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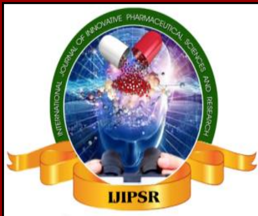

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THERMOSTABLE FUNGAL STRAINS HAVING BIOTECHNOLOGICAL IMPORTANCE PROVED BY HCL METHOD AT WIDE RANGE OF TEMPERATURES

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Abstract
Enzyme analysis of five microbial soil fungal strains like *Trichophyton terrestre, Penicillium camembertii*, *Cladosporium cladosporoides, Aspergillus niger k32* and *A. niger mtc872* were conducted. Activities of the enzyme amylase were evaluated by absorbance and the reaction rate shown by the enzyme in starch-iodine solution through HCL methods at wide range of temperatures from 0^0C to 80^0C. Enzyme reaction at different temperatures would determine at which temperature the enzyme worked most efficiently. Analyzing absorbance of the solutions with spectrophotometry would determine the reaction rate. The dominating characters like thermostability of the enzyme amylase extracted from *Trichophyton terrestre* worked much efficiently at wide range of temperatures.

Keywords: Fungal strains, *Trichophyton terrestre*, HCL Method, enzyme amylases, starch degradation, spectrophotometry

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INTRODUCTION

Soil fungi are prominent sources of enzymes like amylases, vitamins, pigments, lipids, glycolipids, polysaccharides and polyhydric alcohols. From the human perspective, the power of fungal enzymes is Janus-faced. In recent years the potential of using fungal strains as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms [1, 2]. Amylase is a class of enzymes that catalyze the hydrolysis of starch into smaller carbohydrate molecules such as maltose. Two categories of amylases, denoted alpha and beta, differ in the way they attack the bonds of the starch molecules. [3,4,5]. Amylases are one of the most important enzymes because of having great biotechnological importance and are considered a worthy class of industrial enzymes having approximately 25% of the world enzyme market [6]. Among two categories of enzyme amylase, α-amylase tends to be faster-acting than β-amylase and its optimum pH is 6.7–7.0 [7]. Many prominent biotechnological practices are also based on the catalytic power of fungal enzymes: baking, brewing, wine fermentation, production of certain cheeses, and the koji process are ancient examples of the way humans have employed fungi for their own benefit. Hydrolytic enzymes, amylases, glucoamylases, lipases, pectinases, and proteases are involved in the degradation of relatively simple biopolymers such as starch and protein have been purified, characterized, and utilized within industrial settings [8,9]. Amylases are one of the important and widely used enzymes whose spectrum of applications has widened in many sectors such as clinical, medicinal and analytical chemistry. A possible strategy to counteract the negative effect of cold on the activity of an enzyme could be to synthesize more enzymes, but it can be easily understood that this would be energetically expensive. Therefore, the common strategy used to maintain sustainable activity at a permanently low temperature is to produce a cold-adapted enzyme with enhanced catalytic efficiency k_{cat}/K_m [10]. For extracellular enzymes that work at saturating substrate concentrations, adaptation consists mainly of increasing k_{cat} [11,12,13]. Alternatively, for secreted marine enzymes or intracellular enzymes that could face low substrate concentrations, a decrease in K_m (Michaelis–Menten constant) providing a higher substrate affinity could be useful [14,15,16,17]. Production of fungal amylases has been investigated through submerged (SmF) and solid-state fermentation (SSF) [18,19,20]. SmF has been traditionally used for the production of industrially important enzymes because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture [21,22]. Filamentous fungi are suitable microorganisms for solid state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate [23].

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MATERIALS AND METHODS

Culture Condition

The strains of *Trichophyton terrestre, Penicillium camembertii*, *Cladosporium cladosporoides, Aspergillus niger k32* and *A. niger mtc872* used in this study were isolated from the soil of *Juglans regia L.* rhizosphere during the screening programme for amylase production [24]. Fungi were isolated as mono-cultures on three media: potato dextrose agar (PDA, Hi Media), malt-extract agar (Hi Media) and Czapek's agar (Hi Media). The spores of isolated fungal strains were inoculated in liquid Czapak Dox medium in 250 ml Erlenmeyer flasks containing 50 ml growth medium according to the given composition (g/l): sucrose 30; NaNO3 3; KH2PO4 1; MgSO4 ·7H2O 0.5; KCl 0.5; FeSO4 · 7 H2O 0.01 and yeast extract 1 at final pH 7.3. These liquid cultures were incubated for 4 days at 30^0C on a rotary shaker. The liquid culture was filtered through cheese cloth and the filtrate was used as source of enzymes.

