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Research Article

Expression and Induction Optimization of *Mucor Circinelloides* Lipase in *Escherichia Coli*

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Abstract: Microbial lipases serve as versatile biocatalysts for modifying lipid substrates, enabling diverse biotechnological applications spanning food technology, renewable energy production, and pharmaceutical sectors. Genomic analysis of *Mucor circinelloides* WJ11 revealed multiple lipase-encoding genes, with WJ_23 successfully cloned and heterologously expressed in *Escherichia coli*. Since the lipase expression was mainly concentrated in the cell pellets, appropriate induction strategies had been investigated to enhance the extracellular production of the lipase. The results showed that when 0.5 mmol/L Isopropyl-1-thio-β-D-Galactopyranoside (IPTG) and 0.8% Triton X-100 were simultaneously added in the later stage of logarithmic growth at 30°C, the peak extracellular lipase activity attained 2580 U/L, demonstrating a 6.79-fold increase over the wild strain. This study provided a theoretical basis for further improving the extracellular production of other heterogonous enzymes in *E. coli*, and it also laid a foundation for the future application of lipase in *M. circinelloides*.

Keywords: *Mucor circinelloides*, Lipase, Expression, Induction, *Escherichia coli*

Introduction

Lipase, or triacylglycerol acylhyrolase (EC3.1.1.3), can not only catalyze the hydrolysis of glycerol ester into glycerol, diacylglycerol ester, monoacylglycerol ester and fatty acid but also catalyze the hydrolysis, ester synthesis, acidolysis, transesterification, alcoholysis and ammonolysis of glycerides and water-insoluble esters (Chandra *et al.*, 2020; Yao *et al.*, 2021; Nie *et al.*, 2024). Therefore, lipase is widely used in bioenergy, oil industry, food processing, leather silk spinning raw material degreasing, washing industry, medicine and feed industry and many other fields (Coelho and Orlandelli, 2021; El-Metwally *et al.*, 2023; Lu *et al.*, 2024). In recent years, lipases derived from microorganisms have attracted wide attention because of their wide variety, good catalytic activity and stability and low cost (Yao *et al.*, 2021; Patel *et al.*, 2019; Majumder *et al.*, 2024).

The zygomycete filamentous fungus *Mucor circinelloides* was a lipogenic strain that can produce a high content of a valuable ω-6 polyunsaturated fatty acid Gamma-Linolenic Acid (GLA) (Fazili *et al.*, 2022; Zhang *et al.*, 2022). Previous studies have also shown that *M. circinelloides* was a potential lipase producer that can synthesize lipase both in intracellular and extracellular and the immobilized *M. circinelloides* was used for whole-cell catalysis of ester synthesis, transesterification and transesterification (Andrade *et al.*, 2012; 2014; Garcia-Galan *et al.*, 2013). Although lipase from *M. circinelloides* had broad application prospects, the production cost still became the main restriction factor.

In order to increase the production of microbial enzymes, heterologous and efficient overexpression of enzymes by genetic engineering is considered to be one of the most effective ways. *Escherichia coli* has the advantages of rapid growth, a short fermentation cycle and low nutritional requirements, so it is the first choice of host for genetic engineering. In our previous study, potential lipase genes in *M. circinelloides* were identified by extensive bioinformatics analysis. In preliminary studies, RT-qPCR analysis revealed that the WJ_23 gene

exhibits a high level of expression in *M. circinelloides* (Zan *et al.*, 2016). Elevated expression levels suggest this gene is highly active within the cell and may play a pivotal role in cellular metabolic processes. Selecting genes with high expression levels for experimental investigation can enhance the success rate and reliability of the data. Furthermore, the protein encoded by the WJ_23 gene contains a signal peptide, facilitating its efficient secretion outside the cell, enabling it to exert its biological functions intercellularly. This characteristic is of significant importance for studying metabolic regulation between cells (Zan *et al.*, 2016; 2018; 2019). Based on the predicted characteristics, sub-cellular location and expression profiling of these lipase genes, the candidate lipase gene WJ_23 was selected for further detailed study.

In the present study, the selected lipase WJ_23 from the oleaginous fungus *M. circinelloides* WJ11 was involved in cloning and enhancing its expression in *E. coli*. Various cultivation approaches, including optimizing induction concentration, temperature and time, have been implemented to enhance the secretion of recombinant lipase by *E. coli*. Additionally, the effect of surfactant Triton X-100, as well as its interaction with the improvement of lipase yield during the culture period, were also discussed. This study provides a theoretical basis for further improving the production of other heterogonous enzymes in *E. coli* and it also lays a foundation for the future application of lipase in *M. circinelloides*.

