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Study on Improvement of Extracellular Production of Recombinant *Thermobifida fusca* Cutinase by *Escherichia coli*

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Abstract *Escherichia coli* is one of the most commonly used host strains for recombinant protein production. More and more research works on the production of recombinant protein indicate that extracellular production throughout a culture medium is more convenient and attractive compared to intracellular production. In present work, inducing temperature and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration were investigated to decrease the formation of inclusion body and increase the amount of soluble recombinant cutinase initially. Enzyme activity in the culture medium reached to 118.9 U/ml at 64 h of culture, and no inclusion body was detected in cytoplasm under the inducement condition of 0.2 mM IPTG and 30°C. In addition, it was found that a large amount of cutinase had been accumulated in periplasm since 16-h cultivation under the same inducement condition. Therefore, glycine and surfactant sodium taurodeoxycholate (TDOC) were further used to promote the leakage of recombinant cutinase from periplasm. Supplied with 100 mM glycine and 1 mM TDOC, the amount of cutinase in periplasm decreased remarkably, and the activity in the culture medium reached to 146.2 and 149.2 U/ml after 54 h of culturing, respectively.

Keywords *Thermobifida fusca* · Cutinase · *Escherichia coli* · Extracellular production

Introduction

Cutinase is a unique hydrolase named by its capability to degrade water-insoluble cutin [1]. Besides cutin, cutinase can hydrolyze soluble esters, insoluble triglycerides, and various

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types of polyesters. Furthermore, cutinase is capable of catalysis of the esterification reaction and transesterification reaction. These diverse properties of cutinase have widely broadened its application in food, chemical, detergent, and agricultural industries. Significantly, recent studies showed that cutinase could also be utilized in bioscouring of cotton fibers and biomodification of synthetic fibers. Thus, it was regarded as a key enzyme to facilitate clean production in textile industry [2–4].

Cutinase was found in both fungi and bacteria [5, 6]. Present research works on either experimental or practical levels were mainly focused on fungal cutinase [7–10]. However, fungal cutinase exhibits relatively poor thermal stability and is incapable of practical application in textile industries. In our previous studies, a bacterial cutinase which is both thermally stable and catalytically efficient was successfully isolated from *Thermobifida fusca*. Correspondingly, encoding gene of *T. fusca* cutinase (*Tfu_0883*) was successfully identified and expressed in *Escherichia coli* by fusing a secretion peptide PelB in the upstream of cutinase in the vector pET20b(+) [11].

As a Gram-negative bacterium, *E. coli* has both inner and outer membranes. Other researchers reported that recombinant proteins with a periplasmic secretory peptide such as PelB were translocated into periplasm after being synthesized. After cleavage of the PelB peptide, some of the proteins localized and accumulated in the periplasm, meanwhile other proteins passed through the outer membrane and then released into the culture medium by nonspecific leakage [12–17]. The reported mechanism accords with our previously experimental facts of PelB-fused *T. fusca* cutinase expressed in *E. coli* [11].

This study aimed to enhance the extracellular production of recombinant *T. fusca* cutinase by *E. coli*. Initially, we improved the soluble recombinant cutinase in the periplasm and culture medium through varying the inducing temperature and concentration of the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG). Subsequently, based on the fact that recombinant cutinase was accumulated in the periplasm during the culture period, glycine and surfactant sodium taurodeoxycholate (TDOC) were added into the medium to increase cell outer membrane permeability and facilitate the release of recombinant cutinase from periplasm into the culture medium. Through this strategy, the production of recombinant cutinase in culture medium was proposed to increase remarkably.

Materials and Methods

Chemicals

Peptone and yeast powder were purchased from Oxiod Ltd. *p*-Nitrophenyl butyrate (*p*NPB) and TDOC were obtained from Sigma. *N*-Phenyl-1-naphthylamine (NPN) was purchased from Aladdin Reagent Ltd. (Shanghai, China). Other chemical reagents were obtained from Sinopharm Chemical Reagent Co. Ltd.

