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Thermostable Amylase from *Cytobacillus firmus*: Characterization, Optimization, and Implications in Starch Hydrolysis

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HOT springs are known to be rich in diverse microorganisms, including thermophilic bacteria capable of producing industrially important enzymes. This study aimed to isolate, purify, and identify thermophilic bacteria from three hot springs located in the southern region of Saudi Arabia, namely Al-Ma'a Al-Har, Al-Aredhah, and Al-Ahsarai hot spring. The isolated bacterial isolates were screened for amylase enzyme production, and the most potent isolate, *Cytobacillus firmus* OQ834432 isolate OHA8, was selected for large-scale production of the amylase enzyme. The enzyme was then purified using salt precipitation, Sephacryl S-200 chromatography, and DEAE-Sepharose column chromatography. A total of twenty-one bacterial species were identified, with *Cytobacillus firmus* exhibiting the highest amylase activity. The optimal conditions for the amylase enzyme activity were found to be at 50°C and pH 5.0, with a 153% enhancement in activity in the presence of magnesium ions. The enzyme's activity was inhibited by EDTA and was found to be active with starch and amylopectin at 100% and 110.3%, respectively. In conclusion, this study demonstrates the potential of thermophilic bacteria from hot springs to produce industrially important enzymes and provides insights into the optimal conditions for enzyme production and activity. These findings have significant implications for the development of biotechnological applications utilizing thermophilic bacteria in various industrial sectors.

Keywords: α- amylase enzyme, *Cytobacillus firmus,* Hot springs, Industrial applications, Thermophilic bacteria.

Background

Thermophilic bacteria are primarily found in hot springs, which are considered to be hubs of microbial diversity and an excellent source of novel genes, compounds, and hydrolytic enzymes for various applications (Saxena et al., 2016). However, there is a significant gap in knowledge regarding the potential of hot springs as a resource in Saudi Arabia (Narsing Rao et al., 2021). Thermophilic bacteria are highly valued for their species diversity, molecular phylogeny, and generation of secondary metabolites due to their extreme habitat (Fandi et al., 2014; Narsing Rao et al., 2021). Two broad categories of methods are utilized to study bacterial diversity in hot springs: culture-independent and culture-dependent techniques, with the latter being traditionally used for isolating isolates and investigating their properties (Simoes et al., 2007; Samarasinghe et al., 2021).

Extremophiles comprise a group of microorganisms that thrive in severe and extreme environments, such as those characterized by extreme temperatures, pH levels, salinity, and pressure (Singh et al., 2019; Narsing Rao et al., 2021). Specifically, thermophiles and hyperthermophiles are capable of thriving in high-temperature conditions (Berenguer, 2011).

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Natural habitats like hot springs and geysers, where hot groundwater emerges due to the Earth's mantle heat, exhibit distinctive and extreme physicochemical properties influenced by various factors (Bisht et al., 2011; Narsing Rao et al., 2021; Najar, 2022). The potential of hot springs as a reservoir for thermophilic bacteria has been investigated in numerous countries, including Saudi Arabia, China, India, Japan, the Philippines, Indonesia, Iceland, and Kamchatka (Mehta & Satyanarayana, 2013; Selim et al., 2017). However, the diversity and potential of thermophilic bacteria in Saudi Arabian hot springs remain largely unexplored. Therefore, there is an urgent need for further research in this domain to comprehensively comprehend the diversity and potential of thermophilic bacteria in hot springs in Saudi Arabia. This knowledge could potentially lead to the discovery of novel enzymes, genes, and compounds with applications in various biotechnological fields.

In the southwestern region of Saudi Arabia, specifically in Jizan, three potential geothermal locations have been identified. Among these, the initial site is situated in the Al-Ardah area, while the second is found in the southeastern part of the city and includes a prominent hot spring with a surface temperature of 76°C (Rehman & Shash, 2005). Extremophiles, a category of microorganisms capable of enduring harsh environments like high temperatures, have developed unique adaptations to thrive under such extreme circumstances. Enzymes derived from thermophiles, in particular, hold significant commercial and industrial value owing to their robustness and effectiveness in functioning in conditions of extreme heat (Ullah et al., 2020).

The genetic evolution of thermophilic bacteria, involving processes like gene loss, horizontal gene transfer, or gene mutations, potentially underlies their capacity to withstand high temperatures as a response to shifts in their environment (Averhoff & Muller, 2010). Investigating geothermal sites in Jizan offers the prospect of uncovering previously unknown thermophilic bacteria and their enzymes, which hold significant promise for various biotechnological applications. This understanding could play a pivotal role in advancing novel commercial products and processes, particularly in sectors reliant on robust and effective enzymes for high-temperature operations.

Extremophiles have become a focal point of interest for their prospective industrial utility in challenging settings (Geng et al., 2018). Scientists globally are delving into genetic alterations of microorganisms possessing advantageous industrial attributes, specifically focusing on heat-resistant enzymes sourced from thermophilic bacilli, which find diverse applications in the commercial sphere (Margaryan et al., 2018). Hot springs are renowned for housing an abundant reservoir of microbial enzymes, encompassing lipases, proteases, and cellulases (Akanbi et al., 2019).

In recent times, metagenomic techniques have gained prominence as more accurate means of assessing both the microbial variety and functional genomics within hot springs (DeCastro et al., 2016). This investigation seeks to assess the range of bacteria and their enzymatic potential in samples collected from different hot springs located in the southwestern part of Saudi Arabia. This will be achieved through the utilization of a blend of both culture-dependent and culture-independent methodologies.

Extremophiles are microorganisms with the remarkable ability to thrive in conditions of extreme temperature, pH levels, salinity, and pressure (Singh et al., 2019; Narsing Rao et al., 2021). Within this category, thermophiles and hyperthermophiles excel in high-temperature environments (Berenguer, 2011). Natural habitats like hot springs and geysers, where hot groundwater emerges due to the Earth's mantle heat, boast distinctive and extreme physicochemical properties influenced by various factors (Bisht et al., 2011; Narsing Rao et al., 2021; Najar, 2022).