Materials and Methods

Strains and Cultural Conditions

The strain *Mucor circinelloides* WJ11 (CCTCC no. M2014424), possessing well-established lipase activity, was previously isolated and stored in our lab (Zan *et al.*, 2016). The plasmid pMD19T and *E. coli* DH5α were employed for gene cloning purposes. For heterologous expression, the plasmid pET20b (+) and *E. coli* Rosetta (DE3) were utilized to facilitate protein expression in *E. coli*.

The K&R culture medium for *M. circinelloides* WJ11 consisted of yeast extract 1.5 g/L, ammonium tartrate 3.3 g/L, glucose 30 g/L, KH_2PO4 7.0 g/L, Na_2HPO_4 2.0 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L, $Co(NO_3)_2{\cdot}6H_2O$ 0.0001 g/L $FeCl_3{\cdot}6H_2O$ 0.008 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L, $MnSO_4{\cdot}5H_2O$ 0.0001 g/L, $ZnSO_4{\cdot}7H_2O$ 0.001 g/L, $CuSO_4{\cdot}5H_2O$ 0.0001 g/L (Zhang *et al.*, 2022). The Luria-Bertani (LB) broth, featuring yeast powder 5 g/L, peptone 10 g/l and NaCl 10 g/L, maintained a pH of 7.1 and served as the seed medium. The fermentation medium contained yeast powder 24 g/L, peptone 12 g/L, glucose 6 g/L, KH_2PO_4 2.31 g/L, $K_2HPO_4{\cdot}3H_2O$ 16.43 g/L, pH 7.1 (Zhang *et al.*, 2018).

Following a 24-h submerged culture of *M. circinelloides* WJ11 spore suspension in 150 ml K&R medium (30°C, 150 rpm orbital shaking), fungal biomass was harvested via filtration for subsequent lipase gene isolation procedures. *E. coli* cultures were incubated in LB broth at 37°C with 100 μg/mL ampicillin. The recombinant *E. coli* Rosetta (DE3) strain carrying the plasmid was initially cultured in 50 mL of seed medium supplemented with 100 μg/mL ampicillin. This preculture was grown at 37°C with constant shaking at 200 rpm for 8 h. Subsequently, 10% of this starter culture was transferred to a fresh fermentation medium containing the same antibiotic concentration for batch fermentation. At certain intervals of 3 ~ 6 h, samples were collected to monitor both cell growth and lipase activity. Isopropyl-1-thio-β-D-Galactopyranoside (IPTG) and Triton X-100 with different concentrations were prepared as inducers. The induction parameters, including concentration and the initial temperature, etc., are contingent upon the experimental setup. Each value is the average of three separate measurements.

Cloning and Expression of Lipase from M. circinelloides

The lipase coding sequence was PCR-amplified from *M. circinelloides* WJ11 cDNA using gene-specific primers flanked by *Nde*I and *Xho*I restriction sites at their 5' and 3' termini. The forward primer sequence is GCGGCATATGGTCT and the reverse primer sequence is CGCAGGATCCTTAGAGACAGAGACCCTCATT GATA. Thermal cycling conditions consisted of an initial 94°C denaturation step for 30 sec, followed by 32 cycles featuring a touchdown annealing protocol (63 to 55°C, decreasing 1°C per cycle) for 30 sec, with a 72°C extension for 1 min. The resulting amplicon was subsequently ligated into both pMD19T-simple and pET20b (+) vectors via the engineered restriction sites. Positive clones containing the WJ_23 lipase insert were identified through restriction analysis and Sanger sequencing, yielding the final constructs pMD19T/lip and pET20b/lip.

The recombinant *E. coli* DE3 strain carrying pET20b/lip plasmid was inoculated into a 50 mL LB starter culture supplemented with 100 μg/mL ampicillin for primary cultivation at 37°C with 200 rpm agitation (8 h). The culture was incubated at 37°C with shaking at 200 rpm for 8 h. Subsequently, a 10% (v/v) inoculum was transferred into 100 mL of fermentation medium supplemented with equivalent antibiotic concentration. Secondary cultivation proceeded at 30°C with identical agitation until reaching the mid-log phase (OD_{600} 1.5-2.0), whereupon 0.5 mM IPTG was introduced for recombinant protein induction. Following induction for 18 h, biomass was harvested by centrifugation (10,000 rpm, 30 min, 4°C) with subsequent separation of extracellular supernatant and cellular pellet fractions.

