PROGRESS IN BIOLOGICAL SCIENCES

Production of a halothermotolerant α-amylase from a moderately halophilic *Nesterenkonia* strain F.

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Abstract

Production of extracellular amylase was demonstrated under conditions of high salinity in aerobically cultivated culture of a newly isolated moderately halophilic Gram-positive coccus,designated strain F in basal medium containing Peptone from meat, Yeast extract, NaCl (7% w/v) and starch. Biochemical and physiological characterization along with 16S rRNA sequence analysis placed F in the genus *Nesterenkonia*. The enzyme production was synchronized bacterial growth and reached a maximum level during the early-stationary phase in the basal medium. Maximum yield production was observed when the soluble starch, yeast extract and NaCl (1%, 0.75% and 10% w/v, respectively) were used in the fermentation medium with pH 8.0, inoculated with 7% v/v) pre-culture medium and incubated in 30°C for 72 h with aeration of 250 rpm (299.25 U/ml).
The potential of different carbohydrates in the amylase production was in the order: starch> maltose>dextrin>fructose>lactose=glucose>sodium acetate>sodium citrate. The production yield in the presence of different salts was as follows: NaCl> KCl> $NaNO_3$> Na_2SO_4. Maximum activity for enzyme was assayed in 3.0 M NaCl, pH: 5-7, and 40°C. α-amylase hydrolyzed starch, dextrin and glycogen to form maltose, maltotriose and maltotetraose as major products, and has no effect on amylose and pullulan as substrates. These results suggest that the amylase secreted by *Nesterenkonia* sp. strain F is industrially important from the perspective of its tolerance to a broad temperature range and its high tolerance to a wide range of salt concentrations (0–3 M NaCl).

Keywords: amylase production, malto-oligosaccharide, α-amylase, *Nesterenkonia* sp. strain F, moderate halophile.

Introduction

Starch, the main component of maize, potatoes, rice, wheat and cassava, is stored in plant cells as storage material for the organism in the form of water insoluble granules (Synowiecki *et al*., 2007). α-amylase (endo-1,4-α-D-glucanhydrolase EC 3.2.1.1) is an extracellular enzyme which hydrolyses the α-D-1,4 glycosidic linkage of starch and release α-configuration oligosaccharides with varying length, α-limit dextrins, and branched oligosaccharides (Vihinen and Mantsala, 1989; Van der Maarel *et al*., 2002). Amylases can be derived from plants, animals, and microorganisms, but microbial amylases meet industrial demands because of economic bulk production capacity and simple manipulation to find desired properties (Gupta *et al*., 2003). Amylases constitute a class of industrial enzymes having approximately 25 % of the enzyme market (Rao *et al*., 1998). It has a wide range of applications in all industrial

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processes such as glucose and fructose syrup production, anti stalling agent in bakery, brewing and alcohol production, detergents, textile and paper desizing, pharmaceuticals and animal feed (Vihinen and Mantsala, 1989; Gupta, 2003). Halophiles are extremophilic microorganisms that live, grow, and multiply in highly saline environments, and could be a source of halophilic enzymes (Margesin *et al*., 2001).Moderately halophilic bacteria which grow optimally in media containing 3-15 % NaCl, produce halophilic exoenzymes that could be active over a wide range of salt concentrations even in the absence of salt and therefore have potential for use in various biotechnological processes (Ventosa, 1994). The addition of such enzymes in laundry and dishwashing detergents has been of great importance, because they can be used in processes in which the salt or metallic ion concentrations are variable and diverse from very low concentration to almost salt saturation(Ventosa, 1994; Adams, 1987). Amylase production has been reported in eubacterial moderate halophiles such as *Acinetobacter* (Onishi and Hidaka, 1978), *Micrococcushalobius* (Onishi and Sonoda, 1979), *Micrococcusvarians* subsp. *halophilus* (Kobayashi *et al*., 1986), other *Micrococcus* isolates (Khire, 1994; Onishi, 1972), and *Halomonasmeridiana* (Coronado *et al*., 2000). The amylase gene, amyA, was cloned, overexpressed, and purified from *Halothermothrixorenii*, a thermophilic and moderately halophilic anaerobic bacterium (Mijts and Patel, 2002). Among the *Bacillus* group, only two strains viz. halotolerant *Bacillusdipsosauri* (Deutch, 2002) and *Halobacillus* sp. strain MA-2 (Amoozegar *et al*., 2003) have been reported earlier.In the present study, Gram positive coccus, designated as *Nesterenkonia* sp. F, isolated from Aran Bidgol Lake, Kashan, Iran has been investigated, that was highly potent for extra cellular α-amylases production. Screening, production and optimization of extra cellular amylases conducted in this research leading to purification and characterization of two halophilic α-amylases with completely different biochemical properties (Shafiei *et al*., 2010, 2011).

Materials And Methods

Bacterial Strains, Culture Media And Growth Conditions

The strain studied in the present research, *Nesterenkonia* sp. F, was isolated from Aran Bidgol Lake, Kashan, Iran. The basal medium for the growth of the organism was a modification of Luria-Bertani (Gilboa *et al*., 1991) medium (gl^{-1}), peptone from meat, 10.0g; yeast extract, 10.0; NaCl, 70.0 and pH 7.5. Stock cultures were maintained in the same medium supplemented with 1.5 % (w/v) agar slants at 4°C. The amylase production primarily was screened using the solid medium described above containing 1% (w/v) soluble starch from potato. Starch hydrolysis was illustrated by flooding the agar plates with lugol's iodine solution and appearance of halo zone around the bacterial colonies (Amoozegar *et al*., 2003).

