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Original Article

تولید و ارزیابی مشخصات آنزیم آلفا آمیلاز گرمادوست و نمک دوست جدا شده از باکتری *آنوکسی باسیلوس جونیسیس* AT23

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چکیده

سابقه و هدف: آلفا آمیلازهای گرمادوست کاربردهای بسیار زیادی در صنایع مختلف دارند. از این میان می توان به فرآوری نشاسته و تولید شوینده ها اشاره نمود. این مطالعه با هدف جداسازی باکتری های مولد آلفا آمیلاز گرمادوست و نیز تعیین خصوصیت آن ها انجام شد.

مواد و روش ها: پس از نمونه برداری از چشمه آبگرم گروه واقع در استان کرمان، به منظور جداسازی باکتری های مولد آلفا آمیلاز از محیط نشاسته آگار استفاده گردید. سویه مورد نظر به کمک توالی یابی ژن *16S rDNA* شناسایی گردید. خصوصیت آلفا آمیلاز گرمادوست در حضور عوامل مختلفی مانند pH دما، یون های فلزی و حلال های آلی تعیین شد. همچنین پارامترهای کینتیکی آنزیم در حضور غلظت های مختلف نشاسته تعیین گردید.

یافته ها: در این مطالعه سویه برتر مولد آلفا آمیلاز گرمادوست خارج سلولی به عنوان باکتری *آنوکسی باسیلوس جونیسیس* AT23 معرفی گردید. آنزیم آلفا آمیلاز سویه AT23 دارای فعالیت بهینه در pH های ۵ و ٦ بود. یک افزایش هفت برابری در فعالیت آنزیمی آلفا آمیلاز در حضور کلرید سدیم (غلظت ۳ مولار) مشاهده شد. یون های منگنز و روی فعالیت آنزیمی را به ترتیب حدود ۹۵ و ۳۱ درصد افزایش دادند. پارامترهای کینتیکی، *Km* و V_{max} به ترتیب ۱/٦٥٧ (میلی گرم بر میلی لیتر) و ۰/۰۰۵۹ (میلی گرم بر میلی لیتر بر دقیقه) تعیین گردید. همچنین فعالیت آنزیمی در غلظت ۱۰ درصد از حلال های آلی ان-بوتانول و سیکلو هگزانول حدود دو برابر افزایش یافت.

نتیجه گیری: نتایج به دست آمده نشان می دهد که آلفا آمیلاز سویه AT3 یک آنزیم نمک دوست و مقاوم در برابر است. بنابراین می تواند در صنایع مختلف مورد استفاده قرار گیرد.

واژگان کلیدی: *آنوکسی باسیلوس جونیسیس*، آلفا آمیلاز، گرمادوست، نمک دوست، چشمه آبگرم.

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Original Article

Production and characterization of a thermophilic and extremely halotolerant alpha-amylase isolated from *Anoxybacillus gonensis* AT23

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Abstract

Background & Objectives: Thermophilic alpha-amylase can be used in different industries such as starch processing and detergents. This study was performed to isolate alpha-amylase-producing bacteria and characterization of the enzyme.

Materials & Methods: After sample collection from Gorooh hot spring in Kerman province, Iran, thermophilic alpha-amylase- producing bacteria were isolated using the starch-agar medium. 16S rDNA sequencing was used to identify the bacterial strain. Characterization of the thermophilic alpha-amylase was performed in the presence of various factors such as pH, temperature, metal ions, chemical compounds, and organic solvents. Also, kinetic parameters of the enzyme were determined in different concentrations of starch.

Results: *Anoxybacillus gonensis* AT23 was identified as the best thermophilic alpha-amylase-producing strain. The alpha-amylase enzyme showed the optimal activity at pH 5 to 6. Sevenfold increase in the enzyme activity was observed in the presence of NaCl (3M). Mn^{2+} and Zn^{2+} increased the enzyme activity about 95% and 31%, respectively. Kinetic parameters including K_m and V_{max} were estimated about 1.657 mg/ml and 0.0059 mg/ml/min, respectively. Also, enzyme activity was also improved about 2 folds in the presence of organic solvents including n-butanol and 10% cyclohexane.

Conclusion: Our results indicated that AT23 alpha-amylase is a halophile and organic solvent-tolerant enzyme. Therefore, it can be used in different industries.

Keywords: *Anoxybacillus gonensis*, Alpha-amylase, Thermophilic, Halophile, Hot spring.

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Introduction

Alpha-amylases are the most important enzyme in industry, occupying about 30% of enzyme global market (1). They act on α (1-4) bonds in the linear chain of amylose (2).

Utilization of alpha-amylase in various industrial sectors such as starch processing, brewing, pharmacy, paper, biofuel, and detergents industries and also in bread making has been confirmed (3). Industrial applications of alpha-amylases in different fields should be compatible with their biochemical characteristics such as halophilicity, thermostability, and pH profile (4). The good activity of alpha-amylases at high salt concentrations has allowed those enzymes to be used in industries where the presence of solutions with high salt concentrations can avoid enzyme activity (5).

Thermal stability of alpha-amylases provides the possibility for the efficient application of the enzymes in the saccharification process of starch as a suitable alternative for acidic hydrolysis of starch (6). Thermostable alpha-amylases are normally synthesized by thermophile bacteria which are found in geothermal areas such as hot springs and deserts (6). These enzymes represent particular properties for industrial purposes as they display resistance toward denaturant agents such as detergents, chaotropic agents, organic solvents, and high pH (7).

The production of thermostable alpha-amylases during thermophilic fermentation is a beneficial technique from the point of the environmental target of views like reduction of further cooling costs and risk of microbial contamination, a better solubility of substrates, and less viscosity which increases mixing and pumping speed (8). Different *Bacillus* genus including *coagulans*, *stearothermophilus* and *candolyticus,* and a number of thermostable *Actinomyces* including *Thermomonospora* and *Thermoactinomyces* has been found to produce thermostable alpha-amylases (9).

The aim of this study was isolation, production, optimization and biochemical characterization of a thermophilic alpha-amylase whit suitable properties to apply in various industries. This is the first report on the production of thermophilic alpha-amylase with commercialized properties from *Anoxybacillus gonensis*.