SDS-PAGE and Western Blot Assay

SDS-PAGE and Western Blot (WB) were used to identify the protein secretion and expression.

Resuspended cell pellets were lysed in a buffer (pH 8.0) containing 100 mM KH_2PO_4, one mM Dithiothreitol (DTT), one mM benzidine hydrochloride and 20% glycerol (v/v). The purification of the target protein was performed utilizing a HisTrap HP column (GE Healthcare, Sweden). Total protein quantification employed the Bradford assay with Bovine Serum Albumin (BSA) as the calibration standard. SDS-PAGE analysis was executed on a 12% (w/v) acrylamide gel under electrophoretic conditions of 200 V for 45 min. The entire purification procedure was conducted at 4°C to preserve protein stability. To preserve functional integrity, the purified protein underwent cryopreservation at -80°C in 40% (v/v) glycerol solution immediately following purification (Zan *et al.*, 2018).

After electrophoresis, the stacking gel was removed. The resolving gel was assembled with a PVDF membrane (activated in methanol and equilibrated in transfer buffer) and transfer buffer-soaked filter papers. Electroblotting was performed at a constant voltage of 30 V for 30 min (for target proteins ≤50 kDa). Following protein transfer, the PVDF membrane was sequentially rinsed with 1×PBST (three cycles of 5-min washes) under ambient conditions, then incubated with 5% (w/v) non-fat milk blocking buffer prepared in 1×PBST using orbital shaking (40 rpm) for 120 min. After three additional PBST washes, the membrane was incubated with primary antibody (1:2000 dilution in 5% non-fat milk) for one h at room temperature. The primary antibody was subsequently recovered and stored at -20°C. Following three final PBST washes, the membrane was incubated with an enhanced chemiluminescence substrate solution in the dark for 2 min and chemiluminescent signals were captured by the imaging system with exposure times optimized based on signal intensity.

Determination of Bacteria Biomass

Culture density measured at 600 nm (OD_{600}), representing the bacterial biomass, was monitored with a spectrophotometer during cultivation.

Determination of Lipase Activity

Lipase activity was determined using *p*-nitrophenyl palmitate (*p*-NPP) as the hydrolytic substrate. Spectrophotometric quantification of *p*-nitrophenol release was performed at 410 nm by mixing 50 µL cell-free fermentation broth with 100 µL 10 mM *p*-NPP in 3.85 mL Tris-HCl buffer (50 mM, pH 8.0) at 40°C. Enzyme activity Units (U) were standardized as the amount of catalyst required to release one µmoL *p*-nitrophenol per minute under these reaction conditions (Zhang *et al.*, 2017). Each value is the average of three separate measurements.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics 22.0 (IBM, USA). Experimental results are presented as mean ± SEM values derived from triplicate experimental replicates. Comparative analyses of intergroup differences were conducted via Student's *t*-test, with statistical significance defined at $p<0.05$.

Results and Discussion

Cloning and Expression of Lipase WJ_23 in E. Coli

The lipase WJ_23 coding sequence was isolated from *M. circinelloides* WJ11 cDNA and heterologously expressed in *E. coli*. The length of the lipase gene was 1158 bp for encoding a mature protein consisting of 386 amino acids. Following cloning into the pET-20b (+) expression vector, the recombinant construct was transformed into *E. coli* Rosetta (DE3) host cells. After culture for 60 h, the lipase activity of the recombinant strain using *p*-NPP as substrate was 1560 U/L in the culture supernatant, which was 4.1-fold higher than that of extracellular lipases from *M. circinelloides* WJ11. SDS-PAGE and Western Blot analysis demonstrated that the recombinant lipase WJ_23 exhibited a molecular mass of approximately 43 kDa and the expression of the enzyme concentrated in the cell pellets of *E. coli* (Fig. 1). Under the same culture conditions, the *ρ*-NPP hydrolase activity and expression protein bands were not measured in the culture supernatant of the control strain with an empty plasmid pET 20b (+). Several appropriate strategies, such as optimizing induction conditions, were carried out in the following study to target the lipase to the culture medium.

