BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of hyperthermostable α-amylase from *Geobacillus* sp. IIPTN

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Abstract A newly isolated *Geobacillus* sp. IIPTN (MTCC 5319) from the hot spring of Uttarakhand's Himalayan region produced a hyperthermostable α-amylase. The microorganism was characterized by biochemical tests and 16S rRNA gene sequencing. The optimal temperature and pH were 60°C and 6.5, respectively, for growth and enzyme production. Although it was able to grow in temperature ranges from 50 to 80°C and pH 5.5–8.5. Maximum enzyme production was in exponential phase with activity 135 U ml^{-1} at 60°C. Assayed with cassava as substrate, the enzyme displayed optimal activity 192 U ml^{-1} at pH 5.0 and 80°C. The enzyme was purified to homogeneity with purification fold 82 and specific activity 1,200 U mg^{-1} protein. The molecular mass of the purified enzyme was 97 KDa. The values of K_m and V_{max} were 36 mg ml^{-1} and 222 μmol mg^{-1} protein min^{-1}, respectively. The amylase was stable over a broad range of temperature from 40°C to 120°C and pH ranges from 5 to 10. The enzyme was stimulated with Mn^{2+}, whereas it was inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and EDTA, suggesting that it is a metalloenzyme. Besides hyperthermostability, the novelty of this enzyme is resistance against protease.

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Introduction

Among various starch containing biomass, cassava (*Manihot esculenta*) now-a-days is attracting the modified starch industries because of low production cost, cultivation in poor soil, and excellent starch quality. At present, the modified starch industries besides native starch, which is used in the production of glucose and maltose syrups and syrups with different level of dextrose equivalent, also needed cassava for the food, paper, and textile industries, for production of monosodium glutamate, sorbitol, vitamin C, various sweeteners, and alcohol for biofuel, drinking, and industrial purposes (Srinivas 2007). The starch industry is the biggest consumer of enzymes for the hydrolysis of starch (Haki and Rakshit 2003; Sivaramakrishnan et al. 2006).

α-Amylase (EC3.2.1.1, 1,4-α-D-glucan glucanohydrolase and endoamylase) hydrolyze the starch by randomly cleaving internal α-1,4-glucosidic linkages and produce reducing sugar. Amylases from different sources like plant and microbial (bacterial and fungal) source have been used for centuries as food additives and in brewing industry. Among bacterial source, *Bacillus* sp. like *B. subtilis, B. stearothermophilus, B. licheniformis,* and *B. amyloliquefaciens* were potentially exploited for thermostable α-amylase production to fulfill industrial requirements (Sivaramakrishnan et al. 2006). Thermophilic microorganisms and thermostable enzymes have been of great scientific interest for several decades, principally in regard to their potential industrial applications (Kumar et al. 2009a). Thermostability, acidic pH and long-term stability at normal condition is a desired

characteristic for the amylases to fit potentially in starch-processing industry. There are very few commercially available α-amylase that work between 80–120°C and pH 5.0–5.5, e.g., *B. licheniformis* (100°C, pH 6.0–6.5) (Viara et al. 1993), *B. stearothermophilus* (70–80°C; pH 5.0–6.0) (Vihinen and Mantsala 1990).

Therefore, screening of microorganisms that can produce novel amylases with higher stability at extreme condition is necessary (Gupta et al. 2003). In the present paper, we report the purification and characterization of novel hyperthermostable amylase produced by thermophilic strain *Geobacillus* sp. IIPTN (MTCC 5319), which efficiently hydrolyze the cassava and other starch biomass as well as showing resistant to protease degradation.

Material and methods

Isolation, identification, and culture conditions of microorganism

The bacterial strains were isolated from hot spring water of Uttarakhand's Himalayan region (India). The water samples were inoculated into nutrient broth containing (g l^{-1}): peptone, 5.0; yeast extract, 3.0; NaCl, 5.0; glucose, 10.0 (pH 7.0), and incubated at 60°C. Subsequent dilutions of the 24-h old culture were spread on nutrient gelrite starch plates (g l^{-1}: peptone, 5.0; yeast extract, 3.0; NaCl, 5.0; soluble starch, 10.0; gelrite, 10.0; pH 7.0) for further screening of amylase producing strain and incubated at 60°C. After 16-h growth, the plates were flooded with iodine soultion to check the zone of clearance against gelrite starch plate as control. The one isolate, identified as *Geobacillus* sp. IIPTN (deposited in 'Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh (India)' with accession no. MTCC 5319), that showed the clear zone of more than 1.0 cm diameter with iodine test, was selected and maintained routinely on rich medium slants (1% (*w/v*) yeast extract, 1% (*w/v*) peptone, 0.5% (*w/v*) NaCl, 1% (*w/v*) gelrite, and 0.5% (*w/v*) soluble starch, at pH 6.8–7.0). The master culture was preserved in 40% (*v/v*) glycerol at −80°C.

The newly isolated strain *Geobacillus* sp. IIPTN (MTCC 5319) was characterized by partial 16S rRNA gene sequencing. The primers selected for PCR amplification experiments were, forward primer 5′ CAGGCCTAACA CATGCAAGTC and reverse primer 5′ GGGCGGWG TGTACAAGGC. The partial 862-bp 16S rRNA gene sequence was aligned with submitted sequences available in the NCBI database using clustalX software, and the phylogenetic tree was depicted using the TREEVIEW program. The partial 16S rRNA gene sequence has been deposited to the GenBank under the accession no. DQ323407.

