Cloning, sequencing and expression of an α -amylase gene, *amyA*, from the thermophilic halophile *Halothermothrix orenii* and purification and biochemical characterization of the recombinant enzyme

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A recombinant clone expressing an amylase was identified from an *Escherichia coli* generated genomic library of the thermophilic, moderately halophilic, anaerobic bacterium *Halothermothrix orenii* by activity screening, and the gene encoding the enzyme was designated AmyA. The *amyA* gene was 1545 bp long, and encoded a 515 residue protein composed of a 25 amino acid putative signal peptide and a 490 amino acid mature protein. It possessed the five consensus regions characteristic of the α -amylase family and showed the greatest homology to the *Bacillus megaterium* group of α -amylases. The *amyA* gene was expressed in *E. coli* as a hexahistidine-tagged enzyme and purified. The purified recombinant enzyme was optimally active at 65 °C in 5% (w/v) NaCl at pH 7.5, with significant activity retained in the presence of up to 25% (w/v) NaCl. It had a specific activity of 22.32 U mg⁻¹ and required NaCl and CaCl₂ for optimum activity and thermostability. The relatively high proportion of acidic amino acids typically observed for many enzymes from halophiles was absent in *H. orenii* AmyA.

Keywords: alpha-amylase, halophile, thermophile, anaerobe, *Halothermothrix orenii*

INTRODUCTION

Considerable focus has been placed on starch-degrading enzymes from thermophilic and hyperthermophilic prokaryotes as they are likely to produce enzymes with activity and stability characteristics suitable for high-temperature industrial processes required by the starch industry. A great deal of effort has also been invested in finding the basis of their thermostability for enzyme engineering. However, little attention has been given to starch-degrading enzymes from other physiological groups of extremophiles such as halophiles and alkaliphiles. Studies on enzymes from such organisms would not only yield suitable enzymes for catalysing starch hydrolysis under unusual conditions but could also, through comparative analysis with the large number of enzyme amino acid sequences already available in

databases, yield insights into the specific structural properties required for activity under these conditions. Considering the industrial potential of enzymes from halophiles there has been only limited research in this area (Grant *et al.*, 1998). The amylases from the mesophilic extremely halophilic *Archaea*, namely *Halobacterium halobium* (Good & Hartman, 1970) and *Natronococcus amylolyticus* (Kobayashi *et al.*, 1992) and the moderately halophilic mesophilic aerobic bacterium *Halomonas meridiana* (Coronado *et al.*, 2000a) have markedly different enzymic properties and range from being halotolerant enzymes, which are relatively stable and active at low salt concentrations, to extremely halophilic ones, which are inactivated at low salt concentrations. Studies have shown that the enzymes from *Halomonas meridiana* (Coronado *et al.*, 2000b) and *N. amylolyticus* (Kobayashi *et al.*, 1994), have significantly elevated levels of acidic amino acids but this characteristic is much more prevalent in the more halophilic *N. amylolyticus* amylase. In general, the amylase enzymes from mesophilic halophilic organisms may be of limited use in industrial starch-degrading

Abbreviation: DNS, dinitrosalicylic acid.

The GenBank accession number for the sequence reported in this paper is AF442961.

applications as they are not active or stable at high temperatures (above 60 °C). Jana *et al.* (1997) described an enzyme from *Bacillus megaterium* VUMB-109 that was extremely thermostable and halotolerant but the enzyme was not purified or the gene cloned and hence its structure–function characteristics have yet to be elucidated.

Halothermothrix orenii (Cayol *et al.*, 1994) and *Thermohalobacter barriensis* (Cayol *et al.*, 2000) are the only truly thermophilic halophilic prokaryotes that have been reported to date. Here, we report on an α -amylase cloned from *H. orenii*. *H. orenii* is an anaerobic bacterium that is thermophilic (optimum growth at 60 °C) and also a moderate halophile (optimum growth at 10 %, w/v, NaCl) (Cayol *et al.*, 1994). It may therefore be a potential source for enzymes that are uniquely adapted to activity at high temperatures and salt concentrations. We have isolated a gene encoding an α -amylase from a *Halothermothrix orenii* genomic DNA library constructed in *Escherichia coli*. The enzyme was expressed with an N-terminal hexahistidine tag, purified and biochemically characterized. To our knowledge this is the first report of cloning or characterization of an extracellular enzyme from an anaerobic, moderately halophilic, thermophilic bacterium.

