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Characterization of the Thermophilic Starch Degrading *Petrotoga* Strain 64G3 and the Expression of its α-amylase Gene

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Abstract: A starch degrading bacterial isolate 64G3 was taxonomically characterized. Based on physiological properties, 16S rDNA sequence analysis and DNA-DNA hybridization, this isolate was assigned to *Petrotoga mexicana* species. A gene encoding an α-amylase of this strain was cloned and overexpressed in *Escherichia coli*. The gene is 1, 992 bp long encoding a polypeptide of 663 amino acids with a calculated molecular mass of 77, 042 Da. The deduced amino acids sequence shows a high identity of 94% with *Petrotoga mobilis* SJ95 α-amylase but low identities (up to 45%) with other glycosyl hydrolase family 13 enzymes. Recombinant amylase produced in *Escherichia coli* showed hydrolytic activity towards amylose, amylopectin, glycogen and maltooligosaccharides but not pullulan or other types of cyclodextrins. The optimal temperature and pH for the enzyme activity on starch were 45°C and 6.5, respectively. The enzyme cleaved soluble starch in endo-acting manner to release maltose, maltotriose and a minor amount of glucose during hydrolysis.

Key words: α-amylase, thermophilic bacteria, thermotogales, *Petrotoga mexicana*

INTRODUCTION

Starch is a high yielding resource for many industrial productions, such as ethanol, feed proteins and sweetener. Amylolytic enzymes (amylases) form a large group of enzymes which operate on starch and related polysaccharides. Amylases possessing unique and desirable properties are of great significance in many industrial processes (Van der Maarel *et al.*, 2002). Extremophilic micro-organisms that thrive in extreme environments including either high (55-100°C) or low temperatures (-2-15°C), high salinity (2-5 M NaCl), either high (10-12) or low (0-3) pH, high levels of radiation and high pressure (average 38 MPa), are the preferred sources of novel enzymatic properties (Gomes and Steiner, 2004).

The order thermotogales, including the following genera: *Thermotoga, Fervidobacterium, Thermosipho, Thermococcoides, Mesotoga, Geotoga, Kosmotoga, Marinitoga, Thermopallium* and *Petrotoga*, comprise both moderately and extremely thermophilic eubacteria. To date, only a few amylolytic enzymes from the *Thermotogales* have been characterized at the biochemical and genetic level. Remarkably, most attention has only been given to amylases of three extremely thermophilic species. These include *T. maritima* (Ballschmiter *et al.*, 2006; Liebl *et al.*, 1997; Lim *et al.*, 2003), *F. pennavorans* (Koch *et al.*, 1997) and *T. neapolitana* (Park *et al.*, 2010). Together with their thermostability, other notable properties of thermotogales amylases such as α-1-6 glucosidic bond specificity of *F. pennavorans* pullulanase (Bertoldo *et al.*, 1999), the formation of maltose as major product by *T. neapolitana* exo-type α-amylase (Park *et al.*, 2010) and the alkaline resistance of *T. maritima* amylase C (Ballschmiter *et al.*, 2006) have also been reported. However, no attention, as yet, has been paid to enzymes or proteins from the less thermophilic genera of the thermotogales. Members of the genus *Petrotoga* are anaerobic, thermophilic bacteria which have been isolated from the extreme environment, hot oil reservoirs (Miranda-Tello *et al.*, 2007). In this genus, *P. miotherma* was the first species reported for amylase activity but its enzymatic properties have not yet been studied (Davey *et al.*, 1993). It is known that *Petrotoga* produce polysaccharide degrading enzymes because of their capacity to utilize starch (Davey *et al.*, 1993) or xylan (Miranda-Tello *et al.*, 2004) as a carbon source for growth. To our knowledge, however, no characterization of either amylases or other enzymes from this genus have been reported.

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In this study, the starch degrading thermophilic bacterial isolate 64G3 was (1) phylogenetically classified as a *Petrotoga mexicana* species and (2) a novel α-amylase of this strain was identified, overexpressed and characterized.

