# PRODUCTION OF ALPHA AMYLASE USING FUNGUS FROM THE RHIZOSPHERE OF *Moringa oleifera* .

BY

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# CERTIFICATION

I hereby certify that this write up was carried out by **QUARSHIE, DORIS OLUWAKEMI (MCB/14/2337)** under my supervision in the Department of Microbiology, Faculty of Science, Federal University Oye-Ekiti, Ekiti State, Nigeria.

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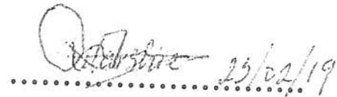

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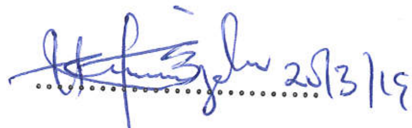

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# DEDICATION

This write up is dedicated to the glory of God Almighty and to my immediate family.

## ACKNOWLEDGEMENTS

My gratitude goes to God Almighty, to Him be praises now and forever and I sincerely appreciate my supervisor DR S.A. ADEGOKE for his support morally and academically, who always challenged me almost every day by prompting me to do more researches about my project topic and for his provision of materials during the course of this write up.

I also appreciated every other person especially my immediate family members for their contributions and encouragement whether financially, morally and spiritually towards the completion of my education

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# ABSTRACT

Alpha amylase enzyme (EC 3.2.1.1) (α-1, 4 glucan-glucanohydrolase) is widely distributed in nature such as plants, animals and microorganisms. Production of extracellular alpha-amylase from fungi was isolated from rhizosphere of Moringa oleifera. Three (3) fungi isolates were selected after serial dilution of the soil samples, four optimization parameters was employed to determine the enzyme activity and they include effect of inoculum size, pH, nitrogen source and surfactant on the enzyme production. *Aspergillius* has the maximum enzyme production at 0.697 U/ml in the presence of 1ml inoculum size, the minimum enzyme production for *Trichoderma* was at 0.045U/ml in the presence of Groundnut cake whereas the stimulatory ability of pH at 7.0 was for *Penicillum*. This study reveals that Fungi is also a best source for the production of alpha amylase especially *Aspergillus* and *Penicillium* specie because of their higher yield of enzyme activity.

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# CHAPTER ONE

## 1:0 INTRODUCTION

The new potential of using microorganism as biotechnological source of industrially relevant enzymes has stimulated interest in exploration of extracellular enzymatic activities in several microorganisms ( Bilinski and Stewart 1995). Enzymes have been used for thousands of years to produce food and beverages, such as cheese, yoghurt, beer and wine. (Renge et al., 2012). Enzymes are protein catalysts synthesized by living systems and are important in synthetic as well as degradative process (Omemu A. M et al., 2005).

Alpha amylase enzyme (EC 3.2.1.1) (α-1,4 glucan-glucanohydrolase) is widely distributed in nature. This extracellular starch degrading enzyme hydrolyses α-1,4 glucosidic linkages randomly throughout the starch molecule producing oligosaccharides and monosaccharide including maltose, glucose and alpha limit dextrin (Bhanja *et al.,* 2007). Alpha-amylase enzymes account 65% of enzyme market in world. Amylases had numerous applications including liquefaction of starch in the traditional beverages, baking and textile industry for desizing of fabrics. Moreover, they have been applied in paper manufacture, medical fields as digestive and as detergent additives. Hence, any substantial reduction in the cost of production of enzymes will be a commercial positive stimulus. Fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential. These enzymes have commercial application in various industries .Many useful enzymes are produced using industrial fermentation belonging to the genus *Aspergillus* (Ugru *et al.,* 1997). In fact *Aspergillus niger* is the largest fungal source of enzymes. α-amylase is widespread in animals, fungi, plants, and is also found in bacteria. Amylases from microbial sources are generally used in industrial processes due to a number of factors including productivity, thermo stability of the enzyme as well as ease of cultivating

microorganisms (Reddy and Seenayya 1999). Alpha ($\alpha$)-amylases are produced commercially in bulk from microorganisms and represent about 25-33% of the world enzyme market.

*Moringa oleifera* is a widely cultivated tree considered as a multi-purpose plant (Fahey, 2005). It includes its use as functional food, cleaning water material, oil extraction for biofuel production, and other applications. In a traditional way, it is used with medicinal purposes around the world due to empiric observations (Goyal *et al.,* 2007). These benefits have been associated with metabolites such as phenolic compounds, vitamins and proteins (Adedapo *et al.,* 2009). Nevertheless, the complete knowledge about what kind of metabolites are present in each organ, and their ecological and biological roles, are poorly elucidated. Some studies about macromolecular characterization have been made in the last years, including a protein with the ability to agglutinate, proteinase inhibitors, lectins, carbohydrates and lipid contents ( Santos *et al.,* 2005). Plant parts, including leaves, stem, roots, seeds and flowers have been reported as source of different biochemical compounds with anti-carcinogenic, anti-inflammatory, anti- diabetic, antioxidant, and antimicrobial effects (Peixoto *et al.,* 2011). *Moringa oleifera* contains essential amino acids, carotenoids in leaves, and components with nutriceutical properties, supporting the idea of using this plant as a nutritional supplement or constituent in food preparation. Some nutritional evaluation has been carried out in leaves and stem. Studies have shown that conventional anti-nutritional factors like trypsin and amylase inhibitors, glucosinolates, cyanogenic glycosides, saponins and The enzymatic profiles for amylases also corresponded with variations among plant parts, with more intensity in signals in stem and roots than in leaves, which could be related with the translocation or utilization of reserves. For example, amylases seemed less abundant in leaves. This very likely indicates the leaves as a source organ, actively producing sugars

which are then translocate to sink organs (i.e., stem, roots) where they are stored as starch, and breakdown by amylases, to provide energy to the sinks (Göttlicher *et al.,* 2006).

Many attempts have been made to optimize culture conditions and suitable strains of fungi. Selection of the microbial source for α-amylase production depends on several features, such as the type of culture (solid-state or submerged fermentation), pH and genotypic characteristic of the strain (Gupta *et al.,* 2009).

