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Full Length Research Paper

Thermostable α-amylase from natural variants of *Bacillus* spp. prevalent in eastern Himalayan Range

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North Eastern Region of India in the eastern Himalayan Range has a rich diversity of microbial flora which has remained unexplored. It is thus, an ideal place to explore the rich and complex microbial diversity for the isolation of novel α-amylase enzyme. In the present study, various bacterial strains were isolated from soils of North-Eastern region of India spread over diverse habitats ranging from an altitude of 24 to 3855 msl. Thirty bacterial isolates were screened for α-amylase production by plate assay method. Sixteen such strains were found to be positive for amylase production on starch agar medium. The amylase activity was assayed by DNSA method at different time intervals (10 - 60 min). Two bacterial strains (MK8 and MA9) were selected for further assays based on their higher enzyme activity. Based on the morphological, physiological and biochemical characteristics and phylogenetic position as determined by 16S ribosomal DNA gene sequencing, the bacterial strains MK8 and MA9 were identified as *Bacillus cereus* and *Bacillus subtilis* respectively. The optimum activity in shake-flask cultures was seen between 30 - 40 min of incubation and the maximum enzyme production was achieved during the stationary phase (36 - 48 h) of the growth of the organism. Bacterial cultures in stationary phase were taken for enzyme activity assay and the incubation time was optimized at 30 min based on the optimal activity. The maximal α-amylase activity was achieved at 60ºC, pH 7 and 80 ºC, pH 6 - 8 for MA9 and MK8, respectively. The amylase of MK8 strain remained stable between 30°C and 80°C, whereas, in case of MA9 strain, the amylase was found to be stable between 30 and 60°C. This indicates that the enzyme produced by MA9 strain is moderately thermostable while that of the MK8 strain is highly thermostable. Ca^{2+} enhanced the α-amylase activity of the MA9 strain whereas in case of MK8 strain, addition of Ca^{2+} did not show any significant increase in amylase activity. Pb^{2+} had higher inhibition of enzyme activity compared to Cd^{2+}, Cr^{3+} and Zn^{2+}. Nearly 100% was recovered with Cu^{2+}addition in case of MA9 strain but only 50% was recovered for MK8 strain.

Key words: α-amylase, screening, thermostable, optimization, *Bacillus.*

INTRODUCTION

North Eastern region of India in the Eastern Himalayan range is one of the biodiversity hotspots of the globe (Myers et al., 2000). It is rich in diverse groups of flora and fauna but the microbial groups have not been explored to a great extent. In the recent years, the importance of microorganisms as producers of industrial enzymes has increased tremendously. One of the many reasons being their biochemical diversity and the ease with which one can increase the enzyme concentration by various manipulations - environmental and genetic. Amylases are a group of starch degrading enzymes with utmost significance in the biotechnology industries and have huge application in food, fermentation, textile and paper production (Pandey et al., 2000). The various sources of amylase are plants, animals and microbes (Kathiresan and Manivannan, 2006). Out of these, microbes are widely preferred because of their vast availability and flexibility. Amylases are produced by both bacteria and fungi. They hydrolyse the α - (1, 4)-glycosidic

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bonds in starch and glycogen, producing short maltooligosaccharides and maltose. Among the amylases, β-amylase is exo-acting whereas α-amylase is endo-acting enzyme.

Thermostable enzymes are more versatile than thermolabile (Fogarth et al., 1974) as they have higher operational stability and a longer shelf life at elevated temperatures (Niehaus et al., 1999). Therefore, there is a need to screen the microorganisms for the production of thermostable α-amylase, which can be used in a wide array of industrial processes. Bacteria belonging to the genus *Bacillus* have been widely used for the commercial production of thermostable α-amylase. Some of the work on thermostable α-amylase from genus *Bacillus* include *Bacillus brevis* (Stefanova et al., 1992), *Bacillus thermooleovorans* NP54 (Malhotra et al., 2000), *Bacillus subtilis* (Asghar et al., 2007), *Bacillus* strain HUTBS71 1F (Al-Quadan et al., 2009). Syed et al. (2009) also reported a newly isolated alkali-thermotolerant strain *Streptomyces gulbargensis* DAS131 which exhibited maximum activity at pH 9.0 and 45°C. In industry, bacterial α-amylases are produced mainly from cultures of *Bacillus subtilis* var. *amyloliquefacien*s (Uhlig 1998). *Bacillus stearothermophilus* and *Bacillus licheniformes* α-amylases are well characterized and heavily used in the starch processing industry. Since thermostability is an important factor in the use of amylolytic enzymes in starch processing, amylases from thermophilic and hyperthermophilic bacteria are of special interest as a source of novel thermostable enzymes (Leveue et al., 1989).

Taking into consideration the extensive applications of the enzyme, attempts have been made in this study to screen, isolate, characterize (by biochemical tests) and identify (by molecular characterization) some amylase positive bacteria from soil of Eastern Himalayan range and also optimize the pH and temperature conditions for the enzyme activity.

