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Enhanced extracellular α-amylase production in *Brevibacillus choshinensis* by optimizing extracellular degradation and folding environment

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Abstract: A strategy for optimizing the extracellular degradation and folding environment of *Brevibacillus choshinensis* has been used to enhance the extracellular production of recombinant α-amylase. First, a gene (*bcp*) encoding an extracellular protease and another encoding an extracellular chaperone (*prsC*) were identified in the genome of *B. choshinensis* HPD31-SP3. Then, the effect of extracellular protein degradation on recombinant α-amylase production was investigated by establishing a CRISPR/Cas9n system to knock out *bcp*. The effect of extracellular folding capacity was investigated separately by coexpressing extracellular chaperones genes from different sources (*prsA*, *prsC*, *prsL*, *prsQ*) in *B. choshinensis*. The final recombinant strain (BCPPSQ), which coexpressed *prsQ* in a genetic background lacking *bcp*, produced an extracellular α-amylase activity of 6940.9 U/ml during shake-flask cultivation. This was 2.1-fold greater than that of the original strain BCWPS (3367.9 U/ml). Cultivation of BCPPSQ in a 3-l fermenter produced an extracellular α-amylase activity of 17925.6 U/ml at 72 h, which was 7.6-fold greater than that of BCWPS (2358.1 U/ml). This strategy demonstrates its great potential in enhancing extracellular α-amylase production in *B. choshinensis*. What's more, this study provides a strategic reference for improving the extracellular production of other recombinant proteins in *B. choshinensis*.

Keywords: α-Amylase, *Brevibacillus choshinensis*, Protease, Chaperone, 3-l Fermenter fermentation

Introduction

α-Amylases (α-1–4-glucan-glucanohydrolase, EC.3.2.1.1) are widely distributed *endo*-acting amylolytic enzymes that can break down α-1,4-glycosidic bonds of starch and related large polysaccharides in an endo fashion and produce glucose or glucose-containing oligosaccharides (Burhanoglu et al., 2020). Currently, α-amylase has a wide range of applications in industry, ranging from the food, brewing, paper, pharmaceutical, and detergent industries to the textile industry (El-Sayed et al., 2019). Although α-amylases can be produced by many species, using microorganisms to produce α-amylases is preferred because of their low culture cost and simple genetic manipulation, which makes them easy for carrying out economical mass production (Li et al., 2018).

Brevibacillus choshinensis (formerly *Bacillus brevis*) is a Gram-positive bacterium obtained from soil by Hiroaki et al. in 1989 (Takagi et al., 1989). Among them, *B. choshinensis* HPD31 (formerly *B. brevis* HPD31) has excellent characteristics. It is nonpathogenic, has low extracellular protease activity, and has high protein synthesis and secretion capacity (Yao et al., 2020). Especially during the culture process, the protein concentration in the extracellular medium of *B. choshinensis* HPD31 can be as high as 30 g/l, which is 1.5-fold greater than that of *Bacillus subtilis* (20 g/l) (Pohl & Harwood, 2010). Thus, *B. choshinensis* HPD31 seems to have greater potential for the production of recombinant target proteins than *B. subtilis*, which has been widely used in recombinant target protein production. In fact, *B. choshinensis* HPD31 is gradually being used for recombinant expression of target proteins (Yao et al., 2020). Currently, many target proteins have been expressed in *B. choshinensis* HPD31, such as single-chain variable fragment (Hu et al., 2017), pullulanase, and β-glucosidase (Ichikawa et al., 2019; Zou et al., 2016).

Although researchers have adopted a series of strategies, including host strain optimization, recombinant expression element optimization and fermentation optimization, to enhance target protein production in *B. choshinensis* HPD31 (Duan et al., 2019; D'Urzo et al., 2013; Kajino et al., 1999; Sagiya et al., 1994; Yao et al., 2020), previous research has focused mainly on the latter two strategies. Hitherto, host strain optimization has been performed mainly to reduce extracellular protease activity (Hanagata & Nishijyo, 2010; Kajino et al., 1999). *B. choshinensis* 31-OK, which could not secrete a 48-kDa extracellular protease, was obtained through spontaneous mutation of *B. choshinensis* HPD31 (Kajino et al., 1999). *B. choshinensis* HPD31-SP3 was obtained by knocking out the spore-forming gene *hos*, the intracellular protease gene *imp*, and the extracellular protease gene *emp* in the *B. choshinensis* HPD31 genome (Hanagata & Nishijyo, 2010). Compared with *B. choshinensis* HPD31, *B. choshinensis* HPD31-SP3 exhibited greatly reduced extracellular protease activity, so *B. choshinensis* HPD31-SP3 was widely used for recombinant target protein production. However, Zou Liang found that when *B. choshinensis* HPD31-SP3 was used for sucrose isomerase recombinant production in a 3-l fermenter, the recombinant strain BCpNapr-SI still exhibited extracellular degradation during the later stage of fermentation (Zou, 2019). Therefore, in order to improve the use of

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Table 1 Strains Used in This Study

Strains	Descriptions	Reference
Escherichia coli JM109	*endA1*, *recA1*, *thi*, *gyrA96*, *supE44*, *hsdR17* Δ(*lac-proAB*)/F′ [*traD36*, *lacIq*, *lacZ*ΔM15, *proAB*+]	Takara
Brevibacillus choshinensis HPD31-SP3	*B. choshinensis* HPD31 derivative, Δ*hos*, Δ*emp*, Δ*imp*	Takara
B. choshinensis Δ*bcm*	*B. choshinensis* HPD31-SP3 derivative, Δ*bcm*	This study
B. choshinensis Δ*bcp*	*B. choshinensis* HPD31-SP3 derivative, Δ*bcp*	This study
BCWPS	*B. choshinensis* HPD31-SP3 containing plasmid pNCamyS	This study
BCMPS	*B. choshinensis* Δ*bcm* containing plasmid pNCamyS	This study
BCPPS	*B. choshinensis* Δ*bcp* containing plasmid pNCamyS	This study
BCWPSA	*B. choshinensis* HPD31-SP3 containing plasmid pNCamyS-*prsA*	This study
BCWPSC	*B. choshinensis* HPD31-SP3 containing plasmid pNCamyS-*prsC*	This study
BCWPSL	*B. choshinensis* HPD31-SP3 containing plasmid pNCamyS-*prsL*	This study
BCWPSQ	*B. choshinensis* HPD31-SP3 containing plasmid pNCamyS-*prsQ*	This study
BCPPSA	*B. choshinensis* Δ*bcp* containing plasmid pNCamyS-*prsA*	This study
BCPPSQ	*B. choshinensis* Δ*bcp* containing plasmid pNCamyS-*prsQ*	This study

B. choshinensis HPD31-SP3 to produce recombinant target proteins, it is necessary to further reduce its extracellular degradation activity.

In addition to extracellular degradation activity, the extracellular folding ability of host strains is also an important factor affecting the expression level of extracellular recombinant target proteins (Zhang et al., 2020). Extracellular chaperones are common folding cofactors and have been widely used in *Bacillus* expression systems to enhance extracellular target protein production (Ane et al., 2019). At present, the most widely studied extracellular chaperone in *Bacillus* expression systems is *B. subtilis* PrsA (Tjalsma et al., 2004). Many reports have described the use of *prsA* overexpression to improve levels of target protein expression in *B. subtilis* (Chen et al., 2015; Yang et al., 2019). However, enhancing recombinant target protein production through *prsA* overexpression in *B. choshinensis* has never been reported. Therefore, overexpression or coexpression of *prsA* or its analogs might also enhance recombinant target protein production in *B. choshinensis*.

