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Purification and characterization of extracellular acidophilic α-amylase from *Bacillus cereus* MTCC 10205 isolated from soil

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Amylase from *Bacillus cereus* MTCC 10205 was purified 20.41 with 11.82% recovery by ammonium sulfate precipitation, gel filtration chromatography through Sephadex G-100 and ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. The final enzyme preparation was pure to near homogeneity as judged by native-polyacrylamide gel electrophoresis (PAGE). The enzyme had a molecular weight of 55 kDa as determined by gel filtration and a single band of 55 kDa as determined by sodium dodecyl sulfate-polycrylamide gel electrophoresis showing it to be a monomer. The purified enzyme had temperature optima of 55°C and pH optima of 5.5. The enzyme retained 72% of its original activity after 90 min of incubation and exhibited gradual loss in activity when incubated at higher temperature. At 60°C after 90 min of incubation, the enzyme was completely inactive. The enzyme appeared to be quite stable at 4°C as it could be stored upto five days with 10% loss in activity, whereas at 35°C, the enzyme lost 28% of its activity just after three days of storage. Inhibition studies revealed SH groups to be involved at the active site of the enzyme.

Key words: Amylase, *Bacillus cereus,* gel-filtration, purification, sodium dodecyl sulphate.

INTRODUCTION

Amylases are of ubiquitous occurrence and are holding maximum market share of enzyme sales (Sivaramakrishanan et al., 2006). These hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965) and hence are used in a wide range of starch industries that is brewing, baking, starch liquefaction and distillery (Souza and Magalhães, 2010). Amylases that are active at acidic pH are generally used in the glucose syrup industry, whereas those active at basic pH are explored in detergent industries (Tonokova, 2006). Although amylases can be derived from several sources such as plants, animals and microorganisms, microbial sources are the most preferred one for large scale production in meeting industrial demands (Rao et al., 2007). The microbial amylases are usually extracellular and are widely distributed in bacteria, actinomycetes and fungi (Sivaramakrishanan et al., 2006). Two major classes of amylases have been identified in microorganisms, namely amylase

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Abbreviations: SM, Starch medium; **DNSA,** 3, 5- dinitrosalicylic acid; **DEAE,** diethyl aminoethyl; **PAGE,** polyacrylamide gel electrophoresis; **PHMB,** para hydroxyl mercuric-benzoate; **PMSF,** phenyl methoxy sulfonyl fluoride; **DTNB,** 5, 5-dithiobis-2-nitrobenzoic acid; **EDTA,** ethylenediaminetetraacetic acid; **β-ME,** β-mercaptoethanol.

α-amylase and glucoamylase. The α-amylases (endo-1,4-α-D glucan glucohydrolase, EC.3.2.1.3) are extra cellular enzymes that randomly cleave 1,4-α-D-glucosidic linkages between adjacent glucose units in linear amylose chain. Glucoamylase (exo-1,4-α-D glucan glucohydrolase, EC.3.2.1.1) hydrolyses single glucose unit from the non-reducing end of amylose and amylopectin in a stepwise manner. We report here purification and characterization of acidic amylase from a new strain of bacteria having potential of being industrially used.

MATERIALS AND METHODS

Reagents

All the chemicals used in the present work were of analytical grade and were purchased from Sigma Chemicals Co., USA, Hi-Media, Sisco Research Laboratories and E. Merck, Bombay.

Bacterial strain and growth conditions

Microorganisms isolated from the soil collected from vegetable and grain market, were screened for amylase production. The most efficient producer was identified as *Bacillus cereus* MTCC-10205 (Institute of Microbial Technology-IMTECH Chandigarh India). This strain was grown at 35°C from an inoculum containing 2.55 x 10^6 cell mL^{-1} in starch medium (SM) for 36 h under submerged conditions. Inoculum was in a proportion of 2%. Starch medium contained soluble starch 10.0 g L^{-1}, yeast extract 5.0 g L^{-1}, peptone 3.0 g L^{-1}, $MgSO_4$ $7H_20$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1} and K_2HPO_4 0.8 g L^{-1} (pH 7.0).

