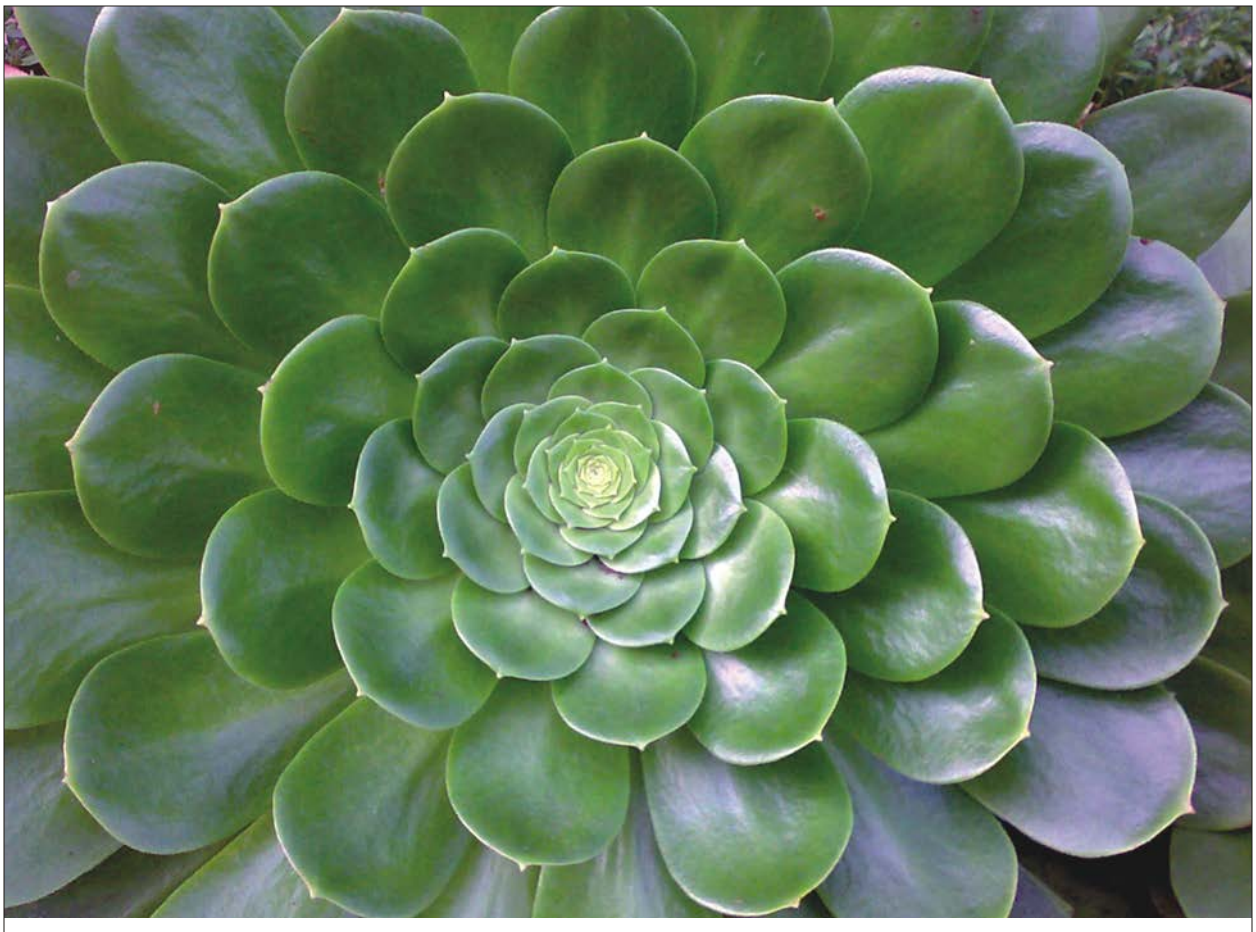

Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

Journal of Plant Sciences 10 (3): 99-107, 2015
ISSN 1816-4951 / DOI: 10.3923/jps.2015.99.107

