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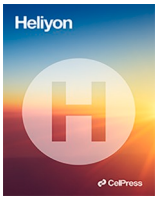

Research article

An impact of N-glycosylation on biochemical properties of a recombinant α-amylase from *Bacillus licheniformis*

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ABSTRACT

Amylases are enzymes that are known to hydrolyze starch. High efficiency of amylolytic enzymes allows them to compete in the industry with the technology of chemical hydrolysis of starch. A *Bacillus licheniformis* strain with high amylolytic activity was isolated from soil and designated as T5. The gene encoding α-amylase from *B. licheniformis* T5 was successfully expressed in both *Escherichia coli* (rAmyT5-E) and *Pichia pastoris* (as rAmyT5-P). According to the study, the recombinant α-amylases rAmyT5-E and rAmyT5-P exhibited the highest activity at pH 6.0 and temperatures of 70 and 80 °C, respectively. Over 80% of the rAmyT5-E enzyme activity was preserved following incubation within the pH range of 5–9; the same was true for rAmyT5-P after incubation at pH 6–9. N-glycosylation reduced the thermal and pH stability of the enzyme. The specific activity and catalytic efficiency of the recombinant AmyT5 α-amylase were also diminished by N-glycosylation.

1. Introduction

Hydrolytic enzymes known as α-amylases (EC 3.2.1.1) break down starch molecules into dextrins and oligosaccharides by cleaving glycosidic bonds [1,2]. α-Amylases belong to the GH 13 family of glycoside hydrolases [3] and are calcium-dependent endoenzymes [4–6]. For the industrial conversion of starch, thermostable microbial α-amylases are of particular interest, which are used for saccharification and liquefaction of starch at 70–80 °C. α-Amylases with increased pH stability in an alkaline pH range (7.0–9.0) are promising for use in detergents [7]. Among bacterial α-amylases, thermostable and alkaline α-amylases are known to be present in *Bacillus licheniformis* and *Bacillus amyloliquefaciens* and have maximum activity at pH 6.0–9.0 [8–10].

Post-translational modifications can significantly change the properties of enzymes. They can affect pH and thermal stability, or increase or decrease resistance to detergents, metal ions, or organic solvents [11,12]. Bacteria do not have a well-developed apparatus for post-translational modifications, but recombinant DNA technology can help to obtain post-translationally modified bacterial enzymes, including α-amylases [13,14]. Among *Pichia pastoris*'s many advantageous features include its ability to undergo post-translational modifications, the most significant of which N-glycosylation, its high protein production rate, its ease of genetic manipulation, and its high cell density [15]. The process of N-glycosylation in yeast by which oligosaccharides bind to particular

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asparagine residues in polypeptides [16]. Glycosylation in *P. pastoris* differently affects such biochemical properties of enzymes as high-temperature activity and thermal stability [17–20]. According to the findings of one study, there is no discernible pattern in the way that glycosylation influences the biochemical properties of enzymes. For instance, in Ref. [21], researchers obtained glycosylated esterase FAEA from *Aspergillus niger*, which turned out to be less thermally stable than the nonglycosylated form of this protein obtained in *Escherichia coli*. Conversely, *Penicillium purpurogenum* β-glucuronidase expressed in *P. pastoris* showed an increase in functional characteristics of the enzyme after glycosylation in comparison with the nonglycosylated enzyme [22].

Although some authors [13,23] have noted an increase in thermal stability of many enzymes upon N-glycosylation, a *Bacillus* sp. recombinant alkaliphilic α-amylase expressed in the yeast *P. pastoris* has proven to be less thermally stable than the natural form of the protein [24].

This study aimed to investigate how N-glycosylation affects the biochemical features of α-amylase from a *B. licheniformis* isolate. For this purpose, the enzyme was expressed as two variants: a nonglycosylated one in *E. coli* and a glycosylated enzyme in *P. pastoris*. An analysis was conducted comparing the biochemical properties of nonglycosylated and glycosylated α-amylases. This included examining optimal pH and temperature ranges, pH and thermal stability, as well as the effects of metal ions, solvents, and detergents on enzyme activity. The substrate specificity and kinetic characteristics of the two different versions of α-amylase were also identified. The main short-chain hydrolysis products were identified.

2. Materials and methods

2.1. Plasmids, host strains, and reagents

Vectors pJET1.2/blunt (Thermo Fisher, USA), pPICZαA (Invitrogen, USA) and pET28c(+) (Novagen, UK), were employed in the construction of yeast shuttle plasmids, bacterial expression, and cloning. Strains *E. coli* DH5α (Thermo Fisher Scientific, USA), ArcticExpress(DE3)RP (Novagen, France), and *P. pastoris* X-33 (Invitrogen, USA) were employed for cloning and expression. The reagents utilized in the study were purchased from Sigma-Aldrich, Thermo Fisher, AppliChem, Promega, or Amresco. Table 1 lists the primers used in the work.

2.2. Growth media

Nutrient agar (HiMedia, India) with 1% (w/v) of starch (Sigma, USA) was utilized to isolate and cultivate *Bacillus* spp. *E. coli* strains were cultured in Lennox broth (LB): 0.5% (w/v) of yeast extract, 1% (w/v) of tryptone, and 0.5% (w/v) of NaCl. *P. pastoris* X-33 cells were cultured on the following media: YEP (1% (w/v) of yeast extract and 2% (w/v) of peptone), YEPD (YEP and 2% (w/v) of glucose), YEPG (YEP and 1% (v/v) of glycerol), and YEPM (YEP and 1% (v/v) of methanol).

2.3. Isolation of strains from soil

Bacterial isolates were extracted from soil in Kazakhstan in Taraz city with coordinates "42.9 N 71.36667 E". Isolation and preparation of pure cultures of the bacteria included the following steps: 1 g of soil was suspended in 9 mL of 0.9% (w/v) NaCl solution, then further diluted by a factor of 10. A 100 μL aliquot of the suspension was spread on nutrient agar plates and incubated at 37 °C for 48 h. The selection of amylolytic isolates was detected by growth on starch nutrient agar plates and further treatment with Lugol's solution. Clear zones around colonies confirmed the amylolytic activity of the isolates. Amylolytic colonies were transferred to a nutrient agar plate, and colony purity was checked by Gram staining and light microscopy.

