Postprint of Optimization of High-Density Fermentation of Recombinant Humicola insolens Cutinase

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Abstract

When employing the previously constructed recombinant strain E.coli BL21(DE3)/pET20b(+)-hic for high-density fermentation to prepare cutinase, it was observed that cell concentration decreased significantly under high induction intensity fermentation. Simultaneously, by measuring the phospholipid hydrolysis activity of the purified recombinant cutinase, the damaging effect of the recombinant enzyme on host cells was investigated and verified. The phosphatidylethanolamine activity of the recombinant enzyme was 9.8 U · mg-1 (with NPB hydrolysis specific activity of 1047.6 U · mg-1), and a distinct reaction zone phenomenon appeared on the egg yolk plate. Based on this, this study further attempted a fermentation strategy combining high cell density with high induction intensity to further improve the expression level of the recombinant enzyme in a 3-L fermenter. The optimized optimal conditions and results were as follows: at an OD600 of 75, lactose solution was continuously fed at a constant rate of 0.8 g · L · h-1, and after 24 h of fermentation, the enzyme activity reached a maximum value of 4788.0 U · mL-1, approximately 28 times that of shake-flask fermentation enzyme activity. Compared with the fermentation strategy induced at an OD600 of 50 with feeding of 0.2 g · L-1 · h-1 (enzyme activity 2233.0 U · mL-1), the improvement was approximately 114.0%, and the fermentation time was shortened by 40.0%.

Full Text

Optimization of High-Density Fermentation for Recombinant *Humicola insolens* Cutinase

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Abstract

During high-density fermentation of cutinase using the previously constructed recombinant strain *E. coli* BL21(DE3)/pET20b(+)-hic, a significant decline in cell concentration was observed under high induction intensity. To verify the damaging effect of the recombinant enzyme on host cells, the phospholipid hydrolysis activity of the purified recombinant cutinase was measured. The phosphatidylethanolamine activity was 9.8 $U \cdot mg^{-1}$ (with a specific activity of 1047.6 $U \cdot mg^{-1}$ for NPB hydrolysis), and a distinct reaction zone appeared on the egg yolk plate. Building on these findings, this study attempted a fermentation strategy combining high cell density with high induction intensity to further improve recombinant enzyme expression in a 3-L fermenter. The optimized conditions and results were as follows: when OD_{600} reached 75, lactose solution was fed at a constant rate of 0.8 $g \cdot L^{-1} \cdot h^{-1}$, and after 24 h of fermentation, the enzyme activity reached a maximum of 4788.0 $U \cdot mL^{-1}$, approximately 28-fold higher than that achieved in shake-flask fermentation. Compared with the strategy of inducing at OD_{600} 50 with 0.2 $g \cdot L^{-1} \cdot h^{-1}$ lactose (yielding 2233.0 $U \cdot mL^{-1}$), this represented an increase of about 114.0% while shortening the fermentation time by 40.0%.

Keywords: *Humicola insolens*; cutinase; phospholipase activity; high-density fermentation

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Introduction

Cutinase (EC 3.1.1.74) is a multifunctional hydrolase that can hydrolyze short- or long-chain fatty acid esters, triglycerides, and plant-derived polymeric cutin. It holds significant potential for applications in textile processing, food industry, chemical synthesis, detergents, environmental protection, and new energy development [**?**]. Currently, cutinase is primarily sourced from pollen and microorganisms, with microbial sources including pathogenic fungi, certain bacteria, and a few actinomycetes [**?**]. Early research focused on cutinases from phytopathogenic fungi such as *Fusarium solani* pisi [**?**] and *Pyrenopeziza brassicae* [**?**], followed by reports on enzymes from actinomycetes like *Thermobifida fusca* [**?**] and *Streptomyces acidiscabies* [**?**], and bacteria such as *Pseudomonas putida* [**?**]. Generally, fungal cutinases exhibit lower optimal temperatures around 30–40 °C and poor thermal stability at elevated temperatures, whereas bacterial cutinases have higher optimal temperatures of 50–60 °C.

Notably, the *T. fusca* cutinase was the first thermostable cutinase gene to be deciphered, with an optimal temperature of 60 °C and long-term stability at

this temperature [**?**]. *Humicola insolens* cutinase demonstrates even better thermostability; at its optimal temperature of 80 °C, the wax layer on cotton fiber surfaces melts, facilitating cutinase binding to cutin. Consequently, *H. insolens* cutinase offers superior application prospects.

