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Characterization of a Neutral Protease Gene of *Bacillus subtilis* Isolated from the Guts of *Bombyx mori*

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ABSTRACT

To explore distribution and probiotics function of enzyme-producing bacteria in gut of the silkworm, a *Bacillus subtilis* strain (No.951NA) was isolated from the guts of the fourth instar larvae of *Bombyx mori*. The strain was separated using NA casein medium and identified by the morphology and the 16S rDNA sequences analysis. In vitro assays, the enzyme activity was 24.2U•mL^{-1}. Meanwhile, a gene encoding a kind of neutral protease was cloned and its prokaryotic expression in *Escherichia coli* was also carried out. The cDNA of this gene was 2644bp encoding a protein with a molecular weight of about 60 kD, which was confirmed by SDS-PAGE. The positive transformant strains were cultured with different concentrations of IPTG and their enzymatic activities of expressed proteins was determined by Folin-phenol method, which demonstrated that the constructed expression system had an efficient expression capacity for the neutral protease NPR. These results provided a foundation for further function study of this neutral protease.

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Authors' Contributions
ZW, WY and CL executed the experimental work. DL and LS analyzed the data. ZW, WY and BZ wrote the article.

Key words
***Bombyx mori, Bacillus subtilis*, neutral protease, 16S r DNA, prokaryotic expression.**

INTRODUCTION

The silkworm is an economically important insect that also acts as an important model in Lepidoptera research (Xia *et al.*, 2004). During the growth stage, the enzymes produced by probiotic bacteria in the silkworm intestine contribute to the digestion of the silkworm's food. These bacteria can also inhibit the growth of pathogenic microorganisms (Washburn *et al.*, 1995; Stoven *et al.*, 2000; Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004). Therefore, the study of the silkworm intestinal flora, enzyme production and biological function will play important roles in the improvement of the intestinal micro-ecological environment of the silkworm and in the development of an artificial diet. Limited studies on the intestinal bacteria of the silkworm have been reported since 1990, however studies of the isolation and identification of gut bacteria and digestive enzymes produced in the silkworm gut (Byeon *et al.*, 2005; Anand *et al.*, 2010; Shi *et al.*, 2010) were conducted, and some microbial flora was found to be closely related to silkworm physiology, metabolism and disease resistance (Campbell, 1989).

B. subtilis is an important probiotic applied to many types of mammalian feed to improve their intestinal microenvironment. The gene for neutral protease of *B. subtilis* has been cloned and expressed in yeast (Stahl and Ferrari, 1984; Sloma *et al.*, 1988, 1990). Furthermore, a genetically modified bacterium with high fibrinolytic activity (Yang *et al.*, 1984; Tran *et al.*, 1991) has been cultivated. *B. subtilis* strains with high-activity proteases from different sources have been reported by several laboratories (Uchida *et al.*, 2004; Gerze *et al.*, 2005; Orhan *et al.*, 2005; Pillai and Archana, 2008; Moradian *et al.*, 2009). A *B. subtilis* strain with protease belonging to a family of serine proteases having strong fibrinolytic protease activity has also been reported (Kim *et al.*, 2006). To develop probiotic feed additives, Sun *et al.* (1996) separated several strains of aerobic and facultative anaerobic microbes from the silkworm intestine and selected a partial composite mixture to feed to the silkworms on the last day of their 4th instar. The experiment showed a clear reduction in morbidity and a great increase in the overall cocoon quantity and number of cocoon layers. As proteins are major components of essential nutrients for silkworms, proteases are important for the improved digestion and absorption of food protein. Silkworm larval digestive juices lack their own digestive enzymes and require probiotics to produce enzymes, which can be added to the artificial feed or smeared on the surface of mulberry leaves. This practice assists the silkworms in fully digesting and absorbing nutrients such as protein and starch, improving the feed efficiency (Anand *et al.*, 2010). To obtain a stronger effect, investigation on enzyme-coding genes from the intestinal flora of the silkworm will be conducive to further

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improving the application potential of microecologics. Furthermore, although probiotics producing protease, such as *B. subtilis*, have been widely used in industrial production, silkworm-originated probiotics have rarely been reported. This paper reports the isolation of a bacterial strain with high protease activity from the intestine of silkworms, and characterization of the neutral protease gene and its expression in *Escherichia coli.*

MATERIALS AND METHODS

Isolation of protease producing bacteria from larva gut

The 4th instar larva of silkworm of the variety *B. mori* 951 provided by the Department of Sericulture, Anhui Agricultural University, was fed on mulberry leaves under normal conditions.

