wrapped

around the cellulose fibrils, it needs to be removed in order to increase the cellulose digestibility. Thus, increase in hemicellulose removal increases the accessibility of the cellulose and its hydrolysis rate (Zabed *et al*., 2016).

Lignin

Lignin is a three-dimensional polymer of 4-propenyl phenol, 4-propenyl-2-methoxy phenol and 4-propenyl-2.5-dimethoxyl phenol, and it is the third most abundant constituent of lignocellulosic biomass. It also provides structural support to the plants, and contributes to the impermeability and resistance against microbial attack (Hendriks & Zeeman, 2009). This compound ties all the constituents of lignocellulosic biomass making. It is insoluble in water, and more difficult to degrade. Due to its properties, lignin is the main obstacle to enzymatic hydrolysis. Moreover, it influences the digestibility of the biomass. High lignin content results in low digestibility of the biomass. Therefore, increasing the lignin removal increases the biomass digestibility (Hendriks and Zeeman, 2009).

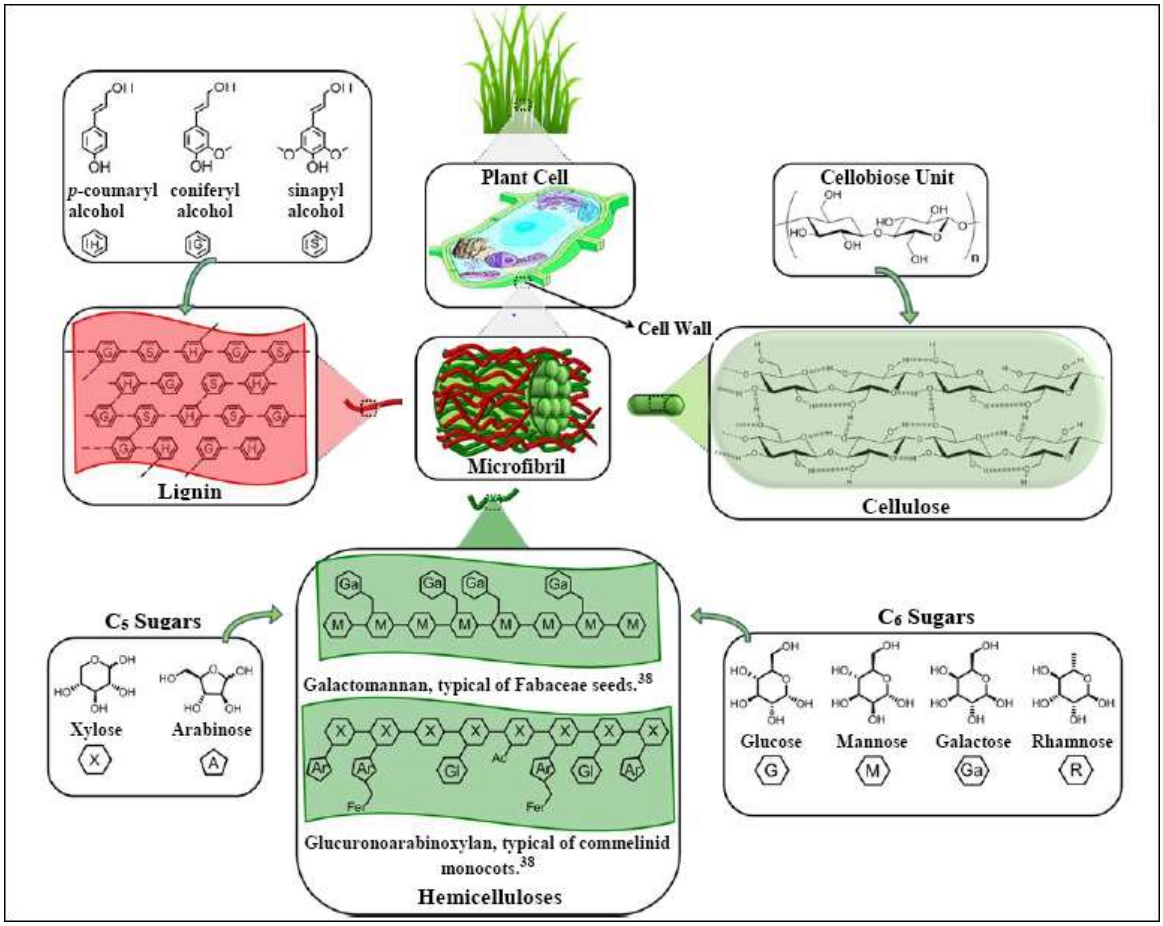

Figure 2: The main components and structure of lignocellulose

2.5. Grass biomass

As report of Clayton *et al*., (2016) shows that, there are around 11,369 accepted grass species that have been known worldwide till now. The habitat of these grasses ranges from infertile land mass to well drained fertile soil in varied climatic conditions (Clayton *et al*., 2016). Grasses are composed primarily of carbohydrate polymers (cellulose, hemicellulose and phenolic lignin) along with other compounds, such as proteins, acids, salts, and minerals. The accumulation of carbohydrate can be attributed to the photosynthetic cycle in plants. The carbohydrate content is not similar for all the types of grasses, and significantly varies due to a lot of factors such as, variety of the grass, developmental stage of the grasses and environmental conditions in which it is grown (Nelson and Moser, 1994).

Among the different varieties of wild and cultivated grasses, bluestems, Indian grass, and switch grass are some of the most common examples of wild grasses (Grman *et al*., 2021). Cultivated grasses such as smooth broom grass, timothy, meadow foxtail are some of the species that are derived from wild species of grasses. They are developed through different breeding methods such as pure line selections, mutants, poly ploids and intergeneric/interspecific hybrids. Both wild and cultivated grasses are considered as suitable plant biomass because of their high carbohydrate content, their longevity, redevelopment after the cut off, and effective capability to tolerate the drought (Wongwatana *et al*., 2012). has been reported that the structural carbohydrates in cell wall increase with the maturity of the grasses, the reverse phenomena is observed for the cell components.

The type of grass variety and the environmental conditions in which it is cultivated play an important role in determining the chemical composition of the grasses and thereby it has potential to be considered as a bioethanol crop (Tye *et al*., 2017).Therefore, attentive considerations of the ecological aspects would serve as an integral part for bioethanol production from grasses.

2.5.1. Potential of grass biomass for bioethanol production

The most favorable biomass resource for biofuel production should be readily available, have high yielding biomass per dry weight, unwavering desirable chemical concentrations and should be economical (Mohapatra *et al*., 2017). Other features like elevated carbon and hydrogen concentrations and minimum concentrations of oxygen, nitrogen and other organic components are also crucial for biomass to be considered for bioethanol production

(Mohapatra *et al*., 2017). It also lies on the fact that the industries using the biomass should produce less effluent and offer low CO_2 emission ability.

Grasses have the advantage of possessing possibly all the above mentioned features. They grow naturally and do not require any special requirements for cultivation, which makes the biomass growth cost effective, as application of fertilizers and pesticides is not a necessity (Tan *et al*., 2008). It also has good aboveground foliage and much denser growth which maximizes the amount of biomass that an acre of land can produce. Additionally, grasses are composed primarily of carbohydrate polymers of cellulose, hemicellulose, lignin and lower concentrations of various other compounds, such as proteins, acids, salts, and minerals (Mohapatra *et al*., 2017).

2.5.2. Sedge grass biomass

All forms of wetlands such as macrophytes, extensive swamp forest complexes and ferns are characterized in Ethiopia. From wetland macrophytes, *S. tabernaemontani* (Syn. *Scirpus validus*) is a species of flowering plant in the sedge family. It is known by different common names such as softstem bulrush, grey club-rush and great bulrush. It can be found throughout much of the world (Tucker, 1987). It also grows in moist and wet habitat, and sometimes in shallow water (NRCS, 2017). As report of Tucker (1987) stated that *S. tabernaemontani* plant is quite variable in appearance, thus explaining the long list of synonyms that have been created over the years. It is a perennial herb producing dense stands of many narrow erect stems reaching 1-3 m in height (Annex 1a).

There are no reports related to sedge grass utilization for ethanol production. But being as grass family it is expected to have lignocellulosic composition and it could be a favorable biomass to bioethanol production. It is abundant ~~i~~n water bodies worldwide including

Ethiopia. The country has several water bodies (lakes). Among these lakes, Lake Hawaasa is well known for growing this plant. It grows with less investment cost, and also not used for human food. Therefore, it would be considered as an appropriate feedstock for ethanol production. There is no information about chemical compositions such as alkaline elements content of this plant. For this reason, study on biomass composition is necessary in order to assess the suitability of the biomass as a fuel resource.

2.6. Processes in Bioethanol Production

Ethanol can be produced in two different ways. Either chemically, by hydration of ethylene, which is derived from crude oil or natural gas, or by fermentation of sugar containing feeds, starchy feed materials or lignocellulosic materials (Sheldon, 2014). another reports indicated that, bioethanol is produced via the 'classical' well-known alcoholic fermentation, where sugar molecules are converted into ethanol via microorganisms by splitting off carbon dioxide (CO_2) and releasing thermal energy (Okoro *et al.,* 2017).

The process of ethanol production depends on the types of feed stocks used. Feed stocks are usually pretreated in order to reduce its size and facilitate subsequent processes. Then, the hemicellulose and cellulose will be hydrolyzed to fermentable sugars. Microorganisms like, Yeast (*S. Cerevisiae*) and *F. oxysporum* are well known to ferment these sugars into ethanol (Azhar *et al.,* 2017). The Major steps in ethanol productions are:

2.6.1. Pretreatment

Pretreatment process is the process requires in reducing the Lignocellulosic biomass size, crystallinity, and providing easier access for hydrolysis (Aditiya *et al.*, 2016). It has a significant effect on the overall process which makes the hydrolysis easier and produces higher amount of fermentable sugars (Azhar *et al.,* 2017). It is required in order changing

the structures of cellulosic materials and to make cellulose more accessible to the enzymes or dilute acids, which convert the carbohydrate polymers in to fermentable sugars (Hailemichael, 2016).

The commonly used methods currently used for pretreatments are physical, chemical, biological and physicochemical. Physical pretreatment uses drying and mechanical milling to ground the substrate. The common chemical pretreatment includes ozonolysis, acid hydrolysis, alkaline hydrolysis and organosoly based process (Wang *et al.*, 2009). The common pretreatment practice in the last two decades has been to incubate lignocellulosic materials in the medium of dilute sulfuric acid (0.5 to 1.5% H_2SO_4) at 100 to 150°C (Wingren *et al*., 2003). Generally, pretreatment must meet the following requirements: Improve the formation of sugars or theability to subsequently form sugars by acidic or enzymatic hydrolysis; avoid the degradation or loss of carbohydrate; avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes (Azhar *et al.*, 2017).