Preparation of a BSA Standard Curve

500 mg of Bovine Serum Albumin (BSA) was dissolved in 50 ml of distilled water (10 mg/ml), from this stock solution 5 ml solution was dissolved in 50 ml distilled water (1 mg/ml) which was used as a working solution.

METHOD

5 ml of the alkaline solution was added to 1 ml of the test solution. After thoroughly mixing it was allowed to stand at the room temperature for 10 minute or longer. After the incubation period, 0.5 ml of diluted Folin-Ciocalteau reagent was added and immediately mixed. After 30 minutes reading was noted against the appropriate blank at 750_{nm}.BSA standard curve (fig 1) was made by using the experimental data (table 1).

Protein Estimation by Folin-Lowry Method

Protein content of the isolated fungi was estimated by using the Folin-Lowry method and optical density of each strain was compared with the BSA standard curve to calculate the amount of protein (mg/ml) present in the supernatant used in amylase and is given in table 2.

Assay of Amylases in Crude Extract by Using HCL Method

The activity of enzyme amylases was evaluated by the method based on the reduction in blue color intensity resulting from the enzyme hydrolysis of starch [25,26].

Procedure

Test tubes were set-up for the experiment and each was filled with 1 ml starch solution and 1 ml water.1 ml of supernatant taken from the harvested mycelium of all the strains used for the experiment was added to their respective test tubes. Test tubes containing starch solution and enzyme extract were incubated at wide range of temperatures (0^0C, 4^0C, 20^0C, 37^0C, 50^0C,

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70^0C and 80^0C) for 10 minutes. After the incubation period of 10 minutes, 0.1 ml of reaction mixture was taken from each and poured into new test tubes then reaction was terminated by the addition of 0.1N 1ml of HCl. Then 0.2 ml from this solution was added to 2 ml iodine reagent containing (0.05%) iodine and (0.5%) KI. The absorbance was measured at 720_{nm}. One unit of activity was defined as the amount of enzyme that reduces the intensity of blue color of starch iodine solution at the assay conditions.

Spectro Zero

0.1 ml water was added to 1 ml of HCl and 0.2 ml of the reaction mixture was added by 2 ml of iodine reagent. Spectro zero was used to set the spectrophotometer at zero absorbance.

Control

1 ml starch solution was added by 2 ml water and incubated. After incubation period 0.1 ml of the solution was add by 1ml HCl. 0.2 ml of this reaction mixture was added by 2 ml of iodine reagent. Optical density of the control was recorded at 720_{nm}.

Table 1: OD_{750} of different dilutions of BSA

Sl. No.	Standard Solution	Distilled Water	BSA Concentration (mg/ml)	Optical density
1	0.0	5.0	0.0	0.00
2	5.0	4.5	0.1	0.09
3	1.0	4.0	0.2	0.12
4	1.5	3.5	0.3	0.17
5	2.0	3.0	0.4	0.21
6	2.5	2.5	0.5	0.24
7	3.0	2.0	0.6	0.31
8	3.5	1.5	0.7	0.32
9	4.0	1.0	0.8	0.39
10	4.5	0.5	0.9	0.43
11	5.0	0.0	1.0	0.45

Table 2: Protein Estimation of five Fungal Strains by Using Folin-Lowry Method

Sl. No.	Fungal strains	Optical density	Protein content (mg/ml)
1	Trichophyton terrestre	0.09	0.16
2	Penicillium camemberttii	0.32	0.58
3	Cladosporium cladosporoides	0.11	0.22
4	Aspergillus niger k32	0.43	0.78
5	Aspergillus niger mtc872	0.48	0.87

Table 3: Starch Degradation (mg) by Enzyme Amylase of five Fungal Strains at wide range of Temperatures by Using HCL Method