Until now, the utilization of hot springs in Saudi Arabia as a reservoir for thermophilic bacteria remains largely untapped. Consequently, there is a pressing need for additional research in this domain to comprehensively grasp the diversity and capabilities of thermophilic bacteria within Saudi Arabian hot springs. Countries like China, India, Japan, the Philippines, Indonesia, Iceland, and Kamchatka also boast similar geothermal resources (Mehta & Satyanarayana, 2013; Selim et al., 2017). This underscores the significance of delving into the microbial diversity and enzymatic potential inherent in hot springs, potentially leading to the identification of novel

enzymes, genes, and compounds with promise for a range of biotechnological applications.

Materials and Methods

Sampling and physiochemical analysis

Hot spring samples were collected from three different areas in the Southern region of Saudi Arabia: Al-Ma'a Al-Har (20.462213494907118, 40.47208486017083) Al-Lith; Al-Aredhah (17.044857739113496, 42.98872929781254) Jazan and Al-Ahsarain (19.024211898476977, 41.71088784138378) Al-Majaridah, Aseer hot spring (Fig. 1). Samples were collected in sterile polythene bags and falcon tissue culture flasks, kept cool during transport, and stored at 4°C until analyzed according to Najar et al. (2018). Temperature and pH were measured on site using an electronic thermometer and pH meter (Orion; Thermo Fisher Scientific, Waltham, MA, USA).

Isolation and identification of bacterial isolates

Bacterial isolates were isolated from the water and sediments of hot springs using the standard serial dilution plating technique on two different media: International Streptomyces Project media (ISP2) and Modified Hagem media (Sujatha & Swethalatha, 2017). The water samples were diluted and spread onto agar plates, which were then incubated at 40.5ºC for 2-4 weeks. The resulting colony-forming units (CFUs) were quantified, and pure cultures were preserved in sterile 20% glycerol at -80ºC for subsequent use (Najar et al., 2018).

Bacterial identification was carried out using the Coverslip culture technique with electron microscopy on both ISP2 and Hagem media agar, as described by Alhelaify et al. (2022). Molecular identification was achieved by sequencing the 16S rRNA gene. The genomic DNA was extracted from each isolate using the DNeasy® Plant Mini Kit from QIAGEN.

Fig. 1. Geographic map generated using Google Earth, which displays the locations of water and sediments samples collected from three distinct hot springs found in Southwestern Saudi Arabia. A: Al-Ma'a Al-Har (20.462213494907118, 40.47208486017083) Al-Lith), B: Al-Aredhah (17.044857739113496, 42.98872929781254) Jazan and C: Al-Ahsarain (19.024211898476977, 41.71088784138378) Al-Majaridah

The 16S rRNA gene of the selected isolates was sequenced by Macrogen, Korea, using the universal primers 27F (5'-AGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The extracted DNA from each sample was quantified using a Nanodrop 8000 spectrophotometer device. Phylogenetic trees of the aligned sequences were constructed using the neighbor-joining method (Saitou & Nei, 1987) and MEGA X software.

Screening of isolated bacterial isolates for enzymatic activity

The ability of bacterial isolates to produce extracellular enzymes, namely amylase, protease, and lipase, was evaluated. Screening was conducted on starch agar plates, Skim milk agar (SMA), and Peptone tween agar (PTA) media, respectively, using the methodology outlined by Armada & Simora (2016).

Optimization of amylase enzyme production

To enhance the cost-effectiveness of α-amylase enzyme production, it is essential to optimize the process parameters. The objective of this study was to investigate the impact of various culture conditions on the maximum production of amylase enzyme by the OHA8 isolate. The critical factors that could influence enzyme production, including the pH of the culture medium, incubation temperature, and incubation period, were systematically varied to determine their optimal values for enzyme production. The effect of pH was evaluated by adjusting the pH of the culture medium within the range of 5.0-9.0 using phosphate buffer (pH 8.0-9.0) and Tris-HCl buffer (pH 5.0-7.0). The impact of incubation temperature was examined at different temperature values ranging from 25°C to 55°C, while the incubation period varied from 24 to 96 hours. The amylase activity was measured under each condition, and the optimal culture conditions were determined based on the highest amylase activity, as described in previous studies (Gupta et al., 2003; He et al., 2021).

Large scale fermentation and enzyme production

The production of amylase enzyme from the OHA8 isolate was carried out using a starch medium consisting of 5.0 gm peptone, 3.0 gm yeast extract, 10 gm soluble starch, 3.0 gm NaCl, 1.0 gm K_2HPO_4, and 0.2 gm $MgSO_47H_2O$ per liter. The amylase activity was quantified by measuring the glucose released from starch hydrolysis using the 3,5-dinitrosalicylic acid (DNSA) method, as described by Karnwal & Nigam (2013). The activity was defined as one unit of α-amylase, which liberates 1µmol of reducing sugar per hour under the experimental assay conditions. The protein content of the active fractions was determined spectrophotometrically at 280/260nm using standard bovine serum albumin.

Purification of amylase enzyme

The amylase enzyme was subjected to a purification process using a salt precipitation method with ammonium sulphate, followed by Sephacryl S-200 chromatography and DEAE-Sepharose column chromatography, as outlined by Silva-Salinas et al. (2021). The subunit molecular weight was estimated using SDS-polyacrylamide gel electrophoresis, with the use of Prestained Protein Marker, Broad Range (11-190kDa) (Vivantis Technologies Sdn. Bhd., Malaysia).