2.6.2. Hydrolysis

Hydrolysis process takes place after pretreatment to break down the feed stocks into fermentable sugar to bioethanol production. The two most commonly used hydrolysis methods are acidic and enzymatic. Enzymatic hydrolysis requires enzymes to hydrolyze the feed stocks into fermentable sugars (Taherzadeh and Karimi, 2007).

Acid hydrolysis is considered as the oldest and most commonly used method (Jeffries and Jin, 2000). This method is effective in solubilizing the cellulosic and hemicellulosic components of biomass when proper combinations of pH, temperature, and reaction time are operated (Alvira *et al*., 2010). The amount of alcohol produced in case of acid

hydrolysis is more than that of alkaline hydrolysis. Even though there are two types of acid hydrolyze, concentrated acid hydrolysis is not preferred. This because concentrated acids are highly corrosive, and are not environmentally and economically viable. On the other hand, dilute acid hydrolysis which is an easy and productive process is more established method and provides less probability of sugar degradation to inhibitors such as 5-HMF or furfural but requires more time and higher temperature to increase the reaction rate for sugar release (Loow *et al*., 2016). These toxic by-products inhibit microbial growth and interface with subsequent ethanol fermentation (Zelelew *et al.,* 2018). Dilute acid hydrolysis is the most commonly used process. However, it generates large amount of inhibitors compared to concentrated acid hydrolysis. Hydrolysis of lignocellulosic biomass is conducted in two-stage process as the pentose sugars degrade more rapidly compared to hexose sugars. Hemicellulose is hydrolyzed in the first stage using dilute acid while cellulose is hydrolyzed in the second stage using concentrated acid. Concentrated acid process generates high sugar recovery (90%) in shorter period of time (Lenihan *et al*., 2010). Enzymatic hydrolysis requires enzymes to hydrolyze the feed stocks into fermentable sugars (Taherzadeh and Karimi, 2007).

2.6.3. Fermentation

Fermentation is a biological process that converts fermentable sugars such as glucose and xylose to cellular energy in the presence of microorganisms which produce waste by-products ethanol and carbon dioxide anaerobically by the metabolic pathways of sugar (Tekalign, 2018). It is conducted under standard fermenting conditions and will utilize all the major biomass (Hailemichael, 2016). Fermentation of biomass to ethanol can be carried out in batch, fed batch, repeated batch or continuous mode (Azhar *et al.,* 2017). Yeast is the most commonly used microorganism in fermentation processes. Yeasts are minute, often

unicellular fungi. The yeasts used are typically brewer's yeasts. Examples of yeast capable of fermenting the decaying biomass include but are not limited to *Saccharomyces cerevisiae* and *Saccharomyces uvarum* (Hailemichael, 2016).

2.6.4. Distillation

The ethanol solution resulted from fermentation process needs to be further processed to remove the water content, giving dry and high quality ethanol product. Removal of water content can be done by the principle of distillation (Aditiya *et al.*, 2016). Generally, distillation is the method used to separate two liquids based on their different boiling points (Tekalign, 2018). However, to achieve high purification, several distillations are required. This is because all materials have intermolecular interactions with each other, and two materials may co-distill during distillation (Hailemichael, 2016). Although the boiling point of ethanol, 78.3℃, is significantly lower than the boiling point of water, 100 ^{0}C, these materials cannot be separated completely by distillation (Tekalign, 2018). Thus, fractional distillation is commonly used to purify bioethanol that is obtained by fermentation process.

2.7. Yeast *S. Cerevisiae* and *F*. oxysporum as Fermenters

S. Cerevisiae has been selected through thousands of years of selective evolution for its ability to grow on different types of glucose-rich hydrolysates for the production of different types of alcoholic beverages (Anasontzis and Christakopoulos, 2014). Despite its obvious advantages, *S. Cerevisiae* has several drawbacks that hinder its use in lignocellulose-based processes; these include its dependence on previous chemical or enzymatic steps in order to break down the plant cell wall biomass to monosaccharides, and also its inability to metabolize pentoses such as xylose (Anasontzis and

Christakopoulos, 2014). Due to that looking filamentous fungus like *F. oxysporum* and other genetically modified microbes is recommendable.

F. oxysporum is probably more commonly known as a plant pathogen, as the pathogenic strains are responsible for vascular wilt disease in more than 120 different species. Even so, it is a ubiquitous microorganism, whose global distribution reveals a wider ecological influence and a recognized ability to survive even without any pathogenic activity (de Lamo and Takken, 2020). This can be partly attributed to the fact that this microorganism has the ability to produce a wide range of biomass degrading enzymes and can generally be used for both hexose and pentose production. *F. oxysporum* consumes the ethanol it produces, and its rate of fermentation is so low that it takes days to complete as opposed to hours by the yeast. Since the first discovery, our understanding of metabolism has improved and *F. oxysporum* has been studied thoroughly, but the fundamental challenges in using such a filamentous fungus in consolidated bioprocessing for ethanol production still remain (Anasontzis and Christakopoulos, 2014).

The probability of success in finding a new organism that can compete with yeast in the fermentation efficiency is quite low. Even so, in order for filamentous fungi, such as *F. oxysporum*, to become advantageous in a realistic sense, a number of improvements would have to be made, either through genetic modification or evolutionary engineering, in combination with process development and optimization (Fan *et al*., 2011).

2.8. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier Transform Infrared Spectroscopy (FTIR) analysis is an analytical technique used to identify organic, polymeric, and in some case inorganic materials. The FTIR analysis method uses infrared light to scan test samples and observe chemical properties (Xu *et al*.,

2019). The FTIR instrument sends infrared radiation of about 10000 to 100 cm^{-1} through a sample, with some radiation absorbed and some passed through. The absorbed radiation is converted in to rotational or vibrational energy by the sample molecules. The resulting signal at the detector presents as spectrum, typically from 4000 cm^{-1} - 400cm^{-1}, representing molecular fingerprint of the sample. Each molecule or chemical structure will produce a unique spectral fingerprint, making FTIR analysis a great tool for chemical identification (Bakare *et al.*, 2019).

As Bodirlau and Teaca (2009) states bioethanol have characteristic of Infrared absorptions associated with the O-H, C-O, and C-H stretching vibrations. When run like a liquid film, the region 3500-3200 cm^{-1} with a very intense and broadband indicated the O-H stretch of alcohols, while the region 1260-1050 cm^{-1} confirms the C-O stretch. The bands at around 2880 and 2930 cm^{-1} were assigned as the symmetric stretching modes of the -CH_2 and-CH_3 groups, respectively.

3. MATERIALS AND METHODS

3.1. Materials and Chemicals

3.1.1. Description of study area

Sample sedge grass was collected during the maturity stage in (November, 2019) from Lake Hawassa which is located 273 km from Addis Ababa. It is found at an altitude of 1750 masl and 7^{o} 3' N latitude with 38^{o} 28' E longitude. The mean annual rainfall is about 971.9 mm, average temperature 20.85 (National Meteorology Agency, 2019).

The experimental analysis of the present study was carried out in the laboratories of School of Chemical Engineering, Institute of Technology, Hawassa University and Veterinary Campus, Mekelle University, Ethiopia.

3.1.2. Materials used

The materials used for the current study include sedge grass, oven (model 100 -800) , grinnder (ZAIBA super blender), sieves (mesh size of 2.0 mm, Sortmks-3332, PFEUFFR, Germany), Digital balances (EP214C), pH Meter (pH meter 3310, JENWAY), autoclave (LaMCS204), distillation set up, centrifuge graduated cylinders, shaker incubator (THZ-300C), water bath (GYROMAX 929), Vacuum Filter (model-BN 3 STAATLICH, Berlin), spectrophotometer (SPECTRO UV-VIS DOUBLE BEAM PC 8 SCANNING AUTO CELL UVD -3200) and Fourier Transform Infrared spectroscopy (Perkin Elmer Spectrum, 65 FTIR) machine.

3.1.2. Chemicals and reagents

Chemicals used during the experiment were sulfuric acid (H_2SO_4, 98%), England), sodium hydroxide (NaOH, min. assay 98% BDH Chemicals Ltd pool England cellulose), Benedict's solution, yeast extracts broth, urea, peptone water, dextrose sugar and $MgSO_4.7H_2O$, potato dextrose agar (PDA), potato dextrose broth (PDB) and potassium dichromate. Some chemicals which were used in the experiment are given in Appendix section (Annex 8a).

3.2. Methods

3.2.1. Plant material collection and preparation

For the present investigation, 2 kg of sedge grass was collected from Lake Hawassa. After the steam part of sample was cutting in to pieces, it was washed with tap water and oven dried at 60 ^{0}C for two days. The dried and ground powder plant material of larger particle size was ground again and again until all particle size became less than 2 mm.Grinding of sedge grass to powder form was aimed to increase the surface area of the sample which enhances the contact between hemicelluloses and cellulose and reduces sample cellulose crystallinity.

3.2.2. Determination of chemical composition

Determination of extractives: 3 g of sedge grass powder at 60 ^{o}C for 72 hours was loaded into the Soxhlet extraction tube as shown in (Annex 2a & 2b) by stabling inside the millimeter paper; 150 ml of acetone was used as solvent for extraction. Residence times for the boiling and rising stages was carefully adjusted to 70^{o}C and 25 min, respectively, on the heating mantle for 4 hours running period at 70^{o}C. After extraction, the extracted residue was air dried at room temperature with in few minutes. The constant weight of the

extracted material was achieved in a convection oven at 105 ^{0}C for 1 hour. The % (w/w) of the extractive was evaluated as the difference in weight between the raw material and extractive free sample (NREL, 2005). For determination of extractives, the reason used acetone rather than other alcohol is due to less boiling point and easly evaporated. The % (w/w) of the extractives content was evaluated using Equation 1.

$$Extractive\ \% = \frac{W1-W2}{W1} \times 100 \quad \text{(Eq. 1)}$$

Where; W1 = oven dried sample; W2 = extracted residue

Determination of hemicellulose: According to NREL, (2005), 1.0 g of extractive free powder sample was transferred into 250 ml of conical flask and 150 ml of 0.5N NaOH was added into 250 ml conical flask held extractive free sample. The mixture of powder sample and NaOH was then boiled at 60 ^{0}C for 3.5 hours in hot plate. After the mixture was boilled and cooled, it was filtered through vacuum filtration and washed until neutral pH was obtained. The residue was dried at 105 ^{o}C for 4 hours in convection oven.The difference between the sample weights before and after this treatment is hemicellulose content (% by w/w) (Equation 2).