Alpha-Amylase from *Rhizopus arrhizus* Fisher

[1]Adekunle Odunayo Adejuwon, [2]Adesola Adetutu Ajayi and [3]Olusola Abiola Ladokun
[1]Department of Microbiology, Faculty of Information Technology and Applied Sciences, Lead City University, Ibadan, Nigeria
[2]Department of Biological Sciences, Covenant University, Ota, Nigeria
[3]Department of Biochemistry, Faculty of Information Technology and Applied Sciences, Lead City University, Ibadan, Nigeria

Corresponding Author: Adekunle Odunayo Adejuwon, Department of Microbiology, Faculty of Information Technology and Applied Sciences, Lead City University, Ibadan, Nigeria

ABSTRACT

Amylases are enzymes that act on starch, converting it into sugars by their catalytic activities. They are highly rated amongst enzymes because of their numerous applications in industries. Microorganisms are the best source of amylase and they are advantageous because they can easily be exploited to produce the enzyme, α-amylase. This research work therefore, employed a strain of *Rhizopus arrhizus* fisher which was capable of growth on 1% flour medium for the production of α-amylase within ten days of incubation. α-Amylase activity was optimum on the 8th day of inoculation and was expressed as 933 U. The crude enzyme was partially purified by a combination of ammonium sulphate precipitation and dialysis. The activity of the partially purified enzyme was optimum at 30°C and pH 6.0. The enzyme was capable of degrading starch with an optimum activity expressed at 1.2 mg mL^{-1} concentration. It was stimulated by NaCl and $CaCl_2$ but inhibited by P-chloromercuric benzoate and Ethylene Diamine Tetraacetic Acid (EDTA). The enzyme was affected by heat with complete loss of activity within 10 min of heating at 70°C. *Rhizopus arrhizus* can therefore be suitably genetically modified and employed for the commercial production of α-amylase.

Key words: Amylase, *Rhizopus arrhizus*, flour medium

INTRODUCTION

Amylases are hydrolytic enzymes with their beneficial uses dating back to several decades (Sundarram and Murthy, 2014). Amylases are enzymes catalyzing the conversion of starch to sugar (Raul *et al*., 2014). They had been isolated from different microbial and plant sources and Bacillus species are the most widely used for commercial production of amylase (Sundarram and Murthy, 2014). *Bacillus subtilis* serves as a good source of α-amylase for industrial purposes (Sani *et al*., 2014). Alkaliphilic α-amylase from *B. subtilis* (A10) was isolated from orchard soils of Kahramanmaras in Turkey. The α-amylase was found to be suitable for waste-paper, starch and bioethanol industries (Aygan *et al*., 2014). The storage of stability of five α-amylase from miswak, (*Salvadora persica*) a medicinal plant which serves as a natural toothbrush was reported. It was discovered that the enzyme in the toothpaste retained 55% of its original activity after ten months of storage at room temperature (Mohamed *et al*., 2014). Adejuwon *et al*. (2013) in an earlier research reported that strains of *Candida albicans* and *Fusarium* sp. were involved in the production of α-amylase. Immobilization can improve the activity of α-amylase and its stability

when compared with free enzyme in solution without hampering the enzymatic reaction rate (Singh *et al*., 2014). This research was therefore, carried out with a view of obtaining α-amylase with appreciable activity from *R. arrhizus* fisher for industrial purposes.

MATERIALS AND METHODS

Source and identification of isolate: The isolate of *R. arrhizus* used for this research was isolated from mouldy bread and identified using techniques contained in the illustrated Handbook of Fungi (Hanlin, 1990; Cannon and Kirk, 2007). The identification was done by observing cultural and morphological characteristics. The isolate was cultured on Potato Dextrose agar. The nature of growth, rate of growth, colony colour and sporulation patterns were carefully observed. Sporulating mature cultures were used in microscopic examination. Fungal samples were taken from advancing margins and centres of the growth regions with the aid of sterile inoculating needle. The samples were smeared on glass slides and stained with lactophenol cotton blue. Macroscopic and microscopic morphological characteristics like arrangement and shape of spores, type of sporangia, type of hyphae and presence or absence of septa on hyphae were examined under the high power objective of a compound binocular microscope.

