Study on Some Properties of Calcium-dependent α-Amylase from *Bacillus subtilis* through Submerged Fermentation of Wheat Bran

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This study reveals the production of α-amylase through *Bacillus subtilis* by submerged fermentation of wheat bran as a substrate. Various conditions were optimized and maximum production was observed at initial medium pH of 7.0, incubation temperature of 35 °C with agitation speed of 140 rpm for 48 h of fermentation period. Peptone, ammonium sulphate and soluble starch favored enzyme production as nitrogen and carbon source, respectively. The crude α-amylase was purified 2.03-fold by column (sephadex G-100) chromatography having specific activity of 480.0 U mg^{-1}. The purified enzyme had molecular mass of 54.18 kDa as determined by 12 % SDS-PAGE. The optimum pH and temperature of the enzyme was 8.5 and 60 °C, respectively. Ca^{2+} played an important role in the stability of the enzyme retaining relative activity of 87 % and 67 % at pH 9.5 and 80 °C, revealing the Ca^{2+} dependency respectively. The enzyme was highly stable towards surfactants and oxidizing agents. Kinetic study indicated that the enzyme exhibit K_m and V_{max} value of 4.4 mg mL^{-1} and 714.2 U mL^{-1} using soluble starch as a substrate respectively.

Key words:
α-amylase, purification, characterization, *Bacillus* sp.

Introduction

α-Amylase (EC 3.2.1.1) is an enzyme that hydrolyses α –1, 4-glucosidic linkage in the starch, and converts it into low molecular products. This enzyme is ubiquitous in nature and found in a wide variety of organisms, like plants, animals, and microbes. This enzyme is of particular interest due to its broad application in industrial sector like food, fermentation, paper, and textile industry[1]. Commercially important α-amylase is produced by microbial sources. α-Amylase is particularly produced from bacterial species, especially the genus *Bacillus,* like *B. subtilis, B. licheniformis, B. stearothermophilus* and *B. amyloliquefaciens*[2]. Mostly industrial processes were preceded at high temperatures and bacterial amylases are known to be more thermostable than fungal amylases[3].

Amylases could be produced through solid state and submerged fermentation processes. However, mostly, submerged fermentation systems were used for production due to the ease in controlling the conditions like pH, temperature, and other environmental conditions. Agricultural wastes were also utilized for amylase production, but for commercial production, synthetic media were used through submerged fermentation[4–7]. In our previous study[8], the metal profile of α-amylase was studied which showed that the enzyme produced by this strain was Ca^{2+} dependent. Presently, saccharification of starch is done by α-amylase which requires calcium ions for its activity and stability. This study aimed to purify and characterize the α-amylase from *Bacillus subtilis* produced from submerged fermentation using wheat bran as a substrate.

Materials and methods

Bacterial strain

Bacillus subtilis was obtained from Microbiology laboratory, Food & Biotechnology Research Center, Pakistan Council of Scientific and Industrial Research Laboratories, complex Ferozepur Road, Lahore Pakistan, 54600, and was used in amylase production in submerged fermentation process.

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Inoculum preparation

A twenty-four-hour-old vegetative cell was used as an inoculum during the present study. Twenty-five milliliters of nutrient broth (Oxoid) was sterilized in 250 mL Erlenmeyer flask at 121 °C for 15 min. After sterilization, the media was inoculated with a loopful of *Bacillus subtilis* and incubated at 35 °C for 48 h with the agitation speed of 140 rpm.

Fermentation methodology

Submerged fermentation technique was used for the production of α-amylase in 500 mL Erlenmeyer flask containing 100 mL of medium (%) 0.5 g peptone, 0.2 g K_2HPO_4, 0.25 g yeast extract, 0.1 g $MgSO_4 \cdot 7H_2O$ and 2 % wheat bran as a carbon source. The pH of the medium was maintained at 7 with 1 mol m^{-3} HCl/NaOH before sterilization at 121 °C for 15 min. After sterilization, the medium was inoculated with 1 ml of vegetative cells and incubated at 35 °C for 48 h.

