

1 Research Paper
2 **Isolation, Identification and Optimization of**
3 **Cultural Conditions of *Bacillus* sp. for Alkalo-**
4 **thermostable Amylase Production**

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ABSTRACT

Aims: To isolate thermostable and alkaline amylase producing bacteria and optimization of cultural conditions for maximum enzyme production

Study design: Optimization of different physiological and nutritional parameters on amylase production was studied.

Place and Duration of Study: Soil Samples: Herbal garden of Amity University Haryana, Gurgaon (Manesar), India, between April 2012 and September 2012.

Methodology: Amylolytic isolates were selected by flooding the nutrient agar plates containing 2% starch with Lugol solution. Isolates selected on the basis of higher ratio of clear zone to colony size were grown in nutrient broth containing 2% starch and the level of amylase was detected in culture filtrate. The selected isolate showing maximum amylase production was identified on the basis of 16S rDNA amplification.

Results: An alkalo-thermostable amylase producing bacterial isolate from soil was identified as *Bacillus* sp. on the basis of 16 S rRNA. It yielded 3.5 U mL^{-1} of amylase in medium containing (%) starch 2.0, beef extract 0.5, NaCl 0.5 at 50 °C, pH 7.0 at 180 rpm after 72 h. The optimum pH and temperature for amylase activity was 8.0 and 50 °C, respectively. The enzyme exhibited 67 % activity after 60 min incubation at 50 °C. At pH 8.0 the enzyme retained 78% activity after 4 h.

Conclusion: These properties made this enzyme its possible use in starch processing and baking industries.

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Keywords: Amylase, alkalo-thermostable, Bacillus sp., optimization, starch

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1. INTRODUCTION

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Amylases (E.C.3.2.1.1) constitute a class of enzymes which hydrolyze starch molecules to relinquish numerous products together with dextrans and increasingly smaller polymers composed of aldohexose units. These enzymes exhibit their potential applications in starch liquefaction, food, textile, paper and brewing industries [1]. Amylases have completely

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replaced chemical hydrolysis of starch in starch processing industry [2]. Around 25% of the enzyme market has been obtained from varied sources like plants, animals, microorganisms [2]. The microbial source of amylase meet the industrial demand because of economical bulk production capacity ,thermostability and the fact that microbes are easy to manipulate to produce the enzymes of desired characteristics [2, 3]. A broad range of microorganisms have been reported to produce amylase such as *Penicillium fellutanum*, *Bacillus subtilis* JS-2004, *Bacillus megaterium* and *Hallobacillus* sp. LY9 [4-6]. The thermostability of enzyme is desirable in many industrial processes such as bioprocessing of starch and the thermophilic organisms are better choice for production of thermostable enzymes [7]. Amylases with alkaline and thermophilic nature are a favorable choice due to the wealth of data currently available about this enzyme family. Alkaline amylases having pH values higher than 8.0 have been used in textile industries and also are an ingredient in detergents for automatic dishwashers and laundries [8]. Therefore, the potential of thermostable and alkaline amylases for industrial applications have prompted the search for microbial isolates expressing amylase activities with desired properties. In the present investigation, thermostable and alkaline amylase producing bacterial isolate identified as *Bacillus* sp., was optimized for maximum enzyme production.

2. MATERIAL AND METHODS

2.1 Screening and identification of amylase producing bacteria

A total of thirty bacterial isolates from soil were screened for amylase production on nutrient agar plate containing (%) soluble starch 2.0, yeast extract 0.3, peptone 0.5, NaCl 0.5, agar 2.0, pH 7.0 at 37 °C. Amylolytic isolates were selected by flooding the agar plates with Lugol solution [9]. Isolates selected on the basis of higher ratio of clear zone to colony size were grown in nutrient broth containing 2% starch and the level of amylase was detected in culture filtrate. The selected isolate showing maximum amylase production was identified as *Bacillus* sp. (accession No.KC533174) on the basis of 16 S rRNA sequencing and maintained by sub culturing after every fifteenth day.

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50 **2.2 Enzyme Production**

51 The nutrient broth medium for enzyme production comprised (%): soluble starch 2.0, yeast
52 extract 0.3, peptone 0.5, NaCl 0.5, pH 7.0. The medium (25mL) was inoculated with 1mL of
53 the inoculum with an optical density of 0.6 at 600 nm and incubated at 37 °C for 96 h.
54 Samples were withdrawn after a regular interval of 24 h and centrifuged at 10,000 rpm for
55 15 min in a Remi centrifuge (CPR-24). Cell free supernatant was used as the crude enzyme.
56 All the experiments were carried out in triplicates and results presented are the mean of
57 three values. The standard deviation was within 5%.

