Turk J Biol
36 (2012) 327-338
© TÜBİTAK
doi:10.3906/biy-1106-7

Thermostable α-amylase from moderately halophilic *Halomonas* sp. AAD21

Kamil Serkan UZYOL[1], Berna SARIYAR AKBULUT[2], Aziz Akın DENİZCİ[3], Dilek KAZAN[2]

[1]Institute for Graduate Studies in Pure and Applied Science, Marmara University, Göztepe Campus, 34722 Kadıköy, İstanbul - TURKEY

[2]Bioengineering Department, Engineering Faculty, Marmara University, Göztepe Campus, 34722 Kadıköy, İstanbul - TURKEY

[3]The Scientific and Technological Research Council of Turkey (TÜBİTAK), Genetic Engineering and Biotechnology Institute, Marmara Research Center, Gebze, Kocaeli - TURKEY

Received: 06.06.2011

Abstract: The moderately halophilic *Halomonas* sp. strain AAD21, which produces extracellular thermostable α-amylase, was isolated from the Çamaltı Saltern area located in İzmir, Turkey. NaCl, carbon, and nitrogen sources in the growth medium were optimized to enhance α-amylase yield. The highest enzyme yield was measured in the presence of 20% NaCl with peptone as the nitrogen and starch as the carbon sources in the fermentation broth. This microorganism was also found to utilize waste potato peel as a carbon source for α-amylase production. Concentrations of carbon and nitrogen sources were optimized using a statistical approach, and α-amylase activity increased from 4.07 U mL^{-1} min^{-1} to 26.25 U mL^{-1} min^{-1}. Maximum α-amylase production was achieved at the end of 48 h of growth in the presence of 20% NaCl, 4.12% starch, 1.0% peptone, 0.2% KCl, 2% $MgSO_4{\cdot}7H_2O$, and 0.03% trisodium citrate pentahydrate. The optimum pH and temperature of the α-amylase were found to be 7.0 and 50 °C, respectively. The α-amylase synthesized by *Halomonas* sp. AAD21 was also thermostable. Crude α-amylase did not lose its original activity after 2 h of incubation at 50 °C and 60 °C, and it retained 70% of its original activity after 120 min of incubation at 90 °C.

Key words: Moderate halophile, *Halomonas* sp. AAD21, α-amylase, thermostable, optimization

Ilımlı halofilik *Halomonas* sp. AAD21'in ısılkararlı α-amilazı

Özet: Isılkararlı α-amilaz üreticisi ılımlı halofilik *Halomonas* sp. AAD21, İzmir'de bulunan Çamaltı Tuzlası'ndan izole edilmiştir. Enzim üretimini arttırmak amacı ile NaCl, karbon ve azot kaynakları optimize edilmiştir. En yüksek enzim aktivitesi azot kaynağı olarak pepton, karbon kaynağı olarak nişastanın kullanıldığı besi yerinde, % 20 NaCl konsantrasyonunda elde edilmiştir. İzole edilen mikroorganizmanın patates kabuklarını da karbon kaynağı olarak kullandığı belirlenmiştir. Karbon ve azot kaynaklarının konsantrasyonu istatistiksel yaklaşımla optimize edilmiş ve α-amilaz aktivitesi 4,07 U mL^{-1} dak^{-1} den 26,25 U mL^{-1} dak^{-1} değerine yükselmiştir. En yüksek α-amilaz üretimi büyümenin 48.nci saatinde % 20 NaCl, % 4,12 nişasta, % 1 pepton, % 0,2 KCl, % 2 $MgSO_4{\cdot}7H_2O$, ve % 0,03 trisodyum sitrat pentahidrat içeren besi yerinde elde edilmiştir. α-Amilazın optimum sıcaklık ve pH değeri sırası ile 50 °C ve pH 7 olarak belirlenmiştir. Ayrıca enzimin yüksek sıcaklıklarda kararlı olduğu da bulunmuştur. Ham enzim 50 °C ve 60 °C sıcaklıklarda iki saat inkübasyonda aktivitesinin tamamını korumaktadır. 90 °C ise 120 dakika inkübasyon sonunda ise aktivitesinin %70'i korunmuştur.

Anahtar sözcükler: Ilımlı halofil, *Halomonas* sp. AAD21, α-amilaz, ısılkararlı, optimizasyon

Introduction

Microorganisms constitute the most important source for the production of industrial enzymes that have great economic potential in many processes, including agricultural, chemical, and pharmaceutical applications. The growing demand for enzymes stable under high salt concentrations has focused attention on halophiles, microorganisms that live under extreme saline conditions. Depending on their salt requirements, halophiles can be classified as slightly halophilic (2%-5% NaCl), moderately halophilic (5%-20% NaCl), or extremely halophilic (20%-30% NaCl) (1,2). Moderate halophiles receive special interest owing to their growth in a wide range of salt concentrations. These microorganisms are attractive sources of enzymes including DNases, lipases, amylases, gelatinases, and proteases, which are capable of functioning under high concentrations of salt and which lead to the precipitation or denaturation of most proteins. Due to their low solubility, halophilic enzymes are commonly used in aqueous/organic and nonaqueous media (3,4).

α-Amylases (endo-1, 4-α-D-glucan glucanohydrolase EC 3.2.1.1), which are extracellular endoenzymes, are one of the most important groups of industrial enzymes. They have applications in the sugar, textile, paper, brewing, baking, and distilling industries, as well as in the preparation of digestive aids and in the production of cakes, fruit juices, starch syrups, and pharmaceuticals (5-8). This class of industrial enzymes constitutes approximately 25% of the total enzyme market (6).

