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Soluble expression of pullulanase from *Bacillus acidopullulyticus* in *Escherichia coli* by tightly controlling basal expression

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Abstract *Bacillus acidopullulyticus* pullulanase (*Ba*Pul13A) is a widely used debranching enzyme in the starch industry. A few details have been reported on the heterologous expression of *Ba*Pul13A in *Escherichia coli* (*E. coli*). This study compares different *E. coli* expression systems to improve the soluble expression level of *Ba*Pul13A. When pET22b(+)/pET28a(+) was used as the expression vector, the soluble expression of *Ba*Pul13A can be achieved by tightly controlling basal expression, whereas pET-20b(+)/pGEX4T2 leads to insoluble inclusion bodies. An efficient process control strategy aimed at minimizing the formation of inclusion bodies and enhancing the production of pullulanase was developed by a step decrease of the temperature in a 5-L fermentor. The highest total enzyme activity of *Ba*Pul13A reached 1,156.32 U/mL. This work reveals that the T7 promoter with *lac* operator and *lac*I gene collectively contribute to the soluble expression of *Ba*Pul13A, whereas either a T7 promoter alone or combined with the *lac* operator and *lac*I gene results in poor solubility. Basal expression in the initial growth phase of the host significantly affects the solubility of *Ba*Pul13A in *E. coli*.

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Keywords *Bacillus acidopullulyticus* · Pullulanase · Inclusion body · Soluble expression · Tightly controlling · Basal expression

Introduction

*Ba*Pul13A is debranching enzyme that specifically hydrolyzes the α-(1,6) glycosidic linkages in pullulan, amylopectin, and related polymers, which is generally used in combination with other amylolytic enzymes in starch processing industry [14, 24]. The current industrial production of *Ba*Pul13A is expressed in *Bacillus licheniformis*. A few details have been reported on the heterologous expression of *Ba*Pul13A in *E. coli*. A previous study in our laboratory indicated that the soluble expression of *Ba*Pul13A is difficult to achieve in *E. coli* with the major challenge being the aggregation tendency and the subsequent formation of inactive inclusion bodies (IBs). Efforts to recover protein from inclusion bodies by refolding enhancement are time consuming, and a high expression rate is rarely guaranteed [2, 21]. More work is thus required to increase the level of soluble expression of *Ba*Pul13A in *E. coli*.

It is not just *Ba*Pul13A, also the production of insoluble recombinant proteins is a common phenomenon in *E. coli*, which still presents a major obstacle in industrial production [3, 4, 7, 20]. IBs are widely considered as the nonspecific aggregation of incompletely folded or partially denatured polypeptides [16, 23, 26]. IBs often occur as a stress response when recombinant protein is expressed at high rates [8, 19, 22]. Limited information is known about the formation mechanism of IBs [9]. Strategies that guide the soluble production of proteins in *E. coli* have become increasingly attractive. Several methods have been proposed for the efficient production of soluble proteins in *E.*

coli, including the use of some mutated host strains, co-production of chaperon and foldase, lowering of the cultivation temperature, and addition of a fusion partner [11, 15, 27]. With all these efforts, a universal approach that can be applicable to resolve this solubility issue in recombinant protein expression has yet to be developed, except for the strenuous trial and error method [1].

This work focuses on a pullulanase from the bacterium *Bacillus acidopullulyticus* and examines the soluble expression of *Ba*Pul13A in *E. coli*. We investigated the effects of promoters and tags on the soluble expression of *Ba*Pul13A through different *E. coli* expression systems without the engineering of *Ba*Pul13A. This work provides a rational option for producing soluble and active recombinant *Ba*Pul13A in *E. coli*.

Materials and methods

Strains, plasmids, and materials

The pullulanase gene from *Bacillus acidopullulyticus* (Accession No. Ax203843.1) was synthesized by Invitrogen (Shanghai, China). The plasmids pET-20b(+), pET-22b(+), pET-28a(+) (Novagen, USA), and pGEX4T4 (Pharmacia, USA) were used as expression vectors. *E. coli* BL21(DE3) and BL21 (Novagen, USA) were the expression hosts. Restriction enzymes, Q5 High-Fidelity DNA Polymerase, and T4 DNA ligase were purchased from NEB (MA, USA). Medium for bacterial culture, antibiotics and expression inducers were purchased from Sangon (Shanghai, China). Scaled-up fermentation was implemented using a 5-L fermentor (Applikon, Holland).

