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Progress in Lipases, Its Immobilization and Application in Biodiesel Preparation with Emphasis on Our Practice

Abstract: Biodiesel is one of the most ideal renewable energies. Among its preparation technologies, biological technology exhibits the most promising prospect. However, high operation cost of lipase and easy inactivation are the main obstacles for large scale industrialization of enzymatic catalyzed biodiesel production. To solve these bottlenecks, such strategies have been employed as producing the cheapest and most tolerable lipases, developing better catalysis techniques, and further bio-refining byproduct glycerin of biodiesel into high-valued products. Eventually, enzymatic catalyzed technology has been succeeded in industrializing with good commercial efficiency. This article reviews a decade endeavor on research and practice of these strategies.

Keywords: Strategy, Enzymatic catalyzed biodiesel production, High-valued product, Biorefinery, Oil

Introduction

With depletion of fossil fuel and deterioration in environmental pollution, renewable and clean energies are imperative for sustainability of mankind. Biodiesel is one of the most ideal renewable and clean energies, which are recently urgent to be explored.[1] As known, among different technologies for its production, biological technology can overcome most shortcomings of chemical technology. However, high price and easy inactivation of the lipases are the main obstacles for large-scale industrialization of enzymatic catalyzed biodiesel production.[2-4] Moreover, biodiesel itself is not a high value-added product, whose price might be greatly influenced by the market price fluctuation of feedstock.[5, 6] Therefore, aiming to solve the above bottlenecks, such strategies have been employed as producing the cheapest and most tolerable lipases, developing better catalysis techniques and further bio-refining byproduct glycerin of the biodiesel into high-valued products. Eventually, under continuous endeavor, enzymatic catalyzed technology has been putting into industrialization with a good commercial efficiency.

1. Widely searching for tolerable, versatile and lower-cost lipases

Lipases (EC 3.1.1.3) can catalyze the hydrolysis of triacylglycerols (TAG) at the oil-water interface.[7] Besides the hydrolytic activity on triglycerides, lipases can also catalyze esterification, transesterification, acidolysis, alcoholysis and aminolysis, etc.[8] Since they are substrate specific, enantiomerically selective, regioselective and able to catalyze various reactions, lipases have been widely utilized in industries, including detergents, oil processing, cosmetics, medicine, food industry and biodiesel production.[9] However, many available lipases can not work well under industrial reaction conditions for practical use where they are required to be thermostable, pH stable and solvent tolerable.[10,11] Therefore, more attention is being focused on identifying and characterizing unique lipases from cultivated or uncultivated microbes, and modifying lipase structure to improve their catalytic properties through

immobilization or protein engineering strategies such as directed evolution and rational design.[12,13]

As most microbiologists do, tolerable and versatile lipases have been searching for from all kinds of microhabitats, such as oil stained soil, sewage water and mud from oil mills, marine mud, hot or cold spring mud. Tow-step method is employed to screen lipase-producing strains. By primary plate screening with Rhodamine B as indicator and second flask fermentation assay.[14] So far, more than 600 lipase-producing strains have been screened, and a microbial lipase strains library has been established.

As known, most strains from natural environment produce very little lipase, which can not match the demand of industrial scale. Many strategies for over-expression of enzymes are adopted to improve lipases production.[15] Till now, more than 40 lipase genes have been cloned in our laboratory, among which some lipase genes have been first reported, such as *Stenotrophomonas maltophilia* lipase,[16] *Aspergillus niger* lipase A and B,[17-19] *Pseudomonas fluorescens* 26-2 lipA,[20] *Candida rugosa* lipase J08 and *Yarrowia lipolytica* Lip365.[21,22] According to hydrolysis activity or transesterification ability, a few lipases were chosen to construct genetic engineering strains. Fourteen recombinants with highly effective expression of lipases were obtained by combined several strategies using homologous and heterologous expression technologies,[23-26] among which seven have been completed fermentation at 3-10-100L scale bioreactors. [27- 29]

