Research Progress on Whole-Cell Lipases - Post-print

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Abstract

Lipases constitute an important class of industrial enzymes with widespread applications across numerous industrial sectors. Compared to free lipases and physically or chemically immobilized lipases, whole-cell lipases exhibit advantages including simple preparation, elimination of protein extraction and purification, natural immobilization, enhanced stability and stress resistance, and reduced preparation and equipment costs. Consequently, the utilization of lipases in whole-cell form is regarded as one of the most promising approaches for reducing biotransformation costs, rendering research on whole-cell lipases a persistent hotspot in the lipase field. This article summarizes and reviews the research progress on whole-cell lipases, encompassing both wild-type and genetically engineered variants, and offers perspectives on future research directions to provide valuable references for subsequent studies.

Full Text

Research Advances in Whole-Cell Lipases

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Abstract

Lipases are important industrial enzymes widely used in numerous industrial fields. Compared with free lipases and physically or chemically immobilized lipases, whole-cell lipases offer several advantages, including simple preparation without protein extraction and purification, natural immobilization, enhanced stability and resistance, and lower preparation and equipment costs. Consequently, utilizing lipases in whole-cell form is recognized as one of the most

promising approaches for reducing biotransformation costs, and research on whole-cell lipases has remained a hot topic in the lipase field. This paper summarizes and reviews research advances in whole-cell lipases, encompassing both wild-type and genetically engineered variants, and prospects future research directions to provide a valuable reference for subsequent studies.

Keywords: Whole-cell lipase; Research advance; Review

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important ester-hydrolyzing enzymes widely present in various organisms. Due to their rich resource diversity, varied catalytic activities, simple genetic manipulation, high yield, stable supply independent of seasonal fluctuations, short production cycles, low production costs, and good stability, microbial lipases have become the primary source of industrial lipases. They are extensively applied in numerous industrial sectors, including food and beverages, oils and fats, detergents, feed, textiles, leather, novel materials, fine chemicals, pharmaceuticals, cosmetics, papermaking, environmental remediation, and bioenergy [1-4].

Lipase-catalyzed reactions exhibit high chemoselectivity, regioselectivity, and/or stereoselectivity. At oil-water interfaces, they catalyze the hydrolysis of fatty acid glycerides into glycerol and fatty acids, while in microaqueous or non-aqueous phases, they can catalyze various chemical reactions such as esterification, transesterification, alcoholysis, aminolysis, and acidolysis [5-7].

Whole-cell lipases offer significant advantages over free lipases: they eliminate cumbersome extraction and purification processes, avoiding substantial enzyme loss and the associated equipment investment and operating costs; they benefit from cellular protection, resulting in better stability and resistance; and they can be easily reused through simple centrifugation and washing [8-10]. Due to this natural immobilization, whole-cell lipases overcome many drawbacks of physical or chemical immobilization, including complex immobilization procedures, loss of enzyme activity and/or physicochemical properties during immobilization, tedious optimization of enzyme-carrier compatibility, and high preparation costs [11, 12]. Therefore, utilizing lipases in whole-cell form represents one of the most promising methods for reducing biotransformation costs [9, 11].

Based on preparation methods, whole-cell lipases can be classified into wild-type whole-cell lipases and genetically engineered whole-cell lipases. A comparison between these two types is presented in Table 1 .

1. Wild-Type Whole-Cell Lipases

Wild-type whole-cell lipases represent the simplest form of whole-cell lipases, with strain screening being the critical step. These strains possess intracellular lipases or cell-bound lipases that can exert catalytic function in whole-cell form, primarily including filamentous fungi, bacteria, and actinomycetes.

1.1 Filamentous Fungi

Filamentous fungi constitute the primary source of wild-type whole-cell lipases. Their mycelia are generally applied as immobilized whole-cell biocatalysts in biotransformation processes. In most cases, mycelial immobilization occurs automatically during fermentation by adding suitable solid particles, such as polyurethane foam materials or polystyrene packaging materials, to the fermentation medium.

