J Appl Biotechnol Rep. 2025 June;12(2):1642-1651

Journal of
Applied Biotechnology Reports

doi 10.30491/jabr.2025.487018.1803

Original Article

Enhanced Production of Thermostable α-Amylase in *Bacillus subtilis*

Askar Kholikov [1], Muhammad Latif Nazirov [1], Khushnut Vokhidov [1], Alexandr Kachan [2], Anatoli N. Evtushenkov [2], Akhmadzhan Makhsumkhanov [1*]

[1] Institute of Microbiology, Uzbekistan Academy of Sciences, Tashkent, Uzbekistan
[2] Department of Molecular Biology, Belarusian State University, Minsk, Belarus

Corresponding Author: Akhmadzhan Makhsumkhanov, Ph.D., Associate Professor, Institute of Microbiology, Uzbekistan Academy of Sciences, Tashkent, Uzbekistan. Tel: +998712626966, E-mail: amakhsumkhanov@gmail.com

Received November 4, 2024; **Accepted** February 22, 2025; **Online Published** June 30, 2025

Abstract

Introduction: The increasing industrial demand for thermostable amylases necessitates the exploration of new sources, especially from understudied regions like Uzbekistan, which may offer novel, reliable, and stable enzyme characteristics. The objective of this study was to screen, identify, and characterize bacterial species producing thermostable α-amylase, clone its corresponding gene, and test *B. subtilis* 168 *htr9* mutant hosts for recombinant enzyme overproduction.

Materials and Methods: Bacterial isolates with amylase-producing capabilities were selected using a plate assay, and their taxonomy was confirmed through 16S rRNA sequencing. The α-amylase activity was measured using the 3,5-dinitrosalicylic acid method. The B6-1-5-amyL vector was specifically engineered to clone and express the α-amylase gene in expression hosts.

Results: This study presents the first isolation and characterization of bacterial strains from the soils of Uzbekistan that produce thermostable α-amylase. Following a screening process, two isolates, 104.K and amyR, displaying high amylolytic activity, were selected and identified as *Bacillus licheniformis* 104.K and *Bacillus velezensis* amyR, respectively. The crude α-amylases from both strains exhibited maximum activity at 90 °C and 70 °C, respectively. The α-amylase gene from *B. licheniformis* 104.K was cloned into a newly developed B6-1-5 shuttle vector. Notably, overexpression of this amylase gene in *Bacillus subtilis* 168 *htr9 ehp241* led to a 1.14-fold and 373-fold increase in amylase activity compared to the *B. subtilis* 168 *htr9* and *B. licheniformis* 104.K strain.

Conclusions: The *B. licheniformis* 104.K strain was selected for its thermostable α-amylase, and the gene was successfully cloned and expressed in *Escherichia coli* and *B. subtilis* 168 strains. These findings hold promise for enhancing the catalytic efficiency of recombinant α-amylase using computational biology methods and promoting *B. subtilis* 168 for large-scale production of recombinant enzymes.

Keywords: Thermostable α-amylase, Isolation, Cloning, Expression, *Bacillus licheniformis, Bacillus subtilis*

Citation: Kholikov A, Nazirov ML, Vokhidov K, Kachan A, Evtushenkov AN, Makhsumkhanov A. Enhanced Production of Thermostable α-Amylase in *Bacillus subtilis.* J Appl Biotechnol Rep. 2025;12(2):1642-1651. doi:10.30491/jabr.2025.487018.1803

Introduction

A selection of natural microbial strains plays a crucial role in achieving high yields of the desired enzyme. Amylases are starch-degrading enzymes that catalyze the cleavage of the α-1-4 glycosidic bonds of starch into products of small molecular masses, including glucose and maltose. Microbial amylases are more widely used in industry than those obtained from plants and animals due to their catalytic properties, large production scale, and stability.[1] Bacteria are the main source of thermostable α-amylases, mostly originating from the genus *Bacillus.*[2] Commonly known bacterial α-amylases are those derived from *Bacillus licheniformis,*[3–6] *Bacillus amyloliquefaciens,*[7–9] *Bacillus subtilis,*[10–12] and *Bacillus velezensis.*[13–15]

Among the various extracellular enzymes, amylase holds high ranks in commercial sectors such as the food, detergent, alcohol, textile, and paper industries. Amylase is also utilized in pharmaceuticals, environmental remediation, biofuel production, and other related biotechnological processes.[16,17] There are two types of amylases: exo-amylases and endo-amylases. Exo-amylases act on the non-reducing end of the starch molecule, breaking it down into smaller sugar molecules. In contrast, endo-amylases break the glycosidic linkages inside the starch molecule, breaking it down into smaller fragments. Both types of amylases play important roles in starch digestion.[18,19]

Thermostable enzymes have considerable potential in various fields, such as agriculture, medicine, and biotech nology, largely due to their ability to operate effectively under high-temperature conditions.[20] Thermostable enzymes possess inherent stability that allows them to maintain their activity even when exposed to extremely high temperatures (usually exceeding 50 °C) without degradation.[21,22] However,

some producers of α-amylase, such as *B. licheniformis* and *B. velezensis*, exhibit enzymes with temperature optima that do not exceed 70 °C and 40 °C, respectively.[23,24] The increasing demand for thermostable amylases in industrial applications underscores the need for new, affordable, cost-effective, and environmentally sustainable sources.[20] This study was motivated by the swift identification of new thermostable amylase producers in geographically understudied regions.

In the past decade, several research groups have isolated amylase producers from soils, cloned their genes into corresponding plasmids, and expressed them in *Escherichia coli* cells.[25,26] Most heterologous proteins are expressed in *E. coli* hosts; however, this system has several limitations. For example, most recombinant proteins are synthesized in the cytoplasm because of the complex arrangement of the *E. coli* cell wall.[27] This is especially true for enzymes, in which the expression products are usually accumulated in *E. coli* cells in the form of inclusion bodies.[25,28,29] Recovery of recombinant proteins from inclusion bodies is a complicated and costly process. In contrast, the *B. subtilis* 168 expression system directly produces recombinant enzymes in the culture medium, is recognized as a safe host organism (does not produce endotoxins), and is commonly used for the expression of industrial proteins in extensive fermentation procedures.[30,31]

Previously, we isolated and studied the highly active α-amylase of the *B. subtilis* 150 strain, which did not show activity at temperatures above 50-60 °C.[12] In this context, the screening and characterization of thermostable α-amylase, as well as obtaining its recombinant form, are essential. We also assessed the expression levels of thermostable α-amylase as a model enzyme using mutant *B. subtilis* 168 *htr9* strains. These mutant strains, developed by Alexander V. Kachan and Anatoly N. Evtushenkov, were specifically engineered to enhance the expression levels of recombinant enzymes.[32] The objective of this study was to screen, identify, and characterize bacterial species producing thermostable α-amylase, clone its corresponding gene, and test *B. subtilis* 168 *htr9* mutant hosts for recombinant enzyme overproduction.

Materials and Methods

Screening

All the strains tested were isolated from soils in Uzbekistan and stored in a lyophilized form at the Enzymology Laboratory of the Institute of Microbiology, Academy of Sciences of Uzbekistan. The nutrient composition of the selected growth medium for initial screening included 10 g of potato starch, 5 g of peptone, 5 g of KH_2PO_4, and 10 g of agar-agar per liter, with the pH adjusted to 7.0. After sterilization, 20 ml of the nutrient mixture was aseptically poured into petri dishes under sterile conditions. Bacterial colonies were incubated at 35 °C for 48 hours and then stained with an iodine solution to evaluate amylase activity.