Culture conditions and preparation of inocula: The isolate was subcultured and maintained on Potato Dextrose agar plates and slants. The fungus was further subcultured into test tubes of the same medium and incubated at 25°C. Ninety-six hours-old cultures of *Rhizopus arrhizus* were used as the inoculum. According to the modified method of Olutiola and Ayres (1973), cultures were grown on 1% flour medium. Conical flasks (250 mL) containing 100 mL flour medium were inoculated with 1 mL of an aqueous spore suspension containing approximately 5×10^5 spores mL^{-1} of isolate. Experimental flasks contained the inoculated sterilized medium while control flasks contained only the sterilized uninoculated medium. Spores were counted using the Neubauer counting chamber (Olutiola *et al*., 1991). Experimental and control flasks were incubated without shaking at 25°C (Olutiola and Nwaogwugwu, 1982).

Flour as a source of carbon: The flour used in this study was obtained for Eagle Flour Mill Company, Ibadan, Nigeria. One gram of the flour was weighed into conical flask containing 100 mL distilled water. The contents of the flask were mixed by swirling and then sterilized at 15 Ib in^{-2} for 20 min. The flour medium were allowed to cool and then used as the growth medium and carbon source.

Enzyme and protein assays

α-amylase: α-amylase activity was determined using the method of Pfueller and Elliott (1969). The reaction mixtures consisted of 2 mL of 0.2% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0 as substrate and 0.5 mL of enzyme. Controls consisted of only 2 mL of the prepared substrate. The contents of both experimental and control tubes were incubated at 35°C for 20 min. The reaction in each tube was terminated with 3 mL of 1 N HCl. Enzyme (0.5 mL) was then added to the control tube. Two millilitre of the mixture from each of the sets of experimental and controls were transferred into new sets of clean test tubes. Three millilitre of 0.1 N HCl were added into the contents of each test tube after which 0.1 mL of iodine solution was added. Optical density readings were taken at 670 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 0.1% reduction in the intensity of the blue colour of starch-iodine complex under conditions of the assay. Specific activity was calculated as enzyme units per milligram protein.

Protein concentration determination: Protein concentration was routinely determined by the method of Lowry *et al.* (1951). The Lowry assay, a colometric protein assay, is based on the reaction of the protein with copper in alkali and the reduction of the phosphomolybdic-phosphotungstic reagent (Folin's reagent) by the copper treated protein. Absorbances of treated samples were taken at 600 nm. Serial dilutions of Bovine serum albumin treated likewise were used to plot a standard graph. The unknown protein value in each test sample was interpolated from the standard calibration graph.

Ammonium sulphate fractionation: The crude enzyme, on the day of optimum activity during samplings, was treated with ammonium sulphate (analytical grade) at 90% saturation (662 g L^{-1}). Precipitation was allowed to continue at 4°C for 24 h. The mixtures were centrifuged at 4,000 rpm for 30 min at 4°C using a high speed cold centrifuge (Optima LE-80K Ultracentrifuge, Beckman, USA) at the Central Laboratory, Obafemi Awolowo University, Ile-Ife, Nigeria. The supernatant was discarded. The precipitate was re-constituted in 0.2 M citrate phosphate buffer, pH 6.0. The protein contents were determined using the Lowry *et al.* (1951) method while α-amylase activity was determined using the method of Pfueller and Elliott (1969).

Dialysis: Using acetylated dialysis tubings (Visking dialysis tubings, Sigma) (Whitaker *et al.,* 1963) and a multiple dialyser, the enzyme preparation was dialysed under several changes of 0.2 M citrate phosphate buffer, pH 6.0 at 4°C for 24 h. The protein content of the dialysed enzyme was determined using the Lowry *et al.* (1951) method while α-amylase activity was determined using the method of Pfueller and Elliott (1969).

Properties of the partially purified enzyme: The effects of temperature, pH, salts and some chemicals on the activity of the partially purified α-amylase from *R. arrhizus* were investigated.

Effect of temperature: The substrate used was 0.2% (w/v) starch in 0.2 M citrate phosphate buffer, pH 6.0. The reaction mixture was 2 mL of substrate and 0.5 mL of enzyme. Incubation was at a range of 20-40°C for 1 h. α-amylase activity was there after determined.