$$Hemicellulose\ \% = \frac{W1-W2}{W1} \times 100 \quad \text{(Eq. 2)}$$

Where W1= oven dried sample; W2= oven dried extracted residue

Determination of lignin: According to National Renewable Energy Laboratory (NREL) protocol, there are two types of lignin: acid-insoluble and acid-soluble lignin (NREL, 2005). In order to get total lignin content, both acid-soluble and acid-insoluble lignin was determined first. Extractive free sample (0.5 g) was placed in a flask and then 3 ml of 72% H_2SO_4 was added. The sample was kept at room temperature for 2 hours with carefully

shaking at 30 min intervals for complete hydrolysis. After that, 84 ml of distilled water was added to mixture of extractive free sample and concentrated H_2SO_4. The second step of hydrolysis was carried out in an autoclave for 1 hour at 121 °C. The slurry was then cooled at room temperature. Hydrolyzates were filtered by vacuum filtration. The acid insoluble lignin was determined by drying the residues at temperature of 105 ^oC for 3 hours in oven and accounting for ash by incinerating the hydrolyzed samples at 575 ^oC for 2 hours in a muffle furnace and it was calculated using Equation 3. Then to determine acid-soluble lignin, the collected Hydrolyzates were neutralized with sodium hydroxide and the absorbance was measured on UV spectroscopy at 205 nm and it was calculated using (Equation 4 and 5), respectively. Therefore, lignin content was calculated using Equation 6 by the summation of acid insoluble lignin and acid soluble lignin.

$$Acid - insoluble\ lignin = \frac{\text{weight of AIR} - \text{weight of ash}}{weight\ of\ ODS} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Where; AIR = acid-insoluble residue, ODS = oven dry sample

$$Acid\ soluble\ lignin\ \% = \frac{d \times v \times Aa}{a \times w \times L} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

Where; d = the dilution (dimensionless) and it was calculated by:

$$d = \frac{volume\ of\ sample\ +\ volume\ of\ diluting\ solvent}{volume\ of\ sample} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

V = the filtrate volume (L), Aa = the average absorbance of the sample (dimensionless)

a = absorptivity of the lignin (L/g.cm), W = the oven-dry mass of the sample (g)

L = path length of UV-Vis cell (cm)

The value of "a" at 205 nm = 110

Therefore, $The\ total\ lignin\% = Acid\ insoluble\ lignin + Acid\ soluble\ lignin \ldots\ldots\ldots\ldots\ldots (6)$

Determination of Cellulose: The (%w/w) of cellulose content was calculated by the difference in summation of biomass components (extractives, hemicelluloses and lignin) from hundred percent as these (Equation7 & 8) respectively indicated (Ayeni *et al*., 2013).

WC + WH +WE + WL = 100……. (7)

WC = 100 -WH - WE -WL ……..... (8)

Where: WC, WH, WE, WL are cellulose, hemicellulose, extractive and lignin content respectively.

3.3. Bioethanol Production Process

3.3.1. Acid pretreatment

Pretreatment of lignocellulosic biomass for biofuel production apply from 0.05 to 2% H_2SO_4 (w/v) at temperatures between 120 and 220^oC for 20 to 90 min by ratio of 1:10 (w/v) sample to solution (Hailemichael, 2016). Thus, in this study, sedge grass sample was pretreated using solution of 1.5 (% v/v) dilute H_2SO_4. The prepared sample solution in the 500 mL flask was pretreated in an autoclave at a temperature of 121^oC for 60 min. Then it was cooled and filtered using vacuum filter. The residue was washed four times with distilled water to remove traces of sulfuric acid from it until the pH reaches to the recommended interval of 6.78 during pretreatment. Then the neutralized solid part was placed in an oven at 60^0C temperature for 24 hrs then it became dry and kept for hydrolysis as in (Annex 1b). The purpose of the pretreatment was to remove lignin, reduce cellulose crystallinity and increase the porosity of the materials. Pretreatment is usually expected to meet the following requirements: improve the formation of sugar, avoid the degradation or loss of carbohydrate and avoid the formation of byproduct inhibitors (Kumar *et al*., 2009).

3.3.2. Dilute acid hydrolysis

Even though there are many types of hydrolyses, dilute acid hydrolysis is an easy and productive process (Warrand and Janssen, 2007). The amount of alcohol produced in the case of acid hydrolysis is more than that of alkaline hydrolysis. A review by Gladyshko, (2011), the dilute acid hydrolysis of different lignocellulosic materials has defined optimal process conditions: temperature 80-200^{O}C, H_2SO_4 concentration 0.25–8 wt%, and reaction time 10-2000min. In this study, the hydrolysis was taken by 1.5, 2.5 and 3.5% (v/v) of dilute H_2SO_4 (Annex 3a, b). The powder of sedge grass was hydrolyzed in the reactor at three levels of temperature (115,125 and 135^{O}C) and three levels of time of 40, 60 and 80 min. Next, solid particles were separated from the liquid hydrolyzate by vacuum filtration (to remove the non-fermentable lignin portion). After separating the solid part, the liquid part was kept for fermentation. The whole hydrolysis procedures are shown in the figures (Annex 4a, b and c).

pH adjustment

Before addition of any micro-organism to the above prepared sample, pH of the sample was adjusted around 4 - 5. Otherwise, the micro-organism would die in hyper acidic or basic condition (Wondale, 2012). The acid hydrolyzed sample solution was, therefore, primarily checked for its pH using digital pH meter until it reaches a pH of 5 by adding 2N prepared NaOH by drop wise (Annex 5b).

Sterilization

The reactor and all the equipment that were used for fermentation purposes were sterilized in autoclave. The sterilization was carried out at a temperature of 121 ^{o}C for 15 min (Hossain *et al*., 2015).

3.3.3. Absorbance reading for standard glucose

The total reduced sugar content produced through acid hydrolysis process was investigated by Benedict's solution method. Before measuring the reduced sugar, mixture of hydrolyzed sample and benedict solution was heated by water bath at 90^0C for 5 min. The color change from Benedict‟s reaction gives a semi-quantitative or a rough estimate of the reducing sugars present within a sample. The amount of reducing sugar present in a sample can be quantified using the following color change: Blue (no sugar), Green (0.5 % sugar), Yellow (1 % sugar), Orange (1.5 % sugar), Red (2 % sugar), Brown (highest level of sugar). This color change existed due to reduction of blue copper (II) ions to brick red copper (I) oxide of hot alkaline solution (benedict's solution) by reducing sugars. So, in this case, absorbance and concentration of standard glucose were recorded (Danmaliki *et al*., 2016).

3.3.3.1. Preparation of standard glucose solution

This standard glucose solution is important to determine the slop and intercept which are important in determination of the glucose concentration of the samples. Thus, standard glucose solution was prepared by adding 0, 0.2, 0.4, 0.6, 0.8 and 1 g of glucose into 6 separate test tubes, Then 5 ml of distilled water was added to each test tube to dissolve the standard glucose. Other 6 test tubes with 5ml of benedict solution to each were prepared. Then 1 ml of each of the standard solutions was pipetted out into test tube which contains the Benedict's solution. The mixture was kept in water bath at a temperature of 90^oC for 5 minutes and after rapid cooling and filtering the mixture, the absorbance of filtrate was recorded at 540 nm using UV-visible spectrophotometer. Then the the slop and intercept were calculated from the standard glucose concentration verses absorbance standard graph using (Equation 9).

$Y = mx + b$ …… (9)

Where; y = absorbance; x = glucose concentration

m = the slop and b is the intercept

The result of standard glucose calibration curve of absorbance at 540 nm plotted from concentration verses absorbance was plotted (Table 3 and Figure 3).

3.3.4. Determination of sample total reducing sugar concentration

The concentrations of unknown sugar samples were determined from standard curve of glucose ($Y = 0.024X - 0.002$; $R^2 = 0.998$) (Figure 4 In section 4). To determine the sugar concentration of unknown samples, their absorbance was determined first. 27 test tubes were prepared with 3 replications and to each tube 5 ml of Benedict's solution were added. Then from each sample reducing sugar, 1ml was pipetted to each 5ml benedict solution held 27 tubes. The mixture was kept in water bath at a temperature of 90 ^{O}C for 5 min and filtered. Then the absorbance of each sample was read at 540 nm using UV-visible spectrophotometer (Annex 6a, b, c). Then the concentration and yields of unknown samples reducing sugar were calculated using Equation10 & 12, respectively.

$$\text{conc. of unknown sample(trsc)} = \frac{\text{absorbance of unknown sample(y)} - \text{y intercept(b)}}{\text{slop(m)}} \ldots\ldots\ldots (10)$$

After the concentration of total reducing sugar (TRS) calculated, the total reducing sugar in gram was calculated using (Equation 11).

$$TRS\ produced\ in\ gram = \frac{TRSC}{milliliter\ of\ solution} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (11)$$

$$TRSY(\%) = \frac{TRS\ produced\ in\ gram}{raw\ material\ used} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (12)$$

Where; TRSY = Total Reducing Sugar Yield; TRSC = Total Reducing Sugar Concentration

$$raw\ material\ used = \frac{gram\ of\ sample\ used}{mililiter\ of\ solution} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (13)$$

3.3.5. Fermentation

The aim of the experiment was to measure ethanol fermented by two different fermenting microbes (*S. cerevisiae* and *F. oxysporum*) at different fermentation days from hydrolyzed sedge biomass. The biocatalysts used for fermentation were taken from Ethiopian Biodiversity Addis Ababa Institute (EBI) (Annex 7a). Before fermentation starts, the best reduced sugar yield (49.83%) was then selected for fermentation by both fungal intermediates. Prepared solution from selected reducing sugar and media 1:10 (media to sample ratio) held flask was covering by aluminum foil. The fermentation was carried out under shaking incubator anaerobically at a temperature of 30 ^{o}C, pH 5.45 with 200 rpm. Aftes sample reducing sugar were fermented using two microbes separatelly, the data were taken at 3 days, 5 days and 7 days respectively (Annex 9a & b). Before conducting fermentation, media for yeast *S. cereviciae* and *F. oxysporum* were prepared under optimum condition for growth. Both Media were prepared by mixing the following nutrients in the proportions discussed below.

Media for yeast *S. cereviciae:* .The media for yeast *S. cereviciae* was prepared by a mixture of the following nutrients in their correct proportion. It was prepared in a 500 mL conical flask from 4g dextrose, 0.5g yeast extract (agar), 4g peptone, 2.5g Urea, 2.5g $MgSO_4.7H_2O$, and 250 mL distilled water. Then the pH of the media was adjusted to 5 and autoclaved at 121 ^{0}C for 15 minutes. 1.5 ml of yeast *S.* c*erevisiae* which obtained from Institute of Biodiversity, Addis Ababa, was dropped in a 350 ml conical flask. The media

solution held conical flask was properly covered with aluminum foil. It was then placed in a shaking incubator for 24 hours, a temperature of 30 ºC and 200 rpm for yeast growth (Annex 8b) (Wang *et al*., 2016).