MATERIALS AND METHODS

Bacterial strains and media: Strain 64G3 was isolated from oil/water mixtures taken from production well-heads of a petroleum reservoir located in Vung Tau city in Vietnam. The *in situ* temperature was about 50°C. The samples were collected anaerobically in sterile glass containers and transported to the laboratory at ambient temperature. Strain 64G3 was routinely grown in a basal medium containing (g L^{-1}): glucose, 5.0; $MgSO_4$, 3.0; $(NH_4)_2SO_4$, 0.5; $CaCl_2$, 0.5; K_2HPO_4, 0.2; NaCl, 30; yeast extract, 1.0. Prior to inoculation, the medium was supplemented with a sterile stock solution of $NaHCO_3$ and $Na_2S.9H_2O$ to obtain final concentrations of 0.04% and 0.1%, respectively. One milliliter of vitamin solution (DSMZ-141, www.dsmz.de) was added to the medium and the pH was adjusted to 7.0 with 10% $NaHCO_3$. The strain was cultured anaerobically in 25 mL Hungate tubes by transferring a 10% (v/v) inoculation to fresh medium and incubating for 3 days at 50°C. Growth experiments were performed in duplicate.

E. coli DH5α and Rosetta (DE3) plysS (Invitrogen) were used for gene cloning (or subcloning) and expression, respectively. *E. coli* strains were aerobically grown in Luria-Bertani (LB) broth or agar containing 1% bacto-tryptone (wt./vol), 0.5% yeast extract (wt./vol), 0.5% NaCl (wt./vol), supplemented with ampicillin (100 μg mL^{-1}) or chloramphenicol (30 μg mL^{-1}) if needed.

Scanning electron microscopy: Cells were fixed in the medium with glutaraldehyde and sodium azide at concentrations of 3% and 25 mM, respectively. After one hour of incubation, cells were harvested by centrifugation and resuspended in a small volume of medium containing 25 mM sodium azide. Cells were immobilized and bound for 10 min onto gold and poly-L-lysine coated glass slides. After washing with a washing buffer (50 mM cacodylate pH 7.4, 25 mM sodium azide, 1 mM $CaCl_2$ and 0.9% NaCl) followed by distilled water, cells were stained for 20 seconds with 1% uranyl acetate and air dried. The glass slides with cells were mounted onto stubs and sputtered with 10 nm gold particle with a Polaron SC7640 sputter coater (VG Microtech, East Sussex, UK) applying 1 kV and 13 mA. The samples were examined in a ZEISS DSM 940A scanning electron microscope (Oberkochen, Germany) at 7 kV and a working distance of 4 mm. Photos were made with equipment from Point Electronic (Halle, Germany).

DNA base composition analysis: The GC-content of the DNA was determined at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). DNA was isolated and purified on hydroxyapatite according to the procedure as described by Cashion *et al.* (1977). DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analyzed by HPLC (Shimadzu, Japan). GC determination was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT), using non-methylated Lambda DNA (GC-content 49.85 mol%) as a reference (Mesbah *et al.*, 1989).

Genomic DNA-DNA hybridization: DNA-DNA hybridization was carried out by a previously described method (De Ley *et al.*, 1970), with some modification (Huss *et al.*, 1983; Escara and Hutton, 1980) using a model 2600 spectrophotometer equipped with the Gilford model 2527-R thermo-programmer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the Transfer. Bas program (Jahnke, 1992). DNA-DNA hybridization was performed at the DSMZ (Germany).

Construction of *Petrotoga mexicana* 64G3 genomic DNA library: A genomic library was constructed using pUC19 as the cloning vector and procedures were conducted as described by Lim *et al.* (2003). Genomic DNA of the strain 64G3 was partially digested with *Sau*3AI and the fragments with sizes ranging from 2 to 5 kb were pooled and purified from agarose gels. The obtained fragments were then ligated to BamHI-digested and dephosphorylated pUC19 (at 16°C, overnight). The ligation mixture was used to transform *E. coli* DH5α by electroporation. The genomic library containing approximately 20,000 transformants was stored in a deep freezer until screening.

