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In Silico Study and Optimization of *Bacillus megaterium* alpha-Amylases Production Obtained from Honey Sources

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Abstract

This study aimed to screen alpha-amylase producing microorganisms from honey as a low water activity medium, a suitable source for selecting stable and cost-beneficial bacterial enzyme production systems. Plackett–Burman method was used to select twelve effective factors including pH, inoculum size, temperature, time, corn starch, KH_2PO_4, peptone, $MgSO_4$, $CaCl_2$, NaCl, glycerin, and yeast extract concentrations on bacterial alpha-amylases production yield. The Box–Behnken method was utilized to optimize the level of selected significant factors. The stability of bacterial alpha-amylases was also determined in low pH and high-temperature conditions. In addition, in silico study was used to create the alpha-amylase structure and study the stability in high-temperature and low water available condition. Among all isolated and characterized microorganisms, *Bacillus megaterium* produced the highest amount of alpha-amylases. The in silico data showed the enzyme 3D structure similarity to alpha-amylase from *Halothermothrix orenii* and highly negative charge amino acids on its surface caused the enzyme activity and stability in low water conditions. Based on Box–Behnken results, the temperature 35 °C, pH 6 and starch 40 g/l were determined as the optimum level of significant factors to achieve the highest alpha-amylases unit (101.44 U/ml). This bacterial alpha-amylases enzyme showed stability at pH 5 and a range of temperatures from 40 to 60 °C that indicates this enzyme may possess the potential for using in industrial processes.

Introduction

Honey plays an important role as a reservoir for a wide range of sugar-tolerant microorganisms, which come from pollen, nectar, digestive tracts of honey bees, air, and other microbial sources [1, 2]. Honey contains water, sugar, several minerals, and about 54 chemical elements such as Na, K, Ca, Mg, P, Cu, Pb, Zn and Mn that provide many nutritions' factors (such as carbon source and macro/microelements) to keep on microorganism [2]. Due to low moisture contents, honey has low water activity (a_w) that makes it a selective culture medium for existing of some hydrolytic enzymes (i.e., alpha-amylase) producing microorganisms (spore-forming bacteria and of sugar-tolerant microorganisms) in the honey environment condition such as *Bacillus* spp. and *Aspergillus* spp. [1, 3]. Alpha-amylase hydrolyzes the branched/unbranched starch through breakage of the alpha-1,4 bonds between glucose subunits. Among all alpha-amylases, microbial alpha-amylases have commonly used in industry because of some beneficial properties such as the best stability in harsh conditions. Easy genetic manipulation and high inexpensive production procedure are advantages of microbial alpha-amylases production [4–6]. Alpha-amylases, produced by *Escherichia* sp., *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Aspergillus oryzae* and *Rhizopus* sp., are used in food, paper, textile, pharmaceuticals, and detergents production industries [7, 8]. The processed-food industry has widely utilized alpha-amylases in a variety of applications such as brewing, baking, production of cakes,

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preparation of digestive aids, starch syrups and fruit juices [9].

The starch industry is one of the most important of alpha-amylase applications in the food industry. Alpha-amylases used in the starch processing must be active and stable in a high-temperature and acidic condition which shows the importance of screening of suitable microbial alpha-amylases [10]. Enormous microbial types were isolated from hot water springs environments where a unique suitable place for thermophilic microorganisms that produce active and stable alpha-amylases in high-temperature conditions. For instance, the *Geobacillus bacterium* has been isolated previously from Manikaran hot springs with the alpha-amylases optimum temperature activity at 80 °C [11].

Starch processing possesses the acidic pH (pH 4.5) condition, and thus the screening of microorganisms producing low pH stable alpha-amylase like *Lactobacillus manihotivorans* can be considered a crucial issue in alpha-amylases production [12, 13].

For the optimization of microbial alpha-amylases production, genetic/media engineering methodologies have been utilized in industry scale [4]. The instability of recombinants occurs in genetically engineered microorganisms through losing their vectors during fermentation due to structural instability of genetically safe expression vectors caused the reduction of the produced recombinant protein [14]. One of the media engineering methods (response surface methodology, RSM) has been considered a practical approach for enzyme overproduction because of reducing the numbers of experimental trials to find the optimized amount of factors and their interactions [4, 15]. The RSM method has been previously exploited to improve alpha-amylase overproduction by *B. amyloliquefaciens* [16], *A. oryzae* S2 [8], *A. oryzae* CBS 819.72 [17] and *A. oryzae* CBS 819.72 [17].

