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ORIGINAL PAPER

Cloning and expression of acidstable, high maltose-forming, Ca^{2+}-independent α-amylase from an acidophile *Bacillus acidicola* and its applicability in starch hydrolysis

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Abstract The α-amylase encoding gene from acidophilic bacterium *Bacillus acidicola* was cloned into pET28a(+) vector and expressed in *Escherichia coli* BL21 (DE3). The recombinant *E. coli* produced a 15-fold higher α-amylase than *B. acidicola* strain. The recombinant α-amylase was purified to homogeneity by one-step nickel affinity chromatography using Ni^{2+}-NTA resin with molecular mass of 62 KDa. It is active in the pH range between 3.0 and 7.0 and 30 and 100 °C with optimum at pH 4.0 and 60 °C. The enzyme is Ca^{2+}-independent with K_m and k_{cat} values (on soluble starch) of 1.6 mg ml^{-1} and 108.7 s^{-1}, respectively. The α-amylase of *B. acidicola* is acidstable, high maltose forming and Ca^{2+}-independent, and therefore, is a suitable candidate for starch hydrolysis and baking.

Keywords *Bacillus acidicola* · Acidstable · α-Amylase · Gene cloning

Introduction

α-Amylase is one of the most important industrial enzymes with potential application in starch saccharification, food, paper, pharmaceuticals and textile industries. Each application of α-amylase needs unique properties like specificity, stability, temperature and pH dependence (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 producing α-amylase with specific properties facilitate the discovery of novel amylases suitable for starch saccharification and other industrial applications in baking, brewing (Gupta et al. 2003).

The α-amylase currently used in starch industries is active at pH 6.5 and requires Ca^{2+} for its activity/stability. As the pH of the native starch is 3.2–4.5, the change in pH and removal of Ca^{2+} from the product streams by using ion exchangers adds to the cost of the products. Maltogenic α-amylases are known to be good anti-staling agents as maltodextrins produced by α-amylase specifically blocks the interaction between starch and gluten (Martin and Hoseney 1991). The search for α-amylases with the desired kinetic properties for diverse applications is encouraged because these will improve the industrial process in terms of economics and feasibility (Martin et al. 1991).

The acidophilic bacterium *Bacillus acidicola* produces high maltose forming, Ca^{2+}-independent and acidstable α-amylase, but the production level of the enzyme was very low for industrial applications (Sharma and Satyanarayana 2010, 2011). In order to enhance enzyme production, cloning and expression of the α-amylase encoding gene from *B. acidicola* was attempted. The kinetic properties of the recombinant α-amylase have been compared with those of the native.

Materials and methods

Organism and culture conditions

Bacillus acidicola was isolated from a soil sample collected from Rameshwaram (Tamil Nadu, India) (Sharma

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and Satyanarayana 2010) and routinely grown on starch/peptone/beef extract/agar (g l^{-1}:10 g starch, 5 g peptone, 3 g beef extract, 20 g agar and pH 4.5) at 37 °C and preserved at −20 °C in glycerol. The culture is deposited at Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India (MTCC-10504). α-Amylase production was carried out in starch-peptone-yeast extract broth (Sharma and Satyanarayana 2011).

Gene cloning and plasmid construction

The genomic DNA of *B. acidicola* was extracted according to Bazzicalupo and Fani (1995). Partial gene sequence was amplified using DAF1 and DAR1 set of primers designed from the highly conserved regions identified by multiple sequence alignment of amino acid sequences of α-amylases from the species of *Bacillus* and *Geobacillus*. PCR amplification was carried out using Thermocycler (Bio-Rad, CA, USA), and the amplicon was cloned into pGEMT-easy vector (Promega, Madison, WI, USA). When the cloned amplicon was sequenced and analyzed by the BLAST, a high homology with α-amylases of *G. stearothermophilus* and *Bacillus* sp. MK716 was recorded. A new set of primer (Forward primer-AamylBamH1F and Reverse primer-AamyXhoR) was designed to obtain the full catalytic domain of α-amylase from *B. acidicola* (Table 1). PCR reaction was performed with the following cycling protocol: initial denaturation at 95 °C for 5 min followed by 29 cycles of 50 s at 95 °C, annealing at 59 °C for 50 s, extension at 72 °C for 1 min followed by final extension of 10 min at 72 °C.

