

Isolation, Identification and Optimization of Cultural Conditions of *Bacillus* sp. strain PM1 for Alkalo-thermostable Amylase Production

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ABSTRACT

Aims: To isolate and optimize the cultural conditions for thermostable and alkaline amylase production from bacteria.

Study design: Optimization of different physiological and nutritional parameters for amylase production and kinetic studies of amylase.

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

Methodology: Amyolytic 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. strain PM1 on the basis of 16 S rRNA. It yielded 3.5 U/ml 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.

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

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1. INTRODUCTION

19 Amylases (E.C.3.2.1.1) constitute a class of enzymes which hydrolyze starch molecules to
20 relinquish numerous products together with dextrans and increasingly smaller polymers
21 composed of aldohexose units. These enzymes exhibit their potential applications in starch
22 liquefaction, food, textile, paper and brewing industries [1]. Amylases have completely
23 replaced chemical hydrolysis of starch in starch processing industry [2]. Around 25% of the
24 enzyme market has been obtained from varied sources like plants, animals, microorganisms
25 [2]. The microbial source of amylase meet the industrial demand because of economical bulk
26 production capacity, thermostability and the fact that microbes are easy to manipulate to
27 produce the enzymes of desired characteristics [2, 3]. A broad range of microorganisms
28 have been reported to produce amylase such as *Penicillium fellutanum*, *Bacillus subtilis* JS-
29 2004, *Bacillus megaterium* and *Hallobacillus* sp. LY9 [4-6]. The thermostability of enzyme is
30 desirable in many industrial processes such as bioprocessing of starch and the thermophilic
31 organisms are better choice for production of thermostable enzymes [7]. Amylases with
32 alkaline and thermophilic nature are a favorable choice due to the wealth of data currently
33 available about this enzyme family. Alkaline amylases having pH values higher than 8.0
34 have been used in textile industries and also are an ingredient in detergents for automatic
35 dishwashers and laundries [8]. Therefore, the potential of thermostable and alkaline
36 amylases for industrial applications have prompted the search for microbial isolates
37 expressing amylase activities with desired properties. In the present investigation,
38 thermostable and alkaline amylase producing bacterial isolate identified as *Bacillus* sp. strain
39 PM1, was optimized for maximum enzyme production.

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2. MATERIAL AND METHODS

2.1 Screening and identification of amylase producing bacteria

42 A total of thirty bacterial isolates from soil were screened for amylase production on nutrient
43 agar plate containing (%) soluble starch 2.0, yeast extract 0.3, peptone 0.5, NaCl 0.5, agar

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44 2.0, pH 7.0 at 37 °C. Amylolytic isolates were selected by flooding the agar plates with Lugol
45 solution [9]. Isolates selected on the basis of higher ratio of clear zone to colony size were
46 grown in nutrient broth containing 2% starch and the level of amylase was detected in
47 culture filtrate. The selected isolate showing maximum amylase production was identified as
48 *Bacillus* sp. strain PM1 (accession No.KC533174) on the basis of 16 S rRNA sequencing
49 and maintained by sub culturing after every fifteenth day.

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, Rajendra Electric Motor Industries, Mumbai, India).
56 Cell free supernatant was used as the crude enzyme. All the experiments were carried out in
57 triplicates and results presented are the mean of three values. The standard deviation was
58 within 5%.

59 **2.3 Enzyme assay**

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

67 **2.4 16S rDNA amplification and sequence analysis**

68 Bacterial genomic DNA was isolated following the protocol of Chen and Kuo [11]. The 16S
69 rDNA was selectively amplified from the genomic DNA by polymerase chain reaction (PCR)
70 using forward primer 5'-GAGAGTTTGATCCTGGCTCAG-3' and reverse primer 5' –

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71 CTACGGCTACCTTGTTACGA-3' [12]. The amplified 16S rDNA gene fragments were
72 cloned into the pGEM-T vector (Promega, Madison, Wis.), transferred into *Escherichia coli*
73 [13] and sequenced using Applied Biosystems 3730 xL DNA analyzer, U.S. The 16S rDNA
74 sequence of the isolated strain was compared and analyzed by an existing gene bank of
75 NCBI (National Centre for Biotechnology Information) .

