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Isolation of  $\alpha$ -Amylase Producing Fungi from South western part of Ethiopia,  
Characterization and evaluation of the enzyme for bio-ethanol Production

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## Lists of Abbreviations

$\alpha$	Alpha
AAUam	Addis Ababa University amyase
$^{\circ}\text{C}$	Degree Celsius
Dd	Distilled
DNS	Dinitrosalicylic acid
GHG	Green House Gas
g	Gram
hr	Hour
L	Liter
mm	Millimeter
OD	Optical Density
%	Percent
PDA	Potato Dextrose Agar
Rpm	Revolution per minute
SHF	Separate hydrolysis and fermentation
SSF	Solid State Fermentation
TLC	Thin Layer Chromatography
WB	Wheat Bran

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## **Abstract**

*Fungi are well known for their ability to excrete enzymes in to the environment. The fungal isolate (Designated as AAUam13) which was isolated from coffee effluent of shesheka (Jimma zone) was the best alpha amylase producer among thirty isolates obtained from south western part of Ethiopia which were screened and evaluated for amylase production. The morphological study of isolate AAUam 13 indicated that the isolate belongs to genus Aspergillus. The optimal initial pH and temperature for amylase production by the same isolate was found to be 5 and 30°C respectively. Yeast extract and soluble starch were found to be the best nitrogen and carbon sources for amylase production respectively. The enzyme alpha amylase was optimally active at 35 °C and pH5. Alpha-amylase production by co-cultivation of the selected isolates showed higher activities than single cultures. The co-culture of isolate AAUam13 and AAUam15 was higher than individual activities of AAUam 13 and AAUam15. Bioethanol production was achieved by separate hydrolysis and fermentation process using the selected fungal isolate and yeast. The bioethanol concentration was measured quantitatively with potassium dichromate and sulphuric acid method. Yeast gave the highest concentration of ethanol as inoculated in the pretreated media than the yeast inoculated in the untreated media after 72 hours.*

**KEY WORDS:** *Aspergillus, Enzymes, Ethanol, Fermentation, Substrate.*

# 1. Introduction

## 1.1. Background of the Study

Amylase is a general name given for starch hydrolyzing enzymes. Starch is a glucose polymer linked together by a linear  $\alpha$ -1, 4 & branched  $\alpha$ -1, 6 glycosidic bonds. Amylases act by hydrolyzing the linear  $\alpha$ -1, 4 and branched  $\alpha$ -1, 6 glycosidic bonds between adjacent glucose units of the polymer (Bernfeld, 1951).

Amylases are classes of industrial enzymes that constitute approximately 25% of the enzyme market (Yasser *et al.*, 2009). The first enzyme produced industrially from a fungal source in 1894, which was used for the treatment of digestive disorder (Crueger and Crueger, 1984). Amylases are produced by variety of organisms, such as bacteria, fungi, plant and animals. Bacteria and fungi secrete amylases to the outside of their cells to carry out extracellular digestion. Amylase is present in human saliva, where it begins the chemical process of digestion. In storage tissues such as seeds, starch a polysaccharide of glucose is a hydrolyzed for utilization by the growing seedlings to meet its energy requirement (Hema *et al.*, 2006).

Alpha-amylase cleaves internal  $\alpha$  -1, 4 glycosidic linkages in starch to produce glucose, maltose, or dextrans. Glucoamylase (amyloglucosidase) in turn hydrolyses  $\alpha$ -1, 4-glycosidic bonds from non-reducing ends of starch molecules, resulting in the production of glucose. To a lesser extent, glucoamylase also has the ability to hydrolyze  $\alpha$ -1, 6 linkages, also resulting in glucose as the end product (Mertens and Skory, 2006). Fungal  $\alpha$ -amylase and glucoamylase may be used together to convert starch to simple sugars, which in turn serve as a feedstock for production of bioethanol or in the production of high fructose syrups (Seviek *et al.*, 2006). Fructose syrups are important sweetener in food processing industries like beverages, bakeries and confectionaries. The use of these sugars for producing bioethanol leads to opportunities for farmers by increasing demand for their products, resulting in a boost in rural economies (Olfert and Weseen, 2007). Bioethanol, is an alternative source of energy that has received special attention over the world due to depletion of fossil fuels. According to United States Department of Energy, for every unit of energy put towards ethanol production, 1.3 units are returned (Hill *et al.*, 2006). Ethanol has the advantages of being renewable, cleaner burning and produces no greenhouse gases (Altintas *et al.*, 2002).

The efficiency of bioethanol production largely depends on the availability of suitable substrate, yeast strain and method employed. Starch, a macromolecular polymer of glucose units, is a significant component of domestic and commercial waste and a useful resource that can be converted into ethanol. Yeast is directly used to ferment sugar cane and sweet sorghum. But, starchy food items need reduction into glucose and oligosaccharides (Biol *et al.*, 1998). This task is achieved by two enzymes:  $\alpha$ -amylase, which hydrolyzes  $\alpha$ -1, 4 linkages and glucoamylase, which hydrolyze  $\alpha$ -1, 4 and  $\alpha$ -1, 6 linkages in starch molecules. Yeast can be cultivated in the fermentation media which was pretreated with fungal amylases to produce ethanol.

In Ethiopia there are around twenty five leather tanning industries which involve different chemicals which are not environmental friendly and cost effective and also there are a number of brewery industries which totally import 40, 000 tone malt or half of the malt they require and costs 40, 000 us dollar annually. All of these processes can be substituted by starch hydrolysates and or enzyme hydrolysis.

This experiment is initiated in order to isolate, identify, screen and evaluate for effective amylase producing fungal isolates from the environment and thus contribute to industries utilizing starch in their processes.

## **1.2. General Objectives**

The study aimed of isolating amylase producing molds from the environment and analyzes their contribution to modern bio technological application.

### **1.2.1. Specific Objectives**

The specific objectives of the study were to:

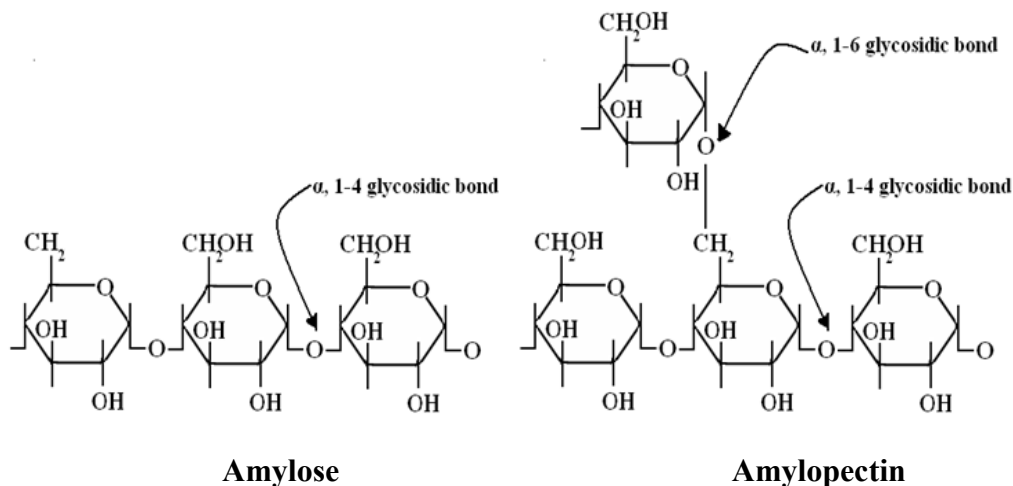
- Isolate, identify and screen fungal isolates that produce  $\alpha$ -amylase and Glucoamylase
- Characterize fungal alpha amylase
- Evaluate the synergism of co-cultivation of the fungal isolates for the production of amylase
- Evaluate the potential of fungal amylase and yeast isolate for the production of ethanol from starch

## 2. Literature Review

### 2.1. Starch

Starch is a homopolymer made up of glucose monomers, which is insoluble in water and stored by all green plants. Plants store starch in different organs (fruit, seeds, rhizomes and tubers) to prepare for the next growing season. It is stored in amyloplasts, which are specialized plastids present in plant storage organs (Tomlinson *et al.*, 1997). The main crops with high starch content are potatoes, sweet potatoes, cassava, maize, sorghum, wheat, rye, triticale, barley, peas and rice (Gordon, 1999). It is a major source of carbon and energy for man and most other organisms. It has many industrial applications like: manufacture of paper, textiles, pharmaceuticals and its hydrolysates have a wide range of importance (Van der Maarel *et al.*, 2002).

Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. Two types of glucose polymers are present in starch: (i) amylose and (ii) amylopectin (Figure 1). Amylose is a linear polymer of D-glucose units linked by alpha -1, 4 glycosidic bonds (Marc *et al.*, 2002). It accounts up to 20-30% of the total starch of plants. Whereas, Amylopectin is the major constituent of starch and consists of large, highly branched molecules. Amylopectin is composed of a linear polymer of D-glucose units which are branched by alpha-1, 6 linked glucose units (Morrison, 1995). Degree of polymerization in the millions depends on the plant species (James *et al.*, 2003). But, its typical value is one per twenty-five glucose units in the unbranched segments (Thompson, 2000). The complete amylopectin molecule contains about 2, 000,000 glucose units and it makes one of the largest molecules in nature (Marc *et al.*, 2002).



**Fig 1.** Amylose and amylopectin chain structures (Amira *et al.*, 2012).

### **2.1.1. Starch digestibility**

Starch is present in microscopic granules of storage structures and highly ordered structure makes quite resistant to penetration by water and or hydrolytic enzymes. Disruption of these ordered structures is possible by a process called gelatinization. Gelatinization initiates important changes in the physical, chemical and biological properties of starch (Collison, 1968).