Amylase assay

Amylase activity was determined through spectrophotometrical measure of dinitrosalicylic acid reduction by reducing sugars released from soluble starch used as substrate (Miller, 1959). The reaction mixture contained 0.80 mL starch (10 g L^{-1} in 0.016 M sodium acetate buffer, pH 4.8) and 200 μL of enzyme solution in a final volume of 1 mL. The reaction was incubated at 40°C for 30 min and stopped by adding 2 ml of 3, 5- dinitrosalicylic acid (DNSA) reagent (1% DNSA, 0.05% sodium sulphide, 30% sodium potassium tartarate and 0.2% phenol in 0.4 N NaOH). The mixture was heated for 5 min in boiling water bath and then cooled to room temperature. Absorbance of sample was measured at 540 nm against the substrate blank. A standard curve of maltose ranging from 0 to 1000 µg/ml was constructed and then the released maltose was determined in the samples from standard curve. One unit of amylase activity was defined as the amount of enzyme that liberated 1 nmol of maltose equivalent under the experimental conditions in 1 min. Amount of soluble protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme purification

B. cereus MTCC-10205 grown in SM for 36 h was filtered through muslin cloth. The filtrate containing amylase was centrifuged at 12,000 rpm for 15 min to obtain cell-free supernatant which was referred to as crude extract. The crude extract was subjected to 45-70% $(NH_4)_2SO_4$ saturation, centrifuged at 12,000 rpm for 15 min and precipitates dissolved in 0.016 M sodium acetate buffer (pH 4.8) and dialyzed against same buffer. The enzyme preparation obtained after ammonium sulphate fractionation was carefully layered over the top of a Sephadex G-100 column (700 x 15 mm) and eluted with 0.016 M sodium acetate buffer pH 4.8, at a flow rate of 10 ml/h. Fractions of 3 ml each were collected and protein content (A_{280}) and enzyme activity analyzed. The fractions with amylase activity were pooled and concentrated by dialyzing against sucrose. The concentrated enzyme was loaded on a diethyl aminoethyl (DEAE)-cellulose column (300 x 30 mm). The column was washed with 0.016 M sodium acetate buffer (pH 4.8) and amylase was eluted by linear gradient of NaCl (0- 0.4 M) in the same buffer. Purity of enzyme was checked by native-polyacrylamide gel electrophoresis (PAGE).

Purity of enzyme by native-PAGE

Purity of the final enzyme preparation obtained after DEAE-cellulose chromatography was checked by native-PAGE (10% gel) on slab gels (M/S Atto, Japan) using anionic system (Davis, 1964).

Sample preparation

200 µl of each enzyme preparation *viz*., crude $(NH_4)_2SO_4$ fraction, Sephadex G-100 fraction and DEAE-cellulose fraction was taken in an eppendorf tube. To it, 0.2 ml glycerol (20%) and 25 µl of 5% bromophenol blue were added and contents mixed thoroughly.

Electrophoresis

The clean plates were sealed by tygon tubing, clamped to make a mould and 10% resolving degassed gel solution was poured. A layer of water was then gently overlaid using a syringe. The assembly was left undisturbed for polymerization of the gel which took about 30 min and was indicated by a sharp interface between water and gel. Water was removed, 3.75% stacking gel solution poured and comb inserted immediately with care so that no air bubble was trapped beneath it. After polymerization, comb and tygon tubing were removed. Gel plates were fixed to the electorphoretic apparatus. Sample wells were rinsed with electrode reservoir buffer and the two reservoirs were filled with electrode buffer. Samples containing 200 μg protein were loaded in separate wells and electrohoresis was carried out at a constant current of 10 mA for first 30 min followed by 20 mA constant current till the tracking dye (bromo phenol blue) reached one cm away from the lower end of the gel.

Gel staining

Gel was removed from glass plates and stained overnight with staining solution (2.5% Coomassie Brilliant Blue containing methanol and acetic acid in the ratio of 40:7). The excess stain was removed by diffusion in destaining solution (7.5 % acetic acid and 5.0% methanol). After complete destaining, gel was transferred to 7% acetic acid and photographed.

Molecular weight determination

The molecular weight of purified amylase was determined by molecular exclusion chromatography through Sephadex G-100

Table 1. Summary of purification of amylase from *Bacillus cereus* MTCC 10205.