Production of α-amylase

Geobacillus sp. IIPTN was grown in basal salt medium containing (g l^{-1}): NaH$_2$PO$_4$ × 2H$_2$O, 1.56; NH$_4$NO$_3$, 5.0; KCl, 0.745; Na$_2$SO$_4$ × 10H$_2$O, 0.644; citric acid, 0.42; MgCl$_2$ × 6H$_2$O, 0.25; soybean meal, 5.0; yeast extract, 2.5; soluble starch, 5.0; trace elements (CaCl$_2$, 2.2×10^{-3}; ZnO, 2.5×10^{-3}; FeCl$_3$ × 6H$_2$O, 2.7×10^{-2}; MnCl$_2$ × 4H$_2$O, 1.0×10^{-2}; CuCl$_2$ × 2H$_2$O, 8.5×10^{-4}; CoCl$_2$ × H$_2$O, 2.4×10^{-3}; NiCl$_3$ × 6H$_2$O, 2.5×10^{-4}; H$_3$BO$_3$, 3.0×10^{-4}; Na$_2$MoO$_4$, 1.0×10^{-3}), 20 ml; pH was adjusted to 6.8–7.0 with 1 M NaOH before sterilization (Santos and Martins 2003). The medium was inoculated with 12-h old culture and incubated at 60°C in orbital shaker at 130 rpm.

The effect of temperature and pH on enzyme production was studied. The bacterial strain was also grown on the different carbon and nitrogen sources. The various carbon sources with concentration 1–5 g l^{-1} used were: maltose, glucose, amylose, amylopectin, and raw starchy substrates like cassava, tapioca root, corn starch, sweet sorghum. To study the effect of nitrogen on enzyme production, different organic source (yeast extract, beef extract, tryptone, casein hydrolysate, peptone, and soybean meal) and inorganic source (ammonium sulfate, ammonium chloride, ammonium nitrate, ammonium acetate, and urea) were used. The concentration of nitrogen source used was 1–5 g l^{-1}. The studies were carried out in Erlenmeyer shake flask.

Enzyme assay

α-Amylase activity was determined by estimating the reducing sugar from 1% (*w/v*) cassava starch as substrate at 80°C and pH 5.0. The amount of released reducing sugars was determined by the dinitrosalicylic acid method (Miller 1959). The D-glucose was used as standard. One unit of α-amylase activity was defined as the amount of enzyme that released 1 µmol of reducing sugars per minute at 80°C.

Purification of α-amylase

Ultrafiltration of the enzyme

The culture broth was centrifuged at 34,000×g for 10 min at 4°C in refrigerated centrifuge SORVALL Evolution$^{®}$. The cell-free supernatant was collected and filtered through Amicon Ultrafiltration unit using 10,000 NMWL regenerated cellulose membrane from Millipore (USA).

Chromatography

Four milliliters of concentrated enzyme (concentration 8 mg ml^{-1}) was applied to a Macro Prep High S support (strong cationic exchanger purchased from BIORAD, USA)

column (1.5×30 cm), pre-equilibrated with 20 mM phosphate buffer pH 6.5. The unbound proteins were eluted by using same buffer at flow rate of 0.5 ml min^{-1}, while the bound proteins were eluted by using linear salt gradient with 1 M NaCl in the same buffer at flow rate of 0.5 ml min^{-1}. The active fractions no 5–15 out of 40 collected fractions (1 ml each) was concentrated in Millipore Amicon Ultra, centrifugal filter device Ultracel-30 K that has 30,000 MNCO regenerated cellulose membrane (Millipore, USA).

The concentrated enzyme (500 μl) was applied to the pre-equilibrated CHT Type I Ceramic Hydroxyapatite binding column (1×10 cm) from BIORAD, USA. The unbound proteins were eluted with 10 mM phosphate buffer pH 6.8 and 0.3 mM CaCl$_2$ at a flow rate of 1 ml min^{-1} (1 ml per fraction) and bound proteins were eluted by using 400 mM phosphate buffer pH 6.8. Among the collected 40 fractions, fractions no. 9–11 (1 ml each) showed the activities which were concentrated and used for sodium dodecyl sulfate-polyacrlyamide gel electrophoresis (SDS-PAGE).

Electrophoresis and molecular mass determination

SDS-PAGE was carried out according to Laemmli (1970) with 7.5% polyacrylamide. Electrophoresis was performed in a PROTEAN II xi cell (BIO-RAD). The molecular weight markers (29 to 205 KD) purchased from SIGMA run along with samples. The protein bands were visualized by staining with coomassie brilliant blue G 250.

Zymogram preparation

The purified enzyme was evaluated in PAGE according to Laemmli (1970). After electrophoresis, the gel was washed with deionized water and immersed in 20-mM acetate buffer of pH 5.0. Then the gel was imbibed in 0.5% (w/v) soluble starch at 4°C for an hour to assure starch penetration into the gel. Gel was then incubated for 2 h at 60°C, after which it was stained with iodine solution. After staining, amylase band was detected as clear yellow zone against dark blue background of non-hydrolyzed stained starch. The gel was immediately photographed using Gel Doc™ XR (BIORAD, USA) (Martínez et al. 2000).

Protein estimation

Protein content was measured by Folin–Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

Blue value determination

The residual starch was determined by blue value method. One milliliter of reaction mixture was removed at various time intervals. The reaction was stopped by adding 1 ml of 0.1 N HCl and then 1 ml iodine solution was added to develop the color. The reduction in optical density was monitored at 660 nm. The reduction in value was determined with the standard graph (Najafi et al. 2005).

Characterization of the enzyme

Influence of temperature and pH on enzyme activity

The temperature profile to achieve maximum activity was determined by incubating the enzyme for 30 min in the temperature range of 20–120°C. After incubation the enzyme was kept at 4°C. The activity was measured by assaying the reducing sugar using 1% (w/v) cassava starch at different temperature. Similarly, the effect of pH on amylase activity was determined at 80°C in the pH ranges of 3.0 to 10.0 with glycine-HCl buffer (3.0–4.0), acetate buffer (5.0–6.0), phosphate buffer (6.0–8.0), and glycine-NaOH buffer (9.0–10.0). The enzyme was incubated with these buffers at 80°C for 10 min without substrate and then activity was determined on 1% (w/v) cassava starch by measuring the reducing sugars produced.