Current international research on cutinase primarily focuses on screening and characterization, crystal structure analysis, and engineered strain construction, with relatively few studies on optimizing fermentation conditions to increase production. Calado, Pio, and Macedo have optimized cutinase expression at both shake-flask and 3-L fermenter levels, yet the yields remained low [**?**]. Domestic research on cutinase production has concentrated mainly on *T. fusca* and *F. solani* pisi sources. For instance, He et al. [**?**] utilized short-chain organic acids as carbon sources for *T. fusca* cutinase production, achieving a maximum activity of 51.0 $U \cdot mL^{-1}$. Liu et al. improved extracellular expression of *T. fusca* cutinase through gene knockout, co-expression of chaperone proteins, and TIR degenerate mutation, reaching a maximum extracellular yield of 80.0 $U \cdot mL^{-1}$. Zhang and Guo [**?**] constructed a *T. fusca* cutinase-cellulose binding domain (CBM) fusion protein and, through medium optimization and secretion pathway modification, obtained a maximum activity of 492.0 $U \cdot mL^{-1}$. Su et al. [**?**] further optimized *T. fusca* fermentation in 3-L fermenters, achieving a maximum activity of 2258.5 $U \cdot mL^{-1}$ with a protein expression level of 5.1 $g \cdot L^{-1}$. For *F. solani* pisi cutinase, Zhang et al. [**?**, **?**] recombinantly expressed it in *Bacillus subtilis* WSHB06-07 and, using a two-stage pH and temperature control strategy, reached a maximum activity of 312.5 $U \cdot mL^{-1}$. Chen et al. [**?**] investigated the effect of fed-batch feeding on *F. solani* pisi cutinase production, achieving a maximum activity of 545.9 $U \cdot mL^{-1}$.

This study employed the previously constructed extracellular secretion strain *E. coli* BL21(DE3)/pET20b(+)-hic for cutinase expression.

Materials and Methods

1.1.1 Strains and Media The recombinant strain *E. coli* BL21(DE3)/pET20b(+)-hic was constructed in our laboratory previously. Ampicillin was purchased from Shanghai Generay Biotech Co., Ltd. Yeast extract and tryptone were from OXOID. Industrial yeast extract powder was from Angel Yeast Co., Ltd. Industrial peptone was from Shandong Xiwang Group. Glycerol, glucose, and other common reagents were from Sinopharm.

LB medium ($g \cdot L^{-1}$): peptone 10.0, yeast extract 5.0, NaCl 10.0.
TB medium ($g \cdot L^{-1}$): glycerol 5.0, peptone 12.0, yeast extract 24.0, KH_2PO_4 2.3, $K_2HPO_4 \cdot 3H_2O$ 16.4.
Trace element solution: $Al_2(SO_4)_3 \cdot 18H_2O$ 2.0, $CoSO_4 \cdot 7H_2O$ 0.75, H_3BO_3 0.5, $MnSO_4 \cdot 7H_2O$ 24.0, Na_2MoO_4 3.0, $NiSO_4 \cdot 6H_2O$ 3.0, $ZnSO_4 \cdot 7H_2O$ 1.0 (all concentrations in $g \cdot L^{-1}$).
3-L fermenter medium ($g \cdot L^{-1}$): industrial peptone 2.4, industrial yeast

extract 4.8, $MgSO_4 \cdot 7H_2O$ 1.4, glycerol 8.0; trace element solution 10.0 mL, pH adjusted to 7.0 with ammonia.
3-L fermenter feed medium: industrial peptone 2.4, industrial yeast extract 4.8, $MgSO_4 \cdot 7H_2O$ 18.4, glycerol 600.0.

1.2 Culture Methods **Seed culture:** 10 L of glycerol stock was inoculated into 10 mL LB liquid medium containing 100 $g \cdot mL^{-1}$ ampicillin and cultured at 37 °C, 200 $r \cdot min^{-1}$ for 8–10 h.

Shake-flask fermentation: 2.5 mL of seed culture was inoculated into 50 mL fermentation medium containing 100 $g \cdot mL^{-1}$ ampicillin and cultured at 33 °C, 200 $r \cdot min^{-1}$ for 48 h.

3-L fermenter scale-up: The glycerol stock was transferred to 50 mL industrial LB medium with 0.1 $g \cdot L^{-1}$ ampicillin at 2% inoculation volume and cultured at 37 °C, 200 $r \cdot min^{-1}$ for 8 h. This seed culture was then transferred to a 3-L fermenter at 10% inoculation volume. Temperature was maintained at 37 °C and dissolved oxygen at 30% using automatic control, with pH maintained at 7.0 by ammonia supplementation. After 5–7 h, when the carbon source in the medium was exhausted and dissolved oxygen rebounded, feed medium was added exponentially until fermentation ended, with specific growth rate controlled at $= 0.18\ h^{-1}$. During the 6–8 h feeding phase, when cell concentration reached a specific OD_{600}, lactose induction began with constant-rate feeding of lactose solution at various concentrations. Samples were taken at intervals to measure cell concentration (OD_{600}) and enzyme activity. Fermentation was terminated when enzyme activity began to decline.