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi (Pandey *et al.,* 2004). Several additional compounds also released apart from the usual products of fermentation called secondary metabolites which, range from several antibiotics to enzymes .The development of techniques such as Solid State Fermentation (SSF) and Submerged Fermentation (SmF) has led to industrial-level production of useful enzymes. Solid-state fermentation (SSF) has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water. The solid matrix could be either the source of nutrients or simply a support supplemented by the suitable nutrients that allows the development of the microorganisms. There are some disadvantages of SSF like difficulties on scale-up, low mix effectively, difficult control of process parameters (pH, heat, moisture, and nutrient conditions), problems with heat build-up, higher impurity product and increasing recovery product costs. Optimization of various parameters is one of the most important techniques used for the production of enzymes in large quantities to meet industrial demands (Pandey *et al.,* 2009).

Production of extracellular alpha-amylase in fungi is known to depend on the growth of mycelium and both morphological and metabolic state of the culture (Carlsen *et al.;* 1996).

The selection of a substrate (agricultural waste) for enzyme production depends upon several factors mainly related with cost and availability of the substrate, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as anchorage for the cells. These agriculture wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of microorganisms. These nutrient sources included orange and mandarin wastes, rice and wheat bran, tea waste, cassava flour, oil palm waste, apple pomade and banana waste.

An increasing trend toward efficient utilization of natural resources has been observed around the world. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bio-converted into different metabolites, with a higher commercial value. Although several investigations were employed on the production of enzymes by fungal strains using different agriculture wastes, only few researches were done studying the production of enzymes by fungal strains using cassava fibre wastes (Tomsen MH. 2005).

**1:1 JUSTIFICATION**

Indigenous production of amylase enzymes would save a lot of foreign exchange and create jobs, hence, this project intends to use local strains of fungus to produce alpha amylases and reduce costs of production of the enzyme via the use of solid substrate fermentation.

**1:2 SCOPE**

To isolate fungi from rhizosphere of *Moringa oleifera*, using solid state fermentation, typing the alpha amylase producing ability of the fungi isolates, optimizing some amylase production parameters such as inoculum size, pH, and nitrogen source and determining the time course of alpha amylase production.

# CHAPTER TWO

## 2.0: LITERATURE REVIEW

### 2.1 Sources of Enzyme

Alpha (α) -Amylase (E.C.3.2.1.1) can be isolated from plants, animals or microorganisms where they play a dominant role in carbohydrate metabolism. The enzyme has been isolated from barley and rice plants (Oboh, 2005). It has been found that cassava mash waste water is a source of α-Amylase which is active in wide range of pH and temperature.

### 2.2 Fungi as Enzyme Source

Fungi that produce α -amylase have been limited to a few species of mesophilic fungi, fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* and *Penicillium* (Kathiresan and Manivannan 2006). The *Aspergillus* species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance (Hernández *et al.*, 2006). Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry. *A. oryzae* has received increased attention as a favourable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. α -amylase (Kammoun *et al.,* 2008). *Aspergillus niger* has important hydrolytic capacities in the amylase production and, due to its tolerance of acidity ($pH < 3$), it allows the avoidance of bacterial contamination (Djekrif-Dakhmouche *et al.,* 2006). Filamentous fungi are suitable microorganisms for solid state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate (Rahardjo *et al.,* 2005). The fungal α -amylases are preferred over other microbial sources due to their more accepted

GRAS (Generally Recognized As Safe) status (Gupta *et. al.,* 2003). The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase. (Jensen *et al.,* 2002) and (Kunamneni *et al.,* 2005) purified α -amylase, proving its thermo-stability. Fungal amylases are widely used in preparation of oriental foods (Popovic *et al.*, 2009).

### 2.2.1 Advantages and Disadvantages of Microbial Enzymes

1) The growth of microorganisms is rapid and this will in turn speed up the production of enzyme.
2) They require lesser space and serve as more cost effective sources.
3) Microorganisms can be easily manipulated using genetic engineering or other means.
4) They can be subjected to strain improvement, mutations and other such changes
5) Microorganisms can be tailored to cater to the needs of growing industries and to obtain enzymes with desired characteristics like thermo stability as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy.
6) When hydrolysis is carried out at higher temperatures, the polymerization of D-glucose to iso-maltose is minimized (Konsoula and Liakopoulou-Kyriakides *et al* ., 2007).

## 2.3 Function of Alpha Amylase

1) They catalyses the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of D-(1-4) glycosidic bonds.
2) They play a major role, in carbohydrate metabolism.

## 2.4 Application / Uses of Alpha Amylases

Amylase, a starch degrading enzyme have gained importance in various industrial process like pharmaceutical, food, brewing, paper, textile and chemicals. It is extensively used in pharmaceutical industries in digestive tonics, for hydrolysis of starch to produce different sugars like glucose and maltose which have several applications. The most widespread applications of α-amylases are in the starch industry, which are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups (Nielsen and Borchert, 2000).

### 2.4.1 Bakery Industry

Amylases are extensively employed in processed-food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Couto and Sanromán, 2006). The α- amylases have been widely used in the baking industry. These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. It generates additional sugar in the dough,, which improves the taste, crust colour through Maillard reactions responsible for the browning of the crust, the development of an attractive baked flavour (Lundkvist *et al.*, 2007) and toasting qualities of the bread. Besides generating fermentable compounds, α - amylases also have an anti-staling effect in bread baking; a slight overdose may result in gummyness of the bread. This is caused due to production of branched dextrins (Chi *et al.,* 2009). In such cases pullulanase is used in combination with amylase resulting in specific hydrolysis of compounds responsible for the gummy nature of amylase treated bread (Kulp *et al.,* 1981) and they improve the softness retention of baked goods, increasing the shelf life of these products (van der Maarel *et al.,* 2002). Currently, a thermo

stable maltogenic amylase of *Bacillus stearothermophilus* is used commercially in the bakery industry (van der Maarel *et al.,* 2002).

### 2.4.2 Textile Industry

Amylases are used in textile industry for desizing process (Ahlawat *et. al.,* 2009) Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be removed quite easily. Starch is later removed from the woven fabric in a wet-process in the textile finishing industry. Desizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. The α -amylases remove selectively the size and do not attack the fibres (Feitkenhauer, H. (2003).

