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Efficient expression of nattokinase in *Bacillus licheniformis*: host strain construction and signal peptide optimization

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Abstract Nattokinase (NK) possesses the potential for prevention and treatment of thrombus-related diseases. In this study, high-level expression of nattokinase was achieved in *Bacillus licheniformis* WX-02 via host strain construction and signal peptides optimization. First, ten genes (*mpr*, *vpr*, *aprX*, *epr*, *bpr*, *wprA*, *aprE*, *bprA*, *hag*, *amyl*) encoding for eight extracellular proteases, a flagellin and an amylase were deleted to obtain *B. licheniformis* BL10, which showed no extracellular proteases activity in gelatin zymography. Second, the gene fragments of P43 promoter, Svpr, nattokinase and TamyL were combined into pHY300PLK to form the expression vector pP43SNT. In BL10 (pP43SNT), the fermentation activity and product activity per unit of biomass of nattokinase reached 14.33 FU/mL and 2,187.71 FU/g respectively, which increased by 39 and 156 % compared to WX-02 (pP43SNT). Last, Svpr was replaced with SsacC and SbprA, and the maximum fermentation activity (33.83 FU/mL) was achieved using SsacC, which was 229 % higher than that of WX-02 (pP43SNT). The maximum NK fermentation activity in this study reaches the commercial production level of solid state fermentation, and this study provides a promising engineered strain for industrial production of nattokinase, as well as a potential platform host for expression of other target proteins.

Keywords Nattokinase · *B. licheniformis* · Recombinant expression · Host construction · Signal peptides optimization

Abbreviations

NK	Nattokinase
aprN	The gene of nattokinase
mpr	The gene of glutamyl endopeptidase Mpr
vpr	The gene of extracellular serine protease Vpr
aprX	The gene of serine protease AprX
epr	The gene of minor extracellular protease Epr
bpr	The gene of bacillopeptidase Bpr
wprA	The gene of protease WprA
aprE	The gene of serine protease AprE
bprA	The gene of bacillopeptidase BprA
hag	The gene of flagellin
amyl	The gene of α-amylase
Svpr	The signal peptide of Vpr
SsacC	The signal peptide of SacC
SbprA	The signal peptide of BprA

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TamyL	The terminator of α-amylase gene
pHY300PLK	*E. coli* and *B. subtilis* shuttle vector

Introduction

Thrombus-related cardiovascular diseases threaten human health severely, e.g. 17 millions of people die from these diseases each year in the world. Excess clot of fibrin is the main reason for formation of thrombus, which will further lead to various cardiovascular complications [8]. To prevent and treat the thrombus-related diseases, multiple fibrinolytic products have been developed to degrade the fibrin. To date, commercially available thrombolytic products include injection grade drugs (urokinase, streptokinase and tissue type plasminogen activator) and nattokinase (NK)-related functional foods. Compared with the injection drugs, NK possesses the advantages of oral convenience, low massive haemorrhage risk, and low cost [3, 29]. Therefore, development of NK products has been gaining increasing interests.

Recombinant expression is an important method to facilitate the production of target proteins. Many genetic strategies have been developed to improve the production of recombinant proteins, such as the optimization of promoters and signal peptides [4, 19], the use of protease-deficient host strains to prevent degradation [5], and the deletion of extracellular protein genes to reduce secretion stress [24]. NK encoded by *aprN* gene was secreted from *Bacillus subtilis* strains [14], which have been broadly applied in the commercial production of natto food by solid state fermentation [20]. Recently, secretory expression of NK has been performed in host strains of *Escherichia coli* [9], *Lactococcus lactis* [10], and *B. subtilis* [2, 15]. In *B. subtilis* DB104, the recombinant expression of NK was enhanced by promoter optimization [33]. Moreover, high-level expression of NK was also achieved using the proteases-deficient host strains, *B. subtilis* WB700 [2], and *B. subtilis* WB800 [15]. Though NK has been expressed successfully in above host strains, developing novel host strains will provide more choices for NK expression.

As a GRAS (Generally Recognized as Safe) strain, *Bacillus licheniformis* shows high protein secretion capacity, and it has been used for production of multiple native and recombinant enzymes [1, 32]. Specifically, *B. licheniformis* WX-02 (CCTCC M208065) is an excellent workhorse for production of various bioproducts [18, 28], and the corresponding genetic manipulation methods have been developed [18]. In this study, a novel *B. licheniformis* host strain BL10 was constructed by deletion of ten genes from WX-02 for the first time, and the signal peptides were also optimized to facilitate the NK expression.