MATERIAL AND METHODS

Isolation and identification of bacterial strains

Bacterial strains were isolated on nutrient agar medium from the soil samples collected from different altitudes (24 to 3855 msl) of North East India. Surface and sub-surface soil samples were collected aseptically from different microhabitats. 10 g of the sample was suspended in 90 ml of sterile double distilled water and shaken vigorously on a magnetic stirrer for 20 - 30 min to obtain uniform suspension. A serial dilution upto 10^{-5} was made and 0.1 ml aliquot from each dilution was inoculated on nutrient agar and incubated at 37ºC for 24 h. Bacterial strains isolated were maintained on nutrient agar slant and also in glycerol stock. The strains which showed promising results were further examined for morphological, physiological and biochemical characteristics with reference to Bergey's Manual of Determinative Bacteriology (Holt et al., 2000).

Identification was carried out by morphological, biochemical and molecular methods.

PCR amplification and 16S rDNA sequencing

Genomic DNA was extracted using bacterial genomic DNA miniprep purification spin kit (QIAGEN, Germany). The universal 16S rDNA primers; forward 27F (5'AGAGTTTGATCCTGGCTCAG3'), (Frank et al., 2008) and reverse 1541R (5'AAGGAGGTGATCCAGCCGCA3') (Loffler et al., 2000) were used for amplification of the 16S rDNA gene. Amplification of DNA was carried out with a 9700 Gold thermal cycler (Applied Biosystems, UK) under the following conditions: initial denaturation at 94ºC for 5 min; 35 cycles of denaturation at 94ºC for 1min, annealing at 55.5ºC for 1min, extension at 72ºC for 2 min, and a final extension at 72ºC for 5 min. The amplified PCR product was analyzed on an agarose gel, amplified DNA was purified using QIA quick® gel extraction kit and sequenced (BigDye terminator, Applied Biosystems, UK).

Nucleotide sequence accession number

The nucleotide sequence data of both the strains, MK8 and MA9 were submitted to GenBank at NCBI, (http://www.ncbi.nlm.nih.gov/) under the accession number HM769816 and HM769817, respectively.

Screening for amylase production by plate assay method

The bacterial strains were tested for amylase activity by employing zone clearing technique using starch agar medium. Inoculated plates were incubated at 37°C for 3 days. The zone of hydrolysis of starch was detected by flooding the plates with iodine solution. The development of blue colour indicated the presence of starch, while the areas around the hydrolytic bacteria appeared clear.

Enzyme production in shake-flask cultures

The medium used for enzyme production was starch urea with the following composition (gL^{-1}): starch 10.0, urea 2.0, K_2HPO_4 0.5, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.1 and pH 7.0. The cultures were inoculated in Erlenmeyer flasks, containing 50 mL of the starch urea medium and cultivated under agitation at 180 rpm for 96 h. After every 24 h, 5 ml sample was taken out from each of the three replicate flasks. The cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant containing amylase was used as the starting material to evaluate enzymatic activity and protein content.

Microbial growth and α-amylase assay

Growth of the bacterial strains was determined spectrophotometrically by measuring the optical density of the culture at 600 nm in a UV-visible spectrophotometer. α-amylase activity was assayed by the addition of 1 mL supernatant sample to a 1%(W/V) starch solution prepared in 0.1 M citrate phosphate buffer of pH 6.5 at 37°C for different periods of time. The reaction was stopped by the addition of 2 mL dinitrosalicylic acid (Bernfeld, 1955). The absorbance was taken at 540 nm in a UV-visible spectrophotometer (Cecil, Aquarius 7000 series, UK). One unit of α-amylase was defined as the amount of enzyme necessary to produce reducing sugars equivalent to 1 µmol of glucose, at 37°C. The specific activity was expressed as units per gram of protein. Protein was estimated by the method of Lowry et al. (1951) using BSA as the standard.