In order to construct expression vector, the amplicon was ligated into the pET28a(+) vector. The construct was confirmed by colony PCR as well as digestion of recombinant plasmid with respective restriction enzymes (*Bam*HI and *Xho*I). The recombinant vector is designated as *amy*-ba pET28a(+). Three positive clones containing α-amylase gene were sequenced at the nucleic acid sequencing facility, University of Delhi South Campus, New Delhi. One of the clones with high enzyme production was used in the production of recombinant enzyme. The nucleotide sequence of the α-amylase encoding gene was submitted to the Genbank database (accession number JN680873).

Table 1 Primers used in the investigation

Primers	Oligomeric sequences (5′–3′)
DAF1	AGCTTTAAGTGGCGCTGGTA
DAR1	GGGGCATCAAACAAAGACAT
AamylBamH1F	CCCGGATCCATGTTTAACGGCACCATGATG
AamyXhoR	CCCCTCGAGTCTAGGAACCCAAACCGA AACCG

Expression of α-amylase encoding gene of *B. acidicola* (amy-ba)

The recombinant vector was isolated from *Escherichia coli* DH5α cell and transformed into *E. coli* BL21 (DE3) for the expression of the recombinant protein. Several clones of *E. coli* BL21 (DE3) were picked from LB-kanamycin plates and grown overnight at 37 °C as a seed culture. The fresh LB-kan medium was inoculated with 1 % seed culture and protein expression was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) to the absorbance value of 0.6 at 600 nm. The culture was harvested after 4–6 h of induction and resuspended in lysis solution [50 mM acetate buffer (pH 4.5), 2.5 M NaCl, 100 mM $MgCl_2$] and sonicated in an Ultrasonic Sonicator (Sonics, Vibra-cell, Newtown, CT, USA) for 2 s on and 2 s off for 5 min to release intracellular protein. The cell-free extract was centrifuged at 10,000 rpm for 15 min to remove cell debris and the supernatant was used in α-amylase assays.

Determination of α-amylase activity and protein concentration

α-Amylase was assayed according to Sharma and Satyanarayana (2010). One unit of saccharogenic α-amylase activity is defined as the amount of enzyme that liberates 1 µmol reducing sugar as maltose min^{-1} under the assay conditions. Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as the standard.

Purification of recombinant α-amylase

The recombinant α-amylase was purified using Ni^{2+}-NTA resin. One ml of Ni^{2+}-NTA resins were equilibrated with solution A (10 mM KH_2PO_4, pH 4.0, 5 mM β-Mercaptoethanol) followed by solution B (buffer A and 20 mM imidazole). The cell-free lysate was allowed to bind to equilibrated resins for 1 h at rocker. The unbound proteins were removed using multistep washings with solution C (10 mM acetate buffer pH 4.5, 5 mM β-Mercaptoethanol, 10 % glycerol and 300 mM NaCl). Purified α-amylase was eluted with a linear imidazole gradient (100–500 mM) and collected in 1 ml fractions.

SDS and native polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed using molecular weight marker (Genei protein molecular weight marker PMWM). The 12 % gel was prepared according to Laemmli (1970), and Coomassie brilliant blue R-250 was used for staining the gel. Non-denaturing gel

electrophoresis was performed using 10 % gel with 0.5 % starch. After electrophoresis, the gel was incubated at 60 °C for 4 h in 50 mM KH_2PO_4 (pH 4.0). Clear zones developed by the action of amylase were visualized by flooding with Lugol's iodine.