α-Amylases from moderate halophiles such as *Acinetobacter* (9), *Micrococcus halobius* (10), *M. varians* subsp. *halophilus* (11), other *Micrococcus* isolates (12,13), *Halomonas meridiana* (14), and *Chromohalobacter* sp. TVSP 101 (4) have been studied. In addition, halotolerant *Bacillus dipsosauri* (15), *Halobacillus* sp. strain MA-2 (16), and moderately halophilic *Bacillus* sp. strain TSCVKK (6) were reported to have amylase production. Recently, a highly efficient raw starch digesting α-amylase from *B. licheniformis* ATCC 9945a (7) and a novel alkali-stable α-amylase from *Chryseobacterium taeanense* TKU001 (8) were reported. Research efforts have been directed mainly toward the production, purification, and characterization of this synthesized α-amylase. There is only limited information about the cost-effectiveness of medium composition, especially in batch cultures. It is crucial to find low-cost substrates for microbial enzyme production if the enzyme is to be produced on a large scale. Recently, many agroindustrial byproducts, including wheat bran, rice bran, molasses bran, barley bran, maize meal, soybean meal, potato peel, and coconut oil cake, have been screened as low-cost substrates for solid state fermentation (17).

Turkey has a vast number of different ecological areas, which gives it a broad microbial diversity. Çamaltı Saltern is one of the most important marine-origin sources of salt production. In the present work, we report the optimum conditions for α-amylase production by a newly isolated moderately halophilic *Halomonas* sp. strain AAD21 from the Çamaltı Saltern area. Since statistical approaches can rapidly and accurately produce the technical information needed to design and produce low-cost, highly reliable products and processes (18), in this study, optimum starch and peptone concentrations in the fermentation broth were determined using a statistical design. Finally, the potential of potato peel as an economical substrate for α-amylase production was investigated in batch fermentation.

Materials and methods

Chemicals

Chemicals used in the bacterial cell cultivation and taxonomic studies were supplied by Merck AG (Darmstadt, Germany). All other chemicals were of analytical grade and supplied by Sigma (St. Louis, USA).

Bacterial strain isolation, identification, and culture conditions

Halomonas sp. strain AAD21 was isolated from salt sediment collected from ponds in the Çamaltı Saltern area in İzmir, located in western Turkey. The isolate was maintained in Brown medium (19) with the following composition (m/v): 0.5% yeast extract, 0.3% trisodium citrate pentahydrate, 2% magnesium sulfate heptahydrate, 0.2% potassium chloride, and 20% sodium chloride at a pH of 7.0. This medium, supplemented with 1% soluble starch, was used as the basal medium for α-amylase production.

The strain was identified based on typical cultural, morphological, and biochemical characteristics and on a 16S ribosomal ribonucleic acid (rRNA) gene sequence.

Gram reactions were performed using a Gram staining kit (bioMerieux) according to the manufacturer's instructions. To determine salt tolerance, isolates were grown in Brown medium containing 0%-25% sodium chloride. The effect of temperature was monitored by allowing growth at temperatures from 10 °C to 65 °C. Oxidase, catalase, DNase, urease, Tween 80, Tween 20, and indole production, in addition to gelatin degradation and starch hydrolyses, were tested using the procedures described earlier (16,20-22). The substrate mass fractions used were $w = 1\%$ gelatin, 1% soluble starch, and 0.1% Tween 80 and Tween 20, respectively. Reduction of nitrate to nitrite was determined in tubes containing 10 mL of liquid medium supplemented with 1% KNO_3. Durham tubes were used to examine the presence of gas and nitrite accumulation (23). Medium with 0.001% phenol red and yeast extract reduced by $w = 0.5\%$ was used to determine acid production from different sugars ($w = 1\%$ final fraction). Sodium citrate was not added to this medium.

Genomic DNA of the strain was isolated with PureLink (Invitrogen), and the universal primers 27f and 1492r were used to amplify the 16S rRNA gene. An amplicon was purified using a Spin Column PCR purification kit (Invitrogen). DNA sequencing was performed with a Beckman CQ8800 sequencer. The 1361 base pairs of 16S rRNA gene sequences of species closely related to strain AAD21 were retrieved from the GenBank database using BLASTN (24). A phylogenetic tree was constructed using MEGA 4 (25) after multiple alignments of the data with ClustalX (26). Distances (distance options according to the Kimura 2-parameter model) (27,28) and clustering were based on the neighbor-joining and maximum-parsimony methods. Tree topology was reexamined using the bootstrap method of resampling using 1000 bootstraps (29). The organism was deposited at our research laboratory at Marmara University, Turkey, and a 16S rDNA sequence of strain AAD21 was deposited in the NCBI database under Accession No. HM352832 (in progress).

Production of α-amylase

α-Amylase production was achieved using an inoculum culture of *Halomonas* sp. strain AAD21, 24 h old, at 1% (v/v) in 50 mL of basal medium retained in 250-mL Erlenmeyer flasks. These flasks were incubated in an orbital shaker at 37 °C and 180 rpm for 48 h. Different carbon and nitrogen sources were tested separately for α-amylase production. Amylase production was tested in a basal medium containing different organic and inorganic nitrogen sources (yeast extract, peptone, and 1:1 mixtures of yeast extract and peptone, yeast extract and NH_4NO_3, yeast extract and $NaNO_3$, and yeast extract and $(NH_4)_2SO_4$). The amounts of inorganic and organic nitrogen sources in the growth medium were kept at 1% and 0.5% (w/v), respectively (30). The effect of various carbon sources on α-amylase synthesis was investigated by replacing the starch found in the basal medium with 1% (w/v) glucose, maltose, sucrose, lactose, or potato peel. In order to determine the extent of improvement that sodium chloride presence could bring to enzyme synthesis, basal media supplemented with (w/v) 5%, 10%, 15%, or 20% sodium chloride were tested. At the end of 48 h, cells were harvested and the supernatants obtained were dialyzed against a 20 mM phosphate buffer (pH 6.9). These were further used for measurement of α-amylase activity. Cell growth was monitored by measuring absorbance at 600 nm.

