Journal of ENVIRONMENTAL Treatment Techniques

J. Environ. Treat. Tech.
ISSN: 2309-1185
Journal web link: http://www.jett.dormaj.com

Screening, Characterization and Production of Thermostable Alpha-Amylase Produced by a Novel Thermophilic *Bacillus megaterium* Isolated from Pediatric Intensive Care Unit

Seyyedeh Narjes Abootalebi[1], Amir Saeed[1], Ahmad Gholami[2*], Milad Mohkam[2], Aboozar Kazemi[3], Navid Nezafat[3], Seyyed Mojtaba Mousavi[4], Seyyed Alireza Hashemi[5], Eslam Shorafa[2*]

[1]Department of Pediatrics, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Biotchnology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[4]Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan
[5]Department of Mechanical Engineering, Center for Nanofibers and Nanotechnology, National University of Singapore, Singapore

Received: 09/03/2020 Accepted: 13/06/2020 Published: 20/09/2020

Abstract

This study aimed to isolate the thermophilic *Bacillus* strain capable of producing a high amount of thermo-stable α-amylase.The screening and isolation of amylase producing bacteria were done on selective media. The identification of bacteria was made using routine biochemical and molecular 16s rRNA techniques. The amylase activity assay was performed by using dinitrosalicylic acid (DNS) method, and finally, the optimum temperature, K_m (Michaelis constant) and maximum rate of reaction (V_{max}) of the enzyme was calculated. Results: The newly thermo-stable amylolytic enzymes of *Bacillus megaterium* designated AGH01was isolated from pediatric intensive care unit through a selective enrichment procedure. This isolate was identified based on biochemical and morphological traits along with 16s rRNA partial sequence analysis. This isolate showed the highest amylolytic activity (19.2 U/mL after 24 h) as compared to other isolates. The optimum temperature for the enzyme activity was achieved at 90°C and pH 7.0. At this condition, K_m of the enzyme for the degradation of starch was 3.36 mg/mL and (V_{max}) was 0.177 mM/min. The enzyme was stable at 90°C and 80°C in 33.4% and 35.7% of its original activity at pH 7.0 after 30 minutes and 9.1% and 48% after 60 minutes of incubation, respectively. Bioinformatics analysis showed the enzyme had no disulfide bond and had a 35 amino acid signal sequence. The aliphatic index (69.14) and grand average of hydropathicity index (-0.399) calculated by ProtParam server indicated that this α-amylase may be stable for a wide range of temperature and has excellent solubility in water.Conclusions: The high-thermal stable enzyme produced by *Bacillus megaterium* AGH01 could be considered merit as an excellent alternative source for bioethanol production and pharmaceutical applications. Moreover, our data provides such beneficial information for a better industrial formulation with proper stability in extreme conditions.

Keywords: Amylase, Amylolytic activity, *Bacillus megaterium*, Thermo-stability

1 Introduction

Amylases take part as an essential class of industrial enzymes having about 25-33% of the world enzyme market. Initially, the term amylase was used originally to designate an extracellular enzyme capable of hydrolyzing α-1,4-glucosidic linkages in polysaccharides containing three or more glucose units (1). The enzyme acts on starches, glycogen and oligosaccharides randomly, leading to liberating reducing groups of sugars. It has potential applications for a large number of industrial purposes such as textile, bread and parchment paper, detergent, food, pharmaceutical and fine-chemical industries (2). However, with the advent of the new frontier in biotechnology, the amylase application has also expanded in numerous other fields such as clinical, analytical and medicinal chemistry(3-6), as well as their extensive usage in the distilling and brewing industries and starch saccharification (7, 8). It is necessary to have more thermo-active and thermostable amylases because these types of enzyme should be applicable at the high temperature of liquefaction (80–90 °C) and gelatinization (100–110 °C) to economize processes (9). Since the geographic region where the microorganisms are isolated, affect and determine enzyme behaviour, many attempts have been made to isolate some potential thermostable amylase producing strains from the harsh ecological area in the world (10-12). Geothermal sites, hot spring, compost, and soils were recognized as suitable habitats for microorganisms which can provide the source of thermostable enzymes (13). Because of degrading the starch

Corresponding authors: (a) Ahmad Gholami, [2]Biotchnology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, E-mail: Gholami@sums.ac.ir, (b) Eslam Shorafa, Biotchnology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, shorafae@sums.ac.ir. Tel. /fax: +98 711 2426729.

constitutes (the highest substrate of these media), amylolytic bacteria are supposed to be usually available in there(14-18). Numerous studies have proven that *Bacillus sp.* found in natural areas (19-21) (especially those grown at harsh conditions) was recognized as the leading producer of exotic amylase with high economic importance (22, 23). This work is focused on the isolation and identification of a bacterial strain, which can generate thermophilic and thermo-stable amylase with excellent characteristics and could be applicable for commercial purposes.

2 Material and methods

2.1 Screening and isolation of microorganisms

During the present investigation, the samples were collected from pediatric intensive care unit, Namazi hospital in Shiraz, Iran. One gram of each sample was suspended in 9 mL of sterile saline (9 g/L NaCl), serially diluted up to 10^{-7} and cultured on starch nutrient agar (25 g/L nutrient agar and 3 g/L starch at pH 7.0) plate. After 24 h of incubation at 37°C and further subculturing to obtain pure culture, particular colonies were isolated, and colonies were measured in diameter.

2.2 Identification of amylase-producing strains

Distinct colonies were identified based on morphological features like size, colour, elevation, shape, margin, gram stain, motility, spore stain and cell shape and then, the amylolytic activity of each isolated strain was measured by adding Gram's iodine reaction mixture (0.2% KI, 0.2% I_2) (24). Colonies with clear and transparent zone were selected as amylase producing strains. The selected colonies were purified by replica plating on starch-nutrient agar slant.

2.3 Selection of desirable enzyme producer strain

In order to select the best thermo-tolerant enzyme producer, the selected bacteria were cultured at different temperatures (30, 37, 42, 45, 50 and 55°C) in the fermentation medium and incubated at 140 rpm.