76 **2.5 Effect of different physiological parameters on amylase production and** 77 **amylase activity**

78 A range of different temperatures and pH were used to study their effects on amylase
79 production and amylase activity. Amylase stability was determined at 50 °C and at pH 8.0 for
80 different time periods.

81 **2.5.1 Effect of incubation temperatures on amylase production and kinetic** 82 **studies of amylase for temperature profile and thermostability determination**

83 Temperatures varying from 37 - 70 °C were examined for their effects on amylase
84 production. The effect of temperature on amylase activity was determined by assaying
85 enzyme activity between 37 - 80 °C for 20 min. Thermostability of the enzyme was examined
86 at an optimum temperature of 50 °C for 20 – 120 min.

87 **2.5.2 Effect of initial medium pH on amylase production and kinetic studies of** 88 **amylase for pH Profile and alkalostability determination**

89 The initial pH of the medium ranging from 5.0 – 10.0 was used to examine its effect on
90 amylase production. The pH of the medium was adjusted using 1N HCl or 1N NaOH. The
91 effect of pH on amylase activity was measured at different pH values by incubating the
92 reaction mixture for 20 min in 50 mM Glycine-NaOH buffer (pH 7.5 –10.0) at 50 °C. pH
93 stability was examined in the Glycine-NaOH buffer (pH 8.0) for 1-4 h.

94 **2.6 Effect of different nutritional parameters on amylase production**

95 Various carbon sources such as maltose, wheat bran, rice bran and maize crush were used
96 as a replacement of soluble starch (2% w/v) in enzyme production medium to evaluate their

97 effect on amylase production. The best carbon source found was further optimized in the
98 range 1–5% (w/v) for maximum enzyme production. Peptone (0.5%) and yeast extract
99 (0.3%) in the production medium were replaced with nitrogen sources (0.5%) viz. tryptone,
100 beef extract, yeast extract and peptone individually. The best nitrogen source observed was
101 optimized in concentrations varying from 0.5 -3 %.

102 **2.7 Effect of agitation on amylase production**

103 The optimized enzyme production medium comprising 2% soluble starch, 0.5% beef extract,
104 0.5% NaCl, pH 7.0 was inoculated with *Bacillus* sp. and incubated at 50 °C under shaking
105 conditions (180 rpm).

106 **3. RESULTS AND DISCUSSION**

107 **3.1 Identification of bacterial isolate by 16S rDNA amplification and sequence** 108 **analysis**

110 A large number of enzymes are produced by the genus *Bacillus*, of which amylases are of
111 considerable interest for industrial purpose [14]. Present study involves the optimization of
112 cultural conditions for amylase production by *Bacillus* sp. strain PM1 and effect of different
113 kinetic parameters on enzyme activity.

114 On the basis of level of amylase production, isolate producing a maximum of 0.53 U/ml of
115 enzyme in 24 h was selected and studied for further detailed investigation. The selected
116 isolate was gram positive, rod-shaped, aerobic, catalase positive and spore forming. The
117 16 S rDNA of 1532 bases was sequenced and on the basis of 16S rDNA sequence analysis,
118 selected bacterial isolate was identified as *Bacillus* sp. PM1 (accession no. KC533174)
119 showing its belongingness to the genus *Bacillus* sp.

