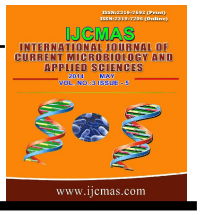
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 5 (2014) pp. 859-868**
http://www.ijcmas.com



Original Research Article

Extracellular amylase from the isolate, *Aspergillus tubingensis*

P.V.Ratnasri*, B.K.M Lakshmi, K.Ambikadevi, and K.P.J Hemalatha

Department of Microbiology, Andhra University, Visakhapatnam-530 003, Andhra Pradesh, India

Corresponding author

Keywords

Amylase, *Aspergillus tubingensis*, Cereal flours, submerged fermentation.

A B S T R A C T

The soil samples were collected from different depths of paddy and sugarcane fields. The samples were primarily screened for isolation of amylase producing fungi. Among the isolated fungi, amylase producing isolates were identified by growing on starch agar media. The isolate (20E) which form the maximum zone of clearance on starch agar media by iodine was identified and it was subcultured on potato dextrose agar (PDA). The isolate (20E) was morphologically characterized by performing cotton blue staining and scanning electron microscopic observations under required magnifications. Molecular characterization of isolate (20E) was performed by ITS/5.8S rRNA gene sequence analysis and it was confirmed as *Aspergillus tubingensis* (MTCC Acc. No. 11398). Optimization of cultural conditions for maximum production of amylase was carried out by different cereal flours, incubation periods, temperatures, nitrogen sources and with different phosphate concentrations. *Aspergillus tubingensis*showed maximum amylase activity (245±0.54/mg protein) when cultured in pearl millet at 35^0C for 54hrs of incubation period.

Introduction

Enzymes are proteins that speed up biochemical reactions. Among the industrial enzymes starch degrading enzymes such as amylases gaining more importance in modern biotechnological era (Prakasham *et al.*, 2007). These enzymes are found in animals (saliva, pancreas), plants (malt), bacteria and fungi (Abu *et al.*, 2005). Amylase was the first enzyme to be found and isolated in 1833 by Scientists AnselmePayen. Amylase is an enzyme that can break down starch and convert into sugars. Amylase enzymes are considered as glycoside hydrolases and they function on α-1, 4-glycosidic bonds. Depending on the type of amylase, starch is degraded to simple sugars like glucose, maltose or to oligosaccharides, or dextrins (Abou-Elela *et al.*, 2009). Solid state fermentation has been used in the industrial production of amylase, and it has great potential applications like

simplicity of operations, low capital cost and high volume productivity (Akpan*et al*., 1999). The microbial amylases are the most important enzymes in present day biotechnology as they meet industrial demands. They have almost completely replaced chemical hydrolysis of starch in starch processing industries and have a great significant demand and they hold approximately 25% of the enzyme market (Sidkey*et al.*, 2010). Microbial amylases replace the chemical hydrolysis in starch saccharification (Gupta *et al.*, 2003). Among the microbial production of amylases fungal amylases are preferred over the bacterial sources mainly because of their more acceptable GRAS(Generally regarded as safe) status (Prakasham *et al.*, 2007).

The fungal origin of amylases are more stable than the bacterial enzymes on a commercial scale (Duochuan *et al*., 1997 and Abu *et al*., 2005). Molds are capable of producing high amounts of amylase (Suganthi *et al*., 2011). Many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. Filamentous fungi are the best adapted for submerged fermentation. The hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates (Saranraj and Stella 2013). Generally the fungus requires simple fermentation media and absence of rigorous control of fermentation parameters, uses less water and produces lower wastewater. It has easier control of bacterial contamination and requires low cost for downstream processing. Today, amylases have the majorworld market share of enzymes. The spectrum of amylase applications has widened in many other fields, such as clinical, medicinal (Giri*et al.,* 1990), and analytical chemistry as well as their widespread application in starch saccharification, food and brewing industries, baking, and preparation of digestive aids. Amylases have also shown to play significant role in textile and detergent industries (Feitkenhauer 2003). Potential industrial application of the amylase is especially in solid waste management. The present work is undertaken for screening of amylase producing *Aspergillus sps.*,from soil and optimization of cultural conditions for efficient production of amylase from cheaply available raw materials with a cost effective manner**.**

Materials and Methods

Collection of soil samples

The soil samples were collected under sterile conditions from different depths *viz.* (10, 15, 20cms) of paddy and sugarcane fields after their harvesting in Pendurthi, in Vijayanagaram District of Andhra Pradesh, India.