Materials and methods

Screening of the microorganisms and culture conditions

In this study, sampling was performed from Gorooh hot spring in Kerman province, Iran to isolate thermostable alpha-amylase-producing bacteria. The water samples were cultivated on the starch agar medium (Merck, Germany) containing (w/v) 1.0% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.1% NaCl, 0.1% $MgSO_4.7H_2O$, and 0.02% $CaCl_2.6H_2O$. Plates were incubated at 50 °C for 72h (10).

The AT23 strain was selected as a good producer of thermostable alpha-amylase after flooding the plates with 1% Gram's iodine solution. Enzyme production was performed in the culture medium consisting (g/l) KH_2PO_4 1.0, $Na_2HPO_4.2H_2O$ 3.13, tryptone 2.0, $(NH_4)_2SO4$ 2.0, $MgSO_4.7H_2O$ 0.05, $CaCl_2.6H_2O$ 0.05, and soluble starch 1.0 (pH 7.0) (11).

Biochemical and molecular identification of

the selected isolate

For identification of this isolate, 16S rRNA fragment sequencing was carried out following by standard biochemical tests described in Bergey's Manual of Systematic Bacteriology. 16S rRNA fragment was amplified through verifying polymerase chain reaction (PCR) program using two general Forward (5′-AGTTTGATCCTGGCTCAG-3′) and Reverse (5′-GGCTACCTTGTTACGACT-3′) primers (12). Bacterial genomic DNA was extracted using phenol-chloroform method (13). Briefly, a PCR reaction contained 2.5 µl of PCR buffer (10X), 1 µl of $MgCl_2$ (10 mM), 0.5 µl of dNTPs mixed (20mM), 0.3 µl of each primer, 1-4 µl template DNA and 0.2 µl Tag DNA polymerase, in a total volume of 25 µl.

PCR amplifications were carried out under the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 94 °C for 45 min, annealing at 51 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 8 min. The sequence was used in Basic Local Alignment Search Tool (BLAST) to determine the homology of this isolate with the other reference strains (11).

Alpha-amylase activity

To determine the alpha-amylase activity, the reaction mixture including 0.5 ml of enzyme solution and 0.5 ml of soluble starch (1%) in 100 mM phosphate buffer (pH 7.0) was incubated at 50 °C for 20 min. The amount of reducing sugars was assayed by adding 1 ml of dinitrosalicylic acid (DNS) and measuring the absorbance at 540 nm in UV visible spectrophotometer (Biowave WPA) (14).

One unit of the alpha-amylase activity is the amount of alpha-amylase that released 1 µmol of glucose per minute under standard assay conditions.

Optimization of enzyme production conditions

Some effective factors on alpha-amylase production such as carbon and nitrogen sources, required metal ions and pH of the culture medium were tested in this research. Effect of various carbon sources on alpha-amylase production was tested by adding 0.1% of starch, glucose, galactose, maltose, and fructose into the basal medium, individually (15).

The basal medium was also supplemented by organic and inorganic nitrogen sources including yeast extract, gelatin, peptone, ammonium chloride, and sodium nitrate, with a concentration of 0.2% individually (15). To optimize the enzyme production of the medium, various ion components such as KCl, NaCl, $MgSO_4.7H_2O$, and $CaCl_2.6H_2O$ was added into the basal medium (free of metal ions), at the concentration of 0.1% (15). The suitable pH for alpha-amylase production was determined by setting up culture medium pH at 5.0, 6.0, 7.0, 8.0, and 9.0 (16).

In all cases, 5 ml of the culture medium containing the grown bacterium was harvested after 48h of incubation, and then centrifuged at 10000 rpm and 4 °C for 10 min. The obtained supernatant was used for alpha-amylase assay as described above.

Partial purification of the alpha-amylase

- Anion exchange chromatography:

To determine molecular mass of AT23 alpha-amylase, *Anxybacillus gonensis* AT23

was cultured in the optimal medium containing 0.5% starch at 50 °C. After centrifugation at 1000 rpm at 4 °C for 10 minutes, supernatant was passed through a Q-sepharose column pre-equilibrated with 50 mM phosphate buffer at pH of 7.8. To elute the purified protein, the column was washed using a linear gradient of NaCl (0.0-5.0 M) in the same buffer at a flow rate of 1.0 ml/min, and fractions were gathered to assess protein content (at 280 nm), and alpha-amylase activity (at 540 nm) (12).

SDS-PAGE and Zymography of alpha-amylase

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli method (17). Activity staining (zymogram) was carried out in non-denatured gel containing 10% polyacrylamide, and 1% soluble starch. Alpha-amylase activity was confirmed by keeping the gel in KI/I2 solution and observing clear bands in dark background (11).

Biochemical characterization of the alpha-amylase:

- Effect of various pHs on the enzyme activity and stability

pH activity and stability of AT23 alpha-amylase was investigated in pH range of 3.0-12.0. For this investigation, various buffers (50 mM) including glycine (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), tris (pH 8.0-10.0) and glycine (pH 11.0-12.0) were used. 0.5 ml of each buffer was added to the mixture containing 0.5 ml of enzyme solution, and 0.5 ml of soluble starch (1%) and then incubated at 50 °C for 20 min (18). To assess alpha-amylase stability, AT23 alpha-amylase was pre-incubated in the same buffers for 1 h at room temperature. Then, the residual activity was measured under standard assay conditions at 540 nm (19).

- Effect of temperature on the enzyme activity and stability

The effect of temperature on enzyme activity was studied by incubating 0.5 ml of the enzyme with 0.5 ml of soluble starch (1%) in 100 mM phosphate buffer (pH 7.0) at different temperatures (30-80 °C). Thermal stability was also evaluated by assaying the residual activity of enzyme solution pre-incubated for 1h in the mentioned temperatures at the standard conditions (15). Thermal inactivation of the enzyme was also investigated at 50 °C and 60 °C for 3h. The enzyme was pre-incubated with and without 20 mM $CaCl_2.6H_2O$ at the relevant temperatures and the sample was picked up at 30 min intervals. Then, the residual activity was assayed in the presence of soluble starch as substrate at standard assay conditions (19).