METHODS

Bacterial strains and plasmids. *Halothermothrix orenii* was obtained from Dr Bernard Ollivier (IRD, Université de Provence). *Escherichia coli* strain DH10B [F^- *mcrA* Δ (*mrr*–*hsdRMS*–*mcrBC*) ϕ 80*dlacZ* Δ M15 Δ *lacX74* *endA1* *recA1* *deoR* Δ (*ara*–*leu*)7697 *araD139* *galU* *galK* *nupG* *rpsL* λ^-] was used for construction of the genomic library in pBluescriptSK+ vector (Stratagene). *E. coli* strain TOP10 [F^- *mcrA* Δ (*mrr*–*hsdRMS*–*mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *endA1* *recA1* *deoR* Δ (*ara*–*leu*)7697 *araD139* *galU* *galK* *nupG* *rpsL*] was used as host for pTrcHis vectors (Invitrogen) in expression experiments.

Screening of *H. orenii* genomic DNA library for starch-degrading activity. Culturing of *H. orenii* and genomic DNA library construction were as described previously (Mijts & Patel, 2001). The *H. orenii* genomic library, which had a mean insert size of 3.5 kb and had been stored in 96-well microtitre plates, was replica-plated on to LB agar plates containing 0.3 % (w/v) soluble starch and 100 μ g ampicillin ml⁻¹ using a 48-colony arrayer, grown at 37 °C for 18 h, sealed in plastic bags and transferred to a 65 °C incubator overnight. Starch-hydrolysing clones were detected by flooding plates with 0.2 % (w/v) KI/0.1 % (w/v) I₂ solution and checking for halo formation.

Sequencing and identification of the *amyA* gene of *H. orenii*. The complete double-stranded nucleotide sequence of the starch-degrading recombinant insert was determined using a primer-walking strategy with an ABI 377 automated DNA sequencer (ABI-Perkin Elmer) at the Griffith University Molecular Biology Facility. DNA sequencing reactions were performed as described previously (Mijts & Patel, 2001). Putative genes were identified using GeneMark software (Besemer & Borodovsky, 1999) and function assigned by BLAST searches (Altschul *et al.*, 1990) against the GenBank nucleotide database (Benson *et al.*, 2000).

Construction of an *amyA* expression vector. PCR amplifica-

tion of the *amyA* coding sequence from the putative signal peptide cleavage site to a region downstream of the stop codon was carried out using the oligonucleotides F2-*Pst*I (5'-TATCT-GTTTTCTCTGTTTCTGCAGACGATTTTCGAAAAAC) and R1-*Kpn*I (5'-GCCTCGTGGAGGGTACCTATGACCTTG) with bases from the template sequence modified in order to generate restriction sites indicated as underlined bases. This PCR product was digested with *Kpn*I followed by *Pst*I, gel-purified, ligated with similarly treated pTrcHisB expression vector (Invitrogen) and transformed into electro-competent cells of *E. coli* strain TOP10 (Invitrogen). A number of clones were sequenced using the primer-walking strategy described above and a clone with the correct sequence was used for all further expression and characterization experiments.