Fermentation

The bacterial isolates were grown for α-amylase production in a culture broth containing (g/L): potato starch-10, yeast extract-1, peptone-1, $(NH_4)_2HPO_4$-10, $MnCl_2*4H_2O$-0.015, $MgSO_4*7H_2O$-0.25, $CaCl_2*2H_2O$-0.05, $FeSO_4*7H_2O$-0.01. α-Amylase production was carried out at 35 °C for 72 h in an orbital shaking incubator set at 150 rpm. The crude culture broth was centrifuged at 13000 rpm for 5 min at 4 °C, and the supernatant was used for the α-amylase activity assay.

B. subtilis 168 *htr9* cells expressing recombinant α-amylase were cultivated in LB medium containing 5 μg/ml chloramphenicol and 1 μg/ml erythromycin at 37 °C for 20 hours with agitation at 200 rpm. The supernatant was separated by centrifugation at 4700 rpm using a bucket rotor (Allegra X-30R, Beckman Coulter) at 4 °C for 30 min, and the expression level was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and amylase activity assay.

Enzyme Assay

The α-amylase activity assay was performed using a slightly modified version of the 3,5-dinitrosalicylic acid (DNS) method.[33] Activity was measured at temperatures ranging from 30 °C to 100 °C in a 0.6 ml reaction mixture. This mixture consisted of 0.54 ml of a 0.5% (w/v) potato starch solution in 100 mM potassium phosphate buffer (pH 7.0) and 0.06 ml of the enzyme solution. After incubating the reaction mixture in a water bath at the specified temperature for 10 minutes, the enzymatic reaction was terminated by adding 1.8 ml of DNS reagent and boiling the mixture for 10 minutes. The mixture was then cooled to room temperature, and the absorbance was measured at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar per minute under the given reaction conditions. A calibration curve using glucose solutions of varying concentrations was constructed to quantify the reducing sugars (p<0.05).

SDS-PAGE and Zymography

The 12% SDS-PAGE resolving gel was prepared according to the Roche Lab Handbook (http://www.Roche-Applied-Science.Com/Labfaqs). After electrophoresis the gel was stained with Coomassie Brilliant Blue (R-250) and destained in boiling water. The zymography of α-amylase activity in a native gel was carried out by Andrades & Contreras.[34]

Identification of Microorganisms

Microscopic Observation: Bacterial isolates were observed under a light microscope using a Gram staining kit (Himedia, India). Biochemical Characterization: Bacterial

isolates were characterized biochemically according to Bergey's Manual.[35] Molecular Genetic Identification: Genomic DNAs of the selected bacteria were isolated using the Marmur procedure,[36] and the 16S rRNA gene region was amplified using the universal primers 27F (5′-AGAGTTT GATCMTGGCTCAG-3′), 533F (5′-GCCAGCAGCCGCG GTAA-3′) and 1492R (5′-TACGGTTACCTTGTTACGAC TT-3′). The polymerase chain reaction (PCR) products were sequenced using the Sanger sequencing method and the obtained sequences were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST).

Effect of Temperature on α-amylase Activity and Stability

The effect of temperature on crude α-amylase activity was measured in various temperature ranges from 30 °C to 100 °C in 10 °C intervals. A phosphate buffer solution (100 mM, pH 7.0) containing 0.5% (w/v) potato starch solution was used as a substrate, and enzyme activity was estimated under standard enzyme assay conditions (p<0.05). The relative activity values are shown as percentages of the maximal activity, which is taken as 100%. Thermostability profiles of the crude enzymes were determined by incubating them in 100 mM phosphate buffer (pH 7.0) containing 5 mM $CaCl_2$ at 70 °C in a dry block heater for 30 min and 60 min, cooling on ice for 10 min and measuring residual α-amylase activities at optimum temperatures under standard enzyme assay conditions (p<0.05). Nonincubated enzyme served as a control, which is taken as 100%.

PCR Amplification

DNAs extracted from the amyR and 104.K strains were used as a template for the PCR amplification of amylase genes. Specific primers targeting the α-amylase gene were designed based on the corresponding nucleotide sequences available in GenBank (Accession Numbers: CP045711.1, CP041192.1, JX081246.1, and CP014842.1) and were procured from IDT, Belgium (Table 1). PCRs were conducted in a total volume of 50 µl with denaturation at 95 °C for 4 min followed by 35 cycles (95 °C for 40 sec, 56 °C for 30 sec, and 72 °C for 1:30 min) and a final extension at 72 °C for 10 min. PCR amplicons were electrophoresed on 0.8% agarose gels and purified from agarose gels using a quick gel extraction kit (Invitrogen, Lithuania).

Table 1. Primers Designed for α-amylase Genes

NO.	Primer name	Sequence (5′ → 3′)	Target organism
1	AmyF_B. amy AmyR_B. amy	ccaGGATCCATGATTCAAAAACGAAAGC ccaCATATGCCTTATTTCTGAACATAAATGG	*Bacillus amyloliquefaciens*
2	AmyF_B. lich AmyR_B. lich	ccaGGATCCATGAAACAACAAAAACGG ccaCATATGGCTCTTCTATCTTTGAACAT	*Bacillus licheniformis*
3	AmyF_B. sub AmyR_B. sub	ccaGGATCCATGATTCAAAAACGAAAGC ccaCATATGCCTTATTTCTGAACATAAATGG	*Bacillus subtilis*
4	AmyF.1_B. vel AmyR.1_B. vel	ccaGGATCCATGTTTGAAAAACGATTCAA ccaCATATGCCATTAATGCGGAAGATAAC	*Bacillus velezensis*
5	AmyF.2_B. vel AmyR.2_B. vel	ccaGGATCCATGATTCAAAAACGAAAGCG ccaCATATGCCTTATTTCTGAACATAAATGG	*Bacillus velezensis*
6	Primer1.For Primer2.Rev	aaaACTAGTCACATTGAAAGGGGAGGAGAATCAT aaaGAGCTCGAGGGCTGATGACACTTTGTTA	*Bacillus licheniformis*

Plasmid Construction

The B6-1-5-amyL vector construct was specifically engineered to express of the thermostable α-amylase in *E. coli* and *B. subtilis* 168 *htr9* strains. The construct incorporates the strong constitutive 1-5 promoter from *Priestia flexa* (unpublished own results). For cloning and selection, the plasmid includes an *E. coli* compatible origin of replication coupled with an ampicillin resistance gene. In *B. subtilis* 168, replication is driven by a *B. subtilis* specific origin of replication, along with a chloramphenicol resistance marker. When necessary, the growth medium was supplemented with antibiotics: ampicillin (100 µg/ml), erythromycin (1 µg/ml for maintaining mutations in the *B. subtilis* 168 *htr9* mutant strains), and chloramphenicol (5 µg/ml). For the cloning procedure, the primer pairs listed as number 6 in Table 1 were utilized. The primer was modified to include additional nucleic acid sequences predicted to serve as signal peptides and stop codon fragments. The plasmid map used in this study is shown in Figure 1 of the supplementary information.