120 **3.2 Effect of incubation temperatures on amylase production and** 121 **determination of temperature profile and stability of amylase**

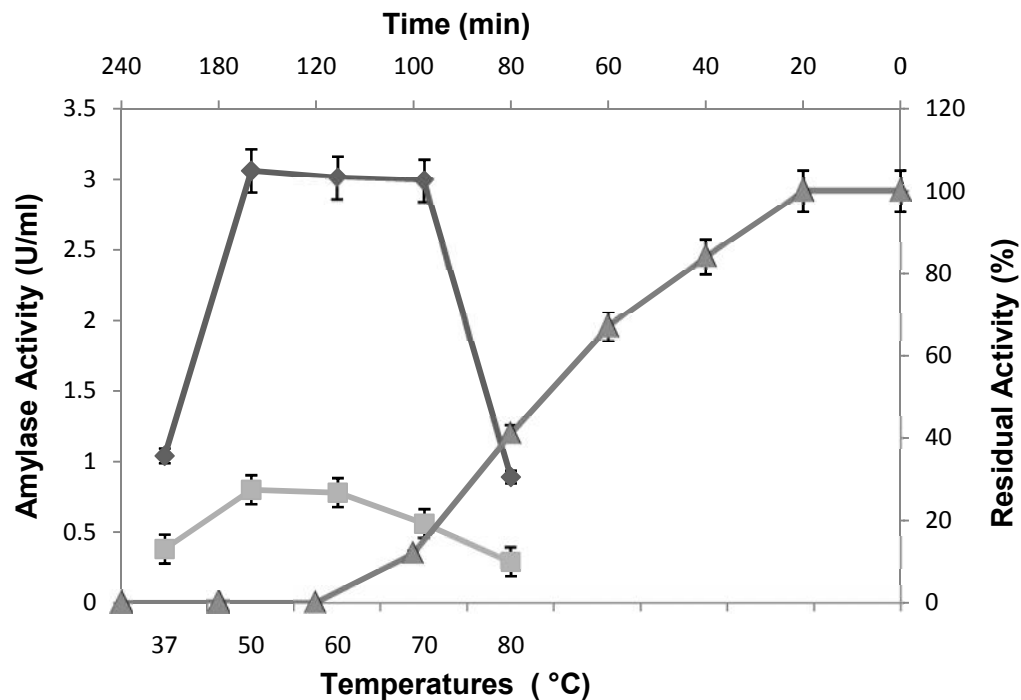
122 Temperature is a crucial environmental factor that controls the growth and production of
123 metabolites by microbes and usually differs from one organism to another [15-16].

Bacterial amylases are produced in broad range of temperatures. In present investigation, *Bacillus* sp. strain PM1 was capable of producing amylase in a temperature range of 37 °C – 70 °C with maximum production at 50 °C (3.07 U/ml). However, enzyme production declined with increase in temperature beyond 50 °C (Fig. 1). Around three times increase in amylase yield was observed at 50 °C compared to 37 °C, confirming the major role of temperature in amylase production from *Bacillus* sp. strain PM1. Similarly, De Souza Teodoro and Martins [17] and Amoozegar et al. [18] reported the optimum temperature of 50 °C for amylase production from *Bacillus* sp. and *Halobacillus* sp. strain MA-2, respectively. Results of temperature range (37°C – 70°C) are in accordance with the findings of amylase production from various *Bacillus* sp. such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus* sp. PN5 that have been reported to produce amylase at temperatures 37–70 °C [14,19-20].

As starch liquefaction is generally performed at higher temperatures of 70–90 °C, the thermostable amylases are of great significance. In this study amylase produced by *Bacillus* sp. strain PM1 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 50-70 °C. A reduction in enzyme activity was observed at temperatures above 70 °C. The data suggest a less sensitivity of enzyme to temperature especially, in the range of temperature between 50-70 °C. The amylase retained around 67% residual activity on incubation at 50 °C for 60 min which decreased with increased incubation time and was completely lost at 120 min. This property encourages its application in processes that require complete inactivation of the enzyme, such as in the baking industry [21]. Amylases working in range of 40 – 80°C have been reported [5, 22]. Our results are in accordance with the results of De Souza Teodoro and Martins [17] and Amoozegar et al. [18], who reported 50 °C to be the optimal temperature for amylase activity.

