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Optimization of pullulanase production in *Escherichia coli* by regulation of process conditions and supplement with natural osmolytes

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HIGHLIGHTS

- Low induction strength and betaine favored the soluble expression of pullulanase.
- The optimized process gave an 8.3-fold improvement in pullulanase activity.
- The pullulanase activity represented the highest yield reported so far.
- The process showed potential application in the production of pullulanase.

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ABSTRACT

In this study, the effects of temperature, IPTG (Isopropyl β-D-1-thiogalactopyranoside) concentration, and osmolytes (proline, K-glutamate, and betaine) on cell growth and soluble pullulanase productivity of recombinant *Escherichia coli* were investigated. The yield of soluble pullulanase was found to be enhanced with decrease in cultivation temperature, lower IPTG concentration, and betaine supplementation in a shake flask. In addition, a modified two-stage feeding strategy was proposed and applied in a 3-L fermentor supplied with 20 mM betaine, which achieved a dry cell weight of 59.3 g L^{-1}. Through this cultivation approach at 25 °C, the total soluble activity of pullulanase reached 963.9 U mL^{-1}, which was 8.3-fold higher than that observed without addition of betaine at 30 °C (115.8 U mL^{-1}). The higher expression of soluble pullulanase in a scalable semisynthetic medium showed the potential of the proposed process for the industrial production of soluble enzyme.

1. Introduction

Pullulanase (EC 3.2.1.41) is a well-known starch-debranching enzyme, which catalyzes hydrolysis of the α-1,6 glycosidic linkages in pullulan and amylopectin, and the α- and β-limit dextrins of amylopectin. This enzyme can be used to break down starch to produce glucose, fructose, maltose syrups, cyclodextrins, and amylose, in conjunction with or without α-amylase, β-amylase, glucoamylase, or cyclodextrin glucosyltransferase, which could dramatically increase the yield of sugars and reduce the reaction time (Chen et al., 2013; Roy and Gupta, 2004). Recently, pullulanase has been used as a processing aid for the production of fuel ethanol, low-calorie beers, resistant starch, maltotriose, etc. (Hii et al., 2012; Shi et al., 2013). With the increasing commercial interest in potential industrial applications, pullulanase production has drawn extensive attention in recent years (Li et al., 2012; Zhang et al., 2013).

Pullulanase was first isolated from a culture of *Klebsiella pneumoniae* by Wallenfels et al. in 1966. Until now, many microorganisms, including mesophiles, thermophiles, and hyperthermophiles, have been identified as pullulanase producers, and numerous pullulanases have been identified and characterized (Ben Messaoud et al., 2002; Gomes et al., 2003). In addition, the crystal structure of the enzyme has been solved (Turkenburg et al., 2009). As it is difficult to directly obtain the desirable pullulanase-producing microbes of high efficiency from natural sources, attempts have been made to overexpress pullulanase by DNA recombinant technology, using *Pichia pastoris* and *Escherichia coli* (*E. coli*) as expression hosts (Ayadi et al., 2008; Bertoldo et al., 2004; Chen et al., 2013; Kang et al., 2011).

However, studies have shown that the yield of recombinant pullulanase is low, and the highest yield in *E. coli* expression system ever reported was 180 U mL^{-1} (Chen et al., 2013). Currently, low yield and productivity is the major limiting factor for large-scale production and widespread application of pullulanase. Previously, an *E. coli* expression system consisting of the *pul*A gene encoding pullulanase, obtained from *Bacillus deramificans*, was

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constructed in our laboratory (Duan et al., 2013). Initial study showed that the recombinant enzyme was mainly found in the insoluble fraction, and that only a small proportion was soluble and active (data not shown).

Generally, the formation of insoluble inclusion bodies (IBs) in the cytoplasm or periplasm upon gene overexpression is the major cause of the low productivity of recombinant protein in *E. coli* expression system (Baneyx, 1999; Jhamb and Sahoo, 2012; Le et al., 2011; Pan et al., 2003). Several strategies have been exploited to reduce the formation of IBs in vivo, including (a) lowering the growth temperature and concentration of the inducer, (b) enabling secretion of recombinant proteins into the periplasm, (c) co-expressing chaperone genes, and (d) increasing the concentration of viscous osmolytes (Diamant et al., 2003).

In the present study, an efficient recombinant *E. coli* process control strategy aimed to reduce the formation of IBs and enhance the production of pullulanase was developed, and the yield of pullulanase obtained represents the highest yield reported so far. The developed optimal feeding strategy may be useful for the efficient production of the soluble form of other recombinant proteins in *E. coli*.

