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 wild-type −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 β-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 *Bam*HI and *Xba*I, 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 μL 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°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°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 lab
BAX-9/pHY-300	BAX-9 harboring the plasmid pHY300PLK	Stored in lab
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 + *bsp-1*+ TamyL	This study
pHY-Psra/BSP-1	pHY300PLK + Psra + *bsp-1*+ TamyL	This study
pHY-Psrs/BSP-1	pHY300PLK + Psrs + *bsp-1*+ TamyL	This study
pHY-PrnpB/BSP-1	pHY300PLK + PrnpB + *bsp-1*+ TamyL	This study
pHY-Pffs/BSP-1	pHY300PLK + Pffs + *bsp-1*+ TamyL	This study
pHY-Phyp/BSP-1	pHY300PLK + Phyp + *bsp-1*+ TamyL	This study
pHY-Ppqq/BSP-1	pHY300PLK + Ppqq + *bsp-1*+ TamyL	This study
pHY-Pscp/BSP-1	pHY300PLK + Pscp + *bsp-1*+ TamyL	This study
pHY-Pcsp/BSP-1	pHY300PLK + Pcsp + *bsp-1*+ TamyL	This study
pHY-P43/BSP-1	pHY300PLK + P43+ *bsp-1*+ TamyL	This study
pHY-PtrnQ/BSP-1	pHY300PLK + PtrnQ + *bsp-1*+ TamyL	This study
pHY-Psrf/BSP-1	pHY300PLK + Psrf + *bsp-1*+ TamyL	This study
pHY-Pitu/BSP-1	pHY300PLK + Pitu + *bsp-1*+ TamyL	This study
pHY-Pfen/BSP-1	pHY300PLK + Pfen + *bsp-1*+ TamyL	This study
pHY-Pbac/BSP-1	pHY300PLK + Pbac + *bsp-1*+ 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°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°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.

Primer name	Sequence of primer (5' to 3')
bsp-1-F	CG**GGATCC**ATGAGAAGCAAAAAATTGTGGAT
bsp-1-R	TTATTGTGCAGCTGCTTGTACG
bsp-1 (Ppqq)-F	ACAGCTTCATTGCGAATGAGAAGCAAAAAATTGTGGAT
bsp-1 (Tamyl)-R	AAGAGCAGAGAGGACTTATTGTGCAGCTGCTTGTACG
TamyL-F	GCAGCTGCACAATAAAAGAGCAGAGAGGACGGATT
TamyL-R	GC**TCTAGA**CGCAATAATGCCGTCGCACT
Psra-F	CG**GAATTC**AACGAAAAGACGCCAAAAG
Psra-R	CG**GGATCC**TTGTTAAGGGTATACGGGAGAT
Psrs-F	CG**GAATTC**GCAGTTTGTTTCTTGAAAATCA
Psrs-R	CG**GGATCC**CACAGCAGATTTTGATTTTCAA
PrnpB-F	CG**GAATTC**GGTCGTATTCGGCGCAT
PrnpB-R	CG**GGATCC**CAAAATAAATATCAAATTTTGATATG
Pffs-F	CG**GAATTC**GGATTATGAAACCTTTCATCAAG
Pffs-R	CG**GGATCC**TAAGAACACTTGTTCATTATAAAGC
Phyp-F	CG**GAATT**CTTAAAATCACACTGACAGCAGAC
Phyp-R	CG**GGATCC**CTGCCATTTGTTCTCACCTC
Ppqq-F	CG**GGATCC**GGCAGGAGCTGTCTCTTTAT
Ppqq-R	TTTTTTGCTTCTCATTCGCAATGAAGCTGTCTTT
P43-F	CG**GAATTC**TGATAGGTGGTATGTTTTCG
P43-R	CG**GGATCC**GTGTACATTCCTCTCTTACCTATAATG
PtrnQ-F	CG**GAATTC**GTCGTCTCTTTTTCCCATTTT
PtrnQ-R	CG**GGATCC**ATATAGACTGCGTTATGAGAACGTC
Pscp-F	CG**GAATTC**CTAAAAAATAGTGATTTTTATCAGG
Pscp-R	CG**GGATCC**ATCTATTCCTCCTTTTCTTTTACTA
Pcsp-F	CG**GAATTC**AACATGTTATTTCGAAAAAAGTTA
Pcsp-R	CG**GGATCC**GAAATTTCCTCCTAAAGCGAC
Psrf-F	CG**GAATTC**AGCGCTCTATGTAAAATAGAGTGC
Psrf-R	CG**GGATCC**ATGTGTGCGCCTCCCCTT
Pitu-F	CG**GAATTC**TAATTTCTGACACAATAATGCCAA
Pitu-R	CG**GGATCC**GAGATTCCTCCGATCATATTGAA
Pfen-F	CG**GAATTC**CAAAAATGGGCGGAATTTT
Pfen-R	CG**GGATCC**AATGGCAGTTTTATCCTCCAG
Pbac-F	CG**GAATTC**ATTCATTCACATCCTCCTTAAGA
Pbac-R	CG**GGATCC**CTGGATTTCCCCGCCTT
pHY300-YF	GTTTATTATCCATACCCTTAC
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℃ 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:

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

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

A

Pre

```