2.4. Strain identification

Strains were identified by proteomic profiling of ribosomal proteins and by sequencing of a conserved DNA locus in accordance with [11,25]. A proteomic analysis was conducted using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry with the BiotyperMicroflex LT instrument (Bruker Daltonics, Bremen, Germany).

Genomic DNA was isolated using Monarch Nucleic Acid Purification Kit (New England Biolabs). 16S rRNA gene fragment was amplified by PCR with standard primers 27F and 1492R. The amplified DNA fragment was sequenced by Sanger method [26] using the BigDye™ Terminator v3.1 (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. Utilizing an ABI 3730xl automatic sequencer (Applied Biosystems, USA), DNA fragments were separated. Chromatograms were examined and compared with a

Table 1
Gene-specific oligonucleotides used for cloning and sequencing.

Name	Oligonucleotide sequence in 5′–3′ direction
AmyT5_F	ATGAAACAACAAAAACGGCTTTAC
AmyT5_R	TCTTGAACATAAATTGAAACCGA
AmyT5_NdeI	GGAATTCCATATGGCAAATCTTAATGGGACGCTG
AmyT5_NotI	TTTCCTTTTGCGGCCGCTCTTTGAACATAAATTGAAAACCG
AmyT5_EcoRI	CCGGAATTCCGGATGAAACAACAAAAACGGCTTTA

reference sequence utilizing the Vector NTI version 11 software package and the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Cloning of the α-amylase (amy) gene into vectors pET28(+)c and pPICZαA

The *amy* gene was amplified from the gDNA of *B. licheniformis* strain T5 (obtained as described in subsections above) using primers AmyT5_F and AmyT5_R (Table 1) and cloned into the pJET1.2/blunt vector (according to the manufacturer's protocol). Plasmid DNA from positive clones was isolated with the GeneJET Plasmid Miniprep Kit (Thermo Scientific), and the *amy* gene (hereafter *amyT5)* was sequenced by Sanger method.

The *amyT5* gene was amplified from pJET1.2/amy using AmyT5_NdeI/AmyT5_NotI and AmyT5_EcoRI/AmyT5_NotI. To construct the pET28/amyT5 and pPICZα/amyT5 vectors, amplified fragments were cloned into pET28c(+) at the *Nde*I and *Not*I sites and pPICZαA at the *Eco*RI and *Not*I sites using appropriate restriction endonucleases.

The accuracy of the insertion in pET28/amyT5 and pPICZα/amyT5 vectors was confirmed by sequencing with primer pairs T7_F/T7_R and AOX1_F/AOX1_R, respectively.

2.6. Expression and purification of a recombinant amylase

Two variants of recombinant AmyT5 amylase — rAmyT5-E and rAmyT5-P — were obtained by intracellular expression in *E. coli* strain ArcticExpress(DE3)RP and secretory expression in *P. pastoris* strain X-33, respectively. The ArcticExpress(DE3)RP competent cells were transformed with the pET28/AmyT5 plasmid by electroporation. 20 ng of plasmid DNA was added to 50 μL of the cells, mixed well and then suspension was pulsed in a 0.2 cm cuvette at 2.5 kV for 4.6 ms with electroporator (MicroPulcer™, Bio-Rad, USA). Kan^R colonies were grown in 500 mL of LB with added kanamycin (50 μg/mL) at 37 °C and 150 rpm. When the bacterial culture reached an optical density of 0.6 at 600 nm ($OD_{600} = 0.6$), cells were chilled on ice for 20 min and 0.5 mM of isopropyl-β-D-1-thio-galactopyranoside (IPTG) was added. Then culture was grown for 16 h at 18 °C. Cells were harvested by centrifugation (at 6000×*g*, 4 °C, 7 min) and suspended in 20 mM Tris-HCl (pH 8.0) containing 500 mM NaCl. Cells were lysed by lysozyme (2 mg/mL), and sonicated. Clarified lysate was obtained by centrifugation (40,000×*g*, 4 °C, 1 h). The target protein was purified by immobilized metal affinity chromatography using AKTA Purifier 10 FPLC (General Electric, USA) on a 1 mL HiTrap Chelating column (General Electric, USA) charged with Ni^{2+} ions. Column was preequilibrated with loading buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, and 20 mM imidazole). Namely, the clarified lysate in volume 5 mL with 10.875 mg of total protein was loaded onto the column, and the rAmy-E protein in amount 0.6 mg was eluted with elution buffer (20 mM Tris-HCl pH 8.0, and 500 mM NaCl) containing imidazole as a linear of 20–500 mM. Flow was 0.5 mL/min, volume of gradient was 12 mL. Size of fractions were 0.5 mL. Fractions were collected and analyzed by SDS-PAGE.

P. pastoris X-33 competent cells were electroporated with the pre-linearized pPICZα/AmyT5 plasmid. 80 μL of cells were mixed with 3 μg of pre-linearized plasmid DNA and electroporated in a 0.2 cm cuvette at 2.0 kV for 5.1 ms using an electroporator. The transformed cells were plated on YEPD-agar with zeocin (100 μg/mL). Genomic DNA was isolated from 20 zeo^R clones, and the insert was checked by PCR using primer pairs AOX1_F/AOX1_R and AmyT5_F/AmyT5_R. Positive clones were analyzed for amylase activity. The most active clone was transferred into 10 mL of YEPG and incubated for 24 h at 30 °C and 250 rpm. The cells were harvested by centrifugation at 3500×*g* for 15 min at room temperature. They were then resuspended in 100 mL of YEPM and incubated at 30 °C at 220 rpm for 120 h, with 1% methanol supplied every 24 h. The cells were collected by centrifugation at 10,000×*g* for 15 min, at 4 °C, and the pellet was wasted. Clarified culture supernatant was passed through a 0.22 μm filter for sterilization and further used to purify α-amylase. Next, 2 mL of Phenyl Sepharose 6 Fast Flow (Cytiva, Sweden) was added to a 5 mL column, and the column was equilibrated with loading buffer (100 mM phosphate buffer pH 6.5 and 1 M ammonium sulfate). The column was loaded with 90 mL of the supernatant mixed with 90 mL of loading buffer. rAmyT5-P was eluted under the force of gravity with 0.1 M phosphate buffer (pH 6.5) containing a decreasing gradient of ammonium sulfate (1000–0 mM). Volume of gradient was 6 mL. Size of fractions were 3 mL. Fractions were tested for α-amylase activity and checked by SDS-PAGE. Fractions containing purified rAmyT5-P were pooled and dialyzed against 20 mM phosphate buffer pH 6.5. 3.75 mg of purified rAmyT5-P was collected.