Amplification of an *amyA* DNA fragment: Based on known conserved regions in glycosyl hydrolase family 13 (GHF-13) (Janecek, 1997), five degenerate primers were synthesized (Table 1). Amplification was done using genomic DNA of strain 64G3 as the template and six alternative combinations of primers listed in Table 1. The sequence of PCR product obtained was used to design a pair of primers for PCR-based synthesis of a DNA probe to be used in Southern hybridization experiments. The probe, approximately 210 bp in length, was PCR labeled

Table 1: Degenerate primers used for amplification of partial *amyA* gene

Primer name	Primer sequence[a]	Specific to conserved region
GA1F	ATTGAYYTIGTIMTYAAYCA	1
GA2F	GTHGATGGITKCMGAMTIGA	2
GA2R	ARCARCRTCIATKCKSAAICC	2
GA4R	GAAGWMICKTKSIWYRTCRTG	4
GA5R	GCCIWKYTCITCICCITRRTA	7

[a]The primer sequence was designed corresponding to the conserved regions in the GHF-13 enzymes (Janecek, 1997)

using the DIG-DNA labeling and detection kit (Boehringer Mannheim, Germany) with the following primers: 5'-TCGACTCGGTTATGAATTAC-3' and 5'-CAGCTAATTTCGTTCGTTGT-3', with strain 64G3 genomic DNA as the template. Plasmids comprising of pUC19 and DNA inserts were immobilized on Hybond N+ membrane and hybridized with the probe at 68°C. Hybridization results led to the selection of positive clones which contained a full or partial sequence of the *amyA* gene.

Sequence data analysis: Searches for sequence similarities and Open Reading Frames (ORF) were conducted with BLAST and ORF finder tools provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). The signal peptide cleavage and a potential transcription termination site were predicted using the program SignalP 3.0 (Nielsen *et al.*, 1997) and Mfold 3.2 (Zuker, 2003), respectively.

Overexpression of *amyA* gene: The DNA corresponding to the mature *RamyA* ORF was obtained by PCR amplification using the following primers, CuAmyEfet22, 5'-AGACGGATCCATATTCGAAAGAAGCGAC -3' (forward primer; *BamH*I site underlined) and CuAmyEret22, 5'-GCCGCTCGAGCTCGCTAACAAGT ACAAGA-3'(reverse primer; *Xho*I site underlined) and *P. mexicana* 64G3 genomic DNA as template. The gene was cloned into the *BamH*I - *Xho*I restriction sites of the vector pET22b(+) (Novagen), creating the pET/*amyA* plasmid. *E. coli* Rosetta (DE3)plysS was transformed with pET/*amyA* to produce the expression host, Rosetta/*amyA*.

Two mL of the overnight culture (Rosetta/*amyA*) was used to inoculate 200 mL of LB broth containing ampicillin (100 μg mL^{-1}) and chloramphenicol (34 μg mL^{-1}) in a 1 L glass flask which was agitated at 37°C until an optical density at 600 nm of 0.5 was reached. Then, 1 mM isopropyl-thio α-D galactopyranoside (IPTG) was added to the culture medium and the induced culture was incubated at 28°C with agitation for an additional 3 h. Cells were harvested by centrifugation (6,000 xg, 10 min,

4°C), then resuspended in 10 mL of binding buffer (40 mM tris-HCl pH 7.9, 0.5 M NaCl and 10 mM imidazole), followed by sonication at 4°C for cell disruption. Clear supernatant and debris fraction were separated by centrifugation (10,000 xg, 20 min, 4°C).

The r*amyA* in the clear supernatant was purified using the His Bind Purification Kit (Novagen, Germany) according to the supplier's instructions. Insoluble r*amyA* in the debris fraction was refolded using the Protein Refolding Kit (Novagen, Germany), followed by purification.

Gel electrophoresis and activity staining: Protein samples were separated by Sodium Dodecyl Sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) with a 12% (w/v) gel (Laemmli, 1970). The gel was stained with Coomassie brilliant blue R-250. Amylase activity staining was performed by non-denaturing PAGE (in the absence of SDS and reducing reagent in the system) of amylase containing samples with the following protocol: after electrophoresis, the gel was rinsed with distilled water for 10 min at room temperature and washed for 30 min in 50 mM sodium phosphate buffer pH 6.5 with gentle agitation. The washed gel was then soaked in fresh buffer containing 0.5% soluble starch and incubation was performed for 5 hours at 45°C. The gel was then stained with iodine solution (0.3% of I$_2$, 5% KI) for 10 min and rinsed with distilled water. The zone of amylase activity was visualized as a light band against a dark blue background of uncleaved starch.