Gelatinization is a phase transition of starch granules from an ordered to a disordered state during heating with excess water (Freitas *et al.*, 2004). Changes in the granule due to gelatinization are swelling, loss of birefringence and leaching of amylose (Blanshard, 1987). It increases the digestibility of starch by making starch accessible for amylase in the human digestive system. The temperature for gelatinization ranges from 70-90°C depending on the botanical source, amylose-amylopectin proportion and amount of moisture available for hydration (Alavi, 2003).

### **2.1.2. Microorganisms utilizing starch**

The potential of using microorganisms as a source of biotechnological enzymes has an interest to study microorganisms for their amylolytic activities (Buzzini and Martini, 2002). Amylases are industrially important enzymes in the starch processing industries which can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand (Pandey *et al.*, 2000).

The major advantages of using microorganisms for production of amylases include economical bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Pandey, 2003). Microbial amylases are broad spectrum as compared to chemical hydrolysis of starch and to those of plant and animal sources (Tanyildizi *et al.*, 2005). Fungal and bacterial sources are predominant with potential industrial applications (Prabakaran *et al.*, 2009). But, fungi have high secretion capacity and are preferable for hydrolysis of starch and liberate its glucose units (Tsukagoshi *et al.*, 2001). It has been observed that certain fungal species such as *Aspergillus niger*, *Aspergillus oryzae* (Aunstrup, 1979), *Penicillium expansum* (Doyle *et al.*,

1989) and *Mucor* (Domsch *et al.*, 1995; Petruccioli and Federici, 1992) are known to produce high levels of amylase.

## **2.2. Amylolytic enzymes**

They are enzymes which hydrolyse alpha -1, 4 and/ or alpha -1, 6 linkages in starch and starch related compounds. Enzymes belonging to amylases: Endoamylases, Exoamylases and Debranching amylases are able to hydrolyse starch. These enzymes are classified according to the manner in which the glycosidic bond is attacked (Coutinho and Henrissat, 1999).

### **2.2.1. Endoamylases**

Endoamylases cleave only the alpha -1, 4 bonds in the inner regions of the starch molecule randomly (Vihinen and Mantsala, 1990). The alpha amylase (EC 3.2.1.1) is the best known endoamylase. There are two types of alpha amylases (Liquefying and Saccharifying). A liquefying alpha amylase hydrolyses 30-40 % and a scarifying alpha amylase hydrolyses 50-60 % of the glycosidic linkage of starch (Vihinen and Mantsala, 1990). Majority of the alpha amylases are found to be extracellular while, a few others are intracellular and enable the producer organisms to utilize maltodextrin or storage polysaccharides during the exponential growth phase (Ballschmiter *et al.*, 2006).

### **2.2.2. Exoamylases**

Exoamylases also cleave the alpha -1, 4 bonds, e.g. beta-amylase (EC 3.2.1.2), but some of them are able to attack both the alpha -1, 4 and alpha -1, 6 bonds, e.g. glucoamylase (EC 3.2.1.3) and alpha-glucosidase (EC 3.2.1.20). These enzymes act externally on substrate bonds from the non-reducing end of starch and hence produce only low molecular weight products from starch. Beta-amylase produces maltose & beta-limit dextrin while glucoamylase and alpha-glucosidase produces glucose (Wind, 1997). Fungal glucoamulase is the most important industrial enzyme; which has wide application in the starch processing industry at acidic pH and has low thermostability (Shenoy *et al.*, 1985).

### **2.2.3. Debranching Amylases**

The two types of starch debranching enzymes are pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68). Both are specific for alpha -1, 6 bonds in starch (amylopectin) and produces long

linear polysaccharides (Wind, 1997). The two types of debranching enzymes greatly differ from each other in substrate specificity: isoamylase can debranch amylopectin and glycogen, while pullulanase (which also have been referred to as R-enzymes) can hydrolyze the alpha -1, 6 bonds from pullulan and amylopectin (Nakamura, 1996).

### **2.3. Solid state fermentation**

The production of  $\alpha$ -amylase by submerged fermentation (SmF) using synthetic media has been used for many decades (Hamilton *et al.*, 1999). It works with synthetic media which are very expensive and uneconomical. Solid-state fermentation involves the cultivation of microorganisms on a solid substrate. The use of agricultural and industrial wastes makes solid state fermentation attractive alternative method. Solid state fermentation (SSF) is better than submerged fermentation (SmF) due to its simple techniques, low capital investment, less effluent generated, lower levels of catabolic repression, better product recovery and value addition of wastes (Pandey *et al.*, 2000). The metabolites so produced are concentrated and purification procedures are less costly (Ellaiah *et al.*, 2002).

Fungi are commonly used in SSF, due to their relatively high tolerance to low water activities and their high potential to excrete hydrolytic enzymes (Rahardjo, 2005). The hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates (Raimbault, 1998). It has been reported that SSF is the most appropriate process in developing countries due to the advantages it offers (Carrizales and Jaffe, 1986).

### **2.4. Properties and uses of ethanol**

Ethanol or ethyl alcohol is a volatile, flammable and colorless chemical compound. It has unique combination properties as a solvent, germicide, beverage, antifreeze, fuel, depressant, and its versatility as a chemical intermediate for other organic chemicals. The molecular formula for ethanol is  $C_2H_5OH$ , with a molecular weight of 46.07 g/mol and boils at 78.5 °C. It is miscible with water in all proportions and ethanol that is completely free of water is called absolute ethanol (Gaur, 2006).

Its production is economical as well as environmentally friendly (Altintas *et al.*, 2002). Ethanol has the advantages of being renewable (the energy is derived from plants that can be

replenished), cleaner burning (is pure substance). Whereas, gasoline is composed of C<sub>4</sub>–C<sub>12</sub> hydrocarbons and has wider transitional properties (Thomas and Kwong, 2001). Ethanol produces no GHG (Balata *et al.*, 2008).

The main use of ethanol is as a motor fuel and fuel additive. Bioethanol is appropriate for the mixed fuel in the gasoline engine due to its high octane and its low cetane number and high heat of vaporization which blocks self-ignition in the diesel engine (Kim *et al.*, 2005). In a flexible-fuel vehicle that can operate on blends of up to 85 % bioethanol (called E85) is used (Malca and Freire, 2006). In Ethiopia, the blending of Ethanol with Benzene was started in September 2008 with 5 % Ethanol and 95 % benzene (MoFED, 2010).

The second major use of ethanol is in alcoholic beverages. Alcoholic beverages vary considerably in their ethanol content. Most alcoholic beverages can be classified as fermented beverages, beverages made by the action of yeast on sugary foodstuffs, or as distilled beverages, beverages whose preparation involves concentrating the ethanol in fermented beverages by distillation. Fermented beverages may contain up to 15–20 % ethanol by volume (Gaur, 2006).

#### **2.4.1. Feedstock's for bioethanol production and generation of ethanol plants**

The feed stocks used for ethanol production vary throughout the world depending upon the climatic conditions and prices. Cereal grains are an attractive feedstock's, because grains contain a high proportion of starch and can be stored dry for many months, allowing year round processing. Maize is used extensively in the USA, Rye is used extensively in German and a hybrid of rye and wheat are used in Sweden (Senn and Pieper, 2000).

feed stocks for ethanol production are classified as starchy materials like: cereal grains, potato, sweet potato, and cassava (Balata *et al.*, 2008), sucrose-containing as sugar cane, either in the form of cane juice or cane molasses and lignocellulosic biomass such as agricultural residues (corn stover, sugarcane bagasse, forest residues), paper mill residues, wood process wastes and other organic components of municipal solid wastes (Wiseloge *et al.*, 1996).

Over 90% of the world's biofuel derives from crops (60% from cane sugar and beet sugar and the remainder from grains (first generation of ethanol plants). It is easy to obtain fermentable sugars. Biofuels in the second generation biofuel plants are either expensive (Szambelan *et al.*,

2004) or difficult because of its complex structure: cellulose, hemicelluloses and lignin, which resist degradation or involve many steps for bioethanol production (Wiseloge *et al.*, 1996).

#### **2.4.2. Ethanol production**

Fermentation is a process by which a chemical changes are brought about in an organic substrate through the action of biochemical catalysts (enzymes), elaborated by specific types of living microorganisms (Paturau, 1989). Bioethanol produced from biomass resources by fermentation is the most promising biofuel (Spencer-Martins and Van Uden, 1977). And, biomass is a potential renewable energy source that could replace fossil energy (Govindaswamy and Vane, 2007).

Ethanol from starchy feed stocks is produced from two major categories of microorganisms. Yeasts are single-cell fungi organisms which are facultative anaerobes and under anaerobic conditions can ferment glucose to ethanol. The isolates which belonging to *Saccharomyces cerevisiae* have been used most commonly in fermentation processes (Laplace *et al.*, 1993). *S. cerevisiae* is ideal for ethanol production due to several properties including fast growth rates, efficient glucose repression, effective ethanol production and tolerance for environmental stresses, such as high ethanol concentration and low oxygen levels (Piskur *et al.*, 2006). They are known to be used directly ferment sugar cane and sweet sorghum. The enzyme ‘‘Invertase’’ contained in the yeast converts the di- saccharides in sugar into mono-saccharides like glucose and fructose. Subsequently, the enzymes ‘‘Zymase’’ contained in the yeast converts the mono-saccharides into ethyl alcohol and carbon dioxide (Rao, 1997). But, starchy food items need reduction into glucose and oligosaccharides. There are two types of processes to hydrolyze the feedstock’s for fermentation into ethanol, acid (dilute and concentrated) and enzymatic hydrolysis (Demirbas, 2005). Enzymatic hydrolysis has advantages like low cost, no corrosion of equipments and production of high and defined products over acid hydrolysis (Duff and Murray, 1996).