58 **2.3 Enzyme assay**

59 The amylase activity was assayed by adding 0.3 ml of culture filtrate, 0.7 ml of 0.5% starch
60 solution in 2 ml of 50 mM glycine-NaOH buffer, pH 8.0 and incubating at 60 °C for 20 min [6].
61 The total amount of reducing sugars in the reaction mixture was determined by following the
62 method of Miller, 1959 [10]. Absorbance was measured at 575 nm using a
63 spectrophotometer (Systronics 106). Maltose (1mg/ml) was used as reference standard.
64 Enzyme activity was expressed in units (micro moles of maltose released per milliliter per
65 minute).

66 **2.4 16S rDNA amplification and sequence analysis**

67 Bacterial genomic DNA was isolated following the protocol of Chen and Kuo [11]. The 16S
68 rDNA was selectively amplified from the genomic DNA by polymerase chain reaction (PCR)
69 using forward primer 5'-GAGAGTTTGATCCTGGCTCAG-3' and reverse primer 5' –
70 CTACGGCTACCTTGTTACGA-3' [12]. The amplified 16S rDNA gene fragments were
71 cloned into the pGEM-T vector (Promega, Madison, Wis.), transferred into *Escherichia coli*
72 [13] and sequenced using Applied Biosystems 3730 xL DNA analyzer. The 16S rDNA
73 sequence of the isolated strain was compared and analyzed by an existing gene bank of
74 NCBI (National Centre for Biotechnology Information).

75 **2.5 Effect of different physiological parameters on amylase production**

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76 Temperatures varying from 37 - 70 °C and pH in the range of 5.0 – 10.0, were examined for
77 their effects on amylase production .The pH of the medium was adjusted using 1N HCl or 1N
78 NaOH.

79 **2.6 Effect of different nutritional parameters on amylase production**

80 Various carbon sources such as maltose, wheat bran, rice bran and maize crush were used
81 as a replacement of soluble starch (2% w/v) in enzyme production medium to evaluate their
82 effect on amylase production. The best carbon source found was further optimized in the
83 range 1–5% (w/v) for maximum enzyme production. Peptone (0.5%) and yeast extract
84 (0.3%) in the production medium were replaced with nitrogen sources (0.5%) viz. tryptone,
85 beef extract, yeast extract and peptone individually. The best nitrogen source observed was
86 optimized in concentrations varying from 0.5 -3 %.

87 **2.7 Effect of agitation on amylase production**

88 The optimized enzyme production medium comprising 2% soluble starch,0.5% beef extract,
89 0.5% NaCl , pH 7.0 was inoculated with *Bacillus* sp. and incubated at 50 °C under shaking
90 conditions (180 rpm).

91 **2.8 Temperature Profile and stability of amylase**

92 The effect of temperature on amylase activity was determined by assaying enzyme activity
93 between 37 - 80 °C for 20 min. Thermostability of the enzyme was examined at an optimum
94 temperature of 50 °C for 20 – 120 min.

95 **2.9 pH Profile and stability of amylase**

96 The effect of pH on amylase activity was measured at different pH values by incubating the
97 reaction mixture for 20 min in 50 mM Glycine-NaOH buffer (pH 7.5 –10.0) at 50 °C. pH
98 stability was examined in the Glycine-NaOH buffer (pH 8.0) for 1-4 h.

99 100 **3. RESULTS AND DISCUSSION**

101 102 **3.1 Identification of bacterial isolate by 16S rDNA amplification and sequence** 103 **analysis**

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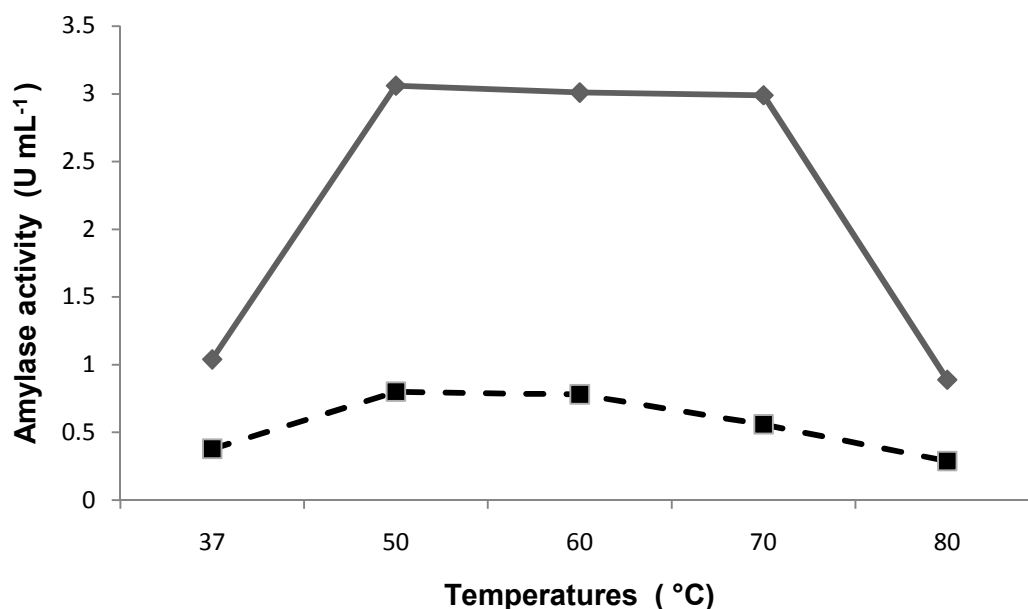
104 On the basis of level of amylase production , isolate producing a maximum of 0.53 U mL⁻¹ of
105 enzyme in 24 h was selected and studied for further detailed investigation. The selected
106 isolate was gram positive, rod-shaped, aerobic, catalase positive and spore forming. The 16
107 S rDNA of 1532 bases was sequenced and 16S r DNA sequence analysis placed bacterial
108 isolate belonging to the genus *Bacillus* sp. (accession no. KC533174).