Plasmid construction

The pMD18-T-Pul13A plasmid with a full-length *Ba*Pul13A gene was used as the template in the polymerase chain reaction to construct the pET-20b(+)-*Ba*Pul13A and pET-22b(+)-*Ba*Pul13A expression plasmids. Primers (Table 1) were synthesized to (i) amplify the entire coding sequence of *Ba*Pul13A, as well as (ii) introduce a *Hin*dIII restriction site at the 5′ end and a *Xho*I site at the 3′ end of the coding sequence. The PCR product was inserted into the *Hin*dIII and *Xho*I site of the expression plasmid. The resulting plasmids were verified by restriction digesting and sequencing from both ends of the inserted fragment. A similar method was performed to construct the pET-28a(+)-*Ba*Pul13A and pGEX4T2-*Ba*Pul13A expression plasmids, except that the 5′ end of the latter two vectors was a *Bam*HI restriction site. The T7 promoter in pET-22b(+)-*Ba*Pul13A was replaced with a Tac promoter to confirm the effect of the T7 promoter in the context of the soluble expression of *Ba*Pul13A. All recombinant plasmids were introduced into BL21(DE3), except for pGEX4T2-*Ba*Pul13A, which was introduced into BL21.

Culture media and conditions

The cells with plasmids were aerobically cultured at 37 °C and 200 rpm circular shaking for 8 h in LB medium with 100 μg/mL ampicillin or 50 μg/mL kanamycin (pET-28a(+)-Pul13A) for seed cultivation. The seed culture was then diluted (volume ratio 1:100) into 50 mL LB media in a 500-mL Erlenmeyer flask. Pre-induction culture was incubated at 37 °C for 2 h, then 30 °C for 1 h, and then 23 °C for 1 h with 200 rpm circular shaking to achieve the OD_{600} biomass ranged at 0.5–1.0 before 0.1 mM of isopropyl-β-D-thiogalactoside (IPTG) was added. Post-induction culture was incubated at 16 °C and 160 rpm circular shaking for 60 h. A modified TB medium was used in a 5-L fermentor. The modified TB medium contains the following (in g/L): tryptone (24.00), yeast extract (48.00), KH_2PO_4 (2.31), K_2HPO_4 (9.85), glycerol (25.00), and pH 7.0 (25 % ammonia water and 17 % H_3PO_4 were used for the pH controlling). The glycerol feeding medium (in g/L): glycerol (714.0), yeast extract (50.0), $(NH_4)_2SO_4$ (25.0). For pullulanase production, 5 % (v/v) seed culture was seeded into the modified TB medium.

Table 1 Nucleotide sequences

Name	Nucleotide sequences (5′ → 3′)[a]
*Hin*dIII-Pul	5′-CCC**AAGCTT**GATTCTACTTCGACTAAAGTTATTGTTC-3′
Pul-*Xho*I	5′-CCG**CTCGAG**TTGTTTGAGAATAAGCGTACTTATAGC-3′
*Bam*HI-Pul	5′-CGC**GGATCC**GATTCTACTTCGACTAAAGTTATTGTTC-3′
T7 → Tac[b]	F: 5′-GATCTCGATCCCGCGAAATTTGACAATTAATCATCGGCTCGTATAATGGGGGAATTGTGAGCGGATAACAATTCCCCT-3′
	R: 5′-CTAGAGGGGAATTGTTATCCGCTCACAATTCCCCCATTATACGAGCCGATGATTAATTGTCAAATTTCGCGGGATCGA-3′