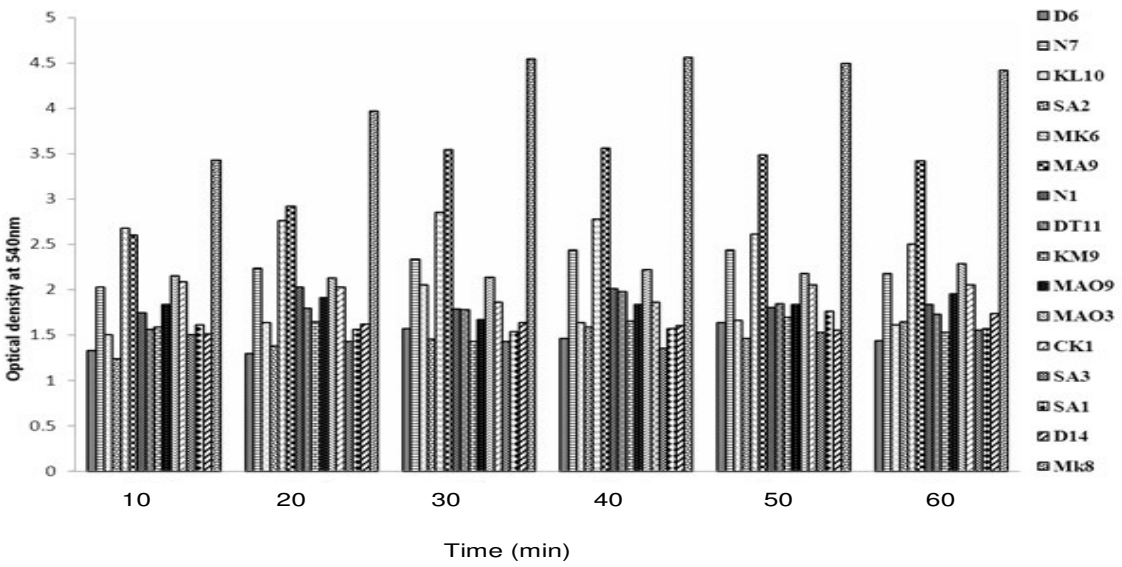

Figure 1. α-amylase activity of different bacterial strains isolated from soil.

Effect of temperature on enzyme activity and stability

The temperature optimum of the enzyme was evaluated by measuring the α-amylase activity at different temperatures (30 - 100°C) in 0.1M citrate phosphate buffer. The stability of the enzyme was determined by incubating the crude enzyme extract for 30 min prior to the enzyme assay in different temperature (30 - 100ºC). Activity was determined at 30ºC and percentage relative activity calculated using the following formula (Saliu, 2009):

Relative activity (%) = (Remaining activity / Initial activity) x 100

The percentage relative activity was plotted against temperature.

Effect of pH on enzyme activity and stability

The pH optimum of the enzyme was determined by varying the pH of the assay reaction mixture using the following buffers 0.1M sodium acetate (pH 4 - 5), sodium phosphate (pH 6.0 - 7.0), Tris-HCl (pH 8 - 9) and glycine-NaOH buffer (pH 10). The stability of the enzyme was determined by incubating the crude enzyme extract for 30 min in different buffers (pH 4-10). Activity was determined at 30ºC and percentage relative activity calculated.

Effect of metal ions on enzyme activity

The effects of different metal ions (Zn^{2+}, Cr^{3+}, Pb^{2+}, Fe^{3+}, Ca^{2+} and Cd^{2+}) on α-amylase activity were determined by the addition of the corresponding ion at a final concentration of 1 to 5 mM to the reaction mixture and the assay was carried out under standard conditions.

RESULTS

Screening of α-amylase producing bacteria

Thirty bacterial strains were screened for their potential amylolytic activity and only sixteen strains are found to be positive for amylase production on starch agar medium. The amylase activity of the sixteen bacterial strains was assayed by DNSA method at different time intervals (10 - 60 min) (Figure 1). The optimum activity was seen between 30 - 40 min of incubation and 30 min was taken as the optimum time of the enzyme activity assay. Two bacterial strains (MK8 and MA9) were selected for further assays. MK8 strain showed higher enzyme activity as compared to MA9 strain in plate assay method (Figure 2).

Characterization and identification of bacterial strains

Morphological and biochemical characterization

On nutrient agar, the MK8 strain is a round, opaque, off white, flat colony with a wrinkled and dry surface. Edge is irregular and the size is about 2 mm in diameter. MA9 strain, on the other hand, is a round, opaque, off white, raised colony with a smooth surface and an even margin. Cells of both the strains are rod shaped and straight, stain Gram positive, have round endospores and are motile with peritrichous flagella. The two selected strains were characterized by various biochemical parameters (Table 1). From the various biochemical tests it was inferred that the selected strains MK8 and MA9 were *Bacillus cereus* and *Bacillus subtilis*, respectively. This was further confirmed by molecular methods.

Molecular identification

The product of PCR amplification using the primers 27F

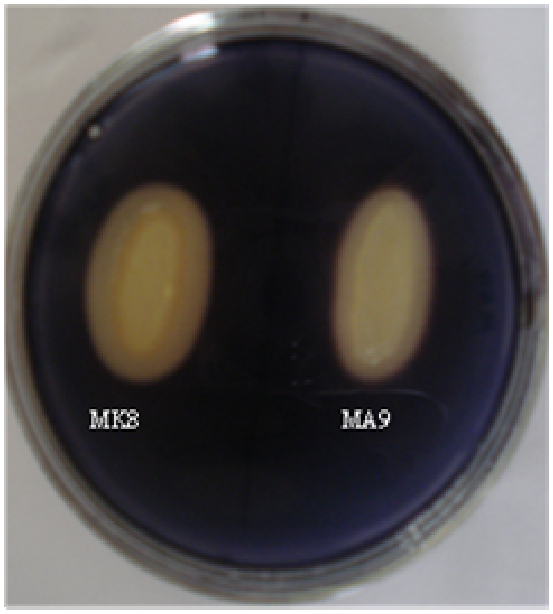

Figure 2. Zone of hydrolysis of starch by α-amylase.

