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CLONING, PURIFICATION AND STUDY OF THE BIOCHEMICAL PROPERTIES OF A-AMYLASE FROM *BACILLUS LICHENIFORMIS* T5 STRAIN

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Abstract

Amylases are rather important enzymes in modern biotechnology. Alpha-amylases hydrolyze starch to form glucose and maltose and are involved in the conversion of starch-containing raw materials. In comparison with α-amylases of eukaryotic organisms, bacterial α-amylases have a number of advantages, namely, the preservation of enzymatic activity at high temperatures, which is important in enzymatic starch hydrolysis technologies. In this work, the α-amylase gene from the Kazakh strain of *Bacillus licheniformis* T5 was cloned into the pET-28c(+) vector. Recombinant α-amylase was obtained by plasmid expression in *Escherichia coli* strain ArcticExpressRP(DE3). Studies have shown that the pH and temperature optimum for the recombinant alpha-amylase was 6.5 and 80°C, respectively. As has been established that α-amylase is a calcium-independent enzyme. The study of the temperature stability of α-amylase showed that the enzyme retains more than 70% of its activity when α-amylase is incubated at 80°C for 30 minutes. The obtained results show a high prospect of using α-amylase from *Bacillus licheniformis* T5 as a thermostable enzyme in the technologies of enzymatic hydrolysis of starch-containing raw materials.

Key words: α-amylase; *Bacillus licheniformis*; recombinant enzyme; plasmid; starch; hydrolysis; amylase activity.

Introduction

Alpha-amylases (EC 3.2.1.1) are endoenzymes that hydrolyze starch (polysaccharide) to form glucose (monosaccharide) and maltose (disaccharide) [1]. For modern biotechnology, α-amylases are one of the important enzymes for the hydrolysis of starch-containing raw materials [2]. Amylolytic enzymes account for about 25% of the market of all industrial enzymes [3]. Although amylases can be obtained from several sources, including plants, animals, and microorganisms, enzymes of microbial origin are the most demanded for industrial use [4]. The use of α-amylases in biotechnology includes the processing of starch-containing raw materials, the production of biofuels, and the use in the food and feed industries. Amylases are used in the paper and textile industries, used as additives to detergents. Amylases are used in bioremediation and medicine [5]. One of the industrially significant parameters of α-amylases is an increased optimum temperature at which α-amylase demonstrates its enzymatic properties, which should be at least 70°C, and thermal stability, at which the enzyme can remain active for a long time [6]. Another important characteristic is the dependence of α-amylases on coenzymes. Most known α-amylases are metalloenzymes that require metal ions (mainly calcium ions) for their activity, structural integrity, and stability [3], while metal-independent α-amylases are more preferable for industrial use.

These parameters correspond to α-amylases of microbial origin, the producers of which are both thermophilic and mesophilic microorganisms [7]. Well known

commercial strains of microorganisms for the production of α-amylase are *Bacillus subtilis, B. licheniformis, B. amyloliquefaciens,* and *Aspergillus oryzae* [5]. However, the ever-increasing demand for various amylolytic enzymes stimulates the search for new sources of enzymes and the search for α-amylases with improved properties. Along with the search for new natural strains-producers of α-amylases, it seems promising to create genetically engineered strains-producers [6, 8].

Among the α-amylases of microbial origin, known bacterial amylases are those derived from *Bacillus subtilis* [9], *Bacillus amyloliquefaciens* [10], *Bacillus velezensis* [11], *Bacillus licheniformis* [12], *Geobacillus stearothermophilus* [13]. However, all of them do not have sufficiently high thermal stability [13, 14]. For extremophiles such as the *Archaea Pyrococcus furiosus*, it was noted that α-amylase is resistant to temperatures of 100°C [15], however, archaea are difficult to industrial cultivation, so it limits their use in industrial biotechnology.

The aim of this work is to study the temperature characteristics of α-amylase from the Kazakh strain of *Bacillus licheniformis* T5 obtained in *Escherichia coli*

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Materials and methods

Strains, vectors, culture media and reagents

The target gene was cloned using the pJET1.2/blunt plasmid cloning kit (Thermo Fisher Scientific, USA). Plasmid pET28c(+) (Novagen, UK) was used to clone the target gene. The *Escherichia coli* DH5α and ArcticExpress(DE3)RP strains were obtained from Thermo Fisher Scientific (USA) and Novagen (Merck4Biosciences, France), respectively.