The enzymatic hydrolysis and the fermentation process can be carried out by separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). In case of SSF, fermentation of starch to ethanol is a one step process using co-cultures of two different strains and has potential application for the direct bioconversion of starch into ethanol (Zeikus, 1979). But, SHF allows the fermentation and hydrolysis to be performed at separate conditions; hence

the fermenting organism and the enzymes can be used at independent optimum temperature and pH which may reduce the efficiency of the hydrolysis (Tengborg *et al.*, 2001).

The scheme below shows the general principle of starch conversion to glucose by enzymes (1) and Ethanol production by the action of yeast (2).

1. Starch → Glucose

2. Glucose → Ethyl alcohol + Carbon dioxide

### 3. Materials and Methods

#### 3.1. Description of the study area

The study was undertaken in southwestern part of Ethiopia (Jimma and Arsi zones).

#### 3.2. Sample collection

A total of 22 samples were aseptically collected from coffee effluents, hot springs, agricultural fields (Barley, Maize and Teff) and refuse dumping grounds, presented in Table 1 and Appendix 1. The debris was swept off from the top of the soil, and hand trowel (sterilized spatula) was used to collect a sample of top soil (about 100 grams), in to a ‘zip loc’ bag. Whereas, water samples were collected using plastic containers and the samples were immediately transported to the mycology laboratory and stored at 4°C until used (Sakthi *et al.*, 2012).

**Table 1.** Summary of samples collected for amylase secreting fungal isolates

<b>No</b>	<b>Locality</b>	<b>Isolated from</b>	<b>No of isolated Pure Cultures</b>	<b>Designation of fungal isolates</b>
1	Jimma Zone, (Shesheka)	Coffee Effluent	1	AAUam1
2	>>	>>	2	AAUam 2, 3
3	>>	>>	3	AAUam 4, 5, 6
4	>>	>>	2	AAUam 7, 8
5	>>	>>	2	AAUam 9, 10
6	>>	>>	3	AAUam 11, 12, 13
7	AAU 5 Killo	Food Dumping (top soil)	1	AAUam 14
8	>>	>> (at 5 cm depth)	1	AAUam 15
9	Shalla Billa Kebele (Lake Shlla)	Water sample	0	
		Soil sample	0	
10	>> (Catchment Area)	Water sample	0	
		Soil sample	0	
11	>> (Hot Spring)	Water Sample	0	
		Top Soil	0	

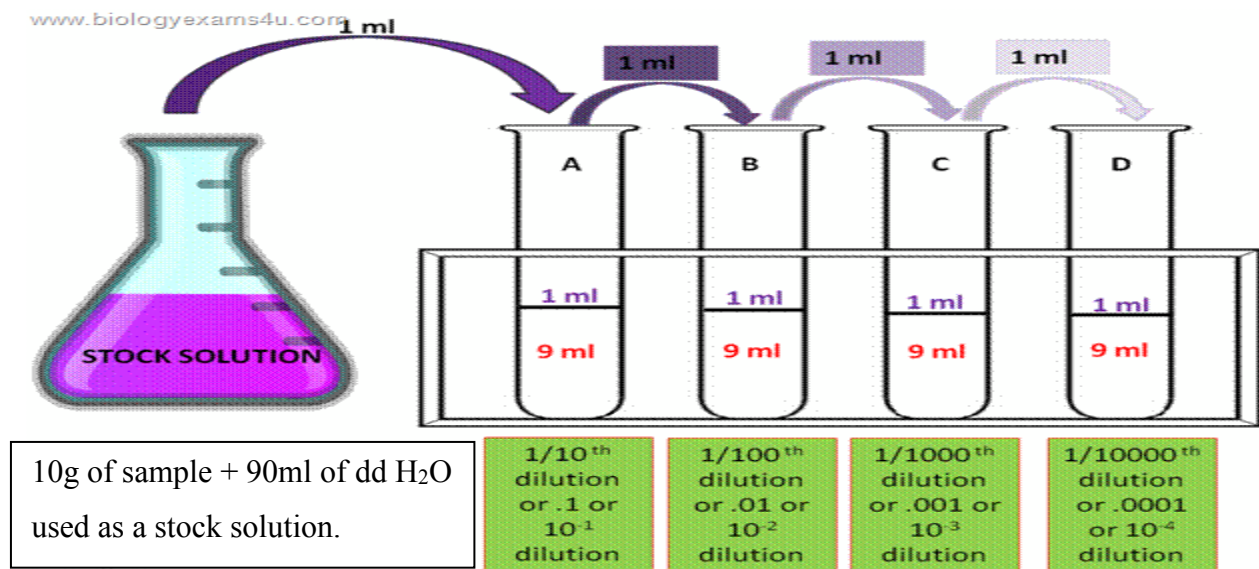
		Bottom Soil	0	
12	>> (Hot Spring)	Water Sample	0	
13	Arsi Zone, Gubeta Arjo woreda	Teff Farm Land	2	AAUam 16, 17
14	Dugda Bora Woreda, Adami Tulu	Barley Farm Land	5	AAUam 18, 19, 20, 21, 22
15	Gerbi Widena Boremo Woreda, Adami Tulu	Maiz Farm Land	4	AAUam 23, 24, 25, 26
16	Wolin Bula Kebele	Maiz Farm Land	1	AAUam 27
17	Digda Woreda, Korki Odi Kebele	Teff Farm Land	2	AAUam 28, 29
18	፩ Koka, 01 Kebele	Water Sample	1	AAUam 30

### 3.3. Preparation of culture media

Thirty nine gram of Potato Dextrose Agar (PDA) was dissolved in Erlenmeyer flask containing 1000 ml distilled water. The flask was covered with cotton-plug and aluminum foil and sterilized by autoclaving at 121°C for 15 minutes. After cooling to 45°C, 20ml of the media was poured in to different Petri dishes and left undisturbed until the agar solidifies under laminar flow at aseptic condition.

#### 3.3.1. Isolation of fungal isolates

Ten gram or (ml) of the samples from different sources was suspended in 90ml of distilled water, followed by serial dilution of each sample into four different flasks. The samples were diluted up to  $10^{-4}$  (Fig 2). A loopful of each sample from dilutes  $10^{-3}$  and  $10^{-4}$  were streaked (pipetted) on the solidified PDA media plates (appendix 2), incubate at 30°C for 72hrs for the fungal growth. From 22 samples collected 30 fungal isolates were isolated. The fungal cultures were purified, incubated at 30°C for 7 days and maintained on PDA slants at 4°C for further identification (Maharjan, 2009).



**Fig 2.** Preparation of serial dilution from the stock sample

### 3.4. Taxonomic identification of promising fungal isolates

#### 3.4.1. Morphological characterization

The fungal isolates were identified by morphological characteristics according to the taxonomic key described by Alexopoulos *et al.* (1996). Both the macroscopic and microscopic characteristics were used to examine and identify the fungal isolates. In microscopic identification, the spores after inoculation period of 7 days at 30°C on PDA media were dispersed in lactose phenol blue solution on a slide and the spore arrangement was examined under compound microscope. The macroscopic examination: color, texture and the shape of spores were scored.

### 3.5. Media and culture conditions for fungal amylases

#### 3.5.1. Screening of fungal isolates for starch hydrolysis

The fungal isolates were screened for their starch hydrolyzing ability. The fungal isolates were inoculated on 1 % starch containing PDA Plate. After 3 days of fungal growth, the plates were flooded with iodine solution (Appendix 3 and 7). Starch reacted with iodine to form a dark blue starch-iodine complex that covered the entire agar. When starch was broken down into sugars,

there were clear zones surrounding streaked lines which indicate starch hydrolysis (Appendix 8), (Alfred, 2007). The zones of hydrolysis formed by each isolate were measured in mm of diameter and the fungal isolates which showed maximum zone of starch hydrolysis were selected for enzyme production.

### **3.5.2. Substrates preference optimization**

Corn Cob, Coffee Hask, Pilled Orange, Sugar Cane Bagasse, Sawdust and Wheat Bran (WB) were prepared in the lab. Substrates were allowed to dry and grounded into coarse powder (about 0.71mm particle size, by using a sieve) with a blender and used as a substrate to identify the optimal substrate preference of fungal isolates.

### **3.5.3. Amylase production from fungal isolates under solid state fermentation**

On the basis of high hydrolysis zone the fungal isolates AAUam 7, AAUam 10, AAUam 13, AAUam 15 and AAUam 28 were adopted for enzyme production under SSF using wheat bran as a substrate. Ten (10)g wheat bran amended with 10 ml of mineral salt solution containing (g/L):  $\text{KH}_2\text{PO}_4$ ; 10,  $\text{MgSO}_4$ ; 2,  $\text{NaCl}$ ; 2 and  $\text{MnSO}_4$ ; 0.5 was taken in 250 ml cotton plugged Erlenmeyer flask, mixed homogenously and sterilized at 121°C for 15 min in an autoclave. Subsequently, the flask were cooled at room temperature and inoculated with 1ml spore suspensions at aseptic condition. The flasks were then incubated at 30°C for 5 days (Muhammad *et al.*, 2012).

### **3.5.4. Inoculum preparation**

Ten ml of sterilized distilled water was added to a sporulated 5 days old PDA culture. An inoculum needle was used to dislodge the spore clusters under sterilized conditions and then it was shaken thoroughly to prepare homogenized spore suspension. 1ml suspension was used as inoculum (Muhammad *et al.*, 2012).