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◆ Amylase production ■ Amylase activity ▲ residual Activity of amylase (%) at 50 °C

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153 **Fig.1. Production of amylase at different temperatures and kinetic studies of amylase**
 154 **showing temperature and thermostability profile**

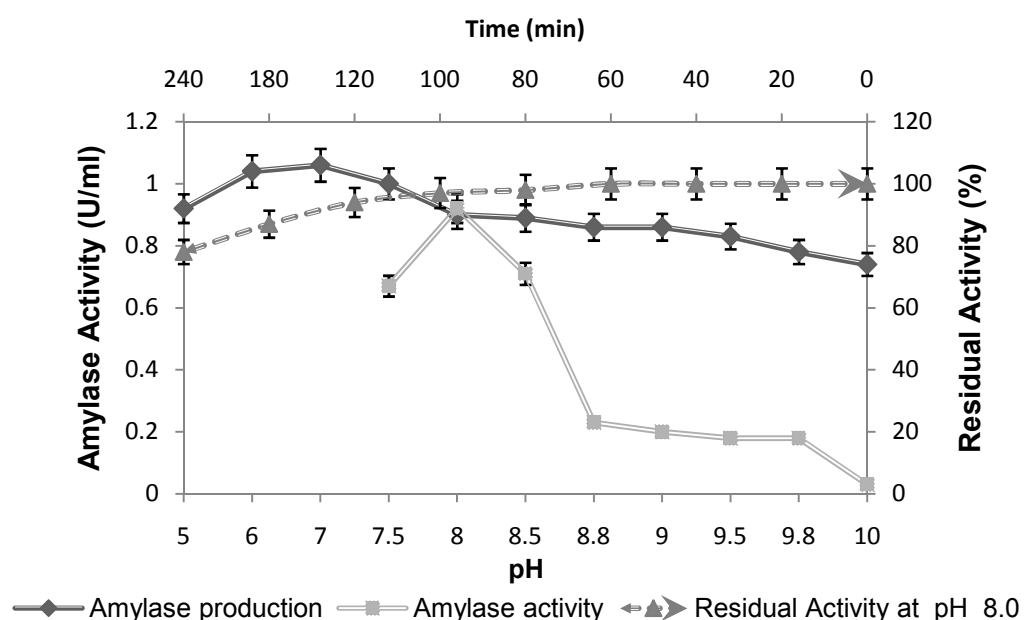
155 **3.3 Influence of initial pH of the medium on amylase production and effect of**
 156 **pH on amylase activity and stability of enzyme**

157 The culture pH affects the enzyme production, thus optimization of medium pH is essential
 158 for the maximum production of enzymes. Stimulation of amylase synthesis was observed
 159 with an increase in pH from 5 to 7 and maximum synthesis (1.06 U/ml) was achieved at pH
 160 7.0 (Fig. 2). Similarly the studies on *Bacillus* sp. by De Souza Teodoro and Martins [17] and
 161 Sankaralingam et al. on *Bacillus licheniformis* [23] revealed the high level of amylase
 162 production at pH 7.0. Although, *Bacillus* sp. strain PM1 produced maximum level of amylase
 163 at pH 7.0 but a pH range of 5-10 also supported the amylase production. Similar
 164 observations in which most of the *Bacillus* strains used commercially for the production of

165 amylases by submerged fermentation (SmF) have an optimum pH between 6.0 - 9.0 for
166 enzyme production [3, 14,20].

167 The effect of pH on amylase activity has been shown in Fig.2. The enzyme was found to
168 have an optimum pH of 8.0. The enzyme activity at pH 8.5 and 8.8 were around 77 % and
169 25 % of that at pH 8.0, respectively. Further increase in the initial pH resulted in decreased
170 activity of enzyme. The enzyme showed stability for 1-4 h on incubation of crude enzyme
171 solution at pH 8.0 and retained 78 % activity even after 4 h. The amylases related to *Bacillus*
172 genus have shown optimum activities at pH values as low as 3.5 or as high as 12 [5, 24].