For isolation of gut, the larva were starved for 24 h, and then fixed in 75% alcohol. The larva was dissected and the guts taken out and mashed in a centrifuge tube. One mL of the intestinal juice obtained after centrifugation was cultured in enrichment medium at 150 rpm and 28°C for 48 h in a shaking incubator. Then the fermentation broth was diluted to 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}. Each concentration of the diluted fermentation broth (0.1m L) was cultured by the streak plate method in Luria- Bertani (LB) medium at 28°C for 48 h till a single colony acquired. When the single colonies appeared, a single colony was picked for streaking to single bacterium. The separation and purification was conducted 3 times to ensure the purity of the strain.

Each purified strain was inoculated on the plates with 1% casein in order to acquire transparent circles on the casein plates in the experimental groups. The protease activity was determined by the Folin-phenol method using 1% casein as substrate (Lowry *et al.*, 1951).

Ribotyping of the target strains

The protease producing bacterial culture (1m L) during log phase was collected and centrifuged for isolation of DNA using a bacterial DNA extraction kit (Shanghai Shenggong Company) according to the manufacturer's instructions.

The isolated DNA was PCR amplified for 16S r DNA using primers
27f-1492r (27f: 5'- AGAGTTTGATCCTGGCTCAG-3'; 1492r: 5'- TACGGYACCTTGTTACGACTT-3').

The PCR reaction mixture (25µL) composed of DNA(1µL), primers (3µL), d NTPs (2µL), PCR reaction buffer (2.5µL, including Mg^{2+}) and Taq DNA polymerase (0.5µL). PCR amplification was followed using thermal cycle of 94°C for 10min, followed by 35 cycles each of 94°C for 35s, 54°C for 40s and 72°C for 90s, the extension was done at 72°C for 10min. The PCR products observed in the agrose gel was excised and then purified using a DNA purification kit (Shanghai Shenggong Company). The purified PCR DNA was checked in the gel and then ligated into the PMD-19T plasmid using DNA ligase (Shanghai Shenggong Company). The recombinant plasmid was used for transformation of the DH5α strain. The positive colonies were picked up. Then the DNA was isolated and gotten sequenced by the Company of Shanghai Invitrogen.

The 16S rDNA was compared with similar sequence in the data bank using BLAST, ClustalW, and Interproscan at NCBI for sequence alignment and homology comparison. The neighbor-joining law for evolutionary trees and the Signal P3.0 Server for signal peptide prediction (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used.

Cloning of the protease gene of B. subtilis

Three pairs of primers were designed according to Primer premier5.0 software as follows:
PrimerZ1: 5'-GCACGTGCTAGGGGCGTTGG-3',
PrimerZ2: 5'-ACGGTCAGCCAGCCTGCTCT-3';
PrimerZ3: 5'-CATTGTGGGTTTAGGTAAGA-3',
PrimerZ4: 5'-CGGGCAGACTGAATGAGA-3';
PrimerZ5: 5'-TGCTGTTTTTGGCCGCTCCGT-3',
PrimerZ6: 5'-CAGACACTCCCGCCAGCAGC-3'.

The PCR was followed using thermal cycle of 94 °C for 5min, followed by 35 cycles each of 94°C for 35s, 59°C for 40s and 72°C for 90s, the extension was done at 72°C for 10min. The PCR products observed in the agrose gel and sequenced by Invitrogen, Shanghai. Other program was as same as described above.

Construction of the prokaryotic expression vector and protein expression

The primers npr-B
(5'-CA*GGATCC*ATGAGTTTATCAATCAGCCT-3')
and npr-X
(5'-GAC*CTCGAG*TTACAATCCAACAGCATTCC-3')
(the italicized sequences correspond to the cutting sites of *Bam*HI and *Xho*I) were designed to amplify the open reading frame (ORF) of the target gene. The PCR conditions were the same as described above. The PCR product and pET-28a vector were ligated after they were both digested with restriction enzymes BamHI and XhoI. The recombinant plasmids (pET-28a-npr) were identified by sequencing and then transformed into *E.coli* BL21 (DE3) cells (Beijing TransGen Co., Ltd., Beijing, China) for protein expression.

