High-Level Expression of *Bacillus naganoensis* Pullulanase from Recombinant *Escherichia coli* with Auto-Induction: Effect of *lac* Operator

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Abstract

Pullulanase plays an important role in specific hydrolysis of branch points in amylopectin and is generally employed as an important enzyme in starch-processing industry. So far, however, the production level of pullulanase is still somewhat low from wide-type strains and even heterologous expression systems. Here the gene encoding *Bacillus naganoensis* pullulanase was amplified and cloned. For expression of the protein, two recombinant systems, *Escherichia coli* BL21(DE3)/pET-20b(+)-*pul* and *E. coli* BL21(DE3)/pET-22b(+)-*pul*, were constructed, both bearing T7 promoter and signal peptide sequence, but different in the existence of *lac* operator and *lacI* gene encoding *lac* repressor. Recombinant pullulanase was initially expressed with the activity of up to 14 U/mL by *E. coli* BL21(DE3)/pET-20b(+)-*pul* with IPTG induction in LB medium, but its expression level reduced continually with the extension of cryopreservation time and basal expression was observed. However, *E. coli* BL21(DE3)/pET-22b(+)-*pul*, involving *lac* operator downstream of T7 promoter to regulate foreign gene transcription, exhibited pullulanase activity consistently without detected basal expression. By investigating the effect of *lac* operator, basal expression of foreign protein was found to cause expression instability and negative effect on production of target protein. Thus double-repression strategy was proposed that *lac* operators in both chromosome and plasmid were bound with *lac* repressor to repress T7 RNA polymerase synthesis and target protein expression before induction. Consequently, the total activity of pullulanase was remarkably increased to 580 U/mL with auto-induction by *lac* operator-involved *E. coli* BL21(DE3)/pET-22b(+)-*pul*. When adding 0.6% glycine in culture, the extracellular production of pullulanase was significantly improved with the extracellular activity of 502 U/mL, which is a relatively higher level achieved to date for extracellular production of pullulanase. The successful expression of pullulanase with *lac* operator regulation provides an efficient way for enhancement of expression stability and hence high-level production of target protein in recombinant *E. coli*.

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Introduction

Pullulanase (pullulan-6-glucanohydrolase, EC 3.2.1.41) is a kind of enzyme acting on branched substrates, generally used for hydrolysis of glycogen and amylopectin, by cleaving the α-1,6-glucosidic linkages in amylaceous polysaccharides [1-3], and hence belongs to the glycosyl hydrolases (GHase) family 13 that is also termed as the α-amylase family [4]. The most important industrial application of pullulanase is the production of glucose and maltose syrups from starch hydrolysis. Because pullulanase specifically hydrolyzes the branch points in the amylopectin, whereas glucoamylase or β-amylase has only to hydrolyze the linear α-1,4-glucosidic linkages, so using pullulanase in combination with glucoamylase or β-amylase during saccharification process would allow for more efficient and rapid conversion reactions [5,6]. As an industrially important enzyme, therefore, pullulanase is generally employed together with other amyloytic enzymes (α-amylase, β-amylase,
High-Level Expression of Pullulanase

Results and Discussion

Construction of PUL expression systems concerning lac operator

The pullulanase gene pul was amplified by PCR with the specific primers based on the pullulanase-encoding gene sequence (GenBank Accession No. JN872757), using genomic DNA of B. naganoensis as the template. The obtained open reading frame was 2,781 bp in length, encoding 926 amino acids with the predicted molecular weight of 101.4 kDa. To make the extracellular production of the target protein more feasible, pET-20b(+) and pET-22b(+) were selected as the candidate vectors both bearing T7 promoter and signal peptide sequence, but different in the existence of lac operator sequence and lac gene encoding lac repressor. Recombinant plasmids harboring the pullulanase gene pul, pET-20b(+)–pul and pET-22b(+)–pul, were constructed by ligation to the vectors at BamHI and Xhol restriction sites, respectively (Figure 1).