2. Methods

2.1. Strain and media

The recombinant *E. coli* BL21 (DE3) harboring plasmid *pul*A/pET20b(+), in which the *pul*A gene encoding the pullulanase (Accession number CAC60157) from *B. deramificans*, was constructed previously in our laboratory (Duan et al., 2013). For seed cultivation, LB media supplemented with 100 μg mL^{-1} ampicillin were used. LB media consisted of (g L^{-1}): tryptone 10, yeast extract 5, and NaCl 10. TB was employed in all shake-flask cultures. The TB medium contained (g L^{-1}): yeast extract 24, tryptone 12, glycerol 5, KH_2PO_4 2.31, K_2HPO_4 16.43, and ampicillin 100 μg mL^{-1}. The medium for pullulanase production in 3 L fermentor was modified semisynthetic medium (Neubauer et al., 2007), which contains (g L^{-1}): tryptone 30.0, NaCl 5.0, yeast extract 20.0, Na_2SO_4 2.0, $(NH_4)_2SO_4$ 2.5, NH_4Cl 0.5, $(NH_4)_2$–H–citrate 1.0, K_2HPO_4 14.6, $NaH_2PO_4{\cdot}2H_2O$ 3.6, $MgSO_4{\cdot}7H_2O$ 2.0, thiamine 100 mg L^{-1}, glycerol 8.0, and trace metal solution 1.0 mL L^{-1}, pH 7.0. The feeding solutions contained (g L^{-1}): glycerol 500, $MgSO_4{\cdot}7H_2O$ 3.4, yeast extract 50, tryptone 50. The induction medium contained 200 g L^{-1} lactose.

2.2. Culture conditions

2.2.1. Shake-flask

To express the enzymes, 100 μL of frozen glycerol stock (kept at −80 °C) was inoculated into 50 mL LB medium containing 100 μg mL^{-1} ampicillin in a 250 mL flask and grown at 37 °C in a rotary shaker at 200 rpm. The overnight seed culture was then diluted (1:25) into 50 mL of TB medium containing 100 μg mL^{-1} ampicillin in a rotary shaker (200 rpm) at 37 °C until an optical density (OD) at 600 nm of 1.0 was reached. IPTG was added to induce the expression of the target protein. Incubation was continued for another 24 h at 25 °C unless otherwise stated. To investigate the effect of osmolytes on the growth of *E. coli* or soluble production of recombinant pullulanase, osmolytes were supplemented to the culture medium (2 h before IPTG addition) unless otherwise stated. At certain time intervals, samples were collected and analyzed for OD_{600}, dry cell weight (DCW) and enzyme activities.

2.2.2. Bioreactor

The seed culture was started by inoculating 100 μL of frozen glycerol stock (kept at −80 °C) and cultured for 8 h in 50 mL of LB medium (containing 100 μg mL^{-1} ampicillin) in a 250 mL shake flask at 37 °C and 200 rpm. A 10% (v/v) seed culture was inoculated into semisynthetic medium (containing 100 μg mL^{-1} ampicillin) for fed-batch cultivation. Fed-batch cultivation was performed in a 3-L fermentor (BioFlo 110, New Brunswick Scientific Co., Ltd) which consisted of three phases.

The first one was characterized by batch phase with an initial glycerol concentration of 8 g L^{-1} and temperature of 37 °C. After inoculation, the DO value decreased suddenly with a concomitant decrease in pH and glycerol. The end of glycerol consumption was detected by a sudden increase in both DO and pH value, and then the second exponential feeding (pre-induction) phase of fed-batch cultivation was started. Exponential feeding was performed as described by Fang et al. (2011).

During the second phase, when a certain DCW of 30 g L^{-1} reached, the inducer was fed at 0.8 g L^{-1} h^{-1} and temperature decreased to 25 °C (unless otherwise stated) for pullulanase production. And finally the third post-induction phase began. During the whole process, the pH was kept at 7.0 by automatic addition of ammonia solution (25%, v/v). Antifoam was added manually when necessary. To maintain the DO level around 30% of air saturation, the agitation speed was varied from 300 to 900 rpm. The air flow rate was 1.8 L min^{-1}. Dissolved oxygen concentration, pH, temperature, and impeller speed were recorded using Advanced Fermentation Software (AFS) from New Brunswick Scientific Co. Inc.