Expression and purification of recombinant AmyA. Cultures (50 ml) of *E. coli* TOP10 cells containing a pTrcHis expression construct were grown overnight at 37 °C in LB Amp medium with and without 1 mM IPTG and harvested by centrifugation (5000 g for 10 min). Cells were resuspended in 5 ml native binding buffer (20 mM phosphate, 500 mM NaCl, pH 7.8), lysozyme was added to a final concentration of 100 μ g ml⁻¹ and the sample was incubated on ice for 15 min. The cell suspension was then sonicated at maximum intensity for three 10 s bursts while kept on ice. Final cell lysis was achieved by three rapid freeze/thaw cycles in liquid nitrogen and a 50 °C water bath. Any insoluble material was removed by centrifugation at 4000 g for 15 min. Heat precipitation (68 °C for 30 min) of the majority of host proteins in the presence of 0.5 M NaCl was used as a technique to partially purify AmyA expressed in *E. coli*. The protein preparation was then transferred to ice for 15 min to maximize *E. coli* protein precipitation and host proteins pelleted by centrifugation at 10000 g for 10 min; the supernatant was retained and stored at –20 °C. The partially purified, polyhistidine-tagged recombinant enzyme was purified using ProBond columns (Invitrogen) according to the manufacturer's instructions. Enzyme elution was performed using the imidazole elution protocol under native conditions.

Characterization of the recombinant amylase. Unless otherwise indicated, the following standard assay was used. Predetermined units of AmyA were added to 50 μ l Tris buffer pH 8.0, 5 % (w/v) NaCl, 10 mM CaCl₂ and 0.5 % (w/v) starch in microtitre plates, which were incubated at 65 °C for 30 min in a Bio-Rad iCycler thermal cycler; the amount of starch hydrolysed was determined by using the starch–iodine assay and/or the sugar released by using the DNS reducing sugar assay (described below). The substrate spectrum that could be used by the enzyme was determined by replacing starch with amylose, amylopectin, pullulan, glycogen and α -, β - and γ -cyclodextrin. The pH optimum for enzyme was determined using the standard assay but with the following pH buffers (100 mM): Bistris/HCl (pH 5.5–7.0), Tris/HCl (pH 7.5–8.5) and glycine/NaOH (pH 8.5–10.5). The NaCl optimum for activity was determined using the standard assay described above but with NaCl at final concentrations up to 25 % (w/v). Temperature optimum was determined using the standard assay in which the buffer was substituted with 100 mM Bistris/HCl pH 7.0 and the incubation temperatures ranged from 37 to 80 °C. The effect of NaCl and CaCl₂ on enzyme thermostability was determined by preincubating enzyme solutions in 200 mM Tris buffer pH 8.0 at 70 °C and removing samples at 0, 60 and 120 min. Pre-incubation samples were set up at 0 % (w/v) NaCl, 0 mM CaCl₂; 10 % (w/v) NaCl, 0 mM CaCl₂; 0 % (w/v) NaCl, 10 mM CaCl₂; and 10 % (w/v) NaCl, 10 mM CaCl₂. Buffered starch/saline solution was then added

Fig. 1. Amino acid alignment of *H. orenii* AmyA and high-matching sequences. HOAMYA, *H. orenii* amylase A; BMAMY, *Bacillus megaterium* α -amylase (Metz *et al.*, 1988); PPPUL, *Paenibacillus polymyxa* pullulanase (Yebra *et al.*, 1999); DTAMY3, *Dictyoglomus thermophilum* α -amylase C (Horinouchi *et al.*, 1988); XCAMY, *Xanthomonas campestris* α -amylase (Abe *et al.*, 1996); TMGLT, *Thermotoga maritima* 4- α -glucanotransferase (Liebl *et al.*, 1992). Signature sequence regions (1-5)

to each sample to re-establish standard assay conditions and activity determined by reducing sugar assays after incubation at 65 °C for 30 min.

Enzyme digest time-course. A starch digest time-course was performed by adding a suitable amount of enzyme to a 1500 μ l solution [100 mM Tris pH 8.0, 5% (w/v) NaCl, 8 mM CaCl₂, 0.5% (w/v) soluble starch], incubating at 65 °C and removing 150 μ l aliquots for up to 480 min for DNS reducing sugar assays (described below) and starch-iodine assays. Reducing sugar production was calculated as a percentage of total starch conversion to sugars assuming maltose as an end product.