The inductive expression of enzyme in the *E.coli* BL21 (DE3) cells was done by using different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM) of

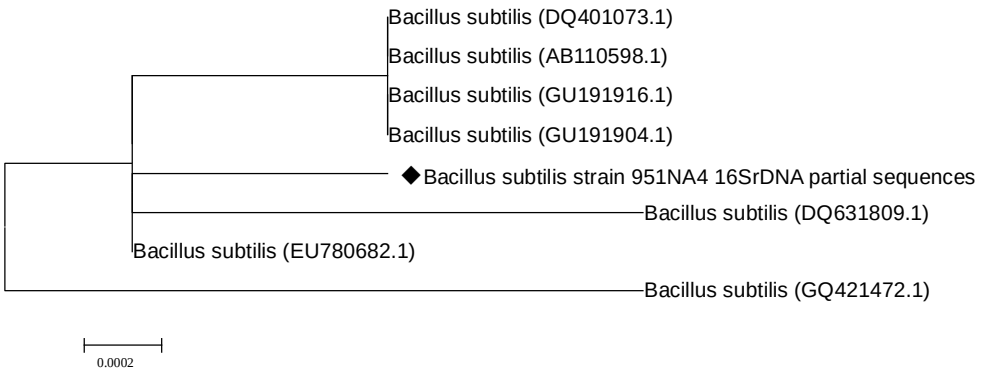

Fig. 1. Phylogenetic tree of the 16SrDNA sequences of the strain of 951NA4 and related strains. The strain of 951NA4 was marked with a

isopropyl-beta-D-thiogalactopyranoside (IPTG). After the host strain was cultured for 12 h at 25 °C in a shaking incubator, the pellets were collected and split by ultrasonic waves (300 W, 20 min, treatment 3 s, interval 2 s) in order to release all the protein within cells. The total protein content of the strains was visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

Characteristics of protease producing B. subtilis

A Gram-positive blunt-ended straight rod bacterial strain (No. 951NA$_4$), showed a high protease activity of 24.2 U/ mL. In the non-log phase, the endospores occurred partially in sporocysts with no obvious swelling or parasporal crystals. The strain (No. 951NA$_4$) grew rapidly on NA medium with basic bacterial colony characteristics such as buff colour of colony surface, non-transparent fold and anomalistic brain similar to *B. subtilis*.

The 16S r DNA sequence from the strain No. 951NA$_4$ showed 97-99% homology with *B. subtilis* strains. Figure 1 shows phylogenetic position of the bacterial isolate with other *B. sublilis* reported in the literature. Based on the morphological observations, sequence analysis of the 16Sr DNA and the phylogenetic tree results, the strain (No. 951NA$_4$) could be identified as *B. subtilis.*

Characteristics of the neutral protease gene

The amplified gene of the protease of 2644 bp showed 77-98% homology with the similar gene of mobA-nprE. The open reading frame was found to be 1521 bp long, encoding 507 amino acids without a signaling peptide (Fig. 2). The enzyme was named NPR which had the expected isoelectric point of 6.895 and molecular weight of 60 k Da. The similarity in overall length between the neutral protease NPR and other enzymes ranges between 91% and 100%, though the genes share approximate 99% similarity. The phylogenetic tree (Fig. 3) also showed that the target protein shares great homology with the strains of mobA-nprE.

According to BLAST analysis in Gen Bank and phylogenetic tree construction, the product of the gene was a neutral protease from *B. subtilis.* It was also displayed in Figure 4 that the target gene shares great homology with the strains of mobA-nprE (AF012285.1).

Expression of the recombinant protein

Figure 4 shows a protein with a molecular weight of 60 k Da which is not induced by IPTG concentration of 0.2 mmol L^{-1} (Table I). The other IPTG concentrations (0.4, 0.6, 0.8, 1.0, 1.2 mmol L^{-1}) had no effect on the yields of the target protein. These results demonstrated that the constructed expression system can efficiently express the neutral protease (NPR).

