Production of a thermostable pullulanase in *Bacillus subtilis* by optimization of the expression elements

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ABSTRACT Pullulanase is a debranching enzyme commonly used in the starch processing industry. Production of a thermostable pullulanase with high activity is desired due to the application of the enzyme to starch processing at high temperature. In this study, a thermostable pullulanase with high activity was overproduced in *Bacillus subtilis.* Via improvement of the plasmid backbones, promoters and ribosome-binding-site (RBS) sequences, we obtained an optimal recombinant strain *B. subtilis* WB800/RBS7 with an extracellular pullulanase activity of 154.9 U/mL in a shake flask, which was 136.8 times higher than that of the wild type. When the strain was cultured in a 5-L bioreactor, the pullulanase activity was significantly improved to 269.1 U/mL (2.74 mg/mL). These results are expected to aid pullulanase production in industrial starch debranching applications.

Keywords Starch debranching enzyme, Pullulanase, *Bacillus subtilis*, Promoter, Protein expression

1. Introduction

Pullulanase (EC 3.2.1.41) is a commonly used starch debranching enzyme that specifically hydrolyses the α-1,6-glycosidic linkages in amylopectin and pullulan and the α- and β-limit dextrins of amylopectin to form linear α-1,4-linked oligomers.[1] Based on differences in specific function and hydrolytic products, pullulanases are divided into type I and II. Type I pullulanase specifically hydrolyses the α-1,6-glycosidic linkages in starch, pullulan, and other polysaccharides to produce maltotriose and linear oligosaccharides. Type II pullulanases are bifunctional enzymes that cleave the α-1,6-glucosidic linkages in pullulan, exhibiting pullulanase activity, and also cleave the α-1,4-glucosidic linkages in starch and related oligosaccharides, displaying α-amylase function.[2] In the food industries, pullulanase has been applied on a large scale in starch saccharification together with saccharifying amylases such as glucoamylase, α-amylase, and β-amylase for preparation of glucose, maltose and fructose syrups.[3] With the developments in biotechnology, the application of pullulanase has been extended to the pharmaceutical chemistry,[4] bioenergy,[5] material and baking industries.[6,7]

Pullulanase was first isolated from a mesophilic bacterium *Klebsiella pneumoniae* by Bender and Wallenfels in 1961.[8] Historically, pullanase was discovered in and

identified in various microorganisms, such as psychrotrophic *Exiguobacterium* sp. SH3,[9] mesophilic *B. cereus*,[10] thermophilic *Geobacillus thermocatenulatus* and *Anoxybacillus* sp. WB42,[11,12] hyperthermophilic *Pyrococcus yayanosii* CH1,[13] etc. However, wild-type strains generally produce pullulanase at low level, leading to limited potential for industrial application.[14] One feasible approach is to optimize the expression elements for improved production of pullulanase in a heterologous host strain.

Bacillus species have been widely used as a type of important strains for the production of industrial enzymes.[15] *B. subtilis* is an attractive expression host due to its desirable features, involving GRAS (generally recognized as safe) status and a naturally efficient secretory system.[16] To improve the extracellular production of heterologous protein in *B. subtilis*, researchers have optimized expression elements such as the plasmid, signal peptide, promoter and RBS sequence.[17,18,19] However, the improvement in protein yield was limited by optimizing only one of these expression elements,[20] and systematic optimization is necessary to supply a more efficient expression system in *B. subtilis.*

Industrial starch conversion involves debranching by pullulanase at 60-65°C,[1] and therefore, thermostable pullulanases are expected for industrial applications. In a

previous study, we screened a novel type II pullulanase (Pul_A) from *Anoxybacillus* sp. WB42, in which the specific activity was high, and its optimum temperature was at 65°C.[12] A mutant pullulanase (Pul_{AC}) with 1.5 times greater specific activity and much higher thermostability than that of the Pul_A was isolated.[21] The thermostability and high activity of the Pul_{AC} predict its high value in industrial applications.

In this study, via stepwise optimization of expression elements including the plasmid backbone, promoter and RBS sequence, we obtained an optimal recombinant strain *B. subtilis* WB800/RBS7 with extracellular Pul_{AC} production of 154.9 U/mL in a 250-mL shake flask. When the strain was cultured in a 5-L bioreactor, the Pul_{AC} production was significantly improved, resulting in activity of 269.1 U/mL (2.74 mg/mL). These results are expected to aid pullulanase production in industrial starch debranching applications.

2. Materials and methods

2.1. Strains, plasmids, and materials

E. coli JM109 (Invitrogen, San Diego, CA, USA) stored in our lab was used as an intermediate host for the construction of recombinant plasmids. *B. subtilis* WB800 (BioVector NTCC Inc, Beijing, China) conserved in our lab was used as a host for the

expression of Pul_{AC}. A previously constructed plasmid pET-22b(+)-*pul*$_{AC}$,[21] bearing the promoter T7 and a pullulanase-encoding gene *pul*$_{AC}$ from *E.coli* BL21 (Novagen, Schwalbach, Germany), was used as a template for PCR. Shuttle plasmids pP43NMK, pMA0911, pSTOP1622 (BioVector NTCC Inc) were used as the expression vectors for Pul_{AC} expression. Primer STAR MAX DNA Polymerase, restriction enzymes, and T4 DNA ligase were purchased from TaKaRa (Dalian, China). The plasmid extraction kit and DNA primers were purchased from Sangon Biotech (Shanghai, China). All other reagents were of analytical grade and obtained from Sangon Biotech.