Effects of metal ions and denaturing chemicals

Effect of metal ions on activity was measured by incubating the enzyme with 5 mM metal solutions of MnSO$_4$, CaCl$_2$, NaCl, CoCl$_2$, MgCl$_2$, FeCl$_3$, HgCl$_2$, KCl, ZnSO$_4$, CuSO$_4$, BaCl$_2$ and 5 mM denaturing agents like EDTA, SDS, urea for 30 min without substrate. Relative enzyme activity was measured on 1% (w/v) cassava at 80°C and pH 5.0. The control was used without any metal ion or denaturing reagents and reported as 100% activity.

Kinetic determinations

The initial rate of reaction for starch hydrolysis was calculated by estimating the reducing sugar by dinitrosalicylic acid method at different starch concentrations of 1 to 10 g l^{-1}. The Michaelis constant (K_m) and the rate of reaction (V_{max}) were determined according to Lineweaver–Burk plot (Lineweaver and Burk 1934).

Substrate specificity

Different starch such as potato soluble starch (SIGMA, USA), corn starch (Hi-Media, India), amylopectin (Hi-Media, India), cassava, tapioca, maize flour, and sweet sorghum were used to determine the substrate specificity of the enzyme. All the substrates were used in gelatinized as well as in native form. The hydrolysis was carried out at 80°C with 1% (w/v) substrate concentration in 20-mM acetate buffer of pH 5.0.

Mode of action

The partially purified enzyme was incubated with 1% (w/v) cassava at 80°C and pH 5.0. The samples were removed at fixed time intervals. The residual starch and reducing sugar determined accordingly. The end products were analyzed by high-performance liquid chromatography (HPLC) using high-performance carbohydrate column (Waters) at 30°C with acetonitrile and water mixture (75:25) as mobile carrier at a flow rate 1.4 ml min^{-1} and detected by a Waters 2414 refractive index detector (Kumar et al. 2009b).

Stability of enzyme against protease

The partially purified amylase was kept at 4°C without adding PMSF. After 6 months, the enzyme tested for its stability. The reaction was carried out at 80°C and pH 5.0 with 1% (w/v) cassava. The enzyme stability was also checked against Proteinase K (SIGMA, USA). The partially purified amylase was incubated with 10 U of proteinase K at room temperature for 30 min. Amylase activity was checked by assaying reducing sugar with 1% (w/v) cassava at 80°C and pH 5.0

Results

Isolation, characterization, and taxonomic classification of geobacillus sp. IIPTN

Geobacillus sp. IIPTN was found potent amylase producer which showed the zone of clearence on starch plate when flooded with iodine solution (Fig. 1). The morphological and physiological characteristics of the isolate was observed as rod shape, Gram positive, catalase positive, indole test negative, positive to fermentation test for glucose and xylose, and thermophilic in nature since it was unable to grow below 50°C.

The phylogenetic tree clearly depicts that the strain was maximum homologous with Geobacillus sp. and thus submitted to genebank as Geobacillus sp. IIPTN (accession no. DQ323407) (Fig. 2).
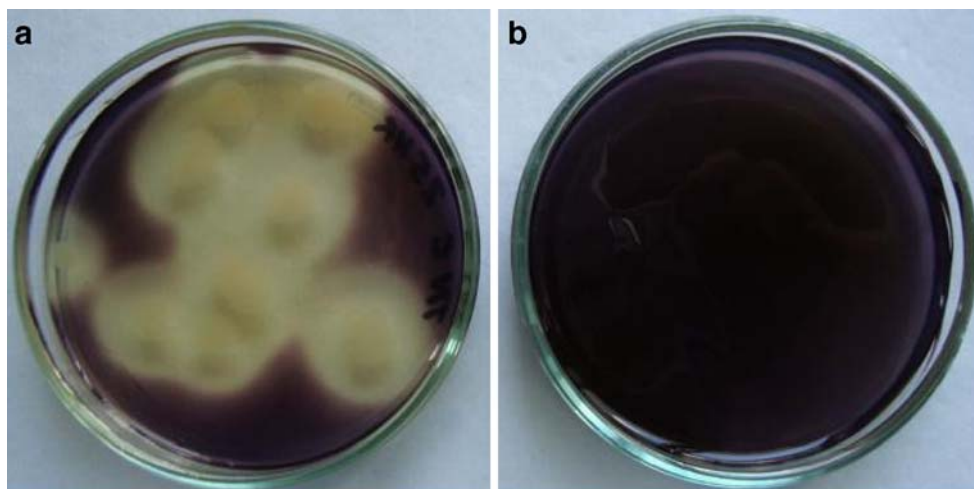
Production of enzyme

Geobacillus sp. IIPTN was able to grow and produce amylase in the temperature ranges from 50°C to 80°C and pH 5.5–8.5 (data not shown) showed maximum production of amylase at its optimum growth temperature 60°C and pH 6.5. Amylase production was found to be growth associated. Enzyme production started at early log phase and reached maximum in exponential phase (Fig. 3). The α-amylase, produced by Geobacillus sp. IIPTN, was named as IIPTN α-amylase.

Among the various carbon sources such as maltose, glucose, amylase, amylopectin, cassava, tapioca root, corn starch, and sweet sorghum, the maximum production of enzyme was observed on the medium containing 0.5% (w/v) cassava. In case of nitrogen sources, 0.5% (w/v) tryptone, yeast extract, soymeal, ammonium chloride, and ammonium nitrate enhanced the amylase production. No growth was observed with ammonium acetate.