2.7. Obtaining anti-rAmyT5 antibodies and western blotting

To obtain polyclonal antibodies, a 6-month-old New Zealand rabbit was immunized with rAmyT5-E 6 times. The rAmyT5-E protein concentration was adjusted to 0.3 mg/mL with 1X PBS. Prior to the start of immunization, 1 mL of blood was drawn from the rabbit's ear vein for a negative control. The first immunization was performed with 300 μg of rAmyT5-E mixed with Freund's complete adjuvant in a ratio of 1:1 (total volume 1 mL). The second immunization was performed 7 days later with 300 μg of rAmyT5-E mixed with incomplete Freund's adjuvant in a ratio of 1:1 (total volume 1 mL). The next 3 immunizations were carried out with an interval of 7 days only rAmyT5-E in the amount of 150 μg. The day after the fifth immunization, 0.5 mL of blood was drawn from the ear vein and western blotting were performed to check for specify and sensitivity of the antibodies using simultaneously along with the negative control. The sixth immunization, also in the amount of 150 μg of rAmyT5-E, was done 3 days after the fifth immunization, and the next day 20 mL of blood was taken for serum and immunoglobulin isolation. Immunoglobulins were precipitated with 33% sulfate ammonium and dialyzed against 1X PBS. The anti-rAmy antibody was stored at −20 °C. The standard methodology [27] for western blotting was followed, with protein samples being separated by SDS-PAGE in a 12% gel and then transferred to a PVDF membrane that had been pre-activated with 96% ethanol. The membrane was blocked in a 5% (w/v) milk solution in Tris-buffered saline

supplemented with 0.05% of Tween 20 (TBST). The aforementioned polyclonal antibody (dilution 1:15,000) was used as the primary antibody to identify proteins, and a secondary antibody (1:10,000) that was coupled to horseradish peroxidase (Sigma-Aldrich Chimie Sarl, Lyon, France) was used to detect rabbit IgGs. The bands were detected ECL chemiluminescent substrate (AppliChem GmbH, Darmstadt, Germany), and X-ray film exposed to the membrane (AgfaPhoto GmbH, Germany).

2.8. Glycosylation prediction and validation

Purified rAmyT5-P was deglycosylated with EndoH (NEB) at 37 °C for 16 h. The deglycosylated protein and untreated protein were analyzed by SDS-PAGE and western blotting.

2.9. Determination of protein concentration

Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay Dye, Munich, Germany) using bovine serum albumin as a standard.

2.10. α-Amylase activity assay

Activity of α-amylase was measured by the method of reducing sugars adapted to α-amylase activity [28,29]. In brief, starch (Sigma-Aldrich, USA) was dissolved in 100 mM phosphate buffer (pH 6.0) to prepare a 1% (w/v) solution. Subsequently, 40 μL of the enzyme was incubated with 1 mL of the starch substrate for 10 min (rAmyT5-E at 80 °C and rAmyT5-P at 70 °C). Then 1.5 mL of the 3, 5-dinitrosalicylic acid (DNSA) reagent was added to the reaction mixture. Reaction mix was then boiled in a water bath for 10 min. The absorbance at a wavelength of 540 nm was determined using a Shimadzu UV-1900i spectrophotometer. Glucose (Sigma-Aldrich, USA) was used as the standard to create a calibration curve. A unit of α-amylase activity corresponds to the enzyme's ability to release 1 μmol of reducing glucose in 1 min. The end result is expressed as the mean of the triplicate measurements that were performed.

2.11. Effects of pH and temperature on α-amylase activity and stability

The pH range of 2.0–10.0 (with an interval of half a unit) was used to determine the effect of α-amylase activity at 80 °C for rAmyT5-E and at 70 °C for rAmyT5-P. The temperature dependence of the enzymatic activity was determined in the temperature range of 20–100°C in 100 mM phosphate buffer (pH 6.0). The maximum enzymatic activity was set to 100% activity, and the results obtained at various pH levels and temperatures were evaluated as a percentage of the maximum.

rAmyT5-E and rAmyT5-P were preincubated in a suitable buffer for 1 h at 80 and 70 °C, respectively, to assess the impact of temperature on enzyme stability. To study pH stability, enzymes were preincubated in buffers having different pH values in the range of 3–12 at 25 °C for 10 h, followed by determination of activity.

The residual activity was computed as a percentage of the initial enzymatic activity, which was initially set at 100%. The 50 mM sodium citrate buffer (pH 3.0–4.5), the 100 mM sodium acetate buffer (pH 4.0–5.5), the 100 mM phosphate buffer (pH 6.0–8.0), and the 100 mM glycine-NaOH (pH 9.0–12.0) buffer systems had been used to provide required pH. The final result is provided as the mean of the triplicate measurements, which were taken.

2.12. Effects of metals, detergents, and organic solvents on the enzymatic activity

The influence of metal ions on α-amylase activity was determined in the presence of one of eight chlorides: ZnCl2, $CaCl_2$, $CuCl_2$, NiCl2, $MnCl_2$, MgCl2, $FeCl_3$, or $CoCl_2$, which were used at a concentrations of 5 and 10 mM. The following detergents and chemicals were tested: 100 and 200 mM urea, 0.5 and 1 % (v/v) Triton X-100, 100 and 200 mM guanidine hydrochloride, 0.5 and 1 % (v/v) β-mercaptoethanol, 10 and 20 mM dithiothreitol, 10 and 20 mM sodium dodecyl sulfate, 5 and10 mM EDTA, ethanol, isopropanol, methanol, and acetone at a concentrations of 5 and 10 % (v/v). Following a 1-h preincubation at room temperature with a test substance, the enzyme's activity was assessed under optimal conditions. The residual activity was calculated as a percentage of the native activity, which was the enzymatic activity in the absence of preincubation with metal ions, detergents, or other compounds (native activity). The percentage of this activity was established at 100%. Measurements were taken in triplicate, and the average of the triplicates is reported as a final result.