### 2.4.3 Sugar and Glucose Industry

High fructose containing syrups is prepared by enzymic isomerization of glucose with glucose isomerase. The starch is first converted to glucose by enzyme liquefaction and saccharification. Maltose is a naturally occurring disaccharide. Its chemical structure has 4-0-α-D-glucopyronosil-D-glucopyranose. It is the main component of maltose sugar syrup (Yakup *et al.*, 2010). Maltose is widely used as sweetener and also as intravenous sugar supplement. It is used in food industry because of low tendency to be crystallized and is relatively non-hygroscopic (Sameh *et al.*, 2011). Corn, potato, sweet potato and cassava starches are used for maltose manufacture (Uma *et al.*, 2007). The concentration of starch slurry is adjusted to be 10-20% for production of medical grade maltose and 20-40% for food grade. Thermostable alpha amylase from *B. licheniformis* and *B. amyloliquefaciens* are used (Archana *et al.,* 2011).

### 2.4.4 Paper Industry

The use of α -amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch (van der Maarel *et. al.,* 2002).The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper. In this application, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α -amylases in a batch or continuous processes. Starch is a good sizing agent for the finishing of paper, improving the quality and erasebility, besides being a good coating for the paper. The size enhances the stiffness and strength in paper (Gupta *et al* ., 2003). Examples of amylases obtained from microorganisms used in paper industry includes Amizyme® (PMP Fermentation Products, Peoria,USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and _-amylase G9995® (Enzyme Biosystems, USA).

### 2.4.5 Alcohol Industry

Bio fuels mainly include ethanol fuel. Ethanol can be derived from renewable resources such as waste generated from the agriculture crops and by products. Enzymes such as alpha amylase and others like glucoamylase and cellulose are important to produce fermentable sugars to produce ethanol (Kirk *et al*., 2002) .Ethanol is the most utilized liquid biofuel. For the ethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (Chi *et al.,* 2009). In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars. The bioconversion of starch into ethanol involves liquefaction and saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as α -amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae* (Öner,

2006). In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* (Chi *et al.*, 2009). Among bacteria, α -amylase obtained from thermo-resistant bacteria like *Bacillus licheniformis* or from engineered strains of *Escherichia coli* or *Bacillus subtilis* is used during the first step of hydrolysis of starch suspensions (Sanchez and Cardona, 2008).

### 2.4.6 Chocolate Industry

Amylases are treated with cocoa slurries to produce chocolate syrup, in which chocolate starch is dextrinizing and thus syrup does not become thick. Cocoa flavoured syrups having a high cocoa content and excellent stability and flow properties at room temperature may be produced by using an amylolytic enzyme and a sufficient proportion of Dutch process cocoa to provide a syrup pH of 5.5 to 7.5. The syrup is made by alternate addition of cocoa and sweetener to sufficient water to achieve a solids content of about 58 to 65 weight per cent, adding an amylolytic enzyme, heating to a temperature of about 175 -185°F for at least 10 to 15 min, raising the temperature to about 200° F. and cooling. The stabilized cocoa flavoured syrups may be added at room temperature to conventional non-acid confection mixes for use in the production of quiescently frozen chocolate flavoured confections (Ismail *et al.*, 1992).

### 2.4.7 Detergent Industry

Detergent industries are the primary consumers of enzymes, in terms of both volume and value. The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe.α-Amylase is used to digest the starch containing food particles such as potatoes, gravies, custard, chocolate, etc. into smaller water soluble oligosaccharides. Starch can attract soil particles on to the clothes.

Hence removal of starch is also important to maintain the whiteness of clothes. The stability of α-Amylase at low temperature and alkaline pH contributes to its extensive use in detergents. Examples of amylases used in the detergent industry are derived from *Bacillus* or *Aspergillus* (Mitidieri *et al.*, 2006).

### 2.4.8 Feed, Building Product Industry

Besides amylases application in food, leather, alcohol, paper etc., spectrum of amylase uses has been expanded to many other fields. These are used in animal feed to enhance digestibility (Marc *et al.*, 2002). Many starches or barley material are present in the feed. So, the nutritional value of the feed can be improved by the addition of alpha amylase. Modified starch is used in the manufacture of gypsum board for dry wall construction. Enzyme modified the starch for the industry use.

## 2.5 Moringa oleifera as Medicinal Plant

*Moringa oleifera* (*MO*) is an aboriginal of Indian subcontinent and has become naturalized in the tropical and subtropical areas around the world. Nearly thirteen species of *Moringa* are included in the family Moringaceae (Nadkarni, 1976). Indians have been using it as a regular component of conventional eatables for nearly 5000 years (Anwar et al., 2005; Anwar and Bhanger, 2003; D'Souza and Kulkarni, 1993). *Moringa* tree can grow well in the humid tropic or hot dry land with average height that ranges from 5 to 10 m. It can survive in harsh climatic condition including destitute soil without being much affected by drought (Morton, 1991). It can tolerate wide range of rainfall requirements estimated at 250 mm and maximum at over 3000 mm and a pH of 5.0 to 9.0 (Palada and Chang, 2003). Its trunk is soft, white, corky and branches bearing a gummy bark. Each tripinnately compound leaves bear several small leaf legs. The flowers are white and the three wings seeds are scattered by the winds. The flowers, tenders leaves and pods are eaten as vegetables. The leaves are rich in iron and

therefore highly recommended for expected mothers. In some part of the world, *MO* is referred to as the 'drum stick tree' or the 'horse radish tree', whereas in others, it is known as the kelor, marango, mlonge, moonga, mulangay, nebeday, saijhan, sajna or Ben oil tree (Anwar and Bhanger, 2003; Prabhu et al., 2011). In India and Pakistan, *MO* is locally known as Sohanjna and is grown and cultivated all over the country (Anwar et al., 2005; Qaisar, 1973). It has been reported by Bureau of plant industry that *Moringa* is an outstanding source nutritional components. Its leaves (weight per weight) have the calcium equivalent of four times that of milk, the vitamin C content is seven times that of oranges, while its potassium is three times that of bananas, three times the iron of spinach, four times the amount of vitamin A in carrots, and two times the protein in milk (Kamal, 2008). Besides, *Moringa* is also suggested as a viable supplement of dietary minerals. The pods and leaves of *Moringa* contains high amount of Ca, Mg, K, Mn, P, Zn, Na, Cu, and Fe (Aslam et al., 2005). Although, minerals content of Moringa shows variation in composition with changes in location (Anjorin et al., 2010). *MO* has enormous medicinal potential, which has long been recognized in the Ayurvedic and Unani system (Mughal et al., 1999). Nearly every part of this plant, including root, bark, gum, leaf, fruit (pods), flowers, seed, and seed oil have been used for various ailments in the indigenous medicine (Odebiyi and Sofowora, 1999), but recent research is also indicating about several active constituents for accepting its applicability in modern medicine.

