RESEARCH LETTER – Biotechnology & Synthetic Biology

Construction of a *Bacillus amyloliquefaciens* strain for high purity levan production

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#These authors contributed equally to this work.

One sentence summary: Deleted $\textit{pgs}$ cluster (for $\gamma$-PGA synthesis) and the $\textit{sac}$ cluster (for levan synthesis) from the NK-1 strain, respectively and constructed high purity levan and $\gamma$-PGA producing strains.

Editor: Alexander Steinbüchel

ABSTRACT

*Bacillus amyloliquefaciens* NK-1 has the potential to produce levan and poly-$\gamma$-glutamic acid ($\gamma$-PGA) simultaneously. However, it is not possible to purify each single product from the same strain because the extraction process is identical. We deleted the $\textit{pgs}$ cluster (for $\gamma$-PGA synthesis) from the NK-1 strain and constructed a $\gamma$-PGA-deficient NK-ΔLP strain. Nuclear magnetic results showed that the NK-ΔLP strain could produce high purity levan product. However, its levan titer was only 1.96 g L$^{-1}$ in the basal medium. Single-factor experimental and response surface methodology was used to optimize the culture condition, leading to levan titer of 13.9 and 22.6 g L$^{-1}$ in flask culture and in a 5-L bioreactor, respectively. The levan purity can reach to 92.7% after 48 h cultivation. Furthermore, the relationship between levanase (LevB) and levan molecular weight was studied. The results showed that LevB resulted in the production of low molecular weight levan and its expression level determined the ratio of high and low molecular weight levan. We also deleted the $\textit{sac}$ cluster (for levan synthesis) from the NK-1 strain and constructed a levan-deficient NK-L strain. The NK-L strain exhibited increased purity of $\gamma$-PGA product from 79.5 to 91.2%.

Keywords: levan; poly-$\gamma$-glutamic acid; response surface methodology; levanase

INTRODUCTION

Microbial levan, which is a fructan biopolymer polymerized by $\beta$-(2→6) glycosidic bonds with occasional $\beta$-(2→1) branching, is found in many plants and microbial products (Han 1990). It is of commercial importance and has a variety of industrial applications in the fields of foods, cosmetics and pharmaceuticals. It is currently used as prebiotic, industrial gums, a blood plasma extender, sweeteners and an antitumor agent (Leibovici and Stark 1985; Poli et al. 2009; Shih, Chen and Wu 2010; Huang et al. 2013). Microbial levans are produced by a variety of microorganisms. It can be synthesized by transfructosylation by a secreted levansucase (EC: 2.4.1.10) from the sucrose substrate (Dedonder 1966;
Kennedy, Stevenson and White 1989). Many Bacillus species have the ability to produce levan.

Bacillus amyloliquefaciens NK-1, a derivative of B. amyloliquefaciens LL3, is a glutamate-independent \( \gamma \)-PGA-producing strain (Cao et al. 2011). In our previous work, we observed that the \( \gamma \)-PGA products from B. amyloliquefaciens NK-1 were of low purity and some impurity peaks were detected from the gel permeation chromatography (GPC) results. The molecular size of the impurities in the \( \gamma \)-PGA product was around 5000–7000 Da, which was similar to previously reported levan molecular size (Shih, Chen and Wu 2010). The genome information indicated that NK-1 strain has the sacB gene encoding the levansucrase (Geng et al. 2011); thus, it has the potential to produce levan and \( \gamma \)-PGA simultaneously. Considering that the purification procedure of levan and \( \gamma \)-PGA was similar, we hypothesized that the impurity in \( \gamma \)-PGA product was levan. To verify our hypothesis and obtain high purity of levan product, we deleted the pgs cluster to block the synthesis of \( \gamma \)-PGA in the NK-1 strain. And we successfully constructed a \( \gamma \)-PGA-deficient NK-\( \Delta \)LP strain which could produce increased levels of highly pure levan.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

All the strains and plasmids used in this work are listed in Table 1. Escherichia coli DH5\( \alpha \) was used for plasmid propagation and transformation. Escherichia coli GM2163 was used as the host for plasmid demethylation.