2.13. Determination of substrate specificity and kinetic parameters of the enzymes

Kinetic parameters of the enzymes were determined by an enzymatic reaction involving starch at concentrations of 1–15 mg/mL for 10 min under optimal conditions. K_M and V_{max} constant values were calculated using the Michaelis–Menten equation [30]. To measure the kinetics parameters, 0.1–1.2% soluble starch substrates were incubated under the chosen reaction conditions. The linear velocity was measured to determine the K_M and K_{cat} constants, which were derived from the one-site binding (hyperbolic) model using GraphPad Prism 8.0.1. Substrate specificity of the enzymes was determined in 100 mM phosphate buffer (pH 6.0) at 70 °C with potato starch (Sigma-Aldrich, St. Louis, MO, USA, product no.S2004), cellulose (Sigma-Aldrich, St. Louis, MO, USA, product no.C6288), carboxymethylcellulose (Sigma-Aldrich, St. Louis, MO, USA, product no.419273), pullulan (Tokyo Chemical Industry, Tokyo, Japan, product no.P0978), beechwood xylan (Megazyme, Ireland, product no. P-XYLNBE), amylose (Megazyme, Ireland, product no.

P-AMYL), β-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA, product no.C4767).

2.14. TLC for the identification of starch hydrolysis products

The detection of hydrolysis products by Thin Layer Chromatography (TLC) was carried out according to Ref. [31]. For this purpose, 1% (w/v) potato starch in 100 mM phosphate buffer (pH 6.0) was incubated with a recombinant enzyme (10 U/mL) at 50 °C for 10 h. The reaction was stopped by heating to 100 °C. The samples were applied to an aluminum plate with silica gel (Silica gel 60 F254, Merck, Germany). Glucose (Sigma), maltose (AppliChem), maltotriose, maltotetraose, and maltopentaose (Megazyme, Ireland) served as standards for the TLC.

A mixture of butanol, glacial acetic acid, and deionized water in a ratio of 2:1:1 (v/v) was used as the mobile phase. The plate was dried at 60 °C for 20 min, then placed in a developing solution containing sulfuric acid and ethyl alcohol in a ratio of 5:95 (v/v) and α-naphthol at a final concentration of 0.5% (w/v). To detect hydrolysis products, the plate was heated to 130°C until the hydrolysis products became visible.

2.15 Software tools, bioinformatics, and statistical analysis

Each experiment was performed independently three times. Means and standard deviations were computed using version 8.0.1 of GraphPad Prism. The means of α-amylase activities and the means followed by standard deviations for other parameters are provided (n = 3). Calculation of protein molecular weights and isoelectric points, analysis of chromatograms after sequencing, and the design of primers were performed utilizing SnapGene Viewer 5.2.4 and Vector NTI Advance 11. Web services BLASTN and BLASTP were used to compare nucleotide and protein sequences with the NCBI nucleotide/protein database, respectively. To predict a signal peptide region the Peptide Signal IP 5.0 online software (http://www.cbs.dtu.dk/services/SignalP/) was employed. NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) sources were used to predict potential glycosylation sites.

3. Results

3.1. Isolation and identification of the amylolytic bacterium

Eight isolates of bacteria were obtained from soil in Kazakhstan via cultivation on nutrient agar. Based on the results of screening on starch agar plates, an isolate with the highest α-amylase activity was chosen. This isolate formed clear zones on a starch-containing medium when stained with iodine. The isolated microorganism exhibits aerobic metabolism and possesses a Gram-positive cell wall structure. Its cellular morphology is oval in shape, and it demonstrates motility. Notably, this microorganism is distinguished by its ability to form spores. According to morphological features and cultivation properties, this isolate was assumed to be a gram-positive bacterium from the genus *Bacillus*. Analysis and comparison of this isolate's proteomic profile with MALDI-TOF Biotyper mass spectrometric databases showed that the isolate is the species *B. licheniformis* (Score 2.104). Sequencing of the 16S rRNA fragment of the bacterial isolate and comparison with the NCBI database revealed that this isolate is *B. licheniformis* strain T5 with an identity and an overlap of more than 99%. The complete genome of the *Bacillus licheniformis* T5 strain has been sequenced and is available for

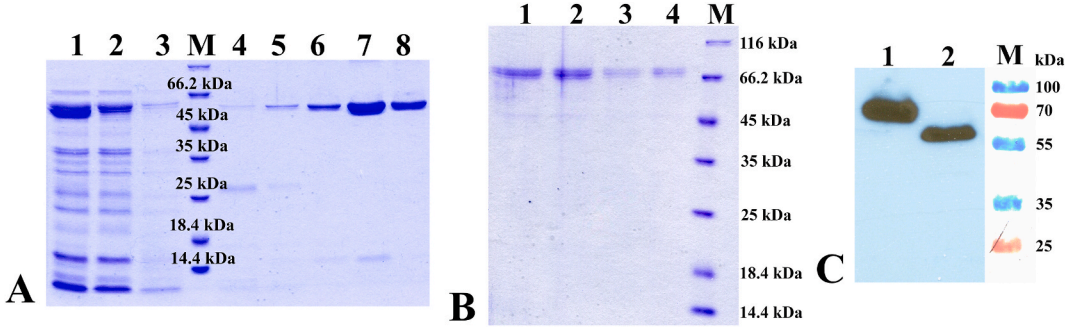

Fig. 1. A. SDS-PAGE after purification of rAmyT5-E protein from the culture of *E. coli* ArcticExpress(DE3)RP_pET28/AmyT5 (IPTG-induced expression). Lane 1: clarified lysate; lane 2: flowthrough; lane 3: wash; lanes 4,5: fraction eluted with 100 mM imidazole; lanes 6–8: fraction eluted with 150 mM imidazole; lane M: protein molecular weight markers (Thermo Fisher Scientific, #26610) (Supplementary materials: Fig. S1A).
B. Chromatographic purification of the recombinant rAmyT5-P protein from the secreted cultural media of the P. pastoris X-33-pPICZα/AmyT5 (methanol-induced expression) strain. Lane 1: culture after expression induction, lanes 2–4: the fraction after chromatographic purification on the Phenyl-Sepharose column, lane M: protein molecular weight markers (Thermo Scientific #26610) (Supplementary materials: Fig. S1B).
C. Western blotting of rAmyT5-P before and after treatment with deglycase EndoH. Lane 1: purified rAmyT5-P; lane 2: EndoH treated rAmyT5-P; lane M: protein molecular weight markers: Broad Range Color Protein Standards (New England Biolabs, #P7712S)(Supplementary materials: Fig. S1C).

use (accession number CP124852) [32].

