Electronic Journal of Biotechnology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

# Improving the expression of recombinant pullulanase by increasing mRNA stability in *Escherichia coli*

Tao Li [a,b], Yatong Ding [a], Jun Zhang [a], Guobao Jiao [c], Lipeng Sun [c], Zhongmin Liu [c], Liyou Qiu [a,*]

[a] College of Life Sciences, Henan Agricultural University, Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, Zhengzhou 450002, China
[b] Medicine and Nursing College, Sanmenxia Polytechnic Institute, Sanmenxia 472000, China
[c] Henan Yangshao Biochemical Engineering Co., Ltd, Mianchi 472400, China

ARTICLE INFO

*Article history:*
Received 19 March 2017
Accepted 3 June 2017
Available online xxxx

*Keywords:*
Amylases
Carbohydrate debranching enzyme
Enzyme activity
Expression vector
Gene expression
Starch hydrolysis
Industrial applications
Pullulanase fermentation
SD sequence
Semi-quantitative RT-PCR
Stem-loop structure

ABSTRACT

*Background:* Pullulanase production in both wild-type strains and recombinantly engineered strains remains low. The Shine-Dalgarno (SD) sequence and stem-loop structure in the 5′ or 3′ untranslated region (UTR) are well-known determinants of mRNA stability. This study investigated the effect of mRNA stability on pullulanase heterologous expression.

*Results:* We constructed four DNA fragments, pulA, SD-pulA, pulA-3t, and SD-pulA-3t, which were cloned into the expression vector pHT43 to generate four pullulanase expression plasmids. The DNA fragment pulA was the CDS of pulA in *Klebsiella variicola* Z-13. SD-pulA was constructed by the addition of the 5′ SD sequence at the 5′ UTR of pulA. pulA-3t was constructed by the addition of a 3′ stem-loop structure at the 3′ UTR of pulA. SD-pulA-3t was constructed by the addition of the 5′ SD sequence at the 5′ UTR and a 3′ stem-loop structure at the 3′ UTR of pulA. The four vectors were transformed into *Escherichia coli* BL21(DE3). The pulA mRNA transcription of the transformant harboring pHT43-SD-pulA-3t was 338.6%, 34.9%, and 79.9% higher than that of the other three transformants, whereas the fermentation enzyme activities in culture broth and intracellularly were 107.0 and 584.1 times, 1.2 and 2.0 times, and 62.0 and 531.5 times the amount of the other three transformants (pulA, SD-pulA, and pulA-3 t), respectively.

*Conclusion:* The addition of the 5′ SD sequence at the 5′ UTR and a 3′ stem-loop structure at the 3′ UTR of the pulA gene is an effective approach to increase pulA gene expression and fermentation enzyme activity.



## 1. Introduction

Pullulanase (EC 3.2.1.41) has been the most extensively studied and industrially applied carbohydrate debranching enzyme. There are two types of pullanases, type I and type II. Type I preferentially and efficiently hydrolyzes the α-(1,6) glucosidic bonds in pullulan and branched polysaccharides, giving maltotriose as the final product [1,2]. Type II, or amylopullulanase, can hydrolyze either α-(1,6) or α-(1,4) glucosidic bonds in pullulan and branched polysaccharides. The final product is maltotriose when hydrolyzing pullulan, whereas the final products are glucose, maltose, and maltotriose when hydrolyzing poly- and oligosaccharides (starch) [3]. Therefore, pullulanase is usually used in combination with α-amylase and glucoamylase to enhance the hydrolysis rate and saccharification rate [4] or with β-amylase to increase the maltose yield [5] in the starch conversion process. However, pullulanase production is low in wild-type strains, and thus, industrially applied pullulanase is solely derived from recombinantly engineered strains.

Heterologous gene expression in engineered bacteria is affected by many factors, such as host strain selection, plasmid copy number, promoter selection, mRNA stability, and codon usage [6]. Many host strains have been used for the heterologous expression of pullulanase, including *Escherichia coli* [7,8,9], *Bacillus flavothermus* [10], *Bacillus licheniformis* [11], *Bacillus subtilis* [12,13], *Brevibacillus choshinensis* [14], *Klebsiella aerogenes* [15], *Pichia pastoris* [16], *Raoultella planticola* [17], and *Saccharomyces cerevisiae* [18], with various plasmids, promoters, or signal peptides. However, improvement in the expression of recombinant pullulanase by increasing mRNA stability has not been reported.