2.3. Determination of biomass

Cell growth was monitored during cultivation by measuring the optical density of the culture broth at 600 nm using a spectrophotometer (BioPhotometer plus, Eppendorf Co., Hamburg, Germany). Samples were approximately diluted with 0.9% (w/v) NaCl at an OD_{600} value exceeding 0.8. To determine the DCW, 5 mL of culture broth was centrifuged at 10,000*g* for 10 min. The pellet was washed with 0.9% (w/v) NaCl and centrifuged at 10,000*g* for 10 min, and dried to constant weight at 105 °C.

2.4. Cell fractionation

Cell fractionation was performed as described by Le et al. (2011) with some modifications. The extracellular fraction was obtained by centrifugation of the culture broth at 10,000*g* for 20 min at 4 °C, and the supernatant was used as an extracellular fraction. The resulting pellets containing cells were harvested and resuspended in 1 mL of 30 mM Tris–HCl solution (pH 7.0) containing 25% (w/v) sucrose and 1 mM EDTA. The cell suspension was incubated on ice for 2 h and pelleted by centrifugation at 10,000*g* for 20 min at 4 °C. The supernatant was collected as a periplasmic fraction. The total soluble pullulanase activity was the sum of periplasmic and extracellular enzyme activity.

2.5. SDS–PAGE gel electrophoresis

The subunit molecular weight of the recombinant enzyme was determined in denaturing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresis was performed with a 12% separating gel. Protein bands were stained with Coomassie Brilliant Blue R-250 dye according to standard procedures.

2.6. Assay of pullulanase activity

Pullulanase activity was measured in 50 mM sodium acetate buffer (pH 4.5) according to Gantelet and Duchiron, with slight modification using pullulan as substrate (Gantelet and Duchiron, 1998). Briefly, a mixture of 1 mL 1% (w/v) pullulan and 0.9 mL 50 mM sodium acetate buffer pH 4.5 was preincubated at 60 °C for 10 min. 0.1 mL enzyme solution (diluted to a final activity of 1.0–2.0 U mL^{-1}) was then added and the mixture was incubated for a further 15 min. The reaction was stopped by the addition of 3 mL 3,5-dinitrosalicylic (DNS) acid solution. Afterwards, the whole mixture was boiled for 7 min and diluted to 15 mL with deionized water before the measurement of the absorbance at 540 nm. One unit of pullulanase activity was defined as the amount of enzyme that released 1 μM of D-glucose reducing sugar equivalents per minute from pullulan, under the defined assay conditions (pH 4.5, 60 °C).

2.7. Transmission electron microscopy

Morphometric analyses were also performed according to Wu et al. (2011) with slight modification using a Hitachi H-7650B (Hitachi, Japan) transmission electron microscope. *E. coli* BL21 (DE3) cells were withdrawn at 24 h of culture, rinsed once by centrifugation at 3000*g*. For transmission electron micrography, 2.5% glutaraldehyde and 2% osmium tetraoxide were used successively to fix the cells. After a graded-ethanol serial dehydration step, the cells were embedded in epoxy resins, sectioned. Then the ultrathin sections were stained by uranyl acetate solution and lead citrate. Lastly, the samples were examined using a Hitachi H-7650B (Hitachi, Japan) transmission electron microscope at an accelerating voltage of 80 kV.

2.8. Statistical analysis

All the experiments were performed at least three times, and the results were expressed as the means ± standard deviation (SD). Statistical analysis was performed with Student's *t*-test. *P* values of <0.01 were considered statistically significant.

3. Results and discussion

3.1. Recombinant pullulanase distribution in E. coli BL21(DE3) harboring pulA gene

In a previous study, the pullulanase encoding gene *pul*A from *B. deramificans* was successfully overexpressed in *E. coli*. In the present study, the production of recombinant pullulanase was induced by the addition of 0.4 μM IPTG at 37 °C. SDS–PAGE revealed that only a small proportion of the pullulanase protein produced was soluble, with extracellular supernatant and soluble periplasmic pullulanase activities of only about 3.2 and 9.98 U mL^{-1}, respectively, and a large amount of insoluble fraction, considered as IBs (Fig. 1). In addition, as shown in Fig. S1A, transmission electron microscope image of the thin-sectioned cells expressing pullulanase clearly revealed newly formed IBs occupying a large portion of the cytoplasm, which were not observed in cells without the expression plasmid (data not shown). This observation clearly confirmed the in vivo formation of IBs of the recombinant enzyme.