- Effect of metal ions, chelators and surfactants on enzyme activity

The activity of AT23 alpha-amylase in the presence of metal ions was measured under standard conditions by incubation of a mixture of 0.5 ml of enzyme solution, 0.5 ml of 1% starch soluble in phosphate buffer, and 0.5 ml of each metal ions at a final concentration of 4 mM. Metal ion salts used were including of $FeSO_4$, $CuSO_4$, $CoCl_2$, $ZnSO_4$, $MnSO_4$, $MgSO_4$, $CaCl_2$, $HgCl_2$, and KCl (7).

Enzyme assay was also carried out in the

presence of chemical compounds including EDTA and 2-mercaptoethanol as chelating agents, H_2O_2 as an oxidizing agent, as well as SDS and Triton X-100 as a surfactant in the same way (19-21). The sample activity with no metal ions or chemical compounds was considered as 100%.

- *Effect of detergents on the enzyme activity*

The effect of detergents on AT23 alpha-amylase activity was determined after the incubation of enzyme solution with 1% starch and 1% solution of solid detergents including Kaf, Shooma, Dioxigeneh, Tage, Darya, Barf, and Banoo in sodium phosphate buffer (50 mM, pH 7.0) under standard assay conditions (7). Enzyme activity of the control (detergent-free) was assumed as 100%.

- *Effect of salt concentration on the enzyme activity*

To test the effect of salt concentration on the enzymatic activity, different concentrations of NaCl, ranging from 0.0 M to 4.0 M (w/v) were incorporated into the mixture of 1% starch and enzyme solution, and the activity was measured at the standard conditions (5).

- *Effect of organic solvents on the enzyme activity*

Alpha-amylase activity in the presence of organic solvents was measured under standard assay conditions by incubating the mixture of 0.5 ml enzyme solution, 0.5 ml substrate (1% starch in 100 mM sodium phosphate buffer (pH 7)), and 0.5 ml various organic solvents including isopropanol, 1-botanol, dimethylformamide, dimethyl sulfoxide (DMSO) (miscible solvents), diethyl ether, cyclohexane, chloroform, and toluene (immiscible solvents), each at a final concentration of 10% (7, 21). All organic solvents were prepared in the sodium phosphate buffer (pH 7, 50 mM), and the pH was finally adjusted to the 7.0.

Determination of kinetic parameters

Starch concentration is one of the effective factors in alpha-amylase activity. The enzyme activity was assayed in the presence of different starch concentrations in the range of 0.5-7.0 mg/ml at standard conditions. The results were interpreted by estimating *K*m and V_{max} values from the Lineweaver-Burk plots.

Statistics analysis

All experimental data are means ± standard deviation (SD) of triplicate determinations. Graphs are drawn from Microsoft Excel and Sigma plot software.

Results

Identification of the isolated strain

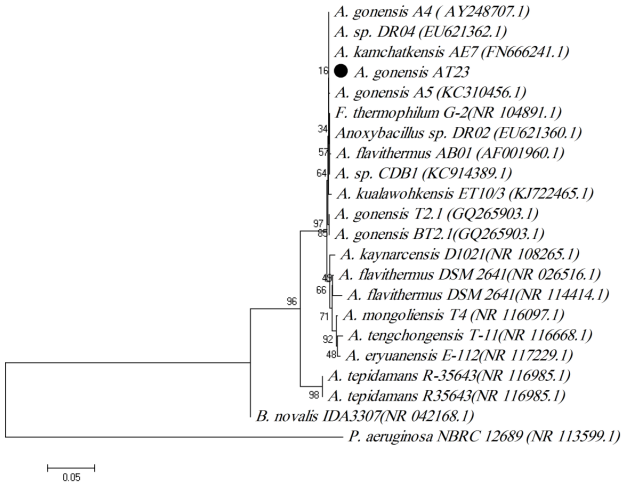

Fig.1. The phylogenetic tree of AT23 isolate and related strains, based on the 16S rDNA sequence.

Screening results of alpha-amylase-producing bacteria on agar-plate showed that AT23 isolate produces the highest clear halo around the colony after 72 h of incubation at 50 °C.

Biochemical and physiological tests and microscopic morphological characteristics showed that this strain is a member of *Bacillus* sp.. More particular characterization by 16S rRNA fragment sequencing explained that this strain is closely related to *Anoxybacillus gonensis* which is deposited in Genbank with the accession number of KT933139. Fig. 1 shows the phylogenic tree of AT23 isolate designed with MEGA4.

Optimization of the alpha-amylase production

In this research, alpha-amylase production was investigated in the presence of various carbon sources including starch, glucose, maltose, fructose and galactose. Except glucose, the enzyme production had an increase in presence of all sources, with an optimum yield (57 U/ml) using soluble starch (Table 1). AT23 also could produce alpha-amylase at media containing various nitrogen sources, but adequate production was achieved by peptone (Table 1). The effect of metal ions on AT23 alpha-amylase production was investigated by assaying alpha-amylase activity in basal media with different metal ions. As shown in Table 1, the most AT23 alpha-amylase production level was measured in divalent ion Ca^{2+}.

The desirable culture pH to produce alpha-amylase by *Anoxybacillus gonensis* AT23 was determined by changing media pH within the range of 5.0 to 9.0. The maximum yield of AT23 alpha-amylase was observed at pH 6.0 and 7.0. The optimum culture medium for alpha-amylase production by *Anoxybacillus gonensis* AT23 was determined after cultivating the isolates in the basal medium containing 0.1% starch and also a culture medium in which all optimum medium

Table 1. Production of AT23 alpha-amylase in the presence of different supplements including carbon and nitrogen sources, metal ions and pHs.

