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

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Authors' contributions

This work was carried out in collaboration between all authors. Author KS performed the experiments related to the production of amylase. Author SB identified the bacterium as Bacillus sp. on the basis of 16S rDNA sequencing. Author SMPK helped in providing the financial assistance for carrying out this study and author UKK supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To isolate and optimize the culture conditions for thermo stable 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: Amylolytic isolates were selected by flooding the nutrient agar plates containing 2% starch with Lugol solution. Isolates were selected on the basis of higher ratio of clear zone to colony size and grown in nutrient broth containing 2% starch. The level of amylase was detected in the 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 16S rRNA. It yielded 3.5 U/ml of amylase in medium containing (%) starch 2.0, beef extract 0.5, NaCl 0.5 at 50ºC, 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 minute incubation at 50ºC.

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At pH 8.0, the enzyme retained 78% activity after 4 h.
Conclusion: The properties of the isolated enzyme are adequate for its use in starch processing and baking industry.

Keywords: Amylase; Alkalo-thermostable; Bacillus sp.; optimization; starch.

1. INTRODUCTION

Amylases (E.C.3.2.1.1) constitute a class of enzymes which hydrolyze starch molecules to relinquish numerous products together with dextrin 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 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, thermo stability 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 *Halobacillus* sp. LY9 [4-6]. The thermo stability of enzyme is desirable in many industrial processes such as bio processing of starch and the thermophilic organisms are better choice for production of thermo stable enzymes [7]. Amylases with alkaline and thermophilic nature are a favourable choice due to the wealth of data currently available about this enzyme family. Alkaline amylases have been used in textile industries and also as an ingredient in detergents for automatic dishwashers and laundries, having pH values higher than 8.0 [8]. Therefore, the potential of thermo stable and alkaline amylases for industrial applications have prompted the search for microbial isolates expressing amylase activities with desired properties. In the present investigation, thermo stable and alkaline amylase producing bacterial isolate identified as *Bacillus* sp. strain PM1 was optimized for maximum enzyme production.

2. MATERIALS AND METHODS

2.1 Screening and Identification of Amylase Producing Bacteria

A total of thirty bacteria were isolated from soil and 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 were grown in nutrient broth containing 2% starch, selected on the basis of higher ratio of clear zone to colony size. The level of amylase was detected in culture filtrate. The bacterium showing maximum amylase production was analyzed and sequenced by 16S rRNA analysis. The selected isolate was identified as *Bacillu*s sp. strain PM1 (accession No.KC533174) and maintained by sub culturing after every fifteenth day.

2.2 Enzyme Production

The nutrient broth medium for enzyme production comprised (%): soluble starch 2.0, yeast extract 0.3, peptone 0.5, NaCl 0.5, pH 7.0. The medium (25mL) was inoculated with 1mL of the inoculums with an optical density of 0.6 at 600 nm and incubated at 37ºC for 96 h. Samples were withdrawn after a regular interval of 24 h and centrifuged at 10,000 rpm for

15 min in a Remi centrifuge (CPR-24, Rajendra Electric Motor Industries, Mumbai, India). Cell free supernatant was used as the crude enzyme. All the experiments were carried out in triplicates and results presented are the mean of three values. The standard deviation was within 5%.

2.3 Enzyme Assay

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

2.4 16S rDNA Amplification and Sequence Analysis

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

2.5 Effect of Different Physiological Parameters on Amylase Production and Amylase Activity

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

2.5.1 Effect of incubation temperatures on amylase production and kinetic studies of amylase for temperature profile and thermo stability determination

Temperatures were examined for their effects on amylase production (37-70ºC) and amylase activity (37 - 80ºC for 20 min).Thermo stability of the enzyme was examined at an optimum temperature of 50ºC for 20 – 120 min.

2.5.2 Effect of initial medium pH on amylase production and kinetic studies of amylase for pH profile and alkalostabilty determination

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

2.6 Effect of Different Nutritional Parameters on Amylase Production

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

2.7 Effect of Agitation on Amylase Production

The *Bacillus* sp. was inoculated in the medium comprising of 2% soluble starch, 0.5% beef extract, 0.5% NaCl at pH 7.0 and incubated at 50°C under shaking conditions (180 rpm).

