

Purification and Biochemical Characterization of α -Amylase from Newly Isolated *Bacillus Cereus* Strain and its Application as an Additive in Breadmaking

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Abstract

Amylase has numerous applications in the processing food sector, including brewing, animal feed, baking, fruit juice manufacturing, starch syrups, and starch liquefaction. Practical applications have been the primary focus of recent research on novel properties of bacterial α -amylases. Many amylolytic-active bacterial isolates were obtained from samples of organic-rich, salinity-rich soil. Morphological and 16S rRNA gene sequence studies clearly revealed that the organism belongs to *Bacillus* sp. and was named *Bacillus cereus* strain GL2 (PP463909.1 (When pH 6.0, 45°C, and 12 hours of incubation were met the optimal growth conditions for the strain produced the highest amount of α -amylase activity.

B. cereus strain GL2 α -amylase isoenzyme was purified to homogeneity using Sephacryl™ S-200 chromatography and ammonium sulfate precipitation. The electrophoretic molecular weight of *B. cereus* α -amylase was 58 kDa. The optimal pH and temperature for measuring α -amylase activity were 50°C and 6.0, respectively. α -Amylase did not change at 50°C. The purified enzyme improves bread texture by reducing stiffness while improving cohesiveness and flexibility. Purified α -amylase was added to the flour, which improved the rheological properties and overall bread quality. As a result, the α -amylase from *B. cereus* strain GL2 can be used to promote bread-making.

Key words: biochemical and molecular bacterial identification, α -amylase production extraction, purification, breadmaking adaptive

Introduction

α -Amylases break down polysaccharides such as starch, amylose, and amylopectin by hydrolyzing their internal 1,4-glucan linkages. These enzymes belong to the glycoside hydrolase families 13 (GH13) and 31 (GH31) and play a crucial role as catalysts in various modern production processes (Ottone et al. 2020; da Costa-Latgé et al. 2021).

The evolution of enzymes should prioritize dynamics due to their close connection with biological function (Petrović et al. 2018). The popularity of amylases has been increasing annually, constituting up to 25% of the global enzyme market. Amylolytic fungi and bacteria found in soil or hot springs can produce amylases. Bacteria in the soil can thrive by utilizing available nutrients, leading to the production of multiple amylase enzymes to cater to various industries (Mahmoud et al. 2022).

Microorganisms are a superior source of α -amylase compared to plants or animals due to their cost-effectiveness, environmental friendliness, and ease of modification and purification (Parmar et al. 2021). Microbe-derived amylase is widely used in commercial enzyme production for its high capacity (Silaban et al. 2020). Microbial amylases have primarily replaced the chemical hydrolysis of starch in the processing industry due to their stability and suitability for various commercial applications. Bacterial production of α -amylase, particularly by *Bacillus* species, is efficient and cost-effective (Farooq et al. 2021).

Bacillus species, such as *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* have been shown to produce thermostable α -amylase in large quantities for commercial use, including in baking (Rasooli et al. 2008; Prakash and Jaiswal 2010; Gazali et al. 2018; Jujavarapu and

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Dhagat 2019). Amylases from *B. licheniformis* (BLA) are particularly well-known for their industrial applications (Kohli et al. 2020). Enzyme activity is significantly influenced by temperature and other factors. α-Amylase functions optimally at pH 7.0. Enzymes have evolved to perform best at temperatures that align with the conditions of the organisms they act (Arcus et al. 2020).

Amylase, glucosidase, and protease are bacterial hydrolytic enzymes that enhance the volume and texture of gluten-free foods. Amylolytic enzymes break down starch into sugars, promoting faster yeast multiplication. Biotechnology primarily utilizes amylase, a bacterial enzyme with diverse practical applications (Goesaert et al. 2009). Industrial applications of enzymes from bacteria and fungi have become more widespread, with amylases having various uses in different industries (Elyasi Far et al. 2020; Giri 2021).

Amylase is used in the processed food sector, brewing, animal feed, baking, fruit juice, starch syrups, and more. Amylases from *B. stearoothermophilus* and *B. licheniformis* can enhance bread quality (Dahiya et al. 2020). Starch-digesting enzymes improve bread properties by breaking down starch in wheat flour to produce maltose for yeast fermentation, leading to rapid and uniform dough fermentation (Atudorei et al. 2021; Sadeghian Motahar et al. 2022). Amylase accelerates fermentation, reduces dough viscosity, enhances bread's flavor, quality, and shelf life, and improves texture, toast ability, and crust color (Jujjavarapu and Dhagat 2019; Farooq et al. 2021).

This research aims to identify new amylolytic strains capable of efficiently producing α-amylase by screening novel strains from various biotopes in Saudi Arabia. The identified strain underwent comprehensive morphological, biochemical, and genomic characterization, then optimization, study, and application of α-amylase in bread making.

Experimental

Materials and Methods

Chemicals. Starch (99% purity) was purchased from Sigma-Aldrich® (Merck KGaA, Germany). All chemicals used for cultivation and enzyme assays are analytical grade from Merck KGaA (Germany). Oligonucleotides were synthesized by the Genome Express (France).

Samples collection for bacterial isolation. The soil samples were collected from the salinity region surrounding Rabigh city (Fig. 1) in the Kingdom of Saudi Arabia. The samples were enriched with various vegetable organic matter and incubated for one week at ambient temperature. The organic soil samples were then transported in sterile plastic bags and stored at 4°C until use.

Culture media. α-Amylase-producing bacteria were cultured using starch nitrate agar plates with the following composition (g/l): 20 starch, 15 agar, 0.5 MgSO₄, 1 KH₂PO₄, 3 CaCO₃, 2 KNO₃, 0.5 NaCl, 1 ml Trace solution and pH 7 (Shirling and Gottlieb 1966). After 48 h of incubation at 37°C, amylolytic activity was detected using the starch-iodine test (Gupta et al. 2003). Thirteen isolates were grown on starch nitrate medium with the following composition (g/l): 20 starch, 0.5 MgSO₄, 1 KH₂PO₄, 0.01 FeSO₄, 3 CaCO₃, 2 KNO₃, 0.5 NaCl, 1 ml Trace solution and pH 7. The Trace solution contains (%): 0.1 FeSO₄, 0.1 MnCl₂, and 0.1 ZnSO₄ (Gupta et al. 2003).