1.3.1 Protein Purification The fermentation broth was centrifuged to obtain the supernatant as crude enzyme solution, which was then subjected to 40% ammonium sulfate precipitation and dialysis. The resulting enzyme solution was purified using a Mono Q anion exchange column with linear gradient elution at 1.0 $mL \cdot min^{-1}$, and fractions were collected in 1.5 mL tubes. Protein concentration was determined using the Bradford method.

1.3.2 Enzyme Activity Assay **pNPB hydrolysis activity:** Enzyme activity was measured using continuous spectrophotometry at 37 °C. The total reaction volume was 1.5 mL, containing 30 L of 50 $mmol \cdot L^{-1}$ *p*-nitrophenyl butyrate (pNPB) substrate, 30 L enzyme solution, and 1440 L Tris-HCl buffer (pH 8.0). The increase in *p*-nitrophenol was recorded at 405 nm for 1 min. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 mol pNPB to produce 1 mol *p*-nitrophenol per minute at 37 °C.

1.3.3 Phospholipase Activity Assay **Borax-egg yolk plate preparation and qualitative analysis:** Borax-egg yolk plates were prepared (NaCl 0.66%, H_3BO_3 1.09%, egg yolk emulsion 2.0%, $Na_2B_4O_7 \cdot 10H_2O$ 0.19%, agar

1.5%, pH 7.2–7.4). A plastic ring was placed on the plate, and enzyme solution was added to prevent diffusion, with heat-inactivated enzyme as control. The plate was incubated at 37 °C for 8–12 h. After removing the ring, the egg yolk reaction zone was observed for preliminary qualitative assessment of phospholipase activity.

Phosphatidylethanolamine substrate solution: 1 g phosphatidylethanolamine was dissolved in 50 mL of 20 mmol · L^{-1} phosphate buffer (pH 8.5) containing 0.5% (w/v) Triton X-100 surfactant by ultrasonication.

Recombinant cutinase phospholipid hydrolysis activity: 10 mL of substrate solution was mixed with recombinant cutinase solution and reacted at 80 °C for 5 min. The reaction was terminated by adding 15 mL of 95% ethanol. A blank control contained 10 mL substrate solution and 15 mL 95% ethanol. Phenolphthalein was added, and the generated fatty acids were titrated with 0.01 mol · L^{-1} NaOH. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the decomposition of 1 mol phosphatidylethanolamine to produce 1 mol fatty acid per minute at 80 °C.

Results and Discussion

2.1 Effect of Induction Intensity on High-Density Fermentation
Given that lactose induction intensity significantly affects target protein expression, optimization was performed by adjusting the lactose feeding rate. When OD_{600} reached 50, the temperature was lowered to 30 °C, and lactose solution was fed at constant rates of 0.1, 0.2, and 0.4 g · L^{-1} · h^{-1} to induce expression. Cell biomass and enzyme activity were measured at various time points. The results are shown in [Figure 1: see original paper].

At a lactose feeding rate of 0.1 g · L^{-1} · h^{-1}, the maximum cell concentration OD_{600} reached 109. As the lactose feeding rate increased, cell biomass gradually decreased. At 0.4 g · L^{-1} · h^{-1}, the maximum OD_{600} was only 72, representing 66% of that at 0.1 g · L^{-1} · h^{-1}. Within a certain range, recombinant cutinase activity increased with lactose feeding rate, but beyond a threshold, activity declined. At 0.1 g · L^{-1} · h^{-1}, enzyme activity was 1731.0 U · mL^{-1}. The highest activity of 2233.0 U · mL^{-1} was achieved at 0.2 g · L^{-1} · h^{-1}, approximately 1.3-fold higher than at 0.1 g · L^{-1} · h^{-1}. At 0.4 g · L^{-1} · h^{-1}, enzyme activity began decreasing after 8 h of induction, reaching a maximum of only 1562.0 U · mL^{-1}—the lowest among the three conditions. SDS-PAGE analysis of the extracellular supernatant at 0.2 g · L^{-1} · h^{-1} is shown in [Figure 2: see original paper], demonstrating that protein expression increased with fermentation time.