Characterization of purified amylase enzyme

The purified amylase enzyme was characterized by evaluating its activity and stability under various conditions. The enzyme-containing fraction was assessed for its activity across a range of pH levels, from 3.0 to 8.0, and at temperatures between 10 and 80 ºC. The impact of different cations, such as K^+, Mg^{2+}, Ba^{2+}, Na^+, Ni^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} on the amylase enzyme activity was investigated. Furthermore, the effect of several inhibitors and activators, including urea, Indole acetic acid (IAA), dithiothreitol (DTT), 1,10-phenanthroline, ethylenediaminetetracetic acid (EDTA), ß-mercaptoethanol, cysteine, and sodium azide, on the enzyme activity was examined, following the methodology outlined by Baltas et al. (2016).

Results

Physicochemical characteristics of sampling sites

The analysis of sampling sites revealed notable disparities in the physicochemical attributes. Specifically, the temperature spanned from 51°C at Al-Ma'a Al-Har hot spring to 75°C at Al-Aredhah hot spring, signifying significant thermal variations among the locations. Furthermore, all the hot springs displayed a

near-neutral pH, with readings consistently falling within the range of 7.1 to 7.6, indicating a relatively steady pH environment.

Isolation and identification of bacterial isolates

A thermophilic bacterial isolate, denoted as OHA8, was successfully extracted from samples of water and sediment. The morphology of the OHA8 isolate was thoroughly examined using electron microscopy, revealing Gram-positive, elongated rods capable of spore formation, each with a diameter measuring less than 0.9µm (Fig. 2). The colonies of this isolate exhibited small to medium size, semi-transparency, and a flat appearance.

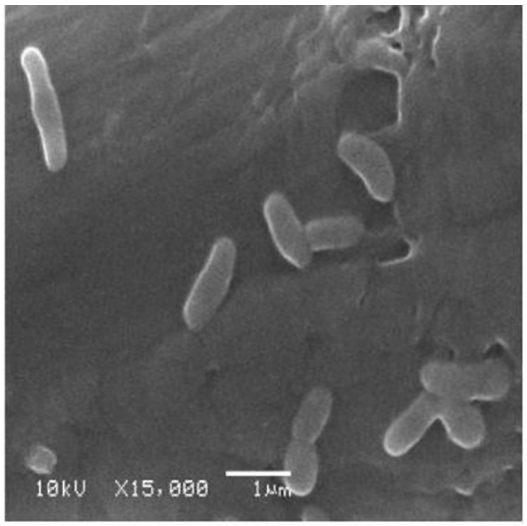


Fig. 2. Electron micrograph of *Cytobacillus firmus* accession # OQ834432 strain OHA8, with a scale bar writing down a length of approximately 100µm

The identification of this bacterial isolate was achieved through a combined approach involving molecular sequencing of the 16S rRNA gene and microscopic analysis. These analyses confirmed that the OHA8 isolate represents a specific species within its genus.

The comprehensive investigation encompassed 21 bacterial isolates; however, for the scope of this study, emphasis will be placed solely on the characterization and comparison of Brevibacillus, Bacillus, and Roseomonas species. Among these, Brevibacillus accounted for 19%, Bacillus for 19%, and Roseomonas for 14.3% of the isolates encountered (Table 1).

Screening the isolated bacterial isolates for their enzymatic activities

A total of twenty-one bacterial isolates were subjected to screening for enzymatic activities in this study, with a particular emphasis on amylolytic, proteolytic, and lipolytic functions. Out of the twenty-one isolates, only three demonstrated amylolytic activity on starch agar medium, while none exhibited protease or lipase activity. The assessment of amylolytic activity was based on the width of the clearing zones, with the isolate *Cytobacillus firmus* showcasing the largest haloforming zone (Fig. 3).

Molecular identification

Utilizing a combined approach involving 16S rRNA molecular sequencing and microscopic analysis of samples from both water and sediment, seven bacterial isolates were discerned, representing seven distinct species spanning six diverse genera. The prevailing genera encompassed Brevibacillus (42.86%), Bacillus (28.57%), Roseomonas (14.29%), and Dietzia (14.29%). Specifically, two bacterial isolates were affiliated with the Bacillus genus (comprising one species), while three isolates were classified within the genera Brevibacillus (encompassing two species), Dietzia (one species), and Roseomonas (one species). Regarding sequence homology and taxonomic categorization, bacterial isolate OQ834432 exhibited varying degrees of genetic similarity, as indicated by percentages of identity when compared to known reference strains based on their 16S ribosomal RNA genes. The analysis revealed a high level of similarity between OQ834432 and several strains, including *Cytobacillus firmus* strain YHSA15, *Cytobacillus oceanisediminis* strain H2, *Cytobacillus firmus* strain NBRC 15306, and *Cytobacillus firmus* strain IAM 12464, all showing identities of 99% or higher. Slightly lower, yet still notable, similarities were observed with *Cytobacillus depressus* strain BZ1, *Bacillus depressus* strain BZ1, *Cytobacillus gottheilii* strain WCC 4585, *Mesobacillus foraminis* strain CV53, *Heyndrickxia shackletonii* strain LMG 18435, and *Mesobacillus subterraneus* strain COOI3B, with identities ranging from 98% down to 97%. This comparative analysis provides insights into the relatedness of isolate OQ834432 to these reference bacterial strains based on their genetic sequences (Table 1 and Fig. 4).