2.2. Construction of optimal plasmids for Pul_{AC} *expression*

Based on the nucleic acid sequence of pET-22b(+)-*pul*$_{AC}$, three common oligonucleotide primer pairs, namely, pP43NMK-F/R, pMA0911-F/R and pSTOP1622-F/R (as listed in Table S1), were designed for the amplification of *pul*$_{AC}$. Three fragments 2.2 kb in size were amplified. Thereafter, the PCR products and the shuttle plasmids pP43NMK, pMA0911, pSTOP1622 were digested with *Kpn* I and *Hin*d III, *Nde* I and *Bam*H I, and *Spe* I and *Bam*H I, respectively. The PCR products were ligated at these restriction sites to obtain recombinant plasmids pP_{43}, p0911 and p1622. The plasmids were transformed into *E. coli* JM109 using the conventional

calcium chloride method. After selection on 100 μg/mL ampicillin, the positive transformants were confirmed via DNA sequencing and transformed into *B. subtilis* WB800. The steps for preparing competent *B. subtilis* WB800 cells and transformation were conducted as described by Anagnostopoulos and Spizizen.[22]

2.3. Substitution of promoter P_{43} *in* pP_{43} *with different strong promoters*

Expression plasmids containing different strong promoters were generated as follows. First, plasmids with three different single promoters were constructed. Common oligonucleotide primer pairs P_{ylb}-F/R, P_{BH4}-F/R and P_{HpaII}-F/R (listed in Table S1) were designed for amplification of promoters P_{ylb}, P_{BH4} and P_{HpaII} based on three templates, namely, the genome of *Bacillus subtilis* 168 and plasmids BSBHGus (conserved in our lab)[23] and pMA0911. The PCR products were extracted by the Gel Extraction Kit (Thermofisher, Shanghai, China) and used as mega-primers[24] in the following PCRs based on template pP_{43}. The second-round PCR products were digested by *Dpn* I and transformed into *E. coli* JM109 for DNA sequencing. The correctly sequenced plasmids pP_{ylb}, pP_{BH4} and pP_{HpaII} were transformed into *B. subtilis* WB800 for Pul_{AC} expression.

To construct plasmids with a dual promoter, common oligonucleotide primer pairs P_{HpaII}-P_{ylb}-F/R, P_{BH4}-P_{ylb}-F/R, P_{43}-P_{ylb}-F/R, P_{ylb}-P_{ylb}-F/R, P_{ylb}-P_{HpaII}-F/R, P_{ylb}-P_{BH4}-F/R, and

P_{ylb}-P_{43}-F/R (listed in Table S1) were designed to amplify the corresponding single promoters. The PCR products were extracted by the Gel Extraction Kit and used as mega-primers in the subsequent PCRs based on template pP_{ylb}. The correctly sequenced recombinant plasmids pP_{HpaII}-P_{ylb}, pP_{BH4}-P_{ylb}, pP_{43}-P_{ylb}, pP_{ylb}-P_{ylb}, pP_{ylb}-P_{HpaII}, pP_{ylb}-P_{BH4} and pP_{ylb}-P_{43} were transformed into *B. subtilis* WB800 for Pul_{AC} expression.

2.4. Modification of RBS sequence to enhance Pul_{AC} *production*

The RBS prediction tool (https://salislab.net/software/) was used to calculate the initial translation rate. Seven RBS sequences with different strengths were designed to replace the wild-type RBS in plasmid pP_{HpaII}-P_{ylb} using mega-primer PCR, yielding seven new plasmids labelled RBS1 through RBS7. Primers RBS1-F/R through RBS7-F/R are listed in Table S1. The PCR products were digested by *Dpn* I and transformed into *E. coli* JM109 for DNA sequencing. The correctly sequenced plasmids were transformed into *B. subtilis* WB800 for Pul_{AC} expression.

2.5. Media and growth conditions

2.5.1 Shake-flask cultivation

The recombinant strains were initially cultured in 5 mL of Luria-Bertani (LB) medium containing 50 μg/mL antibiotic (*B. subtilis* WB800/p1622 with tetracycline, all other *B. subtilis* WB800 transformants with kanamycin) at 37 °C overnight as a seed liquid and subsequently inoculated into a 250 mL shake flask containing 50 mL of LB medium with 1 % (v/v) inoculum size. Cultures were grown at 37 °C with shaking at 220 rpm for 96 h. For transformant *B. subtilis* WB800/p1622, 5 g/L xylose was added into the culture to induce the expression of Pul_{AC} until the optical density at 600 nm reached approximately 1.0.[25]

2.5.2 Bioreactor cultivation

The seed culture of recombinant *B. subtilis* WB800 strain was prepared in 5 mL of the seed medium (pH 7.0) composed of 40 g/L maltose, 10 g/L yeast extract, 20 g/L tryptone, and 6 g/L KH_2PO_4. After overnight incubation at 37 °C with shaking at 220 rpm on a rotary shaker (ZQZY-78CV, Zhichu Instrument Co., Shanghai, China), the seed culture was diluted in a 250 mL shake flask containing 50 mL of fermentation medium at a ratio of 1 % (v/v) and cultured at 37 °C with shaking at 220 rpm for 12 h.

For batch fermentation, a 2 % (v/v) seed culture was used to inoculate the 5-L fermenter (FS-01-A05P-220, Winpact, Major Science Co., USA) containing 3 L of scale-up fermentation medium. During the entire fermentation process, the pH was maintained at 7.0 using 20 % (v/v) H_3PO_4 and NH_4OH.[26] The temperature was maintained at 30 °C, and the dissolved oxygen content (DO) was maintained at 30 % saturation with agitation speed from 250 to 300 rpm. An antifoam agent was manually added to control the foam height. The culture medium was sampled at the given times for each experiment.

The fermentation medium contained 25 g/L maltose, 40 g/L yeast extract, 4 g/L NH_4Cl, 1.125 g/L KH_2PO_4, 1.875 g/L $K_2HPO_4 \cdot 3H_2O$, and 2 g/L $CaCl_2$.[27]

2.6. Expression analyses and activity assay

The enzyme activity assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed to evaluate the expression of Pul_{AC} in *B. subtilis* WB800. The culture broth was collected by centrifugation (12,000 rpm) at 4 °C for 10 min, and the broth supernatant was used to evaluate the enzymatic activities. SDS-PAGE analysis was used to evaluate the production level of Pul_{AC}.