To further confirm the effect of lac operator on the expression of target protein, the lac operator sequence in pET-22b(+)-pul was deleted to generate the mutant plasmid pET-22b(+)–pulΔlac. Specific primers were designed to amplify the DNA fragment (5’-BglII site–T7 promoter–rbs–PelB signal peptide–pul gene–Xhol site–3’) in pET-20b(+)-pul, which contained the same functional sequences as those of pET-22b(+)-pul, except for the lack of lac operator sequence. After digested with BglII and Xhol, the resulted DNA fragment was inserted into pET-22b(+) and the mutant recombinant plasmid pET-22b(+)–pulΔlac was obtained, whose sequence was the same as that of pET-22b(+)-pul, except that the former contained no lac operator sequence (Figure 1).

Expression of PUL in LB medium

When pullulanase gene pul was expressed in LB medium, the phenomenon of unstable expression was firstly observed for the recombinant E. coli BL21(DE3)pET-20b(+)-pul. By the IPTG-induction method in LB medium, the fresh transformant of E. coli BL21(DE3)pET-20b(+)-pul initially expressed PUL with the pullulanase activity of up to 14 U/mL, possessing the optimal temperature of 62.5 °C and optimal pH value of 4.5, which would be an aciduric and thermotolerant enzyme as the wild type and suitable for industrial starch hydrolysis process. However, when using the frozen glycerol stock of E. coli BL21(DE3)pET-20b(+)-pul, less and even no detectable pullulanase activity occurred, namely degeneration of expression strain. From the SDS-PAGE of total protein fractions before and after degeneration (Figure 2), the optimal expression of PUL was obtained when inoculating the recombinant immediately after transformation, while cultivation from the frozen glycerol stock performed the decreased expression level of PUL obviously and even undetected level. In a further research, the pullulanase activity was detected from the recombinant without IPTG induction (data not shown),
indicating the basal expression in *E. coli* BL21(DE3)/pET-20b(+)-*pul*. As reported, leaked expression of target protein frequently happens to *E. coli* expression systems, and basal expression in the pre-induction phase, especially for the T7 expression system, might be detrimental to the host and consequently lead to the issues involving instability of expression system and decreased target protein synthesis [31,32,37].

To further address the contributing factors for decreased expression level of target protein from *E. coli* BL21(DE3)/pET-20b(+)-*pul* with time, a series of the possible issues were taken into account. At first, the recombinant plasmid was recovered from degenerated *E. coli* BL21(DE3)/pET-20b(+)-*pul* strain, followed by restriction enzyme analysis with BamHI and XhoI. Then two DNA fragments were obtained, corresponding to the target gene and vector fragment from pET-20b(+) (Figure 3A). Although the obtained results were not quantitative, they indicated that plasmid loss alone could not account for the reduced level of expression. To address possible plasmid loss more directly, degenerated strains from same stock were plated on LB agar and LB agar containing ampicillin to investigate the ability of forming individual colonies with constant selective pressure for plasmid maintainence, respectively. It was found that the strains formed almost equivalent numbers of colonies on the plates whether or not ampicillin was present. In addition, the plasmid stability of the recombinant strain was also analyzed (Figure 3B). For most of the host cells, the loss of plasmid did not occur during the cryopreservation. Thus, plasmid loss would not be sufficient to be responsible for the observed decrease in protein expression. On the other hand, the recombinant plasmid from the degenerated strain was isolated and reintroduced into the competent *E. coli* BL21(DE3) cells. Then the level of protein production in newly transformed cells was similar to that of the original strain (Figure 3C). Therefore, plasmid loss or mutation would not be a significant cause of decreased target protein production with time.

