J. Microbiol. Biotechnol. (2014), 24(10), 1405–1412
http://dx.doi.org/10.4014/jmb.1404.04039

jmb

Cloning, Characterization, and Production of a Novel Lysozyme by Different Expression Hosts

Haifeng Zhang[1,2†], Gang Fu[1,3†], and Dawei Zhang[1,2,3*]

[1]*Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, P. R. China*
[2]*Key Laboratory of Systems Microbial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, P. R. China*
[3]*National Engineering Laboratory for Industrial Enzymes, Tianjin 300308, P. R. China*

Received: April 22, 2014
Revised: June 28, 2014
Accepted: June 28, 2014

First published online
July 2, 2014

*Corresponding author
Phone: +86-10-24828749;
Fax: +86-10-24828749;
E-mail: zhang_dw@tib.cas.cn

†These authors contributed equally to this work.

pISSN 1017-7825, eISSN 1738-8872

Lysozyme is a protein found in egg white, tears, saliva, and other secretions. As a marketable natural alternative to preservatives, lysozyme can act as a natural antibiotic. In this study, we have isolated *Bacillus licheniformis* TIB320 from soil, which contains a lysozyme gene with various features. We have cloned and expressed the lysozyme in *E. coli*. The antimicrobial activity of the lysozyme showed that it had a broad antimicrobial spectrum against several standard strains. The lysozyme could maintain efficient activities in a pH range between 3 and 9 and from 20°C to 60°C, respectively. The lysozyme was resistant to pepsin and trypsin to some extent at 40°C. Production of the lysozyme was optimized by using various expression strategies in *B. subtilis* WB800. The lysozyme from *B. licheniformis* TIB320 will be promising as a food or feed additive.

Keywords: Lysozyme, *Bacillus licheniformis*, *Bacillus subtilis*, antimicrobial, protein expression system

Introduction

Lysozyme is a ubiquitous enzyme in milk, saliva, tears of most mammals, and avian egg whites [14]. It is an enzyme that degrades the bacterial cell wall by catalyzing the hydrolysis of β-(1,4)-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetyl glucosamine found in the peptidoglycan layer, which is the major component of the cell wall of both gram-positive and gram-negative bacteria [21].

Based on its primary structure, three major classes of animal lysozymes have been identified as the chicken (C) type, the goose (G) type, and the invertebrate (I) type [2]. Although the overall sequence similarity of lysozymes in the different classes is low, they share a similar overall three-dimensional structure, and they have been proposed to have a common ancestry [11].

The major function of lysozymes in most organisms is to provide antibacterial protection, because hydrolysis of peptidoglycan will compromise the integrity of the cell wall and cause cell lysis. Owing to the bactericidal activity and thermal stability, lysozyme has been of interest in medicine, cosmetics, and the food industry. The most important application of lysozyme is usage as a food preservative, such as in cheese, fish, meat, fruit, vegetables, and wine.

Great progress has been made for lysozyme production in previous studies. Bacteriophage T4 lysozyme has been purified using the Ni-chelate affinity chromatography technique from overexpressing *Escherichia coli* cells [6]. Hen egg white lysozyme is produced at the metric ton scale by purification from eggs, and recent advances in transgenic plant technologies have led to similarly low production costs for recombinant human lysozyme [31]. Human lysozyme has also been expressed in *Escherichia coli* [16], *Saccharomyces cerevisiae* [12], and *Pichia pastoris* [28].

E. coli is a preferred host in heterologous protein expression. *E. coli* has the ability to grow rapidly at high density on inexpensive substrates, and it has well-characterized genetics, and a large number of cloning vectors and mutant host strains [27, 33, 34]. However, the *E. coli* expression system still has disadvantages. For

Table 1. Primers used in this study.

Primer name	Sequence(5′-3′)	Used for plasmids
LysoE-F	actgcaCATATGatgggaatcaaaggaatcgac	pET21b-lyso
LysoE-R	actgcaCTCGAGttatctaacacgaattttctgacca	pET21b-lyso
P43-F	actgcaCTCGAGtgtcgacgtgcatgcaggc	pGJ104, pGJ105, pGJ106
P43-R	tataatggtaccgctatcacttta	pGJ104, pGJ105, pGJ106
PsacB-F	taaagtgatagcggtaccattataagttctttaggcccgtagtct	pGJ106
sacB-F	taaagtgatagcggtaccattataatgaacatcaaaaagtttgcaaaac	pGJ105
sacB-R	actgcaGAATTCttctttcgcaaacgcttgagtt	pGJ105, pGJ106
amylaseSP-F	taaagtgatagcggtaccattataatgtttgcaaaacgattcaaaacctc	pGJ104
amylaseSP-R	actgcaGAATTCcgtttcagcactcgcagcc	pGJ104
Lyso-F	actgcaGAATTCatgggaatcaaaggaatcgac	pGJ201, pGJ202, pGJ203
Lyso-R	actgcaGGATCCttatctaacacgaattttctgacca	pGJ201, pGJ202, pGJ203

Note: capital letters represent restriction enzyme sites.

example, it is a pathogenic bacterium and has endotoxins (lipopolysaccharide) [20]; it secretes protein into the periplasm and often into inclusion bodies [3, 35]. As an alternative, *Bacillus* species are among the champions in secreted enzyme production. In biotechnological processes for protein production, *Bacillus subtilis* is the most popular host owing to its excellent fermentation properties, and huge capacity for secreting proteins directly into the growth medium, which offers major advantages in their downstream processing, and is non-pathogenic and free of endotoxins and offers non-biased codon usage [5, 25, 26, 29, 32].