3.2. Expression of the amyT5 gene in E. coli cells and preparation and purification of rAmyT5-E

Transformation of competent cells of the *E. coli* strain ArcticExpress(DE3)RP with the pET28/AmyT5 plasmid resulted in *E. coli* strain ArcticExpress(DE3)RP_pET28/AmyT5, which efficiently produced α-amylase rAmyT5-E. The recombinant AmyT5-E protein was extracted from *E. coli* cells and purified on the HiTrap Chelating 1 mL HP column with Ni^{2+} ions. Fig. 1A shows the results of the chromatographic purification of the rAmyT5-E protein. rAmyT5-E got eluted from the column when imidazole concentration was 150 mM. The estimated molecular weight of 6xHis tagged rAmyT5-E, is 58.8 kDa, which is corresponding with the experimental data obtained by SDS-PAGE (Fig. 1A).

Activity calculation showed that the yield of purified α-amylase rAmyT5-E after purification from *E. coli* cells was 34.4%. Metal affinity purification made it possible to achieve 6.23-fold enrichment of the enzyme. The recombinant nonglycosylated α-amylase rAmyT5-E have a specific activity of 2086 ± 5 U/mg. (Table 2).

3.3. Expression of the amyT5 gene in P. pastoris and production and purification of recombinant α-amylase rAmyT5-P

By electroporation of competent cells of *P. pastoris* strain X-33 with linearized plasmid pPICZα/AmyT5, clones of yeast cells producing rAmyT5-P were obtained. One clone (with the highest α-amylase activity) was chosen as the producer strain of recombinant α-amylase AmyT5-P. In this *P. pastoris* X-33-pPICZα/AmyT5 strain, the recombinant rAmyT5-P protein was produced on the YEPM medium. Purification of the recombinant α-amylase from yeast culture was performed by hydrophobic chromatography on a Phenyl-Sepharose 1 mL column. Fig. 1B shows the outcome of the chromatographic purification of the recombinant AmyT5-P protein.

The results of SDS-PAGE indicated that the hydrophobic chromatography gave high purity of the rAmyT5-P protein, and the yield of the enzyme was 14.27% (Fig. 1B). Fractions 2–4 were combined, the protein concentration was measured (which was 0.2 mg/mL), and this solution was subjected to experiments to assess biochemical parameters of the rAmyT5-P α-amylase.

Bioinformatic analysis of the amino acid sequence of the AmyT5-P protein using the NetNGlyc 1.0 software revealed that the AmyT5-P protein has six potential N-glycosylation sites: Asn-Xaa-Ser/Thr at positions 4, 27, 275, 280, 309, and 455. Treatment of α-amylase rAmyT5-P with EndoH endoglycosidase confirmed glycosylation of the protein. Fig. 1C shows the results of western blotting with the polyclonal anti-rAmyT5-E antibody. The protein's molecular weight decreased by around 10 kDa after deglycosylation (Fig. 1C).

The impact of pH on the α-amylase activity of recombinant enzymes rAmyT5-E and rAmyT5-P was studied next. The optimum pH for both enzymes was found to be 6.0. At this pH value, the highest activity is observed for both enzymes. In the pH range of 5.0–10.0, rAmyT5-E and rAmyT5-P have an activity of more than 50% of the maximum. At pH values below 4.0 and above 11, there was decrease of the activity of both enzymes (Fig. 2A).

B. Effects of pH (after 10 h incubation at 25 °C) on the activity of rAmyT5-E and rAmyT5-P (the pH stability assay).

rAmyT5-E and rAmyT5-P pH stability was evaluated by 10 h incubation of the enzymes pH 3.0–12.0 at RT (25 °C) in appropriate buffer systems, followed by measurement of residual activity. Fig. 2B illustrates the dependence of the residual activity on pH. Based on the data provided, both enzymes showed resistance to an alkaline environment.

3.4. The influence of temperature on the stability of α-amylases rAmyT5-E and rAmyT5-P

Fig. 3A shows the dependence of the α-amylase activity of rAmyT5-E and rAmyT5-P on temperature in the range from 20 °C to 100 °C.

Thermal stability of the recombinant α-amylases AmyT5-E and AmyT5-P was studied at 70 and 80 °C, respectively, at the pH 6.0 for 60 min (Fig. 3B). As displayed in Fig. 3B, more than 70% and 60% of the enzymatic activity is retained by rAmyT5-E and rAmyT5-P, respectively, after preincubation of each enzyme at 70 °C for 60 min. The half-life of rAmyT5-E at 80 °C was 18 min, and that of rAmyT5-P was 9 min.

Table 2
Purification of rAmyT5-E and rAmyT5-P recombinant α-amylases from *E. coli* ArcticExpress(DE3)RP_pET28/AmyT5 and from *P. pastoris* X-33_pPICZα/AmyT5 (induced expression in both strains).

Purification step	α-Amylase activity, U	Total protein, mg	Specific activity, U/mg	Fold enrichment	Recovery, %
rAmyT5-E					
Clarified supernatant	3642	10.875	334.9	1	100
Ni^{2+} affinity chromatography	1252	0.6	2086.67	6.23	34.4
rAmyT5-P					
Culture supernatant	12240	36	340	1	100
Phenyl-Sepharose	1754.5	3.915	448	1.3	14.3
Dialysis	1747	3.75	466	1.4	14.27

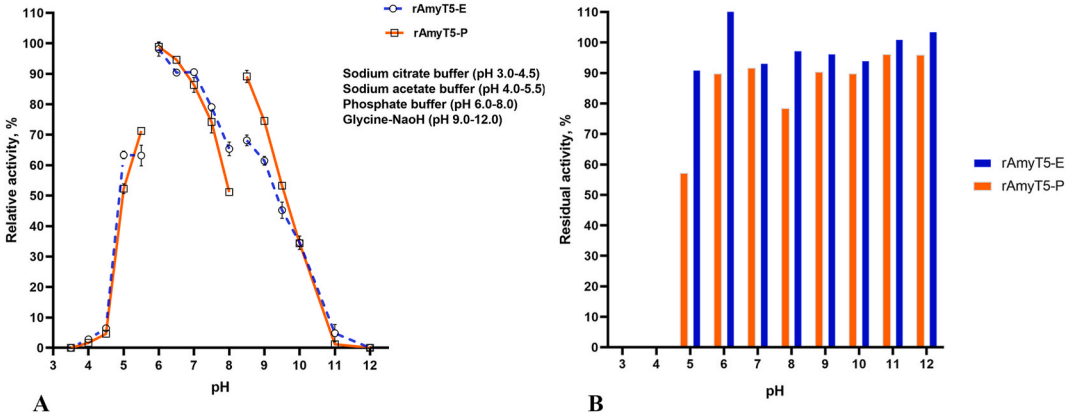

Fig. 2. A. The impact of pH on the rAmyT5-E and rAmyT5-P activity.