For \( \gamma \)-PGA production, B. amyloliquefaciens were cultured at 37°C, 180 rpm for 48 h in \( \gamma \)-PGA fermentation medium: 50 g L\(^{-1}\) sucrose, 6 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 0.6 g L\(^{-1}\) MgSO\(_4\), 6 g L\(^{-1}\) KH\(_2\)PO\(_4\), and 14 g L\(^{-1}\) K\(_2\)HPO\(_4\), 1 mM FeSO\(_4\), 1 mM CaCl\(_2\), 1 mM MnSO\(_4\) and 1 mM ZnCl\(_2\) (Feng et al. 2013). For levan production, B. amyloliquefaciens were cultured at 37°C, 180 rpm for 48 h in optimal levan fermentation medium (\( \rho H \) 6.0), which contains 250.9 g L\(^{-1}\) sucrose, 2.6 g L\(^{-1}\) urea, 0.62 g L\(^{-1}\) MgSO\(_4\), 8.16 g L\(^{-1}\) KH\(_2\)PO\(_4\), 18.24 g L\(^{-1}\) K\(_2\)HPO\(_4\), 3H\(_2\)O, 1 mM FeSO\(_4\), 1 mM CaCl\(_2\), 1 mM MnSO\(_4\) and 1 mM ZnCl\(_2\). Antibiotics were used at the following concentrations as necessary: 100 \( \mu \)g ml\(^{-1}\) ampicillin, 5 \( \mu \)g ml\(^{-1}\) chloramphenicol. 5-fluorouracil (5-FU) was added to the medium at a final concentration of 100 \( \mu \)g ml\(^{-1}\).

Construction of the gene knockout mutant strains

In this study, we aimed to delete the pgs cluster (including pgsB, pgsC and pgsA genes) and the sac cluster (including sacB and levB genes) from B. amyloliquefaciens NK-1.

To construct the deletion vectors, the temperature-sensitive p-\( uu \)pp plasmid was used (Feng et al. 2014). The oligonucleotide primers used in this study were listed in Table S1 (Supporting Information). The strategy for the construction of the deletion vectors pKS\(_7\)-\( \Delta \)pgs and pKS\(_7\)-\( \Delta \)sac was similar to our previously reported method (Feng et al. 2014), except for the use of primers pgs-SF/pgs-SR, pgs-XF/pgs-XR and sac-SF/sac-SR, sac-XF/sac-XR, respectively.

Gene knockout mutant strains were constructed by an upp-based marker-less gene deletion method reported previously (Keller, Bender and Wall 2009; Zhang et al. 2014). Primers pgs-SS/pgs-XX and sac-SS/sac-XX were used to determine the gene deletion mutants by PCR.

The pgs cluster deletion strain was designated B. amyloliquefaciens NK-\( \Delta \)LP and the sac cluster deletion strain was designated B. amyloliquefaciens NK-\( \Delta \)L.

Isolation and identification of levan

The method for the purification of levan from B. amyloliquefaciens NK-\( \Delta \)LP was similar to the \( \gamma \)-PGA purification process (Goto and Kunioka 1992), except for the use of a 3500 Mw dialysis bag after precipitation by the cold ethanol. To identify the product levan, samples dissolved in heavy water (D\(_2\)O) were analyzed using a nuclear magnetic resonance (NMR) spectrometer (Varian Infinity plus 400, USA), and the \( ^{1}H \) NMR and \( ^{13}C \) spectra fingerprints of the sample were compared with that of a levan standard (Sigma).

Single-factor tests and multiple responses optimization

The levan titer was only 1.96 g L\(^{-1}\) in the \( \gamma \)-PGA fermentation medium. To obtain a higher levan production, single-factor tests and response surface methodology (RSM) were used to optimize the medium. In the present study, the Box-Behnken experimental design model was used (Montgomery 2001). Actual values of the factors were selected at three levels, coded as −1, 0 and +1 for low, middle and high values, respectively (Table S2, Supporting Information). The software Design-Expert 8.0.6.1 (Stat-Ease, USA) was used for the experimental design. Table 2 showed the

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**Table 1. Strains and plasmids used in this study.**

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant genotype and characteristics</th>
<th>Source</th>
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<tbody>
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<td><strong>Strains</strong></td>
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<td>E. coli DH5( \alpha )</td>
<td>F(<em>{-})( \gamma )-80dlacZAM1, ( \Delta )SacZYA-argF(</em>{169}), deoR, recA1, endA1, hsdR17(( \gamma )( ^{-}), m(_{2})), phoA, supE44, ( \lambda )-( thi-1), gyrA96, relA1</td>
<td>This lab</td>
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<td>E. coli GM2163</td>
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<td>136 (Str(<em>{R}^{R})) xyl-S mtl-1 dam13::Tn9 (Cam(</em>{R}^{R})) dcm-6 mcrB1 hsdR2 mcrA</td>
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<tr>
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<td>Smith and Youngman (1992)</td>
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<td>pKS(_7)-( \Delta )sac</td>
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Table 2. Experiment design and results of the Box-Behnken central composite design.

