Improvement of levan production in *Bacillus amyloliquefaciens* through metabolic optimization of regulatory elements

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Abstract Levan is a functional homopolymer of fructose
with considerable applications in food, pharmaceutical and
cosmetic industries. To improve the levan production in
Bacillus amyloliquefaciens, the regulatory elements of *sacB*
(encoding levansucrase) expression and levansucrase secre-
tion were optimized. Four heterologous promoters were eval-
uated for *sacB* expression, and the Pgrac promoter led to the
highest level for both *sacB* transcription and levansucrase en-
zyme activity. The levan production in the corresponding re-
combinant strain ΔLP-pHTPgrac reached 30.5 g/L, which
was 114% higher than that of the control strain NK-ΔLP. In
a further step, eight signal peptides were investigated (with
Pgrac as the promoter for *sacB* expression) for their effects
on the levansucrase secretion and levan production. The signal
peptide yncM was identified as the optimal one, with a
secretion efficiency of approximately 90%, and the levan pro-
duction in the corresponding recombinant strain ΔLP-Y
reached 37.4 g/L, which was 161% higher when compared
with the control strains NK-ΔLP. Finally, fed-batch fermenta-
tion was carried out in 5-L bioreactors for levan production
using the recombinant strain ΔLP-Y. A final levan concentra-
tion of 102 g/L was achieved, which is very close to the ever
reported highest levan production level from the literature. To
our best knowledge, this is the first report of the improvement
of levan production through metabolic optimization for *sacB*
expression and levansucrase secretion. The results from this
study provided essential insights for systematically metabolic
engineering of microbial cell factories for enhanced biochem-
ical production.

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Introduction

Levan is a homopolymer of fructose polymerized by β-(2 →
6) glycosidic bonds in a main chain with occasional β-(2 → 1)
linked branching points (Nakapong et al. 2013). Besides the
common features of natural polysaccharide like safety, bio-
compatibility and biodegradability, levan also exhibits many
favorable properties such as moisturizing, anti-obesity, anti-
tumor, anti-oxidant, anti-inflammatory, hyperglycemic inhib-
itor and other biomedical functions (Dahech et al. 2011;
Kucukasik et al. 2011; Dahech et al. 2012, 2013).
Therefore, levan has been widely applied in food, prebiotics,
cosmetics and medicines.

Levan biosynthesis usually involves the enzyme
levansucrase (EC 2.4.1.10), which belongs to the glycoside
hydrolases family 68 according to Carbohydrate-Active

enzymes Database (Alamäe et al. 2012; Li et al. 2015). Levansucrase hydrolyzes sucrose by cleaving β-(1 → 2) linkage and transfers the fructosyl group to an acceptor molecule such as sucrose or fructooligosaccharide chain to generate levan. The molecular weight of the generated levan depends on specific features of the levansucrase (Li et al. 2015). Levansucrase is an bacterial extracellular enzyme, which can be produced by a wide range of bacterial strains, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Zymomonas mobilis*, *Pseudomonas fluorescens* and *Lactobacillus reuteri* (Abdel-Fattah et al. 2005; de Paula et al. 2008; Sims et al. 2011; Jathore et al. 2012; Feng et al. 2015a).

From the literature, there are several strategies that have been employed to increase the production of levan. First, intensive studies have been focused on selection of good natural producers, optimization of fermentation media and improvement of fermentation process design (Silbir et al. 2014; Wu et al. 2013). Second, sucrose metabolic pathways have been engineered to increase the synthesis of levan. The extracellular sucrase gene (*sacC*) disruption strain *Z. mobilis* showed 3-fold higher levansucrase activity than the wild-type strain, and the levan production was improved from 15.5 to 21.2 g/L (Senthilkumar et al. 2004). Similar genetic modification was carried out in *L. reuteri*, and results demonstrated that the deletion of the sucrose phosphorylase gene *scrP* also increased levan production (Teixeira et al. 2013). Recently, protease-deficient strains were constructed to improve the levan production. The *B. amyloliquefaciens* NK-Q-7 strain with six extracellular protease genes that were deleted showed the highest levan production of 31.1 g/L, representing a 102% increase compared to the control strain (Feng et al. 2015b).

In this study, we attempted to achieve enhanced levan production from a different aspect, that is by optimizing the regulatory elements for levansucrase activity, including identifying the optimal strong promoter and signal peptide. The promoter of P43 is a well-characterized strong constitutive promoter in *Bacillus* and has been widely used for enzyme overexpression and metabolic engineering (Zhang et al. 2005, 2011). The inducible promoters Pgrac (IPTG inducible), PxylA (xylose inducible) and PsacB (sucrose inducible) have been successfully applied for the protein expression and claimed to bring about high-level expression of the target protein (Liu and Du 2012; Phan et al. 2012).

Therefore, these promoters were first evaluated for obtaining high levansucrase activity and enhanced levan production. On the other hand, the signal peptides located at the N-terminus of secretory protein play an important role in targeting the protein to the translocation machinery and transporting the protein from cytoplasm into the growth medium (Ng and Sarkar 2013). In the previous research, the secretion systems in *Bacillus* have been extensively investigated and many exceptional signal peptides have been identified and characterized (Zhang et al. 2013). Although the

primary amino acid sequences of *Bacillus* signal peptides are not conserved, they share certain common features. Generally, a typical signal peptide contains three distinct regions, including a positively charged N-terminal domain, a hydrophobic core region (H region) and a C-terminal region with a signal peptidase cleavage site (Song et al. 2015). On the basis of the signal peptidase (SPase) recognition sequence, signal peptides are classified into four major families, and the Sec-dependent signal peptides have attracted the most attention due to its widespread and potential application for the secretion of enzyme production (Chen et al. 2007; Ying et al. 2012). However, the adaptation mechanism between the target protein and the signal peptide was still unclear, which might involve many aspects, such as molecular mass, folding rate and charge of the target protein (Zhang et al. 2013). Therefore, screening the optimal signal peptide was usually carried out by non-rational approaches, including single specific verification, random mutagenesis and high throughput screening (Brockmeier et al. 2006; Caspers et al. 2010). In this study, we attempt to improve levansucrase secretion through screening the optimal signal peptide.

Based on the levan producing *B. amyloliquefaciens* NK-ΔLP strain (Feng et al. 2015a), here, we further optimized the regulatory elements of levansucrase to achieve high-level levan production. Pgrac, PxylA, PA2up and PC2up were evaluated for their effects on the transcriptional level of *sacB* (encoding levansucrase). The strong promoter Pgrac was eventually identified for optimal levan production. Then, eight signal peptides were screened for the enhanced secretion of levansucrase. It was found that when fused with the yncM signal peptide, the levansucrase activity was 19.1-fold higher than the wild-type strain, and the levan production was improved from 14.2 (in control) to 37.4 g/L in flask fermentation. Our results from this study provided valuable reference for boosting bioproduct production in relevant hosts through rational metabolic engineering approaches.