Media for *F. oxysporum:* In order to prepare agar media for growth of *F. oxysporum* wild type f3 strain, 3 petri dishes glasses were prepared first with 90cm diameter and sterilized at autoclave with 121^0c or 15 minutes (Christakopoulos *et al*., 1996). From 2.9 gram of potato dextrose agar (PDA), 75 ml of media solution was prepared by 39 gram to 1 Littre of distilled water ratio. The prepared solution was heated in hot plate and then autoclaved by 121^0C for 15 min. The sterilized media was put to 3 Petri dish glasses by volume of 25 ml for each in sterilized condition (laminar air flow) (Annex 7b). After cooling down the agar, the fungus broth which acquired from Institute of Biodiversity, Addis Ababa was dispensed onto three agar containing petri dish glasses. The microbes were incubated for 3 days in an incubator at the temperature of 28 ºC (Annex 7c). The procedure for preparation of broth media for *F. oxysporum* was similar with the procedure for preparation of agar media. But their difference is only in sample state.100 ml of broth media was prepared in 250 ml conical flask from 3.9 g of potato dextrose broth and distilled water (Christakopoulos *et al*., 1996).

3.3.6. Distillation

The separation process of bioethanol from water was carried out using simple distillation. After fermentation, based on their boiling point difference ethanol were separated from water by simple distillation setup at a temperature of 78ºC with in 3 hours (Tekalign, 2018). The picture in annex 10a & b indicated that, produced ethanol before and after distillation.

3.3.7. Estimation of ethanol content using potassium dichromate solution

Ethanol standard curve preparation: First, potassium dichromate ($K_2Cr_2O_7$) solution was prepared from 325 ml of 96% of H_2SO_4 and 33.8g $K_2Cr_2O_7$ perlittre of distilled water (Annex 10c). Then 1ml of 0, 0.5, 1, 1.5, 2, 2.5, 3 % (v/v) standard ethanol solution was prepared with in 6 test tubes and 9 ml of distilled water was added into each standards by ratio of 1:10 sample ethanol per distilled water. From each 6 standard ethanol solution tubes, 1ml was taken to another 6 tubes and 2 ml of potassium dichromate solution was added to each 1ml standard ethanol solution tubes. After heating the solution in water bath at 60^{o}C for 20 min and rapidly cools down, the absorbance of standard ethanol solution was recorded at 600nm (Singh and Singh, 2015).

3.3.8. Estimation of ethanol content

To estimate sample ethanol concentration, 1ml of dilute sample ethanol solution was prepared from 1 ml of each sample ethanol and 9 ml of water by ratio of 1:10 Ethanol sample per distilled water in 6 tubes with 3replication. To each 1ml solution contained test tube, 2 ml of $K_2Cr_2O_7$ solution was added and heat at 60^{o}C water bath for 20 min. Then the Absorbance mixture was recorded at 600 nm and the concentration of sample ethanol was determined by using prepared calibration curve of standard ethanol. Finally the yield (%) of sample ethanol was calculated using Equation13.

$$\text{Percentage of sample ethanol yield } (\%) = \frac{(\text{Cs})}{\text{Cu}}\frac{(\text{Au})}{\text{As}} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (13)$$

Where; Cs= Concentration of standard ethanol

Cu = Concentration of sample ethanol

Au = Absorbance of standard ethanol

As = Absorbance of sample ethanol

3.3.9. FTIR analysis

After recovering the ethanol from the distillation column, the condensed liquid or distillate was analyzed by using prinks Elmer Spectrum 65 Fourier Transform Infrared (FT-IR). In order to identify the functional groups found in the ethanol, the % of absorbance was recorded in the form of transmittance in % with the wave number region for the analysis was recorded at 4000cm- to 400cm- in IR spectrum (Rubio-arroyo *et al*., 2011). The analysis was conducted in adis ababa university, Faculty of Natural Science, Department of Chemistry, Addis Ababa. Then, the percentage absorbance versus wavenumber was recorded and the spectrum was plotted.

3.3.10. Experimental design

The experimental design of chemical composition determination of sedge grass biomass collected were a complete randomized design (CRD) with three replications. The hydrolysis step experiment was laid out in factorial arrangement using CRD with three replications. The treatments studied include with three levels of temperature (115^{o}C,125^{o}C and 135^{o}C), three levels of time (40 min, 60 min and 80 min) and three levels of H_2SO_4 concentrations (1.5%, 2.5% and 3.5%) in hydrolysis. Whereas in fermentation, the treatments was with two levels of fermenting microbes (S. *cereviciae* and *F*. *oxysporum*) and data were taken at 3, 5 and 7 days respectivelly.

3.3.11. Statistical analysis

The data were subjected to analysis of variance (Proc Mixed model procedure) using SAS software version 9.0 (SAS Institute, 2009) to test treatments effects on dependent variables. Analysis of Variance (ANOVA) was conducted and mean comparison was done using Least Significant Difference (LSD) at 1% level of significance.

4. RESULTS AND DISCUSSION

In this section, the chemical composition (extractives, cellulose, hemicellulose and lignin) of the sedge grass, effect of hydrolysis variables (hydrolysis time, H_2SO_4 concentration and temperature) on total reducing sugar yield were discussed. The produced ethanol yields fermented by *S. cereviciae and F.oxysporum* were compared. Discussions of the results and comparisons with other studies were also given in this section.

4.1. Chemical Composition Analysis

In this study, it has been found that steam part of sedge grass consists of 39.87% cellulose, 42% hemicellulose, 13.07% of lignin and 5.06% extractives (Equation 1-5 in section 3 in figure 3). The result of this study was found to be comparable with the report below about grass biomass components. As report of Marriot *et al*., (2016) indicates, the main constituents of plant secondary cell walls are cellulose, hemicellulose, lignin, and some amount of extractives that consists of nonstructural aromatic compounds such as volatile oils, chlorophyll, fatty acids, and their esters, waxes and resins. These are present in varying proportions in different feedstocks. Estimation of chemical components of lignocellulosic biomass, is important in order to determine the conversion efficiency of biomass to bioenergy and other valuable chemicals (Timung *et.al*., 2016). According to report of Sun and Cheng, (2002), the chemical composition of all grass biomasses are 25 - 40 % cellulose, 35 - 50% hemicellulose, lignin 10 - 30% and 1 - 5% extractive. Another similar study reported on cellulose, hemicellulose and lignin component of grass biomass is 35-45%, 40-50% and 20%, respectively (Marriot *et al*., 2016). The composition of these different components and their proportion varies depending on the type of biomass, part of the plant material and at what time it was harvested or cultivated, which is why a unified

pretreatment technique couldn't be used for all the biomass (Seid and Goulart, 2016; Tye *et al*., 2017). So, the slight difference of the present finding with report of similar work could be attributed to different reasons such as type of grass biomass, the environmental coditions in which it is cultivated and the experimental works. The higher the cellulose and hemicellulose content, the higher glucose and xylose could be obtained. In similar way, the lower the lignin contents, the easer hydrolysis condition, and less formation of toxic chemicals such as, aromatic, polyaromatic, phenolic and inhibitory compounds (Mussatto and Roberto, 2004).

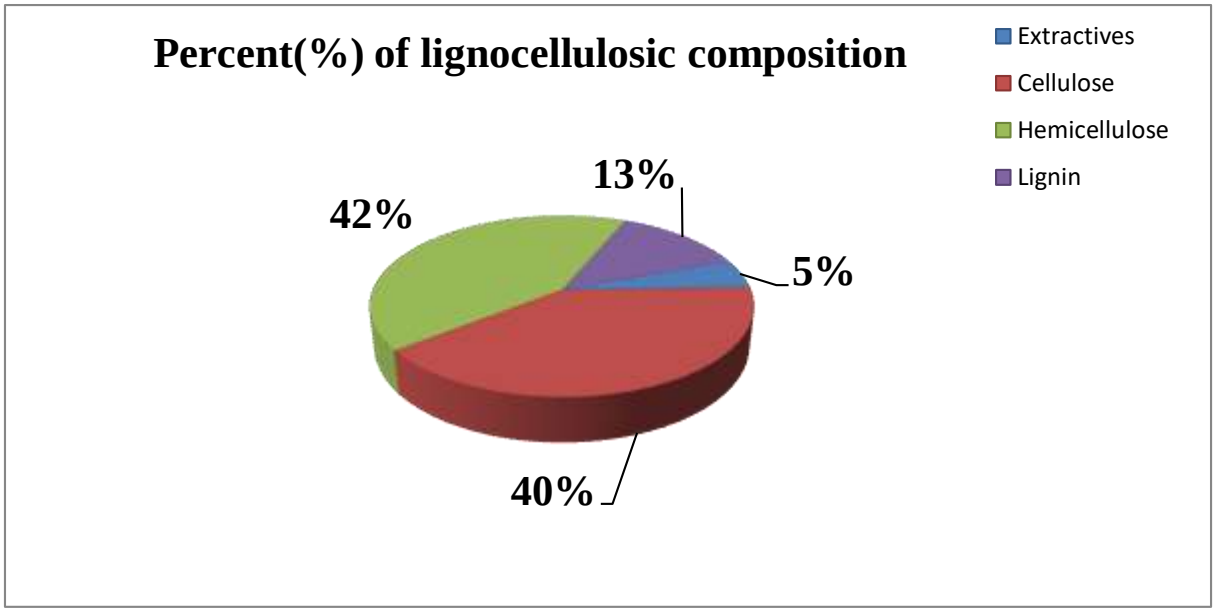

Figure 3: The percent of chemical composition of sedge grass biomass

4.2. Effects of Hydrolysis Factors on Total Reducing Sugar Yield

The yield of total reducing sugars hydrolysed from lignocellulosic biomass is affected by many factors such as solid to liquid ratio, particle size, part of the plant, hydrolysis time, temperature, and acid concentration (Sindhu *et al*., 2014).In present study, the powder sedge grass hydrolysis was investigated at basic parameters such as concentration of H_2SO_4, hydrolysis time and hydrolysis temperature.

The total reducing sugar yield usually increases to a maximum and then decreases due to reversion and degradation of sugars. As a dependent variable, the total reducing sugar yield has significant effect at a time of pretreated biomass hydrolysis. The statistical difference between the main effect of the treatments, two way and three way interactions that compared at 1% probability level ($p < 0.01$) were significantly affected for total reducing sugar yield. The total reducing sugar was determined based on the calibration curve constructed from standard glucose solution using benedict's solution (Singh and Singh, 2015). The calibration curve plotted by standard glucose concentration verses absorbance at 540 nm was also plotted (Figure 4).