According to increase industrial applications of the alpha-amylase enzyme, finding good and novel microbial sources can play an important role to progress the related industries. During the production process, the harsh condition such as acidity and high-temperature condition needs to use the active and stable enzyme [18]. Honey possesses some microorganisms that are stable in acidic and low water activity conditions because of honey low water content and acidic pH [19]. Therefore, several studies have been required to find suitable microbial sources with alpha-amylase production properties from honey. Also, the structure of alpha-amylase, specificity, and catalytic mechanism are essential to carry out possible applications [20]. To study the protein structures, prediction tools look to be appreciated than nuclear magnetic resonance (NMR) and X-ray crystallography due to these methods' time-consuming and difficult processes [21].

This study aimed to screen alpha-amylase producing microorganisms from Iranian collected traditional honey, in silico studies of the alpha-amylase structure, and to screen the most effective factors related to alpha-amylases producing optimization.

Material and Method

Materials

Glucose, $MgSO_4$, $CaCl_2$, KI, agar, KH_2PO_4, NaCl, HCl, iodine and trichloroacetic acid were purchased from Merck (Merck, Darmstadt, Germany).Yeast, beef, malt extract, and agar were provided from Liofilchem (Liofilchem, Roseto d.Abruzzi, Italy) and Biolife (Biolife, Milano, Italy), respectively. Peptone and tryptone were bought from Himedia (Himedia, Mumbai, India). Alpha-amylase and glycerin were purchased from Sigma (Sigma, Steinheim, USA) and Duksan (Duksan, Kyungkido, South Korea), respectively. Corn starch was bought from Sigma (Sigma, microbial grade, Steinheim, USA).

Sample Collection, Isolation, and Screening of alpha-Amylase Producing Microorganisms

Honey samples were collected using sterile plastic flasks from the west of Iran. Enrichment cultures were performed separately using three common culture medium such as nutrient broth, Luria–Bertani broth, and one medium for fungi (contain malt 20 g/l, peptone 1 g/l, glucose 20 g/l, agar 20 g/l; pH 7) that were incubated in 150 rpm at 30 °C for 24 h into shaker incubator (Vision, Cheonan, South Korea). After incubation, the microorganisms were streak cultured in the same enrichment culture medium containing 15 g/l agar for isolation of single colon of enriched microorganisms. For amylolytic activity determination, every single colon separately was streak cultured on starch agar medium (contain corn 10 g/l, beef extract 3 g/l, and agar 15 g/l; pH 7) then plates were incubated at 30 °C for 24 h (Paat-Ariya Co. Sh 2006 model, Mashhad, Iran). The plates were flooded by Gram's iodine solution (0.3% I_2 and 0.6% KI) and the clear zone around the lines of grown microorganisms was considered as starch hydrolysis activity.

Genomic DNA was extracted from the producing alpha-amylase bacterial isolates by CTAB, SDS, and proteinase K method [22]. The PCR amplification was conducted using a thermal cycler Eppendorf (Eppendorf AG Mastercycler gradient, Hamburg, Germany) on extracted genomic DNA of each *Bacillus* bacteria by applying the amplification sets of primers forward 5′-AGA GTT TGA TCT GGC TCAG-3′ and reverse 5′-TACCTTGTTAGGACTTCACC-3′ [23] and for coccus bacteria by applying the amplification sets of primers forward 5′-GTAGCCGTATCGGAAGGTGC-3′ and reverse 5′-GTCGTGCTGGGGATAGAGCATT-3′. The

gel recovered PCR products were sequenced by a Korean sequencing company (Macrogen). For identification and characterization of bacteria, the sequences were analyzed using the BLAST program with the sequences deposited in NCBI and GenBank (https://www.ncbi.nlm.nih.gov/BLAST) and the bacteria with at least 98% homology were considered to the same species.