[a] Nucleotides in bold font show the restriction sites

[b] Underlined fonts show the Tac promoter

Fed-batch cultivation was performed in a 5-L fermentor (Applikon, Holland) consisting of 3 phases. The first phase was a batch phase characterized by the continuous decrease in dissolved oxygen (DO) value and glycerol. The temperature was maintained at 30 °C in this phase. The end of the glycerol consumption was detected by the DO spiking. The feeding phase I (pre-induction) then started. The feeding rate of the glycerol feeding medium was 6.0 mL/(h L). The second phase lasted for 3 h, and the temperature decreased from 30 to 20 °C. When the culture temperature reached 20 °C, 0.1 mM IPTG was injected. The feeding phase II (post-induction) began with a 3.5 mL/(h L) medium flow rate. Antifoam was then added manually. The circular shaking speed was adjusted in real time between 300 and 1,000 rpm at a 1 vvm air flow rate to maintain the DO level at 30 % of saturate air. Fermentation samples in a time series were collected to monitor the OD_{600}, dry cell weight, and enzyme activity.

Cell fractionation

The 1.0 mL culture was harvested through centrifugation at 4,000×*g* and 4 °C for 30 min. The supernatant was collected and defined as the extracellular fraction. The cells were lysed in 1.0 mL 10 mM PBS (pH 7.4) by a sonicator (25 % amplitude, 2 s pulse with a 2 s interval between pulses, 6 min in total) on ice after the freeze–thaw cycle was performed thrice. The soluble fraction was isolated by centrifugation at 12,000×*g* and 4 °C for 10 min. The cell pellet was obtained as the insoluble intracellular fraction and resuspended in 1.0 mL 1 % SDS buffer.

Transmission electron microscopy

Morphometric analyses were performed with a Hitachi H-7650B (Hitachi, Japan) transmission electron microscope (TEM) according to Wu et al. [28]. The cells in the culture broth with 6 h of IPTG incubation were collected and rinsed once with 10 mM PBS (pH 7.4). The samples were processed according to Wu et al.

Enzyme activity

The pullulanase activity was measured in 100 mM sodium acetate buffer (pH 5.0) using 1 % (w/v) pullulan as the substrate at 60 °C for 10 min. The method was modified from Lappalainen et al. [10]. The modifications are as follows: 0.1 mL of the diluted enzyme solution was added to 0.2 mL of 1 % pullulan preheated at 60 °C for 5 min. The mixture was then incubated at 60 °C for a further 10 min. The reaction was stopped by the addition of 0.45 mL of 3,5-dinitrosalicylic acid (DNS) solution. The mixture was then heated for 7 min in a boiling-water bath. The mixture was then placed in an iced-water bath, and up to 15.0 mL of ddH_2O was added. Light absorbance was measured at 540 nm. One unit of enzyme activity is defined as the amount of enzyme required to release 1.0 μmol of aldehyde groups per minute at 60 °C when the pH is 5.0.

Enzymatic properties

The optimum temperature for pullulanase was determined by assaying the enzyme activity in buffers of different pH values ranging 3.0–8.0 at 60 °C. The optimum temperature for pullulanase was determined by assaying the enzyme activity in a 100 mM sodium acetate buffer (pH 5.0) at different temperatures (30–80 °C). k_m was determined by a Lineweaver–Burk plot. k_{cat} was obtained using the equation $k_{cat} = V_{max}/[E]$, where $[E]$ is the molar concentration of the enzyme. The pullulan was prepared at a concentration between 1 and 10 mg/mL in a 100 mM sodium acetate buffer (pH 5.0). *Ba*Pul13A was purified with an AKTA purifier system for detailed analysis. The enzyme concentration was measured using the Bradford method with bovine serum albumin as standard.

Results and discussion

Expression of pET-20b(+)-Pul13A/pGEX4T2-Pul13A leads to insoluble inclusion bodies

