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EXTRACELLULAR EXPRESSION OF THE ALPHA-AMYLASE GENE FROM BACILLUS AMYLOLIQUEFACIENS MDC1974 STRAIN USING BACILLUS SUBTILIS RIK1285 CELLS

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Abstract

The alpha-amylase gene from *Bacillus amyloliquefaciens* MDC1974 strain was molecularly cloned into the *E. coli*/*B. subtilis* pBE-S shuttle vector and subsequently expressed extracellularly by the recipient strain *Bacillus subtilis* RIK1285, which exhibited low protease activity. As a result of optimizing fermentation conditions, a secretion of alpha-amylase with an activity of 1400 units per mL was achieved.

Keywords: *Bacillus amyloliquefaciens, α-amylase, extracellular expression, E. coli/B. subtilis shuttle vector*

Introduction

α -Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that hydrolyze the internal α -1,4-O-glycosidic bonds of polysaccharides preserving the α -anomeric configuration of the products. Most α -amylases are metalloenzymes that require calcium (Ca^{2+}) ions for their activity, structural integrity, and stability. They belong to the glycoside hydrolase enzyme family 13 (GH-13) based on amino acid sequence similarity [1]. Amylases are one of the most important industrial enzymes, which are widely used from the conversion of starch into sugar syrups to the production of cyclodextran for the pharmaceutical industry. They account for 30% of the world production of enzymes [2].

The α-amylase GH-13 family is classified within the GH-H clan of glycoside hydrolases, which also encompasses the GH-70 and GH-77 families. This clan constitutes the largest family of transferases and isomerases, represented by more than 30 different enzyme

forms [3-5]. These enzymes include endoamylases, exoamylases, debranching enzymes, and transferases [5].

In numerous studies, conservative sites of the primary structure of α-amylases, as well as their domain and spatial structures, and catalytic mechanisms, have been extensively investigated [6].

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α-Amylases are ubiquitous enzymes produced by plants, animals, and microbes, where they play a significant role in carbohydrate metabolism. Amylases derived from plants and microbes have been utilized as food additives for centuries. For instance, barley amylases are crucial in beer production, while fungal amylases are commonly employed in various food processing applications. Despite their wide distribution, microbial-origin amylases, particularly from bacteria and fungi (such as *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, and *B. amyloliquefaciens*), are predominantly used in industry due to their cost-effectiveness, ease of production, and adaptability to modification and optimization of the production processes [4, 7-8].

In the application processes of α-amylases, determining the optimal temperature and pH for their activity, as well as assessing their heat stability, are crucial factors. Based on these parameters, the search for and characterization of new α-amylases represent ongoing biotechnological challenges [4, 9-10].

The impact of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, and other metal ions on the activity of α-amylases has been investigated in numerous studies [4, 8].

The substrate specificities of amylases for soluble starch, amylose, amylopectin, glycogen, maltodextrin, cyclodextrins, and other substrates have been investigated in many modern studies [11-12]. The quest for new α-amylases capable of hydrolyzing raw starch (in technologies utilizing them, the energy-intensive stage of starch gelatinization is bypassed) is one of the pressing issues in modern biotechnology [13-15].

Conflict Setting

The demand for α-amylases with diverse physiological and biochemical characteristics across different industries drives the purposeful search for enzymes with novel attributes using recombinant technologies [16-19] and enzyme engineering [20-21]. For instance, through point mutagenesis involving the deletion of amino acids arginine 179 and glycine 180 of the α-amylase from *B. stearothermophilus*, a recombinant enzyme with enhanced properties (such as increased heat resistance, capacity to function at low pH, and reduced calcium dependency) was successfully generated and characterized [22]. Furthermore, by optimizing the signal peptide of the α-amylase from *B. stearothermophilus* and enhancing the expression of the corresponding chaperone, it became feasible to achieve the overexpression of the secreted enzyme (at a level of 9200 units/ml) [23].

Therefore, the acquisition and characterization of recombinant alpha-amylase-producing strains and enzyme variants with novel, enhanced attributes are currently significant issues. These enzymes find widespread use in the processing of starch-containing raw materials, and ongoing efforts in this direction are essential to meet the demands of biotechnological industries.