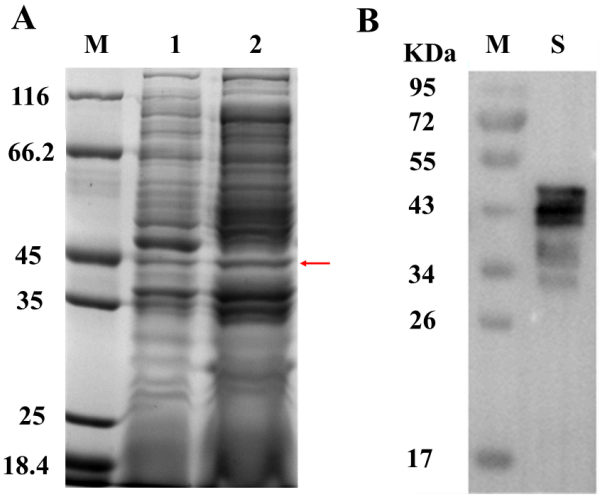

Fig. 1: Expression and identification of SDS-PAGE (A) and WB (B) results. (A) Expression and identification of SDS-PAGE results.

Effect of Induction Concentration on Cell Growth and Extracellular Lipase Yield

IPTG is a highly effective and stable inducer that is not metabolized by bacteria, so it is widely used in the laboratory to induce the expression of target proteins (Xu *et al.*, 2023; Simas *et al.*, 2023). The recombinant *E. coli* was cultured in the seed medium at 37°C overnight and then cultured in the fermentation medium at 30°C until OD_{600} reached 1.5-2.0, IPTG with the concentration of 0-1 mmol/L was added for induction. The effect of IPTG concentration on the process of cell growth and lipase production is shown in Fig. (2). After fermentation for 24 h, the growth of recombinant *E. coli* reached the maximum, then the growth of strain entered the equilibrium stage and the recombinant strain began to decline at 40 h (Fig. 2A). As the concentration of IPTG increased, its negative impact on cell growth became more significant. At an IPTG concentration of 1 mmol/L, the growth of recombinant *E. coli* was significantly inhibited. When the culture was induced with 0.5 mmol/L IPTG, the extracellular lipase activity reached the maximum value of 1620 U/L (Fig. 2B), which was increased by 3.5-fold compared with the control group (no inducer added). Thus, 0.5 mmol/L IPTG was chosen for further study to induce lipase production. It is worth noting that the recombinant protein was also expressed to some extent when IPTG was not added in the control group, which might be due to the presence of lactose-inducible components similar to IPTG in the composite medium. When a certain amount of IPTG was added to the medium, the induced expression of recombinant protein could be enhanced.

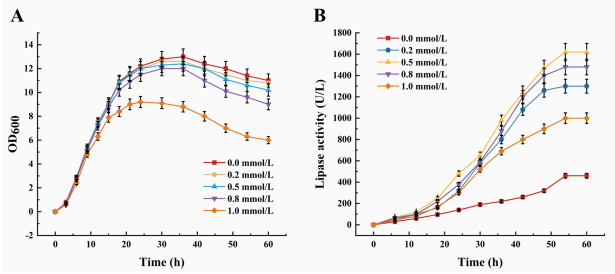

Fig. 2: Cell growth (A) and lipase activity (B) from cultivations by recombinant E. coli when induced with different concentrations of IPTG. The concentrations are 0.0, 0.2, 0.5, 0.8 and 1.0 mmol/L. The induction conditions were evaluated at five concentrations (0.0, 0.2, 0.5, 0.8 and 1.0 mmol/L), with data representing mean ± SEM from triplicate fermentation experiments

Effect of Induction Temperature on Cell Growth and Extracellular Lipase Yield

Cell growth and recombinant protein synthesis exhibited affected temperature dependence, prompting evaluation of three induction temperatures (25, 30, 37°C) as demonstrated in Figure (3). The growth of recombinant *E. coli* was accelerated with the increase of temperature and the maximum biomass was obtained at 37°C (Fig. 3A). Higher biomass did not necessarily correlate with higher extracellular enzyme production. The extracellular lipase activity measured at 37°C was significantly reduced compared to that at other temperatures. The results of this study demonstrated that induction at 37°C strongly inhibited the extracellular secretion of lipase. Figure (3B). Higher temperatures may have accelerated lipase production, resulting in the development of huge quantities of inactive protein aggregates known as inclusion bodies (Cheng *et al.*, 2011). This might happen because, while high temperatures enhance cell membrane fluidity and protein production rate, overexpression frequently leads to protein accumulation within the cell (Rezaei *et al.*, 2020; Chen *et al.*, 2014). In contrast, lowering the induction temperature can slow protein production and modify cytoplasmic membrane fluidity, thereby encouraging appropriate protein folding and preventing aggregation (Schumann, 2000; Schügerl *et al.*, 2000). Appropriate temperature reduction was conducive to the transmembrane transport of recombinant proteins. Based on the results of biomass and lipase activity, 30°C was identified as the optimal temperature for both cell growth and lipase production. Under these conditions, the maximum extracellular lipase activity was attained at 1640 U/L.