2.4 Identification of microorganisms

Various biochemical, physiological and morphological characterizations of the selected amylase-producing isolates were determined by using Bergey's Manual of Determinative Bacteriology (25). Biochemical tests such as catalase and Voges-Proskaeur and oxidase test, oxygen requirement and motility were also studied. Colony morphology such as form, margin, elevation, colour and diameter (mm) after 24 h were observed by light microscopy, and finally, the genus of the selected strain was determined (26). For further identification, the analysis of 16s rRNA gene sequence was done following Gholami et al. method (27). Concisely, the bacterial genomic DNA was extracted by the heat shock method. The universal prokaryotic (16s rRNA) primers, 5'-ACGGGCGGTGTGTAC-3' as the forward primer and 5'-CAGCCGCGGTAATAC-3' as the reverse primer was used to amplifying the partial sequence of 16s rRNA gene of isolates by PCR, which amplify ~ 800 base pair region of the 16s rRNA gene. The PCR products were purified and then determined by CinnaGen Company (Tehran, Iran) and then resulting 16s rRNA gene sequences were aligned and the sequence similarity compared to some known microorganisms in GenBank database of the National Center for Biotechnology Information by using Basic Local Alignment Search Tool (BLAST).

2.5 Inoculum preparation

Purified bacterial strains were selected based on the capability to cultivate in the selective medium containing starch as an individual carbon and energy source at room temperature. The activation media had the following composition: Soluble starch (3.0 g/L) and nutrient broth (25 g/L) at pH 7.0. Ten ml of this activation medium was inoculated by 100µl of 0.5 McFarland of the bacterial strain that grown on starch nutrient agar plates and incubated for 12 h in a rotary incubator shaker at 42°C at 140 rpm (28).

2.6 Seed culture preparation

A two-day-old culture was used for the preparation of cell suspension. 25 ml of seed medium was added to 250 ml Erlenmeyer flask. The medium contained soluble starch (2 g/L), peptone (5 g/L) and yeast extract (3 g/L) at pH 7.0. The flasks were autoclaved at 105 Pa pressure (121 °C) for 20 min and then gradually cooled at 25°C. Under sterile environments, 1ml of inoculum was added to the flask, and the flask was incubated in a rotary incubator shaker (which is kept at 140 rpm) overnight at 37°C.

2.7 Production of amylase in the bioreactor system

Enzyme production was carried out using a 10 L bioreactor (Biotron Inc., South Korea) containing 5L of fermentation medium. A 20% (v/v) microorganism suspension was transferred from seed culture to the fermentation medium containing: NaCl (2 g/L), $(NH_4)_2SO_4$ (2 g/L), K_2HPO_4 (2.5 g/L), KH_2PO_4 (1 g/L), $MgSO_4$.$7H_2O$ (0.01 g/L), $FeCl_3$ (1 g/L), $CaCl_2$ (0.01 g/L), peptone (2 g/L) and soluble starch (2 g/L) at pH 7.0. Subsequently, the bioreactor was set up at 140 rpm at temperatures ranging from 30 till 90 ºC for 12, 24, 36, 48 and 60 h. After the fermentation period, the cells were discarded by centrifugation, and the Cell-free filtrate was used for enzyme assay.

2.8Amylase assay

Amylase assay was performed by using a reaction solution consisting of 0.5 ml of the substrate (20 g/L soluble starch in 50 mM phosphate buffer pH 7.2 and 0.5 ml of the supernatant as a crude enzyme) (8**).** The reaction was done at 90°C for 5 min. The released sugars from starch were estimated using dinitrosalicylic acid (DNS) reagent according to the Miller method. Subsequently, the product was heated to 100°C for 10 min and then cooled. The optical density of samples was taken at 575 nm in a spectrophotometer (Shimadzu, Japan). The activity of the enzyme was measured in units (1 unit was defined as the quantity of enzyme which produces 1mmole glucose under assay condition.

2.9 Characterization of amylase and kinetic properties

0.25 ml of the diluted crude enzyme was combined with a solution of starch (0.25 mL, 0.02%) at 90°C and the quantity of resulting glucose over the time was measured. The effects of temperature, pH and substrate concentration on enzyme activity were considered, and K_m and V_{max} values of the enzyme were measured by using double-reciprocal (Lineweaver-Burk) plot.

2.10 Evaluation of thermo-stability of the enzyme

At 80 and 90 ºC, the stability of the enzyme produced by *Bacillus* sp. was assessed at without any additives at pH 7.0, and the half-life of the enzyme was estimated.

2.11 Bioinformatics Study of α-amylase

Specific primers for α-amylase was designed according to the available α-amylase nucleotide sequences in the NCBI database (Forward: 5'-GTGCTAACGTTTCACCGCATC-3' and Reverse: 5'-CAAGGCCATGCCACCAACCGT-3') and then sequenced. The multiple sequence alignment (MSA) of amylase sequences was performed using the CLC free Workbench, and then the evolutionary tree was constructed by using the Neighbour Joining algorithm (27, 29). Percentages of hydrophobic and hydrophilic residues were computed from the primary structure analysis outcomes. The physicochemical traits, theoretical isoelectric point (pI), molecular weight, extinction coefficient, the total number of positive and negative residues, aliphatic index, instability index and grand average hydropathy (GRAVY) were calculated using the Expasy's Prot Param (http://us.expasy.org/tools/protparam.html) prediction server. The putative amino acid sequence was inspected for a signal sequence by the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). The existence of disulfide bonds was predicted by CYS_REC and DISULFIND servers (27, 30).

3 Results and discussion

It is so necessary to screen bacteria for their amylase production, which could be a significant source of the enzyme after pure culture on a large scale. The productions of enzymes for industrial consistency and cost-effectiveness can be adopted with the selection of proper strain (31). The preliminary screening of environmental bacteria has prime importance as the starch-rich soil and water are the primary sources for varieties of bacteria as well as their amylase producing activity (32). Bacteria that produce amylase may be isolated from locations like vermin compost sites (33), processing firms, fermenting roots of cassava and naturally fermented alfalfa (34) and honey processing areas (35) as well as sewage surrounding mills (36).