The objective of this study is to molecularly clone the α-amylase gene of the *B. amyloliquefaciens* MDC1974 strain into the *E. coli*/*B. subtilis* pBE-S shuttle vector, express the recombinant enzyme extracellularly, and characterize it. Previously, our working group has achieved some success in researching this enzyme. Specifically, we were able to amplify the amylase gene from the complete bacterial genome (selected based on the analogy of the 16S ribosomal RNA gene of both our and host strains) and characterize the corresponding enzyme [24]. Additionally, a phylogenetic analysis of the *B. amyloliquefaciens* MDC1974

strain was conducted based on the 16S ribosomal gene sequence, and the enzyme was further characterized in detail [25].

Materials and Methods

The source of the α-amylase gene used in this study was B. amyloliquefaciens MDC1974 strain, which was provided by the microbial depository center of SPC "Armbiotechnology". The B. subtilis RIK 1285 strain, known for its low protease activity, was utilized as the host for expression vector. The E. coli Top10 strain from Invitrogen was employed for the initial transformation and propagation of the expression vector. The pBE-S shuttle vector from Takara Bio served as the vector for cloning and extracellular expression of the recombinant α-amylase gene.

Bacterial strains were stored either on meat peptone broth agar plates at 4°C or in meat peptone broth supplemented with 50% (v/v) glycerol at -45°C. *E. coli* or *Bacillus* strains were cultured on a Sanyo rotary shaker at 150 rpm, maintained at 37°C for *E. coli* and 33°C for *Bacillus*, respectively, in Luria-Bertani (LB) medium (composed of 1% peptone, 0.5% yeast extract, and 0.5% NaCl). When necessary, LB medium was supplemented with ampicillin at 100 μg/ml for *E. coli* or kanamycin at 10 μg/ml for *B. subtilis*. Overnight bacterial cultures were also grown in LB medium for DNA isolation purposes.

DNA from the *B. amyloliquefaciens* strain MDC1974 was purified using the Monarch Genomic DNA Purification Kit following the manufacturer's instructions. For the Gibson assembly method, the following primer pairs were utilized to clone variants of the target alpha-amylase gene, with and without the signal peptide, into the shuttle pBE-S vector:

For the variant with the signal peptide:

- Forward primer: amy1974_pBE-S_NdeI_F GCCGGTGCACATatgattcaaaaacgaaagcggacag
- Reverse primer: amy1974_pBE-S_XbaI_R ATGGTGATGTCTAGAttatttctgaacataaatggagacggacc

For the variant without the signal peptide:

- Forward primer: amy1974_pBE-S_NdeI_sig_F GCCGGTGCACATATGgtaaatggcacgctgatgcagta
- Reverse primer: amy1974_pBE-S_XbaI_R ATGGTGATGTCTAGAttatttctgaacataaatggagacggacc

PCR amplification of the alpha-amylase gene was conducted under the following conditions: Initial denaturation: 95°C for 2 min; Cycling: 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 2 min; Final extension: 72°C for 5 min.

DNA electrophoresis was conducted using a 0.8% agarose gel (Agarose I™, VWR® tablets) in 40 mM Tris-Acetate-EDTA buffer, pH 8.0, with the gel run at 100 volts for 35 minutes. DNA bands were visualized using "Millipore" GelRed® nucleic acid stain. NEB's TriDye™ 1 kb Plus DNA ladder was employed as a reference for agarose gel sizing.