109 **3.2 Effect of incubation temperature on amylase production**

110 Bacterial amylases are produced in broad range of temperatures. In present investigation,
111 *Bacillus* sp. was capable of producing amylase in a temperature range of 37 °C –70 °C with
112 maximum production at 50 °C (3.07U mL⁻¹). However, enzyme production declined with
113 increase in temperature beyond 50 °C (Fig. 1). Around three times increase in amylase yield
114 was observed at 50 °C compared to 37 °C, confirming the major role of temperature in
115 amylase production from this *Bacillus* sp. Similarly, De Souza Teodoro and Martins [14] and
116 Amoozegar et al. [15] reported the optimum temperature of 50 °C for amylase production
117 from *Bacillus* sp .and *Halobacillus* sp. strain MA-2, respectively. Results of temperature
118 range (37 °C –70 °C) are in accordance with the findings of amylase production from
119 various *Bacillus* sp. such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus*
120 sp.PN5 that have been reported to produce amylase at temperatures 37–70 °C [16-18].

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125 **Fig.1. Production of amylase (—◆—) at different temperatures and temperature profile**
 126 **of amylase activity (-■-)**

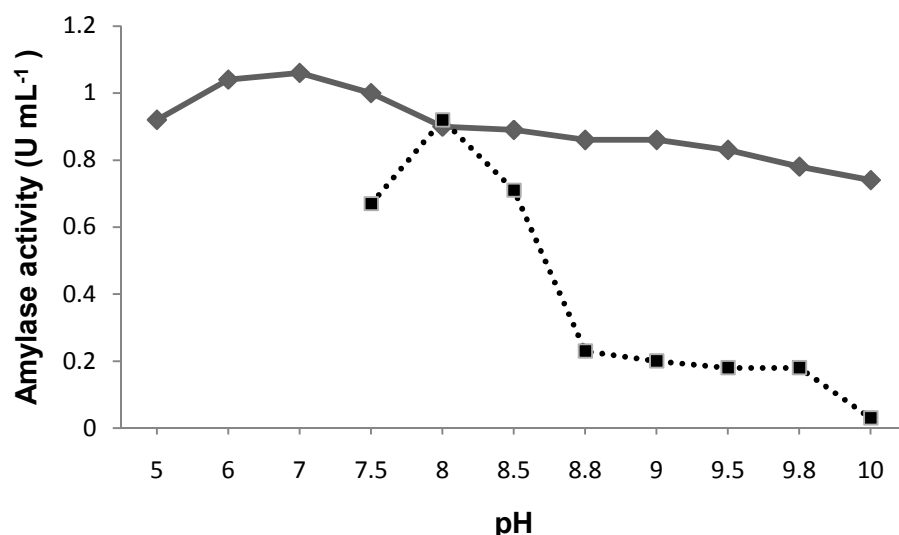
127 3.3 Influence of initial pH of the medium on amylase production

128 The culture pH affects the enzyme production and thus optimization of medium pH is
 129 essential for the maximum production of enzymes. Stimulation of amylase synthesis was
 130 observed with an increase in pH from 5 to 7 and maximum synthesis (1.06 U mL^{-1}) was
 131 achieved at pH 7.0 (Fig. 2). Similarly the studies on *Bacillus* sp. by De Souza Teodoro and
 132 Martins [14] and Sankaralingam et al. on *Bacillus licheniformis* [19] revealed the high
 133 level of amylase production at pH 7.0. Although, *Bacillus* sp. produced maximum level of
 134 amylase at pH 7.0 but a pH range of 5-10 also supported the amylase production. Similar
 135 observations in which most of the *Bacillus* strains used commercially for the production of
 136 amylases by submerged fermentation (SmF) have an optimum pH between 6.0 - 9.0 for
 137 enzyme production [3, 17-18].