Due to their high efficiency and precision, CRISPR systems have gradually become a mainstream gene editing technology (Jiang et al., 2013). The CRISPR/Cas9 system is the most widely used among them (Zhang et al., 2016). Compared with Cas9, the Cas9 nickase (Cas9n), obtained by aspartate-to-alanine (D10A) substitution in the RuvC I domain of Cas9, can effectively reduce the damage caused by Cas9 DNA double-strand cleavage (Cong et al., 2013). However, to the best of our knowledge, there have been no reports on the application of CRISPR systems in *B. choshinensis*.

The aim of this study was to enhance extracellular α-amylase production in *B. choshinensis*. It began with a search of the *B. choshinensis* HPD31-SP3 whole genome sequencing results to identify potential extracellular protease and extracellular chaperone genes. Then, the effects of extracellular degradation activity and folding capacity of *B. choshinensis* on recombinant α-amylase production were investigated separately by (1) establishing a CRISP/Cas9n gene editing system to inactivate the extracellular protease gene identified in the previous step, and (2) coexpressing extracellular chaperones from different sources. By coexpressing extracellular chaperones in a genetic background lacking the extracellular protease gene, the effects of extracellular degradation and folding environment of *B. choshinensis* on recombinant α-amylase production were comprehensively considered. Finally, the resulting *B. choshinensis* recombinant strain was cultured in a 3-l fermenter to verify its ability to produce α-amylase.

Materials and Methods

Strains and Media

The strains used in this study are shown in Table 1. In this study, *Escherichia coli* JM109 and *B. choshinensis* HPD31-SP3 were used to construct recombinant vectors and recombinantly express the *Bacillus stearothermophilus* α-amylase gene (*amyS*), respectively. Luria-Bertani (LB) medium was used to culture *E. coli* JM109. TM medium (Zou et al., 2016) was used for seed culture and shake-flask fermentation of *B. choshinensis* recombinant strains. The basic medium of 3-l fermenter fermentation included (per liter) 15.0 g polypeptone, 15.0 g beef extract, 0.5 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 10.0 g glucose, 1.0 g $FeSO_4{\cdot}7H_2O$, 1.0 g $MnSO_4{\cdot}4H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$, and 2.0 g $MgSO_4{\cdot}7H_2O$. The feeding medium of 3-l fermenter fermentation only included (per liter) 100.0 g glucose.

Plasmid Construction and Transformation

Plasmid construction

The plasmids and related primers used in this study are shown in Table 2 and Supplementary Table S1, respectively. The *amyS* used in this study was obtained from pET-20b-*amy* (Li et al., 2016) with primers F1/R1 and then cloned into vector pNCMO2 (purchased from Takara Bio Inc., Dalian, China) using restriction enzymes *Pst* I and *Hind* III, creating plasmid pNCamyS.

To establish the CRISPR/Cas9n gene editing system, the vector pHYcas9nd (Supplementary Fig. S1) was amplified from pHYcas9d (Zhang et al., 2016) with primers F2/R2. To inactivate *bcm*, the vector pHYcas9ndm, which contains a 20-bp complementary region (N20) corresponding to the *bcm* gene, was obtained from pHYcas9nd with primers F3/R3. When the homology arm length was approximately 300 bp, the upstream and downstream homology arms of *bcm* were obtained from the *B. choshinensis* HPD31-SP3 genome with primers F4/R4 and F5/R5, respectively. When the homology arm length was approximately 500 bp, the upstream and downstream homology arms of *bcm* were obtained from the *B. choshinensis* HPD31-SP3 genome with primers F6/R4 and F5/R6, respectively. When the homology arm length was approximately 1,000 bp, the upstream and downstream homology arms of *bcm* were obtained from the *B. choshinensis* HPD31-SP3 genome with primers F7/R4 and F5/R7, respectively. The homologous repair template of *bcm* was obtained by overlapping PCR fusion of upstream and downstream homologous arms of *bcm*. A portion of the homologous repair template of *bcm* is shown in Supplementary Fig. S2. Then, the homologous repair template of *bcm* was cloned into vector pHYcas9ndm using restriction enzyme *Xba* I,

Table 2 Plasmids Used in This Study

Plasmids	Descriptions	Reference
pNCMO2	Amp^r (*Escherichia coli*), Nm^r (*Brevibacillus choshinensis*), P_2 promoter, SP_{R2L6}	Takara
pET-20b-*amy*	Amp^r (*E. coli*), α-amylase gene *amyS*	(Li et al., 2016)
pNCamyS	pNCMO2 derivative, α-amylase gene *amyS*	This study
pHYcas9d	Amp^r (E. coli), Tet^r (*Bacillus* subtilis and E. coli), PE194 temperature-sensitive replicon, *cas*9, sgRNA of srfC	(Zhang et al., 2016)
pHYcas9nd	pHYcas9d derivative, *cas*9n	This study
pHYcas9ndm	pHYcas9nd derivative, sgRNA of bcm	This study
pHYcas9ndbcm	pHYcas9ndm derivative, repair template of *bcm*	This study
pHYcas9ndh	pHYcas9nd derivative, sgRNA of hwp	This study
pHYcas9ndhwp	pHYcas9ndh derivative, repair template of *hwp*	This study
pHYcas9ndp	pHYcas9nd derivative, sgRNA of bcp	This study
pHYcas9ndbcp	pHYcas9ndp derivative, repair template of *bcp*	This study
pHYYamySP	Amp^r (*E. coli*), Tet^r (*E. coli* and *B. subtilis*), PrsA gene *prsA*	(Yao et al., 2019)
pNCamyS-*prsA*	pNCamyS derivative, PrsA gene *prsA*	This study
pNCamyS-*prsC*	pNCamyS derivative, PrsC gene *prsC*	This study
pNCamyS-*prsL*	pNCamyS derivative, PrsL gene *prsL*	This study
pNCamyS-*prsQ*	pNCamyS derivative, PrsQ gene *prsQ*	This study

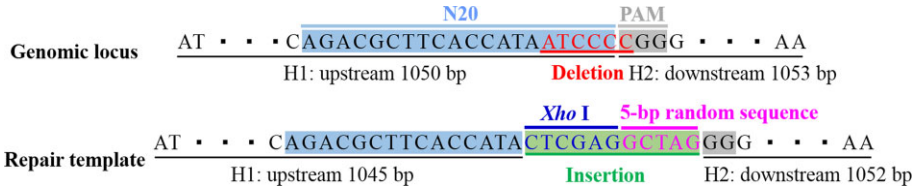

Fig. 1 Partial homologous repair template sequence of *bcp*. N20 is a 20-bp complementary sequence; PAM is a specific protospacer-adjacent motif; H1 is the upstream homology arms of *bcp*; and H2 is the downstream homology arms of *bcp*.

creating knockout vector pHYcas9ndbcM. The primers for *bcm* gene knockout PCR verification were F8/R8. The nucleotide sequences of the homology arms with different lengths are shown in Additional File 1: Sequences in the Supplementary Materials.