Materials and methods

Strains, plasmids and primers

The strains used in this study included *B. licheniformis* WX-02 (CCTCC M208065), *B. subtilis* MBS 04-6 (CGMCC AS 1.107), *B. subtilis* 168 (BGSCID) and genetically engineered strains. The plasmids were derived from T2(2)-ori and pHY-300PLK. The detailed information for strains and plasmids were shown in Table 1, and primers sequences were listed in the supplementary materials (Table S1).

Chemicals and culture media

DNA polymerase, T4 DNA ligase, restriction enzymes, protein and DNA Markers were bought from Takara Biotechnology (Dalian) Co., Ltd. The antibiotics were purchased from Sigma–Aldrich Co. LLC. The other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. LB medium consisted of 1 % tryptone, 0.5 % yeast extract, and 1 % NaCl, with the pH 7.2–7.4. The NK fermentation medium contained 2 % glucose, 1 % peptone, 1 % soy peptone, 1.5 % yeast extract, 0.5 % corn steep liquor, 0.6 % $(NH_4)_2SO_4$, and 1 % NaCl.

Gene prediction

All the annotated gene sequences of the proteases from *B. licheniformis* were selected from the MEROPS database (http://merops.sanger.ac.uk/), and were used to find the corresponding genes in *B. licheniformis* WX-02 by Blast tool (http://blast.ncbi.nlm.nih.gov). The localization of proteases was predicted by the Signal-3L (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3L/) and Cell-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/), and the results were further corrected according to the extracellular proteome data of *B. licheniformis* [22, 23].

Construction of the knockout vectors

The knockout vectors were constructed by a previously reported method [18]. Figure 1a showed the construction procedure for the *hag* gene knockout vector (T2-*hag*). The upstream and downstream homologous arms of *hag* gene, named hag (A) and hag (B), were amplified using the primers of hagKF1/hagKR1 and hagKF2/hagKR2 (Table S1) respectively, and the amplified fragments were further purified and recovered. By splicing overlap extension PCR method (SOE-PCR) using the primers of hagKF1/hagKR2, the hag (A) and hag (B) were fused to form the fragment hag (A + B), which was then cloned into the T2(2)-ori at the *Xba* I and *Sac* I restriction sites. Other knockout vectors were constructed following the same method.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant properties	Source
Strains		
B. subtilis MBS 04-6	NK producing strain (CGMCC AS 1.107)	CGMCC
B. subtilis 168	The strain with pP43 promoter	BGSCID
B. licheniformis WX-02	Wide-type host strain (CCTCC M208065)	CCTCC
B. licheniformis BL10	Δ*hag*; Δ*mpr*; Δ*vpr*; Δ*aprX*; Δ*epr*; Δ*bpr*; Δ*wprA*; Δ*aprE*; Δ*amyL*; Δ*bprA*	This study
BL10 (PHY300PLK)	BL10 with pHY300PLK plasmid	This study
WX-02 (pP43SNT)	WX-02 with pP43SNT plasmid	This study
BL10 (pP43SNT)	BL10 with pP43SNT plasmid	This study
BL10 (pP43SNT-SsacC)	BL10 (pP43SNT) with SsacC	This study
BL10 (pP43SNT-SbprA)	BL10 (pP43SNT) with SaprE	This study
Plasmids		
T2 (2)-ori	*E. coli* and *B. subtillis* shuttle vector; Kan^r	Stored in lab
T2-*amyL*	T2 (ori)-amyL (A + B); to knock out *amyL*	This study
T2-*hag*	T2 (ori)-hag (A + B); to knock out *hag*	This study
T2-*mpr*	T2 (ori)-mpr (A + B); to knock out *mpr*	This study
T2-*vpr*	T2 (ori)-vpr (A + B); to knock out *vpr*	This study
T2-*aprX*	T2 (ori)-aprX (A + B); to knock out *aprX*	This study
T2-*epr*	T2 (ori)-epr (A + B); to knock out *epr*	This study
T2-*bpr*	T2 (ori)-bpr (A + B); to knock out *bpr*	This study
T2-*wprA*	T2 (ori)-wprA (A + B); to knock out *wprA*	This study
T2-*aprE*	T2 (ori)-aprE (A + B); to knock out *aprE*	This study
T2-*bprA*	T2 (ori)-bprA (A + B); to knock out *bprA*	This study
pHY300PLK	*E. coli* and *B. subtilis* shuttle vector; Amp^r, Tet^r	Stored in lab
pP43SNT	PHY300PLK + P43 + Svpr + *aprN* + TamyL	This study
pP43SNT-SsacC	PHY300PLK + P43 + SsacC + *aprN* + TamyL	This study
pP43SNT-SbprA	PHY300PLK + P43 + SbprA + *aprN* + TamyL	This study