Assay for α-amylase

α-Amylase activity was measured using the 3,5-dinitrosalicylic acid (DNS) method described by Bernfeld (31). Namely, 0.5 mL of substrate solution (1% soluble starch dissolved in 20 mM phosphate buffer, pH 6.9) was mixed with 0.5 mL of dialyzed supernatant, and the mixture was incubated at 37 °C for 1 h. The amount of reducing sugar released was quantified using DNS with maltose as the standard. One unit of α-amylase activity was defined as the amount of enzyme releasing one microgram of reducing sugar per minute from soluble starch under the assay conditions.

Effect of pH, temperature, and salinity on α-amylase activity

The effect of temperature on crude enzyme activity was investigated at temperatures between 30 °C and 60 °C at pH 6.9. The optimum pH of the crude preparation was investigated in the pH range of 4.0-8.5 by using different buffers (acetate buffer for pH 4.0 and 5.0, and phosphate buffer for pH values between 6.0 and 8.5). In order to determine the effect of salinity on α-amylase activity, the enzyme was assayed at 37 °C in 20 mM phosphate buffer at pH 6.9, containing 1% soluble starch supplemented with sodium chloride in the range of 0% to 6%.

Thermal stability of the α-amylase

In order to determine the thermal stability of the α-amylase, dialyzed supernatant was incubated at 50, 60, 70, 80, and 90 °C for 2 h. Thermal stability was expressed as percent residual activity, taking the initial enzyme activity at each temperature studied as 100%. Residual enzyme activities at different incubation times were assayed as described above. Results are given as arithmetic averages of multiple experiments with less than 5% standard error.

Optimization of starch and peptone concentrations using a statistical approach

Following the determination of the best carbon and nitrogen sources and optimum salt concentration for α-amylase production, a statistical design was applied to fermentation media in order to find optimum concentrations of the selected carbon and nitrogen sources. In this approach, carbon and nitrogen concentrations were selected as variable parameters and the total enzyme activity measured in units per milliliter (U mL^{-1}) was set as the response. The 2 parameters in the system were varied in the direction of the maximum increase of the response until the response no longer increased. The methodology of experimental Taguchi design arrays was modified to pick different carbon and nitrogen concentrations around the initial operating point, which were 10 g L^{-1} for starch and 5 g L^{-1} for peptone. The levels of all other fermentation parameters, such as temperature, pH, inoculum age and volume, aeration rate, and agitation speed, were kept constant. Starch was coded as $x1$ and peptone was coded as $x2$. These coded variables were normalized using Eq. (1).

$$X_{coded} = (a_i - a_0) / \Delta a, \qquad (1)$$

where X_{coded} is the independent variable coded value, a_i is the real value of the independent variable, a_0 is the real value of the independent variable on the center point, and Δa is the step change.

After obtaining the experimental results for the response variable using the chosen starch and peptone concentrations, a multiple variable linear regression analysis was performed on the data using Excel (Tools-Data Analysis-Regression) to find the steepest ascent. The coded variable with the largest magnitude of slope was chosen as the parameter for which to pick an increment. Based on this selected increment, the increment in the other coded variable was calculated assuring that its movement was in the same direction as the steepest ascent. This vector in the coded variables was then converted back to increments in carbon and nitrogen amounts, and the 2 parameters were incremented simultaneously to measure the response (32,33).

The starch and peptone concentrations that gave the highest response variable in the first set of experiments were used as the initial point for the second round of regression analysis. Since the response variable gets closer to the absolute maximum after each regression step, smaller increments were used for the process variables. The statistical design procedure continued until there was no longer a significant increase in the response.

Results and discussion

Identification of bacterium

A comparison of the similarities and differences in growth and phenotypic characteristics between *Halomonas* sp. strain AAD21 and other closely related *Halomonas* species (34-36) is given in Table 1. The newly isolated strain was oxidase-negative, catalase-positive, and did not reduce nitrate to nitrite. The strain grew at salt concentrations in the range of 3%-20% (w/v) sodium chloride, with optimum growth in the presence of 10% sodium chloride. There was no growth above 20% (w/v) sodium chloride or in the absence of sodium chloride. Cells grew at temperatures between 10 °C and 45 °C and at pH values between 6.5 and 8.5. Under anaerobic conditions, no growth was detected for 7 days of incubation at 37 °C.

Table 1. A comparison of the growth and phenotypic characteristics between strain AAD21 and closely related Halomonas species.

Characteristic	*Halomonas* sp. AAD21	**H. salina* DSM 5928^{T}	**H. halophila* DSM 4770^{T}
Morphology	Rod	Short rod	Short rod
Motility	+	–	+
Exopolysaccharide production	+	+	–
Pigmentation	Dark pink	Yellow cream	Cream
Tyrosine pigment	-	–	+
Oxidase	-	+	+
Salt range (%)	3-20	2-20**	2-25**
Temperature range	10-45	4-45**	4-45**
pH range	6.5-8.5	5-10**	5-10**
Acid from:			
D-Glucose	+	–	+
Maltose	+	–	+
D-Mannose	+	–	+
Hydrolysis of:			
Tween 20	+	+	–
Tween 80	-	–	–
Nitrate reduction	-	**v**	+
Growth on:			
L-Arabinose	+	+	+
D-Xylose	+	+	+
D-Melezitose	**ND**	+	+
Lactose	+	–	–

*Data are from Garcia et al. (34) and Mata et al. (35)
**Data are from Arahal et al. (36)
ND: no data available, v: variable

Comparative 16S rRNA gene sequence analyses showed that the strain AAD21 was related to type strain *Halomonas halophila* DSM 4770^{T} (34). The phylogenetic tree constructed using the neighbor-joining method indicated that strain AAD21 is a member of genus *Halomonas*. Strain AAD21 formed a clade with *H. halophila* DSM 4770^{T} with a bootstrap value of 100% (Figure 1). The phylogenetic tree constructed using the maximum-parsimony method showed essentially the same relations (Figure 2).