Enzyme activity

The activity of α-amylase was assayed as described earlier[8]. Reaction mixture containing 0.5 mL of the enzyme solution and 0.5 mL of soluble starch (0.5 %, w/v prepared in 0.1 mol L^{-1} sodium phosphate buffer, pH = 7) was incubated at 60 ºC for 30 min. After incubation, the reaction was stopped and the reducing sugars released were assayed colorimetrically by the addition of 1 mL of 3-5-dinitrosalicylic acid reagent. One enzyme activity unit (U) was defined as the amount of enzyme releasing 1 μmol of maltose from the substrate in 1 minute at 60 ºC.

Soluble protein estimation

Soluble protein in the cell free extract was estimated by the method of Lowery *et al.,*[9] using bovine serum albumin as standard.

Purification of α-amylase

The enzyme produced by *Bacillus subtilis* in submerged fermentation was subjected to purification by ammonium sulphate fraction followed by dialysis and sephadex G-100 column chromatography. The column size of 1.5 cm × 30 cm was used on FPLC system (Biologic LP, Bio-Rad, USA) with flow rate of 30 mL h^{-1}. Fractions showing amylase activity were pooled and further processed for homogeneity by SDS-PAGE (12 %) by the method of Laemmeli[10]. All the purification procedures were done at 4 °C.

Characterization of α-amylase

Effect of pH on activity and stability of α-amylase

The optimum pH for the enzyme was determined by incubating purified enzyme with substrate (1 % starch) prepared in appropriate buffers; 0.05 mol L^{-1} citrate buffer (pH 3.0 to 6.0), 0.05 mol L^{-1} sodium phosphate buffer (pH 6.0 to 8.0), 0.05 mol L^{-1} Tris-HCl (pH 8.0 to 9.0) and 0.05 mol L^{-1} glycine-NaOH (pH 9.0 to 11.0). Enzyme and substrate was incubated for 30 min at 50 ºC. After incubation, the reaction was stopped by the addition of DNS reagent and absorbance was measured at 550 nm. The pH stability of the purified enzyme was studied in the presence and absence of Ca^{2+} and Mg^{2+} at various pH buffers ranging from 7–10. After that, the enzyme activity was measured by standard assay procedures.

Effect of temperature on activity and stability of α-amylase

The effect of temperature on activity of α-amylase was determined by incubating enzyme mixture in 1 % starch in 0.05 mol L^{-1} sodium phosphate buffer, pH 8, at temperatures between 40 to 90 °C with regular interval of 10 °C. Enzyme activity was assayed by DNS method at different temperatures as described above. Thermostability studies of the enzyme were conducted in the presence and absence of metal ions (Ca^{2+} and Mg^{2+}) by pre-incubating the enzyme solution at 50, 55, 60, 65, and 70 °C for 0 to 140 minutes. After incubation, the enzyme activity was checked by DNS method as described earlier.

Effect of surfactants and oxidizing agents

To study the effect of different surfactants and oxidizing agents, the enzyme was incubated in the presence of surfactants (Tween 80, triton X-100 and SDS with concentrations of 0.5, 1.0, and 2.0 %) and oxidizing agent (H_2O_2 with concentrations of 0.5, 1.0, and 2.0 %) for 2 h at 50 °C. After that, the enzyme activity was checked as described above.

Enzyme kinetics

The K_m and V_{max} values for α-amylase were determined by linear regression analysis by Lineweaver-Burk plot (double reciprocal plot) using various concentrations of starch (5, 10, 15, 20, 25, and 30 mg mL^{-1}). The experiments were carried out in triplicate and the activity measured according to the standard assay conditions.

Statistical analysis

All the data obtained was statistically analyzed by Minitab software version 17 and the values presented were the mean of triplicates.

Results and discussion

Optimization of process parameters for amylase production

Effect of fermentation time

Different fermentation time (12–96 h) was tested for optimum production of amylase from *B. subtilis* in submerge fermentation. Results (Fig. 1) revealed that enzyme production had increased with increase in fermentation period. The peak production was observed at 48 h of fermentation. Further increase in fermentation time resulted in a decline in enzyme production, which might be due to depletion of nutrients or some other secondary metabolites production from bacteria that inhibited enzyme production. Swain *et al.*[11] achieved optimum amylase production in stationary phase (36 h) from *B. subtilis* CM3. de Carvalho *et al.*[12] reported 32 h optimum fermentation period for amylase production from *Bacillus* sp. strain SMIA-2.

