JOURNAL OF PURE & APPLIED MICROBIOLOGY, Nov. 2014. Vol. **8**(Spl. Edn. 2), p. 43-50

Secretion Expression of Recombinant *Stichopus japonicus* Lysozyme in *Bacillus subtilis* WB600

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

School of Biological Engineering, Dalian Polytechnic University, Dalian Liaoning, P.R. 116034, China.

(Received: 20 August 2014; accepted: 30 October 2014)

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*). The growth rate of engineering bacteria was consistent with that of wild type strain, WB600, and the presence of lysozyme did not affect its growth. The stability analysis showed that there was no gene rearrangement and lost of the recombinant plasmid in the bacteria which showed that it has high genetic stability. Soluble SjLys was successfully expressed with 1 mmol/L IPTG treatment for 36, 48 h and the soluble protein showed remarkable inhibitive effect on the growth of the *Vibrio parahaemolyticus*. To our knowledge, this is the first report of the *SjLys* gene authentic heterologous expressed in *Bacillus subtilis* and it may provide a new way for recombinant SjLys production by this secretion system.

Key words: Sea cucumber lysozyme, *Bacillus subtilis*, Genetic engineering bacteria, Antimicrobial activity.

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 past decades[1-2]. However, recently, 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 *Stichopus 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[3-4].

* To whom all correspondence should be addressed. Tel.: +86-411-86323646; Fax: +86-411-86323646; E-mail: alzw@dlpu.edu.cn

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 non-enzymatic activity in some species[5-7]. 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), which was identified mainly in vertebrates including mammals, birds and fish; invertebrate-type lysozyme (i-lysozyme), plant lysozyme, bacterial lysozyme and phagelysozyme[8-10].

Stichopus japonicus relies on their innate immune system to resist invasion of prokaryotic and eukaryotic pathogens. Therefore, the lysozyme would play an important role in the immune, digestive system and autolysis for the sea cucumber which signifies that it is important to both immunity and growth[11-13]. Many comprehensive studies have been performed and showed that the *Stichopus japonicus* lysozyme is belong to i-type lysozyme, which widely existed in every tissue of sea cucumber, even in the gastrovascular cavity liquids. Therefore, the utilization of the sea cucumber i-type lysozyme should be very helpful for sea cucumber farming. Recently, many lysozyme genes have been identified through sequencing or protein purification and some of them were well-studied for their functions in immunity[3,7]. Our previous work showed that the recombinant *Stichopus japonicas* lysozyme protein (rSjLys) in *Escherichia coli* (*E.coli*) has broad-spectrum antimicrobial activity, the enzyme can lyse not only the gram-positive bacteria but also the gram-negative bacterium. Especially, it has obvious effect on the serious aquatic animal diseases caused by pathogenic *vibrio* and *pseudomonas*. Therefore, the recombinant SjLys should play important role on aquatic animal disease control by substituting antibiotics which could cause serious enviromental contamination, and it has aroused attention of the domestic and overseas experts and scholars[3] .

The high cost and quite complex process by the traditional methods for lysozyme production limited lysozyme application to aquaculture. At present, the biological engineering methods for lysozyme production has been thought to be the most possible means to solve this problem. Genetic engineering *E. coli* was successfully used for recombinant lysozyme production in many previous studies. However, the low production efficiency and unstable enzyme activity of recombinant lysozyme by *E. coli* also limited further application for market demand. In addition, most of recombinant lysozyme existed as inclusion body that complicated subsequent active protein purification[14-16]. 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*[17-19]. 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 non pathogenic and does not produce any endotoxins, hence it is generally regarded as safe organism (GRAS)[20-22]. The other advantages of *Bacillus 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[15,23-25]. These further make the *Bacillus subtilis* a potential *bacillus* in genetic engineering and industrial application[26-31].

In this study, the *Stichopus japonicus* lysozyme gene (*SjLys)* was subcloned to *Bacillus subtilis* expression vector pHT43. After transformation of pHT43-*SjLys* to *Bacillus subtilis* strain WB600, *SjLys* was successfully expressed and then secreted to the medium, which showed potential application to i-type lysozymes production for aquaculture.

