Isolation Identification and Characterization of some Penicillium Isolates for Alpha Amylase Activity

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Certificate

This is to certify that the M. Phil. Thesis entitled **"Isolation Identification and Characterization of some Penicillium Isolates for Alpha Amylase Activity"** is the original research work carried out by **Mr. Gowhar Hamid Dar**, as a whole time M. Phil. research scholar in Environmental Science, University of Kashmir, Srinagar. This work has been carried out under our joint supervision and has not been submitted to this University or to any other University so far and is submitted for the first time to the University of Kashmir. It is further certified that this thesis is fit for the submission for the degree of ***Master of Philosophy in Environmental Science*** and the candidate has fulfilled all the statutory requirements for the completion of the M. Phil. Programme.

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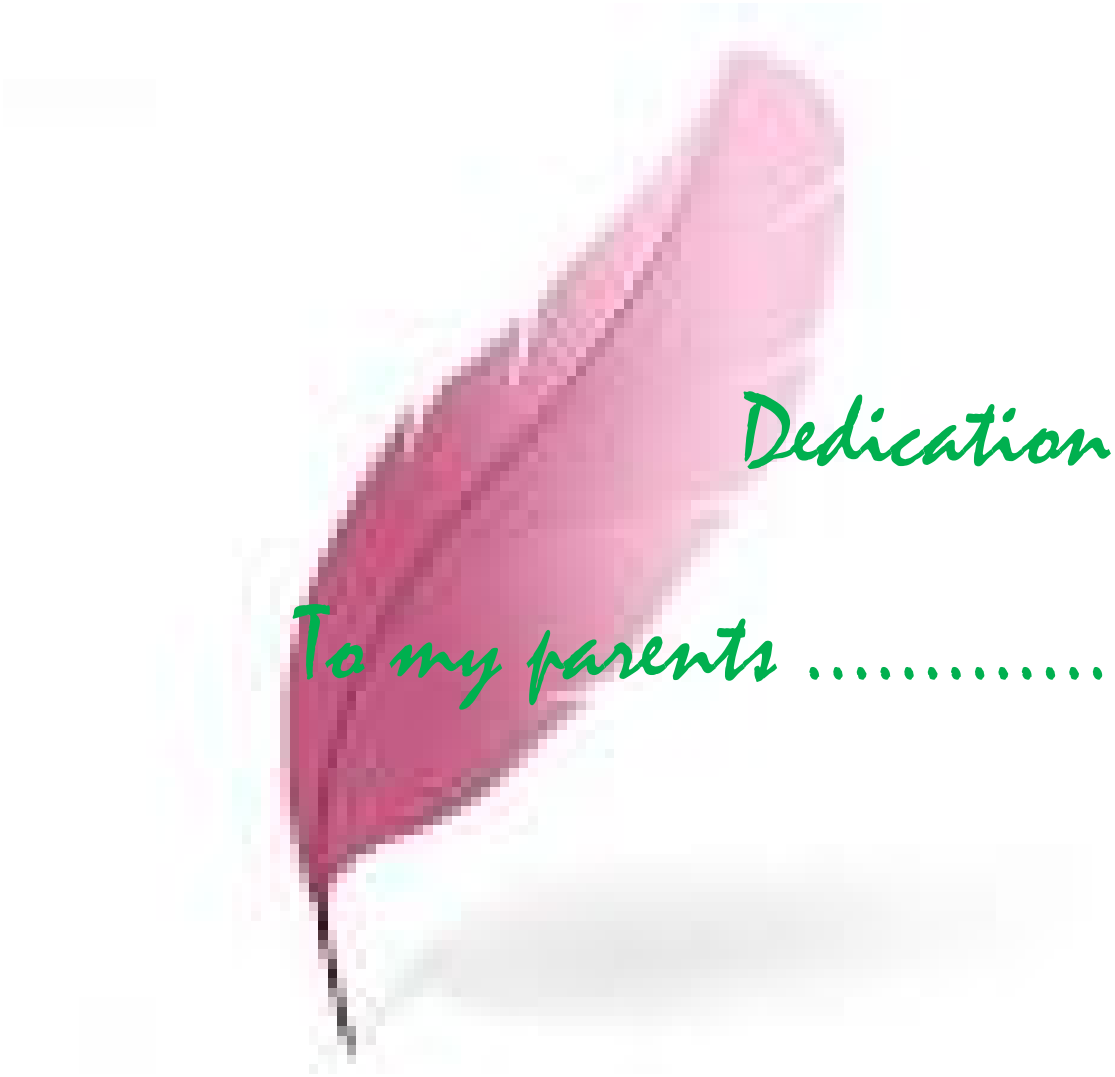

Dedication

To my parents .....

ACKNOWLEDGEMENT

THE POWER OF DEVOTED WORD LEADS ONE FROM SELFISHNESS TO SELFLESSNESS

With limitless humility, I praise and thank Allah, the Almighty, to whom I owe profound gratitude for His merciful and beneficent blessings, especially the courage, confidence and perseverance as the tools of consistent effort for purposeful success in the decided course. With everlasting passion and great pride, I express my deep sense of regards, eternal gratitude and indebtness to my esteemed supervisor **Prof. Azra N. Kamili,** *H.O.D. Department of Environmental Science/Director, C.O.R.D., University of Kashmir, for not only providing me her inspiring guidance but also her constant efforts, constructive criticism and above all her sustained encouragement during the investigation and preparation of the manuscript.*

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GOWHAR HAMID DAR

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List of Abbreviations

PDA	Potato Dextrose agar
RBA	Rose Bengal agar
CZ	Czapek's dox Agar
CYA	Czapek's Yeast Agar
MEA	Malt Extract Agar
WB	Wheat Bran
DNS	Dinitrosalicylic acid
U/ml	Units per ml
SD	Standard deviation
ASL	Above sea level
SmF	Submerged fermentation
CFU	Colony forming units
ºC	Degree Centigrade
Km^2	Kilo meter square
Km	Kilometre
m	Meter

ABSTRACT

An investigation was carried out to determine the presence of amylase producing *Penicillium* species in the soils of Harwan area of Srinagar city. Two study sites were choosen from the area. Soil samples were collected from the study sites for the isolation of *Penicillium* isolates. Pour plate method involving serial dilution was used for the isolation of fungi. The media used for isolation were Potato Dextrose Agar (PDA), Rose Bengal agar (RBA) and Malt Extract Agar (MEA). A total of 4 *Penicillium* isolates were obtained during the study, which showed positive alpha amylase activity, all belonging to division Ascomycota of kingdom fungi. The highest colony forming unit (cfu/g) of 3.5×10^3 was found for *P. chrysogenum* at site II in the month of August. Certain agricultural wastes like mustard oil cake, linseed oil cake and wheat bran were used as crude ingredients in the submerged fermentation process for the production of amylases. The carbon and nitrogen sources were also altered in the culture media for the production of alpha amylases from the isolated *Penicillium* species. The results of this investigation show that *P. chrysogenum* has maximum activity of 550.4 U/ml in medium with linseed oil cake at pH 6 and incubation period of 6 days. *P. purpurogenum* showed maximum activity of 883.93 U/ml in fermented medium with mustard oil cake as crude substrate at pH 6 incubation period of 6 days. *P. caesicolum* showed maximum activity of 1834.8 U/ml in medium with glucose as carbon source at pH 9 and incubation period of 18 days. *P. funiculosum* showed maximum activity of 4254.4 U/ml at pH 6 and incubation period of 18 days with mustard oil cake as crude substrate in the medium. The optimum conditions in the medium which were found best to promote the production of alpha amylase from *Penicillium* species were, when medium was maintained at pH 9 and incubation period of 18 days with glucose as carbon source followed by medium maintained at pH 9, 6 and incubation period of 18 days with yeast extract as nitrogen source and medium maintained at pH 6 and incubation period of 18 days with mustard oil cake in the medium.

A lot of work has been carried out with regard to the isolation, identification and characterisation of the fungal isolates for alpha amylase production, the world over, but no such work has been carried out here in Kashmir Valley so far. Thus an attempt was made here to portray the importance of isolation, identification and characterisation of the *Penicillium* isolates for alpha amylase activity.

Microbes are essential components of every ecosystem, they form the foundation of many food webs. Microbes play an important role in breaking down of dead plant and animal material through a process called decomposition. Most importantly, microbes are involved in the cycling of nutrients and other compounds throughout the environment. For example, microbes in the oceans generate much of the oxygen used by organisms throughout the world (Kennedy and Smith, 1998). Only one percent of microbes that live in soil have been identified and these organisms both the known and unknown ones take part in the formation of soil, and are essential components of these ecosystems. They are responsible for the transfer of nutrients such as carbon, nitrogen and sulphur to plants and animals through various cycles. Some microbes convert nitrogen from a gas to a form that plants can use, like natural fertilizer. Without the microbes, many plants would not be able to grow.

Bacteria and fungi that live in soil feed mostly on organic matter such as other plants and animals. They promote the decomposition of dead material, which releases useful nutrients into the soil. This makes microbes the primary members of the soil food web. These microbes are very sensitive to their local environment. Factors such as the levels of carbon dioxide and oxygen, pH, moisture and temperature all affect the growth of microbes in the soil (Piao *et al.,* 2000).

It is estimated that there are 1.5 million fungal species on earth, of which only about 70,000 have been described till recently (Hawksworth, 2006). *Penicillium* is a large anamorphic Ascomycetous fungal genus with widespread occurrence in most terrestrial environments and many are common soil inhabitants (Kirk *et al.,* 2008). It is a well-known cosmopolitan genus of moulds with more than 300 accepted species. It is one of the dominant fungal genera in soil (Thom, 1930; Christensen *et al*., 2000) where it is mainly responsible for decomposing organic matter and assists in the maintenance of soil nitrogen fertility in concert with other organisms (Seneviratne and Jayasinghearachchi, 2005). They function as decomposers of dead materials and are especially important as post-harvest organisms, where they spoil various food commodities (Janisiewicz, 1987; Pitt and Hocking, 1997; Holmes and Eckert, 1999; Morales *et al*., 2007). It leads to the production of a large group of antibiotics for pharmaceutical industries. *Penicillium* species produce a much diversified array of active secondary metabolites, including antibacterial, antifungal substances, immunosuppressants, cholesterol-lowering agents and also potent mycotoxins (Singh *et al.,* 2008). *Penicillium* and many other terrestrial moulds have been identified till date for the production of various industrially important enzymes including alpha amylases.

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively small polymers composed of glucose units. The history of amylases began in 1811 when the first starch degrading enzyme was discovered by Kirchoff. The major advantage of using micro organisms for the production of amylases is the economic bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Lonsane *et. al.,* 1991). Enzymes from fungal and bacterial sources have dominant applications in industrial sectors. Two major classes of amylases have been identified in microbes,

alpha amylase (E.C.3.2.1.1) hydrolyze 1, 4-alpha-D glucosidic linkages between adjacent glucose units in the amylase chain.

Amylases is the family of hydrolytic enzymes that are wide spread in nature, being found in animals, micro-organisms and plants, with the advent of new frontiers in biotechnology had found a potential application in a number of industrial processes such as bread making, brewing, starch processing, pharmacy, textile, paper industries and bioconversion of solid wastes (Pandey *et al.*, 2000). Amylases constitute a class of industrial enzymes representing approximately 25-33% of the world enzyme market (Nguyen *et al*., 2002; Van der Maarel *et al*., 2002) and have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey *et al*., 2000). They are among the most important products obtained for human needs through microbial sources including Fungi.

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. The *Aspergillus* species produces a large variety of extracellular enzymes, of which amylases and proteases are of significant industrial importance (Pandey *et al.*, 2000). Fungal amylases have not only been used in fermentation processes, but also in processed food industry and in the textile and paper industries (Ellaiah *et al.*, 2002 and Gigras *et al.*, 2002,). In general, data obtained in studies suggest that, amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Mitchell and Lonsane, 1990; Akpan *et al*., 1999). Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand (Pandey *et al*., 2000). A large number of them are available commercially but microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries.

In recent years, the new potential of using micro-organisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extra cellular enzymatic activity in several micro-organisms. Thus the present work dealing with the isolation, identification and characterization of alpha amylase activity from various *Penicillium* species, isolated

from soil of a local habitat from Harwan area of Srinagar district was carried out to focus on the below mentioned objectives:

1. To isolate and characterize various *Penicillium* species from two diverse habitats of Kashmir valley.

2. To study the α-amylase activity of isolated *Penicillium* species on different media.

The valley of Kashmir is very well known for its snow capped peaks, forests, lakes and springs. It lies to the north of the Indian subcontinent between 32° 20´ and 35° 5´ N latitudes and 73° 30´ and 76° 30´ E longitudes with an elevation of 5200 feet above sea level (Bates, 2005). Traversing the valley is the River Jhelum and its tributaries. The Jhelum (also called Vitasta/Vyeth) has been and continues to be the key element of the ecosystem of Kashmir (Raza *et al*., 1978). The shielded valley of Kashmir is characterized by distinct geographic features and snow clad peaks and resembles the mountainous and continental parts of the temperate latitudes. In the regions of Jammu and Kashmir the soils are loamy and there is little clay content in them. Poor in lime but with a high content of magnesia, the soil is treated with chemical fertilisers and enriched with green manure and legume before cultivation. There is sufficient organic matter and nitrogen content in the alluvium of the Kashmir valley as a result of plant residue, crops stubble, natural vegetation and animal excretion (Dar *et al.,* 2000). The valley of Kashmir has many types of soils like: Gurti (clay), Bahil (Loam), Sekil (Sandy), Nambaal (Peats), Surzamin, Lemb, floating garden soils and Karewa soils (Mahapatra *et al.,* 2000).

Harwan area of this beautiful valley was choosen to collect the soil samples for the research. Harwan situated at an altitude of about 1743 m above sea level , with the geographical coordinates 34º 10' 0" N and 74º 54' 0" E, lying in District Srinagar, J&K, India, is a small village set in the heart of mountains and is an ideal picnic spot. The climate of this beautiful area is very bracing and enjoys a subtropical climate. Here the precipitation normally occurs in the form of mild snowfall during the winters. Summers are mild and winters are very cold. (Kausar, 2005).

Two different sites were selected in this area to carry out the present work, the first site was Apple orchard and the second site selected was a Fallow land area.

Site I - Apple orchard Harwan

It was that area which was not affected by the human and animal activities. The grazing rate in this area was negligible. It is renowned for its green pasture and lies between the geographical co-ordinates of 74^{o} 54′ 40.06″ E and 34^{o} 09′ 45.16″ N, at an altitude of about 1687 m above sea level. The site was predominantly occupied by large trees. The vegetation in the site was herbaceous, having *Cynodon* sp., *Trifolium* sp. and other different herbs forming a community structure. The site area was fenced, which prevented it from grazing (**Plate I, Fig. A**).

Site II - Fallow land area Harwan

The fallow land area site was a degraded site influenced heavily by human and animal activities with huge quantity of animal excreta (dung) and carcass of grazing and non-grazing animals. The site lies in between the geographical co-ordinates of 74^{o} 54′ 64.73″ E and 34^{o} 10′ 01.12″ N, at an altitude of about 1687 m above sea level (**Plate I, Fig. B**).

Fig. (A): Site I-Apple orchard Harwan

Fig. (B): Site II-Fallow land area Harwan

Plate I: Study sites

Soil, one of the greatest gifts of nature is such a vital factor for life on which the survival and development of living organisms including man depends. It is the best medium not only for the growth of plants but the micro-organisms as well. Soil is a natural body consisting of layers (soil horizons) of mineral constituents of variable thicknesses, which differ from the parent materials in their morphological, physical, chemical and mineralogical characteristics. Soil microbial population is the key element in the bio-geochemical cycling of nutrients in nature (Pelczar *et al.,* 1993). The cycling of carbon, oxygen, sulphur and nitrogen that take place in terrestrial and aquatic ecosystems is made possible by the different types of microbes dwelling in these ecosystems (Griffiths *et al.,* 2003). More recently microbial diversity has also been recommended as biological indicator of soil quality. Soils that are clayey often have more bacteria than sandy soils because the clay creates lots of small pores (spaces) which offer protection for bacteria. Sandy soils with fewer aggregates and small pores are less suitable habitats for bacteria and fungi unless a large amount of organic matter is added to the soil (Kennedy and Papendick, 1995). Most organisms are found in the top layers of soil, usually the top 2-3 centimetres, since this is typically where most of the organic matter is present. Organisms do occur to depths of several kilometres below the soil surface, but the types of organisms that occur this far down are not the same as those close to the

surface. The organisms in soil are often commonly found close to root surfaces in the rhizosphere, within living and dead roots, on soil particles, or amongst aggregates of soil particles (Fierer and Jackson, 2006).

A fungus is an important group of the soil micro biota typically constituting more of the soil biomass than bacteria, depending on soil depth and nutrient conditions. The role of fungi in soil is extremely complex and is fundamental to the soil ecosystem. They perform ecological services that strongly impact the quality of human life and have enormous potential for providing economic benefits, e.g., the isolation and identification of the soil fungus. Fungi are heterotrophic organisms, requiring organic compounds for nutrition. When they feed on dead organic matter, they are known as saprophytes. Saprophytes decompose complex plant and animal remains, breaking them down into simpler chemical substances that are returned to the soil, thereby increasing its fertility. Fungi are eukaryotic chemo-organotrophic organisms that have no chlorophyll. The thallus or body of a fungus may consist of a single cell as in the yeasts more typically the thallus consists of filaments which are commonly branched (Hawksworth, 1991). Fungi perform important functions within the soil in relation to nutrient cycling; disease suppression and water dynamics, all of which help plants become healthier and more vigorous. The major activity of soil fungi is to disintegrate the complex organic molecules e.g. cellulose, lignin, pectin, hemicelluloses etc. which leads to the addition of nutrients in the soil. Fungi also play an important role in humus formation. Some fungi associate themselves with roots of higher plants in the form of mycorrhizae or fungus roots, which help plants to scavenge essential minerals from nutrient poor soils (Curtis and Sloan, 2005). They also form mutualistic associations with algae and cyanobacteria in the dual organisms known as lichens. Fungi are found wherever there is hard, carbon-rich woody organic matter. This could be dead rotting trees in a forest, leaf litter on the surface of orchard soils, or plant roots. Fungi in soil are present as mycelial bits, rhizomorph or as different spores. Their number varies from a few thousand to a few million per gram of soil. Soil fungi possess filamentous mycelium composed of individual hyphae. The fungal hyphae may be aseptate /coenocytic (Mastigomycotina and Zygomycotina) or septate (Ascomycotina, Basidiomycotina and Deuteromycotina). Most vascular plants could not grow without the symbiotic fungi, or mycorrhizae, that

inhabit their roots and supply essential nutrients. Other fungi provide numerous drugs (such as *Penicillin* and other antibiotics), foods like mushrooms, truffles and morels, and the bubbles in bread, champagne, and beer (Dance, 2008). Fungi also cause a number of plant and animal diseases. In human's ringworm, athlete's foot, and several more serious diseases are caused by fungi. Because fungi are more chemically and genetically similar to animals than other organisms, this makes fungal diseases very difficult to treat. Plant diseases caused by fungi include rusts, smuts, and leaf, root, and stem rots, and may cause severe damage to crops. However, a number of fungi, in particular the yeasts, are important "model organisms" for studying problems in genetics and molecular biology

As observed by Jackson (1975), most commonly encountered genera of fungi in soil are; *Alternaria, Aspergillus, Cladosporium, Cephalosporium Botrytis, Chaetomium, Fusarium, Mucor, Penicillium, Verticillium, Trichoderma, Rhizopus, Gliocladium, Monilia, Pythium,* etc. As these soil fungi are aerobic and heterotrophic, they require abundant supply of oxygen and organic matter in soil. Fungi are dominant in acid soils, because acidic environment is not conducive and suitable for the existence of either bacteria or actinomycetes. The optimum pH range for fungi lies-between 4.5 to 6.5. They are also present in neutral and alkaline soils and some can even tolerate pH beyond 9.0 (Piao *et al.*, 2000).