Characterization of soil

Mineral matter of soil such as sand silt and clay contents were analyzed with the use of different sizes of sieves by the method of Alexander (1969). Soil pH was measured at 1:1.25 soil to water ratio using pH meter. Organic carbon content of soil was determined by Walkely and Black method (1934), and total nitrogen content in soil was measured by the Microkjeldhal method (Jackson 1997). Phosphorous in soil was measured by the method of Chapman and Pratti (1961).

Isolation of fungal isolates from soil samples

One gram of each soil sample was suspended in the 9.0ml of sterile water. This sample was serially diluted by dilution plating method (Warcup 1950). They were poured into PDA (potato dextrose agar) (Hi-Media) with chloramphenicol, a broad spectrum antibiotic used in inhibition of bacterial growth. The plates were incubated at 28^0C for 2-3 days.

Determination of fungal amylase activity

The fungal isolates were tested for amylase production by starch hydrolysis. The modified starch agar media (Soluble starch-2g, Peptone-2g, Yeast extract -1g, Agar-2g, Distilled water -100ml at pH-6) were inoculated with the isolates and incubated for 48hrs at 28^0C. After the completion of incubation period, the petridishes were flooded with the iodine solution, the zone of clearance formed around the microbial growth indicates the production of amylase.

Characterization of fungal isolates

Microscopic identification of fungal isolates

Identification of fungal species was done as per the manuals of Domsch*et al.*,(1980) and Barnett and Hunter (1972). After isolation of fungal isolate it was sub cultured on the PDA slants. Later it was primarily subjected to the Lacto phenol cotton blue staining, and then analyzed the morphology by Scanning Electron Microscope (JSM-6610 instrument model, JEOL/EO format) under required magnifications to observe morphology of mycelium and spore structures.

Molecular characterization of fungal isolates

The molecular characterization of the fungal isolate was done by the gene sequence analysis of ITS/5.8S rRNA and construction of phylogenetic tree based on evolutionary relationship of taxa based on ITS sequence data by Neighbor-Joining method (Saitou and Nei,1987). The bootstrap consensus tree inferred from 100 replicates, which were taken to represent the evolutionary history of the taxa analyzed by method of Felsenstein(1985). The evolutionary distances were computed using the Kimura2-parameter method (1980) and evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Amylase production

The isolate (20E) was subjected to fermentation medium containing (KH_2PO_4-0.14g, NH_4NO_3-1g, KCl-0.5g, $MgSO_4.7H_2O$-0.01g, $FeSO_4.7H_2O$-0.001g, soluble starch-2g, Distilled water-100ml at pH6.5). Erlenmeyer flasks were sterilized by autoclaving at 121^0C for 15mins. Spore suspension (0.5ml) was added and incubated in a shaking incubator for 48hrs, at 200rpm and at 28^0C.

Extraction of amylase from fungal isolates

The fermented broth was centrifuged at 7000rpm for 30mins. The cell free supernatant was used for the estimation of amylase.

Demonstration of enzyme activity/Enzyme assay

One milliliter of culture extract (enzyme) was pipetted intotest tube, and 1.0 ml of

1% soluble starch in citrate phosphate buffer pH 6.5 was added and incubated in water bath at 40^0C for 30mins. Then treated with DNS(Dinitro salicylic acid) and the reaction was stopped by boiling for 5mins, and cooled to room temperature and 20ml of distilled water was added and color intensity was measured at 540nm. One unit of amylase activity is defined as the amount of enzyme that releases 1µmol of maltose per minute under the assay conditions. Specific activity of the enzyme is expressed in units per mg protein.