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175 **Fig.2. Production of amylase at different pH and kinetic studies of amylase showing**
176 **pH and alkalostability profile**

177 3.4 Effect of carbon source on amylase yield

178 Carbon source greatly influences amylase production. In present study, *Bacillus* sp. strain
179 PM1 was capable of utilizing all the carbon sources such as soluble starch, maltose, wheat
180 bran, rice bran and maize crush that supported the amylase production. Different patterns of

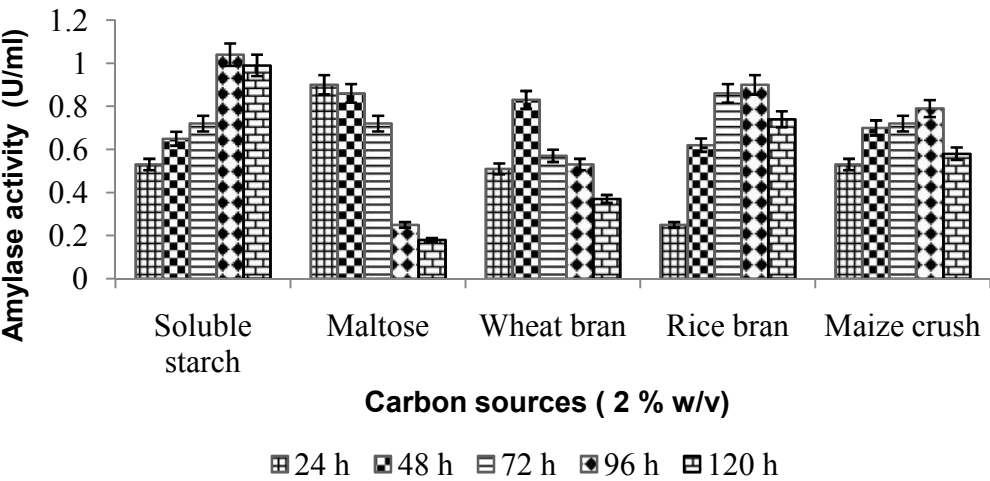
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the amylase induction were obtained when these carbon sources were used in the production medium (Fig. 3). Results showed that amylase level was maximum (1.04 U/ml) on starch followed by maltose and rice bran (0.9 U/ml), wheat bran (0.83 U/ml) and maize crush (0.80 U/ml). The data is in agreement with the reports of Narang and Satyanarayana [25] and the results of Karataş et al. [26] who reported soluble starch as the best carbon source for amylase production. Corresponding with the findings of Busch et al. [27], maltose was also found a good inducer of amylase as considerable levels of amylase (0.90 U/ml) were observed with in 24 h. According to Priest [28], like other extracellular hydrolytic enzymes, amylase production is induced due to the release of soluble products such as maltose from extracellular polymeric substrate. However, a decreasing pattern was observed with maltose with increase in incubation time due to the depletion of substrate. All other carbon sources well supported the amylase production from *Bacillus* sp. strain PM1 which fall in with the results of Narang and Satyanarayana; Ikram-ul-Haq et al. and Anto et al [22, 25, 29] who reported amylase production from many *Bacillus* strains using various carbon sources including different agricultural by-products. In present study *Bacillus* sp. strain PM1 produced 0.83U/ml amylase on wheat bran after 48 h and 0.79 U/ml on maize crush after 96 h. Amylase levels (0.90 U/ml) were found to be similar on maltose (24 h) and rice bran (96 h). These findings indicate that these sources could serve as economical and easily available raw material for the production of high-priced enzymes. Different carbon sources such as starch and other sugars, present in these wastes may prove useful for growth as well as for bacterial production of amylases [30-33].