MATERIALS AND METHODS

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

E.coli DH5$_{\alpha}$, pMD18-T Simple Vector, *Taq* DNA polymerase, T4 DNA ligase, gel extraction and purification Kit, plasmid extraction kit, 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 Vibrio parahaemolyticus* was preserved in our lab.

Primers design and amplification of the target *SjLys* gene

Based on the published cDNA sequence (EF036468) of *SjLys*, a pair of specific primers were designed by using the Primer Premier 5.0 software. The upstream primer and downstream primer are designed respectively as following, HS-Q-P11: 5'-

GCCGGATCCATGCAAGTTCCTTCTG-3', HS-Q-P12: 5'-GCCCGGGAATTCTCAGTTGTTGCTC-3'. The restriction sites of *Bam*HI and *Sma*I were introduced into the amplified fragments by the primers (underline), and the box indicated the start or stop codon.

Amplifications were done using a PTC 225 Peltier Thermal Cycler (MJ Research Inc. USA) with the following conditions: 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, and holding at 4°C.

Construction of the expression vector pHT43-SjLys

The amplified *SjLys* fragment was cloned in vector pMD18T-Simple to yield pMD18T-Simple-SjLys. The plasmid pMD18T-Simple-SjLys and pHT43 were digested with *Bam*HI/*Sma*I, then the *SjLys* fragment and digested pHT43 were ligated by T4 DNA ligase to transform comptent cell ($DH5_{\alpha}$). Positive colony was confirmed by colony PCR and the sequence was verified by plasmid sequencing.

Transformation of *Bacillus subtilis*

The pHT43-SjLys plasmid with a chloramphenicol resistance marker was used for transformation. It was transformed into *Bacillus subtilis* WB600 competent cells using electrotransformation. Transformants were screened on LB agar plates containing 10 µg/mL chloramphenicol. The positive recombinant engineering strain named as pHT43-SjLys/WB600 was identified by PCR analysis, restriction enzyme digestion and sequencing.

The growth curve and genetic stability of the pHT43-SjLys/WB600

Cell growth curve was measured according to Shoham *et al.* [32] with minor modifications. The wild type WB600, pHT43/WB600 and pHT43-SjLys/WB600 (with 10 µg/mL chloramphenicol) were activated in Luria-Bertani medium (LB). Cells were 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. The cell growth was determined by measuring OD_{600} of the cell culture with indicated intervals 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 according to Avsaroglu *et al.*[33] and Leen *et al.*[34]. All assays were performed in triplicates and the results are the means of three independent experiments.

SDS-polyacrylamide gel assay

The recombinant bacterium pHT43-SjLys/WB600 was cultured in LB medium containing 10µg/mL chloramphenicol and induced by 1 mmol/L isopropylthiogalactoside (IPTG) for 12 h, 24 h, 36 h, and 48 h respectively. The fermentation broth was centrifuged at 12,000 rpm under 4°C for 10 min to remove cells, and then run sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) for recombinant protein analysis. Proteins were visualized by Coomassie Brilliant Blue staining.

Antimicrobial activity assay

Antimicrobial activity of the recombinant SjLys was assessed with the radial diffusion assay as described by Cong *et al.*[35]. *Vibrio parahaemolyticus* strain was used as substrates. The bacterial concentration in the fermentation broth was adjusted to OD_{600}=0.1 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 wild type strain WB600 and pHT43/WB600 were used as negative controls.

RESULTS

The fragment of the *Stichopus japonicus* lysozyme gene

The *Stichopus japonicu* lysozyme gene (*SjLys*) was re-amplified 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 Figure 1A. In the Figure 1A, an anticipated 400 bp fragment was clearly observed. The sequencing result revealed that the nucleotide sequence of the re-amplified fragment was identical to the reported cDNA of *Stichopus japonicus* body wall in NCBI (EF036468). These results showed that the *SjLys* gene from *Stichopus*