Table I.- Effect of IPTG (0.2 mM/L) on the protease activity (U/mL) of transformed *B. subtilis* (n=3).

Transetta (DE3)	Positive transformants	
	IPTG (0 mM/L)	IPTG (0.2 mM/L)
4.62±0.25	1.18±0.13	13.66±1.27

```
     1        M  S  L  S  I  S  L  P  G  V  Q  A  A  E  G  H  Q  L  K E
 1          ATGAGTTTATCAATCAGCCTGCCAGGTGTTCAGGCTGCTGAAGGTCATCAGCTTAAAGAG
     21       N  Q  T  N  F  L  S  K  N  A  I  A  Q  S  E  L  S  A  P  N
61         AATCAAACAAATTTCCTCTCCAAAAATGCAATTGCGCAATCAGAACTCTCCGCACCAAAT
     41     D  K  A  V  K  Q  F  L  K  K  N  S  N  I  F  K  G  D  P  S
121  GACAAGGCTGTCAAGCAGTTTTTGAAAAAGAACAGCAACATTTTTAAAGGTGACCCTTCC
     61     K  R  L  K  L  V  E  S  T  T  D  A  L  G  Y  K  H  F  R  Y
181  AAAAGGCTGAAGCTTGTTGAAAGCACGACTGATGCCCTTGGATACAAGCACTTTCGATAT
     81     A  P  V  V  N  G  V  P  I  K  D  S  Q  V  I  V  H  V  D  K
241  GCGCCTGTCGTTAACGGAGTGCCAATTAAAGATTCGCAAGTGATCGTTCACGTCGATAAA
     101    S  D  N  V  Y  A  V  N  G  E  L  H  N  Q  S  A  A  K  T  D
301  TCCGATAATGTCTATGCGGTCAATGGTGAATTACACAATCAATCTGCTGCAAAAACAGAT
     121    N  S  Q  K  V  S  S  E  K  A  L  A  L  A  F  K  A  I  G  K
361  AACAGCCAAAAAGTCTCTTCTGAAAAAGCGCTGGCACTCGCTTTCAAAGCTATCGGCAAA
     141    S  P  D  A  V  S  N  G  A  A  K  N  S  N  K  A  E  L  K  A
421  TCACCAGACGCTGTTTCTAACGGAGCGGCCAAAAACAGCAATAAAGCCGAATTAAAAGCG
     161    I  E  T  K  D  G  S  Y  R  L  A  Y  D  V  T  I  R  Y  V  E
481  ATAGAAACAAAAGACGGCAGCTATCGTCTTGCTTACGACGTGACGATTCGCTATGTCGAG
     181    P  E  P  A  N  W  E  V  L  V  D  A  E  T  G  S  I  L  K  Q
541  CCTGAACCTGCAAACTGGGAAGTCTTAGTTGACGCCGAAACAGGCAGCATTTTAAAACAG
     201    Q  N  K  V  E  H  A  A  A  T  G  S  G  T  T  L  K  G  A  T
601  CAAAATAAAGTAGAACATGCCGCCGCCACTGGAAGCGGAACAACTCTAAAGGGCGCAACT
     221    V  P  L  N  I  S  Y  E  G  G  K  Y  V  L  R  D  L  S  K  P
661  GTTCCTTTGAACATCTCTTATGAAGGCGGAAAATATGTTCTAAGAGATCTTTCAAAACCA
