Chapter 15
Expression of *Stichopus japonicus* Lysozyme Gene in *Bacillus subtilis* WB600

Zhiwen Liu, Xingyu Liao, Lu Sun, Dan Zou, Dan Li and Lina Cong

Abstract In this study, a genetic engineering bacteria *Bacillus subtilis* pHT43-SjLys/WB600 was successfully constructed for the expression of the lysozyme gene from sea cucumber (*Stichopus japonicus*) by the method of recombinant DNA technique. The growth trend of engineering bacteria was consistent with the wild-type strain WB600, and the results demonstrated that insertion of foreign gene did not affect its physiological and biochemical metabolism. In the absence of selection pressure, the analysis of the stability revealed that there was no gene rearrangement and lost of the recombinant plasmid in the bacteria which showed that it has high genetic stability. The SDS-PAGE results demonstrated that pHT43-SjLys/WB600 successfully expressed soluble SjLys protein after incubated for 48 h induced by ITPG. The heterologous expression protein of pHT43-SjLys/WB600 displayed remarkable inhibitive effect on the growth of the *Vibrio parahaemolyticus*. To our knowledge, this is the first report about the *SjLys* gene authentic heterologous expression in *B. subtilis*. It should provide a robust secretion expression system for genetic engineering of *B. subtilis* and was thus proposed a potentially new way for producing recombinant SjLys protein.

Keywords Sea cucumber · Lysozyme · *Bacillus subtilis* · Genetic engineering bacteria · Antimicrobial activity

15.1 Introduction

Sea cucumber (*Stichopus japonicus*) is one of the most important and valuable holothurian species in coastal fisheries and commonly consumed echinoderms because of their good flavor and medicinal value. Aquaculture of sea cucumber has rapidly developed in many Asian countries in recent decades [7, 20]. However, in

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recent years, because of rapid expansion and intensification of cultivation, sea cucumber in China has suffered from frequent disease outbreaks. *Bacteria*, especially *vibrios*, often caused major diseases such as skin ulcer and bacterial ulceration syndrome in *S. japonicus* at breeding, aestivation, and outdoor cultivation stages. It poses a threat to the aquaculture industry and lead to heavy economic losses. In addition, the abuse of antibiotics in aquaculture has accelerated the development of drug-resistant bacteria and more virulent pathogens [6, 12].

Lysozyme (EC3.2.1.17), a well-known bacteriolytic enzyme, is widely distributed in the animal and plant kingdoms. Their key role is to lyse bacteria by hydrolyzing the β-1,4-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (GlcNAc) of peptidoglycan in the bacterial cell walls. Thus, the major function of lysozyme is host defense, as it acts as an antimicrobial and immune-modulating agent, and furthermore, it can display digestive activity or nonenzymatic activity in some species [1, 9]. Based on the differences in their structure, biological functions, catalytic character, and original source, lysozymes are classified into six groups: chicken-type lysozyme (c-lysozyme) present in many vertebrates and insects is the most extensively studied lysozyme; goose-type lysozyme (glysozyme) identified mainly in vertebrates including mammals, birds, and fish; invertebrate-type lysozyme (i-lysozyme), plant lysozyme, bacterial lysozyme, and phagelysozyme [3].

Sea cucumber (*S. japonicus*) relies on their innate immune system to resist invasion of prokaryotic and eukaryotic pathogens, whose digestive system are simple and lack of specific immune. Therefore, the lysozyme would play an important role in the immune, digestive system, and autolysis for the invertebrates sea cucumber which signifies that it is related to both immunity and growth [21, 23]. Many comprehensive studies have been performed and showed that the *S. japonicus* lysozyme is belong to i-type lysozyme, whereas it is widely existed in every organization of sea cucumber, even in the no cell gastrovascular cavity liquids. The further research and utilization of the sea cucumber i-type lysozyme should have important value and significance for the sea cucumber farming, product development, biopharmaceutical, and other marine creatures immune [6]. The i-type lysozyme gene from *Asterias rubens* was the first reported i-type lysozyme gene. Recently, many lysozyme genes have been identified through sequencing or protein purification. Some of them were well-studied for their functions in immunity [24]. Cong et al. [6] previously reported and presented the full-length cDNA sequence encoding lysozyme from the sea cucumber (*S. japonicus*) body wall and registered in the NCBI GenBank under the accession number EF036468. They found that the recombinant *S. japonicus* lysozyme (SjLys) protein in *Escherichia coli* has broad-spectrum antimicrobial activity; the enzyme can dissolve not only the gram-positive bacteria but also the gram-negative bacterium. Especially, it has significant antibacterial effect on the serious aquatic animal diseases caused by pathogenic *vibrio* and *pseudomonas*. Therefore, the recombination SjLys protein should play important role on the prevention and control of the aquatic animal disease and substitute for antibiotic medicines applying in green aquaculture, and it has aroused attention of the domestic and overseas experts and scholars.