The proportion of pullulanases in total cell protein (TCP) kept going up with the increase of cultivation time post-IPTG induction, except for lane 8 in Fig. 1a. But the target protein was not detected in soluble fraction of TCP (Fig. 1b), and no enzyme activity was detected as well. These results suggest that the expression of *Ba*Pul13A in *E. coli* has an extremely low solubility, and the protein is predominantly in IB form. Morphometric analyses were performed with a Hitachi H-7650B TEM to confirm the said speculation. A comparison between BL21(DE3)-pET-20b(+) and BL21(DE3)-pET-20b(+)-*Ba*Pul13A was performed at the same condition. The TEM image of Fig. 2b clearly shows that the newly formed IBs occupy the cell poles, which are hardly observed in Fig. 2a. The results also demonstrate that IB is a dynamic entity within the cell that tends to migrate to the poles. Some TEM images show that several IBs are located in the middle of the cells, whereas most IBs are located at the poles (Fig. 2c). After 54 h of induction, the amount of recombinant enzyme decreased (Fig. 1a, lane 8) as the cells grew, which can be degraded by proteases. These two observations are consistent with Rokney's work [18]. Given that the GST tag is known to promote solubility of heterologous proteins, the BL21-pGEX4T2- *Ba*Pul13A expression system was tested. A high expression level with very low solubility was observed similar to that of BL21(DE3)-pET-20b(+)-*Ba*Pul13A (Fig. 2d).

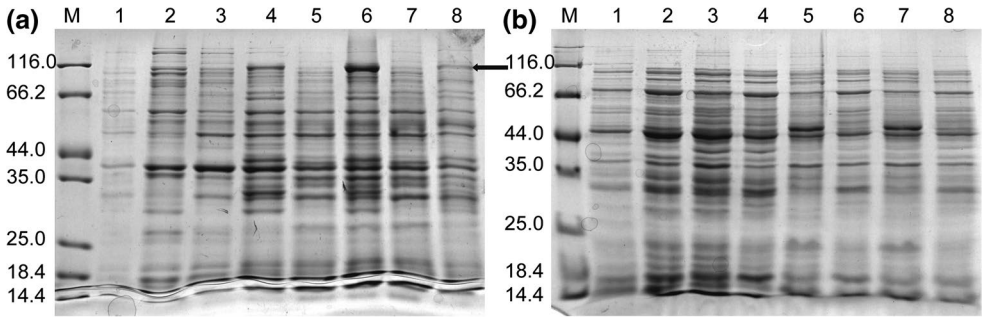

Fig. 1 SDS-PAGE analysis of BL21(DE3)-pET-20b(+)-*Ba*Pul13A. **a** TCP, **b** soluble fraction of TCP; *lanes 1*, *3*, *5*, and *7* BL21(DE3)-pET-20b(+); *lanes 2*, *4*, *6*, and *8*: BL21(DE3)-pET-20b(+)-*Ba*Pul13A; *Lanes 1* and *2*: before induction; *Lanes 3* and *4*: 24 h after IPTG induction; *Lanes 5* and *6*: 48 h after IPTG induction; *Lanes 7* and *8*: 54 h after IPTG induction. The *arrow* indicates the target protein