Cloning was conducted using the Gibson ligation method [26]. Initially, the pBE-S vector was double-digested with NdeI and XhoI restriction enzymes following the manufacturer's instructions provided by NEB (New England Biolabs). In the subsequent step, cells of *E. coli* Top 10 were transformed using the heat shock method with pBE-S_amy1974sig and pBE-S_amy1974 vectors synthesized (directly from the cloning reaction mixtures). Transformed colonies were selected from colonies growing on ampicillin in LB medium using the colony PCR method. Plasmids were isolated using the QIAprep Spin

Miniprep kit from QIAGEN following the manufacturer's instructions and stored at -20°C until further use. (Link to the QIAprep Spin Miniprep kit: https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/plasmid-dna/qiaprep-spin-miniprep-kit)

B. subtilis RIK 1285 strain cells were transformed using the obtained pBE-S_amy1974sig and pBE-S_amy1974 vectors, resulting in the generation of B. subtilis RIK 1285_amy1974sig and B. subtilis RIK 1285_amy1974 strains. Transformations were conducted following the manufacturer's instructions (https://www.takarabio.com/documents/User%20Manual/3380/3380_UM.pdf) provided by "Takara Bio," with certain modifications. The method involves inducing bacterial cells to enter the stage of sporulation through starvation, which coincides with the stage of competence for cell transformation [27].

The selection of fermentation media for recombinant amylase strains was made from 5 fermentation media with the same synthetic composition but different organic nitrogen sources. The synthetic composition of the environments had the following percentage composition: glucose: 1, starch: 1, $(NH_4)_2SO_4$: 0.3, NH_4Cl: 0.2, KH_2PO_4: 0.5, $MgSO_4$: 0.025. The media also contained 40 mg/l of L-lysine and L-tryptophan and 10 mg/l of kanamycin. In addition to the above composition, the T1 medium contained 1% peptone and corn extract each, T5 medium - 1% peptone and yeast extract, T6 medium - 1% tryptone and yeast extract, T7 medium - 1% soybean meal and yeast extract, and T8 medium contained 2 percent yeast autolysate. In the experiments studying the effect of the presence of own signal peptide and the intensity of aeration on amylase output, T9 medium was used, which differed from T8 in that glucose was replaced by sucrose (in order to avoid possible catabolic repression). And in experiments studying the effect of kanamycin on amylase output, T9 medium without starch was used. An overnight culture was grown in LB medium at 33°C on a rotary shaker (140 rpm). The overnight culture (seed material) was introduced into the fermentation medium in a volume of 5%. The fermentation was provided in cotton-stoppered 500 mL wide-mouthed flat-bottomed flasks containing 20 mL each of growth medium (33°C, 220 rpm).

Enzyme activity secreted in the culture fluid was determined by estimating the number of reducing groups formed during starch hydrolysis using Sumner's 3,5-dinitrosalicylic acid (DNS) reagent method [28] with Miller's modification [29]. The reaction medium in the final volume (0.2 mL) contained 1% (w/v) starch, 50 mM acetate buffer at pH 6.0, 1 mM $CaCl_2$, and the required amount of enzyme preparation. After 10 minutes of incubation at 55°C, 1 mL of DNS reagent was added, followed by 15 minutes of incubation in a boiling water bath. Subsequently, 1 mL of 20% Segnet salt solution (sodium-potassium tartaric acid salt) was added. The optical absorption of the solutions was measured at a wavelength of 546 nm.

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of reducing groups in one minute under the specified conditions. The amount of protein was estimated using the method of Groves and Davis [30].

Research Results

The pBE-S shuttle vector utilized in this study is depicted in Fig. 1a. It encompasses the kanamycin and ampicillin resistance sites, the ColE1 and pUB origins, as well as the aprE promoter and signal peptide of the subtilisin protease.

The results of the amplification of the amy1974sig and amy1974 variants of the α-amylase gene of *B. amyloliquefaciens* MDC1974 strain are depicted in Fig. 1b. The process of cloning the amy1974 version of the same gene is illustrated in Figure 1c. Cloning of target genes into

the shuttle vector was accomplished using Gibson's assembly method. For this purpose, the quantities of the linearized vector and the amplified gene introduced into the reaction mixture were calculated: 0.03 and 0.06 picomoles per 10 µl of the reaction mixture. An alpha-amylase gene cloning reaction was then conducted using NEBuilder® HiFi DNA Assembly Master Mix at 50°C according to the manufacturer's instructions provided by NEB. The manufacturer's recommended 15-minute incubation was extended to 40 minutes. As a result, pBE-S_amy1974sig and pBE-S_amy1974 vectors with and without the signal site, respectively, were obtained.

