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# Genetic engineering for enhanced production of a novel alkaline protease BSP-1 in *Bacillus amyloliquefaciens*

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Alkaline protease has been widely applied in food, medicine, environmental protection and other industrial fields. However, the current activity and yield of alkaline protease cannot meet the demand. Therefore, it is important to identify new alkaline proteases with high activity. In this study, we cloned a potential alkaline protease gene *bsp-1* from a *Bacillus subtilis* strain isolated in our laboratory. BSP-1 shows the highest sequence similarity to subtilisin NAT (S51909) from *B. subtilis* natto. Then, we expressed BSP-1 in *Bacillus amyloliquefaciens* BAX-9 and analyzed the protein expression level under a collection of promoters. The results show that the P43 promoter resulted in the highest transcription level, protein level and enzyme activity. Finally, we obtained a maximum activity of 524.12 U/mL using the P43 promoter after fermentation medium optimization. In conclusion, this study identified an alkaline protease gene *bsp-1* from *B. subtilis* and provided a new method for high-efficiency alkaline protease expression in *B. amyloliquefaciens*.

#### KEYWORDS

alkaline protease, *bacillus amyloliquefaciens*, promoter screening, recombinant expression, fermentation optimization

### **1** Introduction

Alkaline protease is found in all living organisms (Gupta et al., 2002). Alkaline protease has a lot of functions and it is widely applied in washing, food, textile, leather, pharmaceutical and other industries (Contesini et al., 2018; Harish and Uppuluri, 2018; Pathak and Rathod, 2018; Tavano et al., 2018; Jahangirian et al., 2019; Sharma et al., 2019; Burchacka et al., 2022). However, in recent years, the yield of alkaline protease fail to satisfy the industrial demand, causing the shortage problem of large-scale industrial enzymes (Barzkar, 2020). Therefore, developing efficient alkaline protease has great industrial prospects.

Many strains produce alkaline protease, Bacillus genus are the main producer of the enzyme in industrial production (Cai et al., 2019). Bacillus have high secretory capacity and become the main producer of the enzyme in industrial production (Westers et al., 2004). However, the yield and activity of alkaline protease in Bacillus is usually low (Zhang et al., 2020; Ouyang et al., 2021; Zhang et al., 2021; Espoui et al., 2022). To achieve high-efficiency expression of target proteins, researchers had reformed expression elements, such as signal peptides, transcription factors, and molecular chaperones (Liu et al., 2019; Suberu et al., 2019; Gong et al., 2020; Neef et al., 2021; Su et al., 2021). For instance, Gang et al. constructed a signal peptide library in Bacillus subtilis, and screened out the amylase activity up to 5086 U/mL (Fu et al., 2018). In addition, by combining the transcriptional activator Spo0A with the regulatory region of Bacillus licheniformis, Zhou et al. successfully achieved 1.46-fold increase in the yield of alkaline protease AprE (Zhou et al., 2020). In addition, since promoter is one of the most critical factors in gene expression regulation (Kumar and Bansal, 2018; Brázda et al., 2021; Cazier and Blazeck, 2021; Jensen and Galburt, 2021), its engineering also serves as an important strategy to increase microbial gene expression and metabolites production. Su et al. substituted the wildtype -10 box and -35 box of aprN promoter, resulting in significantly higher nattokinase production than those previously reported in B. subtilis (Wu et al., 2011). Liao et al. (2018) optimized the ribosome-binding site to improve  $\beta$ -Gal activity in B. amyloliquefaciens. Together, genetic manipulation of the above elements has been widely applied in the production of industrial enzymes.

*B. subtilis* has significant attributes, such as fast growth and development, strong metabolic capacity and rich products, *B. subtilis* has been the preferred organisms for industrial production of a variety of products (Gu et al., 2018). *B. amyloliquefaciens* could secrete recombinant proteins using a variety of signal peptides (Cui et al., 2018), thus it had been an efficient platform for producing various proteases (Uyar and Baysal, 2008; Matkawala et al., 2021; Meng et al., 2021). In this study, we identified an alkaline protease gene *bsp-1* from *B. subtilis*, and then expressed it in *B. amyloliquefaciens* BAX-9. Subsequently, we further analyzed the expression level under different promoters. Finally, the highest expression level of alkaline protease was obtained by using the promotor P43 combined with fermentation optimization.

## 2 Materials and methods

### 2.1 Chemicals

In this study, the *TransStartFastPfu* DNA polymerase was purchased from TransGen Biotech Co., Ltd. (Beijing, China). Restriction enzymes, dNTPs, T4 ligase and RNase were provided by Takara Biotechnology Co., Ltd. (Dalian, China). We purchased the Total RNA Isolation Kit and PrimeScript RT

Master Mix Kit from Vazyme Biotech Co.,Ltd. (Nanjing, China). Standard alkaline protease was purchased from Sigma-Aldrich Co., LLC (St.Louis, United States). All the other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

### 2.2 Recombinant expression of bsp-1 gene

To express BSP-1 in B. amyloliquefaciens BAX-9, we constructed the recombinant strains following the procedure reported in our previous study (Zou et al., 2020). All strains and plasmids involved were listed in Tables 1, 2, listed all designed primers. For example, a pair of primers were designed to amplify the bsp-1 gene from B. subtilis DNA, Ppqq promoter from B. amyloliquefaciens and TamyL terminator from B. licheniformis WX-02 respectively, and then Ppqq promoter, TamyL terminator and bsp-1 gene were fused by Splicing with Overlap Extension PCR (SOE-PCR). The fused fragment and the pHY-300PLK plasmid were digested by restriction enzymes of BamHI and XbaI, and then they were ligated to obtain the expression plasmid pHY-Ppqq/BSP-1. The expression plasmid pHY-Ppqq/BSP-1 was electroporated into BAX-9 to obtain the recombinant strain. In this study, the remaining recombinant strains were constructed using the same procedure.

#### 2.3 Skim milk agar medium assay

Skim milk agar medium contain 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1.5% Agar and 2% skim milk. To screen the protease activity, 20  $\mu$ L of supernatant of the cultures were added to the wells of milk agar plates made by a hole punch. The radius of transparent circles were measured after the plates were cultured at 37°C for 12 h.

### 2.4 Protease fermentation

The *B. amyloliquefaciens* cells were inoculated into 5 ml liquid LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl), and incubated at  $37^{\circ}$ C for 12 h with shaking at 180 rpm. Then, a 3% (v/v) inoculum was transferred into the 50 ml alkaline protease fermentation medium (40 g/L tryptone, 20 g/L yeast extract, and 10 g/L NaCl) and cultured at  $37^{\circ}$ C for 72 h with shaking at 180 rpm.

# 2.5 SDS-PAGE analysis of the expressed proteins

Protein samples were prepared by 100% trichloroacetic acid (TCA) precipitation (Benabdelkamel et al., 2018).

TABLE 1 Strains and plasmids used in this study.

Strains	Characteristics	Source
BAX-9	B. amyloliquefaciens HZ-12 deficient in epr, nprE, aprE, aprX, mpr, bpf, vpr, htrB, yktc1	Stored in lat
BAX-9/pHY-300	BAX-9 harboring the plasmid pHY300PLK	Stored in lat
BAX-9/pHY-Psra/BSP-1	BAX-9 harboring the plasmid pHY-Psra/BSP-1	This study
BAX-9/pHY-Psrs/BSP-1	BAX-9 harboring the plasmid pHY-Psrs/BSP-1	This study
BAX-9/pHY-PrnpB/BSP-1	BAX-9 harboring the plasmid pHY-PrnpB/BSP-1	This study
BAX-9/pHY-Pffs/BSP-1	BAX-9 harboring the plasmid pHY-Pffs/BSP-1	This study
BAX-9/pHY-Phyp/BSP-1	BAX-9 harboring the plasmid pHY-Phyp/BSP-1	This study
BAX-9/pHY-Ppqq/BSP-1	BAX-9 harboring the plasmid pHY-Ppqq/BSP-1	This study
BAX-9/pHY-Pscp/BSP-1	BAX-9 harboring the plasmid pHY-Pscp/BSP-1	This study
BAX-9/pHY-Pcsp/BSP-1	BAX-9 harboring the plasmid pHY-Pcsp/BSP-1	This study
BAX-9/pHY-P43/BSP-1	BAX-9 harboring the plasmid pHY-P43/BSP-1	This study
BAX-9/pHY-PtrnQ/BSP-1	BAX-9 harboring the plasmid pHY-PtrnQ/BSP-1	This study
BAX-9/pHY-Psrf/BSP-1	BAX-9 harboring the plasmid pHY-Psrf/BSP-1	This study
BAX-9/pHY-Pitu/BSP-1	BAX-9 harboring the plasmid pHY-Pitu/BSP-1	This study
BAX-9/pHY-Pfen/BSP-1	BAX-9 harboring the plasmid pHY-Pfen/BSP-1	This study
BAX-9/pHY-Pbac/BSP-1	BAX-9 harboring the plasmid pHY-Pbac/BSP-1	This study
B. subtilis 168	the strain containing P43 promoter	Stored inlab
B.licheniformis WX-02	CCTCC M208065, wild type	Stored inlab
Plasmid	Characteristics	Source
pHY300PLK	E. coli-Bacillusshuttle vector for geneexpression, Apr, Tet	Stored in lab
pHY-PX/BSP-1	pHY300PLK + <i>bsp-1</i> + TamyL	This study
pHY-Psra/BSP-1	pHY300PLK + Psra + <i>bsp-1</i> + TamyL	This study
pHY-Psrs/BSP-1	pHY300PLK + Psrs + <i>bsp-1</i> + TamyL	This study
pHY-PrnpB/BSP-1	pHY300PLK + PrnpB + <i>bsp-1</i> + TamyL	This study
pHY-Pffs/BSP-1	pHY300PLK + Pffs + <i>bsp-1</i> + TamyL	This study
pHY-Phyp/BSP-1	pHY300PLK + Phyp + <i>bsp-1</i> + TamyL	This study
pHY-Ppqq/BSP-1	pHY300PLK + Ppqq + <i>bsp-1</i> + TamyL	This study
pHY-Pscp/BSP-1	pHY300PLK + Pscp + <i>bsp-1</i> + TamyL	This study
pHY-Pcsp/BSP-1	pHY300PLK + Pcsp + <i>bsp-1</i> + TamyL	This study
pHY-P43/BSP-1	pHY300PLK + P43+ bsp-1+ TamyL	This study
pHY-PtrnQ/BSP-1	pHY300PLK + PtrnQ + <i>bsp-1</i> + TamyL	This study
pHY-Psrf/BSP-1	pHY300PLK + Psrf + <i>bsp-1</i> + TamyL	This study
pHY-Pitu/BSP-1	pHY300PLK + Pitu + <i>bsp-1</i> + TamyL	This study
pHY-Pfen/BSP-1	pHY300PLK + Pfen + <i>bsp-1</i> + TamyL	This study
pHY-Pbac/BSP-1	pHY300PLK + Pbac + <i>bsp-1</i> + TamyL	This study

Specifically, 0.90 ml of fermentation supernatant was mixed with 0.10 ml of trichloroacetic acid, inverted 10 times to mix and incubated at 4°C for 12 h. Then, the mixture was centrifuged at 6,000 ×g for 10 min. After that, the supernatant was discarded and the pellet was washed with 0.20 ml of absolute ethanol for three times. After dried at 37°C, the pellet was dissolved with 30 µL of loading buffer containing 8 mol/L urea and 2 mol/L thiourea. At last, the samples were analyzed by SDS-PAGE electrophoresis.