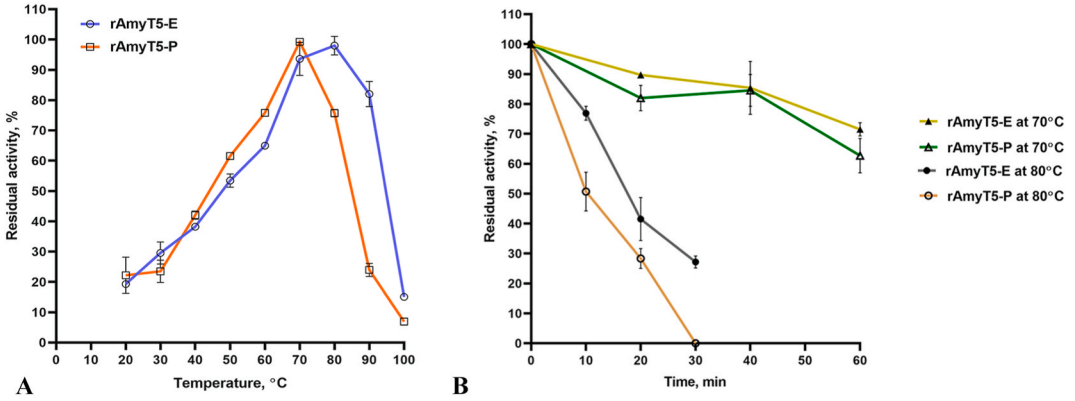

Fig. 3. A - The impact of temperature on the activity of rAmyT5-E and rAmyT5-P.B - The impact of temperature, at 70 or 80 °C, on the stability of rAmyT5-E and rAmyT5-P.

3.5. Effects of inhibitors, detergents, metal ions, and organic solvents on the activity of α-amylases rAmyT5-E and rAmyT5-P

The investigation into the metal ions impact and various chemical agents on the enzymatic activity of rAmyT5-E and rAmyT5-P revealed that the presence of Fe^{3+} ions resulted in the complete inactivation of both enzymes (Table 3). In this study, the impact of Cu^{2+} ions on the activity of rAmyT5-E and rAmyT5-P enzymes revealed that at a concentration of 5 mM, Cu^{2+} ions caused a significant decrease in the activity of rAmyT5-E and rAmyT5-P enzymes by 51% and 72%, respectively. Furthermore, at a higher concentration of 10 mM, Cu^{2+} ions completely inhibited the activity of both forms of the enzyme. The ions Ca^{2+} at concentration 10 mM increase activity of rAmyT5-P by 17%. Mn^{2+} ions at concentrations of 5 and 10 mM increase α-amylase activity of rAmyT5-P by 18% and 24%, respectively. Co^{2+} ions, at concentrations of 5 and 10 mM, respectively, modestly enhance rAmyT5-E activity by 10% and 21%. In the case of rAmyT5-P, adding 5 mM Co^{2+} ions cause a 24% increase in activity. The impact of 5 and 10 mM concentrations of ethylenediaminetetraacetic acid (EDTA) on the activity of rAmyT5-P revealed that the presence of EDTA at these concentrations led to a nearly twofold decrease in the activity of rAmyT5-P. However, it was observed that EDTA had no significant effect on the activity of rAmyT5-E. Selectivity is also shown for sodium dodecyl sulfate (SDS). Pre-incubation with SDS results in complete inactivation of rAmyT5-P, indicating its susceptibility to SDS processing. In contrast, the non-glycosylated form of the enzyme exhibits resistance to SDS treatment. In opposite of Dithiothreitol (DTT), β-mercaptoethanol at concentration of 1% increases the activity of rAmyT5-P by 34%. The activity of rAmyT5-E and rAmyT5-P is not considerably impacted by the presence of organic solvents, chaotropic agents such urea and guanidine hydrochloride and nonionic detergent such Triton X-100.

3.6. Determination of substrate specificity and kinetic parameters of α-amylases rAmyT5-E and rAmyT5-P

rAmyT5-E and rAmyT5-P exhibited high substrate specificity toward starch. No enzymatic activity was detectable when the substrate was carboxymethylcellulose, cellulose, β-cyclodextrin or xylan. Enzyme activity was observed for both enzymes in relation to starch, pullulan and amylose (Table 4).

KM, Vmax, and kcat kinetic parameters of the recombinant enzymes were calculated by examining different amounts of potato

Table 3
Effects of metal cations, detergents, organic solvents, and other chemicals on the α-amylase activity of rAmyT5-E and rAmyT5-P.

Reagent	Residual activity (%)			
	rAmyT5-E		rAmyT5-P	
Metal ions				
Metal ion	Metal ion concentration 5 mM	Metal ion concentration 10 mM	Metal ion concentration 5 mM	Metal ion concentration 10 mM
Control	100 ± 2	100 ± 5	100 ± 0,8	100 ± 1
Ni^{2+}	96 ± 3	91 ± 5	103 ± 2	92 ± 2
Ca^{2+}	101 ± 5	110 ± 5	100 ± 1	117 ± 1
Zn^{2+}	87 ± 3	92 ± 3	80 ± 1	83 ± 1
Co^{2+}	110 ± 4	121 ± 3	124 ± 1	83 ± 1
Cu^{2+}	49 ± 5	0	28 ± 1	0
Mn^{2+}	111 ± 3	100 ± 2.3	118 ± 1	124 ± 1
Mg^{2+}	109 ± 1	100 ± 5	105 ± 1	102 ± 1
Fe^{3+}	0	0	0	0
Chelating agent				
EDTA 5 mM	95 ± 4		57 ± 2	
EDTA 10 mM	97 ± 5		53 ± 4	
Chemicals				
β-mercaptoethanol 0.5 %	87 ± 7		118 ± 6	
β-mercaptoethanol 1 %	100 ± 11		134 ± 2	
Dithiothreitol (DTT) 10 mM	100 ± 5		114 ± 14	
Dithiothreitol (DTT) 20 mM	94 ± 2		102 ± 2	
SDS 10 mM	102 ± 10		0	
SDS 20 mM	85 ± 9		0	
Triton X-100 0.5%	98 ± 5		117 ± 2	
Triton X-100 1%	102 ± 5		117 ± 18	
Urea 100 mM	102 ± 1		113 ± 4	
Urea 200 mM	89 ± 5		107 ± 1	
Guanidine hydrochloride 100 mM	96 ± 7		112 ± 2	
Guanidine hydrochloride 200 mM	80 ± 4		114 ± 15	
Acetone 5%	80 ± 2		101 ± 12	
Acetone 10%	92 ± 9		103 ± 4	
Isopropanol 5%	96 ± 7		108 ± 2	
Isopropanol 10%	97 ± 4		112 ± 9	
Methanol 5%	87 ± 10		106 ± 7	
Methanol 10%	90 ± 13		102 ± 5	
Ethanol 5%	89 ± 5		115 ± 3	
Ethanol 10%	101 ± 2		113 ± 6	