Optimization of cultural conditions

Effect of different cereal flours on enzyme production

The effect of different cereal flours such as sorghum, sammai, finger millet, pearl millet and horse gram on enzyme production was carried out at pH 5.6 at 30^0C for 48hrs.

Effect of temperature

The effect of temperature on enzyme production was investigated by incubating the fermentation medium at 25^0C, 30^0C, 35^0C, 40^0C and 45^0C at pH 5.6 for 90hrs.

Effect of incubation period

The effect of incubation period on enzyme production was investigated by incubating the fermentation medium at regular intervals of 18, 36, 54, 72 and 90hrs at pH 5.6 and at 35^0C.

Effect of different Nitrogen Sources

The effect of different Nitrogen sources on enzyme production was investigated by adding ammonium nitrate, ammonium chloride, ammonium sulphate, and sodium nitrate separately to the fermentation medium and incubating them at pH 5.6 for 90hrs at room temperature.

Effect of different concentrations of Phosphate

The effect of phosphate concentration in the enzyme production was investigated by adding 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1.4% of phosphate in the medium at pH 5.6 for 90hrs of incubation.

Statistical analysis: All the experiments were repeated thrice and standard deviation was calibrated.

Results and Discussion

Soil Sample Analysis

Fungal isolate from soil have beneficial role in industrial areaBergquist*et al.*, (2003), Nevalainen and Teo(2005)and their saprophytic ability mainly depends on the chemistry, texture, salinity, and water holding capacity (Wanwright and Falih 1995, Ortuno *et al.*,1977). Some of the physico chemical characteristics of the soil from which the microorganisms isolated are shown in Table 1.1. Soil pH is one of the most indicative measurements of the soil because it is an important factor for the survival of microorganisms(Durghi and Bottomley 1983). In the present study the pH of the soil was determined to be 7.0.

Isolation and identification of fungal isolates

Morphological characterization of isolates

There are various methods for isolating the fungi (Kelman, 1967 and Stevens,1974)but the simplest one is dilution plate method and the lifting of conidia from sporulating conidiophores.

According to the starch hydrolyzing capacity, five fungal isolates were isolated from soil samples. Among these only one isolate (20E) showed the maximum zone of clearance on starch agar media.The isolate (20E) was characterized morphologically by lactophenol cotton blue staining and scanning electron microscopic analysis, details are presented in Table 1.2. The macro and microscopic images of isolate (20E) were shown in figures 1.2(a) and 1.2(b).

Molecular characterization of isolate (20E)

Molecular characterization of the isolate (20E) was performed by ITS/5.8S rRNA gene sequence analysis. It was confirmed that the isolate as *Aspergillus tubingensis*(MTCC Acc. No. 11398). ITS/5.8S rRNA gene sequence and phylogenetic Evolutionary relationship of taxa based on ITS sequence data of *Aspergillus tubingensis* were shown in figures 2.1(a) and 2.1(b).

Effect of different cereal flours on enzyme production

Addition of different cereal flours like finger millet, pearl millet, sorghum, sammai and horse gram to the fermentation medium, maximum enzyme activity was observed with pearl millet (163±0.6U/mg protein) and minimum with horse gram (107±0.45U/mg protein) by *Aspergillus tubingensis* at 30^0C temperature as shown in figure3(a).

Effect of incubation period

Incubation period varies with the type of enzyme production. Short incubation period offers potential for inexpensive production of enzyme (Sonjoy *et al.*, 1995). As shown in figure 3(b), incubation of fermentation medium with pearl millet as the carbon source at different time intervals 18, 36, 54, 72, and 90 hrs, maximum (215±0.53U/mg protein) and minimum (78±0.54 /mg protein) enzyme activity was observed within 54hrs and 18hrs respectively at 30^0C.