Sl. No.	Fungal strain	0^0C	4^0C	20^0C	37^0C	50^0C	70^0C	80^0C
1	Trichophyton terrestre	0.8	1.4	3.5	7.9	5.5	1.1	0.0
2	Penicillium camembertii	2.1	3.3	4.8	5.5	4.2	0.3	0.0
3	Cladosporium cladosporoides	0.3	0.5	2.7	3.5	2.1	0.2	0.0
4	Aspergillus niger k32	0.9	2.9	3.5	4.4	3.0	0.4	0.0
5	Aspergillus niger mtc872	0.7	2.2	3.5	4.8	3.2	0.3	0.0

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Table 4: OD_{720} of different Concentrations of starch (mg/0.2ml) for starch standard curve

Sl. No.	Concentration of starch (mg/0.2ml)	Optical density of control	Optical density of Reaction mixture
1	0.2	0.25	0.20
2	0.4	0.38	0.32
3	0.6	0.55	0.45
4	1.0	0.68	0.56
5	1.6	0.70	0.60
6	2.0	0.85	0.70
7	3.0	0.88	0.70

Table 5: Amylase Activity (V/mg Protein) of five Fungal Strains

S No.	Fungal Strains	Starch degradation (mg)/ mg protein at wide range of temperatures						
		0^0C	4^0C	20^0C	37^0C	50^0C	70^0C	80^0C
1	T. terrestre	2.2	4.46	21.87	49.37	20.5	3.5	0.0
2	P. camembertii	2.7	5.67	8.25	9.46	4.5	1.1	0.0
3	C. cladosporoides	1.2	2.27	12.25	15.89	6.4	1.5	0.0
4	A. niger k32	1.5	3.71	4.48	5.63	2.2	0.2	0.0
5	A. niger mtc 872	1.1	2.50	3.99	5.47	2.0	0.1	0.0

Table 6: Starch Degradation by Enzyme Amylases (Starch Degraded; mg/0.2ml) of T. Terrestre at Different Substrate Concentrations

Sl. No.	Starch (mg/0.2ml)	Starch after incubation	Starch degraded (mg/0.2ml)
1	0.2	0.16	0.04
2	0.4	0.33	0.07
3	0.6	0.49	0.11
4	1.0	0.82	0.177
5	1.6	1.37	0.23
6	2.0	1.64	0.36
7	3.0	2.38	0.62

After the incubation period of 10 minutes, the amount of starch degraded by the enzyme amylase at different temperatures (0^0C, 4^0C, 20^0C, 37^0C, 50^0C, 70^0C and 80^0C) was calculated (table 3) by using the calculations of starch (1g) dissolved in 100 ml distilled water and thereby taking the optical density of control observed as 0.46 at 720_{nm}.

Starch Degradation Estimation

Amount of starch present after incubation= amount of starch in 1 ml of starch solution X O.D. of reaction mixture/O.D. of control.

Starch degraded = amount of starch in 1ml of starch solution – amount of starch present after incubation. The amount of starch degraded by the amylase of each strain at different temperatures has been represented graphically in Fig 2.

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Preparation of a Starch Standard Curve

Starch standard curve was prepared by using 2% starch solution. 1g of starch was added to 5ml water and final volume was made 50ml by adding further 45ml of boiling water (20mg/ml). The mixture was cooled down to room temperature. Serial dilution was made having different concentrations of starch (g/0.2ml).

0.1ml starch solution + 1.9ml water = 1mg/ml or 0.2mg/0.2ml.

0.2ml starch solution + 1.8ml water = 2mg/ml or 0.4mg/0.2ml.

0.3ml starch solution + 1.7ml water = 3mg/ml or 0.6mg/0.2ml.

0.5ml starch solution + 1.5ml water = 5mg/ml or 1mg/0.2ml.

0.8ml starch solution + 1.2ml water = 8mg/ml or 1.6mg/0.2ml.