To inactivate *hwp*, the vector pHYcas9ndh, which contains a 20-bp complementary region (N20) corresponding to the *hwp* gene, was obtained from pHYcas9nd with primers F9/R9. The upstream and downstream homology arms of *hwp* were obtained from the *B. choshinensis* HPD31-SP3 genome with primers F10/R10 and F11/R11, respectively. The homologous repair template of *hwp* was obtained by overlapping PCR fusion of upstream and downstream homologous arms of *hwp*. The primers for *hwp* gene knockout PCR verification were F12/R12. The nucleotide sequence of the homology arm is shown in Additional File 1: Sequences in the Supplementary Materials.

To inactivate *bcp*, the vector pHYcas9ndp, which contains a 20-bp complementary region (N20) corresponding to the *bcp* gene, was obtained from pHYcas9nd with primers F13/R13. The upstream and downstream homology arms of *bcp* were obtained from the *B. choshinensis* HPD31-SP3 genome with primers F14/R14 and F15/R15, respectively. The homologous repair template of *bcp* was obtained by overlapping PCR fusion of upstream and downstream homologous arms of *bcp*. A portion of the homologous repair template of *bcp* is shown in Fig. 1. Then, the homologous repair template of *bcp* was cloned into vector pHYcas9ndp using restriction enzyme *Xba* I, creating knockout vector pHYcas9ndbcp. The primers for *bcp* gene knockout PCR verification were F16/R16. In this study, the sgRNA for the *bcp* gene knockout was designed using sgRNAcas9 software (version 2.0). To obtain high knockout efficiency, the sgRNA selection parameters were GC content less than 45% and N20 as close as possible to the 5′ end of *bcp* gene. Therefore, the position of the sgRNA started 209 bp downstream of *bcp* gene initiation codon. To improve the editing efficiency of the CRISPR/Cas9n system, the sequences of the upstream and downstream homologous arms of the *bcp* gene were obtained by selecting nucleotide sequences of corresponding length from the 5′ and 3′ ends of PAM (a specific protospacer-adjacent motif), respectively.

The P_{aprE}-*prsA* fragment was obtained from pHYYamySP (Yao et al., 2019) with primers *prsA*-F/*prsA*-R. The pNCamyS fragment (backbone of pNCamyS-*prsA*) was obtained from pNCamyS with primers F17/R17. The plasmid pNCamyS-*prsA* was created by linking the P_{aprE}-*prsA* fragment with the pNCamyS fragment using Vazyme's One Step Cloning kit (Vazyme Biotech Co., Ltd, Nanjing, Chian). The *prsC* gene was obtained from the *B. choshinensis* HPD31-SP3 genome with primers *prsC*-F/*prsC*-R. The *prsL* gene was obtained from the *Bacillus licheniformis* genome with primers *prsL*-F/*prsL*-R. The *prsQ* gene was obtained from the *Bacillus amyloliquefaciens* genome with primers *prsQ*-F/*prsQ*-R. The pNCamyS-P_{aprE} fragment was obtained from pNCamyS-*prsA* with primers F18/R18. pNCamyS-*prsC*, pNCamyS-*prsL*, and pNCamyS-*prsQ* were created by linking the *prsC*, *prsL*, and *prsQ* gene fragments with the pNCamyS-P_{aprE} fragment using Vazyme's One Step Cloning kit.

Plasmid transformation

The plasmid transformation method used in this study was based on the method of Li et al. (2016), with some modifications. Briefly, recombinant plasmids were transferred into *B. choshinensis* by electroporation. To prepare *B. choshinensis* competent for electrotransformation, a single *B. choshinensis* strain clone was transferred to 10 ml TM medium and cultured at 37°C, 200 rpm for 10 h. Then, 200 μl of the above culture medium

was transferred to 20 ml TM medium and cultured for 4.5 h under the above culture conditions. After an ice bath for 10 min, the supernatant of the resulting mixture was removed by centrifugation at 4,000 × *g* and 4°C for 5 min, and the bacterial precipitate was collected. After repeated washing of four times with SHC buffer (1 mM $CaCl_2$, 10% sucrose, 15% glycerol, 16 mM N-(2-Hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid, pH 7.0), the bacterial precipitate was resuspended with 1 ml SHC buffer. For electroporation, 100 μl of the above resuspended bacterial solution was mixed with approximately 1 μg plasmid and an equal volume of 15% polyethylene glycol (PEG) solution. Then, the mixture obtained was transferred to an electroporation cuvette (1 mm) and the electric shock was performed under an 18 kV/cm pulse. After the electric shock, 1 ml TM medium was added immediately and gently mixed. This mixture was cultured at 37°C, 120 rpm for 2 h. Finally, the resuscitated culture solution was spread on a TM plate supplemented with 20 mg/l neomycin and cultured overnight at 37°C. The positive monoclonal strain on the plate was the expected *B. choshinensis* transformant.

Cultivation Methods

Shake-flask culture methods

The seed culture used for shake-flask fermentation was obtained by inoculating 10 ml of TM medium with 20 μl of bacterial solution from a glycerol tube cryopreserved at −80°C and incubating this mixture at 37°C, 200 rpm for 12 h. Then, 250 μl of the seed liquid was transferred to 50 ml TM medium in a shake-flask and incubated at 33°C, 200 rpm for 48 h for shake-flask fermentation. The TM medium was supplemented with 20 mg/l neomycin during shake-flask culture.

3-l Fermenter culture methods

The seed liquid used for 3-l fermenter fermentation was prepared by inoculating 100 ml of TM medium with 200 μl of bacterial liquid from a glycerol tube cryopreserved at −80°C and incubating the mixture at 37°C, 200 rpm for 12 h. Then, 100 ml of the seed liquid was transferred to 900 ml basic medium contained in a 3-l fermenter and incubated at 33°C, 200 rpm. During the 3-l fermenter fermentation process, the dissolved oxygen (DO) level of the fermentation broth was controlled at 30% by adjusting the stirring speed (200–700 rpm). In addition, the pH of the fermentation broth was controlled at approximately 7.0 by 3 M NaOH and 10% (vol/vol) H_2SO_4. When cultured for approximately 13 h, the DO increased suddenly and the stirring speed decreased suddenly, the feeding was started. The fermentation broth in the 3-l fermenter was sampled at regular intervals, and its glucose concentration was measured using automatic biosensors (Sieman Technology Co., Ltd, Shenzhen, China). The glucose concentration of the fermentation broth was controlled at 0–3 g/l by adjusting the feed flow rate (0–5 ml/h). In the process of seed preparation and 3-l fermenter fermentation, the medium was supplemented with 20 mg/l neomycin.

Determination of Bacterial Concentration

The bacterial concentration of the fermentation broth was determined using dry cell weight (DCW). To determine DCW, 10 ml samples of fermentation broth were centrifuged at 12,000 × *g* and 4°C for 10 min, and then the bacterial precipitates were collected. After washing three times with 0.9% (wt/vol) NaCl solution, the bacterial precipitate was dried to constant weight at 105°C. The unit of DCW was g/l.

Determination of α-Amylase Activity

In this study, the α-amylase activity was determined based on our previous report (Yao et al., 2019). First, a mixture consisting of 1 ml 1% soluble starch and 0.9 ml phosphate buffer (20 mM, pH 6.0 NaH_2PO_4-Na_2HPO_4) was incubated at 70°C for 10 min. Then, after adding 0.1 ml of appropriately diluted crude enzyme solution, the resulting mixture was reacted at 70°C for 5 min. Next, 3 ml 3,5-dinitrosalicylic acid (DNS) was added to terminate the reaction, and the resulting mixture was treated in boiling water for 7 min. After an ice bath for 3–5 min, the volume of the resulting mixture was made up to 15 ml with pure water. Finally, the optical density of the resulting mixture was determined at 540 nm. The crude enzyme solution used for determination of α-amylase activity was the fermentation broth supernatant after centrifugation at 12,000 × *g* and 4°C for 10 min.