The traditional methods to produce lysozyme with high cost and complex production process, which cannot meet the increasing market demand. At present, the biological engineering methods to produce lysozyme are the important trend and means. The recombinant lysozyme producing genetic engineering strains of *E. coli* have been successfully constructed in many studies; however, its expressions in *E. coli* have been demonstrated that there were many problems and difficulties, such as low production and unstable enzyme activity, even more the lysozyme protein existed in inclusion body so that the subsequently operation of the protein denaturation and renaturation were very complicated [8, 25]. Although the transformation of gram-negative bacteria such as *E.coli* is routine for most molecular biologists, many laboratories struggle with incorporating foreign DNA into gram-positive bacteria such as *Bacillus* [13, 14]. The *Bacillus subtilis* has been considered as an attractive and ideal host for expression and secretion of heterologous proteins in gene engineering operation and a kind of beneficial probiotics that is widely dispersed in soil, lakes, and oceans. It is nonpathogenic and does not produce any endotoxins and is generally regarded as safe organism (GRAS) [15, 19]. The other advantages of *B. subtilis* include direct secretion of the functional proteins into the medium, no significant bias in codon usage, its well-known genetics, simple fermentation processes, and the development of simplified downstream processing procedures [16, 22]. These further make the *B. subtilis* a potential *bacillus* in genetic engineering and industrial application [4, 11].

To our knowledge, so far, there is no report about the *S. japonicus* lysozyme gene (*Sjlys*) heterologous expression in *B. subtilis*. In this study, the objective was to isolate and clone the *SjLys*, and then it would be inserted into the *B. subtilis* expression plasmid pHT43. At last, a genetics engineering bacteria *B. subtilis* pHT43-SjLys/WB600 for recombinant expression of *Sjlys* gene would be successfully constructed. This study can provide data on the application of i-type lysozymes from sea cucumber.

15.2 Materials and Methods

15.2.1 Materials

Expression vector pHT43 and six protease defect type *B. subtilis* strain WB600 (his Δ nprB Δ nprE18 Δ aprE Δ epr Δ bpf Δ mpr) was selected as the *Sjlys* gene expression host in this study were purchased from Shanghai Genemy Biological co., Ltd.

Escherichia coli $DH5_{\alpha}$, pMD18-T Simple Vector, *Taq* DNA polymerase, gel extraction and purification Kit, plasmid extraction kit, and restriction enzymes *Bam*HI and *Sma*I were purchased from Takara Biotechnology (Dalian) co., Ltd.

Recombinant plasmid pMD18T-SjLys contained the *Sjlys* gene was constructed by our lab, and the indicator *bacteria Staphylococcus aureu*, *Micrococcus lysodeikticus*, *Vibrio parahaemolyticus*, and *Pseudomonas aeruginosa* are preserved in our lab.

15.2.2 Methods

15.2.2.1 Primers Design and Amplification of the Target *SjLys* Gene

Based on the published cDNA sequence (EF036468) from *S. japonicus* lysozyme gene, a pair of specific primers was designed by using the Primer Premier 5.0 software. The upstream primer and downstream primer are designed, respectively, as following, HS-Q-P11: 5'-GCC GGATCCATGCAAGTTCCTTCTG-3', HS-Q-P12: 5'-GCCCGGGAATTCTCAGTTGTTGCTC-3'. The PCR product was approximately 400 bp. The restriction sites of *Bam*HI and *Sma*I were introduced into the amplified fragments by the primers (underline), and the box indicated the initial or stop codon. They were synthesized by Beijing genomics institute (BGI).

The PCR assays were carried out as the following reagent concentrations: 100 ng pMD18T-SjLys plasmid template DNA; 5.0 μl 10 × Taq PCR buffer; 2.0 mM of $MgCl_2$; 125 μM of each dNTP; 1.0 U of Taq DNA Polymerase; 1.5 μM of each upstream and downstream primer pair. The final reaction volume was adjusted to 50 μl with the sterilized ultrapure $dddH_2O$.