The uses of chemical preservatives and antibiotics for feed and food additives are a critical problem to the health and environment. Consumer demand for natural origin, safe, and environmentally friendly food preservatives has been increasing recently. In this study, we have cloned the lysozyme gene of *Bacillus licheniformis*, a generally regarded as safe strain. The lysozyme was purified and characterized. The enzyme activity and antimicrobial spectrum were examined. The production of lysozyme in *Bacillus subtilis* WB800 was optimized.

Materials and Methods

Bacterial Strains and Culture Conditions

E. coli DH5α was used as a host for the construction of expression vector. *E. coli* BL21 (DE3) and *Bacillus subtilis* WB800 were used as a host for lysozyme expression. *Bacillus subtilis* WB800 was kindly provided by Prof. Hui Song (Tianjin Institute of Industrial Biotechnology, CAS). *E. coli* and *Bacillus subtilis* WB800 were cultured at 37°C in Luria–Bertani medium (yeast extract, 5 g/l; peptone, 10 g/l; NaCl, 10 g/l; and agar, 15 g/l) containing suitable antibiotics. The cultures were incubated in 250 ml conical flasks containing 50 ml of culture broth on a rotary shaker at 200 rpm and 37 °C.

Plasmids Construction

The primers and plasmids used in this study are summarized in Tables 1 and 2, respectively. Amplification of the lysozyme gene

Table 2. Plasmids used in this study.

Plasmid name	Genotype/relevant characteristics	Source or reference
pET21b	Ap^R	Lab stock
pET21b-lyso	Ap^R	This study
pGJ103	Cm^R	Dr. JG Yang (BUCT)
pGJ104	P43, amy signal peptide, Cm^R	This study
pGJ105	P43, sacB signal peptide, lyso, Cm^R	This study
pGJ106	P43, PsacB, sacB signal peptide, Cm^R	This study
pGJ201	P43, amy signal peptide, lyso, Cm^R	This study
pGJ202	P43, sacB signal peptide, lyso, Cm^R	This study
pGJ203	P43, PsacB, sacB signal peptide, lyso, Cm^R	This study

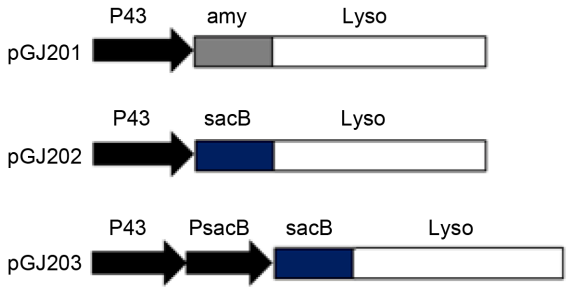

Fig. 1. Schematic diagram elucidating the construction of plasmids applied in this study for expression of lysozyme in *B. subtilis*.

Abbreviations: P43, the P43 promoter; amy, the amylase signal peptide; sacB, the sacB signal peptide; PsacB, the sacB gene promoter; Lyso, the lysozyme gene of *Bacillus licheniformis* TIB320.

from chromosomal DNA of *B. licheniformis* was carried out by polymerase chain reaction (PCR) using gene-specific primers. For expression of lysozyme in *E. coli*, the lysozyme gene was amplified and inserted into the pET21b vector, and the cloning sites of *Nde*I and *Xho*I were introduced into the primers lysoE-F and lysoE-R for ligating into the pET21b-lyso vector.

To produce the lysozyme in *Bacillus subtilis* WB800, promoter P43 and an amy signal peptide, promoter P43 and the sacB signal peptide, and promoter P43 and the PsacB-sacB signal peptide were fused by PCR using primers listed in Table 1, respectively. The plasmid list is shown in Table 2 and the diagram of plasmid construction is shown in Fig. 1. Plasmid pGJ104 was constructed by inserting promoter P43 and amy signal peptide into plasmid pGJ103. Plasmid pGJ105 was constructed by inserting promoter P43 and sacB signal peptide into plasmid pGJ103. Plasmid pGJ106 was constructed by inserting promoter P43, promoter sacB, and the amylase signal peptide into plasmid pGJ103. The lysozyme gene was amplified by using genomic DNA of *B. licheniformis* TIB320 as the template. The cloning sites of *Eco*RI and *Bam*HI were introduced into the primers lyso-F and lyso-R for construction of pGJ201, pGJ202, and pGJ203, respectively.