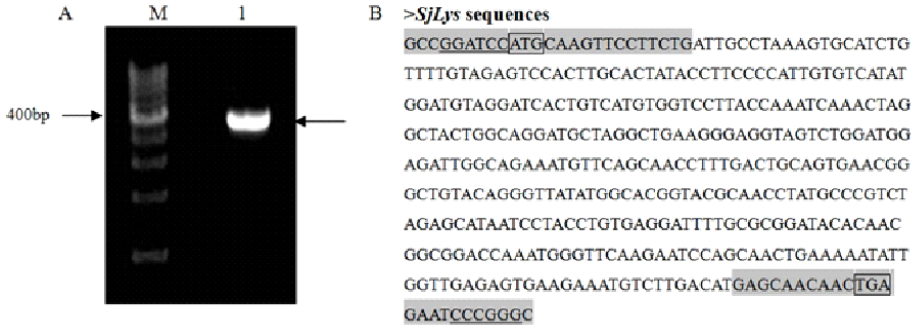

Fig. 1. A: PCR amplification of the target gene *SjLys* (M:100 bp DNA ladder marker, lane 1: target gene *SjLys*.) **B:** Sequence of the re-amplified *SjLys* fragment. (The boxes indicate the start or stop codon, the gray regions represent the primers sequences and the underlines indicate the restrict enzyme sites).

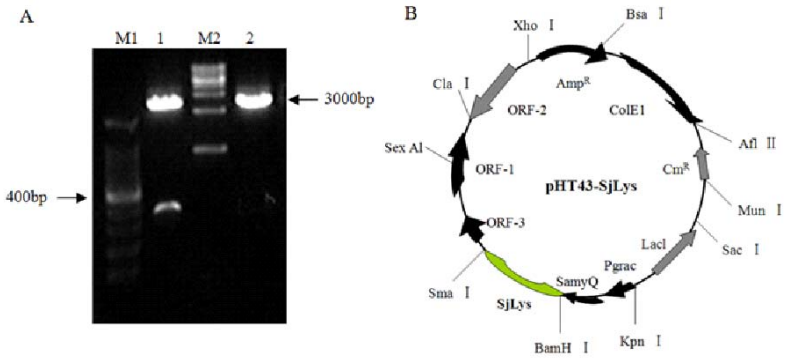

Fig. 2. A: Recombinant plasmid pMD18T-simple-SjLys double digestion verification (M1 and M2: 100 and 1000 bp DNA ladder marker, lane 1: double enzyme digestion, lane 2: pMD18T-Simple-SjLys) **B:** The map of the recombinant expression vector pHT43-SjLys

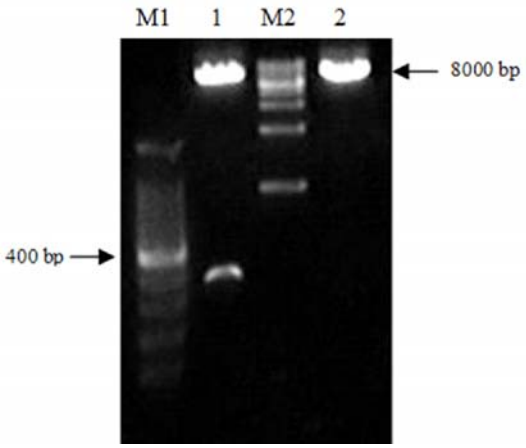

Fig. 3. The double digestion verification from the pHT43-SjLys/WB600 plasmid. (M1 and M2: 100 and 1000 bp DNA ladder marker, lane1: double enzyme digestion, lane 2: pHT43-SjLys).

japonicus has been successfully obtained. Additionally, the analysis of DNA sequence further showed that the lysozyme gene *SjLys* contains 375 bp length fragment that encodes a mature polypeptide of 125 amino acid (Figure 1B).

Construction and identification of the pHT43-SjLys

The target *SjLys* fragment was extracted and purified. The product was then digested with *Sma*I and *Bam*HI, and finally subcloned to vector pMD18T-Simple to yield a recombinant plasmid pMD18T-Simple-SjLys with 3077 bp length that was identified and verified by PCR and restriction enzyme digestion (Figure 2A).