ATG AGA AGC AAA AAA TTG TGG ATC AGC TTG TTG TTT GCG TTA ACG TTA ATC TTT ACG ATG GCG TTC AGC AAC ATG TCT GCG CAG GCT GCC    90
Met Arg Ser Lys Lys Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala Gln Ala Ala    30
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Pro

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GGA AAA AGC AGT ACA GAA AAG AAA TAC ATT GTC GGA TTT AAG CAG ACA ATG AGT GCC ATG AGT TCC GCC AAA AAG GAT GTT ATT TCT    180
Gly Lys Ser Ser Thr Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Ala Met Ser Ser Ala Lys Lys Lys Asp Val Ile Ser    60

GAA AAA GGC GGA AAG GTT CAA AAG CAA TTT AAG TAT GTT AAC GCG GCC GCA GCA ACA TTG GAT GAA AAA GCT GTA AAA GAA TTG AAA AAA    270
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    90

GAT CCG AGC GTT GCA TAT GTG GAA GAA GAT CAT ATT GCA CAT GAA TAT GCG CAA TCT GTT CCT TAT GGC ATT TCT CAA ATT AAA GCG CCG    360
Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Ile Ala His Glu Tyr Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala Pro    120
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Mature

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GCT CTT CAC TCT CAA GGC TAC ACA GGC TCT AAC GTA AAA GTA GCT GTT ATC GAC AGC GGA ATT GAC TCT TCT CAT CCT GAC TTA AAC GTC    450
Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val    150

AGA GGC GGA GCA AGC TTC GTT CCT TCT GAA ACA AAC CCA TAC CAG GAC GGC AGT TCT CAC GGT ACG CAT GTC GCC GGT ACG ATT GCC GCT    540
Arg Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His Gly Thr His Val Ala Gly Thr Ile Ala Ala    180

CTT AAT AAC TCA ATC GGT GTT CTG GGC GTA GCG CCA AGC GCA TCA TTA TAT GCA GTA AAA GTG CTT GAT TCA ACA GGA AGC GGC CAA TAT    630
Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu Asp Ser Thr Gly Ser Gly Gln Tyr    210

AGC TGG ATT ATT AAC GGC ATT GAG TGG GCC ATT TCC AAC AAT ATG GAT GTT ATC AAC ATG AGC TTG GGC GGA CCT ACT GGT TCT ACA GCG    720
Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Thr Gly Ser Thr Ala    240

CTG AAA ACA GTA GTT GAT AAA GCG GTT TCC AGC GGT ATC GTC GTT GCT GCC GCA GCC GGA AAC GAA GGT TCA TCC GGA AGC ACA AGC ACA    810
Leu Lys Thr Val Val Asp Lys Ala Val Ser Ser Gly Ile Val Val Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly Ser Thr Ser Thr    270

GTC GGC TAC CCT GCA AAA TAT CCT TCT ACT ATT GCA GTA GGT GCG GTA AAC AGC AGC AAC CAA AGA GCT TCA TTC TCC AGC GCA GGT TCT    900
Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala Gly Ser    300

GAG CTT GAT GTA ATG GCT CCT GGC GTG TCC ATC CAA AGC ACA CTT CCT GGA GGC ACT TAC GGC GCT TAT AAC GGA ACG TCC ATG GCG ACT    990
Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr    330

CCT CAC GTT GCC GGA GCA GCA GCG CTA ATT CTT TCT AAG CAC CCG ACT TGG ACA AAC GCG CAA GTC CGT GAT CGT TTA GAA AGC ACT GCA    1080
Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala    360

ACA TAC CTT GGA AGC TCT TTC TAC TAT GGA AAA GGG TTA ATC AAC GTA CAA GCA GCT GCA CAA TAA    1146
Thr Tyr Leu Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala Ala Ala Gln End    381
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B

Pre   Pro
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BSP-1 MRSKKLWISL LFALTLIFTM AFSNMS-AQA AGKSSTEKKY IVGFKQTMSA MSSAKKKDVI SEKGGKVQKQ -38
DJ-4  ..G..V.... ....A...... .GST.P.... ....NG.... .........T ..A....... .........