Stability test at 70°C: The effect of heat (70°C) on the stability of the partially purified enzymes at different periods, 0, 2, 5, 10, 20, 25 and 30 min was examined. The activities of the heated enzymes were determined by incubating 0.5 mL of each enzyme with 2 mL of the citrate phosphate buffered 0.2% starch substrate (pH 6.0) at 35°C for 1 h, α-amylase activity was there after determined.

Effect of pH: The substrate used was 0.2% (w/v) starch in 0.2 M citrate phosphate buffer at different pH values ranging from pH 3.0-8.0. The reaction mixture was 2 mL of substrate and 0.5 mL of enzyme. Incubation was at 35°C for 1 h, α-amylase activity was then determined as described.

Effect of substrate concentrations: Different concentrations, 0.2-1.4 mg mL^{-1} starch (Sigma) in 0.2 M citrate phosphate buffer, pH 6.0 were used as substrates. The reaction mixture was 2 mL of substrate and 0.5 mL of enzyme, incubated at 35°C for 1 h, α-amylase activity was afterwards determined.

Effect of salts and some chemicals: The effects of NaCl and $CaCl_2$ at varying concentrations (0, 5, 10, 15, 20 and 30 mM) on the activities of the purified α-amylase were examined. Each salt was prepared in 0.2% starch in citrate phosphate buffer pH 6.0. The reaction mixture, 2 mL of substrate and 0.5 mL of enzyme, was incubated at 35°C for 1 h. α-amylase activity was then determined.

Different concentrations (0, 2, 4, 6 and 10 mM) each of p-chloromercuric benzoate and EDTA were prepared in 0.2% starch in citrate phosphate buffer, pH 6.0. These were used as substrates. α-amylase activity was determined as described.

RESULTS

The results of this investigation revealed that *Rhizopus arrhizus* fisher grew on 1% flour medium. Culture filtrate obtained after eight days of incubation exhibited appreciable α-amylase activity (Fig. 1).

Effect of temperature: α-amylase activity was affected by the temperature of the reaction medium. Optimum activity of the enzyme was observed at 30°C after which there was a gradual decline in the activity of the enzyme (Fig. 2).

Effect of pH: α-amylase activity increased slightly from pH 3.5 and it gradually increased to pH 6.0 after which there was a decline in the enzymes activity (Fig. 3).

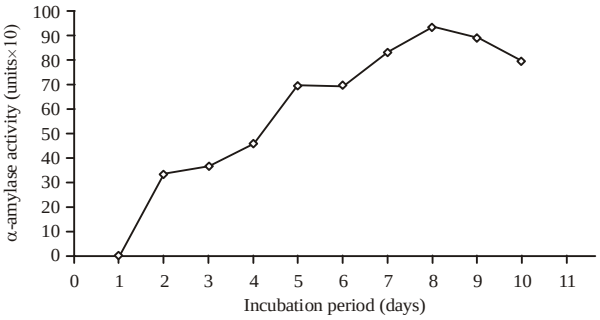

Fig. 1: Effect of days of incubation on α-amylase activity produced by *Rhizopus arrhizus* fisher in 1% flour medium

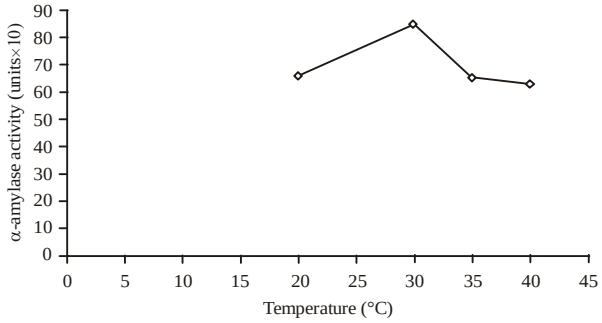

Fig. 2: Effect of temperature on α-amylase activity produced by *Rhizopus arrhizus* fisher

