Kholikov Askar Farhod ugli
PhD Student, Institute of Microbiology,
Academy of Sciences of the Republic of Uzbekistan
Email: asqarxoliqov31@gmail.com
Alimova Barno Khasanovna
Senior Researcher, DSc Institute of Microbiology,
Academy of Sciences of the Republic of Uzbekistan
Pulatova Ozodakhon Mansurovna
Senior Researcher, Institute of Microbiology,
Academy of Sciences of the Republic of Uzbekistan
Makhsumkhanov Akhmadzhan Azamkhanovich
Head of the Enzymology Laboratory, Senior Researcher, PhD
Institute of Microbiology,
Academy of Sciences of the Republic of Uzbekistan
Email: amakhsum@mail.ru
UDC 579.222+577.21

CLONING OF THE THERMOSTABLE α-AMYLASE GENE

Abstract: *This article presents the results of the determination of bacteria of the genus Bacillus, stored in the Laboratory of Enzymology at the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan in order to identify the genes responsible for the synthesis of thermostable α-amylase, which are cloned and expressed in the E. coli system. Preliminary results showed that α-amylases of culture fluid obtained from Bacillus spp.-amyR and Bacillus spp.-104.K isolates demonstrated the highest activity and stability at high temperatures. The Bacillus spp.-amyR isolate showed enzyme activity of 0.96 U/ml at 70 °C, while the Bacillus spp.-104.K isolate showed 0.37 U/ml at 90 °C. Both isolates were identified by the 16S rRNA gene sequence and registered in the NCBI database. Along with these, the corresponding primers for α-amylase genes were designed, amplified by PCR, successfully cloned to the pUC18 cloning vector, and gene expression in host cells on starch-containing Petri dishes was shown.*

Keywords: *Thermostable α-amylase, enzyme activity, Bacillus velezensis, Bacillus licheniformis, 16S rDNA, cloning, PCR amplification, vector, expression.*

Xoliqov Asqar Farhod o’g’li
O‘zR FA Mikrobiologiya instituti tayanch doktoranti.
Alimova Barno Xasanovna
O‘zR FA Mikrobiologiya instituti katta ilmiy xodimi, DSc.
Pulatova Ozodaxon Mansurovna
O‘zR FA Mikrobiologiya instituti katta ilmiy xodimi, b.f.n.
Maxsumxanov Axmadjan A’zamxanovich
O‘zR FA Mikrobiologiya instituti Enzimologiya laboratoriyasi mudiri v.b.,
katta ilmiy xodim, b.f.n.

TERMOSTABIL α-AMILAZA GENINI KLONLASH

Annotatsiya: *Ushbu maqolada O‘zR Fanlar akademiyasi Mikrobiologiya instituti Enzimologiya laboratoriyasida saqlanayotgan Bacillus avlodiga mansub bakteriya izolyatlarining termostabil α-amilaza fermentini sintez qiluvchi genlarini aniqlash, klonlash va E. coli ekspression tizimida ekspressiya qilish bo‘yicha o‘tkazilgan tadqiqotlar natijalari yoritilgan. Dastlabki natijalar shuni ko‘rsatdiki, Bacillus spp.-amyR va Bacillus spp.-104.K izolyatlaridan olingan kultural suyuqlik α-amilaza fermentlari eng yuqori faollikni yuqori haroratlarda namoyish etgan. Bacillus spp.-amyR izolyatining α-amilaza fermenti 70 °C da yuqorti 0.96 Bir./ml faollikni, Bacillus spp.-104.K izolyatida esa 90 °C da 0.37 Bir./ml ni tashkil etdi. Har ikkala izolyat 16S rDNK geni asosida identifikatsiya qilindi va NCBI bazasiga joylandi. Shu bilan birga ularning α-amilaza fermentlari genlariga mos praymerlar tuzildi, PZR-usulda amplifikatsiya qilindi, pUC18 klonlash vektoriga muvaffaqiyatli klonlangan va kraxmalli Petri idishlarida xo‘jayin hujayralarida gen ekspressiyasi ko‘rsatilgan.*

Kalit so‘zlar: *Termostabil α-amilaza, ferment faolligi, Bacillus velezensis, Bacillus licheniformis, 16S rDNK, klonlash, PZR-amplifikatsiya, vector, ekspressiya.*

Холиков Аскар Фарход угли
Аспирант Института микробиологии Академии наук Республики Узбекистан.
Алимова Барно Хасановна
Старший научный сотрудник Института микробиологии АН РУз, DSc.
Пулатова Озодахон Мансуровна
Старший научный сотрудник Института микробиологии АН РУз, кандидат биологических наук.

Махсумханов Ахмаджан Аъзамханович
И.о. заведующего лабораторией энзимологии Института микробиологии АН РУз, старший научный сотрудник, кандидат биологических наук.