Isolation of amylolytic bacteria. One gram of the collected organic soil sample was added to 50 ml of starch nitrate broth medium. The flasks were incubated for 2 days at 37°C. One ml of each broth was spread-plated onto starch nitrate agar plates, which were then incubated at 37°C for 2 days. The screening for amylolytic activity was performed using a starch hydrolysis test on a starch nitrate agar plate (Shirling and

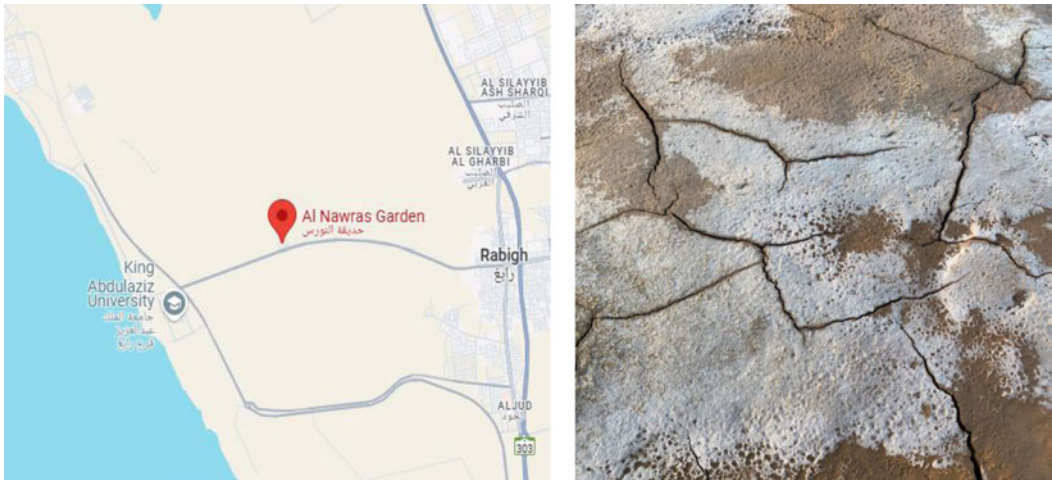


Fig. 1. Type of soil used in this study surrounding Rabigh City in the Kingdom of Saudi Arabia (22.803970N, 38.984341E).

Gottlieb 1966). The pure isolated colonies were inoculated onto starch agar plates with starch as the only carbon source. After incubation at 37°C for 48 hr, the individual plates were submerged with Gram's iodine to produce a blue-colored starch-iodine complex. The appearance of a clearance zone around colonies indicates the presence of α -amylase activity (Gupta et al. 2003). Amylolytic bacteria with the maximum diameter of the clearance zone were retained for further investigations.

Culture conditions. The retained amylolytic strain was precultured at 37°C and 230 rpm for 15 h in 250 ml shaking flasks with 50 ml of starch broth medium. Overnight precultures used as inoculum were cultivated in 1 l shaking flasks with 100 ml of the same culture medium. Cells were grown at 37°C on a rotary shaker at 230 rpm. Growth was monitored by measuring the culture's OD at 600 nm. At the end of the fermentation period, the culture medium was centrifuged at 5,000 rpm for 20 min to obtain the crude extract containing α -amylase activity.

Identification of the selected isolate. Morphological and cultural characteristics. On a starch agar medium, the bacterial isolate was cultured to examine the color and shape of the colonies. Light microscopy was used to conduct morphological analyses (Williams et al. 1989). The following physiological tests, including those for catalase, oxidase, pH, and ideal temperature, were carried out.

Gram staining. Crystal violet was applied, and the bacterial film was allowed to soak for one minute. Then, it was washed off with distilled water after the slide was prepared and the bacterial film was fixed. Ethyl alcohol (95%) was allowed to drip onto the surface of the slide after the iodine was removed from the Gram stain. This process was repeated until the alcohol lost its hue. After being stained with safranin for a minute, the slide was gently cleaned with water. After a brief gentle wash, the slide was dried in the air and inspected with an oil immersion lens while containing a few drops of Cedar oil. Gram-negative bacteria will be red-colored, whereas Gram-positive bacteria will be colored violet.

Catalase test. This examination is performed to detect the catalase enzyme. This enzyme protects against hydrogen peroxide (H_2O_2) toxicity, which can accumulate during aerobic metabolism. A bacterial colony was spread on a glass slide, and a drop of H_2O_2 was added. If bubbles appear, the test is considered successful. If no bubbles are present, it indicates the absence of catalase.

Oxidase test. The oxidase test is conducted based on synthesizing the cytochrome oxidase enzyme. When oxygen is present in the air, the colorless substrate tetramethyl-phenylenediamine dihydrochloride is oxidized by this enzyme to create a dark-purple molecule. The color purple denotes a positive response.

Starch hydrolysis. The oxidase test is conducted based on synthesizing the cytochrome oxidase enzyme. This enzyme oxidizes the colorless substrate tetramethyl phenylenediamine dihydrochloride to create a dark-purple molecule when oxygen is present in the air. The purple color denotes a positive response.