Identification of The Isolates

Morphological and physiological characterizations of the strain were studied in basal culture media containing 7 % (w/v) NaCl. Gram staining, catalase and oxidase activities, nitrate reduction, hydrolysis of esculin, Voges-Proskauer, methyl red and indole production were tested using methods described by Smibert and Krieg (Smibert, 1994). The utilization of carbon sources and acid production were conducted as recommended by Leifson (Leifson, 1963). Other physiological and biochemical tests were conducted as described previously (Ventosa *et al*., 1982; Quesada *et al*., 1984). Genomic DNA of the isolate was extracted and purified according to modified Marmur method described earlier (Ventosa *et al*., 1982). The 16S rRNA genes were amplified using 8F (5′AGAGTTTGATYMTGGCTCAG3′) and 1492R (5′GGTTACCTTGTTACGACTT3′) universal primers. The PCR reaction was performed by initial denaturation at 96°C for 5 min, followed by 35 cycles including 94°C for 60 s, 56°C for 90 s, 72°C for 60 s and final extension in 72°C for 10 min. The purified PCR products were sequenced in both directions using an automated sequencer by SeqLab Laboratory (Germany). The phylogenic relationship of the isolate was determined by comparing the

sequencing data with sequences of related *Nesterenkonia* and *Micrococcus* available in GenBank database of the NCBI.

Basal Medium And Enzyme Production

The preculture medium was prepared of basal growth medium containing 1% (w/v) soluble starch, sterilized at 121°C for 15 min and inoculated with 1.5×10^8 cfu/ml of the fresh culture of the isolate and incubated at 34°C for 24 h with aeration level of 220 rpm.
For α-amylase production, 100 ml Erlenmeyer flasks containing 20 ml of the basal medium was inoculated 1 % (v/v) with preculture and incubated in 34°C for 72 h under aerobic condition (250 rpm). All experiments were performed in triplicate each in three repeats. Bacterial growth was determined by measuring the optical density at 600 nm (OD_{600}).

Analytical Assays

Cell-free supernatant were collected after centrifugation (10000×g) for 20 min at 4°C and used for further assays. α-Amylase activity normally was assayed in a 1.0 ml reaction mixture consisting of 0.5 ml of cell-free prepared supernatantand 0.5 ml of starch solution in the 20 mM phosphate buffer (pH 7.0) and incubated at 40°C for 30 minutes. The amount of reducing sugar was measured by 3,5-dinitrosalicilic acid (DNS) according to Bernfeld method (1955). One unit of α-amylase activity was defined as the amount of the enzyme releasing from 1 μmol of maltose per minute under standard assay conditions.

Effect of NaCl, PH And Temperature On Amylase Production

The strain F was grown at pH 7.5 and 1 % (w/v) soluble starch with different NaCl concentrations (0, 5, 7.5, 10, 15, 20, 25, and 27 %). The effect of initial pH of the medium was tested under standard condition by growing strain F with 10 % (w/v) NaCl at different pH from 4.0 to 10.0 using 100 mM sodium citrate (pH 4.0-5.0), sodium phosphate (pH 6.0-8.0), Tris–HCl (pH 8.0–9.0), glycine-NaOH (pH 9.0-10.0), in the basal medium. Amylase production was studied at different temperatures 20, 25, 30, 35, and 40°C under standard condition (Ventosa *et al*., 2005).

Effect of Carbon And Nitrogen Sources; And Salts On Amylase Production

Effect of different carbon sources including glucose, fructose, maltose, lactose, dextrin, soluble starch, sodium acetate and citrate, were evaluated at concentrations of 0.5, 1.0, and 2.0 % (w/v) on amylase production via substitution of soluble starch in the production medium by them. Yeast extract and peptone 0-1.0 % (w/v) was tested and the optimized nitrogen source was substituted by different organic (casein, urea, soy peptone, meat extract, gelatin) and inorganic (ammonium chloride and ammonium sulfate) nitrogen sources.
Effect of salts on growth and amylase production was tested by substitution of NaCl by KCl (1-4 M), Na_2SO_4 (0.5-2 M), and $NaNO_3$ (0.5-3.5 M).

Effect of NaCl, PH And Temperature On Crude Enzyme

Cell-free supernatant of culture broth of the strain F was used as the crude enzyme solution. The stability of the enzyme in presence of different NaCl concentrations was determined by dialyzing the crude enzyme overnight against 20 mM phosphate buffer, pH 7.0 containing various concentrations of NaCl (0–4.5 M) at 4°C. The residual activity was determined under standard assay conditions.
The optimum pH was determined under standard assay conditions, with 1% soluble starch prepared in appropriate buffers at the concentration of 100 mM in the range of 4.0-10.0. The buffers used were sodium citrate (pH 4.0-5.0), sodium phosphate (pH 6.0-8.0), Tris–HCl (pH 8.0–9.0), glycine-NaOH (pH 9.0-10.0). To determine the optimum temperature for the amylase activity, the assay was performed at various temperatures ranging from 20°C to 60°C (Adams, 1987).