Bacterial Strains and Culture Conditions

E. coli BL21 (DE3) harboring plasmid *Tfu_0883*/pET20b(+) was constructed previously. The primary components of plasmid *Tfu_0883*/pET20b(+) included cutinase gene *Tfu_0883*, T7 promoter, PelB leader encoding sequence, His-tag encoding sequence, and so on [11].

The seed medium (pH 8.0) contained peptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and ampicillin100 mg/L. The fermentation medium (pH 8.0) contained glycerol 5 g/L,

peptone 12 g/L, yeast extract 24 g/L, K_2HPO_4 12.54 g/L, KH_2PO_4 2.31 g/L, and ampicillin 100 mg/L.

E. coli BL21 (DE3) harboring plasmid *Tfu_0883*/pET20b(+) was inoculated into seed medium at 37°C for 8 h. The culture was then diluted to 1:100 with fermentation medium, and the 100-ml culture was carried out in a 500-ml flask on a rotary shaker (200 rpm) at 37° C until value of OD_{600} in the culture reached 0.5. The induction was performed at different temperatures, and then, different amounts of IPTG were added to induce the expression of cutinase gene *Tfu_0883*.

Enzyme Assay

Cutinase activity was shown by esterase activity which was determined by a continuous spectrophotometric assay utilizing *p*NPB as substrate [18]. One unit of enzyme activity is defined as the production of 1 μmol of *p*-nitrophenol (*p*NP) per minute at 20°C under pH of 8.0. The standard assay was measured in a final volume of 1 ml containing *p*NPB (1 mM), enzyme, and the assay buffer (10 mM Tris–HCl and 10 mM NaCl, pH 8.0) at 20°C. The reaction was initiated by the addition of *p*NPB. The hydrolysis of *p*NPB was spectrophotometrically monitored for the formation of *p*NP at 405 nm.

Cell Fractionation

Cell fractionation was performed as follows. After centrifugation of a 1-ml culture broth at 40,000×*g* for 10 min, culture supernatant was collected as extracellular fraction. To obtain the periplasmic fraction, the fully washed and centrifuged cells from the 1-ml culture broth were suspended in 1 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 25% (*w*/*v*) sucrose and 1 mM EDTA. After incubation on ice for 12 h, the mixture was centrifuged (40,000×*g*) for 10 min, and the supernatant was used as a periplasm-located enzyme source. The precipitate was then resuspended in l ml of 20 mM Tris–HCl buffer (pH 8.0) and disrupted by ultrasonication with a Sonifer 450 (Branson, Danbury, CT, USA). After centrifugation at 40,000×*g* for 20 min, the cell debris were collected as insoluble inclusion body fraction. The cell debris were resuspended in 3 μl/OD_{600} of sample buffer (50 mM Tris–HCl, pH 7.6; 100 mM 2-mercaptoethanol; 2% SDS; 5% glycerol; and 0.15 mM bromophenol blue) and boiled for 10 min to solubilize the inclusion bodies.

Assay of Outer Membrane Permeability

Outer membrane permeability was performed using NPN access assay as described previously [19]. Samples of *E. coli* BL21 (DE3) cells were obtained by centrifugation (40,000×*g*), rinsed once, and then suspended in 10 mM sodium phosphate buffer (pH 7.4) to an OD_{600} of 0.5. NPN was added to a total concentration of 10 μM into quartz cuvettes containing 2 ml of cell suspension. The sample was mixed by inverting the cuvette immediately prior to fluorescence monitoring. Fluorescence was measured using a 650–60 spectrofluorometer with slit widths set to 5 mm and excitation and emission wavelengths set to 350 and 428 nm, respectively.

Miscellaneous Methods

OD_{600} was determined using the UV-2450 ultraviolet–visible spectrophotometer from Shimadzu. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was

performed under reducing conditions on a 12% polyacrylamide gel. The gel was visualized with 0.25% Coomassie Brilliant Blue R-250 stain.