starch as a substrate (Table 5).

3.7. Enzymatic hydrolysis of starch and identification of hydrolysis products by TLC

The analysis of starch hydrolysis products by TLC showed that these products are maltooligosaccharides: maltopentaose, maltotetraose, maltotriose, maltose and glucose. Fig. 4 depicts the results of the starch hydrolysis process employing rAmyT5-E (Fig. 4A) and rAmyT5-P (Fig. 4B) enzymes. The TLC hydrolysis products, for both enzymes, have a comparable profile. Despite the fact that

Table 4
Specific activity of rAmyT5-E and rAmyT5-P on different substrates.

Substrate	Specific activity (U/mg)	
	rAmyT5-E	rAmyT5-P
Starch	2086 ± 5	466 ± 1
Carboxymethylcellulose	N/D[a]	N/D[a]
Cellulose	N/D[a]	N/D[a]
β-Cyclodextrin	N/D[a]	N/D[a]
Xylan	N/D[a]	N/D[a]
Pullulan	2 ± 0,06	0.6 ± 0.01
Amylose	2232 ± 1	574 ± 3

[a] N/D - not detected.

Table 5
Kinetic constants of AmyT5-E and AmyT5-P.

Parameter	Values	
	rAmyT5-E	rAmyT5-P
K_M (mg/mL)	0.48 ± 0.05	1.82 ± 0.25
k_{cat} (s^{-1})	118 ± 1	44 ± 1
Catalytic efficiency (k_{cat}/K_M)	244	24

B. licheniformis' α-amylase is an endo-enzyme, maltose and other oligomers are also generated together with glucose.

4. Discussion

The most prevalent post-translational modification of enzymes is glycosylation. Attachment of mannose chains causes a change in the conformation, and depending on the site of attachment of sugar residues, glycosylation leads to a change in the activity of the enzyme and in the binding of the enzyme to a substrate and alters temperature-related properties of the enzyme. In practice, glycosylation is a convenient tool for protein engineering applications, because in this way, it is possible to modify biochemical characteristics of the enzyme. Protein glycosylation has been observed during protein expression in yeasts *Saccharomyces cerevisiae*, P. pastoris, and Kluyveromyces lactis [15,33–36] and in filamentous fungi of genera Aspergillus, Penicillium, and Trichoderma [37,38]. Although *S. cerevisiae* is characterized by abundant glycosylation, the proteins expressed in P. pastoris have moderate glycosylation [39]. It is the yeast P. pastoris that has served as a host strain for modifying N-glycosylation pathways, thereby allowing to obtain humanized glycoproteins [40]. Despite the large number of publications on the preparation of glycosylated enzymes, it is not clear how glycosylation affects properties of glycoside hydrolases.

Table 6 provides information on some native and recombinant glycoside hydrolases obtained in P. pastoris: α-amylase from *Geobacillus stearothermophilus* [41], *Bacillus* [24], *Aspergillus oryzae* [13], and *B. licheniformis* [42]; glucoamylase from *Tricholoma matsutake* [43]; and xylanases from *Thermobifida fusca* and *Bacillus sonorensis* [11,44]. Glycoside hydrolases have different molecular weights from 23.3 kDa for xylanase from Bacillus sonorensis [11] to 68 kDa for β-glucuronidase from Penicillium purpurogenum [22]. α-Amylases, regardless of bacterial or fungal origin, predominantly have a molecular weight of more than 50 kDa [13,24,45]. For α-amylases from Acyclobacillus acidocaldarius and Lactobacillus manihotivorans, molecular masses of 160 and 135 kDa were established, respectively [46,47]. However, α-amylase with a molecular weight of 21 kDa, isolated from B. subtilis strain AS-S01a, has also been described [48]. Glycoside hydrolases are active at acidic and neutral pH and heterologous expression does not affect the pH optimum; only for xylanase from B. sonorensis detected shift of pH from 7 to 6 [11].

Some authors have noticed an increase in thermal stability after N-glycosylation in α-amylase from A. oryzae S2, and the reason is that the protein's structure contains an extra C-terminal loop consisting of 22 residues [13]. Higher thermal stability after glycosylation has been documented for Penicillium purpurogenum β-glucuronidase expressed in P. pastoris [22]. The beneficial effect of N-glycosylation on thermal stability has also been observed in B. sonorensis xylanase and Aspergillus fumigatus xylanase expressed in P. pastoris [11,49].

N-glycosylation is one of the crucial post-translational modifications that plays a significant role in biological processes in eukaryotic cells [50]. The level of N-glycosylation in yeasts varies greatly depending on the species of microorganism, with *P. pastoris* exhibiting mild glycosylation and *Saccharomyces cerevisiae* exhibiting hyperglycosylation of its protein constituents [51]. In the

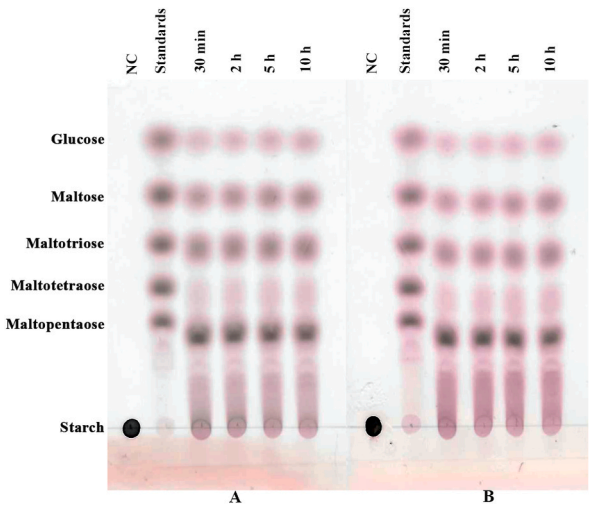

Fig. 4. Hydrolysis of potato starch by rAmyT5-E (**A**) and by rAmyT5-P (**B**).