Analysis of Hydrolytic Products of Amylase

Soluble starch, dextrin, glycogen, amylose and pullulan (all provided by Merck, Germany) as substrate solution in 1 % (w/v) phosphate buffer (pH 7.0) and cell-free supernatant were incubated

at 40°C for 24 h, and the reaction of the mixture was stopped by boiling at 100°C in a water bath. The mixture was applied to silica gel thin-layer chromatogram (TLC) plates (Merck, Germany). The chromatogram was developed with butanol/acetic acid/water (3:1:1) and detected by spraying diphenylamine/aniline/phosphoric acid 85 % and heating at 100°C for 5 min. Maltooligosaccharide kit (Sigma, St. Louis, Mo, USA) containing G1 to G6 was used as standard samples for TLC (Shannon and Creech, 1969).

Result

Characteristics of The Amylase Producing Bacteria

Several strains of moderately halophilic bacteria secreting amylase were isolated from Aran Bidgol Lake, Kashan, Iran. Strain F, showed prominent clear zones around the colonies on starch plates. Considering the physiological, biochemical and molecular studies performed, the strain was tentatively named as ‘‘*Nesterenkonia* sp. strain F. The phylogenetic tree (Fig. 1) constructed by the neighbor-joining method indicated that the isolate F was part of the cluster within the genus *Nesterenkonia*. Among the described species, the closest relative of isolate F was *Nesterenkonia halobia* with 99 % similarity.

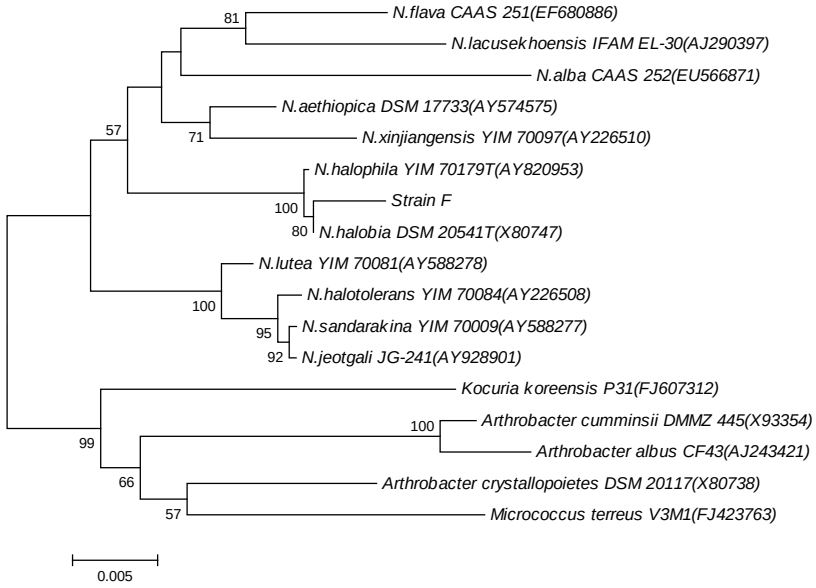

Figure 1. Neighbor-joining tree showing the position of isolate F among members of the halotolerant and halophilic genus *Nesterenkonia*. Numbers on branch nodes are bootstrap values shown as percentages of 1000 replications. Bootstrap values greater than 50% are indicated. Bar, 0.5% sequence divergence.

Optimization of Culture Conditions For Amylase Production

The NaCl concentration, pH, and temperature at which higher amylase production was recorded in broth, were taken to be optimal culture conditions.

NaCl

The obligate halophilic nature of the strain was obvious from the fact that it did not grow in the absence of NaCl (Fig. 3). Maximum growth and amylase production (272.6 U/ml) was obtained at 10% concentration of NaCl in 72 h and decreased above or below this concentration.

PH

The organism grew in a wide range of initial pH from 5.0 to 10.0 (Fig. 2). The highest amylase production, 216.3 U/ml, was observed in 72 h at pH 8.0.

Temperature

Growth and amylase production (299.25 U/ml) were highest in 72 h at 30°C and decreased at 25 and 35°C (Fig. 4).

Carbon And Nitrogen Source And Salts

The growth of the strain F showed an increase in presence of glucose, fructose, and lactose, but amylase was induced at low levels suggesting the inducible nature of the amylase. Maltose 0.5-1.0 % (w/v) produced amylase at near maximum showing a potentially good substrate. Among the polysaccharides tested, soluble starch 1.0 % (w/v) induced highest amylase production followed by dextrin 1.0 % (w/v) (Table. 1). Addition a combination of yeast extract and peptone as nitrogen sources resulted in most biomass production, while maximum amylase production of 231.3 Unit/OD_{600} was observed in the presence of solely yeast extract 0.75 %.

Among the different nitrogen sources tested soy peptone, meat extract, urea, NH_4Cl and NH_4SO_4 induced minimum amylase production. When there was no organic nitrogen source in the medium, accordingly there was no growth and amylase production (Table 2).

The effect of different salts other than NaCl on growth and amylase production was tested. $NaNO_3$ and Na_2SO_4 decreased growth and amylase production (Fig. 5). KCl as 2.0 M concentration produced amylase well but yet at lower levels than NaCl. Maximum amylase production of 255 U/ml was achieved at 2.0 M NaCl.