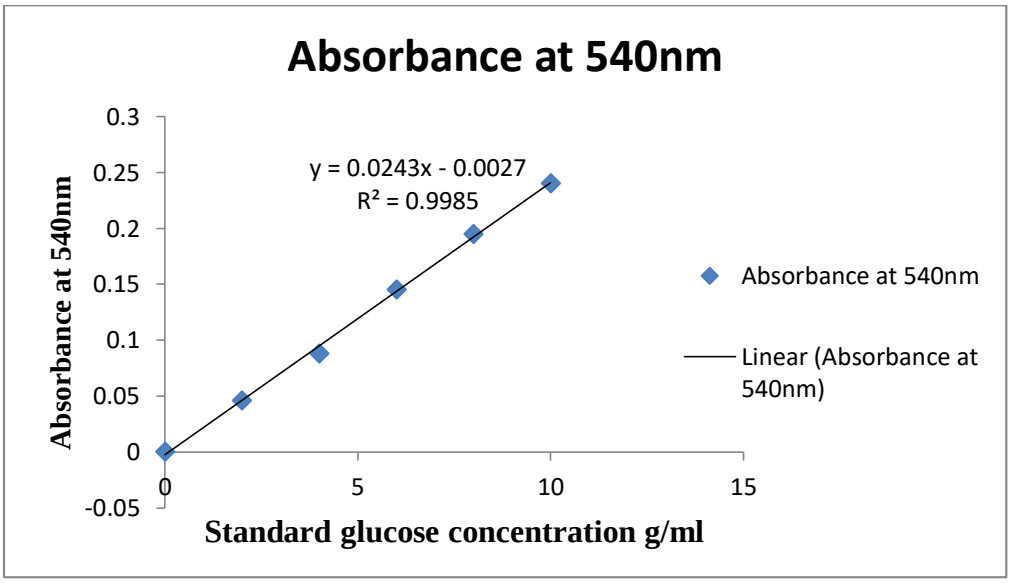

Figure 4: The calibration curve of standard glucose

4.2.1 Main effects of hydrolysis factors on total reducing sugar yield

At constant time 60 min and 1.5% of H_2SO_4, the effect of hydrolysis temperature on the yield of total reducing sugar at constant time and acid concentration of the present study is given below (Table 3). The yield of total reducing sugar was found to increase from 42.8 - 45.02% as temperature increased from 115^oC to 125 ^oC. Maximum yield of total reducing sugar was obtained around 125^oC hydrolysis temperature. Beyond 125^oC hydrolysis temperature, the yield was slightly decreased. The reason that decreases in total reducing

sugar yield at low temperature might be due to insufficient enough to convert cellulose and hemicellulose to glucose and xylose on the hydrolysis stage. Because of the intractable nature of lignocellulose, low temperature may not be able to break its glucosidic hydrogen bond (Balat and Balat 2009). On the other hand, the amount of total reducing sugar yield was also low at high temperature and this is might be due to the reason that lignocellulose was converted to other byproducts rather than to glucose (Mahelete *et al*., 2019). As reported by Taherzadeh and Karimi, (2007), hydrolysis temperature play crucial role on the yield of total reducing sugar in harvesting from lignin contained biomass. In similar way the report of Chandel *et al*., (2012) in brewer`s spent grain states, the reaction temperature dominatly affects the the yield of total reduced sugar which is extracted from lignocellulosic biomass.

Table 3: Effect of temperature, hydrolysis time and H2SO4 concentration on TRSY (%)

Treatments	% TRSY	SE
Temperature	***	
115^0C	42.8c	
125^0C	45.02a	± 0.014
135^0C	43.5b	
LSD	0.04	
Hydrolysis Time	***	
40min	41.89c	
60min	45.5a	±0.014
80min	43.42b	
LSD	0.04	
H_2SO_4 conce.	***	
1.5%	44.51a	
2.5%	44.18b	± 0.014
3.5%	42.63c	
LSD	0.04	
CV	0.168	

Where; TRSY (%) = Total Reducing Sugar Yield in percent, LSD = Least Significant Difference CV= Coefficient of Variation, Values are expressed as mean and means in different letters are statically significant according to LSD test and (***) indicates very highly significance (significant at P<0.01)

The effect of hydrolysis time on the yield of total reducing sugar at constant temperature and acid concentration was also investigated in this study (Table 3). At constant temperature 125^{o}C with 1.5% of H_2SO_4, the yield of total reducing sugar was increased from 41.89 – 44.51% as the hydrolysis time increases from 40 min to 60 min. The higher yield of total reducing sugar was obtained at around 60 min of optimum hydrolysis time. Beyond 60min of hydrolysis time, the total reducing sugar yield is decrease. This result indicated that the composition of cellulose and the branched structures of hemicellulose separated or hydrolyzed efficiently at optimum hydrolysis time 60 min. This result is consistent with the report of Cantero *et al.*, (2015) in the study carried on wheat bran stated that at short reaction time of the hydrolysis happen, the cellulose and hemicellulose is less hydrolysis to sugar efficiently and less recovered.

The result of current study showed that the total reducing sugar yield decreases as the acid concentration increases and reaches maximum in small % of H_2SO_4 for hydrolysis at constant temperature 125^{o}C and time 60 min (Table 3). The maximum reducing sugar yield (44.51%) was produced from hydrolyzed sedge sample by concentration of 1.5% H_2SO_4 followed by 44.18 and 42.63% of reducing sugar yield from concntration of 2.5 and 3.5% dilute H_2SO_4 hydrolyzed sample, respectively. This shows that low concentration of acid hydrolysis is more effective in simple sugar production than higher acid concetration of (2.5 & 3.5 %) H_2SO_4. So, the results were similar with report of Kefale *et al*., (2012) and Mahelete *et al*., (2019) in wet coffee and highland bamboo hydrolysis shown that the amount of sugar obtained was decreases from 44.51%-42.63% as the acid concentration increases from 1.5%-3.5% and reaches maximum in small acid hydrolysis. The decrease of reducing sugar yield in sample treated with increasing of acid concentration may be due to degradation of monomeric sugars (xylose, glucose) to furfural

and HMF (hydroxy methyl furfural) or may be derived from dehydrating or oxidizing by sulfuric acid on glucose. it could also be attributed from the conversion of glucose to levulinic and formic acid which leads to decrease in glucose yield (Loow *et al*., 2016). these excess substances are toxic substances for yeast and can inhibit the yeast growth (Kefale *et al*., 2012).

4.3. Interaction Effects of Hydrolysis Factors on Total Reducing Sugar Yield

A research carried out on the hydrolysis of lignocellulose biomasses showed that there are different factors that affect the total reducing sugar yield (Sun and Cheng, 2002). Besides the effect of single factors on hydrolysis, the interaction effect also influences the total reducing sugar yield. Effects on total reducing sugar are dual and three way interactions. The dual combination effect suchas temperature with acid concentration, temperature with hydrolysis time and hydrolysis time with acid concentration on total reducing sugar yield were found to be significantly affect the total reducing sugar yield at $P<0.01$ as shown in (Annex 11). Like two way interaction, three way interactions of treatments that compared at 1% probability level ($p < 0.01$) were also significantly affected for total reducing sugar yield.

4.3.1. Effect of temperature and time on total reducing sugar yield

The effects of hydrolysis time and temperature on total reducing sugar yield with constant 1.5 % of acid concentration was investigated in this study. The investigation was regarded to interaction effects of two factors including temperature (115, 125 and 135 ^{0}C) with time (40, 60 and 80 min) on total reducing sugar yield (Figure 5). The highest total reducing sugar yield (47.23%) was obtained at a temperature of 125 ^{0}C and 60 min. Whereas, the lowest yield of total reducing sugar (39.91%) was hydrolyzed at a temperature of 115^{0}C

and 40 min of hydrolysis time. Therefore, for the production of highest total reducing sugar, 125 ^{0}C of hydrolysis temperature and 60 min of hydrolysis time were used. However, total reducing sugar yield was found to decrease at increase in temperature and longer hydrolysis time brought. The finding was in agreement with the finding of Timung *et al*., (2016) reported on sugarcane bagasse biomass to ethanol. This is due to sugar degradation to inhibitors such as 5-HMF or furfural but requires more time and higher temperature to increase the reaction rate for sugar release (Loow *et al*., 2016). In similar way, decreasing in both hydrolysis temperature and hydrolysis time resulted in reduced the reducing sugar leve due to small reaction.

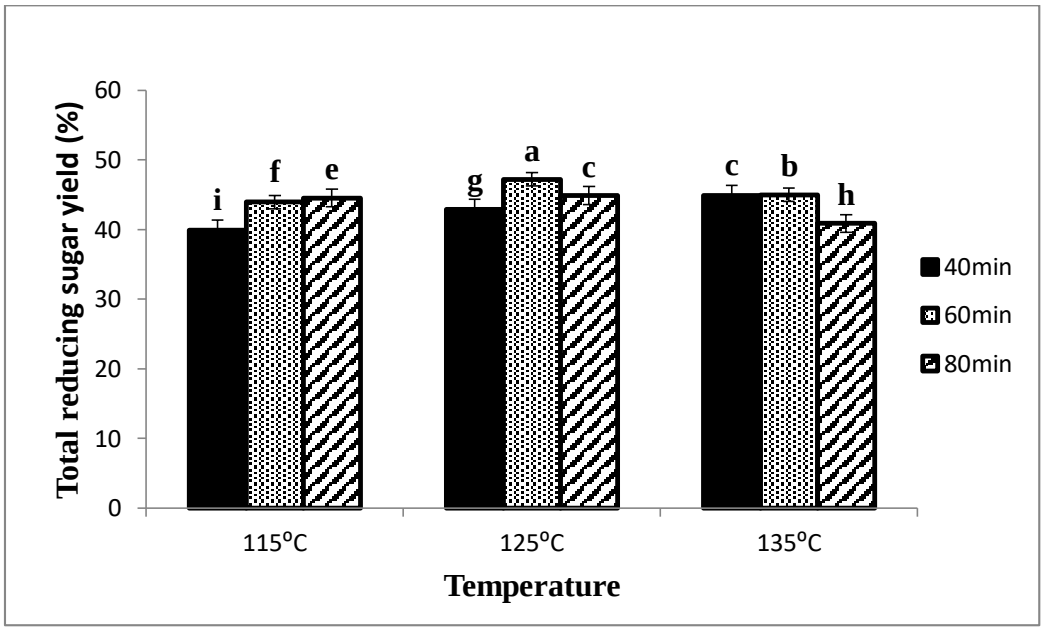

Figure 5: Interaction effect of temperature with hydrolysis time on total reducing sugar yield with constant 1.5% of H_2SO_4 concentration.

4.3.2. Effect of temperature and acid concentration on total reducing sugar yield

As indicated in Figure 6, the interaction of acid concentration and temperature were affected the reducing sugar yield, when hydrolysis time is constant 60 min.

The effect of interaction of temperature with H_2SO_4 concentration on total reducing sugar yield was done at a temperature of (115,125 and 135^{0}C) with 1.5, 2.5 and 3.5% of dilute

H_2SO_4. At combination of 2.5% H_2SO_4 concentration and 125 ^{0}C hydrolysis temperature, the highest total reducing sugar yield (46.22%) was obtained. In similar way at 1.5% H_2SO_4 concentration with high temperature 135 ^{0}C, the reducing sugar yield was also high (46.17%). There is no significatly difference in ($p \leq 0.01$) between these two combinations. Whereas, in combination of 3.5% H_2SO_4 concentration and 135^{0}C hydrolysis temperature, the significantly lower ($p \leq 0.01$) total reducing sugar yield (41.09%) was obtained (Table 6). Therefore, lower temperature and increase in acid concentrations resulted in an increase of total reducing sugar yield. conversely at higher acid concentrations and increase in temperature brought reduced total reducing sugar yield. This report was similar with the reports of Biniyam *et al*., (2016) and Mahelete *et al*., (2019) on *P. juliflora* and bamboo to ethanol indicates that, the increasing in both hydrolysis temperature and acid concentration results in decreased total reducing sugar yield. They also reported on lower acid concentrations and increase in temperature resulted in an increase of total reducing sugar yield. The decrease in the yield of total reducing sugar at high temperature and high acid concentration during hydrolysis leads to sugar conversion into hydroxymethylfurfural (HMF) and methyl furfural (Sindhu *et al*., 2014). the interaction effect of hydrolysis temperature and acid concentration on total reducing sugar yield is presented below (Figure 6).