It is well known that overproduction and accumulation of heterologous protein imposes a metabolic burden on the host cell. Insoluble IBs are formed when the in-house capacity of the cell to fold the protein intermediates is substantially overwhelmed. Findings of a recent study on IBs indicate that proteins with high molecular weight and complicated structure have a high tendency

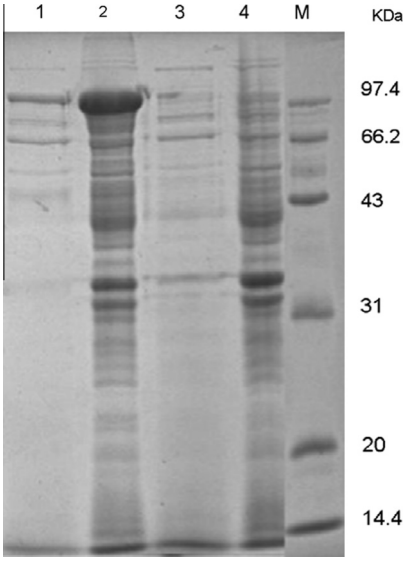

Fig. 1. SDS–PAGE analysis of pullulanase expressed from pET–*pul*A. The distribution was analyzed after 24 h of culture in TB medium. (1) Periplasmic protein of *E. coli* containing pET–*pul*A; (2) insoluble protein of *E. coli* containing pET–*pul*A; (3) periplasmic protein of *E. coli* containing pET-20b(+) without pullulanase encoding gene; (4) insoluble protein of *E. coli* containing pET-20b(+) without pullulanase encoding gene.

to form IBs (Ramshini et al., 2011). In addition, it has been shown that the propensity of proteins to form IBs is sequence-dependent (Ivanova et al., 2004). It is known that the molecular weight of *B. deramificans* pullulanase, composed of six domains, is nearly 101 kDa. Thus, the high propensity of pullulanase to form IBs could possibly be due to the high molecular weight and complicated structure. As low yield is a bottleneck for the production of pullulanase, it is desirable to reduce the insoluble species and enhance production of soluble pullulanase.

3.2. Effect of process conditions on the production of soluble pullulanase in shake flasks

3.2.1. Induction temperature

Induction temperature is an important parameter for recombinant protein production in *E. coli* (Fang et al., 2011; Pinsach et al., 2008). In general, growth of recombinant *E. coli* cells at low temperatures increases the solubility of the recombinant proteins by preventing the formation of IBs. To optimize the temperature for the production of pullulanase, the *E. coli* cells expressing pullulanase were induced at 25 °C, 30 °C, and 37 °C in TB medium. As shown in Fig. 2, high induction temperature had a negative effect on the production of soluble pullulanase, and the adverse effect was more obvious when the cells were cultured at 37 °C. At an induction temperature of 37 °C, the biomass reached 5.6 g L^{-1}, which was 1.5- and 1.3-fold higher than that observed at 25 °C and 30 °C, respectively. However, the extracellular pullulanase activity (2.1 U mL^{-1}) and periplasmic pullulanase activity (10.2 U mL^{-1}) at 37 °C were much lower than those noted at 25 °C and 30 °C. When compared with cells cultured at 30 °C, those grown at 37 °C were observed to form more IBs, indicating a decrease in protein quality at 37 °C, similar to that reported by García-Fruitós et al. (2007). On the other hand, induction at 25 °C attained the highest total pullulanase yield of 36.5 U mL^{-1}, which was 3.0-fold higher than that obtained at 37 °C. These results suggest that low culture temperature facilitates proper folding and thereby improves productivity of pullulanase.

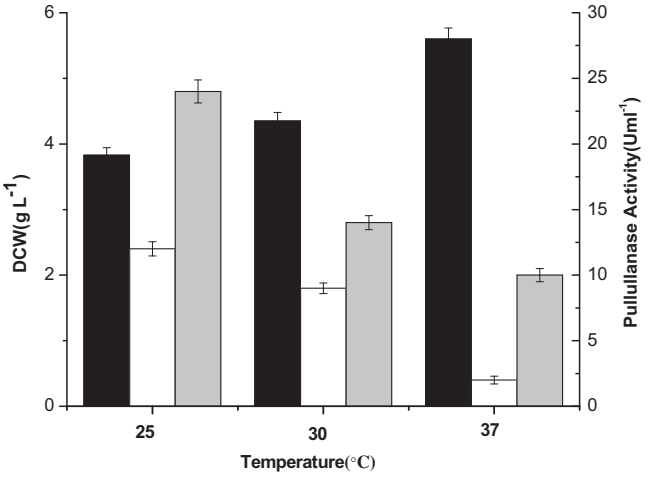

Fig. 2. Comparison of cell growth and pullulanase activity at different temperature. DCW (black), Extracellular pullulanase activity (white), periplasmic soluble pullulanase activity (light gray).