Alpha-amylase Assay

The selected microorganisms with amylolytic activity were subjected to alpha-amylase enzyme assay. Firstly, the microorganisms were cultured in a broth medium containing the following: starch 15, peptone 2.5, NaCl 1.5, yeast extract 2, KH_2PO_4 0.5, $MgSO_4$ 0.5, $CaCl_2$ 0.1 (all g/l) then were incubated into shaker incubator (150 rpm) at 30 °C for 24 h. Following, the cultures were centrifuged at 8000 rpm for 20 min (Beckman Coulter Allegra X-22R model, Krefeld, Germany). 250 µl of each supernatant was added to 0.5 ml acetate buffer (pH 5), along with 1.25 ml of 1% (w/v) soluble starch solution, then was incubated at 50 °C for 10 min. The reaction was stopped by adding of 1 ml dinitrosallicylic acid solution (1 g of 3, 5 dinitrosalicylic acid in 20 ml NaOH (2 M) containing 30 g sodium potassium tartrate by diluting to 100 ml with distilled water) and immediately the absorbance was measured at 575 nm (UV-1800 Shimadzu model, Kyoto, Japan). For calculating the alpha-amylase unit each sample, a standard curve was prepared based on 1–6 different units of alpha-amylase (10–50 unit) and the regression equation was calculated based on the standard curve.

One unit of the alpha-amylase enzyme was defined as the amount of enzyme equivalent to 1 mol glucose release per minute under the different standard assay conditions. The most alpha-amylase producing microorganism was subjected to optimization of alpha-amylase production.

For the study of pH stability, alpha-amylase solutions were tested at several pHs from 4 to 10 at 30 °C for 60 min. The effects of different temperatures on alpha-amylase stability were performed at the range of 20–60 °C for 60 min.

In Silico Study of Alpha-amylase from *Bacillus megaterium*

The alpha-amylase amino acid sequences were achieved from UniProt KB at https://www.uniprot.org [24], and multiple sequence alignment was performed with Clustal X version 2.0 then the results were evaluated by Jalview [25]. Protein Calculator v3.4 web tools (https://protcalc.sourceforge.net/), and Expasy's ProtParam (https://web.expasy.org/protparam/) were utilized to achieve physicochemical properties of the alpha-amylase protein including molecular weight, pI, charge at pH 7, charged residues composition, instability index, and the aliphatic index. HHpred and NCBI-Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) were used to achieve the appropriated PDB structure from the alpha-amylase [26] and Modeller v9.19 program software for modeling [27]. The best-designed models were obtained by discrete optimized potential energy score (DOPE scores) and the high-quality alpha-amylase structure images were designed by PyMOL molecular graphics system (Version1 Del and Scientific LLC) and were validated using Procheck, Errata, Verify 3D, MolProbity, Process, and ProSA web tools.

Optimization of Alpha-amylase Production

Screening of effective medium components was fulfilled using Plackett–Burman design for selected factors such as pH, inoculum size, temperature, time, corn starch, KH_2PO_4, peptone, $MgSO_4$, $CaCl_2$, NaCl, glycerin, and yeast extract concentration. Two levels of effective factors selected by Plackett–Burman screening were shown in Table S1.

Three selected significant factors from Plackett–Burman screening were subjected to the Box–Behnken design with 30 trials at the three different levels (− 1, 0 and + 1), shown in Table S2.

Statistical Analysis

All data were analyzed and experimental matrixes were designed using the Minitab® software version 16.2.4 (Minitab Inc., State College, PA, USA). A one-way analysis of variance [28] was employed for analysis of data and the statistical significance of the factors was determined using the *P*-values ($P \leq 0.05$).