Amylases are hydrolytic enzymes that are widespread in nature, being found in animals, microorganisms and plants. With the advent of new frontiers in biotechnology, the amylase family enzyme finds potential application in a number of industrial processes such as bread making, brewing, starch processing, pharmacy, textile and paper industries. The mycelial growth and amylase production by a mycotoxigenic strain of *Fusarium moniliforme* and *Aspergillus flavus* evaluated in culture medium containing starch, glycerol, wheat bran or corn was studied by Figueira and Hirooka (2000) and reported that the medium composed of milky stage corn supernatant promoted the best mycelial growth and amylase production. Leiter *et al.* (2000) studied that *Penicillium chrysogenum* grown in complex medium possessed an intracellular iron concentration and reported that this iron concentration was sufficient enough to cause growth and antibiotic production by this fungal strain. Amylases have almost completely replaced chemical hydrolysis of starch in starch

processing industry (Pandey *et al*., 2000) and constitute a class of industrial enzymes representing approximately 25-33% of the world enzyme market (Nguyen *et al.*, 2002;Van der Maarel *et al*., 2002).

The isolation, screening, selection and mutation of *Aspergillus oryzae* for α-amylase production showed that mutant strains demonstrated 2.6 fold increased activity over the parental strain in terms of enzyme production (Abdullah, 2005). While as, Balkan and Ertan (2005) studied the fungi and screened their ability to produce α-amylase, *Pencillium chrysogenum* showed high enzymatic activity and α-amylase production by *Pencillium chrysogenum* cultivated in liquid media containing maltose (2%) reached its maximum in 6-8 days at 30^{o}C. However, Kathiresan and Manivannan (2006) studied the effects of pH, temperature, incubation time, salinity, sources of carbon and nitrogen on submerged fermentation process in production of α-amylase by *Pencillium fellutanum* isolated from coastal mangrove soil and reported that the production medium without addition of sea water and with provision of maltose as carbon source, peptone as nitrogen source, incubated for 96 hours maintained with pH of 6.5 at 30°C, was optimal for production of α-amylase. A study carried out by Tiwari *et al.* (2007) on *Pencillium rugulosum* isolated from a soil sample, for production of α-amylase showed that the maximum production of amylase by *Pencillium rugulosum* was observed at 3rd day of incubation with an improvement in its production in the presence of galactose as sole carbon source. It was further observed that the enzyme activity was inhibited in presence of EDTA and enhanced in presence of metal ion Mn^{2+} and Fe^{2+}. While as, Xu *et al.* (2008) worked on optimisation of nutrient levels for the production of *α*-amylase by *Aspergillus oryzae* in solid state fermentation (SSF) with spent brewing grains (SBG), using response surface methodology (RSM) based on Plackett–Burman design (PBD) and Box–Behnken design (BBD). It was found that corn steep liquor (1.8%), $CaCl_2$ (0.22%) and $MgSO_4.7H_2O$ (0.2%) are the most compatible supplements to the substrate SBG to influence α-amylase activity positively.

Metin *et al.* (2010) while studying the extracellular amylase production by *Penicillium citrinum,* reported that amylase exhibited broad substrate specificity because it acted on all the substrates tested and showed that enzyme was activated by Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Ba^{2+}, NH_4^{+} and Al^{3+}, the other ions and EDTA had no effect

on its activity. It was further observed that enzyme activity was inhibited in the presence of phenyl methane sulfonyl fluoride (PMSF), N-bromo succinimide (NBS) and 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide methyl *p* toluene sulphonate (CMC), suggesting that serine, tryptophan residues and carboxyl groups play an important role in the catalytic process. Similarly Raharjo *et al.* (2010) worked on the thermostable α-amylase isolated from selected bacteria from Sonai Hot Spring, South East Sulawesi using sago pith waste as substrate and observed that logarithmic phase reached at 10th hour due to the increasing of amount of bacteria cell and maltose concentration on growth curve. It was further observed that crude enzyme had activity of 0.418 U/mL and the optimal pH, temperature, and substrate concentration were 4.5, 70^{o}C, and 1%, respectively.

Some fungal strains of each of the two filamentous fungi viz, *Aspergillus niger* van Tieghem and *Aspergillus flavus* link ex Gray, were analysed by Shafique *et al*. (2009) for their α-amylase activity and reported that all the test strains exhibited their maximum α-amylase activity after 48 hours of incubation. The wastes from foods and drinks industries were studied by Sidkey *et al.* (2010) focusing on the possibility of using different fermented enviro-agro-industrial wastes as very cheap and available substrates for obtaining microbial α-amylases that are of great industrial importance and isolated seventy three fungi and bacteria from twenty different wastes, e.g. food industrial wastes, daily home wastes, expired food stuff wastes and some agricultural wastes from Al-Madinah, Al-Munawwarah, K.S.A. While as, Oyeleke *et al*. (2010) reported that *Bacillus megaterium* is a good producer of extracellular amylase at high temperatures which could be an indication that amylase produced would be thermostable, while studying a local yam peel dumpsite in Mina, Niger state. The loquat kernel flour (LKF) could serve as a sole source of nitrogen and carbon for the fungus to grow and synthesize α-amylase (Erdal and Taskin, 2010) as the feasibility of waste loquat kernels as substrate in solid state fermentation for α-amylase production by *Pencillium expansum* has been evaluated for it.

The production, purification and characterization of alpha amylase from *Trichoderma harzianum* grown on mandarin peel was studied by Mohamed (2011), and suggested that the enzyme has high affinity towards high-molecular mass substrates. It was further observed that maximum activity of enzyme against soluble

starch was determined at pH 4.5 and 40°C. However, Khokhar *et al.* (2011) reported that filamentous fungi are important due to their high enzyme production potential. Fifteen fungal isolates of three genera, *Aspergilus*, *Penicillium* and *Trichoderma* were examined for their ability to produce amylase. It was found that all isolates exhibited enzymatic potential and reported that *Penicillium*, *Aspergillus raperi* and *Aspergillus speluneus* were hyper active in starch medium and showed the increased growth in starch medium as compared to control. Similarly, Sun *et al.* (2011) studied the feasibility of using banana peel for the production of cellulase by *Trichoderma viride* in solid-state fermentation, indicated that banana peel provided necessary nutrients for cell growth and cellulase synthesis and can be used as a potential substrate for cellulase production by *Trichoderma viride* under solid-state fermentation.

Ahmed (2011), during its study on the fungal strain of *Aspergillus oryzae* used for the production of alpha amylase by solid state fermentation from agro-industrial wastes reported that enzyme production was growth associated and maximum activity (8.23 U/ml) was obtained after 120h when incubated at 30°C on wheat bran with initial moisture content 60% and initial medium pH of 5. It was also found that enzyme activity increased when the solid medium was supplemented with additional nitrogen source. Nwagu and Okolo (2011) worked on the α- amylase producing *Fusarium* sp. isolated from soil at 50 °C and observed that growth and enzyme production occurred at 30, 45 and 55 ^{o}C. It was also reported that Soybean meal at 1% concentration, supplemented with 0.2% NH_4Cl and 2.5% corn starch elicited the highest amylase yield. However, Vijayaraghavan *et al.* (2011) carried out solid state fermentation using banana peel as a substrate for the production of amylase by .*Penicillium* sp. and partially purified enzyme by the combination of ammonium sulphate precipitation, Sephadex G-75 gel filtration chromatography and dialysis, reported that the enzyme showed optimum activity at a pH of 7.0 and incubation temperature 50°C. Similarly, Balkan *et al.* (2011) while studying the production of extracellular α-amylase by *Trichothecium roseum* in solid state fermentation (SSF) examined the effects of wheat bran (WB), rye straw (RS), corncob leaf (CL), sunflower oil meal (SOM) and rice husk (RH) showed that wheat bran (WB) exhibited the highest enzyme production. It was further observed that addition of different metal ions (0.1M) to wheat bran (WB) resulted in better α-amylase

production with $CaCl_2$, while as addition of CuSO4 to wheat bran (WB) resulted in decreased enzyme activities.

The *Aspergillus* strains obtained from IMTECH Chandigarh and from Department of Biotechnology, CDLU, Sirsa by Kumar and Duhan (2011), screened them for their ability to produce amylase on starch agar plates, among the five strains, *A. niger*, showed highest clearing zone on starch agar plates as well as amylase activity in solid state fermentation. Different substrates like wheat bran, rice bran, soybean meal and black gram bran were screened for enzyme production and rice bran was found to be the best substrate for the enzyme production. However, Adejuwon (2011) while studying the *Pencillium* species isolated from decaying apple fruit grown in a synthetic medium containing starch as sole carbon source showed that Culture filtrates exhibited amylase activity, and reported that the presence of cations Mg^{++}, Ca^{++}, K^{+} and Na^{+} stimulated the activity of the enzyme. Use of dried citrus peel and wheat bran as substrate for the production of pectin methyl esterase (PME; EC3.1.1.11) by fungus *Penicillium notatum* Gayen and Ghosh, (2011) showed that maximum enzyme activity was obtained with 1:1 (w/w) substrate ratio, which gives a solid mass of initial pH 5.5, when incubated at 30°C for 120 h at 1:1 (w/v) initial moisture content ratio under static condition.

Bacillus subtilis yielded maximum enzyme at pH 7 and at 37°C and the isolate exhibited higher amylase activity in the presence of organic nitrogen source followed by inorganic nitrogen source (Kumari *et al*., 2011). The *Penicillium* strains from the Howzsoltan lake were studied by Abbas *et al.* (2011) to produce α–amylase and reported that some filamentous fungi can survive and grow in high concentration of salt, analyzed 100 water samples and isolated 65 samples as 9 species of *Penicillium* and showed that solid state fermentation (SSF) medium could increase the α-amylase activity to tenfold, in comparison with subaro broth as submerged fermentation (SmF).While as, the purification and characterization of α-amylase from *Aspergillus flavus*, showed that the activity of the purified α-amylase increased with increasing enzyme concentration and incubation time and the enzyme exhibited maximum acidity at 30^{o}C and pH 6.4 with the optimum starch concentration of 15 mg/ml (Sidkey *et al*., 2011). The production of cellulase and amylase from wild and mutated fungal isolates was studied by Sathyaprabha *et al*. (2011), and it was reported

that mutated fungal strains showed highest production of amylases and cellulases than the wild isolated fungal organisms. However, Sindhu *et al*. (2011) studied the purification and characterization of α-amylase from *Penicillium janthinellum* and its application in detergent industry, concluded that after 96 hours of incubation using wheat bran as substrate for SSF, amylase got purified. The amylase production by *Aspergillus niger* under solid state fermentation using agro industrial wastes was studied by Suganthi *et al.* (2011), reported that *Aspergillus niger* showed the highest production of amylase. They also reported that sucrose and nitrogen improved the yield in the same medium.

Penicillium canescens was cultivated on barley straw hydrolysate as a soluble nutrient source and as inducer for xylanase production by Bakri *et al.* (2012) and it was suggested that xylanase can be produced efficiently by *Penicillium canescens* in continuous culture from an inexpensive source such as barley straw hydrolysate. The best xylanase production (54 U/ml) was observed on hydrolyzed extract from barley straw treated with hot water (100 ºC) for 3 hours. Enzyme production was further improved by scaling up the cultivation process to a 3-L stirred tank bioreactor. Similarly, Ileasanmi *et al.* **(**2012), investigated the production of amylase using *Aspergillus flavus* implicated in the bio-deterioration of starch-based fermented foods and showed that 30^{o}C incubation temperature was optimum for amylase production by this isolate. It was further revealed that an incubation period of 6 days was optimum for amylase production by this isolate. However, Adejuwon *et al*, (2012) grew *Aspergillus niger* in a growth medium with rice as carbon and growth source and in a defined synthetic medium with varying carbon and nitrogen sources at 25^{o}C producing amylase, reported that optimum amylase activity in rice was expressed on the eighth day of incubation as 0.58 Units and in the synthetic growth medium with starch as carbon source and tryptone as nitrogen source, optimum amylase activity was expressed on the seventh day as 0.47 units. Similarly, Sundar *et al.* (2012) investigated *Aspergillus niger* utilizing *Ipomoea batatas*, reported that submerged fermentation holds tremendous potentiality in high biomass yield of alpha-amylase. The effect of varying pH, temperature and nitrogen sources of the medium for the productivity of α- amylase was also studied and it was reported that the maximum

activity of α-amylase was recorded as 450 U/mg after 7 days of submerged fermentation at pH 7.0 and room temperature 28^{o}C.

The culture and nutrient requirements of *Penicillum crysogenum* for production of α- amylase in production media containing different pH, temperature, incubation period, inoculum size, carbon sources, nitrogen sources and metal ions were analyzed under submerged fermentation by Vidya *et al.*, 2012. It was found that the optimum pH, temperature, inoculum size and incubation period for enzyme production were 6, 50°C, 4% and 6th day of incubation. It was also found that minimal medium can be used under submerged fermentation for the maximum production of amylase under controlled conditions. Bhardwaj *et al*. (2012) reported that filamentous fungi have been widely used for the production of amylases under solid state fermentation, wherein certain cultural parameters may provide good growth of microorganisms and thereby better enzyme production and studied the effect of salts of certain metal ions on amylase production by the mangrove isolate of *Aspergillus flavus* and showed that the highest yield of amylase production was obtained by the addition of magnesium sulphate (0.1%) and calcium chloride (0.02%) respectively. Supplementation of the enzyme production medium with non ionic surfactants in general and Tween 80 in particular resulted in an enhanced secretion of the starch hydrolyzing proteins in the medium. The amylase enzyme producing potential of four different *Aspergillus* species was studied. The extracted amylase enzyme was purified by diethyl amino ethyl (DEAE) cellulose and Sephadex G-50 column chromatography and the enzyme activity was measured by using synthetic substrate starch. The partially purified enzyme exhibits maximum activity at the optimum pH (7.0), temperature (60 to 70°C) and substrate concentration (1.5 to 2.0%) under standard assay conditions. Among the four different *Aspergillu*s species examined, *Aspergillu*s *flavipes* showed maximum production of amylase (Doss and Anand, 2012).

The amylolytic enzymes produced by *Aspergillus flavus* isolated from mouldy bread with the aim of establishing some factors that affect its activity shows that *Aspergillus flavus* grows in synthetic medium containing starch as the sole carbon source and synthesize enzymes which exhibited amylolytic activities. The production of the enzyme increases with increase in days of incubation with optimum activity occurring on the tenth day of incubation (Ayansina and Owoseni, 2012). Irshad *et al.*

(2012) investigated the production of α-amylase through solid-state fermentation by *Ganoderma tsuage* by using waste bread as substrate and reported that calcium chloride ($CaCl_2$) increases the activity of α-amylase while as all other compounds inhibit α-amylase activity. It was showed that silver nitrate ($AgNO_3$) was the strongest inhibitor and therefore would not be advised for use in future research against α-amylase production.

Very recently, Alhussaini (2013) worked on the mycobiota of wheat flour to isolate and identify the fungal species, which contaminated the stored flour in Riyadh region in Saudi Arabia. The study revealed that the *Aspergillus* genus was the most active producer of α-amylase and *P. chrysogenum*, *P.crustosum* and *P.olsonii* exhibited high activity in production of L- asparaginase. Adejuwon and Ladokun (2013) worked on the effect of carbon source of growth medium on α-amylase production by strains of *Penicillium solitum* and *Aspergillus rubrum* isolated from yam (*Dioscorea alata*) using potato dextrose agar, rice (*Oryza sativa*) supported fungal growth and α-amylase production. It was found that highest activity was expressed by *Penicillium solitum* on the tenth day of inoculation of the synthetic medium when maltose was carbon source and showed that least activity was expressed by *Aspergillus rubrum* when galactose was carbon source.

The following methodology was followed for evaluating the amylase activity of *Penicillium* isolates during the present study.

A. Materials used

The following materials were used during the study:

3.1. Media used

Rose Bengal Agar (RBA), Czapek's Dox Agar (CZ), Malt Extract Agar (MEA), Czapek's Yeast Agar (CYA) and Potato Dextrose Agar (PDA)

3.2. Chemicals used

Peptone, starch, Fructose, Sucrose, Lactose, Glucose, Yeast Extract, Dinitrosalicylic acid, Acetate buffer, Phosphate buffer, Iodine solution, $MgSO_4$, KCL, KNO_3, NH_4Cl, $NaNO_3$ etc.

B. Methods used

3.3. Sampling

Composite soil samples were collected from the sites under consideration on bi-monthly basis for a year, by digging upto a depth of 5 inches with the help of spade, in a sterile container and brought to the laboratory for further analysis.

3.4. Laboratory analysis

1. Cleaning of glass ware

- All the glass ware used was cleaned with commercial lab. wash to remove oils and organic matter from it.
- It was allowed to drain and dry in an oven at 110^{o}C and then further sterilization procedures were followed.
- Glass ware including test tubes and petridishes were then sterilized by autoclaving them at 121^{o}C and 15lb pressure.