Supplement	Activity (U/ml)
Carbon sources:	
Glucose	14±0.6
Galactose	23±1.2
Maltose	50±1.3
Fructose	44±0.4
Soluble starch	57±0.4
Control	16±0.2
Nitrogen sources:	
Peptone	55±0.3
Yeast extract	21±0.2
Gelatin	30±0.1
$NaNO_3$	27±0.2
NH_4Cl	20±0.2
$(NH4)_2SO4$	22±0.3
Control	23±0.2
Metal ions:	
$CaCl_2$	5±0.1
$MgSO_4$	4.5±0.11
KCl	1.7±0.13
NaCl	1±0.2
Control	5±0.08
Different pHs:	
pH 5.0	57±0.01
pH 6.0	77.6±0.01
pH 7.0	77±0.2
pH 8.0	67±0.2
pH 9.0	54±0.3

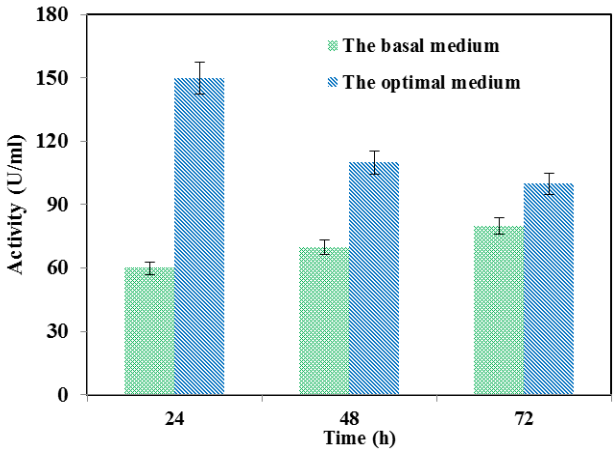

Fig. 2. Investigation of the alpha-amylase production rate by AT23 isolate in the basal culture medium containing 0.1 % soluble starch and a culture medium composed of 0.5% soluble starch, 0.2% peptone and 0.1% Ca^{2+} (pH 6.0). The enzyme assay was performed after 24, 48 and 72h of incubation at 50 °C.

conditions including 0.5% starch, 0.2% peptone, 0.1% $CaCl_2.6H_2O$ (pH 6.0) was met at 50 °C for 72 h.

Fig. 3. Analysis of AT23 alpha-amylase activity by zymography in non-denatured gel.

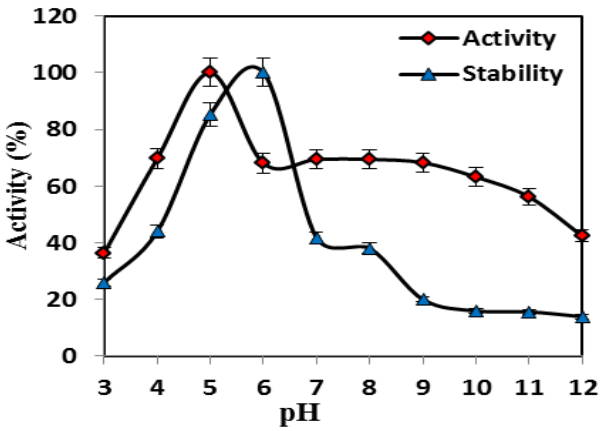

Fig. 4. Influence of the different pH on the activity and stability of AT23 alpha-amylase. Enzyme activity was assayed in the concentration of 100 mM of proportionate buffer systems: glycine (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris (pH 8.0-10.0), glycine (pH 11.0 and 12.0) after 20 min of incubation at 50 °C. pH stability profile was determined using pre-incubation of the enzyme solution in the presence of the same buffers for 1h at room temperature.

After each 24 h interval, 5 ml of both media was harvested and used to measure alpha-amylase activity. Our results in Fig. 2 shows that the higher level of alpha-amylase is secreted in the optimum medium than basal medium, especially after 24 h of incubation.

Partial purification of the alpha-amylase enzyme

The alpha-amylase from AT23 was partially purified by Q-Sepharose column chromatography and serial concentrations of NaCl (0-0.5 M). Fig. 3 shows activity staining of AT23 alpha-amylase in a non-denaturing gel (1%). Clear regions in the dark background of the gel exhibit alpha-amylase activity.

Characterization of AT23 alpha-amylase:

- Effect of pH

Activity and stability of AT23 alpha-amylase were tested in the pH ranges of 3.0-12.0 using the buffer solutions. Fig. 4 shows that the optimum activity and stability of AT23 alpha-amylase is at pH 5.0 and pH 6.0, respectively. It was also found that the enzyme stability slightly declined in alkaline pH of 7.0-11.0, while enzyme activity was kept to more than 60% in this condition.

- Effect of temperature

The results showed that optimum temperature for the enzyme activity and stability was obtained at 50 °C, while a significant decrease in the enzyme activity and stability was observed at temperatures of 60-80 °C. Apart from this, AT23 alpha-amylase showed 68-87% stability at 30°C and 40 °C, while it