DFE   ..G..V.... ....A...... .GST.P.... ....NG.... .........T ..A....... .........
NAT   .......... .......... ....-..... .......... .......... .......... .........
BSF1  .......... .......... ....-..... .......... .......... ..........I...
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Mature
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BSP-1 FKYVNAAAAT LDEKAVKELK KDPSVAYVEE DHIAHEYAQS VPYGISQIKA PALHSQGYTG SNVKVAVIDS 33
DJ-4  ....D..S.. .N........ .......... ..V.QA.... .......V.. ........F. ..........
DFE   ....D..S.. .N........ .......... ..V.QA.... .......V.. ........F. ..........
NAT   .......... .......... .......... .......... .......... .......... ..........
BSF1  .......T.. .N........ ..Q....... .......... .......... .......... ..........
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BSP-1 GIDSSHPDLN VRGGASFVPS ETNPYQDGSS HGTHVAGTIA ALNNSIGVLG VAPSASLYAV KVLDSTGSGQ 103
DJ-4  .........K .A....M... .....F..NN. ..........V. ......V... ........... ...GAD....
DFE   .........K .A....M... .....F..NN. ..........V. ......V... ........... ...GAD....
NAT   .......... .......... .......... .......... .......... .......... ..........
BSF1  .......... .......... .......... .......... .......... ....N..... ..........
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BSP-1 YSWIINGIEW AISNNMDVIN MSLGGPTGST ALKTVVDKAV SSGIVVAAAA GNEGSSGSTS TVGYPAKYPS 173
DJ-4  .......... ..A....... ...S..A ...AA..... A..V..V... ....T..GS. .....G....
DFE   .......... ..A....... ...S..A ...AA..... A....V.... ....T...S. .....G....
NAT   .......... .......... .......... .......... .......... .......... ..........
BSF1  .......... .......... ...S...... ......N... .......... .......... ..........
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BSP-1 TIAVGAVNSS NQRASFSSAG SELDVMAPGV SIQSTLPGGT YGAYNGTSMA TPHVAGAAAL ILSKHPTWTN 243
DJ-4  V......... ........V. .......... .......NK .........S. ......... ....N...
DFE   V......... ........V. .......... .......NK .........S. ......... ....N...
NAT   .......... ........V. .......... .......... .......... .......... ..........
BSF1  .......... ....P..... .......... .......... ...S...... .......... ..........
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BSP-1 AQVRDRLEST ATYLGSSFYY GKGLINVQAA AQ 275
DJ-4  T...SS..N. T.K..DA... .......... ..
DFE   T...SS..N. T.K..DA... .......... ..
NAT   .......... .....N.... .......... ..
BSF1  .......... .....N.... .......... ..
```

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 (mature) were indicated with arrows. "." indicated the same residue 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® 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 SignalP 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

FIGURE 2
Heterologous expression of protease gene *bsp-1* in *B amyloliquefaciens* BAX-9. **(A)** PCR analysis of recombinant strain colonies. M: Marker; 1-2: P BAX-9/pHY-300 and BAX-9/pHY-Ppqq/BSP-1. **(B)** SDS-PAGE of analysis of BAX-9/pHY-Ppqq/BSP-1. M: Marker; 1-2: BAX-9/pHY-300 and BAX-9/pHY-Ppqq/BSP-1.