Media Broth Lennox (LB) Lysogenic Broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5), superoptimal broth with catabolite repression (SOC) (1% tryptone, 0.5 yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM MgSO4, 20 mM glucose, pH 7.5). For the isolation and cultivation of the *Bacillus licheniformis* T5 strain, nutrient agar produced by HiMedia (India) with the addition of 1% starch was used.

The concentration of agar in solid media was 1.5%. Restriction enzymes manufactured by Thermo Fisher Scientific (USA) were used. Taq, Pfu DNA polymerase, T4 ligase, were self-produced. Potato soluble starch (Sigma, St. Louis, USA) was used as a substrate. The chemicals used in this study were of molecular biological or analytical grade and purchased from Sigma-Aldrich (St. Louis, USA) and AppliChem (Darmstadt, Germany).

Isolation of the microorganism

The bacteria were isolated from soil collected near Taraz city. 9 ml of 0.9% NaCl was added to 1 gram of soil, the suspension was diluted 10 times with 0.9% NaCl, and 100 μL of the diluted suspension was spreaded on a Petri plate with nutrient agar. The plate was cultured at 37°C for 48

hours. The purity of the isolates was checked by Gram staining with the light microscopy. Colonies were picked and transferred to nutrient agar plates and cultured at 37° C. for 48 hours. Individual colonies were identified and screened for α-amylase activity by the starch iodine method. The strains were spread on starch agar plates containing 1 g starch, 2 g agar and 1.3 g nutrient broth. Incubation was done at 37°C for 48 hours, every plates was bathed with Gram's iodine solution to confirm starch hydrolysis.

Microorganism identification

Taxonomic analysis and morphological examination of the colonies were performed using light microscopy (Zeiss Primo Star, Germany), the characteristics of each isolate were compared with data from Bergey's Manual of Systematic Bacteriology. The strains were cultured in nutrient broth at 37°C for 24 hours. Cells were harvested by centrifugation (6000×g, 4°C, 7 min). Genomic DNA was isolated from cells using the Monarch Nucleic Acid Purification Kit (New England Biolabs, USA).

The gene encoding the small subunit of the ribosome (16S rRNA) was amplified by PCR and sequenced for identification. Amplification was performed with a universal primer pair: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). PCR (final volume 50 µL) contained 5 µL 10X Taq Buffer (Thermo Fisher), 3 µL 25 mM $MgCl_2$, 5 µL dNTP (2 mM stock solution), 1 µL primer (10 µM stock solution), 100 ng template DNA, 0.2 µL Taq DNA polymerase (5000 U/mL) and nuclease free water.

The following amplification parameters were used: initial denaturation at 95°C for 5 min; then 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; and final elongation at 72°C for 10 min.

Sequencing of target genes was performed on an ABI 3730xl genetic analyzer (Applied Biosystems, USA) using BigDye Terminator v3.1 (Applied Biosystems, USA). Analysis of chromatograms was performed using the VectorNTI version 11 software package. Sequences were compared with GenBank data using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Additionally, the microorganism was identified by the proteomic profile of ribosomal proteins using the Matrix Laser Desorption Ionization (MALDI) and time-of-flight (TOF) mass spectrometry system using BiotyperMicroflex LT equipment (Bruker Daltonics, Bremen, Germany).

Cloning of the α-amylase gene

The amylase gene was amplified from bacterial genomic DNA using PCR primers Amy_fw (5'-ATGAAACAACAAAAACGGCTTTAC-3') and Amy_rv (5'-TCTTGAACATAAATTGAAACCGA-3') and cloned into the pGET1.2/blunt vector according to the manufacturer's recommendations. PCR (final volume 50 µL) contained 25 µL 5X HF Buffer (Thermo Fisher), 3 µL 25 mM $MgCl_2$, 5 µL dNTP (2 mM stock solution), 1 µL each primer

(10 µM stock solution), 50 ng template DNA, 1 µL of Phusion polymerase (1000 U/mL) and nuclease free water. The following amplification parameters were used: initial denaturation at 98°C for 30 s; then 30 cycles of 98°C for 10 s, 55°C for 1 min and 72°C for 1 min; and final elongation at 72°C for 10 min. AmpR clones were screened by insert PCR with forward sequencing primer pJET1.2 (5'-CGACTCACTATAGGGAGAGCGGC-3') and reverse sequencing primer pJET1.2 (5'-AAGAACATCGATTTTCCATGGCAG-3'). Plasmids from 3 positive clones were extracted with the GeneJET Plasmid Miniprep Kit (Thermo Scientific) and the inserts were sequenced.