TABLE 1. Sequence similarity of isolated strains based on 16S rRNA sequences compared to the GenBank database

Bacterial strain	Accessions	Reference strain Genbank Accessions	Description	Identities PCt (%)
OHA1	OQ860765	NR_112926.1	*Brevibacillus nitrificans*	99
OHA3	OQ860766	NR_112926.1	*Brevibacillus nitrificans*	100
OHA4	OQ860767	MK088268.1	*Brevibacillus borstelensis*	98
OHA6	OQ860768	CP012024.1	*Bacillus smithii*	99
OHA8	OQ834432 (current)	NR_112635.1	*Cytobacillus firmus* strain NBRC 15306	99
OHA9	OQ860769	NR_042390.1	*Dietzia cinnamea*	99
OHA12	OQ860770	NR_134161.1	*Roseomonas alkaliterrae*	99

Fig. 3. A clear zone seen on starch agar plates from *Cytobacillus firmus accession* # OQ834432 strain OHA8, showing the enzymatic hydrolysis of starch

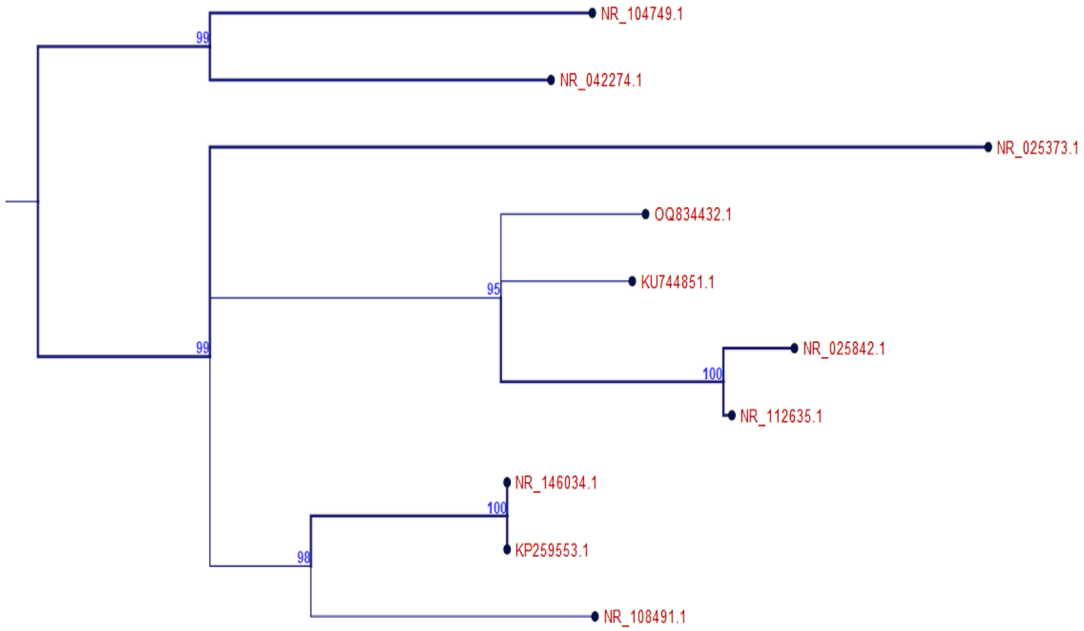


Fig. 4. A neighbor joining tree of OQ834432 isolate OHA8 showing 99% similarity to *Bacillus firmus* (strain YHSA15) accession number KU744851.1

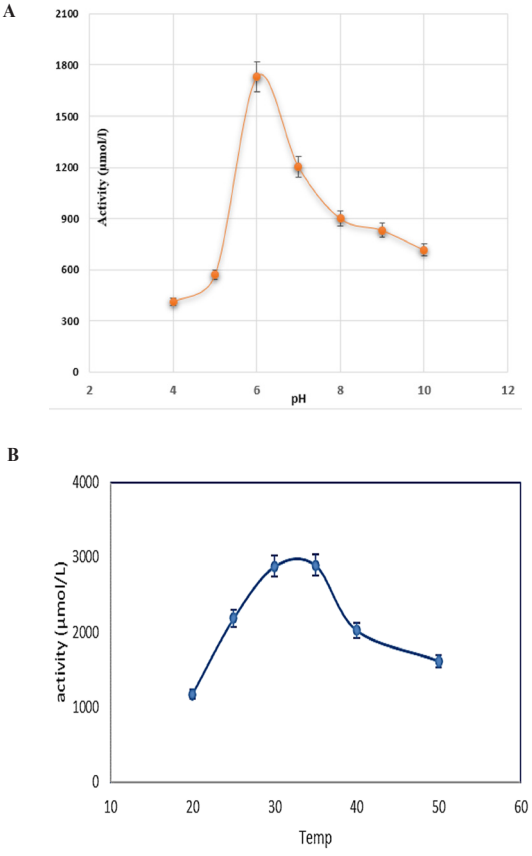
Quantitative assay of amylase activity

The amylase enzyme activity was quantitatively assessed by measuring the absorbance (OD) at 540nm and plotting a standard glucose graph using different concentrations of D-Glucose. Using the given formula, the enzyme activity was determined to be 230U/mL. This calculation involved determining the % Relative activity, which compares the enzyme activity at specific pH, temperatures, or in the presence of certain metal ions to the enzyme activity of the control. This comparison was done by expressing the relative activity as a percentage, calculated as follows:

% Relative activity= (Enzyme Activity (@pH, temperatures/metal ions)/ Enzyme Activity of control) x 100

Optimization of amylase enzyme production

The optimum culture conditions (pH, incubation temperature, and period) to achieve maximum amylase enzyme production by *Cytobacillus firmus* were investigated. The findings revealed that the optimal conditions for maximum amylase enzyme production were at a pH of 6.0, an incubation temperature of 35°C and period of 72h (Fig. 5).