Effect of pH and temperature on α-amylase activity

The starch solutions (0.5 %) and appropriately diluted enzyme solutions prepared in buffers (0.1 M) of pH varying (buffers: glycine-HCl pH 2 and 3, acetate pH 4 and 5, phosphate pH 6 and 7) were used in reaction mixtures and incubated at 60 °C. For determining the effect of temperature on the enzyme activity, the reaction mixtures were incubated at different temperatures (30–100 °C).

The pH stability of the enzyme was studied by incubating the α-amylase in different buffers of pH values (3–7) over a period of 12 h and subsequently assayed at 60 °C. Thermostability of the enzyme was carried out by exposing the enzyme to 60–90 °C over a period of 2 h, followed by enzyme assay at 60 °C.

Analysis of end products by thin layer chromatography

The end products of recombinant α-amylase of *B. acidicola* action on starch were analyzed by thin layer chromatography (TLC). The enzymatic reaction was performed for 15, 30 min, 1 and 2 h. The TLC plate was activated by placing in an oven set at 110 °C for 30 min, and the samples were spotted and developed in butanol:ethanol:water (5:3:2) at room temperature. The plate was then treated with aniline diphenylamine reagent (Hansen 1975) in order to visualize the sugars.

Effect of additives, metal ions, inhibitors and organic solvents on enzyme activity

The effect of various metal ions (1 and 5 mM), inhibitors (1 and 5 mM), surfactants (0.1 and 0.5 %) and organic solvents (10 and 20 %) was studied by incorporating them into the reaction mixtures for assessing their effects on α-amylase activity.

Hydrolysis of raw starch (wheat flour) by the α-amylase

The slurry of 20% (*w/v*) wheat flour was prepared in acetate buffer (10 mM, pH 4.0) and treated with α-amylase (10 Ug^{-1}) of *B. acidicola* for 6 h at 60 °C. Hydrolysis of raw starch granules was followed under scanning electron microscopy (SEM). The SEM analysis of raw starch hydrolysis was carried out by mounting the samples on stubs with silver glue for conduct, and the starch particles were examined under SEM-50B, scanning electron microscope (Philips, Amsterdam, The Netherlands) at Electron Microscope Facility, All India Institute of Medical Sciences, New Delhi.

Results

Cloning and expression of gene encoding α-amylase from *B. acidicola*

The α-amylase gene from *B. acidicola* consists of 1,441 nucleotides with overall G + C content of 50 % that encodes a protein of 479 amino acids with 57 negatively and 45 positively charged residues. The theoretical pI of the recombinant protein is 5.41. The molecular mass of the recombinant α-amylase is 62 KDa on SDS gel. The analysis of amino acid sequence of the enzyme revealed that α-amylase belongs to AmyAc superfamily. AmyAc Superfamily (amylases with 3 domains: A, B, C. A is a (beta/alpha) 8-barrel; B is a loop between the beta 3 strand and alpha 3 helix of A; and C is the C-terminal extension) includes α-amylase, amylosucrase, amylomaltase and others with the triad of catalytic residues (Asp, Glu and Asp). Signal peptide sequence was not detected in the cloned gene by using SignalP 4.0. Four highly conserved regions were identified in this α-amylase by multiple sequence alignment of various α-amylases (Fig. 1). The phylogenetic tree shows a close proximity with that of *B. licheniformis* NH1 (Hmidet et al. 2008), *G. stearothermophilus*, *Bacillus* sp. MK 716, *Geobacillus* sp. SR 74 α-amylase (Fig. 2).

Purification of the recombinant enzyme

The recombinant protein was successfully produced in *E. coli* BL21 (DE3) at 37 °C by induction with 1 mM IPTG under the control of T7 RNA polymerase promoter with 6× His-tag. The recombinant enzyme was purified using Ni^{2+} affinity chromatography. SDS-PAGE electrophoregram indicated that the enzyme is purified and band corresponds to approximately 62 KDa (Fig. 3).