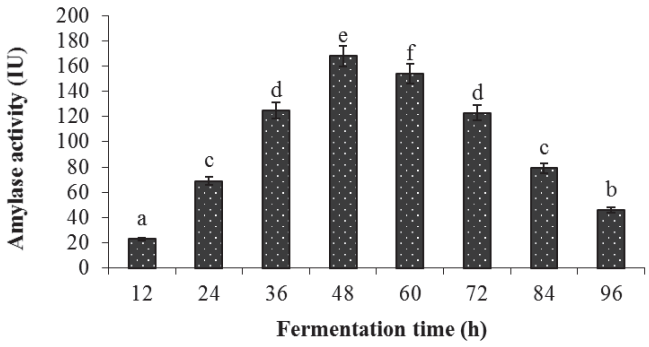

Fig. 1 – *Effect of fermentation time on amylase production in submerged fermentation*

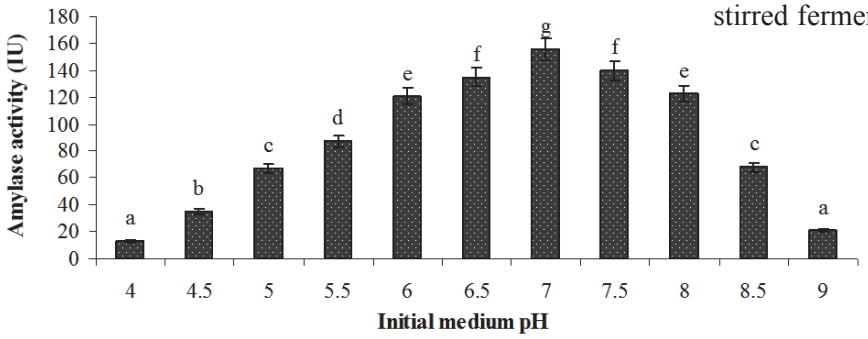

Fig. 2 – *Effect of initial medium pH on amylase production in submerged fermentation*

Effect of initial medium pH

Various initial medium pH (4–9) were tested for maximum production of amylase in submerged fermentation at 48 h. Maximum enzyme production was obtained at initial medium pH of 7 (Fig. 2). Acidic and alkaline medium pH strongly affects the enzyme production. Swain *et al.*[11] reported that initial medium pH in the range of 5.0–9.0 was best for maximum amylase production by *B. subtilis*. Bacterial species like *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* produced maximum amylase at an initial pH of 7.0[13–15].

Effect of incubation temperature

Incubation temperature also plays an important role in microbial growth. Various temperatures (25–45 °C) were employed for maximum production of amylase in submerged fermentation. Results (Fig. 3) showed that enzyme production had increased with increasing in temperature from 25 °C, and peak production was observed at 35 °C. Further increased temperature resulted in a decline in enzyme production. Some strains of genus *Bacillus* like *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* were thermophilic and produced amylase in the temperature range of 37–70 °C[11,15–17].

Effect of agitation speed

Different agitation rates (100–200 rpm) were applied to check the optimum production of amylase in submerged fermentation. Among all these, the agitation speed of 140 rpm was found to be suitable for maximum enzyme production (Fig. 4). Slow agitation speed leads to poor aeration, while increased agitation speed causes cell destruction which reduces enzyme production in submerged fermentation. Jadoon *et al.*[18] reported 150 rpm agitation speed was optimum for maximum amylase production in submerged fermentation. Tanyildizi and Özer[19] reported optimum agitation speed of 150 rpm for amylase production by *Bacillus amyloliquefaciens*. Abdullah *et al.*[20] reported 200 rpm for maximum amylase production by *A. oryzae* in stirred fermenter.

Supplementation of additional sugars

Various sugars (0.50 %) were supplemented to the fermentation medium to check maximum enzyme production by *B. subtilis* in submerged fermentation. Results (Fig. 5) showed that supplementation of soluble starch followed by glucose effectively improved enzyme production as compared to control.