Enzyme assay: Amylase activity was assayed by measuring the amount of reducing sugars released during hydrolysis of soluble starch as described by Miller (1959). A 50 μL volume of the enzyme solution was added in 450 μL of 45°C-prewarmed sodium phosphate buffer 50 mM (pH 6.5) containing 0.5% (w/v) soluble starch and the enzyme reaction mixture was incubated at 45°C for 30 min. The reaction was terminated by cooling on ice, followed by addition of 500 μL of dinitrosalicylic acid (DNS). After boiling for 5 min in a waterbath, the absorbance of the coloured solution was measured spectrophotometrically at 530 nm. One Unit (U) of amylase activity was defined as the amount of the enzyme that released reducing sugar equivalent to 1 nM of maltose per min under the defined conditions. The protein concentration was measured by the Bradford method (Bradford, 1976), with bovine serum albumin as the standard.

Thermostability of the enzyme: Thermostability data were obtained by preincubating the enzyme in the absence of

soluble starch at various temperatures (50-80°C). The enzyme was withdrawn at indicated time intervals (15 min-4 h), cooled on ice and then remaining activity was measured with the standard enzyme assay. Characterization of enzyme was assayed in triplicate and all values were mean of three independent experiments.

Effect of divalent metal ions on *ramyA* activity: Amylase activity was examined after preincubation of the purified *ramyA* with different metal ions at a final concentration of 10 mM at room temperature for 10 min. The residual activity was measured under the standard conditions as mentioned above. The final concentration of metal ions in the reaction solution was 1 mM.

Thin-layer chromatography (TLC) of starch hydrolysates: Reaction products were centrifuged, spotted on Whatman K5F silica gel plates (Whatman, Maidstone, United Kingdom) and developed with isopropyl alcohol-ethyl acetate-water (3:1:1, v/v/v) as the solvent system. For visualization of spots, the plates were dried, sprayed with a solution containing 0.3% (w/v) N-(1-naphthyl) ethylenediamine and 5% (v/v) H_2SO_4 in methanol and then heated for 10 min at 110°C.

Substrate specificity: Substrate specificity of the enzyme was determined by incubating the purified *ramyA* with a variety of polysaccharide substrates (0.5% each), including soluble starch, amylose, amylopectin, three types of cyclodextrins, glycogen and pullulan in the standard assay condition as described above. The sugars released by starch degradation were identified by TLC.

Nucleotide sequence accession number: The nucleotide and deduced amino acid sequences of *amyA* were deposited in the GenBank database under the accession number DQ985807.

RESULTS AND DISCUSSION

Taxonomic characterization of isolate 64G3: A 1,429 bp-DNA fragment of the 16S rDNA gene of strain 64G3 was PCR amplified by using the genomic DNA of the strain as a template and primers Cyaf, 5'-CAGAGTTTGATCCTGGCTCAG- 3' and Cyar, 5'-TACCTTGTTACGACTTCACC- 3'. A BLAST search of the nucleotide database in the GenBank with the sequence of this DNA fragment as the query (accession number JQ417660) retrieved six highly similar 16S rDNA sequences including *P. mexicana* (identity: 99.3%, accession number: NR029058), *P. halophila* (98.6%, AY800102) *P. olearia* (98.1%, NR028947), *P. sibirica*

(97.8%, NR025466), *P. mobilis* (97.4%, CP000879) and *P. miotherma* (96.6%, L10657). The sequence similarities indicated that strain 64G3 is closely related to the genus *Petrotoga*. The GC-content of the DNA was 33.4%, falling into the range of 31 - 36.1% for the six known members of the *Petrotoga* genus (Miranda-Tello *et al.*, 2007). The genomic DNA-DNA relatedness of the strain 64G3 with *P. mexicana* (DSM 14811) (Miranda-Tello *et al.*, 2004) yielded a value of 74.7%, which is above the threshold of 70% recommended for definition of bacterial species (Wayne *et al.*, 1987). Therefore, strain 64G3 was identified as *P. mexicana* species. Morphologically, the 64G3 cells were rod-shaped and varied widely from 1.0 μm up to 60 μm in length and from 0.6 to 1.2 μm in width (Fig. 1a). The cells appeared singly, in pairs or in chains, within a sheath-like structure (a typical characteristic for members of thermotogales) that ballooned over the cell ends (Fig. 1b). The cells were immotile and no flagella were observed. Strain 64G3 grew anaerobically at temperatures
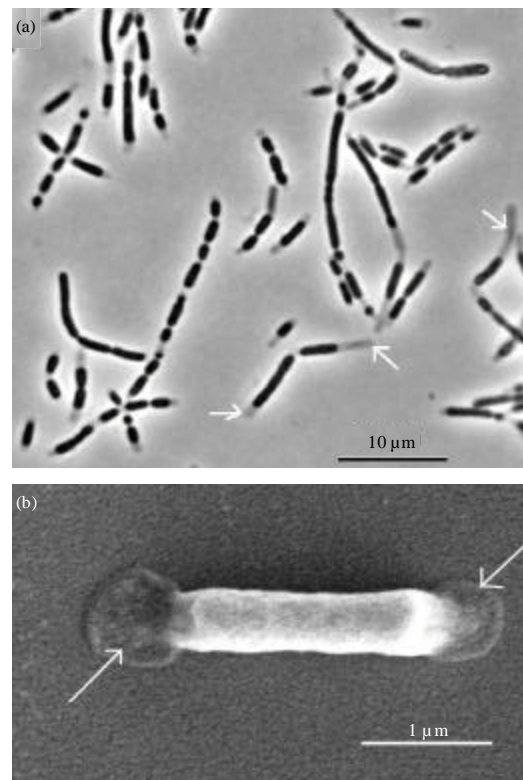