Effect of pH and temperature on enzyme activity and stability

The α-amylase of *B. acidicola* is optimally active at pH 4.0 (Fig. 4). The enzyme retained 100 % activity for 12 h and one hour at pH 4.0 and 3.0, respectively. The enzyme activity increased consistently with the increase in temperature from 30 to 60 °C, and thereafter, it declined (Fig. 5), with optimum at 60 °C. The $T_{1/2}$ values of the enzyme at 80, 90 °C were 30 and 10 min, respectively.

Fig. 1 Nucleotide and deduced amino acid sequences of the *B. acidicola* α-amylase gene. *Underlined regions* **I (DVVFDH)**, **II (GFRLDAVKH)**, **III (EYWS)**, **IV (FVDNHD)** denote highly conserved among amylase. *Bold and double underlined* amino acids (Asp 231; Glu 261 and Glu 328) are involved in catalysis on the basis of proposed 3D structure (PDB ID 1HVX)

FNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGTSRSDVGY
GVYDLYDLGEFNQKGTVRTKYG

I

TKAQYLQTIQAAHAAGMQVYA**DVVFDH**KGGADGTEWVDAVEVNPSDRNQEISG
TYQIQAWTKFDFPGRGNTYSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWD
WEVDTENGNYDYLMYADLDMDHPEVVTELKNWGKWYVNTTNID

II III

GFRLDAVKHIKFSSFPDWLSYVRSQTGKPLFTVG**EYWS**YDINKLHNYITKTNGTMS
LFDAPLHSKFYTASKSGGAFDM

IV

RTLMTNTLMKDQPTLAVT**FVDNHD**TEPVQALQSWVDPWFKPLAYAFILTRQEGY
PCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQHDYLDHSDIIGWTREGVTE
KPGSGLAALITDGPGGSKWMYVGKQHAGKVFYDLTGNRSDTVTINSDGWGEFKV
NGGSVSVWVP

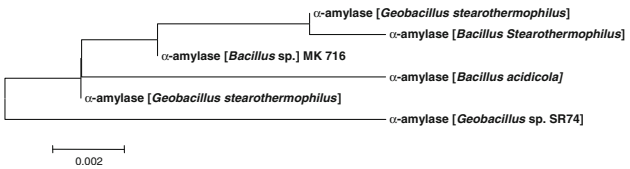

Fig. 2 Phylogenetic tree of recombinant α-amylase of *B. acidicola* with other amylases. Predicted amino acid sequence shared highest homology with α-amylase of *Bacillus sp.* MK 716 and *G. stearothermophilus*. The phylogenetic tree was made using the neighbor-joining method (MEGA 4 software)

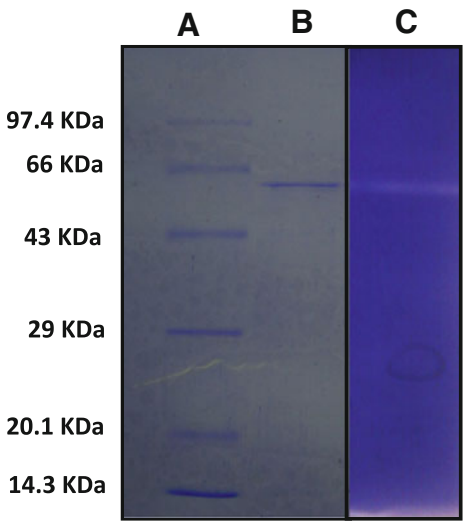

Fig. 3 SDS-PAGE analysis of recombinant α-amylase expressed in pET28a(+) systems (samples were resolved on 12 % polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 and by activity staining). *Lane A* SDS protein marker (Genei PMWM), *lane B* purified recombinant α-amylase obtained from Ni^{2+}-NTA column using imidazole (400 mM), *lane c* gel showing zymogram analysis using Iodine-staining method