It is noted that *Burkholderia cepacia* G63 lipase, an ideal lipase for biodiesel production, has many merits, such as a wide range of oil sources adaptation, high tolerance for temperature and short-chain alcohols. The purified lipase kept stable at a temperature range of 40 - 70 ℃. After incubated at 70 ℃, the optimal temperature of this enzyme, for 10 h it remained 86.1% of its activity. The enzyme was also highly tolerant to a series of organic solution. The remaining activity of the lipase after incubated for 48 h in 50% methanol solution, 98.3 % activity was still retained. The effect of propylalcohol, isopropyl alcohol and amyl alcohol on the activity of lipase G63 is very limited. Incubated for 48 h, the remaining activity was still 93.5 %, 91.7 % and 92.5 %, respectively. Compared with lipase G63, the endurance of lipase PS (a product from Amano) to ethanol, propylalcohol and isopropyl alcohol is rather weak, the remaining activity was only 79.3 %, 73.5 % and 73.6 %, respectively.[14] Lipase G63 has been tested to catalyze transeterification towards a wide feedstock, such as rapeseed oil, cotton oil, soybean oil, olive oil, sewage oil, lard, duck fat, algal oil, etc. 90 – 98 % of biodiesel yield could be obtained within 12 – 48 h reaction.[14, 30, 31] Additionally, *Galactomyces geotrichum* lipase was also cloned and characterized. It exhibited several properties of significant industrial importance, such as pH and temperature stability, wide organic solvent tolerance and broad hydrolysis on vegetable oils. Such a combination of properties makes it a promising candidate for application in non-aqueous biocatalysis, such as biodiesel production, selective hydrolysis or esterification for enrichment of PUFAs.[24]

Y. lipolytica lipase Lip2 (YlLip2), one of the most widely used lipases, heterologously expressed in *Pichia pastoris*, has been achieved with high levels in 3-10-100 L fermentors. The maximal YlLIP2 activity reaches ca 64,600 U/mL with a total protein concentration of 7.9 $g.L^{-1}$ after 154 h incubation, which is the highest ever reported in literature.[27, 28] The investigation on *Geotrichum candidum* recombinant lipase strains have also been completed in 10 L scale fermentation with the highest activity of over 11,000 U/mL,[29] which is also the biggest one as far as we know.

By such operation, we can finally produce several tolerable and versatile lipases, such as *B. cepacia* G63, *Y. lipolytica* lipase Lip2, *G. candidum* lipase, *C. antarctica* lipase B, *Thermomyces lanuginosus* lipase, *Rhizopus*

oryzae lipase, *C. rugosa* Lip1 at lowest cost, less than 150 USD/kg crude enzyme.

2. Immobilization of lipases for enhanced enzymatic properties

It is known to all, free form of lipases usually shows low stability, bad recovery and no reuse, which severely blocks its feasibility in practical use.[32] Thus, immobilized enzyme is often utilized not only for enhancing enzyme stability and improving activity, but also for easy recovery and further reuse so as to achieve a good economical efficiency.[33] So far, there are 6 kinds of lipase immobilization methods, adsorption, covalent attachment, entrapment, encapsulation, cross-linked enzyme aggregates (CLEAs) and cross-linked enzyme crystals (CLECs).[34-37] Moreover, interfacial activation and molecular bioimprinting are novel approaches, which can be effective to enhance enzymatic activity and stability in non-aqueous media.[38]

In order to improve the catalysis of lipases in practical systems, many immobilization technologies have been attempted, such as sol-gel, sodium alginate-gelatin, macroporous resin, magnetic nanoparticles and surface display techniques. Bioimprinting with substrate analogues of fatty acids was employed to improve the esterification activity of *B. cepacia* lipase that had undergone a sol–gel immobilization procedure with methyltrimethoxysilane (MTMS) and tetramethoxysilane (TMOS) as the precursors.[38] The specific activity of the bioimprinted lipase was enhanced 47.9- and 2.5- folds over the free and non-imprinted immobilized lipases, and the bioimprinted lipases exhibited better thermal stability than the free and non-imprinted immobilized ones, and their activity did not change after being incubated at 60.8 ℃ for 12 h.[38]

B. cepacia lipase was adsorbed on macroporous resin NKA (produced from Tianjin Nankai Sci. & Tech. Co. Ltd., Tianjin, China) by combined strategies of bioimprinting and interfacial activation to improve its catalytic performance. The specific activity of the derivative lipase was 211,733.3 U/g-protein, which was 21.7-fold, 19.4 % and 47 % enhancement over the free lipase powder, non-bioimprinted and non-interfacial activation lipase.[39] The derivative lipase also exhibited a satisfactory thermostability over a wide range of temperature (from 30 ℃ to 70 ℃) and a strong tolerance to organic solvents such as methanol, ethanol and acetone with 50 % concentration. After being recycled 50 batches (400 h), the derivative lipase still retained over 92 % of its original activity (methyl esters (ME) yield decreased from 98 % to 90 %). The lipase immobilized on macroporous resin NKA showed a high immobilization efficiency (91 %), high conversion rate of ME yield (97 %), long operational lifespan (over 3000 h), and lowest cost (less than 150 USD per kg). The lipase preparation was probably a promising biocatalyst of satisfactory thermostability, strong solvents tolerance and high operational reusability.[39]