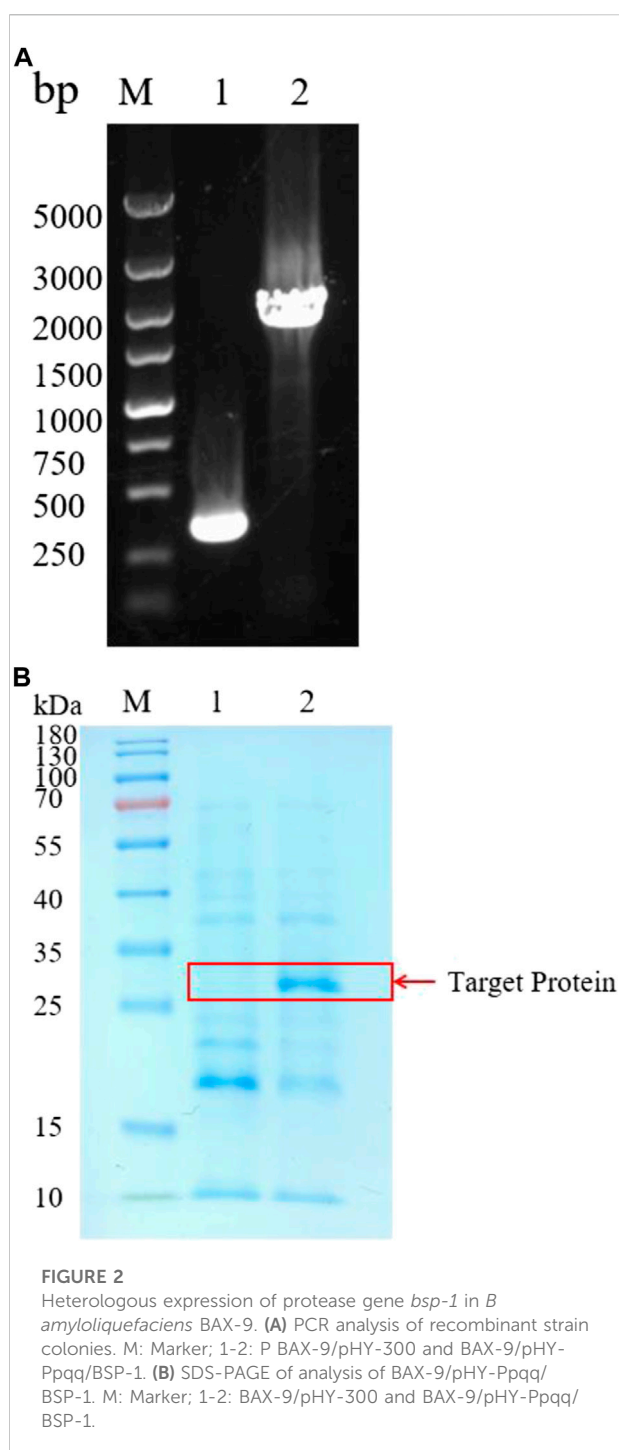
heterologous proteins. Knockout of the protease genes may solve the problem. Therefore, we expressed BSP-1 in *B. amyloliquefaciens* BAX-9, a strain where many protease genes (*epr, nprE, aprE, aprX, mpr, bpf, vpr, htrB, yktc1*) have been knocked out (Chen et al., 2022). At first, the promoter of PQQ-binding-like beta-propeller repeat gene (Ppqq) from *B. amyloliquefaciens* was used to express BSP-

FIGURE 3
Illustration of pHY300-PX/BSP-1, where the promoter sequence can be easily replaced.