2. Sterilization

i. **Dry heat sterilization:** It was used to sterilize materials that can with stand high temperature as well as materials that get damaged by moisture such as:

   a. **Inoculating loops:** Needles, tubes etc which are sterilized by heating in flame before use and after use.

   b. Glass ware, instruments etc. are heated/ dried in hot air ovens at 171^{o}C: 1h; 160^{o}C; 2h; 121^{o}C: 6h, for sterilization.

ii. **Moist heat sterilization:** It was used to sterilize liquids and materials which can't withstand dry heat and get easily charred. Mostly it was used to sterilize gloves, masks, aprons, glassware, instruments like needles, forceps, loops, culture, media, water etc.

iii. **Autoclaving:** Sterilization of media, glassware, instruments and water was done in an autoclave at 121^{o}C, 15psi, for 20 min.

iv. **Radiation**

   Ultra violet radiations were used for killing microbes in inoculation chamber (Laminar Air flow cabinet) and due precautions were taken while using UV radiation.

3.5. Preparation of Medium

Ingredients were first dissolved in distilled water except agar, pH was then adjusted at 7.2 followed by boiling to dissolve agar powder.

3.6. Sterilization of medium

The medium was sterilized in an autoclave at 121^{o} C 15lb/$inch^2$ for 15-20 minutes.

3.7. Dispensing of media

The sterilized medium was dispensed into previously sterilized petriplates. About 15 to 20 ml of medium was poured into each petriplate on a Laminar flow cabinet. The petriplates were incubated over night to check the contamination if any, inside the media.

3.8. Serial Dilution Technique

Since millions of microbes can be found in a single gram of soil, suspension was diluted serially so as to reduce the microbial population by few cells/ml. The sample was diluted to different levels, in order to get the approximate number and density of the fungi, the original sample was diluted upto 5 dilutions viz. (10^{-1}, 10^{-2}, 10^{-3} 10^{-4}, and 10^{-5})

3.9. Inoculation

Spread plate technique was followed for inoculation of sample by spreading (0.1ml) sample from different dilutions over the surface of prepared agar plate by means of a sterilised spreader.

3.10. Incubation

After inoculation, the culture plates were incubated in an incubator in inverted position at a temperature of 28^{o}C for 3-7 days to assess the growth of colonies. Plates containing overlapping or diffused colonies were discarded and only those plates were selected which were showing different types of distinct, individual and well separated colonies.

3.11. Enumeration of colonies

Colonies that develop on agar plates were counted with unaided eyes as per key given by Jhonson and Case (1995). The number of colonies counted was expressed as cfu/g and were calculated by using the formula.

$$\textbf{Cfu/g} = \textbf{n x d}$$

Where n= number of colonies and d = dilution factor

3.12. Isolation of fungi

i. Soil plate method

One gram of soil sample was suspended in 10 ml of double distilled water to make microbial suspensions (10^{-1} to 10^{-5}). Dilution of 10^{-3}, 10^{-4} and 10^{-5} were used to isolate fungi. 0.1 ml of microbial suspension of each concentration were added to sterile Petri dishes containing 15 ml of sterile Potato Dextrose Agar and Czapek,s Dox Agar. One percent streptomycin solution was added to the medium before pouring into petriplates for preventing bacterial growth. The Petri dishes were then incubated at 28±2 ºC in dark. The plates were observed everyday up to three days (Warcup, 1950).

ii. Soil dilution method

About 0.005g of soil was scattered on the bottom of a sterile petri dish and molten cooled (40-45°C) agar medium (PDA) was added, which was then rotated gently to disperse the soil particles in the medium. The Petri dishes were then incubated at 28 ± 2ºC in dark for three days (Waksman, 1922).

iii. Streak plate method

Streak plate technique was used to isolate a single species from a mixed population. The process involved streaking of fungi across an agar plate and was then incubated at 37 ºC. It was used to get the pure cultures.

3.13. Pure Cultures

Pure cultures were obtained by platinum loop through streaking technique (A.P.H.A, 1998). Fungi were transferred from one medium to another by sub culturing for maintaining stock cultures on potato dextrose agar medium.

3.14. Identification of fungi

Identification of fungi was performed on the basis of the micro and macro morphological features, reverse and surface coloration of colonies grown on Czapek's dox Agar (CZ), Malt Extract Agar (MEA), Czapek's Yeast Agar (CYA) and Potato Dextrose Agar (PDA) media. Fungi were identified to genus level using the keys of Barnett and Hunter (1999). Cultures were identified to species level using various mycological texts like *Penicillium* LINK, and Pitt's monograph (2000). These species were grown on various differential media all prepared according to the recipes of Pitt

(2000). Each *Penicillium* culture was inoculated in triplicate on each medium and incubated at three different temperatures (5ºC, 25ºC and 37°C) for a period of 7 days in the dark. Fungal morphology was studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto phenol cotton blue and observed under microscope for the conidia, conidiophores and arrangement of spores.

i. Lactophenol cotton blue staining

The lactophenol cotton blue (LPCB) wet mounts were prepared for the isolated species and the slides were observed under microscope to study their micro morphological features to identify them.

3.15. α-amylase activity

i. Screening of amylase producing fungi

The isolated strains of *Pencillium* were streaked into starch agar plate and incubated at room temperature for 72 hours. After incubation 1% of iodine solution was layered on the agar plates (Hols *et al.,* 1994) and zone of clearance was observed for screening the fungi (Pandey *et al*., 2006).

ii. Submerged fermentation of Amylase

Submerged fermentation was carried out in the Ehlenmeyer flasks by taking 100 ml of amylase production medium (Bernfed, 1951); containing Peptone (6.0g/L), MgSO4 (0.5g/L), KCl (0.5g/L), Starch (1g/L). In addition to this certain agricultural waste products like mustard oil cake, Linseed oil cake and wheat bran were used as a crude substrate ingredient in the submerged fermentation medium. The medium was then maintained at a pH range of 3, 6 and 9, at 30°C on a shaker with 120 rpm for 6 to 18 days (Pandey *et al*., 1999). The medium was also modified with 5 different carbon sources like glucose, sucrose, fructose, lactose and starch and with 5 different nitrogen sources like Peptone, KNO_3, NH_4Cl, $NaNO_3$ and yeast extract.

iii. Enzyme extraction

Crude enzyme was extracted by mixing a known quantity of fermented substrate with distilled water containing 0.1% tween 80 on rotator shaker at 180 rpm/1 hr. The

suspension was then centrifuged at 7000 rpm at 4°C and the supernatant was used for enzyme assay (Pandey *et al*., 2006).

iv. α- amylase assay

α-amylase activity was determined as per the method of Pandey *et al*. (1999). The reaction mixture containing 1.25 ml of 1% soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25 ml of crude enzyme extract was incubated for 10 minutes at 50°C. After incubation the reducing sugar was estimated by Dinitrosalicylic acid (DNS) method (Miller *et al*., 1959). The tubes were kept in boiling water for 5 min to develop the colour, then cooled and absorbance was read at 540 nm. A standard curve of glucose was developed under identical conditions at 575 nm to determine the reducing sugars formed. The enzymatic activity of filtrate was expressed as Units per ml (U/ml), which is defined as the amount of enzyme which liberates 1 μmol of reducing sugar per ml under the assay conditions. Simultaneously a blank without the enzyme extract was run under similar conditions.

Soil samples were collected from the study sites under sterile conditions and processed in the laboratory for isolation of fungal isolates. The soil samples were diluted by serial dilution method and 0.1 ml innoculum was inoculated onto the Potato Dextrose Agar (PDA) plates and incubated for 3-7 days in an incubator at 37º C to develop the fungal colonies. Four *Penicillium* isolates, which showed positive amylase activity were isolated from the study sites. Among all the isolates, *Penicillium chrysogenum* showed the colony forming units of 2.8×10^3 at site I in the month of June and colony forming units of 3.5×10^3 at site II in the month of August. The two sites were having a marked difference in various biotic and abiotic factors.

The identification of *Penicillium* isolates was done on the basis of the micro and macro morphological features, reverse and surface coloration of colonies grown on Czapek's dox Agar (CZ), Malt Extract Agar (MEA), Czapek's Yeast Agar (CYA) and Potato Dextrose Agar (PDA) media and the Fungal morphology was studied macroscopically by observing colony features (Colour and Texture) and microscopically by staining with lacto phenol cotton blue and observed under microscope for the conidia, conidiophores and arrangement of spores.

Pencillium species were isolated and selected for further study. All the isolates were tested for alpha amylase activity on starch agar medium through iodine test (Hols *et al.,* 1994), but only those species of *Penicillium* were selected for further study which showed apparently clear zone around the colonies on starch agar medium when subjected to iodine test (**Plate II, Fig. a&b**). Submerged fermentation (SmF) was used to investigate the effect of pH on enzyme production by these four *Penicillium* species in different substrates by adjusting the pH of basal medium to 3, 6 and 9. The substrates were then incubated for 18 days at 30^0 C in orbital shaking incubator at 120 rpm. The effect of incubation period on enzyme production was investigated by checking the enzyme activity on 6^{th}, 12^{th} and 18^{th} day of incubation in the different medias at pH 3, 6, 9. Carbon and nitrogen sources were altered and modified medium was prepared to find out the rate of production of amylase (**Plate III**). The changing effect of carbon sources on enzyme production was investigated by replacing and supplementing the medium with different carbon sources such as glucose, fructose, lactose and sucrose in place of starch in the basal medium for all the four strains. Similarly, the effect of nitrogen source on enzyme production was also studied by replacing the nitrogen source of medium, with $NaNO_3$, Yeast extract, NH_4Cl and KNO_3, incubated at room temperature for 18 days. Certain agricultural waste products like mustard oil cake, linseed oil cake and wheat bran procured from a local market here, were used as a crude substrate ingredient in the fermentation medium and the effect on amylase production was also studied. The 4 isolates which showed positive amylase activity were present at both the sites (**Plate IV, Fig. a-d**).

During the study the morphological characteristics like appearance, colour, elevation and margins were observed for *Penicillium* species and it was found that *Penicillium* species showed a varied morphology, some were circular, rhizoidal and some were filamentous in appearance. The taxonomic position of four isolated *Penicillium* species indicated that they all belong to division Ascomycota and class Trichocomaceae of kingdom Fungi (**Table 1)**, and the colony forming units (cfu/g) of *Penicillium* species obtained during the study are shown in the **Tables 2-5** and **Fig 1.** On optimising the cultural conditions for the production of alpha amylase from isolated *Penicillium* species**,** the results obtained for the alpha amylase activity and amylase production by *Penicillium chrysogenum* are shown in **Tables 6-8** and **Figs. 2-4**. Similarly the results for the alpha amylase activity and amylase production by

Penicillium purpurogenum are shown in **Tables 9-11** and **Figs. 5-7.** The results of *Penicillium caseicolum* for alpha amylase activity and amylase production are shown in the **Tables 12-14** and **Figs. 8-10** and for *Penicillium funiculosum* are shown in the **Tables 15-17** and **Figs. 11-13**, when grown with different substrates. One unit of amylase activity was defined as the amount of enzyme which released 1µmole glucose under assay conditions.

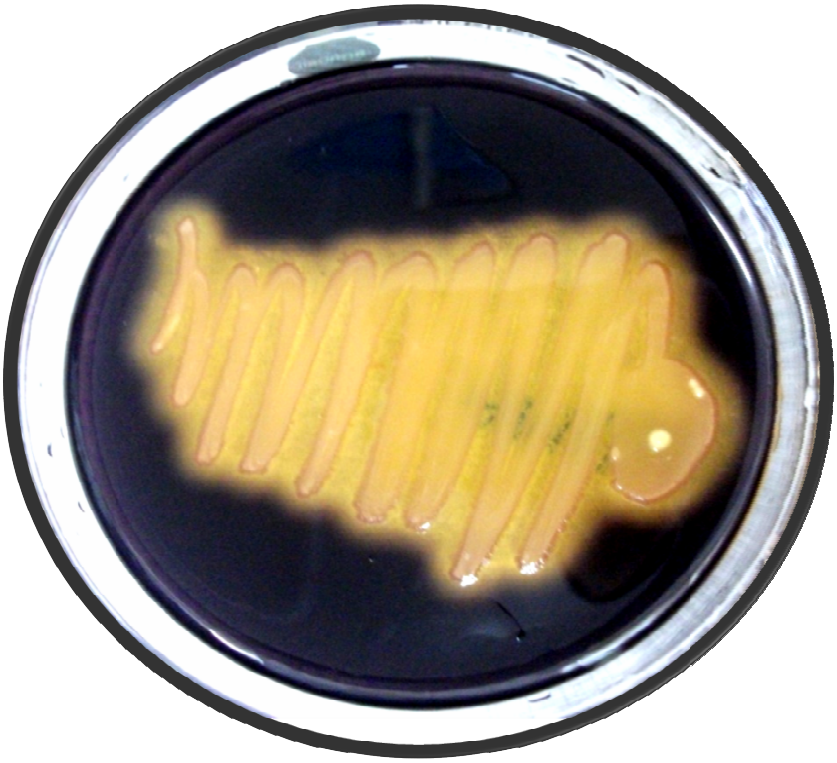


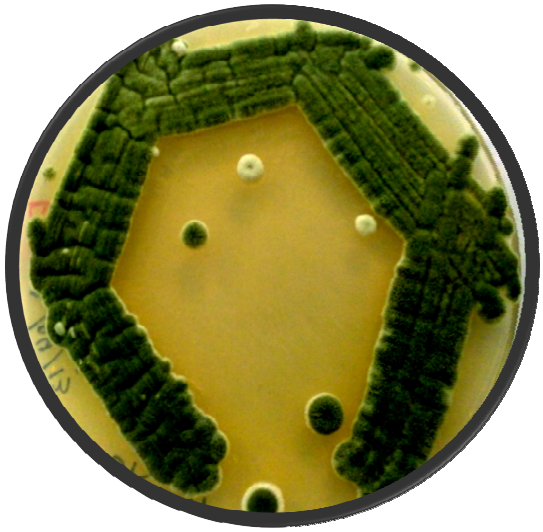
Fig. A: Positive amylase test

Fig. B: Negative amylase test

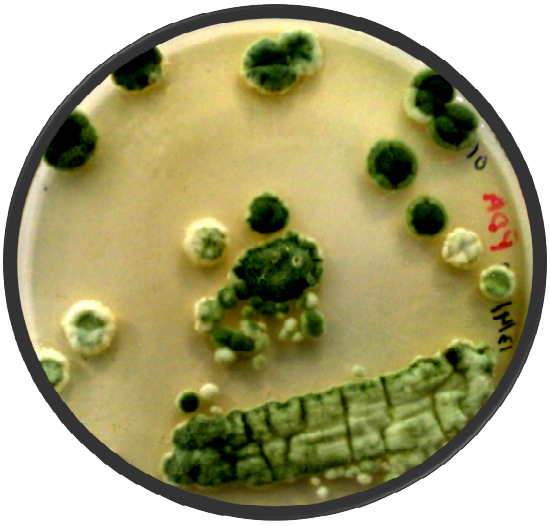
Plate II: Confirmation of amylase activity by *Penicillium* species through iodine test

Plate III:Production of alpha amylase in shaking incubator by submerged fermentation

(a) *P. caesicolum*



(b) *P. chrysogenum*



(c) *P. funiculosum*

(d) *P. purpurogenum*

Plate IV: *Penicillium* species isolated from the soil samples

Table 1: Morphological characteristics and taxonomic position of isolated *Penicillium* species

S.No.	Isolated species	Taxonomic Position				Morphological Characteristics			
		Kingdom	Division	Family	Genus	Appearance	Margin	Elevation	Colour
1.	*Penicillium chrysogenum* Thom	Fungi	Ascomycota	Trichocomaceae	*Penicillium*	Circular	Entire	Flat	Green
2.	*Penicillium purpurogenum* Stoll					Filamentous	Filamentous	Convex	White
3.	*Penicillium caseicolum* Bain					Circular	Filamentous	Convex	Cream
4.	*Penicillium funiculosum* Thom					Rhizoidal	Filamentous	Flat	Green

Table 2: Colony count of isolated *Penicillium* species at Site I

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	0	10	**28**	12	17	9
2.	*P. purpurogenum*	0	12	21	16	12	0
3.	*P. caseicolum*	13	0	18	19	0	5
4.	*P. funiculosum*	14	15	16	17	3	6

Table 3: Colony forming units (cfu/g) of isolated *Penicillium* species at Site I

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	0	1×10^3	**2.8×10^3**	1.2×10^3	1.7×10^3	0.9×10^3
2.	*P. purpurogenum*	0	1.2×10^3	2.1×10^3	1.6×10^3	1.2×10^3	0
3.	*P. caseicolum*	1.3×10^3	0	1.8×10^3	1.9×10^3	0	0.5×10^3
4.	*P. funiculosum*	1.4×10^3	1.5×10^3	1.6×10^3	1.7×10^3	0.3×10^3	0.6×10^3

.