Identification by molecular biology methods. Extraction of DNA from bacterial α -amylase isolate. Bacteria were cultured on a slant and then transferred into 250 ml Erlenmeyer flasks with 30 ml of starch broth. The flasks were incubated at 37°C and 230 rpm for 24 hours. After incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes. Cells were disrupted using liquid nitrogen on a porcelain dish. TE buffer with lysozyme (20 mg/ml) was added to 500 μ l a tube containing bacteria and incubated at 37°C for 30 minutes. Proteinase K and 10% SDS (w/v) were then added and incubated at 55°C for 30 minutes. DNA was precipitated with ethanol, chilled at -20°C for 30 minutes, and centrifuged (10,000 rpm for 10 minutes) to form a pellet. The pellet was washed with ethanol and dissolved in TE buffer. To remove RNA from DNA, the sample was treated with 1 ml of RNase solution (20 g/ml) at 37°C for 1 hour. The DNA was extracted with phenol and chloroform, precipitated, and tested for quality and quantity.

Phylogenetic analysis of 16S rDNA sequence. In a 100 μ l reaction, genomic DNA was amplified using HotStar® Master Mix (2 $\times$) (QIAGEN, Germany). Primers were designed using a conserved region of 16S rDNA from various bacteria. The samples without the primers in the HotStar® Master Mix served as a negative control. Two bacterial primers, 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT-3', were used in polymerase chain reaction (PCR) to amplify the 16S rDNA of the examined bacteria. The DNA sequence was then compared to the GenBank database using the BLAST tool at the National Center for Biotechnology Information (NCBI).

Factors affecting bacterial α -amylase activity of *B. cereus* WL. Effect of incubation periods on α -amylase production. The samples were incubated for two to forty-eight hours. A variety of sterile media was used to fill 250 ml Erlenmeyer flasks. Two ml of preculture was injected into 50 ml flasks. The flasks were kept in a 37°C incubator with a 230 rpm rotation. Bacterial growth and α -amylase production were assessed following each growth phase.

Effect of different incubation temperatures on α -amylase production. Temperature was found to play a role in enzyme synthesis and growth. The amylase broth medium in the 250 ml Erlenmeyer flasks was supplemented with approximately 2 ml of the selected bacterial isolate. The flasks were then incubated for

12 h with shaking at 230 rpm and temperatures ranging from 25 to 60°C. At the end of the incubation period, the same methods were used to evaluate the growth and amylase activity of the inoculated bacteria.

Effect of pH on α -amylase production. The medium was prepared with a pH range from 3 to 9. 250 ml Erlenmeyer flasks were filled with 50 ml sterile medium, and 2 ml of preculture was added. After determining the necessary time, the flasks were placed in an incubator with a speed of 230 rpm and a temperature of 45°C.

Enzyme assay. The activity of α -amylase was measured at pH 6.9 and 25°C. 0.5 ml of the enzyme solution, diluted in 0.1 M phosphate buffer pH 6.5, was incubated with 0.5 ml of 1% soluble starch prepared in 0.1 M phosphate buffer. DNS (3,5-dinitrosalicylic acid) was used to determine the amount of the released reducing sugars. One unit of α -amylase activity was defined as the amount of enzyme that liberates 1 μ mole of reducing sugar (maltose equivalents) per minute at 25°C and pH 6.9 (Salem et al. 2020).

Determination of protein content. The Bradford technique (Bradford 1976) was used to measure the total protein contents in the cell-free supernatant and purified samples. A calibration curve was performed using bovine serum albumin (BSA) as the reference.

Enzyme purification. The selected bacteria were allowed to grow in the medium while being treated with all the optimal conditions found in the literature for producing α -amylase. The culture medium was then centrifuged for 20 min at 4°C and 5,000 rpm. The cell-free filtrate was used as a crude enzyme solution to purify the α -amylase enzyme. The initial α -amylase activity and the protein concentration were measured as described.

Ammonium sulfate precipitation. The obtained supernatant was then treated with solid ammonium sulphate (80% saturation w/v) while being gently stirred at 4°C for 30 min. After that, it was centrifuged for 30 min at 8,000 rpm and 4°C. α -Amylase activity and protein concentration were measured as described previously.

Dialysis. The purification process was used to remove any remaining ammonium sulfate and to eliminate peptides and proteins with small molecular weights. The resulting residue was dialyzed overnight against 2 l of pH 8.5 Tris-HCl 20 mM buffer in a cellophane bag with a 20 kDa molecular mass cutoff. The concentrated cell-free supernatant from dialyzing the original sample is now ready for the following purification steps.

Filtration chromatography. The concentrated dialyzed solution was chromatographed on a Sephacryl™ S-200 column (30 cm $\times$ 1.5 cm; Merck KGaA, Germany). The elution was performed at a flow rate of 30 ml/h followed by measuring the absorption at 280 nm and the α -amylase activity under standard conditions. The active fractions were collected, pooled, and analyzed by SDS-PAGE.

SDS-PAGE analysis. Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described previously (Laemmli 1970). Standard protein markers (low molecular weight 14–60 kDa) were used as standards.

Biochemical characterization of the purified enzyme. Effects of pH and temperature on α -amylase production. To study the effect of the pH of the culture medium on α -amylase production, the selected amylolytic strain was grown in 250 ml shake flasks with 50 ml starch nitrate medium for 48 h at 37°C with different pH levels ranging from 5 to 9. After centrifugation, the α -amylase activity was then measured at pH 6.9 and 25°C. The effect of temperature on amylase production was determined by growing the selected amylolytic strain in 250 ml shaking flasks with 50 ml starch nitrate medium for 48 h at different temperatures ranging from 25 to 50°C. The α -amylase activity was measured at pH 6.9 and 25°C after centrifugation.

Effects of pH and temperature on α -amylase stability. To check the pH stability, the enzyme was incubated with buffers 100 mM: glycine-HCl (pH 4), sodium acetate (pH 5–6), phosphate (pH 7), Tris-HCl (pH 8) and glycine-NaOH (pH 9–12) (in ratio of 1:1) at 25°C for 1 h and assayed under standard assay conditions (pH 6.9 and 25°C). The effect of temperature on α -amylase stability was determined by incubating the enzyme at different temperatures (25 to 100°C) for 30 min. The residual activity was determined after centrifugation under standard conditions (pH 6.9 and 25°C).