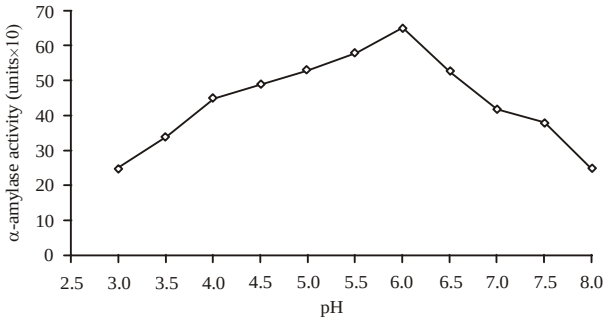

Fig. 3: Effect of pH on α-amylase activity produced by *Rhizopus arrhizus* fisher

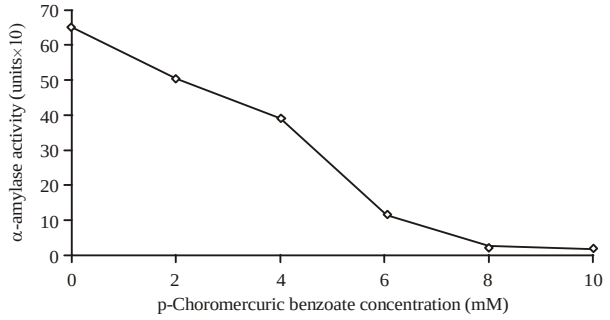

Fig. 4: Effect of p-chloromercuric benzoate on α-amylase activity produced by *Rhizopus arrhizus* fisher

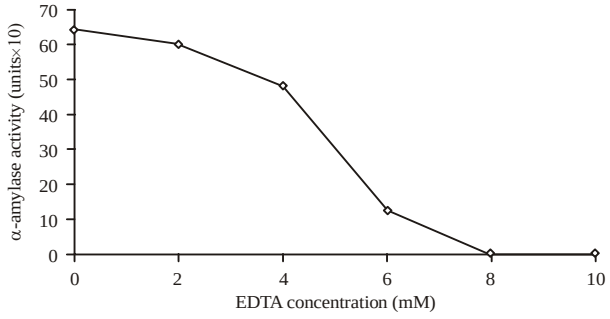

Fig. 5: Effect of EDTA on α-amylase activity produced by *Rhizopus arrhizus* fisher

Effects of chemicals: Enzymes activity decreased gradually with increasing concentrations of p-chloromercuric benzoate and EDTA (Fig. 4 and 5).

Effect of salts: α-amylase activity steadily increased with increasing concentrations of NaCl and $CaCl_2$ (Fig. 6 and 7).

Effect of substrate concentration: There was a gradual increase in amylase activity as the concentrations of substrate (starch) increased. Enzyme activity reached an optimum at 1.2 mg mL^{-1} starch concentrations after which there was a decline (Fig. 8).

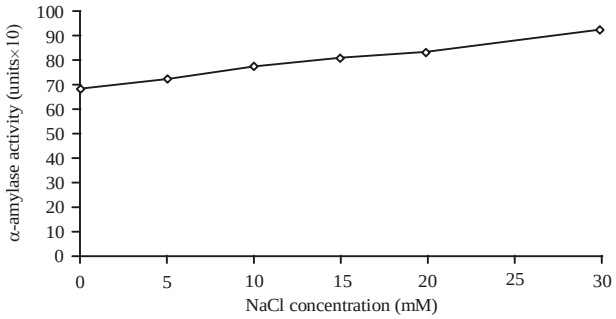

Fig. 6: Effect of NaCl on α-amylase activity produced by *Rhizopus arrhizus* fisher

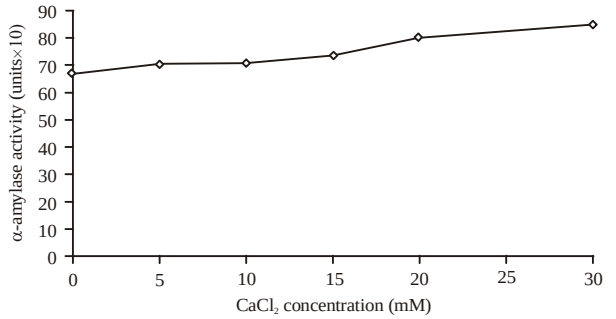

Fig. 7: Effect of $CaCl_2$ on α-amylase activity produced by *Rhizopus arrhizus* fisher

