# High PGL productivity in *Bacillus subtilis* by optimizing the SD sequence

Junjiao Zhang[1,*]

[1] Qilu institute of technology, 250200 Jinan, China

**Abstract.** Alkaline polygalacturonate lyase (PGL, EC 4.2.2.2) can catalyze the cleavage of a-1, 4-glycosidic bonds of polygalacturonate by a trans-elimination reaction and generate an unsaturated oligogalacturonates. As the critical enzyme of many environmental friendly processes, alkaline PGL has been widely used in many fields including paper, textile and beverage industries. At present, *Bacillus subtilis* is an ideal strain for producing PGL, but the yield is too low for industrial production. In this study, the effect of different SD sequences on the production of PGL was comparatively investigated, and the strong SD sequence (AGAGAACAAGGAGGG G) directed efficient PGL secretory expression and increased PGL yield to 264.5 U·mL$^{-1}$ with a high productivity. As a result, the PGL yield in *B. subtilis* was effecively increased and laid the solid foundation for PGL industrial production.

## 1 Introduction

In recent years, due to vigorous development of the chemical industry, global environment has been seriously polluted. Traditional textile wet treatment process includes three steps of desizing, refining and bleaching, which uses water as the medium and reacts under high temperature and high alkali conditions. It not only destroys the structure of cotton and linen fabrics, consumes a lot of heat energy, but also produces a lot of industrial waste water. Biological enzymes represented by PGL have strong alkali resistance, high temperature resistance, and a wide range of sources. They are widely used in the textile industry, paper industry, food industry and other fields, and have huge commercial potential. However, the yield of wild strains screened from nature is generally low[1]. The strains for industrial production of PGL have many problems such as long cycle, high energy consumption, complex process, etc. Therefore, find a simple fermentation condition, short cycle, and safe PGL high-yielding strains have become the focus of current research[2,3].

*B. subtilis*, which has a clear genetic background, a powerful protein secretion system, good safety performance, no obvious codon preference type, clear genetic background research and simple fermentation conditions, is considered the best host choice for the expression of PGL[4]. At present, there have been some reports on the production of PGL by *B. subtilis*, but they are all focused on wild bacterial strain screening and medium optimization[5]. There are no reports on molecular modification. The yield of PGL is generally low and cannot meet the requirements of industrial production. Therefore, systematically optimizing the molecular elements of *B. subtilis* expression vectors and achieving high-efficiency expression of PGL in *B. subtilis* have been the research hotspots[6].

In the previous research, two *BamHI* restriction enzyme sites in the target gene were eliminated through site-directed mutation, and the signal peptide bpr that is most suitable for the secretion and expression of PGL gene was screened, and a strong promoter P43 was connected. The enzyme activity of PGL in *B. subtilis*WB600 was increased from 14.7 U·mL$^{-1}$ to 235.87 U·mL$^{-1}$, and the PGL gene derived from *B. subtilis*WSHB04-02 was successfully expressed in the engineering strain *B. subtilis*WB600. In order to further increase the yield of PGL, this study explored the most suitable SD sequence for PGL gene expression. By screening the most suitable SD sequence, the yield of PGL was further improved and laid a solid foundation for industrial production of PGL.

## 2 Materials and methods

### 2.1 Materials, reagents and instruments

#### *2.1.1 Strains and plasmids*

All strains, plasmids and primers used in this paper are shown in Table 1-1 and Table 1-2.

* Corresponding authors，E-mail: 593183586@qq.com

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

| Strains and plasmids | characteristic |
|---|---|
| **Strains** | |
| *B.subtilis* WB600 | deficient in *npr*E *apr*E *epr bpr mpr npr*B |
| WB43CB | *B. subtilis* WB600 harboring pP43CB |
| WB43CTR | *B. subtilis* WB600 harboring pP43CTR |
| WB43CTA | *B. subtilis* WB600 harboring pP43CTA |
| **Plasmids** | |
| pP43CB | pP43C derivative, bpr, *pgl* gene |
| pP43CTR | pP43C derivative, bpr, *pgl* gene, strong SD |
| PP43CTA | pP43C derivative, bpr, *pgl* gene, consences SD |