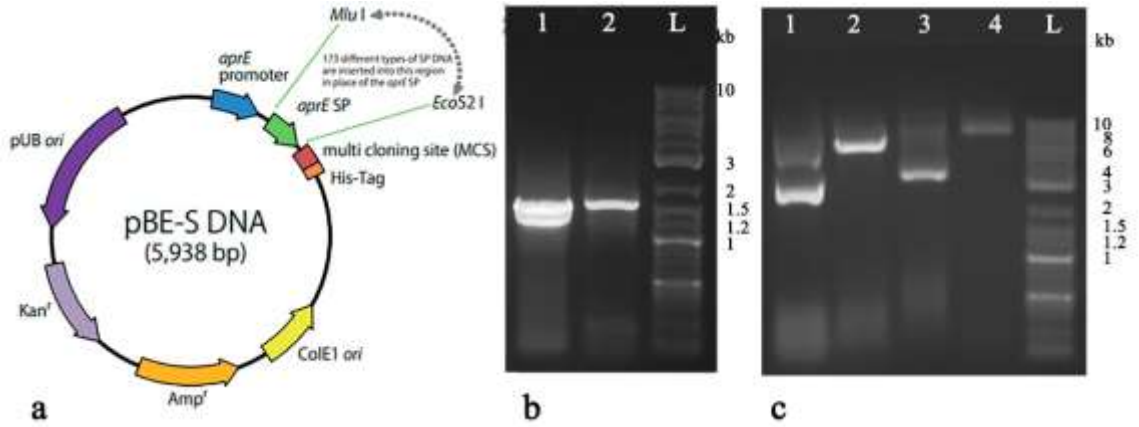

Fig. 1 Molecular cloning process of α-amylase gene of *B. amyloliquefaciens* MDC1974 strain.

(a) - Structural diagram of pBE-S vector. (b) - Flowchart for amy1974 (1) and amy1974sig (2) gene amplification. (c) - Flowchart for amy1974 gene cloning: (1) - pBE-S vector, (2) - pBE-S vector cut with XbaI restrictase, (3) - pBE-S_amy1974 vector, (4) - pBE-S_amy1974 vector cut with XbaI restrictase. In images (b) and (c), DNA ladders are represented by (L).

Transformation of *E. coli* Top 10 cells was employed to select, propagate, and obtain working quantities of the constructed targeting vectors. Transformed colonies were selected from colonies growing on ampicillin in LB medium using the colony PCR method. The transformation efficiency, defined as the total number of colonies per microgram of vector, was found to be 1.5x10^7 and 2.1x10^7 for pBE-S_amy1974sig and pBE-S_amy1974 vectors, respectively.

To obtain an alpha-amylase-secreting strain-producer, *B. subtilis* RIK 1285 strain cells were transformed with pBE-S_amy1974sig and pBE-S_amy1974 vectors propagated in *E. coli* cells. Given that the *B. subtilis* RIK 1285 strain has auxotrophy towards lysine and tryptophan amino acids, the casamino acids recommended by Takara Bio were replaced by L-lysine and L-tryptophan at 40 mg/L each during the stage of obtaining competent cells of that strain.

According to the method, circular plasmid is added to the competent cells and slowly shaken for 90 minutes on a rotary pendulum. Many experiments conducted by us have indicated that transformation occurs only after shaking for 90 minutes and leaving it in static conditions for another 60 minutes. Under these conditions, the efficiency of natural

transformation of the competent strain with pBE-S_amy1974sig and pBE-S_amy1974 vectors was 2.5x10^2 and 2.1x10^2, respectively.

Active transformants carrying pBE-S_amy1974 vectors lacking their own signal peptide gene were utilized to select the composition of the fermentation medium. The media utilized had a constant inorganic salt composition, including the carbon/nitrogen ratio, and contained different sources of organic nitrogen at the same 2% concentration. Considering that the target amylase gene is regulated by the subtilisin protease expression system (the inducibility of this system has no available information), isolated sources of peptides were tested in the study.