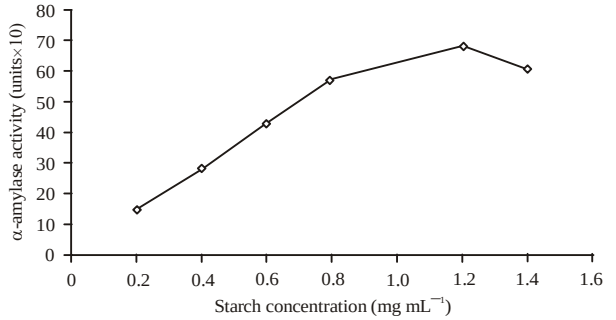

Fig. 8: Effect of starch concentration on α-amylase activity produced by *Rhizopus arrhizus* fisher

Effect of time of heating: Enzyme activity gradually decreased with increase in the time of heating the enzyme at 70°C. The reaction mixture at 0 min exhibited the highest enzyme activity after which there was a gradual decline. The enzyme was completely denatured after 5 min of heating at 70°C (Fig. 9).

DISCUSSION

The result of this investigation revealed that the fungal strain of *Rhizopus arrhizus* fisher used for this research was capable of growth on flour medium with the production of α-amylase within ten days of incubation. α-amylase activity expressed as 933 units was optimum on the eighth day

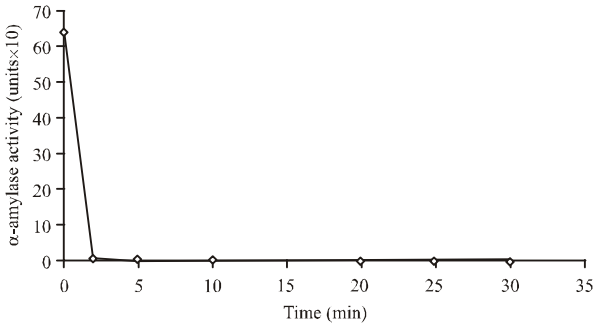

Fig. 9: Effect of heat (70°C) on α-amylase activity produced by *Rhizopus arrhizus* fisher

of inoculation with an optimum temperature of 30°C and pH 6.0. The enzyme activity was stimulated by NaCl and $CaCl_2$ but inhibited by P-chloromercuric benzoate and ethylene diamine tetraacetic acid. According to Pandey *et al*. (2000), most amylases have been produced from soil fungi such as *Aspergillus, Penicillum* and *Rhizopus*. Suganthi *et al*. (2011) using *A. niger* reported that the amylase activity was as high as 22.5 U mg^{-1} on the substrate, gingely oil cake at four days of incubation. The specific activity was 311 U mg^{-1} for the enzyme at six days of incubation in groundnut oil cake substrate. The specific activity was 86 U mg^{-1} for the enzyme at five days of incubation in Black gram bran substrate. For six days of incubation the specific activity was 311 U mg^{-1} which was higher than in any other substrates. In 7 days of incubation in Rice bran the specific activity was 65 U mg^{-1} and for eight days of incubation in coconut oil cake the specific activity was 112 U mg^{-1}. *Aspergillus fumigates* NTCC1222, showed high amylase activity (341.7 U mL^{-1}) at 6 days incubation (Singh *et al*., 2014). Rani (2012) also supported the finding on optimum temperature of amylase enzyme to be 30°C. Maximum amylase activity was recorded at 30°C and further increase in temperature resulted in decrease in activity of amylase (Alva *et al*., 2007). The pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. The pH is known to affect the secretion of α-amylase and its stability (Fogarty, 1983). The effect of initial pH values in the range of 4-7.5 on enzyme production was investigated and initial pH medium of 6.2±0.1 resulted in enhanced α-amylase production (Esfahanibolandbalaie *et al*., 2008). The optimum pH for amylase production was 6.0 (Fig. 3) which is similar to the findings of Patel *et al*. (2005) who reported in *A. oryzae*. The biomass yield was found to be higher in case of pH 5.0 contradictory to the findings of Olama and Sabry (1989) where the amylase activity and the biomass yield was maximum at pH 7.0 in *Aspergillus flavus* and *Penicillium purpurescence*. *Aspergillus oryzae*, *A. ficuum* and *A. niger* were found to give significant yields of α-amylase between pH 5.0-6.0 (Bhimba *et al*., 2011). In this research, the effect of pH on the enzyme activity indicates that the amylase was optimally active in the pH range of 5.5-6.5 with its peak, at 6.0. This suggests that the enzyme would be useful in processes requiring an acidic pH condition.