Enzyme Activity

Amylase activity rarely detected when there was no NaCl in the crude enzyme solution and maximum amylase activity (100%) was obtained with 3.0 M NaCl (Fig. 6). Activity showed an increase with NaCl concentrations from 1.5 to 3.0 M. In 4.5 M NaCl, 84% of the activity was retained. These results clearly indicated the extreme halophilic nature of the enzyme. The enzyme exhibited maximal activity (100 %) at pH 7.0, but the activity was optimally high at near acidic condition (pH, 5.0-6.0) suggesting the preference of activity in lower pH (Fig. 7). The amylase produced by the strain F showed activity within wide range 20–55°C with a maximum at 40°C, but reached zero at 60°C (Fig. 8).

Analysis of Hydrolytic Products

The hydrolytic products generated by the amylase over various incubation periods were analyzed through TLC. A number of spots presumably maltose, maltotriose and maltotetraose appeared at 30 min to 24 h without a change in hydrolysis pattern, while the concentration of the products increased as the reaction proceeded. The same pattern was illustrated for glycogen and dextrin, but the enzyme showed higher activity towards soluble starch than two latter's. Hydrolysis activity was not showed upon pullulan and amylase as substrates, indicating that the enzyme was only able to hydrolyze α-1-4 bonds but not α-1-6 (Fig. 9). These results provided an insight on the internal α-1-4 linkage degradation mode of the enzyme, therefore the enzyme could be considered as α-amylase.

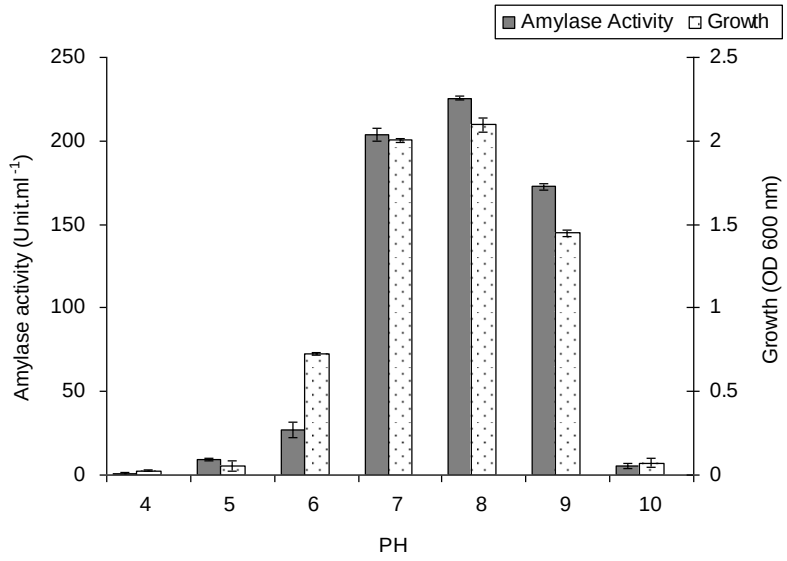

Figure 2. Effect of initial pH on growth and amylase production in the basal medium containing 10% (w/v) NaCl. 100mM citrate buffer (pH 4.0-5.0), 100mM sodium phosphate (pH 6.0-8.0), 100mM Tris–HCl (pH 8.0–9.0), and 100mM glycine-NaOH (pH 9.0-10.0), in the basal medium were used. Values are the average of three independent experiments ± SD under standard condition.

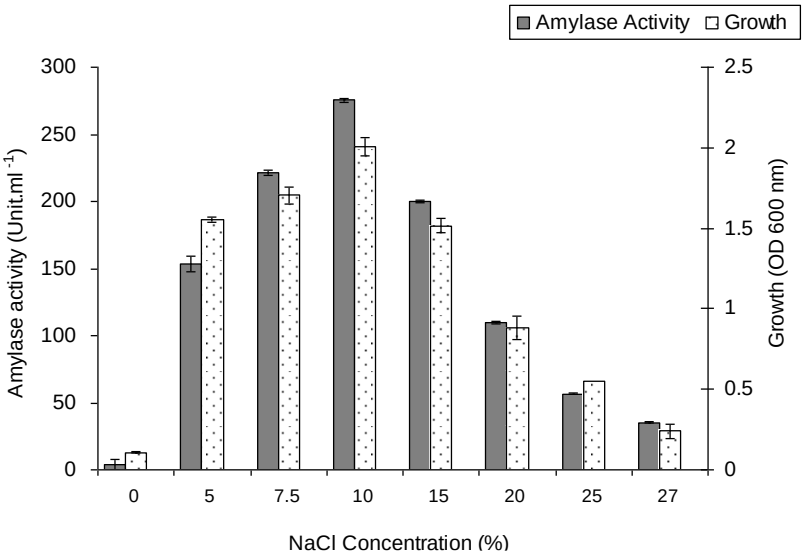

Figure 3. Effect of different NaCl concentrations on growth and amylase production. The culture was grown for production of amylase as described. Different concentrations of Sodium chloride were replaced in the basal medium. pH of the medium was adjusted to 7.5. Values are averages of three independent experiments ± SD.