Bacterial Strains

E. coli TOP10 cells were used for plasmid construction, while the *B. subtilis* 168 *htr9* strain was employed for the expression of recombinant α-amylase. The *B. subtilis* 168 *htr9* strain was specifically engineered with a deleted amylase (*amyE*) gene to enhance the expression of the recombinant amylase. Additionally, several mutant derivatives: *B. subtilis* 168 *htr9 ehp241*, *htr9 cst10*, *htr9 dlt46*, and *htr9 esc11* were analyzed to investigate their roles in recombinant enzyme overexpression. A comprehensive list of the *B. subtilis* 168 *htr9* strains used in this study is provided in Table 1 of the supplementary information.

Statistical Analysis

All experiments were performed in triplicate (n = 3). Data were expressed as means ± standard deviation (SD). Statistically significant differences between experimental

groups were considered when $p \leq 0.05$. The one-way ANOVA was calculated using Microsoft Office Excel.

Results

Selection and Identification of Thermostable α-amylase Producer

Sixty-two bacterial isolates were tested for amylolytic activity on starch-containing media and subsequently stained with iodine solution (data not shown). All isolates showed clear zones of starch hydrolysis (7–12 mm) around their colonies on agar plates after incubation for 48 h at 35 °C. When these isolates were grown in the fermentation medium for 72 h at 35 °C in an orbital shaking incubator, only the amyR and 104.K strains exhibited high amylase activity at high temperatures of 70 °C and 90 °C, with amylase activities of 0.92 ± 0.04 U/ml and 0.37 ± 0.005 U/ml, respectively ($p<0.05$). These strains had starch clearance zones of 10 and 8 mm, respectively, on agar plates (Figure 5A).

Both bacterial strains, amyR and 104.K were Gram-positive according to the gram staining method. A study of their morphological, cultural, and biochemical features showed that they belong to the genus *Bacillus* and display cultural properties very close to those of *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens,* and *B. velezensis*. Both *Bacillus* sp. amyR and *Bacillus* sp.104.K strains 16S rRNA genes were sequenced with 1143 and 1152 base pair fragments, respectively, and aligned to the NCBI database. Based on the partial sequences of 16S rRNA, *Bacillus* sp.104.K strain was identified as *Bacillus licheniformis* 104.K (GenBank: OR781719.1), and *Bacillus* sp. amyR was identified as *Bacillus velezensis* amyR (GenBank: OR781816.1). The phylogenetic trees of these bacteria were developed based on a BLASTN search using the neighbor-joining method (Figure1).

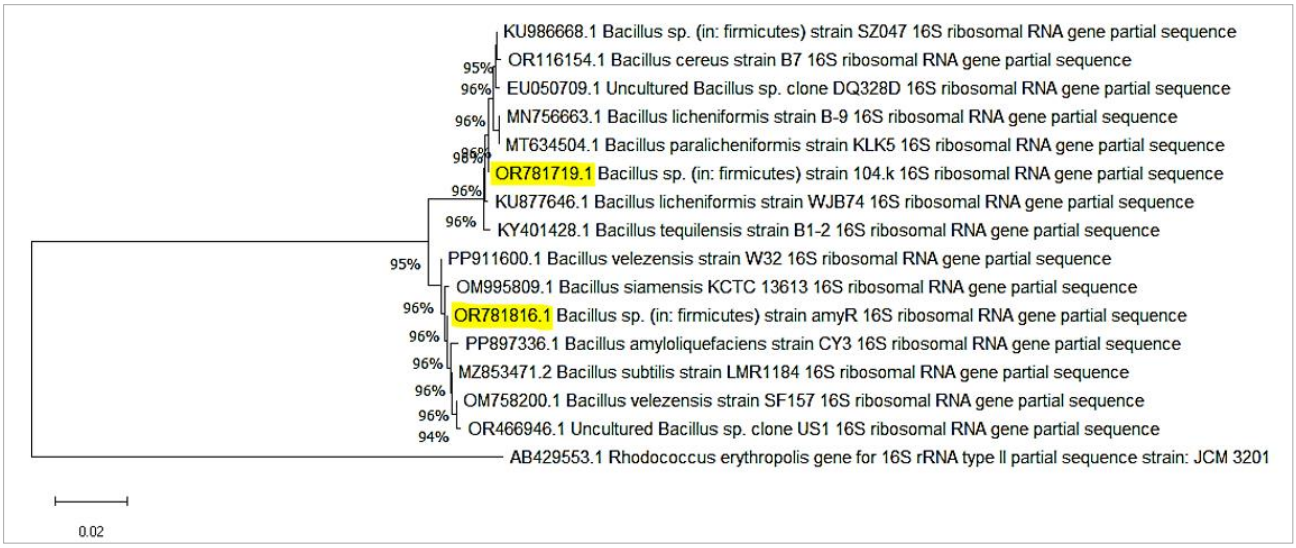

Figure 1. Phylogenetic Analysis of *B. licheniformis* 104.K and *B. velezensis* amyR (highlighted in yellow) based on 16S rRNA Gene Sequencing. The neighbor-joining tree was constructed using MEGA 11 software.

Effect of Temperature and pH on α-amylase Activity

The effect of different temperatures on crude α-amylase activities is presented in Figure 2. It was found that the *B. licheniformis* 104.K strain's crude α-amylase activity, increased at temperatures from 30 °C to 90 °C and slightly decreased at 100 °C. The results showed that *B. licheniformis* 104.K crude α-amylase acts on starch substrates at high temperatures, showing an optimal temperature for enzyme activity of 90 °C. However, determination of *B. velezensis* amyR crude α-amylase activity under the same conditions showed that enzyme activity increased up to 70 °C and rapidly decreased at higher temperature ranges, demonstrating the highest amylase activity at 70 °C, which was optimal for enzyme activity.

Figure 3 demonstrates the thermostability of *B. velezensis* amyR and *B. licheniformis* 104. K crude α-amylases. The α-amylase from *B. licheniformis* 104.K strain was highly stable at 70 °C and retained 82.9 ± 0.5% and 22.5 ± 0.4% of its initial activity after 30 and 60 min of incubation, respectively. In contrast, *B. velezensis* amyR α-amylase is less thermostable, retaining only 5.9 ± 0.3% and 1.7 ± 0.5% of its initial activity after 30 and 60 min of incubation at 70 °C, respectively. These data showed that α-amylase from *B. licheniformis* 104. K is important for further studies to obtain its recombinant form.

Figure 4 illustrates the effect of different pH values on crude α-amylase activity. The maximum activity of both strains amylases was recorded at pH 7.5. Deviations from this optimum within the pH range of 6.5 to 8.5, resulted in a slight decrease in enzyme activity.