Amplifications were done using a PTC 225 Peltier Thermal Cycler (MJ Research Inc. USA). The amplification profile was performed as following: 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 59 °C, 1 min at 72 °C; and 10 min at 72 °C for final extension, 4 °C for holding. 5.0 μl PCR products were resolved using 1.2 % agarose gel electrophoresis for verification of the *SjLys* fragment. The remaining was used for extraction.

15.2.2.2 Construction of the Expression Vector pHT43-SjLys

The target *SjLys* PCR amplified fragment was extracted and purified with the agarose gel DNA fragment recovery Kit Ver.2.0 (Takara), and then cleaved and ligated into vector pMD18T-Simple between the sites of *Sam*I and *Bam*HI to yield positive recombinant plasmid pMD18T-Simple-SjLys.

The products of the plasmid pMD18T-Simple-SjLys and pHT43 digested with the same restriction enzymes *Bam*HI and *Sma*I, were ligated by the isolation buffer to construct the recombinant a express vector in *B. subtilis* and designated as pHT43-SjLys which is confirmed by restriction enzyme analysis and sequencing, the nucleotide sequence was compared to GenBank data using the Blastx (http://www.ncbi.nlm.nih.gov/blast) algorithm and the alignments were done using ClustalW2 (http://www.ebi.ac.uk).

15.2.2.3 Transformation of *Bacillus subtilis*

The pHT43-SjLys plasmid with a chloramphenicol resistance marker was used for integration. It was transformed into *B. subtilis* WB600 competent cells using

electrotransformation. Transformants were screened on LB agar plates containing 10 μg/mL chloramphenicol. Finally the recombinant engineering strains named pHT43-SjLys/WB600 were identified and confirmed by PCR, restriction enzyme analysis, and sequencing.

15.2.2.4 The Growth Curve and Genetic Stability of the pHT43-SjLys/WB600

The growth curve of the strain was measured using a method described by Shoham et al. [17] with minor modifications. The WB600 and pHT43-SjLys/WB600 (with10 μg/mL chloramphenicol) were activated in Luria-Bertani medium (LB). The cells were routinely grown at 30 °C in 50 ml Erlenmeyer flasks with a culture volume of 10 ml in a rotary shaker at 200 rpm for overnight. And then the absorbance value at a wavelength of 600 nm (OD_{600}) at regular intervals of the two bacteria was determined and measured by ultraviolet spectrophotometer. All assays were performed in triplicates and the results are the means of three independent experiments.

The plasmid genetics stability of the recombinant pHT43-SjLys/WB600 for continuous passage culture with chloramphenicol was determined through the method of Avsaroglu et al. [2] and Leen et al. [10]. For the strain, stability experiments were performed in triplicate too.

15.2.2.5 SDS-Polyacrylamide Gel Assay

The recombinant *bacterium* pHT43-SjLys/WB600 was cultured with 10 μg/mL chloramphenicol and induced by 1 mmol/L isopropylthiogalactoside (IPTG) for 12, 24, 36, and 48 h, respectively. The fermentation broth was centrifuged at 12,000 rpm and 4 °C for 10 min to remove cells. Culture supernatant was collected for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of recombinant protein. Proteins were visualized with Coomassie Brilliant Blue.

15.2.2.6 Antimicrobial Activity Assay

Antimicrobial activity of the recombinant SjLys protein was assessed with the radial diffusion assay as described by Cong et al. [5]. Four bacterial strains were used as substrates, including Gram-negative bacteria *P. aeruginosa* and *V. parahaemolyticus*, Gram-positive bacteria *Micrococcus lysodeikticus* and *S. aureus*. The bacterial concentration in the liquid broth was adjusted to an absorbance of 0.1 at 600 nm and poured onto 90 mm LB agar plates. Wells (diameter 5 mm) were cut into the freshly poured plates after the agar solidification. For radial diffusion assays, 10 μl of the recombinant engineering strain was pipetted into individual wells and the agar plates were incubated at 37 °C. After 16 h incubation, the radius of the clearing zone was measured. The original strain WB600 was used as negative control.

