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Effect of Static Magnetic Field on Amylase and Protease Produced by some Fungal isolates using Solid State Fermentation

A thesis

Submitted to the Council of College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قَالَ رَبِّ اشْرَحْ لِي صَدْرِي (٢٥) وَيَسِّرْ لِي أَمْرِي (٢٦)
وَاحْلُلْ عُقْدَةً مِنْ لِسَانِي (٢٧) يَفْقَهُوا قَوْلِي (٢٨)

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Dedications

To the candle that burned to enlighten my way in life

My Mother

To the people who were always encouraging me in my life

My Brothers

To the soul that he was always beside me

Majeed Ali

To the woman who were always encouraging me

in my life

Riyam

Summary

In this study, the effect of static magnetic field on the production of amylase and protease enzymes using solid state fermentations from five different fungal species *Alternaria* sp., *Aspergillus niger*, *Fusarium* sp., *Mucor* sp., and *Penicillium* sp. were investigated. The substrate used for fungi growth was bread only. The above species were exposed to the Northern pole, Southern pole and both poles together (South + North) and their effects were compared with the control treatment (The control of all experiments was the solid medium without the effect of the magnetic field). The results were statistically analyzed by GenStat programm and the Least Significant Differences (LSD) was determined. The results showed that the Northern pole significantly decreased the specific activity of amylase enzyme of *Alternaria* sp., *Fusarium* sp., and *Penicillium* sp. which were 2.50, 2.12, and 3.27 U/mg respectively. The Southern pole significantly increased the amylase specific activity of *Fusarium* sp. (3.84 U/mg) and *Mucor* sp. (3.36 U/mg), while it significantly decreased amylase specific activity of *Alternaria* sp. (2.77 U/mg) and *Penicillium* sp. (3.88 U/mg) compared with control. The effect of both poles was as follow: they were significantly decreased the amylase specific activity of *Alternaria* sp. (2.62 U/mg) and *Penicillium* sp. (2.26 U/mg), on the other hand, they significantly increased the amylase specific activity of *Fusarium* sp. (3.84 U/mg) and *Mucor* sp. (3.83 U/mg). Regarding the effect of the magnetic field on the protease specific activity, Northern pole significantly decreased the protease specific activity of *Alternaria* sp., *Aspergillus niger*, and *Penicillium* sp., which were 16.86, 14.69, 12.03 U/mg respectively. The Southern pole significantly increased the protease specific activity of *Fusarium* sp. (23.04 U/mg) and *Mucor* sp. (12.15 U/mg) except *Alternaria* sp. in which its protease specific activity

was decreased significantly (19.30 U/mg). Both poles significantly increased protease specific activity of *Fusarium* sp. (21.03 U/mg) and *Mucor* sp. (9.75 U/mg), whereas they significantly decreased protease specific activity of *Alternaria* sp. (13.65 U/mg) and *Penicillium* sp., (8.19 U/mg). This study clearly showed that there are significant effect of the electrostatic magnetic field in increasing and decreasing the enzymes activities of the fungal species which could be exploited industrially in increasing the production of important enzymes in industry such as proteases and amylase tested in this study.

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LIST OF ABBERVIATION

Abbreviation	Meaning
T	Tesla
SMFs	Static magnetic fields
EMF	Electromagnetic field /fields
SSF	Solid state fermentation
Hsp	Heat shock protein
DNA	Deoxyribonucleic acid
DNS	Dinitro-sylcilic acid
G	Gauss
LSD	Least significant differences
MF	Magnetic field
mg/ml	Milligram per milliliter
Na/K ATPase	Sodium – potassium Adenosine tri- phosphatase
OMF	Oscillating Magnetic Field
ELF	Extremely low frequency
UV	Ultra-Violette
Bp	Base pair
PDA	Potato dextrose agar
BSA	Bovine serum albumin
Hz	Hertz
Aw	Water activity
DC	Direct current
AC	Alternate current
µm	Micrometer

BC	Before Christ
A.D	Anno Domini
RNA	Ribonucleic acid
mRNA	Messenger RNA
TCA	Trichloroacetic acid
µmole	micromole
Fe Mn	Ferromanganese

Chapter One

Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

Mycology is a term derived from a Greek word *myke* means 'mushroom', and *logos* means 'study'. So, mycology is the study of mushrooms. However, mycology is commonly used to refer to the study of organisms called fungi. Fungi are found in two structural forms. One of them yeast cells which are unicellular. The other is hyphae which made up of thread like structures. A group of hyphae are known as mycelium. Outer cell wall of Fungi are typically made up of a porous made up of chitin. Inner to the cell wall is a cell membrane that is wrapped up in places to increase its surface area for exchange of materials. Heterotrophs can either be saprobes, symbionts or parasites. Reproduction occur both sexual and asexual means. Fungi are important in the food industry. In many counties, mushrooms which considered a delicacy at the dining table. Species such as *Penicillium* sp. are used to add flavour to cheese. The yeasts are important in the fermentation processes of wine and beer manufacturing. Fungi are important in the breakdown of organic matter and organic wastes, it useful in cleaning up of wastes in an ecosystem and contribute to the recycling of nutrients. Some of fungi cause various diseases for plants, but only a few species cause disease in animals and humans, these diseases are permanent (Alexopoulos *et al.*, 1996).

The history of magnetic materials development cannot be denied and it is fabulous. Magnetic objects played an important role in the discovery of the new world and in the development of modern technology. The magnetic properties of materials are important to understand them which led to a deep understanding the main structure of materials. One of the

basic properties of materials is magnetization; these materials appear in different forms, but the studied form known as ferromagnetism. Magnetic field only refers to the elements that exhibit ferromagnetism properties. A magnet is an object that has a magnetic field. "Magnítis líthos", is a Greek word mean magnet which means "magnesian stone". Magnetite has been discovered in Magnesia which is an area in Greece where Magnetite deposits (Peter *et al.*, 2002).

For long time period the effect of magnetic field on biological systems has been an area of interest. Unlike time-varying (electromagnetic) field, which are not related with induced electric currents except during activation and deactivation or when there is motion within the magnetic field. It is effect on the molecular structure of spasmodic membranes, an influence sufficient to alter the role of embedded ion-specific channels.

The effect of the magnetic field energy causes significant changes in the characteristics metabolism of organisms; these changes occur in the exchange of ions through the cell membrane and in the movement of cells. Magnetic field (MF) and electromagnetic field (EMF) that are generated from both external MF and EMF and internal sources natural metabolisms of organisms effect on biological systems. Depending on several studies magnetic and electromagnetic fields have various responses for biosystems such as neuromuscular activity, repair and tissue growth, glandular secretion, and cell membrane function. Electromagnetic field has an effect mostly on charged units and related metabolisms and usually has influence on biochemical reactions that include more than one unpaired electron. The impact of an external magnetic field on enzymatic reaction rates can be determined in the same way classical enzyme kinetic parameters are determined. Enzymes

with chromogenic substrates or products can be followed spectrophotometrically (Koch *et al.*, 2003).

The purpose of the study was to:

Investigate the effect of static magnetic field on amylase and protease production using solid state fermentation.

1.2 Literature Review

1.2.1 Magnetism and Electromagnetism

The force between magnets that pull or push other objects is a physical phenomenon known as magnetism. The materials which exhibit magnetism, some of them are stronger than others. The only form of magnetism strong enough to be felt by people is a ferromagnetism which is permanent magnets, made from iron, which experience the strongest effects (Jiles, 1991).

The force between electrically charged particles defined at the subatomic level as an electromagnetism. It is considered one of the essential interactions of matter. Electromagnetic waves results from oscillating electrical charges. Attraction and repulsion of electrically charged particles resulted from electromagnetism which is related to the electromagnetic force. Which is in nature has been considered one of the essential forces that also include nuclear and gravitational forces. Electrically charged particles, such as electrons, create a magnetic field when they are put into motion and they create electromagnetic radiation when made to oscillate. Depending on the frequency of the oscillation, this can include radio waves, visible light, or x-rays. On a larger scale the creation of a magnetic field from the movement of electrical charges known as electromagnetism. Electrodynamics is the use of electric effect that uses varying magnetic field an electromagnet to induce an electric current (Grant and Phillips, 2008).

1.2.1.1 Magnate and Magnetic Field Energy

Magnetism is the result of electrically charged molecules. Particles in the field can be affected by a magnetic field force which is called Lorentz force. This force depends on three factors namely: particles speed,

magnetic field strength, and the charge magnitude. Though connected, electrical and magnetic fields are different and do different things. Magnetic fields and their lines were first tested by Michael Faraday then by James Clerk Maxwell (Kronmüller, 2007).

Magnetic field is the force surrounding the magnet in which magnetic materials affected by this force. Around a magnet the shape of the magnetic field can be shown as lines. From these lines arrows will point out toward the south and point away from the north to show the direction of the magnetic field. These magnetic force lines do not cross each other. As shown in the Figure (1-1).

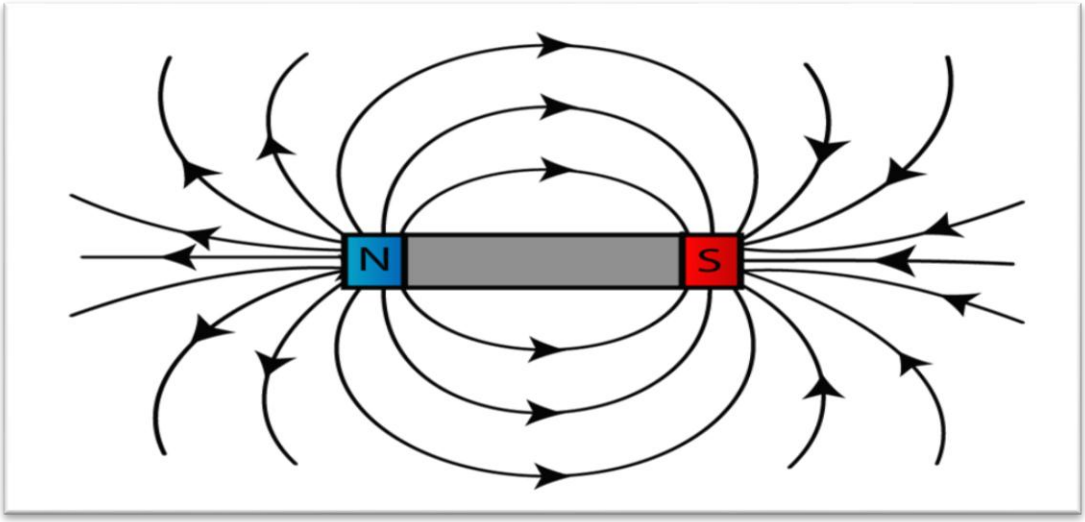


Figure (1-1): The magnetic field is represented by the magnetic field lines.

Magnetic poles are the points where the lines of magnetic fields begin and end. Fields lines move away from each other then it will come close to each other on the poles. These poles are places in which our earth field lines come close to each other. These poles named north and south because of their location on our planet. Every magnetic material has poles and field lines. If a magnet is divided into two pieces, this will make two magnets with new north and south poles. At the fracture point, new north and south poles will be formed (Cullity and Graham, 2008).

1.2.2 The Effects of Magnetic Field on Living Organisms

The influences of magnets and magnetic fields have been mentioned many years ago. Galen, a Greek physician about 200 BC, in book, De Simplicium Medicamentorum Facultatibus, noted about the use of magnets. Also, it had been found that the using magnetism to relieve various disorders in 1000 A.D. In the early 1500s, the great medical doctor, Paracelsus studied several cases on the use of magnet and magnetism. Paracelsus introduced many hints for the nature and use of magnetism, who taught a whole philosophy of universal magnetism (Presman, 1970; Binhi and Savin, 2003).

Several studies have been carried out to examine of the impact of magnetic field on the enzymes activity (Blank *et al*., 1995; Blank and Soo, 1998) and DNA (Ivancsits *et al*., 2002; Nikolai *et al*., 2004). Enzymes are important in the biological processes and cell communication, any change in the enzyme activity may effect on these biological processes.

Some studies mentions that the influence of the magnetic field on cellulolytic microorganisms regarding metabolism. The influence of the magnetic field on the microorganisms was recognized since (1937), when Kimball found that the wine yeasts cells were not affected after exposure for several period of time to the magnetic field (Kimball, 1937). *Staphylococcus aureus* growth rate was increased when exposure to a magnetic field for (3-6) hr, while there was no effect after 7 hr of exposure. The growth rate was unchanged when *Serratia marcescens* was exposed to the magnetic field for 6 hr, but it increased after exposition to a long time (Gerencser *et al.,* 1962). The morphology of *Pseudomonas aeruginosa* was changed by the influences of different levels of static magnetic field. The growth of *Escherichia coli*, was enhanced by static

magnetic field and this enhancement of the growth is proportional to the increase of the magnetic field frequency (Moore, 1979).

The effects of magnetic field on the growth rate of plants have been object of many scientists. The first studies were reported by Savostin, (1930), who found that the increase 100% in seedlings elongation rate by the effect of magnetic field. The strong magneto tropic affection on root development was reported by (Audus, 1960; Pittman, 1977).

Magnetic field has distinctive influence on biological systems. There are many reports about the exposure to magnetic field which effect on different plant functions such as growth (Racuciu and Creanga, 2006), development (Yano *et al.,* 2003; Rakosy-Tican *et al.,* 2005), protein biosynthesis and enzyme activity (Alikamanoglu and Sen, 2011). The mechanism of interaction of fields with the living cells is still unclear (Atak and Emiroglu, 2003). Magnetic field causes an oxidative stress, that may increases the activity and lifetime of the free radicals which are very reactive byproducts of normal metabolism and immune defense (Scaiano *et al.*, 1994). It has been known that the reactive oxygen species which are produced during stress can harm many cellular components like nucleic acids, carbohydrates, proteins and lipids (Halliwell, 1982). Therefore, MF could be altering the activity of antioxidant enzyme (Sahebjamei *et al.,* 2007; Alikamanoglu and Sen, 2011).