Enzyme activity was determined by measuring the reducing sugar released from pullulan, and the 3,5-dinitrosalicylic acid (DNS) method[28] was adopted to measure the pullulanase activity by recording the absorbance at 476 nm using an ultraviolet spectrophotometer (UV-1800, MAPADA, Shanghai, China). In this study, 50 μL of 3 % (w/v) pullulan solution was mixed with 10 μL of 50 mM sodium dihydrogen citrate buffer (pH 6.6) and preheated in a water bath at 65 °C for 5 min. A suitably diluted enzyme sample (40 μL) was added to the preheated solution and incubated at 65 °C for 15 min. Thereafter, 100 μL of DNS was added to terminate the reaction. The reaction was boiled in a water bath at 100 °C for 6 min, and the solution was immediately cooled on ice. The reaction system without an enzyme sample was set as a control. Enzyme activity (U) was defined as the amount of enzyme that released 1 μmol reducing sugar (equivalent to glucose) per minute under specified assay conditions.[29] All values were expressed as the mean ±standard deviation (SD ≤ 5 %) of three independent experiments.

3. Results and discussion

3.1. Selection of plasmid for production of Pul_{AC}

Plasmids pP43NMK, pMA0911 and pSTOP1622 were commonly used in the *B. subtilis* expression system for the expression of heterologous proteins.[30,31,25] In this work, we constructed three recombinant plasmids pP_{43}, p0911 and p1622 and compared the production of Pul_{AC} using *B. subtilis* WB800 as a host.

As shown in Figure 1, the extracellular pullulanase activity of *B. subtilis* WB800/pP_{43} reached 27.8 U/mL at 48 h, significantly higher than that of *B. subtilis* WB800/p0911 (13.5 U/mL) and *B. subtilis* WB800/p1622 (1.1 U/mL). Therefore, plasmid pP_{43} was more suitable for production of Pul_{AC}. The multi-copy feature and the strong promoter P_{43} of pP_{43} might be the reason for the higher production of Pul_{AC}.

Although the *pul*$_{AC}$ gene does not contain any signal peptide sequence according to the prediction from SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/), the Pul_{AC} was secretable in *B. subtilis* WB800 with notably low intracellular pullulanase activities (data not shown). In contrast, when we cloned several commonly used signal peptides from *B. subtilis* (such as AmyE, WapA, Epr, YncM and LipA) before the *pul*$_{AC}$ gene, the Pul_{AC} could not be detected both intracellularly

and extracellularly according to SDS-PAGE analysis (data not shown). The $PulI_{AC}$ from *Anoxybacillus* sp. WB42 was also secretable in its original strain. Several similar reports show that selected proteins could be secreted to the broth supernatant without the aid of a signal peptide using the non-classical secretion pathway.[32] This mechanism could be responsible for the secretion in *B. subtilis* and *Anoxybacillus* without the aid of a signal peptide.

3.2. Substitution of P_{43} *with a stronger promoter to improve production of* $PulI_{AC}$

The strength of the promoter determines the gene transcription strength and affects protein production.[33] Strong constitutive promoters P_{ylb},[34] P_{srfA},[35] and P_{HpaII}[36] cloned from *B. subtilis* and *Staphylococcus aureus* have expression strengths comparable to that of P_{43} and have been used to construct an array of plasmids for the over-production of heterologous proteins. Furthermore, our lab has constructed the 7 bp sequence immediate upstream of the – 10 box of the promoter P_{srfA} via two-round consecutive evolution and obtained a mutant promoter P_{BH4} with a strength approximately 3.0 times higher than that of P_{srfA}.[23] In this work, we substituted the promoter P_{43} in pP_{43} with each of strong constitutive promoters P_{ylb}, P_{BH4}, and P_{HpaII} and thus expected to improve the production of $PulI_{AC}$.

As shown in Figure 2, the extracellular pullulanase activity of *B. subtilis* WB800/pP_{ylb} reached 54.1 U/mL at 48 h, which was 1.9 times higher than that of *B. subtilis* WB800/pP_{43}. The pullulanase production of *B. subtilis* WB800/pP_{BH4} and *B. subtilis* WB800/pP_{HpaII} was lower than that of *B. subtilis* WB800/pP_{43}. Therefore, the protein-expression-level under promoter P_{ylb} was significantly higher than that of promoters P_{43}, P_{BH4}, and P_{HpaII}, especially from the stationary phase, which was consistent with a previous study.[34] Therefore, *B. subtilis* WB800/pP_{ylb} was selected for further optimization.

3.3. Tandem promoter strategy to improve production of $PulI_{AC}$

Tandem combination of multiple promoters is an effective way to improve the production of proteins.[37] For example, Wei et al increased the expression of staphylokinase by tandem promoter P_{32}-P_{lacA}.[38] Kojima et al improved the expression of reporter gene by a factor of approximately 4 by tandem repetition of the *ie1* promoter.[39] Okegawa et al expressed spinach ferredoxin-thioredoxin reductase by tandem promoter T7.[40] Guan et al improved the production of aminopeptidase by dual promoter P_{gsiB}-P_{HpaII}.[41] In this work, we cloned strong promoters P_{HpaII}, P_{43}, P_{BH4}, and P_{ylb} before and after the promoter P_{ylb} in plasmid pP_{ylb}, to construct seven tandem

promoter plasmids pP_{HpaII}-P_{ylb}, pP_{43}-P_{ylb}, pP_{BH4}-P_{ylb}, pP_{ylb}-P_{HpaII}, pP_{ylb}-P_{43}, pP_{ylb}-P_{BH4}, and pP_{ylb}-P_{ylb} to improve the production of Pul_{AC}.