Purification of α-amylase

The crude enzyme sample obtained after ultrafiltration was purified on a cation-exchange chromatography Macro Prep High S support column. The elution pattern showed a major and broad peak of amylase activity (Fig. 4a). The amylase activity was found in unbound protein fractions collected from ion exchanger. Before applying to the hydroxyapatite column, SDS-PAGE was run for the active fractions. SDS-

Fig. 1 *Geobacillus* sp. IIPTN showing the zone of clearance with iodine solution on starch plates, *A Geobacillus* sp. IIPTN; *B* starch plate used as control
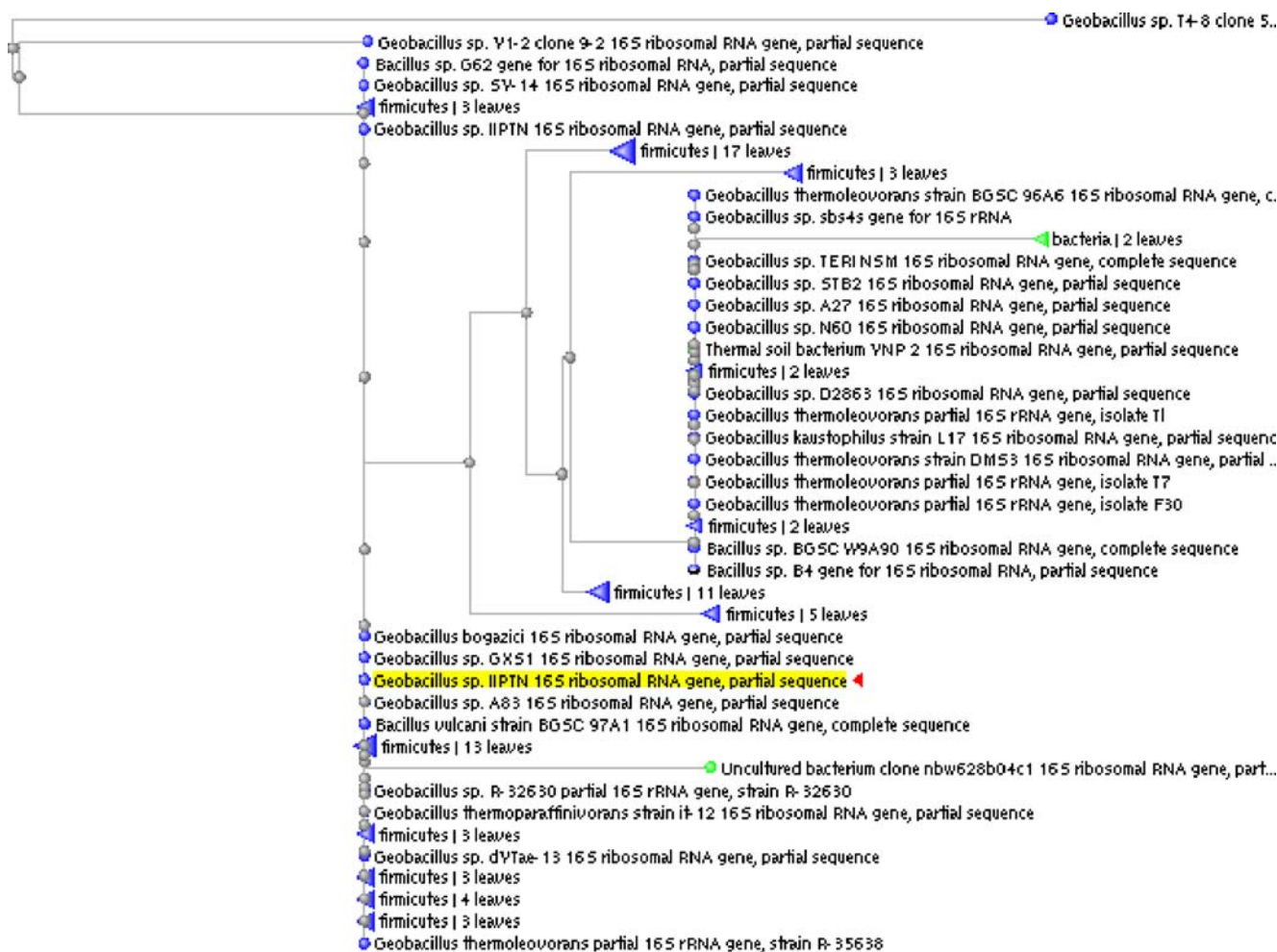
Fig. 2 16S rRNA partial sequence (862 bp) used to create phylogenetic dendrogram, indicating the position of the strain *Geobacillus* sp. IIPTN with *Geobacillus* sp.
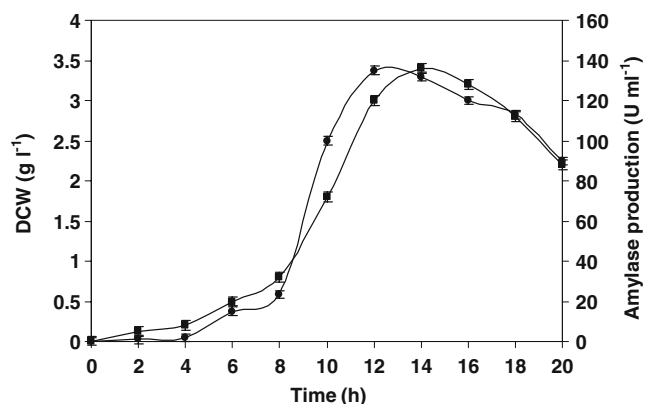

Fig. 3 Growth of *Geobacillus* sp. IIPTN and α-amylase production during growth on basal salt medium at 60°C. Dry cell weight and amylase production was monitored at different interval of time: (*filled square*) DCW (g l⁻¹); (*filled circle*) amylase production (U ml⁻¹) was determined by measuring protein (Folin–Lowry) and activity of enzyme by assaying reducing sugar

PAGE results showed the multiple bands (Fig. 5, Lane 3). The concentrated active fractions were then applied to the hydroxyapatite binding column. A single peak of amylase activity was obtained (Fig. 4b). The SDS-PAGE analysis showed the single protein band (Fig. 5, Lane 4). A summary of the purification is given in Table 1. These procedures yielded a pure amylase with specific activity of 1,200 U mg^{-1} of protein with purification fold 82 and a reasonable yield of 31%. The molecular mass of the purified amylase was found 97 KD on 7.5% SDS-PAGE.