In our research, there was no clear growth disadvantage to *E. coli* BL21(DE3)/pET-20b(+)-*pul* strain, whether degenerated or not, as assessed by monitoring growth over time. Therefore, except for the lost and mutation of recombinant plasmid, mutation to the host cells, resulting in decreased level of functional T7 RNA polymerase, might be the predominant contributory factor for decreased production of target proteins [38]. As reported, even in the uninduced state and at low basal levels, expression of target protein would lead to polymerase mutations and loss of induction capability by resulted detrimental effect [38]. Hence, effective strategies to avoid loss of expression have been proposed previously based on preventing basal expression [38,31,33]. Therefore, the attempt to control the basal expression in the pre-induction phase
should be critical for successful production of target protein [26,34,37]. Compared to *E. coli* BL21(DE3)/pET-20b(+)-pul, the recombinant *E. coli* BL21(DE3)/pET-22b(+)-pul involves a lac operator placed downstream of T7 promoter to regulate the transcription of foreign gene. When cultured in LB medium, *E. coli* BL21(DE3)/pET-22b(+) pul exhibited no obvious pullulanase activity without IPTG induction, suggesting that there was no detected basal expression. Then the expression stability of *E. coli* BL21(DE3)/pET-22b(+) pul was compared with that of *E. coli* BL21(DE3)/pET-20b(+) pul for the cryopreservation stocks at different times. As shown in Figure 4, corresponding to the previous result, *E. coli* BL21(DE3)/pET-20b(+) pul performed remarkable instability of PUL expression, where the PUL expression level reduced continually with the extension of cryopreservation time. By contrast, *E. coli* BL21(DE3)/pET-22b(+) pul showed the expressed pullulanase activity consistently, indicating the stable expression of target protein. Considering the difference of the regulatory elements between these two expression systems, *E. coli* BL21(DE3)/pET-22b(+) pul and *E. coli* BL21(DE3)/pET-20b(+) pul, it was presumed that the expression stability of *E. coli* BL21(DE3)/pET-22b(+) pul might attribute to the lac operator and the lacI gene in the pET-22b(+) vector.

**Effect of lac operator on PUL expression**

As described above, the expression stability and final amount of the target protein in the involved expression systems would be mainly affected by the regulatory elements including lac operator placed downstream of T7 promoter and the lacI gene encoding lac repressor. The single copy chromosome of the host *E. coli* and the multicopy plasmid pET-22b(+) both contain the lacI gene providing constitutively sufficient lac repressor to saturate all of the lac operators in the cell, and lac repressor only bound to lac operator works to interfere with the transcription elongation [31]. Therefore, lac operator would play the role of improving the expression stability of the recombinant system.

To circumstantiate the effect of lac operator, the mutant plasmid pET-22b(+) pulΔlac was constructed without the lac operator sequence, compared to pET-22b(+)-pul. The basal expression levels of the two systems, *E. coli* BL21(DE3)/pET-22b(+) pul and *E. coli* BL21(DE3)/pET-22b(+) pulΔlac, were compared with LB medium under the same cultural conditions without induction. Protein band of PUL was found in the total protein sample of *E. coli* BL21(DE3)/pET-22b(+) pulΔlac (Figure 5), indicating that the deletion of lac operator in expression plasmid would generate detectable basal expression of foreign protein, which confirmed the contribution of lac operator to the tight transcription control of T7 expression.

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**Figure 3. Analysis of loss and mutation of the recombinant plasmid from *E. coli* BL21(DE3)/pET-20b(+) pul.** (A) Restriction enzyme analysis of the recombinant plasmid pET-20b(+)-pul. Lane M: DNA Marker, Lane 1: double digestion of pET-20b-pul; (B) Plasmid stability during cryopreservation; (C) SDS-PAGE analysis of PUL expression in *E. coli* BL21(DE3)/pET-20b(+)-pul strain. Lane M: protein molecular weight marker, Lane 1: total protein of newly transformed strain, Lane 2: total protein of originally constructed strain. doi: 10.1371/journal.pone.0078416.g003
system. The expression stability of frozen glycerol stocks of these two strains was also investigated (Figure 6). *E. coli* BL21(DE3)/pET-22b(+)-pulΔlac lost its ability to synthesize PUL continually, while *E. coli* BL21(DE3)/pET-22b(+)-pul was able to maintain the expression stability, indicating lac operator actually improved the expression stability of *E. coli* BL21(DE3)/pET-22b(+)-pul.

In the host *E. coli* BL21(DE3), T7 gene encoding T7 RNA polymerase is under control of the inducible lacUV5 promoter and the lac operator in the chromosome DNA, while in the vector the gene encoding the desired protein is transcribed by the T7 promoter regulation, which is recognized by T7 RNA polymerase specifically [37]. Theoretically, in the absence of inducer, the binding of lac repressor to lac operator downstream of lacUV5 promoter greatly decreases the frequency of transcription elongation events by *E. coli* RNA polymerase, and hence the T7 gene stays silent in the uninduction phase [32,37]. Actually, however, T7 RNA polymerase would exhibit the basal activity and lead to the expression of target protein, namely the basal expression of target protein [31,37]. For *E. coli* BL21(DE3)/pET-22b(+)-pulΔlac, because lac operator was deleted from the pul gene-inserted plasmid and no obstacle blocked T7 RNA polymerase to move towards the pul gene in the absence of inducer, even leaked expression of T7 RNA polymerase at a low level could initiate the transcription of the pul gene successfully. The