Starch at 2% concentration induced the maximum amylase production (Fig. 4). At higher concentration amylase production declined. This can be because of high viscosity of broth at such concentrations, interfering with oxygen transfer leading to a limitation of dissolved oxygen for the growth of bacteria agreeing with the studies of Malhotra et al [34], who reported the maximum amylase yield at 2% starch concentration. According to Sohail et al. decreased amylase production at higher starch concentrations could be due to the presence

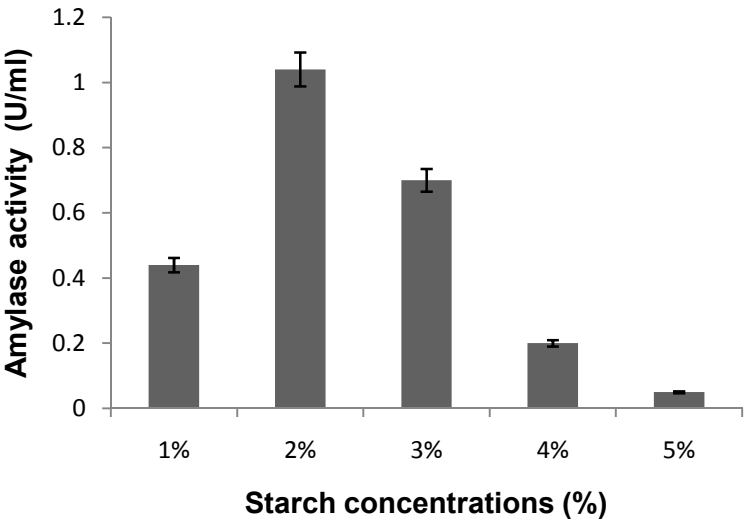
208 of glucose in a complex medium providing an simpler and alternate source of carbon or
 209 starch degradation leads to the formation of oligomers for which enzymes have lesser affinity
 210 [35].

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212

213 Fig.3. Amylase production from *Bacillus* sp. strain PM1 on different carbon sources



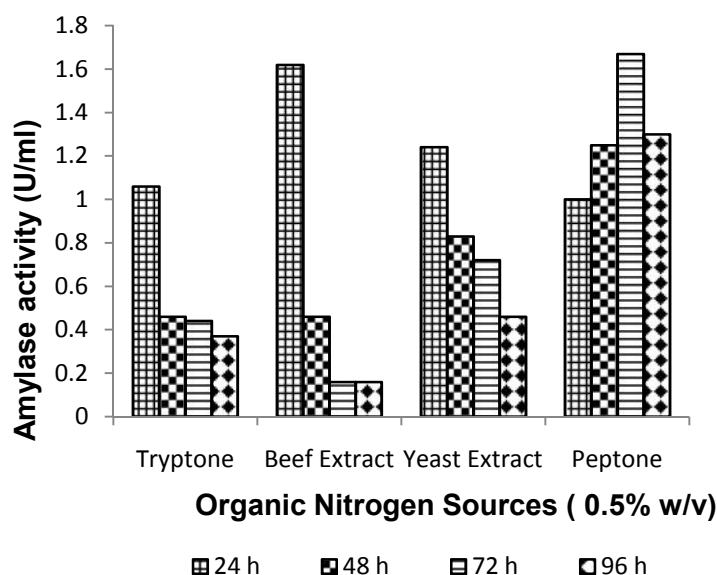
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215 Fig.4. Amylase production from *Bacillus* sp. strain PM1 on different starch
 216 concentrations