As shown in Figure 3, the extracellular pullulanase activity of *B. subtilis* WB800/pP_{HpaII}-P_{ylb} reached 62.3 U/mL at 48 h, which was 1.2-fold higher than that of *B. subtilis* WB800/pP_{ylb}. The pullulanase production of other recombinants was lower than that of *B. subtilis* WB800/pP_{ylb}. Therefore, the stronger tandem-promoter P_{HpaII}-P_{ylb} could be involved in improving protein expression in *B. subtilis*.

3.4. Optimization of RBS sequence to improve production of Pul_{AC}

The RBS sequence determines the translation-initiation rate of mRNA. Optimization of the RBS sequence is an effective way to increase the production of enzyme.[42,43,44,45] In this work, we optimized the RBS sequence following the tandem promoter P_{HpaII}-P_{ylb} to improve the production of Pul_{AC}. As shown in Table S2, seven RBSs with different translation-initiation rates were designed via the Calculator 2.0 website software (https://salislab.net/software/) and were used to substitute the wild-type RBS. The newly constructed plasmids were transformed into *B. subtilis* WB800 to compare the production of Pul_{AC}.

As shown in Figure 4, the extracellular pullulanase activity of *B. subtilis* WB800/RBS7 reached 118 U/mL at 48 h, which was 1.9 times higher than that of *B. subtilis* WB800/pP_{HpaII}-P_{ylb}. Compared with *B. subtilis* WB800/pP_{HpaII}-P_{ylb}, the extracellular pullulanase activity of *B. subtilis* WB800/RBS4, *B. subtilis* WB800/RBS5 and *B. subtilis* WB800/RBS6 was also improved, indicating that optimization of the RBS sequence is an effective way to improve the production of Pul_{AC}. As a result, we successfully established an efficient expression system for the production of Pul_{AC} in *B. subtilis* WB800 through optimizations of the plasmid backbones, promoters and RBS sequences. Compared with integrative expression strategy reported previously,[27] the established plasmid expression system produced much more pullulanase.

3.5. Production of Pul_{AC} *via the optimal expression system*

We produced the Pul_{AC} using the optimal expression system in the 250 mL shake flask and analysed the biomass and extracellular pullulanase activity of *B. subtilis* WB800/RBS7. SDS-PAGE analysis revealed the expression of pullulanase by *B. subtilis* WB800/RBS7 at different cultivation times (Figure 5a), the maximum extracellular pullulanase activity of *B. subtilis* WB800/RBS7 reached 154.9 U/mL (Figure 5b), which was 136.8 times higher than that of the wild type.

3.6. Production of Pul_{AC} *in a 5-L bioreactor*

To produce the Pul_{AC} in a 5-L bioreactor, experiments were performed according to the optimized culture medium and conditions reported by Wang et al.[27] As shown in Figure 6, the maximum extracellular pullulanase activity of *B. subtilis* WB800/RBS7 achieved 269.1 U/mL (2.74 mg/mL), which was 1.7 times higher than that of the shake-flask cultivation. Compared with the pullulanase obtained from recombinant *B. subtilis* WB600 (0.05 mg/mL),[46] *B. subtilis* WB800 (0.13 mg/mL)[27] and *P. pastoris* (0.75 mg/mL),[47] our pullulanase production had a significant advantage. Although the production was still lower than that of the type I pullulanase obtained from *Brevibacillus choshinensis* (4.45 mg/mL),[48] we achieved the highest pullulanase production of type II pullulanase, to the best of our knowledge. Optimization of the scale-up fermentation conditions is needed to improve the production of pullulanase in future studies.

4. Conclusion

In this study, a recombinant plasmid pP_{43} was constructed that harboured the *pul*$_{AC}$ gene and a constitutive promoter P_{43}. Via substitution with the strong promoter P_{ylb}, construction of the tandem promoter P_{HpaII}-P_{ylb} and optimization of the RBS sequence, we obtained an optimal recombinant strain *B. subtilis* WB800/RBS7 with extracellular pullulanase production of 154.9 U/mL in a shake flask. When the strain was cultured in a 5-L bioreactor, the pullulanase production was significantly improved, resulting in an activity of 269.1 U/mL (2.74 mg/mL). Compared with previously reported pullulanase production,[27,46,47] a higher pullulanase production was achieved in this study. Via stepwise optimization of the expression elements, we constructed an efficient expression system in *B. subtilis*. The system had a high pullulanase expression efficiency compared with that of previous reports.

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Compliance with Ethical Standards

The authors confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with the submission.

Conflict of Interest The authors declare that they have no competing interests.

Novelty statement

A thermostable pullulanase was overproduced in *B*acillus *subtilis*.

An efficient pullulanase expression system was established by optimization of the expression elements.

The highest pullulanase production of type II pullulanase was achieved.

References

[1] S. L. Hii, J. S. Tan, T. C. Ling, A. B. Ariff, *Enzyme Res.* **2012**, *2012*, 921362.

[2] J. Xu, F. Ren, C. H. Huang, Y. Zheng, J. Zhen, H. Song, R. T. Guo, *Proteins* **2014**, *82*, 1685.

[3] B. E. Norman, *Starch / Stärke* **1982**, *34*, 340.

[4] H. Guzmán-Maldonado, O. Paredes-López, C. G. Biliaderis, *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 373.

[5] R. S. Singh, G. K. Saini, J. F. Kennedy, *Carbohyd. Polym.* **2010**, *81*, 252.

[6] B. Mikami, H. Iwamoto, D. Malle, H. J. Yoon, E. Demirkan-Sarikaya, Y. Mezaki, Y. Katsuya, *J. Mol. Biol.* **2006**, *359*, 690.

[7] M. Nisha, T. Satyanarayana, *Bioengineered* **2013**, *4*, 388.

[8] H. Bender, K. Wallenfels, *Biochem. Z.* **1961**, *334*, 79.

[9] S. Rajaei, K. A. Noghabi, M. Sadeghizadeh, H. S. Zahiri, *Extremophiles* **2015**, *19*, 1145.

[10] W. Wei, J. Ma, S. Guo, D. Z. Wei, *Process Biochem.* **2014**, *49*, 1893.