     241    T  G  T  Q  I  I  T  Y  D  L  Q  N  R  Q  S  R  L  P  G  T
721  ACAGGCACCCAAATCATCACATATGATTTGCAAAACAGACAAAGCCGCCTTCCGGGCACG
     261    L  V  S  S  T  T  K  T  F  T  S  S  S  Q  R  A  A  V  D  A
781  CTTGTCTCAAGCACAACGAAAACATTTACATCTTCATCACAGCGGGCAGCCGTTGACGCA
     281    H  Y  N  L  G  K  V  Y  D  Y  F  Y  S  N  F  K  R  N  S  Y
841  CACTATAACCTCGGTAAAGTGTACGATTATTTTTATTCAAACTTTAAACGAAACAGCTAT
     301    D  N  K  G  S  K  I  V  S  S  V  H  Y  G  T  Q  Y  N  N  A
901  GATAACAAAGGCAGTAAAATCGTTTCTTCCGTTCACTACGGCACTCAATACAATAACGCT
     321    A  W  T  G  D  Q  M  I  Y  G  D  G  D  G  S  F  F  S  P  L
961  GCATGGACAGGAGACCAGATGATTTACGGTGATGGCGACGGTTCATTCTTCTCTCCGCTT
     341    S  G  S  L  D  V  T  A  H  E  M  T  H  G  V  T  Q  E  T  A
1021 TCCGGCTCATTAGATGTGACAGCGCATGAAATGACACATGGCGTCACCCAAGAAACAGCC
     361    N  L  I  Y  E  N  Q  P  G  A  L  N  E  S  F  S  D  V  F  G
1081 AACTTGATTTATGAAAATCAGCCAGGTGCATTAAACGAGTCTTTCTCTGACGTATTCGGG
     381    Y  F  N  D  T  E  D  W  D  I  G  E  D  I  T  V  S  Q  P  A
1141 TATTTTAACGATACAGAAGACTGGGACATCGGTGAAGACATTACGGTCAGCCAGCCTGCT
     401    L  R  S  L  S  N  P  T  K  Y  N  Q  P  D  N  Y  A  N  Y  R
1201 CTTCGCAGCCTGTCCAACCCTACAAAATACAACCAGCCTGACAATTACGCCAATTACCGA
     421    N  L  P  N  T  D  E  G  D  Y  G  G  V  H  T  N  S  G  I  P
1261 AACCTTCCAAACACAGATGAAGGCGATTATGGCGGTGTACACACAAACAGCGGAATTCCA
     441    N  K  A  A  Y  N  T  I  T  K  L  G  V  S  K  S  Q  Q  I  Y
1321 AACAAAGCCGCTTACAACACCATCACAAAACTTGGTGTATCTAAATCACAGCAAATCTAT
     461    Y  R  A  L  T  T  Y  L  T  P  S  S  T  F  K  D  A  K  A  A
1381 TACCGTGCGTTAACAACGTACCTCACGCCTTCTTCCACGTTCAAAGATGCCAAGGCAGCT
     481    L  I  Q  S  A  R  D  L  Y  G  S  T  D  A  A  K  V  E  A  A
1441 CTCATTCAGTCTGCCCGTGACCTCTACGGCTCAACTGATGCCGCTAAAGTTGAAGCAGCC
     501    W  N  A  V  G  L  *
1501 TGGAATGCTGTTGGATTGTAA
```