**Table 1-2** Oligonucleotides used in this study

| Primer | Sequences |
|---|---|
| PGL-F | ACGCGTCGACGCTGATTTAGGCCACCAGACGT |
| PGL-R | CATGCCATGGGGATCCTTAATTTAATTTACCCGCACCCG |
| P43STR-F | AAACCCGGGAGAGAACAAGGAGGGGAATACAATGAGGAAA AAAACGAAAAACAGACTC |
| P43STR-R | CCCAAGCTTTTAATTTAATTTACCCGCACCCGCT |
| P43STA-F | AAACCCGGGAAAGGAGGTGAAATACAATGAGGAAAAAAAC GAAAAACAGACTC |
| P43STA-R | CCCAAGCTTTTAATTTAATTTACCCGCACCCGCT |

### *2.1.2 Medium*

LB medium (g·L$^{-1}$): peptone 10, yeast powder 5, NaCl 10, pH 7.0.

Seed medium (g·L$^{-1}$): sucrose 20, corn steep liquor 30, peptone 10, $KH_2PO_4$ 6, $K_2HPO_4$ 18.4, pH 7.0.

Fermentation medium (g·L$^{-1}$): corn starch 15, peptone 8, yeast powder 10, $KH_2PO_4$ 3, $K_2HPO_4$ 9.2, pH 7.0.

### 2.2 Operation method

Cell growth was monitored by measuring the optical density (OD) of culture broth using a spectrophotometer at 600 nm after an appropriate dilution. For analyzing the PGL activity, 1 mL of culture was centrifuged (10,000g for 10 min at 4 °C) and the supernatant was used as an extracellular fraction. The resulting pellets resuspended in 500 μL of PBS buffer were then sonicated and centrifuged (10,000g for 10 min at 4°C) to recover the soluble intracellular contents.

The activity of alkaline PGL was determined by measuring the absorbance of unsaturated bonds between $C_4$ and $C_5$ of galacturonic acid at 235 nm. The reaction mixture contained 2 mL of 0.2% (w/v) polygalacturonic acid in 200 mM glycine–NaOH buffer at pH 9.4 (containing 200 mM glycine, 200 mM NaOH, and 60 mM $CaCl_2$) and 20 μL of diluted enzyme solution. The reaction mixture was incubated at 45 °C for 15 min, and then the reaction was terminated by adding 3 mL of 30 mM phosphoric acid. The product was analyzed by a spectrophotometer. One enzyme unit was defined as formation of 1 μmoL unsaturated polygalacturonic acid per minute, with a molar extinction coefficient of 4600 M$^{-1}$ cm$^{-1}$ at 235 nm.

## 3 Results and discussion

### 3.1 Recombinant strain construction

The SD sequence can be recognized and bound by the 3'end of the ribosomal 16 SrRNA to help mRNA start translation. SD sequences of different strengths will have different effects on protein translation. The most conservative part of SD sequences in bacteria is "GGAGG". Changes in conservative sequences can significantly affect the rate of mRNA translation and the effect of protein translation. In order to further improve the production of PGL, this study explored the most suitable SD sequence for PGL gene expression. According to the methods reported in the literature, different SD

sequences were analyzed, and the strongest SD sequence (AGAGAACAAGGAGGGG) with the conservative sequence "GGAGG" and the most conservative SD sequence in *B. subtilis* (AAAGGAGGTGA) were selected. The PGL expression was performed simultaneously with the SD (AAAGGAGCG) sequence used in plasmid pP43CB, and the effects of different SD sequences on PGL gene expression were compared and analyzed. The 1-4 bases downstream of the SD sequence and the bases between SD sequence and start codon ATG have a obviously influence on gene transcription. The transcription efficiency is highest when the 1-4 bases downstream of the SD sequence are A or T. The transcription efficiency is highest when the SD sequence and the start codon are separated by 6-9 bases. Therefore, in the process of constructing recombinant plasmids, select the optimal SD downstream base sequence (A or T), and control the base sequence between the SD sequence and the start codon to be between 6-9.