Materials and methods

Strains and growth conditions

All the strains used in this work are listed in Table 1. *Escherichia coli* DH5α was used for plasmid propagation and transformation. Plasmid demethylation was carried out in *E. coli* GM2163. *B. amyloliquefaciens* NK-ΔLP that was derived from *B. amyloliquefaciens* LL3 (CCTCC M 208109) was used as the starting host strain (Feng et al. 2015a). *E. coil* and *B. amyloliquefaciens* cells were cultured at 37 °C in Luria-Bertani (LB) medium. Selection of *E. coil* was done with ampicillin (100 μg/mL). For *B. amyloliquefaciens*, chloramphenicol (6 μg/mL) was used.

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant genotype and characteristics	Source
Strains		
B. amyloliquefaciens NK-1	LL3 derivative, ΔpMC1, Δ*upp*	Feng et al. (2013)
B. amyloliquefaciens NK-ΔLP	NK-1 derivative, Δ*pgsBCA*	Feng et al. (2015a)
B. amyloliquefaciens ΔLP-01	NK-ΔLP derivative with empty expression plasmid pHT01	This work
B. amyloliquefaciens ΔLP-pHTPA2cup	NK-ΔLP derivative with expression plasmid pHTPA2cup	This work
B. amyloliquefaciens ΔLP-pHTPC2up	NK-ΔLP derivative with expression plasmid pHTPC2up	This work
B. amyloliquefaciens ΔLP-pHTPxylA	NK-ΔLP derivative with expression plasmid pHTPxylA	This work
B. amyloliquefaciens ΔLP-pHTPsacB	NK-ΔLP derivative with expression plasmid pHTPsacB	This work
B. amyloliquefaciens ΔLP- pHTPgrac	NK-ΔLP derivative with expression plasmid pHTPgrac	This work
B. amyloliquefaciens ΔLP-A	NK-ΔLP derivative with expression plasmid pHT-PA	This work
B. amyloliquefaciens ΔLP-B	NK-ΔLP derivative with expression plasmid pHT-PB	This work
B. amyloliquefaciens ΔLP-R	NK-ΔLP derivative with expression plasmid pHT-PR	This work
B. amyloliquefaciens ΔLP-N	NK-ΔLP derivative with expression plasmid pHT-PN	This work
B. amyloliquefaciens ΔLP-E	NK-ΔLP derivative with expression plasmid pHT-PE	This work
B. amyloliquefaciens ΔLP-W	NK-ΔLP derivative with expression plasmid pHT-PW	This work
B. amyloliquefaciens ΔLP-Y	NK-ΔLP derivative with expression plasmid pHT-PY	This work
B. subtilis 168	Wide-type strain	BGSC
E. coli DH5α	F$^-$, φ80d*lacZ*ΔM1, Δ(*lacZYA-argF*)U169, *deoR, recA1, endA1, hsdR17*(r_k^-, m_k^+), *phoA, supE44, λthi-1, gyrA96, relA1*	Lab stock
E. coli GM2163	F$^-$, *ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rpsL 136* (Str^r) *xyl-5 mtl-1dam13*::Tn9 (Cam^r) *dcm-6 mcrB1 hsdR2 mcrA*	Lab stock
Plasmids		
pMD19-T simple	T easy vector for gene cloning; Amp^r	Takara
pUC-A2cup	pMD19-T derivative, PA2cup	Lab stock
pUC-C2up	pMD19-T derivative, PC2up	Lab stock
pUC-sacB	pMD19-T derivative, *sacB* gene	This work
pHT01	Shuttle vector, Amp^r (gram-negative)and Cm^r (gram-positive), IPTG inducible expression vector for *Bacillus*	MoBiTec
pHTPgrac	pHT01 derivative, Pgrac, *sacB* gene	This work
pHTPsacB	pHT01 derivative, PsacB, *sacB* gene	This work
pHTPxylA	pHT01 derivative, PxylA, *sacB* gene	This work
pHTPA2cup	pHT01 derivative, PA2cup, *sacB* gene	This work
pHTPC2up	pHT01 derivative, PC2up, *sacB* gene	This work
pHTPA	pHT-S1 derivative, Pgrac, SPamyE, *sacB* gene	This work
pHTPB	pHT-S1 derivative, Pgrac, SPbglS, *sacB* gene	This work
pHTPR	pHT-S1 derivative, Pgrac, SPbpr, *sacB* gene	This work
pHTPE	pHT-S1 derivative, Pgrac, SPnprE, *sacB* gene	This work
pHTPN	pHT-S1 derivative, Pgrac, SPnprB, *sacB* gene	This work
pHTPW	pHT-S1 derivative, Pgrac, SPwapA, *sacB* gene	This work
pHTPY	pHT-S1 derivative, Pgrac, SPyncM, *sacB* gene	This work

BGSC Bacillus Genetic Stock Center

The genome sequence accession number of *B. amyloliquefaciens* LL3 is CP002634. And the *sacB* gene ID is LL3_RS20055.

DNA manipulation and plasmid construction

All plasmids and primers used in this study are listed in Tables 1 and 2. The *sacB* gene was amplified from the genomic DNA of *B. amyloliquefaciens* NK-ΔLP, using primers sacB-F and sacB-R. The 1.4-kb fragment was digested with *Bam*HI/*Xba*I and cloned into the *E. coli*-*B. subtilis* shuttle vector pHT01, which was digested with same enzymes, resulting in pHTPgrac (Fig. S1). This plasmid has an IPTG-inducible promoter Pgrac and served as a backbone into which four different promoters were inserted individually to drive the expression of levansucrase in *B. amyloliquefaciens* NK-ΔLP. Two constitutive promoters PA2cup and PC2up were amplified from the plasmids pUC-A2cup and pUC-C2up using primer pairs M13F/M13R, respectively (The sequence information of these two promoters is shown in Fig. S1). The xylose-inducible promoter PxylA was cloned from the plasmid pWH1520. The native promoter of *sacB* was amplified from the genomic DNA of *B. amyloliquefaciens* NK-ΔLP. After digested with *Kpn*I and *Bam*HI, these amplified fragments were cloned into the corresponding sites of pHTPgrac, resulting in pHTPA2cup, pHTPC2up, pHTPxylA and pHTPsacB, respectively (Fig. S2).

To investigate the influence of signal peptides on the expression of levansucrase in *B. amyloliquefaciens* NK-ΔLP, the native signal peptide SPsacB of levansucrase was replaced by seven different ones (SPamyE, SPbgls, SPbpr, SPnprB, SPnprE, SPwapA and SPyncM), respectively.