Table 7: Amylase activity (V/mg protein) of *T. terrestre* at various concentration of starch substrate

Sl. No.	Concentration of starch mg/0.2ml	1/S	Amylase activity (V/mg protein)	1/V
1	0.2	5.00	1.25	0.80
2	0.4	2.50	2.18	0.45
3	0.6	1.66	3.43	0.29
4	1.0	1.00	5.53	0.18
5	1.6	0.62	7.18	0.13
6	2.0	0.50	11.25	0.08
7	3.0	0.33	19.37	0.05

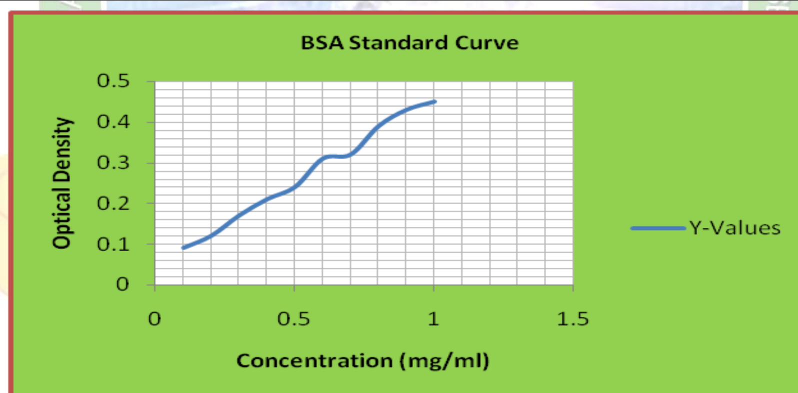

Fig. 1: BSA Standard Curve

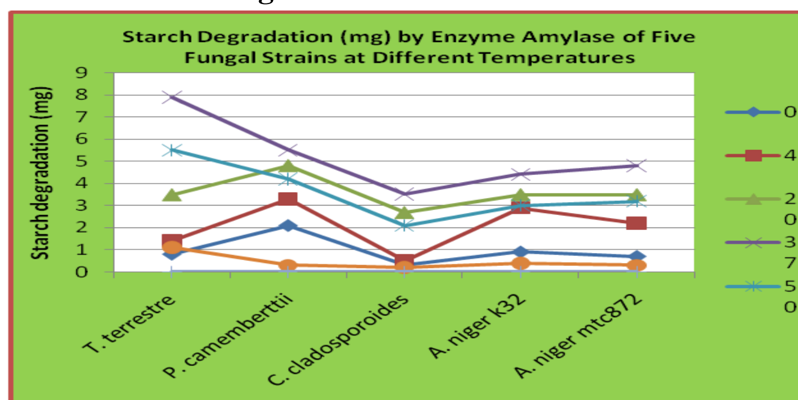

Fig. 2: Starch degradation by amylases of five fungal strains at different temperatures by using HCL method

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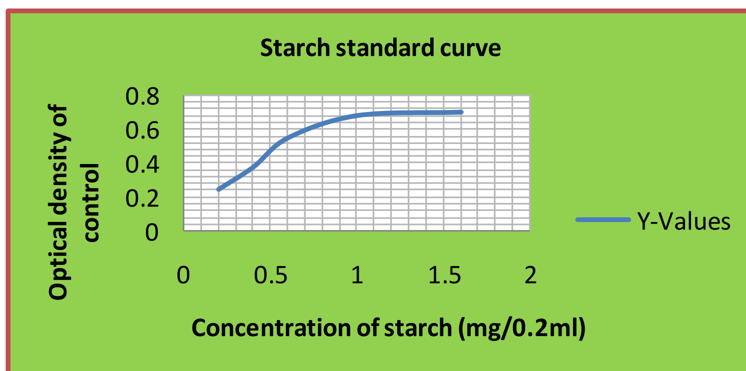

Fig. 3: Starch Standard Curve

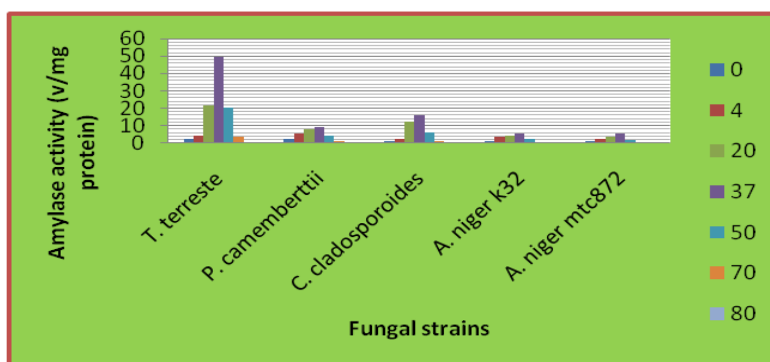

Fig. 4: Amylase activities (V/mg protein) in terms of starch degradation by five fungal strains at different temperatures