3.2.2. IPTG concentration

The impacts of IPTG concentration on cell growth and soluble pullulanase production were investigated at an induction temperature of 25 °C. The results showed that the DCW decreased with the increasing concentration of IPTG, and that the decrease in DCW was rapid when the concentration of IPTG was higher than 0.2 mM L^{-1}. However, the DCW of cultures grown with 0.4 mM IPTG was only 2.5 mg mL^{-1}, which was 62.5% of that of the control.

Previously, it was reported that the levels of soluble protein and IBs varied significantly with the increasing dosage of IPTG (Jhamb and Sahoo, 2012). In order to understand this correlation, the extracellular and periplasmic pullulanase activities were assessed. As shown in Fig. 3, with increasing IPTG concentration from 0 to 0.05 mM, the extracellular and periplasmic pullulanase activities increased and reached the maximum levels of 13 and 23 U mL^{-1}, respectively. However, the activities decreased dramatically once the optimum concentration was crossed. The apparent decrease in soluble protein on induction with 0.4 mM IPTG could be due to the overall high accumulation of the expressed target protein resulting in a crowding effect. On induction with high concentration of IPTG, the folding and transportation of recombinant proteins often failed to match the rate of expression, resulting in aggregation of most of the target proteins into IBs. Thus, lower concentration of the inducer could have a positive effect on the solubility and proper folding of proteins.

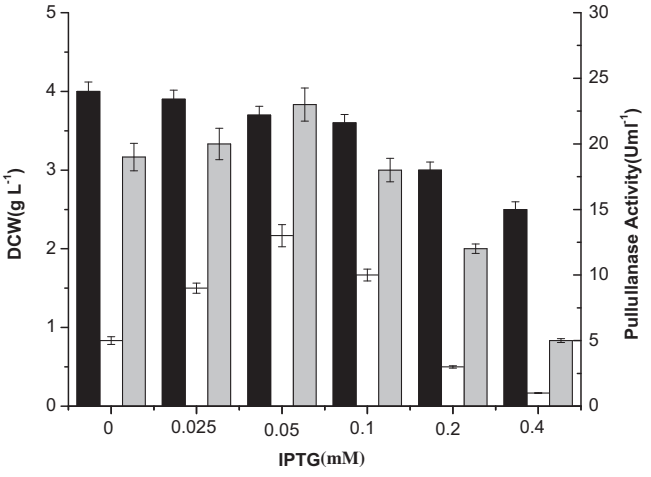

Fig. 3. Comparison of cell growth and pullulanase activity at different IPTG concentration. DCW (black), extracellular pullulanase activity (white), periplasmic pullulanase activity (light gray).

3.2.3. Osmolytes

As shown in Sections 3.2.1 and 3.3.2, pullulanase activity was very low even at low inducer concentration and low temperature, and there were numerous IBs in the cells (data not shown). Previous studies reported that the presence of physiological amounts of osmolytes in the cell can significantly increase the stability of native thermolabile proteins and the yields of the natively folded proteins (De Marco et al., 2005; Ignatova and Gierasch, 2006). In the present study, the effects of osmolytes, proline, K-glutamate, and betaine on the production of soluble pullulanase were investigated.

As shown in Fig. 4A, addition of proline or K-glutamate (with or without NaCl) to the medium led to lower soluble enzyme expression than that observed in the control. Interestingly, the presence of betaine (without NaCl) in the medium resulted in 1.3-fold increase in the amount of total soluble pullulanase (Fig. 4A). The transmission electron microscopy results, presented in Fig. S1B, confirmed that the cellular accumulation of betaine during massive pullulanase synthesis may strongly reduce the insoluble misfolding of the recombinant protein. However, when cultures were supplemented with betaine and NaCl, a slightly lower activity of total soluble pullulanase was noted, when compared with that of the control. This phenomenon may be due to the high content of salts (2.31 g L^{-1} of KH_2PO_4 and 16.43 g L^{-1} of K_2HPO_4) in the TB medium, which is adequate to induce the uptake of betaine into the bacterial cells; however, additional supplementation of NaCl could have inhibited the growth of *E. coli*, resulting in lower level of enzyme expression. The results obtained confirmed that betaine can efficiently favor the native folding pathway of protein in vivo as well as in vitro, similar to that reported by Diamant et al. (2001). Therefore, betaine was chosen for further studies to improve the expression of soluble recombinant pullulanase.