The preparation and characteristics of protein-coated microcrystals (PCMCs) from *B. cepacia* lipase (BS) and K_2SO_4 and their application in biodiesel synthesis, were also investigated via single factorial experiments and response surface methodology (RSM), the optimized PCMC-BS exhibited high activity and stability; the optimal temperature was 60 ℃ (which gave 99.83 % conversion), and it kept fairly high activity after incubation at different temperatures (25-70 ℃). The tolerance towards organic solvents of the PCMC-BS was greatly improved, and significantly reduced ethanol toxicity. When incubated in methanol PCMC-BS showed 67.7 % conversion, while free BS was almost completely inactivated. The unique composition of PCMC-BS probably enabled it to retain some of its enzyme-bound water, due to the existence of the polar microenvironment created by K_2SO_4, while free PS lost large amounts of its essential water. The conversion dramatically increased from 67.7 % to 92.8 % (tetrahydrofuran, logP = 0.49), and remained

stable in the range of 92.8 – 97.4 % (isooctane, logP = 4.5) even as logP increased by 4.01.[25] In addition, lipase from *P. fluorescens* was entrapped by drop-wise addition of an aqueous of sodium alginate-gelatin and immerged in a hardening solution of $CaCl_2$. The optimum immobilized operation conditions were: lipase /sodium alginate ratio 300 U, the diameter of the pinhead 6 (0.5 mm), temperature 55 ℃ and pH 8.0. The immobilized enzyme has strong tolerance to metal ions and short-chain ethanol, which is suitable for the synthesis of biodiesel.[40]

Besides lipases from *B. cepacia*, a combined strategy comprising bioimprinting with dual imprint molecules and a co-solvent coupled to pH tuning, KCl salt activation, lecithin coating and immobilization on macroporous resin was conducted to effectively enhance the activity and operational stability of *Geotrichum* sp. lipase. The modified lipase exhibited 18.4 -fold esterification activity towards methyl oleate synthesis, and retained 90 % activity after being repeatedly used for 10 times.[41] The combined strategy exhibited a significant synergistic effect and was suitable for lipase modification, dramatically enhancing the enzyme activity and operational stability for use in non-aqueous media.[41] Additionally, a dual modification procedure composed of cross-linking and protein coating with K_2SO_4 was employed to modify *Geotrichum* sp. lipase for catalyzing biodiesel production from waste cooking oil. Compared to single modification of protein coating with K_2SO_4, the dual modification of cross-linking and lipase coating (also named CLPCMCs) had improved catalytic properties in terms of thermostablity, organic solvent tolerance, pH stability and operational stability in biodiesel production process. CLPCMCs retained 64 % of relative biodiesel yield after incubation in a pH range of 4–6 for 4 h, 85 % of relative biodiesel yield after incubation in a range of 45 – 50 ℃ for 4 h, and still maintained 83 % of relative biodiesel yield after both treated in polar organic solvent and non-polar organic solvent for 4 h. After five successive batch reactions, CLPCMCs could still maintain 80% of relative biodiesel yield.[42]

However, although immobilization is an optional solution to the problems confronted by free lipases, it first needs a complex purification procedure from the culture medium, and then a time-consuming immobilization process, where the ideal carrier is very difficult to find. Compared with traditional immobilization, surface display of enzymes as whole cell catalysts may be a better way because it is a kind of self-immobilization and needs no purification. Thus, it saves a lot of cost and labor. Actually, so far, many functional enzymes have been genetically immobilized on the cell surface of *Saccharomyces cerevisiae* due to its safety, simplicity of genetic manipulation, and rigidity of cell-wall structure in *S. cerevisiae*.[43-46]