Table 4: Colony count of isolated *Penicillium* species at Site II

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	12	15	23	**35**	13	7
2.	*P. purpurogenum*	9	22	28	30	17	10
3.	*P. caseicolum*	8	30	34	19	13	6
4.	*P. funiculosum*	13	24	27	29	19	14

Table 5: Colony forming units (cfu/g) of isolated *Penicillium* species at Site II

S. No.	Isolated species	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1.	*P. chrysogenum*	1.2×10^3	1.5×10^3	2.3×10^3	**3.5×10^3**	1.3×10^3	0.7×10^3
2.	*P. purpurogenum*	0.9×10^3	2.2×10^3	2.8×10^3	3.0×10^3	1.7×10^3	1.0×10^3
3.	*P. caseicolum*	0.8×10^3	3.0×10^3	3.4×10^3	1.9×10^3	1.3×10^3	0.6×10^3
4.	*P. funiculosum*	1.3×10^3	2.4×10^3	2.7×10^3	2.9×10^3	1.9×10^3	1.4×10^3

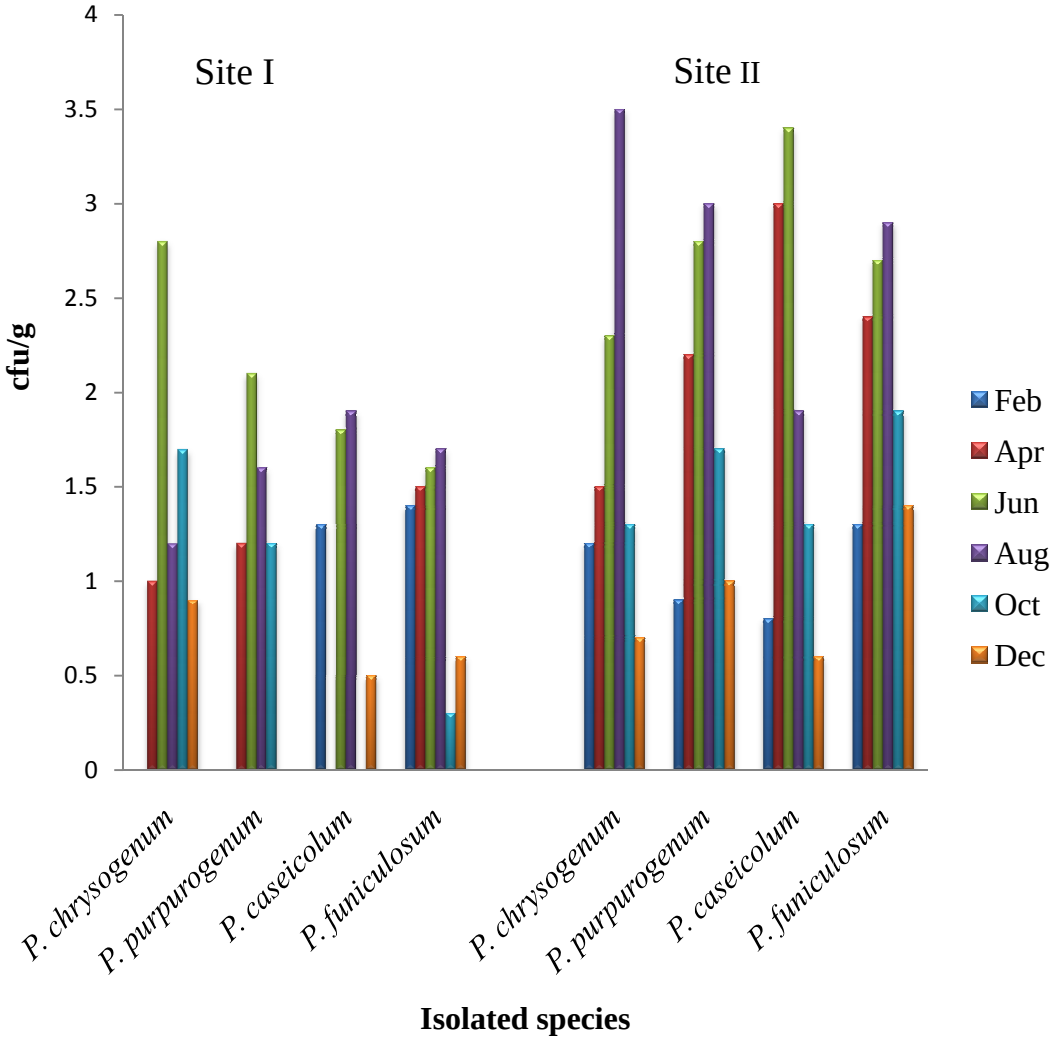


Figure 1: Comparison of colony forming units (cfu/gm) of *Penicillium* species at different sites

The highest colony forming units (cfu/g) was observed for *P. chrysogenum* in the month of August at site II while as no colony forming unit (cfu/g) was recorded in the months of Feb for *P. chrysogenum* and *P. purpurogenum* where as *P. caseicolum* and *P. funiculosum* showed no cfu/g in the months of Dec, Apr and Oct. at site I respectively, but all these species were present at site II. A comparison graph shown in Fig. 1 indicates that the colony forming unit (cfu/g) was highest at site II which was a fallow land area.

During the study the cultural conditions were optimised for production of alpha amylase from *P. chrysogenum* in fermented medium with different agricultural wastes as crude substrate in it. It was found that at pH 6 and incubation period of 6 days *P. chrysogenum* has shown the highest activity of 550.4 U/ml with linseed oil cake in the medium while as lowest activity of 19.19 U/ml was observed for it in the medium with mustard oil cake at pH 9, incubation period of 12 days at pH 3 and incubation period of 12 days with linseed oil cake (Table 6 and Fig. 2). Similarly on altering the carbon source in the basal medium by replacing the starch as shown in table 7 and Fig. 3, it was found that at pH 6, incubation period of 6 days *P. chrysogenum* showed the highest amylase activity of 398.0 U/ml with glucose as carbon source in the medium followed by 354 U/ml at pH 9 and incubation period of 6 days with same carbon source.

Table 6: Amylase production (U/ml) by *P. chrysogenum* on different fermentation medium at variable pH

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Mustard oil Cake	6	37.05 ± 1.26	188.8 ± 3.26	327.2 ± 3.70
		12	45.98 ± 1.64	68.30 ± 1.66	19.19 ± 0.82
		18	32.58 ± 1.21	108.4 ± 2.53	220.0 ± 2.43
2	Linseed oil cake	6	162.0 ± 3.83	**550.4 ± 4.46**	5.80 ± 0.21
		12	19.19 ± 0.89	126.3 ± 2.67	255.8 ± 3.75
		18	184.3 ± 3.42	224.5 ± 3.26	470.0 ± 4.45
3	Wheat bran	6	54.91 ± 1.38	282.5 ± 4.26	63.83 ± 1.23
		12	68.30 ± 1.66	81.69 ± 1.86	505.8 ± 5.24
		18	81.69±1.57	188.8±3.35	162.0±2.65

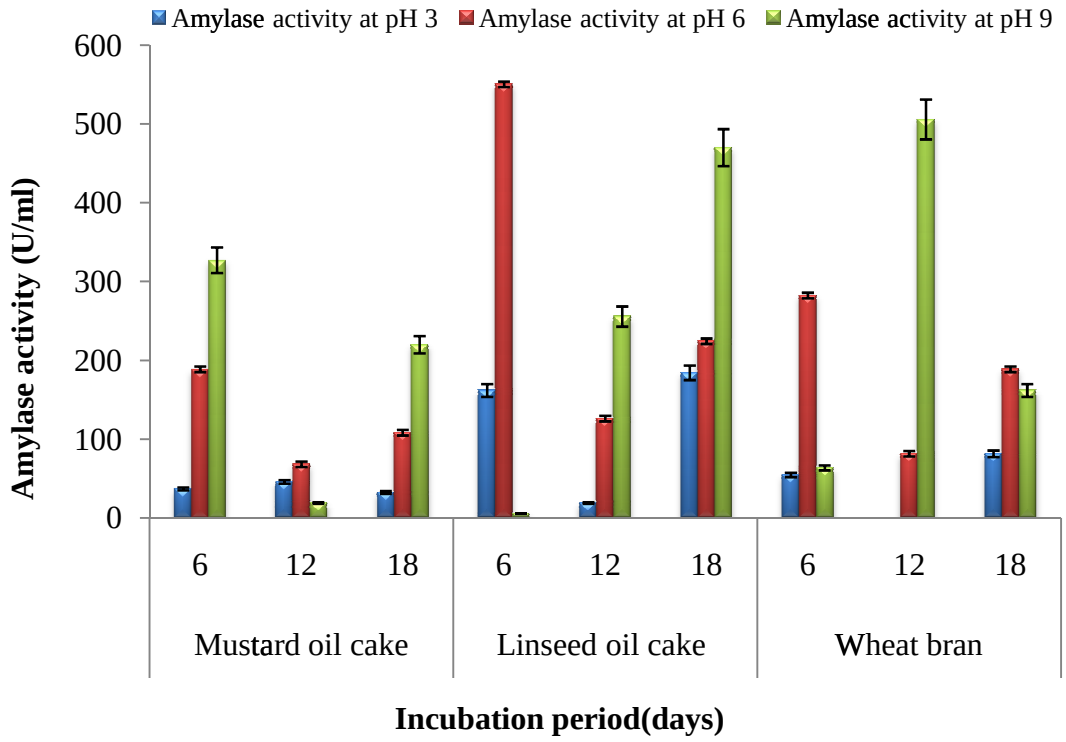


Figure 2: Comparison of amylase activity of *P. chrysogenum* in different fermentation mediums

Table 7: Amylase production (U/ml) by *P. chrysogenum* at variable pH and carbon source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Sucrose	6	32.58 ± 2.06	90.62 ± 4.84	206.6 ± 5.08
		12	255.8 ± 3.02	291.0 ± 4.02	309.0 ± 5.19
		18	99.55 ± 1.08	153.0 ± 2.24	5.80 ± 2.96
2	Fructose	6	90.62 ± 2.20	104.0 ± 3.41	278.1 ± 2.35
		12	166.0 ± 3.30	144.0 ± 2.65	202.0 ± 5.32
		18	41.51 ± 2.56	23.66 ± 2.44	130.8 ± 1.06
3	Glucose	6	19.19 ± 1.85	**398.0 ± 5.29**	354.0 ± 4.26
		12	242.0 ± 1.36	278.0 ± 4.25	162.0 ± 3.25
		18	5.80 ± 2.09	121.0 ± 1.10	14.73 ± 4.23
4	Lactose	6	41.51 ± 2.07	90.62 ± 1.25	121.0 ± 4.36
		12	224.0 ± 2.58	179.0 ± 2.22	278.0 ± 1.03
		18	19.19 ± 3.30	6.38 ± 3.89	19.19 ± 1.32
5	Starch	6	86.16 ± 4.01	112.9 ± 2.09	202.2 ± 2.03
		12	295.0 ± 1.22	166.0 ± 1.02	162.0 ± 4.32
		18	41.50 ± 1.32	95.0 ± 2.88	86.16 ± 3.32

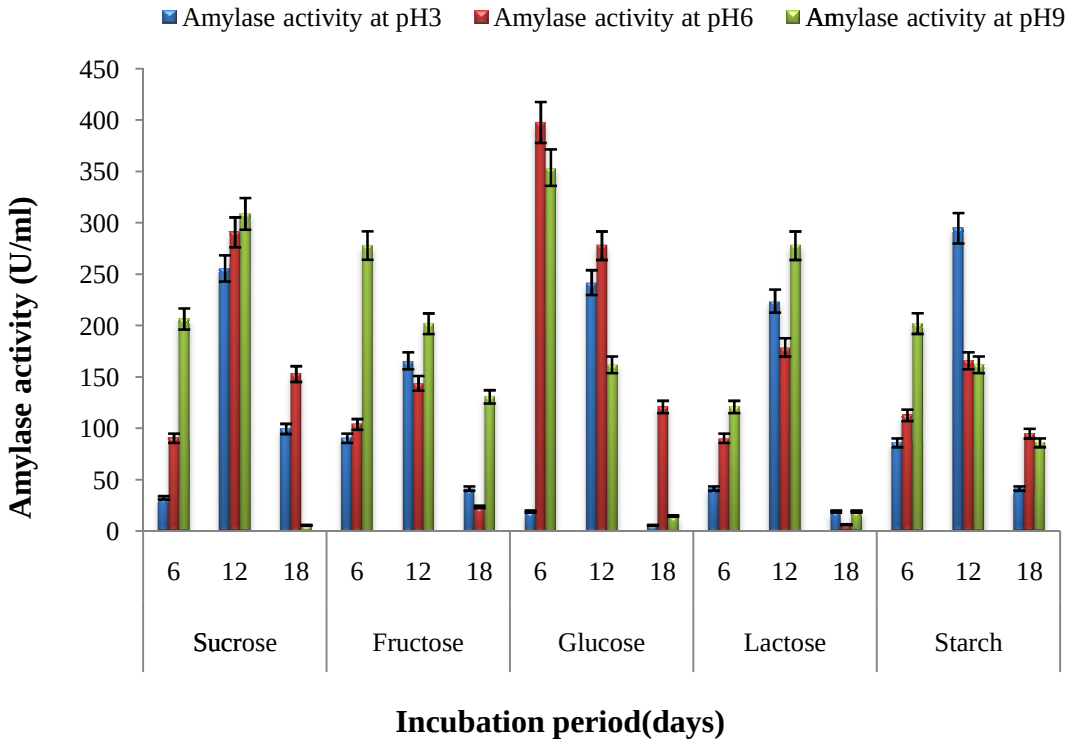


Figure 3: Comparison of amylase activity of *P. chrysogenum* at variable pH and carbon source

On altering the nitrogen source in the basal medium by replacing the peptone in the medium as shown in table 8 and Fig. 4, it was found that from amongst the different mediums prepared, basal medium at pH 6 and incubation period of 12 days *P. chrysogenum* showed the highest activity of 183.3U/ml with peptone as nitrogen source in the medium followed by 144.1U/ml at pH 9 and incubation period of 18 days with potassium nitrate as nitrogen source in the medium.

Table 8: Amylase production (U/ml) by *P. chrysogenum* at variable pH and nitrogen source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	$NaNO_3$	6	86.16 ± 4.00	77.23 ± 2.11	32.58 ± 4.89
		12	19.19 ± 3.53	77.23 ± 3.02	10.26 ± 4.26
		18	10.26 ± 2.32	90.62 ± 3.63	41.51 ± 4.32
2	KNO_3	6	54.91 ± 1.23	90.62 ± 2.65	10.26 ± 3.26
		12	77.23 ± 2.45	10.26 ± 5.32	95.08 ±5.09
		18	50.44 ± 2.12	32.58 ± 4.02	144.1 ± 1.07
3	Peptone	6	1.33 ± 3.22	99.55 ± 2.36	10.26 ± 4.10
		12	32.58 ± 2.14	**184.3 ± 1.21**	14.73 ± 4.23
		18	99.55 ± 5.23	5.80 ± 2.63	77.23 ± 3.20
4	Yeast Extract	6	81.69 ± 4.34	10.26 ± 2.06	37.05 ± 5.23
		12	32.58 ± 1.32	58.03 ± 1.02	92.27 ± 4.01
		18	126.3 ± 3.02	10.26 ±1.11	14.73 ± 2.25
5	NH_4Cl	6	5.80 ± 2.36	14.73 ± 5.36	68.30 ± 2.03
		12	72.76 ± 1.02	45.98 ± 4.21	77.23 ± 1.06
		18	14.73 ± 4.07	95.08 ± 1.09	10.26 ±2.14

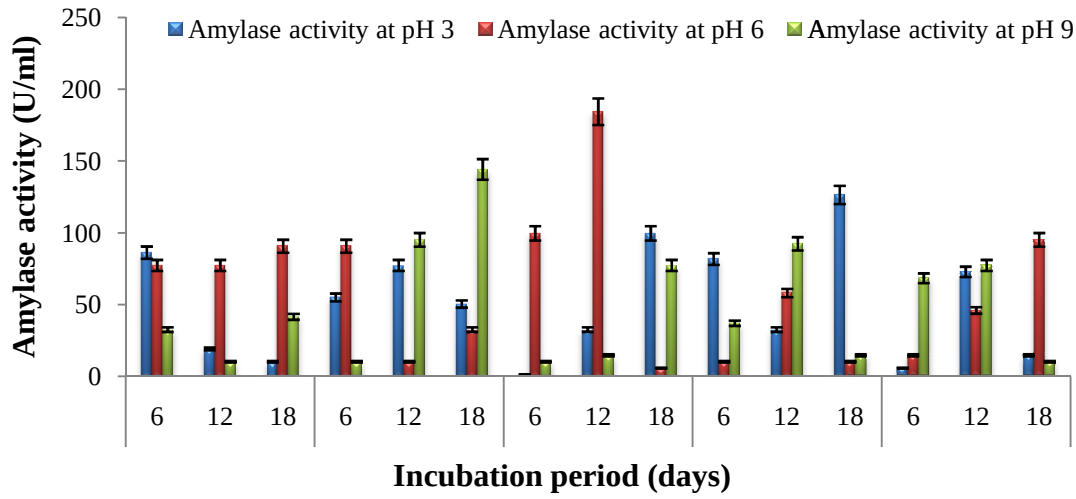


Figure 4: Comparison of amylase activity of *P. chrysogenum* at variable pH and nitrogen source

During the study, *P. purpurogenum* also showed positive amylase activity through iodine test, this was also grown in different fermented medias in order to optimise the cultural conditions for alpha amylase production from it. It was grown in mediums at three different pH's 3, 6 and 9 and the activity was monitored at three different incubation periods for 18 days on 6th, 12th and 18th day of incubation. On growing it in medium fermented with different agricultural wastes as crude substrate, it has shown a highest activity of 883.93 U/ml at pH 6 and incubation period of 6 days with mustard oil cake as crude substrate in the medium as shown in table 9 and Fig. 5 followed by 776.14 U/ml at pH 9 and incubation period of 18 days.