α-Amylase production

Time profiles of enzyme activity and growth are shown in Figure 3. Maximum enzyme production was measured after 48 h of cultivation. *Halomonas* sp. strain AAD21 started α-amylase synthesis in the beginning of the exponential growth phase, which continued until the late stationary phase. Further cultivation caused total α-amylase production to drop sharply. Similar results were reported by Coronado et al. (14) and Prakash et al. (4). They found that α-amylase synthesis in *Halomonas meridian* (14) and *Chromohalobacter* TVSP 101 (4) started during the exponential growth phase and that the highest value was reached during the stationary phase. Amoozegar et al. (16) reported α-amylase production by a similar microorganism, *Halobacillus* sp. strain MA-2, and stated that the production of this enzyme started at the mid-log phase and continued into the stationary phase.

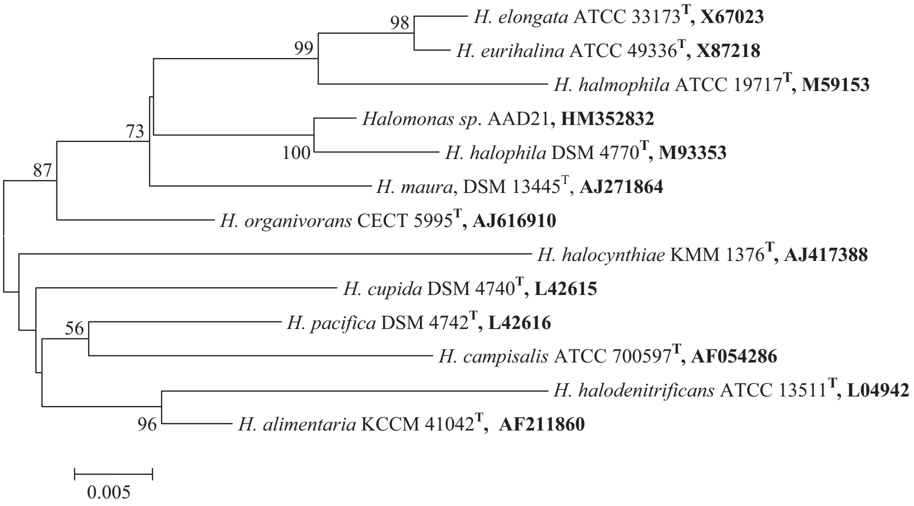

Figure 1. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain AAD21. Bootstrap values are shown as percentages of 1000 replicates. Only values above 50% are shown.

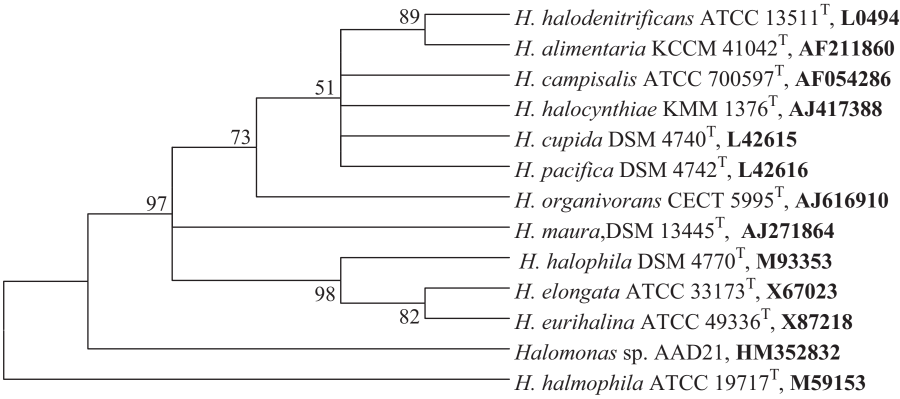

Figure 2. A maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain AAD21. Bootstrap values are shown as percentages of 1000 replicates. Only values above 50% are shown.

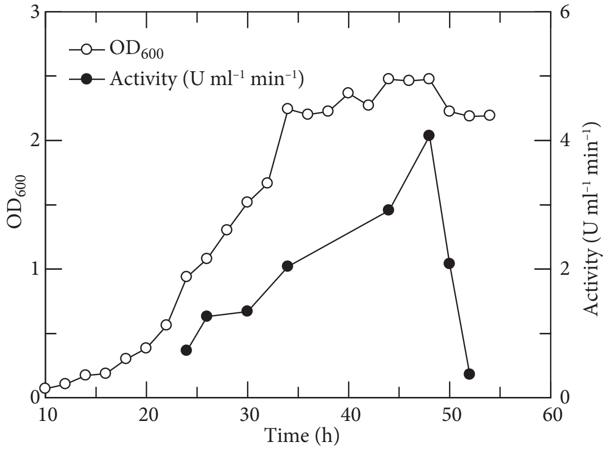

Figure 3. Time profile of growth and α-amylase production.

Since strain AAD21 requires sodium chloride for growth, the effect of sodium chloride concentration on the production of α-amylase was investigated. The highest α-amylase production was measured when cells were cultivated in the presence of 20% sodium chloride (Figure 4). Based on its sodium chloride requirement to survive, *Halomonas* sp. AAD21 was identified as a moderately halophilic microorganism. Amoozegar et al. (16) and Coronado et al. (14) reported similar results for *Halobacillus* sp. strain MA-2 and *Halomonas meridiana*, respectively.