### 2.6 Enzyme activity analysis

1 ml of the sample was transfered to tube A (as blank control) and tube B for activity measurement, respectively. After the samples were heated at  $40^{\circ}$ C for 2 min, A was supplemented with 2 ml of trichloroacetic acid (64.70 g/L), while B was added with 1 ml of casein solution (10 g/L). Both samples were further heated at  $40^{\circ}$ C for 10 min. Then, 1 ml casein solution was added into A, and 2 ml trichloroacetic acid

TABLE 2 Primers used in this study.

<i>bsp-1</i> (Tamyl)-R AAGAGCAGAGGAGGACTTATTGTGCAGCTGCTTGTA	Primer name	Sequence of primer (5' to 3')
beACAGCTTCATTGCGAATGAGAAGCAAAAATTGTGGbep-1 (Tamyl)-RAAGAGCAGAGGAGCTTATTGTGCAGCTGCTTGTAATamyl-FCCAGCTGCACAATAAAAGGCGAGAGAGGAGCGAGTTTamyl-FCCAGCTGCACAATAAAAGGCGCAGAGGAGCGGATTTamyl-RCCGGAATTCAACGCAAAAAGGCGCCAAAAGPars-FCCGGAATTCAACGCACGAGATTGGATTCGGCGCAATAAPars-RCCGGAATTCGCACGTTTGTTCTTGAAAATCAPars-RCCGGAATTCGCACGTTTGGTTCGTGCATTPars-RCCGGAATTCGCACGTTTGGTCAAAATACAPars-RCCGGAATTCGCACGTTTGGTCCACACACAATTGAATTGCAATTGCACTPars-RCCGGAATTCGCACGAATTAGAAACCTTCATCAAAGPars-RCCGGAATTCGCATTTGGATATGGAAACCTTCATCAAGACPars-RCCGGAATTCGCAATAAAATATCAAAATTGAAATTGAAACCTTCATCAAGAPars-RCCGGAATTCGCAATAAAAAATATCAAAACTTCACAAGACGACPirs-RCCGGAATTCGCAATAAAAAATATCAAACCTTCATCAAGACPirs-RCCGGAATTCGCAATAAAAACCTTCATCAAGACCTTCATCAAGACCTTCATCAAGACCTTCATCAAGACCTTCATCAAGACCTCCTCTTACTPirs-RCCGGAATTCGCAATAGAACCTTGTTCATTATAAAGCPirs-RCCGGATCCGCAGGAGGCTGCTCTTTATPirs-RCGGAATTCGCAATAAAATATGGAATTCACATGAACCTCCPirs-RCGGAATTCGCAATAAATATGGAATTCACATTCATAAGAGACCTCCTTTATPirs-RCGGAATTCCTAAAAATATGGAATTTACGAGACCTCPirs-RCCGGATTCCAAAAATATGGAATTTTATCAGAPirs-RCGGAATTCCAAATAATAGGATTTTATCAGAACCTCPirs-RCCGGATTCCAAAATATGGATTTTTATCAGAACCTTTTCTTTTATCAAPirs-RCCGGATTCCAAAATATGGATTTCTCCTCATTATGGAACCTCPirs-RCCGGATTCCAAATATGCAATTTCCTCCTATTAGAGAGCCPirs-RCCGGATTCCAAATTCCTCCTATTAAAATAGGAGCCPirs-RCCGGATTCCAAATTCCTCCATTATGGAAGCCCPirs-R </td <td>bsp-1-F</td> <td>CG<b>GGATCC</b>ATGAGAAGCAAAAAATTGTGGAT</td>	bsp-1-F	CG <b>GGATCC</b> ATGAGAAGCAAAAAATTGTGGAT
bp-1 (Tamyh)-RAAGAGCAGAAGAGGACTTATTGTGCAGCTGCTTGTAATamyh-FGCAGCTGGCACATAAAAGAGCAGAGAGGAGGAGGAGGAGTTamyh-RGCGAGTCGCACAATAAAGGCGGCACATPsra-FCGGATTCAACGAAAAGGCGGCAATAAPsra-RCGGATTCGTGTAAGGGATATCGGGGAGTPsra-RCGGATTCGCGCGTTTGTAAGGGTATACCGGGAGATPsra-RCGGATTCGGCGCTTTGTAAGGGTATACCGGGAGATPmpB-FCGGATTCGGCGCATPmpB-FCGGATTCGGCGTATTGGACGCATPffs-FCGGATTCGGCATTATTGAACGTTCATCAAGPffs-RCGGATTCGGCATTGTTCTCACAGGAGACPhyp-FCGGATTCGGCGCATGCTCACGGAGGACGTGTTPhyp-RCGGATTCGGCGCATGCTGCTCTTTATAAAGCAPhyp-RCGGATTCGTCGCGCATGCTGCTCTTTATPhyp-RCGGATTCGTGCGCATGCGGCAGGCGGTGTCCTTTATPhyp-RCGGATTCGTGGCGCTGTCTCTTATPhyp-RCGGATTCGTGGCGCATGCTGCTCTTTATPhyp-FCGGATTCGTGGCGCTGTCTCTTATPhyp-FCGGATTCGTGGCGCTGTCTCTTATPhyp-RCGGATTCGTGGGCGCTGCTCTTTATPhyp-RCGGATTCGTGGGCGCTGTCTCTTATPhyp-RCGGATTCGTGGCGCTGTCTCTTATPhyp-RCGGATTCGTGGGCGCTCTTTTCGCATTPhyp-RCGGATTCGTGGGCGCTCCTCTTTPhyp-RCGGATTCGTAAGATGGGTATGGTTTTGGPhyp-RCGGATTCGTAAGATGGCGTATGGTTGGCGCTCCCTCTPhyp-RCGGATTCGTAAGATGGCGTATGGGGAGGCPhyp-RCGGATTCGTAAGATGGCGTCTATGGAAGGCPhyp-RCGGATTCGTAAGATGGCGCTCCCTTPhyp-RCGGATTCGTAAGATGGCGTCTATATGGCGCCTCCCTTPhyp-RCGGATTCGTAAGATGCGCGCCCCCCTTPhyp-RCGGATTCGACATATTGCGAAATGGCACPhyp-RCGGATTCGACATATGCCCCTT <tr< td=""><td>bsp-1-R</td><td>TTATTGTGCAGCTGCTTGTACG</td></tr<>	bsp-1-R	TTATTGTGCAGCTGCTTGTACG
TamyL-F   GCAGCTGCACATAAAAAGAGCAGAGAGAGAGCAGGAT     TamyL-R   GCTTAGACGCATAAAGCGCCCACT     Psra-F   CGGAATTCAACGGAAAAGACGCCAAAAG     Psra-F   CGGAATTCACGGAATAATGCGCGCAATAA     Psra-R   CGGAATTCGCACTTTTAAGGGAATACGGGAGAT     Psrs-F   CGGAATTCGCACTTTGTTAAGGGAATACGGGAGAT     Psrs-F   CGGAATTCGCACTTTGTTCTGAAAATCA     Psrs-R   CGGAATTCGGTCCTAAGCACATTTTGATTCAA     PmpB-F   CGGAATTCGGTTATTCGACACTTTTCATCAAG     Pffs-F   CGGAATTCGGATTTGAAACCTTTCATCACAG     Phyp-F   CGGAATTCGGATTATGAACCTTTCATACAAGC     Phyp-F   CGGAATTCCGACGCAGGAGCTGCTCTTTAT     Phyp-F   CGGAATTCCGACGCAGGAGCTGCTCTTTAT     Phyp-R   CGGAATTCCTATAGAACACTTGCTCCTTACT     Phyp-R   CGGAATTCCTACTACGCAATGAAGCTGCTCTTTAT     Phyp-R   CGGAATTCCTACTACTGCACATGAAGCTGCTCTTTAT     Phyp-R   CGGAATTCCTCTCCCCCCCCTTTAT     Phyp-R   CGGAATTCCTACTACTCCTCTTACCCCCTT     Phyp-R   CGGAATTCCTACTACTCTCTCTAAAAAATTCCCATTT     Phyp-R   CGGAATTCCTACTACTCCTCCCATTTTCC     Phyp-R   CGGAATTCCTACTACTCCCCTTTCTTTC     Phyp-R   CGGAATTCCTACTACTCCTCTCTTTCCTTTCCTTTACCATTCT	bsp-1 (Ppqq)-F	ACAGCTTCATTGCGAATGAGAAGCAAAAAATTGTGGAT
TamyLRCCTCTAGACCATAATGCCGTCGCACTPara-FCGGAATTCAACGAAAAGACCCCAAAGPara-RCGGAATCCACGGATATACGGGAGATPara-RCGGAATCCCACAGCAGATTTGTTCTGAAAATCAPars-FCGGAATCCCACAGCAGATTTGATTTCAAPmpB-RCGGAATCCGCACATCGGCCATPmpB-RCGGAATCCGAAAATAATCAAATTTGAATTGAAPfis-FCGGAATCCGAAAATAATCAACTTGATCAGCGACACPhyp-RCGGAATCCGCACAGCAGACTTGTTCTCACCTCPhyp-RCGGAATCCGCACAGCAGACCTTGTTCTCACCTCPhyp-RCGGAATCCGCGCAGAGCGTCTCTTATPhyp-RCGGAATCCGCGCAGAGCGTCTCTTATPhag-RCGGAATCCGCGCAGAGGCGTCTCTTATPhag-RCGGAATCCCGCGCAGAGGCGTCTCTTATPhag-RCGGAATCCGCGCAGAGGGCGTCTCTTATPhag-RCGGAATCCGCGCAGAGGGCGCTCTCTTACCTATAGGGGAGGGGGCGCGTCCCTTTPhag-RCGGAATCCGCGCGCGCGCCCCTTTGTCCCATTAGGAGACGCCPhag-RCGGAATCCGCGCGCCCCTTTTCCCCATTAGGAGACGCCPhag-RCGGAATCCCGCGCCCCCTTTTCCCCATTAGAGAGCGCPhag-RCGGATCCCCCCTTTTCCCATAAGGGACPhag-RCGGATCCCCCCTTTTTCCCAAAAAATGGGACCCCPhag-RCGGATCCCCCCTTTTTCCCAAAAAAGGGACPhag-RCGGATCCCCCCTTTTTCCCAAAAAAGGGACPhag-RCGGATCCCCCCCTTTTTCCCAAAAAGGCACPhag-RCGGATCCCCCCCTTTCTAAAATAGGCACPhag-RCGGATCCCCCCTTTCTAAAATAGGCACPhag-RCGGATCCCCCCTTTCTAAATAGGCACAATAGCCCAATTCCCCCAATTCCCCCCTTPhag-RCGGATCCCCCCCTTTCTAAATAGCACAATAGCCCAATTCCCCCCCTTPhag-RCGGATCCCCCCCTTTCTAAATTCGACAATATTCGACAATATGCAAATAGCACAATAGCCCAAPhag-RCGGATCCCCGGAGATTCCCCCCCCTTPhag-RCGGATCCCCCCCCCTTTCTAAAATAGCACAC	bsp-1 (Tamyl)-R	AAGAGCAGAGAGGACTTATTGTGCAGCTGCTTGTACG