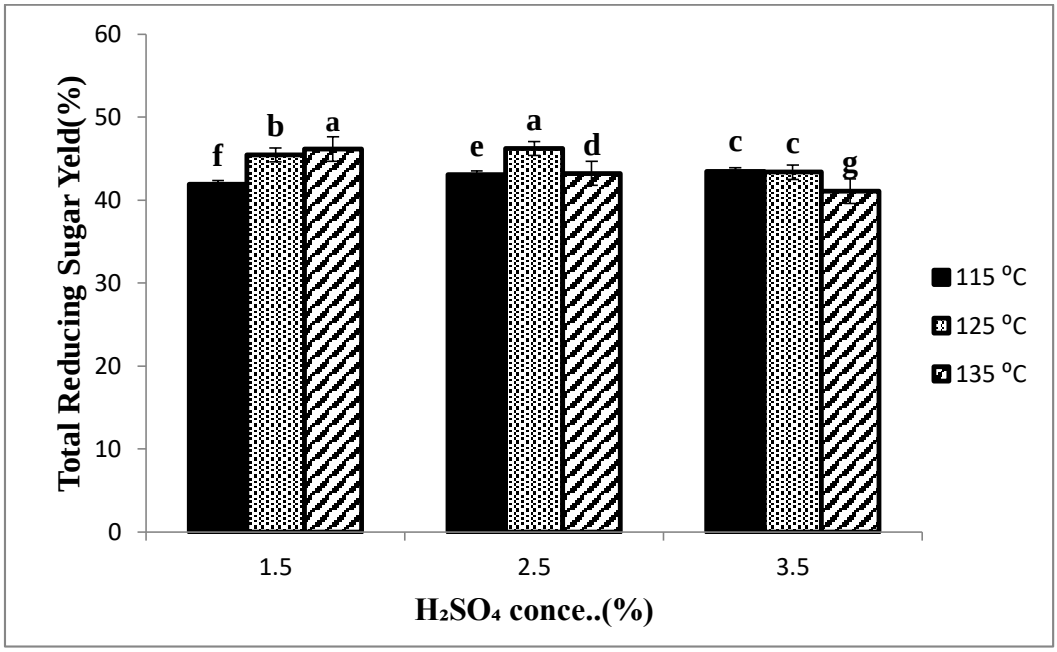

Figure 6: Interaction effect of temperature and H_2SO_4 concentration on total reducing sugar yield with constant hydrolysis time of 60 min.

4.3.3. Effect of time and H_2SO_4 concentration on total reducing sugar yield

The interaction effects of two factors such as H_2SO_4 concentrations with (1.5, 2.5 and 3.5%) and hydrolysis time (40, 60 and 80 min) at constant temperature of 125^oC on total reducing sugar yield was investigated as shown in figure 7. In such combination, significantly higher total reducing sugar yield (46.4%) at $p \leq 0.01$ hydrolyzed with in 60 min and 2.5% of H_2SO_4 concentration was recorded. It had not significantly difference with combination of 1.5% H_2SO_4 concentration and 60 min of hydrolysis time with 46.36% of reducing sugar yield at $p \leq 0.01$. However, significantly lower total reducing sugar yield (41.07%) at $p \leq 0.01$ was obtained in 80 min and 3.5% of H_2SO_4 concentration hydrolysis.

As a result, at lower H_2SO_4 concentration and increase in time resulted in increasing of total reducing sugar yield. Conversely, at higher H_2SO_4 concentrations and increase in time brought a decrease in reducing sugar yield. This is due to the conversion of sugar into

furfural compounds (Biniyam *et al*., 2016). This finding was in line with the report of Hailemichael, (2016) on ethanol production from avocado seed.

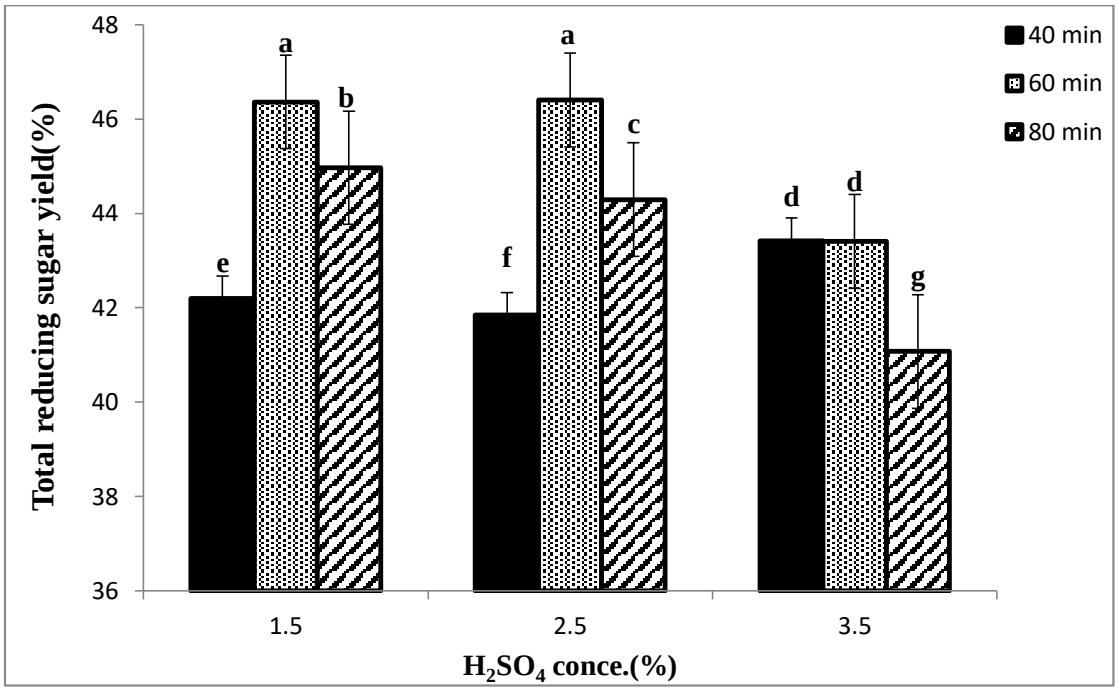

Figure 7: Interaction effect of hydrolysis time and H_2SO_4 concentration on total reducing sugar yield with constant temperature of 125^oC.

4.3.4. Three way interaction effects on total reducing sugar yield

In this section, the effects of three way interaction conditions (i.e., H_2SO_4 concentration, temperature and hydrolysis time) on total reducing sugar yield were studied. Three-way interaction effects of the three treatments on total reducing sugar yield showed that, the total reducing sugar yield were decreased with decrease in time and temperature (Figure 8). It also decreases when high acid concentration at high temperature and long hydrolysis time but increases with optimum temperature, hydrolysis time and H_2SO_4 concentration. Based on this, the maximum total reducing sugar yield were noted on 2.5 % H_2SO_4 concentration, at a temperature of 125 ^0C and 60 min of hydrolysis time (Figure 9). In this condition, the recorded total reducing sugar yield was 49.83%. But the decreasing in total reducing sugar yield was observed at high H_2SO_4 concentration, high temperature and long

hydrolysis time. At that moment 38.44% of total reducing sugar yield was recorded at temperature of 135 ^{0}C, 60 min of hydrolysis time and 3.5% H_2SO_4 concentration. So, the result of the present study was in agreement with the study of Timung *et al*., (2016) on sugarcane bagasse. The report states the lower yield of total reducing sugar was produced in higher hydrolysis temperature, acid concentration and hydrolysis time. This is may be due to formation of fermentative inhibitors like HMF, furfural, formic acid, and acetic acid, which also due to affection of microbial growth during the fermentation process and other value added products (Rajan and Carrier, 2014). On the other hand, the other condition that lowers the total reducing sugar yield was at lower temperature, law H_2SO_4 concentration and short time of biomass haydrolysis. According to report of Lenihan *et al*., (2010), it is due to less converson of lignocellulosic biomasses to five and six carbon sugars, before convertion into ethanol.

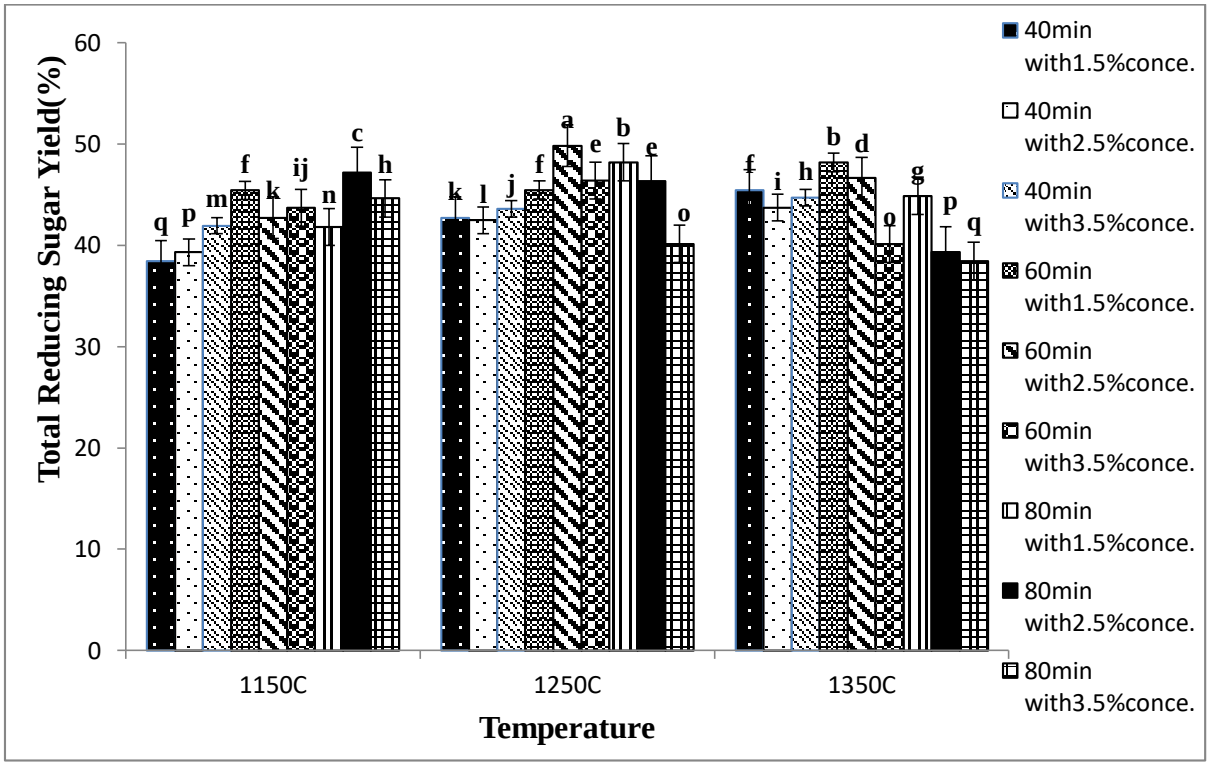

Figure 8: Interaction effect of temperature, hydrolysis time and H_2SO_4 concentration on total reducing sugar yield.