Results and Discussion

Isolation and Screening of Microorganisms Producing Alpha-amylase

Thirty microorganisms were isolated from the honey samples and nine of them were able to produce alpha-amylase, all nine strains were Gram-positive and eight of them were rod-shaped and one was spherical-shaped. *Bacillus megaterium* kp7 (Accession numbers for 16S rRNA gene sequences; MG976764) produced the most alpha-amylases enzyme (Accession numbers for amylase gene sequences; X07261.1) compared to all isolated strains (Table 1). Some studies show similar results about microbial sources for the production of alpha-amylase. The ability to produce a novel group of glycoside hydrolase family 13 alpha-amylases has been previously reported for *Bacillus megaterium* [29]. Many studies' findings have been previously shown the potential/ability of some bacteria regarding

Table 1 Isolated microorganisms producing alpha-amylase

Microorganisms	Strains	Alpha-amylase production (U/ml)
Bacillus endophyticus	KP8	24.66
Bacillus subtilis	Kp6	23.14
Acinetobacter lwoffii	Kp10	23.14
Bacillus subtilis	Kp3	23.65
Arthrobacter crystallopoietes	Kv4	30.73
Bacillus amyloliquefaciens	Kho3	20.1
Bacillus subtilis	Kho2	20.1
Kocuria palustris	Khoi2	20.61
Bacillus megaterium	Kp7	40.86

alpha-amylase production such as *Bacillus subtilis* [30], *Bacillus amyloliquefaciens* [7, 31], and *Arthrobacter crystallopoietes* N-08 with the ability to produce glucoamylase enzyme. Based on our knowledge, we cannot find any report for the production of alpha-amylase by *Kocuria palustris, Acinetobacter lwoffii,* and *Bacillus endophyticus*; thus this is the first time that these bacteria abilities are introduced for alpha-amylase production.

In Silico Studies

The alpha-amylase from the *Bacillus megaterum* sequence (UniProt KB, P20845) showed 51.11% similarity with alpha-amylase A from *Halothermothrix orenii* (PDB: 1WZA_A). Figure 1 illustrates the concluding refined model of the *Bacillus megaterium* alpha-amylase enzyme.

The physicochemical characterization of *Bacillus megaterium* alpha-amylase showed the calculated pI 6.94, the instability index 31.87, and the aliphatic index 72.74 that indicating the thermostability of protein and the enzyme stability under physiological conditions more than 40 h, respectively [32, 33]. The secondary structure of *Bacillus megaterium* alpha-amylase illustrated 22% tern, 38% coil, 27% beta-sheet, and 33% helix. The amount of the alpha-amylase helix, beta-sheet, and coil similarity is comparable with alpha-amylase from *Halothermothrix orenii* (pdb = 1WZA) 31%, 29%, and 38%, respectively, while the tern is similar to alpha-amylase from *Bacillus licheniformis* (pdb = 1BLI). As seen in Fig. 2, the PRoSA Z-score plot from total determined protein chains in the PDB database indicated the alpha-amylase model locates in a typical range of experimentally determined structures [34]. Ramachandran plot showed the acceptable stereochemical quality for the protein created model with MolProbity clash score (11.15) for the model [35].Verify3D resulted in the 3D structure of alpha-amylase possesses good compatibly with its primary sequence because more than 80 percent of the amino acids owns ≥ 0.2 in the 3D/1D profile [36]. The high ERRAT score (99%) also illustrated the high quality of the protein 3D structure in low water availability condition [37].

Solvent exposed regions of the proteins play an important role in protein stability. Halophilic proteins are stable in low water availability conditions because of containing acidic residues on the surface of proteins caused to increase water-binding capacity [38–40] and decrease hydrophobicity at the protein surface [41]. The surface of the alpha-amylase from *Bacillus megaterum* riches of negative charges residues thus it is stable and active in low water availability condition (i.e., the high concentration of starch). Figure 3 displays the electrostatic surface of the alpha-amylase from *Bacillus megaterum*.

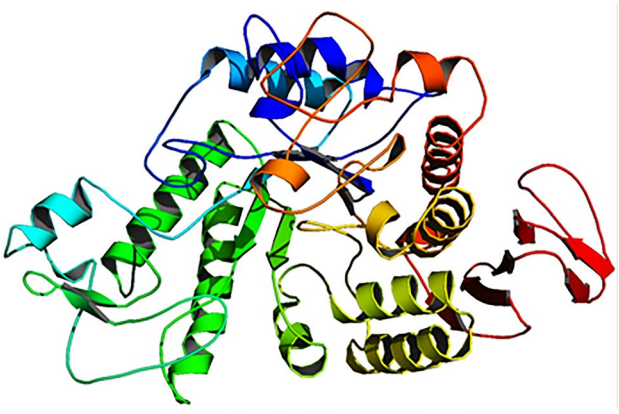

Fig. 1 3D structures of final refined alpha-amylase from *Bacillus megaterium*. Based on their position, the N-terminal and the C-terminal are blue and red, respectively (Color figure online)