Purification step	Volume (ml)	Total activity* (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Fold purification	Yield (%)
Crude extract	485	106908.5	820	130.37	-	100
$(NH4)_2SO_4$fraction (45-70%)	15	24923	44.38	561.58	4.30	23.31
Sephadex G-100	36	20269.61	12.62	1606.15	12.32	18.95
DEAE-Cellulose	42	12639.03	4.75	2660.85	20.41	11.82

*One enzyme unit is the amount of enzyme that liberated 1nmol of maltose equivalent under the experimental conditions in 1 min.

column (650 x 15 mm). The void volume was calculated by passing blue dextran (2 mg mL^{-1}) through the column. The column was calibrated with standard molecular weight markers; cytochrome-C (12.4 kDa), carbonic anhydrase (29.0 kDa), bovine serum albumin (66.0 kDa), alcohol dehdyrogenase (150.0 kDa) and β-amylase (200.0 kDa) and then made protein free by running about 5 bed volumes of 0.016 M sodium acetate buffer (pH 4.8) through the column. The purified enzyme preparation was loaded over the top of the column and eluted with 0.016 M sodium acetate buffer (pH 4.8) at a flow rate of 15 mL h^{-1}. The fractions of 3.0 ml each were collected and analyzed for protein (A280) and amylase activity. The active fractions were pooled and their volume determined. A plot of log of molecular weight versus elution volume gave the molecular weight of the enzyme.

Polypeptide composition and their molecular weight

SDS-PAGE was performed according to Laemmli (1970) to determine the polypeptide composition and their molecular weight. The composition of the gel was same as in case of native-PAGE except that it contained 2% SDS. The samples and the standard proteins were mixed with equal volume of 2X buffer (0.250 M Tris-HCl pH 6.8 containing 4% SDS and 10% β- mercaptoethanol, 20 % glycerol and 0.4% bromophenol blue) and boiled for 5 min. The standard molecular weight protein markers used were galactosidase (175.0 kDa), paramyosin (83.0 kDa) MBP-CBD (62.0 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin B (24.0 kDa), lysozyme (16.5 kDa) and aprotinin (6.5 kDa). Molecular weight of the protein was determined from the standard curve of Rm values against log molecular weight of the standard molecular marker proteins.

$$Rm = \frac{\text{Distance travelled by protein band}}{\text{Distance travelled by tracking dye}}$$

Characterization of purified enzyme

pH, temperature optima and Km value

Optimum pH of the enzyme was determined by carrying out amylase assay at different pH values ranging from 3.5 and 8.5 at 40°C. The buffers used were acetate buffer for pH 3.5 to 5.5, phosphate for 6.0 to 7.5 and Tris-HCl for pH 8.0-8.5. Optimum temperature was determined by amylase activity at different incubation temperatures ranging from 30 to 70°C at pH 5.5. To study the effect of substrate (starch) concentration, enzyme activity was determined over a wide range of starch concentration ranging from 1.0 to 14 g L^{-1}. The Km was determined from reciprocal Lineweaver and Burk (1934) plot.

Thermostability, pH stability and storage ability

To determine enzyme thermostability, the amylase extract was incubated at 40, 50 and 60°C and residual enzyme activity was measured at 15 min interval up to 60 min at optimum conditions of pH and temperature (pH 5.5, 55°C). The amylase stability against pH was analyzed by its residual activity under optimum conditions (pH 5.5, 55°C) after incubating the enzyme in buffers with pH value ranging from 3.5 and 8.5 at 30°C for 1 h. The enzyme stability at storage conditions of 4 and 35°C was assessed by measuring residual activity weekly for 30 days.

Effect of metal ions and other additives

To determine the effect of several metal ions and other additives, the enzyme solution was incubated with 1 and 5 mM solution each of $MgSO_4$, KCl, $MnCl_2$, $MnSO_4$, $ZnSO_4$, $FeCl_3$, $CuCl_2$, $CaCl_2$, SDS, β-ME, iodoacetate, para hydroxyl mercuric-benzoate (PHMB), phenyl methoxy sulfonyl fluoride (PMSF) and 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) at 30°C for 30 min. The enzyme activity was determined as described earlier at optimum conditions (pH 5.5, 55°C).

RESULTS AND DISCUSSION

Purification of amylase

Amylase from *Bacillus cereus* MTCC 10205 was purified using conventional techniques of enzyme purification such as ammonium sulphate precipitation, gel filtration through Sephadex G-100 and ion exchange through DEAE-cellulose. Summary of enzyme purification is given in Table 1. The ammonium sulphate precipitation accounted a purification factor of 4.30 fold and 23% recovery. Concentrated enzyme obtained after $(NH_4)_2SO_4$ fraction was loaded on pre-equilibrated Sephadex G-100 column. The elution profile of the proteins and enzyme activity (Figure 1A) showed a single narrow peak of amylase activity comprising fractions 38-49, which coincided with one peak of protein. This step increased the purification factor to 12.32 fold recovering an 18.95% of the total activity as compared to the crude extract.