Fig. 2. Sequences of nucleotide and amino acid of the neutral protease.

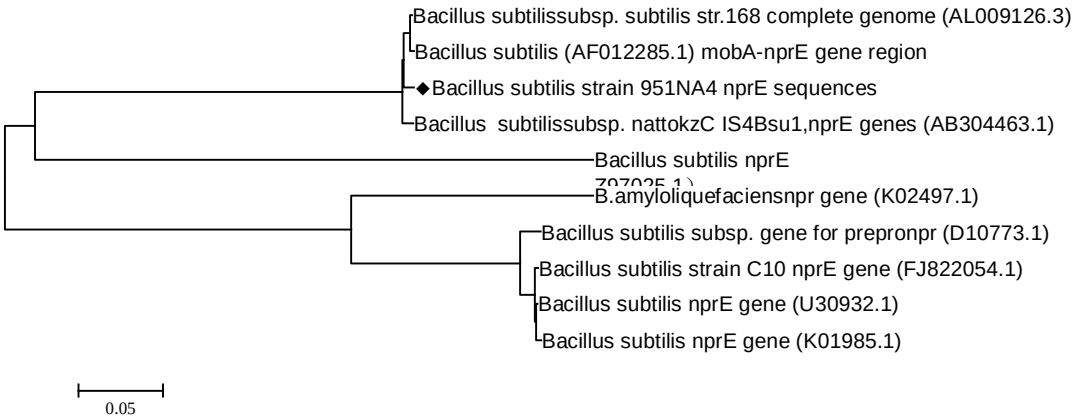

Fig. 3. Phylogenetic position of the nprE sequences of strain (951NA4) and other related taxa
Note: Bar: 0.05 substitutions per nucleotide position. The gene sequence of the neutral protease (NPR) (Genbank accession number: HQ845039) cloned in our test is marked with a ◆
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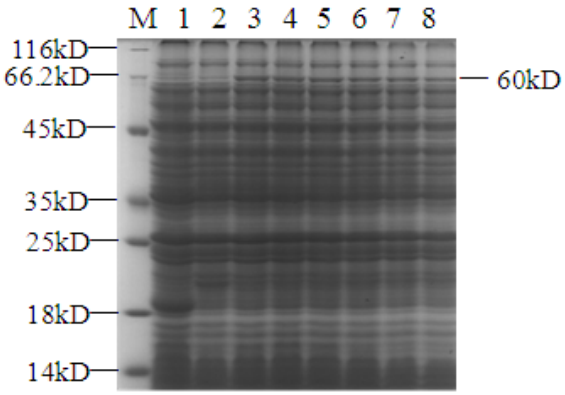

Fig. 4. SDS-PAGE analysis of recombinant proteins. Proteins from *E. coli* Transetta (DE3). Lanes 1-2: without IPTG induction. Lanes 3-8: Recombinant proteins induced by 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM IPTG, respectively. M, Protein marker.

DISCUSSION

A large variety of microbes exist in the intestinal canals of animals. They play important roles in the intestinal system and intimate interact with cells in vital organs to perform physiological functions such as nutrition (Dillon and Charnley, 2002), immunity (Dillon *et al.*, 1996), growth stimulation, etc. In these studies, we screened and identified a particular strain of *B. subtilis* with high enzymatic activity of neutral protease. There has been a kind of *B. subtilis* isolated from the intestine of *Antheraea pernyi* which can produce protease and cellulose (Zou *et al.*, 2011). But it produced protease weekly. Other report only gave the distribution of the microbe isolated from the intestine of some insects without investigation of enzyme secretion.

Many reports showed that *B. subtilis* has been used as a good additive in fodders due to its secreted proteases (Gomez-Gil *et al.*, 1998). The protease of $951Na_4$ has molecular weight 60 kDa which differed from that of other reported proteases from *B. subtilis*, most of which had molecular weights between 27 kDa and 43 kDa. The coding regions of the proteases varied greatly for many rare codons. Compared with the enzymatic activity of the protease induced at 37°C, the protease induced at 25°C showed higher activity, as the expression system was greatly influenced by induction temperature. Although the bacteria grew and expressed products rapidly at 37°C, the expressed products may accumulate in inclusion bodies instead of folding into the correct conformations which result in lower enzymatic activity. The results showed that the gene expression in the prokaryotic *E. coli* expression system was sensitive to environmental temperature.

The isolated strain was proved to have a wide range of function, especially in bacteriostasis (Holzapfel *et al.*, 1998), which can produce enzymes such as proteases, including neutral protease and alkali protease, elastase, fibrinogenase, lipases, cellulases, amylases (Yang and Ferrari, 1984; Tran *et al.*, 1991; Gomez-Gil *et al.*, 1998) and others helpful to animals. In 1905, Metchnikoff (Russian Nobel laureate) firstly suggested that the probiotics had function of nutrition and healthy maintenance, which became the theoretical basis for their

later application (Fuller, 1989). At present, studies of such probiotic strains have been more focused on poultry and fishery (Morishita *et al.,* 1992; Sugita *et al.,* 1997; Huber *et al.,* 2004; Navarrete *et al.,* 2008; Syed *et al.,* 2015) than on sericulture. Therefore, the selected strains in this paper may serve as an effective probiotic preparation for sericulture.

CONCLUSION

One strain producing neutral protease, named No. 951NA_4, was isolated from the intestinal canal of silk worms and identified as a variety of *B. subtilis.* The neutral protease gene sequence was 2644 bp in length (including a complete ORF), encoding a protein of 60 kDa. The inducible expression of the neutral protease in prokaryote cells will provide a solid basis for its further functional analysis and practical application as a microbial ecological preparation for sericulture.

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