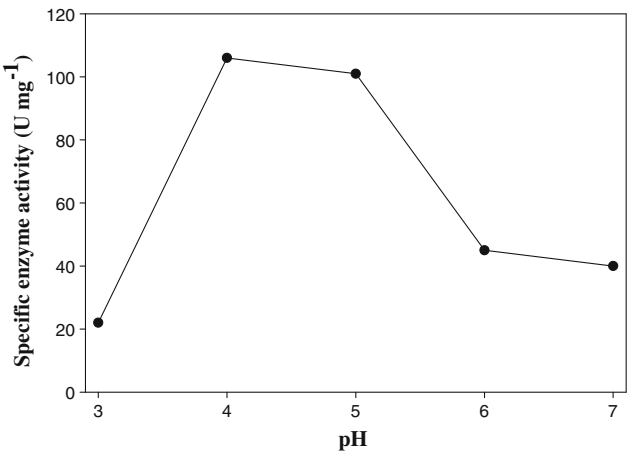

Fig. 4 Effect of different pH on enzyme activity

Effect of metal ions and other chemical reagents on α-amylase activity

Among various metal ions tested (Table 2), the enzyme activity was not significantly affected by Ca^{2+}, Na^{+}, K^{+} at 5 mM. The presence of Co^{2+}, Mg^{2+} and Fe^{2+} stimulated α-amylase activity. The cations Mn^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+} and Ag^{+} inhibited at 5 mM, while Hg^{2+} completely inhibited the enzyme activity at 1 mM. Phenylmethanesulfonyl fluoride (PMSF) and Woodward's Reagent K (WRK) inhibited enzyme activity in a concentration dependent manner. *N*-Bromosuccinimide strongly inhibited enzyme activity even at 1 mM.

Product profile and substrate specificity of the enzyme

The end products of starch hydrolysis are maltose, maltotriose, maltotetraose and maltopentaose (Fig. 6), indicating that the recombinant α-amylase of *B. acidicola* produced

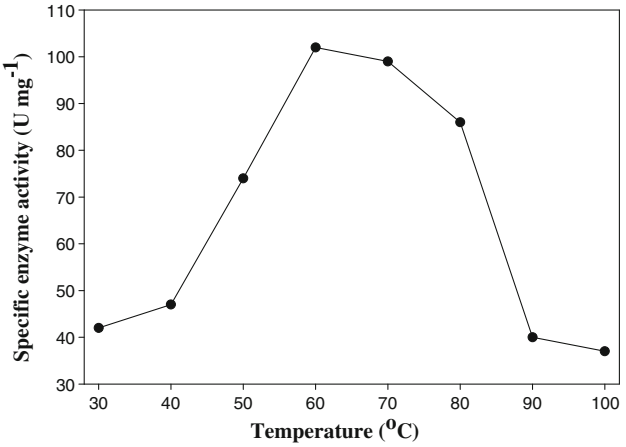

Fig. 5 Effect of different temperature on enzyme activity

by *E. coli* is an endoacting α-amylase. Furthermore, maltose is the main hydrolysis product. The enzyme hydrolyzed soluble starch (100 %), wheat flour (84 %), water chestnut (83 %), rice flour (75 %), amylose (83 %), pullulan (6.6 %), corn starch (68 %), and amylopectin (19 %), but not α- and *β*-cyclodextrins.

Hydrolysis of raw wheat starch by α-amylase

Scanning electron micrographs of raw wheat flour with the enzyme revealed efficient hydrolysis of starch grains (Fig. 7). The initiation of starch hydrolysis was observed after 2 h of enzyme substrate reaction. On further incubation, complete hydrolysis of the raw starch by the enzyme was recorded.

Discussion

The gene encoding α-amylase of *B. acidicola* was cloned in pET28a(+) and expressed in *E. coli* BL21 (DE3). The expression of gene with N and C terminal truncation did not affect the functional expression of the enzyme in *E. coli* as reported in *Bacillus* sp. TS-23, in which α-amylase gene lacking 96 and 294 bp at its 5′ and 3′ ends was successfully cloned in pQE-30 vector and expressed in *E. coli* (Lo et al. 2001). The molecular mass of the native type α-amylase of *B. acidicola* is 66 KDa that confirms the expression of truncated (lacking 37 amino acids from N and 34 amino acids from C terminal) amylase. α-Amylases with similar molecular mass had been reported in *Bacillus* sp. YX1 (56 kDa) (Liu and Xu 2008), *Bacillus* sp. KR-8104 (59 kDa) (Sajedi et al. 2005), *Bacillus* sp. Ferdowsicous (53 kDa) (Asoodeh et al. 2010).