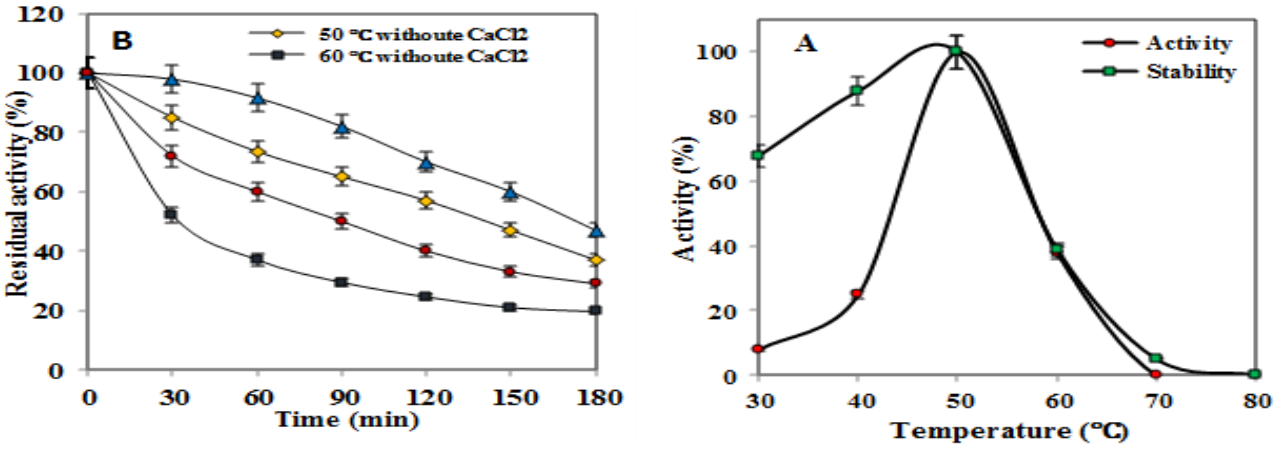

Fig. 5. A) The effect of different temperatures (30-80 °C) on alpha-amylase activity and stability in AT23 strain. Thermal stability of the enzyme was determined after pre-incubation of the enzyme at temperatures ranging from 30 to 80 °C for 1h. Residual activity of the enzyme was measured under standard assay conditions using 1% soluble starch as substrate. B) Thermo-inactivation of AT23 alpha-amylase at 50 and 60 °C in the presence and absence of 20 mM $CaCl_2.6H_2O$. To assess the remaining activity under standard conditions, enzyme solution was incubated at 50 °C and 60 °C, with and without 20 mM $CaCl_2$ for 3h and sample was picked up at 30 min intervals. Remaining enzyme activity at zero time was assumed as 100%.

was active only by 7.0-24.0% in these temperatures (Fig. 5b).

Results of thermo-inactivation in the presence and absence of 20 mM $CaCl_2$ (Fig. 5b) showed that enzyme stability diminishes by increasing the incubation temperature. 20% improvement in thermal stability of AT23 alpha-amylase in the presence of Ca^{2+} after 60 min incubation at 50 °C. Also an increase in its half-life from 30 to 90 min at 60 °C in the presence of Ca^{2+}, explain positive effect of this ion on the stability of the alpha-amylase.

- Effect of metal ions and chemical additives on the enzyme activity

Table 2 shows enzyme activity of AT23 in the presence of metal ions and chemical additives. Not only enzyme activity was maintained in the presence of all tested ions, but also a considerable increasing effect was found in the presence of some of them, as Mn^{2+} resulted in a two-fold increase in alpha-amylase activity.

Table 2. Activity of AT23 alpha-amylase on various metal ions, inhibitors and surfactants.

Metal ions and chemical additives	Activity (%)
Fe^{2+}	105±0.2
Cu^{2+}	113±0.3
Co^{2+}	124±0.5
Zn^{2+}	132±0.6
Mn^{2+}	195±0.5
Ca^{2+}	97±0.2
Hg^{2+}	107±0.2
K^{+}	103±0.4
Mg^{2+}	96±0.2
T.X-100	100±0.4
2-Mercaptoethanol	133±0.1
H_2O_2	103±0.1
SDS	82±0.4
EDTA	107±0.2
Control	100±0.1

Table 3. Activity of alpha-amylase from *Anoxybacillus gonensis* AT23 on some of commercial liquid detergents.

Detergents	Activity (%)
Barf	110±0.6
Shooma	111±0.5
Tage	117±0.5
Banoo	90±0.4
Dioxigeneh	90±0.3
Darya	103±0.1
Kaf	107±0.2
Control	100±0.2

Detergent activity was investigated by incubation of the enzyme in absence and presence of each detergent under standard assay conditions. Activity in absence of the detergents (control sample) was considered to be 100%.

Beside, AT23 alpha-amylase well maintained its activity against protein-denaturing agents including EDTA (107), Triton-X-100 (100) and SDS (82). Enzymes activity was calculated as percent of activity compared to control sample.

- *Effect of solid commercial detergents on the enzyme activity*

The activity of AT23 alpha-amylase was assayed in the presence of some commercial laundry detergents including Kaf, Shooma, Di-oxigeneh, Taghe, Darya, Barf, and Banoo under standard conditions. The results are represented in Table 3 as the percent of alpha-amylase activity compared to the control sample (detergent-free). The enzyme activity showed a slight decrease in (10%) in the presence of Banoo and Di-oxigeneh, while enzyme activity was retained and even increased dramatically in the other detergents.

- *Effect of organic solvents*

AT23 alpha-amylase activity was investigated in the presence of 10% water miscible (isopropanol, 1-butanol, methanol, dimethyl-formamide and dimethylsulfoxide) and water immiscible solvents (toluene, cyclohexane, chloroform and diethyl ether), and also phosphate buffer (100 mM, pH 7) as the control. Enzyme activity in the presence of organic solvents are presented in Table 4.

Among the mentioned organic solvents, the least alpha-amylase activity was observed in

Table 4. Activity of AT23 alpha-amylase in the presence of a number of organic solvents (water miscible and water immiscible).

Organic solvents	Activity (%)
No ion	100±0.1
water miscible solvent:	
Isopropanol	141±0.1
1-botanol	220±0.4
DMF	35±0.3
DMSO	163±0.2
water immiscible solvent:	
Cyclohexane	197±0.1
Chloroform	206±0.6
Toluene	131±0.2
Diethyl ether	88±0.5
Methanol	96±0.5

Activity of the control (organic solvent- free) was taken as 100%.

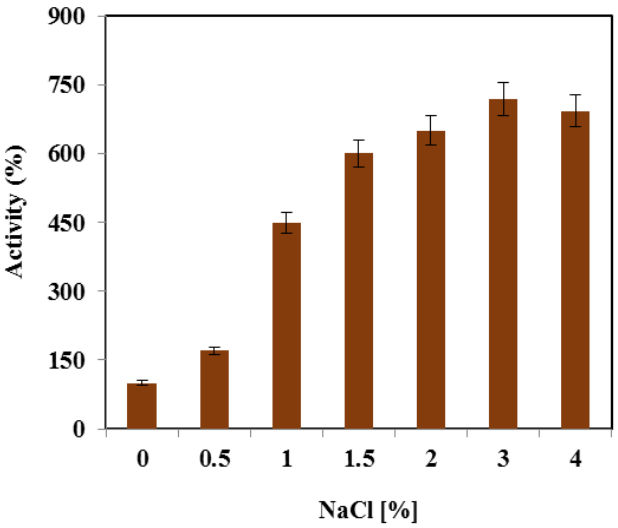

Fig. 6. Salt activity profile of AT23 alpha-amylase. Determination of enzyme activity in different concentrations of NaCl was performed by incubating AT23 alpha-amylase in the presence of 0.0-4.0 M of NaCl salt in standard assay conditions.