Demirkan[21] stated that mesoinositol was the best carbon source for amylase production by *B. subtilis* in submerged fermentation. Anto *et al.*[22] observed maximum amylase production with glucose as carbon source. Some studies suggested that starch and glycerol was a favorable carbon source for amylase production by *Bacillus* sp.[14,23,24]

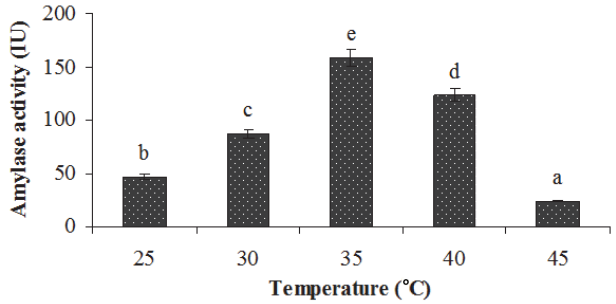

Fig. 3 – *Effect of incubation temperature on amylase production in submerged fermentation*

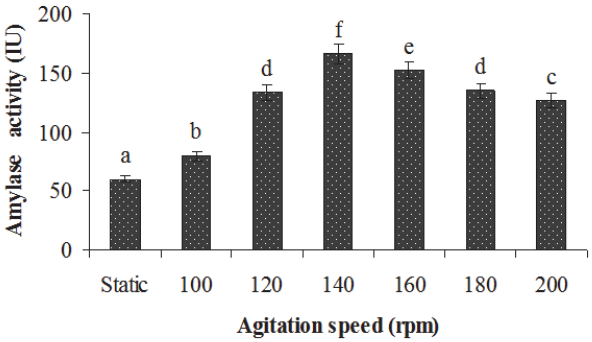

Fig. 4 – *Effect of agitation on amylase production in submerged fermentation*

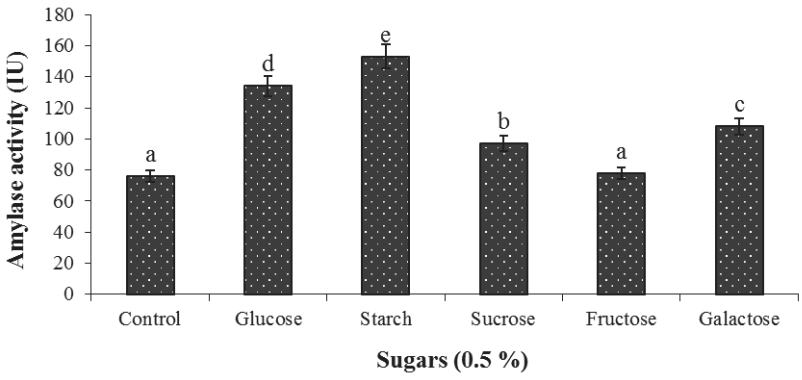

Fig. 5 – *Effect of supplementation of different sugars for amylase production*

Effect of nitrogen source

Various nitrogen sources were tested for maximum amylase production in submerged fermentation. Optimum enzyme production was observed using peptone and ammonium sulfate as organic and inorganic nitrogen sources (Fig. 6). Sodium nitrate and urea gave very low enzyme yield. Malt extract and tryptone were suitable nitrogen sources for amylase production by *B. subtilis*[21]. Yeast extract and ammonium acetate were preferred nitrogen sources for amylase production[11]. Peptone and yeast extract was the best nitrogen sources for amylase production[25,26].

