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Molecular Cloning of an α-Amylase Gene from *Bacillus subtilis* RSKK246 and Its Expression in *Escherichia coli* and in *Bacillus subtilis*

Numan ÖZCAN, Adem ALTINALAN
Department of Animal Science, Faculty of Agriculture, Çukurova University, Adana - TURKEY
M. Sait EKİNCİ
Department of Animal Science, Faculty of Agriculture, Kahramanmaraş Sütçü Imam University, Kahramanmaraş - TURKEY

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Abstract : *Bacillus subtilis* RSKK246 was found to produce approximately a 65-kDa α-amylase enzyme. A gene was isolated encoding α-amylase activity that corresponded to this size and was inserted into pUC18 plasmid which was transferred to *Escherichia coli*. An approximately 1.7kbp fragment, which contains a whole α-amylase gene, was excised and inserted into pUB110 and then transferred into the different *B. subtilis* strains including RSKK246, RSKK243, RSKK244, YB886 and ORBAM. The α-amylase gene was cloned into the plasmids and expressed with its own promoter, and this promoter sequence seemed to function in the *E. coli* and in all *B. subtilis* strains. Specific activity of the cloned enzyme was found to be higher than the native enzyme and molecular weight of the gene product remained the same in all other strains suggesting that it is resistant to the proteolytic attacks of these organisms.

Key Words: Amylase, *Bacillus subtilis*, gene

Bacillus subtilis RSKK246 Şuşundan Bir α-Amilaz Geninin Moleküler Klonlanması ve *Escherichia coli* ve *Bacillus subtilis* Şuşlarında Ekspresyonu

Özet : *Bacillus subtilis* RSKK246 şuşunun yaklaşık 65kDa büyüklüğünde α-amilaz enzimi ürettiği bulundu. 65 kDa büyüklüğünde α-amilaz enzimini kodlayan gen izole edilerek pUC18 plasmidine klonlandı ve *Escherichia coli*'ye transfer edildi. Yaklaşık 1.7-kbp büyüklüğünde tüm α-amilaz genini içeren DNA fragmenti kesilerek pUB110 plasmidine yerleştirildi ve daha sonra çeşitli *B. subtilis* suşlarından RSKK246, RSKK243, RSKK244, YB886 ve ORBAM' ye elektroporasyon ile aktarıldı. α-amilaz geni çeşitli plasmidlere klonlandı ve kendi promoteri ile ekspres edildi. Bu durum α-amilaz geninin promoter sekansının *E. coli* ve Bacillus türlerinde fonksiyonel olduğunu gösterdi. Klonlanmış enzimin spesifik aktivitesinin doğal enzimin spesifik aktivitesinden daha fazla olduğu ve gen ürününün moleküler ağırlığının tüm türlerde aynı kaldığı gözlendi ve bu genin proteolitik enzimlere karşı dirençli olduğunu gösterdi.

Anahtar Sözcükler: Amilaz, *Bacillus subtilis*, gen

Introduction

The food industries, including brewing, baking and jam making, are the main users of starch. Starch hydrolysing enzymes include the α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2), glucoamylases (EC3.2.1.3), isoamylases (EC 3.2.1.68), pullulanases (EC 3.2.1.41) and cyclodextrin glucanotransferases (EC 2.4.1.19) (1). The genus *Bacillus* produces a large variety of extracellular enzymes, some of which are of industrial importance. Some strains have been developed already for the massive production of a particular amylase for industrial purposes (2).