Table 9: Amylase production (U/ml) by *P. purpurogenum* on different fermentation medium at variable pH

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Mustard oil Cake	6	388.39 ± 4.04	**883.93 ± 5.13**	316.96 ± 5.03
		12	187.50 ± 3.23	13.39 ± 4.86	803.57 ± 5.06
		18	40.18 ± 3.20	102.68 ± 3.88	125.00 ± 2.03
2	Linseed oil cake	6	66.96 ± 1.023	348.21 ± 2.06	276.78 ± 3.25
		12	200.89 ± 1.04	508.93 ± 2.36	607.14 ± 4.28
		18	169.64 ± 2.69	406.25 ± 1.08	776.78 ± 3.36
3	Wheat bran	6	35.71 ± 2.35	89.28 ± 3.03	281.25 ± 4.55
		12	214.28 ± 3.56	80.35 ± 2.36	40.18 ± 2.11
		18	147.32 ± 5.02	120.53 ± 3.23	102.68 ± 1.02

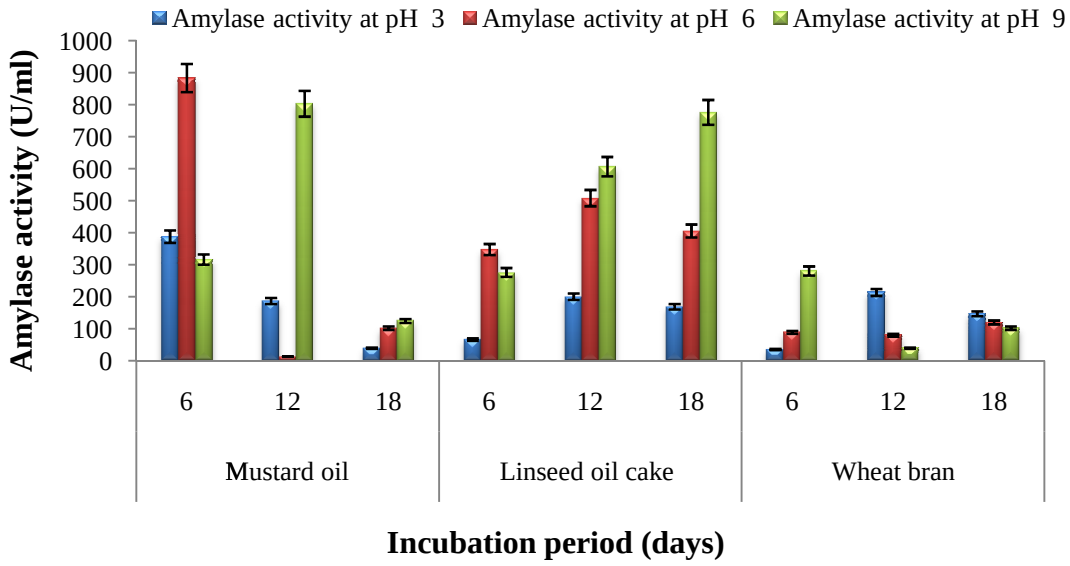


Figure 5: Comparison of amylase activity of *P. purpurogenum* in different fermentation mediums

The effect of different carbon sources on this strain as shown in table 10 and Fig. 6 showed that at pH 9 and incubation period of 6 days, it has shown the highest activity of 366.0 U/ml with glucose as carbon source in the medium followed by 330.3 U/ml at pH 6 and incubation period of 12 days. Similarly, it has been observed, by altering its medium with different nitrogen sources as shown in table 11 and Fig. 6, it was found that at pH 9 and incubation period of 6 days, highest activity of 174.7 U/ml was exhibited by this strain with yeast extract as nitrogen source in the medium followed by 169.6 U/ml at pH 3 and incubation period of 6 days with potassium nitrate as nitrogen source.

Table 10: Amylase production (U/ml) by *P. purpurogenum* at variable pH and carbon source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Sucrose	6	53.57 ± 2.57	75.89 ± 3.89	227.6 ± 5.22
		12	236.60 ± 2.56	285.7 ± 4.48	316.9 ± 4.32
		18	22.32 ± 3.02	8.920 ± 2.21	35.71 ± 2.36
2	Fructose	6	80.35 ± 4.03	93.75 ± 3.20	303.5 ± 3.23
		12	191.96 ± 5.23	125.0 ± 2.20	250.0 ± 1.50
		18	4.464 ± 1.14	40.17 ± 3.21	80.35 ± 4.15
3	Glucose	6	35.71 ± 2.21	330.3 ± 5.25	**366.0 ± 4.02**
		12	281.2 ± 3.22	330.3 ± 5.25	312.5 ± 3.01
		18	102.6 ± 1.23	125.0 ± 2.07	156.2 ± 2.32
4	Lactose	6	49.10 ± 2.01	107.1 ± 1.18	120.5 ± 3.32
		12	223.2 ± 5.21	183.0 ± 3.24	272.3 ± 1.32
		18	31.25 ± 4.23	49.10 ± 2.01	66.96 ± 5.02
5	Starch	6	93.75 ± 2.32	125.0 ± 2.07	205.3 ± 4.02
		12	303.5 ± 1.15	272.3 ± 2.36	294.6 ± 1.06
		18	40.17 ± 1.03	26.78 ± 3.47	53.51 ± 2.22

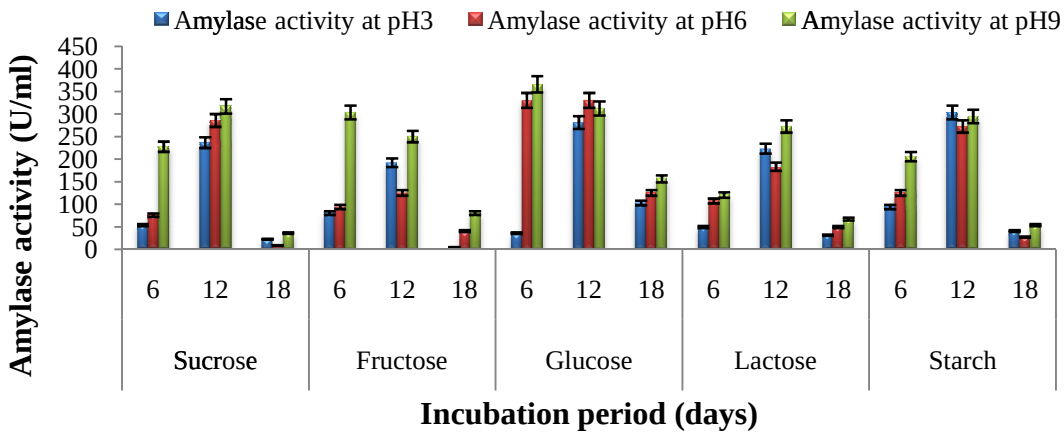


Figure 6: Comparison of amylase activity of *P. purpurogenum* at variable pH and carbon source

Table 11: Amylase production (U/ml) by *P. purpurogenum* at variable pH and nitrogen source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	$NaNO_3$	6	13.39 ± 1.32	71.42 ± 1.71	80.35 ± 2.68
		12	66.96 ± 2.10	31.25 ± 1.80	102.67 ± 1.12
		18	102.6 ± 3.20	22.32 ± 1.48	26.78 ± 1.55
2	KNO_3	6	169.6 ± 3.23	98.21 ± 1.55	44.64 ± 1.62
		12	71.42 ± 1.52	49.10 ± 1.49	58.03 ± 1.32
		18	125.0 ± 1.51	151.7 ± 2.54	102.67 ± 1.18
3	Peptone	6	17.85 ± 1.39	71.42 ± 1.55	53.57 ± 1.35
		12	75.89 ± 1.46	84.82 ± 1.74	98.21 ± 2.30
		18	116.0 ± 3.33	35.71 ± 1.52	151.6 ± 3.21
4	Yeast Extract	6	58.03 ± 1.65	66.96 ± 2.13	**174.7 ± 4.08**
		12	111.6 ± 2.98	40.17 ± 1.25	26.78 ± 1.85
		18	13.39 ± 1.32	89.28 ± 1.02	133.9 ± 1.32
5	NH_4Cl	6	26.78 ± 1.63	80.35 ± 1.42	102.6 ± 1.06
		12	93.75 ± 1.85	71.42 ±1.70	151.7 ± 1.07
		18	17.85 ± 1.42	98.21 ±1.55	89.28 ± 1.65

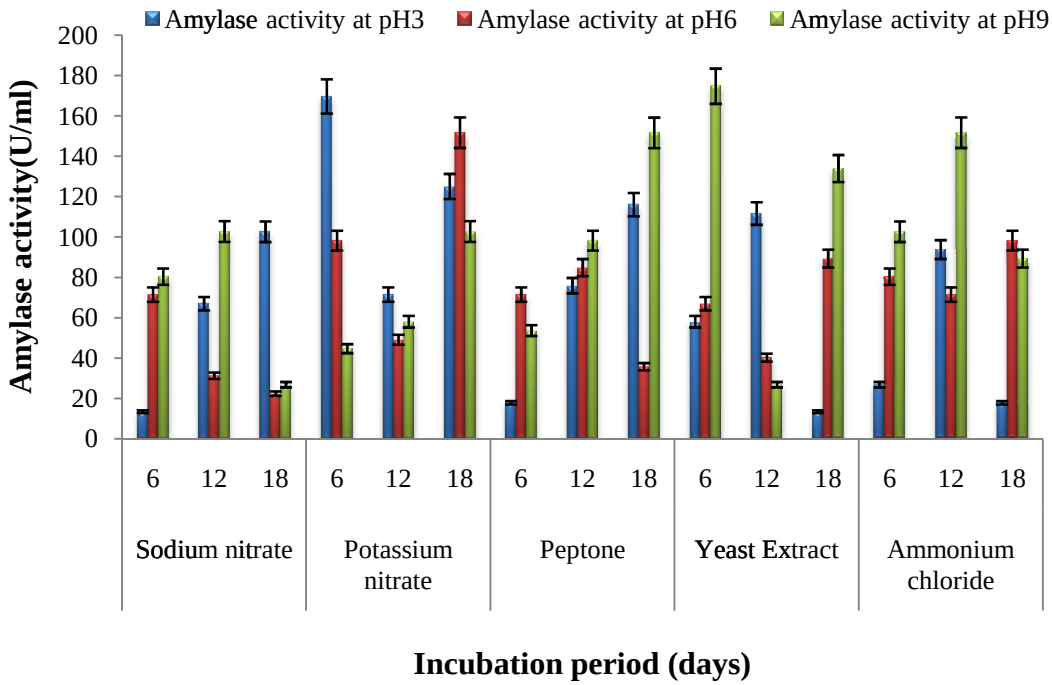


Figure 7: Comparison of amylase activity of *P. purpurogenum* at variable pH and nitrogen source

P. caseicolum showed the highest activity of 397.3 U/ml at pH 6 and incubation period of 18 days when grown with wheat bran as crude substrate in the medium as shown in table 12 and Fig. 8, followed by 366.0 U/ml at pH 3 and incubation period of 12 days with the same crude substrate in the medium. The changing effect of carbon source in the medium as shown in table 13 and Fig. 9, indicates that the glucose in the medium at pH 9 and incubation period of 18 days showed the highest alpha amylase activity of 1834.8 U/ml followed by 1776.7 U/ml at pH 6 and 9 with incubation period of 12 days in the same medium and the effect of nitrogen source on the strain is shown in the table 14 and Fig. 10, indicates that the highest amylase activity of 709.82 U/ml was exhibited by this strain in medium with yeast extract as nitrogen source at pH 9 and incubation period of 12 days followed by the activity of 705.3 U/ml at pH 9 and incubation period of 18 days with the same nitrogen source.

Table 12: Amylase production (U/ml) by *P. caseicolum* on different fermentation medium at variable pH

S. No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Mustard oil Cake	6	169.6 ± 1.31	187.5 ± 1.95	245.5 ± 2.25
		12	147.3 ± 1.23	334.8 ± 2.31	330.3 ± 1.25
		18	272.3 ± 2.60	357.1 ± 3.12	218.7 ±4.02
2	Linseed oil cake	6	13.39 ± 1.23	58.03 ± 1.02	147.3 ±3.78
		12	80.35 ± 1.62	303.5 ± 2.10	312.5 ±4.32
		18	187.5 ± 1.65	214.2 ± 2.26	227.6 ± 2.04
3	Wheat bran	6	227.6 ± 3.23	178.5 ± 2.12	196.4 ± 1.14
		12	366.0 ± 3.06	258.9 ± 2.36	312.5 ± 4.37
		18	272.3 ± 2.65	**397.3 ± 4.03**	303.5 ± 1.78

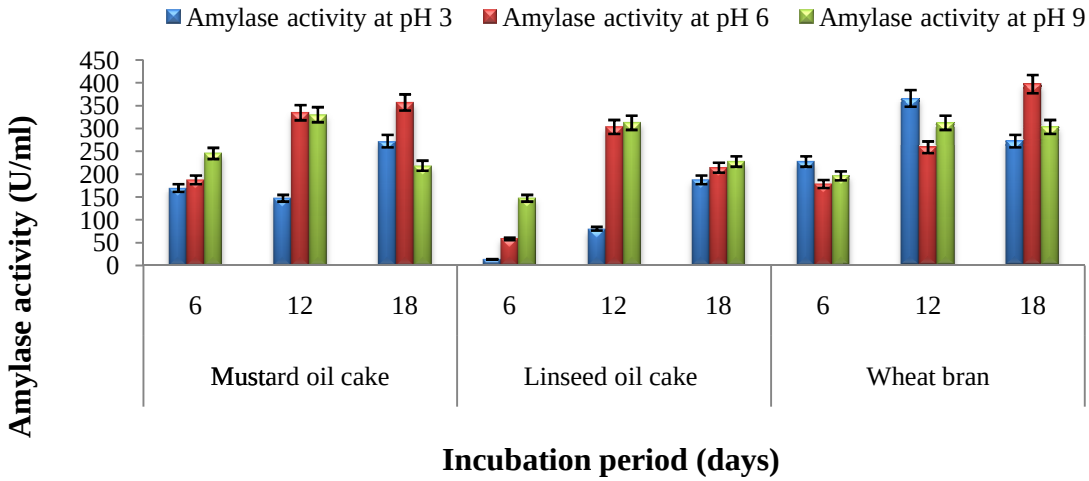


Figure 8: Comparison of amylase activity of *P. caseicolum* in different fermentation mediums

Table 13: Amylase production (U/ml) by *P. caseicolum* at variable pH and carbon source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Sucrose	6	727.6 ±2.35	950.8 ±2.16	803.5 ±2.51
		12	1031.2 ±3.39	1089.2 ± 3.36	1089.2 ± 1.88
		18	1080.0 ± 3.40	1165.1 ± 3.01	1165.1 ± 6.04
2	Fructose	6	776.7 ± 2.21	875.0 ± 4.06	1084.8 ± 3.21
		12	897.3 ± 2.23	1107.1 ± 1.22	1107.1 ± 4.25
		18	1017.8 ± 4.10	1196.4 ± 3.40	1196.4 ± 2.09
3	Glucose	6	1223.2 ± 3.32	1575.8 ± 2.18	1714.2 ± 5.02
		12	1477.6 ± 3.35	1776.7 ± 1.77	1776.7 ± 3.07
		18	1656.2 ± 3.39	1669.6 ± 1.39	**1834.8 ± 3.06**
4	Lactose	6	705.3 ± 2.36	794.6 ± 2.62	861.61 ± 5.33
		12	727.6 ± 2.89	1133.9 ± 3.25	1133.9 ± 1.35
		18	794.6 ± 3.20	950.8 ± 5.02	1196.4 ± 4.21
5	Starch	6	750.0 ± 3.21	772.3 ± 3.02	808.04 ± 2.36
		12	616.0 ± 4.02	660.7 ± 4.03	776.79 ± 1.02
		18	767.8 ± 4.65	772.3 ± 4.07	861.61 ± 1.06

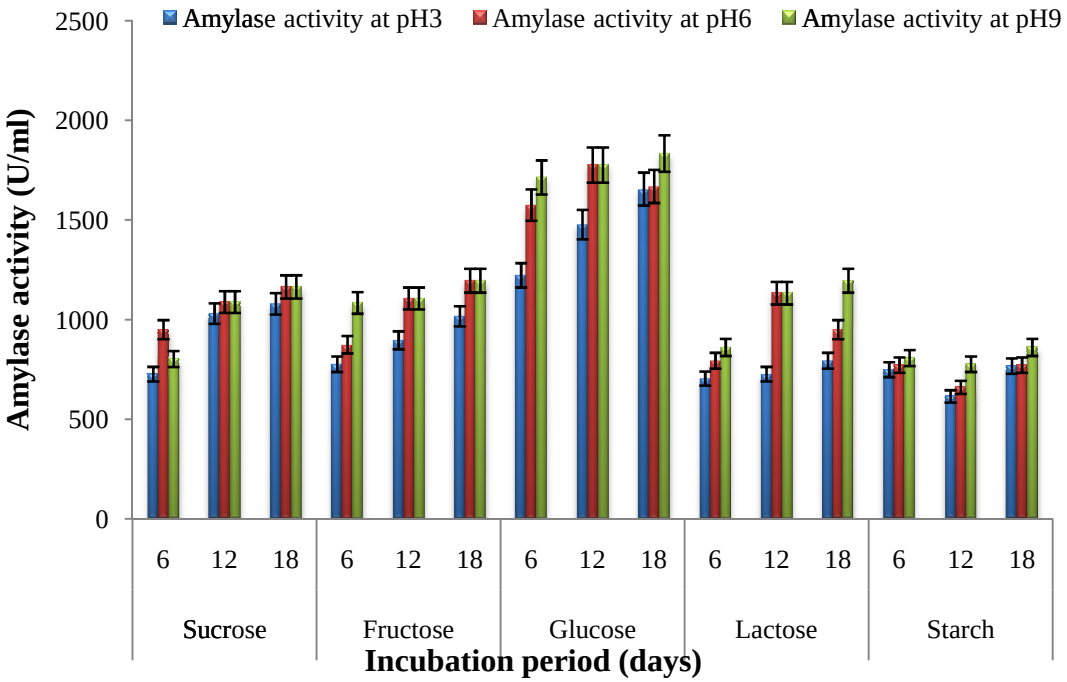


Figure 9: Comparison of amylase activity of *P. caseicolum* at variable pH and carbon source

Table 14: Amylase production (U/ml) by *P. caseicolum* at variable pH and nitrogen source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	$NaNO_3$	6	31.25 ± 2.74	80.36 ± 2.68	53.57 ± 3.37
		12	49.11 ± 2.82	53.57 ± 5.23	84.82 ± 4.24
		18	80.36 ± 3.65	13.39 ± 2.02	49.11 ± 2.20
2	KNO_3	6	80.36 ± 3.65	98.21 ± 3.21	169.6 ± 3.23
		12	62.50 ± 3.25	75.89 ± 1.01	138.3 ± 1.11
		18	8.93 ± 3.33	58.04 ± 2.01	98.21 ± 1.15
3	Peptone	6	49.11 ± 2.52	13.39 ±4.55	35.71 ± 2.32
		12	13.39 ± 2.01	272.32 ± 3.64	80.36 ± 3.35
		18	53.57 ± 3.21	125.00 ± 1.06	187.5 ± 6.60
4	Yeast Extract	6	258.9 ± 2.36	316.96 ± 2.36	415.1 ± 2.30
		12	290.18 ± 2.65	450.89 ± 4.60	**709.82 ± 5.30**
		18	321.4 ± 4.08	500.00 ± 3.21	705.3 ± 1.20
5	NH_4Cl	6	22.32 ± 3.09	44.64 ± 1.63	40.18 ± 1.85
		12	8.930 ± 4.02	40.18 ± 2.30	35.71 ± 1.69
		18	53.57 ± 1.90	276.7 ± 4.01	205.3 ± 1.65