Determination of Protease Activity

In this study, the protease activity of the fermentation broth supernatant was measured using the method described in previous studies (Zou, 2019). First, 1 ml properly diluted crude enzyme solution and 1% casein solution were incubated at 40°C for 10 min, respectively. Then, 1 ml casein solution was added to the enzyme solution and reacted at 40°C for 10 min. Next, 2 ml trichloroacetic acid was added, and the resulting mixture was placed on ice for 10 min. The mixture was centrifuged at 12,000 × *g* and 4°C for 10 min to obtain the supernatant. Then 5 ml sodium carbonate and 1 ml folin reagent were successively added to 1 ml of the supernatant obtained above, and the resulting mixture was incubated at 40°C for 20 min. Finally, the optical density of the mixture was measured at 680 nm. Under the assay conditions described above, one unit of protease activity was defined as the amount of enzyme that released 1 μg of tyrosine per min from casein.

SDS-PAGE Analysis

Crude enzyme solution (20 μl) was mixed with protein loading buffer (5 μl) in a 1.5 ml centrifuge tube, and then placed in a boiling water bath for 10 min. Then, the 1.5 ml centrifuge tube was briefly centrifuged (1,000 × *g*, 30 s) to collect the water vapor that evaporated to the centrifuge tube wall during heating. After mixing the collected mixture with a pipette, an 8 μl sample was taken for electrophoresis. To effectively observe protein bands, the gel was first stained with Coomassie Brilliant Blue R-250 solution and then soaked in an aqueous solution containing acetic acid and ethanol for decolorization.

Statistical Analysis

All data were obtained through three independent experiments and presented as the averages ± standard deviation. The *t* test was used for data significance analysis, and $P < 0.05$ was considered to indicate a significant difference. Statistica 6.0 statistical software was used to perform statistical analysis in this study.

Results and Discussion

Recombinant AmyS Production in *B. choshinensis* HPD31-SP3

To investigate the level of recombinant AmyS expression in *B. choshinensis* HPD31-SP3, the expression vector pNCamyS containing the *amyS* was transferred into *B. choshinensis* HPD31-SP3 to obtain the recombinant strain BCWPS. After shake-flask fermentation for 48 h, the bacterial concentration and the extracellular

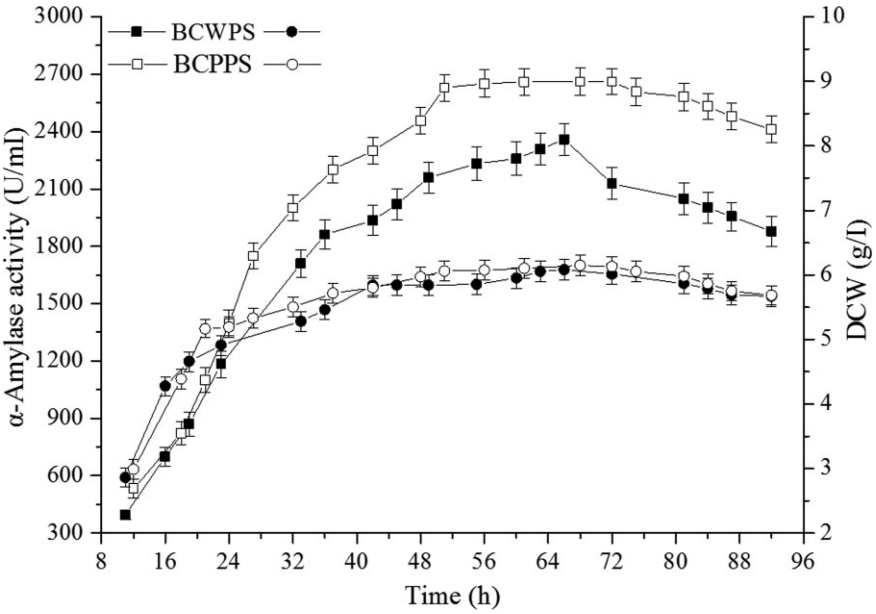

Fig. 2 Scale-up (3-l) fermentation of BCWPS and BCPPS. The squares represent α-amylase activity (U/ml), and the circles represent bacterial concentration (DCW; g/l). Error bars represent standard deviation.

AmyS activity of BCWPS were 2.1 g/l and 3367.9 U/ml, respectively. The extracellular AmyS activity produced by BCWPS was 1.2-fold greater than the highest *B. stearothermophilus* α-amylase activity (2835.1 U/ml) produced by *B. subtilis* in shake-flask culture (Yao et al., 2021), indicating that *B. choshinensis* HPD31-SP3 has great potential for highly efficient AmyS expression.

The bacterial concentration and the extracellular AmyS activity of BCWPS reached their highest values (6.1 g/l and 2358.1 U/ml, respectively) when cultured in a 3-l fermenter for 66 h (Fig. 2). When cultured in a 3-l fermenter, the bacterial concentration of BCWPS was 2.9-fold greater than that obtained using shake-flask fermentation. However, its extracellular AmyS activity was 30% lower. This indicated that there were factors that limit highly efficient recombinant AmyS production in *B. choshinensis* HPD31-SP3.

Although the extracellular protease activity of *B. choshinensis* HPD31-SP3 was already very low (Yao et al., 2020), Zou Liang found that *B. choshinensis* HPD31-SP3 recombinant strains still exhibited extracellular protein degradation during the late stage of 3-l fermenter fermentation (Zou, 2019). As shown in Fig. 3, during the 3-L fermenter fermentation, the BCWPS did not have extracellular degradation similar to that reported by Zou Liang. However, Fig. 2 shows that the extracellular AmyS activity of BCWPS cultured in a 3-l fermenter for 92 h (1878.8 U/ml) was only 80% of that observed at 66 h (2358.1 U/ml). This indicates that extracellular AmyS was still subject to protease degradation during 3-l fermenter fermentation of BCWPS. Moreover, this might be one of the reasons why the extracellular AmyS activity of BCWPS in 3-l fermenter fermentation was lower than that in shake-flask fermentation.

To verify our speculation, the extracellular protease activity of BCWPS during 3-l fermenter fermentation was determined (Table 3). The results showed that the extracellular protease activity was lower in the later fermentation period of BCWPS, but it still existed, which was consistent with our speculation. In addition, the extracellular protease activity of BCWPS gradually increased as the fermentation time was extended. When cultured for 92 h, the extracellular protease activity of BCWPS was 2.08 U/ml (Table 3). This suggests that further reducing the extracellular protein degradation activity of BCWPS might be an effective method to enhance extracellular recombinant AmyS production.

In addition, it is worth mentioning that there are two thick protein bands with molecular weights close to 116 and 45 kDa in the SDS-PAGE gel displayed in Fig. 3. Previous reports made identification of the protein with molecular weight close to 116 kDa straightforward; it corresponds to the cell wall protein of *B. choshinensis* HPD31-SP3, which was encoded by the *hwp* gene (Ebisu et al., 1990). The nucleotide sequence of *hwp* gene is shown in Additional File 1: Sequences in the Supplementary Materials. Although the protein with molecular weight close to 45 kDa has also been reported, its function has not been adequately studied (Tokunaga et al., 2013).