Fig. 1(a-b): Cellular morphology of strain 64G3 (a). Light micrograph of cells, bar 10 μm and (b) Scanning electron micrograph of a cell with toga at each end indicated by arrows, bar 1 μm

ranging from 30 to 65°C with optimum growth at 55°C. Growth was obtained in the pH range of 5.0 to 8.5 with optimum growth at pH 7.0. Strain 64G3 utilized various substrates including glucose, galactose, ribose, xylose, maltose, starch and xylan as sole carbon source. Elemental sulfur and thiosulfate could serve as alternative electron acceptors whereas sulfate could not.

Amylolytic enzymes from strain 64G3: Cell extract of strain 64G3 grown in a basal medium containing 0.5% soluble starch displayed hydrolytic activity towards soluble starch, three types of cyclodextrin and pullulan as measured by the release of reducing sugars. The starch degrading activity of the cell extract was highest at 75°C (Fig. 2). Activity staining of this extract revealed two active bands (Fig. 4b, lane 8), suggesting that strain 64G3 produced at least two amylases. In this study, most amylase activity was found to be inside the cell or associated with the "toga", i.e. the outer sheath of the cell. The culture medium, concentrated 20 fold by ultrafiltration (10,000 molecular weight cut-off Durapore membrane), showed no amylase activity. A study on amylase activity of *Thermotoga maritima* revealed that less than 1% of the amylase activity was detected in growth medium; most activity (85%) was found associated in the "toga" and the remaining activity was distributed inside cells (Schumann *et al.*, 1991).

Cloning and sequence analysis of the *amyA* gene: We used the six alternative combinations of primers listed in table 1 for PCR amplification of DNA fragment encoding GHF-13 enzymes. Use of GA2f and GA5r as forward and reverse primer, respectively, resulted in amplification of a single DNA band of about 420 bp. A 210 bp fragment of the PCR product was labelled and used to screen a genomic library, resulting in the identification of one positive clone. The sequence of the insert (approximately 2,800 bp) was analyzed, revealing one complete ORF of

1,992 bp encoding a polypeptide of 663 amino acid residues (calculated molecular mass of 77,042 Da) flanked by two other incomplete ORFs. The deduced sequence was subjected to BLASTp analysis. The matches with the highest sequence identities were found to a glycosidase (45%) of an uncultured thermotogales strain (GenBank accession number CAJ75719), a cyclomaltodextrinase (41%) of *Laceyella sacchari* (AAX29990) and an amylase (40%) of *Kosmotoga olearia* (ACR80687). The complete ORF also contains sequences coding for four conserved regions that are common in GHF-13 enzymes. The complete ORF and deduced amino acid sequence is presented in Fig. 3.