Effect of temperature

Incubation of fermentation medium at different temperatures (25^0C, 30^0C, 35^0C, 40^0C, and 45^0C) was carried out. Maximum (245±0.54U/mg protein) and minimum (67±0.6U/mg protein) amylase activity was observed at temperatures 35^0C and 25^0C respectively by *Aspergillus tubingensis* at 54hrs of incubation period as shown in figure 3(c).

Effect of different nitrogen sources on enzyme production

Ammonium nitrate, ammonium chloride, ammonium sulphate, and sodium nitrate were used as nitrogen sources separately along with fermentation media. Maximum (228±0.6U/mg protein) and minimum (149±0.54U/mg protein) amylase activity was observed in Ammonium nitrate and ammonium chloride respectively with *Aspergillus tubingensis* at 35^0C for 54hrs of incubation as shown in figure 3(d).

Effect of different concentrations of Phosphate

Incubation of fermentation medium with different concentrations of Phosphate (0.1%, 0.3%, 0.5%, 0.7%, 0.9%, and 1.4%) the maximum (226±0.54U/mg protein) and minimum (43±0.47U/mg protein) amylase activity was observed in 0.9% and 0.1% phosphate respectively with *Aspergillus tubingensis* at 35^0C for 54hrs of incubation as shown in figure 3(e).

Table.1.1 Physico chemical characteristics of the soil sample

Characteristics	Soil sample
Color	Brown
Odour	Normal
pH	7.0
Organic carbon(kg/*A)	68
Phosphorous(kg/*A)	0.67
Potassium(kg/*A)	0.62

*A=Acre

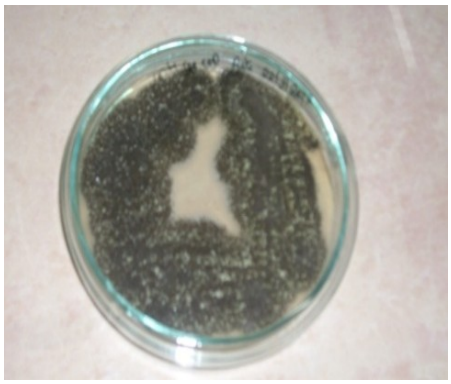
Table.1.2 Macroscopic and microscopic characteristics of fungal isolate (20E)

Parameter	Isolate
Colony color	Black
Colony diameter (cm)	2.0- 7.0
Hyphae	Septate branched
Spore arrangement	Bunch of spores
Spore shape	Round

Fig.1.2 Macro and Microscopic images of isolate (20E)

1.2.(a) isolate(20E)- Macroscopic image

1.2.(b) Isolate(20E)- SEM image



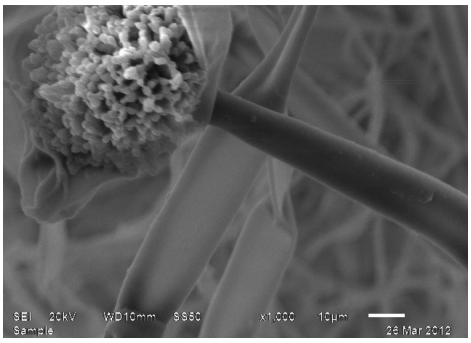


Fig.2.1 (a) *Aspergillus tubingensis*, Strain "(20E)", ITS/5.8S rRNA gene sequence data

TATAGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGGAAAATNGT
TGGAAAACGTCGGCGGCCCNGCCNTCCTATCNTNATTGGTTNTACTACTACCCCTTGA
GGGCTGCACCAGCTCCCTCTCTTTTTCCGGCCCCGCTACCTCAAAACTCCCTATTCCC
AAACCCACTCCCGTCNCTTATTGGTTCTATGCCTCTAAAAGCNNGACGCCCGTANTAC
CTGGGGGCGCANTGTGCGTTCAAAGACTCGATGATTCATTGAATTCTGCAATTCACAT
TAGTTATCGCATTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTG
AAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGGCTTTCAGACAGTGTTC
GTGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGGCAAAGGCGCCCCCCGGCGGCC
GACAAGCGGCGGGCCCGCCGAAGCAACAGGGTATAATAGACACGGATGGGAGGTTG
GGCCCAAAGGACCCGCACTCGGTAATGATATTTCCGCAG