Results

Effect of Inducing Temperature on Extracellular Production of Recombinant Cutinase

Temperature can significantly influence the bacterial growth and protein expression, and thereby, it is necessary to investigate the performances of extracellular production of recombinant cutinase induced in different temperatures. It was found that, with induction at 25°C, 30°C, and 37°C, the highest cell density of culture broth (OD_{600}) reached 8.7, 8.1, and 7.2 with 72, 48, and 40 h of cultivation, respectively (Fig. 1a). Enzyme activity in the culture medium reached 93.6, 95.3, and 71.6 U/ml at 80, 48, and 40 h of culture with

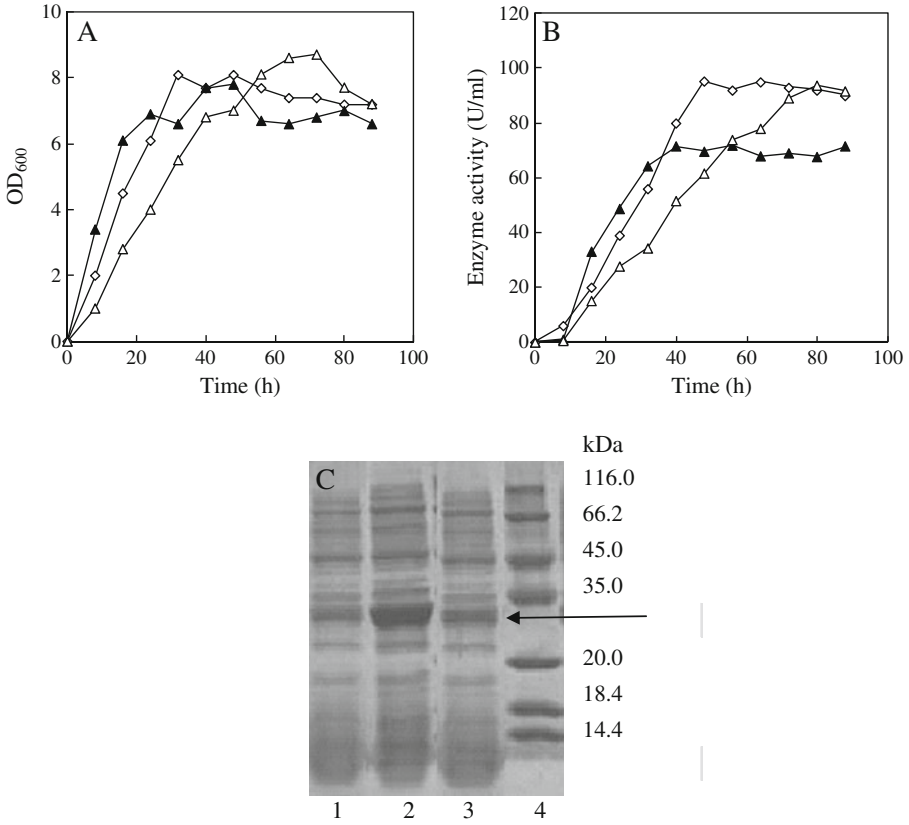

Fig. 1 Effect of inducing temperature on the production of recombinant cutinase. **a** Effect of inducing temperature on the growth of *E. coli*. **b** Effect of inducing temperature on enzyme activity in culture medium; the induction was performed with 0.4 mM IPTG at 25°C (*empty triangle*), 30°C (*empty diamond*), and 37°C (*filled triangle*). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%. **c** SDS–PAGE analysis of soluble recombinant cutinase in culture medium after 48 h of culture under different inducing temperatures. *Lane 1* induction at 25°C, *Lane 2* induction at 30°C, *Lane 3* induction at 37°C, *Lane 4* protein size marker

induction at 25°C, 30°C, and 37°C(Fig. 1b, c). The results indicated that both cell growth and cutinase production in the culture medium induced at 37°C were seriously inhibited compared to those induced at 25°C and 30°C. Moreover, the bacterium induced at 25°C grew much slowly and cutinase producing period was also prolonged compared to induction at 30°C. Therefore, the optimal inducing temperature was determined as 30°C.