To determine the best carbon source for α-amylase production, strain AAD21 was cultivated for 48 h in fermentation media containing different

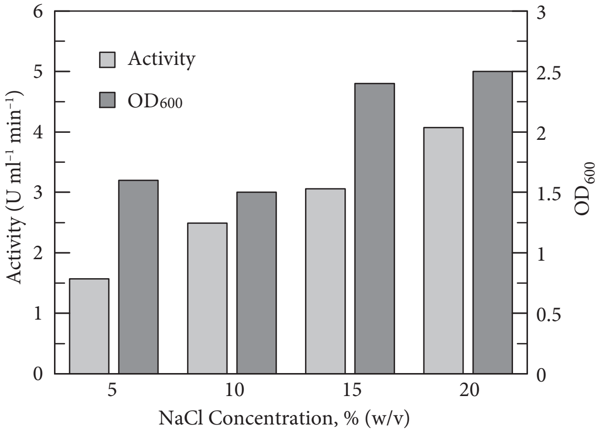

Figure 4. The effect of sodium chloride concentration on growth and α-amylase production.

carbon sources and enzyme activity was measured. Amoozegar et al. (16) investigated α-amylase production by *Halobacillus* sp. strain MA-2 in the presence of starch, dextrin, maltose, sucrose, lactose, and glucose and found that α-amylase production was induced by all of the carbon sources tested. However, the best carbon source for *Halobacillus* sp. strain MA-2 to produce α-amylase was dextrin, and the second best carbon source was starch. Prakash et al. (4) tested glucose, maltose, lactose, mannitol, and potato starch as α-amylase inducers in *Chromohalobacter* TVSP 101. They reported that only maltose, starch, and starch containing flours induced α-amylase production in this microorganism. The findings of Prakash et al. (4) and Amoozegar et al. (16) were taken as the basis to select carbon sources for α-amylase production by the new isolate. Since Shukla and Kar (37) proposed potato peel as a suitable and inexpensive carbon source, potato peel was also tested for α-amylase production. Strain AAD21 produced α-amylase from starch, sucrose, lactose, and potato peel. The relative amounts of α-amylase produced in the presence of these 4 different carbon sources are shown in Figure 5. There was no measurable α-amylase activity when cells were grown in media containing maltose or glucose. With the results obtained, starch was selected as the best carbon source and potato peel was selected as the second best carbon source for α-amylase production by strain AAD21.

In order to determine the best nitrogen source for α-amylase production, the effect of different organic and inorganic nitrogen sources were tested in media with starch as the carbon source. The addition of inorganic nitrogen, ammonium nitrate, sodium nitrate, and ammonium sulfate reduced α-amylase production by the moderately halophilic strain AAD21. This is consistent with the finding of Gupta et al. (5), who reported that organic nitrogen sources were preferred for amylase production. Maximum α-amylase production was achieved with peptone supplement as the nitrogen source (Figure 6). A similar finding was reported by Patel et al. (38). They found that peptone was a good nitrogen source for α-amylase secretion in *Halobacterium salinarium*.

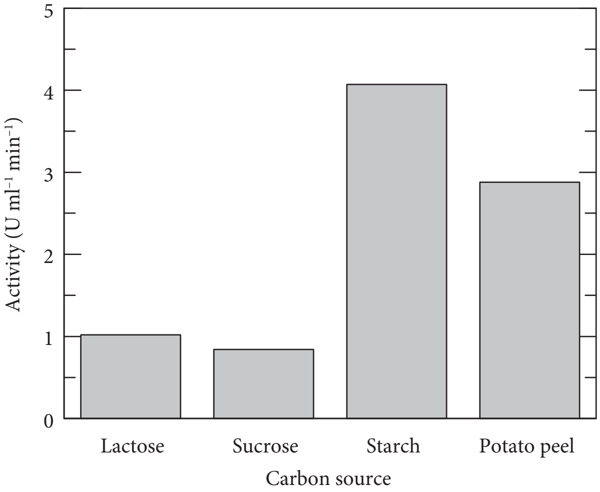

Figure 5. The effect of different carbon sources on α-amylase production.

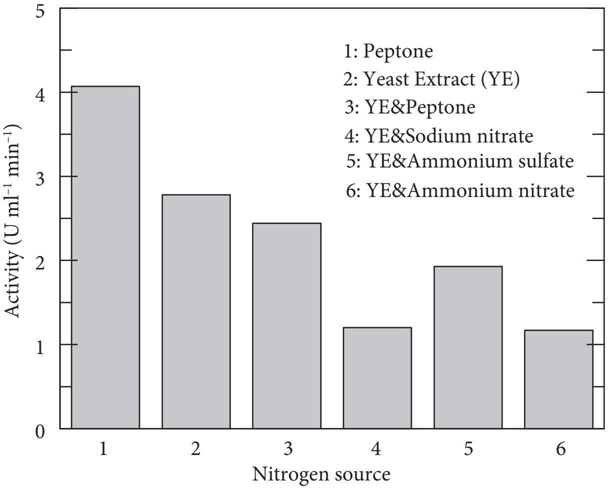

Figure 6. The effect of nitrogen sources on α-amylase production.

A final value of 4.07 U mL^{-1} min^{-1} of activity was obtained from *Halomonas* sp. strain AAD21 in fermentation medium containing (w/v) 1% soluble starch, 0.5% yeast extract, 0.3% trisodium citrate pentahydrate, 2% magnesium sulfate heptahydrate, 0.2% potassium chloride, and 20% sodium chloride at a pH of 7.0.

Medium optimization by statistical design

In the majority of fermentation studies, the basic objective of performing an optimization of a medium composition is to maintain a proper balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. The application of statistical experimental design techniques in the fermentation process development can result in improved product yields, reduced process variability, closer confirmation of the output response to nominal and target requirements, and reduced development time and production economics (18). The advantage of statistical design is that it minimizes the number of necessary runs when it is expensive and/or time-consuming to change parameters in experiments. For this reason, a statistical design procedure was used to increase the total amount of α-amylase produced by strain AAD21.