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142 **Fig.2 Production of amylase (—◆—) at different pH values and pH profile of amylase**

143 **activity**

144 **(-■-)**

145 **3.4 Effect of carbon source on higher amylase yield**

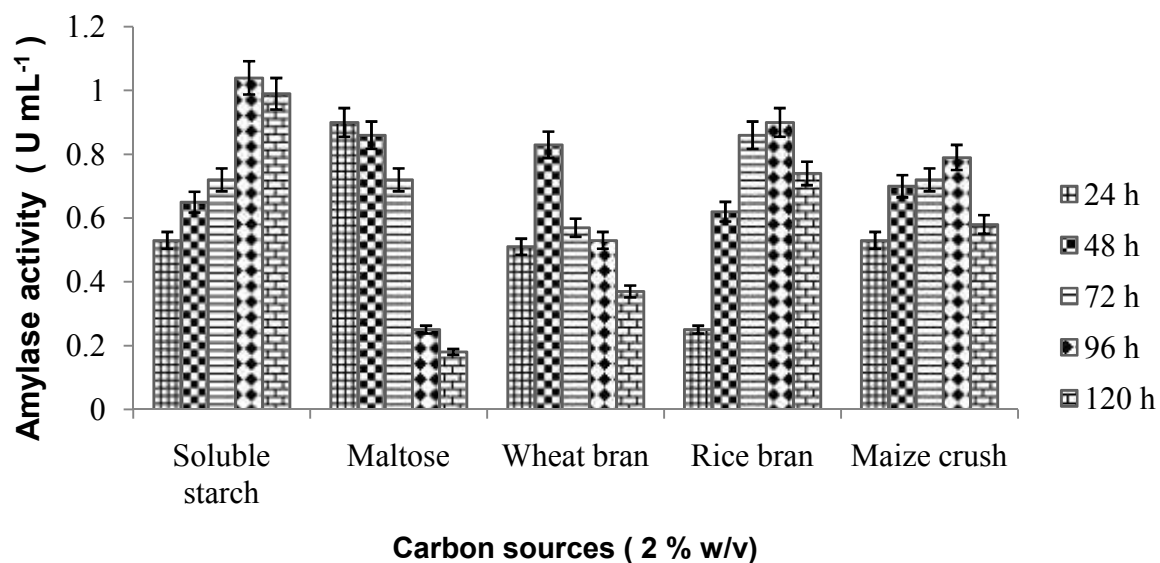
146 Carbon source greatly influences amylase production. In present study, *Bacillus* sp. was
 147 capable of utilizing all the carbon sources such as soluble starch, maltose, wheat bran, rice
 148 bran and maize crush that supported the amylase production. Different patterns of the
 149 amylase induction were obtained when these carbon sources were used in the production
 150 medium (Fig. 3). Results showed that amylase level was maximum (1.04U mL⁻¹) on starch
 151 followed by maltose and rice bran (0.9U mL⁻¹), wheat bran (0.83U mL⁻¹) and maize crush
 152 (0.80U mL⁻¹). The data is in agreement with the reports of Narang and Satyanarayana [20]
 153 and the results of Karataş et al. [21] who reported that soluble starch was the best carbon
 154 source for amylase production. However, amylase has been produced from many *Bacillus*
 155 strains using various carbon sources including different agricultural by-products [20, 22-23].
 156 Starch at 2% concentration induced the maximum amylase production (Fig. 4). At higher

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157 concentration amylase production declined. This can be because of high viscosity of broth at
 158 such concentrations, interfering with oxygen transfer leading to a limitation of dissolved
 159 oxygen for the growth of bacteria as the studies of Malhotra et al. [24] reported the maximum
 160 amylase activity at 2% starch concentration.

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165 **Fig.3. Amylase production from *Bacillus* sp. on different carbon sources**

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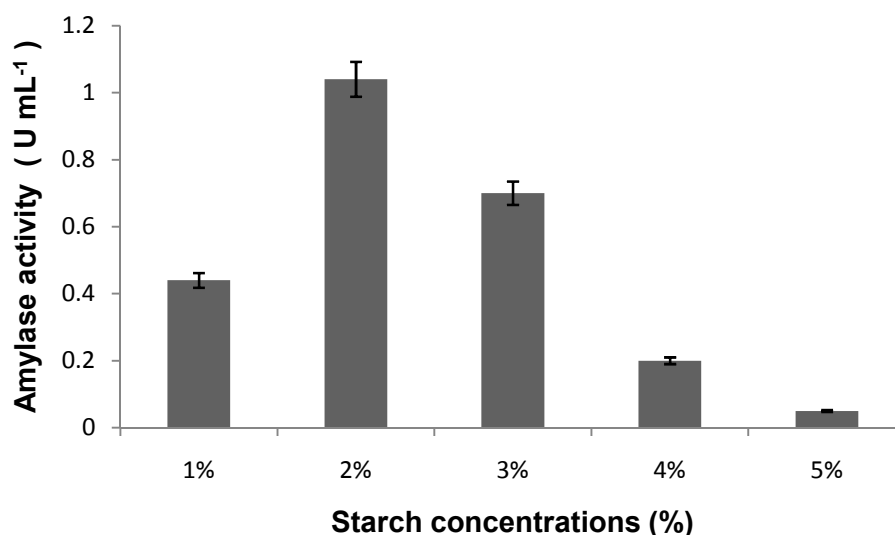