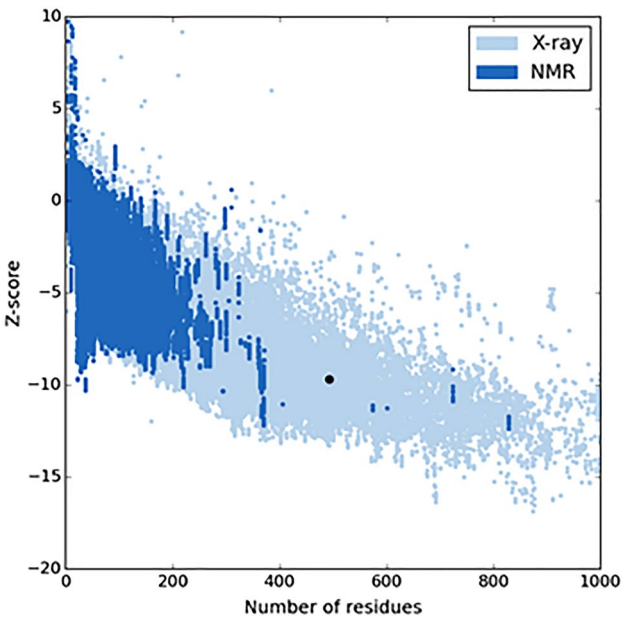

Fig. 2 PRoSA Z-score plots for the alpha-amylase

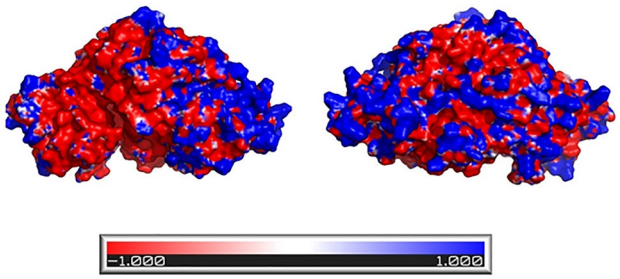

Fig. 3 Electrostatic surface of the alpha-amylase from *Bacillus megaterum*. Blue and red parts represent, respectively positive and negative charged residues (Color figure online)

Finding the Significant Factors by Plackett–Burman Design

Twelve factors (pH, size inoculum, temperature, time, yeast extract, peptone, KH_2PO_4, $MgSO_4$, $CaCl_2$, NaCl, and glycerin) were selected for the screening of significant factors by Plackett–Burman design with 20 runs (Table S3). The three factors including pH, corn starch, and temperature were significant ($P \leq 0.05$) (Fig. 4).

For the optimization of alpha-amylase production by *Aspergillus oryzae*, the inoculum size has shown a significant effect on the alpha-amylase production while in our study it did not show a significant effect on enzyme yield.

Owing to the depletion of nutrients in culture medium and production of toxic material by the microorganisms, the optimum fermentation time is considered as an important factor to produce the maximum yield of alpha-amylase [6]. Based on our findings, the fermentation time did not show a significant effect on enzyme production but time point 24 h was somewhat better than 48, whereas *Streptomyces erumpens* MTCC 7317 has shown the maximum yield of alpha-amylase for 60 h incubation time. These results show the importance of short or long fermentation time effects on bacterial enzyme production yield based on the type of bacteria.

Organic nitrogen sources like yeast extract, soybean proteins and peptone showed a better effect on alpha-amylase production yield than inorganic nitrogen sources such as ammonium chloride, ammonium sulfate and ammonium hydrogen phosphate [6]. The culture medium of *Bacillus* sp. supplemented by soybean meal enhanced the alpha-amylase production yield [4], whereas this factor did not show a significant impact on enzyme production in this study.

