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A Novel Thermostable Alkaline α-Amylase from *Bacillus circulans* PN5: Biochemical Characterization and Production

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ABSTRACT

Amylase (1-4, α-D glucan glucanohydrolase E C 3.2.1.1) hydrolyses α-1,4 glucosidic linkage in starch and related substrates in an endofashion producing oligosaccharides, glucose and α-limit dextrin. In this study, 56 potential bacterial isolates of amylase producer were obtained during the primary screening. Secondary screening of these isolates yielded a highly thermostable alkaline α-amylase producing isolates identified as *Bacillus circulans* PN5. This strain was found to produce 36 U mL^{-1} of thermostable amylase at pH 10. Amylase was further evaluated for its biochemical properties such as pH optima, temperature optima and stability. Results showed that the enzyme was produced in the pH range of 8-11 with pH optima at 10. The enzyme was found to retain more than 60% residual activity in the pH range of pH 7-12. The optimum temperature for enzyme production was 80°C at pH 10. When starch as a carbon and peptone as a nitrogen source were used in production medium, the enzyme yield was increased. These parametric optimization resulted in to the significant increase in the enzyme production with a maximum of 48 U mL^{-1}. The enzyme was highly stable with commercial detergents tested. These studies confirmed the suitability of enzymes for various applications such as starch processing, detergent formulations and many other related industries.

Key words: *B. circulans* PN5, thermostable, alkaline α-amylase, screening, purification

INTRODUCTION

Amylases constitute a class of industrial enzymes, which represent approximately 30% of the world enzyme production (Van der Maarel *et al*., 2002). α-Amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) were classified in family 13 of glycosyl hydrolases and hydrolyzes starch, glycogen and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkage to produce different size of oligosaccharides. They have diverge applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries (Gupta *et al*., 2003). Each application of α-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence (McTigue *et al*., 1995). Although, they can be derived from several sources, such as plants, animals and microorganisms, enzymes from microbial sources generally meet industrial demands. Among the various species of *Bacillus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are two species used most frequently in the commercial production of thermostable amylase. Several amylolytic enzymes with different molecular weight, optimum pH, temperature and specificities have been reported (Burhan *et al*., 2003; Asgher *et al*., 2007; Saxena *et al*., 2007; Hashim *et al*., 2005; Arikan, 2008; Hmidet *et al*., 2008). Screening of microorganisms with higher α-amylase could therefore, facilitate the discovery

of novel amylases suitable for new industrial applications such as bread and baking industries as antistaling agents (Gupta *et al*., 2003). The genus *Bacillus* produces large variety of extracellular enzymes of which amylases and proteases are exploited commercially at large scale. Industrial processes such as starch liquefaction for sweeteners and syrups, textile and paper industries demands the process to be carried out at high temperature so economical application of amylase to such process, its thermostability is of a prime importance. Several reports showed the commercial importance of *B. circulans* for the α-amylase (Takasaki, 1983; Dey *et al*., 2002; Ajayi and Fagade, 2006). Amylase producing strains of *B. circulans* was reported with pH optima of 7 to 8.5 (Hiroshi *et al*., 1989). Also, thermostable alkaline amylase producing *Bacillus* stable at 100°C for 1 h was reported (Saxena, *et al*., 2007). During the screening program for the novel metabolites, strain of *Bacillus circulans* PN5 was isolated. The strain was found to produce alkaline thermostable α-amylase. In present study, the purification and biochemical characterization of extracellular α-amylase produced by novel strain of *Bacillus circulans* PN5 are described.

MATERIALS AND METHODS

Microorganisms: The microorganisms used in this study were obtained from the thermophillic microorganisms from the effluent released from the starch processing industries (Anil Starch Ltd., Ahmedabad). The screening of amylolytic microorganisms was carried onto nutrient agar base containing soluble starch (1% w/v). After incubation at 60°C for 40 h, the plates were flooded with a solution of 0.5% (w/v) I_2 and 5.0% (w/v) KI. Colonies exhibiting holo starch hydrolyzing activity were picked up. The strain *Bacillus circulans* PN5 that produced high level of thermostable alkaline amylase was used for further studies. It was identified as *Bacillus circulans* PN5 according to the method described in Bergey's Mannual of determinative Bacteriology (Claus and Berkeley, 1986). *Bacillus circulans* PN5 strain was routinely maintained on Luria-Bertani (LB)-agar plates and conserved in LB medium added to 30% glycerol at -80°C (Miller, 1972).