15.3 Results

15.3.1 The Fragment of the Stichopus japonicus Lysozyme Gene

The sea cucumber (*S. japonicus*) lysozyme (*SjLys)* gene was reamplified with the specific PCR primers HS-Q-P11and HS-Q-P12 and the plasmid pMD18T-SjLys as the template. The amplification results are shown in Fig. 15.1. In the Fig. 15.1, there was a gene fragment with approximately 400 bp, and it was in accordance with the anticipated objective strap size 400 bp. The sequencing result revealed that the nucleotide sequence of the reamplified fragment was absolutely identical to the reported cDNA of *S. japonicus* body wall in NCBI (EF036468). These results showed that the *SjLys* gene from *S. japonicus* have been successfully reamplified and obtained. Additionally, the analysis of DNA sequence further showed that the inserted lysozyme gene *SjLys* would contain 375 bp length DNA fragment that encodes a mature polypeptide of 125 amino acid (Fig. 15.2).

15.3.2 Construction and Identification of the pHT43-SjLys

The target *SjLys* fragment was extracted and purified with the recovery Kit. The product was then digested with *Sam*I and *Bam*HI, and finally subcloned to vector pMD18T-Simple plasmid to yield a positive recombinant plasmid pMD18T-Simple-SjLys with 3,077 bp length that was identified and verified by PCR and restriction enzyme digestion (Fig. 15.3).

After that, the pMD18T-Simple-SjLys and vector pHT43 were digested with the *Bam*HI and *Sma*I for 4 h at 37 °C. And their products were thus ligated to construct a recombinant expression vector pHT43-SjLys with 8,432 bp length (Fig. 15.4). It was further confirmed and verified by PCR and restriction enzyme digestion.

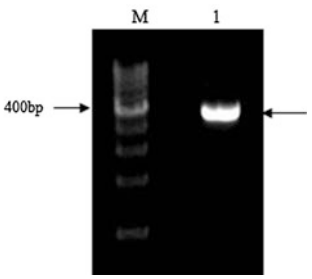

Fig. 15.1 PCR amplification of the target gene *SjLys* (M:100 bp DNA ladder marker, *lane 1*: target gene *SjLys*)

>*SjLys* sequences

GCCGGATCCATGCAAGTTCCTTCTGATTGCCTAAAGTGCATCTGTTTTGTAGAGTCCACTTGCACTATACCTTCCCCAT
TGTGTCATATGGATGTAGGATCACTGTCATGTGGTCCTTACCAAATCAAACTAGGCTACTGGCAGGATGCTAGGCTGA
AGGGAGGTAGTCTGGATGGAGATTGGCAGAAATGTTCAGCAACCTTTGACTGCAGTGAACGGGCTGTACAGGGTTAT
ATGGCACGGTACGCAACCTATGCCCGTCTAGAGCATAATCCTACCTGTGAGGATTTTGCGCGGATACACAACGGCGG
ACCAAATGGGTTCAAGAATCCAGCAACTGAAAAATATTGGTTGAGAGTGAAGAAATGTCTTGACATGAGCAACAAC
TGA GAATCCCGGGC

Fig. 15.2 Sequence of the reamplified *SjLys* fragment. (The *boxes* indicate the initial or stop codon, the *gray* regions represent the primers sequences and the *underlines* indicate the restrict enzyme sites)

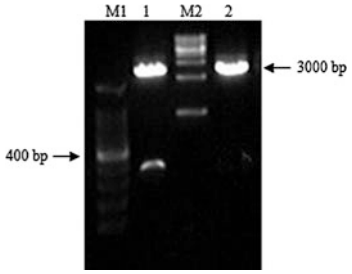

Fig. 15.3 Recombinant plasmid pMD18T-simple-SjLys double digestion verification. (M1 and M2: 100 and 1,000 bp DNA ladder marker, lane 1: double enzyme digestion, lane 2: pMD18T-Simple-SjLys)

15.3.3 Transformation of B. subtilis

The recombinant expression plasmid pHT43-SjLys was selected and transformed to *B. subtilis* WB600. The *B. subtilis* transformants were first selected by antibiotic screening at a concentration of 10 μg/ml chloroamphenicol. As a result, a genetics

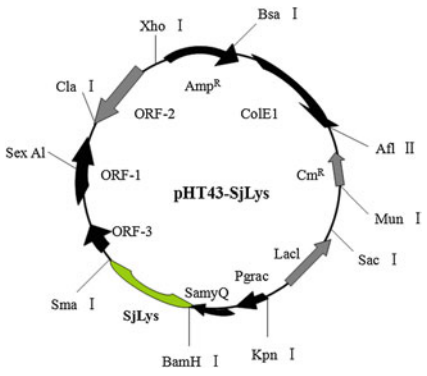

Fig. 15.4 The map of the recombinant expression vector pHT43-SjLys

engineering strain designed pHT43-SjLys/WB600 was successfully obtained by further verification with PCR amplification, double digestion (Fig. 15.5), and sequencing of the *SjLys* gene of its recombinant plasmid extracted from the selected genetics engineering strain.