Transferase activity assay. Transferase activity assays were adapted from a method developed to detect transferase activity in *B. megaterium* α -amylase (Brumm *et al.*, 1996). Starch hydrolysis activity was measured using starch-iodine assays under standard reaction conditions both with and without the addition of various mono-, di- and trisaccharides (fructose, galactose, glucose, maltose, lactose, sucrose, cellobiose, isomaltose, maltitol and maltotriose) at 0.1 M concentration. These carbohydrates are possible acceptor molecules in the transferase reaction and any transferase activity would increase the observed rate of starch hydrolysis.

Reducing sugar assay. DNS assay reagent [50 μ l; 1% (w/v) 1,3-dinitrosalicylic acid, 0.05% (w/v) sodium sulfite, 1% (w/v) sodium hydroxide, 10% (w/v) sodium potassium tartrate] was added to enzyme digests and the resulting samples were incubated at 98 °C for 10 min. Reducing sugar levels were then measured as A₄₉₀ using a Wallac Victor 1420 Multilabel Counter. A series of maltose concentration standards was included with each reducing sugar assay. For experiments that required simultaneous incubation at various temperatures, reactions were performed in 0.2 ml microfuge tubes incubated in water baths at suitable temperatures. Reducing sugar levels were then determined as above.

Starch-iodine assays. Remnant starch was quantitatively determined by measuring the A₆₅₀ of starch-iodine complexes in solution. An iodine stock solution consisting of 0.3% (w/v) I₂, 0.6% (w/v) KI was diluted 1/1000 in 17 mM acetic acid and 950 μ l of this solution added to 50 μ l of sample to be tested. Samples were mixed by vortexing and the A₆₅₀ measured.

RESULTS

Cloning and sequencing of the pSK5A6 insert

A *H. orenii* genomic library with a total of 3360 recombinants with a mean insert size of 3.5 kb was constructed and screened for thermostable starch-degrading activity. A halo-producing recombinant colony was isolated and the plasmid designated pSK5A6. This recombinant produced a relatively indistinct halo compared to other starch-degrading recombinants but was selected for further analysis as the assay conditions may have been suboptimal for enzyme activity. The double-

conserved throughout the α -amylase enzyme family as identified by Nakajima *et al.* (1986) are indicated. Shaded areas indicate regions of high amino acid similarity; black regions indicate regions of 100% similarity.

Table 1. Purification of AmyA

Protein concentration was determined using Bradford assays. Activity was determined using DNS reducing sugar assays under standard assay conditions. One unit of activity was defined as the amount of enzyme that liberates 1 μmol reducing sugar groups (measured as maltose equivalents) min^{-1} .

Step	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Purification factor (-fold)	Recovery (%)
Cell lysate	6.12	28	4.58	1	100
Heat precipitation	1.1	21.3	19.36	4.22	76
Ni-affinity chromatography	0.925	20.65	22.32	4.87	73

stranded nucleotide sequence of the genomic DNA insert of pSK5A6 was determined to be 2894 nucleotides.

Nucleotide sequence analysis of the *amyA* gene

Analysis of the insert DNA sequence with WebGeneMark heuristic approach software (Besemer & Borodovsky, 1999) indicated that the most probable translated open reading frame was from nucleotides 432 to 1977, beginning with a GTG start codon and preceded with a spacing of 8 nucleotides by a putative ribosome-binding site (5'-AAGGATG-3'). BLAST analysis indicated that this gene was homologous to bacterial α -amylase genes and it was designated *amyA*. Although other in-frame ATG and GTG start codons are present in this region these codons lack conserved ribosome-binding sites. A region that is extremely close to the bacterial promoter consensus sequence can be found upstream of the *amyA* gene. This sequence (TTGAAA-N₁₇-TATAAT) begins at nucleotide 376 and differs from the consensus sequence by only one nucleotide.