Construction of the expression vector

As shown in Fig. 1b, the NK expression vector was constructed based on the NK gene (FJ374767.1) from *B. subtilis* MBS 04-6, the P43 promoter (K02174.1) from *B. subtillis* 168, the terminator of *amyL* gene (TamyL) (FJ556804.1) and the signal peptide of *vpr* gene (Svpr) (M76590.1) from *B. licheniformis* WX-02. First, the gene fragments of P43, Svpr, *aprN* gene and TamyL were amplified and purified, respectively. Second, the purified fragments of P43, Svpr and *aprN* gene were fused by SOE-PCR to obtain the P43SN fragment, which was further assembled with TamyL to form the P43SNT fragment by SOE-PCR. Finally, the P43SNT fragment was inserted into the pHY-300PLK plasmid at *Eco*R I and *Bgl* II sites, which was then transformed into *E. coli* DH5α to obtain the expression plasmid pP43SNT.

Gene knockout

The competent cells of *B. licheniformis* were prepared by the method reported [18]. The competent cells were electro-transformed at 2.5 kV with 5 μL knockout plasmid solution (50 ng/μL), maintained in 900 μL recovery medium (LB medium added with 0.5 mol/L sorbitol and 0.38 mol/L mannitol) at 30 °C for 1 h, and then incubated at 30 °C and 100 rpm for 2 h. The cells were spread onto LB plate with 20 μg/mL kanamycin, incubated at 30 °C for 20 h, and the transformants were selected by PCR and plasmid extraction verification. The positive transformants were inoculated into LB liquid medium containing 20 μg/mL kanamycin, cultured at 45 °C for 8 h and plated onto kanamycin LB solid medium, which was further incubated at 45 °C to screen the single-crossover colonies. After PCR verification, the positive clones were subcultured in LB liquid medium without kanamycin at 37 °C for 8 h, and the subculturing was repeated for six times. At last, the cultures were incubated in LB plates at 37 °C for 20 h, and each single colony was inoculated onto a LB plate with kanamycin, as well as one without kanamycin. If a colony grew on the LB plate without kanamycin and was inhibited on LB plate with kanamycin, this strain could be the double crossover one, which would be further verified by PCR.