The seven signal peptides genes were amplified using *B. subtilis* 168 genome as the template, and the levansucrase gene lacking the signal peptide was cloned from *B. amyloliquefaciens* NK-ΔLP. The two DNA fragments (the signal peptides and the truncated levansucrase) were joined by overlap PCR. These seven fusion fragments were digested by *Bam*HI and *Xba*I individually and ligated into the vector pHT01, which was digested with same enzymes, generating pHTPA (SPamyE), pHTPB (SPbgls), pHTPR (SPbpr), pHTPN (SPnprB), pHTPE (SPnprE), pHTPW (SPwapA) and pHTPY (SPyncM), respectively (Fig. S3).

Construction of the recombinant strains

All plasmid propagation was carried out in *E. coli* DH5α, and the constructed plasmids were verified by DNA sequencing. The plasmid DNA was transformed into the host strain *B. amyloliquefaciens* NK-ΔLP by electroporation (Feng et al. 2014), and the empty vector pHT01 was used as the control. The transformed strains were confirmed by PCR using primes pHT-F/R.

Table 2 Primers used in this study (with the restriction enzyme cleavage sites underlined)

Primers	Sequence (5′–3′)
M13F	CGCCAGGGTTTTCCCAGTCACGAC
M13R	CACACAGGAAACAGCTATGAC
SPxyl-F	GGCC*GGTACC*TGAGGCTTAACCTT ACAACG
SPxyl-R	GGCC*GGATCC*TCCCCCTTTGATTT AAG TGAAC
P$_{sacB}$-F	GGCC*GGTACC*CCAAAACACCCCGG ACACCTGCCG
P$_{sacB}$-R	GGCC*GGATCC*GTTCATGTCTCCTT CTGTATGTACT
sacB-F	GGCC*GGATCC*ATGAACATCAAAAA AATTGTAAAAC
sacB-R	CCGG*TCTAGA*TTAGTTGACTGTCA GCTGTCCTTGCTCC
ssAmyE-SF	CCGG*GGATCC*ATGTTTGCAAAACG ATTCAAAACCTC
ssAmyE-SR	TTGGTTATTTTCTTTAGCACTCGCAG CCGCCGGTCCTGCCAG
ssAmyE-XF	GCGGCTGCGAGTGCTAAAGAAAATAA CCAAAAAGCATACAAAG
ssBglS-SF	CCGG*GGATCC*ATGCCTTATCTGAA ACGAGTGTTGCTG
ssBglS-SR	TTGGTTATTTTCTTTAGCTGAGGCAG TAGCAGTGACTGCAAAC
ssBglS-XF	GCTACTGCCTCAGCTAAAGAAAATAA CCAAAAAGCATACAAAG
ssBpr-SF	CCGG*GGATCC*ATGAGGAAAAAAAC GAAAAACAGAC
ssBpr-SR	TTGGTTATTTTCTTTGCCCCGGCTG CTCCCGGAAACAGC
ssBpr-XF	GGAGCAGCCGGGGCAAAAGAAAATAA CCAAAAAGCATACAAAG
ssNprB-SF	CCGG*GGATCC*ATGCGCAACTTGAC CAAGACATCTCTATT
ssNprB-SR	TTGGTTATTTTCTTTAGCTGAGGCAT GTGTTACAAAAACCATT
ssNprB-XF	ACACATGCCTCAGCTAAAGAAAATAA CCAAAAAGCATACAAAG
ssNprE-SF	CCGG*GGATCC*ATGGGTTTAGGTAA GAAATTGTCTGTT
ssNprE-SR	TTGGTTATTTTCTTTAGCCTGAACAC CTGGCAGGCTGATTG
ssNprE-XF	CCAGGTGTTCAGGCTAAAGAAAATAA CCAAAAAGCATACAAAG
ssWapA-SF	CCGG*GGATCC*ATGAAAAAAAGAAA GAGGCGAAAC
ssWapA-SR	TTGGTTATTTTCTTTTGCTAGTACAT CGGCTGGCACTAAT
ssWapA-XF	GCCGATGTACTAGCAAAAGAAAATAA CCAAAAAGCATACAAAG
ssYncM-SF	CCGG*GGATCC*ATGGCGAAACCACT ATCAAAAGGGG
ssYncM-SR	TTGGTTATTTTCTTTAGCGTCTGCCG CGGGTAAACCTGGTAT
ssYncM-XF	CCCGCGGCAGACGCTAAAGAAAATAA CCAAAAAGCATACAAAG
qsacB-F	CGTTTATTCTACACTGACTATTCCG
qsacB-R	TATGGCCTTTGTCTTCAACGTAGTG
RpsU-F	GTCGTTAGAAAAAACGAATCGCTTG
RpsU-R	TTGCGTTTTCTAGCAGCTTCTGACT
pHT-F	GATGACCTCGTTTCCACCGGAATTA
pHT-R	TTCAGTTGCAGACAAAGATCTCCAT

SDS-PAGE analysis

The transformant cell cultures were harvested through centrifugation at 8000 rpm for 20 min at 4 °C. The supernatant was precipitated with 10% trichloroacetic acid (TCA) at 4 °C for 20 min, centrifuged at 12,000 rpm (4 °C) for 10 min, and the pellets were washed twice with 80% acetone. The soluble enzymes were subjected to a 12% (w/v) SDS-PAGE. Protein bands were stained with Coomassie Brilliant Blue R-250 solution.

Real-time quantitative reverse transcription PCR (qRT-PCR) analysis

qRT-PCR was performed to further determine the transcriptional level of sacB with various promoters. Bacterial cultures were harvested after 24 h incubation. Total RNA were extracted using the commercial RNApure Bacteria kit (DNase I) (Cwbio, China), and the samples were treated with RNase-free DNase I (Qiagen), following the manufacturer's protocol. Complementary DNA (cDNA) was obtained by reverse transcription using a HiFi-MMLV cDNA Kit (Cwbio, China). qRT-PCR was performed using the RealMasterMix (SYBR Green) Kit (Cwbio, China). The transcription levels of these genes were normalized against the levels of rspU (Reiter et al. 2011). The primers used in qRT-PCR are listed in Table 2. Samples from five independent experiments were analyzed for each gene. The results were standardized as fold changes compared with that from the wild-type strain.

Levansucrase activity assay

The supernatants were recovered by centrifugation at 8000 rpm for 20 min at 4 °C, and then dialysis through a membrane with a cutoff of 8000 Da were performed for 48 h at 4 °C. Afterwards, the extracellular levansucrase activity of the supernatant was determined. For the intracellular levansucrase activity measurement, the cell pellets were resuspended in a 20 mM potassium phosphate buffer (pH 6.0) containing 1% Triton X-100 and then disrupted by ultrasonication (600 W for 30 min with cycles of sonication of 3 s on and 3 s off). The unbroken cells and cellular debris were precipitated by centrifugation (12,000 rpm for 20 min) at 4 °C, and the intracellular levansucrase extract was recovered for activity determination.