Fig.2.2 (b) Evolutionary relationship of taxa based on ITS Sequence data

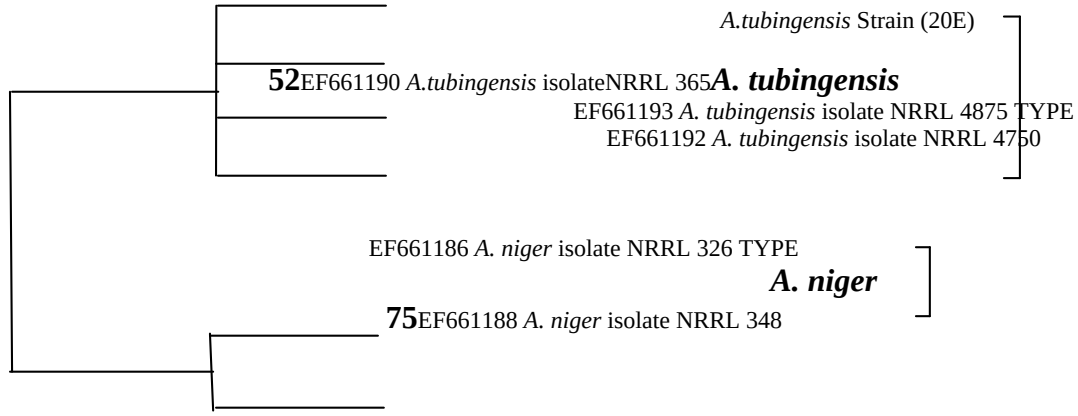
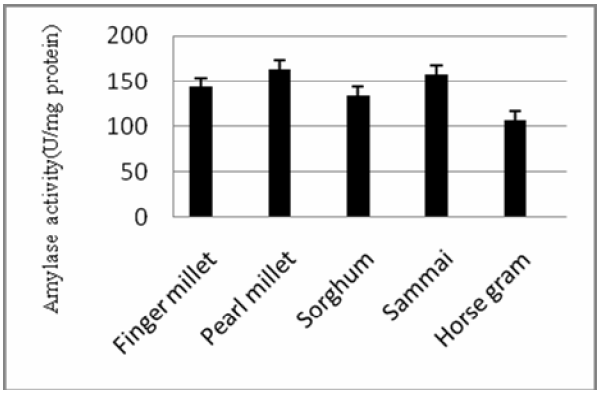
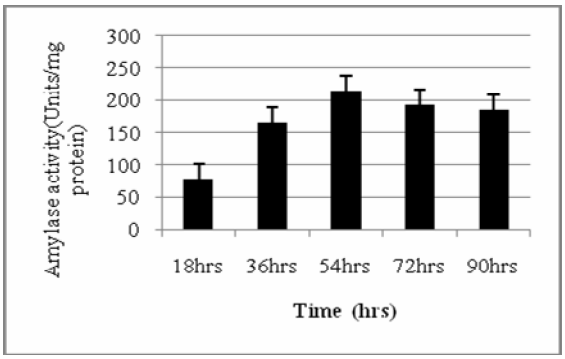