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**Figure 4.** Effect of cryopreservation time of frozen glycerol stocks on expressed PUL activity in different recombinants. *E. coli* BL21(DE3)/pET-20b(+)-pul and *E. coli* BL21(DE3)/pET-22b(+)-pul were induced at 20 °C with 0.5 mM IPTG when cell turbidity (OD$_{600}$ nm) reached 1.2. Fresh transformants were used for expression of the first time, and simultaneously seed culture of the fresh transformants was cryopreserved as frozen glycerol stocks used for the subsequent expressions.

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**Figure 5.** SDS-PAGE analysis of basal expression of PUL in different recombinants. Lane M: protein molecular weight marker; Lane 1: total protein of *E. coli* BL21(DE3)/pET22b(+); Lane 2: total protein of *E. coli* BL21(DE3)/pET22b(+)-pul; Lane 3: total protein of *E. coli* BL21(DE3)/pET22b(+)-pulΔlac.

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resulted basal expression of PUL might be toxic and detrimental to the host, and consequently make the...
recombinant strain degenerated during cryopreservation and even cause growth inhibition in the pre-induction phase, which would lead to negative effects on the expression and accumulation of target protein in the induction phase after exponential growth of recombinant strain. In comparison, for \( E. \) coli \( BL21(DE3)/pET-22b(+)\)-pul, \( lac \) operator sequence following \( T7 \) promoter in “\( T7lac \)” promoter-involved plasmids also provides a binding site for \( lac \) repressor. Therefore, synthesis of \( T7 \) RNA polymerase initiated from chromosome of \( E. \) coli and expression of target protein generated from recombinant plasmid would be both repressed in the pre-induction phase. It was deduced that \( lac \) operator regulation would significantly affect the basal expression of foreign protein in the host strain, which might be detrimental to the host and consequently lead to the problems of system instability and negative effects on the accumulation of target protein. Then the double-repression strategy can be proposed to potentially reduce basal expression before induction and the detriment of foreign protein to the host, and enhance the production of target protein in induced cells (Figure 7) [31,37].

Expression of PUL with auto-induction

The auto-induction bacterial expression method has been proposed in these years and proved to be generally suitable for producing a wide range of proteins to a high yield [32]. Here the auto-induction method was adopted for production of PUL with the engineered strains \( E. \) coli \( BL21(DE3)/pET-22b(+)\)-pul and \( E. \) coli \( BL21(DE3)/pET-20b(+)\)-pul as the expression donators, respectively. As shown in Figure 8, the total activity of expressed PUL from \( E. \) coli \( BL21(DE3)/pET-22b(+)\)-pul increased obviously during the cultivation process with the final total activity of 580 U/mL, more than 40 folds of that in LB medium. By contrast, \( E. \) coli \( BL21(DE3)/pET-20b(+)\)-pul exhibited somewhat poor ability of PUL synthesis, with the final total activity of 23 U/mL merely. The SDS-PAGE analysis of the total proteins after expression (Figure 9) also showed that high-level expression of PUL was achieved in \( E. \) coli \( BL21(DE3)/pET-22b(+)\)-pul with auto-induction, much higher than that of \( E. \) coli \( BL21(DE3)/pET-20b(+)\)-pul.

Auto-induction method is developed from the regulation of bacteria in utilization of carbon and energy sources in the medium, based on the \( lac \) operon regulatory function. During the initial growth period, glucose is preferentially consumed as the carbon source and catabolite repression caused by the presence of glucose inhibits the uptake of lactose, while the depletion of glucose relieves the catabolite repression and leads to a shift in cellular metabolism to the import and consumption of lactose and glycerol, where lactose is converted to allolactose, the natural inducer of the \( lac \) operator,
initiating the expression of target protein [32,39]. When using *E. coli* BL21(DE3)/pET-22b(+)-*pul*, in the early growth phase, glucose in the medium would block the induction by lactose, and double repression derived from the binding interactions between *lac* repressor and *lac* operator in both chromosome and plasmid would almost eliminate the basal expression of foreign protein and its detriment to the host, so that the recombinant strain could maintain its stability in cell growth and subsequent protein synthesis. When glucose was depleted, the utilization of lactose and glycerol would enable the cells to grow continually and induce the production of PUL. Therefore, with the combined strategy involving double-repression and auto-induction, the activity of recombinant pullulanase was significantly enhanced.