Table 1. Biochemical characterization of strains MK8 and MA9.

Biochemical tests	MK8 strain	MA9 strain
Gram staining	+	+
Oxidase	+	+
Catalase	+	+
Indole production	-	-
Methyl Red	-	-
Vogues Proskauer	-	+
Citrate utilization	-	+
Starch hydrolysis	+	+
Nitrate reduction	+	+
Gelatin hydrolysis	+	+
Oxidation and fermentation	Fermentative	Fermentative
Acid mannitol	-	+
Closest relative	*Bacillus cereus*	*Bacillus subtilis*

and 1541R for 16SrDNA genes of MK8 and MA9 was approximately 1500 bp (Figure 3). Multiple sequence alignments were carried out using 16SrDNA sequences of 15 type strains. 16S rDNA sequences from the related species were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and the multiple sequence alignments were carried out with ClustalW. A phylogenetic tree was constructed by the neighbour-joining method using MEGA 4 (Figures 4 and 5). Multiple alignments and the phylogenetic tree showed that the MK8 and MA9 strains were closely related to *B. cereus* and *B. subtilis*, respectively.

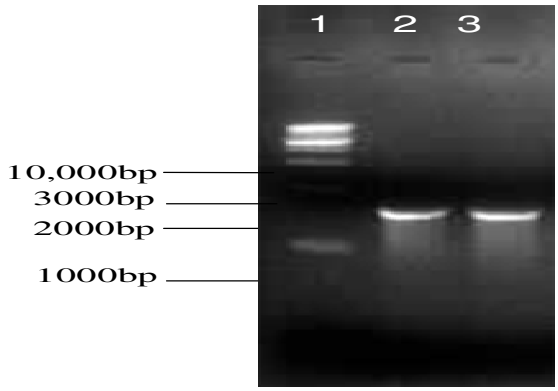

Figure 3. Gel picture of the amplified 16sr DNA; lane 1: 1kb DNA ladder, lane 2: MK8 & lane 3: MA9.

Growth of the strains and enzyme production

Amylase production by the bacterial strains started in the log phase of the growth and maximum enzyme production was achieved during the stationary phase (36-48 h) of the growth of the organism (Figure 6).

Effect of pH and temperature on enzyme activity:

The pattern of amylase activity was influenced by varying pH (Figure 7a). A relatively low level of activity was observed at pH values 4.0 to 5.5. α-amylase activity of MA9 strain increased significantly from pH 6.0 to 7.0 and dropped at pH values above 7.0, whereas the activity of the enzyme of MK8 strain reached its maximum at pH 6 and stabilized upto pH 8. These indicate that the enzyme's activity was optimum at neutral pH.

Activity of the enzyme produced from MA9 and MK8 strains increased with temperature and the maximal α-amylase activity was achieved at 60 and 80ºC, respectively after which there was a decline (Figure 7b).

This result shows that the enzyme produced by MA9 strain is moderately thermostable while that of the MK8 strain is highly thermostable.

Stability of enzyme at different pH and temperature

The amylase of strain MK8 remained stable at a temperature range between 30 and 80°C, above which the stability rapidly declined. The maximum activity was displayed at 80°C by MK8, whereas in case of strain MA9, the amylase was found to be stable at 60°C (Figure 8a). This observation indicates that the amylase produced by both the *Bacillus* strains is thermostable. The amylases of both the strains are quite stable at a pH range between 6 - 8 while that of the strain MA9 showed optimum stability at pH 7 (Figure 8b).