Different concentrations of betaine were used to optimize the production of soluble pullulanase, and the maximum total pullulanase activity (45.3 U mL^{-1}) was achieved at 20.0 mM (Fig. 4B). Higher concentration of betaine failed to increase the enzyme yield, and the enzyme activity was only 22.1 U mL^{-1} when 80.0 mM of betaine was used. In addition, the influence of various betaine supplement times on pullulanase production was further investigated, and the optimum supplement time was found to be the initial time point, at which the soluble pullulanase yield reached 49.4 U mL^{-1} (Fig. 4C).

The presence of high concentrations of osmolytes (called chemical chaperones), such as betaine, proline, and K-glutamate, in the cell can significantly increase the stability of heat-labile proteins during heat stress in vivo, favoring native refolding of the denatured proteins in vitro (Diamant et al., 2001, 2003), and can efficiently improve the yields of natively folded recombinant proteins. This phenomenon could be the result of the direct effect of the viscosity of the osmolytes on slowing down the rates of protein folding and strongly favoring the funneling of early folding intermediates into the native folding pathway (separating misfolding and native folding events) (De Marco et al., 2005). The same mechanism of direct de novo native folding might also occur when the viscosity of the cytoplasm is increased at lower temperatures. In the present study, the three kinds of osmolytes exhibited different effects toward the solubility of pullulanase, and these differential effects could most probably be based on the relative tendency of a given osmolyte to be preferentially excluded from the protein surface, the magnitudes of the osmolyte side chain interactions, and the properties of the particular aggregated species.

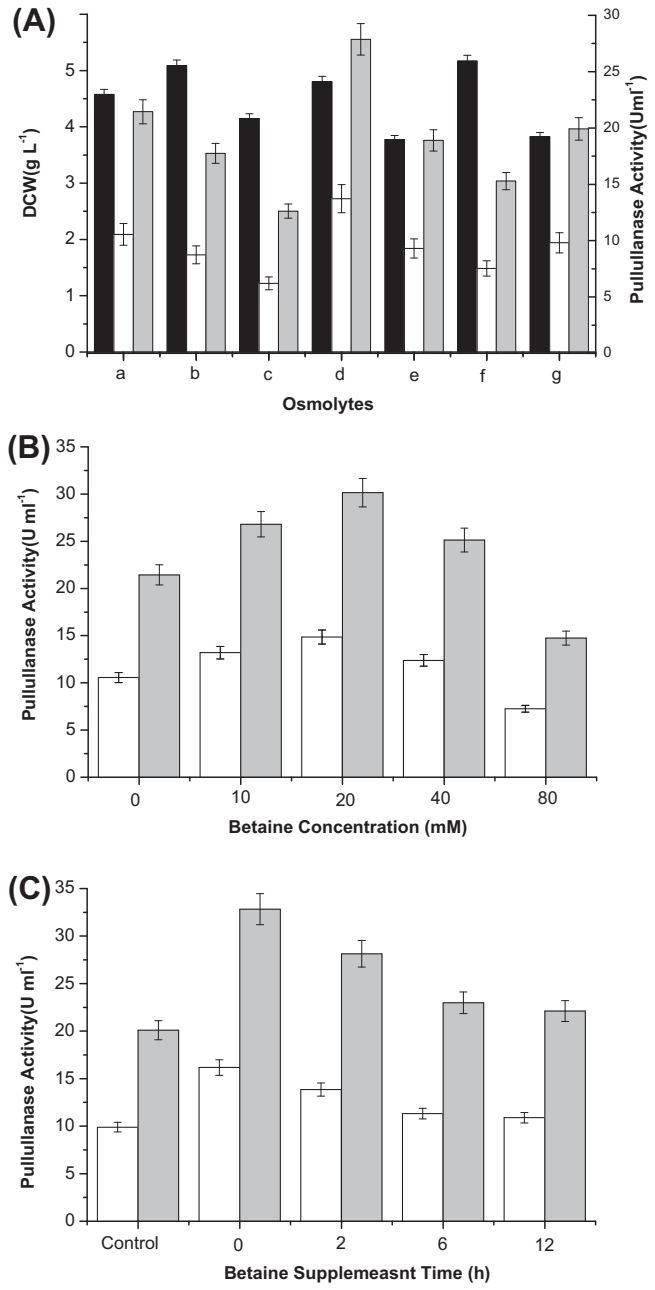

Fig. 4. Comparison of cell growth and total pullulanase activity in different osmolytes (A), betaine concentration (B) and betaine supplement time (C). (a) Control; (b) 20 mM Proline; (c) 20 mM Proline + 0.5 M NaCl; (d) 20 mM Betaine; (e) 20 mM Betaine + 0.5 M NaCl; (f) 20 mM K-glutamate; (g) 20 mM K-glutamate + 0.5 M NaCl. DCW (black), extracellular pullulanase activity (white), periplasmic pullulanase activity (light gray).