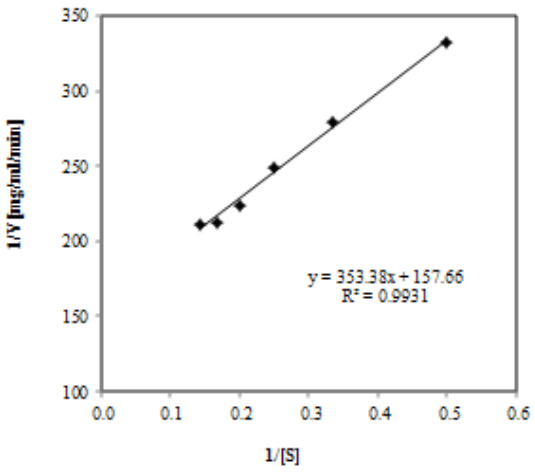

Fig. 7. Line weaver-Burk plot of AT23 alpha-amylase to determine *K*m and V_{max} values. The activity of alpha-amylase was assayed using different concentrations of starch in standard assay conditions.

dimethylformamide, with 35% reduction in enzyme activity. In contrast, the other organic solvents including 1-botanol, chloroform, cyclohexane, dimethylsulfoxide, and toluene resulted in very excellent increase in the enzyme activity, stimulating enzyme activity by 120%, 106%, 97%, 63%, and 31%, respectively. Diethyl ether and methanol had no significant influence on AT23 alpha-amylase activity.

- *Effect of salt concentration on alpha-amylase activity*

The activity of AT23 alpha-amylase was checked in the presence of 0.0-4.0 M NaCl in standard assay conditions. According to the summarized results in Fig. 6, alpha-amylase activity was improved in all NaCl concentrations. Enzyme activity achieved to more than sevenfold at 3 M NaCl concentration as compared to the control (NaCl-free).

Determination of kinetic parameters of the enzyme

Kinetic studies of AT23 alpha-amylase was carried out by assessment of enzyme activity in the presence of 0.0-7.0 mg/ml soluble starch as the substrate. The Michaelis constant (*K*m) and V_{max} values obtained from a Lineweaver-Burk plot (Fig. 7) were 1.657 mg/ml and 0.0059 mg/ml/min, respectively.

Discussion

In present study, we isolated and identified *Anoxybacillus gonensis* AT23, as a thermophilic alpha-amylase producer, from Gorooh hot spring in Kerman. Since the addition of a carbon source such as soluble starch into alpha-amylase medium is essential for its production (21), we investigated AT23 alpha-amylase production in culture media containing various carbon sources.

Our research showed soluble starch (1.0 %) as the best carbon source for AT23 alpha-amylase production. Soluble starch (1.0%) was also reported as the most effective carbon source for alpha-amylase production by *Bacillus sp* (3). The production of the alpha-amylase was also affected by different nitrogen sources.

Different carbon sources and peptone were found to be good inducers of AT23 alpha-amylase production. Similar results were reported by Oziengbe and Onilude (10) in which peptone was identified as the best nitrogen source. Conversely, Asgher and coworkers showed that production of thermophilic alpha-amylase by thermophilic *Bacillus subtilis* JS-2004 is at maximum level in 1.0 % glucose and 1.0% yeast extract (22).

Amongst the various metal ions investigated, optimum production of AT23 alpha-amylase was seen in the presence of calcium ion. The significant production of thermostable alpha-amylase in the presence of calcium ion was mentioned by Prakash et al (23).

The initial pH of the growth medium of the bacterial strain physically traces on the enzyme secretion (22). The highest level of AT23 alpha-amylase production was found at pH 6 and pH 7, respectively. Similarly, adjustment of the medium pH at 7.0 achieved the optimum conditions for enzyme production by *Bacillus subtilis* JS-2004 (22).

AT23 alpha-amylase showed a number of attractive properties which make it appropriate

to be used in industrial processes. pH activity and stability profile of AT23 alpha-amylase showed pH 5.0 and pH 6.0 as optimal pH, respectively. Moreover, alpha-amylase showed a good activity in pH range of 7.0 to 11.0.

However, thermo- and pH-stable alpha-amylase from *Geobacillus caldoxylosilytics* TK4 exhibited a different activity pattern at various pHs, where it was completely inactive at pH range of 3.0-6.0, and optimally activate at pH 7.0 (19).

In contrast, the optimum activity and stability of alpha-amylase from *Chryseobacterium taeanense* were estimated to be at pH of 9.0 and 9-11, respectively (24). Investigation of the pH effect on the activity and stability of the thermophilic alpha-amylases is an important object to the industrial application. Whereas Laundry processes are practicable in alkaline pH (1), thermophilic alpha-amylases such as AT23 alpha-amylase which are active or stable in alkaline pH can be used as additive material in detergents.

The enzyme activity and stability at high temperatures is a considerable property in screening useful alpha-amylases for starch liquefaction industry (8). In this aspect, the activity and stability of AT23 alpha-amylase were studied in various temperatures.

Alpha-amylase was well active at 30-50 °C, with an optimum activity at 50 °C. Optimum stability of this enzyme was also obtained at 50 °C. Similar temperature profile was found for thermostable alpha-amylase from *Geobacillus caldoxylosilyticus* TK4 (19).

Based on these results, thermostable and thermophilic alpha-amylases show activity and stability at a wide temperature range. Thermal stability of AT23 alpha-amylase was also examined at 50 °C and 60 °C, in both presence and absence of 20 mM $CaCl_2$ for 3 h. It was observed that the presence of $CaCl_2$ improves AT23 alpha-amylase thermostability by 20% at 50 °C. Furthermore, $CaCl_2$ elevated half-time of AT23 alpha-amylase to 90 min at 60 °C. A number of reports on the improvement of alpha-amylase thermostability in the presence of Ca^{2+} ion have been published by other researchers (25, 26). The improving effect of Ca^{2+} on temperature stability of alpha-amylase can be due to the removal of hydrophobic residues from enzyme structure by incorporating Ca^{2+} ion into the compact structure of this enzyme (27).