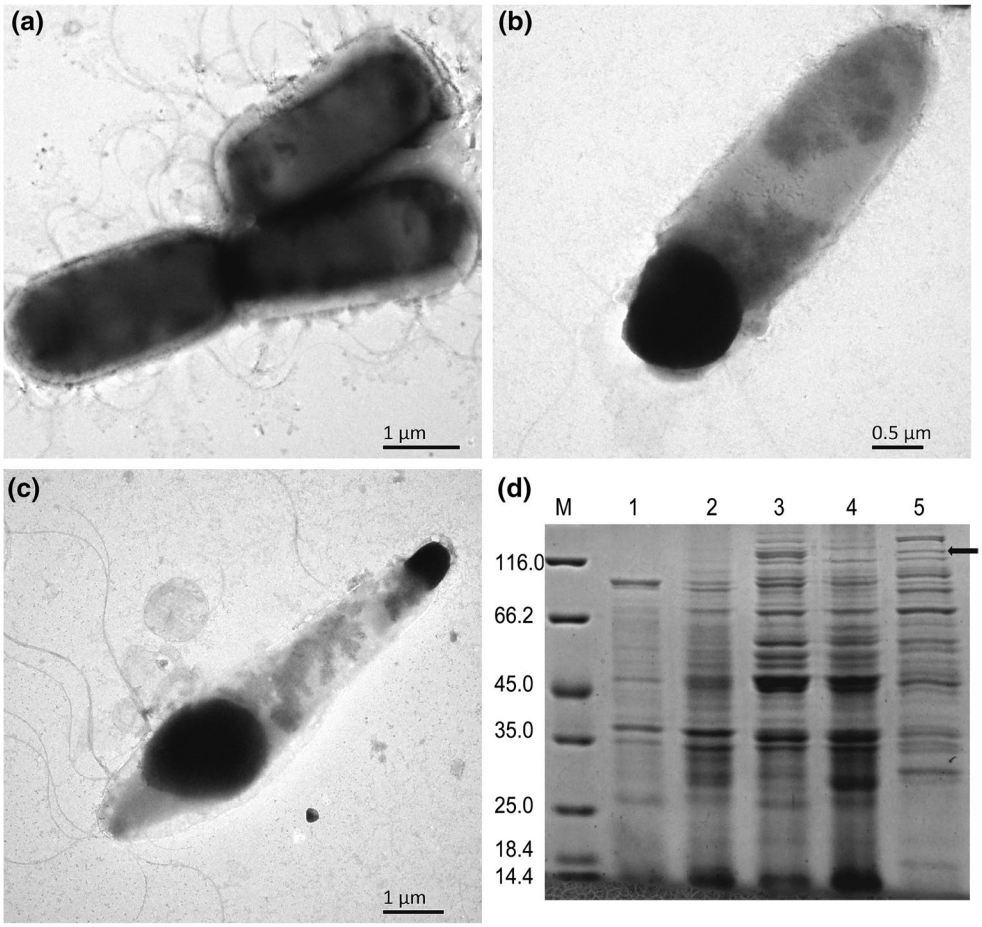

Fig. 2 **a** Morphometric analysis of BL21(DE3)-pET-20b(+), **b** and **c** BL21(DE3)-pET-20b(+)-*Ba*Pul13A, and **d** SDS-PAGE analysis of BL21-pGEX4T2-*Ba*Pul13A. **d** *Lane 3*: TCP fraction after induction for 6 h; *Lane 4*: soluble fraction of TCP; *Lane 5*: insoluble fraction of TCP. The *arrow* indicates the target protein

Expression of pET-22b(+)-*Ba*Pul13A/pET-28a(+)-*Ba*Pul13A leads to sufficient solubility and functional activity

Soluble *Ba*Pul13A was detected when pET-22b(+) or pET-28a(+) was used (Fig. 3). The recombinant *Ba*Pul13A was tested as functionally active, and the activity of the shaking flask culture was 4.07 ± 0.23 U/mL/OD_{600}. The expression level of the BL21(DE3)-pET-28a(+)-*Ba*Pul13A system was comparable with that of the BL21(DE3)-pET-22b(+)-*Ba*Pul13A system.

Unlike pET-20b(+), pET-22b(+) and pET-28a(+) have a T7*lac* promoter and coding sequence for the *lac* repressor (*lac*I) used to control the basal expression. Lane 2 in Fig. 1a shows that in the absence of IPTG, basal expression of the target protein was observed. This result was not