Effect of IPTG Concentration on Extracellular Production of Recombinant Cutinase

The change in concentration of IPTG could affect rate and level of protein expression significantly [20, 21]. Different concentrations of IPTG in a range of 0 to 0.6 mM were added into the culture incubated at 30°C to investigate the expression of recombinant cutinase. As shown in Fig. 2a, cell density decreased slightly as the IPTG concentration was increased. When the culture was induced with 0.2 mM IPTG, the extracellular enzyme activity was detected as 118.9 U/ml at 64 h, which was increased by 24.8% and 42.9% compared to the activity induced with 0.4 and 0.6 mM IPTG, respectively (Fig. 2b).

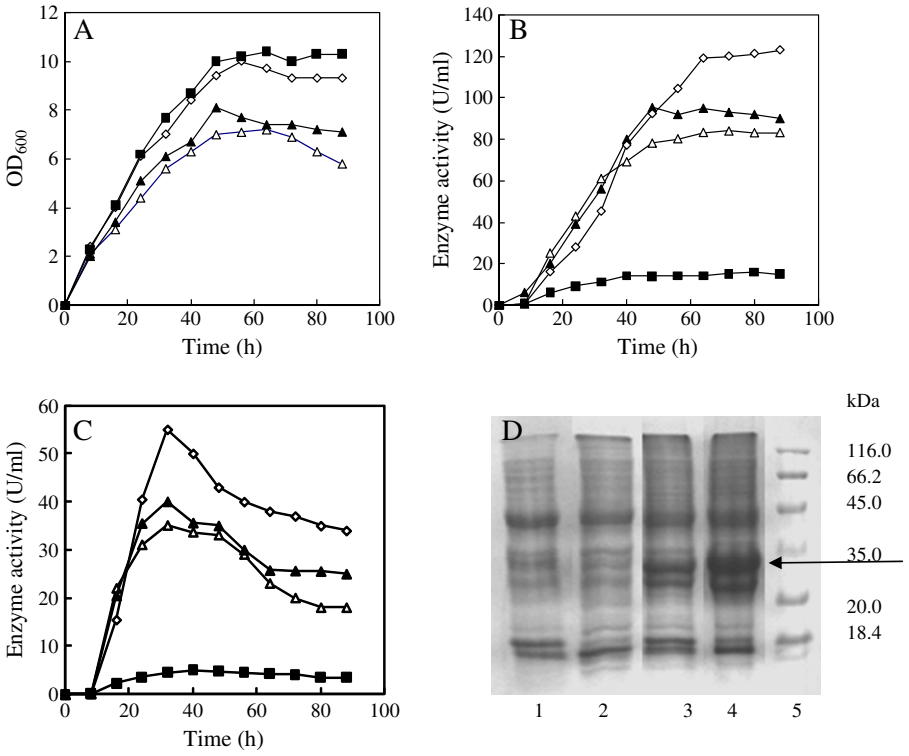

Fig. 2 Effect of IPTG concentration on the production of recombinant cutinase. **a** Effect of IPTG on the growth of *E. coli*. **b** Effect of IPTG on enzyme activity in culture medium. **c** Effect of IPTG on enzyme activity in periplasm. The induction was performed at 30°C with IPTG concentration of 0 mM (*filled square*), 0.2 mM (*empty diamond*), 0.4 mM (*filled triangle*), and 0.6 mM (*empty square*). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%. **d** SDS–PAGE analysis of insoluble inclusion body at different IPTG concentrations. The inclusion body was harvested at 48 h of culture. *Lane 1* 0 mM IPTG, *Lane 2* 0.2 mM IPTG, *Lane 3* 0.4 mM IPTG, *Lane 4* 0.6 mM IPTG, *Lane 5* protein size marker

As shown in Fig. 2c, with induction with 0.2 mM IPTG at 30°C, a large amount of cutinase was found to accumulate in periplasm after 16-h culturing (meanwhile OD_{600} reached to about 8.0). Furthermore, the enzyme activity in the periplasm reached a maximum value of 55.0 U/ml at 32 h and then decreased gradually as the culture time was increased. Finally, after culturing for 88 h, cutinase at the activity of 34.2 U/ml remained in periplasm. SDS–PAGE analysis of insoluble fraction in cytoplasm suggested that no inclusion body existed with induction with 0.2 mM IPTG, while inclusion body was detected when induced by 0.4 and 0.6 mM IPTG (Fig. 2d).