At the present investigation, amylase producing strains were isolated from pediatric intensive care unit in a hospital. Among fifty-five different colony which produced bright halos with iodine solution, only three strains produced amylase at between 10.0 and 20.0 U/ml, and the rest of them produced amylase activity less than 10.0 U/ml. The high amylase producing bacteria labelled as strain N15, strain N32, and strain N7. The halo diameters of the strains N15, N32 and N7, were 31.86, 30.83 and 21.48 mm and their halo diameter to colony diameter ratios were 6.37, 6.17 and 4.3, respectively (Table 1).

Among the three strains, N15 which was isolated from pastry waste produced the highest amylase activity (19.8 U/mL) after 24h at pH 7.0 and 90˚C and indicated the highest value for halo diameter to colony diameter ratio (7.4), which demonstrated highest amylase activities. Therefore, it was chosen for additional evaluations.

3.1 Effect of temperature on enzyme production

The effect of temperature on enzyme production was studied to find the best thermostable enzyme-producing strain, at the temperature ranging from 30 to 55°C (Fig. 1). The best temperature for the highest amylase production of all three strains is 37°C, and the more increase in temperature the less production of amylase was achieved. The results showed that N15 had the highest activity at all temperatures among the three selected strains.

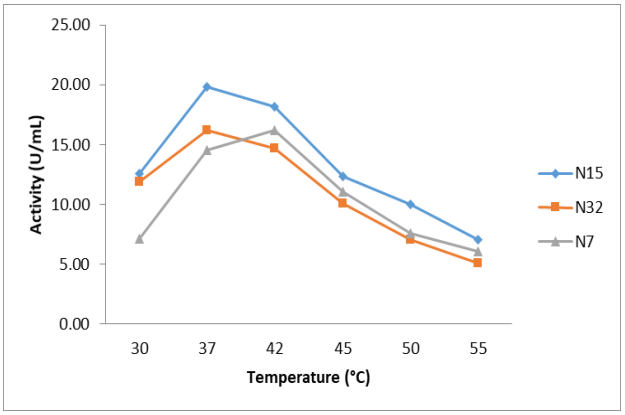

Figure 1: Effect of temperature on amylases production of three selected strains (N15, N32 and N7) after 24h at pH 7.0 in the fermentation medium

3.2 Effect of fermentation time on enzyme production

The suitable fermentation time for the highest enzyme activity was considered. Two strains depicted high enzyme activities after 24 h [N15 (19.2 U/ml), N32 (16.08 U/ml), and one of them produced high amylase activity after 36h (N7 (14.6 U/ml)]) (Fig. 2).

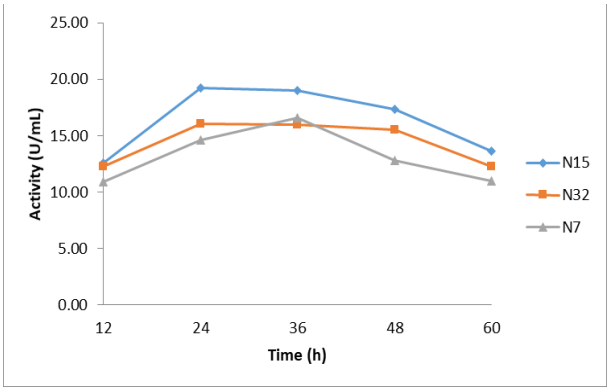

Figure 2: Effect of fermentation time on amylase production of three selected strains (N15, N32 and N7) at pH 7.0and at 37°C

However, the growth rate of three strains showed the same trend and after 12 h reached the maximum growth (Fig. 3). According to the highest enzyme-producing capability at high temperature, the strain N15, which was nominated as the best strain, was further characterized.

Table 1: Comparing the amylase activities of three selected strains (N15, N32 and N7), amylolytic power (diameter of clear halo) and its ratio to colony diameter at pH 7.0 and 90°C after 24 h

Strain	Amylolytic power (mm)	Halo diameter to colony diameter ratio	Amylase activity (U/ml)
N15	37	7.4	19.8
N32	30	6.0	16.3
N7	24	4.8	14.5

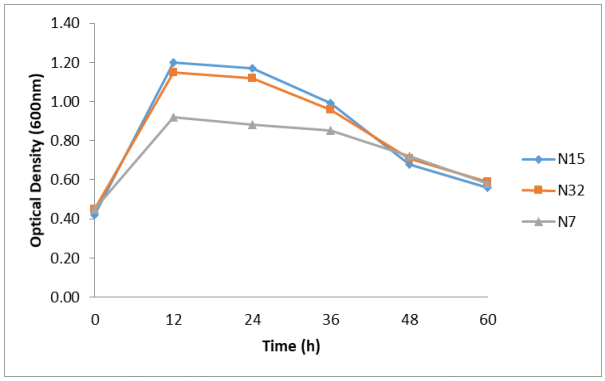

Figure 3: Effect of fermentation on the growth of three selected strains (N15, N32 and N7) at pH 7.0 and 37°C

3.3 Identification of the microorganism

In order to identify the genus of the strain, morphological, cultural and biochemical (Table 2) characteristics were used. The strain N15 was rod-shaped, Gram-positive, motile, and with negative results for oxidase test and positive results for catalase test. According to results, as shown in Table 2, the strain N15 was similar to those of the genus *Bacillus*. Finally, according to the widely used of 16s rRNA gene analysis, we sequenced the 16s rRNA gene of amylase producing strain named as AGH01 (16s rRNA accession no. KF437334). The obtained sequence was blasted against various genomic databases in NCBI, which indicated 99% homology with *B. megaterium*. Therefore, based on perceptions and analysis, strain AGH01 was classified as *B. megaterium* AGH01.