КЛОНИРОВАНИЕ ГЕНА ТЕРМОСТАБИЛЬНОЙ α-АМИЛАЗЫ

Аннотация: *В данной статье представлены результаты определения бактерий рода Bacillus, хранящиеся в лаборатории Энзимологии Института микробиологии Академии наук РУз с целью выявления генов, ответственных за синтез термостабильной α-амилазы, которые клонированы и экспрессированы в системе E. coli. Предварительные результаты показали, что α-амилазы культуральной жидкости, полученные из изолятов Bacillus spp.-amyR и Bacillus spp.-104.K, продемонстрировали наивысшую активность и устойчивость при высоких температурах. Изолят Bacillus spp.-amyR показал активность фермента 0,96 Ед/мл при 70 °C, тогда как изолят Bacillus spp.-104.K – 0,37 Ед/мл при 90 °C. Оба изолята были идентифицированы по секвенсу гена 16S рРНК и зарегистрированы в базе данных NCBI. Наряду с этими, сконструированы соответствующие праймеры к генам α-амилазы, амплифицированы методом ПЦР, успешно клонированы к вектору клонирования pUC18 и показана экспрессия гена в клетках хозяина на крахмалсодержащих чашках Петри.*

Ключевые слова: *Термостабильная α-амилаза, активность фермента, Bacillus velezensis, Bacillus licheniformis, 16S рРНК, клонирование, ПЦР-амплификация, вектор, экспрессия.*

Introduction

The recombinant production of thermostable α-amylase enzymes holds significant importance for both industrial applications and scientific research. Through recombinant production technologies, the structure of the enzyme can be altered, improved, or adapted to specific conditions. These technologies allow for the cloning and expression of genes from various bacterial sources, enabling the mass production of enzymes. Utilizing the local *Bacillus sp.* strains in Uzbekistan for the recombinant production of thermostable α-amylase would facilitate the efficient use of national resources and foster competitive research on an international scale.

Amylases are enzymes that hydrolyze starch by cleaving α-1,4-glycosidic bonds to produce smaller molecular weight products, including glucose and maltose. Due to their catalytic properties, scalability, and stability at high temperatures, microbial amylases are preferred over plant and animal derived amylases for industrial applications [1]. Among bacteria, thermostable α-amylases derived from species of the Bacillus genus are widely used in industry [2]. Common bacterial α-amylases originate from *Bacillus licheniformis* [3-6], *Bacillus amyloliquefaciens* [7-9], *Bacillus subtilis* [10-12], and *Bacillus velezensis* [13-15].

Amylases are extensively employed in various commercial sectors, including food production, detergents, alcohol, textiles, and paper industries. Additionally, they are utilized in pharmaceuticals, environmental remediation, biofuel production, and other biotechnological processes [16, 17]. Amylases can be categorized into two main types: exo-amylases and endo-amylases. Exo-amylases hydrolyze starch molecules to release small sugar units, while endo-amylases cleave internal glycosidic bonds within the starch molecules, breaking them into smaller fragments. Both types play crucial roles in the starch hydrolysis process [18, 19]. Over the past decade, several research groups have successfully isolated amylase-producing bacteria from soil, cloned their genes into suitable plasmids, and expressed them in *E. coli* cells [20, 21].

The aim of this study was to screen thermostable α-amylase producers, identify bacterial strains exhibiting the highest activity and thermostability, and clone their amylase genes.

Materials and methods

To identify bacterial isolates, universal primers used for the 16S rDNA gene amplification, including 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), 533F (5′-GCCAGCAGCCGCGGTAA-3′), and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). Genomic DNA was extracted from bacterial strains using the Marmur method [22].

α-Amylase activity was determined using a modified version of the 3,5-dinitrosalicylic acid (DNS) method [23]. Activity assays were performed at temperatures ranging from 30 °C to 100 °C in a reaction mixture of 0.6 mL containing 0.54 mL of 0.5% (w/v) potato starch solution in 100 mM potassium phosphate buffer (pH 7.0) and 0.06 mL of enzyme solution. The reaction mixture was incubated for 10 minutes at the desired temperature in a water bath, followed by the addition of 1.8 mL of DNS reagent to terminate the reaction. The mixture was boiled for 10 minutes, cooled to room temperature, and absorbance was measured at 540 nm.

Primer design and cloning simulations were performed using SnapGene software [24].