Fig.3(a) Effect of different cereal flours on amylase production



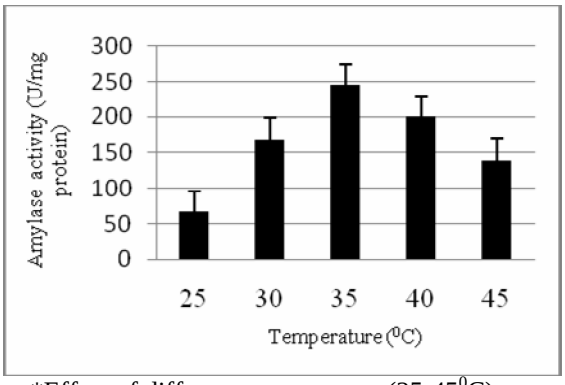
*Effect of different cereal flours on amylase production by *Aspergillus tubingensis* at 30^0C for 48hrs of incubation

Fig.3.(b) Effect of incubation period on amylase production



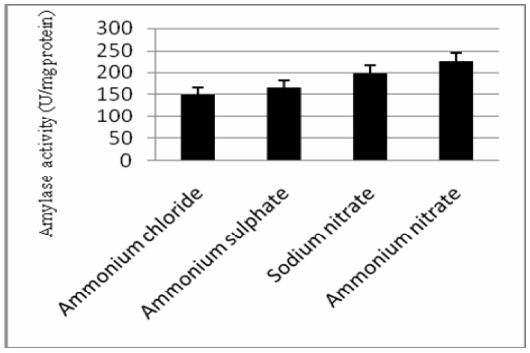
*Effect of different incubation periods (18-90hrs) on amylase production by *Aspergillus tubingensis*

Fig.3(c) Effect of different temperatures on amylase production



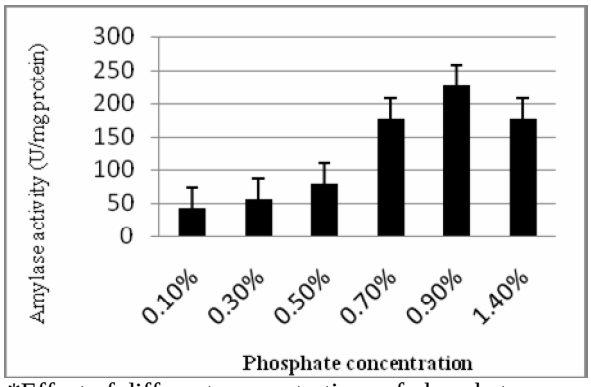
*Effect of different temperatures (25-45^0C) on amylase production by *Aspergillus tubingensis* with pearl millet at 54hrs of incubation

Fig.3(d) Effect of different nitrogen sources on amylase production



*Effect of different nitrogen sources on amylase production by *Aspergillus tubingensis* at 35^0C for 54.

Fig.3(e) Effect of different phosphate concentrations on amylase productions



*Effect of different concentrations of phosphate on amylase production by *Aspergillus tubingensis*at 54hrs of incubation.

Isolation of fungi from soil samples and the rapid screening by plating on starch agar plates led to finding of five fungal isolates capable of producing amylase. Morphological and molecular characterization confirmed the isolate (20E) as *Aspergillus tubingensis.* Different cereal flours used in submerged fermentation, maximum amylase activity (163±0.6U/mg protein) was observed in *Aspergillus tubingensis*with pearl millet at 30^0C for 48hrs of incubation. Gomes (2005) reported that amylase production was maximum (140U/mg protein) with sorghum flour with *Aspergillus niger*at72hrs of incubation period.

Incubation of fermentation medium at different time intervals, the maximum amylase activity (215±0.53U/mg protein) was observed at 54hrs in *Aspergillus tubingensis*. Dhurba Sharma and Shukla (2008) reported the maximum production of amylase (185U/ml) with *Aspergillus fumigatus* for 6 days of incubation at 30^0C. Incubation at different temperatures the maximum amylase activity (245±0.54U/mg protein) was observed at 35^0C by *Aspergillustubingensis*. Got (1998) reported maximum production of amylase (193U/ml) and glucoamylase (200U/ml) by *Aspergillus fumigatus* at 37^0C. By the effect of different nitrogen sources maximum activity of amylase (228±0.6U/mg protein) was observed in *Aspergillus tubingensis* with ammonium nitrate. It was reported that peptone, sodium nitrate and casein hydrolysate were good nitrogen supplements for amylase production in *Aspergillus fumigatus* by Got (1998), in *Aspergillus niger*by Pandey (1994) and in *Aspergillus oryzae* by Pederson and Nielson(2000).