Effect of Glycine on Translocation of Recombinant Cutinase from Periplasm to Culture Medium

The experimental results shown in Fig. 2c indicated that the recombinant cutinase initially accumulated in periplasm to a maximum concentration and then released to the extracellular medium gradually. This fact suggested that the release process of the recombinant cutinase from the periplasm to the culture medium is crucial to the extracellular cutinase production. It is known that either a nonspecific membrane leak or main terminal branch (MTB) secretory pathway can rule translocation protein from periplasm in *E. coli*. However, the MTB secretory pathway is only found in a pathogen. Thereby, it is presumed that the nonspecific membrane leak of cutinase rules the release of the recombinant protein to the medium in our cases [14]. Consequently, it is considered that the improvement of the outer membrane permeability would be an efficient method to accelerate leakage of the accumulated cutinase.

It was reported that glycine could increase the permeability of the outer membrane [16, 22–25]. In the present research, glycine at the concentration range of 0 to 200 mM was supplied into the medium after culturing for 16 h (OD_{600} reached about 4.0), and consequently, its effect on translocation of recombinant cutinase from periplasm was evaluated. The experimental results indicated that in spite of slight inhabitation of bacterial growth (Fig. 3a), the addition of glycine at 50 or 100 mM greatly promoted the cutinase release from periplasm and the accumulation in the culture medium (shown in Fig. 3b, c). With the addition of 100 mM glycine into the culture medium, enzyme activity reached to 146.2 U/ml at 54 h of culture, which was 23.0% higher than culture without glycine. In contrast, while 150 or 200 mM glycine was supplied, bacterial growth was seriously inhibited, and cutinase activity in both the periplasm and medium was also reduced remarkably.

NPN as a hydrophobic fluorescent probe was reported to be used as an indicator to evaluate the permeability of outer membrane [19, 26]. In our cases, the outer membrane permeability of the bacterial cells in the middle exponential and stationary phase was evaluated by the method, respectively. The results shown in Fig. 3d suggested that the outer membrane permeability of cells added with 100 mM of glycine into the culture was notably improved compared with the counterpart without glycine.

Effect of Surfactant on Translocation of Recombinant Cutinase from Periplasm to Culture Medium

Some surfactants were reported to be able to improve the cell membrane permeability [27, 28]. In the present work, TDOC that does not inhibit the activity of cutinase [29], was used to increase the leakage of recombinant cutinase from periplasm to culture medium [30]. TDOC was added into the culture after 16 h of cultivation when the value of OD_{600} reached about 4.0. As shown in Fig. 4a–c, supplement of TDOC at each concentration could