To explore the function of the protein with molecular weight close to 45 kDa in Fig. 3, the corresponding protein band was identified by peptide mass fingerprint (Additional File 2 in the Supplementary Materials). And then, the identified protein sequence was analyzed by NCBI (https://www.ncbi.nlm.nih.gov/). These results indicated that the protein with molecular weight close to 45 kDa might contain the LysM domain. The current studies showed that proteins containing LysM domain could be attached to the cell wall by binding with peptidoglycan, and the proteins containing LysM domain in bacteria were mainly peptidoglycan hydrolase or cell autolysin (Buist et al., 2008). Therefore, it seems reasonable to speculate that the function of the protein with molecular weight close to 45 kDa might be related to the normal growth of the strain. For the convenience of subsequent studies, we named the protein with molecular weight close to 45 kDa BCM (encoded by the *bcm* gene). The nucleotide sequence of *bcm* gene is shown in Additional File 1: Sequences in the Supplementary Materials.

Another thing to note was that there was BCM shown in the fermentation supernatant samples of BCWPS, but without in that of *B. choshinensis* HPD31-SP3 (Fig. 3). The recombinant strain BCWPS was obtained by transferring the expression vector pNCamyS containing the *amyS* into strain *B. choshinensis* HPD31-SP3. Therefore, it was not difficult to speculate that the presence of BCM in the extracellular fermentation supernatant of BCWPS was related to the vector pNCMO2 or *amyS* gene. However, previous

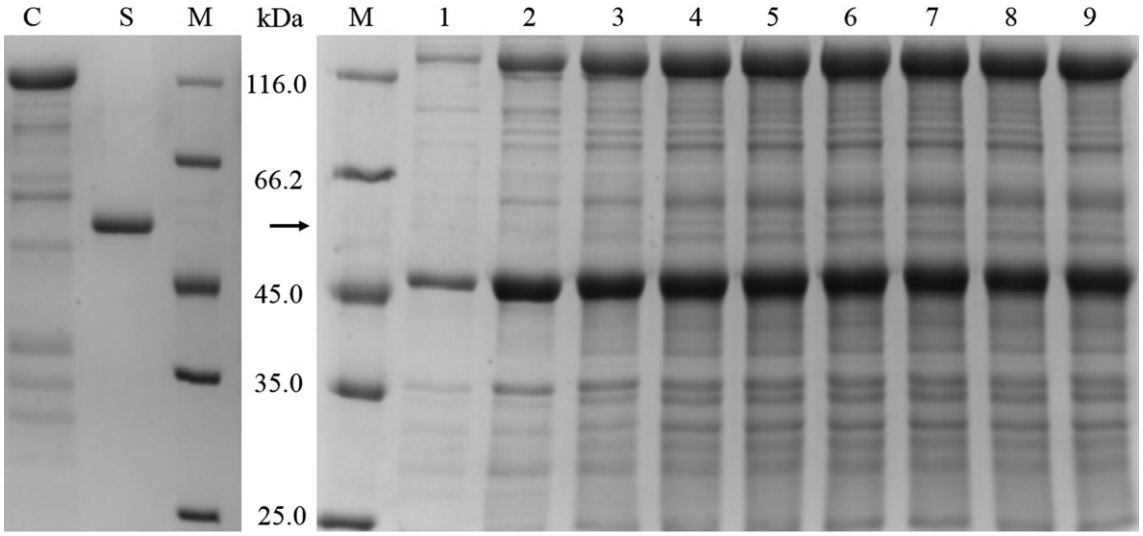

Fig. 3 SDS-PAGE analysis of the supernatant from a 3-l fermenter fermentation of BCWPS. The arrow designates the band at ~55 kDa, which corresponds to the theoretical molecular weight of AmyS. Lanes 1–9: supernatant samples obtained at 12, 24, 36, 45, 49, 57, 66, 84, and 92 h, respectively. Lane M: protein molecular weight markers. Lane S: AmyS standard sample. Lane C: *Brevibacillus choshinensis* HPD31-SP3 sample as a negative control, which was the fermentation supernatant of strain *B. choshinensis* HPD31-SP3 after shake-flask fermentation for 66 h.

Table 3 Extracellular Protease Activity of BCWPS and BCPPS

	Protease activity (U/ml)	
Time (h)	**BCWPS**	**BCPPS**
42	0.38	0.09
48	0.46	0.13
66	0.84	0.24
72	1.64	0.44
92	2.08	0.72

studies showed that there was no major band with molecular weight close to 45 kDa in the extracellular fermentation supernatant of recombinant *B. choshinensis* HPD31-SP3 strains, which contained pNCMO2-derived vectors of different target genes (Duan et al., 2019; Zou et al., 2016; Zou et al., 2019). Therefore, it seems reasonable to speculate that the presence of BCM in the extracellular fermentation supernatant of BCWPS was related to the *amyS* gene. Since the α-amylase gene *amyS* was not expressed recombinantly in the control strain *B. choshinensis* HPD31-SP3, the control lane of SDS-PAGE gel in Fig. 3 was not showing BCM.

Effect of *bcm* and *hwp* Genes' Knockout on Extracellular AmyS Production in *B. choshinensis*

Construction of the B. choshinensis CRISPR/Cas9n gene editing system

In this study, to perform efficient gene editing on *bcm* and *hwp* genes, a CRISPR/Cas9n gene editing system suitable for *B. choshinensis* was constructed based on the CRISPR/Cas9 system of *B. subtilis* (Zhang et al., 2016). The principle used by the CRISPR/Cas9n system established in this study to knock out target genes was to delete a 6-bp nucleotide sequence in the original gene through a homologous repair template, while inserting a 5-bp random nucleotide sequence and an *Xho* I restriction site. Thus, the target gene was inactivated by frameshift mutation.

The editing efficiencies of CRISPR/Cas9 and CRISPR/Cas9n systems were investigated based on *bcm* gene knockout. When the homology arm length was approximately 500 bp, the editing efficiencies of the CRISPR/Cas9n system and the CRISPR/Cas9 system were 16% and 4%, respectively. The editing efficiencies of different systems were represented by the ratio of the amount of positive clones, which were successfully knocked out gene, to that of total clones selected. Thus, the CRISPR/Cas9n system was fourfold more efficient than the CRISPR/Cas9 system. In addition, recent studies have clearly shown that the Cas9n can effectively reduce the damage caused by Cas9-mediated double-strand DNA cleavage (Cong et al., 2013; Li et al., 2018). Therefore, it seems reasonable to speculate that the CRISPR/Cas9n system established in this study could reliably reduce host strain damage, compared with similar CRISPR/Cas9 systems.

In addition, by constructing homologous arms with different lengths, the effect of homologous arm length on CRISPR/Cas9n system editing efficiency was investigated based on *bcm* gene knockout. When the homology arm lengths were approximately 300, 500, and 1,000 bp; the corresponding CRISPR/Cas9n system editing efficiencies were 1%, 16%, and 60%, respectively. Among them, the CRISPR/Cas9n system editing efficiency was the highest when the homology arm length was approximately 1,000 bp. Therefore, in a subsequent study of extracellular protease gene knockout, the homologous arm length in the knockout plasmid was approximately 1,000 bp. To our knowledge, this is the first report on the establishment of a CRISPR/Cas9n system in *B. choshinensis*.