15.3.4 Growth Curve of the pHT43-SjLys/WB600

The growth curves of the pHT43-SjLys/WB600 and WB600 are presented in Fig. 15.6. In the Fig. 15.6, it can be found that the engineering strain and its original strain growth tendency are mostly identical. WB600 entered the logarithmic growth phase after inoculating 1.5 h and ended after 6 h, while the engineering strain were correspondingly 1 and 5 h. After that, the growth rate of these strains was consistent. The growth curve results demonstrated that the heterologous *SjLys* gene insertion did not affect the physiological metabolism and the growth of the engineering bacteria pHT43-SjLys/WB600.

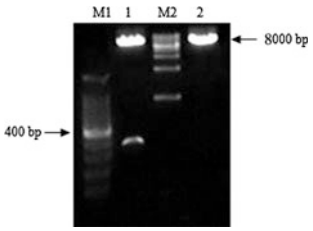

Fig. 15.5 The double digestion verification from the pHT43-SjLys/WB600 plasmid. (M1 and M2: 100 and 1,000 bp DNA ladder marker, *lane1*: double enzyme digestion, *lane 2*: pHT43-SjLys)

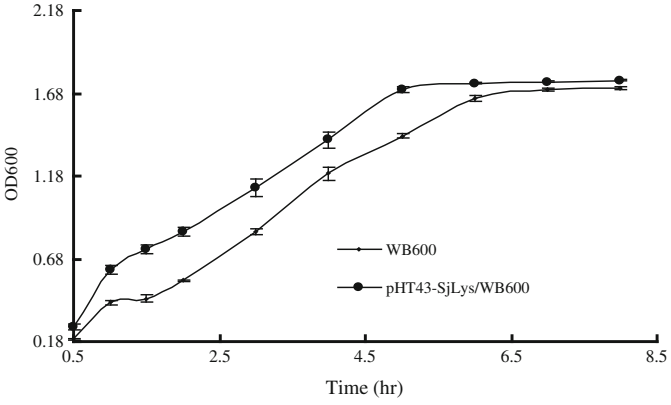

Fig. 15.6 The growth *curves* of *B. subtilis* WB600 and pHT43-SjLys/WB600

15.3.5 The Stability Analysis of the pHT43-SjLys/WB600

To investigate whether the bacterial host affects the stability of the plasmid, the pHT43-SjLys/WB600 strain was continuously cultured in the absence of antibiotics LB medium and the bacterium fluid are measured at each time. Figure 15.7 showed that recombinant plasmid has excellent genetic stability in the absence of selection pressure with approximately 94 % after 5 times (100 generation) continuous cultured while the biology yield were steady.

Plasmid DNA of the pHT43-SjLys/WB600 with 3 and 5 times continuous culture were extracted and digested, respectively. The result demonstrated that the strains grew on the chloramphenicol plates were the same as its initial strain, however, there were no plasmid extraction for the strains that cannot grow on the chloramphenicol plates. There was no rearrangement or lost phenomenon found in the engineered bacteria. These results together suggest that the engineered bacteria have a good genetic stability.

15.3.6 SDS-PAGE Analysis

The recombinant construct pHT43-SjLys/WB600 containing the coding region for the mature SjLys protein, was selected for heterologous expression. After IPTG induction for 12, 24, 36 and 48 h respectively, the recombinant SjLys protein was purified and analyzed by SDS-PAGE on a 12 % gel and autoradiography.

The SDS-PAGE analysis of the rSjlys protein demonstrated that there was a faint band with a molecular mass of approximately 14.0 kDa when it was induced for 36 and 48 h (Fig. 15.8). Furthermore, its molecular mass was accordance with the theoretical size of the fusion protein. These confirmed that the rSjlys protein were successfully expressed but with relatively low level.

