Journal of Molecular Catalysis B: Enzymatic 64 (2010) 129–134

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Expression and characterization of *Bacillus subtilis* PY22 α-amylase in *Pichia pastoris*

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ARTICLE INFO

Article history:
Available online 15 July 2009

Keywords:
Amylase
Bacillus subtilis PY22
Characterization
Overexpression
Pichia pastoris

ABSTRACT

Pichia pastoris is a methanol utilizing yeast which does not naturally produce starch degrading enzymes. In this study, the gene encoding the α-amylase enzyme in *Bacillus subtilis* PY22 was amplified by PCR, sequenced and cloned into *P. pastoris* KM71H host strain using the vector pPICZαA allowing methanol induced expression and secretion of the protein. Recombinant expression resulted in high levels of extracellular amylase production, as high as 22 mg/L in the shake flask culture supernatant. Clones containing various copy numbers of the gene were screened for α-amylase production and two-copy clone was determined to be the best producer at shake flask conditions. The clone capable of the highest production was selected for further study involving the small-scale production and partial purification of the recombinant enzyme. The partially purified enzyme showed the highest activity at 60 °C and pH 7, retaining 78% activity when kept at this temperature and pH for 1 h. The presence of Ca^{2+} ions in the reaction medium resulted in a 41% increase in the amylase activity.

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1. Introduction

Over the past two decades the methylotrophic yeast *Pichia pastoris* has been developed as a commercial expression system for recombinant proteins. It is a common fungal host for the expression of recombinant proteins [1–3]. The tightly controlled and methanol inducible *AOX1* gene, encoding the enzyme responsible for a vast majority of the alcohol oxidase activity in the cell has proven to be a valuable tool in the expression of recombinant proteins in this host organism. Thus, efficient vectors have been designed carrying the *AOX1* promoter region, selectable marker which endows antibiotic resistance and the α-mating factor from *Saccharomyces cerevisiae* which enables signaling of extracellular secretion of the cloned protein.

It is estimated that *Bacillus* sp. enzymes make up about 50% of the total global enzyme market [4]. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquifaciens* are known to be good producers of α-amylase [5]. This enzyme cleaves the α,1-4 glycosidic bonds of starch in an endofashion. The properties of α-amylases such as thermostability and pH profile should match the application. Properties and applications of amylases have recently been reviewed [6,7].

Several studies have been undertaken to express *Bacillus* amylases in *P. pastoris*. *B. licheniformis* α-amylase, which is one of the most thermostable forms of the enzyme, has been expressed in *P. pastoris* [8,9]. Other sources of α-amylase have also been made use such as ripening bananas [10], human pancreatic α-amylase [11] and mouse salivary α-amylase [12]. Chimeric amylase has also been used as a model protein for investigating the effect of methanol feeding methods in the expression in continuous culture [13].

The aim of this study was the isolation and identification of the *amyE* gene from *B. subtilis* PY22 and its expression in *P. pastoris*. The recombinant α-amylase thus produced was also partially purified and characterized.

2. Materials and methods

2.1. Strains, plasmids and culture media

The *B. subtilis* PY22 strain was a generous gift from Dr. Andrew Benson, Food Science and Technology Department, University of Nebraska-Lincoln, Lincoln, NE, USA. *P. pastoris* KM71H and pPicZαA (Invitrogen) were used as host and vector for heterologous expression of the amylase. Chemically competent *Escherichia coli* DH5α was used for plasmid construction. Plasmid Blunt II-Topo (Invitrogen) was used for gene cloning and sequencing. *E. coli* was grown at 37 °C in Luria-Bertani (LB) Lennox medium supplemented with 25 μg/mL of the antibiotic for which the contained plasmid provides tolerance. For amylase activity assays, *B. subtilis* PY22 was

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doi:10.1016/j.molcatb.2009.07.006

grown in a starch containing *Bacillus* medium (10 g/L KH_2PO_4, 10 g/L soluble starch, 2.5 g/L Na_2HPO_4, 2 g/L soytone, 1 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 0.05 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $CaCl_2$) in shake flasks at 37 °C. Batch cultures of *P. pastoris* were grown in baffled shake flasks at 30 °C and 250 rpm orbital shaking, in BMGY or BMMY medium. The BMGY medium contained 10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate buffer (pH 6.0), 13.4 g/L yeast nitrogen base, 4×10^{-4} g/L biotin and 1% (v/v) glycerol. The BMMY medium had the same composition with the exception that the glycerol was replaced with 0.5% methanol for induction.