The screening for a single amylase is difficult because one strain can produce different amylases with different specificities or the amount of amylase produced may be very low. Thus the cloning of one gene directing the synthesis of the desired amylase in a well characterised host like *E. coli* or *B. subtilis* should help greatly in the characterisation of new amylases and also allow a significant yield increase (3-4). To improve production of α-amylase, as well as to construct a recombinant *Saccharomyces cerevisiae* strain for the baking industry, the α-amylase gene of *B. subtilis* RSKK246 was cloned and expressed in both *E. coli* and *B. subtilis*.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Thee strains that were used in this work and their enzymatic properties are listed in Table 1. *B. subtilis* RSKK strains, used throughout the study, were kindly supplied by Refik Saydam Hıfzısıhha Merkezi (Ankara/Turkey). *B. subtilis* ORBAM (amylase negative mutant of *B. subtilis* ORBA) (5) was made in our lab. *B. subtilis* YB886 was supplied from Ohio State University USA. *E. coli* XLI-Blue MRF' was obtained from Stratagene. Plasmid pUC18 (EcoRI, cut BAP+ligaz) was obtained from Pharmacia, and pUB110 from Sigma. *E. coli* strains were routinely grown in LB medium. *E. coli* XLI-Blue MRF' was used as host for pUC based constructs, with 50 μg ml^{-1} ampicillin for the selection of transformed cells (6). The constructs based on pUB110 plasmid introduced into *B. subtilis* strains with 20 mg ml^{-1}kanamycin for selection of transformed cells. *B. subtilis* cultures were grown aerobically overnight at 37ºC in LB-broth medium, and solidified by supplementing with 1.5% (w/v) agar if necessary (Difco).

Table 1. Bacterial strains used for transformation and their native enzyme activities.

Strains	α-amylase	CMCase (Carboxymethl cellulase)
E. coli XL1-Blue MRF'	-	-
B. subtilis YB886	-	-
B. subtilis ORBAM*	-	-
B. subtilis RSKK243	-	+
B. subtilis RSKK244	+	-
B. subtilis RSKK246	+	+

*Mutant type of *B. subtilis* ORBA (3)

Molecular biology procedures

Restriction enzymes, T4 DNA ligase and alkaline phosphatase were obtained from Stratagene and Gibco-BRL. The restriction and ligation reactions were carried out as described by Maniatis et al. (6). Transformation of recombinant plasmid to the *E. coli* cells was performed by the $CaCl_2$ method (7). Transformation of *B. subtilis* cells was done by electroporation (8). Selection and placing of transformed α-amylase positive cells on to LB agar-starch plates was carried out as described previously (9). Size determination of DNA fragments was carried out by the agarose gel technique as described by Maniatis et al. (6) using several restriction enzymes.

Cloning procedure

Chromosomal DNA of *B. subtilis* RSKK246 was prepared by the method of Cutting and Van der Horn (10). Chromosomal DNA was fully digested with *Eco*RI (6), and DNA fragments between 1 and 7 kbp were recovered from the agarose gel by Strata-eluter (Stratagene). DNA solutions were concentrated by prep-A-gene clean kit (Bio-Rad). Purified DNA fragments were ligated with *Eco*RI cut pUC18 plasmid (pUC18/*Eco*RI/BAP+ligaz (Parmacia)); the ligation solution was first incubated at 16ºC for 1h then at 4ºC overnight. Competent *E. coli* cells were prepared and transformed by the methods of Barat-Gueride et al. (11) and Hanahan (7).

Enzyme determinations

Reducing sugars released from polysaccharide substrates were determined by the method of Cotta (9). Supernatant of *B. subtilis* YB886/pBU2 was used for the enzyme assay. The colonies expressing α-amylase activity were detected by exposing the plates to iodine vapours containing appropriate substrate (2), as described previously (12). Proteins from the supernatant of both native and recombinant *B. subtilis* RSKK, YB886 strains and *E. coli* were precipitated with triclora acetic acid (20 w/v) and prepared as described earlier (13). The protein concentration was measured by the method of Lowry (14), using bovine serum albumin as standard. Detection of α-amylase activity following SDS-PAGE was performed as described previously (15-16). Coomassie blue staining of proteins was performed as described by Lee et al. (17). Plate tests for CMCase (Carboxymethyl cellulase), in which plates carrying substrate overlays were stained with Congo red (18), were performed as described previously (19).