Effect of substrate concentration on α -amylase stability. Before the reaction started, the substrate, α -amylase, was introduced to a borate buffer at pH 6.0 and 50°C for one hour at various concentrations. The α -amylase activity assay was then performed under standard conditions.

Enzyme concentration on α -amylase stability. To determine the optimal enzyme concentration, the reaction mixture was prepared with varying concentrations of pure enzyme. The purified enzyme was added to the reaction mixture at concentrations ranging from 0.25 to 3 U/ml and incubated for 1 hour at 50°C and pH 6. Subsequently, the α -amylase activity of the mixture was measured.

Bread making procedure. The bread was made with the following ingredients: 250 g of wheat flour, dry yeast, 3% sugar, 2% salt, 1.25% vegetable oil, and water. After being dissolved in distilled water, 0.5 U/kg and 1.0 U/kg of purified α -amylase were added to the bread mixture. It took 10 min to mix the ingredients. After spending 45 minutes at 50°C, the finished dough was cooked for 45 minutes at 200°C. After cooling for two hours at ambient temperature, the loaves were then cut into slices.

Table I
Gram staining, morphological and physiological characterization
of the isolates having a zone of clearance with iodine solution.

Sample	Gram stain	Morphology under light microscope	Colony color	Colony morphology	Growth on α-amylase agar
M1	+	<i>Bacillus</i>	white	filamentous	++++
M2	+	<i>Bacillus</i>	white	irregular	+++
WL	+	<i>Bacillus</i>	white	irregular	++++
M12	+	<i>Bacillus</i>	creamy	circular	++
M13	+	<i>Bacillus</i>	creamy	circular	++++
L5	–	<i>Bacillus</i>	white	circular	++
L6	+	<i>Bacillus</i>	white	circular	++
L8	+	<i>Bacillus</i>	creamy	irregular	+++
L15	+	<i>Bacillus</i>	creamy	circular	++

+ – low growth; ++ – moderate growth; +++ – high growth; ++++ – very high growth

Results

Screening of α-amylase producing bacteria. This investigation used organic soil from the Rabigh, Saudi Arabia seashore to isolate α-amylase-producing bacteria. Forty-five distinct bacterial amyolytic strains were found. Nine strains exhibited high amyolytic activity when tested for starch hydrolysis using an iodine solution to generate clear zones surrounding their colonies (Fig. 2). Table I provides an overview of the strains’

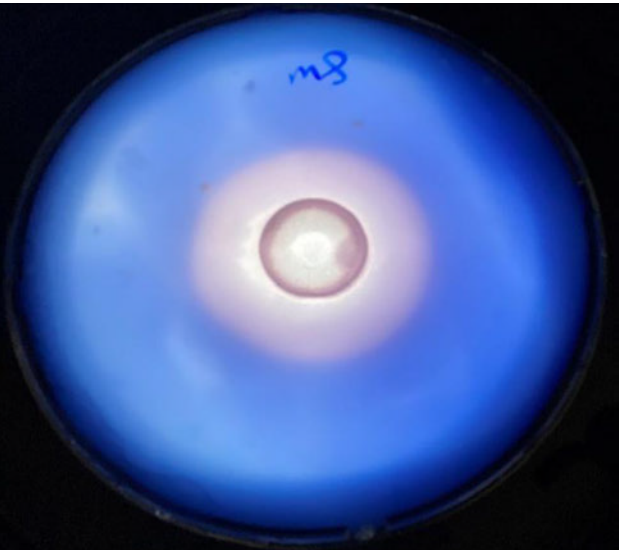


Fig. 2. Isolate WL showing positive starch hydrolysis test in presence of iodine.

morphological, physiological, and Gram stain results. Among the nine identified bacteria, isolate WL had the largest clearance zone on starch agar plates compared to the other isolates. Table II shows the α-amylase activity achieved after cultivating the isolated bacteria for 48 h at 37°C in a starch nitrate medium. The α-amylase activity was determined at pH 6.9 and 25°C, as indi-

cated in the Materials and Methods section. Despite the fact that there were no visible differences between the isolates when producing α-amylase in liquid media, isolate WL was chosen as a potent isolate for identification and α-amylase characterization.

Identification of the selected isolate. Morphological and biochemical characterization. The isolate WL reached its maximum growth on the starch agar medium after 12 h of incubation at 45°C. After Gram staining, a rod-shaped, Gram-positive bacterium was seen. The physiological and biochemical traits of the selected bacterial isolate WL were investigated and are shown in Table III. The results in Table III demonstrate that the selected bacterial isolate WL has a growth temperature range of 35–60°C, with an optimum at 50°C, and a growth pH range of 4–8, with an optimum at pH 6. The WL isolate was determined to be catalase and oxidase-positive. The morphological and biochemical properties suggested that the isolate is close to *Bacillus*

Table II
Screening of α-amylase activity (U/ml) using starch nitrate medium and selecting of WL as a potent isolate for further study.

Isolate code	α-amylase activity (U/ml)
M1	511.08 ± 0.12
M2	572.10 ± 0.14
WL	579.12 ± 0.18
M12	509.98 ± 0.04
M13	499.99 ± 0.02
L5	568.12 ± 0.18
L6	539.08 ± 0.12
L8	568.12 ± 0.12
L15	539.08 ± 0.14

Enzyme activity was measured at pH 6.9 and 25°C.

Table III
Physiological characters of the selected bacterial
Bacillus cereus WL.

Character	WL Strain
Temperature range	35–60°C
Optimum temperature	45°C
pH range	4–8
Optimal pH	6
Catalase	+
Oxidase	+

species and was subjected to molecular biology methods for confirmation and complete identification.

Identification by molecular biology method. The strain’s species identification was supported and then verified using genomic approaches. Furthermore, 16S rRNA phylogenetic research predominated. The WL bacterium’s DNA was extracted first, followed by PCR amplification. The sequencing and analysis of the acquired PCR fragment revealed that the strain is *B. cereus*, and a proposed name is *B. cereus* WL. (Fig. 3). The sequence data was analyzed using the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), using the CLUSTAL W program v1.8 (Thompson et al. 1994) with standard parameters. The phylogenetic tree was constructed using the Neighbor-joining method. The resulting sequence has been deposited in the NCBI Gene Bank under the accession number PP463909.1.