<table>
<thead>
<tr>
<th>Run order</th>
<th>A (g L⁻¹)</th>
<th>B (g L⁻¹)</th>
<th>C (g L⁻¹)</th>
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<th>Predicted (Titer g L⁻¹)</th>
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<tr>
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<td>2</td>
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<tr>
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<td>3</td>
<td>0.8</td>
<td>8.69 ± 0.65</td>
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<tr>
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<td>250</td>
<td>3</td>
<td>0.6</td>
<td>12.73</td>
<td>13.32</td>
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</table>

* A: sucrose; B: urea; C: MgSO₄.
** The results are presented as the means of duplicates.

experimental design and actual experiments that were carried out to develop the model. All 17 runs of the experiment were performed in triplicate.

Levan production via batch culture in a 5-L fermentation tank

For upscaled cultivation, 100-mL seed cultures of B. amyloliquefaciens NK-ΔLP were cultured in the optimized levan fermentation medium in 500-mL shake flasks for 24 h. The 300-mL seed cultures were then transferred to a 5-L fermentation tank cultivation system (Bailun Biological Technology Co. Ltd., China), which contained 3 L of fermentation medium. The pH, agitation speed and sterile air rate were maintained at 6.0, 250 rpm and 8 LPM, respectively. The fermentation proceeded at 37 °C for 48 h and samples were withdrawn at a 6-h interval for further analysis.

γ-PGA production

Single colonies of the B. amyloliquefaciens NK-1 and B. amyloliquefaciens NK-L strains were transferred into 50-mL γ-PGA fermentation medium. After 18 h of incubation at 37 °C and shaking at 180 rpm, 1 mL of each culture was transferred into 500-mL shaking flasks with 100-mL γ-PGA fermentation medium at 37 °C and shaken at 180 rpm for 48 h. γ-PGA was purified by a previously described method (Goto and Kunioka 1992).

Analytical procedures

Cell growth was measured by optical density (OD) using a SHIMADZU UV-1800 spectrophotometer (Kyoto, Japan). The molecular weights of the levan and γ-PGA products were determined by GPC, according to a previously described method and the Shodex Pullulan-82 standards were used to construct the calibration curve (Feng et al. 2013). Relative viscosity was measured by a Brookfield Digital Rheometer (model DV-I, USA) fitted with a spindle S00 code, at a shear rate of 2.5 rpm at 25°C. The purity of γ-PGA (%) was defined as: 1-polysaccharide content (}). The whole polysaccharide content (%) in the γ-PGA product was measured by the phenol-sulfuric acid method (Dubois et al. 1951). To measure the levan product purity, levan product was hydrolyzed by 6 N HCl at 100°C for 15 min. The optical density at 291 nm was measured to determine the concentration of fructose in the hydrolysate (Zhang 2003). The number of the monosaccharide in levan product was defined as: \[ N = \frac{\text{AW(levan)}}{100} \]

The purity of levan (%) = fructose concentration (%)* (\[ N = \frac{\text{AW(levan)}}{100} \]). Real-time quantitative PCR (qRT-PCR) was performed to test leuB gene expression during levan fermentation and it was carried out by a previously described method (Feng et al. 2014). Transcription levels of leuB were normalized against the levels of rpsU (Reiter, Kolstø and Piehler 2011).

RESULTS AND DISCUSSION

Construction of the gene deletion mutants

To our knowledge, levan is another product coproduced with γ-PGA in some Bacillus strains (Shih et al. 2005). Moreover, the purification procedures of these two products are similar and they are both precipitated by cold ethanol (Han 1990; Goto and Kunioka 1992). The whole genome of the NK-1 strain has been sequenced and shown to contain the levansucrase gene sacB (Geng et al. 2011). Combined with the GPC result of γ-PGA product (Fig. 1a), we predicted that strain NK-1 coproduced γ-PGA and levan. The small molecular impurity in Fig. 1a was probably levan. To verify our hypothesis, we deleted the pgs cluster in the NK-1 strain. And we also deleted the sac cluster in the NK-1 strain to determine its effects on γ-PGA production. A method, based on the use of the upp gene and the 5-FU selection, was used for gene markerless deletion (Keller, Bender and Wall 2009; Zhang et al. 2014). Primers pgs-SS/pgs-XX and sac-SS/sac-XX were used to confirm the gene deletion mutant strains (Fig. S1, Supporting Information). The pgs cluster deletion strain was designated B. amyloliquefaciens NK-ΔLP and the sac cluster deletion strain was designated B. amyloliquefaciens NK-L.