**Extracellular production of PUL by adding glycine**

As known, heterologous protein expressed in secretory recombinant *E. coli* system is generally transported to the periplasmic space by the available signal peptide [40]. In this study, although the expression of PUL has been improved significantly, most of the target protein still accumulated in the periplasm fraction. Glycine, a common medium supplement, has been reported to induce modification of peptidoglycan structure in the cell wall and hence increase cell membrane permeability remarkably for enhanced secretion of desired protein from recombinant *E. coli* [41,42].

To obtain the extracellular PUL, glycine was adopted as a kind of additive and supplemented into the culture to improve the extracellular production of PUL, and the effect of glycine concentration on protein secretion was also investigated. From both the respects of enzyme activity (Figure 10) and protein yield (Figure 11), extracellular production level of PUL from the *E. coli* BL21(DE3)/pET-22b(+)-*pul* was significantly improved when glycine was supplemented in the auto-induction culture, compared with that without glycine addition. Of the investigated glycine concentrations, the extracellular activity of PUL reached 502 U/mL with 0.6% glycine supplemented in the culture, almost 10 times of the control in the absence of glycine. To our knowledge, this is a relatively higher level achieved to date for heterologous expression and extracellular production of pullulanase.

**Conclusions**

Pullulanase specifically hydrolyzing the branch points in the amylopectin is industrially important to be employed to efficiently break down biomass into fermentable sugars for generating biofuels and other chemical commodities. In this
study, recombinant systems harboring the \textit{B. naganoensis} pullulanase gene \textit{pul} were constructed with T7 promoter and signal peptide sequence to facilitate the extracellular production of PUL in high yield. By comparing the expression of PUL in the involved recombinant systems, \textit{E. coli} BL21(DE3)/pET-20b(+)\textit{-pul} and \textit{E. coli} BL21(DE3)/pET-22b(+)\textit{-pul}, it was found that \textit{lac} operator regulation would significantly affect the basal expression of foreign protein in the host strain, which might be detrimental to the host and consequently lead to the problems of system instability and negative effects on the accumulation of target protein. Then double-repression strategy was proposed to potentially reduce the basal expression before induction and the detriment of foreign protein to the host. Thus, \textit{E. coli} BL21(DE3)/pET-22b(+)\textit{-pul} was proved to be stable for PUL synthesis and high-level expression of PUL was achieved with auto-induction. In addition, glycine supplementation in culture further enhanced secretion and extracellular production of PUL with the extracellular activity of 502 U/mL. Therefore, this study would provide an efficient approach for enhancement of the expression stability of recombinant \textit{E. coli} system and hence high-level production of the target protein.

Figure 8. Expressed PUL activity profiles of different recombinants with auto-induction. The PUL activities were compared between the two recombinants, \textit{E. coli} BL21(DE3)/pET-20b(+)\textit{-pul} (circle) and \textit{E. coli} BL21(DE3)/pET-22b(+)\textit{-pul} (square).

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Figure 9. SDS-PAGE analysis of PUL expression in different recombinants with auto-induction. Lane \textit{M}: protein molecular weight marker; Lane 1: total protein of \textit{E. coli} BL21(DE3); Lane 2: total protein of \textit{E. coli} BL21(DE3)/pET20b(+)\textit{-pul}; Lane 3: total protein of \textit{E. coli} BL21(DE3)/pET22b(+)\textit{-pul}.

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Materials and Methods

Strains, plasmids and materials

*B. naganoensis* JNB-1 stored in our lab was used as the source of the pullulanase-coding gene. *E. coli* strains JM109 conserved in our lab and BL21(DE3) purchased from the Novagen Company (USA) were used as the host for gene cloning and expression of target protein, respectively. The plasmids of pET-20b(+) and pET22b(+) were purchased from the Novagen Company (USA), both of which are controlled by T7 promoter and contain PelB signal peptide. The polysaccharide of pullulan for determination of pullulanase activity was purchased from Tokyo Kasei Kogyo Co., Ltd (Japan). Restriction endonucleases, DNA polymerase, and ligase were obtained from TaKaRa Biotechnology Co., Ltd (Dalian, China). The DNA primers and Plasmid Mini Kit were obtained from Sangon (Shanghai, China). All other chemicals are of analytical grade.