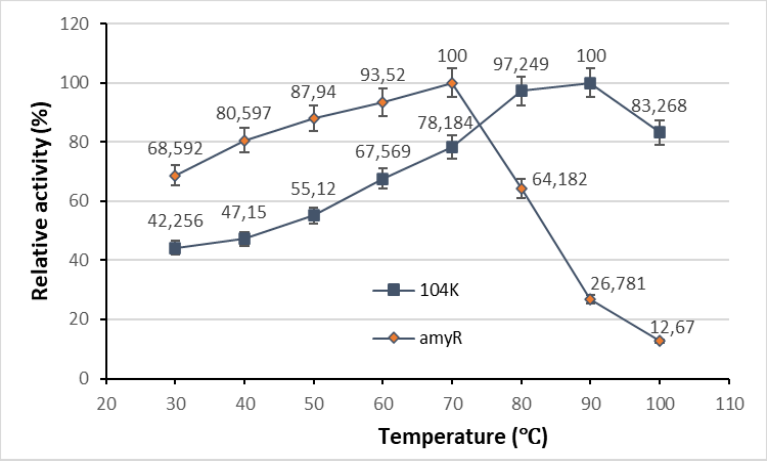

Figure 2. Effect of Temperature on *B. licheniformis* 104.K and *B. velezensis* amyR α-amylase Activity. Crude α-amylase (60 µl) was incubated with 0.54 ml of 0.5% soluble starch in 100 mM phosphate buffer (pH 7.0) at different temperatures. Enzyme activity at the optimal temperature was set as 100%, and relative activities at other temperatures were calculated accordingly. The results represent the mean ± standard deviation (SD), with statistical significance set at $p<0.05$.

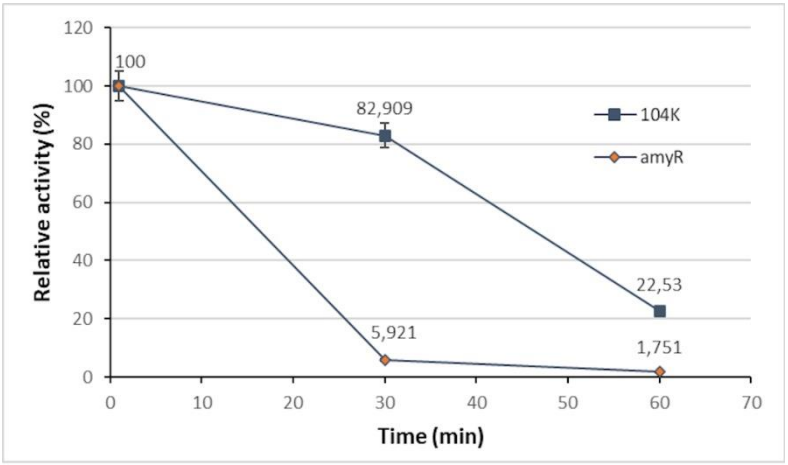

Figure 3. Thermal Stability of *B. licheniformis* 104.K and *B. velezensis* amyR α-amylase. Crude α-amylase was preincubated at 70 °C for different time intervals in 100 mM phosphate buffer (pH 7.0) containing 5 mM $CaCl_2$. Residual enzyme activity was measured at the optimal temperature, with initial activity (time 0) set as 100%. The results represent the mean ± standard deviation (SD), with statistical significance set at $p\leq0.05$.

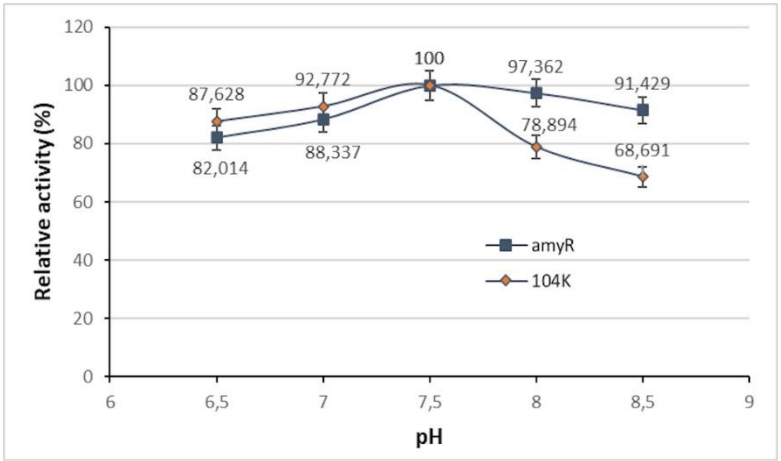

Figure 4. The Effect of pH on *B. licheniformis* 104.K and *B. velezensis* amyR α-amylase Activity. Crude α-amylase activity was measured at the optimal temperature in 100 mM phosphate buffer at various pH values. Enzyme activity at pH 7.5 was considered as 100%, and relative activities at other pH values were calculated accordingly. The results are represented as the mean ± standard deviation (SD), with statistical significance set at $p<0.05$.

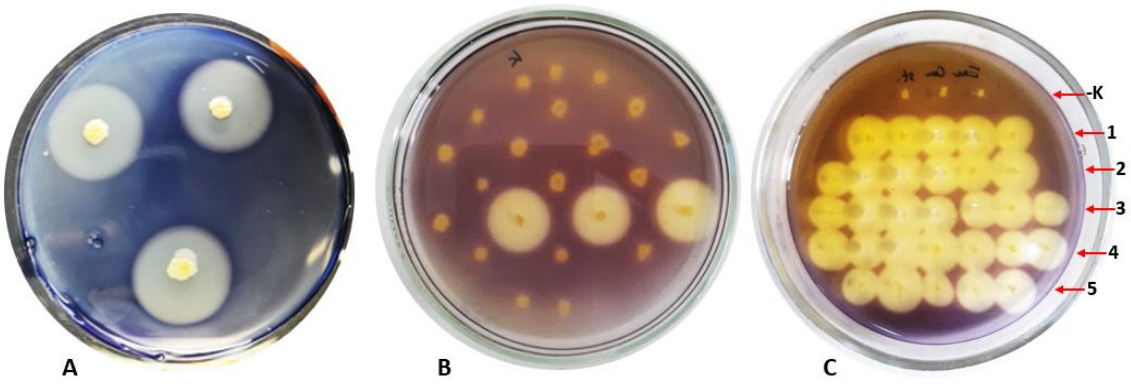

Figure 5. Amylase Activity Assay on Starch-containing Agar Plates. The hydrolysis zones indicate starch degradation by α-amylase production. (**A**) *B. licheniformis* 104.K strain, showing clear starch hydrolysis zones after iodine staining. (**B**) *E. coli* TOP10 transformants expressing recombinant α-amylase, grown on ampicillin-containing plates. (**C**) *B. subtilis* 168 *htr9* transformants expressing recombinant α-amylase, grown on selective plates containing chloramphenicol and erythromycin. The strains tested include: -K – *B. subtilis* 168 *htr9* without vector (negative control), 1) *B. subtilis* 168 *htr9*; 2) *B. subtilis* 168 *htr9 ehp241*; 3) *B. subtilis* 168 *htr9 cst10*; 4) *B. subtilis* 168 *htr9 dlt46*, and 5) *B. subtilis* 168 *htr9 esc11*. All plates were stained with iodine solution (2 g/L KI and 1 g/L I_2) to visualize starch hydrolysis zones.

Cloning and Expression

Only two (*B. licheniformis* and *B. velezensis*) specific primer pairs, Amy_B.lich and Amy.1_B.vel, revealed PCR products with amplicons of 1539 bp and 1980 bp, respectively, as shown in Figure 2 of the supplementary information. The amplified PCR amplicon from *B. licheniformis* 104.K was cloned into the B6-1-5 shuttle vector using restriction enzymes SpeI and SacI. The ligation mixture was transformed into competent *E. coli* TOP10 cells (Figure 5B). The insertion of the amylase gene (*amyL*) was confirmed using double-digest restriction test as shown in Figure 3 of the supplementary information. The functionality of the 1-5 promoter in both hosts was tested on starch-containing agar plates. The results demonstrate that the unique promoter, active in both *E. coli* and *B. subtilis*, allows for the parallel expression of genes in these two organisms (Figure 5B, C).