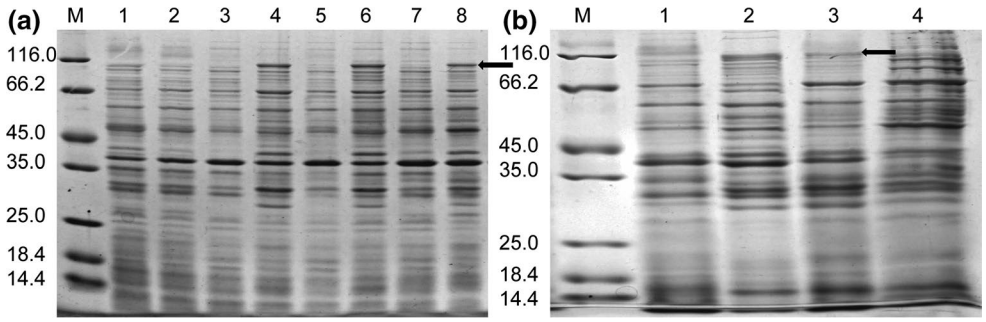

Fig. 3 SDS-PAGE analysis of BL21(DE3)-pET-22b(+)-*Ba*Pul13A. **a** TCP. *Lanes 1*, *3*, *5*, and *7* BL21(DE3)-pET-22b(+); *lanes 2*, *4*, *6*, and *8*: BL21(DE3)-pET-22b(+)-*Ba*Pul13A; *lanes 1*and *2*:before induction; *lanes 3* and *4*: 24 h after IPTG induction; *lanes 5* and *6*: 48 h after IPTG induction; *lanes 7* and *8* 54 h after IPTG induction. **b** (BL21(DE3)-pET-22b(+)-Pul13A). *Lane 1* prior IPTG induction, *Lane 2*: TCP after IPTG induction; *lane 3*: soluble fraction after IPTG induction; *lane 4*: insoluble fraction after IPTG induction. The *arrow* indicates the target protein

observed on lane 2 in Fig. 3a. Most of the induced expressions of the recombinant protein impose additional stress to the host cell and subsequently causes cells to demonstrate different toxication morphologies. Morphometric analyses (Fig. 2a–c of BL21(DE3)-pET-20b(+)-*Ba*Pul13A show a change in cell morphology after induction associated with decreased cell growth rate. This result is evidenced by the cell density measurement at OD_{600}.

Effect of the T7 promoter on the soluble expression of *Ba*Pul13A

The pullulanase gene was unexpressed with solubility in BL21-pGEX4T2-Pul13A, although the pGEX4T2 vector also had a *lac* operator and repressor identical to those of pET-22b(+). The basal expression of *Ba*Pul13A can be caused by the Tac promoter induced by the *E. coli* RNA polymerase. The Tac promoter was used to replace the T7 promoter in BL21(DE3)-pET-22b(+)-*Ba*Pul13A to test the effect of T7 promoter on *Ba*Pul13A solubility. The pullulanase expression predominantly remained insoluble and in IB trapped form. Thus, the T7*lac* operator in pET-22b(+) provided a *lac* repressor binding site, which inhibits T7 RNA polymerase transcription activities in the absence of an inducer. pET-22b(+) also carries a *lac*I gene that provides a *lac* repressor that is effective on T7 promoters on both the vectors and genome, where the lacUV5 promoter regulates the T7 RNA polymerase [6, 13]. The basal expression of *Ba*Pul13A in pET-22b(+) effectively decreased and thus improved the solubility of the protein in fermentation.

However, this phenomenon is somehow protein-dependent, and only applies to a special group of proteins. Previous studies have shown that the target proteins were expressed in soluble form without the formation of IBs when pET-20b(+) were used as vectors [5, 25, 29]. The expression of recombinant proteins usually induces a stress response for host cell. The stress response of host was also provoked by the basal expression of *Ba*Pul13A. This stress response seriously affected the initial growth of the host, which ultimately resulted in the accumulation of target proteins into inclusion bodies in pET-20b(+). The effect of this stress response declined while the expression was induced in the log phase. Thus, pET-28a(+)-BaPul13A had fewer IBs than pET-20b(+)-BaPul13A, because the basal expression was tightly controlled by pET-28a(+) before induction (the initial growth phase of the host). This inference is further supported by the fact that when the expression was induced in the late log phase, the insoluble fraction was less than that of the early log phase. We assume that these sort of stress responses can cause a series of signal transduction within cells, but the mechanism is still unknown.

Effect of hosts and process conditions on the soluble expression of *Ba*Pul13A

The *Ba*Pul13A gene has 51 rare codons. The gene expression containing rare codons can provide translational errors that interfere with the protein biosynthesis [12]. Rosetta(DE3) was tested as a host to remedy the codon bias. Culture conditions, including temperature, IPTG concentration, and chemical chaperons were also optimized. No significant improvement on the soluble expression was observed under these strategies when expressing *Ba*Pul13A in pET-20b(+). On the contrary, the temperature and IPTG concentration significantly altered the expression solubility in the BL21(DE3)-pET-28a(+) system.