Shine-Dalgarno (SD) sequence and stem-loop structure in the 5′ or 3′ untranslated region (UTR) are well-known determinants of mRNA stability. The SD sequence in the 5′ UTR increases mRNA stability in *Bacillus thuringiensis* [19] and *B. subtilis* [20]. The SD sequence complements the 3′-OH end of the 16S ribosomal RNA and increases the affinity between the ribosomal subunits and the mRNA [19]. A ribosomal subunit bound to the 5′ UTR functions as an mRNA stability

* Corresponding author.
*E-mail address:* qliyou@henau.edu.cn (L. Qiu).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2017.07.001


Please cite this article as: Li T, et al, Improving the expression of recombinant pullulanase by increasing mRNA stability in *Escherichia coli*, Electron J Biotechnol (2017), http://dx.doi.org/10.1016/j.ejbt.2017.07.001

inducer [21] to increase the stability of the gene transcripts and improve protein production in *B. subtilis* [22]. The 5′ stem-loop structure at the 5′ UTR or 3′ stem-loop at the 3′ UTR also increases RNA stability in *E. coli* [23], *B. subtilis* [20], and *B. thuringiensis* [24]. A 5′ stem-loop at the 5′ UTR prevents the degradation of the downstream mRNA by RNase E, PNPase, and RNase J [25,26,27], whereas a 3′ stem-loop at the 3′ UTR blocks the processive activities of the two 3′–5′ exoribonucleases PNPase and RNase II and stabilizes the upstream mRNA [28].

We previously isolated a pullulanase-producing bacterial strain of *Klebsiella variicola* [29] and identified the pullulanase expressed by *E. coli* as type I, encoded by the gene *pulA* (GenBank accession number: KJ146839). The optimal pH and temperature for the enzyme reaction were 5.6 and 45°C, respectively [30]. In the present study, we aimed to increase the levels of pullulanase production by adding a 5′ SD sequence at the 5′ UTR and a 3′ stem-loop structure at the 3′ UTR of *pulA* in *E. coli*.

## 2. Materials and methods

### 2.1. Bacterial strains, DNA, and plasmids

*K. variicola* Z-13 is a wild-type pullulanase-producing strain isolated by the Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, Zhengzhou, stored at the China General Microbiological Culture Collection Center (CGMCC), Beijing, and numbered as CGMCC NO.10357. DNA fragment 3t is a stem-loop structure of the *B. thuringiensis* crystal protein gene (GenBank accession number: M37207.1) located at 2504–2982 bp (5′–3′) with a length of 479 bp and was chemically synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The plasmid used to transform and express the *pulA* constructs in *E. coli* BL21(DE3) was the *E. coli–B. subtilis* shuttle vector pHT43 (MoBiTec, Goettingen, Germany).

### 2.2. Expression vector construction

To construct the *pulA* expression vectors and determine the effects of the SD sequence and stem-loop structure on pullulanase expression, four DNA fragments were cloned by PCR (Fig. 1). The pulA fragment was the coding sequence of *pulA* in *K. variicola* Z-13. The PCR template was the genomic DNA of Z-13, and the primers were pul-F and pul-R (Table 1). The SD-pulA fragment was cloned using the genomic DNA of Z-13 as the template and SD-pul-F and SD-pul-R (Table 1) as the primers. Fragments pulA-3t and SD-pulA-3t were cloned by PCR and enzyme ligation. First, the fragments pulAa and SD-pulAa were cloned by PCR using the genomic DNA of Z-13 as the template and the primer pairs pul-F and pul-3t-R and SD-pul-F and pul-3t-R, respectively. Second, the fragment 3tb was cloned by PCR using the DNA fragment 3t as the template and 3t-F and 3t-R (Table 1) as primers. Third, fragments pulAa, SD-pulAa, and 3tb were digested by the restriction enzyme *Spe I* and then ligated by T4 DNA ligase to generate the fragments pulA-3t and SD-pulA-3t.