Characterization of levan produced by B. amyloliquefaciens NK-ΔLP

The product obtained from NK-ΔLP strain (Fig. 1b) was analyzed by a NMR spectrometer. The 1H NMR spectrum and 13C NMR spectrum were compared with that of the levan standard. The 1H NMR spectrum showed six protons between 3.4 and 4.2 ppm, which was almost identical with the levan standard (Fig. 2). The 13C NMR spectrum shows six main resonances at 60.5, 64.1, 75.9, 76.9, 81.0 and 104.9 ppm, which was also comparable with the levan standard and other previously reported levan products (Fig. 2; Table S3, Supporting Information). These results confirmed that the product obtained from the NK-ΔLP strain was levan.

Preoptimization of levan production of B. amyloliquefaciens NK-ΔLP

The levan production in γ-PGA fermentation medium was only 1.97 g L⁻¹. To improve its production, we optimized the levan fermentation medium. The effects of pH, carbon source and nitrogen source on levan production were monitored in shake flask culture. The experiment results showed that the optimum pH for levan production in NK-ΔLP strain was 6.0. As levansucrase uses sucrose as its sole substrate, thus we chose sucrose as the carbon source. In basal medium containing sucrose, different nitrogen
sources were added and the results showed that urea was the best nitrogen source (data not shown). We also tested the effect of mixtures of inorganic and organic nitrogen sources on levan production and found that the medium with only inorganic nitrogen urea exhibited the highest levan production.

We further determined the optimal concentration of the main factors. The optimal concentrations of sucrose, urea, MgSO\(_4\), KH\(_2\)PO\(_4\) and K\(_2\)HPO\(_4\) \(\cdot\) 3H\(_2\)O were 250, 3, 0.6, 8.16 and 18.24 g L\(^{-1}\), respectively. The highest levan titer obtained under this condition was 11.8 g L\(^{-1}\).

**Multiple responses optimization**

RSM is a less laborious and time consuming, but effective statistical technique for analyzing the effects of several independent variables (Myers and Montgomery 1995). This experimental methodology uses quantitative data to evaluate multiple parameters and their interactions by establishing a mathematical model (Brachet et al. 1999). The RSM design was used to optimize the precise factors to increase levan production. The experimental design and results were shown in Table 2. Fig. S2 (Supporting Information) showed the response surfaces of two variables at the center level of other variables, respectively. The non-linear nature of all response surfaces demonstrated that there were considerable interactions between each of the independent variables, and effects of independent variables on the levan product. From these results, we obtained the optimal concentration of the three factors (g L\(^{-1}\)): sucrose 250.9, urea 2.6, MgSO\(_4\) 0.62. Culturing NK-\(\Delta\)LP under this condition in flasks produced levan at was 13.9 g L\(^{-1}\).

**Scaled-up system of levan production in a 5-L fermentation tank**

Levan fermentation in flasks has many limiting factors, such as unstable pH or oxygen limitation. To obtain the best results for levan production, a scaled-up system was carried out in a 5-L fermentation tank. The fermentation tank was set at a constant pH, agitation speed and sterile air rate. As shown in Fig. 3a, cells grew fast during the first 18 h before reaching stationary phase and the DO reached nearly 0% after 12 h cultivation. The levan production increased with time. The maximum levan titer was 22.6 g L\(^{-1}\), which was obtained after cultivation for 48 h. The levan product was of high purity and it reached 92.7% at 48 h (Fig. 4).

The weight-average molecular weights of levan obtained every six hours were determined by GPC (Table S4, Supporting Information; Fig. 4). Consistently, two peaks were observed in the GPC results. One had a molecular weight around 600 K and the other had a molecular weight around 6 K (Table S4, Supporting Information). The peak area of the larger molecule was bigger than the smaller molecule peak at 6 h; the peak areas of the smaller molecule were significantly bigger than that of the larger molecule after 18 h. This phenomenon was also observed by
Figure 2. NMR test results. $^1$H NMR and $^{13}$C NMR spectrums of levan produced by B. amyloliquefaciens NK-ΔLP (a and b); $^1$H NMR and $^{13}$C NMR spectrums of levan standard purchased from Sigma (c and d).

Figure 3. (a) Time curves of process parameters in a 5-L fermentation tank of NK-ΔLP. Cell concentration (OD$_{600}$, filled triangles); levan titer (filled squares); Residual sucrose concentration (inverted filled triangles); dissolved oxygen (DO, filled diamonds). (b) RT-qPCR analysis of levB gene expression. Cells were withdrawn at a 6-h interval and then RNA was extracted and used to analyze the genes expression. Values represent means ± SD.

Shih’s group (2010) and the B. subtilis natto strain can produce two molecular size levan products 2000 and 7 kDa at the same time. The purity of levan products obtained from every interval was also measured and shown in Fig. 4. Combining with the levan purity results and the previously reported work (Shih, Chen and Wu 2010), we confirm that the two peaks in GPC results are levan products.