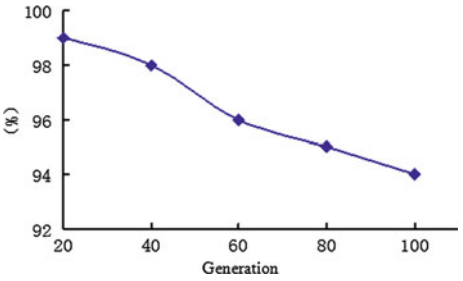

Fig. 15.7 The stability of engineered bacteria pHT43-SjLys/WB600

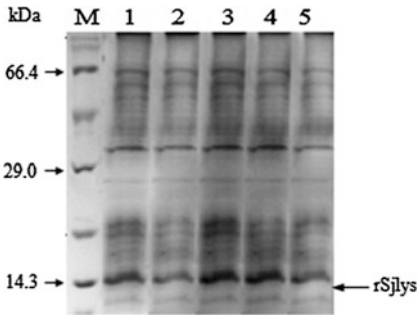

Fig. 15.8 SDS-PAGE analysis of the recombinant SjLys protein secreted by pHT43-SjLys/WB600. (M: Molecular mass markers; *lanes 1, 2, 3* and *4*: Total cellular protein induced by IPTG for 12, 24, 36, and 48 h, respectively; *lane 5*: the control WB600)

15.3.7 Antimicrobial Activities of the Recombinant Proteins

The radial diffusion assay was employed to evaluate the antimicrobial spectrum of the recombinant SjLys proteins against several gram-positive and gram-negative bacteria, including pathogenic bacteria *Vibro parahaemolyticus*, which are commonly present in coastal marine environments and a common finding and the leading agent causing disease of *Stichopus japonicas*. The original *B. subtilis* WB600 was used as control.

Based on the radius of the antimicrobial zone, the recombinant SjLys protein from the pHT43-SjLys/WB600 displayed a remarkable inhibitory effect on the growth of *Vibro parahaemolyticus* (Fig. 15.9). It revealed that rSjLys effectively inhibited the growth of *V. parahaemolyticus*, however, it did not obviously affect the growth of the other strains and inhibit the remaining 3 indicator bacteria. In summary, combined with the results of the 2.6, it is suggested that the heterologous expression of *Sjlys* was successfully realized in *B. subtilis*.

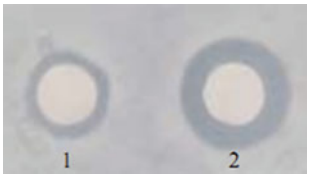

Fig. 15.9 Assay of antimicrobial activity of the recombinant SjLys protein to *V. parahaemolyticus*. (*1* WB600; *2* pHT43-Sjlys/WB600)

15.4 Discussion

Sea cucumber (*S. japonicus*) is one of the economically important farmed echinoderm species in Northern China. However, infectious diseases are becoming a severe problem with increasing culturing. Disease caused by *Vibrio* is most widespread in sea cucumber farming. Antibiotics and chemotherapeutics used to control these diseases can result in the development of drug-resistant bacteria, environmental pollution and unwanted residues in aquaculture. One of the most promising methods for controlling sea cucumber diseases in aquaculture is addition of Lysozyme in feed [18].

To our knowledge, this is the first study about the *S. japonicus* lysozyme gene expression in *B. subtilis*, which provide a new way for *SjLys* gene expression and efficient utilization. Undoubtedly, this knowledge should accelerate the biotechnological application of *B. subtilis* in industry field, and be helpful for the prevention and treatment of the sea cucumber disease [8].

Unfortunately, the protein concentration of genetic engineering strain pHT43-Sjlys/WB600 is not high and can't well meet production's need, which meant that the production of rSjlys protein in *B. subtilis* pHT43-SjLys/WB600 could still be improved. Therefore, it is necessary to improve the genetic engineering strain for effective expression. We are focusing on increasing its expression to achieve more products and taking measures to gradually resolve them, such as optimization of the fermentation conditions and separation and purification technology. Moreover, we are trying to insert the *Sjlys* gene in a new and excellent *B. subtilis* HS-38A for further genetic modification, which it was isolated from the intestine of wild sea cucumber in Dalian sea area [5]. Meanwhile, what the foreign *Sjlys* gene introduced and integrated into *B. subtilis* chromosome for integration expression was ongoing.

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