Expression of Lysozyme in *E. coli* and *Bacillus subtilis* WB800

The recombinant plasmid pET21b-lyso was electrotransformed into *E. coli* BL21 (DE3) and cultured at 37°C in LB medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) containing ampicillin (100 mg/l). The strain culture was incubated with rotary shaking for 12 h, and then 1% of the strain culture was transferred into fresh LB medium. When the culture OD was 0.6, 1 mmol/l (final concentration) isopropyl-/3-D-thiogalactosids (IPTG) was added to the expression culture for inducing the expression of lysozyme, with rotary shaking for 12 h at 30°C. After centrifugation at 12,000 ×*g* for 10 min at 4°C, the cells were collected and crushed for enzyme assays. The lysozyme activity in the supernatant was used to indicate the expression level.

The recombinant plasmids pGJ201, pGJ202, and pGJ203 were electrotransformed into *B. subtilis* WB800 and cultured at 37°C in super-rich medium (25 g/l tryptone, 20 g/l yeast extract, 30 g/l glucose, and 3 g/l K_2HPO_4, pH 7.0) containing chloromycetin (10 μg/ml), respectively. The culture was cultivated for 72 h and samples were collected every 6 h. After centrifugation at 12,000 ×*g* for 5 min at 4°C, the supernatant was used for enzyme assays.

Expression and Purification of Lysozyme

The *E. coli* BL21 (DE3) harboring pET21b-lyso strain was grown in LB medium supplemented with 100 mg/l ampicillin. The cells were grown aerobically in 1 L Erlenmeyer flasks containing 200 ml of medium at 37°C and 200 rpm in an orbital incubator shaker. The cells were induced at ~0.6 OD_{600} with 1 mM IPTG and incubated at 25°C for 16 h. The cells were then harvested by centrifuging at 12,000 ×*g* for 10 min. The pellet was washed twice with 50 mM potassium phosphate buffer (pH 8.0) and resuspended in binding buffer (20 mM sodium phosphate buffer containing 0.5 M NaCl and 20 mM imidazole). The cells were cracked using an Ultrasonic instrument, and the cell lysate was centrifuged at 12,000 ×*g* at 4°C for 30 min to remove the particulate fraction. The soluble fraction was subjected to purification under non-denaturing conditions by Ni-affinity chromatography using a Ni-NTA-HP resin column (GE Healthcare, Sweden). The elution from the column was pooled and dialyzed using a 10 kDa cutoff membrane to remove the salts. The extract was stored at −80°C.

Measurement of Enzymatic Activity of Lysozyme

Lysozyme activity was measured according to Hultmark *et al.* [13]. The enzymatic activity of lysozyme was determined by measuring the clearing of turbidity of *Micrococcus lysodeikticus* suspension (substrate solution) at 570 nm. A decrease in absorbance of 0.001 per minute was defined as 1 U enzymatic activity compared with the control. Standard strains of *Micrococcus lysodeikticus* as the substrate, and the optimum pH and temperature of lysozyme were measured. Firstly, dried *M. lysodeikticus* cells were suspended in 50 mM phosphate buffer (OD_{570} reached about 0.3). Then 1 ml of the lysozyme was added to 2 ml of bacterial suspension in an ice-bath. These were rapidly mixed and transferred to the cuvette, and the absorbance value was read at 570 nm, denoted by A_0. Then the mixture was incubated for 30 min at 37°C, and placed on ice for 10 min to terminate the reaction. The absorbance value was read and denoted by A. The relative enzymatic activity of the lysozyme was obtained: $U_L = (A_0 - A)/A$.

Characterization of Lysozyme

The optimal pH was determined by examining the activity of the enzyme in Tris-HCl buffer. The pH value was regulated by KH_2PO_4 and K_2HPO_4 and was a range of 3–9 in 1 pH increments. Thermostability of the purified enzyme was determined by pre-incubating for 60 min at the temperatures ranging from 20°C to 80°C. Then the residual activity was analyzed.

SDS-PAGE

SDS–polyacrylamide gel electrophoresis was performed in 12% gel according to the method of Laemmli [15]. The resulting gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 45% methanol and 10% glacial acetic acid for 3 h and destained with 45% methanol and 10% glacial acetic acid.

Antimicrobial Spectrum of *Bacillus licheniformis* Lysozyme

For the agar diffusion test [17, 24], 200 µl of bacterial suspension (approximately 5×10^7 cells) was spread on LB agar plates. Freshly mixed specimens (in triplicate) of each test material were placed in holes 5 mm in diameter that were punched into the agar plate. After incubating at 37°C for 24 h, the agar plates were examined for bacterial growth inhibition. The diameter of the halo formed in the bacterial lawn was measured (mm) in two perpendicular locations for each specimen.