Results and Discussion

Isolation of the α-amylase gene from *B. subtilis* RSKK246

A major band of α-amylase activity approximately 65 kDa was detected in *B. subtilis* RSKK246 cultures by SDS

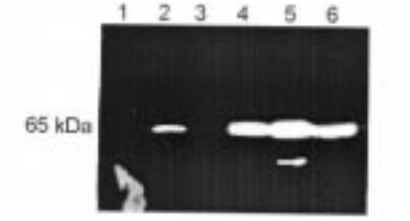

Figure 1. Expression of native and cloned *B. subtitlis* α-amylase, detected by SDS-PAGE zymograms. Lane 1: *B. subtilis* ORBAM. Lane 2: *B. subtilis* ORBAM/pBU. Lane 3: *B. subtilis* RSKK243. Lane 4: *B. subtilis* RSKK243/pBU. Lane 5: *B. subtilis* RSKK246/pBU. Lane 6: *B. subtilis* RSKK246.

zymograms (Fig. 1). Among about 40,000 ampicillin resistant transformants, 3 active colonies showed clear halos detected with iodine vapour; these were found by restriction analysis to have overlapping inserts, and all encoded enzymes were similar in size to the native α-amylase. After purification of recombinant plasmids from *E. coli* cells (20), the size of insert was assumed on agarose gel (6). According to analysis an approximately 1.7kbp *Eco*RI fragment was found to have α-amylase activity and was named pCU2. These transformants produced halos around colonies on LB medium agar plates containing soluble starch when the plasmids were used again for transformation into *E. coli*. Therefore, plasmid pCU2 seemed to contain the amylase gene of *B. subtilis* RSKK246. This α-amylase gene showed a restriction profile different from that of the other cloned *B. subtilis* α-amylase genes (2,17,21,22). In addition, the amylase gene from *B. subtilis* RSKK246 was shown to be stable in *E. coli* without plasmid curing or disappearance of α-amylase production and showed the same size of active enzyme, suggesting that this protein was not digested by *E. coli* proteases. An approximately 1.7kbp fragment was then cut with *Eco*RI and subcloned into *Bacillus* vector pUB110 and the new construct was named pBU2. This new construct was introduced by electroporation into amylase negative *B. subtilis* YB886, RSKK243 and ORBAM, and positive RSKK244 and RSKK246 strains. A band of α-amylase activity was detected in supernatant from culture of *B. subtilis* YB886/pBU2 (especially used for propagation and enzymatic analysis), RSKK243/pBU2, RSKK244/pBU2, RSKK246/pBU2 and ORBAM/pBU2 strains when analysed by SDS-PAGE zymogram (Fig. 1). This band showed the same mobility as the enzyme from the native strain. Having the same size of α-amylase protein of the *B. subtilis* RSKK246 from *B. subtilis* YB886/pBU2, RSKK243/pBU2, RSKK244/pBU2, and ORBAM/pBU2 strains, this enzyme has been suggested to be stable in those species and was not affected by their proteases.

Properties of the *B. subtilis* α-amylase

Supernatant from overnight cultures was used for enzyme determinations. All recombinant Bacillus strains carrying pBU2 gave higher specific amylase activity than the native Bacillus strain RSKK246 (Table 2), suggesting that the promoter region of the amylase gene of *B. subtilis* strain RSKK246 is well recognised by the other *Bacillus* species.

Table 2. Specific α-amylase activity of native and cloned enzyme in *Bacillus* strains.

	Enzyme activity (μmol (mg protein)$^{-1}$ min^{-1})	
	12h incubation	24h incubation
E. coli/pBU2	3.7	5.2
B. subtilis YB886/pBU2	4.6	6.4
B. subtilis ORBAM/pBU2	6.2	5.8
B. subtilis RSKK243/pBU2	5.9	5.6
B. subtilis RSKK246	4.0	6.4
B. subtilis RSKK246/pBU2	5.5	6.0

The pH dependence of the *B. subtilis* supernatant enzyme was determined by preparing 50 mM sodium acetate assay buffers ranging from pH 5.0 to 5.5, with pH values between 5.5 and 7.0 being achieved by the sodium phosphate and pH values between 7.0 and 8.0 obtained with tris-HCl. The pH for optimal α-amylase activity (5.0-6.0) (Fig. 2) was similar to values reported for most bacterial (23-25) and yeast (26) amylases. The optimal temperature was found to be 40ºC (Fig. 3) and enzyme activity was stable at 55ºC (Fig. 4), but 15 min of preincubation at 70ºC destroyed 65% of the enzyme activity. No substantial effect of ions on the amylase activity was observed, while the opposite has been reported in the literature (24,27).