Many studies have shown that the influence of static magnetic fields on biological systems and time-varying magnetic or electromagnetic fields on transport of membrane cations and other functions. These studies show that the influences of exposures to different magnetic fields are not identical. While some reports display an inhibitory effect by the fields, others show activation, while others showed no significant effects on the transport of cation. Many studies indicated to the effect of magnetic field on Ca^{2+} influx across the cell membrane or intracellular

Ca^{2+} movements. Significantly exposed to the time-varying magnetic field suppressed the increase in [Ca^{2+}] and partially inhibited the K^+ influx through Ca^{2+}-dependent K^+ channels, proposing the inhibition of Ca^{2+} influx and/or Ca^{2+} release by the exposure. However, the inhibition of K^+ influx is due to a direct magnetic field exposure on K^+ channels rather than suppression of the increase in [Ca^{2+}] (Ikehara *et al.*, 1997). Liburdy found that the magnetic field affects Ca^{2+} uptake across the cell membrane, but the release of Ca^{2+} from its stores is not affected. This is because eddy current induced by the magnetic field or electric field could not enter the outer cell membrane. The cell membrane is bilayers that act as an electric insulator (Liburdy, 1992). Magnetic fields influence the charge of the cell membrane, which open up the membrane channels. These channels are similar to windows and doors of a house. By opening cell channels, nutrients are capable to enter the cell, and waste is more easily removed from the cell. This would assist to equilibrate and restore optimum cell function.

Also, other studies indicate that the static magnetic fields affect the diffusion of particles in solutions by induction of Maxwell stress or Lorentz force. Lorentz force would influence the diffusion of charged particles like different ions that passes in and out the plasma proteins (Kinouchi, 1988).

1.2.2.1The influence of Magnetic Field on DNA and Cell Division

The interactions between biological activities and electromagnetic fields have focus on different time period exposure to Alternating Circuit fields. Effects on *in vitro* biochemical reactions have been reported .The studies revealed that the DC (static) magnetic fields can interact with living activities at different levels. (Adamkiewicz and Pilon, 1983; Kim,

1976; Markov *et al*., 1992; Richardson *et al.*, 1992; Harkins and Grissom, 1994).

Many researchers have found that the magnetic field affects the growth of bacteria, which includes an increase in mass and cell division. Exposure of *Escherichia coli* to an AC field (0−2 mT, 16 and 50 Hz), shows shortened in generation time (Aarholt *et al.*, 1981). Powerful magnetic fields (5.2 − 6.1 T) caused delayed cell death in stationary cultures of *Bacillus subtilis* (Nakamura *et al.*, 1997). Whether or not an AC magnetic field exerts an inhibitory or else a stimulatory mode of action based on a complex manner on the frequency and the field strength. Moore found high or even no growth rates for *Staphylococci, B. subtilis, Halobacterium, Salmonella typhimurium and Candida albicans,* after exposure to AC frequencies ranging from (0 - 0.3 Hz) and magnetic flux densities of (5−90 mT) (Moore ,1979).

It has been found that the fungus *Physarum polycephalum* responds to weak AC fields (0.2 mT, 60, 75 Hz) with a delay mitotic cycle (Marron *et al.*, 1978; Marron *et al.*, 1975), displayed by an enlarged mitotic cycle span at (0.2 mT and 75 Hz) (Goodman *et al.*, 1976; Goodman *et al.*, 1979). Also, AC fields (0.1 mT, 60 Hz) caused decrease in ATP levels in *Physarum polycephalum*, but no reduction in respiration (Marron *et al*., 1986). Reduced respiration found with (0.2 mT 60 and 75 Hz) (Marron *et al.*, 1975).

Some other studies revealed that weak magnetic fields (0−110 μT) affect DNA-protein conformations in *E. coli*. (Binhi *et al.*, 2001). In *Salmonella,* AC fields (14.6 mT, 60 Hz) do not cause DNA breaks (Williams *et al.*, 2006). Also, AC magnetic fields (120 μT, 50 Hz) cause a reduction in the survival of *Saccharomyces cerevisiae* after UV

irradiation, while sustaining no effect on cell cycle kinetics (Markkanen *et al.*, 2001).

The magnetic fields (AC) can enhance specific sets of genes. In *E. coli*, σ32 mRNA (transcription factor) was increased when exposed to (1.1 mT and 60 Hz) (Cairo *et al.*, 1998). Pulsed square fields (1.5 mT) enhanced the increase in α subunit of RNA polymerase. The translation machinery itself must be magneto sensitive and was not, for example, relying on the existence of a biomembrane. An important observation in this subject was the fact that AC fields can induce translation, even in an in vitro system (Goodman *et al.*, 1993). The weak, alternating, magnetic field (8 and 80 μT, 60 Hz) was able to increase the transcription of human or mouse c-myc genes (Lin *et al.*, 1994). The same response elements were also detected the heat shock gene hsp70 in the promoter region (Goodman and Blank, 2002). This influence depends on the presence of response of specific electromagnetic elements localized between (−353 and −1257 bp) relative to the promoter (Lin *et al.*, 2001).

After exposing *E. coli* to AC fields, there was no alteration in the profile of stress proteins occurred (7.8 − 14 mT, 5 − 100 Hz) (Nakasono and Saiki, 2000). In *Shewanella oneidensis* strong magnetic fields (14.1 T) caused the transcriptional up-regulation of 21 genes and the down-regulation of 4 genes, while at the similar time caused no substantial alterations in growth (Gao *et al.*, 2005). Furthermore, *in S. cerevisiae* no changes in protein profile (2-D gel analysis) or differential gene expression (microarray analysis) were found when exposed to AC magnetic fields (10 − 300 mT, 50 Hz) (Nakasono *et al.*, 2003).

1.2.3 Fungi

Fungi are an essential kingdom of organisms, comprising about 77,000 named species. Mycologists and scientists believe there may be more than 1.2 million fungi species existed on earth. Although fungi have traditionally been classified under the plant kingdom, they do not have chlorophyll (non-photosynthetic) and resemble plants only in their general appearance and lack of mobility (Alexopoulos *et al.*, 1996).

Fungi are eukaryotic organisms classified into yeasts (unicellular microorganisms) or molds (multicellular filamentous fungi). Fungal cell wall differs from that of plant by containing chitin instead of cellulose. The energy reserve of fungi is not starch like plants but it is glycogen like animals. Fungi are aerobic and non-motile. Most are heterotrophic and consume organic matter. Fungi reproduce sexually by mating of two opposite types and fusing of their hyphae. Asexual reproduction by spores or fragmentation (breakage) or binary fission or by budding, hyphae which can produce new mycelium (Terborgh, 1992).

A key characteristic of fungi is the creation of a mycelium (filamentous thallus). A mycelium is composed of branching microscopic tubular cells called hyphae (singular, hypha). These hyphae are typically composed of long chains of cells divided by cross-partitions termed septa (singular, septum). The fungi have unique features which indicate that these microorganisms are not related to other group of organisms. The septa rarely form a complete barrier except when they isolate the reproductive cells. Cytoplasm moves freely throughout the hyphae passing right through major pores in the septa (Harris, 2008).

The mycelium (plural, mycelia) is the vegetative part of a fungus, is a collection of hyphae that grow through and across substrates or food sources, enzymes secreting to break down difficult substrates into simple compounds which can be diffused through the cell wall, resulting in a

unique relationship between the fungi and their environment. Whole components of a fungus are metabolically active continuously interacting with their environment such as soil, plants, or any other materials which enhance the growth of mycelium (Kirk *et al.*, 2008).

Fungi are different from most other organisms in mitosis. The nuclear envelope does not break down and re-form. Mitosis occurs inside the nucleus. Centrioles are lacking in all fungi instead, fungi adjust the construction of microtubules throughout mitosis with relatively small shapeless configurations called spindle plaques. These unique characteristics of fungi suggesting that these microorganisms originated from some unidentified group of unicellular eukaryotes. A spindle apparatus forms there, pulling chromosomes to the poles on each side of the nucleus (Moens and Rapport, 1971).

Fungi are achlorophylls meaning that they do not contain chlorophyll which make them incapable organisms to make their own food like plants do. Fungi based on other organisms for their carbon source. Hence, fungi are heterotrophic organisms. These are fungi that derive their nutrition from other organism protoplasm named the host. Fungi have a distinct mode of nutrition which involves the liberation of enzymes to the surrounding environment to break down the substrates needed for nutrition. Then this substrate is processed outside the cell and absorption of the products (monomer forms) occur through the permeable cell wall and the selectively porous membrane for ultimate digestion by the cells (Raven and Johnson, 1999).

Fungi play important ecological and commercial roles. The organisms that have the capability to decompose lignin (one of the major constituents of wood) are fungi. By breaking down such substances fungi release essential chemical elements like carbon, nitrogen, and phosphorus from the bodies of dead organisms and make these elements accessible to

other organisms. Some fungi attack living plants and animals through in breakdown of organic matter some as a source of organic particles while other fungi invade dead ones (Boerjan *et al.,* 2003).

Two types of mutualistic relations between fungi and autotrophic organisms are important ecologically. Lichens are two organisms associated symbiotically which involved a green algae or less frequently cyanobacteria with a fungus. The fungus gets carbohydrates formed by photosynthesis from the algae or cyanobacteria and the fungus provide them with protection from dryness and ultraviolet light. They are prominent nearly everywhere in different environments around the world particularly in abnormal severe habitats like plain rock. Mycorrhizae, a specialized mutualistic symbiotic association between the fungi and the roots of plants, are typical of about 90% of whole plants kingdom. In each of them, the photosynthetic organisms captured Co_2 from the atmosphere and thus make organic material available to the fungi. The metabolic activities of the fungi consecutively improve the overall ability of the symbiotic association to exist in a particular habitat. The fungal partner accelerates the absorption of important nutrients like phosphorus into the plants (Rodriguez *et al.,* 2009).

Classification of fungi depends on reproductive structures. There are three phyla of fungi. In zygomycetes the hyphae fusion leads to the formation of a zygote, (Phylum zygomycota). (Phylum Ascomycota), in ascomycetes, hyphal fusion leads to constant dikaryons that grow into dense webs of hyphae that form zygotes within a characteristic saclike structure the ascus, mostly ascomycetes that play many important commercial and medical roles. In basidiomycetes, dikaryons also form but zygotes are produced within reproductive structures named basidia, (Phylum basidiomycota). The imperfect fungi have not been observed to

reproduce sexually. Therefore, it cannot be classified into one of the three phyla (Hibbett, 2007).

1.2.3.1 Amylase Enzyme

A group of enzymes known as amylase are secreted by several microorganisms like fungi and bacteria to the outside of the cells to performed extra-cellular digestion (Ellaiah *et al.*, 2003). Source of Fungi is restricted to terrestrial isolates; mostly to *Aspergillus* spp. It has been found that many fungi will be good sources of amylolytic (amylase) enzymes. The highest amylase activity was possessed by *Aspergillus* spp. due to the ubiquitous nature and non-fastidious nutritional requirements of *Aspergillus niger*, studies on fungal amylase particularly in the developing countries have been done mainly on this organism (Abe *et al.*, 1988).

In 1894, amylase was the first enzyme produced industrially from a fungal source, which has been used for the treatment of digestive disorder (Crueger and Crueger, 1984). Amylase is an enzyme that breaks down starch into sugar. In starch processing industries, chemical hydrolysis of starch have been successfully replaced by microbial amylases, they also have potential application in many industrial processes like food, baking, textile, paper, and detergent industries. Besides their use of starch saccharification application of amylase has been extended into many other disciplines like medical, clinical, and analytical chemistry (Pandey *et al.*, 2001).

The solid state fermentation (SSF) processes have been progressively applied for the production of amylases which have been produced by submerged fermentation. By comparison between solid state fermentation and submerged fermentation (SSF), the latter is more simple, has superior productivity, require lower capital, reduce energy requirement, simple

fermentation media and lack of severe control of fermentation conditions, uses less water and produces lower waste water, has easier regulation of bacterial contamination and a low cost is needed for downstream processing (Facciotti *et al.*, 1989).

1.2.3.2 Protease Enzyme

Proteases "previously consider as enzymes of breakdown" are one of the first and major families of enzymes known and are participate in each part of organism's function. They contain a very large and complex group of hydrolytic enzymes that breaks down the peptide bonds of proteins to produce amino acids and other smaller peptides (Mitchell *et al.*, 2007). It was established in all living systems: animals, plants, and microorganisms including viruses. Proteases have a wide range of applications was mostly used in foodstuff and cleaner productions (Yandri *et al.*, 2008). Also, these enzymes vary in their properties like active site, substrate specificity and catalytic mechanism; possess different mechanical stress responses for chemical environment, temperature and pH for stability and activity. Fungal proteases are dynamic over an extensive pH range (pH 4-11) and display broad substrate specificity (Rao *et al.*, 1998).

Proteases work optimally in acidic environments excluding alkaline proteases which has its optimal activity shown in alkaline (basic) pH (Mitchell *et al.*, 2007). They are one of the enzymes classes that have great importance in industry and constitute about 60% of the total world-wide enzyme sales (Barrette and Rawlings, 2003). They are application in several of biotechnological processes such as in pharmaceuticals, food processing, leather industry, and detergent formulations, (Nascimento and Martins, 2004; Beg and Gupta, 2003).

Proteases are one of the main groups of enzymes which secreted by fungi. The submerged as well as solid state fermentation are useful techniques that used for protease production, when cultured in solid-state fermentation usually show better results as compared to bacteria (Pandey *et al.*, 1999). In addition, the conditions in solid state fermentation (SSF) system especially the low moisture content in the system lead to some potential benefits. (i) low moisture content lower the risk of bacterial contamination during the fermentation (ii) SSF conditions favor growth of filamentous fungi since wood, leaves, roots and other organic matter are their natural habitats (iii) The environmental conditions in solid-state fermentation can enhance the microbe to produce enzymes whose features could be dissimilar to those enzymes produced by the identical organism in the submerged fermentation environments.