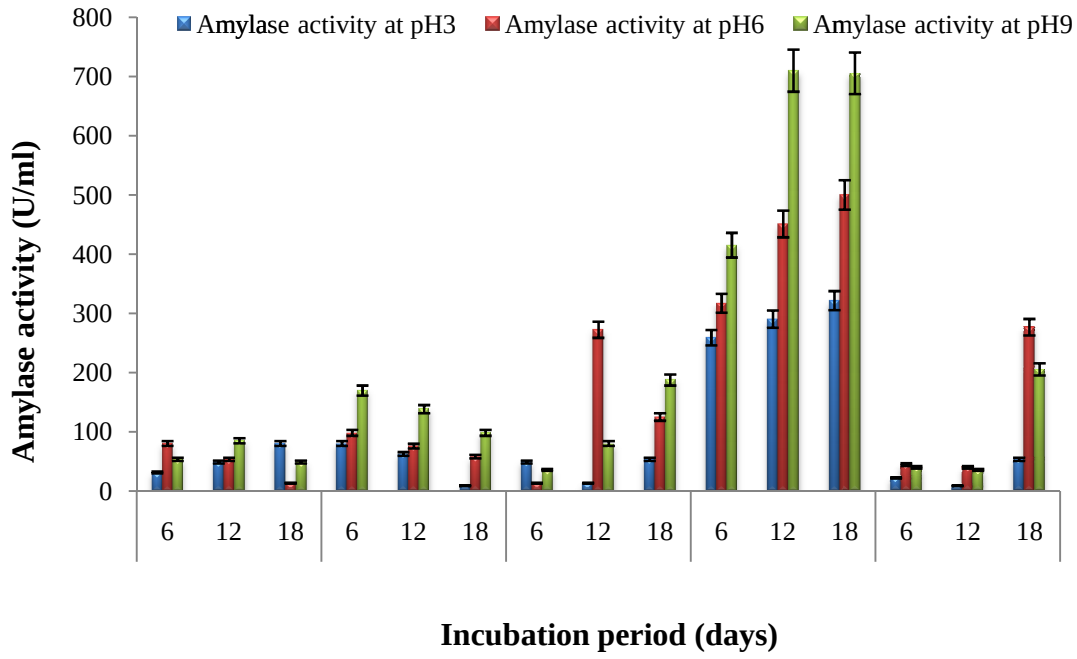


Figure 10: Comparison of amylase activity of *P. caseicolum* at variable pH and nitrogen source

Penicillium funiculosum, like other three species of *Penicillium* isolated during the study also exhibited alpha amylase activity on starch agar medium through iodine test. On optimising the cultural conditions for the production of alpha amylase from this strain by growing it in different fermented mediums, it was found that at pH 6 and incubation period of 18 days, alpha amylase activity of 4254.4 U/ml was observed with mustard oil cake as crude substrate in the medium followed by 4209.8 U/ml at pH 9 and incubation period of 6 days with wheat bran as crude substrate in the medium as shown in table 15 and Fig. 11. Glucose as a carbon source was found to be best for the alpha amylase activity by this strain among other sources as shown in table 16 and Fig. 12, at pH 9 and incubation period of 18 days, highest alpha amylase activity of 477.6 U/ml was recorded followed by 450.8 U/ml at pH 6 and incubation period of 18 days in medium with same carbon source. The nitrogen source which promoted alpha amylase production for this strain was yeast extract as shown in table 17 and Fig. 13 and it was found that at pH 9 and incubation period of 18 days, activity of 330.36 U/ml was recorded for the strain followed by the amylase activity of 308.0 U/ml at pH 6 and incubation period of 18 days.

Table 15: Amylase production (U/ml) by *P. funiculosum* on different fermentation medium at variable pH

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Mustard oil Cake	6	316.9 ± 3.44	816.9 ±4.03	526.7 ± 5.19
		12	348.21 ± 4.22	147.3 ±4.23	187.50 ± 3.89
		18	3361.6 ± 3.21	**4254.4 ± 4.32**	348.2 ± 3.21
2	Linseed oil cake	6	80.36 ± 2.02	35.71 ± 2.31	178.5 ± 3.02
		12	843.7 ± 3.14	933.0 ± 3.21	754.46 ± 1.02
		18	2781.2 ±1.02	3674.1 ± 1.17	3808.0 ± 2.02
3	Wheat bran	6	31.25 ± 1.32	357.14 ± 1.06	4209.8 ± 1.21
		12	1727.6 ± 2.42	549.11 ± 1.88	459.82 ± 4.20
		18	776.79 ± 4.07	325.8 ± 1.96	4165.1 ± 4.09

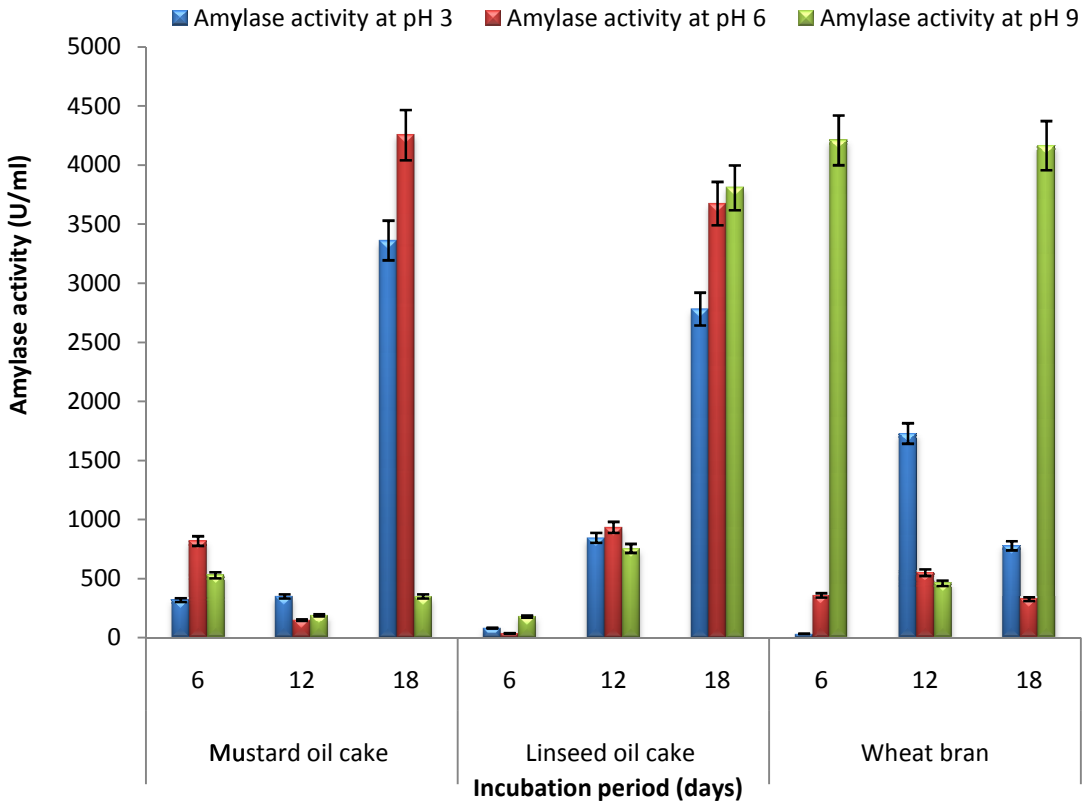


Figure 11: Comparison of amylase activity of *P. funiculosum* in different fermentation mediums

Table 16: Amylase production (U/ml) by *P. funiculosum* at variable pH and carbon source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	Sucrose	6	35.71 ±1.19	58.04 ±1.51	71.43 ±1.08
		12	80.36 ±3.39	4.46 ± 4.32	26.79 ± 2.21
		18	49.11 ± 2.24	31.25 ±5.31	17.86 ± 4.38
2	Fructose	6	80.36 ± 4.36	102.6 ± 4.02	116.0 ± 4.68
		12	93.75 ± 5.24	35.71 ± 1.22	66.96 ± 3.12
		18	102.6 ± 1.25	120.5 ± 1.32	151.7 ± 2.03
3	Glucose	6	370.5 ± 3.31	406.2 ± 5.32	428.5 ± 1.32
		12	383.9 ± 3.65	415.1 ± 4.03	397.3 ± 2.33
		18	437.5 ± 4.25	450.8 ± 1.01	**477.6 ± 4.37**
4	Lactose	6	13.39 ± 4.89	13.39 ± 1.08	35.71 ± 4.35
		12	31.25 ± 3.39	22.32 ± 2.36	44.64 ± 1.36
		18	26.78 ± 6.31	71.43 ± 3.56	13.39 ± 5.64
5	Starch	6	227.6 ± 1.35	281.2 ± 2.54	316.9 ± 6.36
		12	236.6 ± 2.69	267.8 ± 2.65	308.0 ± 1.04
		18	316.9 ± 2.12	339.2 ± 4.35	366.0 ± 2.33

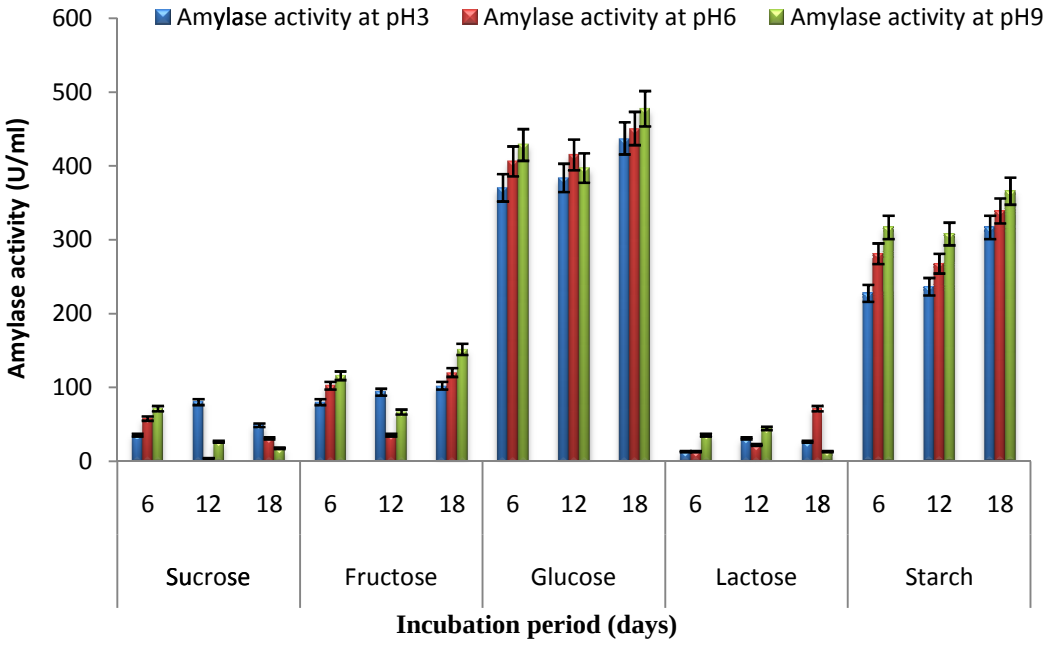


Figure 12: Comparison of amylase activity of *P. funiculosum* at variable pH and carbon source

Table 17: Amylase production (U/ml) by *P. funiculosum* at variable pH and nitrogen source

S.No.	Medium used	Incubation period (Days)	Amylase activity (U/ml) at variable pH		
			pH 3	pH 6	pH 9
1	$NaNO_3$	6	17.86 ± 1.09	40.18 ±3.33	26.79 ± 2.01
		12	84.82 ± 1.03	8.93 ± 2.10	35.71 ± 2.36
		18	138.4 ± 3.21	71.43 ± 2.36	111.6 ± 1.21
2	KNO_3	6	66.96 ± 2.32	53.57 ± 1.32	49.11 ± 4.96
		12	129.4 ± 3.25	80.36 ± 4.04	22.32 ± 5.36
		18	187.5 ± 4.38	102.7 ± 1.30	49.11 ± 3.33
3	Peptone	6	26.79 ± 5.56	31.25 ± 5.21	80.36 ± 4.01
		12	80.36 ± 1.94	22.32 ± 1.21	62.50 ± 2.31
		18	147.3 ± 2.36	214.3 ± 3.34	31.25 ± 5.32
4	Yeast Extract	6	178.5 ± 1.04	218.8 ± 3.98	272.3 ± 1.11
		12	209.8 ± 2.36	276.7 ± 4.25	294.6 ± 1.021
		18	205.3 ± 1.35	308.0 ± 1.44	**330.36 ± 2.44**
5	NH_4Cl	6	142.8 ± 5.36	62.50 ± 4.12	116.0 ± 1.36
		12	40.18 ± 4.36	44.64 ± 2.55	26.79 ± 2.82
		18	125.0 ± 5.32	31.25 ± 3.25	89.29 ± 3.73

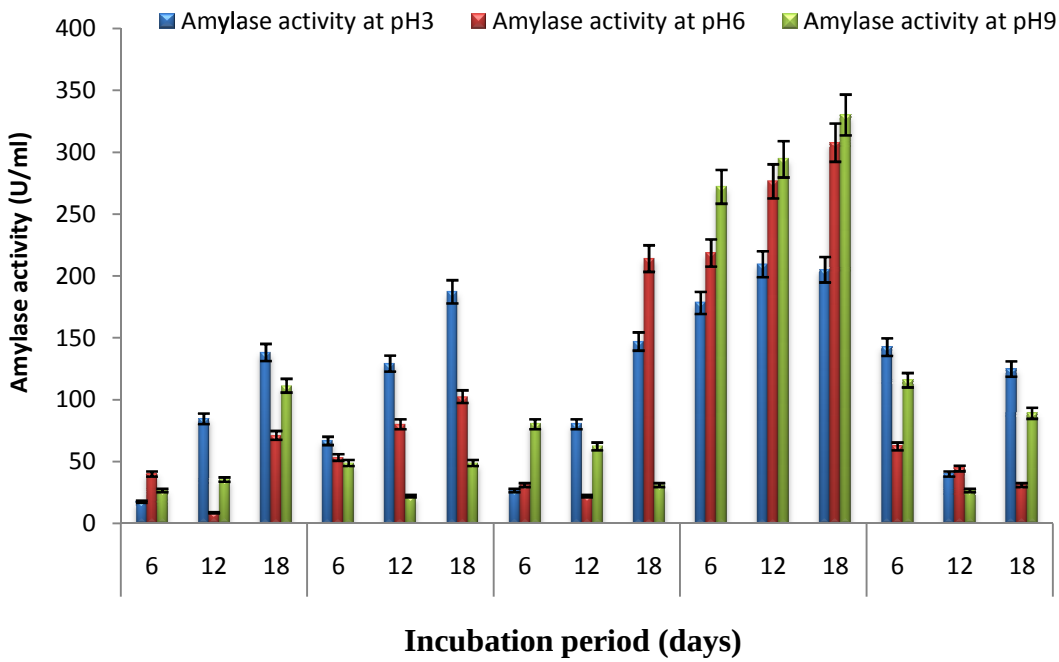


Figure 13: Comparison of amylase activity of *P. funiculosum* at variable pH and nitrogen source

Fungal cultures were isolated from soil sample by serial dilution on Potato Dextrose Agar medium (PDA). Four cultures of *Penicillium* species isolated from soil at the two sites showed positive amylase activity. All the *Penicillium* isolates were tested for positive amylase production by starch hydrolysis through iodine test. When starch agar medium was inoculated with the organism and subsequently flooded with iodine solution, the zone of clearance around the microbial growth indicated the production of amylase. On the basis of the area of clearance, all the four *Penicillium* isolates were selected for further studies on amylase production.

The monthly population (cfu/gm) was found highest for *P. chrysogenum* as 2.8×10^3 and 3.5×10^3 in the months of June and August respectively at the two sites under consideration and the diversity of *Penicillium* species was highest at Site II. This may be attributed to the difference in various biotic and abiotic factors like pH and temperature that have been found to influence the composition and diversity of soil microbial communities and the results obtained are in consonance with the findings of Piao *et al.,* 2000; Fierer and Jackson, 2006, who worked on various biotic and abiotic factors influencing the microbial communities. Since the temperature is found to be highest in the months of June and August in Kashmir valley, which might have increased the reproductive rate of microbial communities. These results are in

accordance with the studies of Murphy, 2000, who evaluated a correlation between temperature changes and microbial communities. The total count (cfu/g) was highest at site II which was dominated by cattle activities. The cattle activities thus might have induced changes in the microbial community structure which is in concurrence with the study carried out by Kohler *et al.,* 2005 in which he has reported the effects of cattle grazing on microbial communities in pastures and has shown that microbial community changes due to simulated effects of cattle grazing.

P. funiculosum and *P. chrysogenum* were obtained in the present study which were also reported by Bandh *et al.*, 2011, from Dal lake and reported that *P. funiculosum* was the most abundant (28.7%) followed by *P. chrysogenum* (27.04%), which also concurs with the present study.

P. chrysogenum showed maximum activity of 550.4 U/ml in fermented medium with linseed oil cake as crude substrate at pH 6 and incubation period of 6 days followed by 398.0 U/ml at pH 6 and incubation period of 6 days with glucose as carbon source and 184.3 U/ml at pH 6 and incubation period of 12 days with peptone as nitrogen source. Similar results were shown by Balkan and Ertan (2005) who studied the fungi and screened their ability to produce α-amylase, *P. chrysogenum* showed high enzymatic activity and α-amylase production by *P. chrysogenum* cultivated in liquid media containing maltose reached its maximum in 6-8 days at 30^{o}C. The study is also in consonance with the study of Vidya *et al.*, 2012 who worked on the culture and nutrient requirements of *P. chrysogenum* for production of α- amylase in production media containing different pH, temperature, incubation period, carbon sources and nitrogen sources under submerged fermentation and found that the optimum pH, temperature and incubation period for enzyme production were 6, 50°C, and 6^{th} day of incubation and showed that minimal medium can be used under submerged fermentation for the maximum production of amylase under controlled conditions.

P. purpurogenum showed maximum activity of 883.93 U/ml in medium fermented with crude substrate, mustard oil cake at pH 6 and incubation period of 6 days, while as *P. caesicolum* showed maximum activity of 1834.8 U/ml in medium with glucose as carbon source at pH 9 and incubation period of 18 days and *P.*

funiculosum showed maximum activity of 4254.4 U/ml at pH 6 and incubation period of 18 days. These results are in consonance with the study of Adejuwon *et al*. (2012) who grew *Aspergillus niger* in a growth medium with rice as carbon and growth source and in a defined synthetic medium with varying carbon and nitrogen sources at 25^{o}C and reported that optimum amylase activity in rice was expressed on the eighth day of incubation as 0.58 Units and in the synthetic growth medium with starch as carbon source and tryptone as nitrogen source.