### **3.5.5. Recovery of enzyme**

Crude enzymes were recovered at specific stages of solid state fermentation; 50 ml of distilled water was added in each flask containing fermented mash and placed on a shaker about 200 rpm

for 60 min. Afterward, the mixture was filtered by cheese cloth and Whattman No 1 filter paper and centrifuged at 8,000 rpm for 15 min. The supernatant were collected contained the crude enzyme (Appendix 4) (Muhammad *et al.*, 2012).

### 3.6. Enzyme assay

Amylase activity was assayed based on the amount of reducing sugar released following the modified dinitrosalicylic acid (DNS) method of Anto *et al.*, (2006) cited in Kindu Nibret Tsegaye and Amare Gessesse, 2014). The DNS reagent was composed of (g/L): phenol, 2; sodium sulfite, 0.5; sodium potassium tartarate (Rochelle salt), 200; sodium hydroxide (NaOH), 10; and dinitrosalicylic acid (DNS), 10.

The substrate was prepared in such a way that a 2% soluble starch was gelatinized on a heater.

The reaction mixture, which was composed of 0.9 ml of 2% substrate in 0.01 M of  $\text{KH}_2\text{PO}_4/\text{K}_2\text{H}_2\text{P}_4\text{O}_7$  buffer and 0.1ml of appropriately diluted crude enzyme, was filled in 20 ml test tubes (Appendix 5, A and B). After 15 min of incubation at  $35^\circ\text{C}$  under water bath the reaction was terminated by adding 2 ml of DNS reagent. The control used was prepared in such a way that the crude enzyme was added after the reaction was stopped by the DNS solution. The reaction mixture was then boiled for exactly 5 min in boiling water ( $92^\circ\text{C}$ ). Finally, the test tubes were cooled to ambient temperature and the optical density of the resulting colored solution was measured at 540 nm against a reagent blank for calibration. Enzyme assay was performed in triplicates and the mean was calculated. One unit of enzyme activity was defined as the amount of enzyme that released 1  $\mu\text{mol}$  of reducing sugar equivalent to glucose per min per ml of enzyme used under the assay condition. It was calculated by the formula:

$$EU = [(13.08 * OD * df + 0.34) * 10 / 15] / \text{ml/min}.$$

Where OD is optical density (absorbance); and df is the dilution factor of the enzyme. The value of df was 10 in the standard assay method, *i.e.*, excluding enzyme dilutions made ahead of the enzyme substrate mixing.

### **3.7. Growth factors and nutritional requirements**

#### **3.7.1. Growth factors**

Various process parameters were optimized for maximal enzyme production such as fermentation period (24-144 hrs), incubation temperature (20-50°C) and initial pH (4-8) as described by Muhammad *et al.* (2012).

#### **3.7.2. Nutritional requirements**

Experiments were also performed to evaluate the influence of different carbon sources (maltose, glucose, galactose, lactose, sucrose, and soluble starch) and nitrogen sources (yeast extract, urea,  $\text{NH}_4\text{NO}_3$ ,  $\text{NH}_4\text{SO}_4$ ,  $\text{NH}_4\text{H}_2\text{PO}_4$  and  $\text{NaNO}_3$  at concentration (0.25%) as described by Muhammad *et al.* (2012).

### **3.8. Effects of co-cultivation of the fungal isolates for the production of amylases**

The five selected fungal isolates were co-cultivated (two isolates in one culture) under SSF to compare the efficacies of mixed and single cultures of the isolates for the production of  $\alpha$ -amylase and glucoamylase (Ominyi *et al.*, 2013).

### **3.9. Enzyme characterization**

#### **3.9.1. Temperature**

Optimum temperature for enzyme activity was determined by conducting the assay at different temperatures ranging from 25°C to 75°C as described by Alva *et al.* (2007).

#### **3.9.2. pH**

Effect of pH on amylase activity was determined by incubating the reaction mixture at pH values ranging from 4.0 to 8.0 by using the following buffer: acetate buffer (4.0-6.5) and  $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$  buffer from (7.0-8.0) as described by Alva *et al.* (2007).

### **3.10. Identification of the type of amylase by sugar analysis on Thin Layer Chromatography (TLC)**

To identify the type of amylase from the fungal isolates based on the starch hydrolysates TLC system as described by Kimura and Horikosh (1989); cited in Gashaw Mamo and Amare Gessesse 1999) was followed. First 0.9 ml of 2% soluble starch mixed with 0.3 ml crude enzyme from the fungal sources was incubated for 30 minutes at 35 °C in the water bath. Then each hydrolysate (reduced sugars released by amylase activity) was spotted on TLC plate along with standard known sugar (glucose, lactose, maltose and soluble starch) solutions using capillary glass tube. The plate was then placed in a TLC separation chamber that had been equilibrated with the mobile phase, which was the mixture of a solvent system buthanol: ethanol: water (at a ratio of 5:3:2, v/v/v) and silica gel as supporting material (solid phase). After a total of 4 ascend air-dry TLC plates were sprayed with a mixture of methanol: H<sub>2</sub>S<sub>0</sub><sub>4</sub> (at a ratio of 1:1, v/v) and heated for 10 minutes at about 100 °C for visualization of the sugars. The dark brown sugar spots appeared was identified by comparing with the standards.

### **3.11. Bio-ethanol production from starchy substrates using fungal amylase and yeast**

Forty g/L of starch was used as a substrate and gelatinized. Then 0.1/ 0.9 ml of the enzyme was added and incubated in water bath at 35 °C for 30-60 min for hydrolysis. After hydrolysis, the flasks containing the hydrolyzed samples, 10g/L of yeast extract and 10g/L of peptone were adjusted to pH 3, covered with cotton wool, wrapped with aluminium foil, autoclaved and cooled at room temperature. Fermentation was carried out in 250 ml Erlenmeyer flask containing 100 ml of distilled water with a 24 hr cell suspension of yeast *S. cerevisiae* (AAUT1) (from Taye Negera, 2014) OD<sub>660</sub> = 0.6 at incubation temperature of 30°C for 3 days (Appendix 5, C), as described by Kumar *et al.* (2011) with some modifications.

#### **3.11.1. Estimation of ethanol by potassium dichromate and sulfuric acid method**

Ethanol assay from sample was tested by the method of Caputie *et al.* (1986). One ml of culture supernatant was taken and make up the volume to 5 ml with distilled water then followed by 1ml of K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> solution and 4 ml of Conc. H<sub>2</sub>SO<sub>4</sub> solution (Appendix 5, D). The intensity of color was read at 660 nm in UV/VIS spectrophotometer. Blank is prepared in the same manner without

ethanol. Alcohol standard was prepared by dissolving absolute ethanol in water to get 10 mg/ml concentration.  $K_2Cr_2O_7$  solution was prepared by dissolving 10 gm of  $K_2Cr_2O_7$  in distilled water in a 100 ml standard flask and make up the volume to mark (Balasubramanian *et al.* , 2011).

## 4. Results

### 4.1. Isolation and screening of fungal organisms

A total of 30 pure isolates were isolated from 22 samples collected from about 18 sampling sites and labeled with code numbers all beginning with a prefix AAUam. The result showed that 9 isolates (30%) were found to be positive for starch hydrolysis (Table 5). Based on the result of high starch hydrolysis zone around the colony five potential isolates (AAUam 7, AAUam 10, AAUam 13, AAUam 15 and AAUam 28) were selected for the production of amylase. It is based on the fact that the wider the diameter of the hydrolysis zone is the higher the amylase is produced by the organism. The clear zone formed around the fungal isolates is depicted in Appendix 8.

**Table 2.** The measurement of clear zones of starch hydrolysis by fungal isolates

<b>Fungal isolates</b>	<b>Hydrolysis zone in mm</b>	<b>Identified as Genus</b>
AAUam7	+ (11.75)	<i>Penicillium</i>
AAUam10	+ (12.75)	<i>Penicillium</i>
AAUam13	+ (12.75)	<i>Aspergillus</i>
AAUam15	+ (11.75)	<i>Penicillium</i>
AAUam28	+ (12.5)	<i>Penicillium</i>

(+) **Amylase Positive**

### 4.2. Taxonomic identification of promising fungal isolates

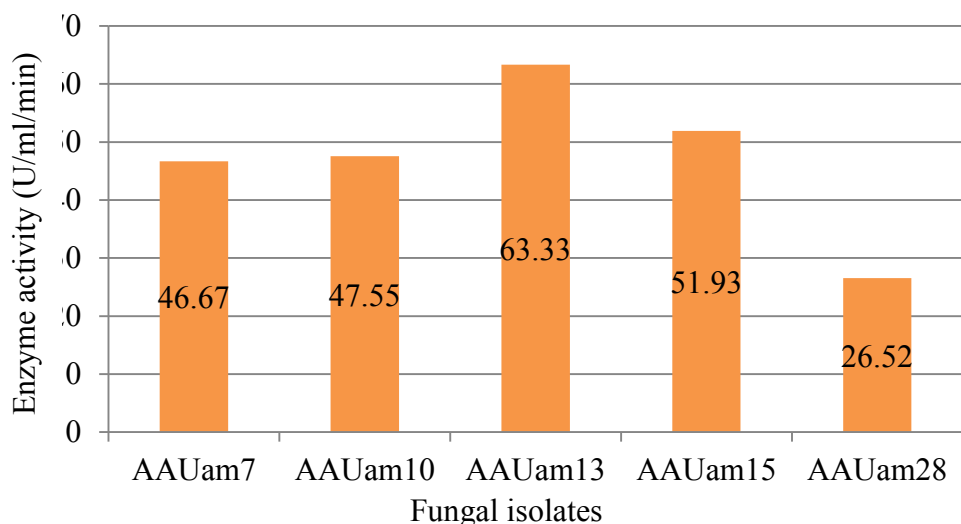
#### 4.2.1. Macroscopic and microscopic characteristics

The macroscopic feature of the fungal isolate AAUam 13 shows a dark brown filamentous growth which sporulates to black powdery growth and later colored to black surface and light yellowish reverse (back side) with rough textures. Whereas, the microscopic study shows as it has long septate hyphae with a conidiophore that bears brown spores. The macroscopic feature of the fungal isolates AAUam 7, AAUam 10, AAUam 15 and AAUam 28 showed dark green color with rough powdery colony on the surface and the back side of colony was greenish cream in color. Based on the above observations the fungal isolate AAUam 13 was found to belong to the

genus *Aspergillus* and the fungal isolates AAUam 7, AAUam 10 AAUam 15 and AAUam 28 were found to belong to the genus *Penicillium*.