1.1.1 *Rhizopus* *Rhizopus* is the main genus of filamentous fungi producing wild-type whole-cell lipases, with *Rhizopus chinensis* and *Rhizopus oryzae* being the primary species exhibiting whole-cell lipase activity. Research on *R. chinensis* has been systematically conducted by Xu Yan' s research group, which investigated the enzymatic properties, fermentation condition optimization, and biotransformation applications of *R. chinensis* whole-cell lipases [13-17].

Regarding *R. oryzae* whole-cell lipases, numerous research groups have employed them as catalysts for biodiesel production, achieving high conversion rates. Tamalampudi et al. utilized immobilized *R. oryzae* mycelia as whole-cell lipases for biodiesel production, achieving a maximum methyl ester yield of 80% from Jatropha oil after 60 hours, with residual esterification activity remaining above 90% after five reaction cycles, outperforming Novozym 435 which yielded only 76% methyl esters after 90 hours [18]. Wang Fei's research group used immobilized *R. oryzae* mycelia to catalyze biodiesel production from Jatropha oil and soybean oil, obtaining maximum methyl ester yields of 82.29% from Jatropha oil (with enzyme deactivation by the fifth cycle) and 94% from soybean oil (maintaining 82% yield after four cycles) [19, 20]. Li Wei et al. cultivated *R. oryzae* using inexpensive industrial-grade whole-fat soybean powder as nitrogen source, and the resulting immobilized whole-cell lipase catalyzed biodiesel production from soybean oil in a tert-butanol system, achieving a 68.5% methyl ester yield within 24 hours [21]. Hermansyah et al. immobilized *R. oryzae* mycelia using two different carriers for transesterification of cooking oil with methyl acetate, achieving fatty acid methyl ester yields of 11-12% and 22-23%, respectively, and subsequently developed a kinetic model for the transesterification reaction [22]. Zhou et al. employed a two-step process using commercial *Candida rugosa* lipase and immobilized *R. oryzae* mycelia to catalyze biodiesel production from unrefined Jatropha oil, achieving maximum yields of 90.3% free fatty acids with the commercial enzyme and 88.6% fatty acid methyl esters with the immobilized whole-cell catalyst, with product yields of 89% and 79% maintained after six cycles, respectively [23]. Bharathiraja et al. compared immobilized pure enzyme and immobilized *R. oryzae* mycelia for biodiesel production from waste cooking oil, evaluating four acyl acceptors. The pure enzyme generally outperformed the whole-cell lipase for all acceptors, with methanol giving the highest biodiesel yield. Under optimal conditions, fatty acid methyl ester yields were 94% and 84% for the pure enzyme and whole-cell catalyst, respectively [24].

1.1.2 *Aspergillus* *Aspergillus* is an important source of wild-type whole-cell lipases with potential applications as catalysts in food and bioenergy fields. Omar et al. isolated an *Aspergillus flavus* strain producing cell-bound lipase and investigated its enzyme production conditions and properties [25]. Yan et al. discovered that *Aspergillus oryzae* mycelia could serve as whole-cell lipases, exhibiting high esterification activity toward a series of short-chain acids and alcohols with strong tolerance to high substrate concentrations, achieving yields exceeding 80% for most heptanoates, some octanoates, and propyl hexanoate after 48 hours [26]. Li et al. screened a novel *Aspergillus niger* strain whose mycelia as whole-cell lipases showed 1,3-selectivity in glycerolysis reactions for 1,3-diglyceride synthesis [27, 28]. Guldhe et al. used immobilized *A. niger* mycelia as whole-cell lipases to catalyze biodiesel production from microalgae oil, finding that stepwise methanol addition improved conversion rates, reaching 80.97% under optimal conditions with no significant decrease after two cycles [29]. Rakchai et al. employed immobilized *Aspergillus nomius* mycelia as whole-cell lipases for a two-step biodiesel production from palm oil, achieving a maximum fatty acid methyl ester yield of 78.23% in the first transesterification step, which increased to 94.77% after the second esterification step, with transesterification and esterification activities maintained at 90.95% and 100% after ten cycles, respectively [30].