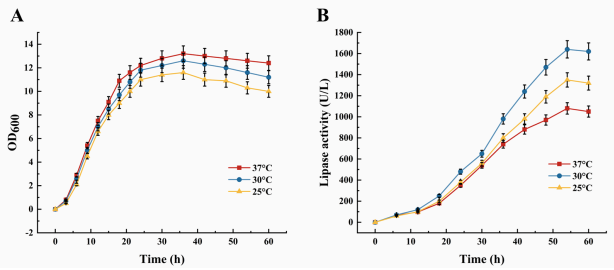

Fig. 3: Cell growth (A) and lipase activity (B) from cultivations by recombinant E. coli at different induction temperatures. Three induction temperatures (37°C, 30°C and 25°C) were compared, with biological triplicates presented as mean ± SEM

Effect of Induction Time on Cell Growth and Extracellular Lipase Yield

Excessive production of recombinant proteins often strains the metabolic capacity of host cells, thereby reducing plasmid stability, protein expression and cell biomass (Zhang *et al.*, 2018; Cheng *et al.*, 2011). In order to explore optimal IPTG induction time, experiments were conducted to add 0.5 mmol/L IPTG to the cultures in the early phase (OD_{600} = 1), middle phase (OD_{600} = 5) and later phase (OD_{600} = 8) of logarithmic growth, respectively (Fig. 4). The results from Fig. (4A) showed that there was no significant impact on cell growth when induced by IPTG at different periods. However, the extracellular expression of lipase was

observably influenced by the induction time. As shown in Fig. (4B), the extracellular production of recombinant lipase induced by IPTG in the late stage reached the maximum activity of 1760 U/L, which was remarkably higher than that induced in the early and middle stages.

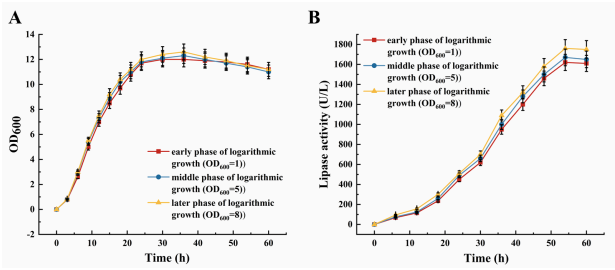

Fig. 4: Cell growth (A) and lipase activity (B) from cultivations by recombinant E. coli when induced at the early phase of logarithmic growth (OD_{600} = 1), middle phase of logarithmic growth (OD_{600} = 5) and later phase of logarithmic growth (OD_{600} = 8). Data are represented as the mean ± SEM of three independent cultures