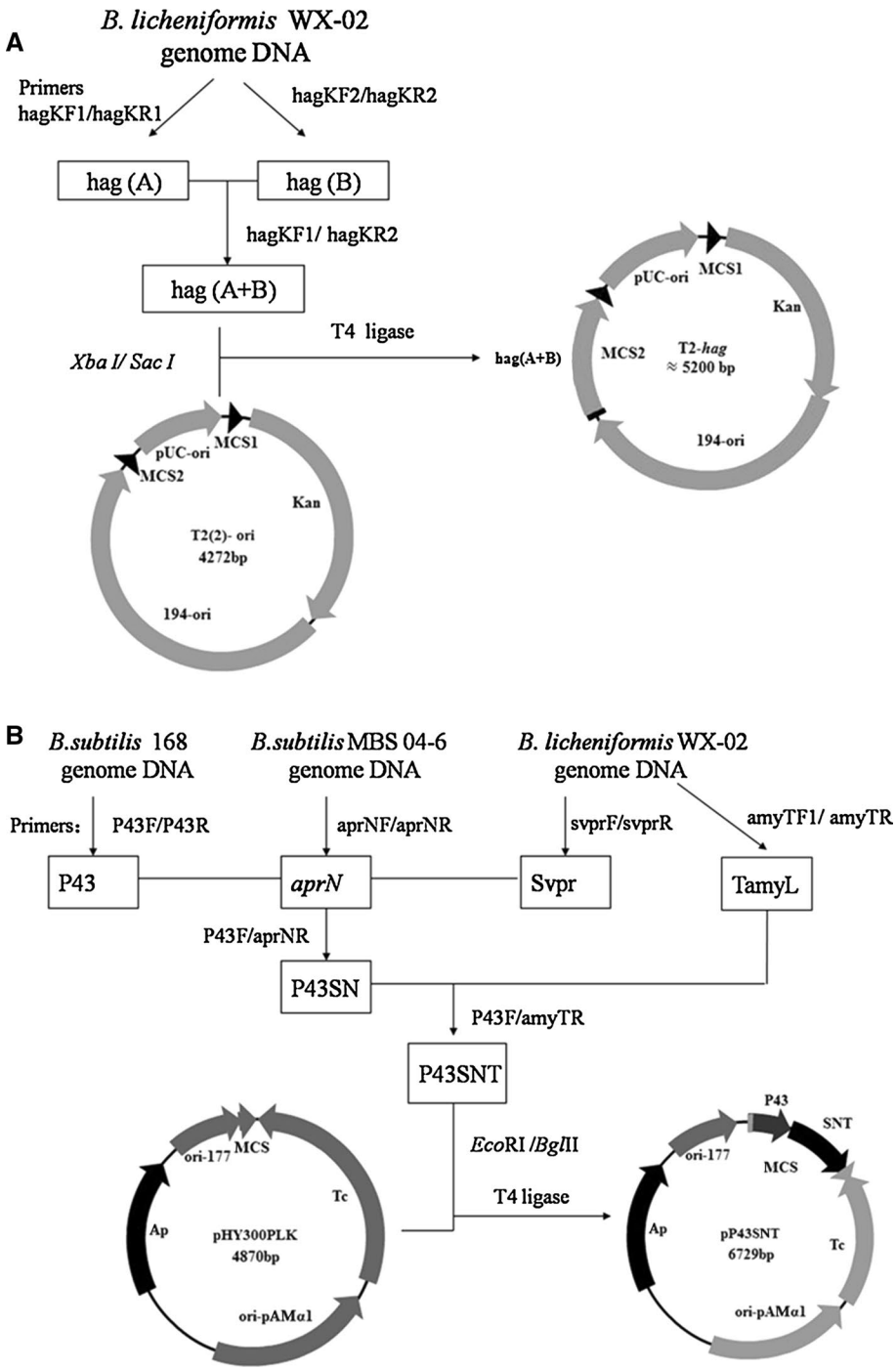

Fig. 1 The construction procedure of the knockout vector (**a**) and expression vector (**b**)

Recombinant expression of NK

The expression vectors were mixed with competent cells (50 ng/μL), and electro-transformation was carried out following the method described above. After electroporation, the cells were incubated in recovery medium at 37 °C for 2 h, then spread onto the LB plate containing 20 μg/mL tetracycline (Tet^r plate), and cultured at 37 °C for 20 h. The single colony was cultured in LB liquid medium (20 μg/mL tetracycline) at 37 °C for 10 h, and then the plasmid was extracted and verified by restriction digestion. The recombinants were inoculated into LB medium supplemented with 20 μg/mL tetracycline, cultured at 37 °C and 180 rpm for 10 h, and then subcultured in LB medium for 10 h as the seed culture. The inoculums (1 %) were transferred into 250 mL flasks containing 50 mL fermentation medium with initial pH 7.0, incubated at 37 °C and 180 rpm for 36 h. The fermentation broth was sampled every 4 h, and the biomass and NK activity were measured.

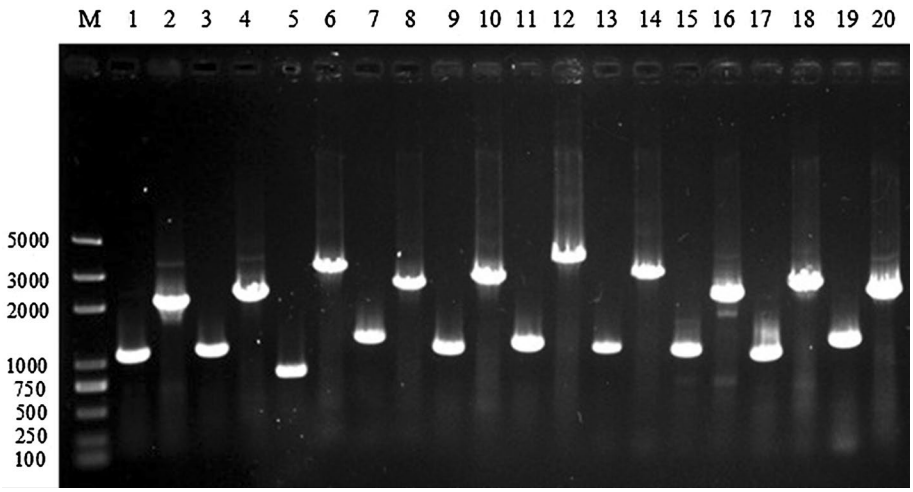

Fig. 2 PCR verification of the BL10 strain. PCR product from BL10 by primers hagKYF/hagKYR (*lane 1*), mprKYF/mprKYR (*3*), vprKYF'/vprKYR' (*5*), aprXKYF/aprXKYR (*7*), eprKYF/eprKYR (*9*), bprKYF/bprKYR (*11*), wprAKYF/wprAKYR (*13*), aprEKYF/aprEKYR (*15*), amyKYF/amyKYR (*17*), bprAKYF/bprAKYR (*19*); PCR product from WX-02 by primers hagKYF/hagKYR (*lane 2*), mprKYF/mprKYRR (*4*), vprKYF/vprKYR (*6*), aprXKYF/aprXKYR (*8*), eprKYF/eprKYR (*10*), bprKYF/bprKYR (*12*), wprAKYF/wprAKYR (*14*), aprEKYF/aprEKYR (*16*), amyKYF/amyKYR (*18*), bprAKYF/bprAKYR (*20*)

Analysis methods

The cell growth was evaluated by measuring the dry cell weight (DCW) [28]. The NK activity was detected by fibrin degradation method [27]. DNA and the proteins were determined by agarose gel electrophoresis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [12], respectively. The activity of extracellular protease was evaluated by gelatin zymography [12].