**Antimicrobial and Anti-Helminthic Effects**

Antimicrobial components of *MO* have been validated after the discovery of inhibitory activity against several microorganisms. In a recent study, aqueous extracts of *MO* was found to be inhibitory against many pathogenic bacteria, including *Staphylococcus aureus, Bacillus*

*subtilis, Escherichia coli,* and *Pseudomonas aeruginosa* in dose dependent manner (Saadabi and Abu Zaid, 2011). *MO* extracts was also found to be inhibitory against *Mycobacterium phlei* and *B. subtilis* (Eilert *et al.,* 1981). Leaf extract of *MO* was found to be effective in checking growth of fungi *Basidiobolus haptosporus* and *Basidiobolus ranarums* (Nwosu and Okafor, 1995). Another study involving aqueous methanolic extract and fixed oil against microorganisms was performed using *Scenedesmus obliquus* (green algae), *E. coli ATCC 13706, P. aeruginosa ATCC10145, S. aureus NAMRU 3 25923, Bacillus stearothermophilus* (bacterial strains) and Herpes Simplex virus type 1 (HSV 1) and Polio virus type 1 (sabin vaccine). Varying degree of antimicrobial activity was observed ranging from sensitive for *B. stearothermophilus* to resistant for *P. aeruginosa* (Ali *et al.,* 2004). Beside antibacterial activity of *MO* oils, it also possess anti-fungal activity (Chuang *et al.,* 2007). Study comparing relative antimicrobial activity of seed extracts against bacteria (*Pasturella multocida, E. coli, B. subtilis* and *S. aureus*) and fungi (*Fusarium solani* and *Rhizopus solani*) revealed that *P. multocida* and *B. subtilis* were the most sensitive strains, and their activity was influenced by cations (Na+, K+, Mg2+ and Ca2+) (Jabeen *et al.,* 2008).

Another relative comparison of antibacterial and antifungal efficacy of *MO* steam distillate observed more inhibition for *E. coli* followed by *S. aureus, Klebsiella pneumoniae, P. aeruginosa and B. subtilis.* In case of fungi, *Aspergillus niger* was strongly inhibited followed by *Aspergillus oryzae*, *Aspergillus terreus* and *Aspergillus nidulans* (Prashith Kekuda *et al.,* 2010).

**Anti-Inflammatory Activity**

*Moringa* plant parts have substantial anti-inflammatory activity. For instance, the root extract exhibits significant anti-inflammatory activity in carrageenan induced rat paw oedema (Ezeamuzie et al., 1996; Khare et al., 1997). The crude methanol extract of the root inhibits

carrageenan induced rat paw oedema in a dose dependent manner after oral administration (Anonymous, 2005). Moreover, n-butanol extract of the seeds of *MO* shows anti-inflammatory activity against ovalbumin-induced airway inflammation in guinea pigs (Mahajan *et al.*, 2009). Amelioration of inflammation associated chronic diseases can be possible with the potent anti-inflammatory activity of *MO* bioactive compounds (Muangnoi *et al.*, 2011).

**Anti-Asthmatic Activity**

It has been reported a long time ago that *Moringa* plant alkaloid closely resembles ephedrine in action and can be used for the treatment of asthma. Alkaloid moringine relaxes bronchioles (Kirtikar and Basu, 1975). The seed kernels of *MO* also showed promising effect in the treatment of bronchial asthma, during a study to analyse efficacy and safety of seed kernels for the management of asthmatic patients with a significant decrease in the severity of asthma symptoms and also concurrent respiratory functions improvement (Agrawal and Mehta, 2008).

**Analgesic Activity**

The analgesic activity of *Moringa* has been reported in several *Moringa* species. In a study using ethanolic extracts of *Moringa concanensis* tender pod-like fruits in experimental animals, a significant analgesic activity was observed (Rao *et al.*, 2008). Furthermore, alcoholic extract of the leaves and seeds of *MO* also possess marked analgesic activity as evidenced through hot plate and tail immersion method (Sutar *et al.*, 2008).

**Antipyretic Activity**

As a result of anti-inflammatory action of *Moringa* bioactive constituents, the antipyretic activity can be hypothesized. A study was designed to assess antipyretic effect of ethanol, petroleum ether, solvent ether and ethyl acetate extracts of *MO* seeds using yeast induced hyperpyrexia method. Paracetamol was used as control during the study. Not surprisingly, ethanol and ethyl acetate extracts of seeds showed significant antipyretic activity in rats (Hukkeri *et al.*, 2006).

**Antihypertensive, Diuretic And Cholesterol Lowering Activities**

*Moringa* leaves contain several bio active compounds; they exert direct effect on blood pressure, and thus these can be used for stabilizing blood pressure. *MO* compounds leading to blood pressure lowering effect includes nitrile, mustard oil glycosides and thiocarbamate glycosides present in *Moringa* leaves (Anwar *et al.,* 2007). In addition, diuretic activity of *Moringa* exists in its roots, leaves, flowers, gum and the aqueous infusion of seeds (Morton, 1991). Moreover, *Moringa* leaves also contain bioactive phytoconstituent, (that is, b-sitosterol) with cholesterol lowering effect. This compound is capable to reduce cholesterol level from the serum of high fat diet fed rats (Ghasi *et al.,* 2000).