The levansucrase activity was assayed by using sucrose as a substrate. The enzymatic reactions were initiated by adding 0.1 mL enzyme solution with 0.4 mL 20 mM potassium phosphate buffer (pH 6.0) and 0.5 mL of 20% sucrose (w/v) in 20 mM potassium phosphate buffer (pH 6.0) preheated at 37 °C, and immediately the mixture was incubated at 37 °C for 20 min. The reaction was stopped by boiling for 10 min. One unit of total levansucrase activity is defined as the amount of the biocatalyst to liberate 1 μM of glucose from sucrose per minute. The concentrations of glucose in the enzymatic reaction were measured using a biosensor (SBA-40D, Biology Institute of the Shandong Academy of Sciences, China). Secretion efficiency was defined as the ratio of extracellular amylase activity to total levansucrase activity (sum of extracellular and intracellular levansucrase activities).

Levan production in flask fermentation

Cell culturing of various B. amyloliquefaciens strains was first started in the 250 mL flask containing 50 mL LB medium and aerobically cultured at 37 °C for 18 h with shaking at 180 rpm. Then, this seed culture was used to be inoculated into 100 mL fermentation medium in shaking flasks at a 3% (v/v) inoculum ratio. The culture was further cultivated at 37 °C and 180 rpm of agitation for 48 h. After growing to an optical density (OD_{600}) of 0.7–0.8, the culture of ΔLP-pHTPgrac and ΔLP-pHTPxylA was induced with 0.1 mM IPTG and 0.5% (w/v) xylose, respectively. All fermentations were repeated for three times.

Batch and fed-batch fermentations for levan production in a 5-L fermenter

For batch fermentation in bioreactor, the seed culture (at an inoculum ratio of 10%, v/v) was inoculated into a 5-L bioreactor (Bailun Biological Technology Co. Ltd., China), containing 2.7 L of fermentation medium (Feng et al. 2015a). The agitation speed and sterile air rate were maintained at 300 rpm and 6 vvm, respectively. The pH was maintained at 6.0 by adding 3 M NaOH. The fermentation proceeded at 37 °C for 48 h, and samples were withdrawn at a 6-h interval for further analysis.

Fed-batch fermentation was carried out in a similar manner except that sucrose substrate was brought back up to 200 g/L at 24 and 36 h, respectively.

Analytical procedures

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). After fermentation, the culture was centrifuged (8000 rpm for 20 min) and the pellets were washed twice with distilled water and dried to a constant weight at 80 °C to determine the biomass of the culture (dry cell weight, DCW). The levan was recovered and purified following the method as described previously (Feng et al. 2015a). The culture was centrifuged at 4 °C, 8000 rpm for 20 min. Fourfold volume of absolute ethanol was added to the supernatant followed by incubation at 4 °C for overnight. The precipitate was resuspended in deionized water; then the solution was dialyzed through a membrane with a cutoff of

3500 Da for 48 h and afterwards lyophilized to obtain levan. The data reported are the average values ± standard deviation (SD) of three replicate experiments. The concentration of sucrose in the culture was measured by a high-performance liquid chromatography (HPLC) equipped with a differential refraction detector (Schambeck SFD) and a Prevail Carbohydrate ES 5u column (4.6 × 250 mm) set at a temperature of 30 °C. Seventy-five percent acetonitrile was used as the mobile phase at a flow rate of 1.0 mL/min. The concentration of the sucrose was estimated by constructing standard curve for sucrose. All assays were run in triplicate.

Results

Cloning of *sacB* gene from *B. amyloliquefaciens* NK-ΔLP

The *sacB* gene encoding levansucrase was amplified from NK-ΔLP genome and cloned into pUC19-simple for DNA sequencing. Sequence analysis showed that the *sacB* contains one 1419-bp open reading frame (ORF) and encodes 472 amino acids. Based on the prediction using the SignalP software (Brockmeier et al. 2006), it was revealed that the

levansucrase consists of a Sec-type signal peptide of 29 aa and a 443 aa mature protein. BLAST search showed that the deduced amino acid sequence of the levansucrase from NK-ΔLP shares the highest similarity of 97% with the levansucrase of *B. amyloliquefaciens* (accession nos. AGJ52124.1 and KMO06910.1), and shares about 75–90% similarity with the levansucrase from other *Bacillus* strains (accession nos. CAA26513.1, CBI68350.1, AGZ16261.1 and ACI15886.1). However, it showed very low similarity with the levansucrase from *Z. mobilis*, *Pseudomonas* and *Leuconostoc mesenteroides*. Figure 1 presents the alignment of the NK-ΔLP levansucrase amino acid sequence with those of other *Bacillus* strains using the Clustal X software.

Effect of different promoters on *sacB* transcription and levansucrase expression