### 3.2 Construction of recombinant plasmids with different strength SD sequences

The construction process of recombinant plasmids with different SD sequences is shown in Fig. 1.

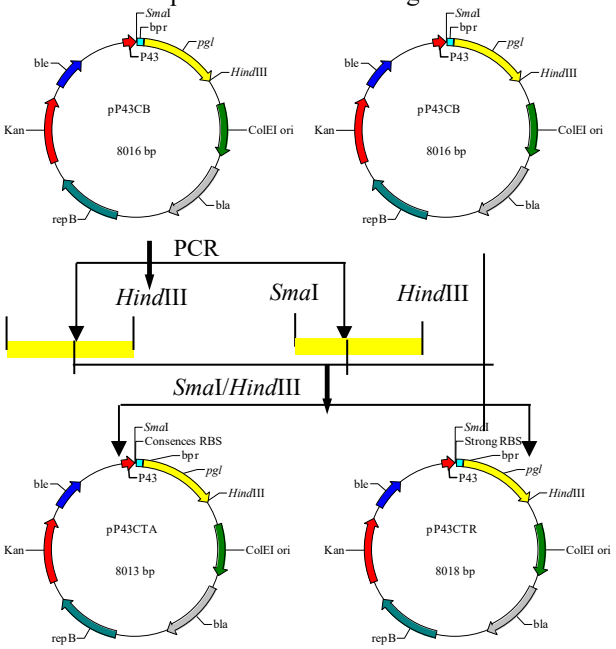


**Fig. 1** Construction of recombinant plasmids pP43CTA and pP43CTR

According to the analysis results, the primers P43STA-R, P43STA-F and P43STR-R, P43STR-F (Table 1-2) were designed. The upstream primers carry the strong SD sequence and the Consences SD sequence respectively, and the downstream 1-4 bases of the SD sequence are A or T. The SD sequence and the start codon are separated by 6 bases. PCR was performed using plasmid pMA0911B as a template to clone two fragments containing strong SD-bpr-pgl and consences SD-bpr-pgl genes. The two fragments were integrated into the plasmid pP43C through the two restriction sites of *SmaI* and *HindIII* to obtain the recombinant plasmids pP43CTR and pP43CTA.

### 3.3 Fermentation of recombinant strains and result analysis

The recombinant plasmids pP43CTA and pP43CTR were respectively transformed into *B. subtilis* WB600 to form recombinant bacteria WB43CTA and WB43CTR. The three recombinant strains WB43CTA, WB43CTR and WB43CB were fermented simultaneously at 37°C, 200 $r \cdot min^{-1}$, and the fermentation results are shown in Fig. 2, Fig. 3 (a), and Fig. 3 (b).

As shown in Fig. 2, PGL mainly accumulates in the logarithmic phase and the stable phase in the fermentation process of the three recombinant bacteria, which is consistent with related reports. After fermentation, the cell concentrations of the three recombinant strains WB43CTA, WB43CTR and WB43CB were ($OD_{600}$): 20.80, 16.64 and 17.45, respectively. The cell concentration of WB43CTA was higher than the other two recombinant strains.