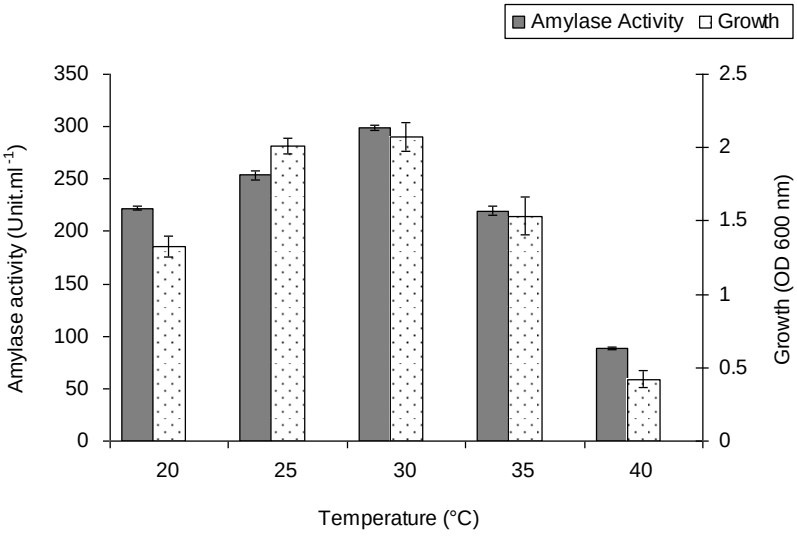

Figure 4. Effect of temperature on growth and amylase production in the basal medium containing NaCl 10% (w/v) and pH 8.0. Basal medium were incubated at different temperatures under standard aerobic condition. Values are the average of three independent experiments ± SD under standard condition.

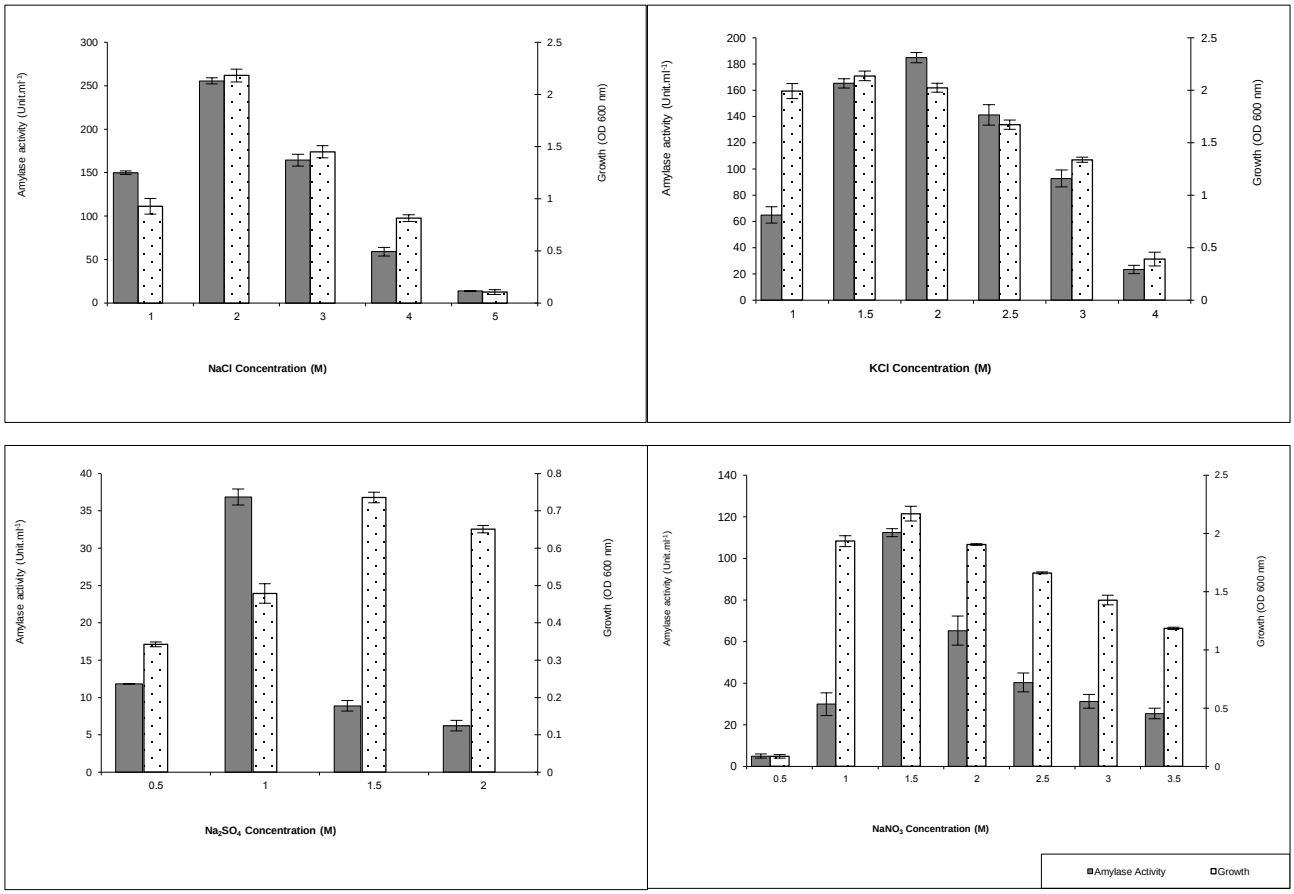

Figure 5. Effect of different salts on growth and amylase production. The culture was grown for production of amylase as described in "Materials and methods". Sodium chloride in the medium was replaced by the various salts in different concentrations. pH of the medium was adjusted to 7.5. Values are averages of three independent experiments ± SD.