Effect of Triton X-100 on Cell Growth and Extracellular Lipase Yield

Triton X-100, also known as polyethylene glycol octyl phenyl ether, is a non-ionic surfactant with a molecular formula of $C_{34}H_{62}O_{11}$ and a molecular weight of 646.86 g/mol. It is known to dissolve lipids to increase permeability to cell membranes (Wang *et al.*, 2013). Thus, we designed concentration-gradient experiments to assess the dual impact of Triton X-100 on recombinant *E. coli* growth kinetics and extracellular lipase production. Specifically, Triton X-100 was added simultaneously with the IPTG inducer during the later stage of fermentation (Fig. 5). The findings indicated that the addition of Triton X-100 into the system resulted in a degree of growth inhibition for the recombinant strain, with increased concentrations of the surfactant corresponding to heightened levels of inhibition (Fig. 5A). However, despite its inhibitory effect on cell growth, Triton X-100 significantly promoted extracellular lipase production in recombinant *E. coli*. This phenomenon can be explained by the fact that surfactants, such as Triton X-100, improve cell membrane permeability and reduce resistance to the transfer of nutrients and oxygen into the cells, thereby enhancing the production of target metabolites during fermentation (Wang *et al.*, 2013). As shown in Fig. (5B), when the concentration of Triton X-100 was 0.8%, extracellular lipase activity reached a maximum of 2580 U/L, which was 1.47-fold higher than that of the control group (no Triton X-100 added). Statistical validation highlighted this measurement as the significant maximum for extracellular lipase activity across all trial groups. The beneficial effect of increasing Triton X-100 may be due to its ability to increase cell membrane permeability, as documented in previous studies (Li *et al.*, 2010). For example, studies have shown that Triton X-100 significantly facilitated the secretion of intracellular pigment and enhanced pigment production from *Monascus purpureus* H1102 (Wang *et al.*, 2013). Similarly, Lin *et al.* reported a 50-fold increase in extracellular alkaline lipase production by *Pseudomonas pseudoalcaligenes* F-111 when Triton X-100 was added to the culture medium (Lin *et al.*, 1995). The addition of Triton X-100 in the medium significantly promoted the production of pullulanase by *E. coli* (Duan *et al.*,2015). A previous investigation demonstrated enhanced extracellular expression of recombinant ZZ protein-alkaline phosphatase (rZZ-AP) in *E. coli* HB101 cultures employing a medium supplemented with 1% glycine, 5% sucrose and 1% Triton X-100. This formulation induced an 18.6-fold increase in rZZ-AP yield compared to additive-free controls (Bao *et al.*, 2016). Taken together, these results highlight the potential of Triton X-100 to enhance the secretion of various intracellular proteins and metabolites, further supporting its utility in optimizing recombinant protein production.

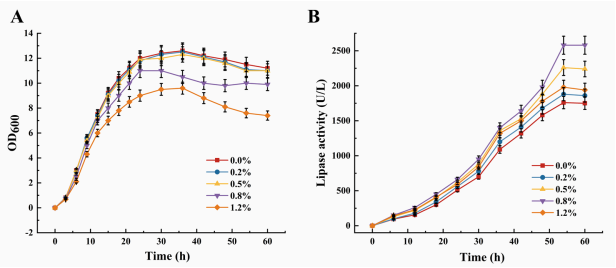

Fig. 5: Cell growth (A) and lipase activity (B) from cultivations by recombinant E. coli with supplementation of Triton X-100 at different concentrations. The concentrations tested were 0.0, 0.2, 0.5, 0.8 and 1.2%, with data presented as the mean ± SEM of three independent fermentation experiments

Conclusion

In conclusion, this study achieved the cloning and successful heterologous expression of the lipase WJ_23 derived from *M. circinelloides* WJ11 using *E. coli* as the host. To address intracellular accumulation of the recombinant lipase, strategic approaches, including induction parameter optimization and cell membrane permeabilization, were systematically evaluated to enhance extracellular secretion. Combinatorial optimization of induction parameters (0.5 mmol/L IPTG and 0.8% Triton X-100) during the later stage of logarithmic growth at 30°C yielded maximal extracellular lipase activity of 2580 U/L, representing a 6.79-fold enhancement over the wild-type strain. These findings not only provide valuable fermentation strategies for improving the extracellular yield of other heterologous proteins expressed in *E. coli* but also lay a solid foundation for scalable industrial lipase production.

Lipase-catalyzed biodiesel production is recognized as an environmentally friendly technology and the demand for efficient enzymes continues to grow. While most lipases are currently expressed in fungal systems, bacterial expression systems, such as the *E. coli* strain used in this study, offer distinct advantages in terms of cost-effectiveness and scalability, particularly when using inexpensive raw materials. This makes our approach highly suitable for economically viable industrial applications.

To further advance this research, future studies should focus on scaling up the production process and exploring broader industrial and biotechnological applications. Specific directions include investigating optimal IPTG concentrations to maximize lipase yield, assessing lipase stability under varying pH, temperature and ionic strength conditions using techniques such as differential scanning calorimetry (DSC) and Circular Dichroism (CD) spectroscopy and applying our findings to practical applications such as waste management and textile recycling. These efforts will not only validate our current results but also expand the potential impact of this research in both academic and industrial contexts.

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Author's Contributions

Yueping Yang: Completed the whole experiment research and manuscript writing.

Yao Zhang: Involved in the study conception, experimental design and execution, data analysis, figures and tables arrangement, result interpretation and review of the final draft.

Xinai Liu, Yan Sun and Zhuo Liu: Participated in the experimental research.

Qing Liu and Yuanda Song: Conceived the study and reviewed the original manuscript.

All authors read and approved the final manuscript.

Ethics

This article is original and contains unpublished material. The corresponding authors confirm that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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