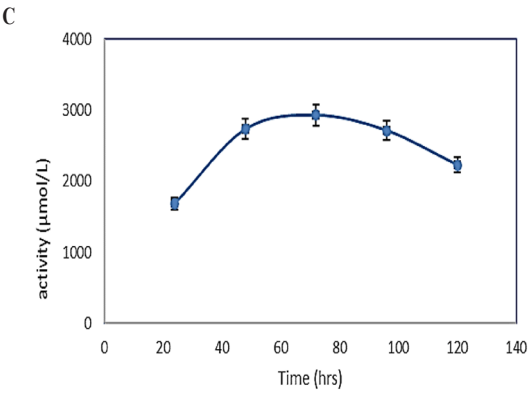


Fig. 5. Effect of (A) pH, (B) temperature, and (C) incubation period on amylase production from *Cytobacillus firmus*

Purification of amylase enzyme

The crude amylase obtained from *Cytobacillus firmus*, which had an activity of 230U/mL, was subjected to precipitation using ammonium sulfate at saturation levels ranging from 40-80%. The outcomes of the purification process, including the activity, protein content, specific activities, and fold purification, are presented in Table 2, as well as in Figs. 6 and 7.

Molecular weight determination

According to the results of the SDS-PAGE analysis, which revealed only one protein band, it was determined that the amylase enzyme isolated from *Cytobacillus firmus* exists as a monomer. The molecular weight of this enzyme was estimated to be around 60kDa, as depicted in Fig. 8.

Characterization of purified amylase enzyme

In our study, we meticulously investigated various factors to determine the optimal conditions that facilitate the highest enzymatic activity of the purified amylase. These assessments involved examining the enzyme's performance across a range of temperatures and pH levels.

Through these experiments, we discovered that the enzyme exhibited its maximum activity at 50°C and pH 5.0, as visually depicted in Fig. 9. The term "maximum activity" refers specifically to the peak level of enzyme function observed under these particular experimental conditions.

To elaborate, "maximum activity" signifies the highest attainable enzymatic performance achieved under optimal pH and temperature conditions. It represents the point where the enzyme demonstrates its most efficient catalytic

capability within the parameters tested. For our purified amylase, this peak in enzymatic function occurred at 50°C and pH 5.0, as indicated by the highest enzyme activity observed in our experimental assays.

Moreover, the enzyme's functionality was affected by EDTA, Tween 80, SDS, and PMSF, resulting in reductions of 18.2%, 59.8%, 77.5%, and 79.3%, respectively (Table 3). Conversely, magnesium ions were found to enhance the enzyme's activity by 153%, whereas EDTA hindered its function (Table 4). The enzyme exhibited full activity with both starch and amylopectin, demonstrating activity levels of 100% and 110.3%, respectively (Table 5).

Discussion

Extremophiles, microorganisms capable of thriving in severe environments, have developed distinctive physiological adaptations to endure their challenging surroundings (Yadav & Singh, 2019). Hot springs, characterized by their unique and complex chemistry, represent an enticing avenue for research into microbial diversity, offering opportunities to uncover novel compounds and genes (Kuddus & Ramteke, 2012).

A comparative analysis was conducted between OQ834432 and several related bacterial strains within the same genus. The analysis was based on the alignment of the 16S ribosomal RNA gene sequences.

TABLE 2. Protein purification steps for α- amylase produced by *Cytobacillus firmus*

Purification step	Total protein (mg)	Total activity (units*)	Specific activity (U/mg protein)	Fold purification	Yield (%)
Culture	6.10	2831	464.1	1	100
Amm. sulfate precipitation	4.20	2378	566.2	1.2	83.9
Sephacryl S-200	2.70	1958	725.2	1.6	69.2
DEAE-Sepharose Amylase I	0.53	1179	2224.5	4.8	41.6
Amylase II	0.27	320	1230.8	2.6	11.3

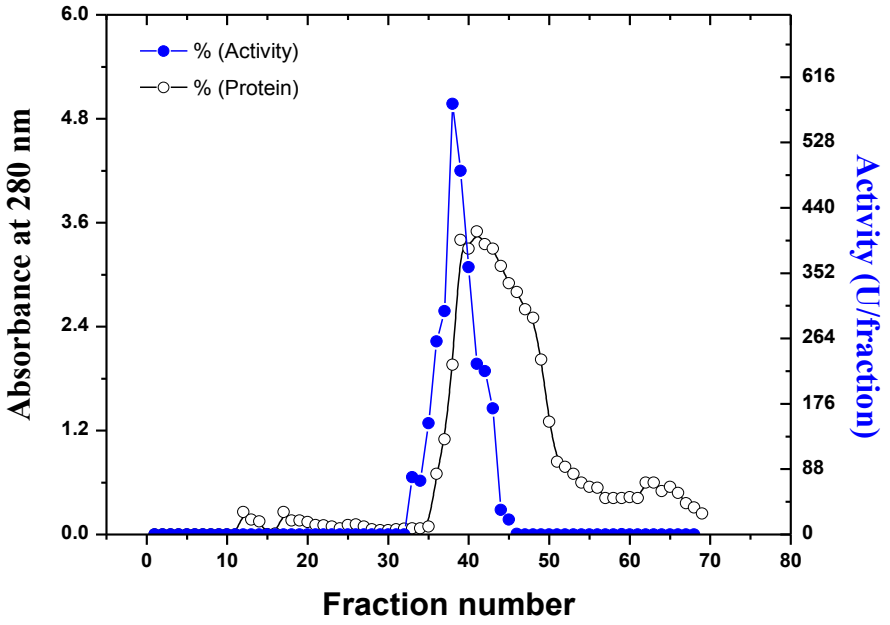


Fig. 6. A standard elution profile for Sephacryl S-200 column chromatography, used for separation and purification of amylase enzyme [The y-axis of the graph shows the absorbance of the sample, while the x-axis stands for the volume of eluent collected during the chromatographic run. The elution profile displays the separation of different biomolecules in the sample based on their molecular size and shape. The elution peaks correspond to different fractions of biomolecules that are eluted from the column in sequence as the eluent flows through the stationary phase]