Results

Construction of the ten genes-deficient BL10 host strain

Ten genes (*mpr*, *vpr*, *aprX*, *epr*, *bpr*, *wprA*, *aprE*, *bprA*, *hag, amyl*) encoding for extracellular proteases, flagellin and α-amylase were selected as the targets for deletion [7, 11, 13]. The upstream and downstream arms (about 500 bp) of each target gene fragment were amplified, and then fused by SOE-PCR. The fused fragment (about 1,000 bp) was ligated into the temperature-sensitive plasmid of T2(2)-ori (4,200 bp), which was then transformed into *E. coli* DH5α. The recombinant plasmids (T2-*hag*, T2-*mpr*, T2-*vpr*, T2-*aprX*, T2-*epr*, T2-*bpr*, T2-*wprA*, T2-*aprE*, T2-*amyL* and T2-*bprA*) (about 5,200 bp) were extracted and used for genes deletion. By overlay knockout of the target genes, 10 mutants were obtained: BL1 (Δ*hag*), BL2 (Δ*hag*Δ*mpr*), BL3 (Δ*hag*Δ*mpr*Δ*vpr*), BL4 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*), BL5 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*Δ*epr*), BL6 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*Δ*epr*Δ*bpr*), BL7 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*Δ*epr*Δ*bpr*Δ*wprA*), BL8 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*Δ*epr*Δ*bpr*Δ*wprA*Δ*aprE*), BL9 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*Δ*epr*Δ*bpr*Δ*wprA*Δ*aprE*Δ*amyL*), BL10 (Δ*hag*Δ*mpr*Δ*vpr*Δ*aprX*Δ*epr*Δ*bpr*Δ*wprA*Δ*aprE*Δ*amyL*Δ*bprA*). The gene deletion was confirmed by diagnostic PCR, and BL10 was taken as an example (Fig. 2). For each target gene, the length of amplified fragment from BL10 was the same as the fused homologous arms fragment, while the fragment length amplified from wild-type WX-02 was equal to the total length of homologous arm and target gene (Fig. 2). These results indicated that ten target genes were successfully deleted from the genome of WX-02.

The extracellular protease activities of BL10

Extracellular proteases activities of the mutants were determined by gelatin zymography method. As shown in Fig. 3a, the protease activities decreased as more genes were deleted, while four residual bands showed that there were still intense protease activities in BL9. According to the molecular weights of the residual bands, corresponding proteases were predicted as: YodJ (30.9 kDa), YdaJ (41.3 kDa), YwaD (48.2 kDa) and BprA (54.2 kDa). After knockout of the *yodJ*, *ydaJ* and *ywaD* genes from BL9, the extracellular protease activities showed no significant difference (data not shown). Surprisingly, the four bands disappeared simultaneously when the *bprA* gene was deleted (Fig. 3b). The mutant BL10 showed no extracellular protease activity in gelatin zymography, which may be beneficial for expression of target proteins [7, 15].

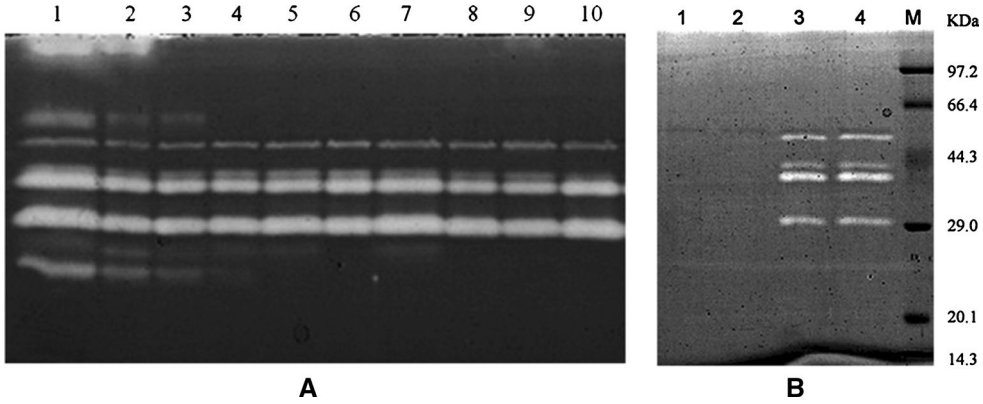

Fig. 3 The gelatin zymography of extracellular proteases. **a** *Lane 1–10* shows the gelatin zymography of extracellular proteases from WX-02, BL1, BL2, BL3, BL4, BL5, BL6, BL7, BL8, BL9. **b** The gelatin zymography of BL10 (*lane 1*, *2*) and BL9 (*lane 3*, *4*)