[11] L. M. Li, F. Y. Dong, L. Lin, D. N. He, W. Wei, D. Z. Wei, *J. Agric. Food Chem*. **2018**, *66*, 10788.

[12] J. F. Wang, Z. M. Liu, Z. M. Zhou, *Starch / Stärke* **2018**, *70*, 1700265.

[13] B. Pang, L. Zhou, W. J. Cui, Z. M. Liu, S. M. Zhou, J. Xu, Z. M. Zhou, *J. Agric. Food Chem*. **2019**, *67*, 9611.

[14] J. Kang, K. M. Park, K. H. Choi, C. S. Park, G. E. Kim, D. Kim, J. Cha, *Enzyme Microb. Technol*. **2011**, *48*, 260.

[15] L. Westers, H. Westers, W. J. Quax, *BBA-Mol. Cell Res*. **2004**, *1694*, 299.

[16] M. Schallmey, A. Singh, O. P. Ward, *Can. J. Microbiol*. **2004**, *50*, 1.

[17] W. Schumann, *Adv. Appl. Microbiol*. **2007**, *62*, 137.

[18] K. Terpe, *Appl. Microbiol. Biotechnol*. **2006**, *72*, 211.

[19] Y. P. Wang, Y. H. Liu, Z. X. Wang, F. P. Lu, *Biotechnol. Lett*. **2014**, *36*, 1783.

[20] K. Zhang, L. Q. Su, X. G. Duan, L. N. Liu, J. Wu, *Microb. Cell Fact*. **2017**, *16*, 32.

[21] B. Pang, L. Zhou, W. J. Cui, Z. M. Liu, Z. M. Zhou, *Appl. Biochem. Biotechnol*. **2020**. https://doi.org/10.1007/s12010-020-03249-2.

[22] C. Anagnostopoulos, J. Spizizen, *J. Bacteriol*. **1961**, *81*, 741.

[23] L. C. Han, W. J. Cui, F. Y. Suo, S. N. Miao, W. L. Hao, Q. Q. Chen, J. L. Guo, Z. M. Liu, L. Zhou, Z. M. Zhou, *Microb. Cell Fact*. **2019**, *18*, 96.

[24] K. Miyazaki, M. Takenouchi, *Biotechniques* **2002**, *33*, 1033.

[25] B. H. Liu, J. Zhang, B. Li, X. R. Liao, G. C. Du, J. Chen, *World J. Microbiol. Biotechnol*. **2013**, *29*, 825.

[26] K. Zhang, L. Q. Su, J. Wu, *Appl. Microbiol. Biotechnol*. **2018**, *102*, 5089.

[27] Y. Wang, S. H. Chen, X. H. Zhao, Y. Zhang, X. Y. Wang, Y. Nie, Y. Xu, *Protein Expres. Purif*. **2019**, *159*, 42.

[28] M. J. Bailey, *Appl. Microbiol. Biotechnol*. **1988**, *29*, 494.

[29] Y. Nie, W. Yan, Y. Xu, W. B. Chen, X. Q. Mu, X. Y. Wang, R. Xiao, *PLoS One* **2013**, *8*, e78416.

[30] Y. Feng, S. Liu, Y. Jiao, H. Gao, M. Wang, G. C. Du, J. Chen, *Appl. Microbiol. Biotechnol*. **2017**, *101*, 1509.

[31] Y. Deng, Y. Nie, Y. Zhang, Y. Wan, Y. Xu, *Protein Expres. Purif*. **2018**, *148*, 9.

[32] L. Q. Zhao, J. Q. Chen, J. B. Sun, D. W. Zhang, *Sci. Rep*. **2017**, *7*, 44023.

[33] S. Yang, Q. T. Liu, Y. F. Zhang, G. C. Du, J. Chen, Z. Kang, *ACS Synth. Biol*. **2018**, *7*, 287.

[34] X. X. Yu, J. T. Xu, X. Q. Liu, X. Y. Chu, P. Wang, J. Tian, N. F. Wu, Y. L. Fan, *Sci. Rep*. **2015**, *5*, 18405.

[35] M. Roggiani, D. Dubnau, *J. Bacteriol*. **1993**, *175*, 3182.

[36] X. X. Gao, Z. M. Liu, W. J. Cui, L. Zhou, Y. P. Tian, Z. M. Zhou, *PLoS One* **2014**, *9*, e92357.

[37] S. Sugimoto, M. Yabuta, T. Seki, T. Yoshida, H. Taguchi, *Appl. Microbiol. Biotechnol*. **1985**, *22*, 336.

[38] W. Z. Wei, H. Xiang, H. R. Tan, *Biotechnol. Lett*. **2002**, *24*, 1669.

[39] K. Kojima, T. Hayakawa, S. Asano, H. Bando, *Arch. Virol*. **2001**, *146*, 1407.

[40] Y. Okegawa, K. Motohashi, *Protein Expres. Purif*. **2016**, *121*, 46.

[41] C. R. Guan, W. J. Cui, J. T. Cheng, R. Liu, Z. M. Liu, L. Zhou, Z. M. Zhou, *New Biotechnol*. **2016**, *33*, 372.

[42] A. Levin-Karp, U. Barenholz, T. Bareia, M. Dayagi, L. Zelcbuch, N. Antonovsky, E. Noor, R. Milo, *ACS Synth. Biol*. **2013**, *2*, 327.

[43] Z. M. Liu, L. Yu, L. Zhou, Z. M. Zhou, *Appl. Environ. Microbiol*. **2019**, *85*, e01327-19.

[44] M. Y. Pan, J. H. Li, X. Q. Lv, G. C. Du, L. Liu, *Enzyme Microb. Technol*. **2019**, *124*, 54.

[45] B. Wang, C. Eckert, P. C. Maness, J. P. Yu, *ACS Synth. Biol*. **2018**, *7*, 276.