4.4. Ethanol Yield

From the experimental work on hydrolysis of sedge grass biomass, the maximum reducing sugar yield produced (49.83%) was selected for fermentation to ethanol. The ethanol yield were determined based on the calibration curve constructed from standard ethanol solution using potassium dichromate method. Figure 9 shows the calibration curve of standard ethanol solutions measured at 600 nm. The yield of ethanol produced was then calculated by using Equation 13.

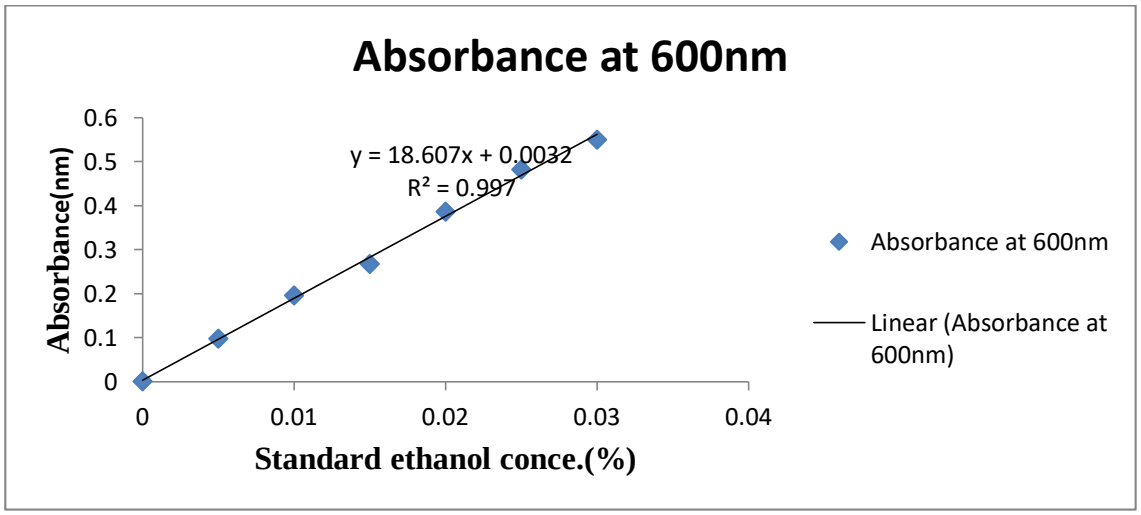

Figure 9: The calibration curve of standard ethanol

4.4.1. Effect of fermenting microbes and fermentation days on ethanol yield

The interaction of fermenting microbes and fermentation days showed a significant ($P \leq 0.01$) influence on ethanol yield % (Annex 12). As the result indicated, *F. oxysporum* gave highest value (32.65%) of ethanol yield while the minimum ethanol yield (20.57%) was recorded from *S.cerevisiae* fermenting treatment (Table 4). The result also revealed that ethanol yield at 3 days of fermentation was highest (30.97%). Whereas the recorded value of ethanol was diminished with the 5 days of fermentation (22.8%) and 7 days of fermentation records better yield of ethanol than 5 day fermentation (26.6%).

Table 4: Effects of fermentation days and fermenting microbes on Ethanol yield (%)

Treatments	EY (%)	SE
Microbes	***	
S.*cerevisiae*	20.57[b]	±0.46
F.*oxysporum*	32.65[a]	±0.46
LSD	1.44	
Fermentation days	***	
3 days	30.97a	± 0.57
5 days	22.8c	± 0.57
7 days	26.06b	± 0.57
LSD	1.76	
CV	2.17	

Whereas EY = Ethanol Yield, LSD = Least Significant Difference CV= Coefficient of Variation, Values are expressed as mean and means in different letters are statically significant according to LSD test and (***) indicates highly significance (significant at P<0.01).

The maximum ethanol yield (51.78%) was obtained from hydrolyzed biomass fermented by *F. oxysporum* at 7 days and the least (0.34 %) of ethanol yield was obtained in *S.cerevisiae* fermenter at 7 days fermentation time. Ethanol yield were decreased with prolonged fermentation days on *S. cerevisiae* fermenter. Whereas fermentation of sample reducing sugar by the help of microbe *F. oxysporum* fermenter were increased with prolonged fermentation days (Figure 10).

The present investigation revealed that, *F. oxysporum* fermenter produces more ethanol due to non-selecting conversion ability. However, it needs long time, at least 7 days to end fermentation. The finding was consistent with report of Anasontzis and Christakopoulos, (2014) that states *F. oxysporum* has the ability to produce a wide range of biomass degrading enzymes and can generally use both hexoses and pentose. However, its rate of fermentation was slow that it takes days to complete. Though, *S. cerevisiae* fermenter can

end the fermentation process at short period of time within 3 days compared to *F. oxysporum*. But less ethanol yield was recoded because it only ferments 6 carbon monomers to ethanol. The result of this study was found to be in line with report of Anasontzis and Christakopoulos, (2014) states that the several drawbacks of *S. cerevisiae* hinder its use in lignocellulose-based processes; these include its dependence on previous chemical or enzymatic steps in order to break down the plant cell wall of the biomass to monosaccharides and its inability to metabolize pentose such as xylose. Thus, looking for filamentous fungus like *F. oxysporum* and other genetically modified microbes are recommendable.

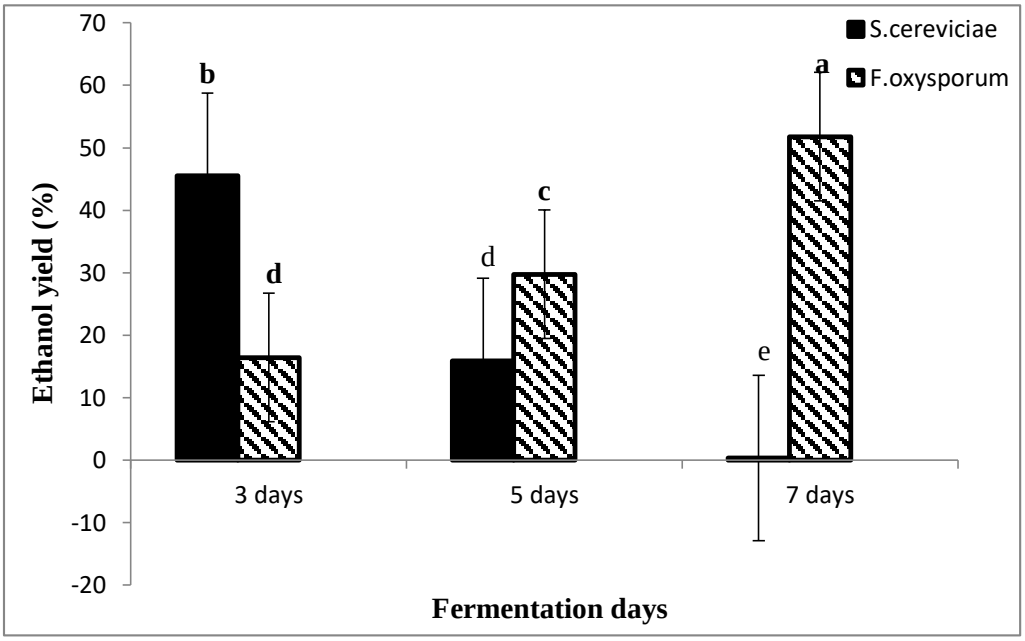

Figure 10: Interaction effect of microbes and fermentation days on ethanol yield

4.5. FT-IR Characterization of Produced Bioethanol

The examination form in region wave number displayed the average-infrared array is 4000-400 cm^{-1}. The O-H, C-H, CO stretching vibrations associated have characteristic IR absorptions of Alcohols. Broadband specified the O-H stretch of alcohol in the region of 3500-3200 cm^{-1} with incredibly concentrated when it runs like a liquid layer, while the region 1260-1050 cm^{-1} proves the C–O stretch. The groups at about 2880-2930 cm^{-1} is allocated as the symmetric elongating modes of the $–CH_2$- CH_3 groups (Coates and Meyers, 2000; Yu *et al*,. 2007). This makes certain that the product obtained from Sedge grass is absolutely ethanol due to the verification of these regions (Figure 11).

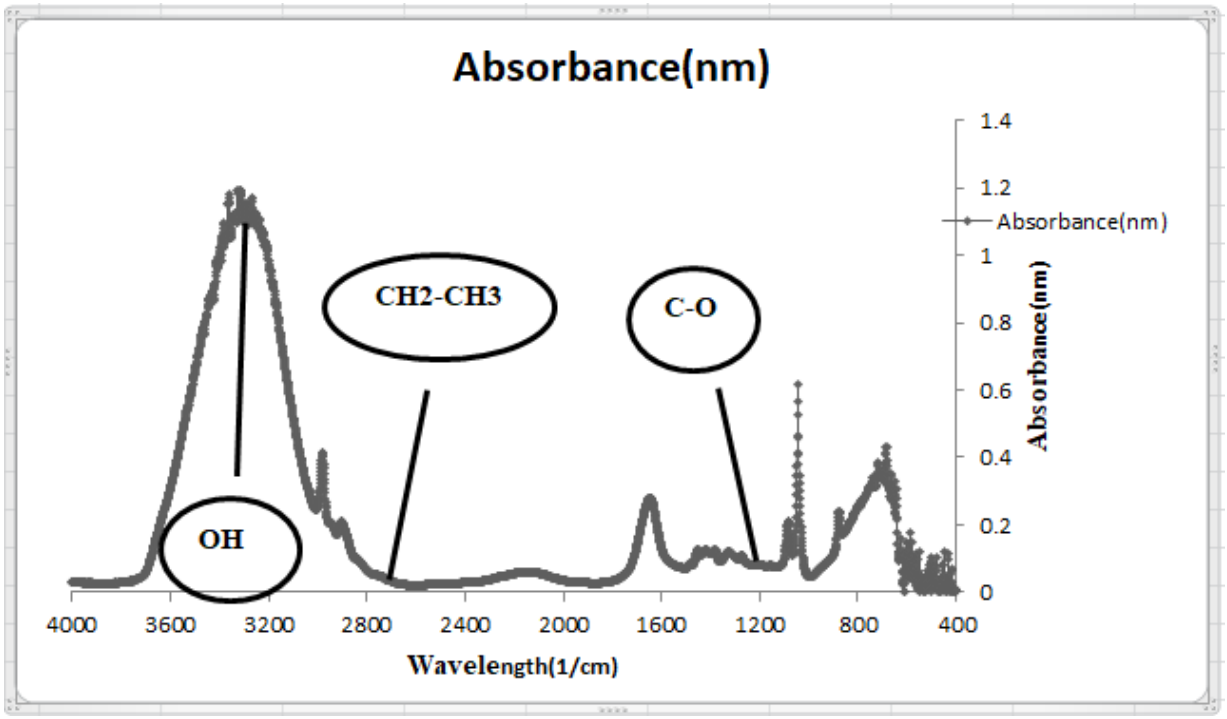

Figure 11: Fourier Transform Infrared spectra of the produced bioethanol from sedge grass.