2.2. Cloning and sequencing of the amyE gene

Isolation of the bacterial and yeast genomic DNA was performed using the MasterPure™ kits (Epicentre, Biotechnologies). Amplification of the *amyE* gene was performed according to [14] using a TGradient (Whatman-Biometra) thermocycler with the primers AmyFPmlI (5′-TCA CGT GGA TGT TTG CAA AAC GAT TCA AAA-3′) and AmyRKpn (5′-AGG TAC CTC AAT GGG GAA GAG AAC C-3′) designed to incorporate PmlI and KpnI restriction sites to the PCR product. *B. subtilis* PY22 chromosomal DNA was used as template for PCR reactions. The expected PCR product was gel purified using the QIAquick Gel Extraction Kit (Qiagen). The PCR product was ligated to Blunt II-Topo vector and transformed into *E. coli* cells. Plasmid isolations were performed using the Strataprep® Kit (Stratagene). The isolated plasmids were sequenced at the Genomics Core Research Facility at the University of Nebraska-Lincoln to ensure that mutations were not introduced during PCR. Quantity measurement of the DNA and plasmids in extracts was achieved by fluorometric detection with Pico Green Quantitation Reagent (Invitrogen) using a TD-700 Fluorometer (Turner Designs).

2.3. Expression of the amyE gene in P. pastoris

All the restriction endonucleases and the Quick Ligation™ Kit were obtained from New England Biolabs. After confirming the DNA sequences pBlunt-II Topo plasmid was double digested with PmlI and KpnI and gel purified using the MinElute PCR purification kit. The cloning vector pPicZαA was also prepared by digesting with PmlI and KpnI and gel purified. The ligation reactions were transformed into *E. coli*. The isolated plasmid was confirmed by checking gel patterns after digestion with various endonucleases and by PCR using the primers AmyEF (5′-GCC ATT CAG ACATCTCCG) and AmyER (5′-CAA ATA AAG CAC TCC CGC) and sequencing using the 5′ and 3′ *AOX1* Sequencing Primers.

The plasmid was linearized with SacI, purified using the MinElute PCR purification kit (Qiagen) and transformed by electroporation using a Gene Pulser II (Bio-Rad) following the manufacturers' instructions. Positive clones were propagated in BMGY medium until they reached an OD_{600} 2–6 and then transferred to BMMY medium for induction by adding 0.5% methanol at 24 h intervals. *P. pastoris* KM71H was used as a control for background expression analysis.

2.4. Southern hybridization analysis

Selected clones were subjected to a Southern blot analysis to determine the plasmid copy number integrated into *AOX1* loci of *P. pastoris*. Approximately 1 μg of each DNA was digested with BstEII, separated by 0.8% agarose gel electrophoresis and transferred onto a Zeta-Probe (Bio-Rad) genomic tested blotting membrane. The target fragment was detected with a digoxigenin (DIG)-labeled probe *AOX1* specific probe prepared using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Germany). The methods and operations were performed according to the manufacturer's instructions and as described by Inan et al. [15].

2.5. SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was preformed in a XCell Sure Lock™ Mini-Cell using NuPAGE® Bis–Tris gels (10%). Culture supernatants or samples obtained throughout the stages of partial purification were prepared as described by the manufacturer in a total volume of 50 μL, and 18 μL of each mixture was loaded in each well unless otherwise specified. Buffer chambers were filled with appropriate mixtures of MOPS buffer and electrophoresis was performed at 200 V for 55 min. The gel was stained with Coomassie Blue R-250 (Merck, Darmstadt, Germany) for 30 min and destained with over an orbital shaker in a solution containing 10% methanol and 7.5% acetic acid for 1–2 h.