From the results of the effect of metal ions on total α-amylase activity, it was deduced that NaCl and CaCl increased the total α-amylase activity in comparison with the control, confirming previous reports which indicated that amylases are mostly metalloenymes and require calcium and manganese ions for its activity, structural integrity and stability (Gangadharan *et al*., 2006; Varalakshmi *et al*., 2009; Michelin *et al*., 2010). Irshad *et al*. (2012) reported that $CaCl_2$ was found to enhance the activity of α-amylase up to 92 U mL^{-1}, while EDTA, TEMED compounds had

inhibitory action to α-amylase activity with $AgNO_3$ being the strongest inhibitor. Rani (2012) also confirmed that NaCl act as activators to enhance the amylase activity in a reaction medium. According to Chung *et al*. (1995), the enzyme activity of amylase from *Thermococcus profundus* DT5432 was slightly stimulated in the presence of dithiothreitol (DTT), β-mercaptoethanol and 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB), indicating that cysteine residue (s) do not take part in catalysis. Also, the enzyme activation by dithiothreitol (DTT) β-mercaptoethanol and 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) could be attributed to the reduction in aggregate size by destroying the intermolecular disulfide linkages and/or by the protection of thiol groups that stabilize the three dimensional structure of enzyme (Khedher *et al*., 2008).

REFERENCES

Adejuwon, A.O., O.E. Abe, B.A. Bamkefa, A.A. Ajayi, K.O. Awojobi, M.A. Adejuwon and O. Ologbosere, 2013. α-amylases by strains of *Candida albicans* and *Fusarium* sp.: Expression and characterization. Rep. Opin., 5: 10-17.

Alva, S., J. Anupama, J. Savla, Y.Y. Chiu and P. Vyshali *et al*., 2007. Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. Afr. J. Biotechnol., 6: 576-581.

Aygan, A., S. Sariturk, S. Kostekci and H. Tanis, 2014. Production and characterization of alkaliphilic alpha-amylase from *Bacillus subtilis* A10 isolated from soils of Kahramanmaras, Turkey. Afr. J. Microbiol. Res., 8: 2168-2173.

Bhimba, B.V., S. Yeswanth and B.E. Naveena, 2011. Characterization of extracellular amylase enzyme produced by *Aspergillus flavus* MV5 isolated from mangrove sediment. Indian J. Nat. Prod. Resour., 2: 170-173.

Cannon, P.F. and P.F. Kirk, 2007. Fungal Families of the World. CAB International, Wallingford, UK., ISBN-13: 9780851998275, Pages: 456.

Chung, Y.C., T. Kobayashi, H. Kanai, T. Akiba and T. Kudo, 1995. Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. Applied Environ. Microbiol., 61: 1502-1506.

Esfahanibolandbalaie, Z., K. Rostami and S.S. Mirdamadi, 2008. Some studies of alpha-amylase production using *Aspergillus oryzae*. Pak. J. Biol. Sci., 11: 2553-2559.

Fogarty, W.M., 1983. Microbial Amylases. In: Microbial Enzymes and Biotechnology, Fogarty, W.M. (Ed.). Applied Science Publishers Ltd., London and New York, pp: 1-92.

Gangadharan, D., S. Sivaramakrishnan, K.M. Nampoothiri and A. Pandey, 2006. Solid culturing of *Bacillus amyloliquefaciens* for alpha amylase production. Food Technol. Biotechnol., 44: 269-274.

Hanlin, R.T., 1990. Illustrated Genera of Ascomycetes. 3rd Edn., American Phytopathological Society Press, Minnesota, USA., ISBN-13: 9780890541074, Pages: 263.