Expression of the α-amylase gene, isolation and purification of recombinant α-amylase

The amyT5 gene was amplified from pGET1.2/amyT5 with PCR primers AmyT5_NdeI (GGAATTCCATATGGCAAATCTTAATGGGACGCTG) and AmyT5_NotI (5'-TTTCCTTTTGCGGCCGCTCTTTGAACATAAATTGAAAACCG-3') and cloned into pET28c(+) at the NdeI/NotI sites, resulting in pET28/amylase plasmid vector. The insertion correction was confirmed by sequencing the T7 locus with T7_fw (5'-TAATACGACTCACTATAGGG-3') and T7_rv (5'-TAATACGACTCACTATAGGG-3'*).* *E. coli* ArcticExpress(DE3)RP cells were electroporated with pET28/amylase T5, and the resulting Kn^R transformants were cultured in 1 L of LB medium with kanamycin (50 µg/mL) at 18°C and 150 rpm. In the middle of the logarithmic growth phase (OD_{600}=0.6), isopropyl-β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and incubated for 16 hours. Cells were harvested by centrifugation at 6000×g for 7 minutes at 4°C, suspended in 20 mM Tris-HCl (pH 8.0) with 500 mM NaCl, and lysed with lysozyme (2 mg/mL) followed by sonication. The recombinant amylase was isolated from the cell lysate by metal affinity chromatography on an AKTA Purifier 10 FPLC chromatograph (General Electric, USA) using a 1-mL HiTrap Chelating column (General Electric, USA) activated with Ni^{2+} ions and previously equilibrated with 20 mM imidazole with 500 mM NaCl and 20 mM Tris-HCl (pH 8.0). The lysate was purified by centrifugation (40,000×g, 1 h, 4°C) and filtration through a 0.45 µm filter. The clarified supernatant was loaded onto the column and the amylase was eluted with 20-500 mM imidazole in 20 mM Tris-HCl (pH 8.0) with 500 mM NaCl using FPLC. Fractions were analyzed by amylase assay, and the most active fractions were subjected to SDS-PAGE. SDS-PAGE was performed according to the Laemmli method [29]. The protein concentration in the fractions was determined. The purity fractions with the highest activity were selected and used in the following experiments.

A-amylase activity assay

The determination of amylase activity was carried out by the starch iodine method based on the interaction between starch and iodine [16]. Briefly, 1 mL of 0.4% water-

soluble starch (in 100 mM phosphate buffer pH 6.5) mixed with 50 µL of the enzyme sample. The solution was incubated at 37°C for 10 min. The reaction was stopped by adding of 0.01 N iodine solution. Next, 4 mL of deionized water was added, the absorption was determined by spectrophotometry at 650 nm. The unit of α-amylase activity was determined as 1 mg of hydrolyzed starch per 1 minute at 37°C.

Electrophoresis and determination of protein concentration

The protein concentration was determined by the Bradford method [17] using bovine serum albumin as a standard. 100 µl of Bradford's reagent (Protein Assay Dye; Bio-Rad, Munich, Germany) was added to 860 µL of 10% PBS with 1% glycerol and vortexed and 40 µL of sample was added. The mixture was incubated for 2 min at room temperature and the optical density was measured on a spectrophotometer at 595 nm. The measurements were carried out in three independent repetitions and the average was taken into account.

Determination of the dependence of α-amylase activity on pH

Amylase activity was measured in the pH range from 2.5 to 11.5 (with half-unit interval) at 37°C. Maximum enzymatic activity was regarded as 100% activity, and the other samples at different pH were assayed for relative activity accordingly. The following buffer systems were used: glycine-HCl (pH 2.0–3.5), acetate buffer (pH 4.0–5.5), phosphate buffer (pH 6, 0–8.5) and glycine-NaOH (pH 9.0-11.5).

Determination of the dependence of α-amylase activity on temperature

Amylase activity was measured in the temperature range of 37–80°C (with 10°C interval) in 100 mM phosphate buffer (pH 6.5). Maximum enzymatic activity was regarded as 100% activity, and the other samples at different temperatures were assayed for relative activity accordingly

Determination of temperature stability

To determine the effect of temperature on enzyme stability, recombinant α-amylase was incubated at 70°C and 80°C in 100 mM phosphate buffer pH 6.5 for 120 minutes. Every 30 minutes, α-amylase activity was measured at 37°C. Initial activity was set to 100%, and residual activity was calculated relative to the initial activity.