Results

Isolated Bacterial Strain and Sequence Analysis of Lysozyme

The strain of *B. licheniformis* TIB320 was isolated from soil in Tianjin, China. The 16S rDNA sequence of *B. licheniformis* TIB320 (1,510 bp) showed high similarity (99.9%) with *B. licheniformis* DSM13/ATCC 14580. The gene encoding lysozyme was amplified by PCR and cloned into the pMD19-T vector. Sequence analysis of the *B. licheniformis* TIB320 lysozyme gene showed that the amino acid sequence of protein shared 90% identity with that from the lysozyme gene of *B. licheniformis* DSM13/ATCC 14580 (KEGG).

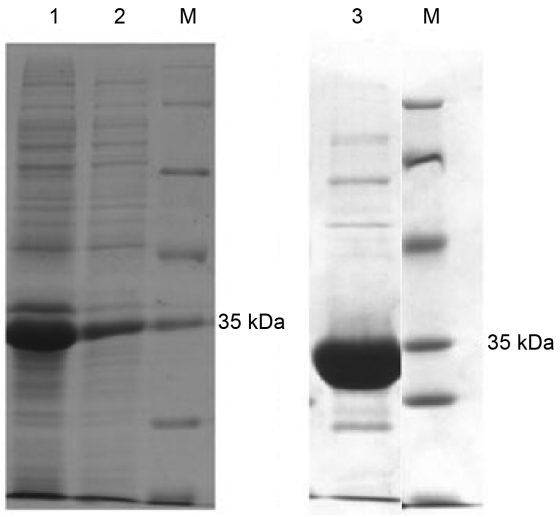

Fig. 2. SDS-PAGE analysis of lysozyme expression in *E. coli*.
SDS-PAGE was performed on a 12% gel and stained with Coomassie Brilliant Blue. Lanes 1, *E. coli* pET21b-lyso whole cell lysate; lanes 2, Soluble fraction of *E. coli* pET21b-lyso cell lysate; lane 3, Purified lysozyme; lane M, maker.

Expression and Purification of *Bacillus licheniformis* Lysozyme in *E. coli*

The lysozyme gene was amplified by PCR without the stop codon and cloned into plasmid pET21b with an C-terminal His-tag fusion. The resulting plasmid was introduced into *E. coli*, and lysozyme expression was induced by the addition of IPTG. The induced *E. coli* BL21(DE3) cells harboring pET21b-lyso were lysed and resuspended in Tris-HCl buffer.

To test the lysozyme expression, whole cell lysate and the soluble fraction of cell lysate were analyzed by SDS-PAGE. From this result, we knew the expressed lysozyme was approximately 70% of the total cellular protein, and the ratio of the soluble to insoluble fractions of lysozyme was about 1:2 (Fig. 2). The his-tagged lysozyme, as determined from the SDS-polyacrylamide gel image, was purified by a Ni-NTA-HP resin column, and the purified protein was observed as a single band on the SDS-polyacrylamide gel (Fig. 2).

Characterization and Activity of *B. licheniformis* TIB320 Lysozyme

The optimal pH value of the lysozyme activity was examined in the range of 3.0 to 9.0 at 40°C. Lysozyme was exposed to various pHs to find out the optimal pH of *B. licheniformis* TIB320 lysozyme. Enzymatic activity assay showed that lysozyme retained maximum activity at pH 6 (Fig. 3A) and 65% of the activity was retained at pH 9. However, the activity was decreased to 63% at pH 3.0 (Fig. 3A). To test the optimal temperature of the lysozyme activity, the temperature was ranged from 20°C to 80°C at the pH of 6. Results showed the optimal temperature was at 40°C (Fig. 3B). To examine if the lysozyme can be used as a feed additive, the lysozyme was incubated with trypsin or pepsin at various concentrations up to 400 mg/l, respectively (Fig. 4). The lysozyme was resistant to the trysin and pepsin to some extent and maintained about 50% of activity at 300 mg/l of trypsin and pepsin (Fig. 4).

Antimicrobial Spectrum of *B. licheniformis* TIB320 Lysozyme

We used the agar diffusion test to analyze the *B. licheniformis* TIB320 lysozyme antimicrobial activity. In the frame of this research survey, various bacteria were examined regarding their susceptibility against the lysozyme. From Table 3, we know that the lysozyme not only can inhibit gram-positive bacteria such as *Micrococcus lysodeikticus*, but also showed inhibition zones against pathogenic gram-negative bacteria, such as *Salmonella* Typhimurium and *Pseudomonas aeruginosa* (Table 3). *Saccharomyces cerevisiae*