The enzyme activity in the presence of Mn^{2+} ion was similar to results reported by Samie et al and Kolcuoğlu et al (11, 19). However, some studies reported complete TKU001 alpha-amylase inhibition in the presence of Mn^{2+} ions (18). Mn^{2+} had also a strong inhibitory effect on the alpha-amylase activity of *Bacillus* sp. DR90, where it reduced the enzyme activity to 52% (7).

The optimum activity of AT23 alpha-amylase in the presence of Mn^{2+} compared to other metal ions proposed the induction of flexible enzyme structure by this ion (11). AT23 alpha -amylase displayed an increase in the activity by 13%, 24% and, 32% when was incubated with Cu^{2+}, Co^{2+}, and Zn^{2+} ions, respectively. But, other metal ions such as Fe^{2+}, Ca^{2+}, Hg^{2+}, and Hg^{2+} had no remarkable influence on the enzyme activity. Yet, there are exceptions for the behaviour of alpha-amylases in the presence of various metal ions. For instance,

Fe^{2+} and Cu^{2+} completely stop enzyme activity in *Chryseobacterium taeanenese* TKU001 (18), while Cu^{2+} was found to be a stimulant agent for AT23 alpha-amylase activity.

Contradictory findings were obtained by Gangadharan et al where Zn^{2+} inhibited the enzyme activity by 56% (25). However, alpha-amylases are usually getting inactivated by metal ions (30).

AT23 alpha-amylase exhibits a good stability in the presence of chelating agents such as EDTA (107%), suggesting that the enzyme is a metal ion independent alpha-amylase (20). The stabilizing effect of EDTA was also reported in case of *Anoxybacillus beppuensis* TSSC-1 (20). Nonetheless, EDTA caused a drastic decrease in the activity of many alpha-amylases such as *Alicyclobacillus acidocaldarius* (2). Due to the remarkable retained enzymatic activity toward chemical additives such as SDS (82%) and Triton X-100 (100%), AT23 alpha-amylase may be considered as a suitable enzyme for detergent industries (28). Interestingly, the activity of AT23 alpha-amylase was protected and even increased in the presence of various commercial detergents.

Bacillus licheniformis NH1 alpha-amylase also retained 65-100% of its initial activity in the presence of solid and liquid commercial detergents such as Nadhif and Arie (25).

In addition, Asoodeh et al revealed high enzyme stability toward a wide spectrum of solid and liquid commercial detergents including Darya, Finish, Shooma, Persil, and Pril (7). Excellent stability of AT23 alpha-amylase toward the tested detergents appears its excellent industrial potential as an additive material in detergents formulation.

Due to the advantages appeared by organic solvents as compared with watery solvents, for instance, enhanced thermal stability and deletion of unfavorable aquatic sideways reactions, organic solvent-stable alpha-amylases are very noteworthy (7).

In this regard, activity of AT23 alpha-amylase was examined in the presence of a number of water-miscible and water-immiscible organic solvents, and results indicated that various chemical compounds including 1-butanol, chloroform, cyclohexane, DMSO, isopropanol and toluene increase enzyme activity by 120, 106, 97, 63, 41, and 31 %, respectively.

Similar to our findings, chloroform and toluene stimulated the enzyme activity up to 1.40 and 1.46 fold, respectively (7). While dimethylformamide significantly reduced AT23 alpha-amylase activity to 35%, alpha-amylase from *Brachybacterium* sp. strain LB25 was partially inactivated by this compound (29).

A salient characteristic of AT23 alpha-amylase was a drastic increase in enzymatic activity toward different NaCl concentrations (0-4 M), as enzyme activity raised up to sevenfold at 3 M NaCl concentration, indicating the necessity of NaCl for favoured hydrolysis of starch by the AT23 alpha-amylase. This activity level induced by NaCl was higher than halophilicity grade observed for other alpha-amylases such as alpha-amylases from *Bacillus licheniformis* AT70 (12), *Chromohalobacter* sp. TVSP 101 (23), and *Nesterenkonia* sp. strain F (5).

Using various soluble starch concentrations (0 -7.0 mg/ml) as substrate, kinetic parameter values including *Km* and V_{max} was measured

as 1.657 mg/ml and 0.0059 mg/ml/min, respectively. *K*m values reported for alpha-amylases from *Anoxybacillus beppuensis* TSSC-1 (20), *Bacillus licheniformis* AT70 (12), *Bacillus amyloliquefaciens* (27), *Nesterenkonia* sp. strain F (5) were 0.5, 1.203, 2.34, and 4.5 mg/ml, respectively.

Although comparison of *K*m and V_{max} values of different alpha-amylases is difficult due to using various substrates and reaction conditions, but *K*m values obtained for the alpha-amylases were reported in the range of 0.35-11.66 mg/ml (30).

Conclusion

Anoxybacillus gonensis AT23 as a thermophilic bacterium produced a moderately thermostable alpha-amylase with attractive enzymatic properties for various industrial applications. The enzyme from this strain displayed a good activity in a broad pH range with optimal activity in pH 5.0.

In addition, the alkalophilicity nature, organic solvent tolerance, and resistance toward mentioned commercial detergents indicated that it can be a candidate enzyme for different industrial applications. Moreover, increased activity of alpha-amylase under a wide range of NaCl concentrations shows its potential application in industrial process containing high salt concentrations.

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References

1. Asoodeh A, Chamani J, Lagzian, M. A novel thermostable, acidophilic alpha-amylase from a new thermophilic "*Bacillus* sp. *Ferdowsicous*" isolated from Ferdows hot mineral spring in Iran. Purification and biochemical characterization. Int J Bio Chromatogr. 2010; 46(3): 289-297.

2. Chandra MS, Mallaiah KV, Sreenivasulu P, Choi YL. Purification and characterization of highly thermostable α-amylase from thermophilic *Alicyclobacillus acidocaldarius*. Biotechnol Bioprocess Eng. 2010; 15(3): 435-440.

3. Gaur D, Jain PK, Bajpai V. Production of extracellular αamylase by thermophilic *Bacillus* sp. isolated from arid and semi-arid region of Rajasthan. India. J Microbiol Biotechnol Res. 2012; 2: 675-684.