ParafiCGGATTCAACGAAAGACCCAAAGParaRCGGATCCATGTTAAGGGATACGGAGATParaRCGGATCCACGAGTTTCTTCTGAAAATCAParsFCGGATCCACACGAGATTTCAATTCAATCAPmBFGCGGATCCCACACGAGATTACGAACCTTTCATTCATCAAGPffsFCGGATTCGGATTATGAAACCTTTCATCAAGACPffsRCGGATCCCAGAAGACCTTGTTCATTAAAACGPhyp-FCGGATCCCGCAGTTGGTCACTCACCACCAGAGAGCPhyp-RCGGATCCGCAGAGCGTGTCTTTATPhq-RCGGATCCGCAGAGCGTGTCTTTATPhq-RCGGATCGCGCAGGAGCTGTCTTTATPhag-FCGGATCGCGCAGGAGCTGTCTTTATPhag-FCGGATCGCTGCATTGGTAAGACGTGTTTGPh3-FCGGATCGCTGCATTGGTAAGTGGTATTGAPhq-RCGGATCGCTGCATTGGTAAGTGGTATTTGPh3-FCGGATCGCTGCATTAGAAGAGGTGTTTTPhag-FCGGATCGCTGTATAGAAAAATGGATTTTATACAGCPhag-FCGGATCCATATGACTGCGTTTTTCCAATTACAGCGCPhag-RCGGATCCATATGACTGCGTTTTTCCAATTACAGCGCPhag-RCGGATCACTGTTTTCCAATTACAGCGCPhag-RCGGATCACTGTTTTTCGAAAAAGTTAPhag-RCGGATCACTGTTTTTCGAAAAAGTTAPhag-RCGGATCACTGTTTTTCGAAAAAAGTTAPhag-RCGGATCACTGTTTTTCGAAAAAAGTTAPhag-RCGGATCACTGTTTTTTCGAAAAAAGTTAPhag-RCGGATCACTGTTTTTTCGAAAAAAGTTAPhag-RCGGATCACTGTTTTTTTTCGAAAAAGTTAPhag-RCGGATCACTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	TamyL-F	GCAGCTGCACAATAAAAGAGCAGAGAGGACGGATT
Para-RCGGATCCTIGTTAAGGGTATACGGAGATPara-RCGGATCCCTIGTTAAGGGTATACGGAGATPars-FCGGATTCGCAGTTIGTTTCTTGAAAATCAParpB-FCGGATCCCAAGCAGATTTGGGTGCGATPmpB-RCGGATTCGGTCGTATTCGGCGCATPffs-FCGGATTCGGATTGGATATCAACCTTGCATCAAGPffs-RCGGAATTCGTAACACTGTGTCATTAAAAGCPhyp-FCGGAATTCGGATGTTGGCGCATCTPhyp-FRCGGATCCTGCCAATGAACACTGTCTTTATPpq-RCGGATCCGCGCAGGAGCGGCGCGCGCGCTCTTTTP43-FCGGAATTCGGATAGCAGTGGTATGTCGCATTGGP43-FCGGAATTCGGCGCGGGATGCTCTTTTATP43-FCGGAATTCGGTAGTGGGTATGTTTCGP43-FCGGAATTCGGTGGTCCTTTTTCGP43-FCGGAATTCGGTGGTCCTTTTTTTP43-FCGGAATTCGGTGGTCCTTTTTCGP43-FCGGAATTCGTGGTCTCTTTTTCGP43-FCGGAATTCGTGGTGTCCTTTTTTCGP43-FCGGAATTCGTGGGGTATGGTTTTCGP43-FCGGAATTCGACATGGGGTTATGAAACGTGP5p-FCGGAATTCCTAAAAATAGGAGATTGTTCGGAAACGTCP5p-FCGGAATTCCAAAAATAGTGATTTTCTTTTTTTTTTTTTT	TamyL-R	GCTCTAGACGCAATAATGCCGTCGCACT
Psrs-FCGGAATTCGCAGTTGTTCTTGAAAATCAPsrs-RCGGGATCCCAAGCAGATTTGGTCGTTTTCAAAPmpB-FCGGAATTCGGTCGTATTGGGCGCATPmpB-RCGGATTCGGATTATGAAACCTTTGTCATCAAGPffs-FCGGAATTCGGATTATGAAACCTTGTCATCAAGPhyp-FCGGGATCCTAAGAACACTGTTCATCAAGAPhyp-RCGGGATCCGGCAGTGGTCTCTTTATPpqq-FCGGGATCCGGCAGGAGCTGTCTCTTATPhqq-FCGGAATTCGTCATGAAGACGTGTCTTTATPhq-FCGGATCCGGCAGGAGCTGTCTCTTATPhqq-FCGGATTCGTCATGCAAGAGAGCGTGTCTTTATPhqq-FCGGATTCGTCATGCGCAGGAGCTGTCTTTATPhqq-FCGGATTCGTCATGCGCAGGAGCGGTATTGTPhq-FCGGATTCGTCATGCGCTGTCTTTATPhqq-FCGGATTCGTCGTCTTTTTCGPhq-FCGGATTCGTCGTCTTTTTCGCAATGAGCGCTPhq-FCGGATTCGTCGTCTTTTTCGCAATGAGACGTCPhq-FCGGATTCGTCGTCTTTTTCCCATTTGPhq-FCGGATTCGTCGTCTTTTTCCCATTTGPhq-FCGGATTCCTCATAAAATGGGTTATTGAGAACGTCPhq-FCGGATTCCTCATAAAATGGGTTATTGGAAAAAGTAPhq-FCGGATTCCTCCTATTCTCCTTTATCTGCAAAAAGGTGCPhq-FCGGATTCACCGCCTTTTTTTTTTATCAGGPhq-FCGGATTCCCCAATTCTCCCCTTTCTTATATGAGGGCPhq-FCGGATTCACCGCCTTCTTATCTCCCTTAAAATAGGTGCPhq-FCGGATTCACCGCCTCCCTTPhq-FCGGATTCACATTTCTGCAAAAAATGGGTGCPhq-FCGGATTCACATTCTCCCATATAGACAATGTTCTCCCAAATAGGCCPhq-FCGGATTCACCGCCTCCCTTPhq-FCGGATTCACATGTGTGGCCCCCCCTTPht-FCGGATTCACATGTTCTCCCATATGGACAATATGCCAAPht-FCGGATTCACACATGTTCTCCGATATAGCCAATATGCCAATATGCCCAATATGCCAATATGCCCAATATGCACATGTTCTCCCATATG	Psra-F	CGGAATTCAACGAAAAGACGCCAAAAG
Psrs-RCGGATCCCACAGCAGTTTGATTTCAAPmpB-FCGGATTCGGTCGTATTCGGCCATPmpB-RCGGATCCCAAATAAATATCAAATTTGATATGPffs-FCGGATCCCAAATAAATATCAAATTTGATAGAPffs-RCGGATCCTAGAACCTTGTCATCAAGAPhyp-FCGGATCCTGCCATTGTTCATCACGCPhyq-RCGGATCCGGCAGGAGCTGTCTTTATPqq-FCGGATCCGTGCATTGTACGAAGCAGTTGTTP43-FCGGATCCGTGTACATTCTCCCTTTATP43-RCGGATCCGTGTACATTCTCCCTTTATPhyq-FCGGATCCGTGTACATTCTCCCTTTATPhyq-FCGGATCCGTGTACATTCTCTCTTATCAAAGAGCTGTCTTTP43-RCGGATCCGTGTACATTCTCCTCTTACCAATAAAGAGTGTPtmQ-FCGGATCCGTGTACATTCTCCCTTTACTAAAGAGCTGCPsq-FCGGATCCATATAGCTGCGTTATTATCAAGAGTAGTPsq-FCGGATTCCTAAAAATAGTGATTTTATCAGAPsq-FCGGATTCCTCTTTCCTTTTCTTTATTACAAGAGTGCPsq-FACGGATCCCTTTTCCTTTTCTTTATTACAAGAGTGCPsq-FACGGATTCCCCATATAGCGCCCCCTTPsq-FACGGATTCCCCATATGTAAATAGAGTGCPsq-FACGGATTCCCCATATGTAAATAGAGTGCPsq-FACGGATTCCCCATATGTAAATAGAGTGCPsq-FACGGATTCCCCATATGTAAATAGAGTGCPsq-FACGGATTCCCCATATGTAAATAGAGTGCPsq-FACGGATTCCCCCTTTTTCTTTACTAATGCCAAPsq-FACGGATTCCCCCTTTTTCTTTATTCCCATATATGCCAAPsq-FACGGATTCCATGTGTGCCCCCCTTPsq-FACGGATTCTAATTTCCCAATAATGCCAAPsq-FACGGATTCATATTTCCCAATAATGCCAAPsq-FACGGATTCATATTTCCCAATAATGCCAAPsq-FACGGATTCATATTCTCCAATAATGCCAAPsq-FACGGATTCATATTCTCCAATAATGCCAAPsq-FACGGATTCAATTTCTCCAATAATGCCAA<	Psra-R	CGGGATCCTTGTTAAGGGTATACGGGAGAT