Lipase Lip2 from *Y. lipolytica* was successfully displayed (self-immobilized) on the cell surface of *S. cerevisiae* using Cwp2 as an anchor protein, which was confirmed by immunofluorescence microscopy and halo assay. $(G_4S)_3$ sequence was further proved to be the best linker to maintain the correct conformation of Lip2. The displayed Lip2 exhibited the highest activity of 7.6 $U.g^{-1}$-dry cell, with an optimal temperature and pH at 40 ℃ and pH 8.0. It did not lose any activity after being treated with 0.1 % Triton X-100 and 0.1 % Tween 80 for 30 min, and retained 92 % of its original activity after incubation in 10% DMSO (Dimethyl sulfoxide) for 30 min, exhibiting a better stability than free Lip2 as reported previously.[44] On the other hand, if a-agglutinin was used as an anchor protein, the displayed Lip2 on *S. cerevisiae* attained an even higher activity (634.9 $U.g^{-1}$-dry cell). Moreover, its thermostability and tolerance to organic solvents were greatly improved. Especially, the displayed Lip2 remained 109.4 % and 98.5 % of its original activity after being treated with 20 % methanol and ethanol, much better than free Lip2, suggesting that it would be more suitable for biodiesel

production.[45]

The lipases Lip7 and Lip8 of *Y. lipolytica* were also displayed on the cell surface of *S. cerevisiae* and *P. pastoris* using a small binding subunit Aga2 of a-agglutinin and Flo1 as anchor protein. The activities towards p-nitrophenyl caprylate of surface-displayed Lip7 and Lip8 with Aga2 reached 283 U g^{-1}-dry cell and 121 U.g^{-1}-dry cell, much higher than those (85 U.g^{-1}-dry cell and 79 U.g^{-1}-dry cell) using Flo1 as anchor protein in *P. pastoris*.[46] The putative signal sequences have significant effect on the activities of the displayed lipases, when deleted, the lipases' activities were significantly declined. The displayed lipases exhibit a high thermal stability. More importantly, to surface-display a target protein, it is recommendable that the structure feature of the protein should be analyzed through bioinformatics methods and then the cell wall proteins with the anchor domain far away from the activity center should be chosen as anchor proteins.[47]

A. niger lipase (ANL) was for the first time displayed on the surface of *S. cerevisiae* using a-agglutinin as an anchor protein. The displayed ANL was confirmed to be active toward tributyrin and p-nitrophenyl caprylate (pNPC). The hydrolytic activity toward pNPC reached 43.8 U/g-dry cell after induction by galactose for 72 h. The ANL displaying cells had good thermostability, retaining almost 80 % of the original activity after incubation at 60℃ for 1 h, and > 80 % of the original activity at 50℃ for 6 h. The displayed lipase showed a preference for medium-chain fatty acid p-nitrophenyl esters, which suggests that the produced whole-cell catalyst is likely to have a wide range of applications.[48]

Geotrichum sp. lipase (GSL), an important enzyme for the enrichment of polyunsaturated fatty acids (PUFAs), was also first displayed on the cell surface of *S. cerevisiae.* The activity of the displayed GSL was higher (43.7 U.g^{-1} dry cell) than that (26.26 U.g^{-1}-dry cell) of *C. antarctica* lipase B and that (4.1 U.g^{-1}-dry cell) of *R. oryzae* lipase. It exhibited higher thermostability than the free lipase, and retained 89 % of the original activity after incubation at 40 ◦C for 3 h, compared with 48 % at 35 ◦C for the free lipase at pH 8.5. Interestingly, the displayed lipase had a wider pH range and better pH stability. It had higher activity at all pH values than the free GSL, and retained 86 % of the original activity in the pH range 9.5 to 10.5, whereas the activity of the free GSL could not be detected at pH 10.[49]

As it is known, each lipase has its own stereo-selectivity. If two or more lipases with complementary region specificities are combined to catalyze transesterification, the synergy would occur,[50] which may offer a potential solution to reduce the total usage of lipases. It have been proved that the immobilized lipases Novozym435 and Lipozyme TLIM to catalyze transesterification of lard generate a markedly positive synergistic action.[51] By following this, two synergistic lipases *C. Antarctica* lipase B and *T. lanuginosus* lipase have been successfully co-displayed on the cell surface of *P. pastoris*, and the derivative lipases exhibited a high synergy for biodiesel preparation, suggesting a promising prospect.[52]

3. Research for more competitive enzymatically catalyzed biodiesel production technology

By using these cheapest lipases, we have developed many enzymatic transesterification technologies for biodiesel preparation based on solvent engineering, using short chain alcohols, methyl acetate and fusel oil as acyl acceptors. Although many authors have reported enzymatic transesterification technologies with different single lipase,[53-58] combined use of two or more lipases with complementary position specificity is a potential way to significantly reduce cost of lipase-catalyzed biodiesel production.[50,51] As mentioned above, the immobilized lipases Novozym435 (non-specific) and Lipozyme TLIM (1, 3-specific) showed positive synergy when catalyzing transesterification reaction. In

solvent free system, when Novozym435 was mixed with Lipozyme TLIM at a ratio of 70 /30, the methyl ester yields of soybean oil were higher than that of only with Novozym435 by 91.5 %. While in *tert*-butanol system, when Novozym435 was mixed with Lipozyme TL IM at the ratio of 60 /40, the methyl ester yields were higher than that of only with Novozym435 by 91. 9 %.[50]