Comparing these results with Wu et al. [**?**], who reported that recombinant maltosyltrehalose hydrolase (MTHase) and maltosyltrehalose synthase (MTSase) from *Sulfolobus acidocaldarius* expressed in *E. coli* showed decreased cell mass after 35 h at 0.4 g · L^{-1} · h^{-1} induction, with maximum cell concentration at ap-

proximately 80% of that at 0.2 $g \cdot L^{-1} \cdot h^{-1}$. Similarly, Zhang et al. [**?**] observed cell mass decline after about 22 h for recombinant trehalose synthase from *Thermus thermophilus*, though the maximum cell concentration remained at 77% of the 0.2 $g \cdot L^{-1} \cdot h^{-1}$ level. In contrast, recombinant *H. insolens* cutinase at 0.4 $g \cdot L^{-1} \cdot h^{-1}$ showed cell mass decline after only 22 h, with maximum cell concentration at just 63.6% of the 0.2 $g \cdot L^{-1} \cdot h^{-1}$ level. Therefore, besides the metabolic pressure from high induction intensity, the low cell concentration at 0.4 $g \cdot L^{-1} \cdot h^{-1}$ may also be attributed to the intrinsic activity of the recombinant cutinase itself.

2.2.1 Purification of Recombinant Cutinase To investigate the hypothesis that cutinase activity affects cell concentration during fermentation, the recombinant enzyme was purified. SDS-PAGE analysis of the purified enzyme is shown in [Figure 3: see original paper], revealing a single protein band with purity suitable for subsequent experiments. The specific activity of the purified recombinant cutinase was 1047.6 $U \cdot mg^{-1}$. The specific activities and yields at each purification stage are summarized in .

2.2.2 Determination of Recombinant Cutinase Phospholipid Hydrolysis Activity As a Gram-negative bacterium, *E. coli* possesses a double membrane structure: the inner membrane composed primarily of phospholipids and membrane proteins, and the asymmetric outer membrane with phospholipids on the inner side and lipopolysaccharides on the outer side. Cutinase belongs to the esterase family and can hydrolyze various ester substrates, including soluble esters, insoluble esters, and polyesters. According to Su et al. [**?**], *T. fusca* cutinase exhibits phospholipid hydrolysis activity against *E. coli* cell membranes, causing host cell damage during fermentation. We measured the phospholipid hydrolysis activity of recombinant cutinase to identify the specific cause of poor cell growth.

Using phosphatidylethanolamine as substrate and acid-base titration (method 1.3.3), the phosphatidylethanolamine activity of recombinant cutinase was determined to be 9.76 $U \cdot mg^{-1}$. Lecithin (phosphatidylcholine) is a major component of egg yolk emulsion. Borax-egg yolk plates prepared with egg yolk emulsion were used for qualitative detection of phospholipase activity. Using heat-inactivated enzyme as control and purified enzyme as sample, overnight reaction results are shown in [Figure 4: see original paper]. The sample treated with recombinant cutinase displayed a milky white halo from substrate hydrolysis, while the control and untreated areas showed no reaction, confirming that recombinant cutinase possesses phospholipid hydrolysis activity.

Combined with the observed low cell concentration during fermentation, these results indicate that recombinant *H. insolens* cutinase causes damage to host *E. coli* cells during expression due to its phospholipid hydrolysis activity. Thus, at 0.4 $g \cdot L^{-1} \cdot h^{-1}$ induction intensity, the rapid cell mass decline and low maximum cell concentration result from both high induction pressure and the enzyme' s

intrinsic phospholipase activity.

2.3 Effect of High Cell Density Combined with High Induction Intensity on Fermentation Based on the results in sections 2.1, 2.2, and 2.3, recombinant *H. insolens* cutinase expression in *E. coli* is affected by both induction intensity and the enzyme's phospholipid hydrolysis activity, with cell growth significantly inhibited at higher induction intensities. However, increased lactose feeding rates substantially enhanced short-term protein expression, suggesting that recombinant enzyme folding remained efficient even under high induction. This characteristic provided an opportunity to explore a novel fermentation strategy. Therefore, we proposed inducing at high cell density while feeding high-concentration lactose solution to reduce the impact of metabolic pressure and phospholipase activity on cell growth while increasing recombinant protein expression and shortening fermentation time.

2.3.1 Optimization of Induction Intensity at OD_{600} 75 When OD_{600} reached 75, the temperature was adjusted to 30 °C, and lactose solution was fed at constant rates of 0.4, 0.8, and 1.2 $g \cdot L^{-1} \cdot h^{-1}$. Cell biomass and recombinant enzyme activity were measured at different time points, with results shown in [Figure 5: see original paper].