Primer Name	Primer Sequence (5′ → 3′)	Restriction Enzyme
AmyF_B. lich	CCAGGATCCATGAAACAACAAAAACGG	BamHI
AmyR_B. lich	CCACATATGGCTCTTCTATCTTTGAACAT	NdeI
AmyF.1_B. vel	CCAGGATCCATGTTTGAAAAACGATTCAA	BamHI
AmyR.1_B. vel	CCACATATGCCATTAATGCGGAAGATAAC	NdeI
AmyF.2_B. vel	CCAGGATCCATGATTCAAAAACGAAAGCG	BamHI
AmyF.2_B. vel	CCACATATGCCTTATTTCTGAACATAAATGG	NdeI

Table 1. Primers used in the study for amplification of *amyL* and *amyV* genes and their associated restriction enzymes.

Results and discussion

Initial screening for thermostable α-amylase producers identified two bacterial isolates with the highest enzymatic activity and thermostability [25]. The amyR isolate displayed an enzyme activity of 0.96 U/ml at 70°C, whereas the 104.K isolate demonstrated an activity of 0.37 U/ml at 90°C (Figure 1). Based on 16S rDNA

sequencing, these isolates were classified as *Bacillus licheniformis* (GenBank: OR781719.1) and *Bacillus velezensis* (GenBank: OR781816.1).

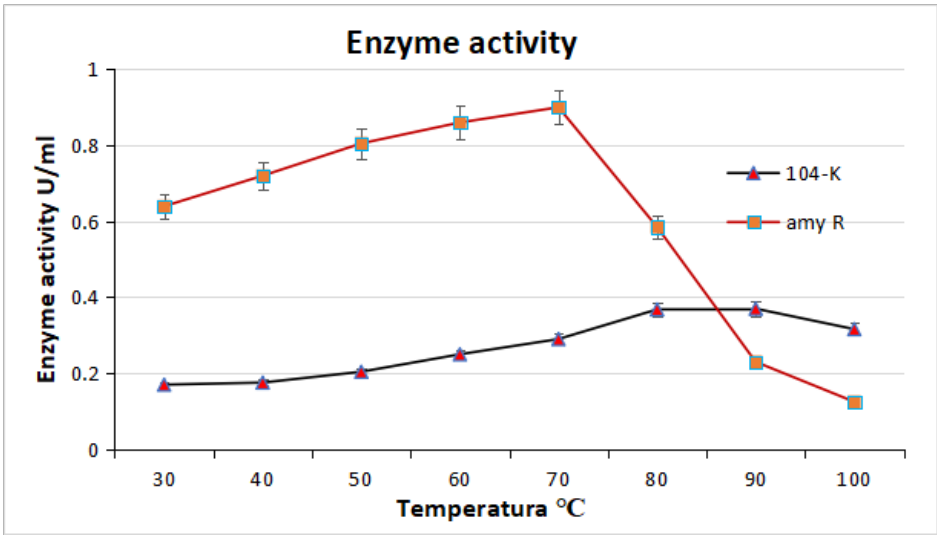

Figure 1. Amylase activity of *Bacillus licheniformis* 104.K and *Bacillus velezensis* amyR strains. The enzyme activity was measured using crude enzyme extracts obtained from the respective strains.

Specific primers targeting the amylase genes were designed based on the genomic DNA sequences of the identified strains (Table 1). PCR amplification yielded *amyL* (1539 bp) and *amyV* (1980 bp) fragments from the bacterial genomic DNA (Figure 2A). These fragments were digested with *BamHI* and *NdeI* restriction enzymes according to the manufacturer's protocol and ligated into the similarly prepared pUC18 vector. The ligation mixtures were then transformed into *E. coli* DH5α competent cells, and transformants were cultured on ampicillin-selective plates. Plasmids isolated from positive colonies were analyzed by double digestion to confirm the successful insertion of *amyL* and *amyV* genes (Figure 2B). The results verified the successful cloning of *amyL* and *amyV* into the pUC18 vector (Figure 3).