The initial medium used for the statistical optimization was (w/v) 1% soluble starch, 0.5% yeast extract, 0.3% trisodium citrate pentahydrate, 2% magnesium sulfate heptahydrate, 0.2% potassium chloride, and 20% sodium chloride at a pH of 7.0. Starch and peptone concentrations were used as coded variables in order to obtain maximum production.

The results of the first round of regression analysis showed that increasing starch concentration from 10 g L^{-1} to 35.8 g L^{-1} and peptone concentration from 5 g L^{-1} to 10.6 g L^{-1} increased the total obtainable α-amylase from 4.07 to 19.08 U mL^{-1} min^{-1} (Table 2). After the second round of regression analysis, total production increased to 23.28 U mL^{-1} min^{-1} with 40.7 g L^{-1} of starch and 10.1 g L^{-1} of peptone (Table 2). Finally, with application of the statistical analysis for the third time, a total of 26.25 U mL^{-1} min^{-1} of enzyme production was achieved in a medium containing 10.01 g L^{-1} peptone and 41.145 g L^{-1} starch (Table 2). Further regression analysis did not improve

Table 2. Statistical design analysis.

	Original parameters		**Response**	**Coded variables**	
	Starch	***Peptone***	***y***	***x1***	***x2***
First round of statistical analysis					
Op. point	**10**	**5**	**4.074**	**0**	**0**
	12	6	7.73	1	1
	12	4	5.64	1	−1
	8	6	4.01	−1	1
	8	4	3.61	−1	−1
Min.	8	4		−1	−1
Max.	12	6		1	1
Range	4	2		2	2
Mid-value	10	5		0	0
Second round of statistical analysis					
Op. point	35.8	10.6	19.08	0	0
	36.8	11.1	20.43	1	1
	36.8	10.1	22.37	1	−1
	34.8	11.1	19.67	−1	1
	34.8	10.1	18.67	−1	−1
Min.	34.8	10.1		−1	−1
Max.	36.8	11.1		1	1
Range	2	1		2	2
Mid-value	35.8	10.6		0	0
Third round of statistical analysis					
Op. point	40.7	10.1	23.28	0	0
	41.2	10.3	24.77	1	1
	41.2	9.9	24.68	1	−1
	40.2	10.3	19.09	−1	1
	40.2	9.9	22.96	−1	−1
Min.	40.2	9.9		−1	−1
Max.	41.2	10.3		1	1
Range	1	0.4		2	2
Mid-value	40.7	10.1		0	0

total enzyme yield. Overall, this statistical design approach helped to enhance α-amylase production almost 7-fold.

Characterization of the α-amylase: optimum pH, temperature, and salinity

The pH profile of the α-amylase produced by strain AAD21 is shown in Figure 7a. The enzyme was active in the pH range of 4.0-7.0. In contrast to this finding, the activity range of the α-amylase from the moderately halophilic *Halobacillus* sp. strain MA-2 was between the pH values of 7.5-8.5 (16). The optimum pH for the α-amylase produced by *Halomonas* sp. AAD21 was found to be 6.5. Coronado et al. (14) found the optimum pH value for α-amylase production from a similar microorganism, *Halomonas meridiana*, to be 7.0. Bozic et al. (7) reported a similar pH optimum as in our findings for α-amylase from *Bacillus licheniformis* ATCC 9945a.

Coronado et al. (14) reported that the optimum temperature of the α-amylase produced by *Halomonas meridiana* was 37 °C, and Amoozegar et al. (16) reported that the optimum temperature for α-amylase production from *Halobacillus* sp. MA-2 was 50 °C. Similar to the findings of Wang et al. (8) and Amoozegar et al. (16), the optimum temperature for α-amylase production from *Halomonas* sp. AAD21 was found to be 50 °C, as shown in Figure 7b.

The effect of sodium chloride concentration on α-amylase activity is shown in Figure 7c. Salt concentration significantly affects enzyme activity. While amylases from *Chromohalobacter* sp. TVSP101 (4), *M. halobius* (10), and *H. meridiana* (14) were active in a broad range of salt concentrations, α-amylase from strain AAD21 was active with only between 0% and 10% sodium chloride. In the presence of 2% sodium chloride, enzyme activity was 1.7 times greater (6.91 U mL^{-1} min^{-1}) than in the absence of sodium chloride (4.07 U mL^{-1} min^{-1}). As the sodium chloride concentration gradually increased above 2%, α-amylase started to lose its activity.

Thermal stability of AAD21 α-amylase

The thermal stability of AAD21 α-amylase is shown in Figure 8. Crude α-amylase from strain AAD21 was highly stable after 2 h of incubation at 50 °C and 60 °C. Following 2 h of incubation at 70 °C and 80 °C, it lost only 5% and 10% of its original activity, respectively.