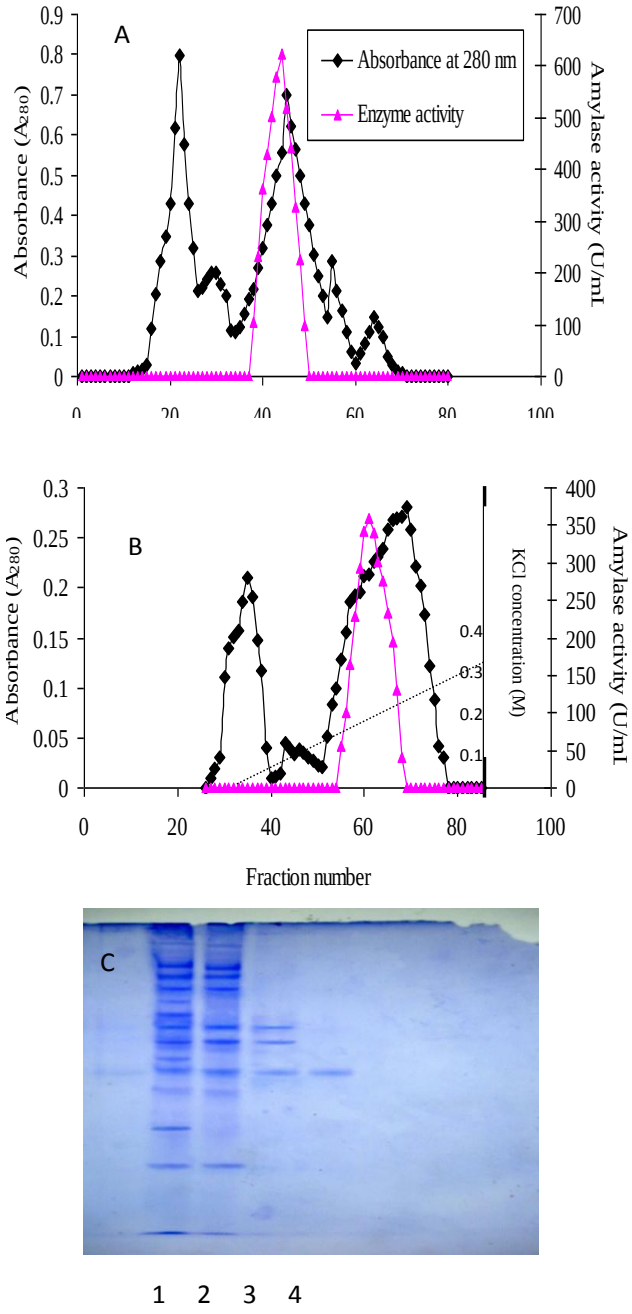

Figure 1. Elution profile of amylase from *Bacillus cereus* MTCC 10205 on Sephadex G-100 [A] and DEAE cellulose [B]. The enzyme activity was shown as -▲- and absorbance at 280 nm (amount of protein) was shown as -♦- which indicated the amount of protein. Lane 1, Electrophoretic pattern of amylase fractions during purification on native-PAGE [C] crude extract; Lane 2, ammonium sulphate fraction; Lane 3, Sephadex G-100 fraction; Lane 4, DEAE-cellulose fraction.

Elution of ion exchange chromatography on DEAE-cellulose also showed a single peak of amylase activity coinciding with one main protein peak between fractions 55-68 (Figure 1B). This purification process resulted in an enzyme preparation 20.40 fold purified with specific activity of 2660.9 U/ mg protein and a