Amino acid sequence analysis and comparison

The first 70 amino acid residues of the predicted ORF were analysed using SignalP version 2.0 signal peptide prediction software (Nielsen *et al.*, 1997) trained on Gram-negative bacterial secretion signal peptide sequences. The results of this analysis indicated that a well-conserved Gram-negative signal peptide 25 amino acids in length was present. Assuming the signal peptide sequence is cleaved as predicted (VYA-NDF) the resulting polypeptide would be 490 amino acids in length with a molecular mass of 56 965 kDa.

The derived amino acid sequence of *amyA* was used to perform a homology search using BLASTP software against the GenBank non-redundant database (Altschul *et al.*, 1990). The results of this search indicated that AmyA showed high homology to a group of five enzymes: α -amylase from *Bacillus megaterium* (Metz *et al.*, 1988), neopullulanase from *Paenibacillus polymyxa* (Yebra *et al.*, 1999), periplasmic α -amylase from *Xanthomonas campestris* K-11151 (Abe *et al.*, 1996) and α -amylase AmyC from *Dictyoglomus thermophilum* (Horinouchi *et al.*, 1988). Lower levels of homology

were also observed for other α -amylases, glucanotransferases and trehalose synthases from thermophilic bacteria.

The amino acid alignment of *H. orenii* AmyA with the five high-matching amino acid sequences of this group of enzymes, and showing the consensus regions commonly found in α -amylases (Nakajima *et al.*, 1986) and also identified in the AmyA amino acid sequence, is presented in Fig. 1. The members of this group share a number of unusual catalytic properties: (i) they are capable of hydrolysing cyclodextrins to varying degrees, despite lacking sequence homology to the pullulanase family, (ii) various degrees of transglycosylating activity are present in each of these enzymes, and (iii) some of the members catalyse the hydrolysis of pullulan to panose. Despite these curious properties, most of these enzymes have only been partially characterized, and in some cases the characterization was from crude protein extracts rather than from purified proteins.

Construction of expression vector and purification of recombinant AmyA

Due to the low level of expression of AmyA observed for plasmid pSK5A6 in *E. coli* an expression vector was constructed to allow purification of the enzyme for further biochemical characterization. A hexahistidine-tagged expression construct was generated in pTrcHis vector as described in Methods. The gene was amplified using PCR from a region close to the predicted signal peptide cleavage site to a region downstream from the stop codon. This PCR product was then digested using restriction enzymes whose target sites had been introduced into the PCR primer sequences and subcloned into similarly digested pTrcHisB vector. A number of clones found to possess thermostable starch-degrading activity were sequenced in order to identify possible PCR errors, and a single recombinant with an insert sequence identical to that of the genomic clone was designated pTH5A6 and used for protein expression, purification and characterization.

The recombinant AmyA protein was purified from *E. coli* TOP10(pTH5A6) as described in Methods. The purification procedure is summarized in Table 1. The recombinant enzyme was judged homogeneous on

Fig. 2. SDS-PAGE gel of AmyA purification steps. Lanes: A, non-IPTG-induced cell lysate; B, IPTG-induced cell lysate; C, heat-precipitated sample; D, Ni-affinity chromatography sample; Std, Bio-Rad low-molecular-mass protein standards.

Fig. 3. Hydrolysis of starch (Δ) and the concomitant production of reducing sugars ($\bullet$) by *H. orenii* AmyA over time. Reducing sugars were measured by the DNS assay and expressed as a percentage: 100% reducing sugar corresponds to the theoretical conversion of all starch to maltose. Starch hydrolysis was measured by the starch-iodine assay; undigested starch in the control samples represents 100%.

the basis of SDS-PAGE (Fig. 2). Very high levels of recombinant AmyA expression were observed in IPTG-induced cultures of TOP10(pTH5A6). Fig. 2 clearly indicates the high levels of AmyA expression observed in IPTG-induced cultures compared with non-induced controls. It is also clear that the majority of *E. coli* host proteins were removed in the heat-precipitation step. However, the additional step of hexahistidine tag affinity chromatography, though not necessary, was performed to ensure that absolute purity of the recombinant enzyme preparation was achieved.

