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Triton X-100 enhances the solubility and secretion ratio of aggregation-prone pullulanase produced in *Escherichia coli*

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HIGHLIGHTS

- *B. deramificans* pullulanase has a strong tendency to form active protein aggregates.
- The insoluble active protein-aggregate fraction can be redissolved *in vitro*.
- Supplementing the medium with surfactant improved the secretion ratio of pullulanase.
- The extracellular enzyme activity was 46.2-fold that of the control in 3-L fermenter.

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ABSTRACT

The pullulanase from *Bacillus deramificans* is an industrially useful starch-debranching enzyme that is difficult to produce in large quantities. In this study, *B. deramificans* pullulanase was found to be an aggregation-prone protein that can be solubilized from the insoluble fraction by surfactants *in vitro*. Studying the effects of various surfactants on pullulanase production in *Escherichia coli* in shake flasks revealed that optimal pullulanase production could be obtained by adding 0.5% Triton X-100 during the later period of fermentation. A modified fed-batch fermentation strategy was then applied to the production of pullulanase in a 3-L fermentor. When supplemented with 0.5% Triton X-100 at 40 h, the maximal extracellular pullulanase production and secretion ratio were 812.4 U mL^{-1} and 86.0%, which were 46.2- and 47.8-fold that of the control, respectively.

1. Introduction

Pullulanase (EC 3.2.1.41) is a well-known starch-debranching enzyme that catalyzes the hydrolysis of the α-1,6 glycosidic linkage in pullulan, amylopectin, and related branched dextrins. It can be used in conjunction with α-amylase, β-amylase, glucoamylase, or cyclodextrin glucosyltransferase to produce glucose, fructose, maltose syrups, cyclodextrins, and amylose (Chen et al., 2014; Roy and Gupta, 2004). Because pullulanase can dramatically increase yield and reduce reaction time, its application has expanded to the production of maltotriose syrup, resistant starch, low-calorie beers, fuel ethanol, and related products (Gohel and Duan, 2012; Singh et al., 2010; Zhang et al., 2013). With this growing interest in potential industrial applications, pullulanase production has drawn sustained attention in recent years (Li et al., 2012; Zhang et al., 2013).

To meet these industrial production requirements, recent research has focused on the overexpression of pullulanase-encoding genes from different types of microorganisms, including *Raoultella planticola* DSMZ 4617 (Hii et al., 2012), *Bacillus* sp. CICIM 263 (Li et al., 2012), *Klebsiella variicola* (Chen et al., 2014), *Bacillus flavothermus* KWF-1 (Shankar et al., 2014), *Bacillus naganoensis* (Nie et al., 2013), and *Bacillus deramificans* (Duan et al., 2013b). However, the production of these pullulanases is still low, particularly because their secretion ratio is low when these enzymes are expressed as secreted proteins in *Escherichia coli* (Zou et al., 2014). *E. coli* is a highly efficient host for heterologous protein expression that been widely used to produce a multitude of enzymes, antibodies, and other valuable proteins. Its ease of culture, unparalleled fast growth kinetics, ability to achieve high cell densities, and highly efficiency protein synthesis make *E. coli* an excellent protein expression host (Choi and Lee, 2004; Jhamb and Sahoo, 2012; Rosano and Ceccarelli, 2014; Sezonov et al., 2007).

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Secretory and extracellular production of recombinant enzymes using *E. coli* offers several advantages over cytoplasmic or periplasmic production, including ease of extraction, simplified purification processes, and improved product yield (Choi and Lee, 2004). However, recombinant proteins often form inclusion bodies (IBs), which dramatically decrease the production of soluble and secreted proteins, in the cytoplasm and/or periplasm of *E. coli* (Le et al., 2011). Several factors contribute to the formation of IBs: the intrinsic nature of the protein, the rate of protein expression, insolubility of the product at the concentrations being produced, and lack of molecular chaperones (Sevastsyanovich et al., 2010; Singh and Panda, 2005). All forms of aggregated protein produced in the cytoplasm and periplasm, including misfolded and dysfunctional polypeptides, have generally been regarded as IBs. However, since 1991, researchers have gradually realized that some of these so-called IBs are still biologically active. These active "IBs" are not really IBs; they are active protein aggregates (Garcia-Fruitos et al., 2007). Active protein aggregates have recently drawn greater attention, and several methods are used to prevent or reduce the formation of protein aggregates: controlling the rate of protein synthesis, co-expression with a molecular chaperone, adding chemical additives, and so on (Pan et al., 2003).

Previously, a recombinant *E. coli* strain was constructed for the production of *B. deramificans* pullulanase. Pullulanase production by this strain was enhanced by optimizing of process conditions, supplementing the medium with betaine, and implementing a glycine feeding strategy (Duan et al., 2013a,b; Zou et al., 2014). Despite these efforts, a substantial portion of the target protein could be found in the insoluble fraction. The results of this study show that recombinant *B. deramificans* pullulanase has a strong tendency to form aggregates, and that most of the recombinant enzyme accumulates in the periplasmic space. To improve the secretory ratio, the medium was supplemented with surfactants, which enabled the highly efficient extracellular production of pullulanase in *E. coli*.