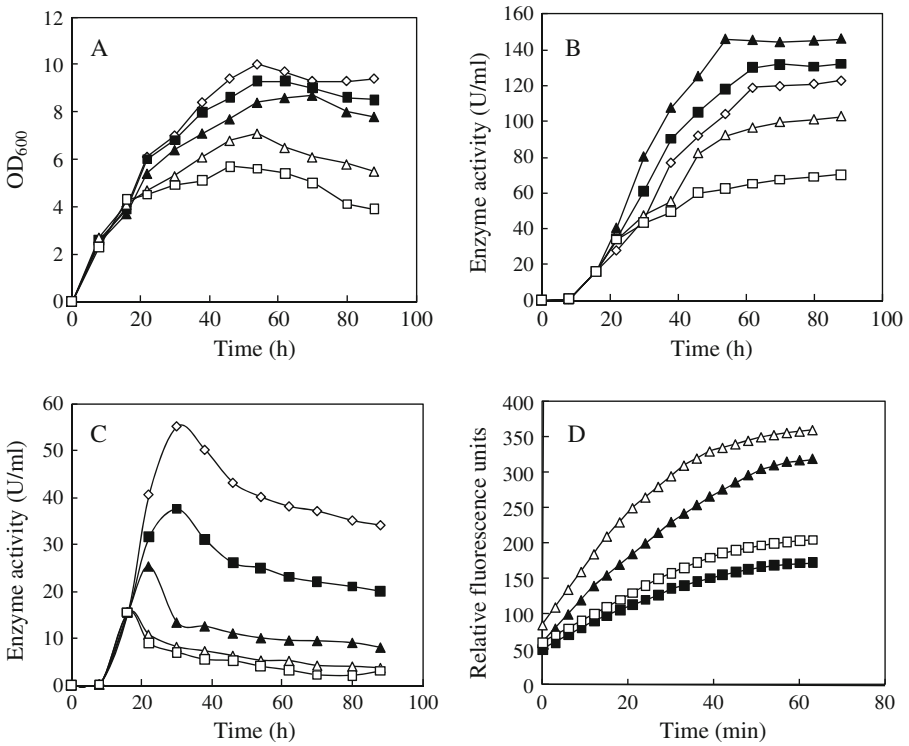

Fig. 3 Effect of glycine on the production of recombinant cutinase. The induction was performed with 0.2 mM IPTG at 30°C. **a** Effect of glycine on the growth of *E. coli*. **b** Effect of glycine on enzyme activity in culture medium. **c** Effect of glycine on enzyme activity in periplasm. Glycine supplement with concentration range of 0 mM (*empty diamond*), 50 mM (*filled square*), 100 mM (*filled triangle*), 150 mM (*empty triangle*), and 200 mM (*empty square*). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%. **d** Effect of glycine on outer membrane permeability of *E. coli*. Samples of *E. coli* cells were obtained by centrifugation and then suspended in 10 mM sodium phosphate buffer to an OD_{600} of 0.5. The bacterial culture was supplemented with 0 mM glycine at 24 h (*filled square*), 100 mM glycine at 24 h (*filled triangle*), 0 mM glycine at 48 h (*empty square*), or 100 mM glycine at 48 h (*empty triangle*). The relative fluorescence units were determined at various time points

facilitate the leakage of cutinase from periplasm to culture medium without apparent inhibition of cell growth. When 1 mM of TDOC was added, the maximum enzyme activity of 149.2 U/ml was achieved after 54 h of culture, which was increased by 25.5% compared to that without addition of TDOC (Fig. 4b). The outer membrane permeability of the middle exponential and stationary phase cells was also significantly improved when 1 mM of TDOC was added into the culture (shown in Fig. 4d).

Discussion

Inducing temperature and IPTG are important factors that can greatly affect not only the cell growth but also the expression of recombinant protein. The present study indicates that under inducement conditions of 30°C and addition of 0.2 mM IPTG, the bacterial cell grows fast, and no inclusion body is detected in the cytoplasm. The result suggests that