3.3. Development of a two-stage glycerol feeding strategy with betaine supplementation

In this study, a modified two-stage glycerol feeding strategy was applied to achieve high-cell-density cultivation of *E. coli* and production of recombinant pullulanase. A combined feeding strategy based on both specific growth rate and amount of glycerol residues after induction was used to control cell growth, acetate production, and glycerol consumption (Cheng et al., 2011). During the pre-induction phase, the glycerol feeding rate was increased exponentially, according to the exponential feeding method (Fang et al., 2011), and the cell growth was controlled at a specific growth rate (μ_{set}) of 0.20 h^{-1}. When the DCW reached 30 g L^{-1}, the post-induction phase began and the feeding rate was changed based on the gradient-decreasing method (Fig. 5A). As the cost of IPTG was high, lactose was used as the inducer in fed-batch fermentations.

The flask experiments, described in Section 3.2, indicated that both induction temperature and betaine (chemical chaperone) are important parameters for the production of soluble recombinant pullulanase in *E. coli*. Based on this observation, a two-stage

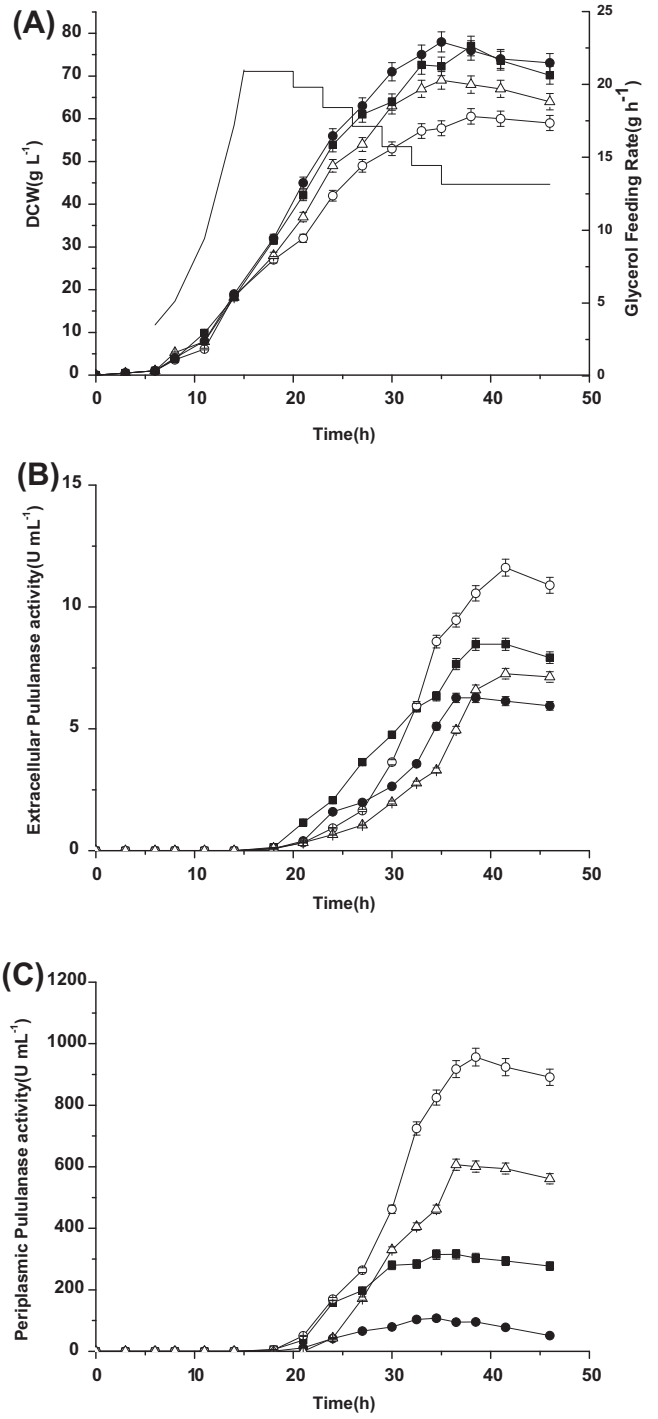

Fig. 5. Comparison of the time profiles for biomass (A), extracellular pullulanase activity (B) and periplasmic pullulanase activity (C) obtained from cultivations induced at 25 °C with 0 mM betaine (■), 30 °C with 0 mM betaine (●), 25 °C with 20 mM betaine (○), and 30 °C with 20 mM betaine (△), glycerol feeding rate (–).