2.6. Purification of the recombinant enzyme

The selected positive clone of *P. pastoris* was used for expression of the α-amylase enzyme in 200 mL cultures in shake flasks. Following a 4-day induction period the cultures were centrifuged at $3250 \times g$ for 15 min to remove cells. All the purification steps were performed at 4 °C. The supernatant was filtered by passing through Supor® membrane disk filters (Pall Life Sciences) of 0.2 μm pore size and brought to 80% saturation with slow addition of ammonium sulfate under constant stirring [16]. The solution was centrifuged at $9500 \times g$ for 30 min to separate precipitated proteins. The pellet was resolubilized in 12 mL of 0.1 M MOPS buffer (pH 6.9) and dialyzed against the same, under constant stirring for 2 h, in a Slide-A-Lyzer® Dialysis Cassettes (Pierce) with 3500 molecular-weight cut off. The dialyzate was then placed in a Centriplus® centrifugal filter device equipped with a YM-50 membrane (Millipore) and centrifuged at $3000 \times g$ for 30 min. The centrifugation step was repeated three more times adding 5 mL of 0.1 M MOPS buffer (pH 6.9) at each interval. The filtrates obtained at each centrifugation step and the final retentate were filter sterilized as described above and kept at 4 °C for further analysis. Protein concentrations were measured spectrophotometrically using Micro-BCA Protein Assay Reagent (Pierce) with BSA as standard.

2.7. Amylase activity assays

Plate assays of amylase activity were performed by growing cultures on MM starch agar (3.4 g/L yeast nitrogen base, 4×10^{-4} g/L biotin, 0.5% (v/v) methanol, 15 g/L agar, 4 g/L soluble starch). In order to induce protein expression, 100 μl of methanol was placed on the plate covers after 24 and 48 h of incubation at 30 °C. The developed plates were then flooded with Lugol solution. Clear zones surrounding the colonies indicated amylase activity. Amylase activity of culture supernatants were assayed either with a fluorometric method using the EnzCheck Amylase Assay Kit (Invitrogen) or with a spectrophotometric method using the dinitrosalicylic acid (DNSA) reagent [17]. One unit of enzyme activity is equivalent to the amount of enzyme required to liberate 1 mg of maltose from starch in 3 min at 20 °C and pH 6.9.

2.8. Enzyme properties

The activity of the enzyme at various pH values was determined by the DNSA method mentioned above. The samples of partially purified enzyme were diluted at a ratio of 1:40 with the appropriate 0.1 M phosphate buffer adjusted to the respective pH values ranging from 5 to 9. Optimum temperature assays were performed after 1:40 dilution of the samples in 0.1 M MOPS buffer (enzymatic grade,

Table 1
Comparison of AmyE protein and the encoding DNA sequences for *B. subtilis* PY22 with *B. subtilis* strain 168 showing regions where there are differences.

Strain/protein	Position	Open frame reading 5′ → 3′
B. subtilis 168/AmyE	501	TTKAVYQINNGPDDRRLRMEINSQSEKEIQFGKTYTIMLK
		||||||||||| |||||||||
B. subtilis PY22/AmyE	501	TTKAVYQINNGPETAFKDGDQFTIGKGDP.FGKTYTIMLK
Strain/gene	**Position**	**DNA sequence 5′ → 3′**
B. subtilis 168/*amyE*	1531	GGACCAGACGACAGGCGTTTAAGGATGGAGATCAA
	1566	TTCACAATCGGAAAAGGAGATCCAATTTGGCAAAA
B. subtilis PY22/*amyE*	1531	GGACCAGAGAC..GGCGTTTAAGGATGGAGATCAA
	1566	TTCACAATCGGAAAAGGAGATCC.ATTTGGCAAAA

Sigma) of pH 6.9 at temperatures ranging from 20 to 80 °C. The temperature stability was measured by determining activity readings after the enzyme dilutions were kept at the respective temperatures for 1 h. The effect of calcium ions on activity was determined by measuring sample activity in a 1:40 dilution of 0.1 M $CaCl_2$, 0.1 M MOPS solution. For the deglygosylation study, the recombinant enzyme was denatured in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) at 100 °C for 10 min. It was then incubated overnight at 37 °C with *Endo* H enzyme in G5 buffer (50 mM sodium citrate, pH 5.5 at 35 °C). The samples were run in an SDS-PAGE for analysis.

3. Results and discussions

3.1. Cloning and sequence analysis of the amyE gene

The *amyE* gene of *B. subtilis* PY22 was amplified with forward and reverse primers that introduced PmlI and KpnI sites, respectively. The PCR fragment was first subcloned into pCRII-TOPO vector and sequenced. Sequencing of two different plasmids revealed that the isolated *amyE* gene was 99% identical to the gene encoding α-amylase in *B. subtilis* strain 168 of which the complete genome has been sequenced [18]. The sequence comprising 1980 bp encoding 660 amino acids that was cloned into *P. pastoris* differs from that of *B. subtilis* strain 168 only in that there is a change in 3 bp and a deletion of 3 bp which has the effect of a frame shift in expression involving a string of 17 amino acids (Table 1). The *amyE* gene was then subloned into *Pichia* expression vector, pPICZαA in frame with alpha mating factor of *S. cerevisiae*.