Molds of the genera *Aspergillus, Penicillium* and *Rhizopus* are especially important for producing proteases. Several species of these genera are generally considered safe (Devi *et al.*, 2008). Among fungi, the ability of many species of *Aspergillus* to produce proteases is well known. *Aspergillus oryzae* produces neutral, acid, and alkaline proteases (Godfrey and West, 1996). *Aspergillus clavatus* ES1 has been recently discovered as a producer of an extracellular decolorizing constant alkaline protease (Hajji *et al.*, 2008). Protease is an important group of enzymes which occupy an essential place with respect to their application in both biological and industrial fields (Pastor *et al.*, 2001; Ward, 1985).

1.2.4 Solid-State Fermentation

The fermentation technique in which microorganisms can grow on solid substances without the presence of free liquid is called solid-state fermentation (SSF) (Cannel and Moo-Youn, 1980).The idea of using solid substrates is probably the oldest method used by man to make

microorganisms work for him. In solid-state when fungi are used, unlike other microorganisms, fungi naturally grow in environment on solid substrates like a pieces of wood, seeds, stems, roots and dried parts of animals like bones, skin, and fecal matter that is low in moisture (Hesseltine, 1977).

In SSF, the moisture important for microbial growth appears in an absorbed state or in complex with solid matrix. However, SSF differs from solid substrate fermentation. In solid substrate fermentation the substrate itself acts as a carbon source and occurs in absence or near absence of free water. However, the process occurs in absence or near absence of free water by employing an inactive substrate or a natural substrate as solid support is called solid-state fermentation (Kumar *et al.*, 2003). The concept of SSF is to bring cultivated fungi or bacteria in tight contact with the insoluble substrate and to achieve the maximum nutrient concentration from the substrate needed for fermentation. This method so far is to run only on a small scale, but has a benefit over submerged fermentation. Solid-state fermentation processes can also be classified according to whether the seed culture for fermentation is pure or mixed. In pure culture SSF, individual strains are used for substrate consumption and with varied culture, different microorganisms are utilized for the bioconversion of agro-industrial remains at once (Oojikaas *et al*., 2000).

1.2.4.1 Moisture and Water Activity in SSF

The idea of water availability in substrate becomes important. Water activity (*Aw*) activity is defined as the ratio of the vapor pressure of water in substances to the vapor pressure of pure water at the same temperature (Oriol *et al.,* 1988). During solid-state, the low level of moisture inside the substrate reduces the growth and metabolism of microorganisms when compared to submerged fermentation. Water activity is a very

important parameter for testing water potential, characterizing the energetic state of water (Scott, 1953).

Water activity of substrates has a very important influence on microbial activity and it is determines type of organisms which can grow in solid state fermentation. Aw of the medium has been attributed as a basic parameter for mass transfer of water throughout the microscopic cells. The control of parameter of Aw could be used to modify microbial metabolic production and its excretion (Pandey *et al.*, 1999).

The study of Grevais and Molin, (2003) showed that the effect of water in solid culture medium on fungal physiology, like radial growth rate and cellular mechanisms. They found that the radial extension rate of mycelia associated to the water activity value. The optimum water activity was found to be (0.99) for *Trichoderma viride* and below (0.90), no fungal development occurred. For *Penicillum roquefortii* the optimal water activity was (0.97).

Water activity has also an effect on the fungal spore production. Maximum sporulation value of *Penicillum roquefortii* was obtained at water activity of (0.96). However, at lower water activity maximum sporulation occurs. The impact of low water activity value on fungal fermentation is not well understood. There is loss of essential nutrients for fungal growth (Charlang and Horowitz, 1971; Charlang and Horowitz, 1974).

Production of secondary metabolites also based on water activity. It was found that there is a direct relationship between the quantities of enzyme produced versus Aw. In a solid culture medium of *Trichoderma viride*, biosynthesis of polygalactouronase, beta-galactosidase and D-xylanase enzyms was influenced by water activity of the substrate. It was found that maximum Aw of polygalactouronase and D-xylanase production is

(0.99). However, beta-galactosidase production was optimum between Aw of (0.96) and (0.98) (Grajek and Grevais, 1987).

Moreover, higher Aw also promoted substrate conversion to fungal biomass and reduced water activity causes lower mass transfer and little water availability for microorganisms (Oriol *et al.,* 1988). Grajek and Grevais, (1987) observed that the reduction in biomass production and protein content of culture medium due to the decrease of water activity by (0.01) (equivalent to 1 % of relative air humidity). Narahara, (1977) noticed that optimum Aw for *Aspergillus* spp. was between (0.97) and (0.99). Fungus was unable to grow below Aw of (0.97). These data prove that fungal growth and their secondary metabolite production during SSF are strongly influenced by Aw of substrate (Grajek and Grevais, 1987; Narahara, 1977; Grevais and Molin, 2003).

1.2.4.2 Temperature and Heat Transfer

In SSF the fungal growth and secondary metabolite production are greatly affected by temperature and heat transfer processes in the substrate bed. A large amount of heat generated through the SSF which is proportional to the activities of metabolisms of the microorganism. Nevertheless, fungi can grow on a broad range of temperatures (20°C to 55°C). Nevertheless, ideal temperature for fungal growth might be different from that required for product formation (Yadav, 1988).

The substrates used for SSF have low thermal conductivities that decrease heat removal and increase its accumulation. Therefore, the important issues in SSF are heat removal, and hence most studies are focused on maximizing heat removal. The heat transfer out or in of an SSF technique is related closely with aeration of the fermentation system and microbial metabolic activity. The fungal germination, metabolites

production and sporulation are effected by high temperature (Raghavarao *et al.*, 2003).

Coupled control of temperature and moisture is also an important issue for consideration. In SSF heat transfer and temperature control would be difficult because of the low moisture and poor thermal conductivity of the substrate. Solving heat transfer problems; is possible only in small-scale solid-state fermentation by minimizing the substrate bed height or by good mixing of substrate with sparged oxygen. Moreover mixing also ensures an effective heat and mass transfer not only aids in the homogeneity of the bed. In large-scale SSF continuous mixing along with addition of water is beneficial for control of temperature and moisture (Raghavarao *et al.*, 2003).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Instruments and Chemicals

2.1.1 Instruments

Table (2-1): Instruments used in this study.

Instruments	**Company**
Autoclave	Express (Germany)
Centrifuge	Heraeus (England)
Cooled Incubator	Sony (Japan)
Electric Oven	Gallenkamp (England)
Electric Sensitive Balance	Delta Range (Switzerland)
Light Microscope	Olympus (Japan)
Magnetic Stirrer	Stuart (England)
pH meter	Radiometer (Denmark)
UV-Visible Spectrophotometer	Mercury (China)
Water Bath	GLF (Germany)

2.1.2 Chemicals

Table (2-2): Chemicals used in this study.

3,5-Dinitrosalicylic Acid	Himedia
Bovine Serum Albumin	Sigma
Calcium Acetate	Himedia
Casein	Oxoid
Coomassie Brilliant Blue G-250	Sigma
Ethanol	Sigma
Folin & Ciocalteu's phenol	Himedia

Hydrochloric Acid	Sigma
• K_2HPO_4	BDH
KH_2PO_4	BDH
Lacto phenol cotton blue	Himedia
L-Tyrosine	Himedia
Maltose	Fluka
Methanol	Sigma
• Phosphoric Acid	Sigma
Sodium Acetate, Trihydrate	Fluka
Sodium Carbonate	Himedia
Sodium Chloride	BDH
Sodium Hydroxide	BDH
Sodium Phosphate	Himedia
Sodium Potassium Tartrate	Sigma
Starch	Oxoid
Trichloroacetic acid	Sigma

2.2 Culture Media

2.2.1 Ready to use media

Medium used in this study Potato Dextrose Agar – PDA was prepared according to the instruction on the containers of their manufacturing companies.

2.3 Buffers and Reagents

2.3.1 Potassium phosphate dibasic (K_2HPO_4, 1 M)

It was prepared by dissolving 174.18 g of K_2HPO_4 in 800 ml distilled water and completed to 1 L with DW. This buffer was stored at 4 °C.

2.3.2 Potassium phosphate monobasic (KH_2PO_4, 1 M)

It was prepared by dissolving 136.09 g of KH_2PO_4 in 800 ml DW and completed to 1 L with DW. This buffer was stored at 4 °C**.**

2.3.3 Potassium phosphate buffer (0.1 M, pH 7.0) (Green and Sambrook, 2012)

It was prepared by mixing 61.5 ml of K_2HPO_4 and 38.5 ml of KH_2PO_4.This buffer was stored at 4 °C.

2.3.4 Soluble Starch Solution (1%)

It was prepared by dissolving 1 g of soluble starch in 100 ml of DW. Solubilization of starch was facilitated by heating the solution in a glass beaker directly on a heating-stirrer plate using constant stirring. The solution was brought to boil for 15 minutes. The solution was permit to cool to room temperature with stirring and the volume was completed to 100 ml using DW.

2.3.5 Sodium Potassium Tartrate Solution

It was prepared by weighing 12 g of Sodium Potassium Tartrate, Tetrahydrate and added in 8 ml of 2 M NaOH which was previously heated to (50 - 70) °C. The solution was dissolved by heating in a water bath with constant stirring.

2.3.6 3,5-Dinitrosalicylic Acid Solution (96 mM)

It was prepared by adding 0.438 g of 3,5-Dinitrosalicylic Acid in 20 ml of DW which was previously heated to (50 - 70) °C. The solution was dissolved by heating in a water bath with constant stirring.

2.3.7 Colour Reagent Solution

It was prepared by slowly adding with stirring Sodium Potassium Tartrate solution followed by 3,5-Dinitrosalicylic Acid solution to 12 ml of DW which was previously heated to (50 - 70) °C, and mixed. The solution should be stored in an amber container at room temperature.

2.3.8 Maltose Standard Solution (0.2%)

It was prepared by weighing 0.2 g of Maltose and dissolved in 100 ml of DW.

2.3.9 Potassium Phosphate Buffer (50 mM, pH 7.5)

It was prepared by dissolving 1.14 g of potassium phosphate dibasic, trihydrate in 100 ml of DW. pH was adjusted to 7.5 with 1M HCl.

2.3.10 Casein solution (0.65%)

It was prepared by mixing 0.65 g in 100 ml of the 50 mM potassium phosphate buffer. The temperature of solution is gradually increased with gentle stirring to 80-85 °C for about 10 minutes.

2.3.11 Trichloroacetic acid solution (1 N)

It was prepared by dissolving 16.33 g of Trichloroacetic acid in 80 ml of DW. The volume was completed to 100 ml with DW. A 100 ml of 110mM of this solution was prepared by mixing 11 ml of 1 N solution with 89 ml DW.

2.3.12 Sodium Carbonate solution (500 mM)

It was prepared by dissolving 5.3 g of anhydrous sodium carbonate in 90 ml. The volume was completed to 100 ml with DW.

2.3.13 L-tyrosine Standard Stock Solution (1.1 mM)

It was prepared by mixing 0.02 g of L-tyrosine in 100 DW and heated gently until the tyrosine dissolves. The L-tyrosine solution was allowed to cool to room temperature.

2.3.14 Bovine Serum Albumin Stock Solution (2mg/ml)

Bovine Serum Albumin (BSA) stock solution was prepared by dissolving 0.02 g of BSA in 10 ml of 0.1 M phosphate buffer.

2.4.1 Coomassie Brilliant Blue G-250 (Bradford, 1976)

Prepared by dissolving 100 mg of Commassie in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added. The volume was then completed to 1000 ml with DW. The reagent was filtered through Whatman No.1 filter paper just before use.

2.2 Methods

2.2.1 Fungal species

The fungi species, *Penicillium* sp., *Alternaria* sp., *Fusarium* sp., *Mucor* sp., and *Aspergillus niger* were obtained from the Department of Biology/ College of Science/ University of Baghdad. The fungi species were identified after growing on Potato Dextrose Agar (PDA) medium by observing the growth characteristics (color, texture appearance and diameter of colonies) and microscopic (microstructure) (Baijal and Mehrotra, 1980; Bisset, 1991). All the cultures were maintained on PDA slants. Then, stored in refrigerator and sub-cultured regularly at an interval of three months.

2.2.1.1 Slide Culture Preparations (Riddle, 1950)

A simple modification of this method using a single agar plate is described below:

- A sterile blade was used to cut out an agar block, small enough to fit under a cover slip.
- The block was flip onto the surface of the agar plate.
- Four sides of the agar block inoculated with spores or mycelial fragments of the fungus to be grown.
- A flamed coverslip was placed centrally upon the agar block.
- The plate was incubated at 28 °C until growth and sporulation have occurred.
- The cover slip removed from the agar block.
- A drop of 95% alcohol was applied as a wetting agent.
- The cover slip gently lowering on to a small drop of Lactophenol cotton blue on a clean glass slide.

- The slide is then left overnight to dry, later sealed with fingernail polish.
- When sealing with nail polish was used a coat of clear polish followed by one coat of red coloured polish.

2.2.2 Static magnetic field

A special magnetic bar of thickness (2.9 cm) with single field strength of 100 Gauss which was measured by a Gaussmeter. The magnetic bars (North Pole, South Pole, or both poles) were put on the side of the cultured flasks using adhesive tape.