Effects of temperature and pH on enzyme activity

Influence of temperature on enzyme activity was observed by incubating the enzyme at different temperatures. The results showed that the activity increased sharply with gradual increase in temperature up to 80°C (specific activity 83.5 U mg^{-1}), and it declined gradually with further rise in temperature (Fig. 6a). The enzyme was 75 % active at 120°C.
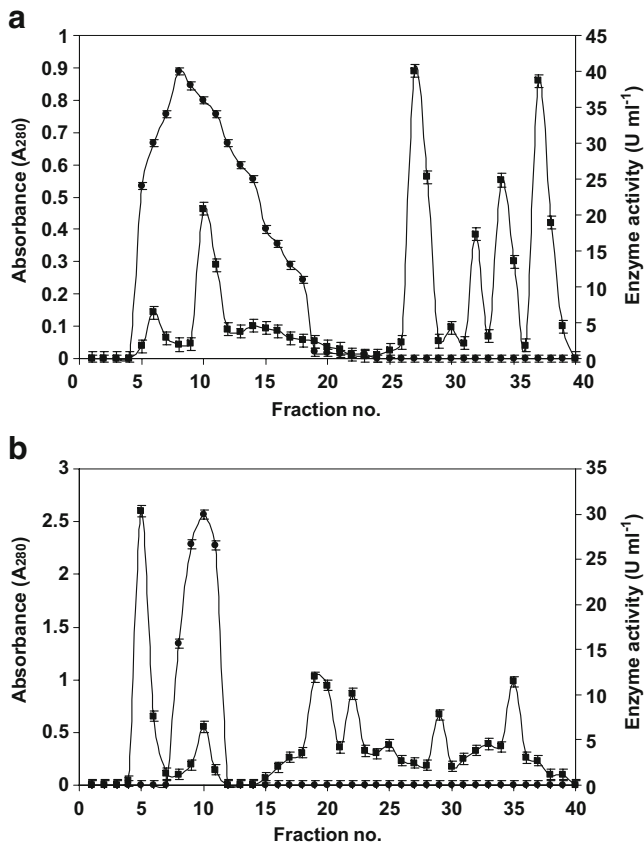
Fig. 4 Eution profile of *Geobacillus* sp. IIPTN α-amylase from **a** cationic exchanger; **b** CHT type I Hydroxyapatite column: (*filled square*) protein absorbance at 280 nm; (*filled circle*) enzyme activity

The effect of pH on IIPTN amylase activity was a bell shaped curve with an optimal activity at pH 5.0 (Fig. 6b). It was active at 4.5 and 9.5 with 19% and 36% of maximum activity, respectively.

Effects of metal ions and denaturing chemicals

Effect of metal ions on IIPTN amylase activity was determined at pH 5.0 and 80°C (Table 2). Enhancement in the activity of amylase on different metals at a final concentration of 5 mM was in following order: $Mn^{2+}> Ca^{2+}>Ba^{2+}>Co^{2+}>Na^+>Fe^{3+}>K^+$. However, inactivation effect of Mg^{2+} and Zn^{2+} was found to be 33% and 50% of original activity, respectively. Mn^{2+} enhanced the amylase activity 91% more than the original activity. The sulfhydryl oxidant metals like Cu^{2+} and Hg^{2+} totally inhibited the enzyme activity. The enzyme was completely deactivated in presence of chelating agent EDTA, suggesting that it is a metallozyme. The effect of SDS and urea on the amylase activity was examined and found that both the chemicals enhanced the activity 42% and 47% more than the original activity, respectively, at the concentration of 5 mM.

Kinetic constants

Apparent K_m and V_{max} values were determined from Lineweaver–Burk plot (Fig. 7) as 36 mg ml^{-1} and 222 μmol mg^{-1} protein min^{-1}, respectively, with cassava starch as substrate.

Substrate specificity

IIPTN α-amylase was examined for its ability to hydrolyze different gelatinized and raw starch by taking the hydrolysis rate of soluble starch as 100%. It efficiently hydrolyzed the gelatinized cassava starch, corn starch, soluble starch, tapioca root, amylopectin, sweet sorghum, and corn flour with relative hydrolysis rate 129%, 107%, 100%, 88%, 86%, 62%, and 58%, respectively. IIPTN α-amylase also hydrolyzed the raw starch substrates with relative hydrolysis rate for cassava, 106%; corn starch, 68%; soluble starch, 67%; amylopectin, 56%; and tapioca root, 50% (Table 3).

The end product of starch hydrolysis was analyzed by HPLC (data not shown). The major end products were maltose, maltotriose, and glucose. The rapid reduction in blue value and production of reducing sugars suggested the endo mode of action (Fig. 8).