Purification of amylase

The enzyme produced by *Bacillus subtilis* was purified using ammonium sulfate fractionation followed by gel filtration chromatography. Maxiumum specific activity of 274.5 ± 1.53 U mg^{-1} was achieved at 60 % ammonium sulfate fractionation (Table 1). After that, the enzyme solution was subjected to dialysis yielding specific activity of 292.8 U mg^{-1}. The dialyzed enzyme suspension was loaded on to column of sephadex G-100. The elution profile of the enzyme showed (Fig. 7) that fractions from 20 to 30 detected specific activity of 480.0 U mg^{-1} with purification fold of 2.03. In this whole purification procedure, the specific activity was increased from 236.1 ± 1.84 U mg^{-1} to 480.0 ± 2.59 U mg^{-1} with enzyme yields of 56.4. These results illustrated that gel filtration chromatography efficiently purified the amylase produced by *B. subtilis* in submerged fermentation. This purification fold was less than that from other strains of *Bacillus* which used DEAE-cellulose[27] and gel filtration chromatography[28] for purification, respectively. Bano *et al.*[29] purified α-amylase from *Bacillus subtilis* having specific activity of 13011 U mg^{-1} with purification fold of 96.3.

Table 1 – *Purification profile of α-amylase from Bacillus subtilis*

Purification steps	Total activity (IU)	Total protein (mg mL^{-1})	Specific activity (U mg^{-1})	Purification folds	Yield (%)
Crude enzyme	170	0.72	236.1	1.00	100.0
Amm. Sulphate ppt.	140	0.51	274.5	1.16	82.3
Dialysis	123	0.42	292.8	1.24	72.4
SephadexG-100	96	0.20	480.0	2.03	56.4

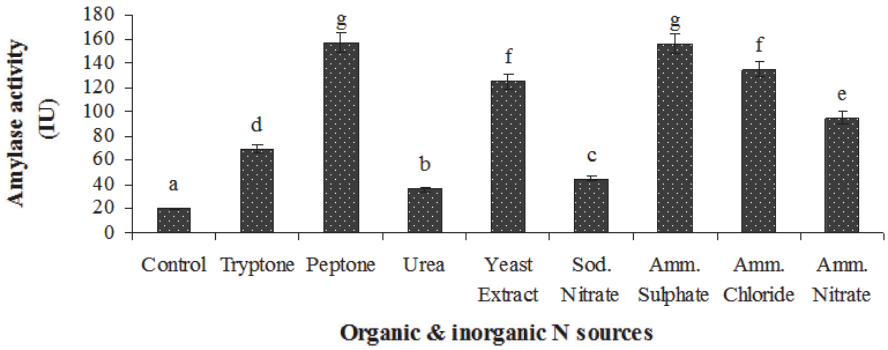

Fig. 6 – *Effect of different nitrogen sources on amylase production in submerged fermentation*

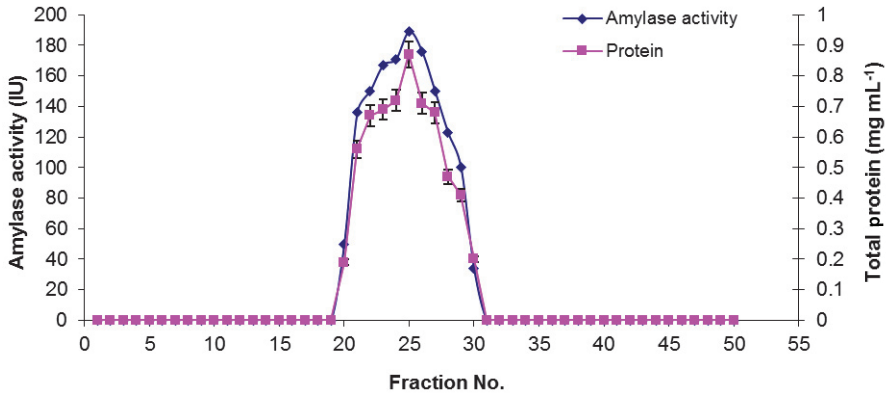

Fig. 7 – *Elution profile of the α-amylase from sephadex G-100 column*

Molecular weight determination of the α-amylase

The molecular weight of the purified α-amylase was found to be 54.18 kDa based on linear regression as it indicated a single band on SDS-PAGE revealing a homogeneous preparation (Fig. 8). This indicated that this protein was monomeric. Molecular weight of this α-amylase was very close to that of other strains of *B. subtilis* which have molecular weight of 56 kDa[21,29] and *B. subtilis* PKTH 10 having molecular weight of 55 kDa[30]. Some strains of *B. subtilis* DM 03, *Bacillus* sp. TS23 and *Bacillus* sp. MNJ23 produced low molecular weight α-amylase of 42.8 kDa, 42.0 kDa and 25kDa, respectively[27,31,32]. But some strains of *Bacillus subtilis* produced α-amylase having high molecular weight of 93, 68, 63[28,33,34].