2.2.3 Spores' suspension preparation

Spores' suspension was prepared according to (Faraj, 1990) with slight modifications as follows:

- Plates containing PDA medium inoculated with fungal isolates were incubated at 28 °C for 3-4 days.
- Spores were harvested by adding 5 ml of sterile DW on the plate.
- The spores' suspension was transferred by a micropipette to a flask containing sterilized bread and incubated at 28 °C for 5 days.
- One hundred ml of DW was added to the flask and mixed vigorously by hand.
- The suspension of spores was filtered through sterile cotton wool.
- The suspension was centrifuged at (3000 rpm for 5 min). Then, the supernatant was discarded and the spores then washed twice by DW and further centrifugation.

- Then, 1 ml of DW was added to the deposit and mixed vigorously.

2.2.4 Spores Counting

Spores were counted according to (Sambrook and Russell, 2001) as follows:

- The hemocytometer was set up by placing it flat on the surface with the glass coverslip on top of the grids.
- The spores' suspension was vortexed for 5 s.
- By using sterile tips, ten microliter of the suspension was transferred to the divot on the hemocytometer.
- The total number of spores was counted in each of the five small squares.
- The following equation was used to estimate the number of spores /ml in original suspension:

Spores /ml = (Average no. of spores / 5) × (25) × (10^4) × (Dilution Factor).

2.2.5 Effect of magnetic field poles on enzyme activity using solid state fermentation

To test the effect of the magnetic field on the fungi cultures under solid state fermentation the following steps were carried out:

- Bread loafs were cut into pieces (approximately 1 cm^3) using bread loaf knife.
- Twelve flasks 500 ml were loaded with 30 g of bread pieces and autoclaved at 121 °C for 15 min.

- Each flask was inoculated with 10^8 spores/ml of fungus.
- The moisture content was constant.
- The twelve flasks were divided into four groups as follow:

Three flasks used as control, three flasks were put under the effect of northern pole, three flasks were put under the effect of southern pole and three flasks were put under the effect of both poles.

- The flasks were incubated at 28 °C for 7 days with shaking every day.

2.2.6 Crude enzyme extraction

After 7 days of fungal fermentation, extraction of crude enzyme was done as follow:

- To each culture flask, 100 ml of phosphate buffer (pH 7) was added and shake vigorously for 15 min to suspend the bread pieces.
- The whole content of the flask was filtered through sterile cotton wool and centrifuged at (3000 rpm for 5 min).
- The supernatant was assembled and filtered by filter paper Whatman No.1 under vacuum.
- The filtrate was stored at 4 °C.

2.2.7 Determination of protein concentration by Bradford method (1976)

The concentration of protein was determined by using the Bradford method, as follows:

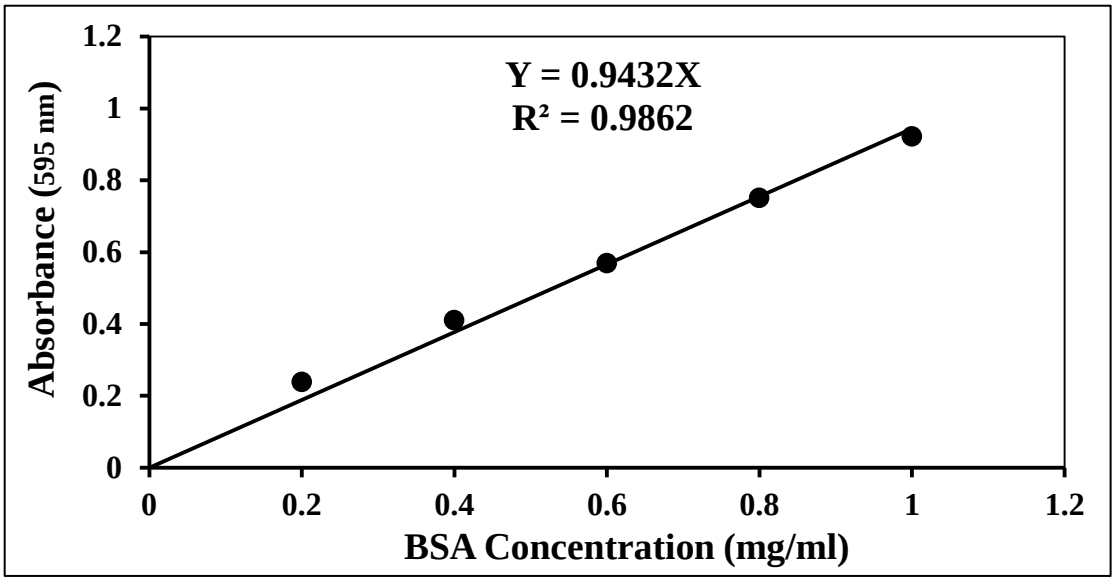
- The Bradford reagent was gently mixed and brought to room temperature.

- A protein standard was prepared ranging from (0.2 to 1 mg/ml). Dilutions are given in Table (2-3) below. Each concentration was done in triplicate.
- The unknown samples were prepared in a similar way to make the final volume (0.1 ml).
- To all the tubes, (3.0 ml) of Bradford reagent were added with continuous mixing, then incubated at room temperature for 5 min.
- The absorbance was measured at 595nm using spectrophotometer.

Table (2-3): Preparation of Bovine serum albumin standard curve.

BSA standard (µl)	Dilution buffer (µl)	Final concentration (mg/ml)	Absorbance (595 nm)
10	90	0.2	0.238
20	80	0.4	0.410
30	70	0.6	0.568
40	60	0.8	0.750
50	50	1	0.921

Figure (2-1): Bovine serum albumin standard curve.



2.2.8 Protease specific activity estimation

2.2.8.1 Preparation of tyrosine standard curve

Tyrosine standard curve was prepared as follow:

- From the tyrosine stock solution, five concentrations, ranging from (0.055 to 0.553 µMole) were prepared. Each concentration was done in triplicate. A test tube containing a blank solution was also prepared.
- The volume was brought up to (2 ml) in each test tube, including the test tube by DW containing the blank solution.
- In each tube, (5 ml) of sodium carbonate were added to regulate any pH drop due to the addition of the Folin's reagent.
- One ml of Folin's reagent was added immediately. The tubes were mixed by swirling and incubated at 37 °C for 30 min, blue colour is development.
- The absorbance of our samples was measured by a spectrophotometer at 660 nm.

- A graph with the concentration of tyrosine on X axis against absorbance at 660 nm on Y axis was plotted.

Tables (2-4): preparation of tyrosine standard curve.

Tyrosine standard (ml)	DW (ml)	Tyrosine Final concentration (µmol/ml)	Absorbance (660 nm)
0.05	1.95	0.0275	0.050
0.1	1.9	0.055	0.124
0.2	1.8	0.110	0.277
0.4	1.6	0.220	0.603
0.5	1.5	0.275	0.736

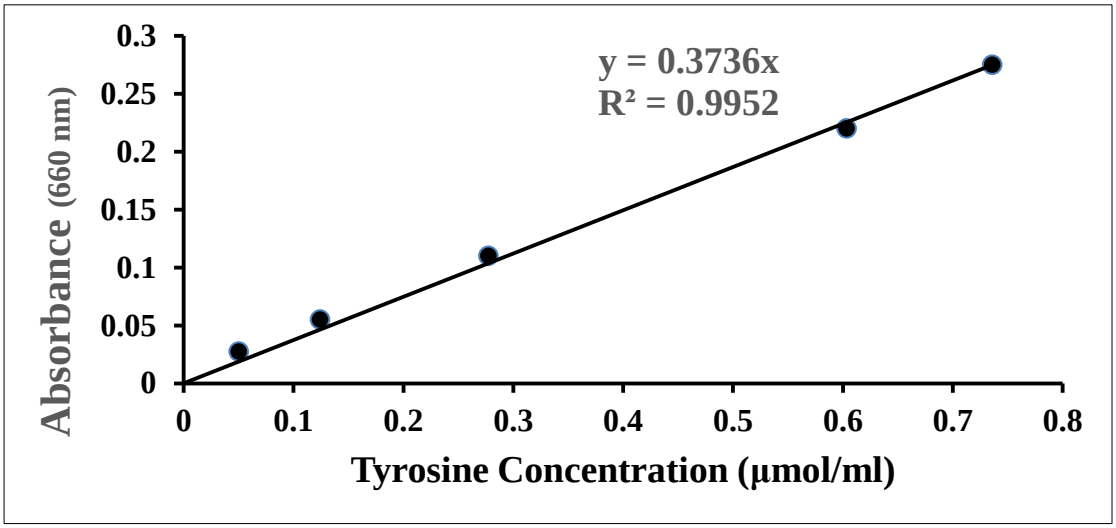


Figure (2-2): Tyrosine standard curve for protease activity determination.

2.2.8.2 Assay of Protease activity

The activity of protease was assayed using casein as a substrate according (Anson, 1938):

- Thirteen sterile test tubes were prepared. One tube was used as a blank and the others were used to assay the activity of protease.

- In each tube, (5 ml) of 0.65% casein solution was added and these tubes were placed in a water bath at 37°C for about 5 min.
- In each tube, (1 ml) of the enzyme extract was added, except the blank in which 1 ml of DW was added, mixed by swirling and incubated at 37 °C for exactly 10 min.
- Five milliliters of TCA reagent were added in each tube to stop the reaction and incubated at 37 °C for 30 min.
- In each tube, (5 ml) of sodium carbonate were added to regulate any pH drop created by the addition of the Folin's reagent.
- One ml of Folin's reagent was added immediately. The tubes were mixed by swirling and incubated at 37 °C for 30 min, Folin's reagent react with protein to produce a blue color.
- The absorbance was measured by a spectrophotometer at 660 nm.

2.2.8.3 Protease specific activity (U/mg)

- Absorbance values obtained from protease activity were put in the standard curve equation (Figure 2-1) to get the amount of tyrosine in micromoles.
- From the values generated above, total activity of protease samples can be calculated in terms of Units, which is the amount of tyrosine released from casein in micromoles per minute, according to the following equation:

$$\textbf{Protease activity (Units/ml)} = \frac{(\textbf{µmol/ml of tyrosine released}) * (\textbf{11})}{(\textbf{1}) \textbf{ x } (\textbf{10})}$$

Where:

- 11 = Assay total volume (ml)
- 1 = Volume of enzyme used (ml)
- 10 = Assay time (min) as the Unit definition

- Finally the enzyme specific activity was estimated by dividing the enzyme activity on the protein activity obtained from bovine serum albumin standard curve (Figure 2-1) according to the following equation:

$$\textbf{Protease specific activity (U/mg)} = \frac{\textbf{Protease activity } (\textbf{U/ml})}{\textbf{Protein concentration } (\textbf{mg/ml})}$$

2.2.9 Amylase specific activity estimation

2.2.9.1 Preparation of maltose standard curve

Maltose standard curve was prepared as follow:

- From the maltose stock solution, seven concentrations, ranging from (0.2 to 1 mg/ml) were prepared. Each concentration was prepared in triplicate. A test tube containing a blank solution was also prepared.
- Using DW, the volume was brought up to (2 ml) in each test tube, including the test tube containing the blank solution.
- To each tube, (1 ml) of DNS reagent was added.
- The contents of the tubes were heated in a boiling water bath for 5 min.
- The test tubes were cooled to room temperature, after taking them out of the water bath.

- To each test tube, (9 ml) of DW were added and mixed well.
- From each test tube, the mixture was transferred into cuvettes and the absorbance was measured at 540 nm.
- A graph with the concentration of maltose on X axis against absorbance at 540nm on Y axis was plotted.

Tables (2-5): Preparation of maltose standard curve.

Maltose standard (ml)	DW (ml)	Maltose Final concentration (mg/ml)	Absorbance (540 nm)
0.2	1.8	0.2	0.065
0.4	1.6	0.4	0.106
0.6	1.4	0.6	0.145
0.8	1.2	0.8	0.190
1	1	1	0.221

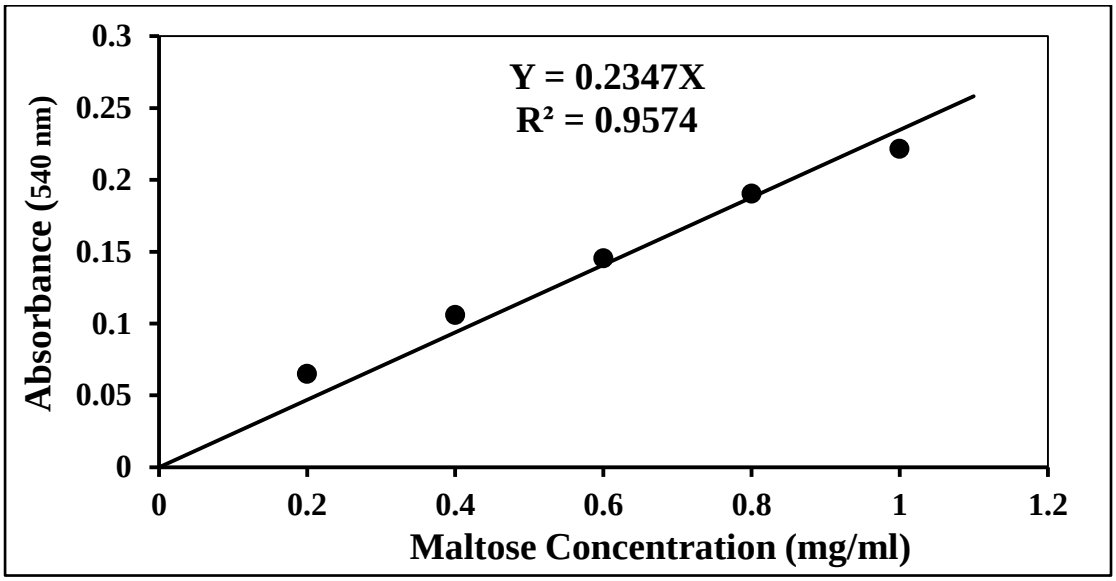


Figure (2-3): Maltose standard curve for amylase activity determination.