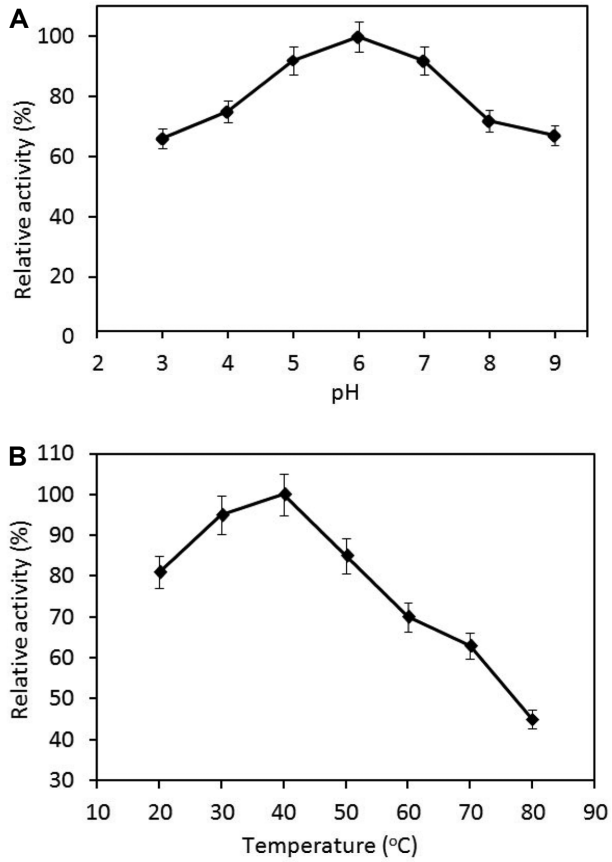

Fig. 3. Effect of pH (**A**) and temperature (**B**) on the activity of lysozyme.

was only slightly sensitive to the lysozyme, as was *Aspergillus oryzae* (Table 3). *Bacillus subtilis* seemed to tolerate higher concentrations of the lysozyme probably because of its peptidoglycan modification. It has many advantages of protein expression by using *Bacillus subtilis* as a host, so we expressed the *B. licheniformis* TIB320 lysozyme in *Bacillus subtilis*.

Expression of *Bacillus licheniformis* Lysozyme in *Bacillus subtilis* WB800

Expression of lysozyme in *E. coli* showed a high amount of production, but a certain amount of protein was inclusion body. To solve this problem, *Bacillus subtilis* was implemented as a secretory expression host. As described above, plasmid pGJ201 was constructed by fusing the P43 promoter, amylase signal peptide, and lysozyme gene in order with overlap PCR and then inserted into plasmid pGJ103 (Fig. 1). The selected *Bacillus subtilis* transformants were cultured in a shake flask and the lysozyme activity in the medium was measured (Table 4). After culture for 12 h at 37°C, lysozyme activity was obviously observed, whereas no activities were measured in the controls. We analyzed the expression of lysozyme by the Hultmark method. As depicted in Table 4, the activity of lysozyme increased with culture growth.

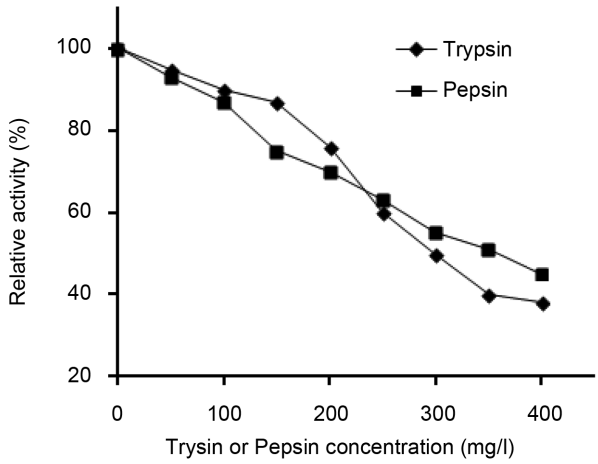

Fig. 4. Effect of trypsin/pepsin concentration on the activity of lysozyme at 40°C for 1 h.

Table 3. Antimicrobial activity of the lysozyme against various strains.

Microorginism	Inhibition zone (mm)[a]
Aspergillus oryzae	5 ± 1
Bacillus subtilis 168	5 ± 1
Bacillus sereus	9 ± 1
Clostridium sporogenes	12 ± 1
Micrococcus luteus	14 ± 2
Micrococcus lysodeikticus	20 ± 2
Pseudomonas aeruginosa	10 ± 1
Salmonella Typhimurium	10 ± 1
Saccharomyces cerevisiae	6 ± 1
Staphyloccocus aureus	8 ± 1
Streptococcus pneumoniae	12 ± 1

[a]Inhibition zone including the 5 mm hole punched into the agar plate.

Table 4. Yield of lysozyme expression in *B. subtilis* (U/ml).

Strains	6 h	12 h	18 h	24 h	30 h
B. subtilis	0	0	0	5 ± 1	5 ± 1
B.subtilis (pGJ201)	5 ± 1	14 ± 1	25 ± 2	33 ± 2	35 ± 2
B.subtilis (pGJ202)	8 ± 1	21 ± 3	35 ± 2	47 ± 3	49 ± 2
B.subtilis (pGJ203)	10 ± 2	29 ± 3	50 ± 1	75 ± 3	78 ± 3

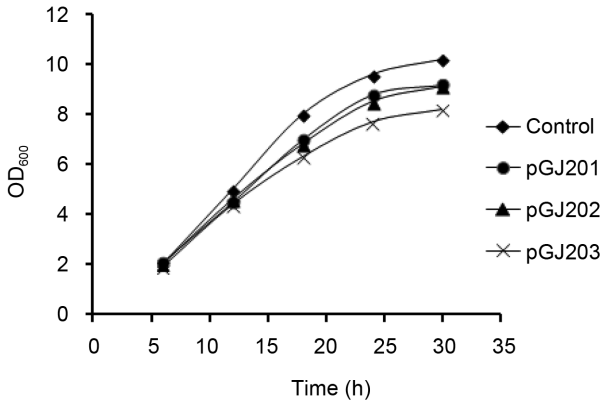

Fig. 5. Growth curve of *B. subtilis* expressing various plasmids.