[46] W. Song, Y. Nie, X. Q. Mu, Y. Xu, *Protein Expres. Purif*. **2016**, *124*, 23.

[47] B. Xu, Y. J. Yang, Z. X. Huang, *J. Microbiol. Biotechnol*. **2006**, *16*, 1185.

[48] C. Zou, X. G. Duan, J. Wu, *J. Ind. Microbiol. Biotechnol.* **2016**, *43*, 495.

Figure legends

Figure 1. Comparison of pullulanase production by *B. subtilis* WB800 recombinants harbouring different plasmids. (a) SDS-PAGE analysis of extracellular pullulanase production at 48 h. M: protein marker, line 1: pP_{43}, line 2: p0911, line 3: p1622. (b) Extracellular pullulanase activity of *B. subtilis* WB800 recombinants at 48 h.

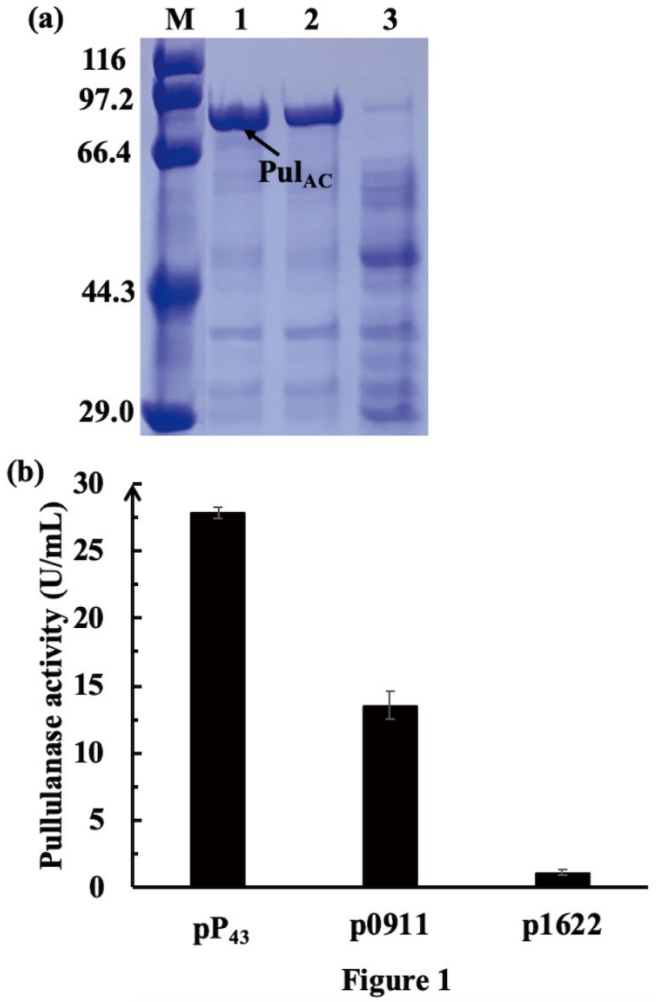


Figure 2. Comparison of pullulanase production by *B. subtilis* WB800 recombinants harbouring different strong promoters. (a) SDS-PAGE analysis of extracellular pullulanase production at 48 h. M: protein marker, line 1: pP_{ylb}, line 2: pP_{BH4}, line 3: pP_{43}, line 4: pP_{HpaII} at 48 h. (b) Extracellular pullulanase activity of *B. subtilis* WB800 recombinants at 48 h.

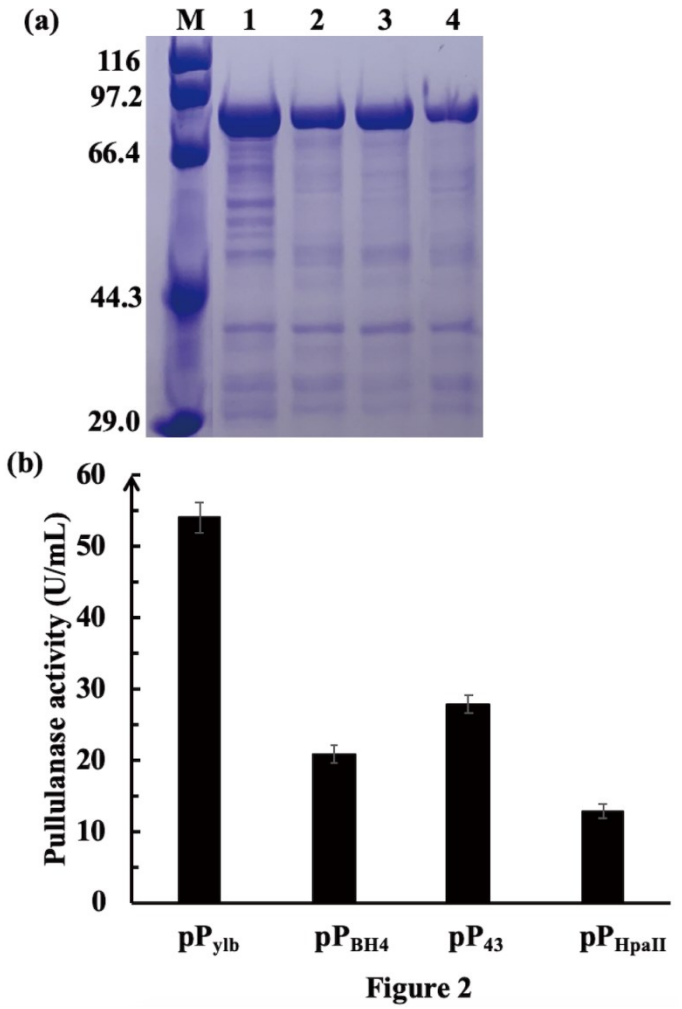


Figure 3. Comparison of pullulanase production by *B. subtilis* WB800 recombinants harbouring different dual promoters. (a) SDS-PAGE analysis of extracellular pullulanase production at 48 h. M: protein marker, line 1: pP_{ylb}, line 2: pP_{HpaII}-P_{ylb}, line 3: pP_{43}-P_{ylb}, line 4: pP_{BH4}-P_{ylb}, line 5: pP_{ylb}-P_{HpaII}, line 6: pP_{ylb}-P_{43}, line 7: pP_{ylb}-P_{BH4}, line 8: pP_{ylb}-P_{ylb}. (b) Extracellular pullulanase activity of *B. subtilis* WB800 recombinants at 48 h.