2.2.9.2 Assay of amylase activity

Amylase activity was assayed as described by (Bernfeld, 1955):

- Thirteen test tubes were prepared. One tube was used as a blank and the others were used to assay amylase activity.
- To each tube, (1 ml) of starch solution was added.
- To each tube, (1 ml) of enzyme extract was added, except the blank to which (1 ml) of DW was added, the tubes were mixed by swirling and incubated at 37 °C for exactly 3 min.
- To each tube, (1 ml) of colour reagent (DNS) was added and boiled in water bath for 15 min.
- The tubes were cooled on ice for 3 min.
- To each tube, (9 ml) of DW were added with mixing.
- The absorbance for all the test tubes was measured at 540 nm using spectrophotometer.

2.2.9.3 Amylase specific activity (U/mg)

One unit of amylase enzyme can be defined as the amount needed for liberation of (1.0 mg) of maltose from starch in 3 min at pH 6.9 at 20°C.

- The absorbance of all sample tests obtained above were put in the slope of the standard curve (Figure 2-3) to give the liberated maltose from starch in mg.
- Total activity of amylase in unit/ml can be obtained using the following equation:

$$\textbf{Amylase activity (U/ml)} = \frac{\textbf{Maltose released (mg)} * \textbf{df}}{(\mathbf{1})}$$

Where:

df = Dilution Factor

1 = Enzyme volume (ml)

- Ultimately, the enzyme specific activity was estimated by dividing the enzyme activity on the protein activity obtained from bovine serum albumin standard curve (Figure 2-1) according to the following equation:

$$\textbf{Amylase specific activity (U/mg)} = \frac{\textbf{Amylase activity } (\textbf{U/ml})}{\textbf{Protein concentration } (\textbf{mg/ml})}$$

2.10 Statistical Analysis

All the experiments were performed in triplicates. The statistical analysis was done using GenStat program. Least significant difference (LSD) was determined for each fungus to both enzymes (Amylase and Protease). The mean of specific activity for each treatment was calculated and subtracted from the control of this treatment manually. If the result of this subtraction is more than LSD then the difference is significant. Alternatively the difference will be not significant if the subtraction results are less than LSD.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Fungal Species Identification

Five species of different genera of filamentous fungi were characterized based on their morphological and microscopic observations which are *Alternaria* sp., *Aspergillus niger*, *Fusarium* sp., *Mucor* sp. and *Penicillium* sp. The growth of fungi was studied morphologically on potato dextrose agar (PDA) at 28°C for 4-5 days.

The identifying features of *Alternaria* sp. in culture shows a white growth with profuse aerial mycelium which gradually turned into greenish grey. Microscopic characters are dark greenish-black surface with gray periphery; black on reverse side; chain of macroconidia (Mmbaga *et al*., 2005), as shown in Figure (3-1 A, B).

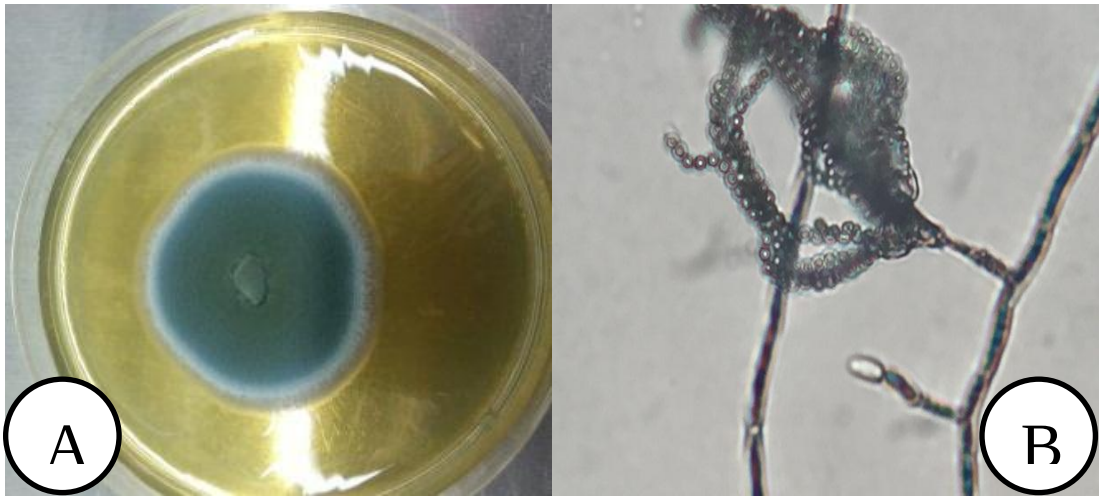


Figure (3-1 A, B): (A) *Alternaria* sp. Morphology on (PDA) agar at 28 °C for 4 days. (B) Microscopic characterization.

Results illustrated in Figure (3-2 A, B) showed *Aspergillus niger* is wooly initially white, quickly becoming black and speed grows rate with conidial production. Reverse side of Petri dish is white to pale yellow and growth may form a radial fissures in the agar, microscopic characters are bluish-green with sulfur-yellow areas on the surface (Fröhlich *et al.*, 2000), as shown in Figure (3-2 A, B).

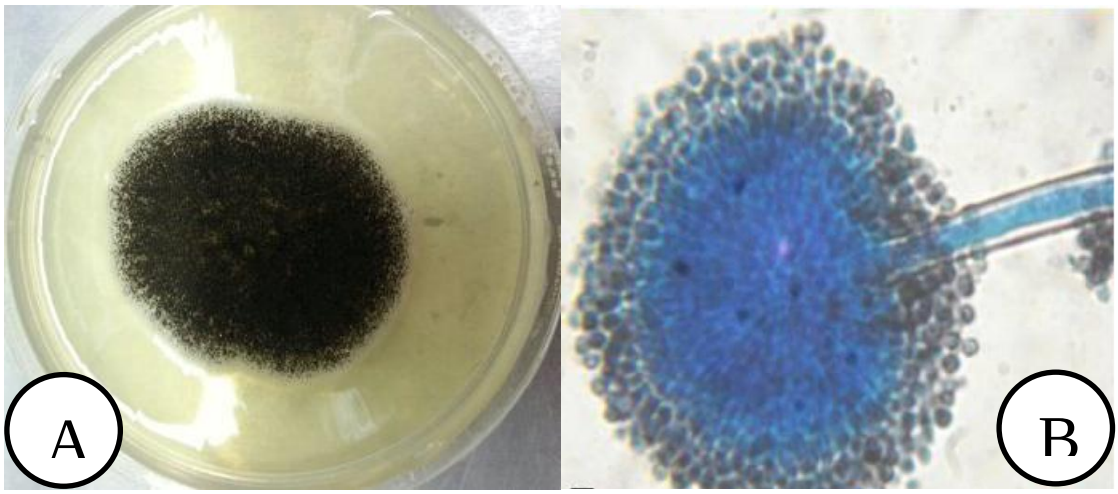


Figure (3-2 A, B): (A) *Aspergillus niger* morphology on (PDA) agar at 28 °C for 4 days. (B) Microscopic characterization.

Fusarium sp. colonies are fast growing, pale or brightly colored and may have a cottony aerial mycelium. The color of the thallus varies from white to yellow, brown, pink, red or lilac shades, different microscopic characteristics can be observed such as yellow, purple, orange and red colonies; sickle shaped macroconidia, as shown in Figure (3-3 A, B). *Mucor* sp. colonies are fast-growing, white to beige or grey and colonies on culture medium may grow to several centimeters in height, microscopic characters are sporangia with slimy texture spores with dark pigment (Domsch *et al*., 1980), as shown in Figure (3-4 A, B).

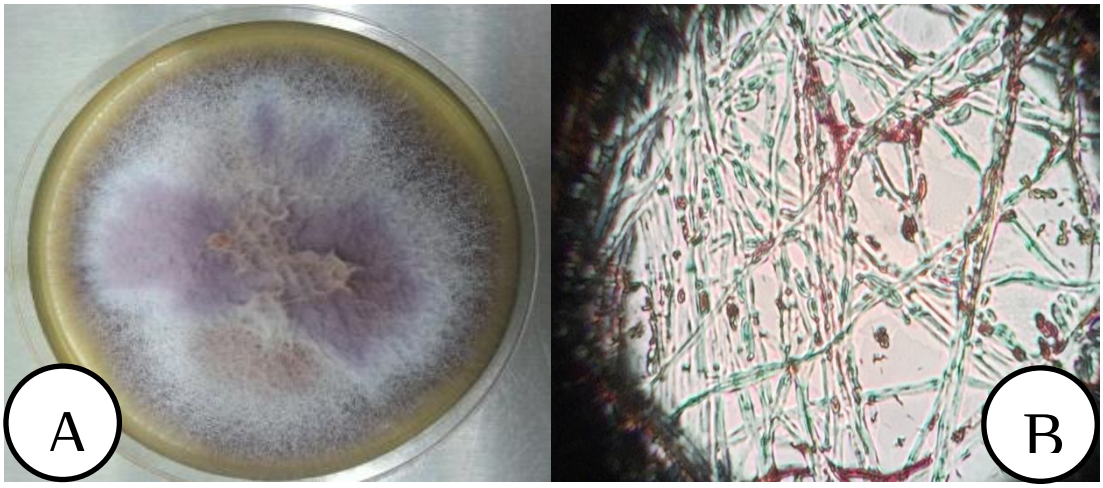


Figure (3-3 A, B): (A) *Fusarium* sp. Morphology on (PDA) agar at 28 °C for 4 days. (B) Microscopic characterization.

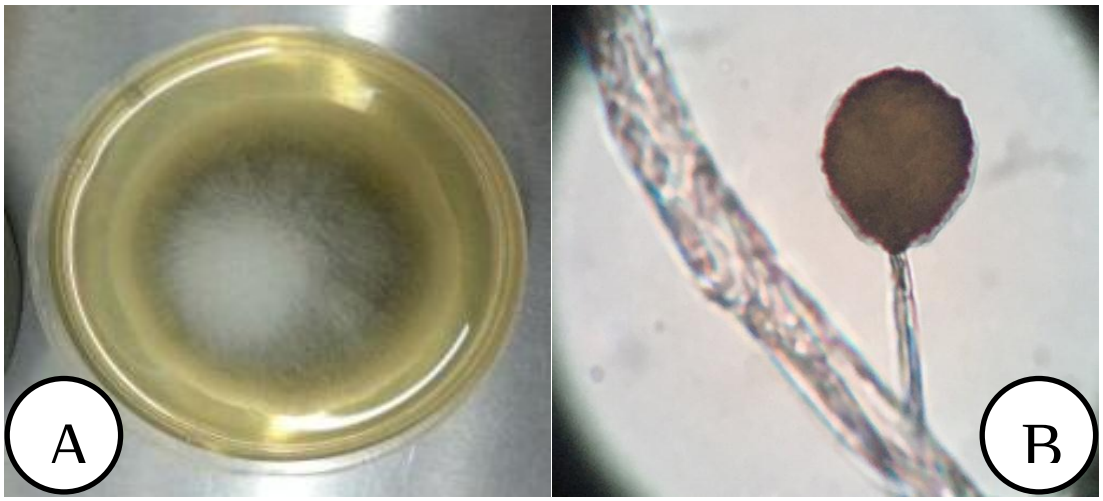


Figure (3-4 A, B): (A) *Mucor* sp. Morphology on (PDA) agar at 28 °C for 4 days. (B) Microscopic characterization.

Suryanarayanan *et al.*, (2000), reported that *Penicillium* sp. colonies are fast growing in shades of green or white, mostly composed of a dense felt of conidiophores, microscopic characters are bluish-green brush arrangement of phialospores, as shown in Figure (3-5 A, B).

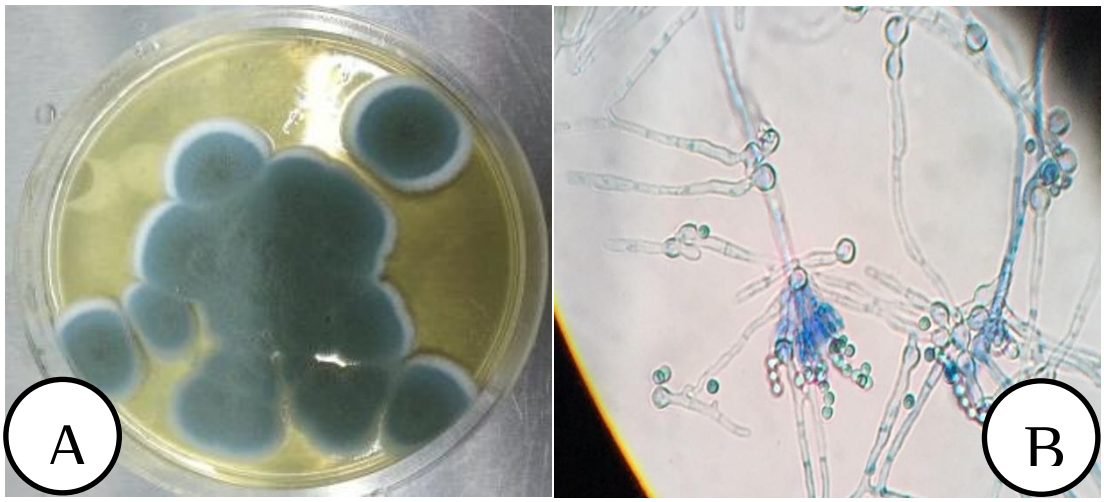


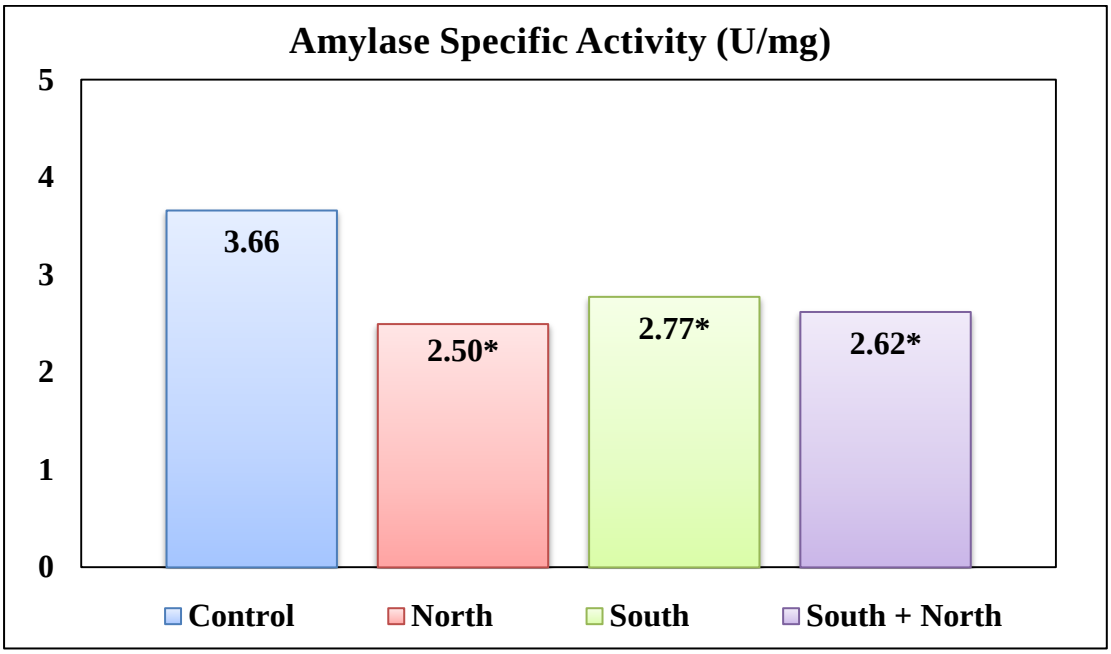
Figure (3-5 A, B): (A) *Penicillium* sp. Morphology on (PDA) agar at 28 °C for 4 days. (B) Microscopic characterization.