3. RESULTS AND DISCUSSION

3.1 Identification of Bacterial Isolate by 16S rDNA Amplification and Sequence Analysis

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

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

3.2 Effect of Incubation Temperatures on Amylase Production and determination of Temperature Profile and Stability of Amylase

Temperature is a crucial environmental factor that controls the growth and production of 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 37ºC – 70ºC with maximum production at 50ºC (3.07 U/ml). However, enzyme production declined with the increase of temperature beyond 50ºC (Fig. 1). Around three times increase in amylase yield was observed at 50ºC compared to 37ºC, 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 50ºC for amylase production from *Bacillus* sp. and *Halobacillus* sp. strain MA-2, respectively. Results of temperature range (37ºC – 70ºC) 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 37–70ºC [14,19-20].

As starch liquefaction is generally performed at higher temperatures of 70–90°C, the thermo stable 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 70ºC, the enzyme activity was 70% compared to the optimum enzyme activity at 50ºC, indicating the range of amylase activity from 50-70ºC. A reduction in enzyme activity was observed at temperatures above 70ºC. The data suggest a less sensitivity of enzyme to temperature especially, in the range of temperature between 50-70ºC. The amylase retained around 67% residual activity on incubation at 50ºC for 60 minute which decreased with increased incubation time and was completely lost at 120 minute. This property encourages its application in processes that require complete inactivation of the enzyme, such as in the banking industry [21]. Amylases working in range of 40 – 80ºC 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 50ºC to be the optimal temperature for amylase activity.

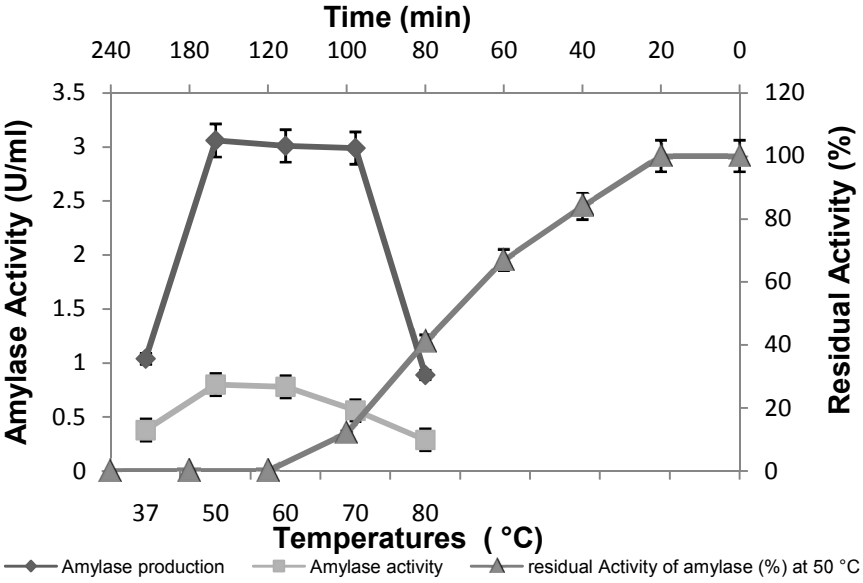

Fig. 1. Production of amylase at different temperatures and kinetic studies of amylase showing temperature and thermo stability profile

3.3 Influence of Initial pH of the Medium on Amylase Production and Effect of pH on Amylase Activity and Stability of Enzyme

The culture pH affects the enzyme production, thus optimization of medium pH is essential for the maximum production of enzymes. Stimulation of amylase synthesis was observed with an increase in pH from 5 to 7 and maximum synthesis (1.06 U/ml) was achieved at pH 7.0 (Fig. 2). Similarly the studies on *Bacillus* sp. by De Souza Teodoro and Martins [17] and Sankaralingam et al. on *Bacillus licheniformis* [23] revealed the high level of amylase production at pH 7.0. Although, *Bacillus* sp. strain PM1 produced maximum level of amylase at pH 7.0 but a pH range of 5-10 also supported the amylase production. Similarly most of the *Bacillus* strains have an optimum pH between 6.0 - 9.0 for enzyme production, used commercially for the production of amylases by submerged fermentation (SmF) [3,14,20].