Fig.4 Amylase production from *Bacillus* sp. on different starch concentrations

3.5 Effect of supplementation of nitrogen sources on amylase production

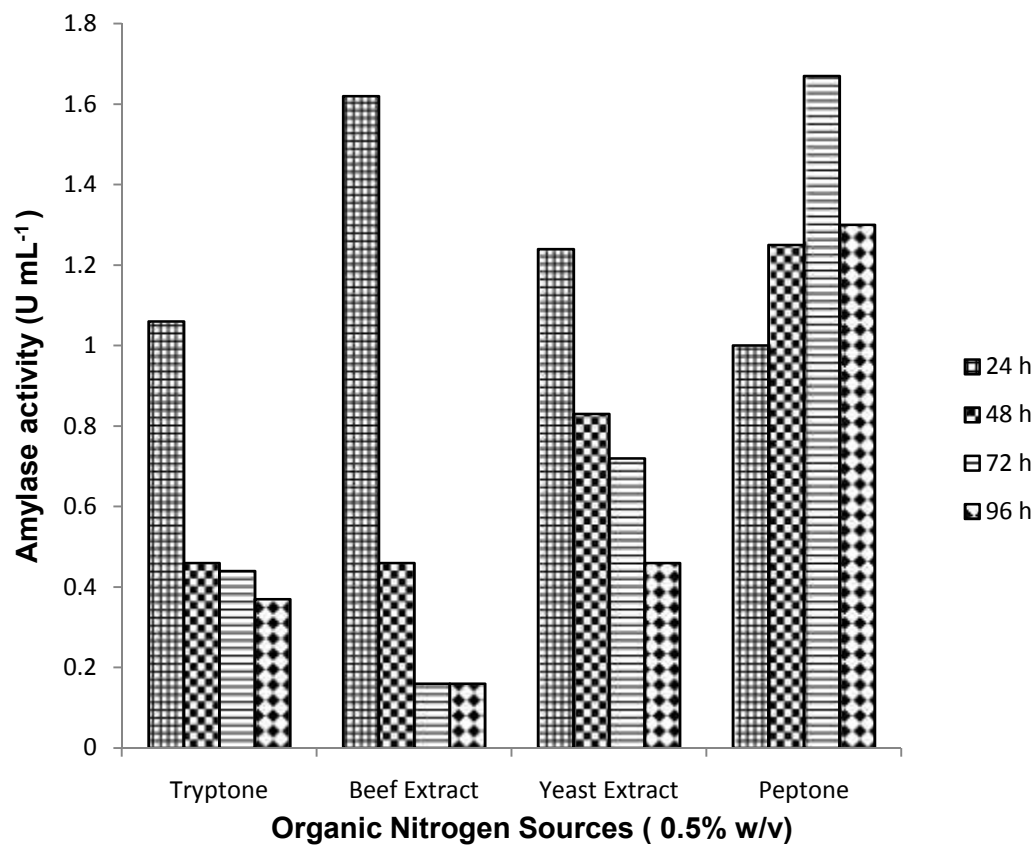
Among the different organic nitrogen sources viz. yeast extract, tryptone, beef extract and peptone, amylase production was achieved almost in equal levels with beef extract (1.62 U mL⁻¹) and peptone (1.67 U mL⁻¹) as represented in Fig. 5. However, *Bacillus* sp. gave maximum amylase yield (1.62 U mL⁻¹) within 24 h in production medium supplemented with beef extract compared to peptone which produced 1.67 U mL⁻¹ of amylase after 72 h. Around 1.55 times increase in amylase level was evaluated in beef extract supplemented medium. Using beef extract as an organic nitrogen source, enhanced levels of amylase from *Geobacillus thermodenitrificans* HRO10 [9], *Bacillus brevis* MTCC 7521 [25] and *Streptomyces erumpens* MTCC 7317 [26] have been reported. However, higher concentrations of beef extract resulted in a sharp fall in production levels. (Fig. 6). Similar trend of declined production of amylase by *Bacillus coagulans* B49 in the presence of increasing concentrations of yeast extract was observed by Babu and Satyanarayana [27]. The decrease in amylase production at higher concentrations of yeast extract could be due

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185 to the lowering of pH or the induction of proteases resulting in destruction of amylolytic
186 enzymes.