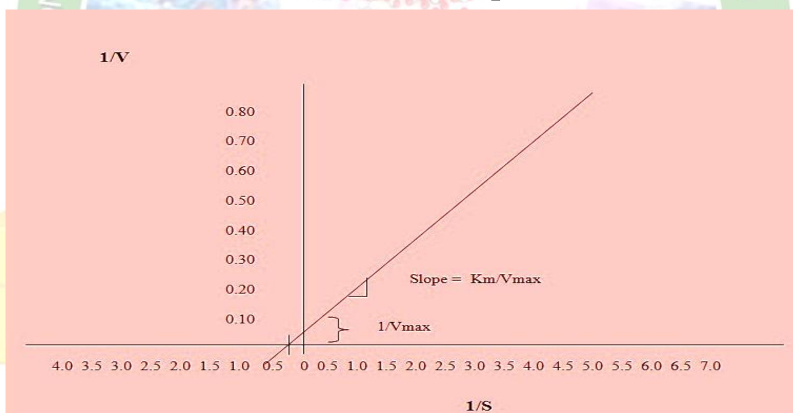

Fig 5: Km value of *Trichophyton terrestre* at different substrate concentrations.

RESULT & DISCUSSION

Specific Activity of Amylase

The amylase activities (starch degraded/mg protein/time) of all the five soil fungal strains at wide range of temperatures was estimated in terms of starch degradation/mg protein/time. It has been estimated and represented in the table 5 and fig 4.

KM DETERMINATION OF AMYLASES

Amylase in the crude extract was assayed as above in the presence of different substrate (starch) concentration. A graph of 1/V max Vs 1/S was plotted (Line-Weaver plot) and Km value was determined.

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Enzyme Kinetic Studies

For enzyme kinetic studies usually lasers are used through microscope to monitor changes in enzymes during catalytic reaction. The change in fluorescence of cofactors during enzyme reaction is recorded during the movement that occurs through catalysis [27]. These studies are providing a new view of the kinetics and dynamics of single enzymes, as opposed to traditional enzyme kinetics, which observes the average behaviour of populations of millions of enzyme molecules [28,29] . Although elementary statistical methods such as the calculation of means and their standard errors are commonly employed, application of the statistical methods of regression analysis in enzyme kinetic studies has received little attention. In estimating the kinetic parameters of the Michaelis - Menten equation [30], for instance, graphical methods such as the double-reciprocal plot [31] are generally used, without supplementary statistical calculations. These methods have been reviewed by Dixon & Webb [32]. Km (Michelis Menten constant): Km value of an enzyme is the finger print of that enzyme. It is the most important criterion to evaluate the enzyme for various uses. From the enzyme studies of different strains *Trichophyton terrestre* was found to yield the most active amylases. Hence this organism was selected for further kinetic characterization of the enzymes.

Starch Degradation by Amylase of *Trichophyton terrestre*

Starch degradation by the enzyme amylases extracted from *Trichophyton terrestre* at different starch-concentrations was observed. By calculating the amount of starch degraded by the enzyme amylase at different substrate concentrations, amylase activity (V/mg protein) was observed.

Km Value of Enzyme Amylase Isolated from *Trichophyton terrestre*

The enzyme kinetics was carried out at room temperature and pH 6.5. The kinetics of the enzyme was estimated by using Michealis Menten kinetics.

Michaelis Menten kinetics

$V = V_{max} S / K_m + S$

The graph was plotted between 1/V and 1/S (fig 5). The slope (Km/Vmax) was 0.12 and intercept (1/Vmax) 0.03 as found out. The maximum reaction rate (Vmax) was 33.34 and Michaelis Menten constant Km was 4.16.