Optimization of *B. cereus* growth and α-amylase production.

The optimal culture medium was used to grow the *B. cereus* strain at various incubation times (2–34 h). After each incubation time, the growth and the α-amylase activity were assessed. The greatest bio-mass production was obtained after 12 h of incubation at 50°C and pH 6. At the same incubation time, *B. cereus* strain produced a maximal α-amylase activity of about 1.386 U/ml (Fig. 4). To maximize the synthesis of the enzyme under various growing conditions, 12 h were chosen since the *B. cereus* produced the maximal α-amylase activity of 1.386 U/ml.

Purification of the enzyme. *B. cereus* strain was cultivated at 50°C, pH 6, and 230 rpm in 250 ml flasks with 50 ml of starch broth medium. After 12 h of incubation, the culture medium was centrifuged at 5,000 rpm for 20 min. The obtained supernatant was subjected to 80% saturation ammonium sulfate precipitation. The precipitated proteins were resuspended in 5 ml Tris-HCl 20 mM, pH 8.5 buffer, and dialyzed against the same buffer using a 20 kDa-MWCO cellophane bag for 24 h.

The obtained dialyzed proteins were loaded onto a gel filtration Sephacryl™ S-200 column. Elution of α-amylase was performed with Tris-HCl 20 mM, pH 8.5 buffer at 30 ml/h. The fractions containing α-amylase activity (eluted at 1.7 void volume) were presented (Fig. 5). The results of the SDS-PAGE analysis of the pooled fractions of this Sephadex G-100 (Merck KGaA,

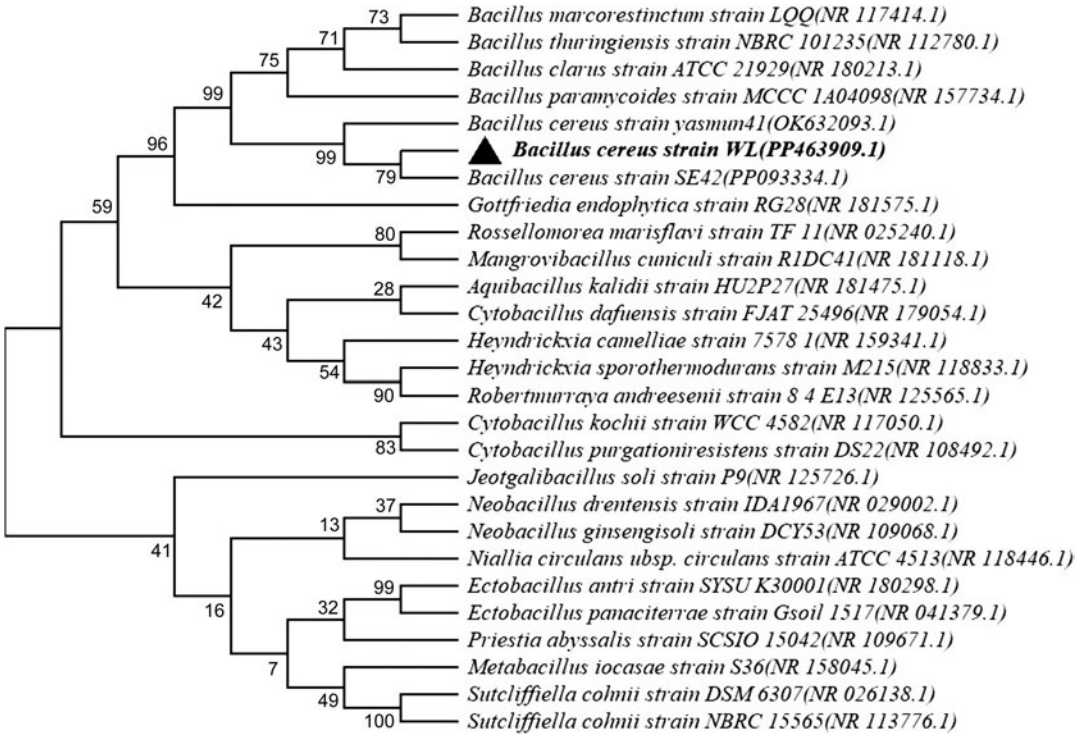


Fig. 3. The phylogenetic tree of *Bacillus cereus* WL with the accession number PP463909.1 and its close relatives based on the 16S rRNA sequence. The tree was constructed using the Neighbor-Joining method in MEGA software v11. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

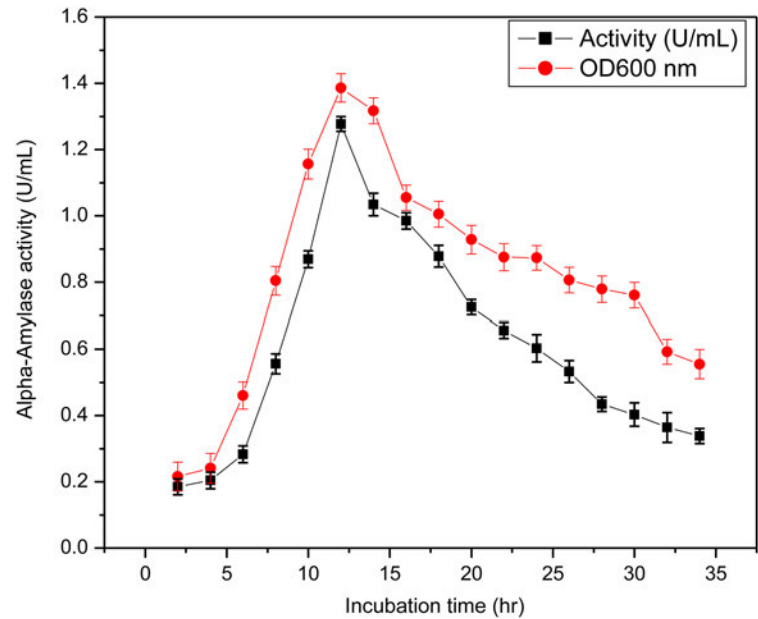


Fig. 4. Effect of incubation time on the growth and the α -amylase activity from *Bacillus cereus* WL.