Signal peptide is an important factor to target the purpose protein to the membrane, and it also affects the protein secretion efficiency. To secrete more lysozyme to the medium in *B. subtilis* WB800, amylase signal peptide was replaced by sacB signal peptide. From Table 4, we can see that sacB signal peptide was better than amylase signal peptide in secretion of lysozyme. Therefore, sacB signal peptide was used for the following experiments. Besides the signal peptide, the transcriptional level is also a major factor to determine the protein amount. To enhance the lysozyme production by increasing its gene transcript, we constructed pGJ203 by fusing the P43 promoter and sacB promoter upstream of the lysozyme gene to enhance the transcription. After fermentation for 24 h, the lysozyme production amount in *B. subtilis* (pGJ203) was significantly higher than that from *B. subtilis* (pGJ202) (Table 4). It was obvious that the expression of lysozyme was improved by the P43-sacB tandem promoter strategy, increasing the transcript of the lysozyme gene [4].

Regards comparison of the growth of *Bacillus subtilis* WB800 with various expression vectors, respectively, all strains could grow well in the medium (Fig. 5). The growth rates of *Bacillus subtilis* WB800 harboring different expression vectors were slightly slower than that of the strain with empty vector, but the production of the lysozyme did not affect the strain growth clearly (Fig. 5). Therefore, *Bacillus subtilis* WB800 was a suitable strain to produce this lysozyme.

Discussion

Lysozyme is not only the most powerful antibacterial agent found in various tissues and secretions of organisms, but is also present in a number of bacteria. Most antibacterial agents had limited antimicrobial spectra. For getting a better antimicrobial enzyme, we have conducted an extensive screening and isolated a strain that contains a lysozyme gene. Identification of the strain by 16S rDNA indicates it belongs to *Bacillus licheniformis*, which is generally regarded as safe.

Food market globalization, new manufacturing processes, and the growing demand for minimally processed, fresh-cut, and ready-to-eat products may require a longer and more complex food chain, increasing the risk of microbiological contamination. Among alternative food preservation technologies, special attention has been paid to biopreservation to extent the shelf-life and to improve the hygienic quality. Biopreservation rationally exploits the antimicrobial potential of naturally occurring micro-organisms in food and/or their metabolites with a long history of safe use. Lysozyme falls in this concept.

From the results of this article, the lysozyme of *Bacillus licheniformis* TIB320 had good effects against both gram-positive and gram-negative bacteria, such as *Micrococcus lysodeikticus*, *Clostridium sporogenes*, *Pseudomonas aeruginosa*, *etc*. Compared with egg white lysozyme [7], which can mainly inhibit gram-positive organisms, *Bacillus licheniformis* TIB320 lysozyme had a broader antimicrobial spectrum. In particular, *Salmonella* Typhimurium is a pathogenic gram-negative bacterium predominately found in the intestinal lumen. Its toxicity is due to an outer membrane consisting largely of lipopolysaccharides that protect the bacteria from the environment. *Pseudomonas aeruginosa* is a gram-negative, rod-shaped bacterium and is an opportunistic human pathogen. It is always listed as one of the top three most frequent gram-negative pathogens [30]. Inhibition activity of lysozyme to these pathogenic bacteria indicated a potential application of the enzyme as a food and feed preservative. This new *Bacillus licheniformis* lysozyme can maintain sufficient activity in a wide range of pH and temperature values, from pH 3 to 9 and 20°C to 60°C, respectively. The features of maintaining high activity at low pH and resistance to pepsin and trypsin would be a promising application for food and feed additives.

Industrial production of hen egg white lysozyme benefits large-scale purification of thousands of kilogram of chicken eggs [31]. However, the production of natural lysozymes in bacteria is limited by the inefficient protein expression system. Previous works in *E. coli* have produced inactive small quantities of enzyme or have been hampered by the isolation of inclusion bodies and refolding, which is inefficient [3, 9, 16, 23]. Several studies have been performed in yeast-based systems and moderate yields have been described in

some of these hosts [8, 18].

Because eukaryotic proteins as well as hen egg white lysozyme formed inactive material in insoluble inclusion bodies when expressed in *E. coli* [10, 22], it has been suggested that a lack of mammalian post-translational modifying enzymes and foldases causes the formation of inclusion bodies in *E. coli*. Recent work described a novel and efficient expression system capable of producing folded, soluble, and functional human lysozyme in *Escherichia coli* cells by simultaneously co-expressing multiple protein folding chaperones as well as the lysozyme inhibitory protein [16].