Software and statistical analysis

All measurements were carried out independently three times. Means and standard deviations were calculated using GraphPad Prism, version 8.0.1. Enzymatic activity is presented as mean value and other parameters as mean value ± standard deviation (n = 3). Calculation of the molecular weight and isoelectric point of the protein, sequencing, primer design, and other manipulations were performed using the Vector NTI Advance 11 and SnapGene Viewer 5.2.4 programs. Nucleotide and protein sequences were compared to the NCBI nucleotide/protein database using the online programs BLASTN and BLASTP, respectively. Peptide Signal IP 5.0 online software (http://www.cbs.dtu.dk/services/Signa

lP/) was used to predict the signal peptide region.

Results

Based on the sequence of the conserved 16S rRNA locus (99.9% identity) and MALDI-TOF Biotyper data (score 2.106), the T5 isolate isolated from the soil was identified as *Bacillus licheniformis*.

Screening showed that only the *Bacillus licheniformis* T5 strain has α-amylase activity. *Bacillus licheniformis* T5 displayed α-amylase activity by forming a clear zone with an average diameter of 2-3 mm around the colonies (Figure 1a).

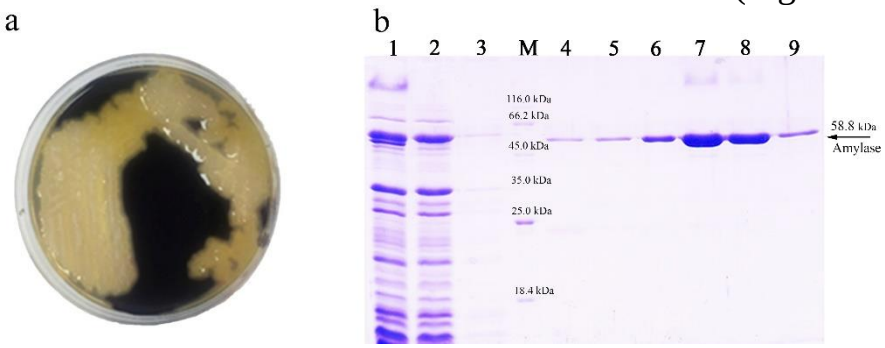

Figure 1 - Amylase activity of *Bacillus licheniformis* T5 strain in starch iodine test (a) and results of chromatographic purification of recombinant α-amylase (b): water-soluble lysate before the column (lane 1), water-soluble lysate after the column (lane 2), fractions (lanes 4-9), protein marker (lane M)

Oligonucleotides were selected and α-amylase gene was successfully amplified from the genomic DNA of the *Bacillus licheniformis* T5 strain. Sequencing of the α-amylase gene and analysis using the Peptide Signal IP 5.0 program showed that the first 29 amino acid residues encode a secretory peptide (MKQQKRLYARLLTLLFFALIFLL PHSAAAA), which is used by the bacterium to transport the enzyme into the medium.

The gene starting from G^{88} was amplified and cloned into the pET-28c(+) plasmid vector. In this vector, the α-amylase gene was inserted under T7 promoter. The open reading frame contains the protein with 515 amino acid residues. At the amide and carboxyl ends, the recombinant protein contains a hexahistidine tag (6His) fused to α-amylase.

The calculated mass of the recombinant protein is 58.8 kDa. Transformation of competent *Escherichia coli* cells of the ArcticEspress(DE3)RP strain with this vector resulted in a strain-producer of recombinant α-amylase. Recombinant α-amylase was successfully purified from the induced cell lysate of the strain by metal affinity chromatography using a HiTrap Chelating HP 1mL (GE) column with Ni^{2+} ions (Figure 1b). Recombinant α-amylase was eluted from the column with 150 mM imidazole. Fractions 6,7,8 were pooled and dialyzed. The concentration of purified recombinant α-amylase was 0.5 mg/mL. The yield of purified recombinant α-amylase is 1.5 mg per 500 mL. The activity of recombinant α-amylase under the

standard conditions (pH 6.5 and 37°C) was 293.3 U/mg.

The study of the relative dependence of the α-amylase activity of the recombinant enzyme on pH showed that the enzyme is active at a level of 80% of the maximum value in the pH range of 4.5-7.5 with a maximum at 6.5 (Figure 2a). In the range from 8.0 to 9.5, the activity decreases from 76.8% to 22.6%.