Table 6
Biochemical properties of glycoside hydrolases expressed in *E. coli* and *P. pastoris* as native (N) or recombinant (R) proteins, respectively.

Enzyme	Strain	Molecular weight, kDa	pH optimum N/R	Half life N/R	References
α-amylase AmyT5	*Bacillus licheniformis*	58	6.0/6.0	18/9 min (80 °C)	Present work
α-amylase AmyA	*Aspergillus oryzae* S2	54	5.6/5.6	10/45 min (60 °C)	[13]
α-amylase ABA	*Bacillus* sp.	55	6.0/6.0	>80/60 min (68 °C)	[24]
glucoamylase *Tm*GLA	*Tricholoma matsutake*	61.5	5.0/5.0	30/30 min (60 °C)	[43]
β-glucuronidase GUS	*Penicillium purpurogenum*	68	5.0/5.0	80/>120 min (65 °C)	[22]
xylanase XynT6	*Bacillus sonorensis*	23.3	7.0/6.0	40/120 min (55 °C)	[11]
xylanase Af-XYNA	*Aspergillus fumigatus*	39	5.0/5.0	10/25 min (70 °C)	[49]

endoplasmic reticulum, oligosaccharide transferase (OST) attaches the oligosaccharide (Glc3Man9GlCNAc2) to the Asn residues in the regions of Asn-X-Ser/Thr (where X is any amino acid other than Pro) of the polypeptide chain. This process is known as N-glycosylation and is carried out on secretory proteins in yeasts. Oligosaccharides attached to peptides are gradually broken down by α-glucosidase I and II. After an endoplasmic reticular chaperone-assisted folding process, the target proteins are transported by vesicles to the Golgi apparatus for further glycosylation, which leads to the secretion of the glycoproteins outside the cell [12]. The protein structure and enzyme activity are impacted by N-glycosylation. N-glycosylation of proteins produced in *P. pastoris* is noted, and this is followed by an increase in molecular weight [27,52]. In the present work, the effect of N-glycosylation on the α-amylase activity and thermal stability of α-amylase from B. licheniformis was determined, and the half-life at 80 °C is twofold shorter for glycosylated enzyme than for non-glycosylated analog. A similar negative influence of N-glycosylation on thermal stability has been observed in the alkaliphilic α-amylase from Bacillus; the researchers attribute this phenomenon to the glycosylation of the recombinant protein [24]. On the other hand, no alterations of the biochemical properties of α-amylase from B. licheniformis were detected in Ref. [42]; those authors explained this finding by the fact that glycosylation sites are located in an inflexible region of the protein.

We have also found that glycosylation enhances the recombinant α-amylase AmyT5's sensitivity to metal ions: Co2+ ions at a concentration of 5 mM increases glycosylated enzyme activity by 24%, and Mn2+ ions at a concentration of 10 mM increases glycosylated α-amylase activity by 24%. Co2+ ions have been shown to have a positive impact on the α-amylases of B. licheniformis [10] and A. oryzae [53]. The inhibitory effect of Cu2+ and Fe3+ ions was noted by the authors for α-amylase from B.licheniformis B4-423 [54]. With the exception of Cu2+ and Fe3+ ions, which are potent inhibitors of rAmyT5 activity, α-amylase from B. licheniformis T5 is tolerable to the effects of other metal ions. The enzyme's sensitivity to EDTA and SDS was increased by N-glycosylation We have shown that the presence of EDTA affects rAmyT5 activity, indicating that α-amylase is a metal-dependent enzyme. It was also found here that N-glycosylation reduced the specific activity and catalytic efficiency of α-amylase rAmyT5 by 4.47- and 10-fold, respectively. The properties of the enzyme are likely to be affected by the degree of glycosylation. Comparative analysis of glycosylated α-amylase from B. licheniformis T5, which has 6 potential N-glycosylation sites, with glycosylated xylanase from B. sonorensis T6, which has only 3 [11], confirms this assumption. If the enzymatic activity of xylanase from B. sonorensis T6 did not change as a result of glycosylation, then the specific activity of α-amylase from B. licheniformis T5 decreased by 4.47 times.

5. Conclusion

The α-amylase gene from *B. licheniformis* T5 was expressed in *E. coli* (rAmyT5-E) and in *P. pastoris* (rAmyT5-P) in nonglycosylated and glycosylated forms, respectively. The two recombinant α-amylase variants are high-temperature enzymes with maximum activity at 80 and 70 °C for rAmyT5-E and rAmyT5-P, respectively. It was found that N-glycosylation does not affect the pH optimum, worsens thermal stability, and diminishes the specific activity and catalytic efficiency of the rAmyT5 α-amylase. The results on the effects of N-glycosylation improve the understanding of the significance of post-translational modifications for biochemical properties of α-amylases.

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Declarations

Ethics statement

The research received approval from the National Center for Biotechnology's Institutional Animal Care and Use Committee

(IACUC) (IRB00013497 National Center for Biotechnology IRB #1). It is affirmed by the authors that every experiment was conducted in adherence to pertinent guidelines and regulations. All laboratory animal research was conducted in adherence to widely recognized ethical principles regarding animal treatment, as outlined in standard operating procedures that abide by the European Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes.

Data availability statement

The data that support the findings of this study are openly available in NCBI GenBank at [https://www.ncbi.nlm.nih.gov/nuccore/CP124852.1/], accession number CP124852.

CRediT authorship contribution statement

Assel Kiribayeva: Writing – original draft, Methodology, Investigation. **Dmitriy Silayev:** Methodology, Investigation. **Zhiger Akishev:** Writing – original draft, Investigation. **Kairat Baltin:** Resources, Funding acquisition. **Saniya Aktayeva:** Investigation. **Yerlan Ramankulov:** Supervision, Methodology, Conceptualization. **Bekbolat Khassenov:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28064.

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