With different phosphate concentrations, maximum amylase activity (226±0.54U/mg protein) was observed in 0.9% of phosphate concentration by *Aspergillus tubingensis* at 35^0C for 54hrs of incubation period.Nguyen*et al.*,(2000) reported that *Thermomyces lanuginosus* showed the maximum amylase activity (190U/mg) with 0.7% of phosphate concentration at 30^0C of incubation. Peixoto(2003) elicited same rate of amylase activity with *Rhizophusmicrosporus,* a thermotolarent fungi when compared with *Aspergillus fumigatus* at 0.5%of phosphate concentration.

References

Abou-Elela, G.M., El-Sersy, A.N.andWefky, S.H. 2009. Statistical optimization of cold adapted α-amylase production by free and immobilized cells of *Nocardiopsisaegyptia.J. Applied Sci. Res.*, 5: 286-292.

Abu,E.A., Ado, S.A., James, D.B. (2005). Raw starch degrading amylase production by mixed culture of *Aspergillus niger and Saccharomyces cerevisiae* grown on *Sorghum pombae*. *Afr.J.Biotechnol*., 4: 785-790.

Akpan, I., Bankole, M.O., Adesemowo, A.M., Latunde-Dada, G.O. (1999). Production

of amylase by *A. niger* in a cheapsolid medium using rice bran and Agricultural materials. Tropical Science 39: 77-79.

Alexander, M. (1961). In: Introduction to Soil microbiology, Wiley Eastern Ltd., New Delhi.

Barnett, H.L. and Hunter, B.B. (1972).Illustrated genera of fungi Imperfect. 3rdEdn, Burgess published company, Minneapolis,MN, USA, 331-339.

Bergquist, P.,Teo, V., Gibbs, M., Curach, N.and Nevalainen, K.,(2003).Recombinant bleaching enzymes from Thermophiles expressed in fungal hosts. In: Mansfield, S.D. Saddler, J.n (Eds). Applications of enzymes to lignocellulosics.*Amer.Chem.Soc.Sympos ium* series: 855, 435- 445.

Chapman, H.D., and Pratti, P.J. (1961).In: Method of analysis for soils, plants and water. University of California, Division of agricultural sciences.

Dhurba Sharma and ShuklaA.k. (2008). Starch hydrolysis and Amylase activity of *Aspergillus* and *Chaetomium.Asian Journal of Biochemistry*. 3: 284-289.

Domsch, K.W., Games, Anderson, T.H.(1980).Compendium of soil fungi. Academic press, London, ISBN-10:0122204018: 22-23.

Duochuan,L., Yijun,Y., Youliang,P., Chongyao,S., Peijin,Z., and Yicum,H. (1997). Purification and properties of a thermostable alpha amylase from thermophilic fungus,*Thermomyceslanugionosus*.Acta-*Microbiol*SIN., 37: 107-114.

Durghi, H.M. and Bottomley, P.J. (1983).Effect of acidity on the composition of an indigenous of *Trifoliumsbterraneum. L., App. Environmen. Microbiol.*,46: 1207-1213.

Feitkenhauer, H., (2003). Anaerobic digestion of desizing wastewater: influence of pretreatment and anionic surfactant on degradation and intermediate accumulation. *Enzyme and Microbial Technology*. 33(2-3):250–258.

Felsenstein, J. (1985). Confidence limits on phylogeneis: An approach using the bootstrap. *Evolution* 39: 783-791.

Giri, V.N., Mohan, A.R., Rao, L.V., Rao. C.P. (1990).Immobilization of α-amylase complex in detection of higher oligosaccharides on paper. *Current Science*.59:1339–1340.