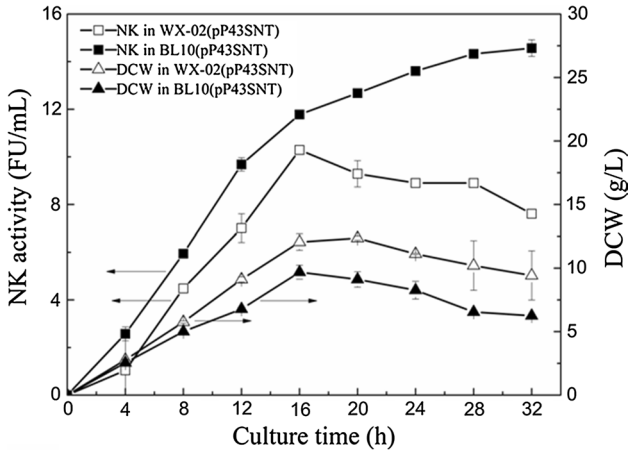

Fig. 4 Time profiles of the nattokinase production and cell growth in BL10 (pP43SNT) and WX-02 (pP43SNT)

Effect of ten-genes-deletion on the NK expression

The expression plasmid (pP43SNT) was transformed into BL10 and wild-type WX-02 strains, respectively, to obtain the recombinant strains BL10 (pP43SNT) and WX-02 (pP43SNT). The cell growth and NK production profiles of these two strains were compared. As shown in Fig. 4, NK was synthesized along with cell growth for each strain. Though BL10 (pP43SNT) grew slower than that of WX-02 (pP43SNT), the former showed higher NK fermentation activity during the whole fermentation process. In WX-02 (pP43SNT), the maximum NK fermentation activity (10.29 FU/mL) was obtained at 16 h, while the activity decreased obviously after 16 h, indicating that NK was degraded by the extracellular proteases. In contrast, the NK activity increased constantly until 28 h in BL10 (pP43SNT), and the maximum activity reached 14.33 FU/mL, which was 39 % higher than that of WX-02 (pP43SNT). In BL10 (pP43SNT), the NK product activity per unit of biomass reached 2,187.71 FU/g, which increased by 156 % compared to WX-02 (pP43SNT).

The extracellular proteins were further evaluated by SDS-PAGE analysis at different fermentation time points (Fig. 5). In WX-02 (pP43SNT), the most intense NK band was observed at 16 h, and then the intensity decreased obviously at 20 h (Fig. 5a), which was probably due to the degradation by extracellular proteases. In BL10 (pP43SNT), the intensity of NK band increased after 20 h (Fig. 5b), and no significant difference was observed even after 28 h (Fig. 5b). These results showed that the deletion of protease genes could prevent protein degradation, which effectively improved the NK production.

Effect of signal peptide replacement on the NK production

The signal peptide of pP43SNT (Svpr) was replaced by the signal peptides of SacC (X05649.1) and BprA (NZ_AHIF00000000.1), which were named SsacC and SbprA [4]. The corresponding recombinant strains of BL10 (pP43SNT-SsacC) and BL10 (pP43SNT-SbprA) were then obtained. Compared with the strain harboring Svpr, higher NK fermentation activities were obtained in the strains with SsacC and SbprA (Fig. 6a). The maximum NK fermentation activity was obtained in BL10 (pP43SNT-SsacC). Figure 6b showed the time profile of NK production by BL10 (pP43SNT-SsacC). The NK activity increased as the fermentation time prolonged before 20 h, and there was no significant difference until 48 h, indicating that NK was highly stable due to the successful deletion of extracellular proteases. At 20 h, the NK fermentation activity and product activity per unit of biomass reached 33.83 FU/mL and 4,528.78 FU/g, respectively, increased by 229 and 431 %, compared to those of WX-02 (pP43SNT). Meanwhile, the productivity of BL10 (pP43SNT-SsacC) reached 1.69 FU/mL h, which was 164 % higher than that of WX-02 (pP43SNT).