CONCLUSION

The comparative study of enzyme amylases of *Trichophyton terrestre* to other isolates shows the efficiency of the enzyme and its thermostability. The activity (v/mg protein) of the enzyme amylase extracted from the soil fungus *T. terrestre* was recorded highest among all the

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species. The activity was measured over a wide range of temperature (0^0C, 4^0C, 20^0C, 37^0C, 50^0C, 70^0C and 80^0C). It was recorded maximum 49.37v/mg protein at its optimum temperature 37^0C. The enzyme amylase activity of *T. terrestre* was recorded decreasing by 41.52% and 17.2% from 37^0C to 50^0C and 70^0C respectively. Its activity at 70^0C was 3.5v/mg protein. Kinetically it also shows suitable Km value. All these properties qualify this fungal species to be used as a source of industrial amylases. Similarly at 0^0C amylase from *Penicilium camemberttii* showed highest activity 2.7v/mg among all the species. Its optimum temperature was also recorded as 37^0C where the enzyme activity was 9.46v/mg protein. The amylase from *P. camemberttii* showed a gradual decrease of 47% and 24.44% activity from 37^0C to 50^0C and 70^0C respectively [33].

REFERENCES

1. Akpan, I., Bankole, M. O., Adesemowo, A. M., Latunde-Dada, G. O. Production of amylase by A. niger in a cheap solid medium using rice band and agricultural materials. Trop. Sci.1999b, 39: 77-79.
2. Abu, E. A., Ado, S. A., James, D. B. Raw starch degrading amylase production by mixed culture of Aspergillus Niger and Saccharomyces cerevisae grown on Sorghum pomace. Afr. J. Biotechnol. 2005, 4(8): 785-790.
3. Gupta R., Gigras P., Mohapatra H., Goswami V.K., Chauhan B. Microbial α-amylases: a biotechnological perspective. Process Biochem. 2003, 38:1599–1616.
4. Kandra L. α-Amylases of medical and industrial importance. Journal of Molecular Structure (Theochem). 2003, 666–667.
5. Rajagopalan G., Krishnan C. Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresour Technol. 2008, 99:3044–3050. [PubMed].
6. Reddy N.S., Nimmagadda A., Sambasiva Rao K.R.S. An overview of the microbial α-amylase family. Afr. J. Biotechnol. 2003, 2:645–648.
7. "Effects of pH (Introduction to Enzymes)". worthington-biochem.com. Retrieved 17 May 2015.
8. Berka, R. M., N. Dunn-Coleman, and M. Ward. Industrial enzymes from Aspergillus species, 1992, p.155-2l4. In J. W. Bennett and M. A. Klich (ed.), Asperglllus. Biology and Industrial Application. Butterworth-Heinemann, Boston, Mass.
9. Bigelis, R. Food enzymes, 1992, p. 361-4l5. In D. B. Finkelstein and C. Ball (ed.), Biotechnology of Filamentous Fungi. Butterworth-Heinemann, Boston, Mass.

DOI: 10.21276/IJIPSR.2018.06.03.672
10. Feller, G. & Gerday, C. Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol. Life Sci. 1997, 53, 830–841.

11. Feller, G., Arpigny, J. L., Narinx, E. & Gerday, C. Molecular adaptations of enzymes from psychrophilic organisms. Comp. Biochem. Physiol.1997,118, 495–499.

12. Narinx, E., Baise, E. & Gerday, C. Subtilisin from psychrophilic antarctic bacteria: characterization and site-directed mutagenesis of residues possibly involved in the adaptation to cold. Protein Engng 10, 1997, 1271–1279.

13. Petrescu, I., Lamotte-Brasseur, J., Chessa, J. P., N tarima, P., Claeyssens, M., Devreese, B., Marino, G. & Gerday, C. Xylanase from the psychrophilic yeast Cryptococcus adeliae. Extremophiles 4, 2000, 137–144.

14. Kim, S. Y., Hwang, K. Y., Kim, S. H., Sung, H. C., Han, Y. S. & Cho, Y. J. Structural basis for cold adaptation. Sequence, biochemical properties, and crystal structure of malate dehydrogenase from a psychrophile Aquaspirillium arcticum. J. Biol. Chem. 1999, 274 (11) 761–11 767.

15. Bentahir, M., Feller, G., Aittaleb, M., Lamotte- Brasseur, J., Himri, T., Chessa, J. P. & Gerday, C.Structural, kinetic, and calorimetric characterization of the cold-active phosphoglycerate kinase from the Antarctic Pseudomonas sp. TACII18. J. Biol. Chem. 2000, 275, 11 147–11 153.