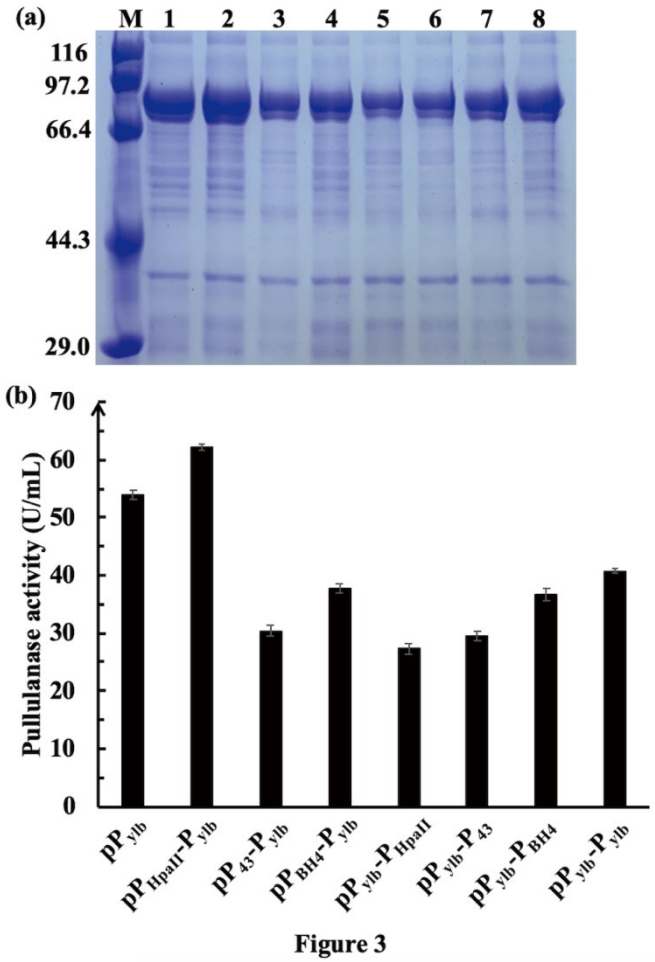


Figure 4. Comparison of pullulanase production by *B. subtilis* WB800 recombinants harbouring different RBS sequences. (a) SDS-PAGE analysis of extracellular pullulanase production at 48 h. M: protein marker, line 1: pP_{HpaII}-P_{ylb}, line 2: RBS1, line 3: RBS2, line 4: RBS3, line 5: RBS4, line 6: RBS5, line 7: RBS6, line 8: RBS7. (b) Extracellular pullulanase activity of *B. subtilis* WB800 recombinants at 48 h.

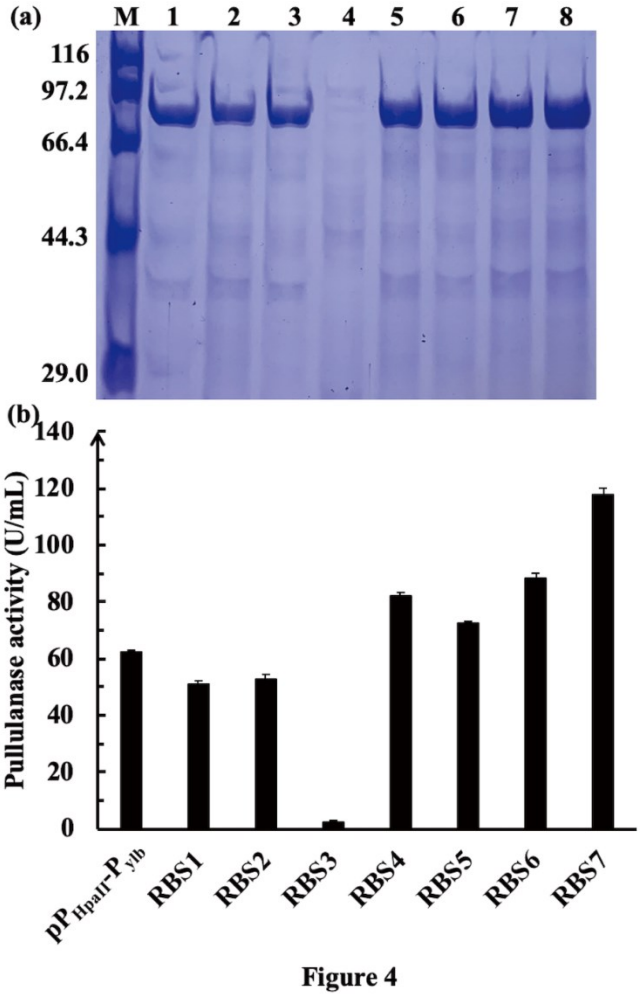


Figure 4

Figure 5. Analysis of pullulanase production by *B. subtilis* WB800/RBS7 in a 250 mL shake flask. (a) SDS-PAGE analysis of extracellular pullulanase production by *B. subtilis* WB800/RBS7 during shake-flask cultivation. M: protein marker, line 1: 12 h, line 2: 24 h, line 3: 36 h, line 4: 48 h, line 5: 60 h, line 6: 72 h, line 7: 84 h, line 8: 96 h. (b) Biomass and extracellular pullulanase activity of *B. subtilis* WB800/RBS7 at different cultivation times.