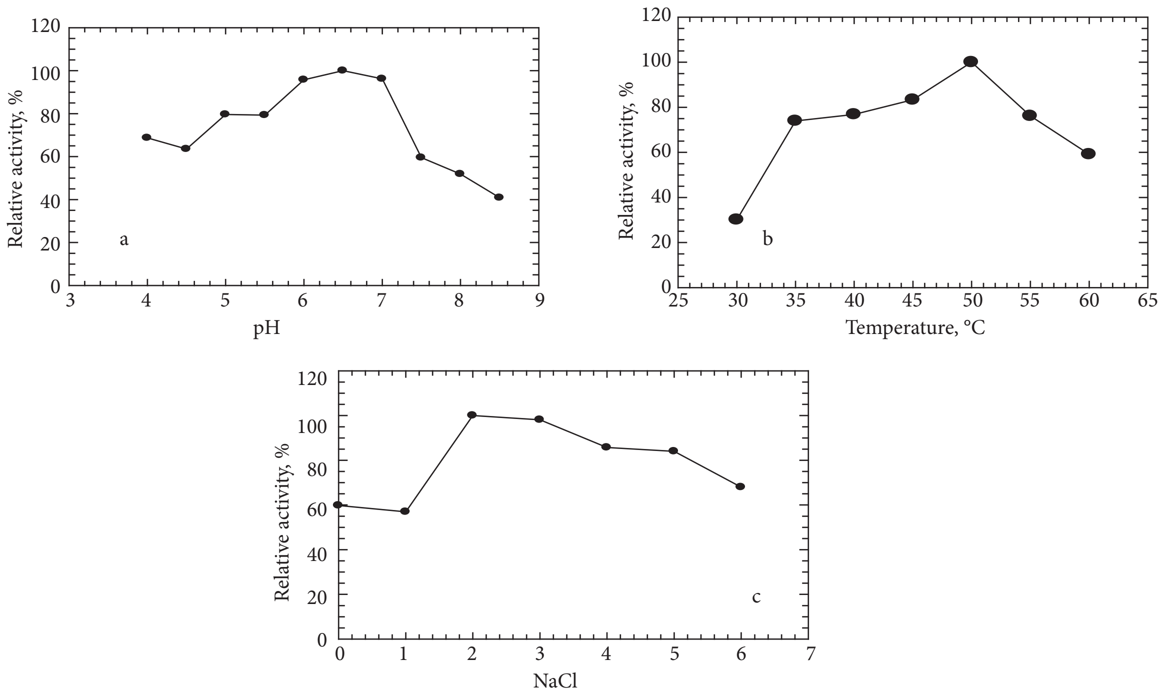

Figure 7. The effect of pH (a), temperature (b), and NaCl (c) on α-amylase activity.

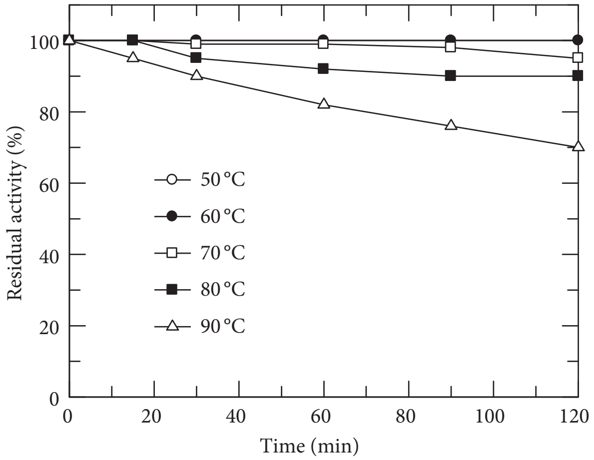

Figure 8. Thermal stability of AAD21 α-amylase.

Table 3. Comparison of the thermal stability of α-amylase from AAD21 with those from the literature.

Organism	Temperature range (°C)	Residual activity (%)	Temperature optimum (°C)	Reference
***Halomonas* sp. AAD21**	**35-60**	**70 (90 °C for 2.0 h)**	**50**	**This work**
Chromohalobacter sp. *TVSP 101*	30-85	50 (80 °C for 1.0 h)	65	(4)
Moderately halophilic *Bacillus* sp. TSCVKK	40-70	10 (55 °C for 5 min)	55	(6)
H. meridiana	25-95	20 (65 °C for 15 min)	37	(14)
Halobacillus sp. MA-2	10-70	50 (70 °C for 30 min)	50	(16)
Lactobacillus manihotivorans	50-60	70 (50 °C for 1.0 h)	55	(39)
Bacillus sp. I-3	65-100	50 (80 °C for 2.5 h)	70	(39)
Thermobifida fusca NTU22	50-60	70 (60 °C for 3 h)	60	(39)
Cryptococcus flavus	50-60	60 (60 °C for 60 min)	50	(39)
Aspergillus tamarii	50-60	90 (65 °C for 3 h)	55	(39)
Scytalidium thermophilum	55-65	50 (55 °C for 25 min)	60	(39)

It retained 70% of its original activity after 2 h of incubation at 90 °C. Comparing the thermal stability of the α-amylase from strain AAD21 with others cited in the literature (Table 3), the temperature range reported for strain AAD21 is significantly higher than those reported for α-amylases from halophilic microorganisms (4,6,14,16) and for other microorganisms studied (39). The thermostability of α-amylase is desirable in a number of processes, such as the thinning and liquefaction of starch.

Based on the above findings, it can be concluded that the α-amylase from *Halomonas* sp. strain AAD21 has its own merits in terms of thermostability; hence, it is a good candidate to be used for the thinning and liquefaction of starch.

Acknowledgments

This work was supported financially by the Marmara University Research Foundation through Project FEN-YLS-290506-0115, BSE-071/131102, and the Turkish Scientific and Technological Research Council (TÜBİTAK) through Project TBAG 103T069.

Corresponding author:
Dilek KAZAN
Bioengineering Department, Engineering Faculty,
Marmara University, Göztepe Campus,
34722 Kadıköy, İstanbul - TURKEY
E-mail: dkazan@marmara.edu.tr