Some crucial salts required for microorganisms growth such as NaCl, $MgSO_4$, KH_2PO_4, and $CaCl_2$ can increase the alpha-amylase production by enhancing the growth rate of microorganisms through increasing the count of producing bacteria [10]. Besides, some enzymes are a metal enzyme and their structures possess metal ion that directly affects enzyme activity. Among these enzymes, alpha-amylase is a metal enzyme with possessing calcium ion on its structure and this ion required for enzyme activity [6]. *Bacillus amyloliquefaciens* culture medium supplemented with $CaCl_2$ has shown a significant increase in alpha-amylase production [16], while *Bacillus* sp. Ps-7 culture medium supplemented by $MgSO_4$ caused to increase significantly alpha-amylase yield [42]. These findings show the importance of these kinds of salts on metal contain enzymes production yield and their activity, but this factor did not affect significantly alpha-amylase production in this study. Glycerin, as a polyol, can play a great role in the formation of metabolic energy through converting to dihydroxyacetone via the glycolytic pathway [4], thus can increase the production of *Bacillus* sp. alpha-amylase [9]. In addition, glycerol improves the stability of alpha-amylase by extending the half-life of alpha-amylase by stabilization against ionic interactions and thermal denaturation [10]. However, the glycerin did not show a significant effect on enzyme production in this research.

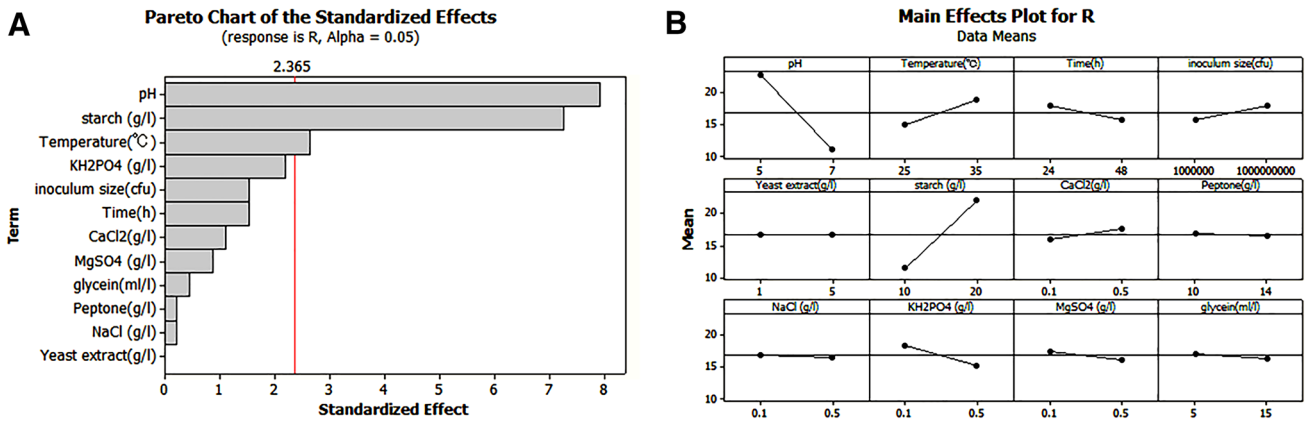

Fig. 4 **a** Pareto chart indicating the effect of factors on alpha-amylase production by the isolated *Bacillus megaterium*, **b** main effects plot for R (alpha-amylase U/ml)

Among the screened factors in this investigation, pH showed a major impact on increasing the alpha-amylase production and selected for Box–Behnken design. Optimum pH is directly related to microbial stability against hydrogen ions concentration, thus can help microorganisms to synthesize and secrete alpha-amylase and increase the enzyme production yield [10].

Corn starch, as a carbon source, causes to increase the population of alpha-amylase producing bacteria and thus can improve enzyme production [43]. Some studies have reported the great role of starch to produce of alpha-amylase by *Bacillus* sp. and *Bacillus amyloliquefaciens* [4, 31], which string along with our results and also selected as the main factor for Box–Behnken design.

Temperature, as a physical factor, can affect directly the growing range of microbial source and production of alpha-amylase [6]. Other investigation results showed the significant impact of temperature on bacterial alpha-amylase production and selected as the main factor through Plackett–Burman design to increase alpha-amylase production by microorganisms [17]. The variance analysis of Plackett–Burman design is represented in Table 2.