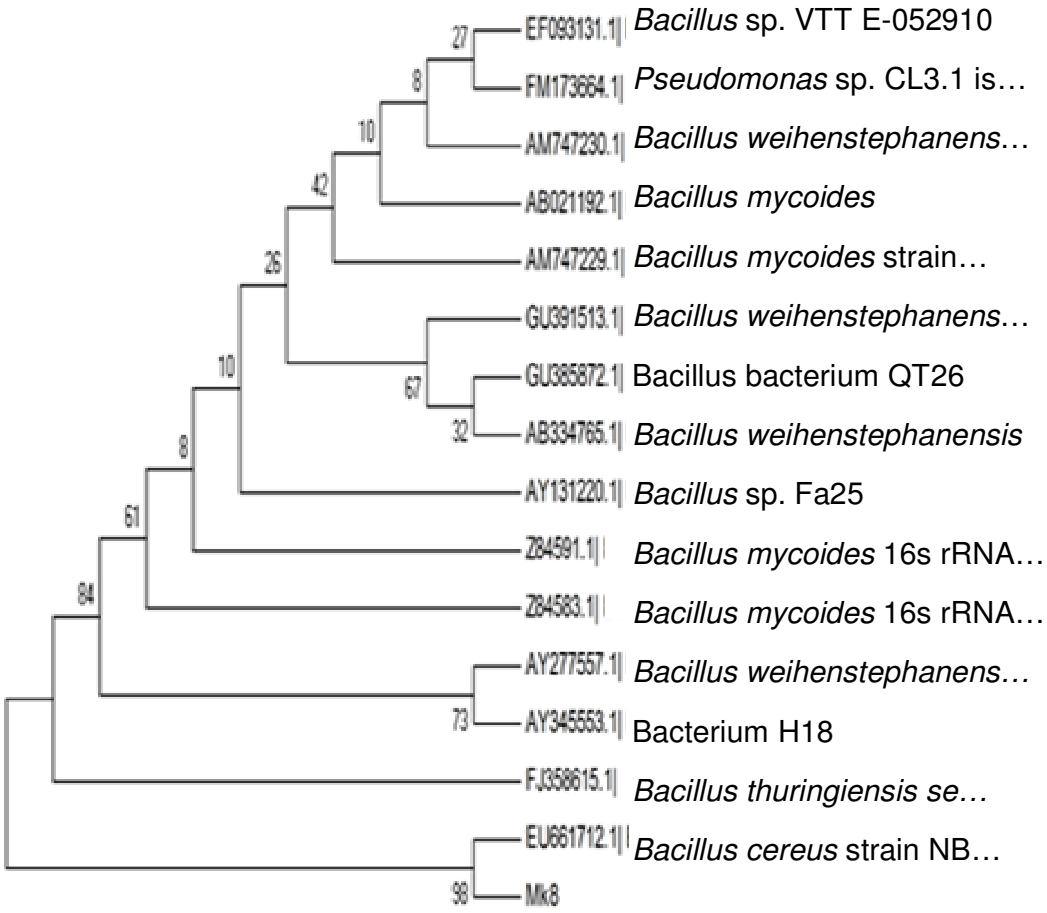

Figure 4. Phylogenetic tree based on the 16SrDNA sequences of the MK8 strain and the related strains.

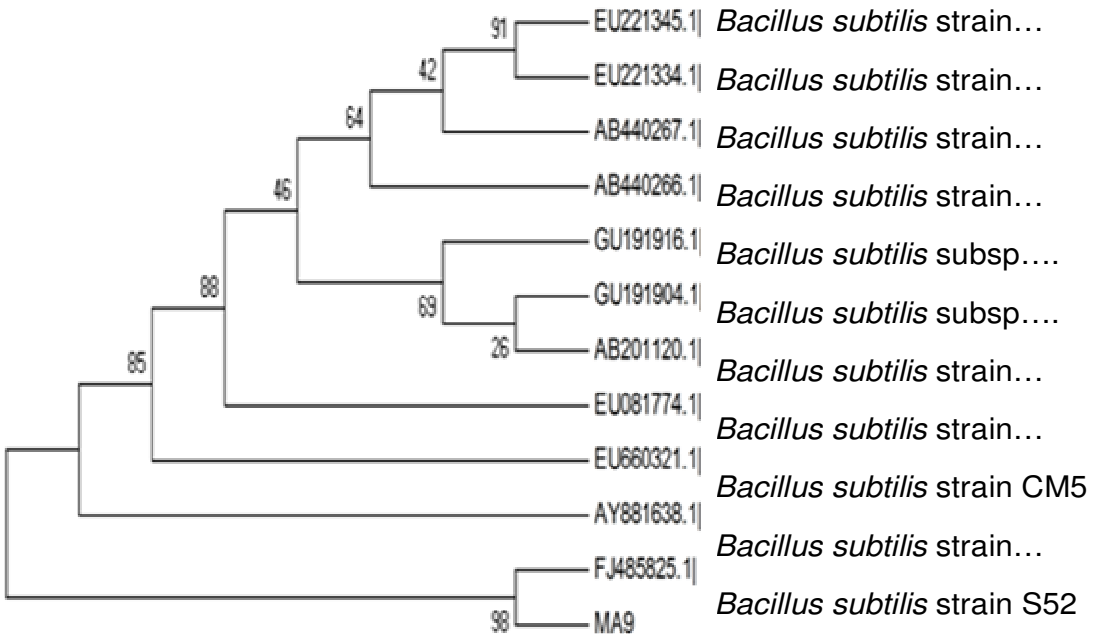

Figure 5. Phylogenetic tree based on the 16SrDNA sequences of MA9 strain and the related strains.