Construction of recombinant plasmids

The open reading frame of the pullulanase-encoding gene was amplified using genomic DNA from *B. naganoensis* JNB-1 as the template. The specific pair of primers, PUL-F1 (5'-GAACAGGATCCAGATGGGACAACCAAAAC-3') and PUL-R1 (5'-ATTCCCTCGAGTTTACCACATGGGCT-3'), were used to amplify the target DNA fragment. The amplified DNA fragment was then ligated into the pET-20b(+) and pET22b(+) plasmids to construct the recombinant plasmids pET20b(pul) and pET22b(pul), respectively.

**Figure 10.** Effect of glycine concentration on extracellular activity of expressed PUL from *E. coli* BL21(DE3)/pET22b(+)pul. Glycine was added in the auto-induction culture to the final concentration of 0 (star), 0.6% (circle), 0.9% (triangle), and 1.2% (square).

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**Figure 11.** SDS-PAGE analysis of extracellular PUL production from *E. coli* BL21(DE3)/pET22b(+)pul with glycine at different concentration. Lane M: protein molecular weight marker; Lane 1: extracellular fraction secreted without glycine; Lane 2: extracellular fraction secreted with 0.6% glycine; Lane 3: extracellular fraction secreted with 0.9% glycine; Lane 4: extracellular fraction secreted with 1.2% glycine.

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synthesized based on the nucleotide sequence (GenBank Accession No. JN872757). The restriction sites BamHI and XhoI were incorporated into the forward primer PUL-F1 and the reverse primer PUL-R1, respectively. The condition for PCR was as follows: one cycle at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 60 °C for 30 s, and 72 °C for 2 min 30 s, with an extra extension step at 72 °C for 10 min. Consequently an approximate 2.8 kb fragment was amplified. The PCR product was digested with BamHI and XhoI, and then inserted into the vectors pET-20b(+) and pET-22b(+), resulting in the recombinant plasmids pET-20b(+)-pul and pET-22b(+)-pul, respectively.

For the construction of pET-22b(+)-pulΔlac, the DNA fragment was amplified using a pair of primers PUL-F2 (5’-GAAACAGATCTCGATCCCAGC-3’) and PUL-R2 (5’-ATTCCCCTCGAGTTTACCACGATGCGT-3’), containing BglII and XhoI restriction sites, respectively. Using the plasmid pET-20b(+)-pul as the template, the PCR product of approximate 2.9 kb fragment was obtained, comprising the resulting DNA fragment was inserted into vector pET-22b(+) to generate the desired recombinant plasmid pET-22b(+) -pulΔlac.

The obtained recombinant plasmids, pET-20b(+)-pul, pET-22b(+)-pul, and pET-22b(+)-pulΔlac, were transformed into the expression host E. coli BL21(DE3) for pullulanase expression. The positive transformants were confirmed by PCR.

**Media and growth conditions**

*B. naganoensis* JNB-1 was cultured at 30 °C for 2 days in the medium containing 0.25 g/L CaCl₂, 0.5 g/L MgSO₄·7H₂O, 0.2 g/L (NH₄)₂SO₄, 2 g/L yeast extract, 5 g/L glucose, and 3 g/L KH₂PO₄, with the pH adjusted to 5.0. Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) was used for *E. coli* cultivation and pullulanase expression for the study of system stability. Detection of basal expression was performed in LB medium without IPTG addition. A modified auto-induction medium [25,32], containing 10 g/L β-lactose, 0.5 g/L glucose, 50 g/L glycerol, 6.8 g/L KH₄PO₄, 0.25 g/L MgSO₄, 10 g/L tryptone, 5 g/L yeast extract, 7.1 g/L Na₂HPO₄, 0.71 g/L Na₂SO₄, and 2.67 g/L NH₄Cl, with the pH adjusted to 7.5-8.0, was used for high-level production of pullulanase. If necessary, ampicillin was added to media with the final concentration of 100 μg/mL.