The slide cultures technique permits fungi to be studied virtually in situ with as less disturbance as possible. It is a fast method to prepare fungal colonies for examination, identification and preserves the morphological features (Riddle, 1950).

3.2 Effect of Magnetic Field on Amylase Specific Activity

3.2.1 Effect of Magnetic Field on the Amylase Specific Activity of *Alternaria* sp.

The effect of magnetic field on specific activity of amylase of *Alternaria* sp. was investigated. It was measured after 7 days of fermentation on solid medium (bread cubes) at 28 °C. The Control of all experiments was the solid medium without exposure to the effect of the magnetic field. The crude extract of the Control flasks was assayed and the specific activity was (3.66 U/mg). Least Significant Differences (LSD) was (0.375). As shown in the Figure (3-6), the Northern, Southern, and both Poles significantly decreased the amylase specific activity (2.50 U/mg), (2.77 U/mg), and (2.62 U/mg) respectively when compared to the Control.



***LSD is 0.375 and the mean difference is significant at the 0.05 level.**

Figure (3-6): Effect of magnetic field on amylase specific activity of *Alternaria* sp.

3.2.2 Effect of Magnetic Field on the Amylase Specific Activity of *Aspergillus niger*

The effect of magnetic field on the specific activity of amylase of *Aspergillus niger* was studied. After 7 days of fermentation on solid medium (bread cubes) at 28 °C the study revealed that the specific activity of the Control was (4.23 U/mg). The LSD was (1.322). Figure (3-7) showed that all treatment for the Northern, Southern, and both Poles decreased the specific activity which were (2.96 U/mg), (3.74 U/mg) and (3.28 U/mg) respectively. However, the decrease was non-significant when compared to the Control.

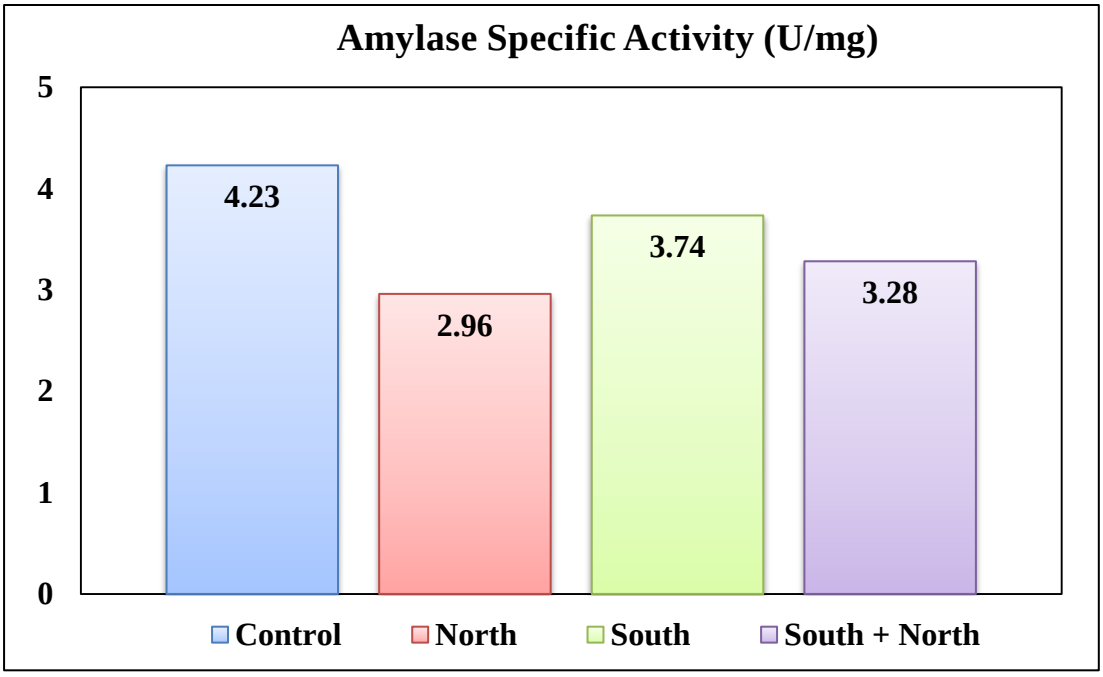
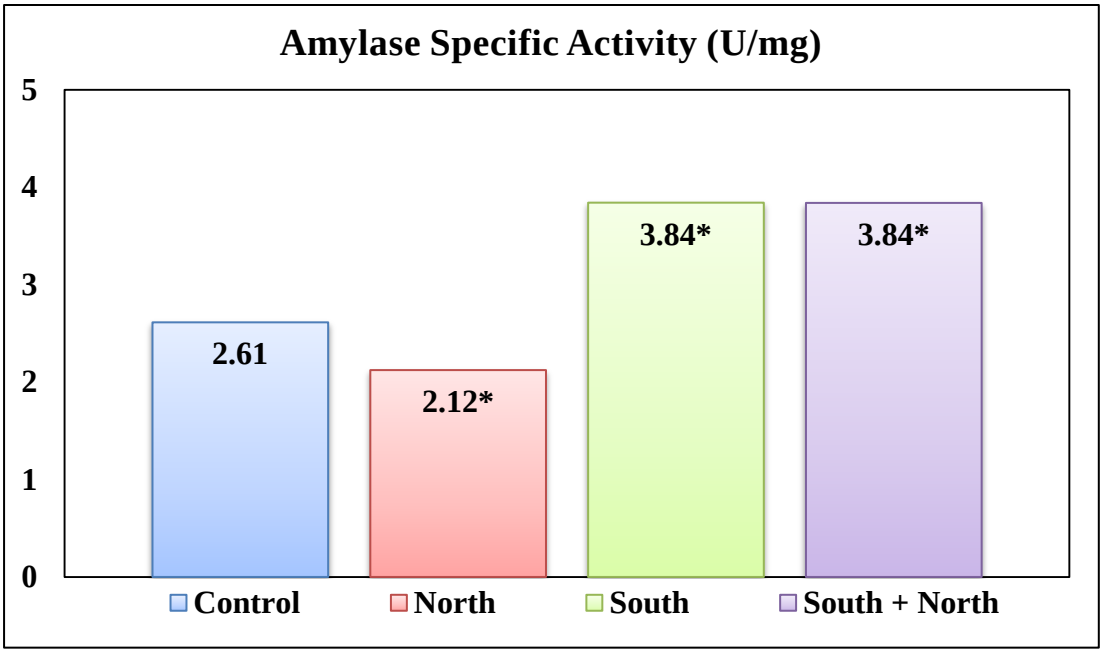


Figure (3-7): Effect of magnetic field on amylase specific activity of *Aspergillus niger*.

3.2.3 Effect of Magnetic Field on the Amylase Specific Activity of *Fusarium* sp.

The effect of magnetic field on amylase specific activity of *Fusarium* sp. was studied. The results in figure (3-8) showed that after 7 days of fermentation on solid medium (bread cubes) at 28 °C that there were significant increase of amylase specific activity when exposed to Southern Pole (3.84 U/mg) as well as the two poles together (South + North) (3.84 U/mg). On the contrary, the Northern Pole showed significant decrease of amylase specific activity (2.12 U/mg) compared with the Control (2.61 U/mg). The LSD of this experiment was (0.459).

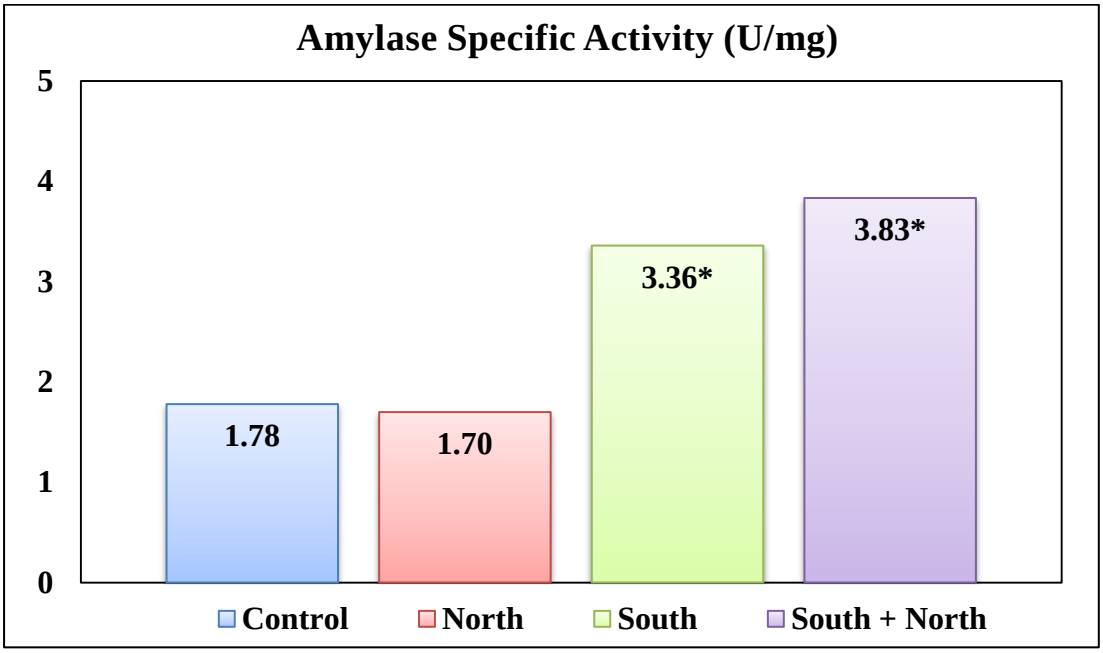


***LSD is 0.459 and the mean difference is significant at the 0.05 level.**

Figure (3-8): Effect of magnetic field on amylase specific activity of *Fusarium* sp.

3.2.4 Effect of Magnetic Field on the Amylase Specific Activity of *Mucor* sp.

The effect of magnetic field on specific activity of amylase of *Mucor* sp. was measured. The results after 7 days of fermentation on solid medium (bread cubes) at 28 °C were indicated. In the Figure (3-9) the specific activity of the Control was (1.78 U/mg). The LSD was (0.565). The Southern and Both Poles significantly increased the specific activity (3.36 U/mg) and (3.83 U/mg) respectively. On the other hand, a non-significant decrease of the specific activity was obtained when the fungus exposed to the Northern pole (1.70 U/mg) when compared with the Control.

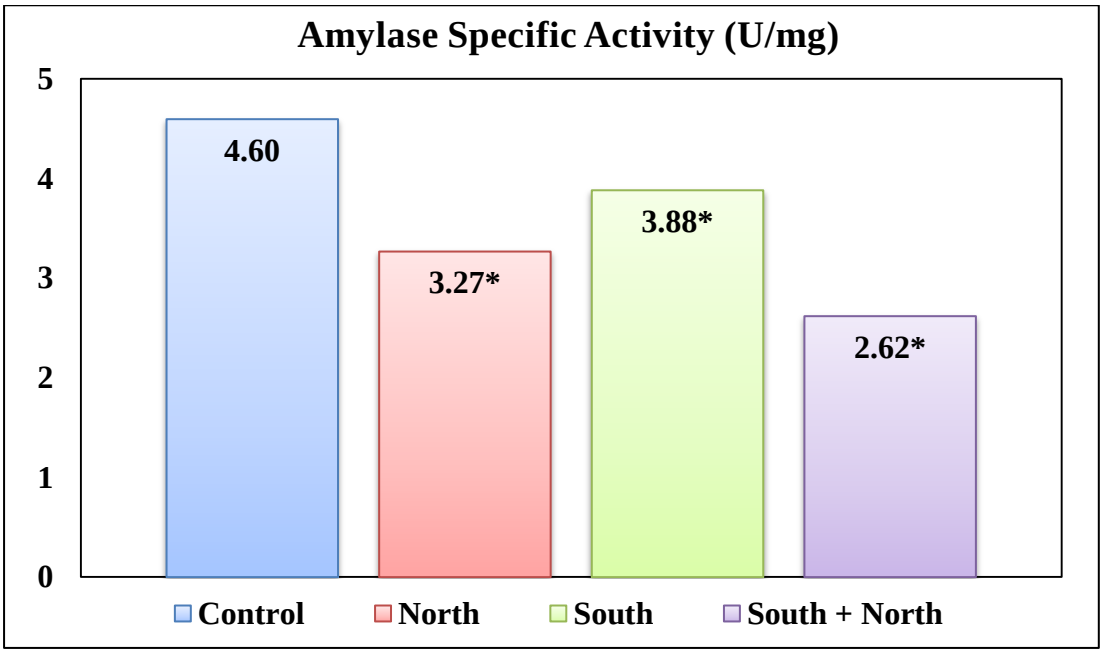


***LSD is 0.565 and the mean difference is significant at the 0.05 level.**

Figure (3-9): Effect of magnetic field on amylase specific activity of *Mucor* sp.