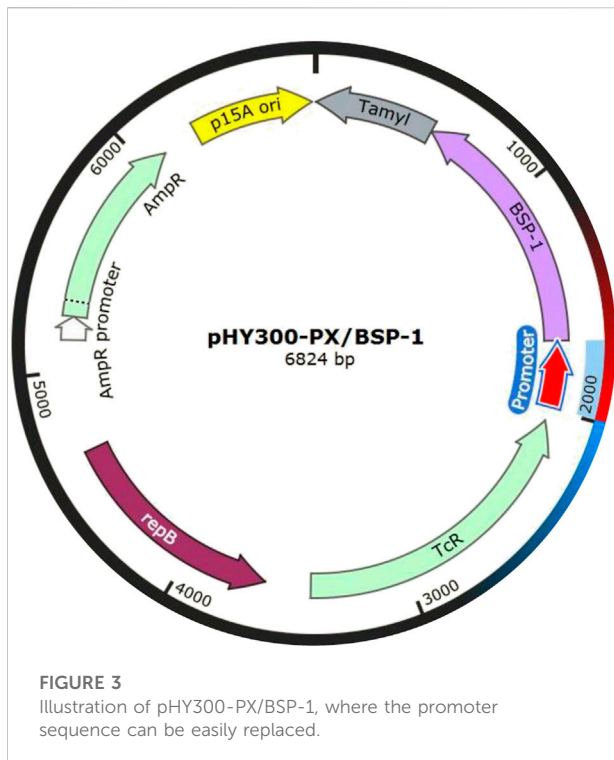
1. The constructed expression plasmid, pHY-Ppqq/BSP-1, was electroporated into *B. amyloliquefaciens* BAX-9, generating the strain BAX-9/pHY-Ppqq/BSP-1 (Figure 2A). Subsequently, the fermentation broth of BAX-9/pHY-Ppqq/BSP-1 and the control strain (BAX-9/pHY-300) were used for SDS-PAGE analysis. As 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
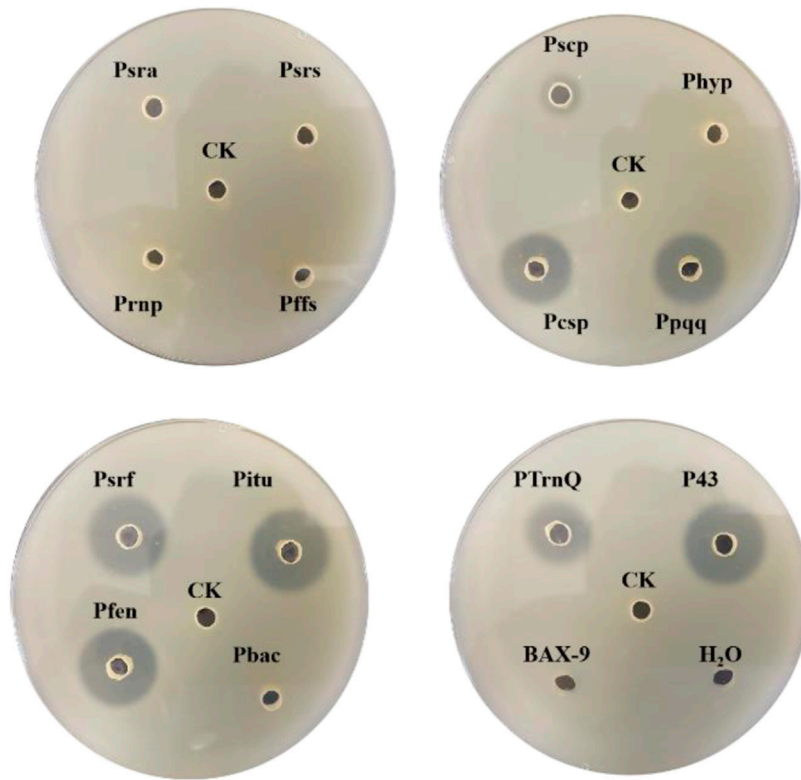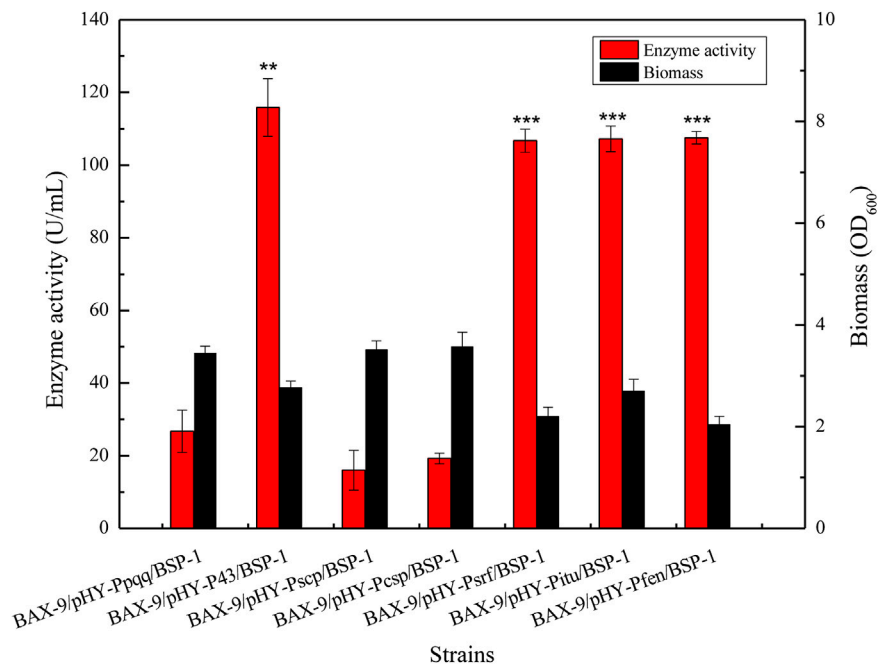
FIGURE 4
Skim milk agar plates of the fermentation supernatant of different strains. CK: BAX-9/pHY-300, Psra: BAX-9/pHY-Psra/BSP-1, Psrs: BAX-9/pHY-Psrs/BSP-1, Prnp: BAX-9/pHY-Prnp/BSP-1, Pffs: BAX-9/pHY-Pffs/BSP-1, Pcsp: BAX-9/pHY-Pcsp/BSP-1, Ppqq: BAX-9/pHY-Pscp/BSP-1, Pscp: BAX-9/pHY-Pscp/BSP-1, Phyp: BAX-9/pHY-Phyp/BSP-1, Psrf: BAX-9/pHY-Psrf/BSP-1, Pitu: BAX-9/pHY-Pitu/BSP-1, Pfen: BAX-9/pHY-Pfen/BSP-1, Pbac: BAX-9/pHY-Pbac/BSP-1, P43: BAX-9/pHY-P43/BSP-1, PtrnQ: BAX-9/pHY-PtrnQ/BSP-1.
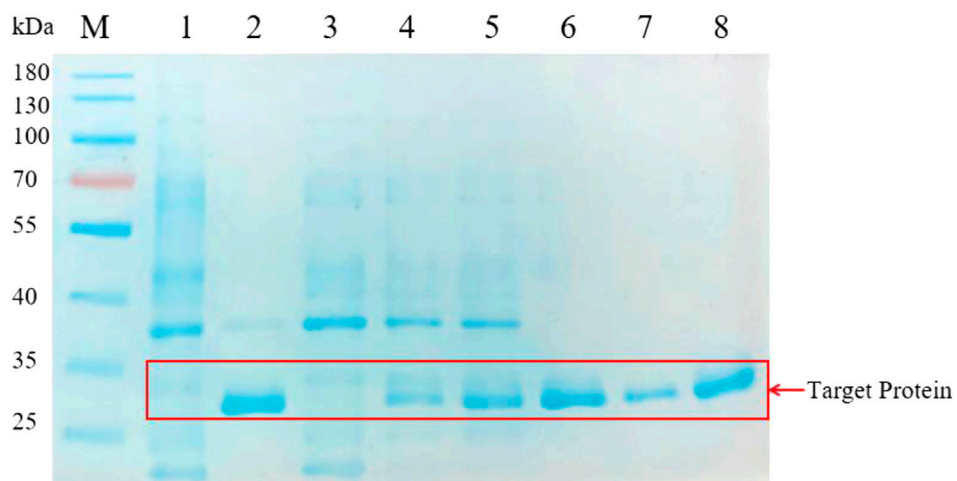
FIGURE 5
SDS-PAGE analysis of the fermentation supernatant of different strains. M: Marker; 1–8: BAX-9/pHY-300, BAX-9/pHY-P43/BSP-1, BAX-9/pHY-Pscp/BSP-1, BAX-9/pHY-Pcsp/BSP-1, BAX-9/pHY-Ppqq/BSP-1, BAX-9/pHY-Psrf/BSP-1, BAX-9/pHY-Pitu/BSP-1, BAX-9/pHY-Pfen/BSP-1.