Enzymic properties of AmyA

CaCl_2 was required for optimal AmyA starch hydrolysis activity. The pH optimum for AmyA activity was found to be 7.5; activity was detected between pH 6 and 9.5. AmyA was active at temperatures between 37 °C and 75 °C and was optimally active at 65 °C, which is consistent with the optimum growth temperature of the native organism of around 60 °C. The enzyme was shown to be halophilic: it required 5% (w/v) NaCl for optimum activity although it retained 45% activity even when no NaCl was present in the reaction. It maintained remarkably high levels of activity even at very high salt concentrations and was 90% active at 25% (w/v) NaCl. A starch digestion time-course (Fig. 3) indicated that AmyA rapidly reduced starch iodine staining capacity and rapidly generated reducing sugar ends, which is consistent with the sequence-based classification of this enzyme as an endo-acting α -amylase.

Starch, and its constituent polysaccharides amylose and amylopectin, were hydrolysed readily by AmyA. The relative activities on each substrate were as follows: soluble starch 100%, amylose 97% and amylopectin 52%. No significant activity was observed on pullulan, glycogen and α -, β - and γ -cyclodextrins. This activity profile indicates activity only on α -1,4 glycosidic linkages. This is consistent with the sequence-based classification of this enzyme as an α -amylase.

The purified AmyA was found to be stabilized at 70 °C by both NaCl and CaCl_2 (Table 2). In the absence of both NaCl and CaCl_2 , activity was rapidly lost by

Table 2. Effect of NaCl and CaCl_2 on the thermostability of AmyA

Activity was determined by DNS reducing sugar assays under standard assay conditions after pre-incubation in the solutions described at 70 °C for 60 and 120 min.

Pre-incubation conditions	Relative activity (%) after pre-incubation at 70 °C	
	60 min	120 min
0% NaCl, 0 mM CaCl_2	3.3	3.8
10% NaCl, 0 mM CaCl_2	29.8	10.6
0% NaCl, 10 mM CaCl_2	74.2	24.4
10% NaCl, 10 mM CaCl_2	81.4	42.2

incubation at 70 °C. Individually, 10% NaCl and 10 mM CaCl₂ each improved the thermostability of the enzyme and optimal thermostability was observed when both 10% NaCl and 10 mM CaCl₂ were present. AmyA was also found to be more stable in the presence of starch substrate (results not shown).

No increase in hydrolytic activity was detected when various di- and monosaccharides were included in starch digestion reactions, indicating that enzyme lacked transferase activity, as observed for the *B. megaterium* amylase. However, strong inhibition of enzyme activity was observed when both maltose (90.9% inhibition) and maltotriose (98.8% inhibition) were included in starch digestion reactions. This is presumably the result of product inhibition and was also observed for *B. megaterium* amylase.

DISCUSSION

The specific activity of purified recombinant AmyA (22.32 U mg⁻¹) is relatively poor compared to those of most bacterial α -amylases, which typically range between 14 and 1700 U mg⁻¹, although specific activities as high as 3800 U mg⁻¹ have been observed (Liebl *et al.*, 1997). Comparison of this specific activity to closely related amylases is difficult as most have been only partially purified or characterized poorly but the specific activity of purified *X. campestris* periplasmic α -amylase was significantly higher at around 250 U mg⁻¹. The relatively poor specific activity of recombinant AmyA is consistent with the indistinct zone of clearance observed for this enzyme during activity screening of the *H. orenii* genomic DNA library.

Some enzymes with high amino acid homology to AmyA are able to hydrolyse unusual substrates such as pullulan and cyclodextrins to varying degrees. These activities are not present in AmyA, with activity being observed only on starch, amylose, amylopectin and glycogen. Also lacking in *H. orenii* AmyA was any transferase activity as detected in the closely related *B. megaterium* α -amylase (Brumm *et al.*, 1996). No increase in AmyA starch hydrolysis reaction rates was observed in the presence of a variety of potential transferase acceptor saccharides. However, as for *B. megaterium* amylase, strong inhibition of starch-degrading activity by maltose and maltotriose was observed for *H. orenii* AmyA. The lack of transferase activity and activity on unusual substrates is less surprising considering the already considerable catalytic diversity observed within this relatively small cluster of enzymes. It is clear that relatively minor changes in important residues of these enzymes result in significant alterations in catalytic diversity.