Growth condition and amylase production: Inocula was routinely grown in LB broth medium composed of (g L^{-1}): Peptone 10.0, yeast extract 5.0, NaCl 5.0 and initial pH was adjusted to 10 (Miller, 1972). The growth medium used for α-amylase production by *B. circulans* PN5 strain was composed of (g L^{-1}): KH_2PO_4 1.5, K_2HPO_4 2.2, $MgSO_4.7H_2O$ 0.0025, yeast extract 0.5, to which soluble starch 20 g was added. The medium pH was adjusted to 10.0 before sterilization at 121°C for 20 min. Samples were taken at interval for microbiological and biochemical analysis (i.e., pH, biomass (OD at 620 nm) and amylase activity). Starch concentration in medium was determined by withdrawing 0.5 mL of culture at intervals followed by addition of 2 mL of 0.1 M H_2SO_4, Iodine solution (5 mL containing 0.3% I_2 and 3% KI) and the absorbance was measured at 600 nm (UV-1800 Spectrophotometer, Shimadzu make, Japan) against the water/iodine blank.

Enzyme assay: A 1 mL reaction mixture containing 0.5% (w/v) soluble starch (Sigma) and 50 μL of the enzyme sample in 50 mM sodium phosphate buffer (pH 10) was incubated at 80°C for 10 min and the amount of reducing sugars released was measured using 2,5-dinitrosalicylic acid (Analytical grade) reagent (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme that librated 1 μmol of reducing sugar as glucose equivalents in 1 min under the assay condition. Protein was determined by Bradford method using bovine serum albumin as standard protein (Bradford, 1976). Amylase activities represent the mean of values of at least two values carried out in duplicate. The difference between values did not exceed 5.0%.

Purification of α-amylase: For the preparation of large amounts of the enzyme, bacteria were cultivated for 24 h in series of 1 L Erlenmeyer flasks each containing 200 mL of mineral salts medium supplemented with soluble starch and the cultures combined. All steps of purifications were carried out at 4°C.

Step 1: The fermented broth was centrifuged at 12000 g for 30 min to remove bacterial cells. The supernatant was concentrated by a tangential flow device with a 10 kDa cut off membrane (Filtron Technology Corp, Northborough, MA, USA). The peristaltic pump used for this operation was a Watson-Marlow 603 U (Falmouth, UK), at a cross-flow rate of 500 mL min^{-1} and a filtrate flow of 19 mL min^{-1} with a back pressure of 12-20 psi. The concentrated supernatant was fractionated with ammonium sulphate (20-60% saturation) and allowed to stand overnight with gentle mixing. The precipitate formed was collected by centrifugation (12 000 g, 30 min) and stored in a minimal volume of 3 M ammonium sulphate dissolved in 0.05 M K_2HPO_4.

Step 2: For ion-exchange chromatography, the partially purified enzyme was dissolved and dialysed overnight against 0.02 M Tris-HCl buffer (pH 8.0). The enzyme sample was then applied to a column of DEAE-Sephadex A-50 anion exchanger gel (250 cm) that was previously equilibrated with 0.02 M Tris-HCl buffer (pH 8.0). The column was eluted (18 mL h^{-1}) with a linear NaCl concentration gradient from 0-1 M in the same buffer. The amylase fractions were pooled, concentrated using a Vivaspin centrifugal concentrator (Vivascience Ltd., Lincoln, UK) and subjected to further purification by gel filtration.

Step 3: The enzyme obtained from ion exchange chromatography was loaded onto an Ultragel ACA-34 (pH 8.0) active fractions were pooled, concentrated by ultrafiltration and used to study the properties of the enzyme.

Effect of pH and temperature on α-amylase: α-Amylase activity and stability of purified enzyme was measured at different pH using various buffers (50 mM sodium acetate, pH 4.0-5.5; 50 mM potassium phosphate, pH 5.5-8.0 and 50 mM sodium carbonate, pH 8.0-10.0). To determine the pH stability, the purified enzyme was incubated in different buffers (pH 4.5 to 9.0 at 4°C for 24 h) and residual α-amylase activity was measured at 80°C as described. Effect of temperature was studied by measuring enzyme activity between 30 and 120°C at pH 10.0. For thermal stability, enzyme at pH 10 was incubated between 50 to 120°C, samples were removed at 5 min intervals, cooled in ice bath and measured the residual α-amylase activity.