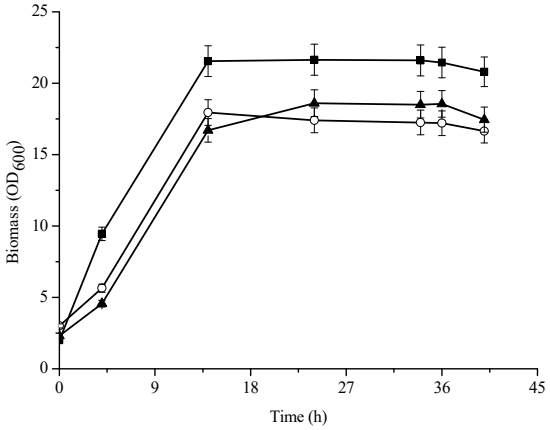


**Fig. 2** Biomass ($OD_{600}$) of recombinant strains WB43CTR, WB43CTA and WB43CB
WB43CTR (○), WB43CTA (■), WB43CB (▲).

The extracellular enzyme activity of the three recombinant strains is shown in Fig. 3 (a). The extracellular enzyme activity of WB43CB with the original SD sequence is 235.87 $U \cdot mL^{-1}$. The PGL yield of the recombinant strain WB43CTR with strong SD sequence reached 264.50 $U \cdot mL^{-1}$ with a high productivity, higher than that of the other two recombinant strains. The extracellular enzyme activity of the recombinant strain WB43CTA using the Consences SD sequence is 70.11 $U \cdot mL^{-1}$, which is much lower than the extracellular enzyme activity of the other two recombinant strains. This may be related to the SD sequence and other promoter.

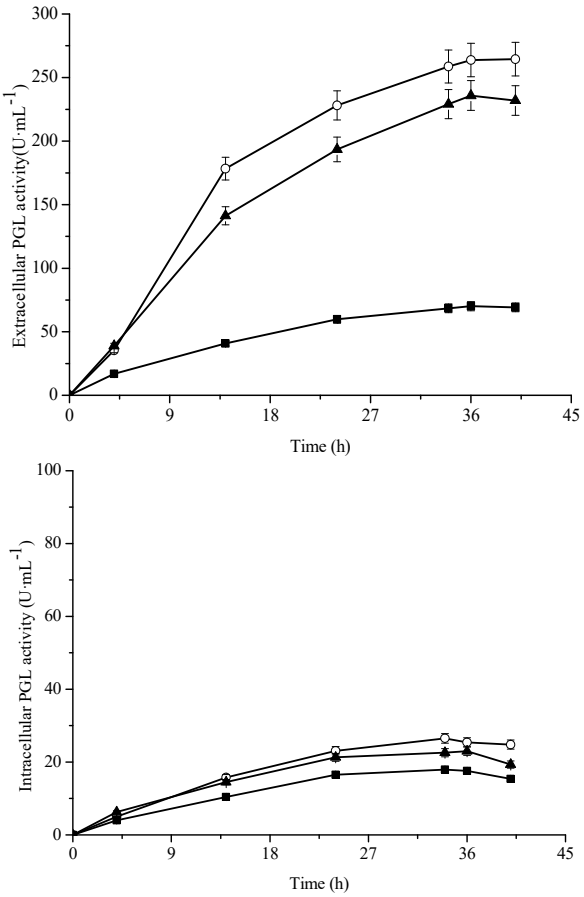


**Fig. 3** Comparison of PGL activity in recombinant strains WB43CTR, WB43CTA and WB43CB
(a): Extracellular PGL activity; (b): Intracellular PGL activity.
WB43CTR (○), WB43CTA (■), WB43CB (▲).

As shown in Fig. 3 (b), the intracellular enzyme activities of the three recombinant strains WB43CTA, WB43CTR and WB43CB are little difference, all of which are less than 30 $U \cdot mL^{-1}$, which is consistent with the previous study.

## 4 Conclusion

Applying suitable SD sequence screen approach, three SD sequences were screened and explored for PGL production in *B. subtilis*. The strong SD sequence (AGAGAACAAGGAGGGG) showed the best secretion efficiency and resulted in a high titer of 264.50 $U \cdot mL^{-1}$ with a high productivity.

## References

1. Wang Z H, Wang Y, Zhang D X, et al. Bioresour Technol. **101**, 1318-1323 (2010).
2. Wang Q, Fan X R, Hua Z Z, et al. Biochem Eng J. **34**, 107-113 (2007).
3. Fang S Y, Li J H, Liu L, et al. Bioresour Technol. **102**, 10671-10678 (2011).
4. Gao T, Wong Y K, Ng C, et al. Bioresour Technol. **121**, 105-110 (2012).
5. Zhang X Z, Sathitsuksanoh N, Zhu Z G, et al. Metab Eng. **13**, 364-372 (2011).
6. Zhang X Z, Zhang Y H P. Microb Biotechnol. **4**, 98-105 (2011).