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 the *SjLys* gene was

ligated to pHT43 for a recombinant expression vector, pHT43-SjLys, construction with 8432bp length (Figure 2 B).

Transformation of *Bacillus subtilis*

The recombinant expression plasmid pHT43-SjLys was selected and transformed to *Bacillus subtilis* WB600. The *Bacillus subtilis* transformants were selected by 10 µg/ml of chloroamphenicol. The genetic engineering strain designed pHT43-SjLys/WB600 was successfully obtained by further verification with PCR, double-digestion (Figure 3) and sequencing analysis.

Growth curve of the pHT43-SjLys/WB600

The growth curves of the pHT43-SjLys/ WB600, pHT43/WB600 and WB600 are presented in Figure 4. It showed that the engineering strain pHT43-SjLys/WB600, pHT43/WB600 and wild type WB600 growth tendency are very identical. WB600 entered the logarithmic growth phase after 1.5 h inoculation and ended after 6 h, while the engineering strain were correspondingly 1 h and 5 h. After logarithmic growth phase, the growth rate kept consistent in each strain. The growth curves demonstrated that the presence of expressed *SjLys* did not affect the growth of the engineering bacteria pHT43-SjLys/WB600.

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 cultured in the absence of antibiotics LB medium and the bacterium was further used for plasmid stability analysis. The results showed that recombinant plasmid has excellent genetic stability in the absence of

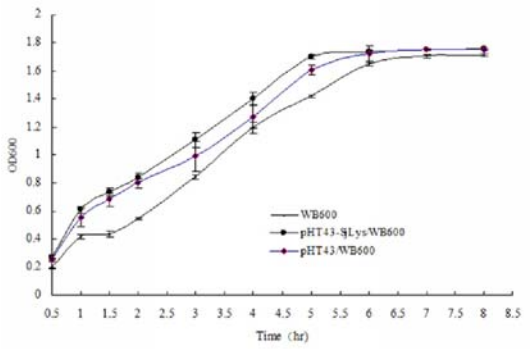

Fig. 4. The growth curves of the *Bacillus subtilis* WB600, pHT43/WB600 and pHT43-SjLys/WB600

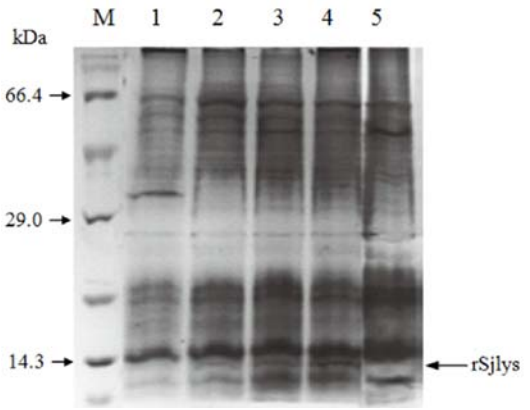

Fig. 6. SDS-PAGE analysis of the recombinant SjLys protein secreted by pHT43-SjLys/WB600. (M: Molecular mass markers; lanes 1, 2, 3 and 4: Total cellular protein induced by IPTG for 12, 24, 36, 48 h respectively; lane 5: the control WB600)

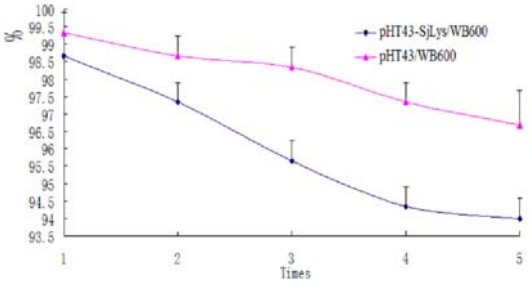

Fig. 5. The stability of engineered bacteria pHT43-SjLys/ WB600

selection pressure with approximately 94% after 5 times (100 generation) continuous passage culture (Figure 5).

Transformed plasmid DNA in pHT43-SjLys/WB600 with 3 and 5 times continuous passage culture were extracted for digestion analysis. The result demonstred that no rearrangement or lost of plasmid DNA was found in the engineering bacteria.