Effect of pH

To study the effect of pH on purified amylase activity, the starch solution (1 %) was prepared in different buffers pH ranging from 4–10. Results (Fig. 9) showed that the enzyme activity had increased with increase in pH, and optimum enzyme activity was observed at pH 8.5. As the pH increased up to 10, the enzyme activity decreased. Mostly the amylase produced by various strains of *Bacillus subtilis* was optimally active in the pH range of 6.0–9.5, as shown in the Table 2. The pH stability of the enzyme was also studied by incubating the enzyme solution in different pH at room temperature (30 °C) for 2 h. Results indicated that Ca^{2+} played an important role in the stability of amylase over the pH range of 8.0–9.0. The enzyme retained 97 % and 94 % activity at pH 9 using 10 mmol L^{-1} Ca^{2+} and 5 mmol L^{-1} Ca^{2+}, respectively. This indicated that the enzyme was Ca^{2+} dependent. Various studies reported that the α-amylase produced by *Bacillus* species was Ca^{2+} dependent and its activity was enhanced and stabilized by the addi-

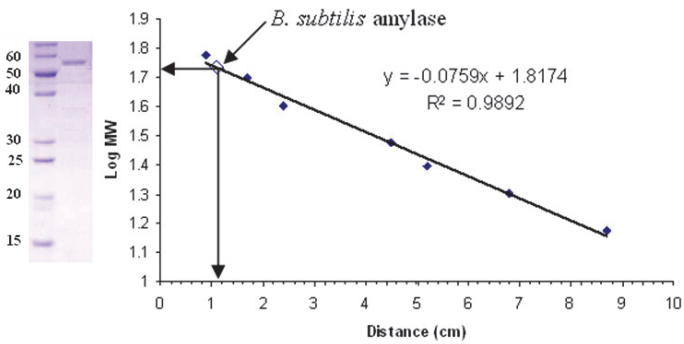

Fig. 8 – *SDS-PAGE and molecular weight determination of the α-amylase produced from B. subtilis in submerged fermentation*

tion of Ca^{2+} due to its stronger affinity[21,28,35,36]. Almost all reported α-amylase have conserved Ca^{2+} binding sites[37–39]. The binding of Ca^{2+} to the enzyme increases the α helical structure thus leading to the stability of the enzyme[40].

Effect of temperature

The purified α-amylase produced by *Bacillus subtilis* under submerged fermentation was tested for optimum temperature determination by incubating the reaction mixtures at various temperatures ranging from 40 °C to 90 °C. The enzyme had optimum temperature of 60 °C and the activity was about zero at 90 °C. These results were comparable to other studies, as shown in Table 2. The purified α-amylase was tested for thermostatbility for 2 h by incubation in the absence and presence of 5 and 10 mmol L^{-1} Ca^{2+} and Mg^{2+}. Results (Fig. 10) indicated that the purified α-amylase retained 67 % and 48 %

Table 2 – *Optimum pH and temperature from different strains of Bacillus subtilis*

Microroganism	Opt. pH	Opt. temp (°C)	Reference
Bacillus subtilis	8.5	60	This study
Bacillus subtilis	6.0	50	45
Bacillus subtilis BMT4i	8.0	40	46
Bacillus subtilis DR8806	9.5	70	47
Bacillus subtilis KC3	6.5	50	48
Bacillus subtilis KIBGE HAS	7.5	50	29
Bacillus subtilis BS5	6.0	50	28
Bacillus subtilis	6.0	45	21
Bacillus subtilis	7.0	37	49
Bacillus subtilis	7.0	37	50