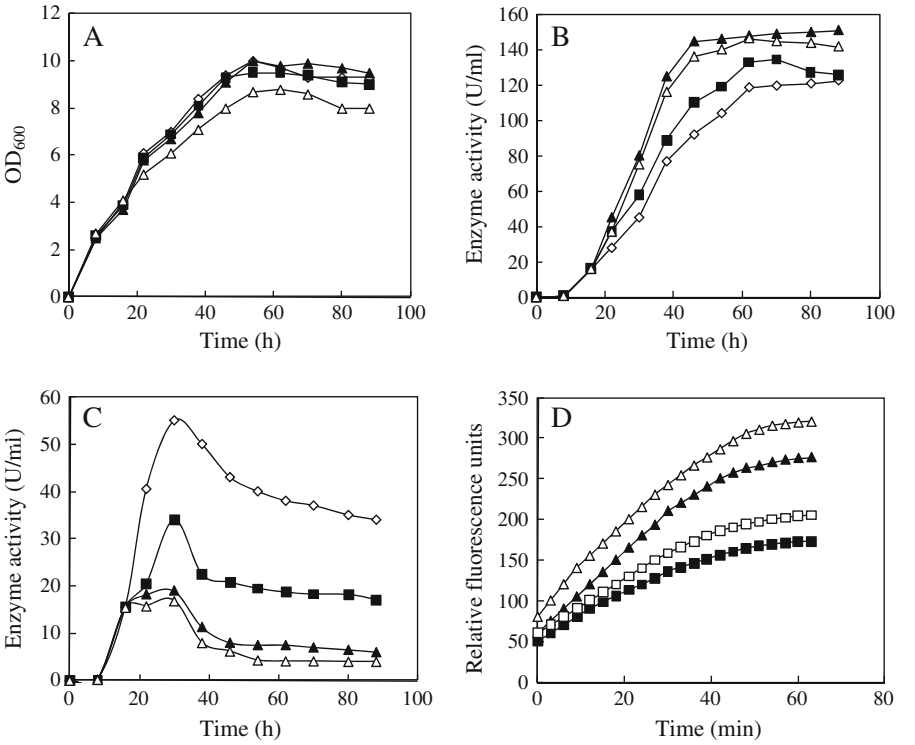

Fig. 4 Effect of TDOC on the production of recombinant cutinase. The induction was performed with 0.2 mM IPTG at 30°C. **a** Effect of TDOC on the growth of *E. coli*. **b** Effect of TDOC on enzyme activity in culture medium. **c** Effect of TDOC on enzyme activity in periplasm. TDOC supplement with 0 mM (*empty diamond*), 0.5 mM (*filled square*), 1 mM (*filled triangle*), or 2 mM (*empty triangle*). Each value represents the mean of three independent measurements, and the deviation from the mean is below 5%. **d** Effect of TDOC on outer membrane permeability of *E. coli*. Samples of *E. coli* cells were obtained by centrifugation and then suspended in 10 mM sodium phosphate buffer to an OD_{600} of 0.5. Supplement with 0 mM TDOC at 24 h (*filled square*), 1 mM TDOC at 24 h (*filled triangle*), 0 mM TDOC at 48 h (*empty square*), or 1 mM TDOC at 48 h (*empty triangle*). The relative fluorescence units were determined at various time points

most of the recombinant cutinase is expressed in the form of soluble protein under the conditions. When the IPTG concentration or the inducing temperature is increased, protein synthesis is accelerated correspondingly, whereas freshly synthesized polypeptides can hardly pass through the inner membrane in time, and thus accumulate in the cytoplasm, existing as a part of inclusion body. However, it is considered that the containment of a large amount of inclusion body is not beneficial to the bacterial growth. Therefore, based on the above results, it is hypothesized that an appropriate balance between protein synthesis rate and protein transportation rate is important to optimization of extracellular production of recombinant cutinase.

It is also observed that PelB-fused recombinant cutinase is accumulated in the periplasm initially at earlier stage of culture and then released into the culture medium at a later stage. The accumulation of recombinant protein in periplasm may cause an osmotic pressure build up to drive selective transport across the outer membrane [31]. Increase of outer membrane permeability is considered to be an effective method to facilitate protein translocation to culture medium on the premise of minimum inhibition of cell growth.

The present work proves that glycine could prompt the leakage of recombinant cutinase from periplasm to culture medium. Higher concentration of glycine, however, may severely impair the outer membrane and thereby affect the growth of bacterial cells, and even leads to cell lysis. Therefore, an appropriate concentration of glycine is desired to maximize the leak of protein as the cost of minimum inhibition of cell growth.

In addition, surfactants have also been reported to be able to block the synthesis of fatty acid and then lead to formation of the cell membrane containing a low concentration level of phospholipid or peptidoglycan. Therefore, the addition of the surfactants would significantly increase outer membrane permeability [23]. Corresponding with our presumption, the experimental results suggest that addition of the surfactant TDOC remarkably improves outer membrane permeability and prompted the leakage of recombinant cutinase to culture medium. Meanwhile, TDOC slightly inhibits cell growth.

Conclusion

In the present work, cutinase activity in the culture medium reached to 118.9 U/ml at 64 h of culture under the inducement conditions of 0.2 mM IPTG and 30°C. With the addition of 100 mM glycine or 1 mM TDOC, the enzymatic activity in the culture medium was achieved, with as high as 146.2 and 149.2 U/ml after 54 h of culture, respectively. It is concluded that, in the T7-driven expression system of *E. coli*, the synthesis rate control of recombinant enzyme and the increase of the outer cell membrane permeability could improve the extracellular enzyme production.

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