The recombinant plasmids pHTPA2cup, pHTPC2up, pHTPxylA, pHTPgrac and pHTPsacB (containing the selected four promoters PA2cup, PC2up, PxylA and Pgrac and the native promoter of levansucrase PsacB, respectively) were transformed into NK-ΔLP. The desirable transformants were screened and identified with PCR. After growing to an optical
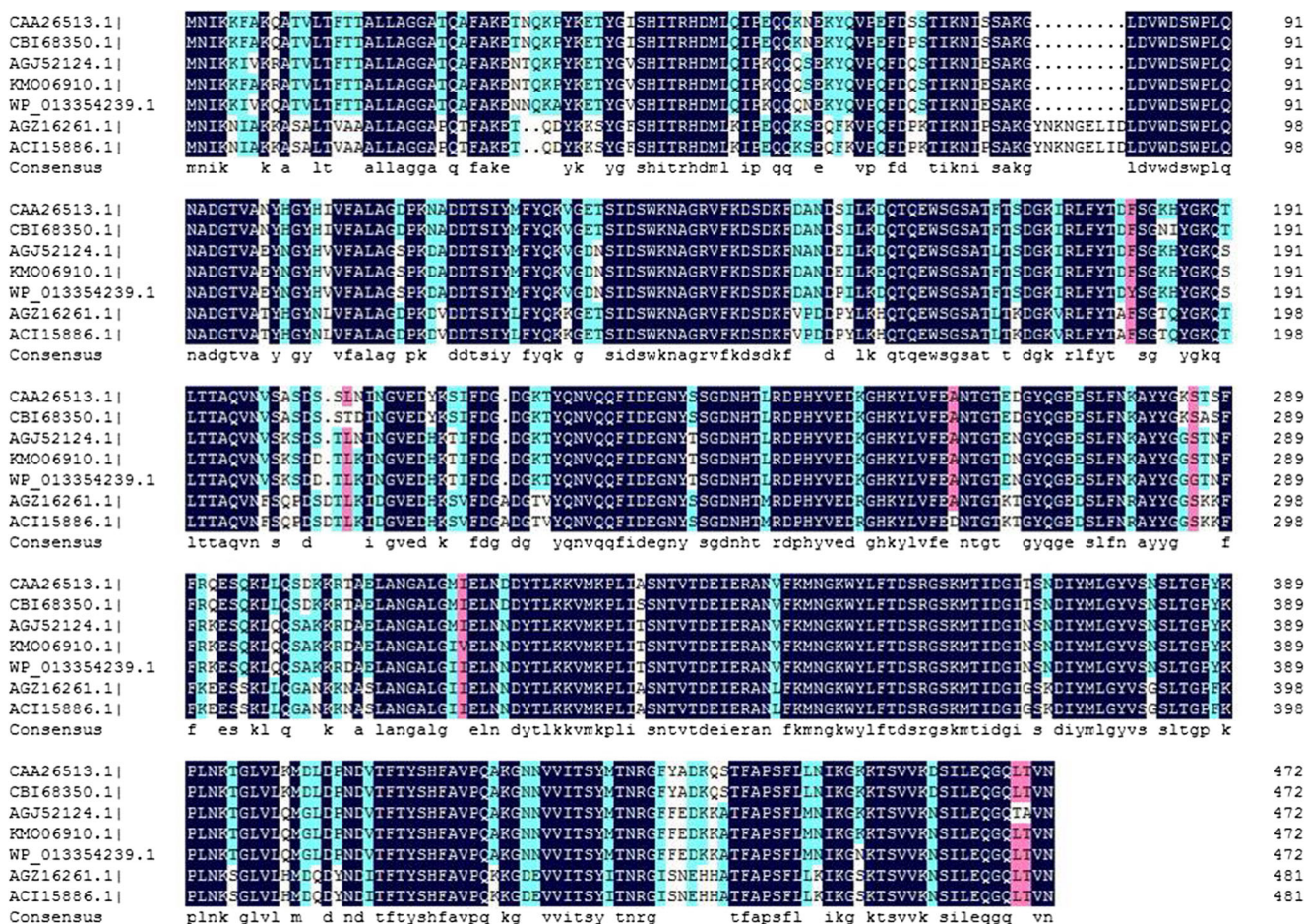

Fig. 1 The alignment of the amino acid sequences of levansucrase from NK-ΔLP with six previously reported levansucrase from other *Bacillus* strains

density (OD_{600}) of 0.7–0.8, the culture of ΔLP-pHTPgrac and ΔLP-pHTPxylA was induced with 0.1 mM IPTG and 0.5% (*w/v*) xylose, respectively. The transcription level of *sacB* in all these strains was determined with qRT-PCR after 24 h of incubation in a levan fermentation medium.

As shown in Fig. 2a, the transcription level of *sacB* in ΔLP-pHTPgrac was significantly higher than that in other strains, which was about 22.1- and 7.21-fold of the control strain NK-ΔLP and the strain ΔLP-pHTPsacB, respectively. The transcription level of *sacB* in ΔLP-pHTPC2up strain was about 6.34-fold of that in the control strain. The *sacB* transcription levels in other three recombinants were comparable. The results demonstrated that the promoter Pgrac is much more efficient than promoters PA2cup, PC2up, PxylA and PsacB in driving transcription of *sacB* in *B. amyloliquefaciens* NK-ΔLP.

To verify the transcription efficiency of *sacB* using the selected promoters, the extracellular levansucrase activity of the recombination strains was measured after 48 h of incubation in the levan fermentation medium (Fig. 2b). Obviously,

the recombinant strain ΔLP-pHTPgrac generated the highest extracellular levansucrase activity, which was about 5–8-fold higher than that of the other strains. In addition, SDS-PAGE analysis was carried out to confirm the levansucrase expression. As shown in Fig. 2c, distinct bands with a molecular mass of about 52 kDa were observed, which is in good agreement with the predicted value. The recombinant strain ΔLP-pHTPgrac generated a much thicker band than others. This was consistent with the result of enzyme activity measurement. Therefore, Pgrac was identified as the optimal promoter to drive *sacB* expression in this study.

Effect of different promoters on levan production

The five recombinant strains as well as the control strain were cultured in flask to investigate the effect of different promoters on levan production and DCW. As shown in Fig. 3, the levan production of ΔLP-pHTPgrac reached 30.5 g/L, and it was 114% higher than that of the control strain ΔLP-pHT01 (14.2 g/L). The recombinant strains ΔLP-pHTPsacB, ΔLP-