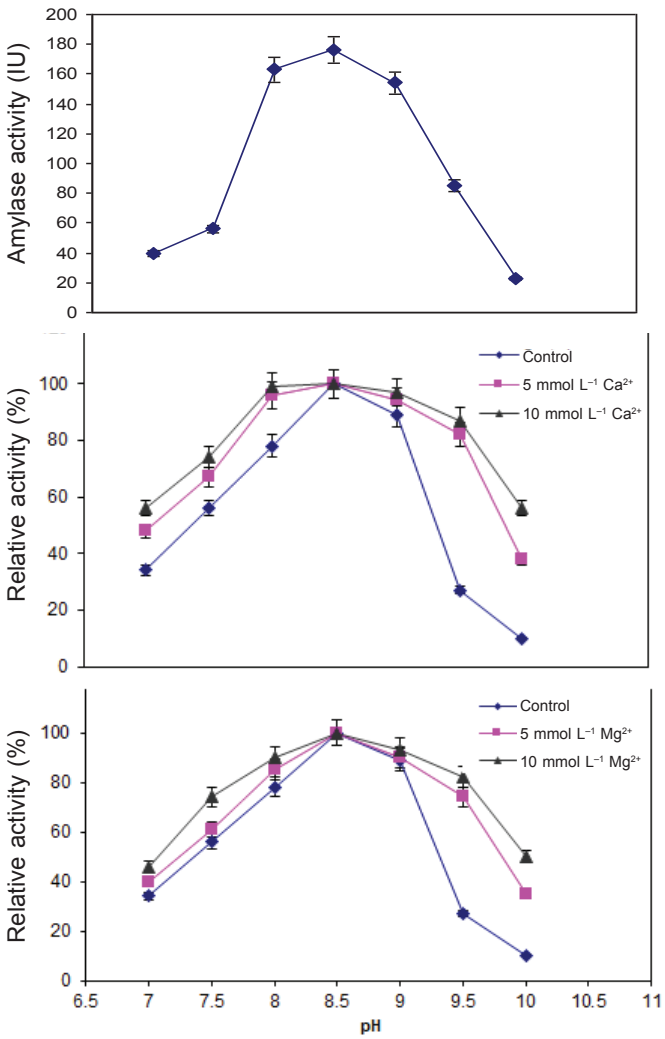

Fig. 9 – *Effect of pH on activity and stability of amylase produced from B. subtilis*

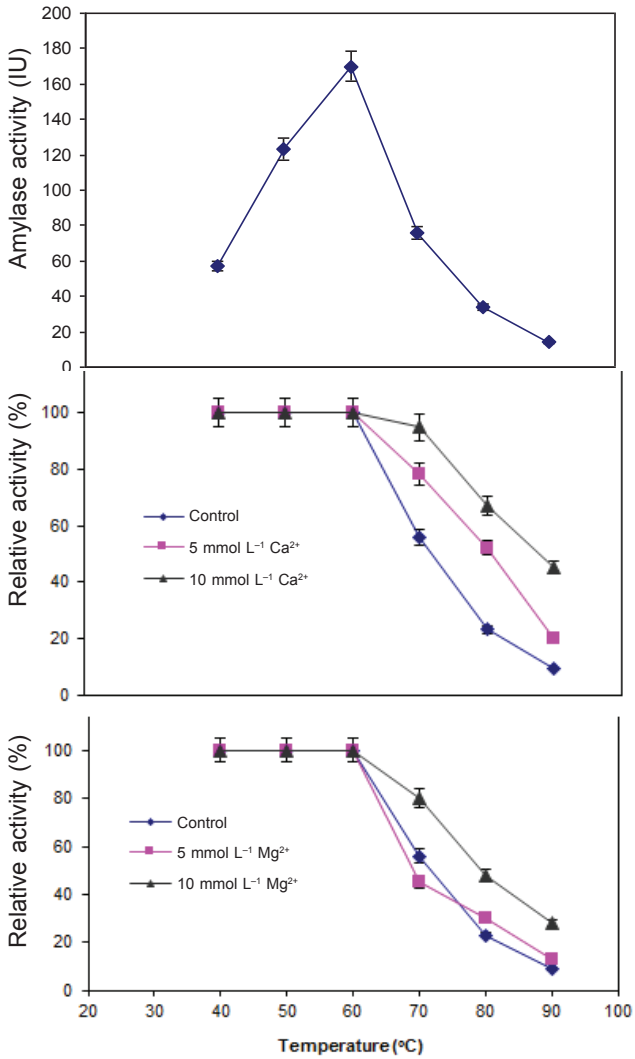

Fig. 10 – *Effect of temperature on activity and stability of amylase produced from B. subtilis*