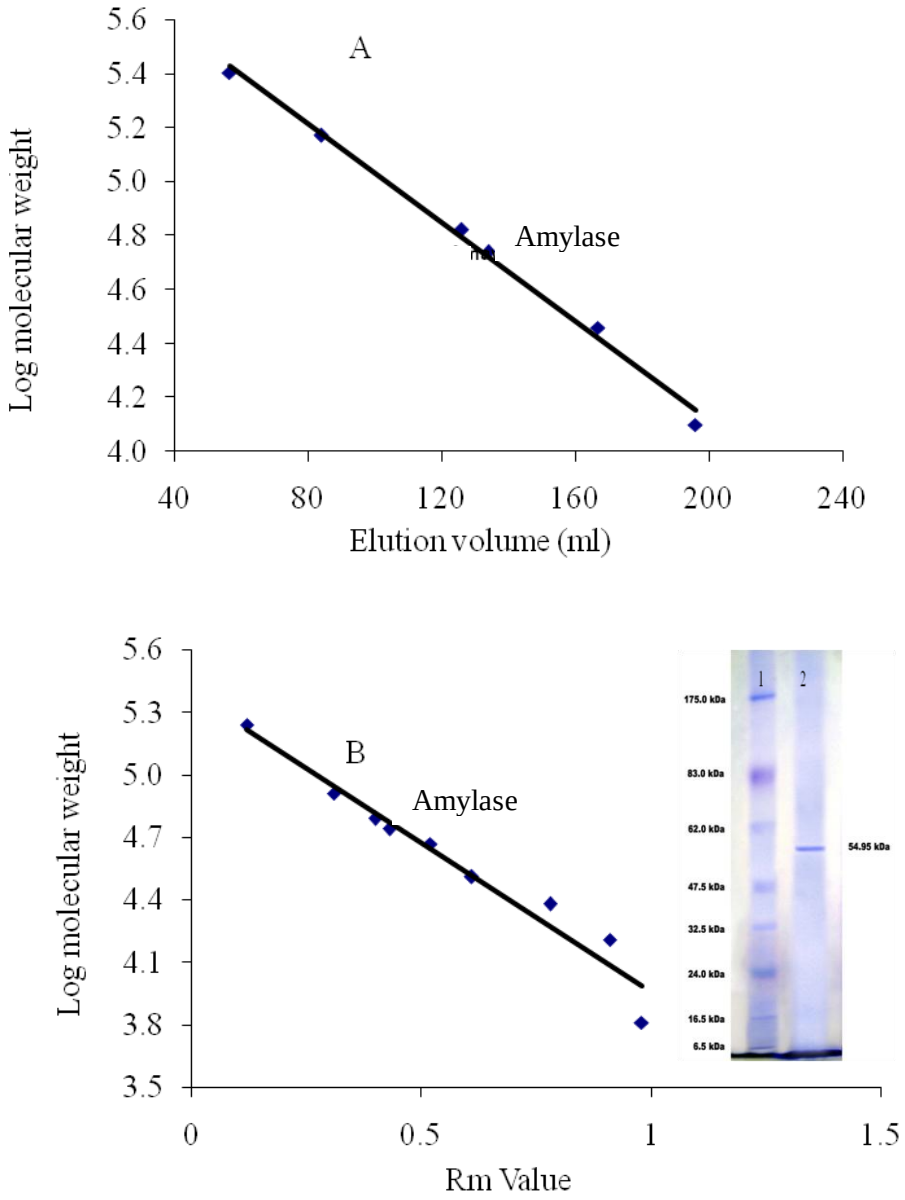

Figure 2. Determination of molecular weight and sub unit composition of purified amylase from *Bacillus cereus* MTCC 10205 using gel filtration through Sephadex G-100 [A] and SDS-PAGE [B]. Lane 1, molecular mass markers: β-galactosidase (175kDa), paramyosin (83.0 kDa), MBP-CBD (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin B (24 kDa), lysozyme (16.5 kDa) and aprotinin (6.5 kDa). Lane 2, amylase purified from *B.cereus.*

yield of 11.82% (Table 1). The purity of the enzyme at each step of purification was analyzed by native polyacrylamide slab gel electrophoresis (Figure 1C). Sixteen major bands were detected in crude preparation whereas only 11 bands were observed after ammonium sulphate fractionation. Sephadex G-100 fraction gave 3 bands while final purified enzyme obtained after DEAE-cellulose column gave one major band suggesting that the enzyme was purified to near homogeneity.

Amylases have been purified from various microorganisms by similar purification processes to that followed in this work. The purification degree of amylase achieved in the present study was similar to that reported by Bano et al. (2009) from *Bacillus subtilis* KIBGE-HAS in a two step process including ammonium sulfate precipitation and ultrafiltration (purification factor: 19.2 fold;