The process of biodiesel production from lard catalyzed by the combined use of Novozym435 and Lipozyme TLIM was optimized by response surface methodology (RSM). Under the optimal conditions: lipase/oil (w/w) 0.04, Novozym435 /total lipases (w/w) 0.49, tert-butanol/oil (v/v) 0.55, methanol/oil (mol/mol) 5.12, and reaction time 20 h, the methyl ester (ME) yield of 97.2 % was attained, very close to the predicted value (97.6 %). Further test proved that the optimal conditions could be extrapolated to other similar reactions with plant oils and animal fats, the obtained ME yields were higher than 95 %. The lipases could be continuously reused for 20 cycles without any loss of activity, exhibiting very high operational stability.[51]

Preparation of biodiesel from waste cooking oil catalyzed by combined lipases in *tert*-butanol medium was further investigated. Such parameters affecting biodiesel yield as dosage of the combined lipases (Novozym435 and Lipozyme TLIM), weight ratio of the lipases, loading of *tert*-butanol, reaction temperature, and molar ratio of oil to methanol were optimized via RSM. Under the optimized conditions, the highest biodiesel yield was up to 83.5 %. The combined lipases exhibited high-operational stability. Their activity maintained 85 % of its original activity after 30 cycles (300 h) successively running. A reaction kinetic model was proposed to describe the system, and the calculated activation energy was 51.71 J/mol.[59]

However, immobilized lipases can easily be inactivated by methanol and depressed by the adsorption of glycerol onto the surface of immobilized vector, which limit their usage in industrialization of biodiesel production. Solvent engineering method was employed to ease the above blockages in the reaction of biodiesel preparation from *stillingia* oil with methanol catalyzed by Novozym 435 and Lipozyme TLIM. This operation would enhance the solubility of methanol in oil and dissolve glycerol, which helps to maintain lipase activity. The results bore out that the yields of biodiesel in co-solvent exceeded those in the pure organic solvents. The mixture system of co-solvent with 60 % acetonitrile and 40 % t-butanol (v/v) was proved to be an optimal one, and the reaction parameters were further optimized with RSM. The optimal conditions were: methanol/oil molar ratio 6.4:1, compound-lipase 4.3 % (wt/wt) and molecular sieve 5.5 % (wt/wt), with the highest ME yield of 96.4 %. There was nearly no loss in activity of the compound-lipases after being recycled for 30 times. Other oils were also examined in this mixture system, and the similar results were observed, which indicated that the mixture system could be an ideal prospective medium applied to biodiesel production.[60]

Biodiesel synthesis catalyzed by immobilized lipases (Novozym 435, Lipozyme TLIM and Lipozyme RMIM) in solvent-free and *tert*-butanol media was also investigated. The effect of biocatalyst type and different alcohols (methanol, ethanol, propanol, isopropanol, isobutanol, isoamyl alcohol and fusel oil-like alcohol mixture) on conversion rate was comprehensively addressed. It was shown that each lipase presented a different kinetic pattern depending on the monohydric alcohols. In addition, a reaction kinetics model was developed for the methanolysis of waste baked duck oil using combined lipases of Novozym 435 and Lipozyme TLIM in solvent-free system. The kinetic parameters were estimated by fitting experimental data and deduced to be a pseudo-third-order reaction, and the activation energy was 31.65 kJ/mol.[61]

The above researches were then combined into a comprehensive technology, where the activity of the compound lipases remained

95 % of its original activity after being repeated 300 cycles (ca. 2400h) in pilot trial. Additionally, many researches confirmed that the technology could suitable for a wide variety of feedstock, such as *jatropha* oil, palm oil, *stillingia* oil, soybean oil, sunflower seed oil, olive oil, camellia oil, corn oil and rapeseed oil, algal oil, fungal oil, lard, duck fat, chicken fat and waste cooking oil,[31, 51, 62] which suggests that the compound lipases catalyzed process shows a good prospect for large-scale industrialization. Actually, in 2006, a 20,000 - ton scale demonstration assembly line has been established and continuously run for 4 years.