Fig. 3: The complete nucleotide sequence of *P. mexicana* 64G3 α-amylase gene and its deduced amino acid sequence

Fig. 2: Effect of temperature on the amylolytic activity of the strain 64G3 extract

Three catalytic residues Asp417, Glu446 and Asp511 corresponding to Asp206, Glu230 and Asp297 of Taka amylase A (Oyama *et al.*, 1996) were contained in conserved regions 2, 3 and 4, respectively (Fig. 3). A potential ribosome binding site (RBS), 5'-AGGGGG-3', is located nine basepairs upstream of the translational initiation codon, ATG. To identify a putative signal peptide, the sequence of the first 50 amino acid residues was processed by Signal P 3.0. The result indicated a putative signal sequence of 25 residues in length with cleavage between Leu25 and Tyr26. The 60 and 15 bp non-coding regions upstream and downstream the ORF of the *amyA* gene contained no potential promoters or transcription terminator motifs. Thus, the *amyA* gene and the two adjacent ORFs maybe located within one operon (all three ORF have the same polarity). During the characterization of the recombinant *amyA*, the sequence of a complete DNA genome of *Petrotoga mobilis* JS95 was deposited (YP_001568457). A very high identity of 94% between deduced *amyA* of the isolate 64G3 with *P. mobilis* SJ95 α-amylase was observed, reflecting a close phylogenetic relationship between the two species.

Gene expression and purification of r*amyA*: Three hours after induction of Rosetta/*amyA* culture with 1 mM IPTG, the supernatant and debris fractions from the host cell lysate were subjected to SDS-PAGE analysis. The results showed the appearance of an approximately 77 kDa protein band (Fig. 4a, lane 2) which was absent from the uninduced culture (Fig. 4a, lane 1). Identification of this protein band as r*amyA* was confirmed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS). The measurement detected a 77,345 Da protein, matching the deduced sequence of the *amyA*. This indicated that r*amyA* was produced in *E. coli*. However, most of the enzyme

(estimated to be about 90%) aggregated into the debris fraction (known as inclusion body) (Fig. 4a, lane 3). The r*amyA* in both supernatant and debris fractions was purified to homogeneity as a single protein band with mobility corresponding to a molecular mass of about 77 kDa (estimated on an SDS gel, Fig. 4a). The purified r*amyA* in the supernatant fraction showed specific activity of 500 U mg^{-1} towards starch, a value higher than that of purified r*amyA* after refolding from the debris fraction (180 U mg^{-1}). This meant that r*amyA* was actively refolded *in vitro* but optimal folding conditions were required to obtain full enzymatic activity. The mass ratio of r*amyA* purified from the supernatant to debris fractions was 9:91. The total yield of purified r*amyA* was 14.3, 100 mg^{-1} soluble cellular protein produced in the host. Active staining of the purified r*amyA* from both fractions revealed similar active bands in regards to molecular mass (Fig. 4b). In comparison, the active band of r*amyA* was distinct from those of the other two native amylases of the 64G3 cell extract (Fig. 4b). This implied that r*amyA* and the other two unidentified amylolytic enzymes in the 64G3 extract are different enzymes. In this study, one of the GHF-13 enzymes of 64G3 strain was heterologous produced in *E. coli*.

Effects of temperature and pH on activity: The temperature optimum of r*amyA* was studied within the range 30-65°C in 50 mM sodium phosphate buffer pH 6.5. The enzyme was active at temperatures between 30 -55°C, with an optimum at 45°C (Fig. 5a). Activity rapidly declined at 55°C and no activity was detected at 60°C. Thermostability results showed that the enzyme was relatively stable at 55°C, with 80% of its activity remaining after 1 h of treatment. Incubation of the enzyme at 60°C for 30 min caused the loss about 45% of its activity. The enzyme was completely inactive after being treated at 65°C for 15 min. However, the presence of 10 mM Ca^{2+} in the enzyme solution could regain 36.1% of activity. The optimal temperature for r*amyA* was 45, 30°C below that of starch hydrolysis by strain 64G3 extract (maximal activity at 75°C, Fig. 2). This result is ascribed to the activation of other extremely thermophilic amylase(s) in the crude extract. The detection of amylolytic activity which was optimal under extreme thermophilic conditions proves that members of *Petrotoga* could be the source of thermostable enzymes. Multiple amylases which differed remarkably in optimum temperatures were also observed in a related species, *T. maritima*, which produced amylase B (Lim *et al.*, 2003) and C (Ballschmiter *et al.*, 2006) with maximum activities at 70 and 90°C, respectively. The effect of pH on the enzyme activity was studied in 50 mM sodium phosphate buffer (pH 5.5-8.0) and in
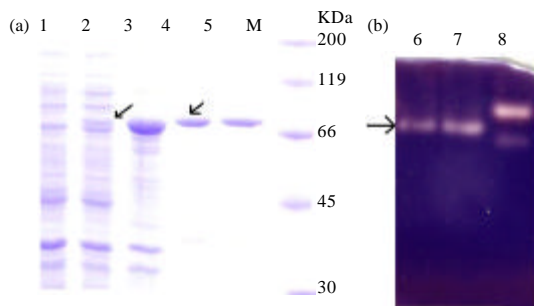