#### 4.3. Comparison of fungal isolates for amylase production

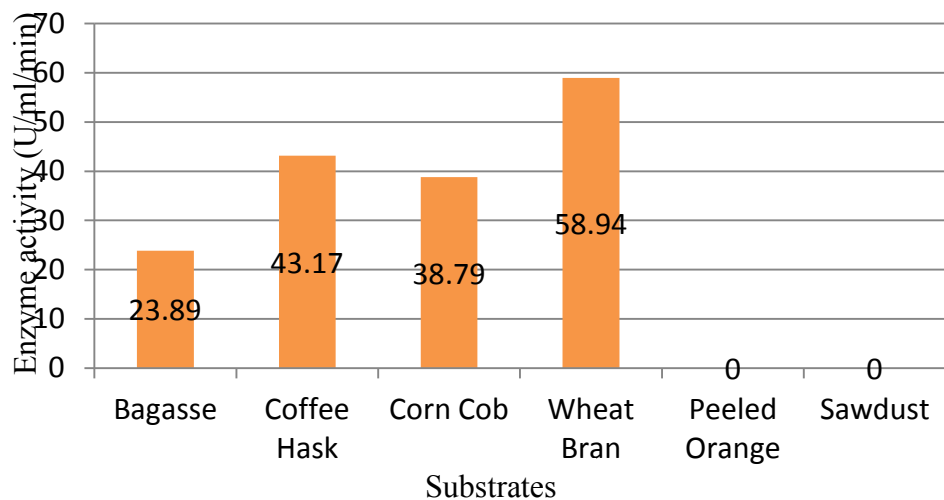
Five fungal isolates (AAUam 7, AAUam 10, AAUam 13, AAUam 15 and AAUam 28) were compared for their amylase production activity at SSF by using wheat bran as a substrate. The result in Figure 3 showed that maximum amylase was produced by a fungal isolate (Coded here as AAUam 13). The rest of the isolates were found relatively producing less amount of the same enzyme. Thus the fungal isolate AAuam 13 was selected for further study.



**Fig 3.** Enzyme production by fungal isolates

#### 4.4. Substrates for enzyme production

Substrate fermentation was carried out with 10g of any one of the substrates for the production of amylase at 30 °C using AAUam 13 for 4 days by using distilled water as moistening agent. Wheat bran and sugar cane bagasse gave the highest and lowest amylase activity of (58.94 IU) and (23.89 IU) respectively (Fig 4). While, Sawdust and Peeled orange have showed no result. Thus, WB was selected as substrate for further study.



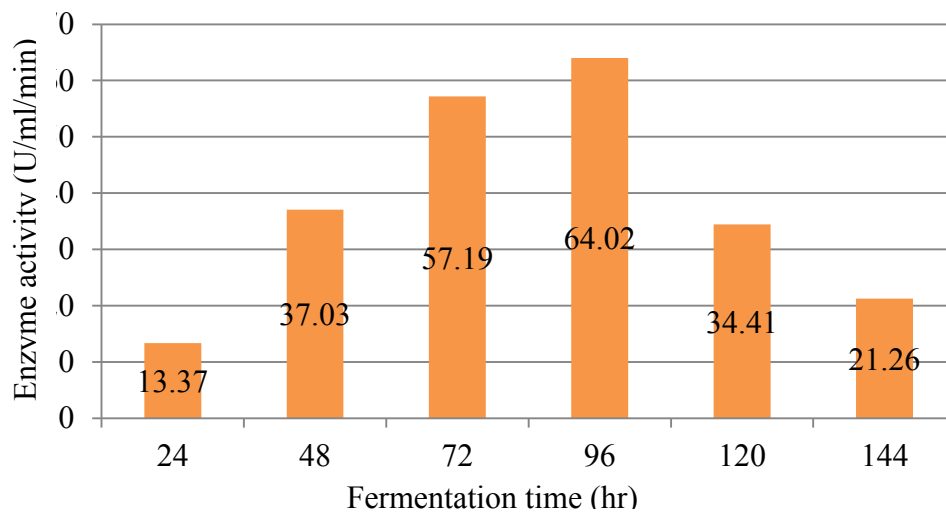
**Fig 4.** Effect of different substrates on amylase production under SSF. Incubation period 4 days, initial moisture level 50 % (w/v), incubation temperature 30°C.

#### 4.5. Effects of cultural and nutritional parameters on the production of amylase

##### 4.5.1. Cultural parameters

##### 4.5.1.1. *Time course study*

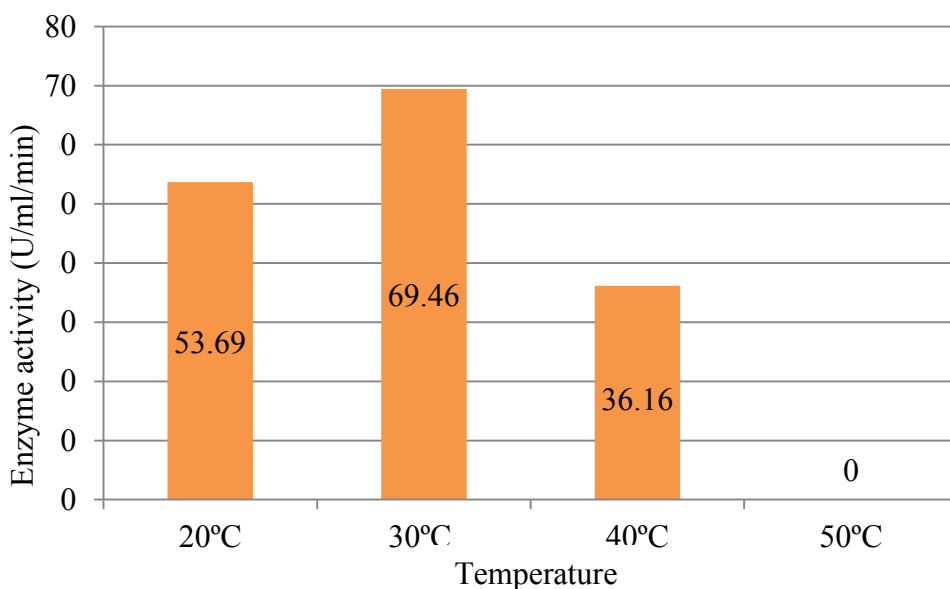
The time course study from (24-144 hr) for the production of amylase by AAUam 13 was studied using SSF. From the result of this experiment, the optimum fermentation time of 96 hr was found to be the best (64.02 IU). Further increase or decrease in the incubation time decreased the amylase secretion with the least amylase production of (13.37 IU) at 24 hr. Therefore, incubation period of 96 hr was selected as optimum time for the production of amylase in the subsequent experimental work (Fig 5).



**Fig 5.** Effect of different fermentation time on amylase production

#### 4.5.1.2. *Temperature*

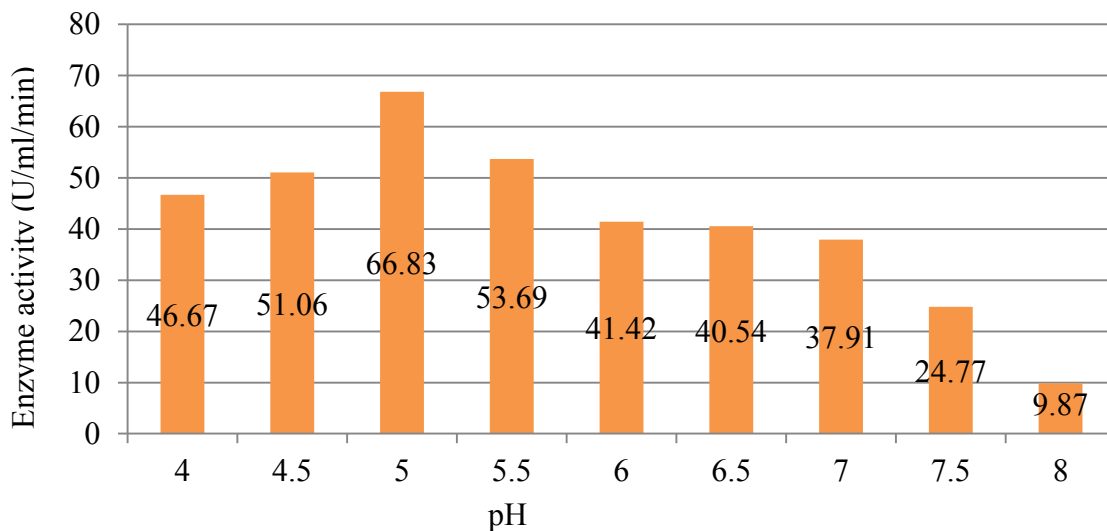
The effect of varying incubation temperature (20-50°C) on the production of amylase by AAUam 13 was studied using SSF. Maximal amylase production of (69.46 IU) was obtained in fermentation flask which was incubated at 30°C and the least amylase production of (36.16 IU) was showed at 40°C. Whereas, there was no amylase production as the fermentation flask was incubated at 50°C (Fig 6).