Fig. 2 The effects of different promoters on *sacB* transcription and expression. **a** qRT-PCR analysis of *sacB* gene transcription. **b** Extracellular levansucrase activity measurement. **c** SDS-PAGE analysis of levansucrase production in culture supernatants. Samples 1–7 correspond to NK-ΔLP, ΔLP-pHT01, ΔLP-pHTPA2cup, ΔLP-pHTPC2up, ΔLP-pHTPxylA, ΔLP-pHTPsacB and ΔLP-pHTPgrac, respectively. Lane M—molecular weight marker. Data are means ± SD from three replicates
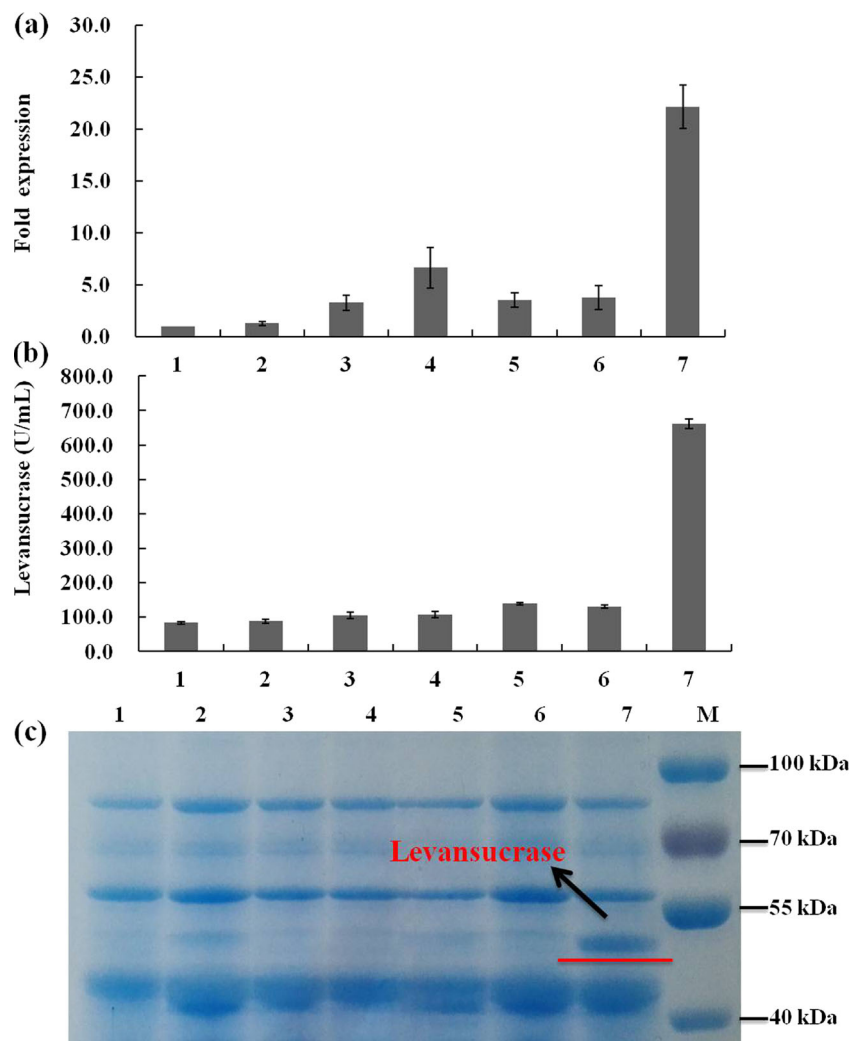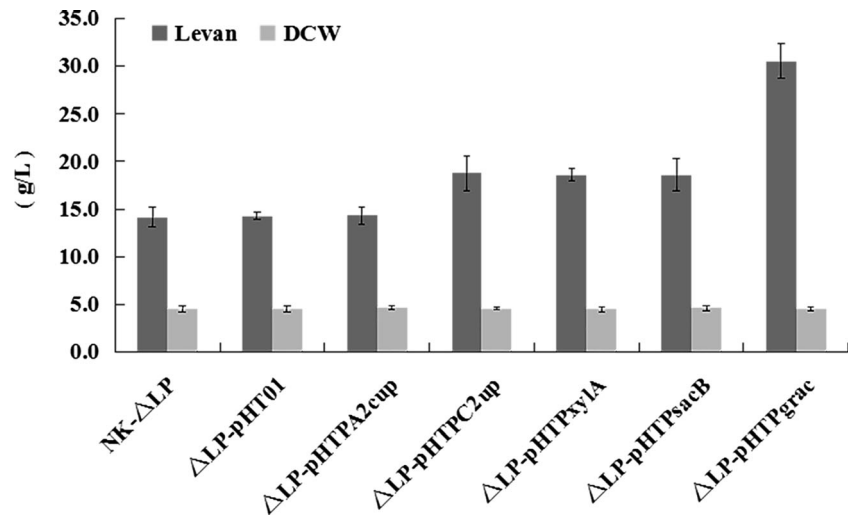
Fig. 3 Levan fermentation results with recombinant strains containing various promoters. Values represent the means ± SD

pHTPxylA and ΔLP-pHTPC2up produced 18.6, 18.6 and 18.8 g/L levan, respectively, which was about 30.3% higher than that of the control strain. The levan production of the ΔLP-pHTPA2cup was comparable with the control strain. On the other hand, interestingly, the DCW of the five recombinant strains were approximately the same as that of the control strain ΔLP-pHT01 and that of the wild-type NK-ΔLP. It was thus concluded here that the different enzyme activities and levan production were resulted from promoter strength not from cell growth.

Effect of different signal peptides on the secretion of levansucrase

An appropriate signal peptide is a critical factor for the extracellular protein expression. So far, although numerous signal peptides have been investigated and characterized, there has been no specific rational approach or algorithm to identify the optimal signal peptide for a target protein. To find the best signal peptide for levansucrase, 14 candidate signal peptides from *B. subtilis* 168 were compared based on their compositions, especially the positively charged N-domain, the hydrophobic H-domain and the C-domain (for recognition and cleavage) (Table 3). Based on this preliminary analysis, seven different signal peptides amyE, bglS, bpr, nprB, nprE, wapA and yncM were chosen for further evaluation to improve the levansucrase secretion. We used Pgrac to drive the expression of levansucrase fused to different signal peptides. The seven plasmids pHTPA (SPamyE), pHTPB (SPbglS), pHTPR (SPbpr), pHTPN (SPnprB), pHTPE (SPnprE), pHTPW (SPwapA) and pHTPY (SPyncM) were constructed and transformed into NK-ΔLP, generating recombinant strains ΔLP-

Table 3 Comparison of different signal peptides used for levansucrase production

Protein	Signal peptide	Charge N region	Hydrophobicity (%)
AmyE	**MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAASA**	**3**	**78.79**
Bpr	MRKKTKNRLISSVLSTVVISSLLFPGAAGA	5	56.67
BglS	**MPYLKRVLLLLVTGLFMSLFAVTATASA**	**2**	**71.43**
Csn	MKISMQKADFWKKAAISLLVFTMFFTLMMSETVFA	3	62.86
Epr	**MKNMSCKLVVSVTLFFSFLTIGPLAHA**	**2**	**62.96**
LipB	MKKVLMAFIICLSLILSVLAAPPSGAKA	2	71.43
Mpr	MKLVPRFRKQWFAYLTVLCLALAAAVSFGVPAKA	3	67.65
NprE	**MGLGKKLSVAVAASFMSLSISLPGVQA**	**2**	**70.37**
NprB	**MRNLTKTSLLLAGLCTAAQMVFVTHASA**	**2**	**57.14**
WapA	**MKKRKRRNFKRFIAAFLVLALMISLVPADVLA**	**8**	**65.63**
SacB	**MNIKKFAKQATVLTFTTALLAGGATQAFA**	**3**	**62.07**
YncM	**MAKPLSKGGILVKKVLIAGAVGTAVLFGTLSSGIPGLPAADA**	**4**	**76.19**
YobV	MKLERLLAMVVLLISKKQVQA	2	61.90
YolA	MKKRITYSLLALLAVVAFAFTDSSKAKA	3	60.71

The signal peptides highlighted in bold were chosen for further evaluation. The cleavage sites of signal peptides were underlined

A, ΔLP-B, ΔLP-R, ΔLP-N, ΔLP-E, ΔLP-W and ΔLP-Y, respectively.

The extracellular levansucrase activity was analyzed for each strain after 48 h incubation in the levan fermentation medium. As shown in Fig. 4a, the strain ΔLP-Y produced the highest extracellular levansucrase titer, which was about 19.1-fold of the control strain NK-ΔLP. Moreover, the extracellular levansucrase activity of the recombinant strain ΔLP-B was secondly higher among all, which was 11.2-fold higher than the control strain. In addition, SDS-PAGE assay was carried out to further confirm the levansucrase secretion. As shown in Fig. 4b, the strains ΔLP-Y and ΔLP-B showed apparently deeper bands than others, which was consistent with the results of enzyme activity assay. Then, it was concluded that the signal peptide SPyncM was an excellent candidate for levansucrase secretory expression.