PmpB-F   CGGAATTCGGTCGTATTCGGCGCAT     PmpB-R   CGGGATCCCAAAATAAATATCAAATTTGATATG     Pfis-F   CGGAATTCGGATTATGAAACCTTGTCATCAAG     Phyp-F   CGGAATTCTAAAAACCACTGACAGCAGAC     Phyp-R   CGGGATCCCGGCAGGAGCTGTCTCTTAT     Pqq-F   CGGAATTCTGATAGGAGCAGTGTCTTTAT     P43-F   CGGAATTCGGCAGGAGCTGTCTCTTAT     P43-F   CGGGATCCGGCAGGAGCTGTCTCTTAT     P43-F   CGGGATCCGTGTGACATTCGCAATGAAGCTGCTTT     P43-F   CGGGATCCGTGTGCCTTTTTCCCATTAT     P43-F   CGGGATCCGTGTGCCTTTTTCCCATTAT     Psq-F   CGGAATTCGTCGTGTGCTTTTTCG     P43-F   CGGGATCCTAAGAAAAAGTGGCT     Psq-F   CGGAATTCGTCGTTTTTCCCATTTT     PtmQ-F   CGGAATTCGTCGTTATTGAAAAAGTGA     Psq-F   CGGAATTCCTAAAAAATAGTGATTTTTATCAGG     Psq-F   CGGAATTCCAAAAAATAGTGATTTTTATCAGG     Psq-F   CGGAATTCCAACATGTTATTCGAAAAAGTA     Psq-F   CGGAATTCCAACATGTATTCGCCCTTTTTAT     Psq-F   CGGAATTCAACATGTTATTCGAAAAAGTAG     Psq-F   CGGAATTCAACATGTTATTCGCAATAATAGAGTGC     Psq-F   CGGAATTCAACATGTTATTCGACAATAATGAGTGC     Psq-F   CGGAATTCAACACGTGTTAGTAAAATAG	Psrs-F	CG <b>GAATTC</b> GCAGTTTGTTTCTTGAAAATCA
PmB-RCGGGATCCAAATAAATATCAAATTTGATATGPf5-FCGGAATCCGATTAGAAACCTTGCATCAAGAPf5-RCGGGATCCTAAGAAACCTTGTCATTATAAAGCPhyp-FCGGAATCCTAAGAACACTGTCAAGACAGCAGACPhyp-RCGGGATCCCGCCATTGTTCCAACCCPpqq-FCGGGATCCGGCAGGAGCTGTCTTTATPpqq-RCGGAATTCTGATAGGTGGTATGTTTCGP43-FCGGGATCCGTGCATTGCTCTTACCTCCTATAGAPtmQ-FCGGAATTCGTGTGCTCTTTTTCCCATTTGPtmQ-FCGGGATCCGTGTACATTCCTCCTATAGAGPsp-FCGGAATTCCTAAAAATAGTGATTTTAACAGGPsp-FCGGAATTCCTAAAAATAGTGATTTTAACAGGPsp-FCGGAATTCCAACATGTTATTCGAAAAAGTAPsp-FCGGAATTCCACACTGTTATTCGAAAAAGTAPsp-FCGGAATTCCACACTGTTATTCGAAAAAGTAPsp-FCGGAATTCCACACTGTTATTCGAAAAAGTAGTPsp-FCGGAATTCACACTGTTATTCGAAAAAGGACPsr-FCGGAATTCACACTGTTATTCGAAAAAGGACGCPsr-FCGGAATTCACACGCCTCATGTAAAAAGGAGCPsr-FCGGAATTCCACACTGTGTGCGCCCCCCTPtm-FCGGAATTCCACATTATTCGCACAATAATGCAAAPtm-FCGGAATTCCACATTATTCCGCACCATAATGCCAAPtm-FCGGAATTCCAATTTCCTCCGATCATTTCGCAACAATATGCCAAPtm-FCGGAATTCCAATTTCCTCCGATCATTTCGAACAATAGCCAAPtm-FCGGAATTCCAATTCCTCCGATCATTTCGCACCAATAATGCCAAPtm-FCGGAATTCCTCCGATCATTTCGCACCAATATGCCAAPtm-FCGGAATTCCTAATTCCTCCGATCATTTCGCACCAATATGCCAAPtm-FCGGAATTCCTAATTCCTCCCGATCATTTCGAACAATATGCCAAPtm-FCGGAATTCCTAATTCCTCCGATCATTTCGACCAATATGCCAAPtm-FCGGAATTCCTAATTCCTCCCGATCATTTCGAACAATATGCACAAPtm-FCGGAATTCCTAATTCCTCCGATCATTTCGACCAATATGCCAA<	Psrs-R	CG <b>GGATCC</b> CACAGCAGATTTTGATTTTCAA
Pris-FCGGATTCGGATTATGAAACCTTCATCAAGPfis-RCGGGATCCTAGAAACATTGTTCATATAAAGCPhyp-FCGGATCCTAGCACTGGCAGGAGCGGCGCGCAGGAGCAGCAGCAPhyp-RCGGGATCCGGCAGGAGCTGTCTTATPpqq-FCGGATCGGCAGGAGCTGTCTTATPhqq-RCGGATTCGATAGGAGCAGTAGCTTCGP43-FCGGATCGGCAGTGACATTCCTCTACCTATAGAPtnQ-FCGGATCGGTGACATTCCTCTTACCTATAGAGPtnQ-FCGGATCGTGTACTTCTCCCATTTTPtnQ-RCGGATCGTGTACATTCCTCTTACCAATGAPsp-FCGGATCCTATAGACTGGTTATGAAACTGTPsp-FCGGATCCATATAGATGGTTTTCGAAAAATGTAPsp-RCGGATCCATATTCCTCTAAAAAGTACPsp-RCGGATCCATATGACCGCTTATGAAAAGTGCPrif-FCGGATCCATGTGTGCGCCCCCTTPitu-FCGGATCCATATTCTGACAATAATGCCAAPitu-RCGGATCCATGTTCTCCGATCATATGCCAAPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTTPitu-RCGGATCCATGTGTGCGCCCCCTT	PrnpB-F	CGGAATTCGGTCGTATTCGGCGCAT
Pfs-RCGGATCCTAAGAACACTTGTTCATTATAAGCPhyp-FCGGATCCTAAGAACACTGACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA	PrnpB-R	CG <b>GGATCC</b> CAAAATAAATATCAAATTTTGATATG
Phyp-F   CGGAATTCTTAAAATCACACTGACAGCAGCA     Phyp-R   CGGGATCCCTGCCATTTGTTCTCACCTC     Ppqq-F   CGGGATCCGGCAGGAGCTGTCTCTTAT     Ppqq-R   TTTTTTGCTTCCATTCGCAATGAAGCTGTCTTT     P43-F   CGGGATCCGTGTACATTCCTCTTTACCTATAGT     PtnQ-F   CGGGATCCGTGTACATTCCTCTTTACCTATAGT     PtnQ-F   CGGGATCCGTGTACATTCCTCCTTTACCTATAGT     Pscp-F   CGGGATCCATATAGACTGCGTTATGAGAACGTC     Pscp-F   CGGGATTCCAAAAAATAGTGATTTTACTAATG     Pscp-F   CGGGATTCCATATTCCTCCTTTTCTTACTAATGA     Pscp-F   CGGGATTCCAACATGTTATTTCGAAAAAAGTTA     Pcsp-F   CGGGATTCCACGCGCTCATATAGACGCC     Psrf-F   CGGGATTCCACGCGCTCCTATGTAAAATAGGAGC     Psrf-F   CGGGATTCACGCGCTCCCTT     Ptru-F   CGGGATTCCATGTGTGCGCCTCCCTT     Ptu-F   CGGAATTCTAATTTCTGACAAATAATGCCAA     Ptu-R   CGGAATTCTAATTTCTGACACAATAATGCCAA	Pffs-F	CG <b>GAATTC</b> GGATTATGAAACCTTTCATCAAG
Phyp-R   CGGGATCCCTGCCATTGTTCTCACCTC     Ppqq-F   CGGGATCCCGGCAGGAGCTGTCCTTTAT     Ppqq-R   TTTTTGCTTCCATTGCCAATGAAGCTGCTCTT     P43-F   CGGGATCCGTGTACATTGCTCTCTTACCTATAATG     PtnQ-F   CGGGATCCGTGTCCTTTTTCCCATTGT     PtnQ-F   CGGGATCCGTGTCCTTTTTCCCATTTT     PtnQ-F   CGGATCCATATAGACTGCGTTATGAGAACGTC     Psqp-F   CGGGATCCATATAGACTGCGTTATGAGAACGTC     Psqp-F   CGGGATCCATCTATTCCTCCTTTTCTATACAG     Psqp-F   CGGGATCCATCTATTCCTCCTTTTCTTACTAA     Psqp-F   CGGGATCCATGTATTTCGAAAAAGTAA     Psqp-F   CGGGATCCATGTATTTCGAAAAAAGTAA     Psqp-F   CGGGATCCATGTATTCCTCCTATAGGAACGC     Psqp-F   CGGGATCCATGTATTTCGAAAAAAGTAA     Psqp-F   CGGGATCCATGTATTCCTCCTAAAGCGAC     Psqp-F   CGGGATCCATGTATTTCGAAAAAAGTAA     Psqp-F   CGGGATCCATGTATGTAAAAAAGGAAC     Psqp-F   CGGGATCCATGTATGTAAAAAAGGAAC     Psqp-F   CGGGATCCATGTATGTAAAATAGAGGAC     Psqp-F   CGGGATCCATGTGTGCCCCCTT     Psqp-F   CGGAATTCTAATTTCTGACAAATAATGCCAA     Psqp-F   CGGAATTCAATTTCTGACAAATAATGCCAA     Psqp-F   CGGAATTCTAATTTCTG	Pffs-R	CG <b>GGATCC</b> TAAGAACACTTGTTCATTATAAAGC