SDS-PAGE analysis

The recombinant pHT43-SjLys/WB600 was used for heterologous *SjLys* expression. After IPTG induction for 12, 24, 36 and 48 h, the recombinant SjLys was purified and analyzed by running 12% SDS-PAGE. The SDS-PAGE analysis of the rSjLys demonstrated that there was a faint band with a molecular weight of approximately 14.0 kDa after induced for 36 and 48 h (Figure 6). These

results confirmed that the rSjlys was successfully expressed but with low-level.

Antimicrobial activities of the recombinant proteins

The radial diffusion assay was employed to evaluate the antimicrobial spectrum of the recombinant SjLys against pathogenic bacteria *Vibro parahaemolyticus*, which is commonly present in coastal marine environments and causing disease of *Stichopus japonicas*. The wild type WB600 and pHT43/WB600 were used as the controls.

Based on the radius of the antimicrobial zone, the recombinant SjLys from pHT43-SjLys/WB600 displayed a remarkable inhibitory effect on the growth of *Vibro parahaemolyticus* (Figure 7). Based on above experimental results, it is suggested that the heterologous expression of *Sjlys* was successfully achieved in *Bacillus subtilis*.

DISCUSSION

Sea cucumber (*Stichopus japonicus*) is economically important farmed echinoderm species in Northern China. However, infectious diseases are becoming a severe problem need to be solved for aquaculture. Disease caused by *Vibrio* is the most wide-spread in sea cucumber farming. Antibiotics and chemotherapeutics used to control these diseases can result in 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 should be adding of Lysozyme in feed[36-38].

To our knowledge, this is the first study about the *Stichopus japonicus* lysozyme gene expression in *Bacillus subtilis*, which provide a new way for *SjLys* gene expression and efficient utilization. Undoubtedly, this should accelerate the biotechnological application of *Bacillus subtilis* in industry field, and be helpful for the prevention and treatment of the sea cucumber disease[14].

Fig. 7. Assay of antimicrobial activity of the recombinant SjLys protein to*Vibrio parahaemolyticus*

Unfortunately, the concentration of expressed protein by genetic engineering strain pHT43-Sjlys/WB600 is not high and can't well meet market's need, and the production process of rSjlys protein in pHT43-SjLys/WB600 still needs to be further optimized. We are trying to insert the *Sjlys* gene in a new *Bacillus subtilis* strain, HS-38A, for further genetic modification, which it was isolated from the intestine of wild sea cucumber in Dalian sea area[35].

ACKNOWLEDGEMENTS

This research was supported by the Natural Science Foundation of China (Grant no. 31072224) and Natural Science Foundation of Liaoning Province of China (Grant no. 20102009).