Optimal activity for *H. orenii* AmyA was found to occur in conditions similar to those at which the source organism grows optimally in culture. The NaCl optimum for growth of *H. orenii* is around 10% (Cayol *et al.*, 1994) and AmyA retained high levels of activity under these conditions. Although not as active or stable

in the absence of NaCl, AmyA was still 44% active when NaCl was not present. Additionally, over 90% activity was observed at the remarkably high salt concentration of 25%. This type of extreme halotolerance has been observed in extracellular amylases from other halophilic organisms including the moderate halophiles *Micrococcus halobius* (Onishi & Sonoda, 1979) and *Halomonas meridiana* (Coronado *et al.*, 2000a) and the extreme halophile *Halobacterium halobium* (Good & Hartman, 1970). However, most enzymes from extreme halophilic *Archaea*, such as amylase from *Natronococcus* sp. strain Ah36, are completely unstable and inactive at submolar salt concentrations. AmyA was found to have an optimum temperature for starch hydrolysis of 65 °C. This is very close to the optimum growth temperature of *Halothermothrix orenii* of around 60 °C. AmyA was found to be relatively thermostable in the absence of starch when both CaCl₂ and NaCl are present. CaCl₂ binding has been reported to increase the overall structural integrity and thermal stability of α -amylases (Violet & Meunier, 1989). AmyA had a pH optimum for activity of around pH 7.5. This is consistent with the optimum pH for growth of the source organism of around pH 7.

As with a previous report on randomly selected sequence tags generated from the *H. orenii* genomic library (Mijts & Patel, 2001), *H. orenii* AmyA also shows no significant excess of acidic amino acids. A significant acidic amino acid excess is a biochemical trait thought to be necessary for activity and stability of enzymes from some halophilic organisms. It is found in most enzymes from extreme halophiles of domain *Archaea* and in extracellular enzymes from moderate halophiles of domain *Bacteria* (Coronado *et al.*, 2000b). The extreme halotolerance of *H. orenii* AmyA suggests that this characteristic is not strictly necessary to maintain enzyme activity and stability at high salt concentrations.

REFERENCES

- Abe, J., Shibata, Y., Fujisue, M. & Hizukuri, S. (1996). Expression of periplasmic α -amylase of *Xanthomonas campestris* K-11151 in *Escherichia coli* and its action on maltose. *Microbiology* **142**, 1505–1512.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., Rapp, B. A. & Wheeler, D. L. (2000). GenBank. *Nucleic Acids Res* **28**, 15–18.
- Besemer, J. & Borodovsky, M. (1999). Heuristic approach to deriving models for gene finding. *Nucleic Acids Res* **27**, 3911–3920.
- Brumm, P. J., Hebeda, R. E. & Teague, W. M. (1996). Purification and characterisation of the commercialized, cloned *Bacillus megaterium* α -amylase. Part II: transferase properties. *Starch/Sterke* **43**, 319–323.
- Cayol, J.-L., Ollivier, B., Patel, B. K. C., Prensier, G., Guezennec, J. & Garcia, J.-L. (1994). Isolation and characterization of *Halothermothrix orenii* gen. nov., sp. nov., a halophilic, thermophilic, fermentative, strictly anaerobic bacterium. *Int J Syst Bacteriol* **44**, 534–540.