Ppqq-F   GGGATCCGGCAGGAGCTGTCTTTAT     Ppqq-R   TTTTTTGCTTCCATTCGCAATGAAGCTGTCTTT     P43-F   CGGAATTCTGATAGGTGGTATGTTTTCG     P43-R   CGGGATCCGTGTACATTCCTCTTTACCTATAGTG     Ptrq-F   CGGAATTCGTCGTCTTTTTCCCATTT     Ptrq-R   CGGAATTCGTCGTCTTTTTCCCATTT     Ptrq-F   CGGAATTCGTCGTCTTTTTCCCATTTT     Ptrq-R   CGGAATTCCTCAAAAAATAGGAACGTC     Pscp-F   CGGAATTCCTAATAGACTGCTTTTTCTACTAA     Pcsp-F   CGGAATTCCACATTTTTCGAAAAAAGTAA     Pcsp-F   CGGAATTCCACATGTTATTTCGAAAAAAGTAA     Psr-F   CGGAATTCCACATTGTAAAATAGGACC     Psr-F   CGGAATTCCACATTGTAAAATAGAGTGC     Psr-F   CGGAATTCACACTGTTATTCGAAAAATAGAGTGC     Psr-F   CGGAATTCACACTGTGTGCCCCCTT     Psr-F   CGGAATTCAACATGTGTGGCCCCCCTT     Psr-F   CGGAATTCAATTTCTGACACAATAATGCCAA     Psr-F   CGGAATTCAATTTCTGACACAATAATGCCAA     Psr-F   CGGAATTCAATTCTGACACAATAATGCCAA     Psr-F   CGGAATTCAATTTCTGACACAATAATGCCAA     Psr-F   CGGAATTCAATTTCTGACACAATAATGCCAA     Psr-F   CGGAATTCAATTTCTGACACATTATTCTGACACATAATGCCAA     Psr-F   CGGAATTCAATTTT	Phyp-F	CG <b>GAATT</b> CTTAAAATCACACTGACAGCAGAC
Ppqq-RTTTTTGCTTCTCATTGCAATGAAGCTGTCTTTP43-FCGGAATTCTGATAGGTGGTATGTTTCGP43-RCGGGATCCGTGTACATTCCTCTTACCTATAATGPtrqFCGGAATTCGTCGTCTTTTTCCCATTTTPtrqRCGGAATTCCTAAAAATAGTGATTTTAACAGGPscp-FCGGAATTCCATATAGACTGCGTTATGAAAAATAGTGATTTTAACAGGPscp-FCGGAATTCCAACATGTTATTTCGAAAAAATAGTAAPcsp-FCGGAATTCCAACATGTTATTTCGAAAAAAGTTAPcsp-RCGGAATTCCAACATGTTATTTCGAAAAAAGTAAPsrf-FCGGAATTCCAACATGTTATTCCTCCTAAAGCGACPsrf-FCGGAATTCAACGCGCTCTATGTAAAATAGAGTGCPsrf-RCGGAATTCCAATGTGTGGCGCCTCCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGAATTCCGAGATTCCTCCGATCATATTGCAAAAATGCCAA	Phyp-R	CG <b>GGATCC</b> CTGCCATTTGTTCTCACCTC
P43-FCGGAATTCTGATAGGTGGTATGTTTTCGP43-RCGGGATCCGTGTACATTCCTCTTACCTATAATGPtmQ-FCGGAATTCGTCGTCTTTTTCCCATTTTPtmQ-RCGGGATCCATATAGACTGCGTTATGAGAACGTCPscp-FCGGGATCCATCTATTCCTCCTTTTCCTATACAGPscp-FCGGAATTCCAACATGTTATTTCGAAAAAGTTAPcsp-RCGGGATCCGAAATTTCCTCCTAAAGCGACPsrf-FCGGGATCCATGTGTGCGCCTCACTTPsrf-FCGGGATCCATGTGTGGCGCCTCCCTTPitu-FCGGAATTCTAATTTCGACAATAATGCAAPitu-RCGGAATTCCAACTGTTATTCGACACATATGCCAA	Ppqq-F	CG <b>GGATCC</b> GGCAGGAGCTGTCTCTTTAT
P43-RCGGGATCCGTGTACATTCCTCTTACCTATAATGPtmQ-FCGGAATTCGTCGTCTTTTCCCATTTTPtmQ-RCGGGATCCATATAGACTGCGTTATGAGAACGTCPscp-FCGGAATTCCTAAAAATAGTGATTTTATCAGGPscp-RCGGAATTCAACATGTTATTCGAAAAAGTTAPcsp-RCGGAATTCCACGCGCTCATGTAAAATAGAGGCCPsrf-FCGGAATTCAACGCGCTCTATGTAAAATAGAGTGCPsrf-RCGGATTCCATGTGTGCGCCTCCCTTPitu-FCGGAATTCAACTGTTATTCGAAAAAAGCAAPitu-RCGGAATTCCACGAATATAGCAAATAGCAAA	Ppqq-R	TTTTTTGCTTCTCATTCGCAATGAAGCTGTCTTT
PtrnQ-F CGGAATTCGTCGTCTTTTTCCCATTTT   PtrnQ-R CGGGATCCATATAGACTGCGTTATGAGAACGTC   Pscp-F CGGAATTCCTAAAAATAGTGATTTTTATCAGG   Pscp-R CGGAATTCCATCTATTCCTCTTTTCTTTTACTAA   Pcsp-R CGGAATTCCAACATGTTATTCGAAAAAGTTA   Pcsp-R CGGAATTCCACACTGTATTCGAAAAAGGTAC   Psrf-F CGGAATTCCACGCGCTCATGTAAAATAGAGTGC   Psrf-R CGGATTCAACTGTGTGCGCCTCCCTT   Pitu-F CGGAATTCTAATTTCGAAAATAGCAA   Pitu-R CGGAATTCCACGACTATTTCGACAATAATGCCAA	P43-F	CG <b>GAATTC</b> TGATAGGTGGTATGTTTTCG
PtrnQ-RCGGGATCCATATAGACTGCGTTATGAGAACGTCPscp-FCGGAATTCCTAAAAATAGTGATTTTTATCAGGPscp-RCGGGATCCATCTATTCCTCTTTTCTTTTACTAPcsp-FCGGGATCCAACATGTTATTTCGAAAAAGTTAPcsp-RCGGGATCCGAAATTTCCTCCTAAAGCGACPsrf-FCGGGATCCACTGTGTGGCGCCTCCCTTPtru-FCGGGATCCATGTGTGGCGCCTCCCATATGCAAAAAGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATGCAA	P43-R	CG <b>GGATCC</b> GTGTACATTCCTCTTACCTATAATG
Pscp-FCGGAATTCCTAAAAATAGTGATTTTTATCAGGPscp-RCGGGATCCATCTATTCCTCTTTTTTTACTAPcsp-FCGGAATTCAACATGTTATTTCGAAAAAAGTTAPcsp-RCGGGATCCGAAATTTCCTCCTAAAGCGACPsrf-FCGGAATTCAGCGCTCTATGTAAAATAGAGTGCPsrf-RCGGGATCCATGTGTGCGCCTCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	PtrnQ-F	CGGAATTCGTCGTCTCTTTTTCCCATTTT
Pscp-RCGGGATCCATCTATTCCTCTTTTCTTTACTAPcsp-FCGGAATTCAACATGTTATTTCGAAAAAAGTTAPcsp-RCGGGATCCGAAATTTCCTCCTAAAGCGACPsrf-FCGGGATCCACGTCTATGTAAAATAGAGTGCPsrf-RCGGGATCCATGTGTGCGCCTCCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	PtrnQ-R	CG <b>GGATCC</b> ATATAGACTGCGTTATGAGAACGTC
Pcsp-FCGGAATTCAACATGTTATTTCGAAAAAAGTTAPcsp-RCGGGATCCGAAATTTCCTCCTAAAGCGACPsrf-FCGGAATTCAGCGCTCTATGTAAAATAGAGTGCPsrf-RCGGGATCCATGTGTGCGCCTCCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	Pscp-F	CG <b>GAATTC</b> CTAAAAAATAGTGATTTTTATCAGG
Pcsp-RCGGGATCCGAAATTTCCTCCTAAAGCGACPsrf-FCGGAATTCAGCGCTCTATGTAAAATAGAGTGCPsrf-RCGGGATCCATGTGTGCGCCTCCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	Pscp-R	CG <b>GGATCC</b> ATCTATTCCTCCTTTTTACTA
Psrf-FCGGAATTCAGCGCTCTATGTAAAATAGAGTGCPsrf-RCGGGATCCATGTGTGCGCCCTCCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	Pcsp-F	CG <b>GAATTC</b> AACATGTTATTTCGAAAAAAGTTA
Psrf-RCGGGATCCATGTGTGCGCCTCCCCTTPitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	Pcsp-R	CG <b>GGATCC</b> GAAATTTCCTCCTAAAGCGAC
Pitu-FCGGAATTCTAATTTCTGACACAATAATGCCAAPitu-RCGGGATCCGAGATTCCTCCGATCATATTGAA	Psrf-F	CG <b>GAATTC</b> AGCGCTCTATGTAAAATAGAGTGC
Pitu-R CG <b>GGATCC</b> GAGATTCCTCCGATCATATTGAA	Psrf-R	CGGGATCCATGTGTGCGCCTCCCCTT
	Pitu-F	CG <b>GAATTC</b> TAATTTCTGACACAATAATGCCAA
Pfen-F CG <b>GAATTC</b> CAAAAATGGGCGGAATTTT	Pitu-R	CG <b>GGATCC</b> GAGATTCCTCCGATCATATTGAA
	Pfen-F	CG <b>GAATTC</b> CAAAAATGGGCGGAATTTT
Pfen-R CG <b>GGATCC</b> AATGGCAGTTTTATCCTCCAG	Pfen-R	CG <b>GGATCC</b> AATGGCAGTTTTATCCTCCAG
Pbac-F CG <b>GAATTC</b> ATTCATTCACATCCTCCTTAAGA	Pbac-F	CG <b>GAATTC</b> ATTCATTCACATCCTCCTTAAGA
Pbac-R CG <b>GGATCC</b> CTGGATTTCCCCGCCTT	Pbac-R	CG <b>GGATCC</b> CTGGATTTCCCCGCCTT
pHY300-YF GTTTATTATCCATACCCTTAC	pHY300-YF	GTTTATTATCCATACCCTTAC
PHY300-YR CAGATTTCGTGATGCTTGTC	pHY300-YR	CAGATTTCGTGATGCTTGTC