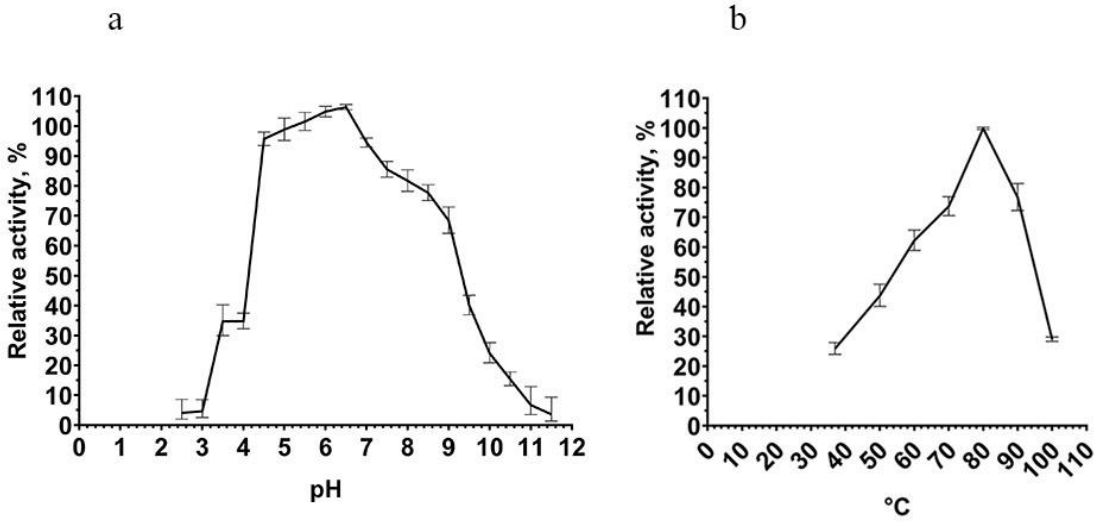

Figure 2 – The influence of pH (a) and temperature on the activity of recombinant α-amylase

At pH values of 2.5 and 11.5, the activity of α-amylase is 4.1% and 3.4%, respectively.

The study of the dependence of the activity from temperature of recombinant α-amylase showed that the enzyme is active at high temperatures (Figure 2b). The activity of α-amylase begins to exceed 60% only at a temperature of 60°C and reaches a maximum at 80°C. At 90°C, α-amylase shows 80% activity, and at 100°C, the activity is 29% of the maximum. It seems interesting to study the temperature stability of recombinant α-amylase. The residual activity of the enzyme after incubation of recombinant α-amylase at 70°C and 80°C was studied. 120 min incubation of the enzyme at 70°C had little effect on its activity, residual activity was 80% of the initial activity. When the enzyme was incubated at 80°C for 30 min, the activity was 70.5% and after 60 min - 56%. After 120 min of incubation, the residual activity was 34.5%.

The effect of Ca^{2+} ions on the activity of recombinant α-amylase was studied. It was found that the addition of $CaCl_2$ at a concentration of 5-10 mM does not affect the activity of the enzyme, which indicates that α-amylase is a calcium-independent enzyme.

Discussion

Sequence analysis of α-amylase from *Bacillus licheniformis* T5 shows that the enzyme is secreted by the bacterium and the strain produces it in the presence of appropriate carbon sources - starch [4]. This phenomenon is typical for evolutionarily advanced microorganisms such as *Bacillus*,

which have the developed enzymatic system [2].

In general, it can be concluded that α-amylase from *Bacillus licheniformis* T5 is a neutral and alkaline enzyme. The study of the dependence of the activity from temperature indicate that the α-amylase from *Bacillus licheniformis* T5 is a high-temperature and thermostable enzyme. Comparison of the results with other enzymes shows that, α-amylase from *Bacillus licheniformis* T5 has advantages over α-amylases from other *Bacillus* species. Thus, α-amylase from *Bacillus alcalophilus* has an optimum of 50°C [18]. In *Bacillus subtilis*, the optimum is slightly higher, 55°C, but the enzyme also loses activity at 80–90°C [14]. Amylase from *Bacillus velezensis* also does not have high thermal stability, its optimum is no more than 55°C [11], as well as α-amylase from *Geobacillus stearothemophilus* [13]. The amylolytic *Bacillus amyloliquefaciens* strain produces a more thermostable enzyme, which has an optimum at 60°C, which is also a calcium independent α-amylase [19], but it is still significantly lower than that of α-amylase from *Bacillus licheniformis* T5. The results obtained allow us to consider α-amylase as a promising enzyme with a good potential for application in starch conversion technologies.