Figure 6. Effect of NaCl concentrations on the amylase stability. For determining the stability, the crude enzyme solutionwas dialyzed against 20 mM phosphate buffer, pH 7.0 containing various concentrations of sodium chloride and the remaining activity was determined under standard assay. The relative activity was defined as the percentage of the maximum activity detected in each assay.

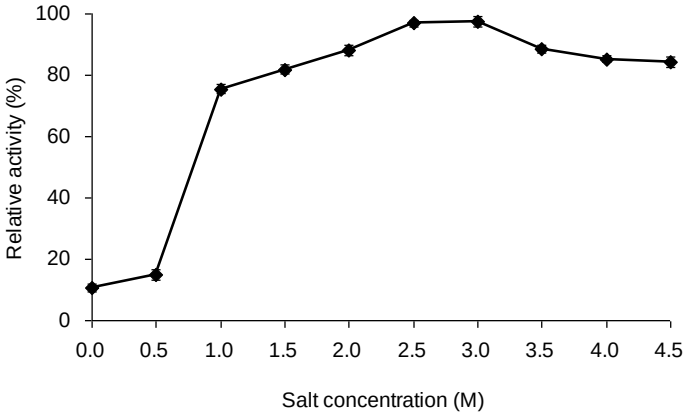

Table 1. Effect of various carbon sources on amylase production by *Nesterenkonia* sp. strain F.

Carbon sources (%)	Cell growth (O.D $_{600\ nm}$)	Activity (Unit/ml)	Amylase Production (Unit/ O.D $_{600nm}$)
Glucose			
0.5	1.4	96.9 ± 9.4	69.2
1.0	1.5	113.4 ± 3.8	75.6
2.0	1.6	39.5 ± 6.0	24.7
Fructose			
0.5	1.5	129.5 ± 6.3	86.3
1.0	1.6	132.4 ± 0.9	82.75
2.0	1.7	8.6 ± 0.6	5.1
Maltose			
0.5	1.4	249.4 ± 12.8	178.1
1.0	1.5	265.3 ± 4.3	176.9
2.0	1.5	133.8 ± 6.0	89.2
Lactose			
0.5	1.3	105.6 ± 5.1	81.2
1.0	1.4	115.0 ± 6.0	82.1
2.0	1.4	38.9 ± 9.5	27.8
Soluble Starch			
0.5	1.4	244.2 ± 11.2	174.4
1.0	1.4	267.0 ± 4.2	191.0
2.0	1.6	163.5 ± 2.9	102.2
Dextrin			
0.5	1.4	208.2 ± 3.3	148.7
1.0	1.5	252.8 ± 10.2	168.5
2.0	1.5	197.1 ± 16.3	131.4
Sodium Acetate			
0.5	1.0	49.6 ± 4.3	49.6
1.0	1.0	52.4 ± 7.4	52.4
2.0	0.9	64.6 ± 5.3	71.8
Sodium Citrate			
0.5	1.1	25.7 ± 4.0	23.4
1.0	1.2	26.4 ± 3.3	22
2.0	0.7	18.8 ± 3.0	26.8

The culture was grown for production of amylase as described in "Materials and Methods". Soluble starch in the medium was replaced by other carbon sources as listed. Sugars were added in 0.5, 1, and 2% to the basal medium. pH of the medium was adjusted to 8.0. Values are the average of three independent experiments ± SD.

Table 2. Effect of various nitrogen sources on amylase production by *Nesterenkonia* sp. strain F.

Nitrogen sources (%)	Cell growth (O.D $_{600nm}$)	Activity (Unit/ml)	Amylase Production (Unit/ O.D. $_{600nm}$)
Peptone			
0.25	0.5	53.8 ± 5.6	107.6
0.5	0.7	78.0 ± 7.1	111.4
0.75	0.9	93.4 ± 6.6	103.8
1.0	1.2	169.6 ± 0.7	141.3
Yeast extract			
0.25	0.7	106.3 ± 7.0	151.8
0.5	1.1	129.1 ± 1.8	117.4
0.75	1.2	277.6 ± 0.1	231.3
1.0	1.3	265.3 ± 0.6	204.1
Peptone 1.0 + Y.E 1.0	1.5	261.6 ± 5.2	174.4
Gelatin 0.75	0.3	51.6 ± 9.9	172.0
Casein 0.75	0.8	154.0 ± 2.9	192.5
Meat extract 0.75	1.2	165.1 ± 3.1	137.6
Urea 0.75	0.5	81.5 ± 13.3	163.0
Ammonium chloride 0.75	0.2	4.1 ± 0.6	20.5

The culture was grown for production of amylase as described in " Materials and Methods". Peptone and yeast extract in the medium was replaced by other nitrogen sources as listed. Peptone and yeast extract were added in 0.25, 0.5, 0.75, and 1.0% to the basal medium, but other sources in 0.75%. pH of the medium was adjusted to 8.0. Values are the average of three independent experiments ± SD.