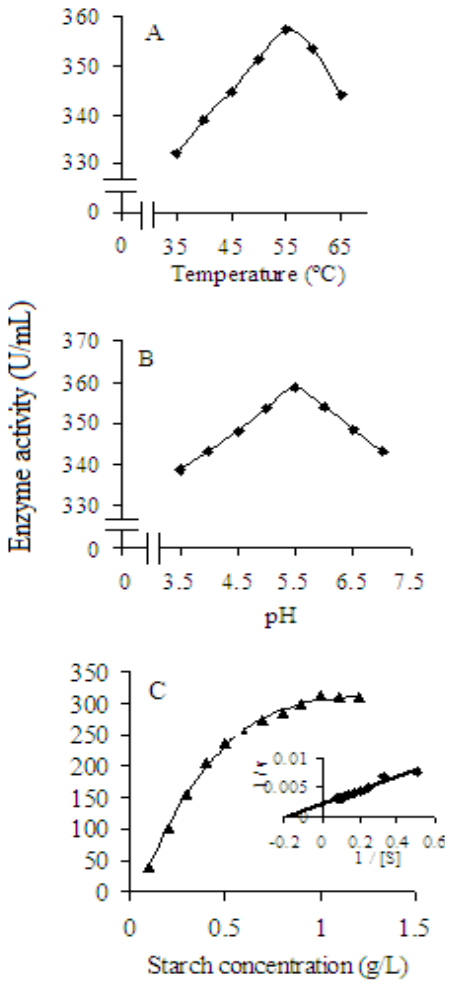

Figure 3. Characterization of purified amylase from B. cereus MTCC 10205 for optimum temperature [A], optimum pH [B] and Km value [C].

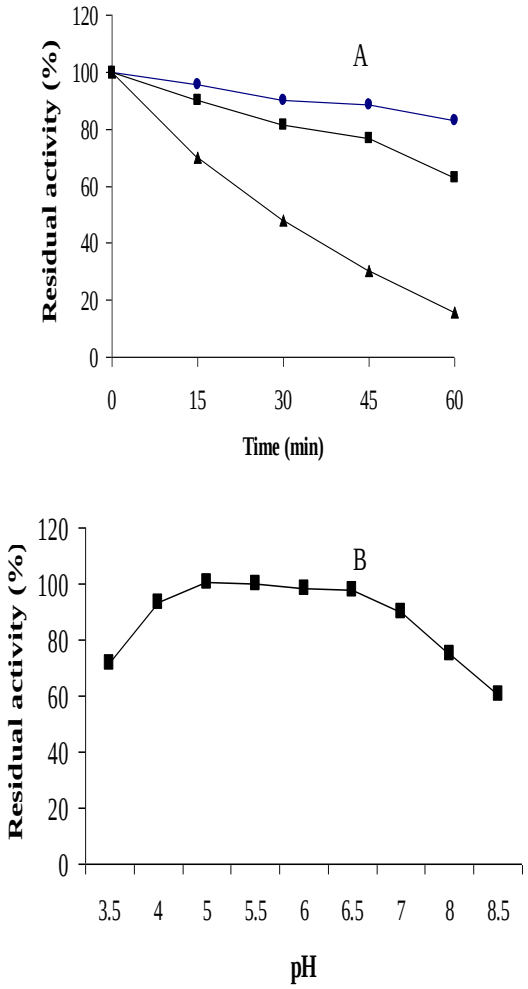

Figure 4. Thermostability (A) and pH stability (B) of the amylase purified from Bacillus cereus MTCC 10205. The enzyme was incubated at 40°C (–●–), 50°C (–■–) and 60°C (–▲–) for its thermostability.

specific activity: 4195 U/ mg protein). Similarly amylase from *B. subtilis* US 116 was purified to near homogeneity by using a combination of acetone preci-pitation, size exclusion and ion-exchange chromatogram-phy (Messaoud et al*.,* 2004). However, the amylase from *B. licheniformis* was purified 20..3 fold with 23.62% yield by ion-exchange chromatography on DEAE-cellulose and gel filtration on BioGel P100 column (Adeyanju et al., 2007).

Molecular weight

From gel filtration results, the molecular weight of native amylase was estimated to be 55.0 kDa (Figure 2A). Electrophoresis of purified amylase in SDS-PAGE/showed a single band with similar molecular weight (54.95 kDa). These results indicate that amylase from *B. cereus* is a monomer (Figure 2B). Amylases of almost similar molecular weight have been reported from *B. cereus* NY-14 (Yoshigi et al., 1985), *Bacillus subtilis* (Uyar et al., 2003), and *Bacillus* sp. AB 68 (Aygan et al., 2008). Endoamylases are almost exclusively single subunit proteins, however, some amylases especially those having large molecular weight are found to possess more than one subunit as reported in *Pyrococcus furiosus* (Laderman et al., 1993) and *Bacillus* sp. A3-15 (Arikan , 2008).