- Cayol, J. L., Ducerf, S., Patel, B. K. C., Garcia, J.-L., Thomas, P. & Ollivier, B. (2000). *Thermohalobacter berriensis* gen. nov., sp. nov., a thermophilic, strictly halophilic bacterium from a solar saltern. *Int J Syst Evol Microbiol* **50**, 559–564.
- Coronado, M., Vargas, C., Hofemeister, J., Ventosa, A. & Nieto, J. J. (2000a). Production and biochemical characterization of an α -amylase from the moderate halophile *Halomonas meridiana*. *FEMS Microbiol Lett* **183**, 67–71.
- Coronado, M. J., Vargas, C., Mellado, E., Tegos, G., Drinas, C., Nieto, J. J. & Ventosa, A. (2000b). The α -amylase gene *amyH* of the moderate halophile *Halomonas meridiana*: cloning and molecular characterization. *Microbiology* **146**, 861–868.
- Good, W. A. & Hartman, P. A. (1970). Properties of the amylase from *Halobacterium halobium*. *J Bacteriol* **104**, 601–603.
- Grant, W. D., Gemmell, R. T. & McGenity, T. J. (1998). Halophiles. In *Extremophiles: Microbial Life in Extreme Environments*, pp. 93–132. Edited by K. Horikoshi & W. D. Grant. New York: Wiley.
- Horinouchi, S., Fukisumi, S., Oshima, T. & Beppu, T. (1988). Cloning and expression in *Escherichia coli* of two additional amylases of a strictly anaerobic thermophile, *Dictyoglomus thermophilum*, and their nucleotide sequences with extremely low guanine-plus-cytosine contents. *Eur J Biochem* **176**, 243–253.
- Jana, M., Chattopadhyay, D. J. & Pati, B. R. (1997). Thermostable, high salt-tolerant amylase from *Bacillus megaterium* VUMB-109. *Acta Biochim Biophys Hung* **44**, 281–289.
- Kobayashi, T., Kanai, H., Hayashi, T., Akiba, T., Akaboshi, R. & Horikoshi, K. (1992). Haloalkaliphilic maltotriose-forming α -amylase from the archaeobacterium *Natronococcus* sp. strain Ah-36. *J Bacteriol* **174**, 3439–3444.
- Kobayashi, T., Kanai, H., Aono, R., Horikoshi, K. & Kudo, T. (1994). Cloning, expression, and nucleotide sequence of the α -amylase gene from the haloalkaliphilic archaeon *Natronococcus* sp. strain Ah-36. *J Bacteriol* **176**, 5131–5134.
- Liebl, W., Feil, R., Gabelsberger, J., Kellermann, J. & Schleifer, K.-H. (1992). Purification and characterization of a novel thermostable 4- α -glucanotransferase of *Thermotoga maritima* cloned in *Escherichia coli*. *Eur J Biochem* **207**, 81–88.
- Liebl, W., Stemplinger, I. & Ruile, P. (1997). Properties and gene structure of the *Thermotoga maritima* α -amylase *amyA*, a putative lipoprotein of a hyperthermophilic bacterium. *J Bacteriol* **179**, 941–948.
- Metz, R. J., Allen, L. N., Cao, T. M. & Zeman, N. W. (1988). Nucleotide sequence of an amylase gene from *Bacillus megaterium*. *Nucleic Acids Res* **16**, 5203.
- Mijts, B. N. & Patel, B. K. C. (2001). Random sequence analysis of genomic DNA of an anaerobic, thermophilic, halophilic bacterium, *Halothermothrix orenii*. *Extremophiles* **5**, 61–69.
- Nakajima, R., Imanaka, T. & Aiba, S. (1986). Comparison of amino acid sequences of eleven different α -amylases. *Appl Microbiol Biotechnol* **23**, 355–360.
- Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int J Neural Syst* **8**, 581–599.
- Onishi, H. & Sonoda, K. (1979). Purification and some properties of an extracellular amylase from a moderate halophile, *Micrococcus halobius*. *Appl Environ Microbiol* **38**, 616–620.
- Violet, M. & Meunier, J. C. (1989). Kinetic study of the irreversible thermal denaturation of *Bacillus licheniformis* α -amylase. *Biochem J* **263**, 665–670.
- Yebra, M. J., Blasco, A. & Sanz, P. (1999). Expression and secretion of *Bacillus polymyxa* neopullulanase in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **170**, 41–49.

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