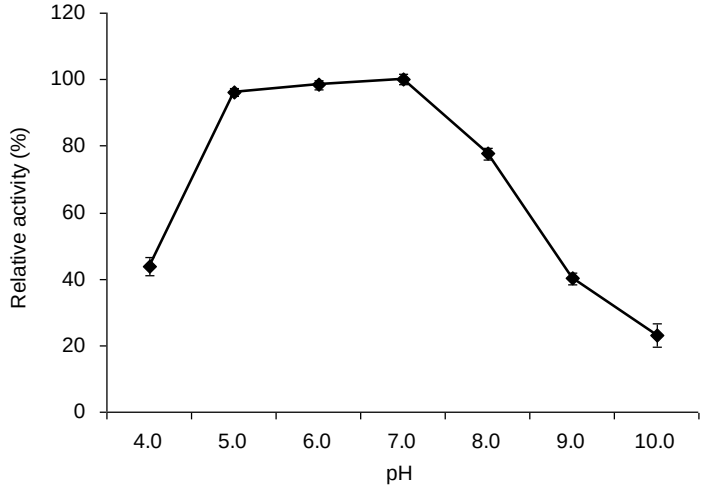

Figure 7. Effect of pH on the amylase activity. Relative activity was defined as the percentage of maximum activity detected in the assay.

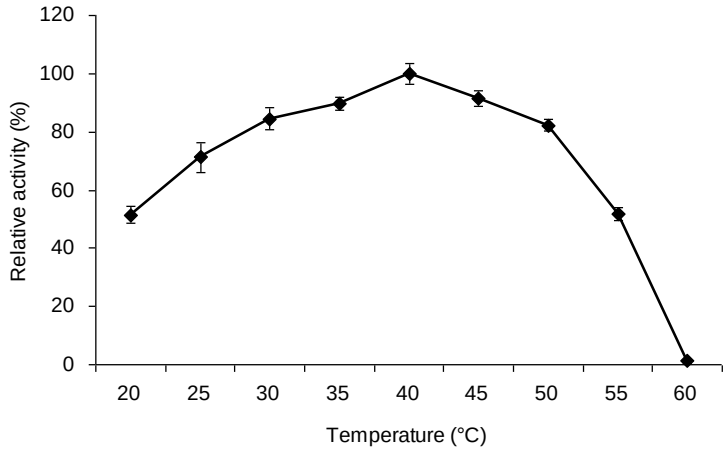

Figure 8. Effect of temperature on the activity of the amylase. Relative activity was defined as the percentage of maximum activity detected in the assay.

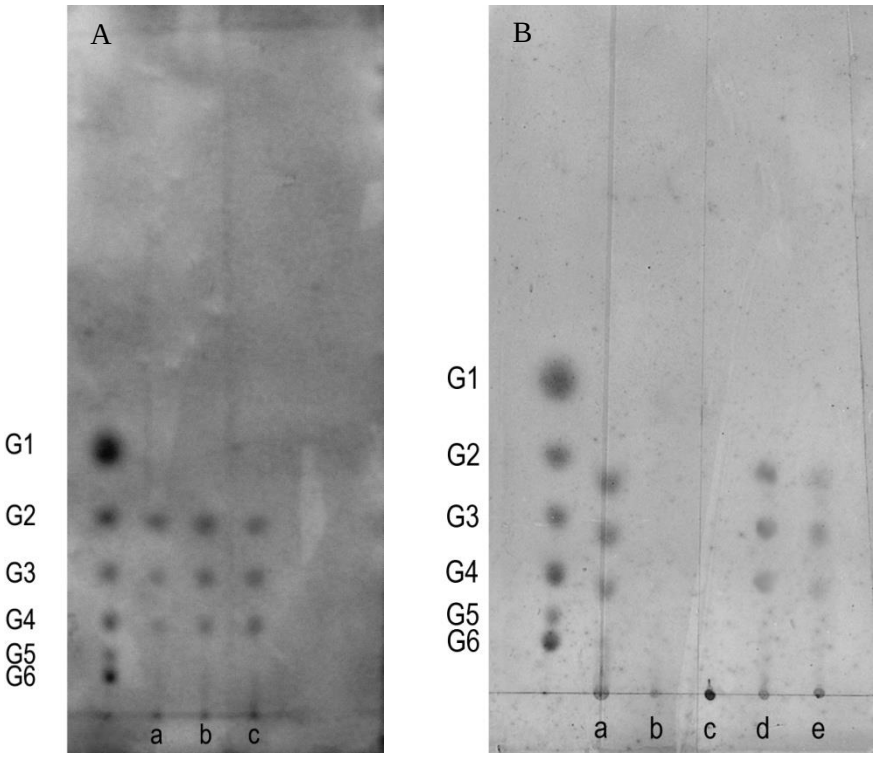

Figure 9. Thin-layer chromatography analysis of the products from starch hydrolysis at different reaction times (A) and hydrolysis pattern of other substrates after 24h by the amylase (B). **A.** Reaction products after 30 min (a), 60 min (b), and 1 h (c). **B.** Reaction products for soluble starch (a) compared to amylose (b), pullulan (c), dextrin (d), and glycogen (e). Malto-oligosaccharide kit indicates the standard sugars as follow:glucose (G1), maltose (G2),maltotriose (G3), maltotetraose (G4), maltopentaose (G5) and maltohexaose (G6).