Geobacillus sp. IIPTN amylase enzyme was stable more than 6 months at 4°C without adding PMSF. There was no loss detected in original activity (192 U ml^{-1}), and the enzyme was found fully active after proteinase K treatment.
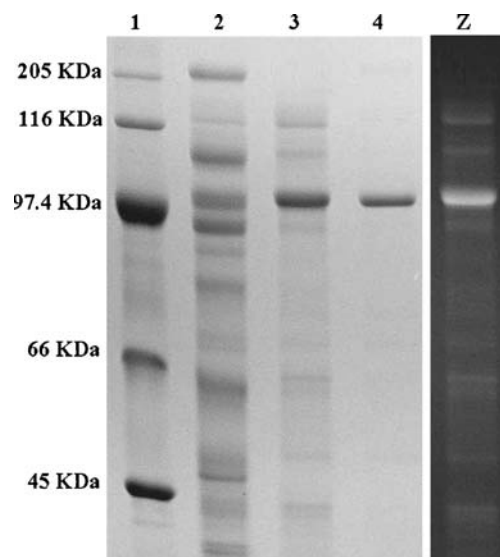

Fig. 5 SDS-polyacrylamide gel electrophoresis of the purified *Geobacillus* sp. IIPTN α-amylase showing: *Lane 1*, molecular markers; *Lane 2*, crude enzyme; *Lane 3*, active fractions after cationic exchange chromatography; *Lane 4*, purified amylase after hydroxyapatite chromatography; *Z* zymogram of purified amylase

Table 1 Purification of the extracellular α-amylase of *Geobacillus* sp. IIPTN

S. No.	Purification step	Total activity (U)	Amount of protein (mg)	Sp. activity (U. mg^{-1})	Yield (%)	Purification fold
1	Culture supernatant	145,000	1,000	14.5	100	1
2	Ultrafiltered	14,000	250	56	96	3.8
3	Cation-exchange chromatography	12,000	42.857	280	82	19.3
4	Hydroxyapatite binding chromatography	4,500	3.75	1,200	31	82

Discussion

The strain *Geobacillus* sp. IIPTN is a hyperthermophilic Gram-positive rod. The partial 16S rRNA gene sequence of strain *Geobacillus* sp. IIPTN falls within the radiation of the genus *Geobacillus*. The α-amylase production during growth of *Geobacillus* sp. IIPTN in mineral salt medium supplemented with 0.5% (*w/v*) cassava substrate was studied. Maximum amylase production was observed at 12 h with enzyme activity 135 U ml^{-1}, and it was found to have declined gradually after the 20th hour (90 U ml^{-1}) (Fig. 3). It was observed that the maximum amylase was

Fig. 6 Effect of **a** temperature at different range 20–120°C with 1% (*w/v*) cassava as a substrate, **b** pH on *Geobacillus* sp. IIPTN α-amylase activity at 80°C with 1% (*w/v*) cassava. The buffers used were glycine-HCl (3.0–4.0), acetate (5.0–6.0), phosphate (6.0–8.0), and glycine-NaOH (9.0–10.0)

produced by *Geobacillus* sp IIPTN when cell population reached at the exponential phase. The production of enzyme was growth associated (Santos and Martins 2003). The amylase production has been reported parallel with growth in *B. subtilis* (Stephenson et al. 1998), *B. licheniformis* TCRDC-B13 (Bajpai and Bajpai 1989), and *Bacillus subtilis* JS-2004 (Asgher et al. 2007). The amylase was produced by *Geobacillus* sp. IIPTN at its optimum growth temperature 60°C. The microorganism could grow the temperature ranges from 50°C to 80°C but the maximum amylase production was observed at 60°C. A wide range of temperature has been reported for optimum growth and α-amylase production using bacteria (Burhan et al. 2003; Konsula and Liakopoulou-Kyriakides 2004; Malhotra et al. 2000). Even the hyperthermophiles like *P. furiosus* and *P. woesei* have been reported to produce α-amylase at their optimum growth temperature of 100°C (Koch et al. 1990; James and Simpson 1996). The strain could not grow in the culture medium having pH below 5.0 and above 8.0. The enzyme production was increased with an increase in pH from 5 to 7 and higher enzyme synthesis
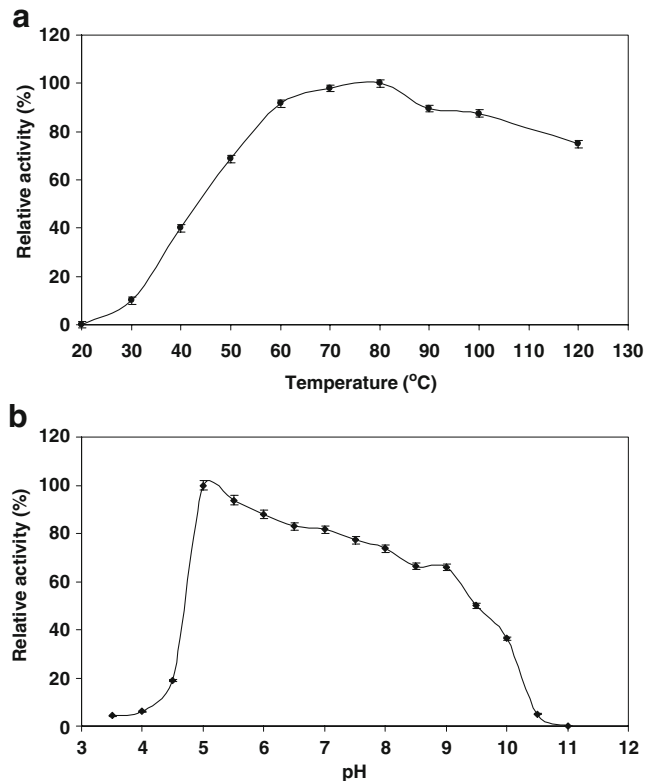
Table 2 Effect of 5-mM metal ions and other chemicals on α-amylase activity was observed at 80°C and pH 5.0