The B6-1-5 amyL vector construct was transformed into *B. subtilis* 168 *htr9* mutant strains using the Spizizen Minimal Medium (SMM) procedure[37] (Figure 5C). The *B. subtilis* strain carrying the B6-1-5 amyL vector construct was grown at 37 °C for 20 h after inoculation of 1 ml overnight culture, and samples were taken every 5 hours from the culture supernatant to detect recombinant amylase expression. The resulting protein band exhibited a molecular

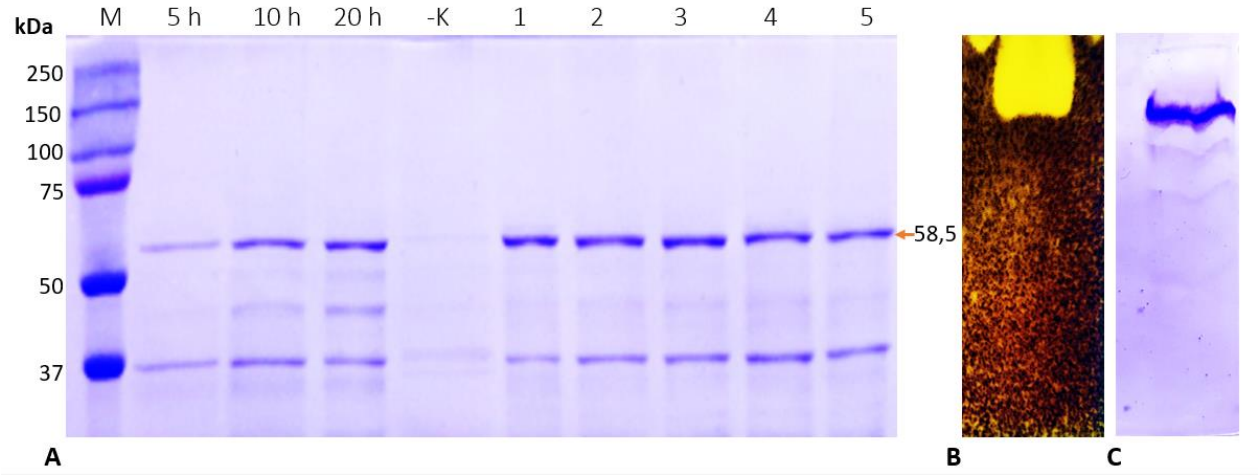

Figure 6. (**A**) 12% SDS-PAGE Analysis of Recombinant α-amylase Expressed in *Bacillus subtilis* 168 *htr9* Mutant Strains. Supernatant samples were collected at different time points (5 h, 10 h, and 20 h) from *B. subtilis* 168 *htr9* (wild-type, WT). *-K* represents the negative control (host cells without the amylase gene). Lanes 1–5 correspond to supernatants from the following strains incubated for 20 hours: 1) *B. subtilis* 168 *htr9* (WT); 2) *B. subtilis* 168 *htr9 ehp241*; 3) *B. subtilis* 168 *htr9 cst10*; 4) *B. subtilis* 168 *htr9 dlt46*, and 5) *B. subtilis* 168 *htr9 esc11*. The molecular weight marker (M) is a dual-color protein ladder (#161-0374, Bio-Rad), with the recombinant α-amylase band observed at approximately 58.5 kDa. (**B**) Zymogram analysis of recombinant α-amylase from *B. subtilis* 168 *htr9* (WT), showing enzymatic activity detected using iodine staining (2 g/L KI and 1 g/L I_2) after separation on 10% native-PAGE. (**C**) Native-PAGE performed in parallel with the zymogram experiment, with the gel stained using Coomassie Brilliant Blue to visualize recombinant α-amylase on the gel.

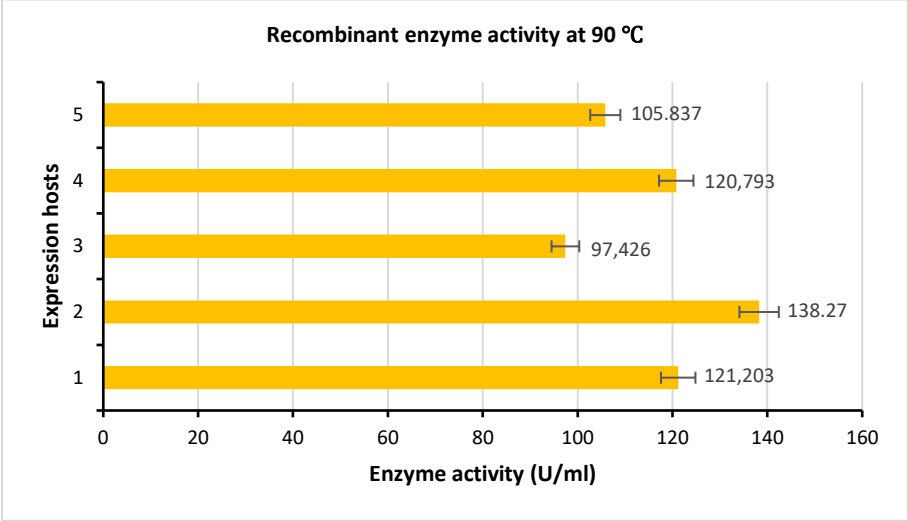

Figure 7. Recombinant Thermostable α-amylase Activity at 90 °C after 20 h of Expression in Different *Bacillus subtilis* 168 Mutant Strains. Enzyme activity (U/ml) was measured using a standard assay and is presented as the mean ± standard deviation (SD) of three independent experiments. The tested strains include: 1) *B. subtilis* 168 *htr9* (wild-type, WT); 2) *B. subtilis* 168 *htr9 ehp241*; 3) *B. subtilis* 168 *htr9 cst10*; 4) *B. subtilis* 168 *htr9 dlt46*, and 5) *B. subtilis* 168 *htr9 esc11*. Statistical significance was determined using one-way ANOVA (p<0.05).

mass of approximately 58.5 kDa, which is consistent with the expected mass of the *B. licheniformis* 104.K α-amylase (Figure 6A). Moreover, recombinant amylase activity was confirmed by native-PAGE and zymogram analysis (Figure 6B, C). The assay demonstrated that the enzyme was catalytically active and successfully expressed in a heterologous host. Both crude and recombinant α-amylase exhibited the same optimal temperature (90 °C) and thermal stability as the native enzyme (data not shown).

The expression level of thermostable α-amylase in *B. subtilis* 168 *htr9 ehp241* was significantly higher, exhibiting an enzyme activity of 138.27 ± 0.3 U/ml, compared to *B. subtilis* 168 *htr9*, which showed an activity of 121.2 ± 0.3 U/ml. In contrast, the mutant strains *B. subtilis* 168 *htr9 cst10*, *B. subtilis* 168 *htr9 dlt46*, and *B. subtilis* 168 *htr9 esc11* demonstrated lower enzymatic activities of 97.4 ± 0.5 U/ml, 120.79 ± 0.3 U/ml, and 105.8 ± 0.2 U/ml, respectively (p<0.05). These results indicate that while the *B. subtilis htr9 ehp241* mutant showed enhanced α-amylase expression, the other mutants exhibited reduced expression relative to the wild-type strain (Figure 7).