The recombinant α-amylase from *B. acidicola* was optimally active at pH 4.0 and 60 °C. α-Amylase from *Bacillus* sp. Ferdowsicus (Asoodeh et al. 2010), *Bacillus*

Table 2 Effect of metal ions, detergents and inhibitors and organic solvents on α-amylase activity

Effect	Relative enzyme activity (%)	
Control	100	
Additives	1 %	2 %
Dextran	88.5 ± 0.63	85.0 ± 0.91
Glycerol	100 ± 0.5	97.4 ± 0.77
PEG 4000	97.3 ± 0.63	106 ± 0.91
PEG 8000	101 ± 0.49	103 ± 0.35
Detergents	0.1 %	0.2 %
Tween 20	97.7 ± 0.35	112 ± 1.2
Tween 40	112 ± 2.8	116 ± 0.9
Tween 80	101 ± 1.4	104 ± 0.8
Triton X-100	96.4 ± 1.6	92.8 ± 1.2
Cholic acid	100 ± 0.8	106 ± 1.4
SDS	94.3 ± 0.8	86.7 ± 1.0
Divalent cations	1 mM	5 mM
Ba^{2+}	93.2 ± 2.4	84.5 ± 2.1
Mg^{2+}	99.2 ± 1.13	134 ± 1.76
Co^{2+}	267.5 ± 2.1	229.5 ± 0.7
Pb^{2+}	87.3 ± 0.6	52.0 ± 1.4
K^{+}	99.4 ± 0.8	89.9 ± 3.2
Mn^{2+}	21 ± 1.4	0
Cu^{2+}	91.7 ± 0.3	20.2 ± 0.6
Hg^{2+}	0	0
Fe^{2+}	175 ± 1.4	130 ± 1.4
Ca^{2+}	100 ± 1	100 ± 0.8
Ag^{+}	94.4 ± 0.8	64.9 ± 1.5
Ni	68.7 ± 0.4	67.4 ± 0.8
Sn	86.1 ± 1.2	88.3 ± 0.9
Cd	88.3 ± 0.9	80.1 ± 1.2
Na	91.6 ± 1.9	88.5 ± 0.7
Zn	90.5 ± 2.6	77.2 ± 1.13
Inhibitors	1 mM	5 mM
Dithiothreitol	104.5 ± 0.7	90.8 ± 1.13
β-Mercaptoetanol	91.6 ± 0.5	88.7 ± 0.3
Iodoacetic acid	90 ± 1.4	84 ± 1.41
Phenylmethanesulfonyl fluoride	77.7 ± 1.6	0
N-Bromosuccinamide	0	0
Woodwards Reagent K	42 ± 1.2	40 ± 0.9
EGTA	100 ± 0.8	100 ± 0.6
EDTA	85.5 ± 0.7	61.3 ± 1.4
Organic solvents	10 %	20 %
Acetone	109 ± 1.4	93.3 ± 0.9
Methanol	106.8 ± 0.21	105.4 ± 0.7
Butanol	114 ± 1.4	73.5 ± 2.12
Ethanol	116.7 ± 0.9	115 ± 1.4
Isoamylalcohol	110.5 ± 0.7	118.2 ± 1.9
Hexane	105.7 ± 0.4	112 ± 1.4