Disruption of bcm and hwp genes using the CRISPR/Cas9n system

Using the established CRISPR/Cas9n system, the knockout vector pHYcas9ndbcm with the homology arm length of approximately 1,000 bp was transferred into *B. choshinensis* HPD31-SP3 to achieve knockout of *bcm* and obtain strain *B. choshinensis* Δ*bcm*. When guided by an sgRNA containing a specific N20 sequence, the Cas9n protein could cut a single DNA strand at specific locations in the *B. choshinensis* HPD31-SP3 genome, and then perform homologous repair under the action of a homologous repair template. Because the homologous repair template introduced an *Xho* I restriction site, successful construction of *B. choshinensis* Δ*bcm* could be

Table 4 Database Comparison Results of Identified Extracellular Protease Gene and Extracellular Chaperone Gene

Gene names/database annotations	*bcp*	*prsC*
COG database	Serine protease, subtilisin family;	Parvulin-like peptidyl-prolyl isomerase; chaperones
GO database	Serine-type endopeptidase activity; proteolysis	Isomerase activity
KEGG database	Thermitase	ND
Swiss-Prot database	Thermophilic serine proteinase OS = *Bacillus sp.* (strain AK1) PE = 1 SV = 1	Foldase protein PrsA OS = *Caldanaerobacter subterraneus subsp. tengcongensis* (strain DSM 15242/JCM 11007/NBRC 100824/MB4) GN = *prsA* PE = 3 SV = 1

Note. 1, evidence at the protein level; 3, inferred from homology.
GN: gene name; ND: no information was detected; OS: organism species; PE: protein existence; SV: sequence version.

verified through *Xho* I digestion of PCR validation products. The results of *Xho* I digestion and DNA sequencing of the PCR validation products of *B. choshinensis* Δ*bcm* are shown in Supplementary Fig. S3. However, although we made many attempts to knockout the *hwp* gene, we failed to obtain a Δ*hwp* strain. Therefore, we suspected that loss of this gene might be lethal to the strain.

Recombinant AmyS production in B. choshinensis Δbcm

The expression vector pNCamyS containing *amyS* gene was transferred into *B. choshinensis* Δ*bcm* to obtain recombinant strain BCMPS. After shake-flask fermentation for 48 h, although the extracellular AmyS activity of BCMPS (3884.7 U/ml) was 1.15-fold greater than that of BCWPS (3367.9 U/ml; *t* test, $P < 0.05$), the bacterial concentration of BCMPS (1.2 g/l) was lower than that of BCWPS (2.1 g/l; *t* test, $P < 0.05$)—only 57.1% of the bacterial concentration of BCWPS. These results indicated that the function of the *bcm* gene was closely related to the normal growth of strain, so we retained this gene in subsequent studies.

Identification of Extracellular Protease and Extracellular Chaperone Genes in the *B. choshinensis* HPD31-SP3 Genome

As it is a commonly used protein expression system, the extracellular protease and extracellular chaperone genes of *Bacillus* have been studied extensively. Among them, the extracellular proteases of *B. subtilis* and *B. licheniformis* have been relatively more studied, and their main extracellular proteases are serine proteases (Millet, 1970; Stahl & Ferrari, 1984; Wei et al., 2015). The relatively extensively studied extracellular chaperone protein of *Bacillus* is *B. subtilis* PrsA. PrsA has *cis-trans* isomerase activity and its precursor protein is attached to the outside of the cell membrane by a lipoprotein signal peptide (Tjalsma et al., 2004). Because *Brevibacillus* and *Bacillus* share more than 50% similarity (Takagi et al., 1993), it was highly likely that analogs corresponding to the main extracellular protease and chaperone genes of *Bacillus* could be found in the *B. choshinensis* genome. Therefore, the potential extracellular serine protease genes in the *B. choshinensis* HPD31-SP3 genome, as well as genes encoding proteins with *cis-trans* isomerase activity and their corresponding precursor proteins containing lipoprotein signal peptides, were selected for further research in this study.

In previous study, we obtained the whole genome sequence of *B. choshinensis* HPD31-SP3 (GenBank: CP069127) and completed a whole genome-wide gene functional annotation based on comparative analysis of databases, such as the Swiss-Prot database, the Cluster of orthologous groups of proteins (COG) database, the Gene Ontology (GO) database, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Additional File 3 in the Supplementary Materials). Based on the results of gene function annotation in the COG, GO, KEGG, and Swiss-Prot databases, as well as signal peptide prediction using SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/), one gene encoding an extracellular serine protease and one gene encoding an extracellular chaperone protein were identified (Table 4 and Supplementary Table S2). For convenience of subsequent research, the predicted gene encoding the extracellular protease was named *bcp*, and the predicted gene encoding the extracellular chaperone protein was named *prsC* (Table 4 and Supplementary Table S2). Although this study did not provide experimental data demonstrating that PrsC is an extracellular chaperone, functional annotation and signal peptide prediction results strongly suggest that PrsC has *cis-trans* isomerase activity and a lipoprotein signal peptide (Table 4 and Supplementary Table S2) very similar to that of PrsA. Therefore, it was reasonable to think that *prsC* of *B. choshinensis* HPD31-SP3 encoded an extracellular chaperone protein. Nucleotide sequences and signal peptide prediction results for *bcp* and *prsC* are shown in Additional File 1: Sequences and Supplementary Table S2 of the Supplementary Materials, respectively.

Effect of Extracellular Protease Knockout on Extracellular AmyS Production in *B. choshinensis*

Disruption of extracellular protease gene using the CRISPR/Cas9n system

Using the established CRISPR/Cas9n system, the knockout vector pHYcas9ndbcp was transferred into *B. choshinensis* HPD31-SP3 to achieve knockout of extracellular protease gene *bcp* and obtain strain *B. choshinensis* Δ*bcp*. The results of *Xho* I digestion and DNA sequencing of the PCR validation products of *B. choshinensis* Δ*bcp* are shown in Supplementary Fig. S4.

Recombinant AmyS production in B. choshinensis Δbcp

The expression vector pNCamyS containing *amyS* gene was transferred into *B. choshinensis* Δ*bcp* to obtain recombinant strain BCPPS. After shake-flask fermentation for 48 h, although the bacterial concentration of BCPPS (1.9 g/l) was lower than that of BCWPS (2.1 g/l; *t* test, $P < 0.05$), the extracellular AmyS activity of BCPPS (4102.2 U/ml) was 1.22-fold greater than that of BCWPS (3367.9 U/ml; *t* test, $P < 0.01$).