Discussion

α-Amylase is encoded by different numbers of nucleotides, which varies depending on the type of microorganism.[38] Specifically, the *B. amyloliquefaciens* α-amylase gene contains 1542 bp nucleotides.[39] *B. stearothermophilus* α-amylase confirmed that the reading frame of this gene consists of 1644 bp.[40] An open reading frame encoding the α-amylase *B. subtilis* (natto) IAM1212 and *B. subtilis* 2633 comprises 1431 bp nucleic acid residues.[41] In our case, the amylase gene of the *B. licheniformis* 104.K strain consists of 1539 bp, a result that aligns with the findings of Niu et al.[42] However, the results of Yuukt et al. differ, reporting the gene as consisting of 1948 bp nucleotides.[43] The amplicon of the *B. velezensis* amyR strain amylase gene was 1980 bp, which is consistent with the results reported by Zhang et al.[15] These results indicate that the amylase gene of the *Bacillus* genus might be horizontally transferred between *Bacillus* species during evolutionary processes. The transfer of genetic material is remarkably adaptable, happening not only within the same species but also between different species. Emamalipour et al. showed that this process has a significant impact on the genetic, physiological, and ecological characteristics of host organisms.[44]

Industrial applications of α-amylase require thermo- and pH-stable enzymes because most industrial processes are performed at elevated temperatures and pH values.[45-47] The *B. licheniformis* 104.K strain amylase reached its maximum activity at 90 °C (Figure 2) and similar results were demonstrated for *B. licheniformis* α-amylases.[48,49] However, different results have been reported by Suthar et al.,[23] Abdel-Fattah et al.[50] and Wu et al.,[51] in which optimal temperatures for *B. licheniformis* α-amylases were 70 °C and 100 °C, respectively. This unique enzyme characteristic in a specific species is attributable to horizontal gene transfer and the adaptation of microorganisms to distinct geographic environments.[52]

On the other hand, the *B. velezensis* amyR strain α-amylase showed its maximum activity at 70 °C, with similar results confirmed by Zhang et al.[15] Both *B. licheniformis* 104.K and *B. velezensis* amyR strains exhibited high amylase activities over a pH range of 6.5-8.5, with the optimal pH for both enzymes being 7.5 (Figure 4). Notably, similar results were reported by Hu et al.[53] and Divakaran et al.[54]

Using the *B. subtilis* expression system for recombinant

enzyme production on an industrial scale offers several advantages such as: Generally Recognized as Safe (GRAS) status, which is considered safe for use in food and pharmaceutical industries,[55,56] a fast growth rate and high cell density. Additionally, *B. subtilis* naturally secretes proteins into the culture medium, facilitating downstream processing and purification of recombinant enzymes. This can lower production costs and improve efficiency.[57] *B. subtilis* does not produce endotoxins, making recombinant enzymes safer for pharmaceutical and food applications.[58] Thus, the B6-1-5 shuttle vector was successfully constructed with a new 1-5 constitutive promoter. Inducers like isopropyl β-D-1-thiogalactopyranoside (IPTG) are unsuitable for industrial fermentation due to their high cost. Instead, constitutive promoters are commonly employed as the standard practice for gene expression in industrial scale.[59]

The α-amylase gene from *B. licheniformis* 104.K was cloned into the B6-1-5 shuttle vector and its expression levels were evaluated across various *B. subtilis* 168 *htr9* mutant strains (Figure 6A). Notably, the *B. subtilis* 168 *htr9 ehp241* strain, which contains an inserted hybrid *prsA* gene, demonstrated a significant positive impact on the overproduction of thermostable α-amylase (Figure 7). These results highlight the potential for developing novel expression hosts tailored for industrial applications. Conversely, the inactivation or replacement of multiple operons regulating two-component stress-responsive systems in the *B. subtilis* 168 membrane negatively influenced recombinant amylase overexpression, aligning with findings previously reported by Zweers et al.[60] Nevertheless, we believe that the thermostable α-amylases of the *B. licheniformis* 104.K and *B. velezensis* amyR strains isolated and studied in Uzbekistan can be utilized as biocatalysts for the hydrolysis of starch-containing substrates, bioethanol, and detergent production.

Conclusion

In this study thermostable α-amylase producers *B. licheniformis* 104.K and *B. velezensis* amyR were selected, identified, and their enzyme catalytic properties were studied. The newly constructed B6-1-5 shuttle vector, featuring the constitutive 1-5 promoter, which is functional in both *E. coli* and *B. subtilis*, is expected to enable the parallel expression of recombinant α-amylase in both organisms. The *amyL* gene from *B. licheniformis* 104.K was successfully cloned and expressed in mutant strains of *B. subtilis* 168 *htr9*. Notably, we tested the overexpression of the thermostable α-amylase in *B. subtilis* 168 *htr9* through various mutations in the two-component stress response operon and the insertion of a hybrid *prsA* gene. We are confident that our findings will contribute to the further improvement of the catalytic properties of the recombinant α-amylase from the *B. licheniformis* 104.K strain using bioinformatic tools, as well as the use of *B. subtilis* as a cell factory for the over production of recombinant enzymes.

Authors' Contributions

Investigation, methodology, data curation, and formal analysis was performed by AK, MLN, KV, and AK; Writing of original draft, reviewing, and editing by AK, AK, and AM; Conceptualization, supervision, funding acquisition, methodology, and project administration by AM and ANE. All authors have read and approved the submitted version of the manuscript.

Funding

This work was supported by The Agency of Innovative Development of the Republic of Uzbekistan (Grant number IL-4821091620).

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

References

1. Yassin SN, Jiru TM, Indracanti M. Screening and Characterization of Thermostable Amylase-Producing Bacteria Isolated from Soil Samples of Afdera, Afar Region, and Molecular Detection of Amylase-Coding Gene. Int J Microbiol. 2021;2021(1):5592885. doi:10.1155/2021/5592885
2. Takasaki Y, Furutani S, Hayashi S, Imada K. Acid-stable and thermostable α-amylase from *Bacillus licheniformis* α. J Ferment Bioeng. 1994;77(1):94-6. doi:10.1016/0922-338X(94)90216-X
3. Lee S, Oneda H, Minoda M, Tanaka A, Inouye K. Comparison of starch hydrolysis activity and thermal stability of two *Bacillus licheniformis* α-amylases and insights into engineering α-amylase variants active under acidic conditions. J Biochem. 2006;139(6):997-1005. doi:10.1093/jb/mvj113
4. EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Lambré C, Barat Baviera JM, Bolognesi C, Cocconcelli PS, et al. Safety evaluation of the food enzyme α-amylase from the non-genetically modified *Bacillus licheniformis* strain T74. EFSA J. 2023;21(8):e08160. doi:10.2903/j.efsa.2023.8160
5. Fraiture MA, Gobbo A, Guillitte C, Marchesi U, Verginelli D, De Greve J, et al. Pilot market surveillance of GMM contaminations in alpha-amylase food enzyme products: A detection strategy strengthened by a newly developed qPCR method targeting a GM Bacillus licheniformis producing alpha-amylase. Food Chem: Mol Sci. 2024;8:100186. doi:10.1016/j.fochms.2023.100186
6. Usul SK, Binay B, Soydan AM, Aslan A. A newly synthesized magnetic nanoparticle coated with glycidyl methacrylate monomer and 1, 2, 4-Triazole: Immobilization of α-Amylase from *Bacillus licheniformis* for more reuse, stability, and activity in the presence of H2O2. Bioorg Chem. 2024;143:107068. doi:10.1016/j.bioorg.2023.107068
7. Yuan S, Yan R, Lin B, Li R, Ye X. Improving thermostability of *Bacillus amyloliquefaciens* alpha-amylase by multipoint mutations. Biochem Biophys Res Commun. 2023;653:69-75. doi:10.1016/j.bbrc.2023.02.064