**Fig 6.** Effect of varying temperatures on amylase production

#### 4.5.1.3. pH

The effect of different initial pH values of (4.0-8.0) on amylase production by fungal isolate AAUam 13 using SSF was studied. The maximum and minimum amylase production of (66.83 IU) and (9.87 IU) was recorded as the fermentation media was adjusted at pH value of 5.0 and 8.0 respectively (Fig 7).

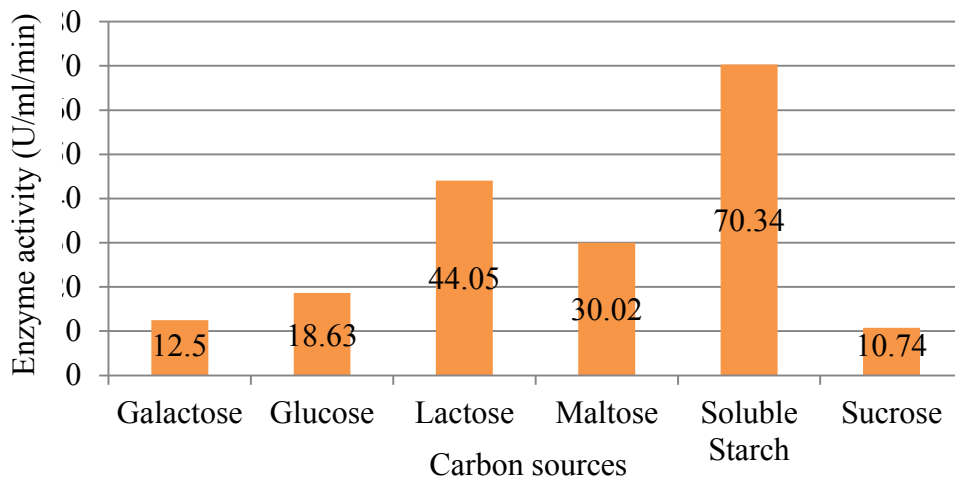


**Fig 7.** Effect of different pH on amylase production

#### 4.5.2. Nutritional parameters

##### 4.5.2.1. Carbon sources

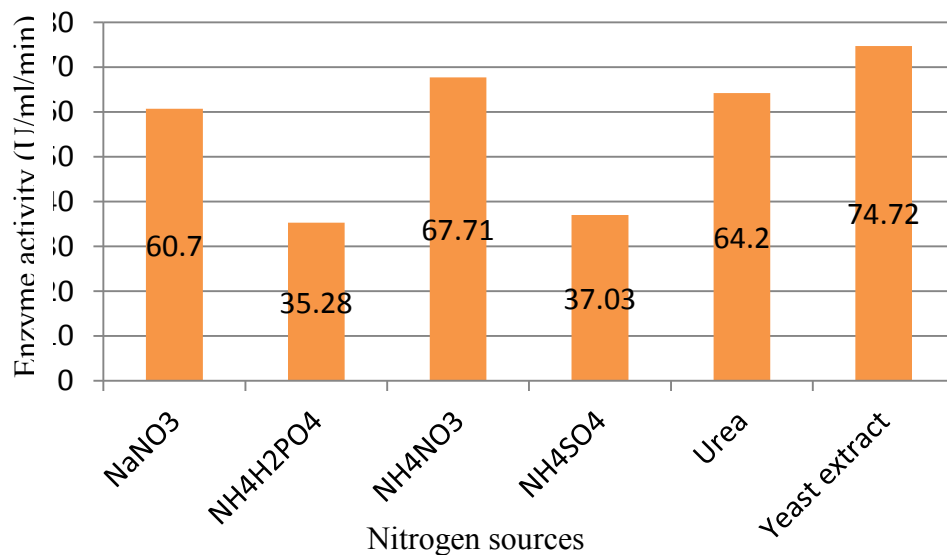
Different carbon sources i.e. galactose, glucose, lactose, maltose, soluble starch and sucrose were evaluated for their effects in the production of amylase (Fig 8). Soluble starch was found to be the best carbon sources for amylase production of (70.34 IU) by the fungal isolate AAUam 13 followed by Lactose (44.05 IU) and a relatively minimum amylase production was recorded by rest of carbon sources.



**Fig 8.** Effect of different carbon sources on amylase production

#### 4.5.2.2. Nitrogen sources

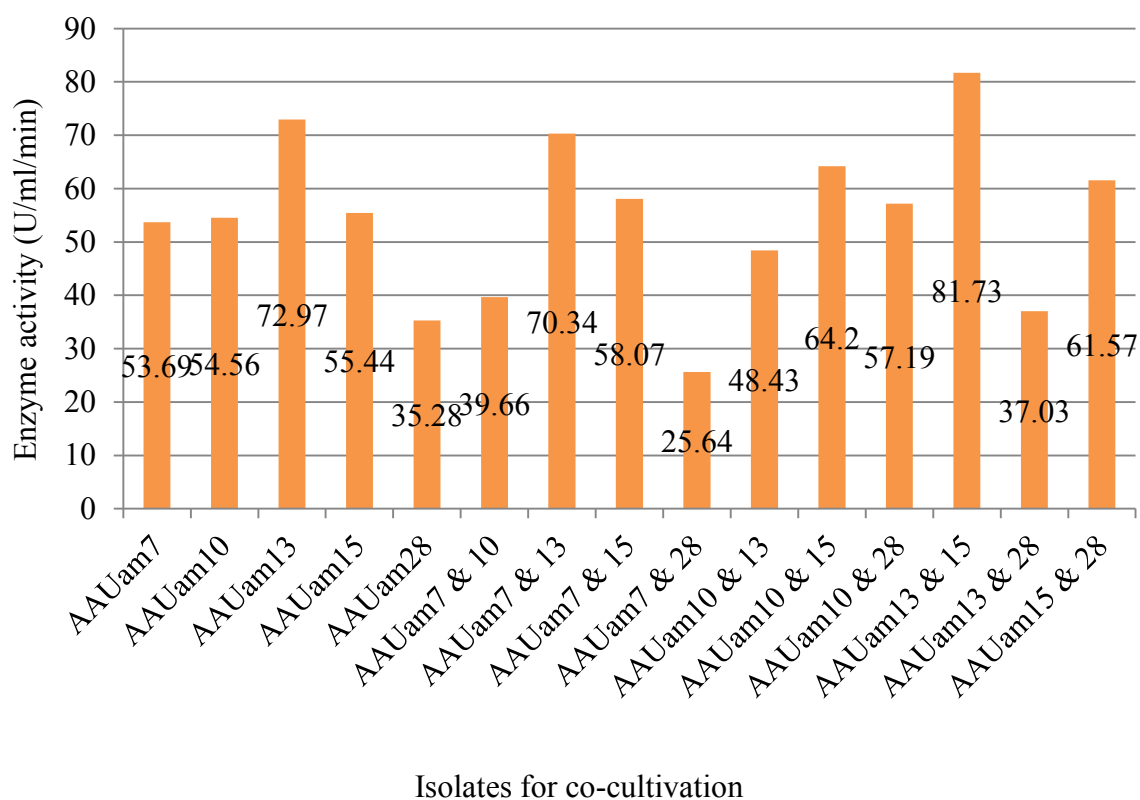
The effect of nitrogen sources ( $\text{NaNO}_3$ ,  $\text{NH}_4\text{H}_2\text{PO}_4$ ,  $\text{NH}_4\text{NO}_3$ ,  $\text{NH}_4\text{SO}_4$ , urea and yeast extract) on the production of amylase by the fungal isolate AAUam 13 were studied using SSF (Fig 9). Among all the selected nitrogen sources maximum amylase production of (74.72 IU) was obtained when yeast extract (0.25%, w/v) was used in the medium. While,  $\text{NH}_4\text{H}_2\text{PO}_4$  (0.25%, w/v) showed the least amylase production of (35.28 IU).



**Fig 9.** Effect of different nitrogen sources on amylase production

#### 4.6. Co-cultivation

The selected five fungal isolates (AAUam 7, AAUam 10, AAUam 13, AAUam 15 and AAUam 28) were evaluated for their single and synergetic effects in the production of amylase (Fig 10). Among the combinations, maximum amylase activity of (81.73 IU) was recorded as the fungal isolates AAUam 13 and AAUam 15 were inoculated together than their corresponding single cultures. While, the co-culturing of fungal isolates AAUam 7 and AAUam 28 showed the least amylase activity of (25.64 IU).