Note: Restriction sites highlight in bold.

was added into B. The mixtures were placed at room temperature for 20 min. Subsequently, A and B were filtered and obtained supernatant, and added 5 ml of sodium carbonate solution and 1 ml of folin phenol reagent to the supernatant, mix well and heat at 40°C for 20 min, and measure the absorbance at 680 nm wavelength. The activity 1) of the dilution of sample was obtained from the standard curve. The enzymatic activity of the sample was calculated as follows:

Enzyme activity (U/mL) =  $\frac{a \times 4 \times N}{10}$  (N: Dilution factor of sample)

# 2.7 Transcriptional analysis during the alkaline protease fermentation

The recombinant engineered strains were cultured for 12 h, and then the cells were collected by centrifugation and washed for

ATG	AGA	AGC	ААА	ААА	TTG	TGG	ATC	AGC	TTG	TTG	TTT	GCG	TTA	ACG	TTA	ATC	TTT	ACG	ATG	GCG	TTC	AGC	AAC	ATG	FCT	GCG	CAG	GCT	Pro GCC	90
Met	Arg	Ser	Lys	Lys	Leu	Trp	Ile	Ser	Leu	Leu	Phe i	Ala	Leu	Thr	Leu	Ile	Phe	Thr	Met	Ala	Phe	Ser	Asn	Met	Ser	Ala	Gln	Ala	Ala	30
							AAA Lys																							
Glu	Lys	Gly	Gly	Lys	Val	Gln	Lys	Gln	Phe	Lys	Tyr	Val	Asn	Ala	Ala	Ala	Ala	Thr	Leu	Asp	Glu	Lys	Ala	Val	Lys	Glu	Leu	Lys	Lys	270 90
																														360 120
							ACA Thr																							450 150
							CCT Pro																							540 180
							CTG Leu																							630 210
																														720 240
																														810 270
																														900 300
																														990 330
							GCG Ala																							1080 360
							TAC Tyr																							
	г	re												Pro																
BSP	-1	MRS	SKK.	LWI	SL	LFA	ALTI	LIF	TM	AFS	NMS	S-A	QA	AGK	ISSI	rek	кү	IVG	FK	QTΜ	SA	MSS	AKF	(KD	νı	SEK	GGI	KVÇ	ξKQ	- 38
DJ-							A																							
DFE		0		v			.A			0	ST.	Ρ.			.NC	G					.т	A								
NAT					• •																									
BSF	1				• •	•••			• •		• • •	· - ·	• •		•••				• •		• •		• • •	• •	• •		• •	.I.	• •	
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DJ-							• • •																							
DFE							•••																						ŀŀ	
NAT							• • •																			• • •	• •	•••	ŀŀ	
BSF	1	•••	•••	т	• •	.N.	• • •	• • •	• •	Q	•••	•••	••	•••	•••	٠ŀ	• •	•••	•••	•••	••	•••	•••	• •	• •	• • •	• •	•••	Ŀŀ	
	1																													
BSP	- T	GID	SS	HPD	LN	VRG	GAS	SFV	PS	ETN	PYÇ	2DG	ss	HGT	HV	AGT	IA	ALN	NS:	IGV	LG	VAF	SAS	LY	AV :	KVI	DS	TGS	GQ	103
							GGA:						I																	103
DJ-	4				.K	.A.		.м.	• •		.F.	N	Ν.	. · ·	•••	•••	v.		•••	<i>v</i>	• •						GAI	D		103
DJ- DFE	4	 	 	 	.к .к	.A.		.м. .м.	 	 	.F.	.N	N. N.	.  .	· · ·	· · · ·	v. v.	 	••• •••	v v	 	 	 	 		 	GAI GAI	D D	 	103
DJ- DFE NAT	4	 	· · · · ·	  	.к .к	.A. .A.	•••	.м. .м.	  	  	.F. .F.	. N	N. N.	.  .	 	· · · ·	v. v.	  	· · · ·	V V 	  	  	 	 	  	  	GAI GAI	D D	 	103
DJ- DFE NAT BSF BSP	4	  YSW	 	 	.K .K 	.A. .A. 	SNNI	.м. .м.	  	 	.F.	. N .N	N. N. 				v. v.  Av	 	,	V V 	  	 	 N	 	 	 	GAI GAI	D D 	  	
DJ- DFE NAT BSF BSP DJ-	4 1 -1 4	  YSW		 	.K .K  EW	.A. .A.  AIS	· · · ·	. M . . M .  MDV	  IN	  MSI	. F . . F . 	.N .N PTG	N. N.  ST .A		TVV		V. V.  AV	  ssc	  	V V VAA	  .AA	 	 N GSS .T.	  	  		GAI GAI	D D  AKY G	 	
DJ- DFE NAT BSF BSP DJ-	4 1 -1 4	 YSW	 	 	.K .K  EW	.A. .A.  AIS 	SNNI A	.M. .M. 	  IN		.F.	PTG S.	N. N.  ST .A .A	ALK	TVV AA AA		V. V.  AV	  ssc a	  	V V VAA .V.	  .AA	GNE	 N XGSS .T. .T.	 	rs 5.		GAI 	D D  AKY G G	 	
DJ- DFE NAT BSF DJ- DFE NAT	4 1 -1 4	 YSW	VIII		.K .K  EW	.A. .A.  AIS 	5NNI A	. M . . M .  MDV	  	 	.F.	N N PTG S	N. N.  ST .A .A	ALK	TVV AA AA	VDK.	V. V.  AV	  ssc a	 	V V VAA .V.	  	GNE	 N GSS .T. .T.	  	rs '	TVG	GAI GAI	D D  AKY G G	 	
DJ- DFE NAT BSF DJ- DFE NAT BSF	4 -1 4	YSW	VIII	NGI	.K .K  EW	.A. .A.  AIS 	5NNI A	. M . . M .  MDV	 	 MSI	.F.	N .N .N .S. .S.	N. N.  ST .A .A	ALK	TVV AA AA	/ DK	V. V. AV	 SSG A A	 	V V VAA .V .V.	  	GNE	 N GSS .T. .T.	 GGS <sup>r</sup> .G	 	TVG	GAI GAI	D D AKY G G	 	173
DJ- DFE NAT BSF DJ- DFE DFE BSF	4 1 -1 4	YSN	VII)	 NGI 	.K .K  EW 	.A. .A.  AIS  NQF	5000 A RAS1	.M. .M.  MDV	    AG	MSI	. F . . F .   	N  	N. N.  ST .A  GV	ALK			V. V.  AV  GT	 SSG A A .N.		V V VAA .V .V. 	   	GNE	 N N  	    GS	  5 	   	GAI GAI 	D D AKY G G	   	173
DJ- DFE NAT BSF DJ- DFE NAT BSF BSP DJ-	4 1 4 1 1 -1 4	YSW  TIP	1VG.	NGI	.K .K  EW   SS	.A. .A.  AIS  NQF	5NNI A RASI	.M. .M. MDV	    AG	MSI	. F . . F .   	N 	N. N.  ST .A  GV	ALK		VDK	V. V.  AV  GT	SSG A A .N.		V V VAA .V .V. 	    MA	GNE	N N .T. .T.	 GGS <sup>2</sup> .GS   GSAAI	  5 	TVG	GAI GAI     GKHI	D D AKY G G PTW .N.	    	173
DJ- DFE NAT BSF DJ- DFE NAT BSF BSP DJ- DFE	4 1 4 1 -1 4	YSN  TIP V	1VG.	NGI	.K .K  EW  .SS	.A. .A.  AIS  NQF	5000 5000 5000 5000 5000 5000 5000 500	.M. .M.  MDV  	   AG V.	MSI	.F.	N 	N. N.  ST .A  GV 	ALK		JDK.	V. V. AV  GT NK	SSG A A .N. YGA		V V VAA .V. .V. 	    	GNE       	 N CGSS .T. .T. 		  5 5 AL	TVG	GAI GAI	D D AKY G G PTW .N. .N.	     	173
DJ- DFE NAT BSF DJ- DFE NAT BSF	4 1 4 1 -1 4	YSN  TIP V	VIII	NGI	.K .K  EW  	.A. .A. AIS 	5NNI A RASI	.M. .M. MDV	    AG V. V. V.	MSI	.F. .F. 	N 	N. N. .N. ST .A .A  GV 	ALK		JUDK	V. V.  AV  GT NK NK	SSC A N YGA		V V VAA .V .V. 	    	GNE    TPH S S	 N CGSS .T. .T. 	    	TS 5.	TVG	GAI (GA) 	D D AKY G G PTW .N .N.	     	173
DJ- DFE NAT BSF DJ- DFE BSF DJ- DFE NAT BSF	4 1 -1 4 1 -1 4	YSW  TIP V V	VIII	NGI	.K .K  EW  .SS  	.A. .A.  AIS  NQF	5000 A RASI	.M. .M. MDV	  IN   AG V. V. V. V.	MSI    SEI  P	.F.	N 	N. N. ST .A .A GV 	ALK	AA AA	VDK.	V. V.  AV  GT NK NK	SSC A N YGA		V V VAA .V .V. 	    	GNE    TPH S S	 N CGSS .T. .T. 	    	TS 5.	TVG	GAI (GA) 	D D AKY G G PTW .N .N.	     	173
DJ- DFE NAT BSF DJ- DFE BSF DJ- DFE NAT BSF	4 -1 4 1 -1 4 -1 4 -1 -1	YSW  TIP V AQ	VIII 	NGI	.K .K  EW     	. A . . A .  AIS       	SNNI A RASI	.M. .M. MDV  FSS 	    AG V. V. V. V. V. FYY	MSI   SEI  P	. F . . F .   	N 	N. N. ST .A .A GV  	ALK SIQ		VDK.	V. V.  AV  GT NK NK	SSC A N YGA		V V VAA .V .V. 	    	GNE    TPH S S	 N CGSS .T. .T. 	    	TS 5.	TVG	GAI (GA) 	D D AKY G G PTW .N .N.	     	173
DJ- DFE NAT BSF DJ- DFE DAT BSF DJ- ESF BSF BSF	4 1 -1 4 1 -1 4 -1 4 -1 4	YSW  TIA V AQ <sup>Y</sup> T	VIII 	NGI	.K .K  EW  .SS   	.A. .A. AIS  .AIS  .AIS  .AIS 	SNNN A RASI	.M. .M. MDV  FSS  	 IN  AG V. V. V. FYY	MSI   SEI  P	. F . . F . 	N 	N. N. ST .A .A GV 	ALK	25T1	VDK.	V. V.  AV  GT NK NK	SSC A N YGA		V V VAA .V .V. 	    	GNE    TPH S S	 N CGSS .T. .T. 	    	TS 5.	TVG	GAI (GA) 	D D AKY G G PTW .N .N.	     	173
DJ- DFE NAT BSF DJ- DFE BSF DJ- BSF DJ- ESF DJ- BSF DJ- BSF DJ-	4 1 -1 4 1 -1 4 1 -1 4	YSW  TIP V V AQ <sup>7</sup> T T	VIII 	NGI   AVN  PRLE S S	.K .K  EW        	.A. .A. AIS  NQF  AT T. T.	SNNI A RASI	.M. .M. MDV  FSS  GSSS DA DA	 IN  AG V. V. V. FYY	MSI   SEI  P	.F.	N 	N. N. ST .A .A GV 	ALK	25T1	VDK.	V. V.  AV  GT NK NK	SSC A N YGA		V V VAA .V .V. 	    	GNE    TPH S S	 N CGSS .T. .T. 	    	TS 5.	TVG	GAI (GA) 	D D AKY G G PTW .N .N.	     	173