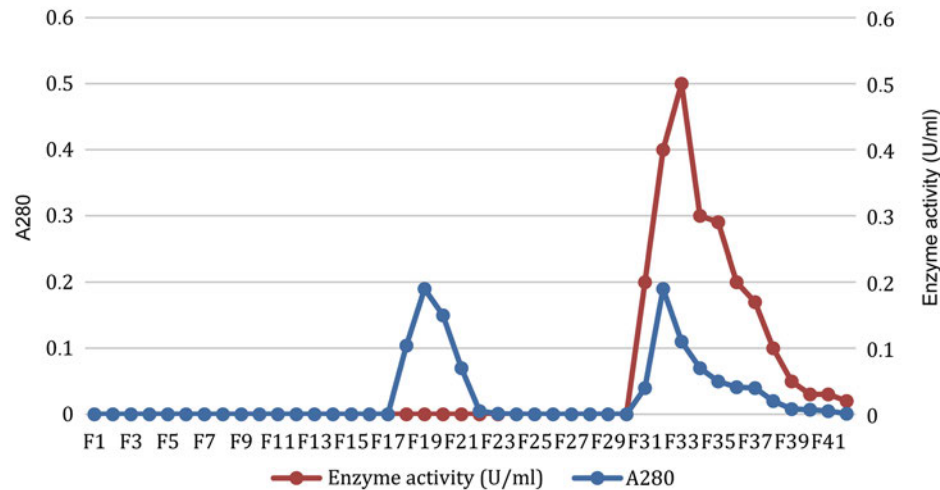


Fig. 5. Chromatography of *Bacillus cereus* WL on Sephacryl™ S-200.

Germany) chromatography are assumed in Fig. 6. This figure shows that the α -amylase of *B. cereus* is present as a major band with a molecular mass of 58 kDa. The purification flow sheet (Table IV) shows that the purified *B. cereus* α -amylase was purified 3.7-fold with a specific activity of 818 U/mg.

Characterization of the purified *B. cereus* α -amylase. Effect of temperature on α -amylase activity and stability. The activity of *B. cereus* α -amylase was measured at different temperatures ranging from 25 to 100°C. The maximal α -amylase activity was measured at 50°C (Fig. 7). In order to investigate the thermal

Table IV
Flow sheet of the *Bacillus cereus* WL purification.

Purification steps	Total activity (U)	Total protein ^a (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Crude enzyme	180	0.817	220	–	1
Participate by amm. sulfate (80%)	90	0.225	400	50	1.8
Sephacryl S-200	45	0.055	818.18	25	3.7

^a – Protein were estimated by Bradford methods. The experiments were conducted three times.

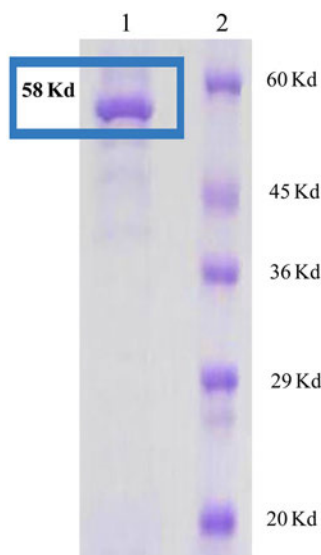


Fig. 6. The SDS-PAGE (12%) analysis of the *Bacillus cereus* WL. Lane 1 – 20 µg of partially purified amylase obtained after Sephacryl™ S-200 chromatography; Lane 2 – Molecular mass markers.

stability, the partially purified α-amylase was incubated for 30 min at different temperatures. Our results show that the enzyme was extremely stable between 25 and 60°C. When incubated at temperature higher than 60°C, the activity decreased rapidly and the enzyme was inactivated after few minutes at 80°C (Fig. 7).

Effect of pH of α-amylase activity and stability. The activity of *B. cereus* amylase was measured at different pH levels ranging from 3 to 9. The maximum amylase activity of the purified enzyme was observed at pH 6 (Fig. 8). To investigate the pH stability of the enzyme, the partially purified α-amylase was incubated

for 1 h at 25°C at different pH levels. Our results show that the enzyme was stable between pH 5 and 7. 60% residual activity was obtained at extreme pH levels of 3 and 9 (Fig. 8).

Effect of different enzymes and substrate concentrations on α-amylase activity. The α-amylase activity was measured using various enzyme concentrations. Different amounts of the enzyme (0.25–3 U/ml) were added to the reaction mixture and incubated at 50°C. The results showed that the maximum α-amylase activity was reached for quantities greater than or equal to 0.5 U/ml (data not shown).

α-Amylase activity was measured using different substrate concentrations (0.25–3 mg/ml) added to the reaction mixture and incubated at 50°C. The results also show that as the substrate concentration progressively increased from 0.25 to 1.75 mg/ml, α-amylase activity also increased. The maximum α-amylase activity was reached at quantities equal to or greater than 1.75 mg/ml (data not shown). The results in Fig. 9 showed that the amylase kinetic constants, K_m and V_{max} , were determined using the Lineweaver-Burk plot and linear regression. The enzyme activity (V) was plotted against the substrate concentration $[S]$ of starch to calculate K_m and V_{max} . The Lineweaver-Burk plot showed a linear relationship, allowing for the estimation of the kinetic constants. The V_{max} value was approximately 1.49 U/mg proteins. The K_m value, representing the substrate concentration needed for half of the maximum enzyme velocity, was found to be 0.05 mM. A lower K_m value indicates a higher affinity of the enzyme for the substrate.