Evaluation of stability and compatibility: α-Amylase activity and stability of purified enzyme was evaluated with five domestic detergent formulations and widely used oxidant agents. The purified enzyme was exposed for 30 min to all detergent formulations (50% w/v conc.). The oxidizing agents were exposed to 30 min and 1 h in two separate set. After exposure the residual enzyme activity was determined.

RESULTS

Screening of α-amylase producing microorganisms About 76 isolates were plated individually onto NA-soluble starch medium. Total of 56 colonies with large holos upon treatment with iodine solution were selected for amylase production in Mineral Salt Medium (MS) containing starch in

shaking condition. The secondary screening was carried out based on the α-amylase productivity among the 56 isolates. Finally the most efficient isolate was selected which produced thermostable α-amylase at highest level. Physiological and biochemical tests were carried out for the selected isolate as described in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986). Colonies appeared circular, translucent, butyrous, wrinkled and knotted branching pattern on NA. Pigmentation was nil and spore were oval with swollen sporangia. The organisms was aerobic and rod-shaped (0.7-0.8 μm width; 1.8-3.0 μm length), Gram positive and motile. Citrate was assimilated and it was positive by the Voges-Proskauer test but negative by the methyl red test. Catalase was produced, casein hydrolysed and gelatin liquefied. It reduced nitrate to nitrite and the pH of Voges-Proskauer broth was 7.2. The organisms grew at 35-65°C, pH from 5.5 to 11 and NaCl concentration of 0-5%. Acid was produced from glucose, arabinose, xylose, mannitol, but no gas production was observed. Starch was hydrolysed on starch agar plate. The selected isolate was identified as *B. circulans* PN5, according to these data and using the scheme of Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986; Smith *et al.*, 1952).

Growth and amylase synthesis by *B. circulans* PN5 on soluble starch: *Bacillus circulans* PN5 grew well in mineral salt medium supplemented with soluble starch (Fig. 1) and reached the stationary phase after 32 h. Growth was paralleled by the production of α-amylase, the activity of enzyme peaked at 28 U mL^{-1} at pH 10. The growth of *B. circulans* PN5 on starch showed a long lag phase that corresponds to a dramatic increase in enzyme activity. This lag phase was considerably reduced by serial transfer of medium in same medium maintaining organisms in exponential phase, before inoculation into production medium. Starch is complex polysaccharide and unless it is broken down into smaller, more soluble compounds, it cannot permeable to cell walls. This lag phase is possibly a reflection of time required to hydrolyze the starch into metabolizable subunit (Annous and Blaschek, 1990). It is suggested that this may be achieved by the continuous secretion of low basal level of hydrolyzing enzymes (Fogarty and Kelly, 1996; Priest, 1984). The biosynthesis of α-amylase by *B. circulans* PN5 appeared to be growth related since the enzyme in this isolate is primarily produced during exponential phase. This observation is similar to the pattern of amylase synthesis by *Clostridium acetobutylicum* (Annous and Blaschek, 1991).

Effect of different carbon and nitrogen sources on amylase synthesis: Various carbon sources at 2% concentration were evaluated as replacement soluble starch. *Bacillus circulans* PN5 were first serially transfer in respective media until the mid-exponential phase before final inoculation. All tested carbon sources supported good growth of selected strain of *B. circulans* PN5. The growth curve is similar to that obtained in the soluble starch medium (Fig. 1). The α-amylase production (in U mL^{-1}) was in order of wheat bran (36) > starch (33)>xylose (19) > galactose (20) > fructose (18) > maltose (29) > glucose (16) > sucrose (16) > lactose (16). Soluble starch (2%) was found to be the best carbon source with maximum enzyme activity (36 U mL^{-1}) as well specific activity (70 IU mg^{-1} protein) (Fig. 2). The superiority of amylase activity with complex substrates has been earlier reported (Saxena *et al.*, 2007). Among the artificial media, the rate of amylase synthesis was greater with soluble starch rather than maltose or glucose was the sole source of carbon. Repression of amylase synthesis by glucose in *Aspergillus niger* and *Clostridium* sp., has previously been reported (Annous and Blaschek, 1990; Ferniksova *et al.*, 1965; Melasniemi, 1987).