3.4 Characterization of amylase and kinetic properties

The pattern of the kinetics of the resulting enzyme from *B. megaterium* in the first 5 min presented zero-order patterns and for this reason, in the subsequent investigation, the reaction time was established on 5 min (Fig. 4). In order to assess the activity of the enzyme produced by *B. megaterium*, different temperatures ranging from 30-95ºCwas studied. The optimal temperature for amylase activity of this strain was 90 ºC, at pH 7.0 (Fig. 5). In this study, amylase showed 92.7, 96.4 and 97.0% of its maximal activity at 80, 95 and 85°C, respectively, at pH 7.0. In contrast, Hayashida et al. obtained the maximum activity of amylase for *B. subtilis* 65 at 60°C and pH 6.0 (37). Therefore, our result showed that this strain produced more heat-stable amylase than previous similar works.

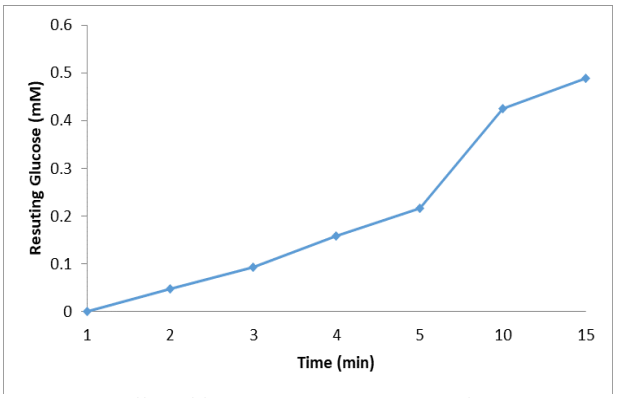

Figure 4: Effect of fermentation time on activity of the enzyme produced by B. megatriumwith soluble starch

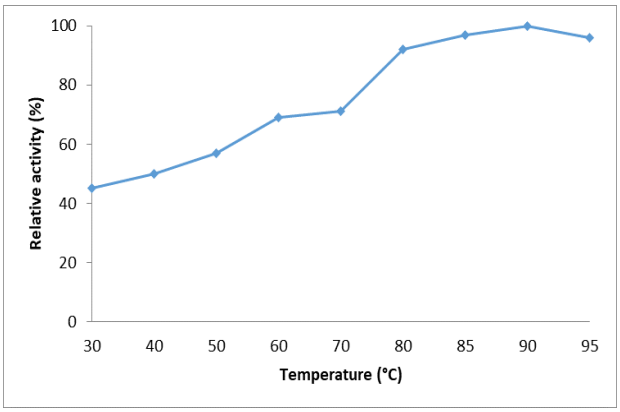

Figure 5: The activity of amylase obtained from B.megatriumat different temperature at pH 7.0

The activity of enzyme achieved from *B. megaterium* at pH ranging from 6.0 to 10.0 was assessed. The optimum pH for activity of amylase was 7.0 (Fig. 6). Enzyme obtains from *B. megaterium* displayed 72.1 and 85.2% of its maximum activity at pH 8.0 and 6.0, respectively.

Table 2: Morphological, cultural and biochemical characteristics of N15

Properties	Response of the strain	Biochemical test	Response of the strain
Color	Pale	Anaerobic growth	_
Form	Irregular	Growth in air	+
Margin	Irregular	Growth at 55ºC	+
Surface	Moist, shiny	Growth in 7% NaCl	+
Opacity	Opaque	Haemolysis on blood agar	β- haemolysis
Elevation	Flat	Lactose fermentation	_
Gram staining	Positive	Hyrolysis of tyrosine	_
Motility	Actively motile	KIA pattern	Acid slant, Acid butt, H2S -, Gas -
Diameter of clear halo (mm)	31.86	Nitrate reduction test	+
Diameter of colony after 24 h (mm)	5	Urease test	+
Shape of vegetative cell	Rod	Oxidase production	+
Spore formation	Positive	Citrate production	+
		Indole production	_
		Catalase production	+
		Voges-Proskaeur test	+

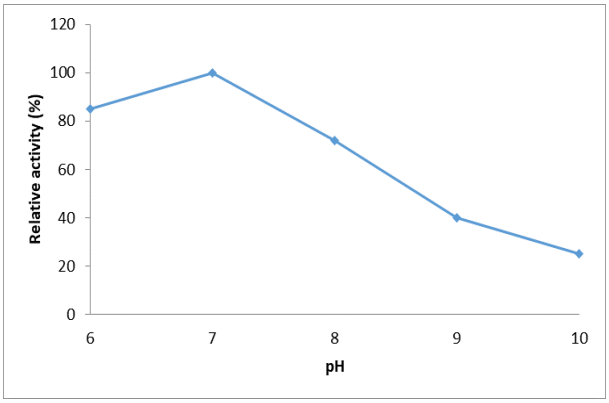

Figure 6: The activity of the enzyme obtained from B. megaterium at different pH at 90°C

Our results were in agreement with other works that indicated the optimal pH of amylase activity obtained from various *Bacillus* spp. such as *B. subtilis* KIBGE HAS (38), *B.licheniformis* (39), and *B.amyloliquefaciens* (40) *and B.coagulans* (41) is neutral. In order to estimate the K_m and V_{max}, the activity of amylase was measured by the rate of substrate consumption. The activity became greater than 0.160 mM/min since the concentration of substrate was raised from 5 to 30 mg/mL at pH 7.0 and at 90°C (Fig. 7).

Figure 7: The activity of the enzyme obtained from B. megaterium with different concentration of substrate at 90°C and pH 7.0

The K_m (Michaelis constant) of the enzyme for the degradation of starch was 3.36 mg/ml, and maximum rate of reaction (V_{max}) was 0.177 mM/min at pH 7.0 and 90°C (Fig. 8). Inferior values of K_m designate superior affinity of the enzyme for the substrate (42). In comparison to previous studies, our research clearly showed the lower Km values attributed to a higher affinity of the enzyme (40, 42). Because of variation in obtaining Km and Vmax values is completely related to the type of substrate and reaction condition, it is difficult to compare them. However, a similar work by Dragomirescu et al. showed the Km and Vmax values of 12.28 mg/ml and 0.82 mM/min at 37°C, respectively, for *B. amyloliquefaciens* (40).