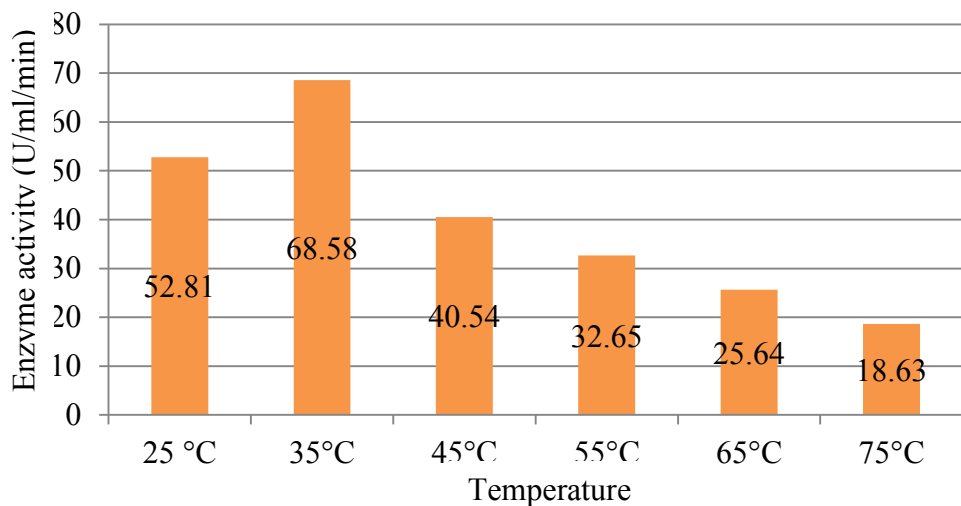
**Fig 10.** Effect of co-cultivation of the fungal isolates on production of amylase

#### 4.7. Enzyme characterization

##### 4.7.1. Effect of different temperature on amylase activity

The activity profile of the fungal amylase which was obtained from fungal isolate AAUam 13 was evaluated at temperature values of (25-75°C). The enzyme showed its optimum activity of (68.58 IU) at 35°C and as temperature increased from 35-75°C the activity of the enzyme

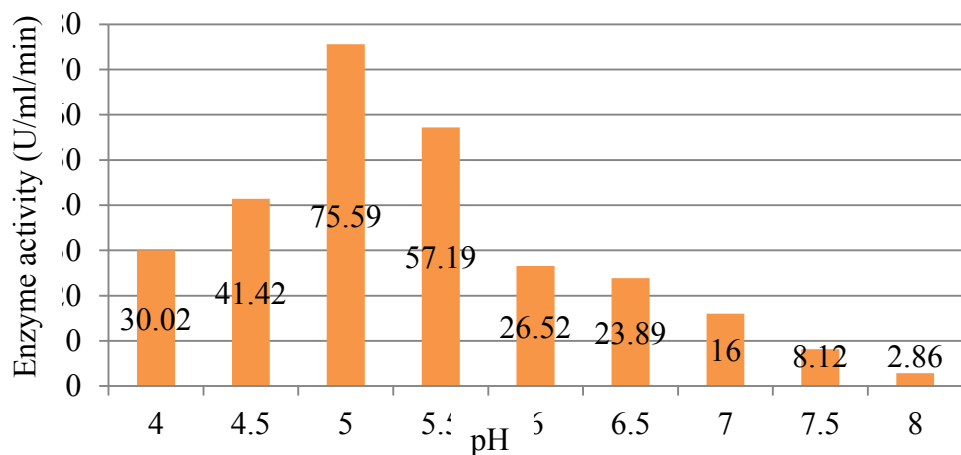
showed a declining trend. While its' least amylase activity of (18.63 IU) was recorded at 75°C (Fig 11).



**Fig 11.** Effect of temperature on amylase activity

#### 4.7.2. pH

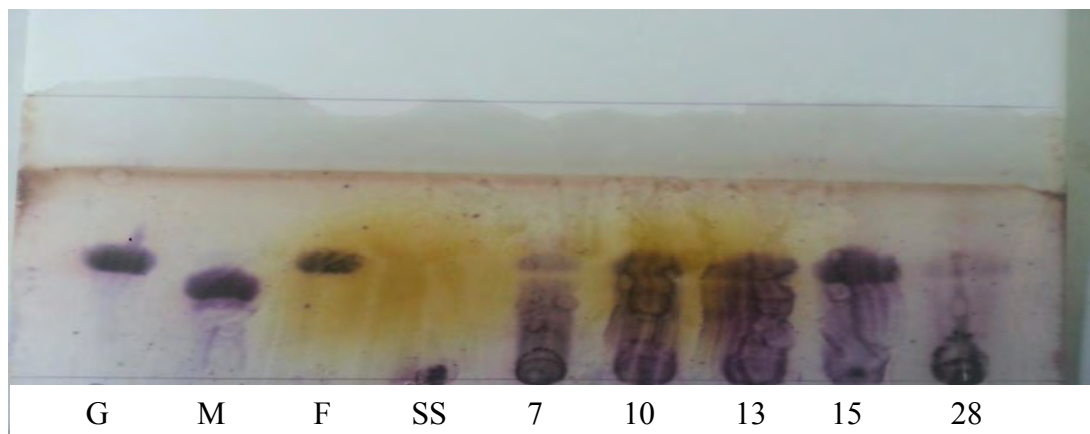
The optimum pH for amylase activity was evaluated at 35°C over the pH ranges from 4.0-8.0. The favorable pH range for amylase activity of the fungal isolate AAUam 13 was found between 4.5-5.5 with an optimum activity of (75.59 IU) at pH 5.0 and a significant drop in amylase activity was observed at pH value of 4.0 and pH values above 5.5 (Fig 12).



**Fig 12.** Effect of pH on amylase activity

#### 4.8. Identification of the type of amylase by sugar analysis on thin layer chromatography (TLC)

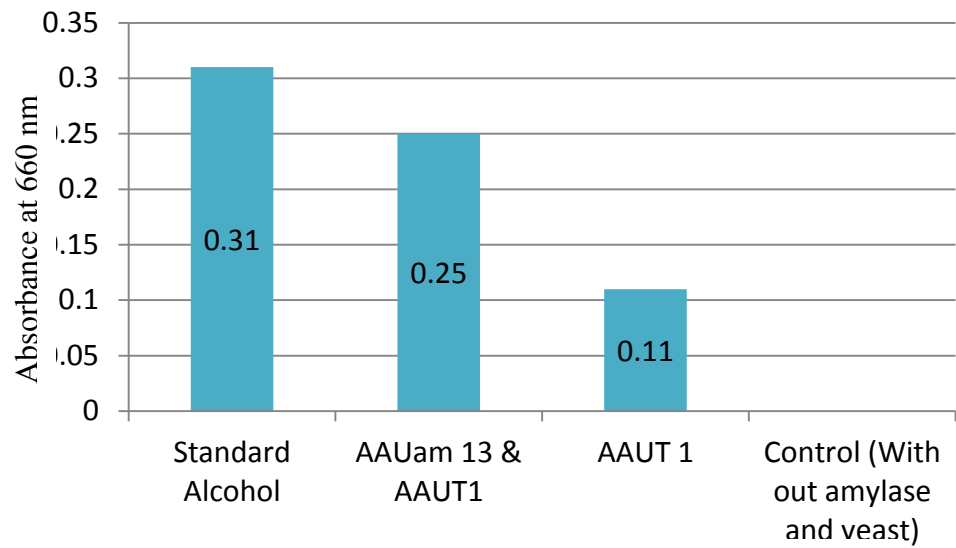
The starch hydrolysates produced by the fungal amylases from selected fungal isolates (AAUam 7, AAUam 10, AAUam 13, AAUam 15 and AAUam 28) and the standard sugar solutions (glucose, maltose, fructose and soluble starch) were run on TLC plate. The major spot developed by the fungal amylases AAUam 7, AAUam 15 and AAUam 28 was glucose indicating that it is glucoamylase; fungal amylases of AAUam 10 and AAUam 13 were glucose and maltose indicating that it was alpha amylase (Fig 13).



**Fig 13.** Analysis of starch hydrolysis products on a TLC plate. Lane g, m, f and s are standard sugars of glucose, maltose, fructose and soluble starch respectively. Lane 7, 10, 13, 15 and 28 are starch hydrolysates by the action of amylases of the fungal isolates AAUam 7, AAUam 10, AAUam 13, AAUam 15 and AAUam 28 respectively.

#### 4.9. Estimation of ethanol production by potassium dichromate and Sulphuric acid method

In this study the efficiency of amylase which was obtained from the fungal isolate AAUam 13 and the yeast (AAUT1) were evaluated for the production of ethanol (Fig 14). The result showed that the highest concentration of ethanol (0.25 OD) was achieved as the yeast (AAUT1) was inoculated in the fermentation medium which was pretreated with the fungal amylase of AAUam 13 and there was a minimum ethanol production (0.11 OD) as the yeast was inoculated in the production medium which was not pretreated with the fungal amylase as compared to the standard alcohol (0.31 OD) and control.



Standard alcohol and production from isolates

Fig 14. Ethanol production by the fungal amylase and yeast.

## 5. Discussion

Ethiopia is a country that follows green economy which later followed by industrial economy. Biotechnological industries are one amongst and they manipulate different enzymes like amylase in their processes. Amylases are a class of enzymes that hydrolyze starch: an agricultural raw material as well as an end product in milling industries. Amylases can be derived from a variety of sources. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Pandey, 2003). The fungal isolate that belongs to the genus *Aspergillus* (AAUam 13) was selected and used in this study to evaluate the cultural and nutritional conditions for enzyme production and enzyme activity. The environmental genera called *Aspergillus* is known in amylolytic activities (Zyska, 1997). The underlying reason why AAUam 13 was positive for amylase positive may be associated with the adaptation of the organism to primarily depend on starch as carbon source.

Different culturing conditions greatly shown affecting the production of amylase (Cherry *et al.*, 2004). The degree or amount of enzyme production by AAUam 13 was compared by growing it in SSF medium using variety of substrates. The use of agricultural and industrial wastes makes solid state fermentation attractive alternative method (Pandey *et al.*, 2000). The maximum amylase production was achieved by using wheat bran as a substrate (Fig 4). Varalakshmi *et al.* (2009) also reported maximum alpha amylase production (74 IU/mg protein) by *A. niger* using wheat bran as a substrate in SSF. The highest activity in wheat bran may be due to its high carbohydrate content (Constitutes 65% of the bran) (Slavin, 2003) and suitable texture (Kumar and Duhan, 2011). In addition to these  $\alpha$ -amylase is an extracellular enzyme and its production is increased by its substrate: proteins are substrate specific (Chimata *et al.*, 2010 and Varalakshmi *et al.*, 2009).