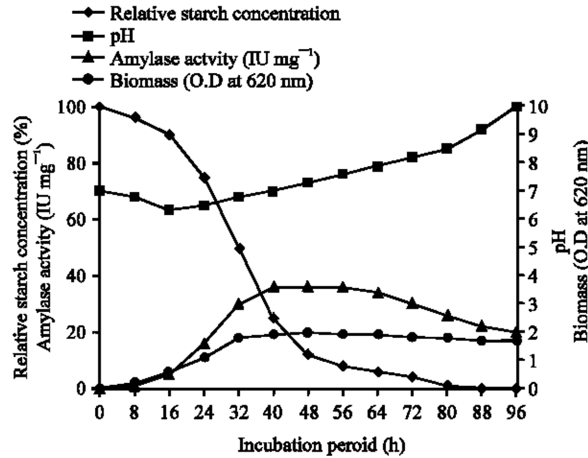

Fig. 1: Time course of growth and α-amylase synthesis by *Bacillus circulans* PN5 in mineral salts supplemented with soluble starch as carbon source

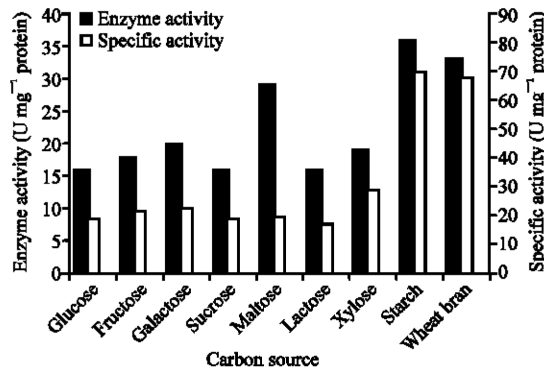

Fig. 2: Effect of various carbon sources on α-amylase production from *Bacillus circulans* PN5

After a 72-96 h incubation period, the starch was completely exhausted. This is an indication of easy degradability of starch and high amylase activity with the cheap local carbon substrates suggests possible utilization of these substrates in industrial fermentation processes.

Among the 11 different nitrogen sources tested, maximum amylase productivity (in U mL^{-1}) obtained was in order of peptone (48) > beef extract (39) > yeast extract (33) casein hydrolysate (31) > casein (28) > sodium nitrate (16) > ammonium chloride (9) > ammonium nitrate (8) > potassium nitrate (6) > urea (4) > ammonium sulphate (2) (Fig. 3). Peptone along with soluble starch (2%) was found to support maximum amylase productivity (48 U mL^{-1}) and also gave best specific activity (82 U mg^{-1} protein) (Fig. 3). Media selection has increased the overall 25% increase in the α-amylase activity.

Purification α-amylase: The culture filtrate of *B. circulans* PN5 grown in mineral salts supplemented with soluble starch and peptone was concentrated by ultrafiltration, precipitated with ammonium sulphate and applied to a DEAE-Sephadex column. The elution pattern showed a peak of α-amylase activity (data not presented) which was heterogeneous by SDS-PAGE analysis.

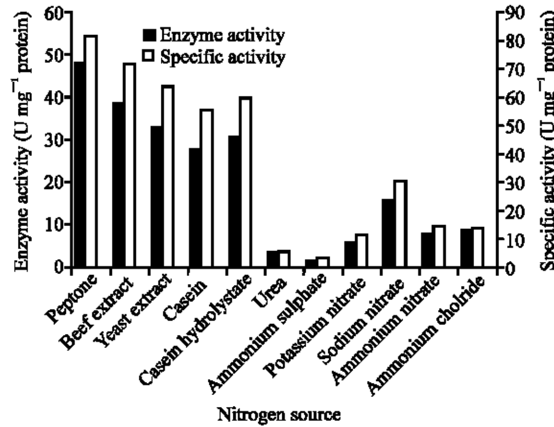

Fig. 3: Effect of various nitrogen sources on α-amylase production from *Bacillus circulans* PN5