The obtained results are depicted in Fig. 2a. It is evident from the figure that T7 and T8 environments provide significantly higher enzyme activity compared to other environments. The volumetric activity of amylase in T8 medium at 48 hours reaches an absolute value of 740 units/ml.

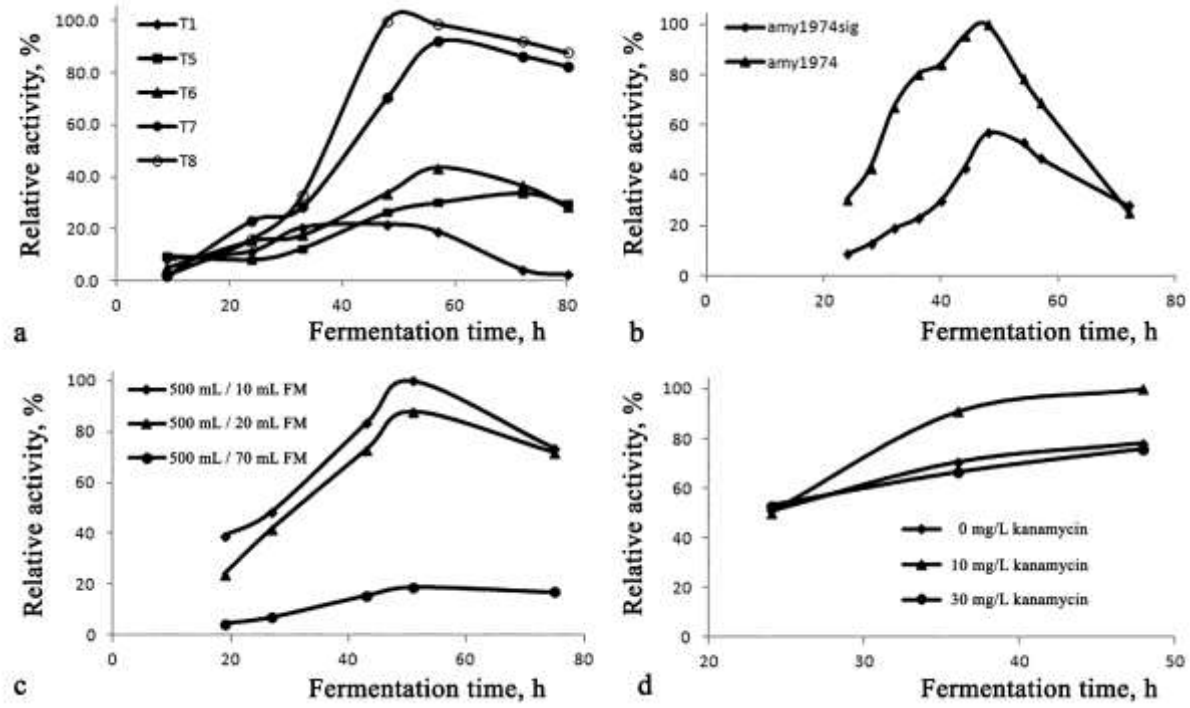

Fig. 2 Dependence of α-amylase expression of *B. amyloliquefaciens* MDC1974 strain.

(a) – on the nature of the nitrogen source of the fermentation medium, (b) – on the presence of its own signal peptide in the cloned gene, (c) – on the intensity of aeration of the fermentation medium and (d) - on the concentration of kanamycin in the medium.

(a) – 100% activity corresponds to Amy1974 volumetric activity of 740 units/ml, (b) – 100% activity corresponds to Amy1974 volumetric activity of 1100 units/ml, (c) – 100% activity corresponds to Amy1974 volumetric activity of 1300 units/ml, (FM: fermentation medium) (d) – 100% activity corresponds to a volumetric activity of Amy1974 of 1410 units/ml.

Additionally, it can be observed from the results that in all investigated environments, the maximum enzyme activity is observed near the 48th hour, gradually decreasing later.

Furthermore, it is evident from Fig. 2a that, under other equivalent conditions, the tryptone-based medium (containing relatively larger peptides) has an advantage over the peptone-based medium. In the subsequent parts of the study, the T8 medium based on yeast autolyzate, which provides maximum expression of the enzyme, was selected as the basis.