3.5 Evaluation of enzyme stability

The thermal stability of amylase was assayed without any additives. After 30 min of incubation at 90°C, amylase obtained from *B. megaterium* kept 33.4% of its original activity at pH 7.0, and after one hour it decreased to 9.1% of its original activity. In comparison, at 80°C after 30 min of incubation, the enzyme retained 35.7% of its original activity at pH 7.0, and after one h, it reduced to 48% of its activity (Fig. 9). Asgher et al. reported that the amylase produced by *B. subtilis* JS-2004 was relatively stable at 60 and 70°C for one h, whereas at 80 and 90°C, 12% and 48% of its original activity was lost, respectively (43). In contrast, amylase obtained from *B. subtilis* AX20 in another study exhibited 60% and 35% of maximal activity at 40 and 70ºC, respectively (44). In comparison to the above mentioned reports, our findings showed that the enzyme obtained from *B. megaterium* AGH01 had superior stability under the harsh conditions.

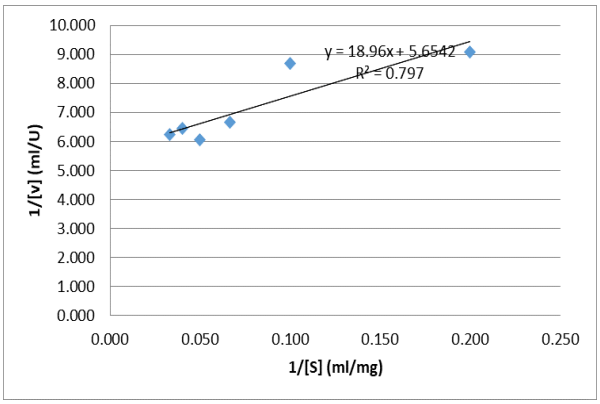

Figure 8: Double-reciprocal plot (Lineweaver-Burkplot) for enzyme produced by B. megaterium

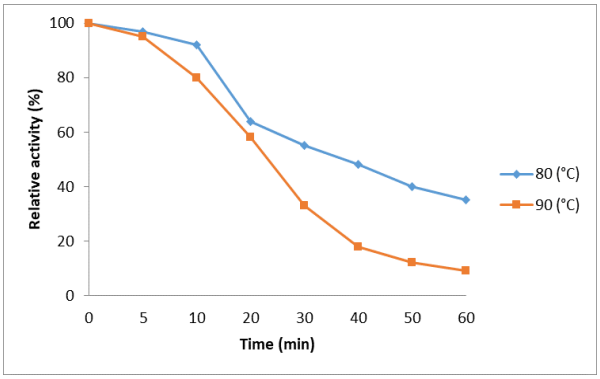

Figure 9: Evaluation of thermo-stability of amylase produced by B. megaterium at 80°C, and 90°C without any additives at pH 7.0

3.6 Bioinformatics Study of α-amylase

α-amylase gene-specific primers were designated as detailed in materials and methods section. The sequences achieved from NCBI was analyzed using the BLASTP search, which disclosed only one large open reading frame, composed of 1647 base pairs and 549 amino acid residues. BLAST searches of the α-amylase protein sequence depicted 89% identity at the amino acid sequence level with *Geobacillus stearothermophilus*. However, it also showed inferior identity (24-86 %) with other bacterial α-amylase (data not shown). The amino acid sequence of α-amylase was introduced to CLC free Workbench software. Then multiple sequence alignment (MSA) (Fig. 10) and phylogenetic tree (Fig. 11) were drawn in order to reveal the taxonomical variation in α-amylase from various *Bacillus* species (45). The multiple sequence alignment of these protein sequences revealed conserved regions at different stretches, namely, from 113 to 150, 246 to 274 and 298 to 310. The constructed phylogenetic tree revealed three distinct clusters (Fig. 11).