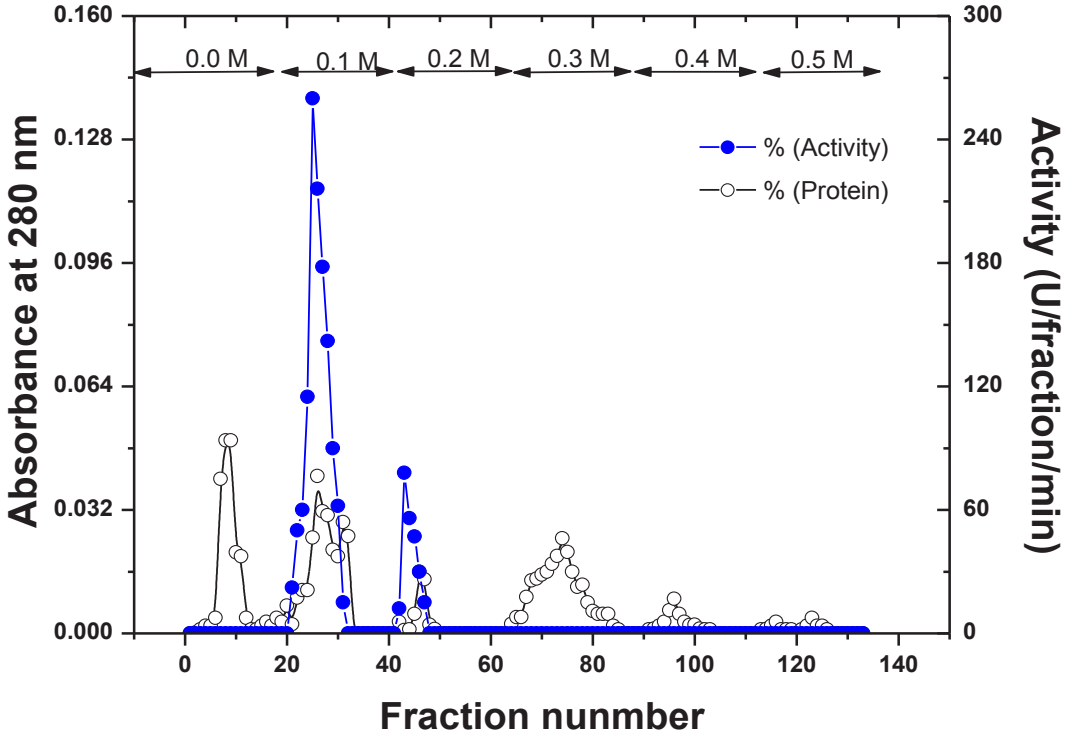


Fig. 7. Elution profile for DEAE-Sepharose column chromatography, used for separation and purification of Amylase enzyme [The y-axis of the graph shows the absorbance of the sample, while the x-axis shows the volume of eluent collected during the chromatographic run. The elution profile reflects the separation of biomolecules based on their charge properties, specifically their net charge and distribution of ionizable groups. The elution peaks correspond to different fractions of biomolecules that are eluted from the column in sequence as the eluent passes through the stationary phase]

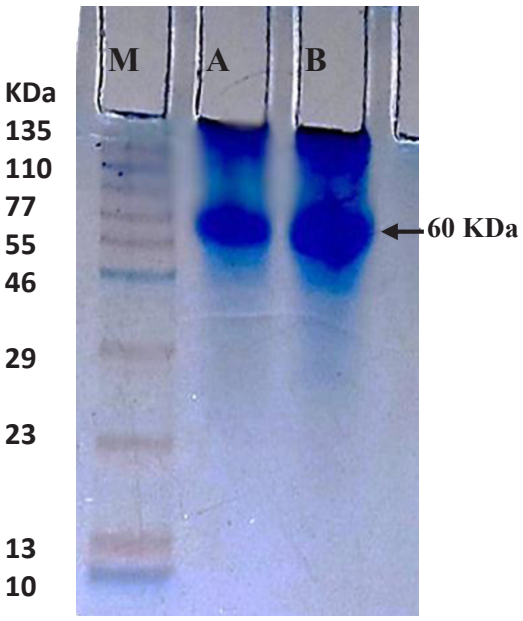


Fig. 8. 10% SDS–PAGE analysis of purified amylase enzyme from *Cytobacillus firmus.* Lane M shows the protein ladder with molecular weight markers indicated [Lanes A and B represent purified samples of the amylase enzyme. It is important to note that due to the high concentration of the purified samples, the bands appear relatively thick. This is consistent with the concentrated nature of the isolated enzyme]