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191 **Fig.5 Amylase production from *Bacillus* sp. on different nitrogen sources**

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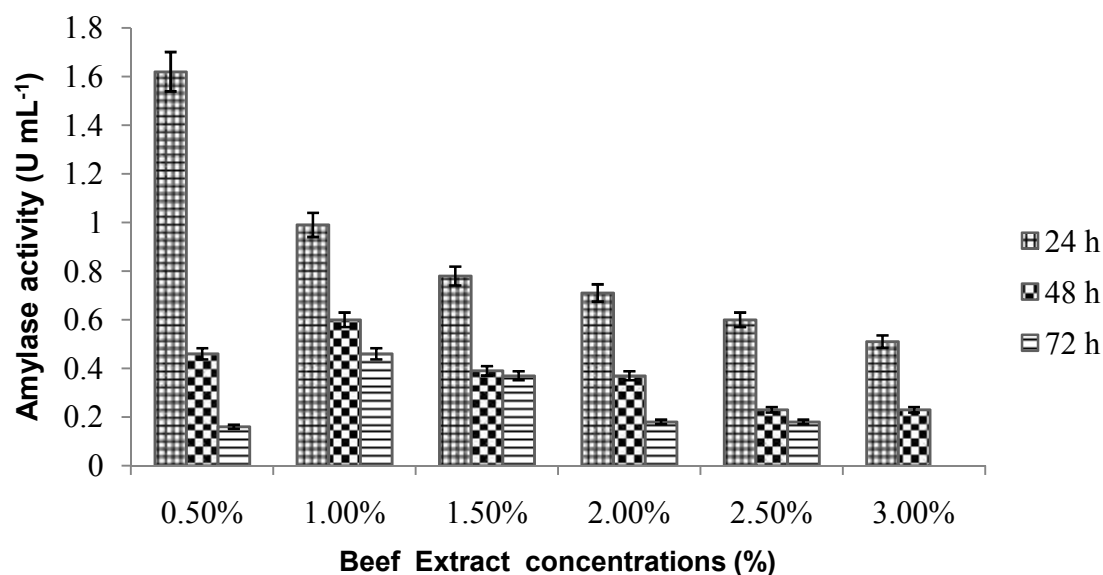


Fig.6. Amylase production from *Bacillus* sp. on different concentrations of beef extract

3.6 Production of amylase under shaking conditions

In this study, *Bacillus* sp. produced amylase both under static and shaking conditions. However, bacterium yielded 3.5 U mL^{-1} of amylase at 180 rpm after 72 h at 50°C . Two times increase in amylase level was detected under shaking conditions compared to static (1.62 U mL^{-1}) indicating the aerobic nature of the bacterium. Agitation intensity influences the mixing and oxygen transfer rate in many microbial fermentations, and thus influences the morphology and product formation [28]. Agitation rates in the range of 100 to 250 rpm have been reported for the production of alpha amylases [9, 29-30].

3.7 Effect of temperature on amylase activity and thermostability of enzyme

As starch liquefaction is generally performed at higher temperatures of $70\text{--}90^\circ\text{C}$, the thermostable amylases are of great significance. In this study amylase produced by *Bacillus* sp. showed considerable enzyme activity in the ranges from lower to higher temperatures as shown in Fig.1. At 70°C , the enzyme activity was 70 % compared to the optimum enzyme activity at 50°C indicating the range of amylase activity from $50\text{--}70^\circ\text{C}$. A reduction in

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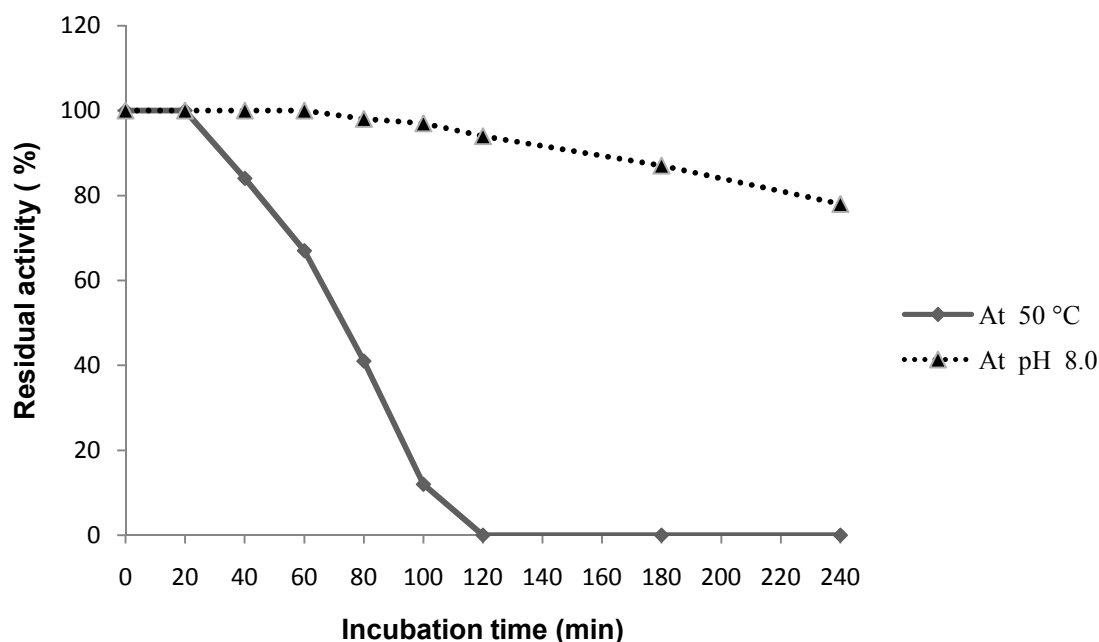
212 enzyme activity was observed at temperatures above 70 °C. The data suggest a less
213 sensitivity of enzyme to temperature especially, in the range of temperature between 50-70
214 °C. The amylase retained around 67 % residual activity on incubation at 50 °C for 60 min
215 that decreased with increased incubation time and was completely lost at 120 min (Fig. 7).
216 This property encourages its application in processes which require complete inactivation of
217 the enzyme, such as in the baking industry [31]. Amylases working in range of 40 – 80°C
218 have been reported [5, 23]. Our results are in accordance with the results of De Souza
219 Teodoro and Martins [14] and Amoozegar et al. [15] , who reported 50 °C to be the optimal
220 temperature for amylase activity.