Fig. 4(a-b): (a) SDS-PAGE of the proteins after the expression and purification of r*amyA* and (b) Activity staining of r*amyA*
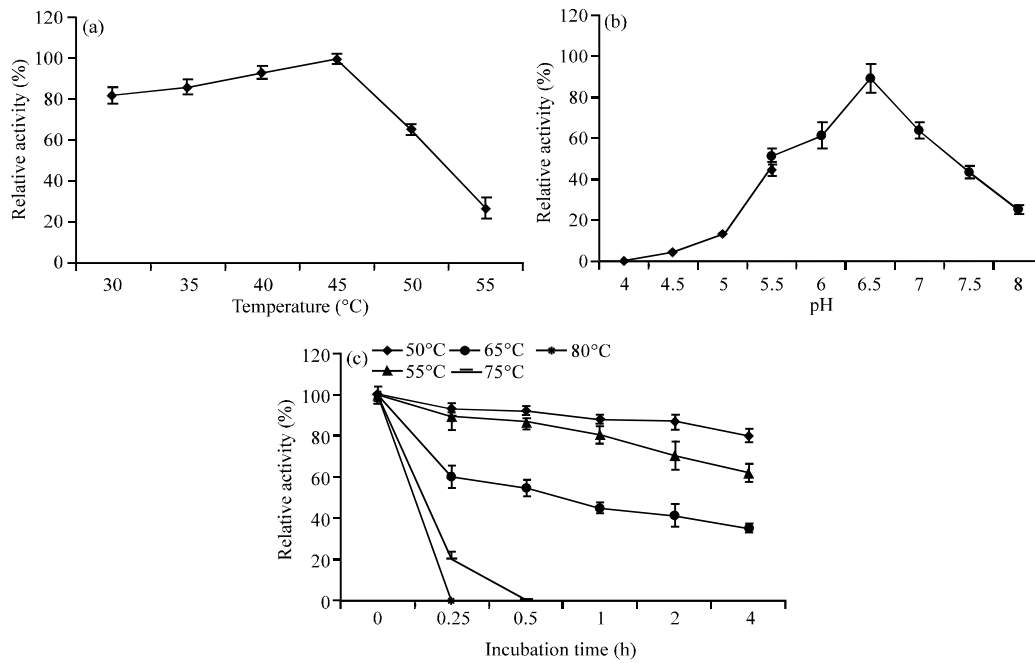
Fig. 5(a-c): Biochemical characteristics of r*amyA* determined by starch hydrolysis

50 mM acetate buffer (pH 3.0 -5.5). The enzyme functioned over a pH range from 4.5-8.0, with an optimum at pH 6.5 (Fig. 5b). Its activity rapidly decreased at pH below 5.0 and pH over 8.0.

Effect of different divalent ions on the r*amyA* activity: The enzyme activity was measured in presence of various divalent ions at a final concentration of 1 mM in the reaction solution. Of all the metal ions tested, Cu^{2+} and Ni^{2+} were remarkable because they inhibited the enzyme activity by 76.4 and 52.9%, respectively (Table 2). Ca^{2+}, Mn^{2+}, Fe^{2+} and Co^{2+} showed stimulating effects of 19.6, 23.5, 37.2 and 58.8%, respectively. Addition of Mg^{2+} and Zn^{2+} had no effect on the activity. Unlike other metal-sensitive amylases of several bacteria such as *P. furiosus* (Landerman *et al.*, 1993), *T. maritima* (Liebl *et al.*, 1997), *T. neapolitana* (Park *et al.*, 2010) and *P. woesei* (Frillingos *et al.*, 2000), r*amyA* was not inhibited by most of the ions tested (except for Ni^{2+} and Cu^{2+}). Treatment of the enzyme with 5 mM or 20 mM EDTA, an inhibitor of metallo-enzymes, caused a similar loss of 58.8%. However, the presence of 10 mM Ca^{2+} or Mg^{2+} in the EDTA treated enzyme reaction retained 85 and 62% of activity, respectively. This demonstrated that r*amyA* is a metal ion(s) dependent amylase, like most other known bacterial amylases.