When cultured in a 3-l fermenter for 68 h, the bacterial concentration and extracellular AmyS activity of BCPPS reached the highest values (Fig. 2). The highest bacterial concentration of BCPPS was 6.2 g/l, which showed no significant change compared with that of the original strain BCWPS (6.1 g/l; *t* test, $P > 0.05$). This suggests that the *bcp* was not necessary for the normal growth of *B. choshinensis* HPD31-SP3. The highest extracellular

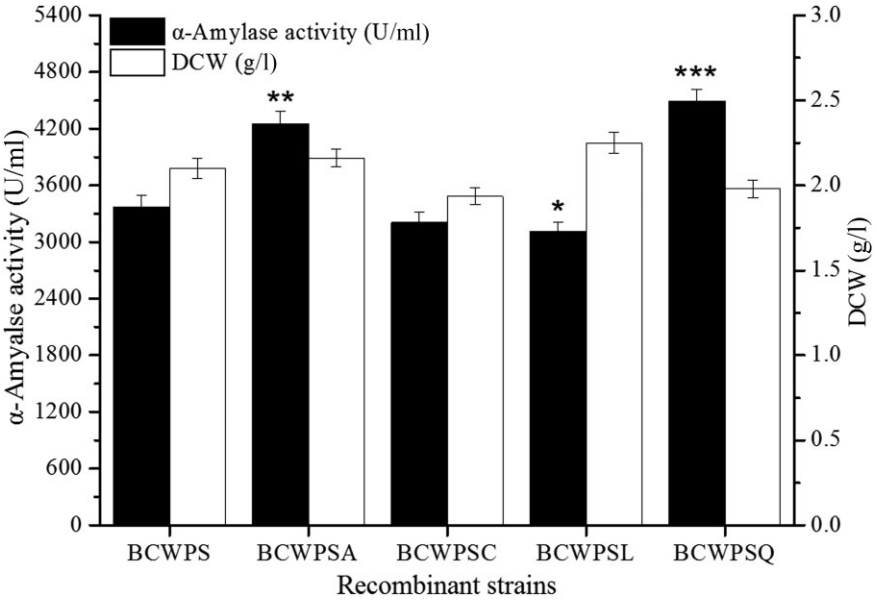

Fig. 4 Effect of extracellular chaperone gene coexpression on extracellular AmyS production. Error bars represent the standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate a statistically significant difference between recombinant strains and BCWPS. The recombinant strain BCWPS as a negative control.

AmyS activity of BCPPS was 2660.9 U/ml, which was 1.13-fold greater than that of BCWPS (2358.1 U/ml; *t* test, $P < 0.01$). In addition, when the fermentation time was extended to 92 h, the extracellular AmyS activity of BCPPS was still as high as 2411.3 U/ml, which was 91% of that at 68 h (2660.9 U/ml). Therefore, during the 3-l fermenter culture, the residual rate of extracellular AmyS activity of BCPPS (91%) was increased by 11% compared with that of BCWPS (80%; *t* test, $P < 0.05$).

To verify whether the increase of extracellular AmyS activity of BCPPS was related to the decrease of its extracellular protease activity compared with BCWPS, the extracellular protease activity of BCPPS during the 3-l fermenter fermentation was determined (Table 3). As shown in Table 3, the extracellular protease activity of BCPPS was significantly lower than that of BCWPS, only 23.7–34.6% of the protease activity of BCWPS. Therefore, knockout of the *B. choshinensis* HPD31-SP3 *bcp* gene reduced extracellular protease activity and decreased extracellular AmyS degradation, thereby increasing extracellular AmyS production in *B. choshinensis*.

Effect of Coexpressing Extracellular Chaperones on Extracellular AmyS Production in *B. choshinensis*

In this study, the endogenous extracellular chaperone gene *prsC* was identified using the genome sequencing results of *B. choshinensis* HPD31-SP3. Previous studies have shown that overexpressing heterologous chaperones in expression host strains can improve the production of recombinant target proteins (Ane et al., 2019). Therefore, the effects of extracellular chaperones derived from *B. subtilis* (PrsA), *B. licheniformis* (PrsL), and *B. amyloliquefaciens* (PrsQ) on the recombinant AmyS production in *B. choshinensis* were also investigated.

The expression vectors pNCamyS-*prsA*, pNCamyS-*prsC*, pNCamyS-*prsL*, and pNCamyS-*prsQ* that coexpressed *prsA*, *prsC*, *prsL*, and *prsQ* were transferred into *B. choshinensis* HPD31-SP3 to obtain recombinant strains BCWPSA, BCWPSC, BCWPSL, and BCWPSQ, respectively. After shake-flask fermentation for 48 h, the extracellular AmyS activities of BCWPSA, BCWPSC, BCWPSL, and BCWPSQ were 4246.6, 3203.8, 3105.0, and 4489.7 U/ml, respectively (Fig. 4). Among them, the extracellular AmyS activities of BCWPSA and BCWPSQ were 1.26- and 1.33-fold greater than that of BCWPS (3367.9 U/ml), respectively. Thus, coexpressing *prsQ* derived from *B. amyloliquefaciens* offered the greatest enhancement of recombinant AmyS production in *B. choshinensis*.

Many studies have shown that the effect of extracellular chaperone overexpression on extracellular target protein production is related to the characteristics of the target protein itself (Vitikainen et al., 2005). Overexpressing an extracellular chaperone can increase, reduce, or have no effect on extracellular target protein production. For example, when amylase genes from *Bacillus sonorensis*, *Geobacillus stearothermophilus*, and *B. amyloliquefaciens* were produced as extracellular proteins in *B. subtilis*, overexpressing the extracellular chaperone from *G. stearothermophilus* increased extracellular *B. sonorensis* amylase activity by 72%, had no significant effect on extracellular *G. stearothermophilus* amylase activity, but decreased extracellular *B. amyloliquefaciens* amylase activity by 71% (Ane et al., 2019). Thus, extracellular chaperones seem to exhibit substrate specificity. In this study, coexpressing extracellular chaperones from different sources resulted in different extracellular AmyS activities in *B. choshinensis*. These results may have been caused by the substrate specificity of the extracellular chaperones. Since coexpressing *prsQ* improved extracellular AmyS activity the most, it seems reasonable to speculate that AmyS is a better match with PrsQ than with PrsA, PrsC, or PrsL.

Similarly, Ane et al. separately investigated the effect of overexpressing extracellular chaperones from six different *Bacillus* sources on the heterologous expression of amylases from various sources in *B. subtilis* (Ane et al., 2019). They found that overexpression of only the extracellular chaperone from *B. amyloliquefaciens* could improve the extracellular activities of amylases from all sources in *B. subtilis* to varying degrees (118–241%) (Ane et al., 2019). These results suggest that the substrate specificity of the *B. amyloliquefaciens* chaperone is relatively weak, so overexpressing this chaperone protein could increase the extracellular activities of amylases from all sources. It also