1.1.3 Other Filamentous Fungi Jacobsen et al. and Druet et al. successively discovered that mycelia of *Geotrichum candidum* and *Penicillium cyclopium* possess cell-bound lipases [31, 32]. Yan et al. screened a *Geotrichum* strain whose mycelia as whole-cell lipases achieved a 94% yield of methyl oleate from methanol and oleic acid in a microaqueous phase within 24 hours, retaining 70% relative hydrolytic activity and 69% methyl oleate yield after eight cycles [33].

1.2 Bacteria and Actinomycetes

Yu et al. employed *Burkholderia cepacia* as a whole-cell lipase to resolve DL-menthyl acetate, achieving 50% conversion of L-menthol with 96% optical purity under optimal conditions [34]. Shu et al. screened a broad-spectrum organic-solvent-tolerant strain *Burkholderia* sp. ZYB002, and after optimizing the production medium, the whole-cell lipase activity increased 5.1-fold, exhibiting good thermal stability and organic solvent tolerance [8]. Zha et al. discovered that *Pseudomonas protegens* Pf-5 possesses intracellular lipase and can be used as a whole-cell lipase with excellent enzymatic properties [35]. Lai Xueneng et al. prepared six high-yield lipase strains as whole-cell biocatalysts and identified two strains capable of catalyzing esculin propionylation: *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*, with the latter showing higher catalytic activity, achieving 47.9% product conversion and 99% regioselectivity under optimal enzyme induction conditions [36].

dos Santos et al. identified *Streptomyces clavuligerus* as a whole-cell lipase and investigated its fermentation conditions, enzymatic properties, and potential

applications as a biocatalyst in hydrolysis and esterification reactions. This whole-cell lipase shows significant potential for hydrolyzing long-chain fatty acid glycerides, esterifying short-chain fatty acids, and formulating detergents [37].

2. Genetically Engineered Whole-Cell Lipases

Generally, wild-type whole-cell lipases suffer from low expression efficiency, limited reaction types, and poor reaction activity and stability. Therefore, the overexpression of superior natural lipases or protein-engineered variants through genetic engineering technology has increasingly become the mainstream approach for whole-cell lipases. The construction of genetically engineered whole-cell lipases primarily employs two strategies: intracellular expression and surface display.

2.1 Intracellular Expression

Intracellular expression-based genetically engineered whole-cell lipases involve the active overexpression of superior lipases in suitable host cells, which are then prepared as whole-cell biocatalysts for biotransformation. Due to protection by the cell wall and membrane, intracellularly expressed lipases face permeability limitations in biotransformation applications, which can be overcome through cell permeabilization. Permeabilization methods 主要包括 solvent treatment (ethanol, isopropanol, toluene, ether, chloroform), detergent treatment (Triton X-100, CTAB), salt treatment (NaCl), freeze-thaw cycles, electroporation, and other physicochemical methods (air-drying, EDTA, alkaline treatment) [38].

Matsumoto et al. used *Saccharomyces cerevisiae* MT8-1 as a host to intracellularly overexpress *Rhizopus oryzae* lipase ROL, achieving a maximum whole-cell lipase activity of 474.5 IU/L. After air-drying permeabilization, the biocatalyst was used to catalyze biodiesel production from soybean oil, attaining a 71% methyl ester yield after 165 hours [39]. Yan et al. intracellularly overexpressed *Thermomyces lanuginosus* lipase Tll in *Pichia pastoris* GS115, achieving hydrolytic activity twice that of the commercial immobilized enzyme Lipozyme TLIM and significantly better tolerance to short-chain alcohols. As a whole-cell biocatalyst, it produced biodiesel from waste cooking oil with an 82% methyl ester yield after 84 hours, maintaining 78% yield after three cycles [12].