The effects of pH, incubation time, sources of carbon and nitrogen on enzyme activity by four *Penicillium* isolates in different agricultural wastes are also in concurrence with the study of Kathiresan and Manivannan (2006) who studied the effects of pH, incubation time, sources of carbon and nitrogen on submerged fermentation process in production of α-amylase by *Pencillium fellutanum* isolated from coastal mangrove soil and reported that the production medium without addition of sea water and with provision of maltose as carbon source, peptone as nitrogen source, incubated for 96 hours maintained with pH of 6.5 at 30°C, was optimal for production of α-amylase. The present study also confirms the results of Tiwari *et al.* (2007) on a fungal strain *P. rugulosum* isolated from a soil sample, for production of α-amylase and showed that the maximum production of amylase by *P. rugulosum* was observed on 3rd day of incubation with an improvement in its production in the presence of galactose as sole carbon source. Similarly, Sidkey *et al.* (2010) focused on the possibility of using different fermented enviro-agro-industrial wastes as very cheap and available substrates for obtaining microbial α-amylases that are of great industrial importance.

Various agricultural wastes and the alteration of different carbon and nitrogen sources were brought in the culture medium during the present study The results obtained are in consonance with the studies of various researchers who also used various agricultural wastes and different carbon, nitrogen sources like Adejuwon (2011) who while studying the *Pencillium* species isolated from decaying apple fruit grown in a synthetic medium containing starch as sole carbon source showed that culture filtrates exhibited amylase activity. Similarly, Gayen and Ghosh, (2011) studied the use of, dried citrus peel and wheat bran as substrate for the production of pectin methyl esterase by fungus *P. notatum* and showed that maximum enzyme

activity was obtained at pH 5.5, when incubated at 30°C for 120 h. The prolonged incubation period has also been found to influence the enzyme activity during the present study. Similar results were shown by Sindhu *et al*. (2011) who studied the purification and characterization of α-amylase from *Penicillium janthinellum* and its application in detergent industry concluded that after 96 hours of incubation using wheat bran as substrate for SSF, amylase got purified and the amylase production by *Aspergillus niger* under solid state fermentation using agro industrial wastes was studied by Suganthi *et al.* (2011), and reported that *Aspergillus niger* showed the highest production of amylase and also observed that sucrose and nitrogen improved the yield in the same medium which is also confirmed by the present study.

In order to study the alpha amylase activity of *Penicillium* species, the fungal cultures were incubated with different medias for 18 days in orbital shaking incubator and the activity was observed after 6^{th}, 12^{th}, and 18^{th} day of incubation and it was found that amylase activity obtained was highest at 6^{th} day of incubation followed by the 18^{th} day of incubation. Therefore it is clear that, each substrate was utilized better in different days of incubation. The enzyme production varied with incubation period. In the present study, six days of incubation gave promising results for the production of amylase by *Penicillium* species. These results are in consonance with Sonjoy *et al.,* 1995 who reported that short incubation period offers potential for inexpensive production of enzyme who studied the Cellular activity of *Trichoderma ressei* on municipal solid waste. Similarly, Ely *et al.,* 2002 showed that the mycelial growth on starch reachs a maximum after five days and maximum amylase activity is produced after two days of cultivation. The decreased activity in the later phase of growth was probably due to catabolite repression by glucose released from starch hydrolysis, that is in agreement with the results reported by Gupta *et al.,* 2008 in *Humicola grisea* and *H. Brevis,* but different from *Papulasporia thermofilia* in which the maximum amylase activity was recorded during the period of fungus autolysis and reported the maximum production of amylase enzyme at five days of incubation period at 30ºC. The present study also confirms the study carried by Ileasanmi *et al.,* 2012 who investigated the production of amylase using *Aspergillus flavus* implicated in the bio-deterioration of starch-based fermented foods and showed that 30^{o}C was optimum for

amylase production by this isolate and also revealed that an incubation period of 6 days was optimum for amylase production by this isolate.

Penicillium species were inoculated into different substrates and incubated for 18 days maintained at different pH. The enzyme was extracted and the activity of the amylase produced at different pH and in different substrates was recorded. The maximum yield of amylase was found in the medias with pH 6 followed by medias with pH 9. Gupta *et al.,* 2008 reported that amylase production is high at pH 5, thus the present study is confirmed. *P. caesicolum* showed maximum activity of 1834.8 U/ml in medium with glucose as carbon source at pH 9. These results are in consonance with the study of Varalakshmi *et al.,* (2009) who reported the maximum enzyme activity of 75 U/mg of protein at pH 9.5. In the present study the effect of pH on the enzyme activity indicates that the amylase is active in the pH range 6-9, both neutral and alkaline. This suggests that the enzyme would be useful in processes that requires wide range of pH change from neutral to slightly alkaline range and vice versa. Similarly multiple pH optima were observed for amylolytic activities in the crude amylase preparation in various literatures (Yamasaki *et al.,* 1997; Bergmann *et al.,* 1998; Hayashida *et al.,* 1998).

Addition of different carbon sources like glucose, maltose, lactose, and starch did not show much remarkable change in the yield. Among all, glucose showed a better activity (1834U/ml). As reported by Varalakshmi *et al.,* 2009, the glucose and sucrose supplementation resulted in the repression of enzyme production. Similar results of catabolite repression of enzyme production by glucose for *A. Niger* and for *Aspergillus* sp. (Alva *et al.,* 2007; Mukerjee and Majumdar, 1993).

$NaNO_3$, Yeast extract, peptone, NH_4Cl and KNO_3 were used as nitrogen source in the basal medium. There was remarkable increase in the production of amylase in medium with yeast extract as nitrogen source (709.82 U/ml at pH 9 and incubation period of 12 days). Similarly the supplementation with nitrogen sources (organic or inorganic) to amylase production by fungi was done with successfully increasing the yield of the enzyme in SSF (Pandey, 2005). The results are also in agreement with the study of Anupama and Ravindra, 2001, who found that the nitrogen supplementation enhances the production of the organism and increased the

biomass production. During the study, peptone was found to increase the enzyme activity which is in consonance with some previous findings where it has been observed that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production in *A. Fumigates* (Got *et al.,* 1998), *A .niger* (Pandey *et al.,* 1994) and *A. oryzae* (Pederson and Neilson, 2000).

P. funiculosum was found to be the best amylase producer and activity of amylase produced by this strain was 4254 U/ml in submerged fermentation with mustard oil cake as substrate in the medium. Similar values of enzyme production have been reported for *Aspergillus niger* isolates by Varalakshmi *et al.,* 2009. Balkan *et al.* (2011) while studying the production of extracellular α-amylase by *Trichothecium roseum* in solid state fermentation (SSF) examined the effects of wheat bran (WB), rye straw (RS), corncob leaf (CL), sunflower oil meal (SOM) and rice husk (RH) showed that wheat bran (WB) exhibited the highest enzyme production, which proves the highest activity of 397.3 U/ml at pH 6 and incubation period of 18 days with wheat bran as substrate in the medium by *P. caesicolum* in the present study.

Thus in the present study, the isolated *Penicillium* species when grown with different culture medias for the production of alpha amylases, the optimum conditions evaluated for the production of alpha amylases were at pH 9 and incubation period of 18 days with glucose as carbon source. This study can be employed to set up Textile industries, Paper industries, Pharmaceutical industry, and Starch degrading industry in Kashmir valley, which will not only boost our economy but will also generate employment opportunities.

The study carried out with respect to the alpha amylase activity of some *Penicillium* species lead to following conclusions:

- Four *Penicillium* isolates were obtained from soil samples during the study, which showed positive alpha amylase activity, all belonging to division Ascomycota of kingdom fungi.
- The highest cfu/gm of 3.5×10^3 was found for *P. chrysogenum* at site II in the month of August.
- *P. chrysogenum* showed maximum activity of 550.4 U/ml in medium with linseed oil cake at pH 6 and incubation period of 6 days followed by 398.0 U/ml at pH 6 and incubation period of 6 days with glucose as carbon source and 184.3 U/ml at pH 6 and incubation period of 12 days with peptone as nitrogen source.
- *P. purpurogenum* showed maximum activity of 883.93 U/ml in medium fermented with mustard oil cake at pH 6 incubation period of 6 days followed by 366.0 U/ml with glucose as carbon source at pH 9 and incubation period of 6 days and 174.7 U/ml at pH 9 and incubation period of 6 days with yeast extract as nitrogen source.
- *P. caesicolum* showed maximum activity of 1834.8 U/ml in medium with glucose as carbon source at pH 9 and incubation period of 18 days followed by

709.82 U/ml at pH 9 and incubation period of 12 days with yeast extract as nitrogen source and 397.3 U/ml at pH 6 and incubation period of 18 days with wheat bran as substrate in the medium.

- *P. funiculosum* showed maximum activity of 4254.4 U/ml at pH 6 and incubation period of 18 days with mustard oil cake as substrate in the medium followed by 477.6 U/ml at pH 9 and incubation period of 18 days with glucose as carbon source and 330.36 U/ml at pH 9 and incubation period of 18 days with yeast extract as nitrogen source in the medium.
- Among all the species isolated activity showed this order *P. funiculosum>P. caesicolum>P. chrysogenum>P. purpurogenum*
- The optimum conditions in the medium which were found best to promote the production of alpha amylase from *Penicillium* species were when medium was maintained at pH 9 and incubation period of 18 days with glucose as carbon source followed by medium maintained at pH 9, 6 and incubation period of 18 days with yeast extract as nitrogen source and medium maintained at pH 6 and incubation period of 18 days with mustard oil cake in the medium.

Abbas, A.S., Ali, N., Mohaddeseh, L. and Bahman, N. 2011. Isolation of halotolerant *Pencillium* strains from the HowzSoltan lake to produce α-amylase. *Middle East Journal of Scientific Research*, **7**(3): 407-412.

Abdullah, R. 2005. *Studies on the production of α-amylase by Aspergillus oryzae using submerged fermentation*. PhD Thesis, University of Lahore.

Abe, K., Gomi, K., Hasegawo, F. and Machinda, M. 2006. Impact of *Aspergillus oryzae* genomics on industrial production of metabolites. *Mycopathologia*, **162**(3): 143-153.

Adejuwon, A.O. 2011. Synthetic Production of Amylase from *Penicillium species* Isolated from Apple Fruit. *World Applied Sciences Journal*, **13** (3): 415-418.

Adejuwon, A.O. and Ladokun, O.A. 2013. The effect of carbon source of growth medium on α-amylase production by strains of *Penicillium solitum* and *Aspergillus rubrum* isolated from yam (*Dioscorea alata*). *Report and Opinion*, **5**(2). 169-174.

Adeniran, H.A. and Abiose, S.H. 2011. Partial purification, characterization and hydrolytic activities of amylases from *Bacillus licheniformis* and *Aspergillus niger* cultured on agricultural residues. *African Journal of Biotechnology*, **11**(6). 1465-1477.

Ahmed, S.A. 2011. *Alpha Amylase Production by Aspergillus Oryzae Using Solid State Fermentation*. Applied Science Department, University of Technology/Baghdad.

Akpan, I., Bankjole, M.O., Adesermowo, A.M. and Lantunde, D. 1999. Production of α-amylase by *Aspergillus niger* in a cheap solid medium using rice bran and agricultural material. *Trop.sci*, **39**(3): 77-79.

Alhussaini, M.S. 2013. Mycobiota of wheat flour and detection of α- Amylase and L-Asparaginase. *Enzymes Life Science Journal*, **10**(1): 233-240.

Alva, S., Anupama, J., Salva, J., Chiu Y.Y., Vyshali, P., Shruthi, M., Yogeetha, B.S., Bhavya, D., Purvi, J., Ruchi, K., Kumudini, B.S. and Varalakshmi, K.N. 2007. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *African Journal of Biotechnology*, **6** (1): 576 – 581.

Anupama and Ravindra. 2001. Studies on production of single cell protein by *Aspergillus niger* in solid state fermentation of rice bran. *Braz. Arc. Biol. Tec.*, **44**(3): 79-88.

APHA. 1998. *Standard methods for the examination of water and wastewater*, 20th edn. American Public Health Association, Washington, DC.

Arunsasi, Manthirikani, S., Jegadeesh, G. and Ravikumar, M. 2010. Submerged fermentation of amylase enzyme by *Aspergillus flavus* using cocos nucifera meal. *Kathmandu University Journal of Science, Engineering and Technology*, **6**(11):75-87.

Ayansina, A.D.V. and Owoseni, A.A. 2010. Studies on amylolytic enzyme synthesized by *Aspergillus flavus* associated with mouldy bread. *Pakistan Journal of Nutrition*, **9** (5): 434-437.

Bakri, Y., Akeed, Y. and Thonart, P. 2012. Comparison between continuous and batch processing to produce xylanase by *penicillium canescens* 10-10c**.** *Brazilian Journal of Chemical Engineering*, **29**(3): 135-139.

Bakri, Y., Magali, M. and Thonart, P. 2009. Isolation and identification of a new fungal strain for amylase biosynthesis. *Polish Journal of Microbiology*, **58**(3): 269-273.

Balkan, B. and Ertan, F. 2005. Production and properties of α-amylase from *Pencillium chrysogenum* and its application in starch hydrolysis. *Biochemical Biotechnology*, **35**(20):169-78.

Balkan, B., Balkan, S. and Ertan, F. 2011.Optimization of parameters for alpha amylase production under solid state fermentation by *Trichothecium roseum. Romanian Biotechnological Letters*, **16**(5): 2011.

Bandh, S.A, Kamili A.N. and Ganai B.A. 2011. Identification of some *Penicillium* species by traditional approach of morphological observation and culture. *African Journal of Microbial. Research*, **5**(21): 3493-3496.

Bansode, S.D. 2010. Screening of Nutritional components for α–amylase production in submerged fermentation by bacteria isolated from soil using Placket-Burman Design. *International journal of pharmacy and pharmaceutical sciences*, **2**(1): 93-98.

Barnett, H.L. and Hunter, B.B. 1999. *Illustrated genera of imperfect fungi* (4th edn.). APS Press, St. Paul, Minnesota, USA.

Bates, C.E. 2005. *A Gazetteer of Kashmir*. Gulshan Books, Residency Road, Srinagar Kashmir.

Bergmann, F.W., Abe J. and Hizukuri, S. 1998. Selection of microorganisms which produce raw starch degrading amylases. *Appl. Microbiol. Biotechnol*, **2**(87):

443-446.

Bernfed, P.C. 1951. Enzymes of starch degradation and synthesis. *Advance in Enzymology*, **12**(6): 379-481.

Bhardwaj, S., Vedamurthy, A.B., Bhattacharya, S. and Das, A. 2012. Effect of inorganic salts and surfactants on the production of α-Amylase by a Mangrove isolate of *Aspergillus flavus* using solid-state fermentation. *Journal of Chemical, Biological and Physical Sciences*, **2**(3): 1390-1397.

Bruns, T. 2006. Evolutionary Biology: A kingdom revised. *Nature*, **443**(7113): 758-761.

Christensen, M., Frisvad, J.C and Tuthill, D.E. 2000. *Penicillium* species diversity in soil and some taxonomic and ecological notes. In: Samson, R.A. and Pitt, J.I. (eds), *Integration of modern taxonomic for Penicillium and Aspergillus classification*. Harwood Academic Publishers, Amsterdam, pp. 309–320.

Curtis, T.P. and Sloan, W.T. 2005. Exploring microbial diversity-A vast below. *Science,* **309**(2): 1331–1333.

Dance, A. 2008. What lies beneath. *Nature*, **455**(112): 724–725.

Dar, M.A., Singh, N., Ganai, M.R. and Bhat, A.R. 2000. Depth-wise distribution of micronutrients in cherry orchard soils of Kashmir. *Applied Biological Research*, **2**(2): 91-95.

Dayalan, S.A.J., Darwin, P. and Prakash, S. 2011. Comparative study on production, purification of *Pencillin* by *Pencillium chrysogenum* isolated from soil and citrus samples. *Asian pacific journal of tropical bio-medicine,* **2**(3): 15-19.

Doss, A. and Anand, S.P. 2012. Purification and characterization of extracellular amylolytic enzyme from *Aspergillus* species. *African Journal of Biotechnology*, **11**(83). 14941-14945.

Ellaiah, V., Adinarayana, K., Bhavani, Y., Padmaja, P. and Srinivasulu, B. 2002. Optimization of process parameters for glucoamylase production under solid-state fermentation by a new isolated *Aspergillus* species. *Process Biochemistry,* **38**(5): 615–620.

Ely, N., Mirela and Waldermarin. 2002. Control of amylase production and growth characteristics of *Aspergillus ochraceus*. *African Journal of Biotechnology*, **44**(2): 4-10.

Erdal, S. and Taskin, M. 2010. Production of α –amylase by *Pencillium expansum* MT-1 in solid state fermentation using water loquat (*Eriobotrya Japonica*

lindley) kernels as substrate. *Romanian biotechnological letters,* **15**(3):5342-5350.

Fierer, N. and Jackson, R.B. 2006. The diversity and biogeography of soil bacterial communities. *PNAS,* **103**(3): 626-631.

Figueira, E.L.Z. and Hirooka, E.Y. 2000. Culture medium for amylase production by toxigenic fungi. *Brazilian Archives of Biology and Technology,* **43**(5):461-467.

Garland, J.L. and Mills, A.L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology*, **57**(3): 2351–2359.

Gayen and Ghosh. 2011. Pectin-methylesterase production from mixed agro-wastes by *Penicillium notatum* NCIM. 923 in solid-state fermentation. *Journal of Bioremediation and Biodegradation*, **2**(1): 2.

Gigras, P., Sahai, V. and Gupta, R. 2002. Statistical media optimization and production of ITS alpha-amylase from *Aspergillus oryzae* in a bioreactor. *Curr. Microbiol*, **45**:203– 208.

Gillman, C. J. 1959. *A manual of Soil Fungi.* Biotech books Oxford and IBH publishing company, Calcutta, pp. 192-193.

Gillman, C. J. 1998. *A manual of soil fungi.* Biotech books, New Delhi, pp. 235.

Got, C.E., Barbosa, E.P., Kistner., L.C.I., Gandra, R.F., Arrias, V.L. and Peralta, R.M. 1998. Production of amylase by *Aspergillus fumigatus*. *Revistade Microbiologia*, **28**(2): 99-103.