3.2. Overexpression of the amyE gene in P. pastoris

Transformation of the SacI digested plasmid pPICZαAmyE into electrocompetent host cells of *P. pastoris* KM71H resulted in over one hundred colonies on YPDS (Zeocin 100 mg/L). Eight of these colonies were randomly chosen for amylase activity screening. Plate cultures on MM starch agar showed clearance zones around all the selected strains except for clones #2 and #3 and the host control (Fig. 1). Although clones #7 and #8 gave larger zones on the plate assay, this did not correlate with shake flask data. However, this was a quick assay for testing the positive clones.

The results of Southern blot analysis showed that copy number of the clones ranged from 1 to 4 including two untransformed colonies (Fig. 2). The host and untransformed clones showed a 2.4 kb band of *AOX1* loci digested with BstEII restriction enzyme. Since the BstEII enzyme does not cut within the pPICZαAmyE plasmid, insertion of the plasmid into the *AOX1* loci increases the band size by addition of plasmid size (5.6 kb). Clone #1 had an 8 kb band expected for one-copy clones. Similarly, clones #4 and #5 had expected 13.6 kb for two-copy clones. The clone #6 had three copies; the clones #7 and #8 had four copies of the plasmid as determined by band size on the Southern blot.

Production of amylase by the clones carrying various copy numbers of the amylase gene was monitored by fluorometric amylase assay of culture supernatants following methanol induction (Fig. 2). Clones labeled #4 and #5, each containing two copies of the plasmid insert produced significantly higher enzyme activity in culture supernatants. Increasing the plasmid copy number more than two decreased total secreted amylase at tested conditions (Fig. 3, clones #6–#8). This has also been observed for other secreted products [15,19]. Increasing gene copy number of a recombinant protein may reduce secreted product by saturating the secretion pathway. Therefore, for optimum secretion of products, a range of copy number of the clones should be screened rather than investigating only high copy number clones. It was calculated using activity/protein concentration data of standard amylase (Sigma 6380) that clone #4 produced about 22 mg/L of the recombinant enzyme (44.34 U/mL). This clone was selected for further amylase production, purification and characterization studies.

B. subtilis PY22 strain was also grown in shake flasks containing starch media. The production of extracellular α-amylase by *B. subtilis* PY22 in shake flask cultures was determined with the flu-

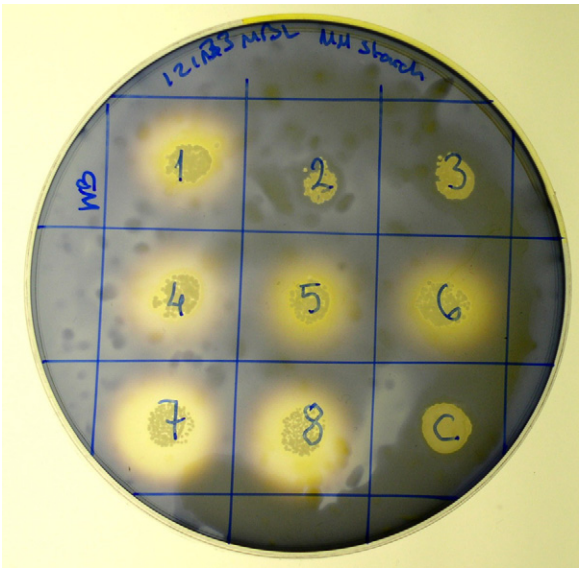

Fig. 1. Test of amylase activity of eight selected clones of the pPICZαAmyE transformation into *P. pastoris*. The clones labeled #2 and #3 and the colony labeled "C" of non-transformed control host KM71H cells show no clearance zones indicating the absence of amylase activity.