#### FIGURE 1

Sequence analysis of *bsp-1* gene and deduced amino acids. (A) Nucleotide (upper line) and deduced amino acid (lower line) sequences of the protease BSP-1. The predicted signal peptide, propeptide and mature peptide are marked with arrows. (B) Amino acid sequence alignment of the protease BSP-1, with subtilisin (DJ-4, DFE) from *B. amyloliquefaciens* and subtilisin (NAT, BSF1) from *B. subtilis*. The signal peptide, pro-peptide, and mature peptide as the first sequence. The catalytic center residues (Asp-32, His-64, and Ser-221) were boxed. The initial amino acid of the mature peptide was numbered as + 1.

transcription analysis, according to the description of Total RNA Isolation Kit and PrimeScript RT Master Mix Kit. DNA removal and RNA reverse transcription were performed simultaneously in one system. The reaction conditions which included EasyScript<sup>®</sup> RT/RI and gDNA Remover were 25°C for 10 min, 42°C for 15 min, and 85°C for 10 s. RT-PCR amplification was performed using three-steps method. Pre-denaturation: 1 cycle (95°C for 5 min); PCR reaction: 40 cycles (95°C for 30 s, 60°C for 30 s) and dissolution curve (1 min at 95°C, 1 min at 65°C). Relative quantitative analysis was performed with 16 s RNA as the internal reference gene.

### 2.8 Statistical analysis

Each group of experiments was designed with three independent replicates. SPSS 20.0 was used for statistical analysis, calculating the means and standard deviations, and evaluating the significance. Origin 8.5 was used to process the data and make the graphs.

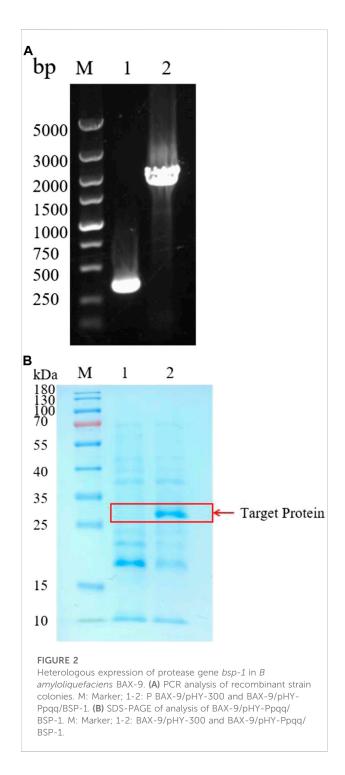
# 3 Results and discussion

# 3.1 Identification of an alkaline protease from a new *B. subtilis* strain

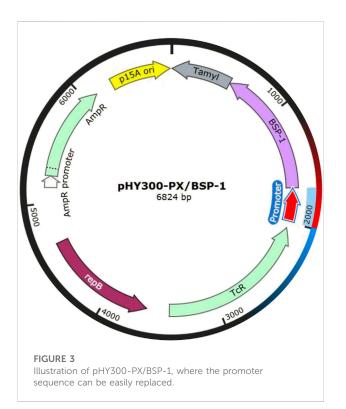
We isolated a B. subtilis strain with high protease activity using skim milk plates. Then the new B. subtilis strain was named B. subtilis D7. In order to identify an alkaline protease from B. subtilis D7, we designed a primer set using the gene sequence of subtilisin NAT (S51909) from B. subtilis. With the primers, we amplified a gene fragment from B. subtilis D7. Sequencing of the gene fragment indicates that it encodes an alkaline protease (BSP-1) containing 381 amino acids (aa) (Figure 1A). Analysis of the protein sequence using Signal P 5.0 indicates a 29-aa predicted signal peptide, while the 30-77 aa and 78-275 aa are the propeptide and mature peptide, respectively (Figure 1A). We aligned the sequence of BSP-1 with four well-characterized proteases (Choi et al., 2004; Yong et al., 2004; Agrebi et al., 2009; Ku et al., 2009), indicating that it has the highest similarity to subtilisin NAT (S51909) and fibrinolytic enzyme BSF1(FJ517584) (>96% similarity) and less similar to subtilisin DJ-4 and subtilisin DFE (Figure 1B). In addition, all the proteases contain the conserved catalytic triad, including Asp-32, His-64, and Ser-221. Together, the data indicates that BSP-1 belong to the subtilisin family of serine proteases (Wei et al., 2011).

# 3.2 Heterologous expression of BSP-1 in *B. amyloliquefaciens* BAX-9

*B. amyloliquefaciens* is considered as a safe strain that have been widely used to express industrial enzymes. *B. amyloliquefaciens* is rich in proteases, and can degrade



heterologous proteins. Knockout of the protease genes may solve the problem. Therefore, we expressed BSP-1 in *B. amyloliquefaciens* BAX-9, a strain where many protease genes (*epr, nprE, aprE, aprX, mpr, bpf, vpr, htrB, yktc1*) have been knocked out (Chen et al., 2022). At first, the promoter of PQQ-binding-like beta-propeller repeat gene (Ppqq) from *B. amyloliquefaciens* was used to express BSP-



1. The constructed expression plasmid, pHY-Ppqq/BSP-1, was electroporated into B. amyloliquefaciens BAX-9, generating BAX-9/pHY-Ppqq/BSP-1 (Figure the strain 2A). Subsequently, the fermentation broth of BAX-9/pHY-Ppqq/ BSP-1 and the control strain (BAX-9/pHY-300) were used for SDS-PAGE analysis. As shown in Figure 2B, compared to the control strain (BAX-9/pHY-300), the target protein (30 kDa) was found in the fermentation broth of BAX-9/pHY-Ppqq/ BSP-1. This result demonstrates that the bsp-1 gene was significantly expressed in BAX-9. The thickness of the band corresponds to the amount of protein present, the thicker band of the target protein also shows the feasibility and great potential of expressing the heterologous protein in B. amyloliquefaciens.

# 3.3 Promoter screening enhanced BSP-1 expression

To further improve the expression of BSP-1 in *B. amyloliquefaciens*, we screened different promoters driving the expression of BSP-1. The promoters include two reported high-strength promoters (P43 (Wang and Doi, 1984), PtrnQ (Song et al., 2016)) from *B. subtilis* and a promoter collection from *B. amyloliquefaciens* HZ-12. We selected the promoters of potentially highly-expressed genes in HZ-12, including *ssrA* (NC\_014551.1: 3271400-3271038), *ssrS* (NC\_014551.1:

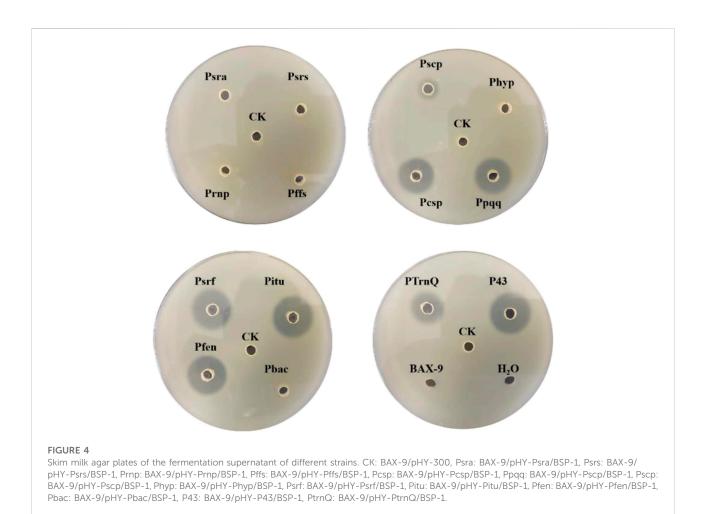
2609656-2609464), *rnpB* (NC\_014551.1: 2202786-2202405), *ffs* (NC\_014551.1: 27641-27905), cold-shock protein gene (NC\_014551.1: 1029767-1029567), PQQ protein gene (NC\_014551.1: 3859752-3858532), spore coat protein gene (NC\_014551.1: 1272412-1271912), hypothetical protein gene (NC\_014551.1: 1272819-1272505). In addition, since *B. amyloliquefaciens* is highly productive of macromolecular substances such as lipopeptides, the promoters of four gene clusters (Surfactin, Iturin A, Fengycins, Bacillaene) producing lipopeptides, i.e., Psrf, Pitu, Pfen, Pbac, respectively, were also used in the study.