**Antidiabetic Activity**

*MO* leaves significantly decrease blood glucose concentration in Wistar rats and Goto-Kakizaki (GK) rats, modeled type 2 diabetes (Ndong *et al.*, 2007). Another study indicated that the extract from *Moringa* leaf is effective in lowering blood sugar levels within 3 h after ingestion (Mittal *et al.*, 2007). As a mechanistic model for antidiabetic activity of *MO*, it has been indicated that dark chocolate polyphenols (Grassi *et al.*, 2005) and other polyphenols

(Al-Awwadi *et al.,* 2004; Moharram *et al.,* 2003) are responsible for hypoglycemic activity. *Moringa* leaves are potent source of polyphenols, including quercetin-3- glycoside, rutin, kaempferol glycosides, and other polyphenols (Ndong *et al.,* 2007). Thus, potential antidiabetic activity of *MO* can be commercialized through the development of suitable technology with achieving anti-diabetic activity up to conventional drugs.

**Antioxidant Activity**

*MO* is a rich source of antioxidant (Chumark *et al.,* 2008). It has been reported that aqueous extracts of leaf, fruit and seed of *MO* act as an antioxidant (Singh *et al.,* 2009). During a study reporting antioxidant property of freeze dried *Moringa* leaves from different extraction procedures, it was found that methanol and ethanol extracts of Indian origin *MO* have the highest antioxidant activity with 65.1 and 66.8%, respectively (Lalas and Tsaknis, 2002; Siddhuraju and Becker, 2003). It was also reported that the major bioactive compounds of phenolics, such as quercetin and kaempferol are responsible for antioxidant activity (Bajpai *et al.,* 2005; Siddhuraju and Becker, 2003). During another study, quercetin and kaempferol have shown good antioxidant activity on hepatocyte growth factor (HGF) induced Met phosphorylation with IC50 value for 12 and ~6 μM/L, respectively (Labbe *et al.,* 2009).

# CHAPTHER THREE

## 3.0: MATERIALS AND METHODS

### 3.1. Sourcing of The Seeds Of *Moringa oleifera*

Dry seeds of Moringa oleifera was collected from various locations in Ekiti and was subsequently sun dried for a period of 2 weeks before planting.

### 3.2. Planting on Natural Soil

Moringa oleifera seeds were then planted on a heap of natural soil for 3-4 weeks to encourage the growth of various microorganisms.

### 3.3. Harvesting and Collection Of Samples

The young Moringa plant were allowed to photosynthesize then harvested by collecting the rhizosphere as far as 10 inches down the soil with soil samples surrounding it.

### 3.4. Dilution Process

10g of soil samples was suspended into 90ml sterile distilled water and mixed on the vortex mixer to dissolve the soil particles, 1ml of the stock culture is dispensed into 9ml of sterile distilled water and serially diluted by dilution plating method (Warcup, 1950). 0.5ml was poured onto Sabouraud dextrose agar (SDA) with Streptomycin for the inhibition of bacterial growth and the plate was incubated at room temperature (25°C) for 2-5 days.

### 3.5. Identification of Fungal Isolates

Fungal isolate were characterized and identified on the basis of morphological feature and microscopy using lactophenol cotton-blue stain. Among the characteristics used are colonial characteristics such as surface appearance, texture and colour of the colonies. In addition, microscopy revealed vegetative mycelium including presence or absence of cross-walls, diameter of hyphae.

### 3.6. Solid Substrate Treatment

Fibrous by-products from crushing and sieving (pulp waste) generated from cassava (garri) was collected from cassava processing enterprise in Oye-Ekiti, the sample was sun dried for 6 hours then oven dried for 1 hour at $80^{o}C$ for uniform moisture level.

### 3.7. Development of the Inoculum

For the development of inoculum, culture were transferred from stock onto a SDA plate by inoculating the spores by spot inoculation and allowed to grow for 2-3 days then with the use of sterile swab stick the spores were collected and sub-cultured into 10ml sterile distilled water and shaken, the number of spores per ml was standardized with haemocytometer.

### 3.8. Confirmation of Alpha Amylase Production

Alpha-Amylase production was confirmed on starch agar plates, amylase production was detected after gently flooding the plates with Iodine solution and waiting for 2 minutes. Iodine reacts with starch medium and change the colour of the medium, presences of blue black colour indicates a positive test around the growth area.

### 3.9. Solid State Fermentation

- 5g of solid substrate was taken into 250 ml conical flasks. 15ml of basal medium $(NH_4)_2SO_4$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $KH_2PO_4$) w/v was added, Each flask was inoculated with 0.5ml of inoculum and subsequently incubated for 7 days, At the end of solid state fermentation, the solid substrates were mixed thoroughly with 50 ml of 0.1M phosphate buffer (pH 7.4) and allowed to stand for 1 hour, then filtered using nylon sieve, the filtrate was centrifuged at 10,000 gravity for 10 minutes using refrigerated centrifuge. The filtrate was then used as crude enzyme which was used to test for amylase.

### 3.10. Amylase Assay

0.2ml of the crude enzyme were taken into a test tube, 0.2ml of 1% soluble starch was added, the reaction was allowed to last for 10 minutes in a water bath at 40°C, and 0.4ml of DNSA was added and placed into the water bath for 15 minutes, the absorbance was read with a spectrophotometer at 540nm, readings were compared with the positive and negative control. Bertrand *et al.*, (2004).

### 3.11. Optimization of Process Parameters

Various process parameters affecting α-amylase production in solid state fermentation were optimized. The strategy was to optimize each parameter independently and subsequently optimum conditions were employed in each experimental run. The tested process parameters for this study were;

- Percentage of Inoculum size (0.2, 0.4, 0.6, 0.8 and 1ml),
- Percentage of pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0),

- Different Surfactants ( Tween 80, Tween 60, Triton X100, SDS, Sodium deoxycolate)
- Different Nitrogen sources: Organic (groundnut cake, yeast extract and cow blood meal), Inorganic : $(NH_4)_2SO_4$, $NH_4Cl$, $NH_4NO_3$

5g of solid substrate was taken into 250 ml conical flasks. 15ml of basal medium $(NH_4)_2SO_4$, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $KH_2PO_4$) w/v was added, Each flask was inoculated with various optimization parameters and subsequently incubated for 7 days, At the end of solid state fermentation, the solid substrates were mixed thoroughly with 50 ml of 0.1M phosphate buffer (pH 7.4) and allowed to stand for 1 hour, then filtered using nylon scarf, the filtrate was centrifuged at 10,000 gravity for 10 minutes using refrigerated centrifuge. The filtrate was then used as crude enzyme which was used to test for starch. Bertrand *et al.*, (2004).