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

One of the most important factors to provide the maximum yield of fermentable sugar is determination of lignocellulosic composition and suitable conditions in acidic hydrolysis. In this study, effects of hydrolysis factor (temperature, acid concentration and hydrolysis time) on total reducing sugar hydrolyzed from Lignocellulosic sedge biomass and effects of fermentation time and microbes (*S. cerevisiae* and *F. Oxysporum*) on ethanol yield was investigated. Those all three treatments (115 ^{0}C, 125 ^{0}C and 135 ^{0}C), time (40min, 60min and 80min) and H_2SO_4 concentration (1.5%, 2.5% and 3.5%) were found to be the significant variables for the yield of total reducing sugar.

The higher hemicellulose and cellulose with lower lignin content was recorded in sedge grass biomass with 42% hemicellulose, 39.87% cellulose, 13.07% lignin and 5.06% extractives. At a temperature of 125 ^{0}C, 2.5% concentration of H_2SO_4 and 60 min of hydrolysis time, hydrolyzed sedge grass biomass gave the highest total reducing sugar yield with 49.83%. Besides that, the highest ethanol was produced from fermented biomass by *F.oxysporum* in 7 days fermentation. Due to the low lignin content of this biomass, hydrolysis of cellulose and hemicellulose to reduced sugar at low H_2SO_4 concentration was done in short period of time and easily processed. As the FTIR spectrum of the end product indicated the presence of O-H, C-H, and C-O, the fermentation product of sedge biomass collected from Lake Hawassa was ethanol. High acid concentration, low and very high temperature and very high and low retention time have negative effect on the yield of total reducing sugar. In conclusion, the present investigation revealed that sedge grass contains

high cellulose and hemicellulose with low lignin content, and found to be a potential feedstock for bioethanol production.

5.2. Recommendation

Based on the result of the present study, the following recommendations are forwarded:

- Sedge grass contains high cellulose and hemicellulose with law lignin content should be preferable feedstock for bioethanol production.
- Research on fermentation of other similar grasses by the help of *F. oxysporum* fermenter to convert zylose and pentose to ethanol completely had better be required.
- Sedge grass biomass for the present study is collected from one place and the experiment conducted in a single season. The work needs to be repeated by taking the different factors in to consideration.

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7. APPENDICES

Appendix Figures and Tables

(Photo taken by Freweyni, January 2020)

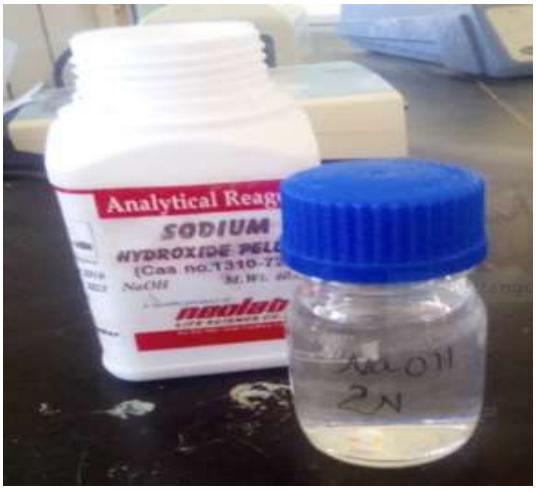
Annex 1: (a) Sedge grass from Lake Hawassa (b) H_2SO_4 pretreated powder sample

Annex 2: (a) Soxhlet extraction setup (b) Extractive free sampe

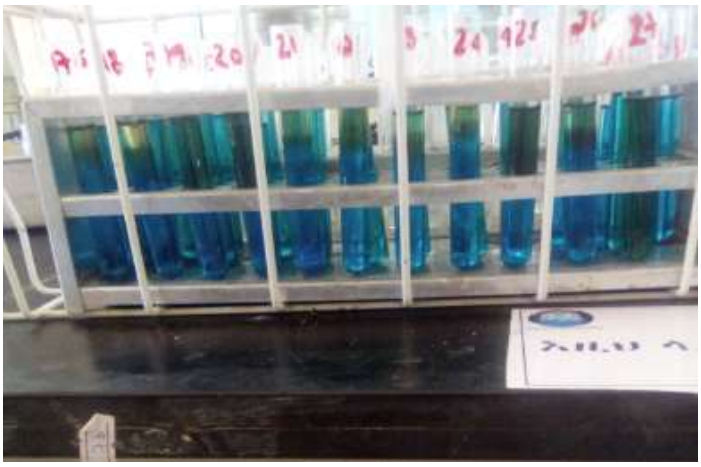
Annex 3: Prepared dilute H_2SO_4 Solution

a,  **b,** 

Annex 4: (a) Sample with diluted H_2SO_4 solution ready for hydrolysis (b) hydrolysis sample solution in autoclave.

a,  **b,** 

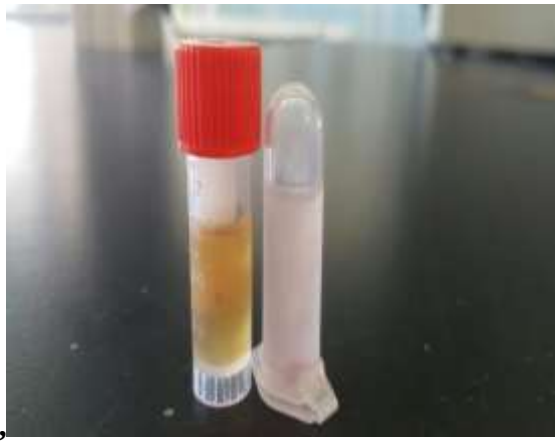
Annex 5: (a) Hydrolyzed and filtered sample reducing sugar (b) Sodium hydroxide solution preparation.

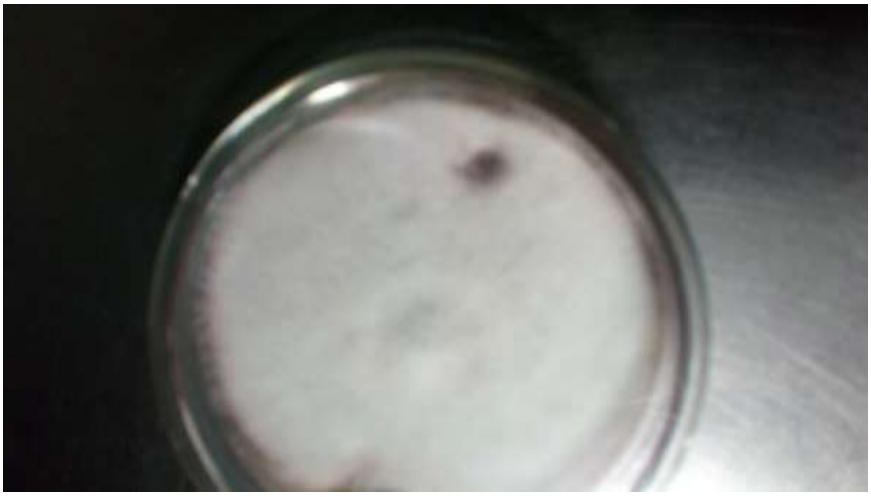
a, 

b, 

c, 

Annex 6: (a) mixture of benedict solution and hydrolyzed sample (b) heated mixture in water bath (c) Absorbance reading by spectrophotometer.

a,  **b,** 

c, 

Annex 7: (a) *F.oxysporum* taken from EBI Adisababa (b) preparation of agar media for *F.oxysporum*(c) *F.oxysporum* cultured in potato dextrose agar media.

Annex 8: (a) the whole chemicals and reagents used (b) broth media for *S.cereviciae* growth

a,  **b,** 

Annex 9: (a) Sample ready for fermentation (b) Fermentation in shaking incubator

a, 

b,  **c,** 

Annex 10: (a) fermented solution before distillation (b) Produced ethanol (c) prepared potassium dichromate solution.

Annex 11: ANOVA table for total reducing sugar yield (%) under different factors (temperature, time and H_2SO_4 concentration)

SV	DF	SS	MS	F Value	P Value
Temperature	2	69.548	34.77	5793.77	<.0.001***
Time	2	118.064	59.03	9835.35	<.0.001***
Acid	2	54.108	27.05	4507.49	<.0.001***
Temperature*time	4	172.089	43.02	7168.00	<.0.001***
Temperature*acid	4	113.566	28.39	4730.34	<.0.001***
Time*Acid	4	89.217	22.03	3716.13	<.0.001***
Temperature*Time*Acid	8	145.239	18.15	3024.81	<.0.001***
Error	54	0.324	0.006		
Total	80	762.157			
CV					

*Where, *** indicates significant difference at P ≤ 1% probability levels, ANOVA= analysis of variance, SV = Source of Variation, DF = Degree of Freedom and SS = Sum Square, MS = Mean Square, DF= Degree of Freedom and CV = Coefficient of Variance.*

Annex 12: ANOVA table for ethanol yield in % under different microbes and fermentation days

SV	DF	SS	MS	F Value	P Value
Microbes	1	656.608	656.6	333.39	<.001***
Fermentation	2	203.263	101.63	51.60	<.001***
Microbes *Fermentation day	2	4875.579	2437.78	1237.79	<.001***
Error	12	23.633	1.969		
Total	17	5759.08			
CV					

*Where, *** indicates significant difference at P ≤ 1% probability levels, ANOVA= Analysis of Variance, SV = Source of Variation, DF = Degree of Freedom and SS = Sum Square, MS = Mean Square, DF= Degree of Freedom and CV = Coefficient of Variance.*

8. BIOGRAPHICAL SKETCH

The author, Freweyni Hailu was born on May 2, 1994 GC at Fatsi, East Zone, Tigray regional state, Ethiopia. She attended her primary education (1-8 grade) at Fkada elementary school and completed her secondary and preparatory education (9-12 grade) at Yemane secondary school in Fatsi Adigrat.

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