S. No.	Metal ions/denaturing agent	Relative activity (%)
1	Control	100
2	MnSO$_4$	191
3	CaCl$_2$	180
4	BaCl$_2$	163
5	CoCl$_2$	162
6	NaCl	142
7	FeCl$_3$	133
8	KCl	131
9	MgCl$_2$	67
10	ZnSO$_4$	50
11	CuSO$_4$	0
12	HgCl$_2$	0
13	EDTA	10
14	Urea	142
15	SDS	147

Fig. 7 Lineweaver–Burk plot

Fig. 8 Hydrolysis of starch estimated by assaying reduction in blue value of starch iodine complex and reducing sugar (by Miller) production using 1% (*w/v*) cassava at 80°C and pH 5.0: (*filled square*) residual starch; (*filled circle*) reducing sugar produced
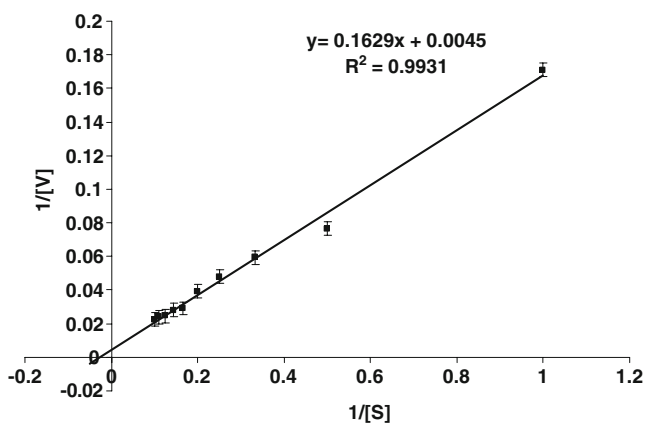
was at pH 6.5, which was a result of enhanced bacterial growth. Most of the *Bacillus* strains are used for the commercial production of α-amylases that have an optimum pH between 6.0 and 9.0 for growth and enzyme production (Burhan et al. 2003; Jin et al. 1999). In this study, *Geobacillus* sp. IIPTN strain showed optimum growth and maximum α-amylase yield at pH 6.5. Amylase production from *Geobacillus* sp. IIPTN was inductive since the enzyme synthesis was high in the presence of cassava (135 U ml^{-1}) and soluble starch (126 U ml^{-1}). *Geobacillus* sp. IIPTN showed comparatively low enzyme production (50 U ml^{-1}) when glucose was used as carbon source. This might due to glucose effect that repressed the enzyme production. It has been reported that the synthesis of carbohydrate-degrading enzymes in most species of the genus *Bacillus* is subject to catabolic repression by readily metabolizable substrates such as glucose (Santos and Martins 2003). Similar results were also reported to the hyperthermophilic archaeon *Sulfolobus solfataricus* in which glucose repressed production of α-amylase, demonstrating that a classical glucose effect was operative in this organism (Haseltine et al. 1996). Amongst various nitrogen sources, the isolate showed the highest amylase synthesis in the presence of soyabean meal and ammonium nitrate. Soya
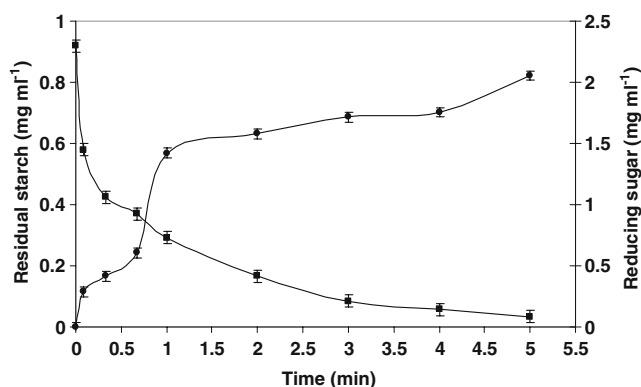
bean meal was found as the best nitrogen source for α-amylase production by *Bacillus* sp. I-3 (Sodhi et al. 2005). The combination of ammonium chloride and tryptone also showed the good amylase synthesis. Ramachandran et al. (2004) reported that peptone (1% *w/v*) and ammonium nitrate (1% *w/v*) enhanced the enzyme yield in SSF using coconut oil cake as substrate when supplemented individually. Malhotra et al. (2000) reported that tryptone was found good nitrogen source for amylase production.

The enzyme was purified by cation exchange and hydroxyapatite binding chromatography. The molecular mass of purified amylase was 97 KD. High molecular mass amylase was reported in *Bacillus* spp. (97 KD) (Kim et al. 1995), amylase from *B. brevis* had molecular mass of 205 KD (Ray et al. 2008). Temperature and pH are the critical parameters for starch hydrolysis. IIPTN α-amylase was optimally active at 80°C. Extracellular and cell-bound hyperthermophilic enzymes are optimally active at temperatures above or far above the host organism's optimum growth temperature and found highly stable (Vieille and Zeikus 2001). The enzyme was active in the broad range of temperature (40–120°C) (Fig. 6a). Few *Bacillus* sp. has

Table 3 Substrate specificity of α-amylase on (1% *w/v*) gelatinized and non-gelatinized substrates was carried out at 80°C and pH 5.0

S. No.	Substrate	Relative activity on gelatinized starch (%)	Relative activity on non-gelatinized starch (%)
1	Soluble potato starch	100	67
2	Corn starch	107	68
3	Cassava beads	129	106
4	Tapioca root	88	50
5	Amylopectin	86	56
6	Sweet sorghum	62	ND
7	Corn flour	58	ND