8. Deb P, Talukdar SA, Mohsina K, Sarker PK, Sayem SA. Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. SpringerPlus. 2013;2:154. doi:10.1186/2193-1801-2-154
9. Abd-Elhalim BT, Gamal RF, El-Sayed SM, Abu-Hussien SH. Optimizing alpha-amylase from *Bacillus amyloliquefaciens* on bread waste for effective industrial wastewater treatment and textile desizing through response surface methodology. Sci Rep. 2023;13(1): 19216. doi:10.1038/s41598-023-46384-6
10. Bano S, Qader SA, Aman A, Syed MN, Azhar A. Purification and characterization of novel α-amylase from *Bacillus subtilis* KIBGE HAS. AAPS PharmSciTech. 2011;12:255-61. doi:10.1208/s12249-011-9586-1
11. Cui J, Fan Y, Lian D, Wang S, Wang M, Du Y, Li Y, Li L. Interaction of narcissoside with α-amylase from *Bacillus subtilis* and *Porcine pancreatic* by multi-spectral analysis and molecular dynamics simulation. Luminescence. 2024;39(2):e4637. doi:10.1002/bio.4637
12. Normurodova KT, Nurmatov SK, Alimova BK, Pulatova OM, Akhmedova ZR, Makhsumkhanov AA. Isolation and characteristics of highly active α-amylase from *Bacillus subtilis*-150. Chem Nat Compd. 2007;43:454-7. doi:10.1007/s10600-007-0159-1
13. Li C, Li S, Dang G, Jia R, Chen S, Deng X, et al. Screening and characterization of *Bacillus velezensis* LB-Y-1 toward selection as a potential probiotic for poultry with multi-enzyme production property. Front Microbiol. 2023;14: 1143265. doi:10.3389/fmicb.2023.1143265
14. Nie M, Khalid F, Hu Q, Khalid A, Wu Q, Huang S, et al. Site-Directed Mutagenesis: Improving the Acid Resistance and Thermostability of *Bacillus velezensis* α-Amylase and Its Preliminary Feed Application. J Agric Food Chem. 2024;72(18):10487-96. doi:10.1021/acs.jafc.4c01630
15. Zhang X, Li C, Chen X, Chio C, Shrestha S, Qin W. *Bacillus velezensis* identification and recombinant expression, purification, and characterization of its alpha-amylase. Fermentation. 2021;7(4):227. doi:10.3390/fermentation7040227
16. Pinto ÉS, Dorn M, Feltes BC. The tale of a versatile enzyme: Alpha-amylase evolution, structure, and potential biotechnological applications for the bioremediation of n-alkanes. Chemosphere. 2020;250: 126202. doi:10.1016/j.chemosphere.2020.126202
17. Gopinath SC, Anbu P, Arshad MM, Lakshmipriya T, Voon CH, Hashim U, Chinni SV. Biotechnological processes in microbial amylase production. Biomed Res Int. 2017;2017(1):1272193. doi:10.1155/2017/1272193
18. Kamon M, Sumitani JI, Tani S, Kawaguchi T, Kamon M, Sumitani J, et al. Characterization and gene cloning of a maltotriose-forming exo-amylase from *Kitasatospora* sp. MK-1785. Appl Microbiol Biotechnol. 2015;99:4743-53. doi:10.1007/s00253-015-6396-5
19. Farooq MA, Ali S, Hassan A, Tahir HM, Mumtaz S, Mumtaz S. Biosynthesis and industrial applications of α-amylase: a review. Arch Microbiol. 2021;203:1281-92. doi:10.1007/s00203-020-02128-y
20. Jaiswal N, Jaiswal P. Thermostable α-Amylases and Laccases: Paving the Way for Sustainable Industrial Applications. Processes. 2024;12(7):1341. doi:10.3390/pr12071341
21. Vieille C, Zeikus GJ. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev. 2001;65(1):1-43. doi:10.1128/mmbr.65.1.1-43.2001
22. Hussian CH, Leong WY. Thermostable enzyme research advances: a bibliometric analysis. J Genet Eng Biotechnol. 2023;21(1):37. doi:10.1186/s43141-023-00494-w
23. Suthar S, Joshi D, Patel H, Patel D, Kikani BA. Optimization and purification of a novel calcium-independent thermostable, α-amylase produced by *Bacillus licheniformis* UDS-5. World J Microbiol Biotechnol. 2024;40(12):385. doi:10.1007/s11274-024-04188-4
24. Esawy MA, Karam EA, Hassan ME, Kansoh AL, Gamal AA. Comparative study between the free and immobilized cells of *Bacillus velezensis* AMA2 P164707 and *Bacillus atrophaeus* AMA6 OP225343 for acidic β, α amylases production and applied experiment. Int J Biol Macromol. 2025;284:138087. doi:10.1016/j.ijbiomac.2024.138087
25. Rashid N, Farooq A, Akhtar M. Insoluble but enzymatically active α-amylase from *Bacillus licheniformis*. Biologia. 2009;64:660-3. doi:10.2478/s11756-009-0132-5
26. Roy JK, Borah A, Mahanta CL, Mukherjee AK. Cloning and overexpression of raw starch digesting α-amylase gene from *Bacillus subtilis* strain AS01a in *Escherichia coli* and application of the purified recombinant α-amylase (AmyBS-I) in raw starch digestion and baking industry. J Mol Catal B Enzym. 2013;97:118-29. doi:10.1016/j.molcatb.2013.07.019
27. Liu SL, Du K, Chen WZ, Liu G, Xing M. Effective approach to greatly enhancing selective secretion and expression of three cytoplasmic enzymes in *Escherichia coli* through synergistic effect of EDTA and lysozyme. J Ind Microbiol Biotechnol. 2012;39(9):1301-7. doi:10.1007/s10295-012-1136-7
28. Ghasemi A, Ghafourian S, Vafaei S, Mohebi R, Farzi M, Taherikalani M, et al. Cloning, expression, and purification of hyperthermophile α-amylase from *Pyrococcus woesei*. Osong Public Health Res Perspect. 2015;6(6):336-40. doi:10.1016/j.phrp.2015.10.003
29. Grzybowska B, Szweda P, Synowiecki J. Cloning of the thermostable α-amylase gene from *Pyrococcus woesei* in *Escherichia coli*: isolation and some properties of the enzyme. Mol Biotechnol. 2004;26:101-9. doi:10.1385/MB:26:2:101
30. Palva I. Molecular cloning of α-amylase gene from *Bacillus amyloliquefaciens* and its expression in *B. subtilis*. Gene. 1982;19(1):81-7. doi:10.1016/0378-1119(82)90191-3
31. Su Y, Liu C, Fang H, Zhang D. *Bacillus subtilis*: a universal cell factory for industry, agriculture, biomaterials and medicine. Microb Cell Fact. 2020;19:173. doi:10.1186/s12934-020-01436-8
32. Kachan AV, Evtushenkov AN. The CssRS two-component system of *Bacillus subtilis* contributes to teicoplanin and polymyxin B response. Folia Microbiol. 2025;70(1):83-99. doi:10.1007/s12223-024-01179-8
33. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426-8. doi:10.1021/ac60147a030
34. Andrades A, Contreras LM. Amylase zymography. Zymography: Methods and Protocols. 2017:301-8. doi:10.1007/978-1-4939-7111-4_29
35. Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, et al., editors. Bergey's Manual of Systematic Bacteriology: Vol. 3. The Firmicutes. New York: Springer; 2009. pp. 21-128. doi:10.1007/978-0-387-68489-5
36. Marmur J. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol. 1961;3(2):208-IN1. doi:10.1016/S0022-2836(61)80047-8
37. Spizizen J. Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonucleate. Proc Natl Acad Sci USA. 1958;44(10):1072-8. doi:10.1073/pnas.44.10.1072

38. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Microbial α-amylases: a biotechnological perspective. Process Biochem. 2003;38(11):1599-616. doi:10.1016/S0032-9592(03)00053-0
39. Takkinen K, Pettersson RF, Kalkkinen N, Palva I, Söderlund H, Kääriäinen L. Amino acid sequence of alpha-amylase from *Bacillus amyloliquefaciens* deduced from the nucleotide sequence of the cloned gene. J Biol Chem. 1983;258(2):1007-13.
40. Ihara H, Sasaki T, Tsuboi A, Yamagata H, Tsukagoshi N, Udaka S. Complete nucleotide sequence of a thermophilic α-amylase gene: homology between prokaryotic and eukaryotic α-amylases at the active sites. J Biochem. 1985;98(1):95-103. doi:10.1093/oxfordjournals.jbchem.a135279
41. Emori MA, Takagi MA, Maruo BU, Yano KE. Molecular cloning, nucleotide sequencing, and expression of the *Bacillus subtilis* (natto) IAM1212 alpha-amylase gene, which encodes an alpha-amylase structurally similar to but enzymatically distinct from that of *B. subtilis* 2633. J Bacteriol. 1990;172(9):4901-8. doi:10.1128/jb.172.9.4901-4908.1990
42. Niu DD, Xu M, Ma JS, Wang ZX. Cloning of the gene encoding a thermostable alpha-amylase from *Bacillus licheniformis* CICIM B0204 and functional identification of its promoter. Acta Microbiol Sin. 2006;46(4):576-80.
43. Yuukt T, Nomura T, Tezuka H, Tsuboi A, Yamagata H, Tsukagoshi N, et al. Complete nucleotide sequence of a gene coding for heat-and pH-stable α-amylase of *Bacillus licheniformis*: comparison of the amino acid sequences of three bacterial liquefying α-amylases deduced from the DNA sequences. J Biochem. 1985;98(5):1147-56. doi:10.1093/oxfordjournals.jbchem.a135381
44. Emamalipour M, Seidi K, Zununi Vahed S, Jahanban-Esfahlan A, Jaymand M, Majdi H, et al. Horizontal gene transfer: from evolutionary flexibility to disease progression. Front Cell Dev Biol. 2020;8:229. doi:10.3389/fcell.2020.00229
45. Karim KM, Husaini A, Sing NN, Sinang FM, Roslan HA, Hussain H. Purification of an alpha amylase from *Aspergillus flavus* NSH9 and molecular characterization of its nucleotide gene sequence. 3 Biotech. 2018;8:204. doi:10.1007/s13205-018-1225-z
46. Leuschner C, Antranikian G. Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. World J Microbiol Biotechnol. 1995;11:95-114. doi:10.1007/BF00339139
47. Lim SJ, Oslan SH, Oslan SN. Purification and characterisation of thermostable α-amylases from microbial sources. BioResources. 2020;15(1):2005-29. doi:10.15376/biores.15.1.2005-2029
48. Morgan FJ, Priest FG. Characterization of a thermostable α-amylase from *Bacillus licheniformis* NCIB 6346. J Appl Bacteriol. 1981;50(1):107-14. doi:10.1111/j.1365-2672.1981.tb00875.x
49. Wang JR, Li YY, Liu DN, Liu JS, Li P, Chen LZ, et al. Codon optimization significantly improves the expression level of α-amylase gene from *Bacillus licheniformis* in *Pichia pastoris*. Biomed Res Int. 2015;2015(1):248680. doi:10.1155/2015/248680
50. Abdel-Fattah YR, Soliman NA, El-Toukhy NM, El-Gendi H, Ahmed RS. Production, Purification, and Characterization of Thermostable α-Amylase Produced by *Bacillus licheniformis* Isolate AI20. J Chem. 2013;2013(1):673173. doi:10.1155/2013/673173
51. Wu X, Wang Y, Chen X, Chen J. Purification and biochemical characterization of a thermostable and acid-stable alpha-amylase from *Bacillus licheniformis* B4-423. Int J Biol Macromol. 2018;109:329-37. doi:10.1016/j.ijbiomac.2017.12.004
52. Marasco R, Fusi M, Coscolín C, Barozzi A, Almendral D, Bargiela R, et al. Enzyme adaptation to habitat thermal legacy shapes the thermal plasticity of marine microbiomes. Nat Commun. 2023;14(1):1045. doi:10.1038/s41467-023-36610-0
53. Hu X, Yuan X, He N, Zhuang TZ, Wu P, Zhang G. Expression of *Bacillus licheniformis* α-amylase in Pichia pastoris without antibiotics-resistant gene and effects of glycosylation on the enzymic thermostability. 3 Biotech. 2019;9:427. doi:10.1007/s13205-019-1943-x
54. Divakaran D, Chandran A, Pratap Chandran R. Comparative study on production of α-amylase from *Bacillus licheniformis* strains. Braz J Microbiol. 2011;42:1397-404. doi:10.1590/S1517-83822011000400022
55. Dergham Y, Le Coq D, Bridier A, Sanchez-Vizuete P, Jbara H, Deschamps J, et al. *Bacillus subtilis* NDmed, a model strain for biofilm genetic studies. Biofilm. 2023;6:100152. doi:10.1016/j.bioflm.2023.100152
56. Lenz P, Hilgers F, Burmeister A, Zimmermann L, Volkenborn K, Grünberger A, et al. The iSplit GFP assay detects intracellular recombinant proteins in *Bacillus subtilis*. Microb Cell Fact. 2021;20:174. doi:10.1186/s12934-021-01663-7
57. van Dijl J, Hecker M. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. Microbial cell factories. 2013;12:3. doi:10.1186/1475-2859-12-3
58. Wenzel M, Müller A, Siemann-Herzberg M, Altenbuchner J. Self-inducible *Bacillus subtilis* expression system for reliable and inexpensive protein production by high-cell-density fermentation. Appl Environ Microbiol. 2011;77(18):6419-25. doi:10.1128/AEM.05219-11
59. Lee SY, Kim HU. Systems strategies for developing industrial microbial strains. Nat Biotechnol. 2015;33(10):1061-72. doi:10.1038/nbt.3365
60. Zweers JC, Wiegert T, van Dijl JM. Stress-responsive systems set specific limits to the overproduction of membrane proteins in *Bacillus subtilis*. Appl Environ Microbiol. 2009;75(23):7356-64. doi:10.1128/AEM.01560-09