Temperature and pH optima and Km value

The maximum activity of purified amylase from *B. cereus* (357.48 U/ mL) reached at 55°C (Figure 3A). Higher incubation temperatures of reaction led to a gradual loss

of activity. The temperature optima observed during the present investigations is in accordance with the temperature optima reported in *B. cereus* (Yoshigi et al*.,* 1985). Thermostable amylases having temperature optima between 50 to 60°C have also been isolated from a number of sources including *Bifidobacterium adolescentis* (Lee et al., 1997), *Aspergillus oryzea* (Ramachandran et al., 2004), *B. subtilis* (Bezerra et al., 2006) and *Bacillus* sp. AB68 (Aygan et al., 2008). The themostable alpha amylases isolated from various species of Bacillus have been preferred for use in starch processing industry (Nigam and Singh, 1995)

Figure 3B depicts that enzyme had maximum activity at pH 5.5 (358.84 U/ mL). At pH above and below 5.5, the activity decreased. The optimum pH for amylase from *B. cereus* MTCC-10205 was different from that of *Alicyclobacillus acidocaldarius* which have been reported to have acidic pH optima of 3.0 (Schwermann et al., 1994) and from that of the alkaline amylases from *B. cereus* (Annamalai et al., 2011), *Bacillus cohnii* US 147 (Ghorbel et al*.*, 2009) and *Bacillus* KSM K-38 (Hagihara et al*.*, 2001) with pH optima of 8, 9 and 10, respectively. However, amylases with stability in a narrow range have also been reported in *Halomonas meridiana* (Coronado et al. 2000). The amylases working in the pH range of 5.0-6.0 are preferred for starch industry as this pH range could eliminate unwanted side reactions during starch processing (Vieille and Zeikus, 2001).

With increasing concentration of substrate (1-14 g L^{-1}), the enzyme showed a typical hyperbolic velocity saturation curve (Figure 3C) revealing that it followed Michaelis-Menten kinetics. The enzyme activity increased with increase in starch concentration attaining maximum value at 10 g L^{-1} starch, above which the enzyme activity remained almost constant suggesting that the enzyme got fully saturated at this concentration. From the double reciprocal Lineweaver Burk plot, the Km of the enzyme was found to be 5.37 g L^{-1}. The Km value observed during the present study reveals higher affinity for the substrate than that observed for amylase from *B. cohnii* US 147 whose Km value is reported to be 7.0 mg/mL (Ghorbel et al., 2009). Adeyanju et al*.* (2007) however, reported that amylase from *Bacillus licheniformis* had sigmoidal kinetics with a Km for soluble starch of 1.097% starch.

Thermostability, storage stability and pH stability

The enzyme showed progressive loss in activity with temperature and with time of incubation (Figure 4A). Amylase retained 83% of its original activity at 40°C after 60 min. At 50 and 60°C after 15 min of incubation, enzyme retained 90 and 70.1% of its original activity whereas after 60 min of incubation, the enzyme showed only 62.90 and 15.6% activity, respectively. The enzyme was stable over a broader pH range retaining >90% of its initial activity after incubation at 30°C in buffers of pH 4.0-7.0 for 1 h (Figure 4A). Thermostable enzymes including amylases, proteases and lipases offer major biotechnological advantages over mesophillic enzyme (McMohan et al., 1999). Bacterial α-amylases possessing high heat resistance have been reported earlier from *Alicyclobacillus* sp.A4 (Bai et al., 2012) *Bacillus* sp. AB-68 (Aygan et al*.*, 2008) and *B. subtilis* KIBGE-HAS (Bano et al., 2009). *Alicyclobacillus* sp.A4 has also been shown to have broader pH stability (Bai et al., 2012).

The purified amylase appeared to be quite stable at 4°C because it could be stored up to 5 days with only 10% loss in activity (Table 2). After that, the activity declined gradually causing 50% loss in activity after 30 days of storage. Storage of the purified amylase at 35°C for 3 days resulted in 28% loss in activity. Further increase in storage period at this temperature led to rapid inactivation of the enzyme showing only 15% of activity after 22 days of storage. However, complete loss in activity was observed after 30 days of storage. In agreement with our results, storage of amylase from *B. subtilis* KIBGE-HAS at 4°C for 124 days retained 70% activity while the storage at 37°C for 25 days resulted in complete loss of activity (Bano et al., 2009).

Effect of metal ions and other additives

Metal ions like K^+ and Zn^{2+} at 1 mM concentration stimulated amylase activity by 31 and 18%, respectively, while at 5 mM concentration, they inhibited activity by 51 and 45% (Table 3). Ca^{2+} however, was stimulatory at both concentrations (1 and 5 mM) whereas other metal ions such as Cu, Mn, Mg, Fe were inhibitory. Ethylene-diaminetetraacetic acid (EDTA) also inhibited amylase activity, suggesting that metal ions were required for the amylase activity.