REFERENCES

1. Dong, X.P., Zhu, B.W., Sun, L.M., *et al*. Changes of collagen in sea cucumber (*Stichopus japonicas*) during cooking. *Food. Sci. Biotechnol.,* 2011; **20**(4): 1137-1141.
2. Wu, H.T., Li, D.M., Zhu, B.W., *et al*. Characterization of acetylcholinesterase from the gut of sea cucumber *Stichopus japonicus*. *Fish Sci.,* 2013; **79**: 303-311.
3. Cong, L.N., Yang, X.J., Wang, X.X., *et al*. Characterization of an i-type lysozyme gene from the sea cucumber *Stichopus japonicus*, and enzymatic and nonenzymatic antimicrobial activities of its recombinant protein. *J. Biosci. Bioeng.*, 2009; **107**(6): 583-588.
4. Liu, H. Z., Zheng, F.R., Sun, X.Q., *et al*. Construction of cDNA library from intestine, Mesentery and Coelomocyte of *Apostichopus japonicus* selenka infected with *Vibrio sp.* and a preliminary analysis of immunity-related genes. *J. Ocean Univ. China*, 2012; **11** (2): 187-196.
5. Alma, B.P., Adriana, T.M., Aldo, A.A., *et al*. Shrimp invertebrate lysozyme i-lyz: Gene structure, molecular model and response of c and i lysozymes to lipopolysaccharide (LPS). *Fish Shellfish Immun.*, 2012; **32**: 230-236.
6. Gao, F.Y., Qu, L., Yu, S.G., *et al*. Identification and expression analysis of three c-type lysozymes in *Oreochromis aureus*. *Fish Shellfish Immun.,* 2012; **32**: 779-788.
7. Zhang, H.W., Sun, C., Sun, S.S., *et al*. Functional analysis of two invertebrate-type lysozymes from red swamp crayfish, *Procambarus clarkia*. *Fish Shellfish Immun.,* 2010; **29**: 1066-1072.
8. Abdou, A.M., Higashiguchi, S., Aboueleinin, A.M., *et al*. Antimicrobial peptides derived from hen egg lysozyme with inhibitory effect against *Bacillus species*. *Food Control*, 2007; **18**: 173-178.
9. Bathige, S.D., Umasuthan, N., Kasthuri, S. R., *et al*. A bifunctional invertebrate-type lysozyme from the disk abalone, Haliotis discus: Genome organization, transcriptional profiling and biological activities of recombinant protein. *Dev. Comp. Immunol.,* 2013; **41**: 282-294.
10. Radka, J., Marcela, S., Petra, P., *et al*. Identification and cloning of an invertebrate-type lysozyme from *Eisenia andrei*. *Dev. Comp. Immunol.,* 2009; **33**: 932-938.
11. Yang, A.F., Zhou, Z.C., Dong, Y., *et al*. Expression of immune-related genes in embryos and larvae of sea cucumber *Apostichopus japonicus*. *Fish Shellfish Immun.*, 2010a; **29**: 839-845.
12. Yue, X., Liu, B.Z., Xue, Q.G. An i-type lysozyme from the Asiatic hard clam *Meretrix meretrix* potentially functioning in host immunity. *Fish Shellfish Immun.*,2011; **30**: 550-558.
13. Yue, X., Wang, H.X., Huang, X.H., *et al.*) Single nucleotide polymorphisms in i-type lysozyme gene and their correlation with vibrio-resistance and growth of clam *Meretrix meretrix* based on the selected resistance stocks. *Fish Shellfish Immun.,* 2012; **33**: 559-568.
14. Fatma, M.M., Carlos, D.D., Marc, B. High transformation efficiency of *Bacillus subtilis* with integrative DNA using glycine betaine as osmoprotectant. *Anal. Biochem.,* 2012; **424**: 127-129.
15. Zhang, A.L., Liu, H., Yang, M.M., *et al*. Assay and characterization of a strong promoter element from *B. subtilis*. *Biochem. Bioph. Res. Co.,* 2007; **354**: 90-95.
16. Zou, M.Y., Li, X.Z., Shi, W.J., *et al*. Improved production of alkaline polygalacturonate lyase by homologous overexpression *pelA* in *Bacillus subtilis*. *Process Biochem.*, 2013; **48**: 1143-1150.
17. Liu, Y.H., Lin, S., Zhang, X.Q., *et al*. A novel approach for improving the yield of *Bacillus subtilis* transglutaminase in heterologous strains. *J. Ind. Microbiol. Biotechnol.,* 2014; **41**: 1227-1235.
18. Mu, L., Wen, J.P. Engineered *Bacillus subtilis* 168 produces L-malate by heterologous biosynthesis pathway construction and lactate dehydrogenase deletion. *World J. Microbiol. Biotechnol.,* 2013; **29**: 33-41
19. Sharipova, M.R., Shagimardanova, E.I., Chastukhina, I.B., *et al*. The expression of

Bacillus intermedius glutamyl endopeptidase gene in *Bacillus subtilis* recombinant strains. *Mol. Biol. Rep.*, 2007; **34**: 79-87.