The low temperature in induction significantly improved the observed enzyme activities (Fig. 4a), which implies that the protein is better folded and more soluble. The pullulanase activity reached 3.27 U/mL/OD_{600} at an induction temperature of 16 °C. A high induction

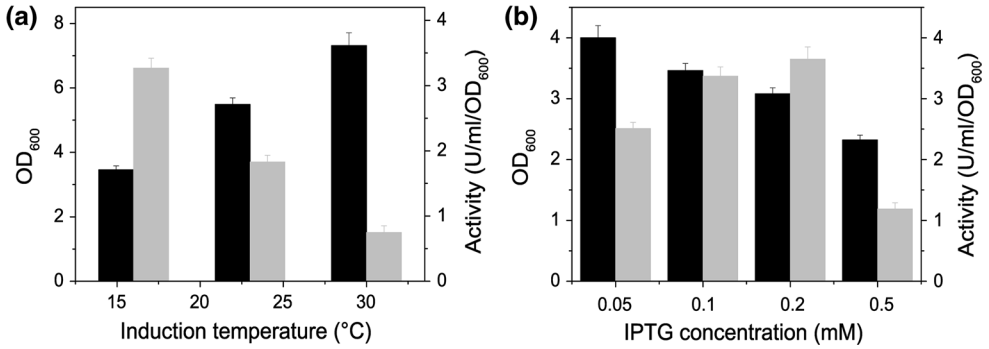

Fig. 4 Effect of the temperature and IPTG concentration on the solubility of *Ba*Pul13A in BL21(DE3)-pET-28a(+)-*Ba*Pul13A. OD_{600}(*black*), pullulanase activity (*gray*). **a** Comparison of cell growth and pullulanase activity at different temperatures. The *E. coli* culture was induced when $OD_{600} = 0.6$ with 0.1 mM IPTG for 20 h. **b** Comparison of the cell growth and pullulanase activity at different IPTG concentrations. The *E. coli* expression was induced at 16 °C and $OD_{600} = 0.6$ for 20 h

temperature facilitated the high biomass, and also affected the soluble expression of *Ba*Pul13A. The total activity of *Ba*Pul13A reached the highest level of 11.30 U/mL at 16 °C than at other temperatures. Figure 4b shows that the biomass (OD_{600}) decreased with the increasing IPTG concentration. The total activity reached the highest level at 0.1 mM IPTG, whereas the best IPTG concentration was 0.2 mM.

Expression of the soluble active form of *Ba*Pul13A was facilitated by a step decrease in temperature in a 5-L fermentor

BL21(DE3)-pET-28a(+)-*Ba*Pul13A was used to scale up the fermentation. The His-tag was recombined in both flanks of the target protein sequence to aid in the purification. The direct use of the glycerol stocks of BL21(DE3)-pET-22b(+)-*Ba*Pul13A was sometimes unstable. New plasmid extraction and electroporation processes had to be performed to produce *Ba*Pul13A in this case. The supernatant expression is significantly affected by the permeability of the *E. coli* membrane (which may be influenced by the freezing process). This observation is consistent with the work of Reetz et al. [17].

Figure 5 shows that the maximum dry cell concentrations in the fermentor reached 42.3 g/L and the corresponding OD_{600} was 87.6. The *Ba*Pul13A expression continuously increased and reached the maximum yield of 1,156.32 U/mL after induction for 28 h. Pullulanase activity then dropped rapidly for cell autolysis. Low yield, which is mainly caused by the formation of IBs upon gene overexpression, is the major limiting factor for the large-scale production of pullulanase. In this work, an efficient recombinant *E. coli* process control strategy that attempts to decrease the formation of IBs and enhance the production of pullulanase was developed. The developed feeding strategy can be useful for the production of a soluble form of other recombinant proteins in *E. coli.*