# CHAPHTER FOUR

## 4.0: RESULTS

### 4.1. Identification of Fungi Isolates

Different fungi colonies were isolated from the soil sample by serial dilution plating method, and screened for amylase activity by plate assay method. Three (3) fungi isolates were selected and characterized physically and identified as *Aspergillus*, *Penicillium* and *Trichoderma* sp. appropriate references were made using mycological identification keys and taxonomic descriptions (David *et al.*, 2007). And sub-cultured on sterile PDA plate by point inoculation and incubated at 28°C for 48 hours to obtain a pure culture.

| Isolate code | Colony morphology | Microscopic observation | Probable identity |
|---|---|---|---|
| FIS 1 | Velvety, Black creamy and white with time | Septate hyphae, brush-like conidiophores chain conidia | *Aspergillus niger* |
| FIS 2 | Green colour colonies on PDA plates and yellowish with time | Mycelium septate, conidiophores with vesicle-like tips | *Penicillum* sp. |
| FIS 3 | Fluffy green colony with whitish coloration at the edges and dark green with time | | *Trichoderma sp* |

*TABLE 1: MORPHOLOGY OF FUNGI ISOLATES*

## 4.2. Optimization of Cultural Conditions

### Effect of Different Inoculum Size on Enzyme Activity

Addition of different percentage of inoculum size i.e. 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml to the fermentation medium for 7 days and the enzyme activity of each isolates were observed.

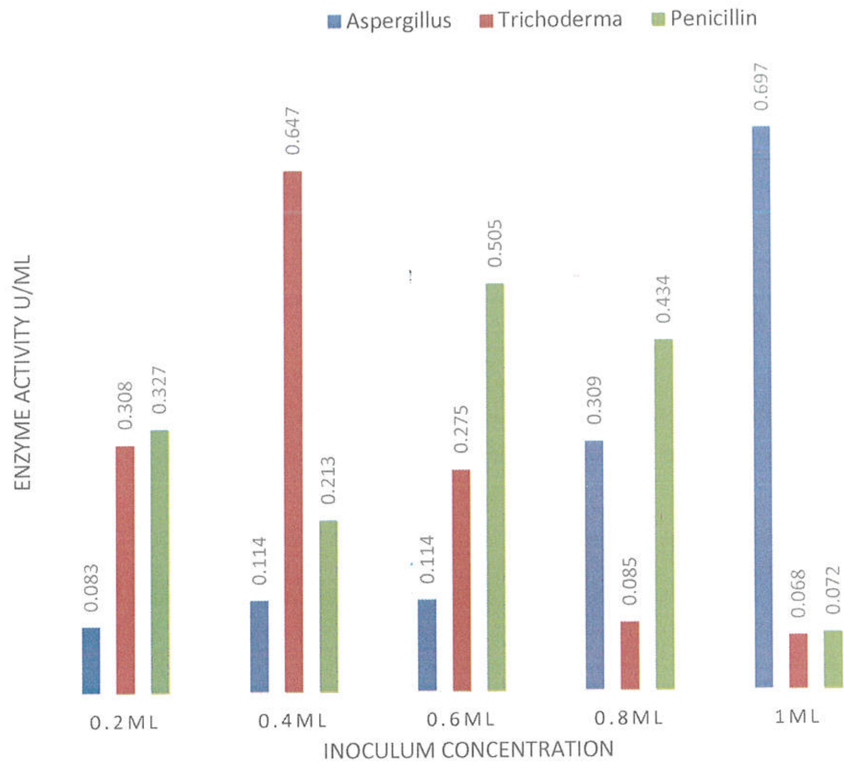


**Figure 1: EFFECT OF INOCULUM SIZE ON AMYLASE ACTIVITY**

**Effect of Different pH on Enzyme Activity**

The effect of pH on amylase activity of the different fungal isolates were observed by varying the pH value from 3.0 to 9.0.