3.2.5 Effect of Magnetic Field on the Amylase Specific Activity of *Penicillium* sp.

The effect of magnetic field on specific activity of amylase of *Penicillium* sp. was tested. After 7 days of fermentation on solid medium (bread cubes) at 28 °C, the specific activity was measured. The LSD of this experiment was (0.508). The Control specific activity was (4.60 U/mg). As seen in the figure (3-10), all treatments were significantly decreased the specific activity. The Northern, the Southern, and the two poles together (South + North) specific activities were (3.27 U/mg), (3.88 U/mg) and (2.62 U/mg) respectively as compared with the Control.



***LSD is 0.508 and the mean difference is significant at the 0.05 level.**

Figure (3-10): Effect of magnetic field on amylase specific activity of *Penicillium* sp.

The statistical analysis of the results in Figures (3-(6, 7, 8, 9, 10)) showed that the Southern and Both Poles treatment significantly increased the amylase specific activity of *Fusarium* sp. and *Mucor* sp. and conversely significantly decreased amylase specific activity of

Alternaria sp. and *Penicillium* sp. compared with the Control. On the other hand, the Northern Pole treatment significantly decreased the amylase specific activity of *Alternaria* sp., *Fusarium* sp., and *Penicillium* sp.

It is well known that there are many factors affect the enzyme activity. These include temperature, pH, substrate concentration and the presence of activator or inhibitor.

The effect of the magnetic field of the Southern pole seems to potentiate the action of (Ca^{2+}) ion leading to increase in amylase specific activity in *Fusarium* sp. and *Mucor* sp. It seems that other fungi (*Alternaria* sp., and *Penicillium* sp.) respond to the action of the southern pole of the magnetic field differently which could be explained by the difference of the amino acid composing these proteins' active sites which led to this decrease of their activity by the effect of the magnetic field.

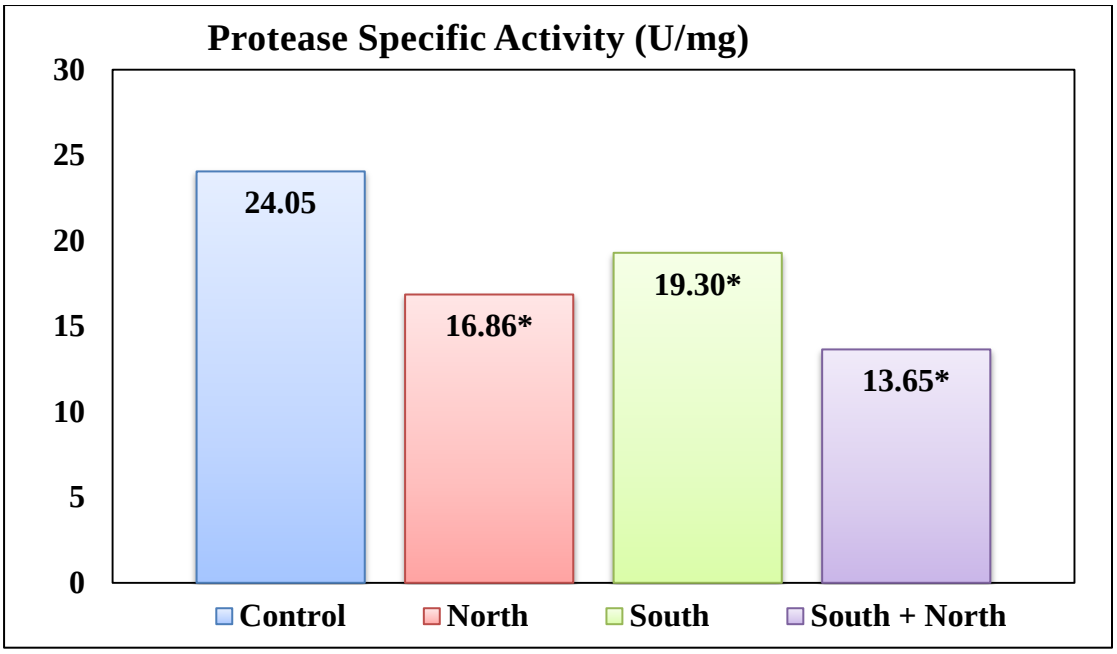
The action of both poles of the magnetic fields which led to increase the specific activity of amylase of *Mucor* sp. and *Fusarium* sp. could be due to the Southern pole only as it competes with Northern pole and suppressed its effect.

The Moving Charge Interaction (MCI) model p suggest that electrons motion affect enzyme activity. Theoretical models have depicted existence of low frequency (ELF) magnetic field interactions with biosystems at ion cyclotron resonance which is, at frequencies corresponding to charge to mass ratios of ions such as Ca^{2+}, Mg^{2+} and K^{+} (Bruce *et al.,* 1992). Experimental investigations detect that low frequency electromagnetic fields affects living systems through the calcium signaling pathways and systolic calcium oscillator (Galvanovskis and Sandblom, 1998). The theories on the effects of MFs on activities of enzyme refer to changes of motion of ions at the active site (Edmonds, 1993).

3.3 Effect of Magnetic Field on the Protease Specific Activity

3.3.1 Effect of Magnetic Field on the Protease Specific Activity of *Alternaria* sp.

The effect of magnetic field on the specific activity of protease of *Alternaria* sp. was tested. After 7 days of fermentation on solid medium (bread cubes) at 28 °C. The LSD of this experiment was (2.644). All treatments significantly decreased the protease specific activity (Figure 3-11). The Northern and Southern Poles as well as the two poles together (South + North) specific activities were (16.86 U/mg), (19.30 U/mg), and (13.65 U/mg) respectively when compared to the Control (24.05 U/mg).

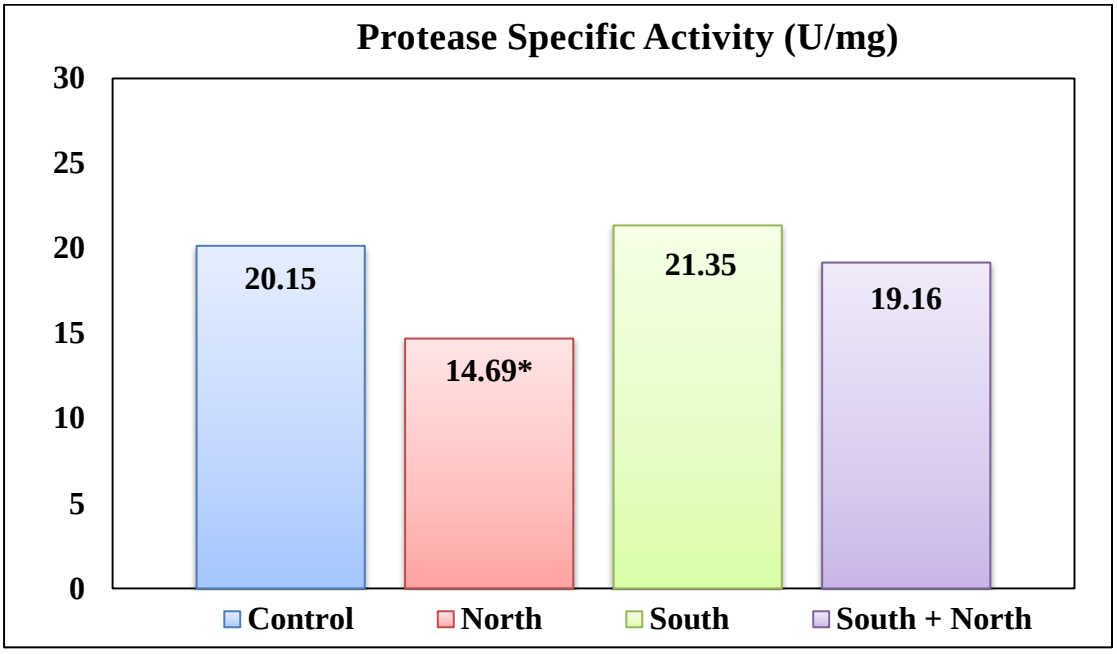


***LSD is 2.644 and the mean difference is significant at the 0.05 level.**

Figure (3-11): Effect of magnetic field on protease specific activity of *Alternaria* sp.

3.3.2 Effect of Magnetic Field on the Protease Specific Activity of *Aspergillus niger*

The effect of magnetic field on the specific activity of protease of *Aspergillus niger* was studied after 7 days of fermentation on solid medium (bread cubes) at 28 °C. The LSD of this experiment was (2.932). The study showed that the specific activity of the Control was (20.15 U/mg) (without MF), the Northern Pole significantly decreased specific activity (14.69 U/mg). The two poles together (South + North) non-significantly decreased the specific activity (19.16 U/mg). On the other hand, the Southern Pole increased specific activity (21.35 U/mg), however, the increase was non-significant (Figure 3-12).

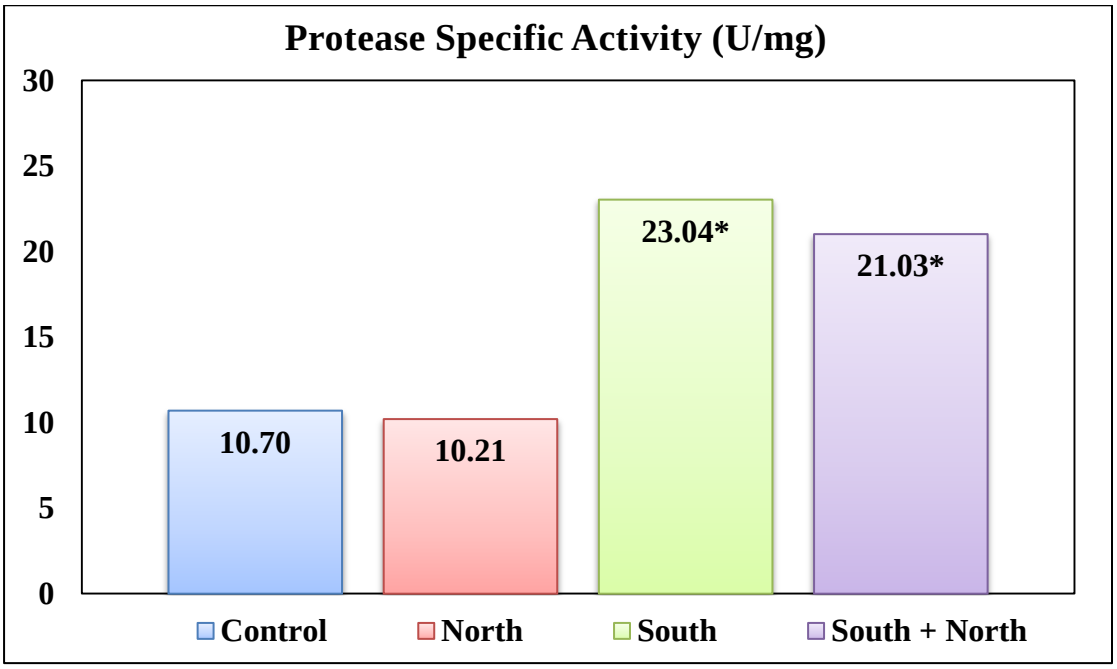


***LSD is 2.932 and the mean difference is significant at the 0.05 level.**

Figure (3-12): Effect of magnetic field on protease specific activity of *Aspergillus niger*.

3.3.3 Effect of Magnetic Field on the Protease Specific Activity of *Fusarium* sp.

The effect of magnetic field on specific activity of protease of *Fusarium* sp. was studied. The results after 7 days of fermentation on solid medium (bread cubes) at 28 °C indicated that the Control specific activity was (10.70 U/mg). The LSD of this experiment was (3.478).The Southern Pole significantly increased specific activity (23.04 U/mg) as well as the two poles together (South + North) (21.03 U/mg). The Northern Pole non- significantly decreased specific activity (10.21 U/mg) (Figure 3-13).