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

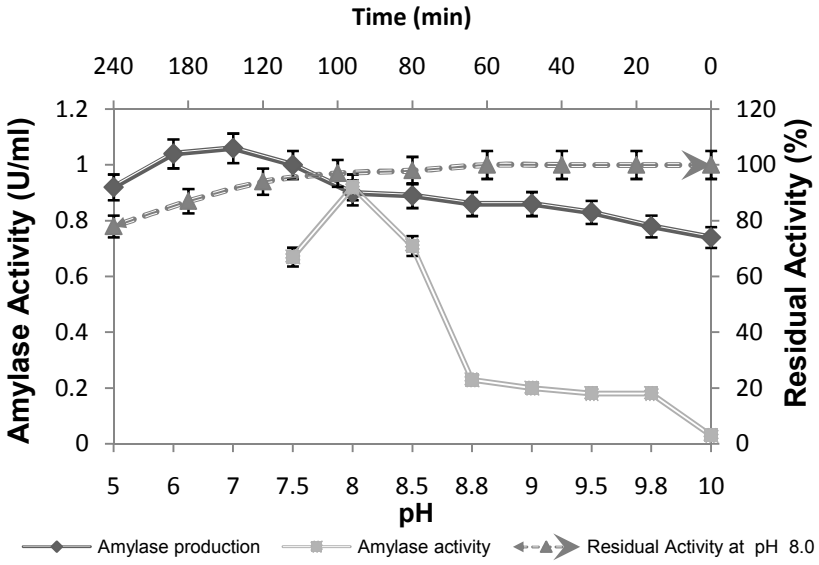

Fig. 2. Production of amylase at different pH and kinetic studies of amylase showing pH and alkalostabilty profile

3.4 Effect of Carbon Source on Amylase Yield

Carbon source may greatly influences amylase production. In present study, *Bacillus* sp. strain PM1 was capable of utilizing all the carbon sources such as soluble starch, maltose, wheat bran, rice bran and maize crush that supported the amylase production. Different patterns of the amylase induction were obtained when these carbon sources were used in the production medium (Fig. 3). Results showed that maximum amylase level (1.04 U/ml) was found 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], showing 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, showing similarity with the results of Narang and Satyanarayana; Ikram-ul-Haq et al. and Anto et al. [22,25,29]. Amylase production was reported from many *Bacillus* strains using various carbon sources including different agriculture 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 (24h) 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 may prove useful for growth as well as for bacterial production of amylases present in these wastes [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 in accord 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 of glucose in a complex medium providing a simpler and alternate source of carbon or starch degradation leads to the formation of oligomers for which enzymes have lesser affinity [35].

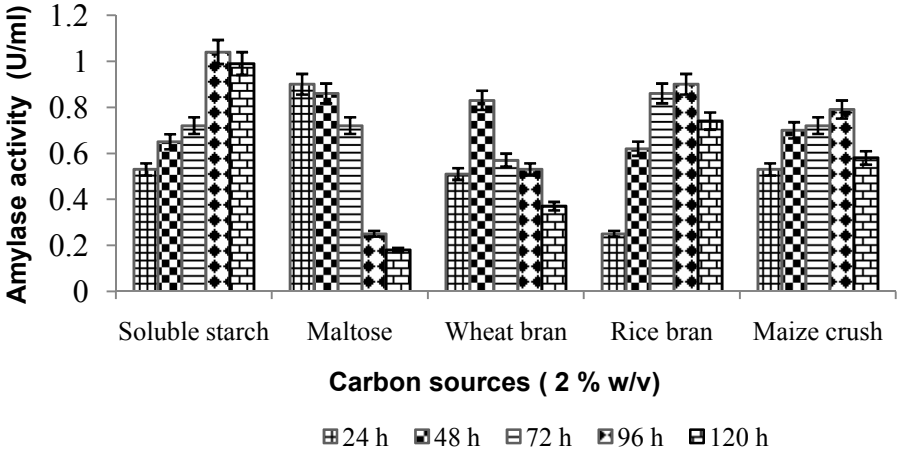

Fig. 3. Amylase production from *Bacillus* sp. strain PM1 on different carbon sources

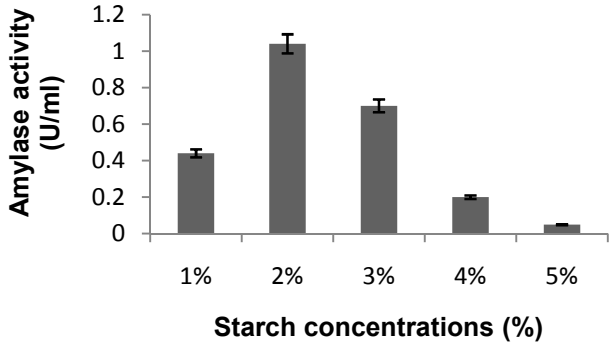

Fig. 4. Amylase production from *Bacillus* sp. strain PM1 on different Starch concentrations