Adachi et al. intracellularly overexpressed *Candida antarctica* lipase CALB in *Aspergillus oryzae* mycelia. The immobilized recombinant *A. oryzae* mycelia as whole-cell lipases were used for a two-step biodiesel production process, achieving over 90% methyl ester yield within 6 hours, with no significant loss of esterification activity after 20 cycles, maintaining methyl ester yields above 90% [40].

Amoah et al. intracellularly overexpressed *Fusarium heterosporum* lipase FHL in *A. oryzae* mycelia. The immobilized recombinant *A. oryzae* was used to catalyze biodiesel production from high-phospholipid oils, achieving over 90% biodiesel

yield after process optimization. Compared with free lipases, this whole-cell lipase demonstrated excellent reaction stability [41, 42].

Zha et al. intracellularly overexpressed *Pseudomonas protegens* Pf-5 lipase LipA in *Escherichia coli* Top10, and the resulting whole-cell lipase exhibited excellent enzymatic properties [35].

2.2 Surface Display

Surface-displayed whole-cell lipases represent an effective method to circumvent cell permeability limitations, as they can be directly applied as catalysts in biotransformation after simple centrifugation and washing. Based on the expression host, lipase surface display can be categorized into bacterial surface display and yeast surface display.

2.2.1 Bacterial Surface Display Pan' s research group used ice nucleation protein (INP) as an anchoring protein to display *Pseudomonas fluorescens* SIK W1 lipase TliA on the surface of *Escherichia coli* JM109 and *Pseudomonas putida* GM730. The *E. coli* surface display system was applied for high-throughput screening of TliA random mutation libraries, while the *P. putida* surface display system was used for three typical lipase-catalyzed reactions: olive oil hydrolysis, triacylglycerol synthesis, and chiral resolution [43, 44]. Lee' s research group employed outer membrane protein OprF as an anchor to display TliA on *E. coli* XL10-Gold and *P. putida* KT2442 surfaces, applying both systems to the chiral resolution of (±)-1-phenylethanol. Compared with the *E. coli* system, the *P. putida* surface display system demonstrated superior performance in enzyme activity, organic solvent tolerance, thermal stability, and chiral resolution capability [45, 46].

Kim et al. used the 8 peptide of autotransporter EstA as an anchoring motif to display *Staphylococcus haemolyticus* L62 lipase on *E. coli* BL21(DE3) surface, applying it to biodiesel production from olive oil with an 89.4% methyl ester yield after 96 hours [47]. Li Xiaojun et al. employed the hybrid peptide Lpp-OmpA as an anchoring motif to display *Burkholderia cepacia* XYU-6 lipase BCL on *E. coli* BL21(DE3) surface, achieving 3.9-fold higher lipase activity than the wild-type strain [48]. Li Chunhua' s research group successfully displayed *Proteus* sp. lipase LipA on *E. coli* Rosetta Blue(DE3) surface using the C-terminal domain of superfolder green fluorescent protein (sfGFP) as an anchor, demonstrating excellent enzymatic properties and high biodiesel conversion rates [49, 50].

Chen Huayou' s research group used spore coat protein CotB as an anchor to display *Thermotoga maritima* MSB8 lipase Tm1350 on *Bacillus subtilis* spore surfaces. The displayed lipase exhibited higher optimal temperature and pH, as well as better thermal and pH stability than the free enzyme, and showed good reusability. Based on this work, they systematically investigated the effects of different linker peptides on spore-surface-displayed lipase activity [51, 52]. Kim

utilized spore coat protein CotE to display *B. subtilis* lipases LipA and LipB on *B. subtilis* spores, with LipB showing higher display activity than LipA. The spore-displayed LipB demonstrated better organic solvent tolerance than free LipB [53].