16. Georlette, D., Jonsson, Z. O., Van Petegem, F., Chessa, J., Van Beeumen, J., Hubscher, U. & Gerday, C. A DNA ligase from the psychrophile Pseudoalteromonas haloplanktis gives insights into the adaptation of proteins to low temperatures. Eur. J. Biochem. 2000, 267, 3502– 3512.

17. Hoyoux, A., Jennes, I., Dubois, P., Genicot, S., Dubail, F., Francois, J. M., Baise, E., Feller, G. & Gerday, C. Cold-adapted betagalactosidase from the Antarctic psychrophile Pseudoalteromonas haloplanktis. Appl. Environ. Microbiol. 2001, 67, 1529–1535.

18. Carlsen, M., Sphor, A.B., Nielsen, J. and Villadesn, J. Morphology and physiology of an @-amylase producing strain of Aspergillus oryzae during batch cultivation. Biotechnol Bioeng., 4, 1996, 266-276.

19. Francis, F., Sabu, A., Nampoothiri, K.M., Ramachandran, S., Ghosh, A., Szakacs, G. and pandey, A. 2003.

20. Pandey, A., Selvakumar, P., Soccl, C.R. and Nigam, P. Solid state fermentation for the production of industrial enzymes, Bioresource technology. 1999, 77: (1), 149-162.

DOI: 10.21276/IJIPSR.2018.06.03.672
21. Couto S.R., Sanromán M.A. Application of solid-state fermentation to food industry- A review. Journal of Food Engineering. 2006,76:291–302.

22. Gangadharan D., Sivaramakrishnan S., Nampoothiri K.M., Sukumaran R.K., Pandey A. Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*. Bioresour Technol. 2008, 99:4597–4602. [PubMed]

23. Rahardjo Y.S.P., Weber F.J., Haemers S., Tramper J., Rinzema A. Aerial mycelia of *Aspergillus oryzae* accelerate α-amylase production in a model solid-state fermentation system. Enzyme Microb. Technol. 2005, 36:900–902.

24. Domingues, C. M. and Peralta R. M. Production of amylase by soil fungi and partial biochemical characterization of amylase of a selected strain (Aspergillus fumigates Fresenius). Can. J. Microbiol. 1993, 39, 681–685.

25. Bajpai, P. High temperature alkaline amylase from Bacillus licheniformis TCRDCB13. Biotech. Bioeng.1989, 33: 72-78.

26. Teodoro, C. E. S., Martins, M. L. L. Culture conditions for the production of thermostable amylase by Bacillus sp. Brazil J. Microbiol. 2000, 31: 298- 302.

27. Xie XS, Lu HP . "Single-molecule enzymology". J. Biol. Chem. 1999, 274 (23): 15967–70. doi:10.1074/jbc.274.23.15967. PMID 10347141.

28. Lu H. "Single-molecule spectroscopy studies of conformational change dynamics in enzymatic reactions". Current pharmaceutical biotechnology. 2004, **5** (3): 261–9. doi:10.2174/1389201043376887. PMID 15180547.

29. Schnell J, Dyson H, Wright P. "Structure, dynamics, and catalytic function of dihydrofolate reductase". Annual Review of Biophysics and Biomolecular Structure. 2004, 33: 119–40. doi:10.1146/annurev.biophys.33.110502.133613. PMID 15139807.

30. Michaelis L. and Menten M.L. *Kinetik der Invertinwirkung* Biochem. Z. 1913, 49:333–369 English translation Accessed 6 April 2007.

31. Lineweaver, H. & Burk, D. J. Amer. chem. Soc.1934, 56, 658.

32. Dixon, M. & Webb, E. C. Enzymes. London: Longmans, Green and Co. Ltd.1958.

33. Tapan Kr. DUTTA, Malabendu Jana, Priti R. Pahari, Tanmay Bhattacharya. The Effect of Temperature, pH, and Salt on Amylase in Heliodiaptomus viduus (Gurney) (Crustacea: Copepoda: alanoida) Turk J Zool. 2006, 30: 187-195.