Influence of *B. cereus* α-amylase incorporation on dough properties. The breadmaking performance of the

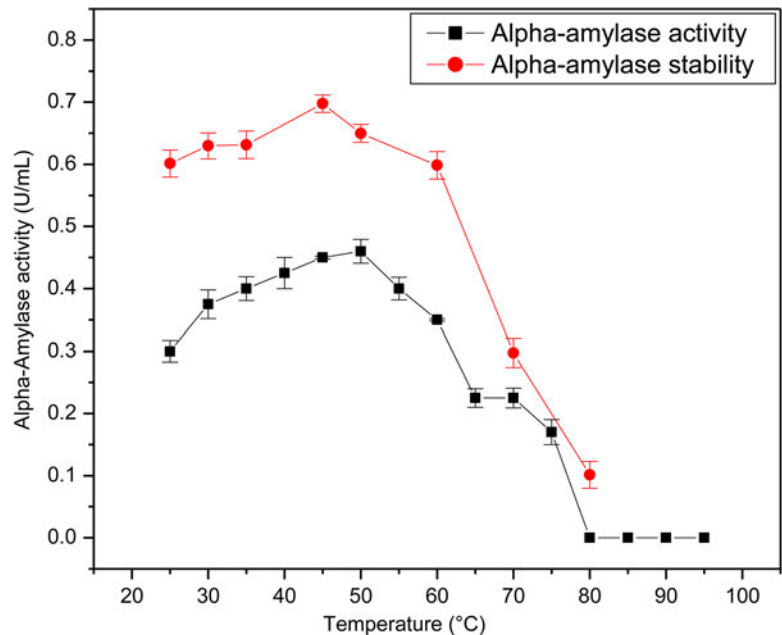


Fig. 7. Effect of temperature on the activity and the stability of the purified *Bacillus cereus* WL. α-Amylase was measured under stander conditions as described in Material and Methods section.

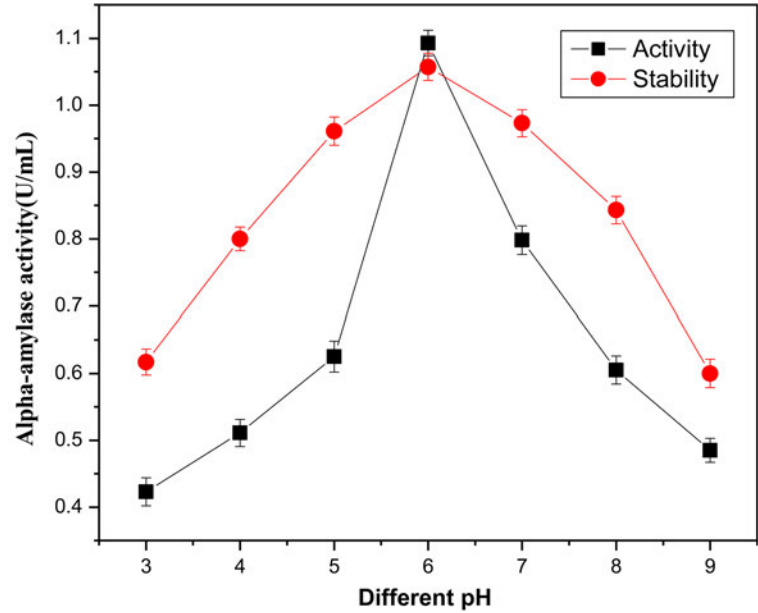


Fig. 8. Effect of pH on the activity and the stability of the purified *Bacillus cereus* WL. α -Amylase was measured under standard conditions as described in Material and Methods section.

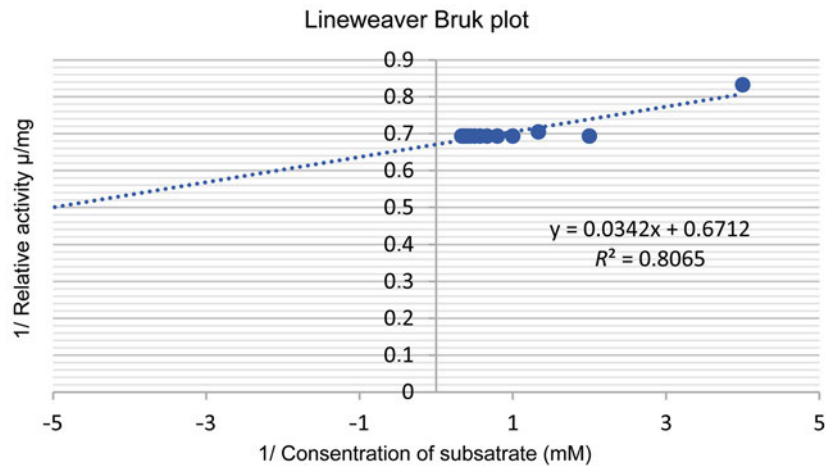


Fig. 9. Lineweaver-Burk plot (1/Relative activity (v) vs. 1/Concentration of substrate (S)) for α -amylase activity.

α -amylase was tested using two enzyme concentrations: 1 U/kg (Fig. 10b) and 2 U/kg (Fig. 10c). The results showed a marked improvement in the textural properties of bread supplemented by the *B. cereus* α -amylase. It was observed that the addition of the enzyme increased the bread volume with more crumb porosity compared to the control (Fig. 10a).

Discussion

This study on *B. cereus*, isolated from the soil near Rabigh city in Saudi Arabia, advances our understanding of microbial amylases and their potential industrial applications, particularly in the food industry. The initial screening step, which resulted in the isolation

of 45 bacterial isolates capable of hydrolyzing starch, demonstrates the soil’s high microbial diversity and potential as a source of industrially relevant enzymes. Among these isolates, the WL isolate showed higher amylolytic activity, laying the foundation for identifying potent enzyme producers crucial for biotechnological applications.