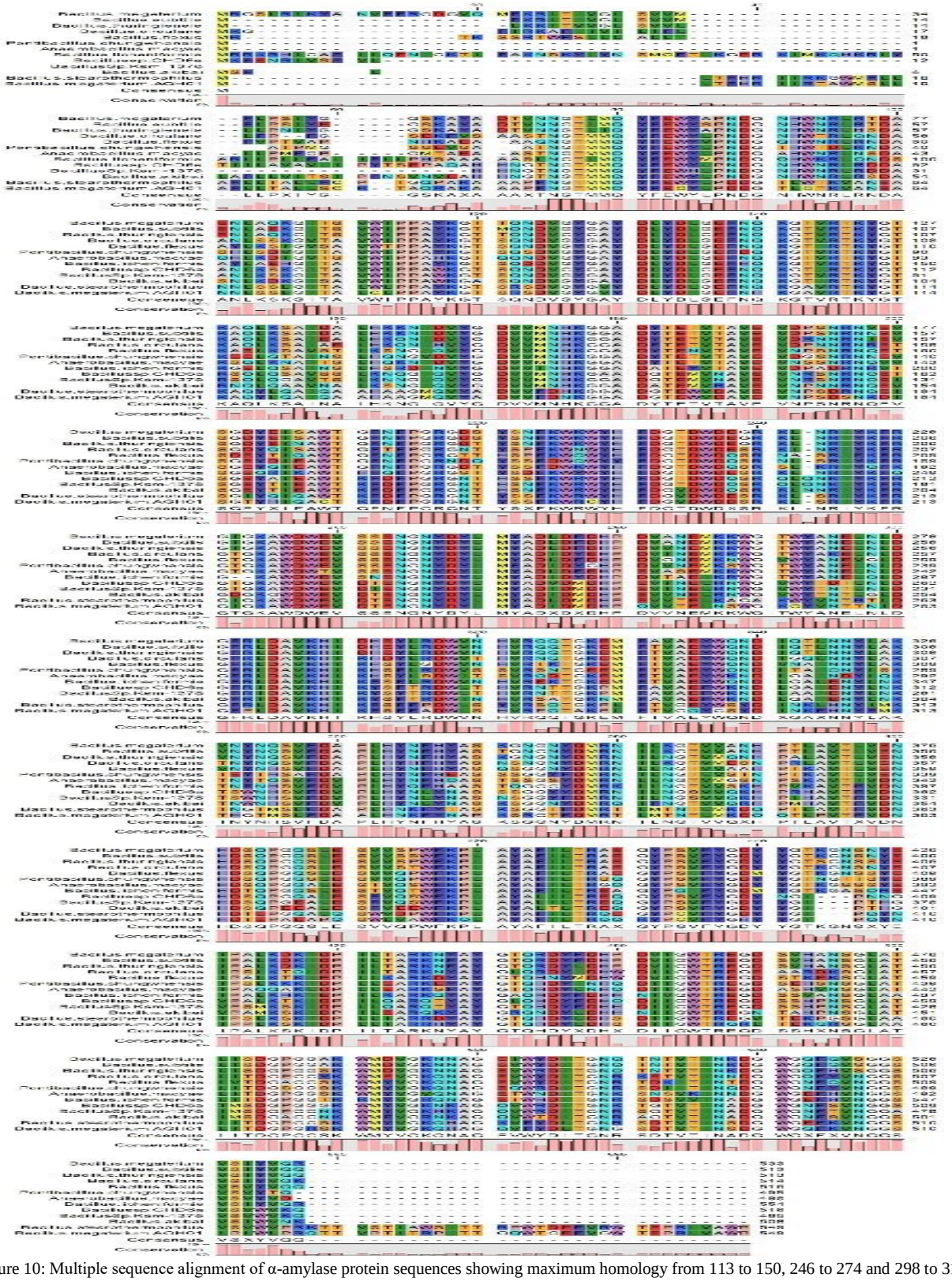

Figure 10: Multiple sequence alignment of α-amylase protein sequences showing maximum homology from 113 to 150, 246 to 274 and 298 to 310 amino acid residues

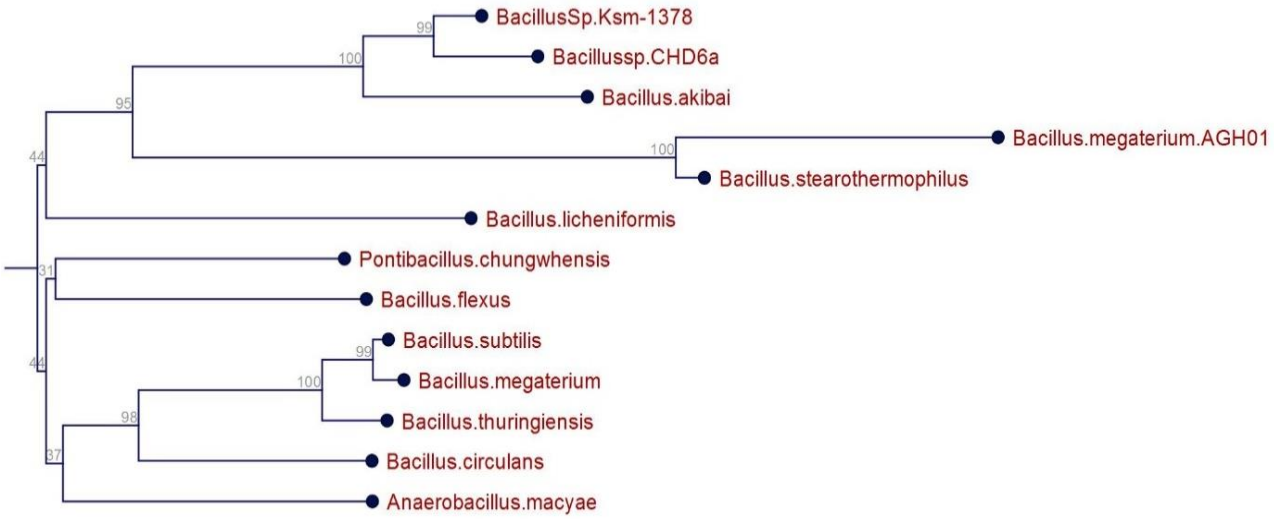

Figure 11: Phylogenetic trees of α-amylase representing common source organisms constructed by the NJ method

Table 3: In-silico physicochemical properties of recombinant α-Amylase obtained from ProtParam tool

No.	Parameters	α-Amylase
1	Sequence length	549 aa
2	Molecular weight	62371.4 D
3	Theoretical pI	7.98
4	-R*	52
5	+R*	54
6	Extinction coefficients**(M-1cm-1 at 260 nm)	141680- 141180
7	Instability index	39.21
8	Aliphatic index	69.14
9	GRAVY index	-0.399

* –R: total number of negative residues. +R: total number of positive residues.
** First value is based on the assumption both cysteine residues form cystines, and the second assumes that both cysteine residues are reduced.

The cluster I consist *B. subtilis*, *B. megaterium*, *B. thuringiensis*, *B. circulans* and *Anaerobacillus maycae*. The cluster II consists of *Pontibacillus chungwhensis* and *B. flexus*. The cluster III consists *B. stearothermophilus*, *B. megaterium* AGH1 (our isolate), *B. akibai* and two other *Bacillus* species. The computed physicochemical traits of α-amylase (by using the ProtParam tool) are demonstrated in Table 3. The calculated isoelectric point (pI value) for α-amylase was 7.98, which indicates its semi alkaline nature. The computed extinction coefficient was 141680, that could be detectable by UV spectrophotometer. The theoretical value for instability index was 39.21, which is stable in a test tube conditions (the index less than 40 are considered stable in the test tube). The aliphatic and GRAVY indexes were 69.14 and -0.399, respectively which implied that this α-amylase was almost thermostable and had excellent solubility in water (the negative GRAVY indicates solubility in water whereas a positive value suggests a hydrophobic nature). These data are inconsistent with our experimental data which confirmed that this enzyme is thermostable and could tolerate a wide range of pH, including alkaline. The result achieved from SignalP 4.0 server signified that α-amylase had signal sequences with 35 amino acids. The functional characterization revealed that α-amylase had seven cysteines (and one cysteine residue in signal peptide); however, our analysis did not show any disulfide bond in this protein (checked with DISULFIND and CYS_REC tools). This might be attributed to nature of Gram-positive bacilli secretion pathways which most of the secretory proteins are exported from the cytoplasm in an unfolded state that do not have disulfide bonds (REF A disulfide bond-containing alkaline phosphatase triggers a BdbC-dependent secretion stress response in *Bacillus subtilis* AND Bacillus subtilis as a cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism).