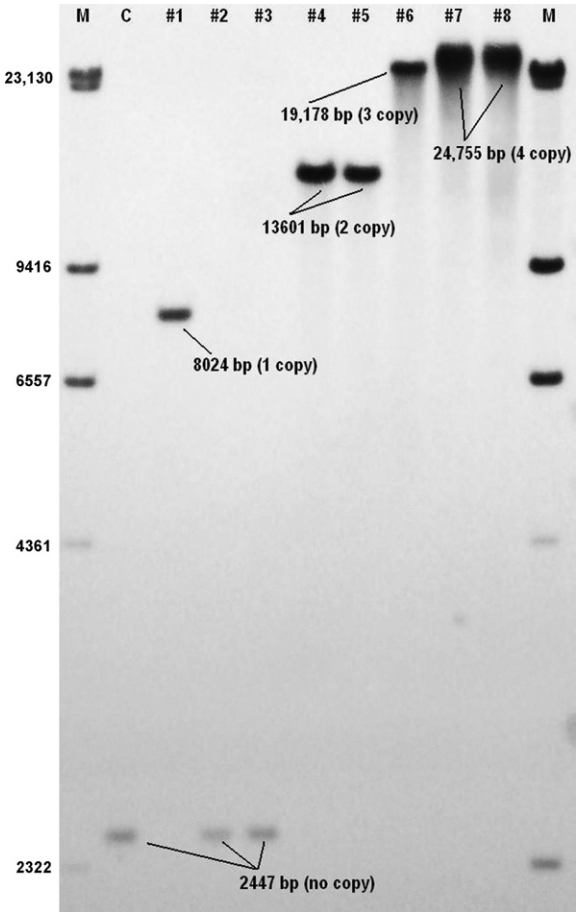

Fig. 2. Southern blot analysis of pPICZαAmyE copy numbers. Lane M: molecular marker (λ DNA/HindIII Fragments, size of bands in bp on left side); lane C: control strain KM71H; lanes #1–#8: selected strains of from cloning procedure.

orometric assay throughout a fermentation period of 72 h (Fig. 4). During this time the maximum amylase activity measured in culture supernatant was 120 mU/mL. A direct comparison between gene donor cell and host cell production of the α-amylase is difficult, however it can be pointed out that the α-amylase activity of the recombinant enzyme in cell free culture following 72 h of induction was found to be roughly 370-fold that of the donor cell after 72 h of fermentation.

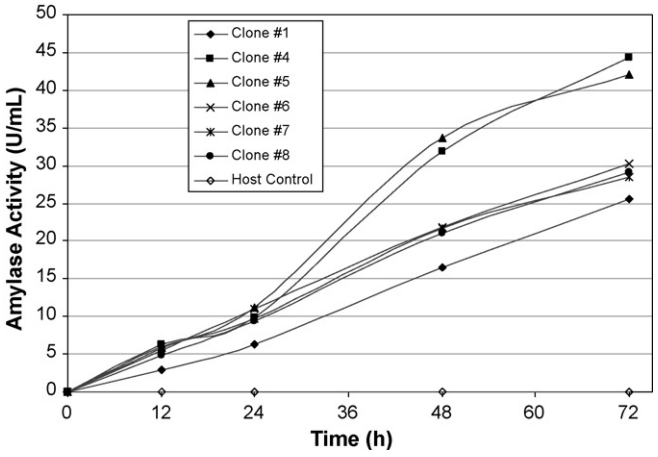

Fig. 3. Amylase activities in methanol induced culture supernatants of *P. pastoris* pPICZαAmyE clones.

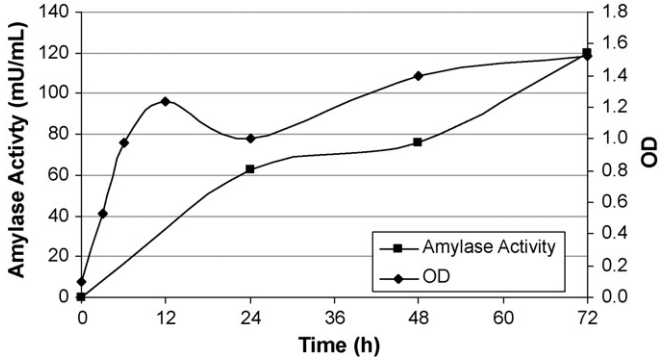

Fig. 4. Amylase activities of culture supernatant of *B. subtilis* PY22 and optical densities throughout fermentation in shake flask cultures.