Table 1
Comparison of parameters for recombinant pullulanase production in shake flask and 3-L fermentor.

Mode of cultivation	Temperature (°C)	Betaine concentration (mM)	Total pullulanase activity (U mL^{-1})	Productivity (U mL^{-1} h^{-1})	Specific productivity ($\times 10^3$ U g^{-1})
Shake flask	30	0	23.0 ± 0.6	0.9 ± 0.1	6.1 ± 0.3
	25	0	36.1 ± 0.8	1.3 ± 0.1	8.2 ± 0.3
	25	20	49.3 ± 0.7	1.8 ± 0.1	10.2 ± 0.5
3-L fermentor	30	0	115.8 ± 9.1	3.0 ± 0.2	1.5 ± 0.1
	30	20	618.3 ± 40.6	16.3 ± 1.2	8.0 ± 0.6
	25	0	321.8 ± 21.5	8.5 ± 0.6	4.6 ± 0.3
	25	20	963.9 ± 69.4	25.4 ± 1.5	16.0 ± 1.1

temperature fermentation strategy was developed, in which two different induction temperatures (25 °C and 30 °C) were investigated with or without betaine in a 3-L fermentor. As depicted in Fig. 5A, the final biomass reached 78.3 g L^{-1} at an induction temperature of 30 °C, which was 1.01-, 1.32-, and 1.13-fold higher than that observed at 25 °C, 25 °C (with betaine), and 30 °C (with betaine), respectively. However, a high level of biomass did not lead to a high yield of soluble protein. Fig. 5A shows that the extracellular pullulanase activity at 25 °C, 30 °C, 25 °C (with betaine), and 30 °C (with betaine) was 8.4, 6.1, 11.6, and 7.4 U mL^{-1}, respectively. Although no significant difference was observed in the extracellular pullulanase activity when induced at 30 °C, the soluble periplasmic pullulanase yield was low, reaching only 107.4 U mL^{-1}. On the other hand, when the cells were induced at 25 °C, the soluble periplasmic pullulanase activity reached 315.7 U mL^{-1}. In the case of cells induced at 30 °C (with betaine), the enzyme activity increased to 606.7 U mL^{-1}. Furthermore, when the cells were induced at 25 °C and supplemented with 20 mM betaine, a higher soluble pullulanase activity (956.5 U mL^{-1}) was found (Fig. 5C), and the average pullulanase productivity reached 25.4 U mL^{-1} h^{-1}.

As specific productivity is an important parameter in the industrial production of enzyme, it was calculated in the present study. As shown in Table 1, the specific productivity of fed-batch fermentation at 30 °C was 1.5×10^3 U g_{cell}^{-1}, which was only 30.0% of that observed at 25 °C (4.6×10^3 U g_{cell}^{-1}). Lower temperature was assumed to reduce the rate of cell growth and protein synthesis as well as decrease the misfolding of the recombinant protein, and consequently enhance the solubility of pullulanase. However, due to the metabolic burden caused by the expression of high amounts of recombinant protein, the specific productivity of the fed-batch fermentation at 25 °C was approximately 86.5% of that of the shake flask fermentation induced at the same temperature. This could be attributed to the insufficient amount of native chaperone produced at high cell densities to promote folding of pullulanase, because most of the pullulanase was observed in the insoluble protein fraction (data not shown). However, when betaine was added to the medium, highest specific productivity (16.0×10^3 U g_{cell}^{-1}) was obtained when induced at 25 °C in a 3-L fermentor, which was 3.4-, 11.0-, and 2.0-fold higher than that noted at 25 °C, 30 °C, and 30 °C (with betaine), respectively. The reason for the obvious improvement in the specific productivity might be the combined effect of low induction temperature and addition of chemical chaperone (betaine).

4. Conclusion

In this work, the supplement of osmolytes and process optimization strategies were combined to reach the overproduction of pullulanase in *E. coli*. A modified two-stage glycerol feeding strategy was developed to reach the soluble production of pullulanase. Through this cultivation approach, the total soluble activity, productivity, and specific productivity of pullulanase reached 956.5 U mL^{-1}, 25.4 U mL^{-1} $h^{-1}$and 16.0×10^3 U g^{-1}, respectively, which represent the highest reported so far. Betaine being non toxic to microbes and inexpensive, is expected to be an excellent chemical additives in overproduction of many enzymes economically at a large scale.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.07.074.

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