Gomes, E., Souza, S.R., Grandi, R.P. and Silva, E.D. (2005). Production of thermostable glucoamylase by newly isolated *A.flavus*. 1.1 and*Thermomyceslanuginosus* A 13.37. *Braz. J. Microbiol*., 36: 75-82.

Got, C.E., Barbosa, E.P., Kistnera, C.L., Moreira, F.G., Lenartovicz, V. and Peralta RM. (1998).Production of amylase by *A.fumigatus*using methyl-D-glucoside, asynthetic analog of maltose, as substrate.*FEMS Microbiol*., 167: 139-143.

Gupta,R., Gigras,P., Mahapara,H., Goswani,V.K. and Chuhan,B. (2003). Microbial α-amylases: A *Biotechnological* prospective process *Biochem.*, 38: 1599-1616.

Jackson, M.A. (1997).Optimizing nutritional conditions for the liquid culture production of effective fungal biological control agents.*J.MicrobiolBiotechnol.*, 19: 180-187.

Kelman,A.(1967).*In*: Source book of laboratory exercises in plant pathology. Source book committee of American Phytopathological Society. W. H Freeman and Co., San Francisco, 180.

Kimura, M. (1980).A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111-120.

Nevalainen, H. and Teo, V.S.J. (2005).Enzyme production in industrial fungi.Role of Molecular Genetics. In: Arora D.K.(Ed.), *Applied Mycology and Biotechnology.*, 3, Fungal Genomics, Elesevier science 468-474.

Nguyen, Q.D, Rezessy-Szabo, J.M and Hoschke, A. (2000). Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyceslanuginosus* ATCC

34626.*Food Technol. Biotechnol.,* 38: 229-234.

Ortuno, A., Noguere, J., Hernansaez, A., Armero, T. (1977).Phosphoric metabolism of *Aspergillus niger* in calcareous and saline. *Microbiol.,* 30: 101-112.

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

Pederson, H. and Nielson, J. (2000). The influence of nitrogen sources on amylase productivity of *A.oryzae* in continuous cultures. *Appl.Microbiol.Biotechnol.,* 53: 278-281.

Peixoto, S.C., Jorge, J.A., Terenzi, H.F. and Polizeli,T.M. (2003). *Rhizopusmicrosporus* var. *rhizopodiformis*: A thermotolarent fungus with potential for production of thermostable amylases. Int. *Microbiol.,* 6: 269-273.

Prakasham,R.S., Rao,C.S., Rao,R.S., and Sarma,P.N. (2007).Enhancement of acid amylase production by an isolated *A.awarmori*. J. *Applied Microbiol*., 102: 204-211.

Saitou, N. and Nei, M. (1987).The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.

Saranraj, P and Stella, D. (2013).Fungal Amylase - A Review *International Journal of Microbiological Research* 4 (2): 203-211, 2013.

Sidkey,N.H., Abo Shadi,H.A., Al-Mutrafy,A.M., Sefergy,F., Al-Reheily,N., (2010). Screening of microorganisms is isolated from some enviro-Agro-Industrial wastes in Saudi Arabia for Amylase production. *J. Americ.Sci*., 6(10): 926-939.

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

Stevens, R.B.(1974).*In*: Mycology Guidebook. University of Washington Press, Seattle, 532.

Suganthi,R., Benazir,J.F., Santhi,R., Ramesh Kumar,V., AnjanaHari, Nidhiya,K.A., Kavitha,G., Lakshmi,R.,(2011). Amylase production by *Aspergillus niger* under solid state fermentation using agro industrial wastes.*International Journal of Engineering science and Technology.* 3(2): 1756-1763.

Tamura.K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*2731-2739.

Walkely, A.J. and Black, I.A. (1934).Estimation of soil organic carbon by Chromic acid titration method. *J.Soil*sci., 37: 29-38.

Wanwright, M. and Falih, A.M.K. (1995). In: An introduction to fungal Biotechnology.Wiley Chichester.

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