4. Karakaş B, İnan M, Certel M. Expression and characterization of *Bacillus subtilis* PY22 alpha-amylase in Pichia pastoris. J Mol Catal B Enz. 2010; 64(3): 129-134.

5. Shafiei M, Ziaee AA, Amoozegar MA. Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F. Process Biochem. 2010; 45(5): 694-699.

6. Jiang T, Cai M, Huang M, He H, Lu J, Zhou X, Zhang Y. Characterization of a thermostable raw-starch hydrolyzing a-amylase from deep-sea thermophile *Geobacillus* sp. Protein Expr

Purif. 2015; 114: 15-22.

7. Asoodeh A, Emtenani S, Emtenani S, Jalal R, Housaindokht MR. Molecular cloning and biochemical characterization of a thermoacidophilic, organic-solvent tolerant alpha-amylase from a *Bacillus* strain in *Escherichia coli*. J Mol Catal B Enz. 2014; 99: 114-120.

8. Ahmadi A, Ghobadi S, Khajeh K, Nomanpour B, Badoei-Dalfard A. Purification of alpha-amylases from *Bacillus* sp. GHA1 and its partial characterization. J Iran Chem Soc. 2010; 7(2): 432-440.

9. Mojsov K. Microbial alpha-amylases and their industrial applications. a review. Management, IT and Engineering. 2012; 2(10): 583-609.

10. Oziengbe EO, Onilude AA. Production of a thermostable alpha-amylase and its assay using *Bacillus Licheniformis* isolated from excavated Land Sites in Ibadan, Nigeria. BAJOPAS. 2012; 5: 132-138.

11. Samie N, Noghabi KA, Gharegozloo Z, Zahiri HS, Ahmadian G, Sharafi H, Behrozi R, Vali H. Psychrophilic alpha-amylase from *Aeromonas veronii* NS07 isolated from farm soils. Process Biochem. 2012; 47: 1381-1387.

12. Afrisham S, Badoei-Dalfard A, Namaki-Shoushtari A, Karami Z. Characterization of a thermostable, CaCl 2-activated and raw-starch hydrolyzing alpha-amylase from *Bacillus licheniformis* AT70: Production under solid state fermentation by utilizing agricultural wastes. J Mol Catal B Enz. 2016; 132: 98-106.

13. Sambrook JR, Russel DW. 2001. Molecular cloning: A laboratory manual. 2001.

14. Bernfeld P. Amylases, α and β. Methods Enzymol. 1955; 1: 149-158.

15. Sivakumar T, Shankar T, Vijayabaskar P, Muthukumar J. Nagendrakannan E. Amylase production using *Bacillus cereus* isolated from a vermicompost site. Int J Microbiol Res. 2012; 3(2): 117-123.

16. Aullybux AA, Puchooa D. Alpha-amylase production on low-cost substrates by *Naxibacter* sp. isolated from Mauritian soils. Br Microbiol Res J. 2013; 3: 478-491.

17. Laemmli, UK. Most commonly used discontinuous buffer system for SDS electrophoresis. Nature. 1970; 227: 680-685.

18. Wang SL, Liang YC, Liang TW. Purification and characterization of a novel alkali-stable alpha -amylase from *Chryseobacterium taeanense* TKU001 and application in antioxidant and prebiotic. Process Biochem. 2011; 46: 745-750.

19. Kolcuoğlu Y, Colak A, Faiz O, Belduz A.O. Cloning, expression and characterization of highly thermo- and pH-stable maltogenic amylase from a thermophilic bacterium *Geobacillus caldoxylosilyticus* TK4. Process Biochem. 2010; 45: 821-828.

20. Kikani BA, Singh SP. The stability and thermodynamic parameters of a very thermostable and calcium-independent α-amylase from a newly isolated bacterium, *Anoxybacillus beppuensis* TSSC-1. Process Biochem. 2012; 47: 1791-1798.

21. Demirkan ES, Mikami B, Adachi M, Higasa TU, tsumi S. Alpha-amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in

E. coli. Process Biochem. 2005; 40: 2629-2636.

22. Aiyer PD. Effect of C: N ratio on alpha-amylase production by *Bacillus licheniformis* SPT 27. Afr J Biotechnol. 2005; 3: 519-522.

23. Prakash B, Vidyasagar M, Madhukumar MS, Muralikrishna G, Sreeramulu K. Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable apha-amylases from *Chromohalobacter* sp. TVSP 101. Process Biochem. 2009; 44: 210-215.

24. Hmidet N, Bayoudh A, Berrin JG, Kanoun S, Juge N, Nasri M. Purification and biochemical characterization of a novel α-amylase from *Bacillus licheniformis* NH1: cloning, nucleotide sequence and expression of *amyN* gene in *Escherichia coli*. Process Biochem. 2008; 43: 499-510.

25. Gangadharan D, Nampoothiri KM, Sivaramakrishnan S, Pandey A. Biochemical characterization of raw-starch-digesting alpha amylase purified from *Bacillus amyloliquefaciens*. Appl Biochem Biotechnol. 2009; 158: 653-662.

26. Saxena R, Singh R. Amylase production by solid-state fermentation of argo-industrial westes using *Bacillus* sp. Braz J Microbiol. 2011; 42: 1334-1342.

27. Božić N, Ruiz J, López-Santín J, Vujčić Z. Production and properties of the highly efficient raw starch digesting α-amylase from a *Bacillus licheniformis* ATCC 9945a. Biochem Eng J. 2011; 53: 203-209.

28. Doukyu N, Yamagishi W, Kuwahara H, Ogino H, Furuki N. Purification and characterization of a maltooligosaccharide-forming amylase that improves product selectivity in water-miscible organic solvents, from dimethylsulfoxide-tolerant *Brachybacterium* sp. strain LB25. Extremophiles. 2007; 11: 781-788.

29. 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. 2008; 77: 1023-1031.

30. Najafi MF, Kembhavi A. One step purification and characterization of an extracellular α-amylase from marine *Vibrio* sp. Enz Microb Technol. 2005; 36: 535-539.