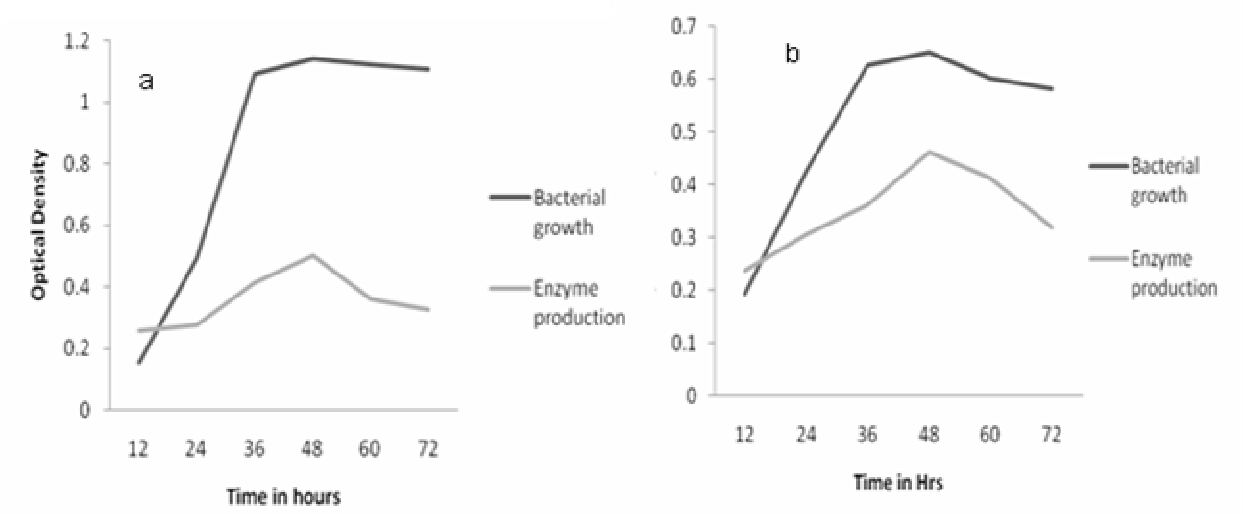

Figure 6. Bacterial growth and α-amylase production by (a) MA9 strain and (b) MK8 strain at different incubation period.

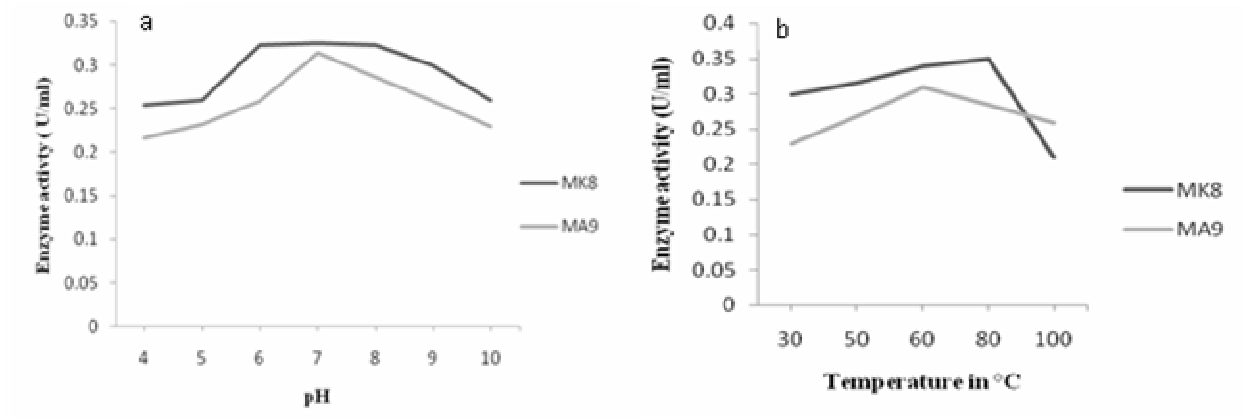

Figure 7. Effect of the (a) pH and (b) temperature on the activity by the crude amylase obtained from the bacterial strains MK8 and MA9.