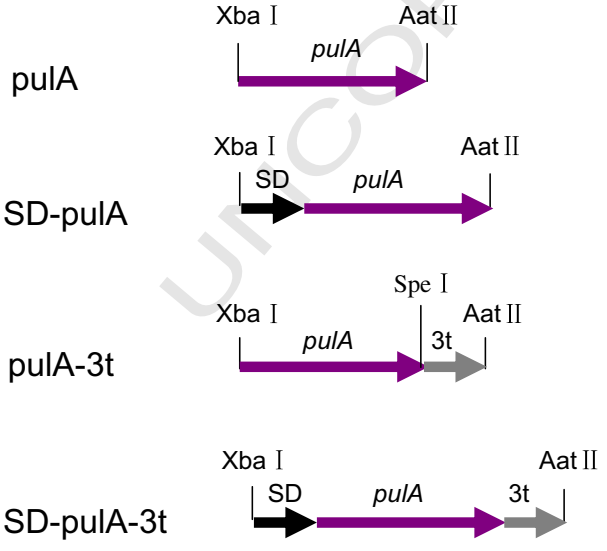


**Fig. 1.** The four DNA fragments cloned into plasmid pHT43 to construct the pullulanase gene *pulA* expression vectors. pulA, coding sequence of the pullulanase gene in *Klebsiella variicola* Z-13; SD, Shine-Dalgarno (SD) sequence; 3t, stem-loop structure of *Bacillus thuringiensis* crystal protein gene.

The four DNA fragments pulA, SD-pulA, pulA-3t, and SD-pulA-3t were cloned into the expression vector pHT43 by enzyme digestion and ligation to construct four pullulanase expression vectors: pHT43-pulA, pHT43-SD-pulA, pHT43-pulA-3t, and pHT43-SD-pulA-3t, respectively.

### 2.3. Transformation and transformant identification

The four pullulanase expression vectors were used to chemically transform competent *E. coli* BL21(DE3) for expression. To screen for the transformants, single colonies growing on LB agar containing 100 mg ampicillin $l^{-1}$ were picked and subcultured on LB agar for five passages. The transformants were identified by colony PCR using the primer pairs pul-F and pul-R (Table 1).

### 2.4. Pullulanase fermentation and enzyme assay

Transformants were inoculated into a 50-ml LB shake flask and cultivated at 200 rpm, 37°C for 2–3 h until $OD_{600}$ reached 0.6; IPTG was added to a final concentration of 0.5 mM to induce the expression of the recombinant pullulanase. Incubation was continued for another 16 h at 20°C, and then the cells and supernatants were separated by centrifugation at 6500 *g* for 15 min at 4°C. The supernatants were stored at 4°C. The cell pellet was suspended in distilled water and sonicated (pulsed on for 3 s, off for 4 s) for 30 min in an ice bath. The crushed cell suspension was centrifuged at 6500 *g* for 30 min at 4°C, and then the supernatants were collected and adjusted to a final volume of 50 ml with distilled water. The protein contents of the crude enzyme extracts were measured by the Bradford method [31]. Pullulanase activity was assayed depending on the concentration of reducing sugars liberated after incubating the pullulanase solution with 0.5% pullulan at 45°C for 30 min. Reducing sugars were quantified by the 3,5-dinitrosalicylate method [32]. One unit of pullulanase activity was defined as the amount of enzyme required to release 1 μM reducing sugars per minute from pullulan under the specified assay conditions [33].

**Table 1**
Primers used in this study.

| Primers | Nucleotide sequence (5′–3′)[a] |
|---|---|
| pul-F | GCTCTAGA**ATG**CTCAGATATACCTGTCATGCCC |
| pul-R | GCCGACGTCAATTATTTACTGCTCACCGGCAGG |
| SD-pul-F | GCTCTAGA**GGAGG**ACAGCT**ATG**CTCAGATATACCTGTCATGCCC |
| SD-pul-R | GCCGACGTCAATTATTTACTGCTCACCGGCAGG |
| pul-3t-R | GCCGACGTCAGGACTAGTCCTTATTTACTGCTCACCGGCAGG |
| SD-pul-3-F | GCTCTAGA**GGAGG**ACAGCT**ATG**CTCAGATATACCTGTCATGCCC |
| SD-pul-3-R | GCCGACGTCAGGACTAGTCCTTATTTACTGCTCACCGGCAGG |
| 3t-F | GGACTAGTCCATTAACTAGAAAGTAAAGAAGTAGTGACC |
| 3t-R | GCCGACGTCAAGCTTACAGAGAAATACACGAGGGC |
| gapA-F | ATGACTATCAAAGTAGGTATCA |
| gapA-R | TTATTTGGAGATGTGAGCGATCAG |

[a] The SD mRNA stabilizing sequence and start codon are indicated in bold, and the *Xba* I, *Aat* II, and *Spe* I restriction enzyme sites are underlined.



Please cite this article as: Li T, et al, Improving the expression of recombinant pullulanase by increasing mRNA stability in *Escherichia coli*, Electron J Biotechnol (2017), http://dx.doi.org/10.1016/j.ejbt.2017.07.001