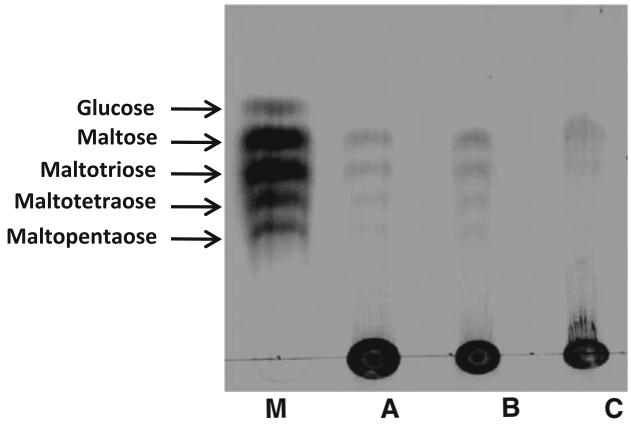

Fig. 6 Analysis of hydrolysis products. *Lane M* showing the sugar standards. *Lanes A–C* oligosaccharide profile obtained from the hydrolysis of starch (sigma) from recombinant α-amylase of *B. acidicola* after 15, 30 min and 2 h, respectively

sp. YX1 (Liu and Xu 2008), *B. stearothermophilus* US100 (Khemakhem et al. 2009), *Bacillus* sp. KR-8104 (Sajedi et al. 2005) exhibited pH and temperature optimum at 4.5, 5.0, 5.6, 4.0–6.0 and 70, 40–50, 80 and 75–80 °C, respectively. The $T_{1/2}$ of the recombinant α-amylase at 90 °C was 10 min. The acidstable α-amylase from *Bacillus* sp. Ferdowsicus (Asoodeh et al. 2010) and *Bacillus* sp. YX1 (Liu and Xu 2008) exhibited $T_{1/2}$ of 48 min at 80 °C and >1 h at 60 °C, respectively.

The lack of any noticeable effect on enzyme activity in the presence of Ca^{2+} and EGTA confirmed that the enzyme is Ca^{2+}-independent. In starch industry, the Ca^{2+}-independent α-amylase is in demand as Ca^{2+} ions inhibit glucose isomerase, and thus, removal of Ca^{2+} from the product streams in the subsequent stages is necessary that adds to the process operating costs (Antranikian 1992). The 100 % inhibition of enzyme activity by Hg^{2+} confirms the presence of carboxyl groups in enzyme molecule (Dey et al. 2002). Further, Hg^{2+} is known to oxidize indole ring and to interact with aromatic ring present in tryptophan (Zhang et al. 2007; Liu et al. 2010). NBS exerted very strong inhibitory action as in β-D-xylanase of *B. halodurans*, indicating the catalytic role of tryptophan (Kumar and Satyanarayana 2011). While strong inhibition by PMSF suggested the involvement of seryl hydroxyl group in enzyme catalysis. The inhibition of α-amylase by WRK signifies the chemical modification of aspartic and glutamic acid residues involved in the active site (Paoli et al. 1997). The inactivation of enzyme by WRK also indicates the involvement of acidic amino acids in the active site of the enzyme (Chauthaiwale and Rao 1994; Komissarov et al. 1995).

High affinity of the enzyme towards starch and amylose and low affinity towards α and β-cyclodextrin was also reported by Asoodeh et al. (2010). The activity of enzyme