Substrate specificity and mode of starch degradation: The hydrolysis of various substrates by the enzyme is shown

Table 2: Effect of metal ions on r*amyA* activity

Metal ions	Relative activity (%)
None	100.0
Ca^{2+}	119.6
Mg^{2+}	102.1
Fe^{2+}	137.2
Mn^{2+}	123.5
Co^{2+}	158.8
Zn^{2+}	100.0
Ni^{2+}	47.1
Cu^{2+}	24.6

Table 3: Substrate specificity of r*amyA*

Substrate	Relative activity (%)
Starch	100
Amylopectin	110
Amylose	74
Glycogen	30
Pullulan	1
α-Cyclodextrin	0
β-Cyclodextrin	0
γ-Cyclodextrin	0

in Table 3. The enzyme hydrolyzed soluble starch (Fig. 6) to produce maltose as the major end-product, maltotriose and glucose to a lesser extent. Maltotetraose and longer oligosaccharides were not recognized on the TLC plate. The hydrolysis pattern of r*amyA* was quite different from those of other typical α-amylases, such as amylase from *B. licheniformis* and *P. furiosus* which hydrolyzed starch in an endo-acting fashion to release oligosaccharides of different degrees of polymerization (Dong *et al.*, 1997). Additionally, the enzyme formed final products differing from those of starch hydrolysis by maltogenic amylases
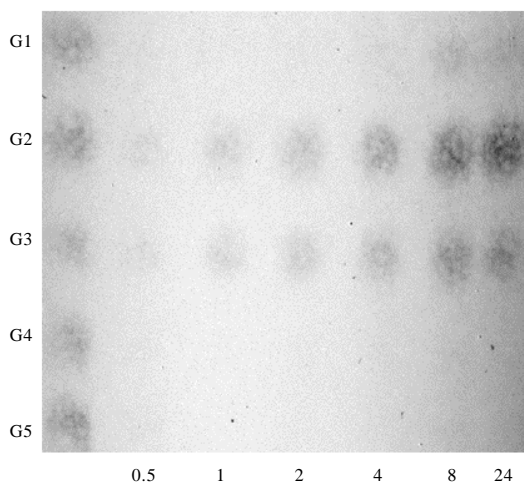
Fig. 6: Hydrolysis patterns on soluble starch, analyzed by TLC

(EC.3.2.1.133) and maltotriose producing amylases which produce maltose and maltotriose as the major product, respectively. Unlike maltogenic amylases (Cho *et al.*, 2000), *ramyA* did not hydrolyze pullulan and cyclodextrins, whereas most maltogenic amylases could. Other oligosaccharides from maltotriose to maltoheptaose were hydrolyzed by *ramyA* to form shorter oligosaccharides (data not shown). The hydrolysis pattern of *ramyA* was similar to that of the related *T. neapolitana* exo-amylase in that both enzymes hydrolyzed maltotriose and formed maltose as the major product of starch hydrolysis. However, *T. neapolitana* amylase did not produce maltotriose during starch hydrolysis. Pullulan and cyclodextrin were also poor substrates for both enzymes. α- Amylase of *A. awamori* KT-11 degraded starch almost in the same manner as that of *ramyA* to produce maltose, maltotriose as the major products and a less amount of glucose (Matsubara *et al.*, 2004). The *ramyA* did not cleave maltose or pNPG (p-nitrophenyl-α-D- glucopyranoside), substrates of α-glucosidase and glucoamylase. Glucose released during hydrolysis of starch by *ramyA* was due to the subsequent cleavage of maltotriose. On the basis of the primary structure, the substrate specificities and the hydrolysis pattern, *ramyA* was classified as an endo-acting α-amylase (EC. 3.2.1.1).

CONCLUSION

In conclusion, we have isolated and classified our strain 64G3 as *Petrotoga mexicana* species. This strain

produces starch degrading enzymes and one gene encoding α-amylase was cloned and functionally overexpressed in *E. coli*. The enzyme attacks starch and other related polysaccharides in a rare manner that is different from most α-amylases, by releasing maltose and maltotriose but no larger sugars during hydrolysis. The nucleotide and deduced amino acid sequences were deposited in the GenBank database under the accession number DQ985807.

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