221 **3.8 Effect of pH on amylase activity and stability of enzyme**

222 The effect of pH on amylase activity has been shown in (Fig.2). The enzyme was found to
223 have an optimum pH of 8.0. The enzyme activity at pH 8.5 and 8.8 were around 77 % and
224 25 % of that at pH 8.0, respectively. Further increase in the initial pH resulted in decreased
225 activity of enzyme. The enzyme showed stability for 1-4 h on incubation of crude enzyme
226 solution at pH 8.0 and retained 78 % activity even after 4 h (Fig.7). The amylases related to
227 *Bacillus* genus have shown optimum activities at pH values as low as 3.5 or as high as 12 [5,
228 32].

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233 **Fig.7. Thermostability (—) and pH stability (···▲···) of amylase at different**
 234 **incubation**

235 **times (min)**

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237 **4. CONCLUSION**

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239 Many of the amylases reported were thermostable but not alkaline active, whereas some of
 240 them were alkaline active but not thermostable. But the amylase from *Bacillus* sp. in our
 241 present study was thermostable (50°C) and also alkaline active (pH 8.0) suggesting its
 242 application in starch processing and baking industries.

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248 **AUTHORS' CONTRIBUTIONS**

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250 'Author A' performed the experiments related to the production of amylase, 'Author B'
 251 identified the the bacterium as *Bacillus* sp. on the basis of 16S rDNA sequencing , 'Author

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C' managed the literature searches and 'Author D' designed the study, managed the analyses of the study and wrote the manuscript. All authors read and approved the final manuscript."

REFERENCES

1. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D et al. Advances in microbial amylases. *Biotechnol Appl Biochem*. 2000; 31: 135-152.
2. Souza PM, Magalhães PO. Application of microbial α -amylase in industry-a review. *Braz J Microbiol*. 2010; 41: 850-861.
3. Burhan A, Nisa U, Gokhan C, Omer C, Ashabil A, Osman G. Enzymatic properties of a novel thermophilic, alkaline chelator resistant amylase from an alkalophilic *Bacillus* sp. isolate ANT-6. *Process Biochem*. 2003; 38: 1397–1403.
4. Kathiresan K, Manivannan S. α -Amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. *Afr J Biotechnol*. 2006; 5: 829-832.
5. Asgher M, Asad MJ, Rahman SU, Legge RL. A thermostable α -amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J Food Engin*. 2007; 79: 950–955.
6. Li Xin, Yu HY. Extracellular production of beta-amylase by a halophilic isolate, *Halobacillus* sp. LY9. *J Ind Microbiol Biotechnol*. 2011; 38: 1837–1843.
7. Adeyanju MM, Agboola FK, Omafuvbe BO, Oyefuga OH, Adebawo OO. A thermostable extracellular α - amylase from *Bacillus licheniformis* isolated from cassava steep water. *Biotechnol*. 2007; 6: 473-480.
8. Kim TU, Gu BG, Jeong JY, Byun SM, Shin YC. Purification and characterization of a maltotetraose forming alkaline α -amylase from an alkalophilic *Bacillus* sp. GM8901. *Appl Environ Microbiol*. 1995; 61: 3105–3112.
9. Ezeji TC, Wolf A, Bahl H. Isolation, characterization, and identification of *Geobacillus thermodenitrificans* HRO10, an α -amylase and α -glucosidase producing thermophile. *Can J Microbiol*. 2005; 51: 685-693.