Effect of different signal peptides on levan production

As shown above, different signal peptides led to various levels of levansucrase secretion. We thus further investigated their effects on levan production. The recombinant strains as well as the control strain were cultured in flask. As shown in Fig. 5, all the recombinant strains exhibited higher levan production than the control strain ΔLP-pHT01, and ΔLP-Y generated the highest levan titer of 37.4 g/L, which was about 161% improvement compared with ΔLP-pHT01. The levan production of ΔLP-B strain reached 32.7 g/L, which was about

130% higher than that of ΔLP-pHT01. This result was well consistent with the result of enzymatic assay.

Scale-up of levan production in a 5-L fermenter

To obtain more practical parameters for industrial levan production, the scale-up fermentation was conducted for the recombinant ΔLP-Y strain in a 5-L fermenter. As shown in Fig. S4, it took about 18 h for the strain to reach the logarithmic growth phase, and the highest levan production of 51.2 g/L was achieved at 30 h, which was 3.58-fold higher than that was obtained from NK-ΔLP strain in flask. Based on previous reports, levansucrase has dual roles of hydrolysis and transfructosylation activities. The ratio of hydrolysis and transfructosylation activities is highly dependent on the sucrose concentration. High concentration of sucrose can promote transfructosylation activity, but when sucrose is close to depletion, hydrolysis tends to become predominant (Öner et al. 2016). Therefore, the depletion of sucrose at 30 h in our fermentation may hinder the further levan synthesis. We thus further carried out the fermentation in a fed-batch mode by supplementing sucrose to the concentration of 200 g/L at 24 and 36 h, respectively. As shown in Fig. 6, the highest levan production of 102 g/L was obtained at 48 h, which was 7.12-fold higher than that obtained from NK-ΔLP strain in flask. So far, the highest levan production reported is 112 g/L by a *B. subtilis* (natto) CCT7712 strain. Our result is very close to this value, indicating that the strain that we generated

Fig. 4 Comparison of levansucrase production in recombinant strains containing different signal peptides. **a** Extracellular enzyme activity. Values represent the means ± SD. **b** SDS-PAGE analysis of levansucrase secretion. Samples 1–9 corresponds to ΔLP-pHT01, ΔLP-A, ΔLP-B, ΔLP-R, ΔLP-N, ΔLP-E, ΔLP-W, ΔLP-Y and ΔLP-pHTPgrac, respectively. Lane M—molecular weight marker
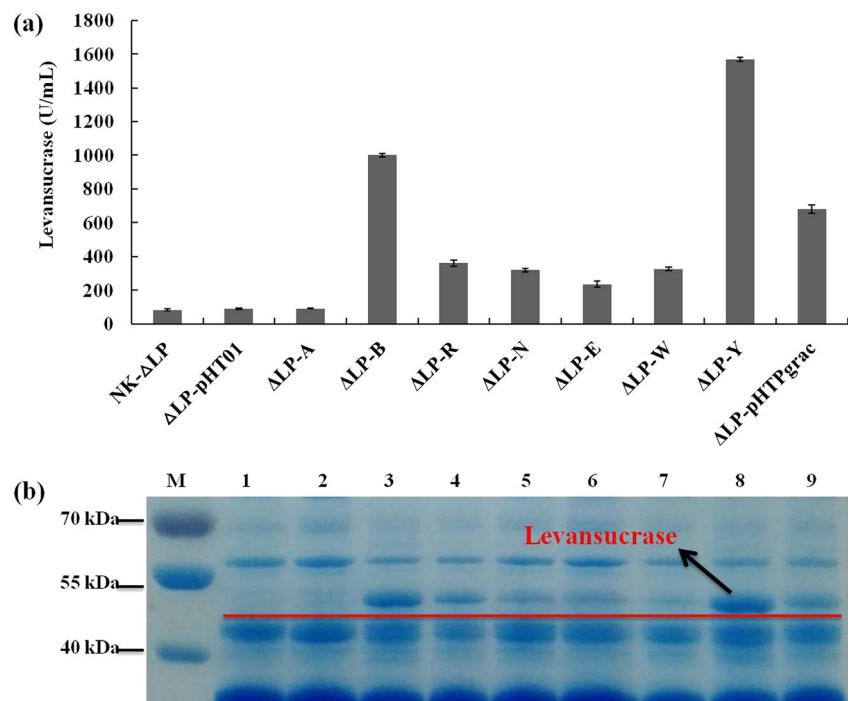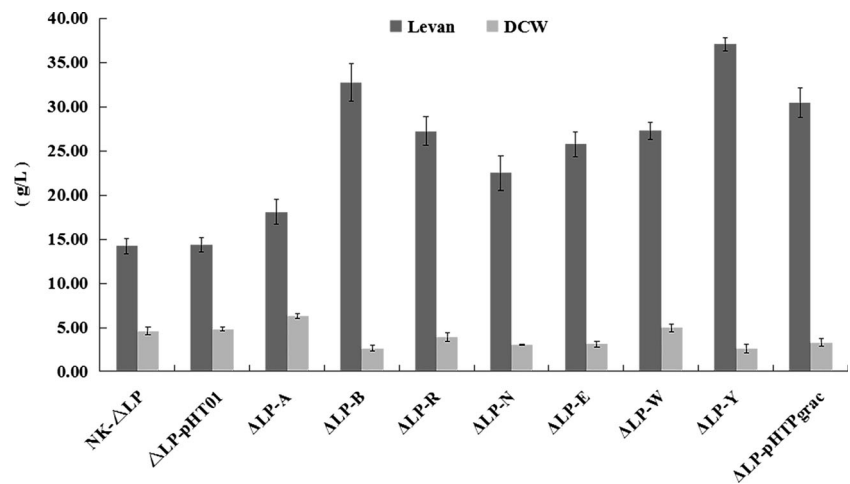
Fig. 5 Levan fermentation results with recombinant strains containing different signal peptides. Values represent the means ± SD

in this study has a great potential for industrial scale levan production (Öner et al. 2016).

Discussion

In our previous study, we generated the NK-ΔLP strain, which can produce levan at the molecular weight around 5 kDa that is significantly lower than other reported levan products (Feng et al. 2015a). The levan of low molecular weight from NK-ΔLP can potentially be used as a prebiotic compound (Feng et al. 2015a). However, the levan production was only about 13.9 g/L (flask fermentation), which is far from the desirable level for an economic industrial scale production. Therefore, in this study, we attempted to enhance the levan production in this strain through rational metabolic engineering approaches.