In this context, the selection of *B. cereus* WL is particularly notable. As highlighted by Carroll et al. (2022) *Bacillus* species are recognized for their ability to produce a variety of important industrial enzymes. The α -amylase-producing *B. cereus* is preferred due to its robustness, efficiency in enzyme production, and the ability to produce enzymes with desirable characteristics such as high stability and activity under various industrial process conditions.

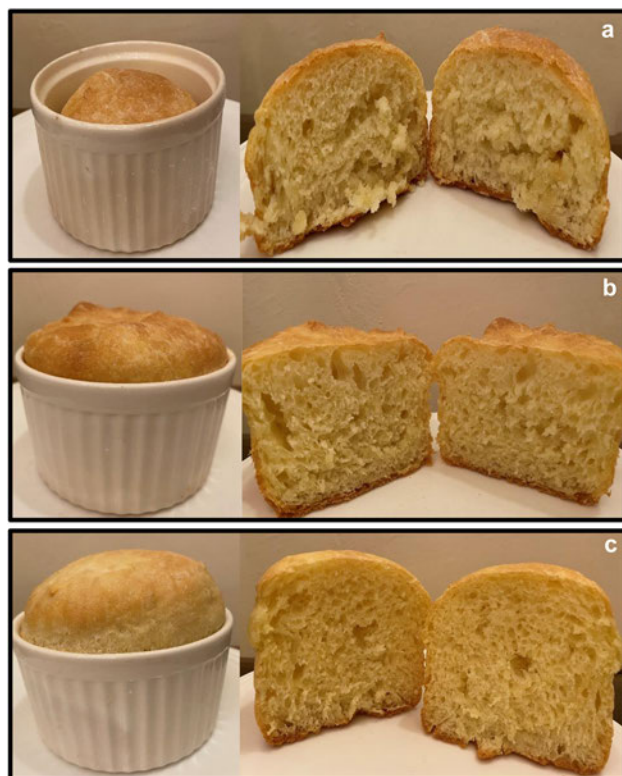


Fig. 10. Effect of the addition of α -amylase on the volume of bread.

a – bread without enzyme; b – bread with 1 U/kg enzyme;
c – bread with 2 U/kg enzyme.

Identifying isolate WL as *B. cereus* WL adds an important dimension to this study. As noted by Kumari and Sarkar (2016) the *B. cereus* group is known for its genetic diversity. This group includes both pathogenic and non-pathogenic strains, offering various applications in various fields. The non-pathogenic *B. cereus*, like the one identified in this study, is particularly beneficial for industrial applications due to its safety profile and effectiveness.

Moreover, the optimization of bacterial growth and α -amylase production revealed that the WL produced the highest α -amylase activity after 12 h of incubation at 50°C and pH 6. This specific temperature and pH range, as discussed by Timilsina et al. (2020) is optimal because they support the most favorable enzyme conformation and activity, which are crucial for industrial processes. This finding is significant as it suggests a tailored approach to optimizing enzyme production, enhancing the efficiency and cost-effectiveness of the process.

The purification process, involving ammonium sulfate precipitation, dialysis, and gel filtration chromatography, resulted in a 3.7-fold increase in specific activity, aligning with the findings of Ndochinwa et al. (2021) and Immonen et al. (2021) who emphasized the importance of enzyme purity for industrial applications. The SDS-PAGE analysis confirmed the enzyme's

purity, which is essential for its application in food and other industries.

Characterization of the purified *B. cereus* α -amylase revealed optimal activity at 50°C and stability between 25°C and 60°C, with the enzyme being most active and stable at pH 6. This temperature and pH stability, echoing the adaptability and resilience of microbial enzymes reported by Shirodkar et al. (2020) enhance the enzyme's potential utility in various industrial applications, including those that require high-temperature operations.

The study also explored the effects of different enzyme and substrate concentrations, finding that optimal α -amylase activity was achieved at enzyme concentrations greater than or equal to 0.5 U/ml and substrate concentrations up to 1.75 mg/ml. The application of the enzyme in bread-making, demonstrating improvements in bread volume and crumb porosity, is particularly noteworthy. These results align with the research by Dahiya et al. (2020) and Sadeghian Motahar et al. (2022), suggesting that enzymes from *Bacillus* species could be viable alternatives to chemical additives in the food industry. The use of microbial enzymes in the food industry, driven by consumer demand for natural and sustainable products, positions α -amylase from *B. cereus* as a natural and efficient alternative to chemical additives.

This study not only corroborates previous findings regarding the potential of soil-derived *Bacillus* species in enzyme production but also contributes new insights, particularly regarding the optimization of growth conditions and the practical application of these enzymes in the food industry. The findings reinforce that microbial enzymes, particularly those from diverse and adaptable *Bacillus* species, hold significant promise for industrial applications, in line with the broader trends noted in microbial enzyme research.

Conclusion

Recent research on bacterial α -amylases with new characteristics has mostly focused on practical applications. Soil samples rich in organic matter and exposed to high salinity levels yielded several bacterial isolates with amylolytic activity. Using phenotypic and genotypic (16S rRNA sequencing) approaches, the strain identified as the most significant α -amylase producer was *B. cereus* WL. The bacteria exhibited the highest α -amylase activity at pH 6.0, 45°C, and after 12 h of incubation. The α -amylase isoenzyme from *B. cereus* WL was consistently purified using ammonium sulfate precipitation and Sephacryl™ S-200 chromatography. The primary α -amylase had an electrophoretic molecular weight of 58 kDa. α -Amylase showed the

highest anti-starch activity at pH 6.0 and 50°C, remaining stable at 50°C. The purified enzyme improved bread texture characteristics by reducing stiffness and increasing cohesion and elasticity values. When added to flour, the purified α -amylase enhanced the bread's rheological properties and overall quality, resulting in increased bread volume and crumb porosity compared to the untreated control bread. The purified enzyme can be considered a potent dough improver and a bread quality enhancer.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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