- 282 10. Miller GL. Use of dinitrosalicylic reagent for determination of reducing sugar. Anal
283 Chem. 1959; 31: 426-428.
- 284 11. Chen WP, Kuo TT. A simple and rapid method for the preparation of gram-negative
285 bacterial genomic DNA. Nucleic Acids Res. 1993; 21: 2260.
- 286 12. Srivastava S, Thakur IS. Biosorption and biotransformation of chromium by *Serratia*
287 sp. isolated from tannery effluent. Environmental Technol. 2012; 33: 113–122.
- 288 13. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd
289 ed. New York: Cold Spring Harbor Laboratory Press; 1989.
- 290 14. Teodoro CED, Martins MLL. Culture conditions for the production of thermostable
291 amylase by *Bacillus* sp. Braz J Microbiol. 2000; 31: 298-302.
- 292 15. Amoozegar MA, Malekzadeh F, Malik KA. Production of amylase by newly isolated
293 moderate halophile, *Halobacillus* sp. strain MA-2. J Microbiol Methods. 2003; 52:
294 353-359.
- 295 16. Syu MJ, Chen YH. A study on the α -amylase fermentation performed by *Bacillus*
296 *amyloliquefaciens*. Chem Eng J. 1997; 65: 237–247.
- 297 17. Swain MR, Kar S, Padmaja G, Ray RC. Partial characterization and optimization of
298 production of extracellular alpha-amylase from *Bacillus subtilis* isolated from
299 culturable cow dung microflora. Pol J Microbiol. 2006; 55: 289-96.
- 300 18. Saxena RK, Dutt K, Agarwal L, Nayyar P. A highly thermostable and alkaline
301 amylase from a *Bacillus* sp. PN5. Biores Technol. 2007; 98: 260-265.
- 302 19. Sankaralingam S, Shankar T, Ramasubburayan R, Prakash S, Kumar C.
303 Optimization of culture conditions for the production of amylase from *Bacillus*
304 *licheniformis* on submerged fermentation. American-Eurasian J. Agric. & Environ.
305 Sci. 2012; 12: 1507-1513.
- 306 20. Narang S, Satyanarayana T. Thermostable α -amylase production by an extreme
307 thermophile *Bacillus thermooleovorans*. Lett Appl Microbiol. 2001; 32: 31–35.

- 308 21. Karataş H, Uyar F, Tolan V, Baysal Z. Optimization and enhanced production of α -
 309 amylase and protease by a newly isolated *Bacillus licheniformis* ZB-05 under solid-
 310 state fermentation. Ann Microbiol. 2013; 63: 45-52.
- 311 22. Ikram-ul-Haq, Ashraf H, Iqbal J, Qadeer MA. Production of alpha amylase by
 312 *Bacillus licheniformis* using an economical medium. Bioresour Technol. 2003; 87:
 313 57–61.
- 314 23. Anto H, Trivedi U, Patel K. Alpha Amylase Production by *Bacillus cereus* MTCC
 315 1305 using solid-state fermentation. Food Technol Biotechnol. 2006; 44: 241–245.
- 316 24. Malhotra R, Noorwez SM, Satyanarayana T. Production and partial characterization
 317 of thermostable and calcium independent alpha amylase of an extreme thermophile
 318 *Bacillus thermooleovorans* NP54. Lett Appl Microbiol. 2000; 31: 378-384.
- 319 25. Ray RC, Kar S, Nayak S, Swain MR. Extracellular α -amylase production by *Bacillus*
 320 *brevis* MTCC 7521. Food Biotechnol. 2008; 22: 234-246.
- 321 26. Kar S, Datta TK, Ray RC. Optimization of thermostable α - Amylase production by
 322 *Streptomyces erumpens* MTCC 7317 in solid-state fermentation using cassava
 323 fibrous residue. Braz Arch Biol Technol. 2010; 53: 301-309.
- 324 27. Babu KR, Satyanarayana T. Extracellular calcium inhibited α -amylase of *Bacillus*
 325 *coagulans* B49. Enz Microb Technol. 1993; 15: 1066-1069.
- 326 28. Humphrey A. Shake flask to fermenter: What have we learnt? Biotechnol Progress.
 327 1998; 14: 3-7.
- 328 29. Santos EO, Martins MLL. Effect of the medium composition of amylase by *Bacillus*
 329 sp. Braz Arch Biol Technol. 2003; 46: 129-134.
- 330 30. Liu J, Zhang Z, Liu Z, Zhu H, Dang H, Lu J et al. Production of cold-adapted
 331 amylase by marine bacterium *Wangia* sp. C52: optimization, modeling, and partial
 332 characterization. Mar Biotechnol. 2011; 13: 837-844.

- 333 31. Coronado M, Vargas C, Hofemeister J, Ventosa A, Nieto JJ. Production and
334 biochemical characterization of an alpha-amylase from the moderate halophile
335 *Halomonas meridiana*. FEMS Microbiol Lett. 2000; 183: 67-71.
- 336 32. Horikoshi K. Production of alkaline enzymes by alkalophilic microorganisms. Agric
337 Biol Chem. 1971; 234: 556-561.