**Expression of PUL in LB medium**

For protein expression in LB medium, *E. coli* BL21(DE3) harboring recombinant plasmid was inoculated into 5 mL LB medium in the presence of ampicillin (100 μg/mL) and incubated at 37 °C and 200 rpm overnight. Then the *E. coli* culture (0.5 mL) was transferred into a 250 mL Erlenmeyer flask containing 50 mL LB medium supplemented with ampicillin (100 μg/mL). The recombinant cells were cultured at 37 °C and 200 rpm, and when the culture turbidity (OD₆₀₀ nm) reached 1.2, 0.5 mM IPTG was added to induce the heterologous expression. The culture was incubated for another 16 h at 20 °C and 200 rpm for the expression of target protein. For the investigation of basal expression, the cultivation and expression conditions were the same as the above, except for no IPTG added into the culture.

**Expression of PUL in auto-induction medium**

For protein expression in auto-induction medium, *E. coli* BL21(DE3) harboring recombinant plasmid was inoculated into 5 mL LB medium in the presence of ampicillin (100 μg/mL) and incubated at 37 °C and 200 rpm overnight. Then the *E. coli* culture (2 mL) was transferred into a 250 mL Erlenmeyer flask containing 50 mL auto-induction medium supplemented with ampicillin (100 μg/mL). After cultivation at 37 °C and 200 rpm for the first 2 h, the culture was incubated at 20 °C and 200 rpm for another 70 h to produce the target protein. For comparing the expression level between the two recombinant systems *E. coli* BL21(DE3)/pET-20b(+) -pul and *E. coli* BL21(DE3)/pET-22b(+)-pul, fresh transformants were used for each cultivation batch to ensure the initial expression ability.

**Expression analysis and activity assay**

The amount of target protein and the expression level were evaluated by combining the results involving activity assay and SDS-PAGE analysis of target protein. The samples for both activity assay and SDS-PAGE analysis were prepared with the same dilution so as to compare the amount of expressed target protein.

The culture was harvested by centrifugation at 12,000 rpm for 10 min at 4 °C, and the supernatant was collected as the extracellular crude sample. The precipitate was washed twice with physiological saline and re-suspended in the same volume of physiological saline as that of the original culture. The suspension was subjected to ultrasonic for 15 min using a VCX750 cell sonifier. The insoluble debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C, and the supernatant was collected as the intracellular crude sample. The molecular mass and the amount of the recombinant enzyme were estimated by 9 % (w/v) sodium dodecyl sulfated-polyacrylamide gel electrophoresis (SDS-PAGE).

Pullulanase activity was assayed by measuring the aldehyde groups released during enzymatic reaction from a mixture consisting of pullulan solution and the diluted enzyme sample [13,14]. The reaction mixture, containing 0.1 mL 2 % (w/v) pullulan in 0.1 M sodium acetate buffer (pH 4.5) and 0.1 mL enzyme solution diluted with 0.1 M sodium acetate buffer (pH 4.5), was incubated at 60°C for 20 min. Then, the amount of released aldehyde groups was assayed with dinitrosalicylic acid (DNS) method by measuring the absorbance at 540 nm spectrophotometrically. One unit of pullulanase activity was defined as the amount of pullulanase that releases 1 μmol of aldehyde groups per min under the reaction conditions. Total activity was defined as the sum of extracellular and intracellular activity. Besides the standard method, the recombinant PUL was diluted with 0.1 M acetate buffer (pH3.0-6.0) to investigate the influence of pH values on the enzymatic activity. For the effect of temperature, enzyme samples were incubated at temperatures ranging from 40 to 70 °C, respectively, for 20 min. All the values of enzymatic activities shown in figures were
averaged from three replicates with standard deviations, and significant differences (p<0.05) were measured.

Analysis of plasmid stability

Analysis of plasmid stability was performed by calculating the ratio of colonies grown on selective and non-selective LB plate medium [43].

Effect of glycine on extracellular production of PUL

To enhance the secretion of pullulanase, glycine was added into the culture when the cultivation temperature was changed from 37 °C to 20 °C. The effect of glycine concentration on extracellular production of PUL was investigated by adding glycine into the culture with the final concentration of 0.6%, 0.9%, and 1.2%, respectively. The culture without glycine addition was set as the negative control.

Author Contributions

Conceived and designed the experiments: YN YX RX. Performed the experiments: WY WBC. Analyzed the data: YN YX RX. Contributed reagents/materials/analysis tools: YN XQM. Wrote the manuscript: YN WY.

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