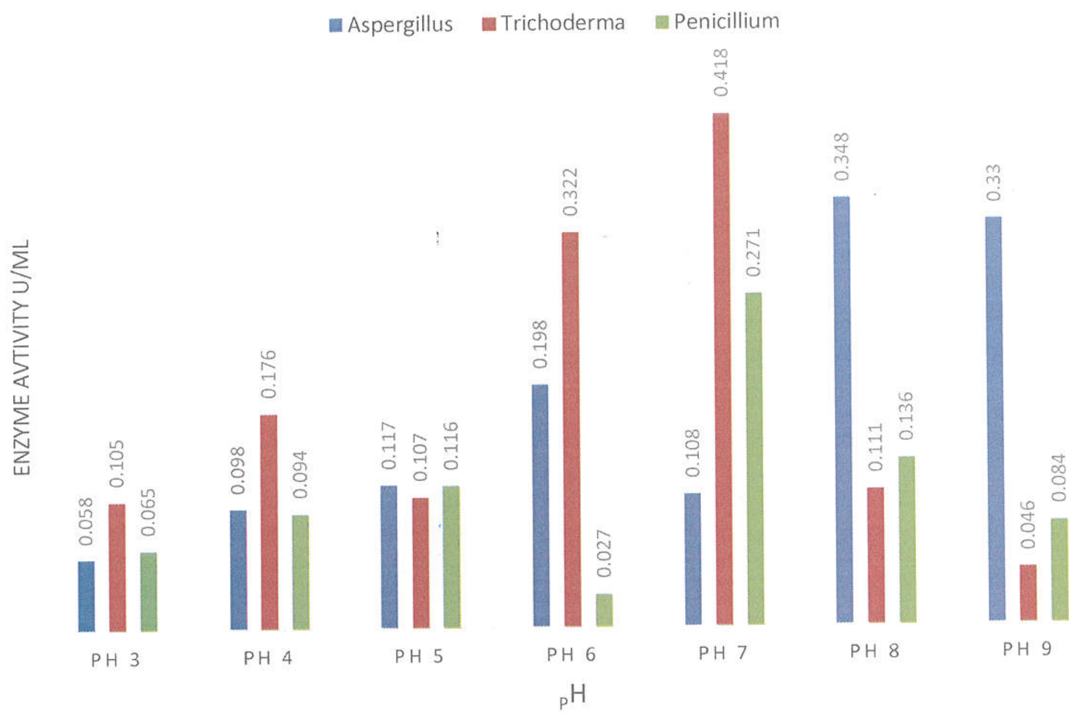


**Figure 2: EFFECT OF DIFFERENT pH ON AMYLASE ACTIVITY**

### Effect of Different Nitrogen Sources on Enzyme Activity

Ammonium Nitrate, Ammonium Chloride, Ammonium Sulphate, Cow blood meal, Groundnut cake and Yeast extract were used as nitrogen source and was added to the fermentation medium separately and incubated for 7 days later observed for amylase activity.

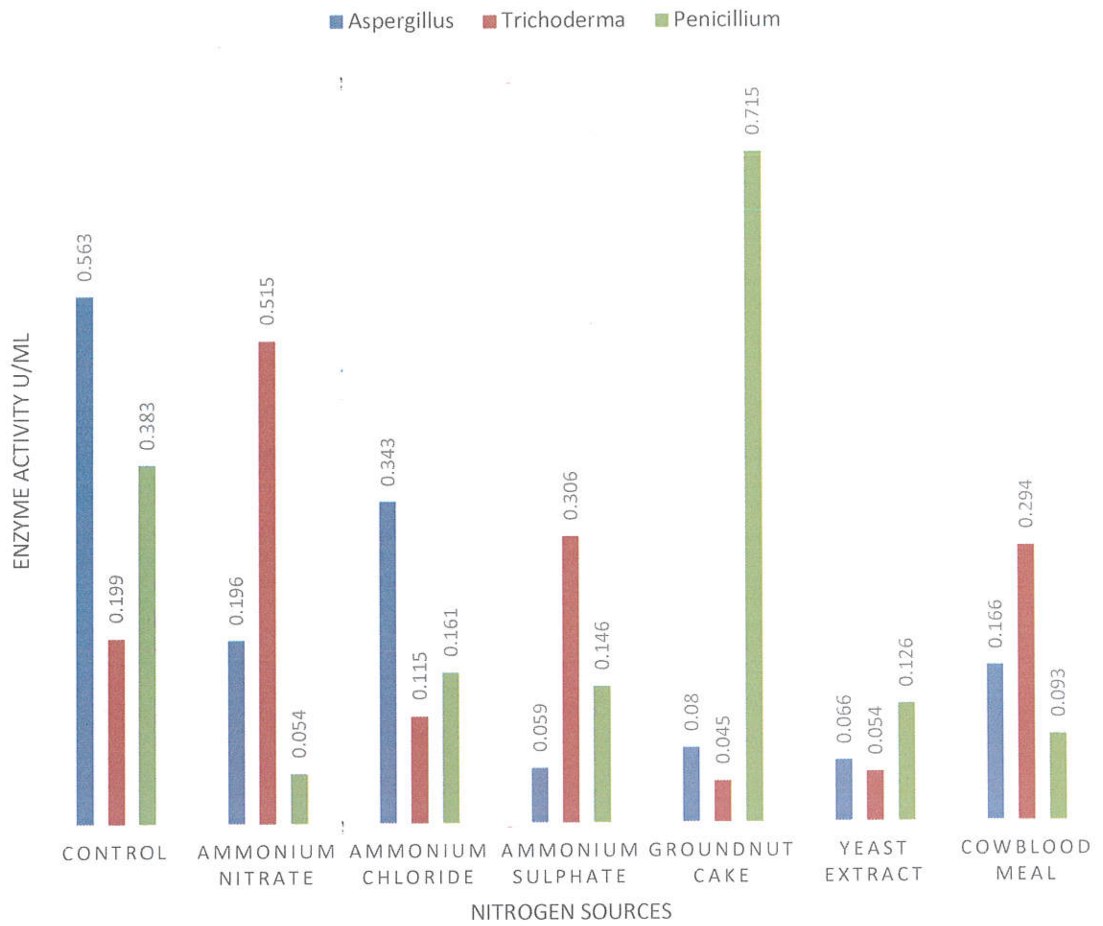


**Figure 3: EFFECT OF DIFFERENT NITROGEN SOURCES ON AMYLASE ACTIVITY**

**Effect of Different Surfactant on Enzyme Activity**

Tween 40, 60, 80, SDS and Sodium deoxycolate were used as surfactant and added separately to the fermentation medium with different fungi isolates.