2. Methods

2.1. Strain and media

E. coli BL21(DE3) carrying plasmid *pul*A/pET20b(+), which contains the *B. deramificans pul*A gene, was used as the pullulanase production strain in this work (Duan et al., 2013b). Luria–Bertani (LB) medium supplemented with 100 mg L^{-1} ampicillin was used for seed cultivation. LB medium contained NaCl 10 g L^{-1}, yeast extract 5 g L^{-1}, and peptone 10 g L^{-1}. Modified Terrific Broth (TB) medium was employed in shake-flask cultures. Modified TB medium contained tryptone 12.0 g L^{-1}, yeast extract 24.0 g L^{-1}, K_2HPO_4 16.4 g L^{-1}, KH_2PO_4 2.3 g L^{-1}, glycerol 5.0 g L^{-1}, and ampicillin 100 mg L^{-1}. The modified semisynthetic medium used to produce pullulanase in a 3-L fermentor contained glycerol 8.0 g L^{-1}, tryptone 30.0 g L^{-1}, yeast extract 20.0 g L^{-1}, NaCl 5.0 g L^{-1}, NH_4Cl 0.5 g L^{-1}, Na_2SO_4 2.0 g L^{-1}, $(NH_4)_2SO_4$ 2.5 g L^{-1}, $(NH_4)_2$-H-citrate 1.0 g L^{-1}, K_2HPO_4 14.6 g L^{-1}, $NaH_2PO_4{\cdot}2H_2O$ 3.6 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 2.0 g L^{-1}, thiamine 100 mg L^{-1}, and trace metal solution 1.0 mL L^{-1}, pH 7.0. The trace metal solution consisted of: $FeSO_4{\cdot}7H_2O$ 10.0 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 5.3 g L^{-1}, $CuSO_4{\cdot}5H_2O$ 3.0 g L^{-1}, $MnSO_4{\cdot}4H_2O$ 0.5 g L^{-1}, $Na_2B_4O_7{\cdot}10H_2O$ 0.23 g L^{-1}, $CaCl_2$ 2.0 g L^{-1}, and $(NH_4)_6Mo_7O_{24}$ 0.1 g L^{-1}. The feeding medium contained: glycerol 500 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 3.4 g L^{-1}, peptone 50 g L^{-1}, and yeast extract 50 g L^{-1}. The induction solutions contained 200 g L^{-1} lactose.

2.2. Shake-flask culture

To produce a seed culture, 50 mL of LB medium containing 100 mg L^{-1} ampicillin in a 250 mL shake flask was inoculated with 100 μL of frozen glycerol stock (kept at −80 °C) and cultured at 37 °C in a rotary shaker at 200 rpm for 8 h. A portion of the seed culture was then diluted (1:25) into 50 mL of modified TB medium containing 100 mg L^{-1} ampicillin in a 250 mL shake flask and shaken in a rotary shaker (200 rpm) at 37 °C until the optical density at 600 nm (OD_{600}) reached 1.0. Then, IPTG was added to a final concentration of 0.04 mM to induce the expression of the recombinant pullulanase. To investigate the effect of surfactant on the growth of *E. coli* or secretion of pullulanase, surfactant was added to the culture medium 24 h after the addition of IPTG, unless otherwise stated. At the designated time intervals, samples were collected and analyzed for OD_{600} and enzyme activities.

2.3. Bioreactor fermentation

Fed-batch cultivation was performed in a 3-L fermentor (BioFlo 110, New Brunswick Scientific Co., Ltd). During the entire fermentation process, the pH was maintained at 7.0 by automatic addition of a 25% (v/v) ammonia solution. Antifoam was added manually, when necessary. To maintain the dissolved oxygen concentration (DO) at 30% air saturation, a cascading impeller speed was employed and the air was supplemented with oxygen. The DO, pH, temperature, and impeller speed were recorded using Advanced Fermentation Software (AFS) from New Brunswick Scientific Co. Inc.

The seed culture was prepared in a 250 mL shake flask by inoculating 50 mL of LB medium supplemented with 100 mg L^{-1} ampicillin with 100 μL of a frozen glycerol stock. This culture was shaken at 200 rpm for 8 h at 37 °C. Fermentation was initiated by inoculating the 3-L fermentor, which contained the modified semisynthetic medium described in Section 2.1, with 10% (v/v) seed culture. Betaine (20 mM) was also added at this point, unless otherwise stated.

Then, the fed-batch cultivation was performed in four phases. The first phase after inoculation was a batch fermentation that was conducted with an initial glycerol concentration of 8 g L^{-1}, at a temperature of 30 °C. The dissolved oxygen (DO) concentration decreased rapidly after inoculation, with a concomitant decrease in both pH and the concentration of glycerol. The end of glycerol consumption was identified by a sudden increase in both DO and pH. The second, exponential feeding phase of the fed-batch cultivation was started at this point. Exponential feeding was performed as previously described (Fang et al., 2011). When the dry cell weight (DCW) reached 30 g L^{-1}, pullulanase production was initiated by feeding an inducer at 0.8 g L^{-1} h^{-1} and decreasing the temperature to 25 °C. This began the third, post-induction phase. In this phase, the feeding rate was changed based on the gradient-decreasing method (Zou et al., 2014). After 40 h of culture, the fermentation entered its fourth phase. The aeration was turned off and the stirring speed decreased to 100 rpm. Tween 80 (0.5%) was added to the broth, then the culture continued for several hours. During this phase of the fermentation, aggregated intracellular protein was disaggregated and the soluble pullulanase was secreted.

2.4. Biomass assay

Cell growth was monitored by measuring the optical density of the culture broth at 600 nm using a spectrophotometer. Samples were diluted with 0.9% (w/v) NaCl to an OD_{600} value within the 0.2–0.8 range. To determine the dry cell weight (DCW), 5 mL of culture broth was centrifuged at 13,800 *g* for 10 min. The pellet was washed with 0.9% (w/v) NaCl and then centrifuged again at 13,800 *g* for 10 min. After the clear supernatant was discarded, the cell pellet was scraped into a weighing pan, the centrifuge tube was rinsed with a few mL of water to collect the remaining solids,