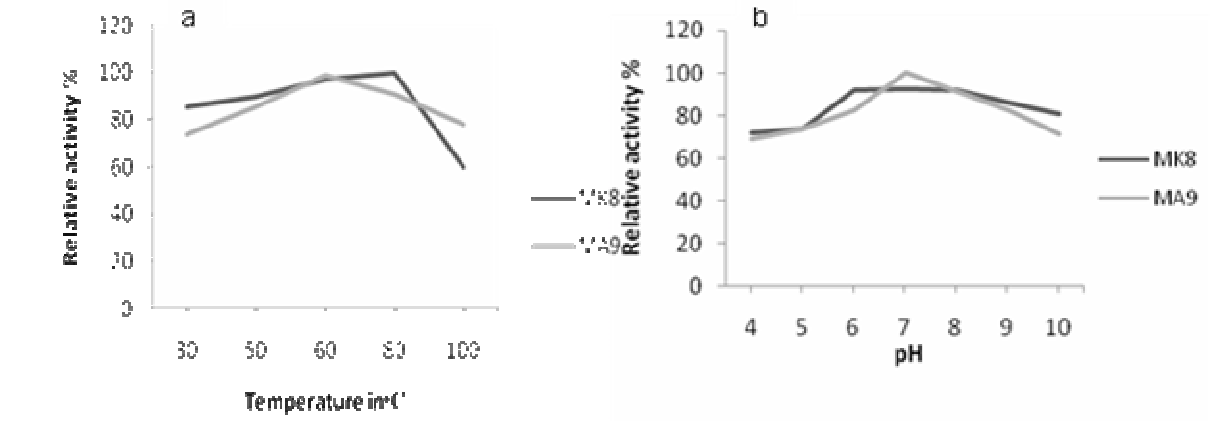

Figure 8. Effect of the (a) temperature and (b) pH on the stability of the crude amylase obtained from the bacterial strains MK8 and MA9.

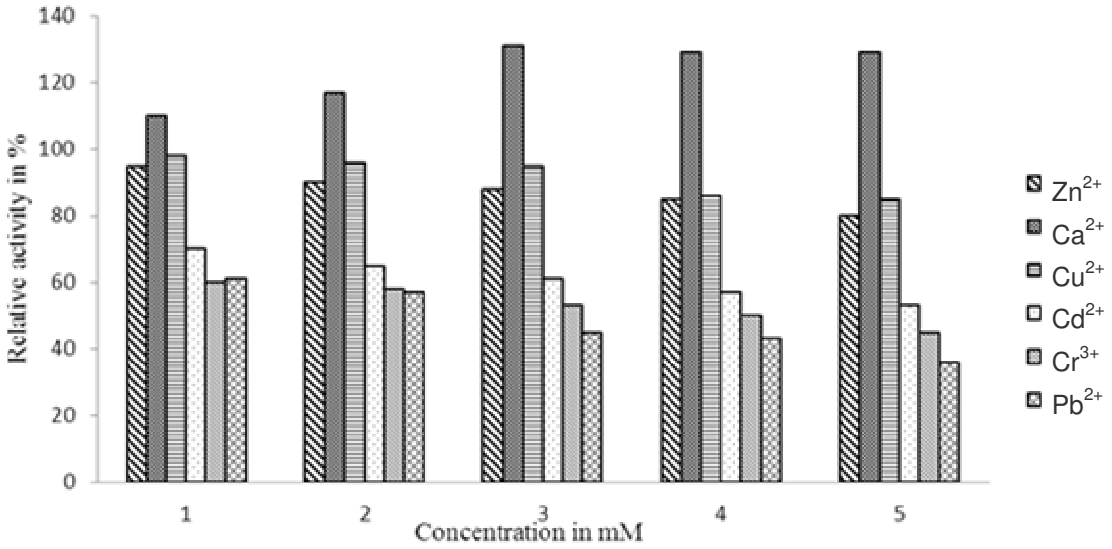

Figure 9. Effect of metal ions on α-amylase activity of MA9 strain.

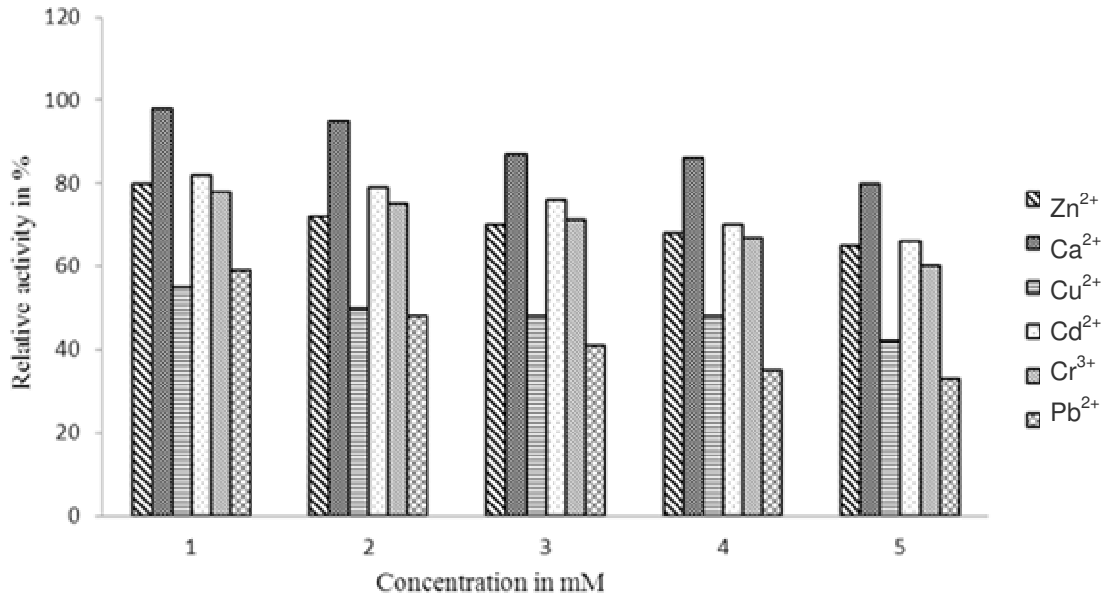

Figure 10. Effect of metal ions on α-amylase activity of MK8 strain.