Griffiths, R.L., Whitely, A.S., O'Donnell, A.G. and Bailey, M.J. 2003. Influence of depth and sampling time on bacterial community structure in an upland grassland soil. *FEMS Microbiological Ecology,* **43**(3):35-43.

Gupta, A., Gupta, V.K., Modi, D.R.Y. and Adava, L.P. 2008. Production and characterization of α-amylase from *Aspergillus niger*. *Biotechnology*, **7**(4): 551-556.

Hawkins, K.M. and Smolke, C.D. 2008. Production of benzylisoquinolone alkaloids in *Saccharomyces cerevisiae. Nature Chemical Biology,* **4**(9): 564-573.

Hawksworth, D.L. 2006. The fungal dimensions of biodiversity: Magnitude, significance and conservation. *Mycological research*, **95**(6): 641-655.

Hayashida, S., Teamoto, Y. and Inove, T. 1998. Production and characteristics of raw potato Starch digesting amylase from *Bacillus subtilis*. *Appl. Environ. Microbiol*, **54**: 1516-1522.

Hegde, S.V., Ramesha, A. and Srinvas, C. 2011. Optimization of amylase production from an endophytic fungi *Discosia* sp. isolated from *Calophyllum inophyllum. Journal of Agricultural Technology*, **7**(3): 805-813.

Holmes, G.J. and Eckert, J.W. 1999. Sensitivity of *Penicillium digitatum* and *P. italicum* to post harvest citrus fungicides in California. *Phytopathology*, **89**:717–72.

Hols, P., Ferain, T. and Garmyn, D. 1994. Use of expression secretion signals and vector free stable chromosomal integration in engineering of *Lactobacillus plantarum* for α- amylase and levanase expression. *Applied Environmental Microbiology*, **60**: 1401-1403.

Huang, B., Guo, J., Yi, B., Sun, L. and Chen, W. 2008. Helterologous production of secondary metabolites as pharmaceuticals in *Saccharomyces cerevisiae*. *Biotechnology letters*, **30**(7): 1121-1137.

Ileasanmi, F., Oluwaseun, F. and Garuba, E. 2012. Amylase Production by *Aspergillus flavus* associated with the bio-deterioration of starch based fermented foods. *New York Science Journal*, **5**(1): 265-266.

Irfan, M., Nadeem, M. and Syed, Q. 2012. Media optimization for amylase production in solid state fermentation of wheat bran by fungal strains. *Journal of Cell and Molecular Biology*, **10**(1): 55-64.

Irshad, M., Anwar, Z., Gulfraz, M., Butt, H.I., Ejaz, A. and Nawaz, H. 2012. Purification and characterization of α-amylase from *Ganoderma tsuage* growing in waste bread medium. *African Journal of Biotechnology*, **11**(33): 8288-8294.

Jackson, C. K., Krichevsky, M. I. and Portyrata, D. 1975. Diagnostic probability matrix for identification of slowly growing mycobacteria in clinical laboratories. *J. Clin. Microbiol*, **20**:722-729.

Janisiewicz, W.J. 1987. Post harvest biological control of blue mold on apples. *Phytopathology*, **77**:481–485.

Johnson, T.R and Case, C. L. 1986. *A Laboratory experiments in Microbiology.* Tata McGraw Hill Pub.Co.Ltd.

Jorgensen, T.R. 2007. Identification and toxigenic potential of the industrially important fungi. *Journal of Food Protection*, **70**(12): 2916-2934.

Kathiresan, K. and Manivannan, S. 2006. α –amylase production by *Pencillium fellutanum* isolated from mangrove rhizosphere soil. *African journal of Biotechnology*, **5**(10): 829-932.

Kausar, S. 2005. *Meaning of Mughal Landscape*. 12-31.

Kennedy, A.C. and Papendick, R.I. 1995. Microbial characteristics of soil quality. *Journal of Soil and Water Conservation*, **50**: 243–248.

Kennedy, A.C. and Smith, K.L. 1998. Soil microbial diversity and the sustainability of agricultural soils. *Plant and Soil*, **170**: 75–86.

Khokhar, I.B., Irum, M. and Sobia, M. 2011. Isolation and screening of amylolytic filamentous fungi. *Journal of Applied Science Environment and Management*, **15** (1): 203 – 206.

Kirk, P.M., Cannon, P.F., Minter, D.W. and Stalpers, J.A. 2008. *Dictionary of the Fungi*, 10th edn. Wallingford, UK.

Kohler, F., Hamelin, J., Gillet, F., Gobat J.M. and Buttler, A. 2005. Soil microbial community changes in wooded mountain pastures due to simulated effects of cattle grazing. *Plant and Soil*, **278** (1-2): 327-340.

Kumar, A. and Duhan, J.S. 2011. Production and characterization of amylase enzyme isolated from *Aspergillus niger* MTCC-104 employing solid state fermentation. *International Journal of Pharma and Bio Science*, **3**(2): 250-258.

Kumar, R., Singh, S and. Singh, O.V. 2008. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. *Journal of Industrial Microbiology and Biotechnology*, **35**(5): 377-391.

Kumari, B.L., Sesha-sairam, C.V., Kumar, T.S., Sudhakar, P., Vijetha, P. and Satyasree, K.P.N.Y. 2011. Screening and isolation of thermostable α–amylase producing bacteria and optimization of physio-chemical parameters for increasing the field. *International Journal of Pharmacy and Technology*, **3**(1): 1570 -1583.

Laloknam, S., Sirisopana, S., Attaphinyo, P., Poohuarai, S. and Phornphisutthimas, S. 2009. Detection of amylase activity from fruit and vegetables in an undergraduate classroom. *Asian Journal of food and agro-industry*, **2**(3): 402-411.

Leiter, E., Emri, T., Nagy, I., Mrepocsi., Winkelmann, G. and Istvan, P. 2000. *Penicillin* production by *Penicillium chrysogenum* in the presence of Fe_3^+ and in low-Iron culture medium. *Folia Microbiology*, **46**(2):127-132.

Lin, L.L., Hsu, W.H. and Chu, W.S. 1997. A gene encoding for α-amylase from thermophilic *Bacillus* sp. strain TS-23 and its expression in *Escherichia coli*. *Journal of Applied Microbiology*, **82**: 325-334.

Lonsane, B.K. and Ramesh, M.V. 1991. *Advances in Applied Microbiology.* Academic press, New York, pp. 35:1-56

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin-phenol reagents. *Journal of Biological Chemistry*, **48**: 17 – 25.

Mahapatra, S.K., Walia, C.S., Sidhu, G.S., Rana, K.P.C. and Tassem, L. 2000. Characterization and classification of soils of different physiographic units in the sub-humid ecosystem of Kashmir region. *J. Indian Soc. Soil Science*, **48**(3): 575-577.

Mahmoud, A.M.Y. and Asfour, H.Z. 2012. Improved production, purification and some properties of α-amylase from *Streptomyces clavifer*. *African Journal of Biotechnology*, **11**(80): 14603-14611.

Manvi, F.V. 2010. Isolation and morphological characterization of antibiotic producing actinomycetes. *Tropical Journal of Pharmaceutical Research*, **9** (3): 231-236.

Metin, K., Oznur K.Z., Burcu, B.A.L. and H Halil. 2010. Purification and characterization of amylase produced by *Penicillium citrinum* HBF62. *African Journal of Biotechnology*, **9**(45): 7692-7701.

Miller, G.L. 1959. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Analytical Chemistry*, **31**(2): 426-429.

Mishra, S. and Behera, N. 2008. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. *African Journal of Biotechnology*, **7** (18): 3326-3331.

Mitchell, D.A. and Lonsane, B.K. 1990. In: Doelle, H.W and Rolz, C. (eds.), *General principles of Solid State fermentation*, Monogram, Oxford London.

Mohamed, S.A., Azhar E.I., Morooj, M.B., Tashkandy, N.R. and Taha, A.K. 2011. Production, purification and characterization of amylase from *Trichoderma harzianum* grown on mandarin peel. *African Journal of Microbiology*

Research, **5**(9): 1018-1028.

Morales, H., Marin, S., Rovira, A., Ramos, A.J. and Sanchis, V. 2007. Patulin accumulation in apples by *Penicillium expansum* during post harvest stages. *Letters in Applied Microbiology*, **44**(1):30–35.

Muhammad, I., Zahid, A., Muhammad, G., Hamama, I.B., Amir, E. and Haq, N. 2012. Purification and characterization of α-amylase from *Ganoderma tsuage* growing in waste bread medium. *African Journal of Biotechnology*, **11**(33): 8288-8294.

Mukerjee, S.K. and Majumdar, S.K. 1993. Fermentative production of alpha amylase by *Aspergillus flavus*. *Indian J. Exp. Biology*, **11**(2): 436-438.

Murphy, R.Y. 2000, Pathogenic lethality validation in ready-to-eat meat products. *Presented at advanced foods company*. Enid, UK.

Nguyen, Q.D., Rezessy-Szabo, J.M., Claeyssens, M., Stals, I. and Hoschke, A. 2002. Purification and characterization of amylolytic enzymes from thermophilic fungus strain ATCC 34626. *Enzyme Microbial Technology*, **31**(2): 345–352.

Nwagu, T.N. and Okolo, B.N. 2011. Extracellular amylase production of a thermotolerant *Fusarium species* isolated from Eastern Nigerian soil. *Brazilian Archives of Biology and Technology*, **54**(4):649-658.

Osfar, S. and Tri, A. 2011. Extracellular amylase activity of amylolytic bacteria isolated from Quail's (*Coturnix japonica*) intestinal tract in corn flour medium. *International Journal of Poultry Science*, **10** (5): 411-415.

Oyeleke, S.B., Auta, S.H. and Egwim, E.C. 2010. Production and characterization of amylase produced by *Bacillus megaterium* isolated from a local yam peel dump site in Minna, Niger state. *Journal of Microbiology and Antimicorbials*, **2**(7): 88-92.

Pan, A., Lorenzotti, S. and Zoncada, A. 2008. Registered and investigational drugs for the treatment of *Staphylococcus aureum* infection. *Recent patents on Anti-infective Drug Discovery*, **3**(1): 10-33.

Pandey, A. 1999. Aspects of fermenter design for solid state fermentation. *Process Biochemistry*, **26**(1): 335-361.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V.T., Singh, D. and Mohan, R. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochemistry*, **31**(2): 135-152.

Pandey, A., Nigam, P., Soccol, V.T., Soccol, C.R., Singh, D. and Mohan, R. 2006. Advances in microbial amylases. *Biochemistry*, **31**(2): 35-152.

Pandey, A., Selvakumar, P. and. Ashakumary, L. 1994. Glucose production by *Aspergillus niger* on rice bran is improved by addition of nitrogen sources. *World J. Microbiol. Biotechnology*, **10**(1): 348-349.

Pandey, A., Soccol, C.R. and Mitchell, D. 2000. New developments in solid state fermentation process. *Biochemistry*, **35**(1): 1153 – 1169.

Pandey, A., Webb, C., Soccol, C.R.L. and Arroche, C. 2005. Rice bran as a substrate for proteolytic enzyme production. *Enzyme Technology*. Asiatech Publishers New Delhi, pp.197.

Pederson, H. and Neilson, J. 2000. The influence of nitrogen sources on alpha amylases productivity of *Aspergillus oryzae* in continuous cultures. *Appl. Microbiol. Biotechnol*, **53**(1): 278-281.

Pelczar, M.J., Chan, E.C.S. and Kreig, N.R. 1993. *Microbiology*. Tata McGraw Hill Pub Co.Ltd.

Piao, H.C., Hong, Y.T. and Yuan, Z.Y. 2000. Seasonal changes of microbial carbon related to climated factors in soils from karst areas of Southwest China. *Biol. Fertil.*, **30**(2): 294-297.

Pitt, J.I. and Hocking, A.D. 1999. *Fungi and Food Spoilage*, (2nd edn). Aspen Publishers, Gaithersburg, MD.

Pitt, J.I. and Hocking, A.D. 1997. *Fungi and food spoilage*. Cambridge, UK: Cambridge Univ. Press. 593.

Pitt, J.I. and Samson, R.A. 2000. In: *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification*, (R.A. Samson and J.I. Pitt, Eds.), Hardwood Academic Publishers, Reading, UK, pp.51-72.

Raharjo, S., Ardiansyah., Endang, P. and Tien. 2010. Isolation of thermostabl α-amylase from local thermophilic bacteria for liquefaction. *Proceedings of the third International Conference on Mathematics and Natural Sciences*.

Raza, M., Ahmad, A. and Mohammad, A. 1978. *The Valley of Kashmir, a Geographical Interpretation*. Vikas Publication House, New Delhi.

Sabat, J. and Gupta, N. 2010. Nutritional factors affecting the antifungal activity of *Penicillium steckii* of mangrove origin. *African Journal of Microbial Research*, **4** (3): 126-135.

Saravanakumar, K. and Kaviyarasan, V. 2010. Seasonal distribution of soil fungi and chemical properties of montane wet temperate forest types of Tamil Nadu. *African Journal of Plant science,* **4**(6):196-198.

Sathyaprabha, G., Panneerselvam, A. and Muthukkumarasamy, S. 2011. Production of cellulase and amylase from wild and mutated fungal isolates. *E-Journal of life sciences,* **1**(1):39-45.

Seneviratne, G. and Jayasinghearachchi, H.S. 2005. A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil. *Soil Biol Biochem.*, **37**(2): 1975–1978.

Shafique, S., Bajwa, R. and Shazia, S. 2009. Screening of *Aspergillus niger* and *Aspergillus flavus* strains for extra cellular α-amylase activity. *Pakistan Journal of Botany*, **41**(2): 897-905.

Sidkey, N.M., Abo-shadi, M.A., Balahmar, R., Sabry, R and. Badrany, G. 2011. Purification and characterization of α-amylase from a newly islolated *Aspergillus flavus* F_2Mbb. *International research Journal of microbiology*, **2**(3): 096-103.

Sidkey, N.M., Al-Rahman Abo-shadi, M.A., Al-Mutrafy, A.M., Sefergy, F. and Al-Reheily, N. 2010. Screening of micro-organisms isolated from some enviro-Agro-Industrial wastes in Saudi Arabia for α–amylase production. *Journal of American Science*, **6**(10):926 -939.

Simmon, B., Denk, U., Poll, V., Rid, R. and Breitenbach, M. 2008. The spectrum of fungal allergy. *International Archives of Allergy and Immunology*, **145**(1): 58-86.

Sindhu, R., Suprabha, G.N. and Shashidhar, S. 2011. Purification and characterization of α-amylase from *Penicillium janthinellum* (NCIM 4960) and its application in detergent industry. *Biotechnol. Bioinf. Bioeng.*, **1**(1):25-32.

Singh, P., Rathinasamy, K., Mohan, R. and Panda, D. 2008. Microtubule assembly dynamics: an attractive target for anticancer drugs. *IUBMB Life,* **60**(6): 368–75.

Sonjoy, S., Bill, B. and Houston, K.H. 1995. Cellular activity of *Trichoderma ressei* (RUT-C30) on municipal solid waste. *Appl. Biochem.Biotechnol*, **15**: 145-153.

Suganthi, R., Benazir, J.F., Santhi, R., Ramesh, K.V., Anjana, H., Nitya, M., Nidhiya, K.A., Kavita, G. and. Lakshmi, R. 2011. Amylase production by

Aspergillus niger under solid state fermentation using agro industrial wastes. *International Journal of Engineering and technology,* **3**(2):1756-1763.

Sun, H.Y., Li, J., Zhao, P. and Peng, M. 2011. Banana peel: A novel substrate for cellulase production under solid-state fermentation. *African Journal of Biotechnology*, **10**(**77**):17887-17890.

Sundar, R., Liji.T., Rajila,C. and Suganyadevi, P. 2012. Amylase production by *aspergillus niger* under submerged fermentation using *Ipomoea batatas. International journal of applied biology and pharmaceutical technology,* **3**(2):175-182.

Thom, C. 1930. *The Penicillia*. Baltimore: Williams & Wilkins Co**.** Maryland.

Tiwari, K.L., Jadhav, S.K. and Fatima, A. 2007. Culture condition for the production of thermostable Amylase by *Penicillium rugulosum*. *Global journal of biotechnology and bio-chemistry,* **2**(1): 21-24.

Tochukwu, N.N. and Okolo, B.N. 2011. Extracellular amylase production of a thermotolerant *Fusarium* sp. isolated from Eastern Nigerian soil. *Brazilian Archives Biology Technology,* **54**(4):649-658.

Van der Marrel, M.J.E.C., van der Veen, B., Uitdehaag, J.C.M., Leemhuis, H. and Dijkhuizen, L. 2002. Properties and applications of starch-converting enzymes of the α-amylase family. *Journal of Biotechnology*, **94**: 137-155.

Varalakshmi, K.N., Kumudini, B.S., Nandini, B.N., Solomon, J., Suhas, R., Mahesh, B. and Kavitha, A.P. 2009. Production and characterization of α-amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. *Polish Journal of Microbiology*, **58**: 29-36.

Vidya, B., Gomathi, D., Kalaiselvi, M., Ravikumar, G. and Uma, C. 2012. Production and optimization of amylase from *Penicillium chrysogenum* under submerged fermentation. *World journal of pharmaceutical research*, **1** (2):1116-1125.

Vijayaraghavan, P., Devi, V.S.L. and Vincent, S.G.P. 2011. Bio-Processing of banana peel for amylase production by *Penicillium* Sp. *Asian journal of experimental biological science*, **2**(2): 257-264.

Waksman, S.A. 1922. A method of counting the number of fungi in soil. *Journal of Bacteriology,* **7**:339-341.

Warcup, J.H. 1950. The soil plate method for isolation of fungi from soil. *Nature,* **166**:117-118.

XY, Li., Zhang, J.L. and Zhu, S.W. 2011. Improved thermostable α-amylase activity of *Bacillus amyloliquefaciens* by low-energy ion implantation. *Genetics and Molecular Research,* **10** (3): 2181-2189.

Xu, H., Sun, L., Zhao, D., Zhang, B., Shi, Y. and Wu, Y. 2008. Production of *α*-amylase by *Aspergillus oryzae* As 3951 in solid state fermentation using spent brewinggrains as substrate. *Journal of the Science of Food and Agriculture*, **88**:529–535.

Yamasaki, Y., Tsuboi, A. and. Suzuki, Y. 1997. Two forms of glucoamylase from *Mucor rouxianus*: Properties of two glucoamylases. *Agric. Biol.Chem*., **41**: 2139-2148.