The production and activity of alpha amylase in the medium is affected by a variety of physicochemical factors (Espargaroet *et al.*, 2008).

Regarding to the incubation period, many investigators have found that extracellular alpha amylase production is growth associated (Abou Dohara *et al.*, 2011; Asoodeh *et al.*, 2010; Murthy *et al.*, 2009). Maximum accumulation of  $\alpha$ -amylase occurs during stationary phase. It might be due to the deficiency of nutrients, accumulation of toxic substances and proteolysis

(break down) of  $\alpha$ -amylase in to polypeptides or amino acids as interpreted by (Chamber *et al.*, 1999 and Shafique *et al.*, 2009). In this study enzyme production by the fungal isolate AAUam 13 was examined between 24-144 hrs. Fermentation time of 96 hr was recorded as best for enzyme production. Muhammad *et al.*, (2012) have also reported fermentation period of 96 hr best for both *Aspergillus niger*-ML-17 ( $4.7 \pm 0.14$  IU) and *Rhizopus oligosporus*-ML-10 ( $2.7 \pm 0.08$  IU) using wheat bran as a substrate in SSF. Further increase in the incubation period decreased the enzyme secretion (Fig 5). In the contrary, it has been reported that maximum amylase production by *Aspergillus* species was obtained after an incubation period of 120 h (Sidkey *et al.*, 2010; Zambare 2010; Bhattacharya *et al.*, 2011 and Farid and Shata, 2011). From the above observations it can be concluded that the isolate AAUam 13 was fast grower and being fast grower is an advantage in that it reduces production time.

Among the physical parameters, the temperature and pH of the medium play an important role in  $\alpha$ -amylase production and activity. Generally, the influence of temperature on amylase production is related to the growth of the organism (Gupta *et al.*, 2003). In this study maximum enzyme production was achieved at fermentation temperature of 30°C and initial medium pH of 5. Subsequently as the temperature and pH values increased the amount of  $\alpha$ -amylase production was shown to decrease (Fig 6 and 7 respectively). Many other researchers have also reported 30°C as optimum temperature for the fungal growth and enzyme production. Alva *et al.* (2007) found specific activity of (11 U/mg of protein) by *Aspergillus* sp. JGI 12 at 30°C. This is because the enzyme production is growth associated and 30°C is optimum temperature for fungi and subsequently  $\alpha$ -amylase production (Shafique *et al.*, 2009 and Dakhmouche *et al.*, 2006). Muhammad *et al.* (2012) have also achieved the optimal  $\alpha$ -amylase production (4.5 IU) by *A. niger* at pH value of 5.0. This might be because the requirement of slightly acidic pH for optimum growth of fungi (Liu and Xu, 2008; Sun *et al.*, 2009). And, Alpha Amylase production by microbial strains strongly depends on the extracellular pH as it influences many enzymatic reactions as well as the transport of various components across the cell membrane (Ellaiah *et al.*, 2002).

The accumulation of sugars in the medium is well documented to inhibit the enzyme production (Dona *et al.*, 2010 and Wang *et al.*, 2006). Of the different carbon sources used as sole carbon sources soluble starch supported maximum enzyme secretion. This may be due to its

polysaccharide nature. Lactose is a disaccharide (Composed of glucose and galactose) which supports maximum enzyme secretion next to soluble starch. While, other tested carbon sources supported gave comparatively less enzyme production which were supplemented to the fermentation medium at concentration of (0.25%, w/v) (Fig 8). Varalakshmi *et al.* (2009) have also reported soluble starch as the best carbon source for the production of alpha amylase (40.43 U/mg of protein). The effect of simple sugars on alpha amylase production is as a result of catabolic repression. Because, the fungal isolates will get free sugars and can directly use it their biological processes i.e. they do not undergo amylase production or it has no role on simple sugars like glucose. Alpha amylase production is also subject for catabolic repression by glucose and other sugars like most other inducible enzymes that are affected by substrate hydrolytic products (Morkeberg *et al.*, 1995).

Nitrogen source as a basal component of the medium is a major factor affecting  $\alpha$ -amylase production (Abou Dohara *et al.*, 2011). The increased  $\alpha$ -amylase production by organic nitrogen sources (yeast extract, Fig 9) could be attributed to the high nutritional amino acids and vitamin content. Oshoma *et al.* (2010) have also reported maximum amylase production (amylase activity of 643 U/ml) by using yeast extract as an organic nitrogen source. However, Hashemi *et al.* (2012) obtained maximum  $\alpha$ -amylase production (140 U/g) with  $\text{NH}_4\text{NO}_3$  at a level of 1% (w/v).

The mixed cultures were more effective in production of  $\alpha$ -amylase and glucoamylase than their component single cultures as indicated by Ominyi *et al.* (2013). Higher amylase productivities were achieved mostly with mixed culture when compared with their component single cultures. For instance, the fungal isolates (AAUam 7 & 15, AAUam 10 & 15, AAUam 10 & 28, AAUam 13 & 15 and AAUam 15 & 28) were co-cultured and proved more productive than their corresponding single isolates. Whereas, the fungal isolates with other combinations have showed the least amylase activity than their corresponding single isolates (Fig 10). This showed that some of the isolates have acted synergistically in their enzyme activities (Ueda, 1981 and Ominyi *et al.*, 2013).

In addition to growth factors, enzyme activity was also evaluated at varying temperature and pH values to understand its application. The temperature optimum for amylase activity of the fungal isolate AAUam 13 was found to be 35°C (Fig 11) and it is related to the growth of the fungal

organism as described by (Mac Gregor *et al.*, 2001). Similarly Varalakshmi *et al.* (2009) has reported 35°C optima for enzyme activity (% Relative activity of 90) for *Aspergillus niger*. JGI 24.

Among the physical parameters pH is the one that affect enzyme activity: a physicochemical denaturant and results also proteolysis. In this study the favorable pH range for amylase activity for AAUam13 was found between 4.5-5.5 with an optimum activity at pH 5.0 (Fig 12). Each microorganism possesses a ph range for its growth and activity with an optimum value (Sodhi *et al.*, 2005). A significant drop in enzyme activity was observed at pH 4.0 and pH values above 5.5. The enzyme behavior clearly indicates that it is more suitable for any application at the pH range of 4.5-5.5 and it is an advantage in which it substitutes acid hydrolysis in starch processing industries. Similarly, Yasser *et al.* (2009) have also reported (% relative amylase activity of 100) at pH 5 for *Aspergillus flavus* FSS60. The AAUam 13 amylase was found to have an optimum pH of 5 indicating that the enzyme is acidophilic.

The highest concentration of ethanol was achieved as the yeast (AAUT1) was inoculated in the fermentation medium which was pretreated with the fungal amylase than as the yeast inoculated alone (Fig 14) and this might be as a result of enzymatic activities in the fermentation media. As the enzyme from starch hydrolyzing fungi release the sugars, the yeast then convert sugars into ethanol. Similarly, Mariam *et al.* (2009) reported initial sugar concentration has been found to determine the amount of alcohol.

## **6. Conclusion**

An agricultural waste like wheat bran which was used in this study was the best substrate for amylase production. Using wheat bran as a substrate for enzyme production is one way of value addition of wastes as well as value addition of wastes.

The fungal alpha amylase from isolate AAUam 13 can be a potential candidate to be applied in starch hydrolysis process at thermal activity of 35<sup>0</sup>C as well its pH activity at 5.0.

## **7. Recommendation**

Based on the current investigation the following recommendations are forwarded:

- The enzyme should be characterized further, purified and tested for small scale production to improve its hydrolysis capacity.
- Enzyme stability should be evaluated for application.
- Co-culturing of fungal isolates AAUam 13 and AAUam 15 to achieve maximum amylase production.
- More work should be undertaken to optimize the production of bioethanol using glucose as a standard to evaluate the effect of hydrolysis.
- Molecular characterization of the fungal isolate should be done to identify the isolate up to species level.

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## Appendix



A



B



C



D

**Appendix 1.** Sample collection from Hot spring (A & B), Lake shalla (C) and Barley farm land from south western part of Ethiopia



**Appendix 2.** Inoculating samples on PDA media for fungal isolation



**Appendix 3.** Screening of isolates for starch hydrolysis activity after inoculation on 2% starch PDA media.



A



B



C



D



E

**Appendix 4.** Enzyme Extraction (fermentation flasks (A), Shaking fermented flask on shaker (B), The researcher extracting the crude enzyme by cheese cloth (C), Extracting the crude enzyme by whattman No 1 filter paper (D) and Crude enzyme (E)).

A



B



C

**Appendix 5.** Preparation of reaction mixture (A), Incubating the reaction mixture in water bath at 35°C (B), Fermentation flask for Ethanol production (C) and Estimation of ethanol by potassium dichromate and



D

**Appendix 6.** Dry weight determination

Dry weight of the substrate was determined by drying in a hot air oven at 100°C for 24 hr.

**Appendix 7.** Preparation of Lugol's iodine

It was prepared by mixing 1% Iodine with 2% potassium iodide

**Appendix 8.** Halo zone formed around fungal isolates after flooding the plates with Lugol's iodine reagent.



AAUam 7

AAUam 10

AAUam 13

AAUam 15

AAUam 28