We expressed *B. licheniformis* TIB320 lysozyme in *E. coli* and the lysozyme activity was detected in the soluble cytosolic fraction. However, a certain amount of inclusion bodies were also observed in the insoluble form by SDS-PAGE. One reason for the formation of inclusion bodies could be the high expression rate of the protein, which does not have enough chaperones or foldases to form the native structure and correct folding of the polypeptide chain.

Motivated by the excellent fermentation properties, high product yields, and the complete lack of toxic byproducts of *Bacillus subtilis*, we sought to develop an efficient expression system for lysozymes in this bacterial host. Moreover, *Bacillus subtilis* vegetative cells have evolved mechanisms to evade or subvert the lysozyme threat by peptidoglycan modification [36]. In contrast to the secreted production of homologous proteins, the secretion of heterologous proteins is usually inefficient. The most immediate bottleneck of lysozyme expression in *B. subtilis* is the transcriptional level and protein secretion pathway. To overcome these barriers, the P43 promoter and amylase signal peptide were fused to the lysozyme gene and then expressed in *B. subtilis*. The activity of lysozyme was successfully detected, which indicated that heterogeneous expression of the enzyme was promising in *B. subtilis*. The signal peptide is a critical factor to target the enzyme to the membrane, and the protein secretion efficiency varies greatly owing to different signal peptide components. To improve the protein secretion level, amylase signal peptide was replaced by sacB signal peptide and the secretion level of enzyme was increased to some extent. In an effort to improve the production and secretion of lysozyme yields further, we constructed a tandem promoter by fusing promoter P43 and promoter sacB. The lysozyme activity was significantly increased in the *B. subtilis* (pGJ203) with the tandem promoter compared with that in the strain with a single promoter. From these results, we know that the lysozyme was expressed well in the strain with plasmid pGJ203. It is suitable for expression of the microbial lysozyme gene by a food-grade host such as *B. subtilis*.

The secretion of lysozyme showed slight effect on the growth of *B. subtilis* in this study. A lysozyme inhibitor can sequester the lysozyme's inherent bactericidal activity and protect the expression host [1, 19]. To further increase the yield of lysozyme and protect our *B. subtilis* host from the toxic effect of lysozyme expression, co-expression of a lysozyme inhibitor could be a solution to this problem in future work.

Acknowledgments

This research was supported by grants from the State Key Development Program for Basic Research of China (973 Program, 2013CB733600), National Nature Science Foundation of China (31200036, 31370089), and Key Projects in the Tianjin Science & Technology Pillar Program (12ZCZDSY12700, 11ZCZDSY08500, 11ZCZDSY08400). The authors gratefully acknowledge the support of the K. C. Wong Education Foundation, Hong Kong.

References

1. Abergel C, Monchois V, Byrne D, Chenivesse S, Lembo F, Lazzaroni JC, Claverie JM. 2007. Structure and evolution of the Ivy protein family, unexpected lysozyme inhibitors in gram-negative bacteria. *Proc. Natl. Acad. Sci. USA* **104:** 6394-6399.
2. Callewaert L, Michiels CW. 2010. Lysozymes in the animal kingdom. *J. Biosci.* **35:** 127-160.
3. Casaite V, Bruzyte S, Bukauskas V, Setkus A, Morozova-Roche LA, Meskys R. 2009. Expression and purification of active recombinant equine lysozyme in *Escherichia coli*. *Protein Eng. Des. Sel.* **22:** 649-654.
4. Chiang CJ, Chen PT, Chao YP. 2010. Secreted production of *Renilla* luciferase in *Bacillus subtilis*. *Biotechnol. Prog.* **26:** 589-594.
5. Dong H, Zhang D. 2014. Current development in genetic engineering strategies of *Bacillus* species. *Microb. Cell Fact.* **13:** 63.
6. During K. 1993. A tightly regulated system for overproduction of bacteriophage T4 lysozyme in *Escherichia coli*. *Protein Expr. Purif.* **4:** 412-416.
7. Ellison RT 3rd, Giehl TJ. 1991. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest.* **88:** 1080-1091.
8. Ercan D, Demirci A. 2013. Production of human lysozyme in biofilm reactor and optimization of growth parameters of *Kluyveromyces lactis* K7. *Appl. Microbiol. Biotechnol.* **97:** 6211-6221.