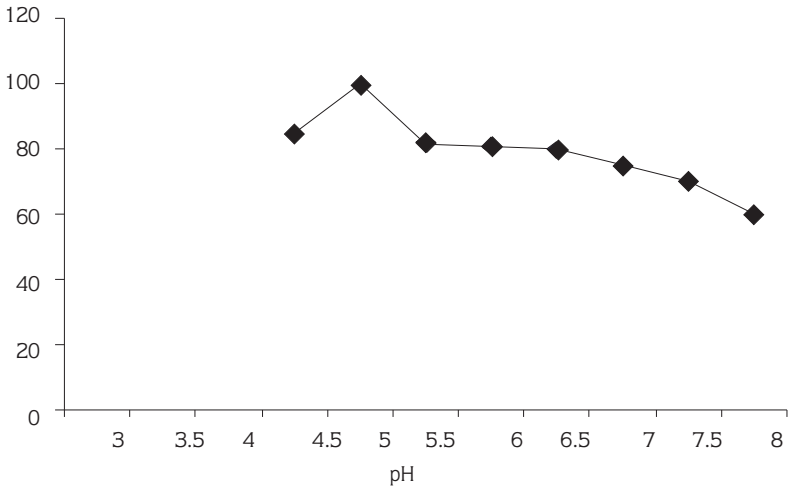

Figure 2. pH optimum of *Bacillus subtilis* YB886/pBU.

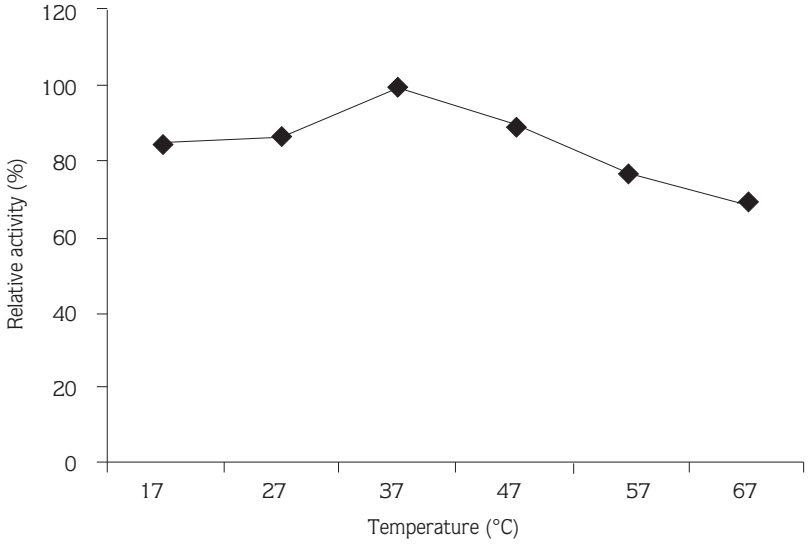

Figure 3. Temperature optimum of *Bacillus subtilis* YB886/pBU.

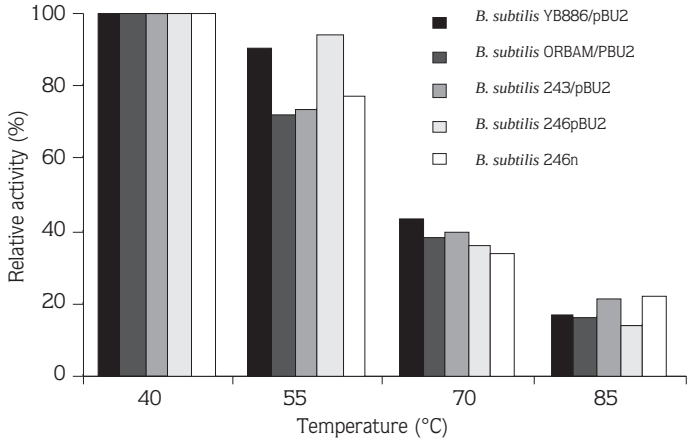

Figure 4. Temperature stability of amylase in different *B. subtilis* strains.

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