All conditions reached maximum cell concentration 4–6 h after induction, followed by a noticeable decline. The decrease was particularly pronounced at 0.8 and 1.2 $g \cdot L^{-1} \cdot h^{-1}$, with OD_{600} dropping below 60 after 8 h of induction. Higher lactose concentrations promoted recombinant enzyme production. At 0.8 $g \cdot L^{-1} \cdot h^{-1}$, maximum enzyme activity of 4778.0 $U \cdot mL^{-1}$ was achieved at 26 h, approximately 2.5-fold higher than at 0.4 $g \cdot L^{-1} \cdot h^{-1}$. However, further increasing induction intensity to 1.2 $g \cdot L^{-1} \cdot h^{-1}$ exacerbated the inhibitory effects on cell growth, shortening the fermentation cycle to 24 h and reducing maximum activity to 3588 $U \cdot mL^{-1}$ (75% of the 0.8 $g \cdot L^{-1} \cdot h^{-1}$ level). SDS-PAGE analysis of the extracellular supernatant at 0.8 $g \cdot L^{-1} \cdot h^{-1}$ ([Figure 6: see original paper]) showed that the characteristic cutinase band intensified with fermentation time, confirming that increased enzyme activity resulted from protein accumulation.

At low induction intensity (0.4 $g \cdot L^{-1} \cdot h^{-1}$), although fermentation lasted 28 h, enzyme activity remained low. Conversely, at 1.2 $g \cdot L^{-1} \cdot h^{-1}$, while activity increased within the same timeframe, the fermentation period was too short. By balancing induction intensity and fermentation duration, the optimal condition was determined to be induction at OD_{600} 75 with a lactose feeding rate of 0.8 $g \cdot L^{-1} \cdot h^{-1}$.

When lactose feeding rates were 0.8 or 1.2 $g \cdot L^{-1} \cdot h^{-1}$, enzyme activity decreased after reaching its peak, possibly because some recombinant proteins secreted in large quantities were not properly folded. Although these proteins showed activity during sampling, they gradually degraded over time, causing activity loss. To assess recombinant protein stability, a sample from 24 h fermentation at 0.8 $g \cdot L^{-1} \cdot h^{-1}$ was stored at 4 °C for 20 days, yielding an activity of 4313

$U \cdot mL^{-1}$, similar to the post-peak value at 26 h. Temperature and pH stability parameters of this sample were comparable to those of purified recombinant enzyme, confirming that the fermented recombinant protein was structurally intact and stable.

2.3.2 Optimization of Induction Intensity at OD_{600} 95 Building on section 2.3.1, we further increased both cell density and induction intensity to achieve higher protein expression and shorter fermentation time. When OD_{600} reached 95, the temperature was lowered to 30 °C, and lactose solution was fed at 0.8, 1.2, and 1.6 $g \cdot L^{-1} \cdot h^{-1}$. Results are shown in [Figure 7: see original paper].

Under all conditions, both cell concentration and enzyme activity peaked at 24–26 h. The maximum activity of 2077 $U \cdot mL^{-1}$ was obtained at 1.2 $g \cdot L^{-1} \cdot h^{-1}$. During 3-L fermenter cultivation, continuous feeding of lactose and medium maintained cell growth and enzyme production. However, when induction began at OD_{600} 95, cells had already entered late exponential phase with reduced vitality, slowing biomass increase and heterologous protein expression capacity. Combined with the effects of inducer and cutinase phospholipase activity, cell biomass declined after only 2 h of induction, resulting in insufficient recombinant protein accumulation. Consequently, enzyme activity at OD_{600} 95 induction was significantly lower than at OD_{600} 75.

The strategy combining high cell density with high induction intensity significantly improved recombinant enzyme expression and shortened fermentation time. The optimized conditions were: induction at OD_{600} 75 with constant lactose feeding at 0.8 $g \cdot L^{-1} \cdot h^{-1}$ for 24 h total fermentation time, maintaining pH 7.0 throughout. Under these conditions, recombinant cutinase activity reached a maximum of 4778.0 $U \cdot mL^{-1}$, approximately 28-fold higher than shake-flask fermentation and 114% higher than the strategy of inducing at OD_{600} 50 with 0.2 $g \cdot L^{-1} \cdot h^{-1}$ lactose (2233.0 $U \cdot mL^{-1}$), while reducing fermentation time by 40%.

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Note: Figure translations are in progress. See original paper for figures.