ND Not determined

been reported which work optimally at 70–100°C, e.g., amylases from *B. licheniformis* (100°C), *B. stearothermophilus* (70–80°C), *B. amyloliquefaciens* (70°C), and *B. stearothermophilus* (70°C) (Haki and Rakshit 2003). The optimum pH of IIPTN α-amylase was 5.0, and it was stable over a wide range of pH, 5.0 to 9.0 (Fig. 6b). At pH below 4.5, there was complete deactivation of enzyme; this could be due to the structure of functional groups in the active site which seems to be basic and resulted into acid hydrolysis of peptide bonds. Acid hydrolysis of peptide bonds at low pH was reported by Vieille and Zeikus (2001), which explained that it occurred most often at C-terminal side of Asp residues, with the Asp-Pro bond being the most susceptible. This may be due to the facts that the nitrogen of proline is more basic than that of other residues, and Asp has an increased propensity for α-β isomerization when linked on the N side of a proline. The natural pH of the starch slurry is approximately 4.5. In present starch-processing methods, the pH of the starch slurry adjusted to 5.8 or above for starch liquefaction. The higher pH values cause by-products and color formation which is not desirable in starch industries. Very few hyperthermostable amylases are reported that works up to 100°C and pH 5.0 like *Thermococcus profundus* with optimal activity at 80°C, pH 4.0–5.0, *Pyrodictium abyssi* with optimal activity at 100°C, pH 5.0, *Staphylothermus marinus* optimal activity at 100°C, pH 5.0 (Vieille and Zeikus 2001). *Geobacillus* sp. IIPTN novel α-amylase worked up to temperature of 120°C and pH 5.0.

Most of the amylases used in industry are reported to be metal ions dependent like, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} (Sivaramakrishnan et al. 2006). *Geobacillus* sp. IIPTN α-amylase was also found a metallozyme since the highest relative activity (191%) was observed in the presence of Mn^{2+} ion (Table 2). It has been reported that α-amylases specifically bind to Ca^{2+}. The α-amylase catalytic site is located in a cleft between two domains (an $[α/β]_8$ barrel and a large loop). Coordinated by ligands belonging to these two domains, Ca^{2+} is essential for the enzyme's catalytic activity and thermostability (Vieille and Zeikus 2001). But in case of *Geobacillus* sp. IIPTN α-amylase, we can assume that Mn^{2+} in place of Ca^{2+} played the role in catalytic activity. EDTA inhibited the amylase activity which confirmed that it is metallozyme. The relative activity was also enhanced by Ca^{2+} (180%), Ba^{2+} (163%), Co^{2+} (162%), Na^+ (142%), Fe^{3+} (133%), K^+ (131%). The effect of Cu^{2+} and Hg^{2+} was found completely inhibitory which suggested that a cysteine residue could be involved in the enzyme activity (Goyal et al. 2005).

Apparent values of K_m and V_{max}, determined from Lineweaver–Burk plot, were 36 mg ml^{-1} and 222 μmol mg^{-1} protein min^{-1}, respectively, with cassava starch as substrate. *Geobacillus* sp. IIPTN showed substrate affinity towards cassava and corn starch with hydrolysis rate 129% and 107%, respectively, taking soluble starch hydrolysis rate as 100% (Table 3). It has been reported that natural starch such as raw sago starch, corn starch, and wheat starch increased α-amylase activities. It could hydrolyzed the wide variety of starch in the order cassava>corn starch> soluble starch>tapioca root>amylopectin>sweet sorghum>corn flour. Similar findings on digestibility of various starch by α-amylase of *A. niger* from rotting cassava bagasse reported were of the order maize starch>cassava starch>sorghum starch>soluble potato starch (Sivaramakrishnan et al. 2006). This enzyme could hydrolyzed the raw starch with hydrolysis rate in the order: cassava (106%)>corn starch (68%)>soluble starch (67%)>amylopectin (56%)>tapioca root (50%). Enzyme showed the affinity for raw cassava starch that is made up of tapioca root but in case of tapioca root the rate of hydrolysis was 50%. The possibility might be, some cultivars have high content of cyanogens and such type of cultivars can be used after cooking (Balagopalan 2002). The cyanogens might have affected the enzyme; therefore, the cooked tapioca root showed 88% hydrolysis rate than uncooked which was 50%. Raw starch hydrolysis also depends upon the C-terminal domain within the enzyme, which has been reported to be necessary for degradation of granular starch by mould glucoamylases. However, in case of bacterial amylases, binding to starch granules is not necessary requirement for the hydrolysis of the raw starch (Goyal et al. 2005). Another factor that counts is the ratio of amylose and amylopectin. The higher facility for water entrance in cassava starch is due to high content of amylopectin and the low molecular density. This property made the enzyme to access the substrate, therefore, raw cassava in this study showed 106% hydrolysis rate (Moore and Amante 2005).

Mostly industrial starch processes involve starch hydrolysis into glucose, maltose, or oligosaccharides. These sugars are then used in fermentation to produce a variety of chemicals e.g., ethanol, lysine, and citric acid. Starch bioprocessing generally involves two steps, liquefaction and saccharification, which run at high temperatures. During liquefaction, starch granules are gelatinized in a jet cooker at 105°C to 110°C for 5 min in aqueous solution (pH 5.8 to 6.5) and then partially hydrolyzed at α-1,4 linkages with a thermostable α-amylase at 95°C for 2 to 3 h. The α-amylases typically used from *B. licheniformis* and *B. stearothermophilus* are inactivated at temperature beyond 105°C. There is need of α-amylases which can operate above 100°C at acidic pH values for improved starch processing. The isolated novel IIPTN α-amylase can efficiently work up to 120°C at pH 5.0. The high-temperature stability, acidic pH, and raw starch degradability of IIPTN α-amylase revealed

that this enzyme has the potential to fit into industrial requirements.

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