4 Conclusion

Based on a clear zone on selective medium containing starch as sole carbon and energy source, 55 bacterial strains were found that showed amylolytic activity. Among them, the highest enzyme producer was recognized as *B. megaterium* AGH01. The maximum amount of amylase production was achieved in 24 h after inoculation in the bioreactor. The results illustrated that the optimum condition for the activity of this enzyme was the temperature of 90°C and pH 7.0. The K_m of the obtained amylase (Michaelis constant) to solubilize the starch was 3.36 mg/mL, and the maximum rate of reaction (V_{max}) was 0.177 mM/min. These properties especially the high thermal stability of the enzyme makes *B. megaterium* AGH01 an excellent candidate for industrial production of amylase in textile, detergent, pharmaceutical and fine chemical industries. In future by purification and study of biochemical traits of AGH01 isolate could determine further its potential for industrial application.

Acknowledgments

This work was supported by research council of Shiraz University of Medical Sciences, Shiraz, Iran.

References

1. Pham HT, Boger MC, Dijkhuizen L, van Leeuwen SS. Stimulatory effects of novel glucosylated lactose derivatives GL34 on growth of selected gut bacteria. Applied microbiology and biotechnology. 2019;103(2):707-18.
2. Souza PMd. Application of microbial α-amylase in industry-A review. Braz J Microbiol. 2010;41(4):850-61.
3. Mousavi SM, Hashemi SA, Jahandideh S, Baseri S, Zarei M, Azadi S. Modification of phenol novolac epoxy resin and unsaturated polyester using sasobit and silica nanoparticles. Polymers from Renewable Resources. 2017;8(3):117-32.
4. Mousavi S, Hashemi S, Zarei M, Amani A, Babapoor A. Nanosensors for Chemical and Biological and Medical Applications. Med Chem (Los Angeles). 2018;8(8):2161-0444.1000515.
5. Bahrani S, Hashemi SA, Mousavi SM, Azhdari R. Zinc-based metal–organic frameworks as nontoxic and biodegradable platforms for biomedical applications: review study. Drug metabolism reviews. 2019;51(3):356-77.
6. Mousavi SM, Zarei M, Hashemi SA, Babapoor A, Amani AM. A conceptual review of rhodanine: current applications of antiviral drugs, anticancer and antimicrobial activities. Artificial cells, nanomedicine, and biotechnology. 2019;47(1):1132-48.
7. Priyadarshini S, Pradhan SK, Ray P. Production, characterization and application of thermostable, alkaline α-amylase (AA11) from *Bacillus cereus* strain SP-CH11 isolated from Chilika Lake. International Journal of Biological Macromolecules. 2020;145:804-12.
8. Kazemi A, Lorpour M, Gholami A, Karimi-Ghavamabadolya SR, Safari A, Solhjoo K, et al. High Level Amylase Production by Pseudomonas stutzeri ML-18; Isolation, Identification and Media Optimization in a Fermentation System using Response Surface Methodology (RSM). Journal of Pure and Applied MicrobiologyVol 9 No Special Edition Nov 2015 2015;9(Special Edition 2):127-33.
9. Aladejana OM, Oyedeji O, Omoboye O, Bakare M. Production, purification and characterization of thermostable alpha amylase from *Bacillus subtilis* Y25 isolated from decaying yam (Dioscorea rotundata) tuber. Notulae Scientia Biologicae. 2020;12(1):154-71.
10. Akbarzadeh-Jahromi M, Aslani FS, Omidifar N, Amooee S. Adenoid cystic carcinoma of bartholin's gland clinically mimics endometriosis, A case report. Iranian journal of medical sciences. 2014;39(6):580.
11. Safaei A, Shokripour M, Omidifar N. Bone marrow and karyotype findings of patients with pancytopenia in southern iran. Iranian journal of medical sciences. 2014;39(4):333.
12. Mousavi S, Zarei M, Hashemi S. Polydopamine for Biomedical Application and Drug Delivery System. Med Chem(Los Angeles). 2018;8:218-29.
13. Sonune N, Garode A. Isolation, characterization and identification of extracellular enzyme producer *Bacillus licheniformis* from municipal wastewater and evaluation of their biodegradability. Biotechnology Research and Innovation. 2018;2(1):37-44.
14. Hoseini M, Hekmatnia A, Hashemipour M, Basiratnia R, Omidifar N, Rezazade A, et al. Sonographic assessment of congenitally hypothyroid children in Iran. Endokrynologia Polska. 2010;61(6):665-70.
15. Adibi A, Jaberzadeh-Ansari M, Dalili A-R, Omidifar N, Sadeghi M. Association between Nonalcoholic Fatty liver disease (NAFLD) and coronary artery disease (CAD) in patients with Angina Pectoris. Open Journal of Medical Imaging. 2013;2013.
16. Sadeghi H, Jahanbazi F, Sadeghi H, Omidifar N, Alipoor B, Kokhdan EP, et al. Metformin attenuates oxidative stress and liver damage after bile duct ligation in rats. Research in Pharmaceutical Sciences. 2019;14(2):122.
17. Mousavi SM, Hashemi SA, Zarei M, Bahrani S, Savardashtaki A, Esmaeili H, et al. Data on cytotoxic and antibacterial activity of synthesized Fe3O4 nanoparticles using Malva sylvestris. Data in brief. 2020;28:104929.
18. Mousavi SM, Zarei M, Hashemi SA, Ramakrishna S, Chiang W-H, Lai CW, et al. Gold nanostars-diagnosis, bioimaging and biomedical applications. Drug Metabolism Reviews. 2020:1-20.
19. Mousavi SM, Hashemi SA, Amani AM, Esmaeili H, Ghasemi Y, Babapoor A, et al. Pb (II) Removal from Synthetic Wastewater Using Kombucha Scoby and Graphene Oxide/Fe3O4. Physical Chemistry Research. 2018;6(4):759-71.
20. Mousavi SM, Hashemi SA, Ghasemi Y, Amani AM, Babapoor A, Arjmand O. Applications of graphene oxide in case of nanomedicines and nanocarriers for biomolecules: review study. Drug metabolism reviews. 2019;51(1):12-41.
21. Tech JET. Investigating the Activity of Antioxidants Activities Content in Apiaceae and to Study Antimicrobial and Insecticidal Activity of Antioxidant by using SPME Fiber Assembly Carboxen/Polydimethylsiloxane (CAR/PDMS). Journal of Environmental Treatment Techniques. 2020;8(1):214-24.
22. de Paulo Farias D, Neri-Numa IA, de Araújo FF, Pastore GM. A critical review of some fruit trees from the Myrtaceae family as promising sources for food applications with functional claims. Food chemistry. 2020;306:125630.
23. Lorpour M, Kazemi A, Gholami A, Dabbagh F, Abbaszadeh S, Safari A, et al. Isolation and Phylogenetic Analysis of Some Lipase Producing Bacteria from a Saline Lake. Journal of Pure and Applied Microbiology. 2015;9(1):497-502.
24. Liu B, Wang Y, Zhang X. Characterization of a recombinant maltogenic amylase from deep sea thermophilic *Bacillus* sp. WPD616. Enzyme Microb Tech. 2006;39(4):805-10.
25. Montazeri-Najafabady N, Ghasemi Y, Dabbaghmanesh MH, Talezadeh P, Koohpeyma F, Gholami A. Supportive Role of Probiotic Strains in Protecting Rats from Ovariectomy-Induced Cortical Bone Loss. Probiotics Antimicrob Proteins. 2019;11(4):1145-54.
26. Kapilan R, Arasaratnam V. A novel *Bacillus pumilus* strain for alkaline xylanase production at and above 40° C. Ceylon J Sci (Biological Sciences). 2010;39(1):61-70.
27. Gholami A, Shahin S, Mohkam M, Nezafat N, Ghasemi Y. Cloning, characterization and bioinformatics analysis of novel cytosine deaminase from *Escherichia coli* AGH09. International Journal of Peptide Research and Therapeutics. 2015;21(3):365-74.
28. Ebrahimi N, Gharibi S, Ghoshoon MB, Karimi Z, Gholami A, Nezafat N, et al. Selective Isolation and Identification of Arginine Degrading Bacteria; the Optimized Arginine Deaminase Production by Enterobacter sp. sgn1 as a New Source of This Potentially Anti-Tumor Enzyme. Journal of Applied Pharmaceutical Science. 2016;6(09):093-101.
29. Irajie C, Mohkam M, Nezafat N, Hosseinzadeh S, Aminlari M, Ghasemi Y. In silico analysis of glutaminase from different species of Escherichia and Bacillus. Iranian journal of medical sciences. 2016;41(5):406.
30. Dorosti H, Eslami M, Negahdaripour M, Ghoshoon MB, Gholami A, Heidari R, et al. Vaccinomics approach for developing multi-epitope peptide pneumococcal vaccine. J Biomol Struct Dyn. 2019;37(13):3524-35.
31. Bahadure R, Agnihotri U, Akarte S. Assay of population density of amylase producing bacteria from different soil samples contaminated with flowing effluents. Int J Parasit Res. 2010;2(1):9-13.
32. Mishra S, Behera N. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. Afr J Biotechnol. 2008;7(18).
33. 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):117-23.
34. Reddy G, Altaf M, Naveena B, Venkateshwar M, Kumar EV. Amylolytic bacterial lactic acid fermentation—a review. Biotechnol Adv. 2008;26(1):22-34.
35. Nwagu TN, Okolo BN. Extracellular amylase production of a thermotolerant *Fusarium* sp: isolated from Eastern Nigerian soil. Braz Arch Biol Techn. 2011;54:649-58.
36. Yang C, Wang Z, Li Y, Niu Y, Du M, He X, et al. Metabolic versatility of halotolerant and alkaliphilic strains of *Halomonas* isolated from alkaline black liquor. Bioresource Technology. 2010;101(17):6778-84.
37. Hayashida S, Teramoto Y, Inoue T. Production and characteristics of raw-potato-starch-digesting α-amylase from *Bacillus subtilis* 65. Appl Environ Microb. 1988;54(6):1516-22.