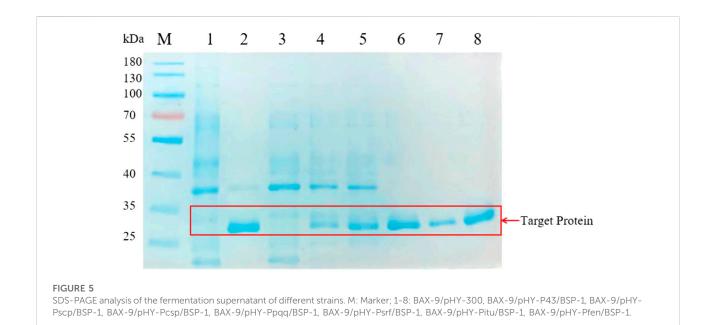
For efficient insertion of the promoters, we constructed a plasmid with replaceable promoter sequence by inserting the fusion fragment of bsp-1 gene and TamyL terminator into pHY300PLK, resulting in the plasmid pHY300PLK-PX/BSP-1 (Figure 3). Then, the sequences of the promoters were amplified and then inserted into pHY300PLK-PX/BSP-1. The resulting plasmids were transformed into BAX-9 to generate engineered strains for further analysis. Engineered strains containing different promoters (Psra, Psrs, Prnp, Pffs, Ppqq, Pcsp, Pscp, Phyp, Psrf, Pitu, Pfen, Pbac, P43, PtrnQ) and the control strain (BAX-9/pHY-300, BAX-9) were fermented. And the enzyme activities of these strains were preliminarily analyzed by using skim milk agar plates. As shown in Figure 4, the supernatant of the fermentation broth of the engineering strains containing P43, Pitu, Pfen, Ppqq, Psrf, Pcsp, PtrnQ and Pscp produced transparent circles of different radius (7, 7, 7, 6.50, 6, 6, 4, 1.50 mm, respectively), while other promoters (Psra, Psrs, Prnp, Pffs, Phyp, Pbac) did not produce transparent circles. The size of the transparent circle reflects the activity of BSF-1, the size of the transparent circle is positively correlated with the activity.

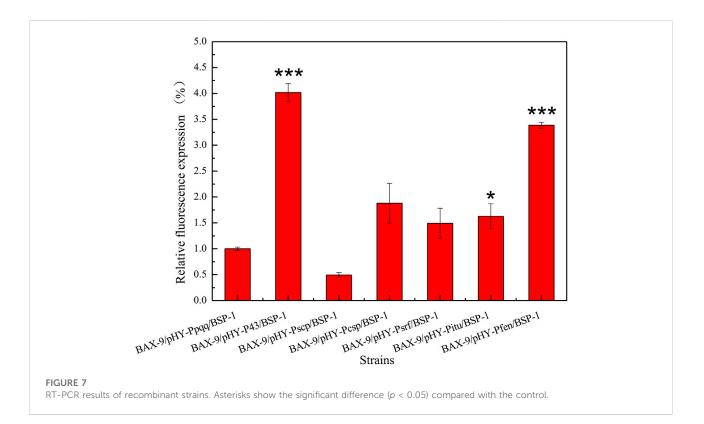
Accorded to the result of the transparent circle, we selected the Pscp, Pcsp, Ppqq, Psrf, Pitu, Pfen promoters from *B. amyloliquefaciens* and the P43 promoter from *B. subtilis* for further analysis. We analyzed the proteins in the supernatant of BAX-9/pHY-Pscp/BSP-1, BAX-9/pHY-Pcsp/BSP-1, BAX-9/ pHY-Ppqq/BSP-1, BAX-9/pHY-Psrf/BSP-1, BAX-9/pHY-Pitu/ BSP-1, BAX-9/pHY-Pfen/BSP-1 and BAX-9/pHY-P43/BSP-1 by SDS-PAGE. As shown in Figure 5, BSP-1 was successfully expressed in all recombinant strains, and exhibited the highest level under the promoter P43, Psrf and Pfen, which was consistent with the result of the transparent circle. The data indicates that a high-strength promoter facilitates the protein expression level and thus increase the activity outcome of protein.

# 3.4 Effects of different promoters on the activity of BSP-1

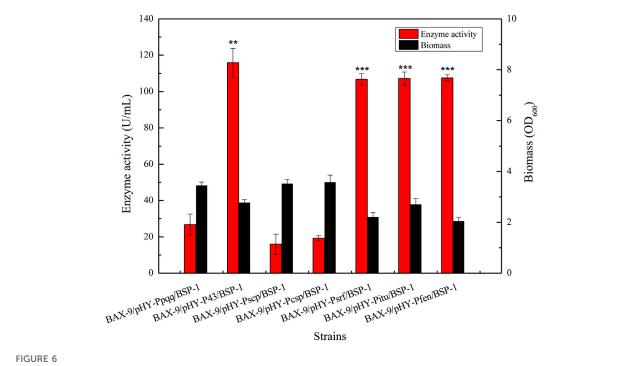
To further analyze the effect of different promoters on the expression level of protease gene *bsp-1*, we measured the activity

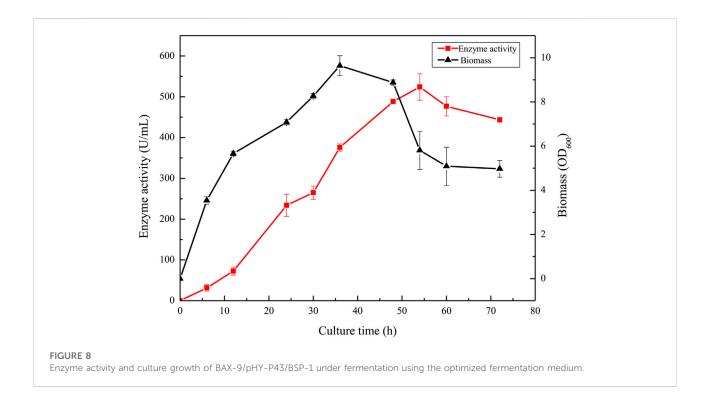












of BSP-1 and the biomass of the seven cultures at 48 h. As shown in Figure 6, the activity of BSP-1 expressed by the promoter of P43, Psrf, Pitu, and Pfen reached 115.87, 106.71, 107.25, and 107.52 U/mL respectively, improving by 4.3-fold, 4.0-fold, 4.0fold and 4.0-fold compared to the Ppqq promoter (26.71 U/mL). The data indicated protomer sceening efficiently enhanced the activity outcome of BSP-1. In addition, the biomass of Ppqq promoter was significantly higher than that of P43, Psrf, Pitu and Pfen promoter, indicating that the enzyme activity and the biomass are not necessarily positively correlated.

# 3.5 Verification of the transcription of *bsp-1* drived by different promoters

To verify the effect of promoter on gene transcription level, we established a microbial fermentation model. The total RNA of different recombinant strains was extracted and analyzed. As shown in Figure 7, the transcription levels of *bsp-I* in BAX-9/pHY-P43/BSP-1, BAX-9/pHY-Pscp/BSP-1, BAX-9/pHY-Pcsp/BSP-1, BAX-9/pHY-Psrf/BSP-1, BAX-9/pHY-Pitu/BSP-1, BAX-9/pHY-Pfen/BSP-1 significantly increased compared to that in BAX-9/pHY-Ppqq/BSP-1. Among them, BAX-9/pHY-P43/BSP-1 showed the highest transcription level, 4 folds than that in BAX-9/pHY-Ppqq/BSP-1. The results of RT-PCR were consistent with the results of SDS-PAGE and enzyme activity, revealing that the efficient transcription by P43 generated high protein level and enzyme activity. The above results provide a reference to select the optimal promoter for protein expression.

### 3.6 The optimization of fermentation for BSP-1 production in BAX-9/pHY-P43/ BSP-1

To further improve the protease yield of the recombinant strain BAX-9/pHY-P43/BSP-1, an optimized medium (40 g/L tryptone, 20 g/L yeast extract, and 10 g/L NaCl) was selected for fermentation (Supplementary Figure S1). During the fermentation process, the activity of BSP-1 protease and the biomass of strains were determined. As shown in Figure 8, in the initial 36 h, the enzyme activity increased as the culture was growing. With the continuation of fermentation, the biomass began to decrease, and the enzyme activity reached a maximum of 524.12 U/mL. Finally, with the continuous decrease of biomass, the enzyme activity declined and tended to be stable. The results indicate that the accumulation of the enzyme was delayed compared to culture growth.

# 4 Conclusion

In conclusion, the BSP-1 protease gene from *B. subtilis* was identified in this study, and explored possibility of high-efficiency

expression system via *B. amyloliquefaciens*. The enzyme activity and transcription level under the expression of different promoters Ppqq, Pscp, Pcsp, Psrf, Pitu, Pfen and P43 were analyzed, and we found P43 was the optimal promoter for heterologous expression of BSP-1 protease. Nevertheless, Psrf and Pfen show comparable expression strength to P43 and thus can be used as high-strength promoters in future. After fermentation optimization, the highest enzyme activity of the engineering strain BAX-9/pHY-P43/BSP-1 reached 524.12 U/ mL. Moreover, monitoring the fermentation process also confirmed no direct correlation between enzyme activity and biomass. This study enriched the originals and hosts for protein expression, and it identified a new resource for the industrialized production of proteases.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## Author contributions

Conceptualization, CJ and WH; Methodology, CJ; Software, CJ and CY; Validation, CJ, CY, and YL; Formal analysis, KH and XJ; Investigation, XW; Resources, XW and WH; Data curation, CJ and YL; Writing—original draft preparation, CJ; Writing—review and editing, CJ, XW, WH, DZ, and LL; Visualization, CY; Supervision, XW; Project administration, XW; Funding acquisition, XW and WH.

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## **Conflict of interest**

YL was employed by GeneMind Biosciences Company Limited. CY and KH were employed by China National Tobacco Corporation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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### Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022. 977215/full#supplementary-material

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