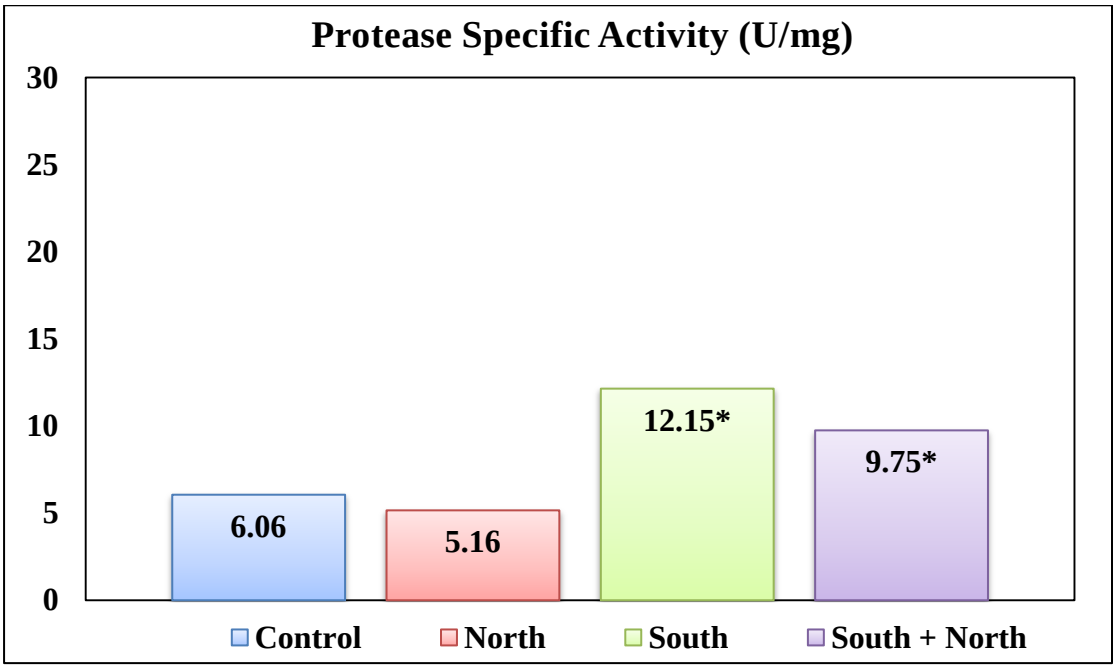


***LSD is 3.478 and the mean difference is significant at the 0.05 level.**

Figure (3-13): Effect of magnetic field on protease specific activity of *Fusarium* sp.

3.3.4 Effect of Magnetic Field on the Protease Specific Activity of *Mucor* sp.

The effect of magnetic field on specific activity of protease of *Mucor* sp. was measured. After 7 days of fermentation on solid medium (bread cubes) at 28 °C, the results displayed that the specific activity of the Control was (6.06 U/mg). The LSD of this trial was (2.424). As seen in the Figure (3-14), the Southern Pole significantly increased specific activity (12.15 U/mg) as well as the two poles together (South + North) (9.75 U/mg), while the Northern Pole non-significantly decreased specific activity (5.16 U/mg).

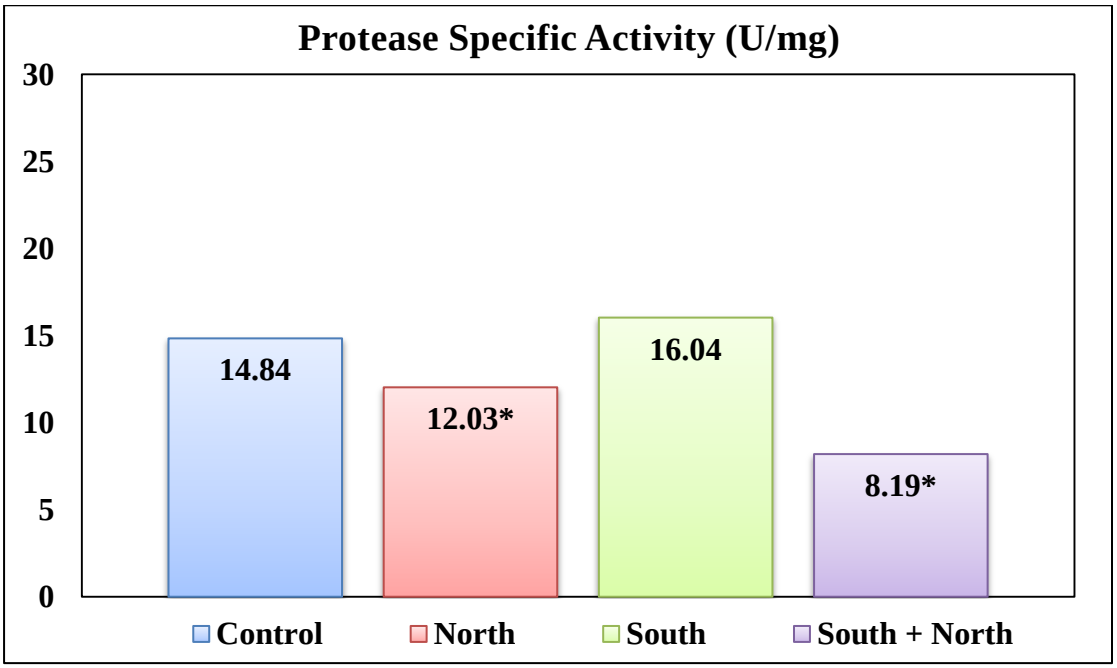


***LSD is 2.424 and the mean difference is significant at the 0.05 level.**

Figure (3-14): Effect of magnetic field on protease specific activity of *Mucor* sp.

3.3.5 Effect of Magnetic Field on the Protease Specific Activity of *Penicillium* sp.

The effect of magnetic field on protease specific activity of *Penicillium* sp. has been investigated. The Figure (3-15) represent after 7 days of fermentation on solid medium (bread cubes) at 28 °C that the Control specific activity was (14.84 U/mg). The LSD of this test was (2.684). The Northern Pole significantly decreased the specific activity (12.03 U/mg) as well as the two poles together (South + North) (8.19 U/mg). On contrary, the Southern Pole non-significantly increased the specific activity (16.04 U/mg).



***LSD is 2.684 and the mean difference is significant at the 0.05 level.**

Figure (3-15): Effect of magnetic field on protease specific activity of *Penicillium* sp.

The statistical analysis of the results in Figures (3-(11, 12, 13, 14, 15)) showed that the Southern Pole treatment significantly increased the protease specific activity of *Fusarium* sp. and *Mucor* sp., while it significantly decrease protease specific activity of *Alternaria* sp. Both Poles treatment significantly increased protease specific activity of *Fusarium* sp., *Mucor* sp. whereas decreased protease specific activity of *Alternaria* sp. and *Penicillium* sp. The Northern Pole significantly decreased the protease specific activity of *Alternaria* sp., *Aspergillus niger*, and *Penicillium* sp.

Regarding the Southern pole magnetic field effect on protease specific activity, it is logical to expect the increase effect of the enzyme activity due to the probable potentiation of the positivity of cations. However, it is not easy to explain the peculiar decrease of protease activity in the case of the fungus *Alternaria* sp.

The most reasonable explanation of the decrease of enzyme specific activity (protease and amylase) after exposing the fungi to the Northern the magnetic field is attributable to the probable interface of the negative charge of magnetic field with the release of the stimulating effect of (Ca^{2+}).

Blank and Soo (2001), demonstrated the EMF interaction mechanism with Na^{+}- K^{+} ATPase and suggested to be due to acceleration of the electron regardless of direction the electrons move regularly and

the threshold force producing the effect on the enzyme . Therefore, it is very likely that the influence of the field on the acid phosphatase could include electron density at the anti-ferromagnetically coupled binuclear Fe(III)-Mn(II) center and the active site.

A second theory concluded that the effect of SMF and OMF on calcium ions bound in calcium-binding proteins, like calmodulin. The calcium ions always vibrate about an equilibrium position in calmodulin binding site. A static magnetic field to calmodulin causes the level of vibration to rotate, or forward in the direction of the magnetic field at a frequency that equal to the cyclotron frequency of the bound calcium. Adding a "wobbling" MF at the cyclotron frequency disturbs the accuracy to such an extent that it loosens the bond between the calmodulin and the calcium ion (Pothakamury *et al*., 1993).

The ELF EMF influence enzyme activity irrespective of temperature, pH and substrate concentration. Optimal pH and temperature are very important for the activity of enzymes. Changing in pH and temperature affect the shape of an enzyme and change the charge or shape properties of the substrate so that the substrate cannot bind to the active site or undergo catalysis. The studies search for whether ELF EMF cause specific changes in the optimal pH and optimal temperature. However, when the samples were exposed to EMF at various pH and temperature the OD value was changed, which indicates there was a

change in the activity of enzyme. But there was no significant effect of ELF EMF on optimal pH and temperature (Prashanth *et al.*, 2008).

Conclusions and Recommendations

Conclusions

- Magnetic field poles (100 gauss) showed different effects on protease and amylase specific activity produced from *Alternaria* sp., *Aspergillus niger*, *Fusarium* sp., *Mucor* sp., and *Penicillium* sp.
- The Northern pole had a negative effect on the amylase specific activity of *Alternaria* sp., *Fusarium* sp., and *Penicillium* sp. as well as on the protease specific activity of *Alternaria* sp., *Aspergillus niger*, and *Penicillium* sp.
- The Southern pole had a positive effect on both amylase and protease specific activity of *Fusarium* sp., and *Mucor* sp. While it had a negative effect on amylase specific activity of *Alternaria* sp. and *Penicillium* sp. and protease specific activity of *Alternaria* sp. as compared with the Control under the same conditions.
- The two poles together (South + North) showed negative effects on both amylase and protease specific activity of *Alternaria* sp. and *Penicillium* sp. While they showed positive effects on amylase and protease specific activity of *Fusarium* sp. and *Mucor* sp. as compared with the Control under the same conditions.

Recommendations

- Demonstrate the effect of magnetic field poles on the gene expression of amylase and protease enzymes of *Alternaria* sp., *Aspergillus niger*, *Fusarium* sp., *Mucor* sp., and *Penicillium* sp.

- Study the optimum conditions for maximum production of amylase and protease enzymes from the five fungal genera.

- Purification of amylase and protease enzymes of fungal source and show the effect of MF poles on them.

- Study the effect of MF poles on other enzyme like cellulase that produced from these fungi.

- Study the effect of MF poles on the amylase and protease produced from other fungi like Rhizopus.

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الخـــــــــلاصة

في هذه الدراسة، أختبر تاثير المجال المغناطيسي الثابت على إنتاج انزيم الأميليز و البروتييز لخمسة أنواع فطرية عائدة لأجناس مختلفة، والتي هي *Alternaria* sp., *Aspergillus niger*, *Fusarium* sp., *Mucor* sp. and *Penicillium* sp. بواسطة تخمرات الحالة الصلبة. أستخدم الخبز فقط كوسط لنمو الفطريات. تم تعريض هذه الفطريات إلى المجال المغناطيسي للقطب الجنوبي والشمالي والاثنين معا، ثم قورن تاثير المجال المغناطيسي على الفعالية الانزيمية مع معاملة السيطرة (التي لم تتعرض لنفس للمجال). تم تحليل النتائج احصائيا بواسطة برنامج (GenStat) وحدد الاختلاف الاقل المعنوي (LSD). اظهرت النتائج بأن الفعالية النوعية لانزيم الاميليز تقل معنويا بتاثير القطب الشمالي للمجال المغناطيسي للفطريات *Alternaria* sp., *Fusarium* sp. and *Penicillium* sp. والتي كانت ٢.٥٠، ٢.١٢، ٣.٢٧ وحدة/ملغم على التوالي. تزداد الفعالية النوعية لانزيم الاميليز بتاثير القطب الجنوبي لفطر *Fusarium* sp 3.84 وحدة/ملغم ولفطر *Mucor* sp. 3.36 وحدة/ملغم ، بينما قللت معنويا الفعالية النوعية لانزيم الاميليز لفطر *Alternaria* sp. 2.77 وحدة/ملغم ولفطر *Penicillium* sp. 3.88 وحدة/ملغم مقارنة مع السيطرة. كان تأثير كلا القطبين كالأتي: وكلا القطبين خفظا معنويا الفعالية النوعية لانزيم الاميليز لفطر*Alternaria* sp. 2.62 وحدة/ملغم ولفطر *Penicillium* sp. 2.26 وحدة/ملغم، وفي المقابل، زادا معنويا الفعالية النوعية لانزيم الاميليز لفطر *Fusarium* sp. 3.84 وحدة/ملغم وفطر *Mucor* sp. 3.83 وحدة/ملغم . فيما يخص تأثير المجال المغناطيسي على الفعالية النوعية لانزيم البروتييز، القطب الشمالي قلل معنويا من الفعالية النوعية لانزيم البروتييز لفطر *Alternaria* sp. وفطر *Aspergillus niger* وفطر *Penicillium* sp. والتي كانت 12.03, 14.69, 16.86 وحدة/ملغم على التوالي. القطب الجنوبي زاد معنويا من الفعالية النوعية لانزيم البروتييز لفطر *Fusarium* sp. (23.04) وحدة/ملغم ولفطر *Mucor* sp. (12.15) وحدة/ملغم ماعدا فطر *Alternaria* sp. الذي قلت فيه الفعالية النوعية لانزيم البروتييز معنويا (19.30) وحدة/ملغم. كلا القطبين زادا معنويا الفعالية النوعية لانزيم البروتييز لفطر *Fusarium* sp. (21.03) وحدة/ملغم وفطر *Mucor* sp. (9.75) وحدة/ملغم، بينما قللا معنويا من الفعالية النوعية لانزيم البروتييز لفطر *Alternaria* sp. (13.65) وحدة/ملغم وفطر *Penicillium* sp. (8.19) وحدة/ملغم .هذه الدراسة اوضحت بان هناك تأثير معنوي للمجال المغناطيسي الثابت في زيادة وتقليل الفعالية

الانزيمية لانواع الفطريات والتي من الممكن استغلالها صناعيا في زيادة انتاج الانزيمات المهمة صناعيا مثل البروتييز والاميليز التي اختبرت في هذه الدراسة.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين / كلية العلوم
قسم التقانة الأحيائية

تاثير المجال المغناطيسي الثابت على أنزيمي الاميليز و البروتيز المنتجة من بعض العزلات الفطرية باستعمال تخمرات الحالة الصلبة

رسالة

مقدمة الى مجلس كلية العلوم – جامعة النهرين
وهي جزء من متطلبات نيل درجة ماجستير في علوم التقانة الاحيائية

من قبل

مروة صباح هاشم

بكالوريوس تقانة احيائية - كلية العلوم - جامعة النهرين -٢٠١٣

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حزيران ٢٠١٦