217 3.5 Effect of supplementation of nitrogen sources on amylase production

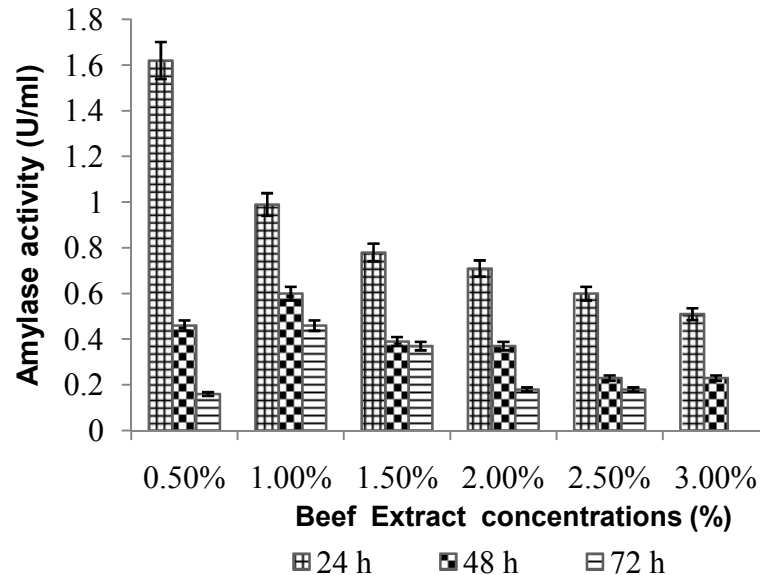
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218 Among the different organic nitrogen sources viz. yeast extract, tryptone, beef extract and
 219 peptone, amylase production was achieved almost in equal levels with beef extract (1.62
 220 U/ml) and peptone (1.67 U/ml) as represented in Fig. 5. However, *Bacillus* sp. strain PM1
 221 gave maximum amylase yield (1.62 U/ml) within 24 h in production medium supplemented
 222 with beef extract compared to peptone which produced 1.67 U/ml of amylase after 72 h.
 223 Around 1.55 times increase in amylase level was evaluated in beef extract supplemented
 224 medium. Using beef extract as an organic nitrogen source, enhanced levels of amylase from
 225 *Geobacillus thermodenitrificans* HRO10 [9], *Bacillus brevis* MTCC 7521 [36] and
 226 *Streptomyces erumpens* MTCC 7317 [37] have been reported. However, higher
 227 concentrations of beef extract resulted in a sharp fall in production levels (Fig. 6). The
 228 decrease in amylase production at higher concentrations of beef extract could be due to the
 229 lowering of pH or the induction of proteases resulting in destruction of amylolytic enzymes.
 230 Similar trend of declined amylase production by *Bacillus coagulans* B49 was observed by
 231 Babu and Satyanarayana [38] with increasing yeast extract concentrations.



233
 234 **Fig.5. Amylase production from *Bacillus* sp. strain PM1 on different nitrogen sources**

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236

237 **Fig.6. Amylase production from *Bacillus* sp. strain PM1 on different concentrations of**
 238 **beef extract**

239 3.6 Production of amylase under shaking conditions

240 In this study, *Bacillus* sp. strain PM1 produced amylase both under static and shaking
 241 conditions. However, bacterium yielded 3.5 U/ml of amylase under shaking conditions(180
 242 rpm) at 50°C temperature after 72 h. Two times increase in amylase level was detected
 243 under shaking conditions compared to static (1.62 U/ml), indicating the aerobic nature of the
 244 bacterium. Agitation intensity influences the mixing and oxygen transfer rate in many
 245 microbial fermentations, and thus influences the morphology and product formation [39].
 246 Agitation rates in the range of 100 to 250 rpm have been reported for the production of alpha
 247 amylases [9, 40-41].

248 4. CONCLUSION

249

250 The cultural conditions for optimal production of amylase from *Bacillus* sp. strain PM1 has
 251 been developed in this study. Amylase synthesis was affected by carbon and nitrogen
 252 sources and maximal yield was attained with 2% starch and 0.5% beef extract. The optimum

enzyme production by the bacterial isolate was found at 50°C and at pH 7.0. Many of the amylases reported were thermostable but not alkaline active, whereas some of them were alkaline active but not thermostable. But the amylase from *Bacillus* sp. strain PM1 in present study was found to be thermostable (50°C) and alkaline active (pH 8.0), suggesting its application in starch processing and baking industries. It can be concluded that, *Bacillus* sp. strain PM1 can be a potential producer of extracellular amylase which could find applications in industry. Due to the importance of these findings, further studies need be carried on in order to commercialize the production process.

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AUTHORS' CONTRIBUTIONS

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

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