Effect of metal ions on enzyme activity

Effect of some metal ions on the α-amylase activity was studied by assaying the enzyme in the presence of metals (Figures 9 and 10). Nearly 100% activity was recovered with Cu^{2+} in case of MA9 whereas only around 50% was recovered in case of MK8. Cd^{2+}, Cr^{3+} and Pb^{2+} inhibited the enzyme activity by around 30 to 50%. It was found that 1 to 5 mM Ca^{2+} enhanced the amylase activity of the MA9 strain but in case of MK8 strain addition of Ca^{2+} did not show any significant increase in the activity, though almost 100% activity was recovered with 1mM concentration.

DISCUSSION

In the present study, the maximum enzyme production

was achieved during the stationary phase (36 - 48 h) which corresponds to the earlier reports, that is *Bacillus amyloliquefaciens* (Roychoudhary et al., 1989), *B. thermooleovorans* (Malhotra et al., 2000), *B. subtilis* (Baig et al., 1984; Najafi et al., 2005; Swain et al., 2006), *B. amyloliquefaciens* (Gangadharan et al., 2006) and *B. brevis* MTCC 7521 (Ray et al., 2008).

Amylase enzyme isolated from *Bacillus* can have a wide range of pH optimum for activity. Most of the *Bacillus* amylases have optimum pH values from 5.0 - 8.5. (Mamo et al., 1999; Malhotra et al., 2000; Gupta et al., 2003; Rasooli et al., 2008). The amylase enzymes from MK8 and MA9 strains showed optimum activity at pH 7 and pH 6 – 8, respectively and remained stable at a pH range from 6 - 8.

It has been reported that Ca^{2+} increases the amylase production and also the stability of enzyme (Tonkova, 1991; Syed et al., 2009). Stefanova and Emanuilova (1992) also earlier reported a thermostable α-amylase by *B. brevis* that required Ca^{2+} or Na^{+} ions for enzyme thermostability. There had been reports on Ca^{2+} independent α-amylase from *B. thermooleovorans* (Malhotra et al., 2000) and *B. subtilis* (Swain et al., 2006). However in the present study, enzyme activity of the crude enzyme extracted from MA9 was found to be enhanced by 110% with Ca^{2+} addition. This shows that the amylase from MA9 is a calcium dependent metalloenzyme (Gupta et al., 2003). Ca^{2+} addition did not show any significant effect in case of MK8 which indicates that the amylase from MK8 strain is a calcium independent enzyme.

Amund and Ogunsina (1987) reported temperature optimum for enzymes obtained from *B. subtilis, B. licheniformis* and *B. cereus* as 30, 37 and 80ºC, respectively. It had been observed by Bose and Das (1996) that amylase activity in starch degrading bacteria is non-growth related. This could be a possible explanation for optimum growth of the presently reported strains at 37ºC whereas maximum enzyme activity of the strains MA9 and MK8 was observed at 60 and 80ºC, respectively. This observation shows that MK8 and MA9 could be a potential source for the production of thermostable α-amylase for various industrial applications.

Conclusion

The *Bacillus* strains merit further importance as potential producers of α-amylase enzyme. Among the two strains, the *B. cereus* strain is a better producer of the enzyme than the *B. subtilis* strain. Also the activity of the enzyme produced by the former strain is higher than the latter. Enzymes from both the strains are thermostable. The amylase of strain MK8 remained stable at a temperature range between 30 and 80°C, above which the stability rapidly declined whereas in case of strain MA9 the amylase was found to be stable at 60°C. Their activity is recovered up to more than 60% in the presence of various metals except Pb^{2+}. Enzyme activity of the crude enzyme extracted from MA9 was found to be enhanced by Ca^{2+} addition but had no significant effect in MK8 strain indicating amylase of MK8 strain to be calcium independent enzyme. Ca^{2+} independent amylase can be of importance in starch liquefaction, especially in the manufacture of fructose syrup, where Ca^{2+} is a known inhibitor of glucose isomerase (Tonkova, 2006). The two amylases also have a pH optimum of pH 7 but are fairly stable at a large range of pH 4-10. These characteristics make the two strains suitable for application in detergent industry, starch processing and other food industries. Further optimization for enhanced enzyme production for commercialized process is underway.

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