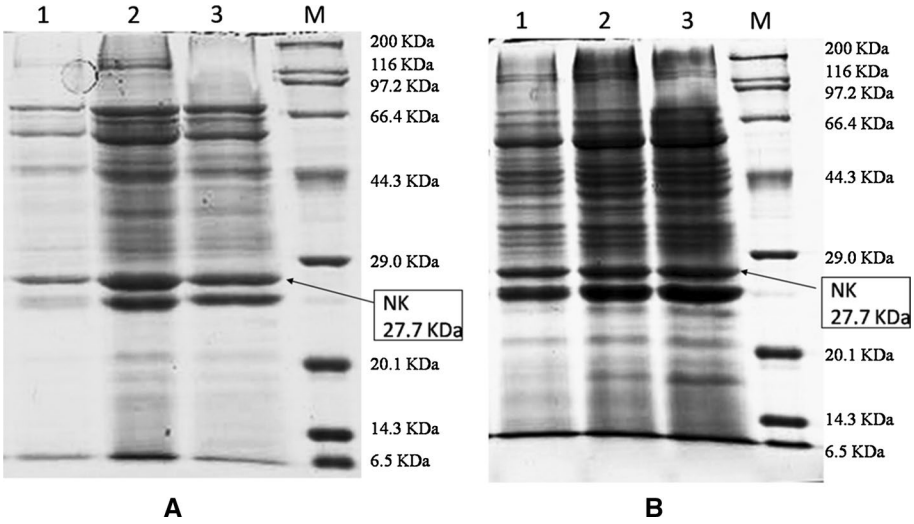

Fig. 5 SDS-PAGE analysis of the extracellular proteins of WX-02 (pP43SNT) and BL10 (pP43SNT) at different fermentation time. **a** The extracellular proteins of WX-02 (pP43SNT) at 12 h (*lane 1*), 16 h (*lane 2*), 20 h (*lane 3*), and Takara Premixed Protein Marker (*lane M*). **b** The extracellular proteins of BL10 (pP43SNT) at 20 h (*lane 1*), 24 h (*lane 2*), 28 h (*lane 3*), and Takara Premixed Protein Marker (*lane M*)

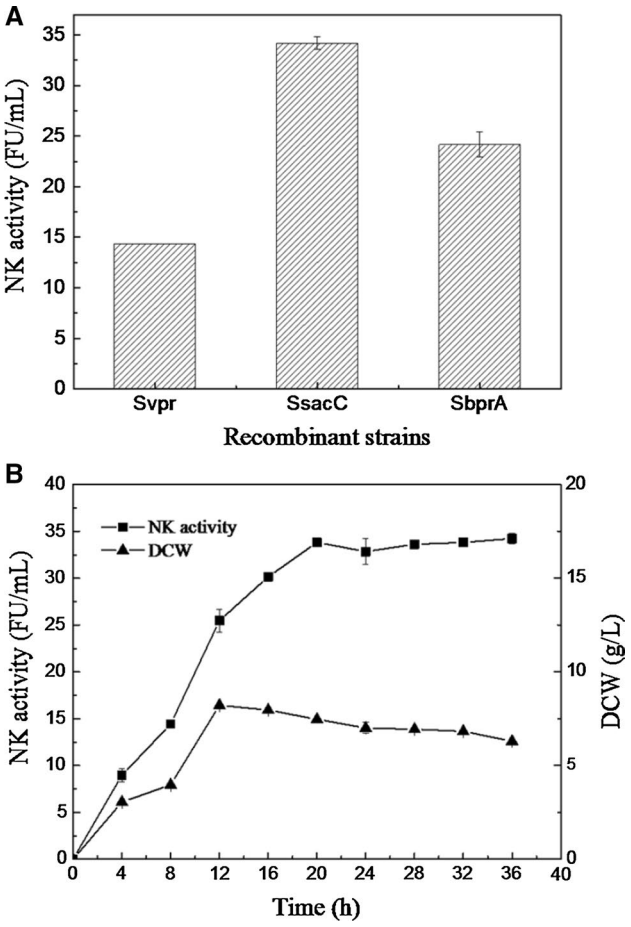

Fig. 6 Effects of different signal peptides on nattokinase production. **a** Nattokinase fermentation activities under different signal peptides. **b** Time profiles of nattokinase biosynthesis and cell growth in BL10 (pP43SNT-SsacC)