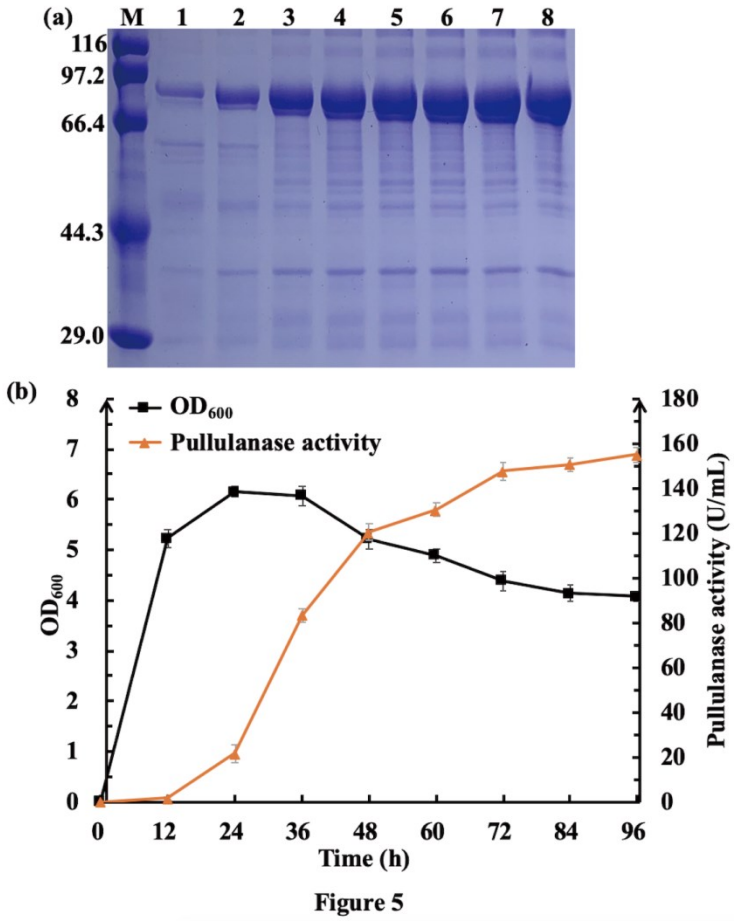


Figure 6. Analysis of pullulanase production by *B. subtilis* WB800/RBS7 in a 5-L fermenter. (a) SDS-PAGE analysis of extracellular pullulanase production during scale-up cultivation. M: protein marker, line 1: 12 h, line 2: 24 h, line 3: 36 h, line 4: 48 h, line 5: 60 h, line 6: 72 h, line 7: 84 h, line 8: 96 h. (b) Biomass and extracellular pullulanase activity of *B. subtilis* WB800/RBS7 at different cultivation times.

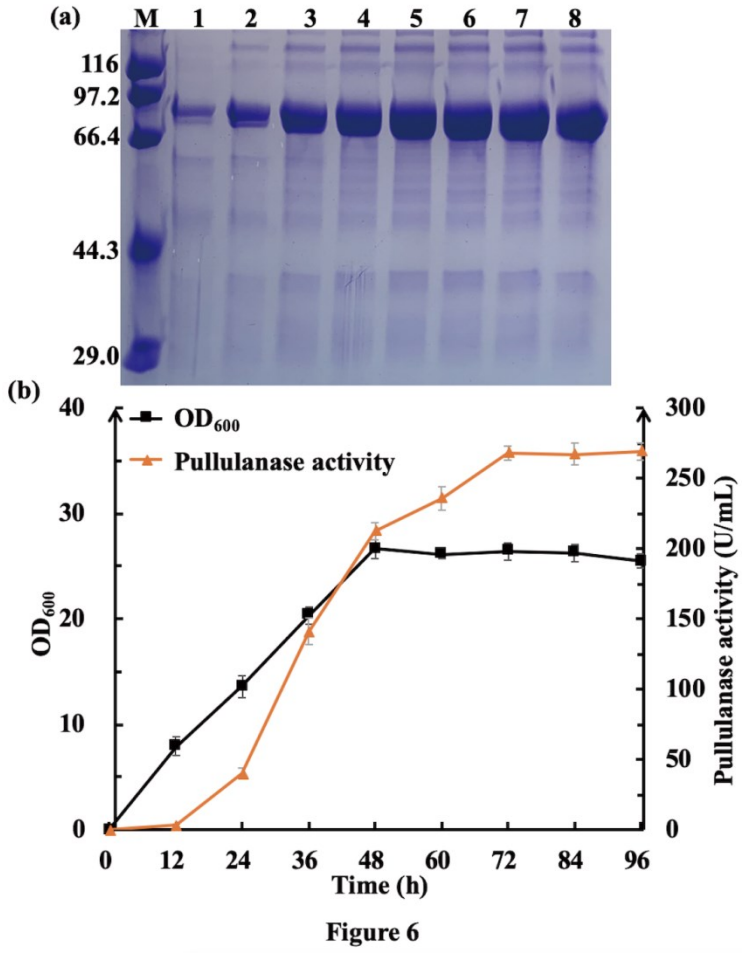
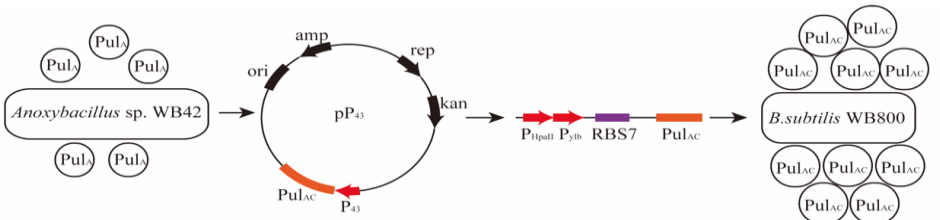


Figure 6

Via optimization of expression elements, a thermostable pullulanase with high activity derived from *Anoxybacillus* sp. WB42 was overproduced in *B. subtilis* WB800, which will be helpful for industrial starch debranching applications.



Graphical abstract