Conclusion

A strain with amylolytic activity was isolated from the soil of South Kazakhstan by morphological, molecular genetic and proteomic characteristics, was identified as *Bacillus licheniformis* T5. The α-amylase gene was cloned and the recombinant strain was obtained. The recombinant α-amylase was purified and the biochemical characteristics were studied. The α-amylase has good thermal stability and does not require the addition of calcium ions as a coenzyme. The results indicate to the prospects of using this α-amylase from *Bacillus licheniformis* T5 as an amylolytic enzyme in technologies for processing starch-containing raw materials.

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BACILLUS LICHENIFORMIS Т5 ШТАММЫНЫҢ А-АМИЛАЗАСЫНЫҢ БИОХИМИЯЛЫҚ ҚАСИЕТТЕРІН ЗЕРТТЕУ, КЛОНДАУ ЖӘНЕ ТАЗАЛАУ

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Түйін

Амилазалар заманауи биотехнологияның маңызды ферменттерінің бірі болып табылады. Альфа-амилазалар крахмалды глюкоза мен мальтозаға дейін ыдыратып, құрамында крахмалы бар шикізаттың түрленуіне қатысады. Эукариотты организмдердің α-амилазаларымен салыстырғанда бактериялық α-амилазалардың бірқатар артықшылықтары бар. Оның бірі крахмалдың ферменттік гидролизі технологияларында маңызы зор болып келетін температураның жоғарғы көрсеткіштерінде ферменттік белсенділікті сақтау қабілеті.

Бұл жұмыста *Bacillus licheniformis* T5 штамының α-амилазы гені pET-28c(+) векторында көбейтілді. *Escherichia coli* клеткаларының ArcticExpressR P(DE3) штамында плазмидалы экспрессия жолымен рекомбинантты α-амилаза алынды. Зерттеулер рекомбинантты α-амилаза үшін pH пен температураның оңтайлы көрсеткіштері 6,5 және 80°C, сәйкесінше, екенін көрсетті. α-амилаза кальцийдан тәуелсіз фермент екені анықталды. α-амилазаның температуралық тұрақтылығын зерттеу жұмыстары фермент 80°C-та 30 минут бойы инкубацияланғаннан кейін де өз белсенділігінің 70%-ын сақтайтынын көрсетті. Алынған нәтижелер *Bacillus licheniformis* T5-тің α-амилазасын крахмал мен құрамында крахмалы бар шикізаттың ферменттік ыдырауы технологияларында термотұрақты фермент ретінде қолдануының жоғары болашағын көрсетеді.

Кілт сөздер: α-амилаза; *Bacillus licheniformis*; рекомбинантты фермент; плазмида; крахмал; гидролиз; амилаза белсенділігі.

КЛОНИРОВАНИЕ, ОЧИСТКА И ИЗУЧЕНИЕ БИОХИМИЧЕСКИХ ХАРАКТЕРИСТИК А-АМИЛАЗЫ ИЗ ШТАММА *BACILLUS LICHENIFORMIS* T5

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Аннотация

Амилазы являются одними из важных ферментов в современной биотехнологии. Альфа-амилазы гидролизуют крахмал до образования глюкозы и мальтозы и участвуют в конверсии крахмалсодержащего сырья. В сравнении с α-амилазами эукариотических организмов бактериальные α-амилазы имеют ряд преимуществ, заключающиеся в сохранении ферментативной активности при высоких значениях температуры, что важно в технологиях ферментативного гидролиза крахмала. В данной работе ген α-амилазы из казахстанского штамма *Bacillus licheniformis* T5 был клонирован в векторе pET-28c(+). Путем плазмидной экспрессии в клетках *Escherichia coli* штамма ArcticExpressRP(DE3) была получена рекомбинантная α-амилаза. Исследования показали, что pH и температурный оптимум для рекомбинантной α-амилазы составил 6,5 и 80°C, соответственно. Установлено, что α-амилаза является кальций-независимым ферментом. Изучение температурной стабильности α-амилазы показало, что фермент сохраняет более 70% активности при инкубации α-амилазы при 80°C в течение 30 минут. Полученные результаты показывают высокую перспективу применения α-амилазы из *Bacillus licheniformis* T5 в качестве термостабильного фермента в технологиях ферментативного гидролиза крахмала и крахмалсодержащего сырья.

Ключевые слова: α-амилаза; *Bacillus licheniformis*; рекомбинантный фермент; плазмида; крахмал; гидролиз; амилазная активность.