Since levan is the catalytic product of the secreted levansucrase, we hypothesized that we can improve levan production by increasing the expression level of levansucrase in the host strain. Therefore, the transcriptional and translational regulations of levansucrase were primarily targeted. Up

to now, the most economic and effective approach for regulation modification still remains the optimization of promoters at transcriptional level (Song et al. 2015). The activity of the promoter directly influences the production efficiency of the target protein. In our previous work, when a strong promoter P43 from *B. subtilis* 168 replaced the *sacB* native promoter, the transcription level of *sacB* was not increased but was reduced by ten times, and the levan production has remarkably declined (Fig. S5). Through the promoter engineering, we identified a few promoters better than P43, including PA2cup, PC2up and PxylA (unpublished data), and thus we further evaluated them for levansucrase expression in this study. The promoters PA2cup and PC2up are both strong and constitutively expressed promoters from *B. subtilis* phage Φ29 that is recognized by sigma A factor (Meijer and Salas 2004). Pgrac promoter, a widely used IPTG-inducible promoter, is a combination of the *B. subtilis* groESL promoter, the *E. coli* lac operator and the *gsiB* Shine-Dalgarno sequence (Phan et al. 2012). The promoter PxylA, originating from *Bacillus megaterium*, is a strong xylose-inducible promoter (Radha and Gunasekaran 2008). As a control, the native promoter PsacB of levansucrase from *B. amyloliquefaciens*

Fig. 6 Fermentation profiles from a fed-batch cultivation of the recombinant strain ΔLP-Y in a 5-L fermenter. Cell growth (*filled diamond*); levan production (*filled triangles*); dissolved oxygen (*DO, filled cycle*); residual sucrose (*filled square*)
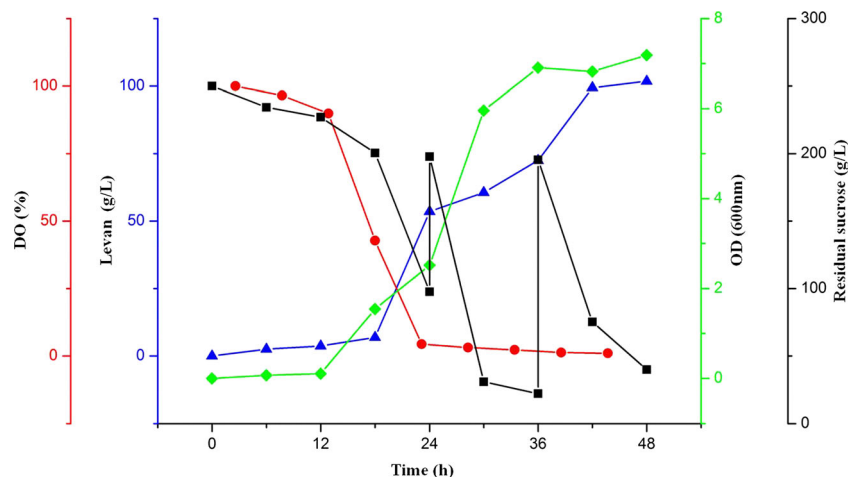
NK-ΔLP was also tested. The result suggested that Pgrac promoter is the most effective promoter in driving the transcription of *sacB* in *B. amyloliquefaciens* NK-ΔLP. To our surprise, the strong xylose-inducible promoter PxylA produced only modest amount of levansucrase. Although the transcriptional level of the promoter PC2up was doubled than that of PsacB promoter, the levansucrase activity and levan production of strain ΔLP-pHTPC2up were very similar to that of the strain ΔLP-pHTPsacB. The results indicate that not all strong promoters are suitable for levansucrase expression in NK-ΔLP. The expression of the target protein may be influenced by other factors, such as characteristics of the protein, promoter type, promoter-gene interactions and type of inducers (Ying et al. 2012).

For many years, *B. subtilis* and related *Bacilli* are known to naturally produce various proteins and secreted into the medium with high concentrations. In addition, *Bacillus* strains are generally classified as generally recognized as safe (GRAS) organisms (Pohl and Harwood 2010). Thus, people paid tremendous attention to them aiming to exploit them as major "cell factories" for secreted heterologous protein of interest (van Dijl and Hecker 2013). Although many studies have focused on the effect of different signal peptides on heterologous proteins expression (Wei et al. 2015; Ying et al. 2012; Zhang et al. 2013), few efforts have been made to study their effects on endogenous proteins production. On the other hand, it was reported that the signal peptide structure itself affects the secretion efficiency. Zanen et al. (2005) reported that the hydrophobicity of the signal peptide was critical for the early stage recognition of the signal peptide by signal recognition particle (SRP) in *B. subtilis*. By modifying the signal peptide with higher positive charge in the N-terminal region and higher hydrophobicity in the core H region, the yield of tuna growth hormone (tGH) was increased by 10-fold (Sagiya et al. 1994). This means that the appropriate signal peptide structure can improve the efficiency of protein secretion. To screen a compatible signal peptide for the levansucrase secretion, seven different signal peptides were selected and evaluated from *B. subtilis* 168. As described previously, the signal peptide of SPyncM can greatly improve the levansucrase secretion and levan production, which has never been reported in levan producing strain before.

Moreover, the intracellular levansucrase activity of the mentioned recombinant strains was characterized in the levan fermentation medium (Fig. S6a), and it was showed that the strain ΔLP-Y possessed the highest secretion efficiency of 89.9% (Fig. S6b). In addition, the different signal peptides have resulted in different levansucrase production rates (both extracellularly and intracellularly), indicating that the secretory expression of the protein has a close relationship with transportation rate directed by signal peptide. In fact, many previous studies have proposed that the balance between the amount of translocated proteins emerging from the trans-side

of the membrane and their extracytosolic folding capacity played an important role on the secretory expression of the target protein (Beena et al. 2004; Brockmeier et al. 2006; Zhang et al. 2013). This could also be the reason for us to achieve the highest levansucrase secretion with appropriate combination of promoter and signal peptide.

In summary, in order to improve the levan production in strain NK-ΔLP, the regulatory elements for levansucrase expression were investigated and optimized. Five promoters were tested, and the strong promoter Pgrac was eventually identified as the optimal. Then eight signal peptides were screened and evaluated for their effects on the levansucrase secretory expression and the levan production. The signal peptide yncM showed the highest secretion efficiency and resulted in the high production of levansucrase and levan in flask fermentation. Moreover, fed-batch fermentation was carried out with the optimized recombinant strain ΔLP-Y, and a very high production of levan 102 g/L was achieved after 48 h of fermentation; this level is comparable to the previous reported highest levan production. This is the first report about improving levan production by optimizing of the regulatory elements for levansucrase. The levan production has been significantly increased. We are trying additional efforts to further improve the levan production in order to meet the industrial scale levan production purpose.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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