Discussion

Bacillus species are usually used as the host strains for recombinant expression of heterologous proteins. However, the host strains can also secrete abundant autologous extracellular proteases, which can degrade the target heterologous proteins to reduce their production [21]. Therefore, deleting extracellular proteases of host strain may enhance the production of heterologous proteins by preventing their degradation. The multiple proteases-deficient host strains have been constructed, such as *B. subtilis* WB600, WB700 and WB800 [5, 15]. The deficiency of proteases genes (*nprE*, *nprB*, *mpr*, *vpr*, *aprX*, *epr*, *bpr*, *wprA*, *aprE*) in *B. subtilis* improved the expression of target proteins obviously [5, 15], and thus, the proteases genes (*mpr*, *vpr*, *aprX*, *epr*, *bpr*, *wprA*, *aprE*) existed in *B. licheniformis* WX-02 genome were selected as the targets to be deleted to prevent the degradation. Furthermore, flagellin (Hag) and α-amylase (Amyl) are among the most abundant proteins in *Bacillus* [11, 13], which may induce the proteins secretion stress response [31]. Therefore, *hag* and *amyl* genes were also chosen as the targets to be deleted to reduce the protein secretion stress. Collectively, seven extracellular proteases genes (*mpr*, *vpr*, *aprX*, *epr*, *bpr*, *wprA*, *aprE*), the flagellin gene (*hag*) and the α-amylase gene (*amyl*) were deleted to obtain the mutant BL9.

According to the gelatin zymography, extracellular proteases activities reduced obviously by the knockout of nine genes, which agreed with a previous report [34]. However, four residual bands indicated that intense proteases activities still existed in BL9. Surprisingly, further deletion of the *bprA* gene resulted in the disappearance of these four bands.

In *B. licheniformis* genome, the *bprA* gene was predicted to encode for the bacillopeptidase F, while the gene sequence showed no similarity with another bacillopeptidase F gene *bpr* (M29035.1) in *B. subtilis*. Several proteins were reported to possess the self-cleaving activities, which cleaved the proteins to form small fragments [16, 17]. The BprA may also have self-cleaving ability to produce the four fragments, and the detailed mechanism is being investigated.

Secretory expression of NK was achieved in ten-genes-deficient *B. licheniformis* BL10. In BL10 (pP43SNT), NK fermentation activity and product activity per unit of biomass increased by 39 and 156 % compared to those of WX-02 (pP43SNT), respectively, indicating that deletion of these ten genes are efficient to enhance NK production in *B. licheniformis*. Similar results were also reported in *B. subtilis* WB800 [15]. BL10 showed almost no extracellular proteases activity, which can prevent the degradation of target protein. Time profiles analysis of NK fermentation activity and protein concentration confirmed that deletion of extracellular proteases enhanced NK production by inhibiting its degradation. Meanwhile, it was verified that deletion of these genes also improved recombinant expression of α-amylase (data not shown). *B. subtilis* WB800 has been used for recombinant expression of multiple proteins, such as nattokinase [15], phospholipase C [5], and methyl parathion hydrolase [35]. Like *B. subtilis* WB800, this study also provides a novel platform host *B. licheniformis* BL10, which has the potential for expression of different proteins.

The secretory expression of a protein is affected obviously by signal peptides [4]. The signal peptide of SacC (SsacC) from *B. suntilis* was confirmed to be efficient to induce the expression of subtilisin BPN' [4]. NK is highly similar with subtilisin BPN' [30], and thus SsacC may have similar effect on the secretory expression of NK. Based on our results (Fig. 3b), BprA showed intense extracellular protease activity in *B. licheniformis*, indicating that its signal peptide (SbprA) may be efficient to mediate the protein secretion. Therefore, SsacC and SbprA were used to replace Svpr. Our results confirmed that replacement of Svpr with SsacC and SbprA could improve NK secretion obviously. The NK fermentation activity by BL10 (pP43SNT-SsacC) was 229 % higher than that of WX-02 (pP43SNT). The signal peptides can influence the protein secretion by regulating the secretion pathways [6], and investigation of the secretion mechanism of NK in *B. licheniformis* is underway. In this study, the maximum NK fermentation activity (33.83 FU/mL) was much higher than previous reports [25, 26], and it reaches present commercial production level (20–40 FU/g) in natto food by solid state fermentation [20]. The productivity of 1.69 FU/mL h in this study also reaches the commercial level (about 1 FU/g h) in solid state fermentation. Therefore, the BL10 (pP43SNT-SsacC) shows potential application for industrial production of NK.

Conclusion

In this study, the ten-genes-deficient strain *B. licheniformis* BL10 was obtained by overlap knockout of ten genes from the wild-type WX-02, and almost no extracellular protease activity was detected in BL10. Our results showed that the deletion of ten genes enhanced the recombinant expression of nattokinase obviously. By host strain construction and signal peptides optimization, the maximum NK fermentation activity of 33.83 FU/mL was achieved. This study provides a valuable strain with the potential application for industrial production of NK, as well as a platform host strain for expression of other proteins.

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