Box–Behnken Design

As shown in the Box–Behnken design outcomes represented in Table S4 and surface plots represented in Fig. 5, the optimal levels for alpha-amylase production were achieved at a pH 5, corn starch at 40 g/l, and temperature at 35 °C. The results for Box–Behnken design was significant due to Lack of fit and P-Value were 0.089 and 0.001, respectively. The calculated regression equation represented as:

$$R = 2756 - 68.0\,\text{pH} + 7.20\,\text{starch} - 141.3\,\text{temperature} - 6.33\,\text{pH} \times \text{pH} + 0.0967\,\text{corn starch} \times \text{starch} + 1.785\,\text{temperature*temperature} + 0.600\text{pH} \times \text{starch} + 3.067\,\text{pH} \times \text{temperature} - 0.3600\,\text{starch} \times \text{temperature}$$

In the *Bacillus* family including *Bacillus amyloliquifaciens* SH8, *Bacillus* sp. SJC B03, and *Bacillus* SY134D, pH 5 has been previously reported as the best pH for the production of alpha-amylase that similar to this study results [44–46] while pH 6.13 was reported as an optimum pH for *Kluyveromyces marxianus* IF0 0288 to produce this enzyme.

Some amylase producing strains of *Bacillus subtilis* (*Bacillus sp.* I-3, *Bacillus sp.* PS-7, and *Bacillus subtilis* IMG22) [4, 42, 47], and *Bacillus stearothermophilus* can consume corn starch, and soluble starch as a carbon source by producing alpha-amylase enzyme [48]. Upon our findings, *Bacillus megaterium* kp7 possesses the ability to produce a maximum yield of alpha-amylase in high starch concentration (40 g/l). Similar to our results, *Aspergillus niger* WLB42 can produce the optimum amount of alpha-amylase at 40 g/l starch concentration in culture medium content [30] whereas the best concentration of corn starch for *Bacillus* sp. CFR-67 has been previously reported 30 g/ml.

Several types of *Bacillus* family, including *Bacillus aquimaris* VITP4 and *Bacillus* sp. MB6 produced optimum levels of alpha-amylase at 37 °C [49–51] whereas the optimum temperature for our isolated *Bacillus* was achieved 35 °C by Box–Behnken design while *Bacillus licheniformis* ATCC 9945a showed 40 °C as an optimum temperature [52]. These findings show different bacteria having a diverse optimum temperature for growth and enzyme production.

Table 2 Analysis of variance for Plackett–Burman design

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Main effects	12	1420.2	1420.29	118.357	11.16	0.002
pH	1	663.55	663.55	663.552	62.57	0.000
Temperature (℃)	1	73.73	73.73	73.728	6.95	0.034
Time (h)	1	25.09	25.09	25.088	2.37	0.168
Inoculum size (cfu)	1	25.09	25.09	25.088	2.37	0.168
Yeast extract (g/l)	1	0.00	0.00	0.000	0.000	0.000
Starch (g/l)	1	557.57	557.57	557.568	52.57	0.000
$CaCl_2$ (g/l)	1	12.80	12.80	12.800	1.21	0.308
Peptone (g/l)	1	0.51	0.51	0.512	0.05	0.832
NaCl (g/l)	1	0.51	0.51	0.512	0.05	0.832
KH_2PO_4 (g/l)	1	51.20	51.20	51.200	4.83	0.064
$MgSO_4$ (g/l)	1	8.19	8.19	8.192	0.77	0.409
Glycein (ml/l)	1	2.05	2.05	2.048	0.19	0.674
Residual error	7	74.24	74.24	10.606		
Total	19	1494.53				