2.2.2 Yeast Surface Display Since Washida et al. first successfully displayed lipase on yeast cells in 2001 [54], yeast surface display has increasingly become the primary strategy for constructing whole-cell lipases. Throughout its development, yeast surface display systems for lipases have evolved from *Saccharomyces cerevisiae* systems to *Pichia pastoris* systems, with several other yeast display systems also being developed. Due to the numerous advantages of *P. pastoris* [55, 56], lipase surface display systems have gradually formed a trend dominated by *P. pastoris* surface display with other systems as supplements.

Li et al. displayed *Rhizopus oryzae* lipase ROL on *P. pastoris* GS115 surface using cell wall protein Sed1p as an anchor, demonstrating broad temperature and pH stability with optimal conditions at 40 °C and pH 7.5 [57]. Moura et al. displayed *Candida antarctica* lipase CALB on *P. pastoris* X-33 surface using flocculin Flo9 or internal repeat protein Pir1 as anchors, with optimal temperature and pH at 45 °C and 7.0, respectively, showing better thermal stability and organic solvent tolerance than the free enzyme [58]. Wang et al. co-displayed CALB with hydrophobins SC3 or HFBI on *P. pastoris* GS115 surface using cell wall protein GCW61 as an anchor, investigating the effects of hydrophobins on co-displayed CALB activity. Results showed that HFBI significantly enhanced CALB activity while SC3 significantly inhibited it [59].

Zhang Rui displayed ROL on *S. cerevisiae* EBY100 surface using agglutinin protein Aga2-Aga1 as an anchor, with optimal temperature and pH at 40 °C and 7.5, respectively, demonstrating good stability and organic solvent tolerance. The sodium alginate-immobilized whole-cell lipase achieved 81.2% biodiesel yield, maintaining transesterification rates above 80% after seven cycles [60]. Yuzbasheva et al. displayed *Yarrowia lipolytica* lipase Lip2p on *Y. lipolytica* Po1f surface using cell wall protein YlPir1p as an anchor. Compared with free lipase, the surface-displayed lipase exhibited higher thermal stability, organic solvent tolerance, and surfactant tolerance. As a whole-cell catalyst for biodiesel production, it achieved 84.1% methyl ester yield after 33 hours in the first cycle and 71% after 45 hours in the second cycle [61].

Yan Yunjun' s research group systematically investigated the effects of anchor proteins, linker sequences, promoters, and expression hosts on lipase surface display, as well as lipase co-display and display order on co-display efficiency, enzymatic properties, fermentation optimization, and applications as biocatalysts in biotransformation. Their work on yeast surface display of lipases has also evolved from *S. cerevisiae* systems to *P. pastoris* systems [54, 55, 62-66].

Future Perspectives

Although whole-cell lipases are complex enzyme systems based on cells that may produce by-products due to the presence of other enzymes, and cellular contents may leak during catalysis to contaminate final products, resulting in higher impurity levels and more cumbersome purification processes with increased costs, their advantages—including simple preparation without protein extraction and purification, natural immobilization, good stability and resistance, and reusability—make them one of the most promising methods for reducing industrial costs in biotransformation. Consequently, they have remained favored by lipase researchers. To date, numerous studies on whole-cell lipases have been conducted with fruitful results. However, extensive and in-depth research is still required in several directions: mining natural whole-cell lipase resources, constructing intracellular expression whole-cell lipases, developing surface-displayed whole-cell lipases, and expanding applications in biotransformation. Specific research prospects are outlined in Table 2 .

Table 2. Future research directions of whole-cell lipases

Research directions	Research contents
Mining natural whole-cell lipase resources	Screening natural whole-cell lipases with excellent enzymatic properties from extreme environments
Construction of intracellular expression whole-cell lipases	Mining host resources, mining lipase gene resources, optimizing expression vector construction, developing cell permeabilization technologies
Construction of surface-displayed whole-cell lipases	Mining host resources, developing anchor proteins, investigating linker peptide effects, developing multi-protein co-display technologies, optimizing display vectors
Applications in biotransformation	Expanding reaction types and optimizing reaction conditions

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