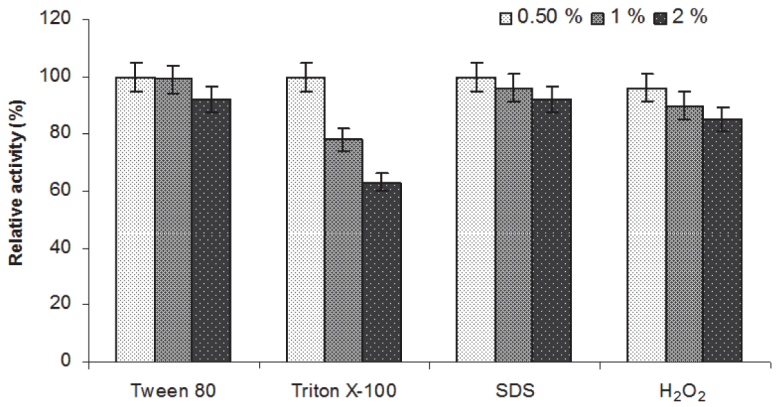

Fig. 11 – *Effect of surfactants and oxidizing agent on activity of amylase produced from B. subtilis*

activity at 80 °C in the presence of 10 mmol L^{-1} Ca^{2+} and Mg^{2+}, while 23 % activity in the absence of these ions. These results suggest that metal ions like Ca^{2+} and Mg^{2+} play an important role in the stability of the enzyme activity. Some amylases from *Bacillus* strains were extremely thermophilic having optimum temperature of 100 °C[41,42]. These Ca^{2+} play an important role in the thermal inactivation of amylases produced by *Bacillus* species[43,44].

Effect of surfactants and oxidizing agent

The purified α-amylase enzyme was tested for stability towards surfactants and oxidizing agents. The surfactants (Tween 80, SDS, and Triton X-100) and oxidizing agents (H_2O_2) were used in three concentrations (0.50 %, 1.0 % and 2.0 %) with incubation of 6 h. Results (Fig. 11) showed that enzyme retained 100 % activity at 0.5 % concentration of surfactants and oxidizing agents. Enzyme activity had slightly decreased in the case of Triton X-100 retaining 63 % relative activity with concentration of 2 %. These findings indicated that the enzyme was highly stable in the presence of surfactants and oxidizing agents which was supported by various reports[47,51–53]. The stability of enzyme towards surfactants and oxidizing agents revealed its potential utilization in detergent industries. A good enzyme used in detergent industries must retain maximum stability with other surfactants.

Kinetics of α-amylase

Kinetic parameters of the purified enzyme revealed that the enzyme exhibited K_m value of 4.14 mg mL^{-1} and V_{max} value of 714.2 ± 1.25 U mg^{-1}, respectively. The K_m value in this study was in agreement with the K_m value in other studies on *Bacillus* species, which ranged from 1.08 to 16.67 mg mL^{-1} [21,28,29,45,46,54]. The V_{max} value in the present study was higher than that of 47.62 U mg^{-1} proteins[45] and lower than that of 5000 U[46] and 1773 U mL^{-1} [29]. Demirkan[21] reported the V_{max} value of 100 and 151 U mL^{-1} from parent and mutant strains of *Bacillus subtilis* respectively. In another study, Femi-Ola and Olowe[28] reported the V_{max} value of 3.8 mg/mL/min from *Bacillus subtilis* BS5. In a recent study, amylase produced from *Bacillus* sp MB6 had K_m and V_{max} values of 5.45 mg mL^{-1} and 24.15 mg/mL/min, respectively[55]. α-Amylase from locally isolated *Bacillus* sp strain B-10 showed K_m and V_{max} value of 1.4 mg mL^{-1} and 6.2 U mL^{-1}, respectively[56].

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