These results are in accordance with those reported by Kaneko et al. (2005), who observed an increase in amylase activity in the presence of Ca^{2+}. The alpha amylase preparations used for starch liquefaction have been reported to show highest activity at pH 5.5-6.0 and require addition of calcium ion for stability (Vieille and Zeikus, 2001). Similarly, the activity of amylase from *Bacillus* sp. AB-68 has been reported to be inhibited by EDTA by 34% thus indicating the requirement of metal ions (calcium) for its activity (Aygan et al., 2008).

Complete inhibition of enzyme activity in the presence of p-hydoxyl-mercuribenzoic acid (PHMB) and 5,5-dithio-bis(2-nitrobenzoate) (DTNB), and stimulation by β-ME indicated the involvement of SH-group(s) at the active site of the enzyme. Iodoacetate also inhibited the amylase activity further confirming the sulfhydryl residue to be essential for catalytic activity of amylase (Table 3). The present observations are similar to the results obtained from the studies of Ezeji and Bahl (2006), Afifi et al. (2008) and Aygan et al. (2008). However, the enzyme

Table 2. Storage ability of purified amylase from *Bacillus cereus* MTCC 10205.

Storage ability	Enzyme activity* (U/ mL)	
Days of storage	Storage temperature	
	4°C	35°C
0	361.11(100)	361.11(100)
1 (day)	360.31 (99.77)	323.96 (89.71)
2 (days)	357.48(99.01)	282.07 (78.11)
3 (days)	350.35(97.02)	259.98 (72.00)
4 (days)	339.48(94.00)	232.35 (64.34)
5 (days)	325.32(90.11)	205.18 (56.82)
6 (days)	315.70(87.42)	173.13 (47.94)
7 (days)	292.82(81.08)	140.86 (39.00)
8 (days)	279.80(77.48)	112.44 (31.13)
15 (days)	252.40(69.90)	81.20 (22.48)
22 (days)	209.48(58.00)	54.91 (15.20)
30 (days)	182.08(50.42)	-

*****One enzyme unit is the amount of enzyme that liberated 1nmol of maltose equivalent under the experimental conditions in 1 min. ******Values in parentheses indicate % of the control values.

Table 3. Effect of different metal ions and additives on purified amylase from *Bacillus cereus* MTCC 10205.

S/N	Metal ions/additives	Enzyme activity* (U/mL)	
		1 mM	5 mM
1	None	359.52 (100)	359.52 (100)
2	$MgSO_4$	347.63 (96.70)	280.82 (78.10)
3	KCl	470.94 (131.00)	184.23 (51.24)
4	$MnCl_2$	280.71 (78.07)	217.52 (60.50)
5	EDTA	104.17 (28.97)	44.16 (12.28)
6	$MnSO_4$	323.51 (89.98)	161.70 (44.97)
7	$ZnSO_4$	424.18 (117.98)	148.11 (41.19)
8	$FeCl_2$	321.02 (89.29)	297.58 (82.77)
9	$CuCl_2$	287.27 (80.00)	152.75 (42.48)
10	$CaCl_2$	404.47 (112.50)	470.94 (130.99)
11	β-ME	371.75 (103.40)	482.15 (134.10)
12	PMSF	345.48 (96.10)	332.57 (92.50)
13	Iodoacetate	101.00 (28.09)	14.83 (4.12)
14	DTNB	77.34 (21.51)	21.96 (6.11)
15	PHMB	51.06 (14.20)	16.98 (4.72)

*****One enzyme unit is the amount of enzyme that liberated 1nmol of maltose equivalent under the experimental conditions in 1 min. ******Values in parentheses indicate % of the control values.

from *B. adolescentis* (Lee et al., 1997) was reported to have groups other than sulfhydryl groups at the active site because the enzyme was not inhibited by iodoacetate. Addition of PMSF-a protease inhibitor had no effect on amylase activity indicating final enzyme preparation to be protease free. Contrarily, Arikan (2008) reported that PMSF inhibited amylase activity from *Bacillus* sp. A3-15.

From the results, it is clear that the enzyme has the ability to work in a wider temperature and pH range and has high thermostability suggesting that it can be used for starch hydrolysis at temperature which restricts microbial growth. The reported pH value indicates it to be similar to those required for efficient starch liquefaction. Therefore,

this enzyme can be used in industrial sector.

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