20. Ranjita, B., Masaru, Y., Hideki, N., *et al*. Enhanced production of 2,3-butanediol by engineered *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.*, 2012; : 651-658.
21. Wang, X.Y., Chen, W.J., Tian, Y.L., *et al*. Surface display of *Clonorchis sinensis* enolase on *Bacillus subtilis* sporespotentializes an oral vaccine candidate. *Vaccine*, 2014; **32**: 1338-1345.
22. Yang, M.M., Zhang, W.W., Chen, Y.L., *et al*. Development of a *Bacillus subtilis* expression system using the improved *Pglv* promoter. *Microb. Cell Fact.*, 2010b; 9: **55**: 1-8.
23. Heinzmann, S., Entian, K., Stein, T. Engineering *Bacillus subtilis* ATCC 6633 for improved production of the lantibiotic subtilin. *Appl. Microbiol. Biotechnol.*, 2006; **69**: 532-536.
24. Reza, P., Ebrahim, V. F., Seyed, A.S., *et al*. Induction of *Bacillus subtilis* expression system using environmental stresses and glucose starvation. *Ann. Microbio.*, 2014; **l64**: 879-882.
25. Yuan, Y., Feng, F., Chen, L., *et al*. Surface display of *Acetobacter pasteurianus AdhA* on *Bacillus subtilis* spores to enhance ethanol tolerance for liquor industrial potential. *Eur. Food Res. Technol.*, 2014; **38**: 285-293.
26. Bahri, D.O., Numan, O. Expression of thermostable-amylase gene from *Bacillus stearothermophilus* in various *Bacillus subtilis* strains. Ann. Microbiol., 2008; **58** (2): 265-267.
27. Chittibabu, G., Ma, C., Netterm H.J., *et al*. Production, characterization, and immunogenicity of a secreted form of Plasmodium falciparum merozoite surface protein 4 produced in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.*, 2014; **98**: 3669-3678.
28. Kim, C.K., Oh, S.D., Rhee, J., *et al*. Expression and purification of recombinant human bone morphogenetic protein-7 (rhBMP-7) in *Bacillus subtilis*. *Biotechnol. Genet. Eng.*, 2010; **15**: 830-836.
29. Li, S.S., Wen, J.P., Jia, X.Q. Engineering *Bacillus subtilis* for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression. *Appl. Microbiol. Biotechnol.*, 2011; **91**: 577-589.
30. Liu, Y.H., Lu, F.P., Li, Y., *et al*. Characterisation of mutagenised acid-resistant *alpha- amylase* expressed in *Bacillus subtilis* WB600. *Appl. Microbiol. Biotechnol.*, 2008; **78** :85-94.
31. Pan, Z.W., Cunningham, D.S., Zhu, T., *et al*. Enhanced recombinant protein production in pyruvate kinase mutant of *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.*, 2010; **85**: 1769-1778.
32. Shoham, Y., Israeli, E., Sonensheim, A. L., *et al*. Inhibition of growth of *Bacillus subtilis* by recombinant plasmid pCED3. *Arch. Microbiol.*, 1991; **156**: 204- 212.
33. Avsaroglu, M.D., Buzrul, S., Sanlibaba, P., *et al*. A kinetic study on the plasmid stability of three Lactococcus lactis strains. *J. Ind. Microbiol. Biotechnol.*, 2007; **34**(11):729-37.
34. Leen, D.G., Jos, M.P., Paul, J., *et al*. Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. *Microbiology*, 2007; **153**: 452-463.
35. Cong, L.N., Ruan, Q.K., Liu, Z.W., *et al*. Isolation of an antimicrobial marine strain HS-A38 and purification of its bioactive substances. *J. Pure Appl. Microbio.*, 2013; **7**(1): 379-385.
36. Qi, H., Dong, X.P., Cong, L.N., *et al*. Purification and characterization of a cysteine-like protease from the body wall of the sea cucumber *Stichopus japonicus*. *Fish Physiol. Biochem.*, 2007; **33**:181-188.
37. Sun, L.M., Zhu, B.W., Wu, H.T., *et al*. Purification and characterization of *Cathepsin B* from the Gut of the Sea Cucumber (*Stichopus japonicas*). *Food Sci. Biotechnol.*, 2011; **20**(4): 919-925.
38. Wang, T.T., Sun, Y.X., Jin, L.J., *et al*. Enhancement of non-specific immune response in sea cucumber (*Apostichopus japonicus*) by *Astragalus* membranaceus and its polysaccharides. *Fish Shellfish Immun*., 2009; **27**: 757-762.