Table 1: Purification of α-amylase from *Bacillus circulans* PN5

Step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1} of protein)	Yield (%)	Purification
Culture supernatant	5800	226200	2285	98.99	100.00	1.00
Tangential flow ultracentrifugation	1550	204600	1607	127.32	90.45	1.29
20-60% $(NH4)_2SO_4$ precipitation and dialysis	285	133950	435	307.93	65.47	3.11
Ion-exchange chromatography on DEAE-Sephadex A-50	228	63840	89	717.30	47.66	7.25
Gel filtration on ultragel ACA 34	105	21250	10	2625.00	33.29	26.52

Therefore, fractions of α-amylase activity were combined, concentrated and fractionated on an Ultragel ACA-34 column. A summary of the purification is shown in Table 1. The purified α-amylase was adjudged to be homogenous based on two criteria: protein and activity profiles coincided in the gel filtration (data not presented); and a single band was obtained when the purified enzyme was subjected to SDS-PAGE.

Effect of pH and temperature on α-amylase activity and stability: The pH activity profile approximates to a bell-shaped curve with a pH optima of 10. To determine the pH stability, the enzyme was incubated in the following buffers: McIlvaine citrate-phosphate buffer (0.02 M, pH 2.8-7.6), Sorensen phosphate buffer (0.02 M, pH 7.8) and Clark borate (0.02 M, pH 8.0-10) for 24 h at 4°C as described by Hayashida *et al.* (1988). The activity was assayed normally at pH 10. The enzyme was stable between pH 8.0 and 11. At lower and higher pH values of 7 and 12, with more than 80% o f the enzyme activity was retained (Fig. 4). The pH activity profile of the enzyme is in agreement with the characteristic single pH peaks shown by most α-amylases (Odibo *et al.*, 1992; Paquet *et al.*, 1991; Morgan and Priest, 1981).

The enzyme was equilibrated at different temperatures for 10 min at pH 10 before assay at the same temperature for 5 min. The α-amylase displayed a peak of activity at 80°C (Fig. 5). The test for thermostability was conducted in thin-walled test-tubes at different temperatures for 30 min according to the method described by Kocchar and Dua (1990). Thereafter, the tubes were promptly chilled in ice and the residual activity assayed at 80°C as described earlier. The enzyme retains 89% activity at 100°C for 6 h and 84% activity at 105°C for 20 min confirming it a novel thermostable alkaline α-amylase (Fig. 5). The optimal temperature for activity and stability of the α-amylase

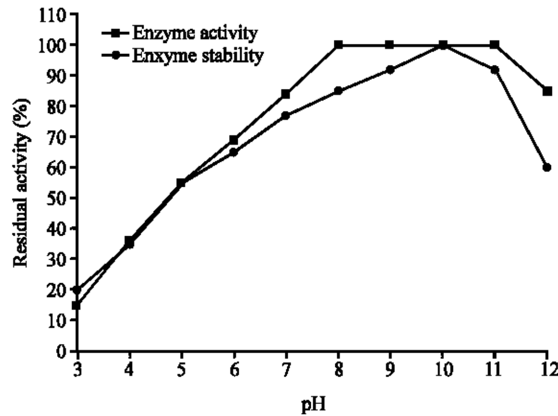

Fig. 4: Effect of pH on the activity and stability of purified α-amylase from *Bacillus circulans* PN5

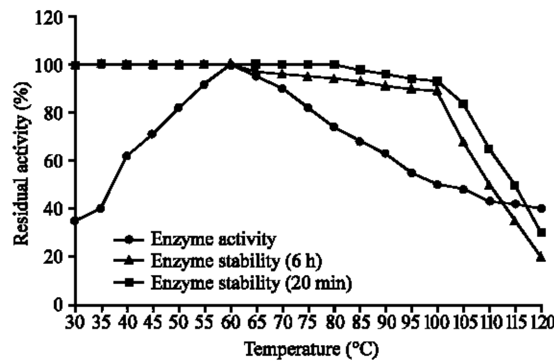

Fig. 5: Effect of temperature on the activity and stability (6 h; 20 min) of purified α-amylase from *Bacillus circulans* PN5

was quite high and comparable with other thermostable α-amylases (Rehana and Nand, 1989; Jin *et al.*, 1990; Saxena *et al.*, 2007). The activity and stability of our sample were higher than those reported for *B. subtilis* (Hayashida *et al.*, 1988). The reason for this high thermostability in our enzyme sample is under investigation.