3.5 Effect of Supplementation of Nitrogen Sources on Amylase Production

The different organic nitrogen sources were used such as yeast extract, tryptone, beef extract and peptone. Amylase production was achieved almost in equal levels with beef extract (1.62 U/ml) and peptone (1.67 U/ml) (Fig. 5). However, *Bacillus* sp. strain PM1 yielded maximum amylase (1.62 U/ml) within 24 h in beef extract supplemented production medium. While it produced 1.67 U/ml of amylase in peptone after 72 h. Amylase level was increased around 1.55 times 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 [36] and *Streptomyces erumpens* MTCC 7317 [37] have been reported. However, higher concentrations of beef extract resulted in a sharp fall in production levels (Fig. 6). The decrease in amylase production at higher concentrations of beef extract could be due to the lowering of pH or the induction of proteases resulting in destruction of amylolytic enzymes. Similar trend of declined amylase production by *Bacillus coagulans* B49 was observed by Babu and Satyanarayana [38] with increasing yeast extract concentrations.

3.6 Production of Amylase under Shaking Conditions

In this study, *Bacillus* sp. strain PM1 produced amylase both under static and shaking conditions. However, bacterium yielded 3.5 U/ml of amylase under shaking conditions (180 rpm) at 50ºC temperature after 72 h. Two times increase in amylase level was detected under shaking conditions as compared to static (1.62 U/ml), showing 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 [39]. Agitation rates in the range of 100 to 250 rpm have been reported for the production of alpha amylases [9,40-41].

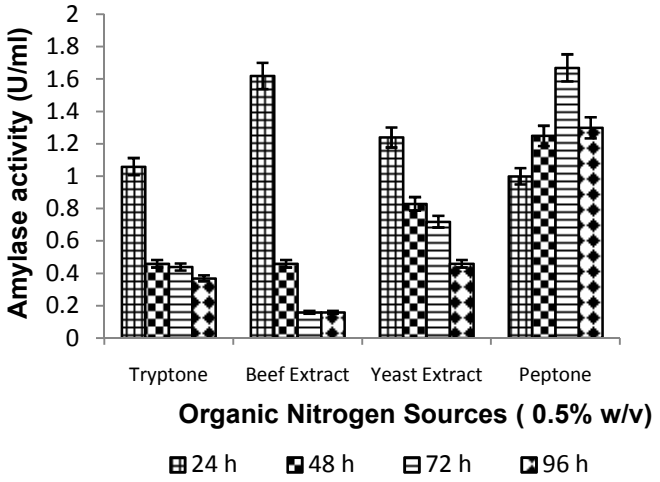

Fig. 5. Amylase production from *Bacillus* sp. strain PM1 on different nitrogen sources

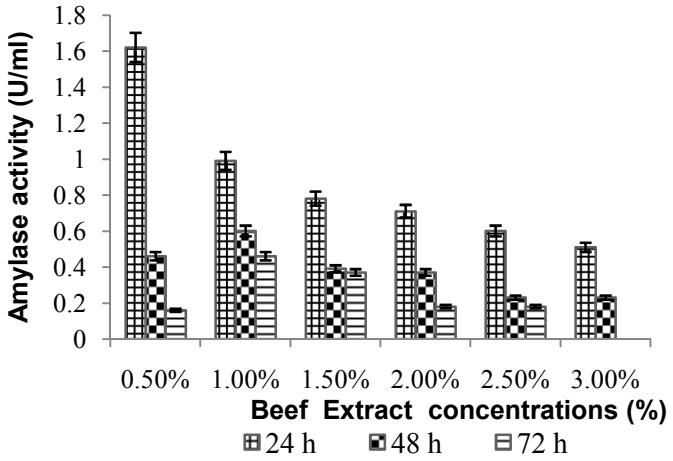

Fig. 6. Amylase production from *Bacillus* sp. strain PM1 on different concentrations of Beef extract

4. CONCLUSION

The culture conditions for optimal production of amylase from Bacillus sp. strain PM1 has been developed in this study. Amylase synthesis was affected by carbon and nitrogen 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 50ºC and at pH 7.0. Many of the amylases were reported as thermo stable but not alkaline active, whereas some of them were alkaline active but not thermo stable. But the amylase from *Bacillus* sp. strain PM1 in present study was found to be thermo stable (50ºC) 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 to be carried out in order to commercialize the production processes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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