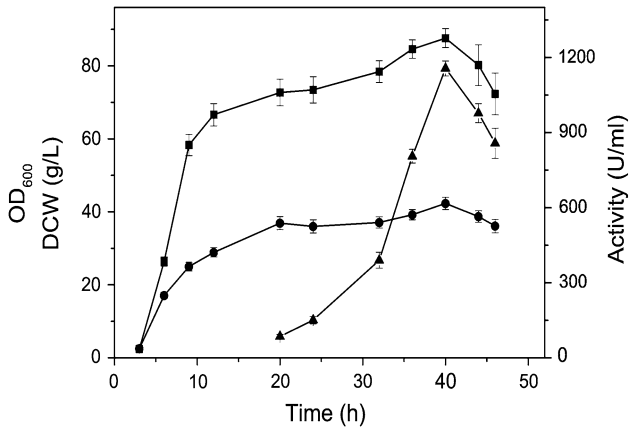

Fig. 5 Time profiles for the fed-batch cultivation of *Ba*Pul13A in a 5-L fermentor. OD_{600} (*closed square*), dry cell weight (*closed circle*), enzyme activity (*closed triangle*)

Characterization of *Ba*Pul13A

The following assays were performed with purified enzyme at a fixed starting level of 40 U/mL. *Ba*Pul13A displayed optimum activity at 60 °C, and the optimum pH was 5.0 (Fig. 6). The kinetic parameters are shown in Table 2.

Conclusion

This work reports that basal expression can lead to *Ba*Pul13A accumulation into IBs in pET-20b(+). The soluble expression of *Ba*Pul13A in *E. coli* can be achieved by tightly controlling the basal expression in pET-28a(+). This difference is caused by the different effects of basal

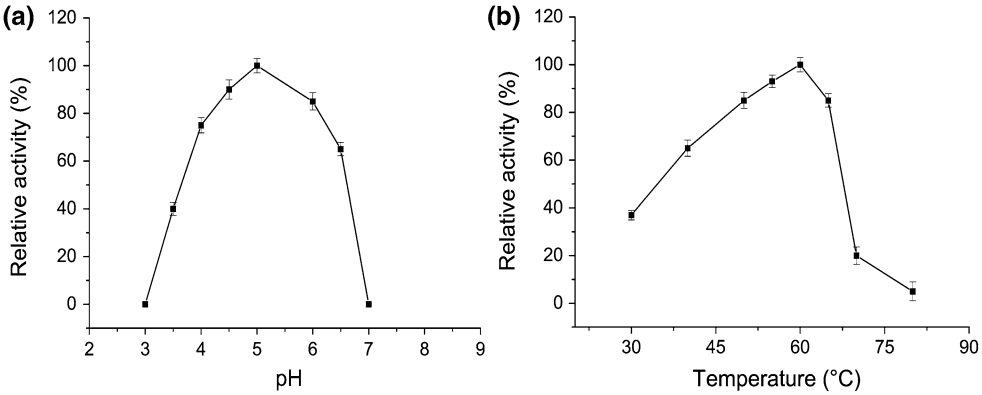

Fig. 6 Enzymatic properties of *Ba*Pul13A. **a** Optimum pH. Buffers used were 50 mM acetate buffer (pH 3.0–6.0) and 50 mM phosphate buffer (pH 6.0–8.0). **b** Optimum temperature. Enzyme activities were expressed as percentages, and the highest activity was defined as 100 % in (**a**) and (**b**). *Error bars* correspond to the standard deviations from three independent measurements

Table 2 Kinetic parameters of *Ba*Pul13A

Enzyme	k_m (mg)	k_{cat} (s^{-1})	k_{cat}/k_m (mg/(ml s))	Specific activity (U/mg)
*Ba*Pul13A	0.45 ± 0.01	3,050.7 ± 102.3	6,770.7 ± 253.8	240.8 ± 12.3

expression on different growth phases. To our knowledge, this study is the first report to illustrate the relationship between basal expression and IB formation. The cultivation process was optimized to reach the overproduction of soluble pullulanases in *E. coli*. The yield was highest at 16 °C and 0.1 mM IPTG in a shake flask. The cultivation process was controlled by a step decrease in temperature to further enhance the production of pullulanases in 5-L fermentor. The highest total enzyme activity of *Ba*Pul13A reached 1,156.32 U/mL.

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Conflict of interest The authors declare that they have no conflict of interest.

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