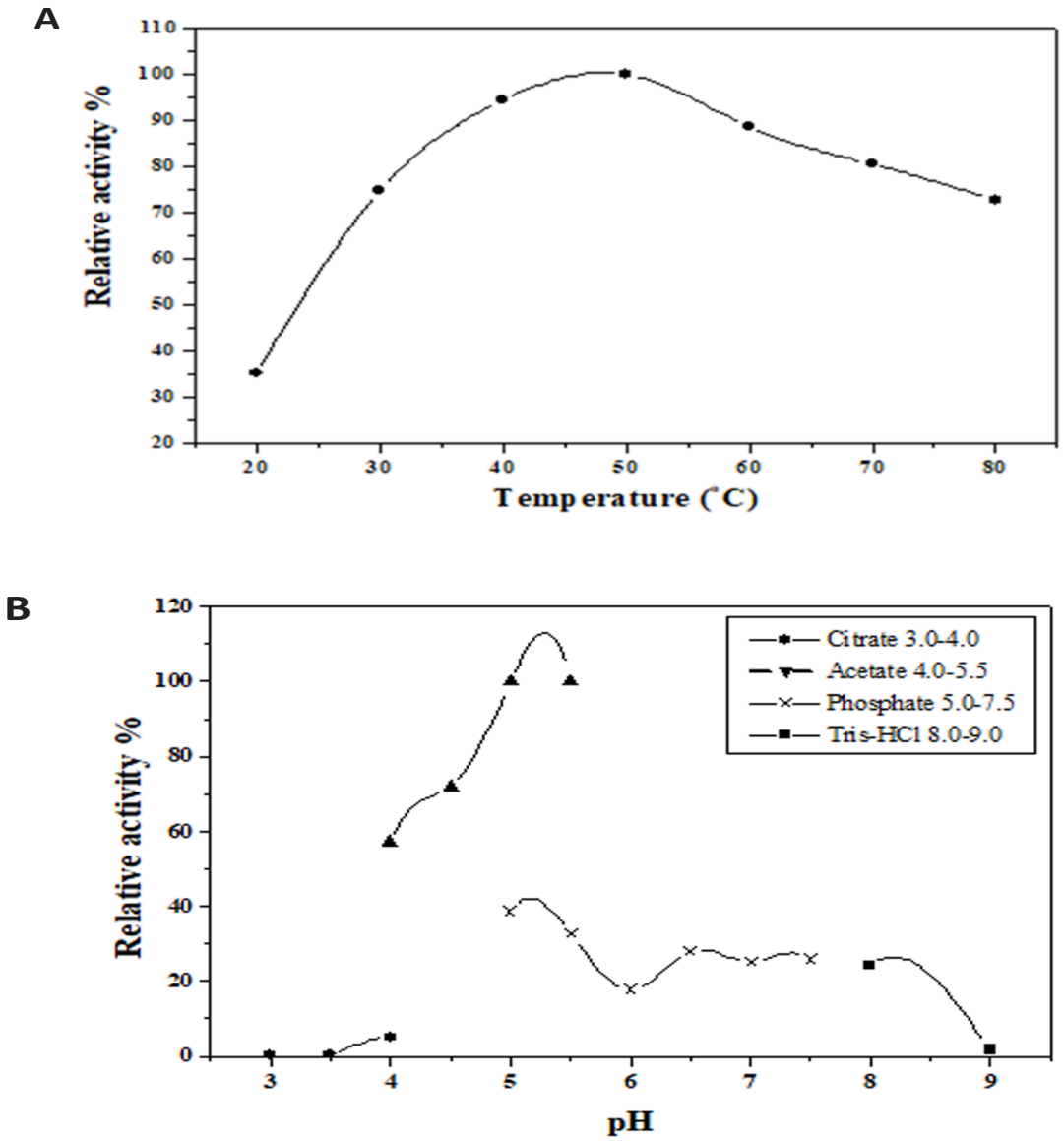


Fig. 9. The optimal conditions for the activity of the purified *Cytobacillus firmus* amylase enzyme with respect to temperature and pH [(A): The optimal temperature required for the enzyme to exhibit maximum activity, (B): The optimal pH level that promotes maximum activity of the enzyme]

TABLE 3. Effect of different compounds on α-amylase activity of *Cytobacillus firmus*

Compound	Relative activity (%)
Non	100
EDTA	18.2
L-Cysteine-HCl	128.1
DTT	126.9
SDS	77.5
Tween-80	59.8
PMSF	79.3

The enzyme was pre-incubated for 30 min at 37°C with the listed compounds individually at a final concentration of 5mM prior to substrate addition. Activity in absence of compounds was taken as 100%. Each value represents the average of two experiments.

TABLE 4. Effect of different metal ions on α-amylase activity from *Cytobacillus firmus*

Metal ions	Relative activity (%)
Non	100
Mg^{2+}	153.7
Co^{2+}	110.9
Ca^{2+}	137.2
Ba^{2+}	130.8
Mn^{2+}	84.3
Zn^{2+}	62.4
K^{+}	91.6
Ni^{2+}	70.9
Na^{2+}	72.1
Cu^{2+}	53.7
Hg^{2+}	2.8

The enzyme was pre-incubated for 30min at 37°C with the listed metal cations individually at a final concentration of 10mM prior to substrate addition.

TABLE 5. Substrate specificity of amylase activity from *Cytobacillus firmus*

Substrate	Relative activity (%)
Starch	100
Glycogen	72.1
Amylose	53.8
Amylopectin	110.3
Xylan (Oat spelts)	3.2
Xylan (Beechwood)	7.9
Xylan (Birchwood)	5.7
Maltose	17.4

The percentage identities between OQ834432 and the following strains were determined: *Cytobacillus firmus* strain YHSA15 (99.50%), *Cytobacillus oceanisediminis* strain H2 (99%), *Cytobacillus firmus* strain NBRC 15306 (99%), *Cytobacillus firmus* strain IAM 12464 (99%), *Cytobacillus depressus* strain BZ1 (98%), *Bacillus depressus* strain BZ1 (98%), *Cytobacillus gottheilii* strain WCC 4585 (98%), *Mesobacillus foraminis* strain CV53 (97%), *Heyndrickxia shackletonii* strain LMG 18435 (97%), and *Mesobacillus subterraneus* strain COOI3B (97%).

This comparative analysis revealed varying degrees of sequence similarity between OQ834432 and the mentioned strains within the genus. Notably, OQ834432 exhibited high sequence similarity (above 99%) with several strains, such as *Cytobacillus firmus* strain YHSA15 and *Cytobacillus oceanisediminis* strain H2, indicating close evolutionary relationships.

In our investigation, only *Cytobacillus firmus* and *Bacillus smithii* exhibited amylase activity. Remarkably, *Cytobacillus firmus* demonstrated the most prominent halozone, indicating heightened α-amylase activity and positioning it as a promising candidate for the production and refinement of thermostable α-amylase enzyme. These findings align with a prior study on Bacillus species isolated from Malaysian hot springs, which similarly showcased the production of thermostable α-amylase enzyme (Msarah et al., 2020).