38. Bano S, Qader SAU, Aman A, Syed MN, Azhar A. Purification and Characterization of Novel α-Amylase from *Bacillus subtilis* KIBGE HAS. AAPS PharmSciTech. 2011;12(1):255-61.
39. Vaseekaran S, Balakumar S, Arasaratnam V. Isolation and identification of a bacterial Strain producing thermostable α-amylase. Tropical Agri Res. 2010;22(1):1-11.
40. Dragomirescu M, Vintilă T, Preda G. Influence of immobilization on biocatalytic activity of a microbial (*Bacillus amyloliquefaciens*) alpha-amylase. Rom Biotechnol Lett. 2012;17(3):7253.
41. Medda S, Chandra AK. New Strains of *Bacillus licheniformis* and *Bacillus coagulans* producing Thermostable α-Amylase Active at Alkaline pH. J Appl Microbiol. 1980;48(1):47-58.
42. Gangadharan D, Nampoothiri KM, Sivaramakrishnan S, Pandey A. Biochemical characterization of raw-starch-digesting alpha amylase purified from *Bacillus amyloliquefaciens*. Appl Biochemi Biotechnol. 2009;158(3):653-62.
43. Asgher M, Asad MJ, Rahman S, Legge R. A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. J Food Engin. 2007;79(3):950-5.
44. Najafi MF, Deobagkar D, Deobagkar D. Purification and characterization of an extracellular α-amylase from *Bacillus subtilis* AX20. Protein Expres Purif. 2005;41(2):349-54.
45. Hajighahramani N, Eslami M, Negahdaripour M, Ghoshoon MB, Dehshahri A, Erfani N, et al. Computational design of a chimeric epitope-based vaccine to protect against Staphylococcus aureus infections. Molecular and cellular probes. 2019;46:101414.