The expression levels of levanase gene levB were also monitored during fermentation (Fig. 3b). It showed that levB expression levels were relatively low at the first 18 h, thereafter it highly increased and the highest expression level was obtained at 48 h. The levB gene expression level at 48 h was almost 37.2-fold higher than that at 6 h. These results were consistent with the GPC results (Fig. 4) and our hypothesis. The NK-ΔLP strain can synthesize high molecular weight levan at the beginning. During the fermentation process, it synthesizes levanase (LevB) to hydrolyze the high molecular weight levan to lower molecular weight levan. During the first 6 h, LevB expresses at very low level, and levan in medium is mostly the higher molecular weight form (Fig. 4). Subsequently, the LevB expression level increases and higher molecular weight levan is hydrolyzed with a corresponding increase of the hydrolyzed lower molecular weight levan. After 48 h of cultivation, the lower molecular weight levan become the major product in the medium. The levan molecular weight produced by NK-ΔLP strain was mostly 5–7 K. Huang et al. (2013) had demonstrated that the low molecular weight levan had the prebiotic effect; thus, levan produced from NK-ΔLP strain can be used as a prebiotic product in the future. Although it has many favorable features such as high
purity and low molecular weight, the levan titer was still too low when compared with the highest titer 145.94 g L\(^{-1}\) produced by \textit{B. licheniformis} FRI MY-55 strain (Huang et al. 2013) and more metabolic engineering works must be done to improve levan production in the NK-\(\Delta\)LP strain in future.

**Comparison of \(\gamma\)-PGA fermentation between \textit{B. amyloliquefaciens} NK-1 and \textit{B. amyloliquefaciens} NK-L**

We also determined the \textit{Sac} cluster deletion on \(\gamma\)-PGA production. As shown in Fig. 1d, the \(\gamma\)-PGA titers from NK-L and NK-1 strains were 3.53 and 3.61 g L\(^{-1}\), respectively. The two strains showed comparable \(\gamma\)-PGA production. GPC results showed that their molecular weights were almost the same (Fig. 1a and c). \(\gamma\)-PGA molecular weight from NK-1 was 418.3 K and was 425.5 K from NK-L strain. The molecular weights of commercial \(\gamma\)-PGA are mostly ranging from 200 to 10 000 K (Ashiuchi 2013). Different molecular size of \(\gamma\)-PGA has different applications. The \(\gamma\)-PGA products obtained from NK-1 and NK-L strains can be used in the field of cosmetics and pharmaceutics. The impurity peaks around 20 min were almost absent in the NK-L strain (Fig. 1a and c). The purity of \(\gamma\)-PGA products from NK-L strain was 91.2%, which was higher than that of the NK-1 control strain (79.5%). The increased purity led to a significant change in broth viscosity. Broth viscosity of NK-L was 152.0 cP, which was almost 5.52-fold higher than that of the NK-1 strain (27.5 cP). Taken together, these results confirmed that the deletion of \textit{Sac} cluster significantly increased the \(\gamma\)-PGA purity but had little effect on the \(\gamma\)-PGA production.

**CONCLUSIONS**

In summary, we constructed a \(\gamma\)-PGA-deficient NK-\(\Delta\)LP strain from the NK-1 strain. After optimizing its culture conditions, the NK-\(\Delta\)LP can produce 13.9 and 22.6 g L\(^{-1}\) levan in flask culture and in a 5-L bioreactor, respectively. The levan purity reached 92.7%
at 48 h, which made the strain NK-ΔLP having the potential to be a favorable levan-producing strain. A levan-deficient NK-L strain was also constructed from the NK-1 strain. The NK-L strain can produce γ-PGA with increased purity of 91.2%.

SUPPLEMENTARY DATA
Supplementary data are available at FEMSLE online.

FUNDING
This work was supported by the National key Basic Research Program of China (“973”-Program)2012CB725204, the National Key Technology Support Program 2015BAD16B04, the Natural Science Foundation of China Grant Nos. 31470213, 31170030 and the Project of Tianjin, China (13JCYBJC27000, 13JCYBJC24900, 13TXSYJC40100 and 14CZDSF00009) and the Ph.D. Candidate Research Innovation Fund of Nankai University.

AUTHOR CONTRIBUTIONS
JF and CJS designed the experiments. JF, YYG, LFH, KXB and YFQ performed the experiment. CY, SFW, WZ and WXG analyzed the data. JF, YYG, LFH, KXB and YFQ wrote the paper.

Conflict of interest. None declared.

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