3.3. Characterization of the recombinant enzyme

In order to proceed with the characterization of the recombinant enzyme, the amylase produced by *P. pastoris* pPICZαAmyE (clone #4) was partially purified (Table 2). The partial purification involving ammonium sulfate precipitation at 80%, dialysis and membrane filtration was not very efficient with a yield of 11%. It is highly likely that much of the protein of interest was lost during the precipitation phase since no amylase was present in filtrates of centrifugal ultrafiltration (SDS-PAGE data not shown). The partial purification resulted in a 32.48-fold purification of the enzyme which was comparable to values obtained in other α-amylase purification studies [20,21]. Samples of the cell free culture broth and the partially purified enzyme were analyzed with SDS-PAGE (Fig. 5). The bands observed confirmed increased concentration of the α-amylase.

Lane 3 in Fig. 5 shows the deglycosylated recombinant AmyE protein where multiple bands observed in lane 1 and 2 were reduced to a single band. In lane 4, a single band is observed at 50 kDa for *B. amyloliquefaciens* α-amylase as expected. The AmyE protein has 11 potential N-glycosylation sites. Since the deglycosylated form of the protein appeared within the range of 50–64 kDa (at approximately 60 kDa) the enzyme with a theoretical molecular weight of 72.28 kDa deduced from whole gene sequence and

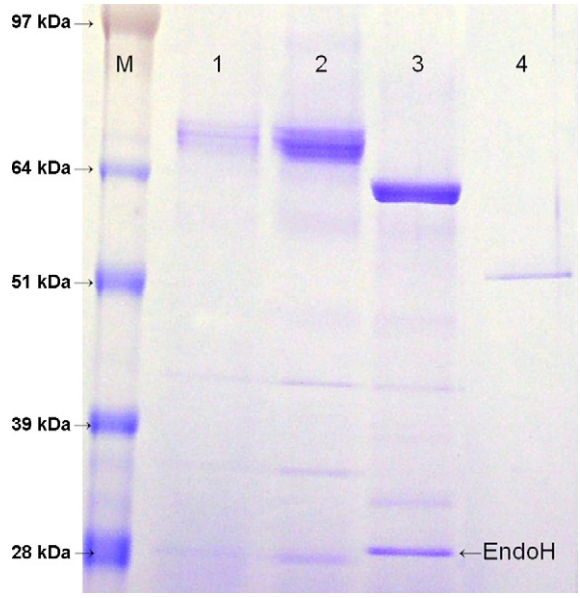

Fig. 5. SDS-PAGE of various α-amylase samples; (1) cell culture supernatant, (2) partially purified recombinant α-amylase, (3) *Endo* H degylosylated recombinant α-amylase and (4) 500 ng load of α-amylase standard protein (Sigma 6380).

Table 2
Purification of the recombinant α-amylase secreted by *P. pastoris*.

	Volume (mL)	Amylase activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Total activity (U)	Yield (%)
Culture supernatant	200	8.93	15.39	0.58	1786	100
Purified sample	7.5	26.38	1.4	18.84	197.85	11

67.91 kDa deduced from whole sequence minus signal sequence [22] is thought to have been cleaved for excretion.

N-linked glycosylation is a common posttranslational modification of membrane and secretory proteins in eukaryotes. Multiple bands observed on the SDS-PAGE gels showed that the enzyme was processed within the yeast cell for secretion and was N-glycosylated. Glycosylated forms of proteins that are not glycosylated in their native state are often less susceptible to thermal inactivation [2]. However, in the case of *P. pastoris* expressed *B. licheniformis* α-amylase, it was shown that deglycosylation resulted in a slight increase in activity and did not improve stability [9].

The optimum working pH of the recombinant amylase was determined at 20 °C, over a pH range of 5–9 (Fig. 6a). Use of phosphate buffer to cover this range was decided on after trials using combinations of buffer solutions. These trials showed that the choice of buffer also affected activity (data not shown) and therefore use of a single buffer system to cover the entire range was preferred. The partially purified recombinant α-amylase showed highest activity at neutral pH. Optimum temperatures for α-amylase activity were determined over a range of 20–80 °C at pH 7 (Fig. 6b). It could be expected that, *B. subtilis* PY22, being a moderate thermophile, would produce an amylase which is also moderately thermophilic. The recombinant enzyme showed peak activity at 60 °C (Fig. 6b), which was slightly higher than the determined optimum growth temperature of 50 °C for *B. subtilis* PY22 (data not shown).