Irshad, M., Z. Anwar, M. Gulfraz, H.I. Butt, A. Ejaz and H. Nawaz, 2012. Purification and characterization of α-amylase from *Ganoderma tsuage* growing in waste bread medium. Afr. J. Biotechnol., 11: 8288-8294.

Khedher, I.B.A., P. Bressollier, M.C. Urdaci, F. Limam and M.N. Marzouki, 2008. Production and biochemical characterization of sclerotinia sclerotiorum α-amylase $scamy_1$: Assay in starch liquefaction treatments. J. Food Biochem., 32: 597-614.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Michelin, M., T.M. Silva, V.M. Benassi, S.C. Peixoto-Nogueira and A.L.B. Moraes *et al*., 2010. Purification and characterization of a thermostable α-amylase produced by the fungus *Paecilomyces variotii*. Carbohydr. Res., 345: 2348-2353.

Mohamed, S.A., Y.Q. Almulaiky, Y.M. Ahmed, O.A. Al-Bar and I.H. Ibrahim, 2014. Purification and characterization of α-amylase from Miswak *Salvadora persica*. BMC Complement. Altern. Med., Vol. 14. 10.1186/1472-6882-14-119

Olama, Z.A. and S.A. Sabry, 1989. Extracellular amylase synthesis by *Aspergillus flavus* and *Penicillium purpurescense*. J. Islamic Acad. Sci., 24: 272-276.

Olutiola, P.O. and P.G. Ayres, 1973. Utilization of carbohydrates by rhynchosporium secalis. I. Growth and sporulation on glucose, galactose and galacturonic acid. Physiologia Plantarum, 29: 92-96.

Olutiola, P.O. and R.I. Nwaogwugwu, 1982. Growth, sporulation and production of maltase and proteolytic enzymes in *Aspergillus aculeatus*. Trans. Br. Mycol. Soc., 78: 105-113.

Olutiola, P.O., O. Famurewa and H.G. Sonntag, 1991. An introduction to General Microbiology: A Practical Approach. Heidelberger Verlagsanslait Druckerei, Heidelberg Germany, Pages: 267.

Pandey, A., P. Nigam, C.R. Soccol, V.T. Soccol, D. Singh and R. Mohan, 2000. Advances in microbial amylases. Biotechnol. Applied Biochem., 31: 135-152.

Patel, A.K., K.M. Nampoothiri, S. Ramachandran, G. Szakacs and A. Pandey, 2005. Partial purification and characterization of α-amylase produced by *Aspergillus oryzae* using spent-brewing grains. Indian J. Biotechnol., 4: 336-341.

Pfueller, S.L. and W.H. Elliott, 1969. The extracellular α-amylase of *Bacillus stearothermophilus*. J. Biol. Chem., 244: 48-54.

Rani, K., 2012. Comparative study of kinetic parameters of bacterial and fungal amylases. J. Biol. Innov., 1: 48-57.

Raul, D., T. Biswas, S. Mukhopadhyay, S.K. Das and S. Gupta, 2014. Production and partial purification of α amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. Biochem. Res. Int. 10.1155/2014/568141

Sani, I., A. Abdulhamid, F. Bello, M. Yahaya and A.I. Bagudo, 2014. Isolation, partial purification and characterization of α-amylase from *Bacillus subtilis*. J. Microbiol. Biotechnol. Res., 4: 49-54.

Singh, S., S. Singh, V. Bali, L. Sharma and J. Mangla, 2014. Production of fungal amylases using cheap, readily available agriresidues, for potential application in textile industry. Biomed. Res. Int. 10.1155/2014/215748

Suganthi, R., J.F. Benazir, R. Santhi, V.R. Kumar and A. Hari *et al*., 2011. Amylase production by *Aspergillus niger* under solid state fermentation using agroindustrial wastes. Int. J. Eng. Technol., 3: 1756-1763.

Sundarram, A. and T.P.K. Murthy, 2014. α-amylase production and applications: A review. J. Applied Environ. Microbiol., 2: 166-175.

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

Whitaker, D.R., K.R. Hanson and P.K. Datta, 1963. Improved procedures for preparation and characterization of myrothecium cellulase: Part 2. Purification procedures. Can. J. Biochem. Physiol., 41: 671-696.