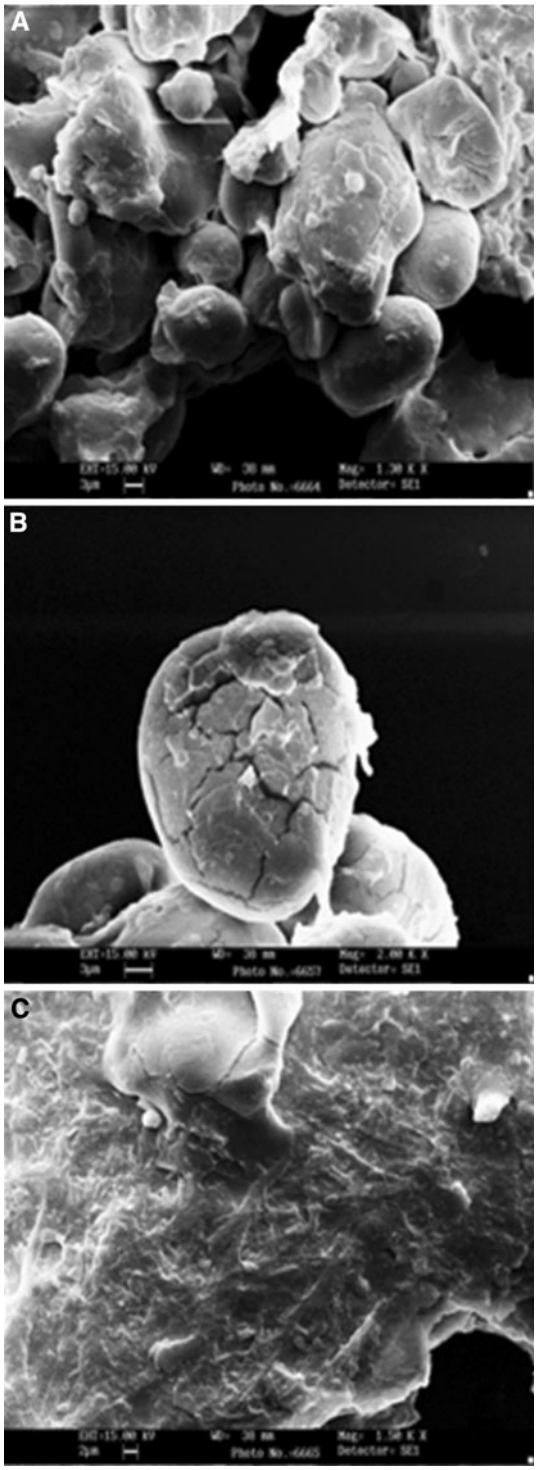

Fig. 7 Scanning electron micrograph showing the hydrolysis of raw wheat starch granules by the α-amylase of *B. acidicola*: **a** untreated starch granules, **b** a hydrolyzed portion of the granule in 2 h, **c** almost completely hydrolyzed starch granule after 6 h

on various starch substrates suggests that the enzyme has been correctly and independently folded in order to retain the tertiary structure that allows a normal substrate recognition and catalytic activity (Lo et al. 2001).

Table 3 Comparison of the characteristics of recombinant α-amylase of *B. acidicola*

Properties	α-Amylase (wild type)	Recombinant α-amylase
Specific enzyme activity (U mg^{-1})	80	1,166
Molecular weight	66	62
Optimum pH	4	4
Optimum temperature (°C)	60	60
Thermostability ($T_{1/2}$ at 90 °C)	5	5
K_m (mg ml^{-1})	1.6	1.6
V_{max} (μM ml^{-1} min^{-1})	4.76	5.26
k_{cat} (S^{-1})	100.3	108.7
k_{cat}/K_m (μM^{-1} S^{-1})	62.68	67.9

Bacterial amylases such as α-amylases from *B. stearothermophilus* NCA 26 (Kim et al. 1989) and *G. thermoleovorans* (Rao and Satyanarayana 2007) are known to digest raw starch, but these enzymes are not acidstable. As the pH of the native starch is 3.2–4.5, the acidstable α-amylase from *B. acidicola* appear to be a suitable enzyme in starch hydrolysis as well as baking.

The native and recombinant α-amylases of *B. acidicola* displayed similar kinetic properties (Table 3). The concentration of the enzyme expressed by *E. coli* was 15-fold higher than that produced in the original host *B. acidicola*.

The cloning and expression enabled us to attain 15-fold higher α-amylase than the wild strain. The activity and stability of α-amylase at low pH, high temperature and Ca^{2+}-independent starch hydrolysis mechanism makes the enzyme applicable in starch saccharification and baking (Sharma and Satyanarayana 2010). Further studies are called for improving the thermostability of the enzyme by site-directed mutagenesis and/or directed evolution.

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