References

1. Ventosa A, Nieto JJ, Oren A. Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 62: 504-544, 1998.
2. DasSarma S, Kennedy SP, Berquist B et al. Genomic perspective on the photobiology of Halobacterium species NRC-1, a phototropic, phototactic and UV-tolerant haloarcheon. Photosynth Res 70: 3-17, 2001.
3. Gomes J, Steiner W. The biocatalytic potential of extremophiles and extremozymes. Food Technol Biotechnol 42: 223-235, 2004.
4. Prakash B, Vidyasagar M, Madhukumar MS et al. Production, purification and characterization of two extremely halotolerant, thermostable, and alkali-stable α-amylases from *Chromohalobacter* sp. TVSP 101. Process Biochem,44: 210-215, 2009.
5. Gupta R, Gigras P, Mohapatra H et al. Microbial α-amylases: a biotechnological perspective. Process Biochem 38: 1599-1616, 2003.
6. Kiran KK, Chandra TS. Production of surfactant and detergent-stable, halophilic, and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK. Appl Microbiol Biotechnol 77: 1023-1031, 2008.
7. Bozic N, Ruiz J, Lopez-Santin J et al. Production and properties of the highly efficient raw starch digesting α-amylase from a *Bacillus licheniformis* ATCC 9945a. Biochem Eng J 53: 203-209, 2011.
8. Wang SL, Liang YC, Liang TW. Purification and characterization of a novel alkali-stable α-amylase from *Chryseobacterium taeanense* TKU001, and application in antioxidant and prebiotic. Process Biochem 46: 745-750, 2011.
9. Onishi H, Hidaka O. Purification and some properties of amylase produced by a moderately halophilic *Acinetobacter* sp. Can J Microbiol 24: 1017-1023, 1978.
10. Onishi H, Sonada K. Purification and some properties of an extracellular amylase from moderate halophilic *Micrococcus halobius*. Appl Environ Microbiol 38: 616-620, 1979.
11. Kobayashi T, Kamekura M, Kanlayakrit W et al. Production, purification and characterization of an amylase from the moderate halophile *Micrococcus varians* subspecies *halophilus*. Microbios 46: 165-177, 1986.
12. Onishi H. Halophilic amylase from a moderately halophilic *Micrococcus*. J Bacteriol 109: 570-574, 1972.
13. Khire JM. Production of moderately halophilic amylase by newly isolated *Micrococcus* sp. 4 from a salt-pen. Lett Appl Microbiol 19: 210-212, 1994.
14. Coronado M, Vargas C, Hofemeister J et al. Production and biochemical characterization of an α-amylase from the moderate halophile *Halomonas meridiana*. FEMS Microbial Lett 183: 67-71, 2000.
15. Deutch CE. Characterization of a salt tolerant extracellular α-amylase from *Bacillus dipsosauri*. Lett Appl Microbiol 35: 78-84, 2002.
16. Amoozegar MA, Malekzadeh F, Malik KA. Production of amylase by newly isolated moderate halophile *Halobacillus* sp. strain MA-2. J Microbiol Methods 52: 353-359, 2003.
17. Mukherjee AK, Borah M, Rai SK. To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. Biochem Eng J 43: 149-156, 2009.
18. Rao RS, Kumar CG, Prakasham RS et al. The Taguchi methodology as a statistical tool for biotechnological applications: a critical appraisal. Biotechnol J 3: 510-523, 2008.
19. Grant WD, Kamekura M, McGenity TJ et al. Class III. Halobacteria, Order I. Halobacteriales. In: Garrity GM. ed. Bergey's Manual of Systematic Bacteriology. Vol 1: The Archaea and the Deeply Branching and Phototrophic Bacteria. Springer; 2001: pp. 294-334.
20. Gerhardt P, Murray RGE, Costilow RN et al. Manual of Methods for General Bacteriology. American Society for Microbiology. Washington, D.C.; 1981.
21. Prescott LM, Harley JP, Klein DA. Microbiology. Wm. C. Brown. Dubuque, IA, USA; 1993.
22. Gonzalez C, Gutierrez C, Ramirez C. *Halobacterium vallismortis* sp. nov. An amylolytic and carbohydrate-metabolizing, extremely halophilic bacterium. Can J Microbiol 24: 710-715, 1978.
23. Brenner DJ, Krieg NR, Staley JT et al. eds. Bergey's Manual of Determinative Bacteriology, Vol. 2. Williams and Wilkins. Baltimore; 1986.
24. Altschul SF, Madden, TL, Schaffer AA et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402, 1997.
25. Tamura K, Dudley J, Nei M et al. MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599, 2007.
26. Thompson JD, Gibson TJ, Plewniak F et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882, 1997.
27. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequence. J Mol Evol 16: 111-120, 1980.
28. Kimura M. The Neutral Theory of Molecular Evolution. Cambridge University. Cambridge; 1983.
29. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791, 1985.

30. Mehrotra S, Pandey PK, Gaur R et al. The production of alkaline protease by a *Bacillus* species isolate. Bioresour Technol 67: 201-203, 1999.
31. Bernfeld P. Amylases, alpha and beta. Methods Enzymol 1: 149-158, 1955.
32. Dobrev GT, Pishtiyski IG, Stanchev VS et al. Optimization of nutrient medium containing agricultural wastes for xylanase production by *Aspergillus niger* B03 using optimal composite experimental design. Bioresour Technol 98: 2671-2678, 2007.
33. Wejse PL, Ingvorsen K, Mortensen KK. Xylanase production by a novel halophilic bacterium increased 20-fold by response surface methodology. Enzyme Microb Technol 32: 721-727, 2003.
34. García MT, Mellado E, Ostos JC et al. *Halomonas organivorans* sp. nov., a moderate halophile able to degrade aromatic compounds. Int J Syst Evol Microbiol 54: 1723-1728, 2004.
35. Mata A, Canovas JM, Quesada E et al. A detailed phenotypic characterisation of the type strains of *Halomonas* species. System Appl Microbiol 25: 360-375, 2002.
36. Arahal DR, Vreeland RH, Litchfield CD et al. Recommended minimal standards for describing new taxa of the family *Halomonadaceae*. Int J Syst Evol Microbiol 57: 2436-2446, 2007.
37. Shukla J, Kar R. Potato peel as a solid state substrate for thermostable α-amylase production by thermophilic *Bacillus* isolates. World J Microbiol Biotechnol 22: 417-422, 2006.
38. Patel S, Jain N, Madamwar D. Production of α-amylase from *Halobacterium halobium*. World J Microbiol Biotechnol 9: 25-28, 1993.
39. Sivaramakrishnan S, Gangadharan D, Nampoothiri KM et al. α-Amylases from microbial sources - an overview on recent developments. Food Technol Biotechnol 44: 173-184, 2006.