The stability tests of the recombinant amylase were performed at the determined optimum pH (Fig. 7). It was observed that the enzyme retained 78% of its activity when kept at the determined optimum temperature of 60 °C. A sharp decline in stability was observed at higher temperatures.

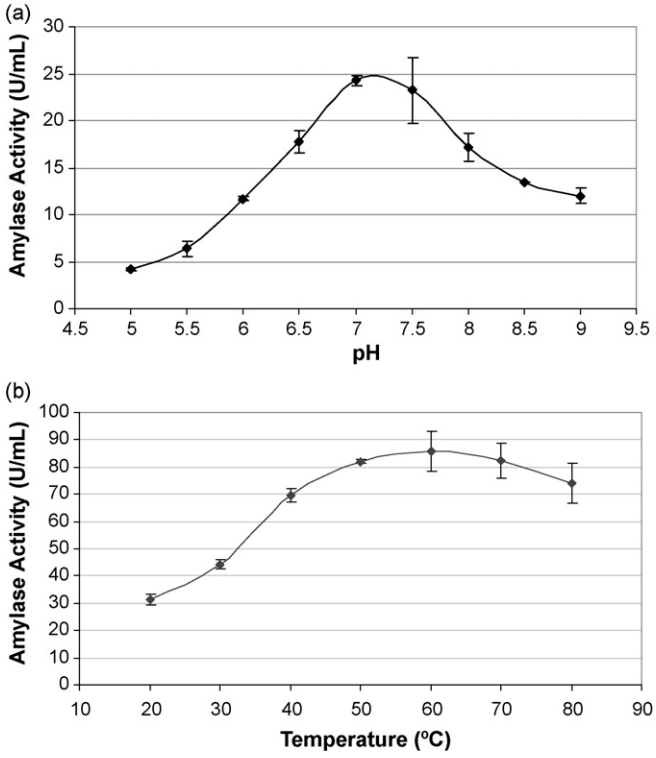

Fig. 6. Effect of pH on α-amylase activity (a) and the effect of temperature on α-amylase activity at pH 7 (b).

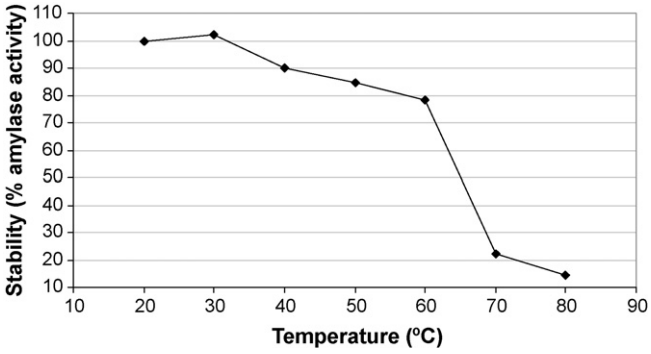

Fig. 7. Temperature stability measurements of the recombinant α-amylase determined by assaying amylase activity following exposure to the respective temperatures for 1 h at pH 7.

α-Amylases are known to be metallo-enzymes with varying dependence on Ca^{2+} [6]. Replacing the reaction medium with 0.1 M Ca^{2+} supplemented buffer showed that the enzyme activity was significantly influenced by the presence of the ion. The results of the DNSA reagent assay confirmed an increase in activity of 41% in the presence of Ca^{2+}.

4. Conclusions

Recombinant expression resulted in high levels of extracellular amylase production, as high as 22 mg/L in the culture supernatant. The partially purified enzyme was Ca^{2+} dependent and showed the highest activity at 60 °C and pH 7. Expression in *P. pastoris* has not only increased the yield of production, but also potentially eased purification. Further fermentation and purification studies could prepare the recombinant organism as a commercial strain. Future application of this enzyme could be considered as a saccharifying enzyme in the processing of starch to syrup. Another alternative use could be as a dough additive in the baking industry to improve yeast growth where heat stability of the amylase is not a requisite and where small amounts that remain in bread could serve as an anti-staling agent.

Acknowledgements

B. Karakaş was financially supported with a PhD research scholarship from the Scientific and Technological Research Council of Turkey (TUBITAK). This work was performed at the Biological Process Development Facility of University of Nebraska-Lincoln, Lincoln, NE, USA.

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