Fig. 2b illustrates the expression of amylase depending on the presence of its own signal peptide in the cloned gene. The results obtained demonstrate that in the presence of the own signal peptide (where signal peptides from the vector and the amylase gene are simultaneously present in the amplified gene), the expression of the enzyme decreases by about two times. This decrease could be attributed either to the complexity of processing two different signal peptides in the same secretion system, or to the activation of different secretion systems by the host organism for different enzymes.

Fig. 2c illustrates the dependence of alpha-amylase expression on aeration intensity. The obtained results indicate that the intensity of aeration substantially enhances the expression of recombinant alpha-amylase of B. amyloliquefaciens MDC1974 strain, reaching 1300 units/ml at the 51st hour in the case of 10 ml fermentation medium in a 500 ml flask.

Fig. 2d depicts the dependence of alpha-amylase expression on kanamycin concentration. In the presence of 10 mg/L kanamycin in the medium, 1410 units/ml of amylase activity is observed at 48 hours. In the absence of kanamycin and with 30 mg/L kanamycin, more than 75% of this activity is maintained, indicating the high segregation and structural stability of the pBE-S vector-based expression system, as evidenced by Takara Bio.

Conclusion

Through cloning by Gibson's assambly method, a stable and high-activity secretory alpha-amylase strain-producer of *B. amyloliquefaciens* MDC1974 strain was successfully obtained utilizing the pBE-S vector from "Takara Bio", with some characteristics of the enzyme synthesis studied. Moving forward, the plan involves introducing the 173 signal peptides from "Takara Bio" into the resulting system and obtaining multiple more active strains of amylase through the selection of active variants.

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BACILLUS AMYLOLIQUEFACIENS MDC1974 ՇՏԱՄԻ ԱԼՖԱ-ԱՄԻԼԱԶԻ ԳԵՆԻ ԱՐՏԱԲՋՋԱՅԻՆ ԷՔՍՊՐԵՍՈՒՄ *BACILLUS SUBTILIS* RIK1285 ԲՋԻՋՆԵՐԻՑ

Տ.Մ. Սողոմոնյան
ՀՀ ԳԱԱ «Հայկենսատեխնոլոգիա» գիտաարտադրական կենտրոն

Bacillus amyloliquefaciens MDC1974 շտամի ալֆա-ամիլազային գենը մոլեկուլային մակարդակում կլոնավորվել է *E. coli*/*B. subtilis* pBE-S մաքոքային վեկտորում և արտաբջջային ձևով էքսպրեսվել ցածր պրոտեազային ակտիվությամբ *Bacillus subtilis* RIK1285 ռեցիպիենտ շտամից: Ֆերմենտման պայմանների օպտիմալացման արդյունքում ստացվել է արտազատվող ալֆա-ամիլազի 1400 միավոր/մլ ակտիվություն։

Բանալի բառեր. *Bacillus amyloliquefaciens,* α-ամիլազ, էքսպրեսում արտազատմամբ, *E. coli*/*B. subtilis* մաքոքային վեկտոր

ВНЕКЛЕТОЧНАЯ ЭКСПРЕССИЯ ГЕНА АЛЬФА-АМИЛАЗЫ ИЗ ШТАММА BACILLUS AMYLOLIQUEFACIENS MDC1974 С ИСПОЛЬЗОВАНИЕМ КЛЕТОК BACILLUS SUBTILIS RIK1285

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Ген альфа-амилазы штамма *Bacillus amyloliquefaciens* MDC1974 был клонирован на молекулярном уровне в *E. coli*/*B. subtilis* pBE-S челночный вектор и экспрессован внеклеточно из штамма-реципиента *Bacillus subtilis* RIK1285 с низкой протеазной активностью. В результате оптимизации условий ферментации была достигнута секреция альфа-амилазы с активностью 1400 ед./мл.

Ключевые слова: *Bacillus amyloliquefaciens*, α- амилаза , экспрессия с секрецией, *E. coli*/*B. subtilis* челночный вектор

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