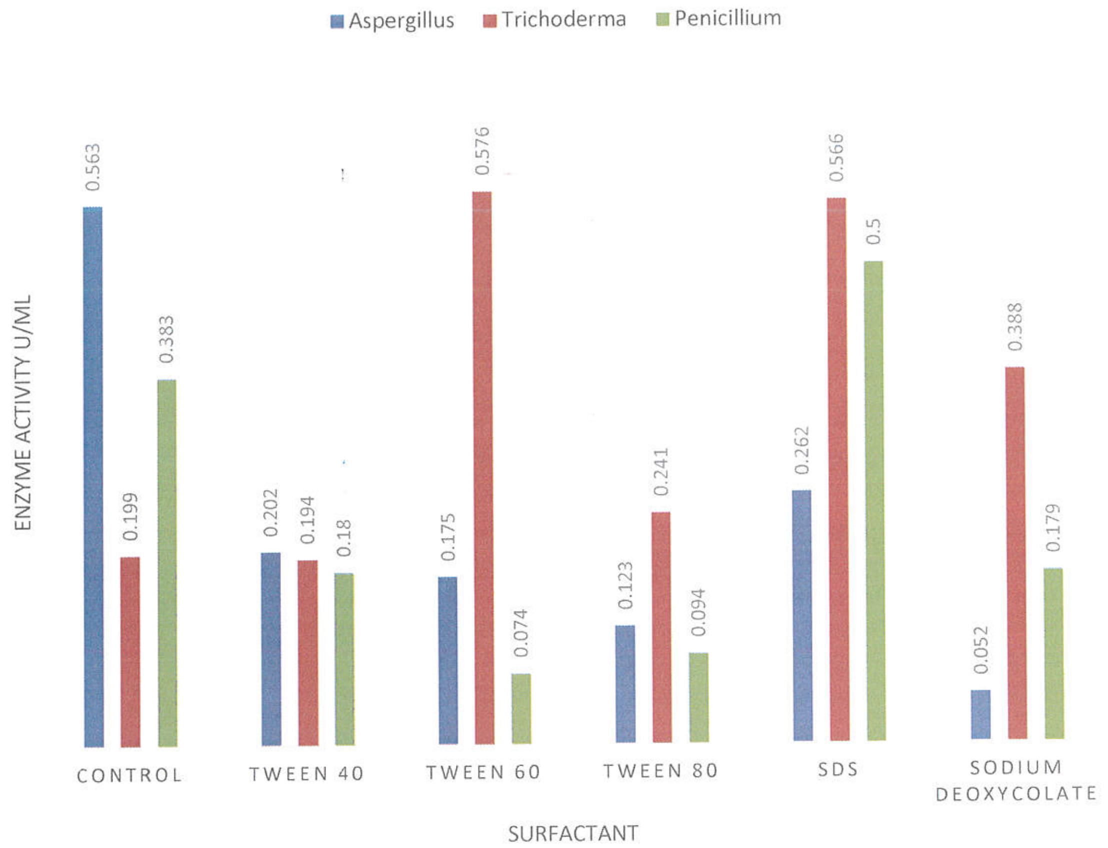


**Figure 4: EFFECT OF SURFACTANT ON AMYLASE PRODUCTION**

# CHAPTER FIVE

## 5.0: DISCUSSION AND CONCLUSION

### 5.1: DISCUSSION

Microbial amylases have successfully replaced chemical hydrolysis in starch processing industries, they are found potential application in a number of industrial processes such as in food, baking, brewing, and detergent, textile and paper industries. With advances in modern biotechnology the spectrum of amylase application has expanded into many other fields such as clinical, medical and analytical chemistry. (Pandey *et al.,* 2000)

In this present study, three (3) fungi isolates were selected and characterized physically and identified as *Aspergillus*, *Penicillium* and *Trichoderma* spp which is similar to a study carried out by Sahoo *et al.,* (2014) also recorded *Aspergillus* and *Penicillium* species as the dominant organisms. Appropriate references were made using mycological identification keys and taxonomic descriptions. These isolates were further used for extracellular amylase production under solid state fermentation.

The result of the effect of different inoculum size of the three selected fungi isolates on the production of alpha amylase is displayed on Fig 1, For *Aspergillus* spp a gradual increase was recorded as the inoculum concentration increases with a maximum amylase activity of 0.697 U/ml which is in agreement with Ramachandran *et al.,* 2004 and Ali *et al.,* 2017. As for *Trichoderma* spp, there was a sharp increase in amylase activity followed by a gradual decrease with the maximum amylase activity of 0.647u/ml while *Penicillium* spp shows an

initial increase and decrease followed by an ensuing increase with a gradual decrease in amylase activity with the maximum activity at 0.505 U/ml.

Fig. 2 shows the effect of pH on alpha amylase activity, *Aspergillus* spp indicates a slow rise in amylase activity with the maximum amylase activity at pH 8 of 0.348 U/ml which is in agreement with Sethi and Gupta (2015), *Trichoderma* spp displays an initial increase then a decline with subsequent increase with maximum amylase activity at pH 7 of 0.418 U/ml in contrast to Sethi and Gupta (2015) also *Penicillium* spp is in contrast with Sethi and Gupta (2015) with maximum amylase activity at pH 7 of 0.271 U/ml. In the course of this study, the varied changes in the wide range of pH is due to the uptake of cations or anions in the medium as a result of the utilization of some compounds in the culture medium.

The effect of different nitrogen sources as shown in Fig 3 in the production of alpha amylase indicates a maximum amylase activity at NH4Cl of 0.343 U/ml and minimum yield at $NH_3SO_4$ of 0.059 U/ml for *Aspergillus* spp, a maximum at $NH_4NO_3$ of 0.515 U/ml and a minimum at groundnut cake of 0.045 U/ml for *Trichoderma* spp and a maximum at groundnut cake of 0.715 U/ml and a minimum at $NH_4NO_3$ of 0.054 U/ml for *Penicillium* spp. Yeast extract has the lowest yield of amylase activity in organic nitrogen sources which is in contrast with Sethi and Gupta (2015), Muhammad *et al.,* (2012), Oshoma *et al.,* (2010), Valaparla (2010), Anto *et al.,* (2006), Pederson and Neilson (2000), while NH4Cl is the best source for alpha amylase production which is similar to Reya *et al.,* (2015). According to this study inorganic nitrogen sources has the best yield for alpha amylase production in contrast with studies carried out by Vahidi *et al.,* (2004) and Akhilesh *et al.,* (2010).

The result of the effect of surfactant on alpha amylase production on Fig 4 shows that *Aspergillus* spp has the maximum amylase activity at SDS of 0.262 U/ml and minimum at sodium deoxycolate of 0.052 U/ml, while *Trichoderma* spp has a maximum at Tween 60 of

0.576 U/ml and minimum at Tween 40 of 0.194 U/ml and *Penicillium* spp has a maximum yield at SDS of 0.500 U/ml with a minimum at Tween 60 of 0.074 U/ml. As indicated by this study SDS is the best source of surfactant used in the production of alpha amylase.

## 5.2: CONCLUSION

The result of this research work shows that fungi has the ability of utilizing various carbon and nitrogen source as good substrate for their growth as well as the production of amylase in solid state fermentation . Fungi being heterotrophic obtain their nutrient from organic matter in the environment and are able to utilize complex carbon sources as their energy source.

Although the results of these investigations are based on experiments conducted in flasks, they provide valuable information for the production of α-amylase by solid state fermentation process at a larger scale especially applying agro-industrial residues as substrates.

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