9. Fischer B, Perry B, Phillips G, Sumner I, Goodenough P. 1993. Physiological consequence of expression of soluble and active hen egg white lysozyme in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **39:** 537-540.
10. Fischer B, Perry B, Sumner I, Goodenough P. 1992. A novel sequential procedure to enhance the renaturation of recombinant protein from *Escherichia coli* inclusion bodies. *Protein Eng.* **5:** 593-596.
11. Goto T, Abe Y, Kakuta Y, Takeshita K, Imoto T, Ueda T. 2007. Crystal structure of *Tapes japonica* lysozyme with substrate analogue: structural basis of the catalytic mechanism and manifestation of its chitinase activity accompanied by quaternary structural change. *J. Biol. Chem.* **282:** 27459-27467.
12. Hayano T, Hirose M, Kikuchi M. 1995. Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell. *FEBS Lett.* **377:** 505-511.
13. Hultmark D, Steiner H, Rasmuson T, Boman HG. 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*. *Eur. J. Biochem.* **106:** 7-16.
14. Jolles P, Jolles J. 1984. What's new in lysozyme research? Always a model system, today as yesterday. *Mol. Cell. Biochem.* **63:** 165-189.
15. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680-685.
16. Lamppa JW, Tanyos SA, Griswold KE. 2013. Engineering *Escherichia coli* for soluble expression and single step purification of active human lysozyme. *J. Biotechnol.* **164:** 1-8.
17. Lewinstein I, Matalon S, Slutzkey S, Weiss EI. 2005. Antibacterial properties of aged dental cements evaluated by direct-contact and agar diffusion tests. *J. Prosthet. Dent.* **93:** 364-371.
18. Maullu C, Lampis G, Desogus A, Ingianni A, Rossolini GM, Pompei R. 1999. High-level production of heterologous protein by engineered yeasts grown in cottage cheese whey. *Appl. Environ. Microbiol.* **65:** 2745-2747.
19. Monchois V, Abergel C, Sturgis J, Jeudy S, Claverie JM. 2001. *Escherichia coli* ykfE ORFan gene encodes a potent inhibitor of C-type lysozyme. *J. Biol. Chem.* **276:** 18437-18441.
20. Petsch D, Anspach FB. 2000. Endotoxin removal from protein solutions. *J. Biotechnol.* **76:** 97-119.
21. Qasba PK, Kumar S. 1997. Molecular divergence of lysozymes and alpha-lactalbumin. *Crit. Rev. Biochem. Mol. Biol.* **32:** 255-306.
22. Sawano H, Koumoto Y, Ohta K, Sasaki Y, Segawa S, Tachibana H. 1992. Efficient *in vitro* folding of the three-disulfide derivatives of hen lysozyme in the presence of glycerol. *FEBS Lett.* **303:** 11-14.
23. Schlorb C, Ackermann K, Richter C, Wirmer J, Schwalbe H. 2005. Heterologous expression of hen egg white lysozyme and resonance assignment of tryptophan side chains in its non-native states. *J. Biomol. NMR* **33:** 95-104.
24. Schwartzman B, Caputo AA, Schein B. 1980. Antimicrobial action of dental cements. *J. Prosthet. Dent.* **43:** 309-312.
25. Simonen M, Palva I. 1993. Protein secretion in *Bacillus* species. *Microbiol. Rev.* **57:** 109-137.
26. van Dijl JM, Hecker M. 2013. *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microb. Cell Fact.* **12:** 3.
27. Wang F, Qu H, Zhang D, Tian P, Tan T. 2007. Production of 1,3-propanediol from glycerol by recombinant *E. coli* using incompatible plasmids system. *Mol. Biotechnol.* **37:** 112-119.
28. Wei JT, Tang CD, Wu MC, Liu GL, Shi HL, Li JF. 2012. Cloning and functional expression of a human lysozyme gene (*hly*) from human leukocytes in *Pichia pastoris*. *Mol. Med. Rep.* **6:** 173-178.
29. Westers L, Westers H, Quax WJ. 2004. *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. *Biochim. Biophys. Acta* **1694:** 299-310.
30. Wiehlmann L, Wagner G, Cramer N, Siebert B, Gudowius P, Morales G, *et al.* 2007. Population structure of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **104:** 8101-8106.
31. Wilken LR, Nikolov ZL. 2011. Process evaluations and economic analyses of recombinant human lysozyme and hen egg-white lysozyme purifications. *Biotechnol. Prog.* **27:** 733-743.
32. Yeh CM, Yeh CK, Hsu XY, Luo QM, Lin MY. 2008. Extracellular expression of a functional recombinant *Ganoderma lucidium* immunomodulatory protein by *Bacillus subtilis* and *Lactococcus lactis*. *Appl. Environ. Microbiol.* **74:** 1039-1049.
33. Zhang D, Shan SO. 2012. Translation elongation regulates substrate selection by the signal recognition particle. *J. Biol. Chem.* **287:** 7652-7660.
34. Zhang D, Shrestha B, Li Z, Tan T. 2007. Ubiquinone-10 production using *Agrobacterium tumefaciens dps* gene in *Escherichia coli* by coexpression system. *Mol. Biotechnol.* **35:** 1-14.
35. Zhang D, Sweredoski MJ, Graham RL, Hess S, Shan SO. 2012. Novel proteomic tools reveal essential roles of SRP and importance of proper membrane protein biogenesis. *Mol. Cell. Proteomics* **11:** M111 011585.
36. Zipperle GF Jr, Ezzell JW Jr, Doyle RJ. 1984. Glucosamine substitution and muramidase susceptibility in *Bacillus anthracis*. *Can. J. Microbiol.* **30:** 553-559.