The most favorable conditions for amylase synthesis by *Cytobacillus firmus* were determined to be a temperature of 35°C, a pH of 6.0, and an incubation period of 72 hours. In contrast, Sen et al. (2016) discovered that thermophilic bacteria isolated from hot springs exhibited optimal α-Amylase production at a slightly alkaline pH. Additionally, the highest extracellular amylase yield from *Anoxybacillus thermarum* was observed at a pH of 7.0 over a 48-hour duration (Baltas et al., 2016). Various studies have explored the optimum culture conditions for amylase synthesis. For instance, Hiteshi et al. (2018) noted that *Bacillus licheniformis* achieved maximal amylase production at a temperature of 50°C, a pH of 8.0, and an incubation period of 48h.

Enzyme purification stands as a crucial procedure in which a pure enzyme fraction is extracted from an initial impure enzyme source. Salt precipitation, a widely employed method for protein purification, involves a gradual escalation of salt concentration. Among the salts employed for this purpose, ammonium sulfate ($(NH3)_2SO_4$) is the most commonly utilized (Lim et al., 2020). Employing the 40-80 % ammonium sulfate precipitation technique, the extracellular α-amylase of *Cytobacillus firmus* was purified to a state of homogeneity, yielding 83.9% and achieving a purification fold of 1.2. Similarly, Xie et al. (2014) documented a 70.8% yield of α-amylase from *B. methylotrophicus* P11-2, resulting in a 2.3-fold purification and a specific activity of 57.6U/mg.

In this study, the α-amylase enzyme derived from *Cytobacillus firmus* underwent an initial separation process through chromatographic partitioning on a Sephacryl S-200 column, followed by subsequent purification utilizing a DEAE-Sepharose column. DEAE Sepharose is the prevailing ion-exchange column employed for α-amylase purification, with commercially available options like HiTrap DEAE Sepharose FF and HiTrap Q Sepharose FF being commonly utilized (Duong-Ly & Gabelli, 2014). The α-amylase enzyme presents a spectrum of characteristics, encompassing substrate preference, sensitivity to pH and temperature, resistance to high temperatures, pH stability, and responsiveness to various factors like metal ions, chelating agents, inhibitors, activators, and kinetic constants. The enzyme exhibited its highest activity at a temperature of 50°C and a pH of 5.0, experiencing a 153% enhancement in activity with the presence of magnesium ions, while being hindered by EDTA. Divalent metal ions, including Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+}, play essential roles in the functionality of most amylases.

The substrate specificity profile of α-amylase plays a pivotal role in determining the efficiency of starch degradation. The findings of this current study align with previous research by Asgher et al. (2007) on crude α-amylase sourced from *B. subtilis* JS-2004, which indicated that the enzyme's peak activity was recorded at pH 8.0 and 70°C. The identification of EDTA as the most potent inhibitor of the enzyme implies that the amylase derived from *Cytobacillus firmus* is a metalloenzyme. Conversely, α-amylase TfAmy48 from *Tepidimonas fonticaldi* isolate HB23 exhibited the highest relative activity towards soluble potato starch, corroborating findings from other studies (Allala et al., 2019, 2020).

Conclusion

The hot springs found in the southern region of Saudi Arabia represent a valuable reservoir of thermophilic and thermotolerant microorganisms. Our investigation revealed that the most prevalent genera found from the isolation sites were Brevibacillus (19%), Bacillus (19%), and Roseomonas. Notably, the hot springs were found to harbor a significant population of bacteria capable of producing thermostable amylase, which holds great potential for industrial applications. The findings presented in this study contribute to a deeper understanding of the microbial community structure in hot springs of the southern region of Saudi Arabia and provide a basis for future comparative studies with other geothermal systems.

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عزل وتوصيف وتحسين انزيم الأميليز المتحمل للحرارة من *Cytobacillus firmus* وتأثيره على التحلل المائي للنشا

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من المعروف أن الينابيع الساخنة غنية بالكائنات الحية الدقيقة المختلفة، بما في ذلك البكتيريا المحبة للحرارة القادرة على إنتاج إنزيمات مهمة صناعيا. هدفت هذه الدراسة إلى عزل وتنقية وتعريف البكتيريا المحبة للحرارة من ثلاثة ينابيع حارة تقع في المنطقة الجنوبية من المملكة العربية السعودية، وهي العين الحارة في مركز غميقة بمحافظة الليث، والعين الحارة بالعارضة، والعين الحارة بقرية الأحسرين بمحافظة المجاردة. تم فحص العزلات البكتيرية المعزولة لإنتاج إنزيم الأميليز، وتم اختيار العزلة الأكثر فعالية، *Cytobacillus firmus* OQ834432 السلالة (OHA8)، لإنتاج إنزيم الأميليز بكفاءة على نطاق واسع. وقد تم تنقية الإنزيم باستخدام salt precipitation, Sephacryl S-200 chromatography, and DEAE-Sepharose column chromatography. تم تحديد 21 نوعًا بكتيري، وأظهرت *Cytobacillus firmus* أعلى نشاط للأميليز. تم تحديد الظروف المثلى لنشاط إنزيم الأميليز عند 50 درجة مئوية ودرجة الحموضة 5.0، مع تعزيز النشاط بنسبة 153% في وجود أيونات المغنيسيوم. تم تثبيط نشاط الإنزيم بواسطة EDTA ووجد أن الإنزيم نشط في وجود النشا والأميلوبكتين بنسبة 100% و110.3% على التوالي. وأخيرا، توضح هذه الدراسة قدرة البكتيريا المحبة للحرارة من الينابيع الساخنة على إنتاج إنزيمات مهمة صناعيًا وتوفر نظرة ثاقبة للظروف المثالية لإنتاج الإنزيم ونشاطه. هذه النتائج لها آثار كبيرة على تطوير تطبيقات التكنولوجيا الحيوية باستخدام البكتيريا المحبة للحرارة في مختلف القطاعات الصناعية.