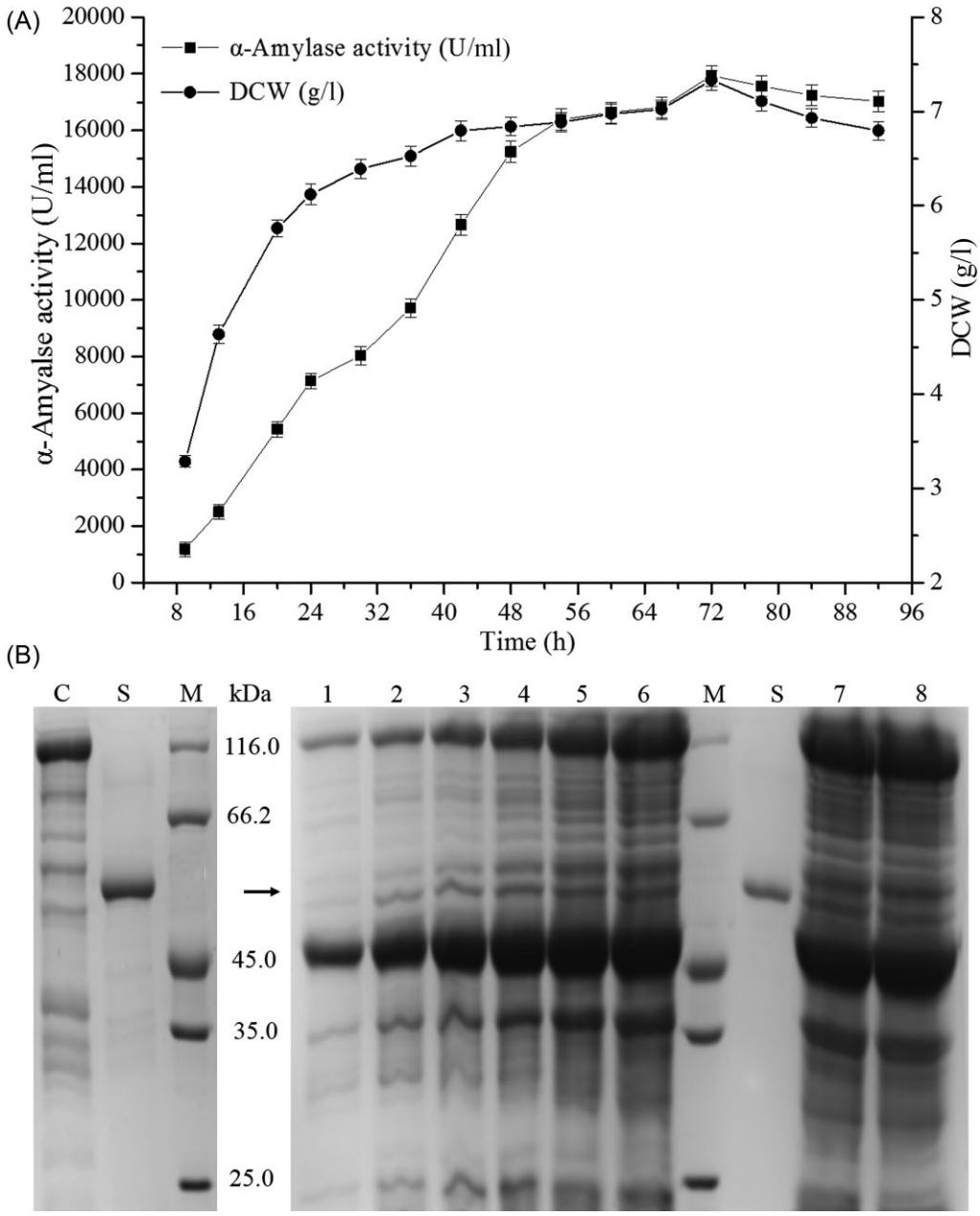

Fig. 5 Scale-up (3-l) fermentation of BCPPSQ. (A) Fermentation curve of AmyS production by BCPPSQ. Error bars represent the standard deviation. (B) SDS-PAGE results of the fermentation supernatant of BCPPSQ at different fermentation times. Lanes 1–8: supernatant samples obtained at 12, 24, 36, 48, 60, 72, 78, and 92 h, respectively. Lane M: protein molecular weight markers. Lane S: AmyS standard sample. Lane C: *Brevibacillus choshinensis* Δ*bcp* sample as a negative control, which was the fermentation supernatant of strain *B. choshinensis* Δ*bcp* after shake-flask fermentation for 72 h.

seems reasonable to speculate that coexpressing *B. amyloliquefaciens* extracellular chaperone might be a general strategy to improve extracellular target protein production in *Bacillus* or *Brevibacillus*.

Effects of Extracellular Protease Knockout and Extracellular Chaperone Coexpression on Extracellular AmyS Production in *B. choshinensis*

To explore whether coexpressing the extracellular chaperone genes *prsQ* or *prsA* within a genetic background lacking the extracellular protease gene *bcp* could further enhance extracellular AmyS production in *B. choshinensis*, the expression vectors pNCamyS-*prsA* and pNCamyS-*prsQ* were transferred into *B. choshinensis* Δ*bcp* to obtain recombinant strains BCPPSA and BCPPSQ, respectively. Compared with BCWPS, BCPPSA and BCPPSQ provided an extracellular environment with lower protein degradation activity and higher protein folding ability for recombinant AmyS production.

After shake-flask fermentation for 48 h, the extracellular AmyS activities of BCPPSA and BCPPSQ (5763.4 and 6940.9 U/ml, respectively) were 1.7- and 2.1-fold greater than that of BCWPS (3367.9 U/ml; *t* test, all $P < 0.001$) and were 1.4- and 1.5-fold greater than those of BCWPSA (4246.6 U/ml; *t* test, $P < 0.001$) and BCWPSQ (4489.7 U/ml; *t* test, $P < 0.001$), respectively. In addition, the extracellular AmyS activity of BCPPSQ with *prsQ* coexpression was still higher than that of BCPPSA with *prsA* coexpression (*t* test, $P < 0.001$). Therefore, only BCPPSQ was selected for subsequent studies.

When cultured in a 3-l fermenter for 72 h, the bacterial concentration and extracellular AmyS activity of BCPPSQ reached maximum values of 7.3 g/l and 17925.6 U/ml, respectively (Fig. 5A). Although the extracellular AmyS activity of BCPPSQ was 7.6-fold greater than that of BCWPS (2358.1 U/ml), the bacterial concentration of BCPPSQ was only 1.2-fold greater than that of BCWPS (6.1 g/l). SDS-PAGE analysis showed that the protein band at approximately 55 kDa, which corresponds to the expected protein molecular weight of AmyS, became increasingly obvious with increasing fermentation time (Fig. 5B). Therefore, it was reasonable to consider that the characteristics of BCPPSQ, including lower extracellular degradation activity and higher extracellular folding ability, were the main reasons why its extracellular AmyS activity was higher than that of BCWPS.

In this study, the highest bacterial concentration of BCPPSQ was only 7.3 g/l, which was far lower than those of other commonly used microbial expression systems, such as the *B. subtilis* and *B. amyloliquefaciens* expression systems (Wang et al., 2019; Yao et al., 2019). The bacterial concentration was related not only to the physiological characteristics of the strains themselves but also to the external culture environment (Yao et al., 2020). Therefore, regulating strain apoptosis at the genomic level or optimizing the culture medium composition and culture conditions to increase bacterial concentration of BCPPSQ might further enhance extracellular AmyS production in *B. choshinensis*.

Conclusion

This report describes a strategy to enhance extracellular α-amylase production in *B. choshinensis*. This strategy comprehensively optimized the extracellular degradation and folding environment of *B. choshinensis* by knocking out the extracellular protease gene *bcp* with the CRISPR/Cas9n system and coexpressing the extracellular chaperone *prsQ*. The extracellular AmyS activity of the strain (BCPPSQ) obtained using this strategy was 6940.9 U/ml in shake-flask culture and 17925.6 U/ml in 3-l fermenter fermentation. These values were 2.1- and 7.6-fold greater than those of the original strain BCWPS (3367.9 and 2358.1 U/ml, respectively), respectively. Therefore, optimizing the extracellular degradation and folding environment of *B. choshinensis* was an effective strategy to enhance extracellular α-amylase production. This strategy might also be useful for improving the production of other extracellular proteins in *B. choshinensis* or other related *Brevibacillus* species.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

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Conflict of Interest

No potential conflict of interest was reported by the authors.

Data Availability

All the required links or identifiers for the data are present in the manuscript as described.

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