Discussion

The adaptation and survival abilities of halophilic microorganisms in a wide range of salinities (0.5-2.5 %) offer potential applications in various areas of biotechnology. Among several bacterial isolates from Aran Bidgol lake, Kashan, Iran, isolate strain F was selected for further studies because it appeared to be the best producer of extracellular amylase in both liquid and solid media. On the basis of biochemical and partial 16S rRNA gene sequencing, the strain was identified as *Nesterenkonia* sp.

The strain F does not secrete amylase in a medium containing meat extract as nitrogen source and sodium chloride salt (in the absence of carbohydrates source), whereas maximum amylase production was achieved in the presence of 1 % of soluble starch. In *Micrococcus* strains 4, *Halobacillus karajensis* and *Bacillus* strain *TSCVKK* enzyme production in the presence of dextrins as a carbon source has been reported more than starch. This indicates that the expression of amylase production is inductive in this strain.

Similar results by other moderate halophilic bacteria has been reported which all of them are inductive enzymes that do not produce carbohydrates in the absence of carbon sources. A decrease in enzyme production was observed when glucose was added to the fermentation medium. This indicates that the synthesis of the amylase in the strain F is repressed by readily metabolizable substrate, glucose (Lin *et al*., 1998). Catabolic repression by glucose has been reported in Gram-negative as well as in Gram-positive bacteria and correlates either with gene repression or activation (Coronado *et al*., 2000). While TSCVKK amylase bacterium did not produced any amylase in the presence of a 1% concentration of glucose (Kanthi Kiran, 2008), the increase of amylase production by the strain F was observed when the concentration of glucose was increased from 0.5 to 1 %. This data indicates that the glucose is not a strong catabolite repressor for the amylase production by the strain. According to the higher production in the absence of peptone and higher efficiency in the concentration of 0.75 % yeast extract, the presence of yeast extract without peptone was appropriate for the design of the basic medium.Similar behavior was reported in *Bacillussubtilis*strain JS-2004 (Asgher *et al*., 2007). In contrast, the amylase production in TSCVKK was stimulated with the presence of complex nitrogen sources (Kanthi Kiran, 2008).

As shown, the salt strength of the bacteria medium is strongly effective on the growth and enzyme production in halophile strains. The highest production was observed in the 10% concentration, which is approximately equivalent to 0.2 M sodium chloride, and this result is similar to the other moderate halophiles. Production of extracellular amylase by strains *Halomonas meridiana* and *Halobacillus karajensis* isolated from saline soil in Iran (Amoozegar *et al*., 2003) and *Bacillus* strain TSCVKKis maximum in a medium containing 10 % NaCl. But the enzyme production has also been observed in 27 % NaCl concentration, which is unique between the moderate halophiles, and so far has not been reported. This result is in concordance with the physiological data of this strain, since it is able to grow in 30 % NaCl concentration. In the case of potassium chloride salt, the maximum growth rate and production was obtained in the 1.5 M potassium chloride concentration, but in other moderate halophile species, only *Acinetobacter sp*. and *Nesterenkonia halobia* have the highest amylase production in the presence of 1 to 2 molar NaCl or KCl, but in the rest, potassium chloride always reduces growth and enzyme production (Onishi and Hidaka, 1978; Onishi and Sonoda, 1979).

This strain is unable to grow in alkaline pH, and other enzyme activity data are also in consistent with these results. In acidic pH, in this bacteria, although the enzyme shows good activity, but the growth and thus the enzyme production are not reliable. Similar results on amylase production have also been reported in *Halobacillus karajensis* and *Bacillus* strain *TSCVKK*. Optimum amylase activity was observed in the pH range of 5.0 to 7.0 and the maximum activity was observed in pH 7.0. Due to the stability of the enzyme in pH 6.0 and the reduction of activity in pH higher than 8.0, this enzyme has been classified under the category of neutral-like acid-bearing enzymes, that causes limitations in its industrial applications in detergent industry. Similar features have also been reported earlier in *Halomonas meridian* and *Nesterenkonia*

*halobia*enzymes (Onishi and Sonoda, 1979; Coronado *et al*., 2000). Except for some amylases produced by different species of *Bacillus*, most α-amylases produced by non-halophilic microorganisms show an optimum activity in pH within the acidic range (5-7), being rather unstable at alkaline pH (Onishi and Sonoda, 1979; Coronado *et al*., 2000).

The highest growth and production was obtained in the temperature of 30°C, while the maximum enzyme activity was obtained in the temperature of 40°C. Together these results indicated that the influence of temperature on amylase production is related to the growth of the microorganism, while optimum temperature of the enzyme activity was independent of the microorganism growth temperature conditions. In the moderate halophiles, these results have been reported only in *Bacillus* strain TSCVKK and the rest had their maximum enzyme activity in the temperature of 50°C.

Degradation patterns of this enzyme are also independent of the time of treatment, and only spots intensity goes darker over time. In different periods before treatment of starch materials, from 30 min to 24 h, the pattern of degradation is the same and is mainly along with the release of maltose, maltotriose and maltotetraose.

In the case of amylase enzyme in *Halomonas meridiana* (Coronado *et al*., 2000)and *Bacillus* strain *TSCVKK* (Kanthi Kiran, 2008), starch and dextrin hydrolysis releases glucose along with maltose and malto-oligosacharides. This enzyme has no effect on pullulan and amylose, in other words this enzyme does not have the ability to break α (1-4) linkage in amylose and pullulan linear chains.

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