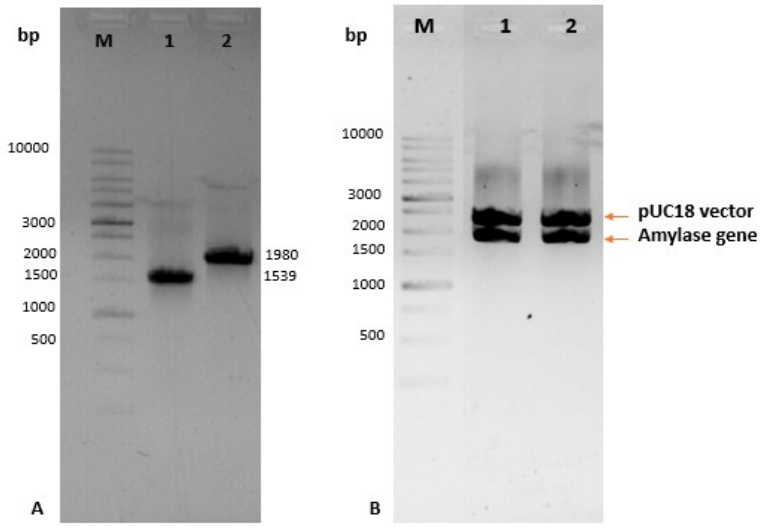

Figure 2. A) Amplification of *amyL* and *amyV* genes from the genomic DNA of *Bacillus licheniformis* 104.K and *Bacillus velezensis* amyR. The amplified *amyL* (1) and *amyV* (2) gene fragments are visualized on a 1% agarose gel. M: 1 kb DNA marker. **B)** Confirmation of the successful cloning of *amyL* and *amyV* genes into the pUC18 vector. The recombinant plasmids containing *amyL* (1) and *amyV* (2) genes were double-digested with *BamHI* and *NdeI*, and the digested fragments were analyzed on a 0.8% agarose gel. M: 1 kb DNA marker.

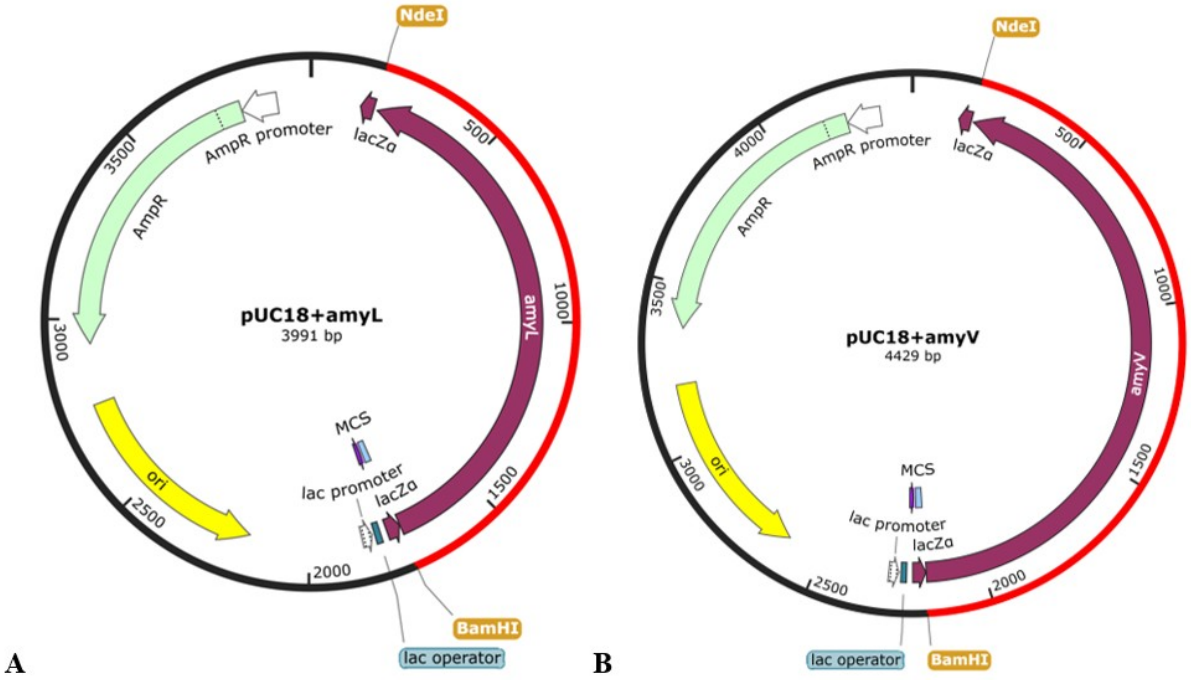

Figure 3. A) Map of the recombinant plasmid containing the *amyL* gene inserted into the pUC18 vector. **B)** Map of the recombinant plasmid containing the *amyV* gene inserted into the pUC18 vector. Plasmid maps were generated using SnapGene software.

The amylase gene from the *B. licheniformis* 104.K strain is 1539 bp in length, a result that is consistent with the findings of Niu et al. [26]. The amplicon of the amylase gene from the *B. velezensis* amyR strain is 1980 bp, which aligns with the results reported by Zhang et al. [27]. Recombinant α-amylase activity detected by iodine solution method on starch containing agar plate (Figure 4).

Figure 4. This image shows the amylase activity screening of *E. coli* DH5α transformants carrying the pUC18+amyV construct on LB agar supplemented with 1% soluble starch. After 24 hours of incubation at 37 °C, the plate was flooded with Lugol's iodine solution to visualize starch hydrolysis.

Conclusion

This study successfully identified *Bacillus licheniformis* 104.K and *Bacillus velezensis* amyR strains as producers of thermostable α-amylase and designed primers specific to their amylase genes. The *amyL* and *amyV* genes were amplified and cloned

into the pUC18 vector. Future studies will focus on transferring these genes into expression vectors and characterizing the properties of the recombinant enzymes.

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