Thin layer chromatography of digests demonstrated the production of glucose, maltose and maltotriose including some small amounts of G4-G6 from starch prepared from several sources. The results were consistent with the action of an endoenzyme which released saccharides with a polymerisation degree of 1-6 (Hayashida *et al.*, 1988). The simultanous liberation of all the maltooligosaccharides indicated a random attack of substrate chain by α-amylase. The simultaneous longer incubation, glucose, maltose and maltotriose were the end products of hydrolysis (data not presented). The yield of α-amylase from *B. circulans* PN5 was better than reported for α-amylase from *B. subtilis* (Odibo *et al.*, 1992) and *C. acetobutylicum* (Paquet *et al.*, 1991). This suggests that the enzyme is of the saccharifying type and contrasts with the liquefying α-amylase of *B. amyloliquefaciens* and *B. licheniformis* which produces predominantly maltosaccharides during starch hydrolysis (Kukn *et al.*, 1982; Matsuzaki *et al.*, 1974; Nakajima *et al.*, 1986).

Compatibility as detergent: Enzyme is highly stable with the commercially available detergents retaining more than 90% activity. Purified α-amylase was evaluated for its stability and

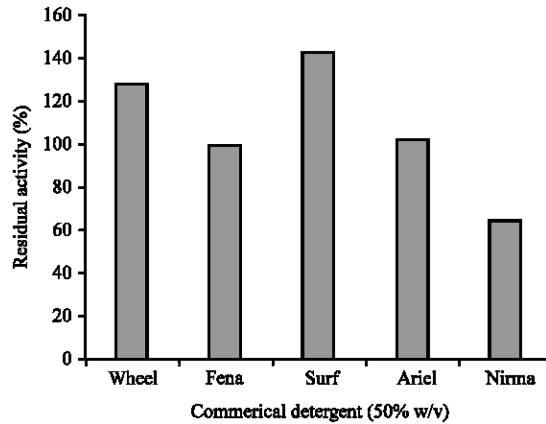

Fig. 6: Enzyme stability with different commercial detergents after 60 min of exposure

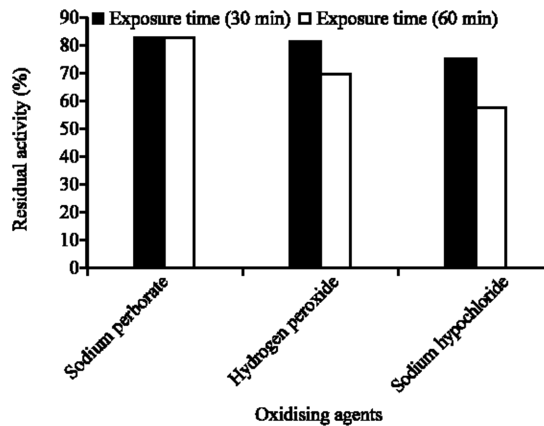

Fig. 7: Enzyme stability with oxidising agents after 30 min and 60 min of exposure

compatibility with five domestic commercial detergent formulations. The studies showed that enzyme retains more than 90% activity after exposure for 30 min to all tested detergent formulations (Fig. 6). These results revealed the suitability of enzyme in detergent formulations. Also, many newer application of thermostable enzymes need to evaluate its stability and compatibility with oxidizing agents. These studies were carried on the three oxidizing agents (Fig. 7). The purified enzyme is also stable with the different oxidizing agents tested and retained more than 75% activity after exposure for 30 min (Fig. 7) with tested oxidizing agents. Even after 1 h exposure the residual activity was more than 56%. This confirmed the suitability of enzymes for the process which has to be carried out in presence of these oxidizing agents.

CONCLUSION

Newly isolated *Bacillus circulans* PN5 produces as novel α-amylase offering interesting hydrolytic properties since the enzyme was active at pH 7-12 with 70-80% activity. Beside pH tolerance the most striking feature of the enzyme is its thermostability. The enzyme was highly thermostable retaining 89% activity at 100°C till 6 h and 74% activity at 105°C for 20 min. Its compatibility with domestic detergent formulations makes it ideal for the detergent industries. Also, significant thermostability of enzyme make it potential for industrial applications such as starch

liquefaction for sweeteners and syrups, textile and paper industries, which demands the process to be carried out in multiple steps at high temperature.

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