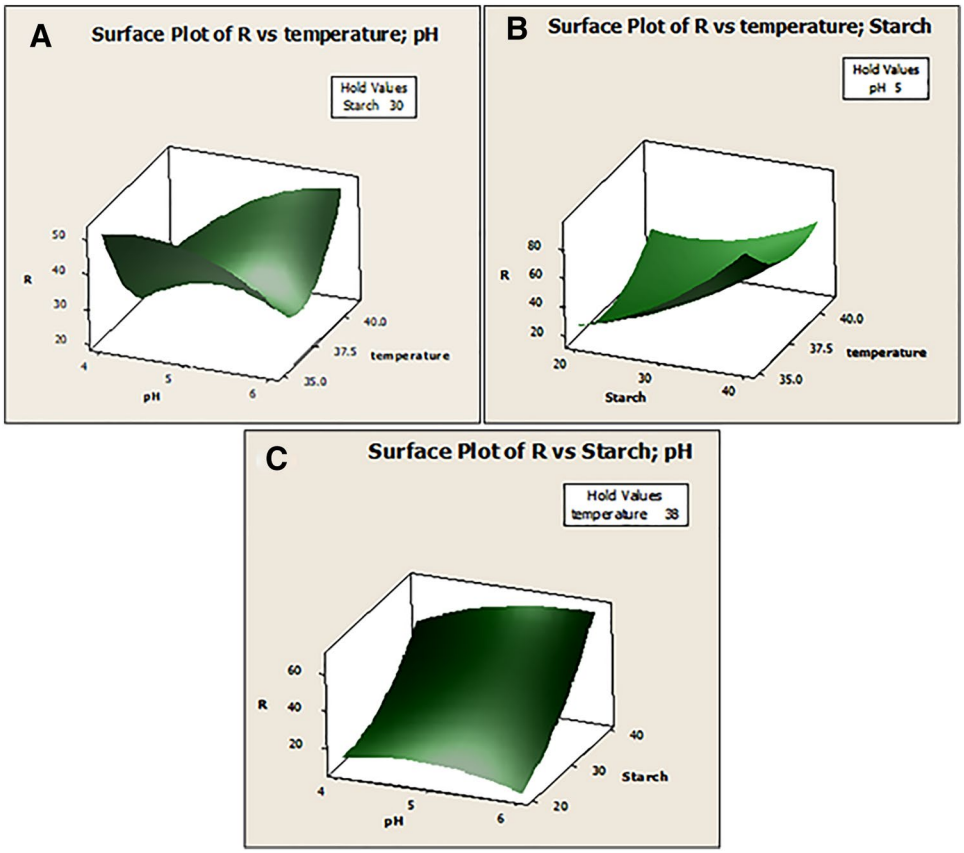

Fig. 5 Surface plot the interaction between initial pH, corn starch concentration, and temperature

Effect of Temperature and pH on Amylase Enzyme Stability

The alpha-amylase produced by the isolated *Bacillus megaterium* was stable at pH 5 and temperature range 40–60 °C. Moreover, the result for temperature stability showed a good correlation to the in silico results (AI = 72.74).

Bacillus sp. I-3 and *Bacillus* sp. PS-7 produced alpha-amylase stable at pH 5 [42, 47] whereas alpha-amylase from *Bacillus* sp. ANT-6 was stable at a pH range of 9–13 [53]. *Thermobifida fusca* NTU22 alpha-amylase was stable at a temperature range of 50–60 °C similar to our findings [54] while *Bacillus* sp. I-3 alpha-amylase was stable at 65–100 °C [47].

Conclusion

Some bacteria isolated from the honey were able to produce alpha-amylase and the results showed that the isolated *Bacillus megaterium* produce alpha-amylase more than other isolated bacteria. In addition, the best temperature for the production of alpha-amylase by *Bacillus megaterium* was 35 °C, starch concentration 40 g/l, and pH of 5. The isolated alpha-amylase was stable at acidic pH and high temperature (60 °C). Based on the findings, the amount of alpha-amylase production was dependent on several effective factors and enzyme-producing bacteria potential. Besides, the in silico results indicate a good correlation to experimental thermostability results from the *Bacillus megaterium* alpha-amylase. Due to highly negative surface charges of the alpha-amylase, the enzyme is active and stable in low water availability conditions.

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Author Contributions BEF performed the study and drafted the manuscript. AD revised the manuscript. AYK designed the study, edited and approved the final version of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest. All authors designed the experiment, conducted and wrote the manuscript. All authors have approved the final article.

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