FIGURE 6
Determination of enzyme activity and biomass of recombinant strains. Asterisks show the significant difference ($p < 0.05$) compared with the control.
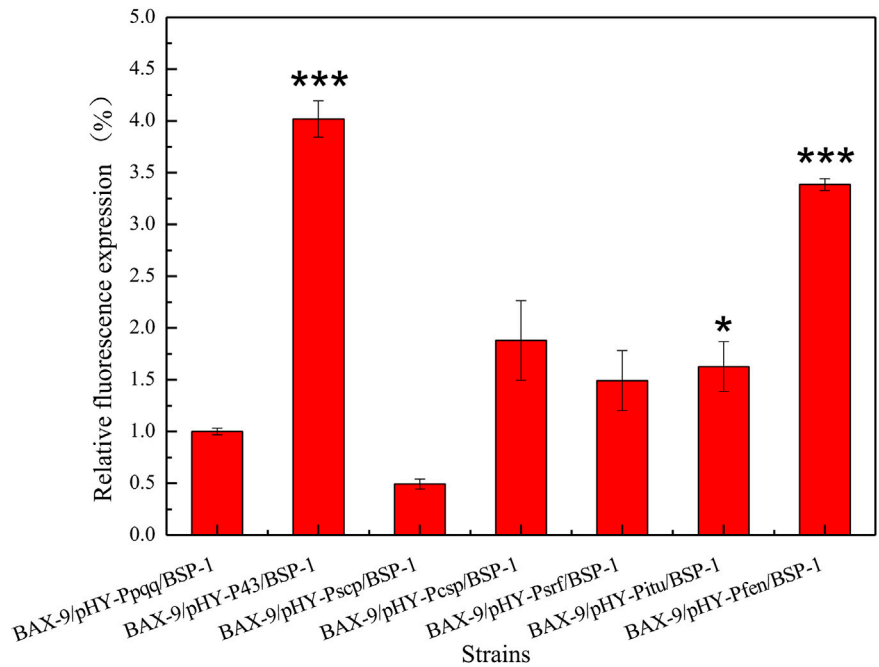
FIGURE 7
RT-PCR results of recombinant strains. Asterisks show the significant difference ($p < 0.05$) compared with the control.
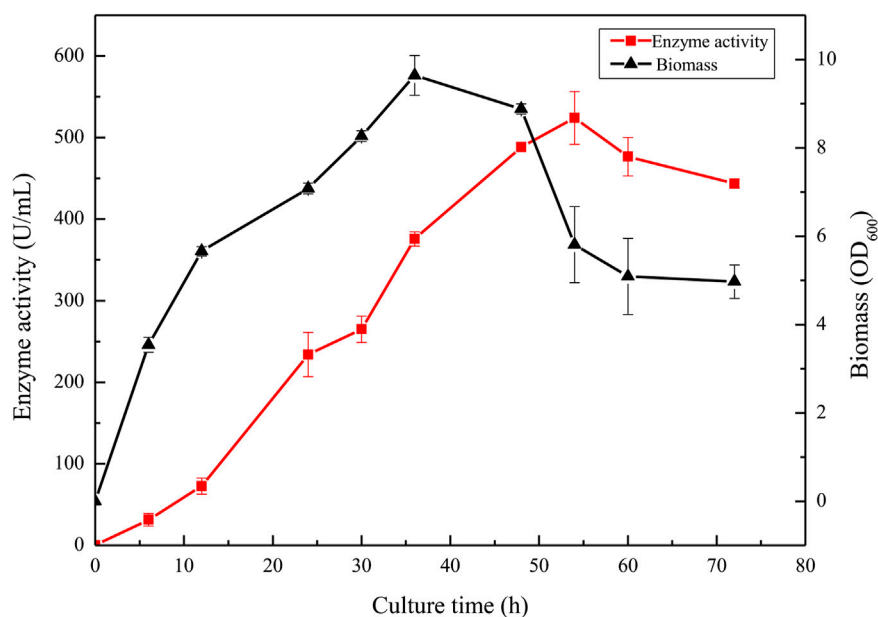
FIGURE 8
Enzyme activity and culture growth of BAX-9/pHY-P43/BSP-1 under fermentation using the optimized fermentation medium.

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, 4.0-fold 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-1* 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 continued to increase until 54 h. 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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