Meanwhile, the lipase from *B. cepacia* adsorbed on macroporous resin NKA was also investigated to catalyze transesterification reaction, after being used of 50 successive batches (400 h), the lipase still retained over 92 % of its original activity (methyl esters yield decreased from 98 % to 90 %). The derivative lipase was probably a promising alternative biocatalyst of satisfactory thermostability, strong solvents tolerance and high operational reusability.[39] Furthermore, solvent engineering method was also employed to examine enzymatic biodiesel production in isooctane systems using *B. cenocepacia* lipase (BCL) instead. A total of 98 % biodiesel yield was obtained under the optimal conditions of methanol/oil molar ratio 4:1 with the addition of methanol in three steps at intervals of 2 h, enzyme dosage 2.5 wt % (based on the oil weight), moisture concentration 7 wt % (based on oil weight), reaction temperature 40 ℃, reaction duration 8 h, stirring rate 300 rpm, and isooctane amount 50 wt % (based on the oil weight). Compared to *tert*-butanol and solvent free systems, the highest biodiesel yield was achieved in the isooctane system. BCL-NKA showed high operational stability with no obvious loss in lipase activity for transesterification in the isooctane system even after 50 cycles (400 h) of repeated usage.[63]

Biodiesel preparation from *Sapium sebiferum* oil catalyzed by immobilized *B. cepacia* G63 lipase was further examined. Several crucial parameters affecting biodiesel yield were successively optimized with Plackett-Burman and Box-Behnken designs, and a second-order polynomial equation was regressed. The optimal conditions for biodiesel synthesis were: 4 : 1 methano /oil molar ratio, 2.7 % (w/w) lipase, temperature 41 ℃ with a ME yield of 96.2 % (R^2 = 98.2 %). No loss nearly in the immobilized lipase activity was observed after being repeatedly used for 20 cycles under the optimum conditions. Two kinetic models were developed to describe the reaction process. One was proposed as the function of ethanol concentration with competitive inhibition, the other was explored on basis of the decrease of substrate concentration (including the oil substrate and the methanol). The proposed kinetic models had a satisfactory coefficient of R^2 (> 99.0 %) and the kinetic parameters were fitted from the experimental data.[64]

Investigations on biodiesel synthesis and conformation of *B. cepacia* lipase (BCL) were also conducted in 19 different ionic liquids (RTLLs) with a range of cation and anion structures. Overall, anions had greater influence on biodiesel conversion than cation. RTILs containing Tf_2N^- and PF_6^- anions were observed to be the suitable reaction media, while RTILs with strong water miscible properties showed very low biodiesel yields. Further studies demonstrated that the activity of BCL was greatly improved in co-solvent mixtures media of ionic liquids-organic solvents. Therefore, a mixture co-solvent engineering was probable a high-effective strategy to enhance the activity of lipase for non-aqueous enzymological reaction. An ultrasound-assisted bio-catalyzed process in RTILs was employed to improve mass transfer rate, leading to 83 % reduction of the reaction time for biodiesel production. Conformational analysis by Fourier transform-infrared spectrometry (FT-IR) and circular dichroism (CD) revealed that higher biodiesel conversion in non-aqueous media was correlated with low tendency in α-helix content of BCL.[65]

The protein-coated microcrystals (PCMCs) from *B. cepacia* lipase (BS) and K_2SO_4 were preliminarily applied in biodiesel synthesis. When catalyzed by PCMC-BS, above 83 % biodiesel yield was obtained for most of the seven oils (soybean oil, *Stillingia* oil, sunflower seed oil, olive oil, camellia oil, corn oil, rapeseed oil). The PCMC-BS activity remained relatively stable after 8 batch reactions, with only a 15.7 % reduction in the conversion (from 99.0 % to 83.3 %).[25]

Actually, a pilot trial of 200-ton per year has been successfully carried out with enzymatic catalysis technology of the lipase from *B. cepacia*. Besides, a dual modification procedure composed of cross-linking and protein coating with K_2SO_4 was employed to modify *Geotrichum* sp. lipase for catalyzing biodiesel production from waste cooking oil. CLPCMCs exhibited a sharp rise in biodiesel yield of 72 % compared to 29 % of free lipase. The modified lipase preparation also dramatically enhanced initial reaction rate and greatly shortened the time required for achieving the highest biodiesel yield compared to the free lipase.[42]

In addition, investigations were conducted to synthesis biodiesel directly by the whole fermented solid of *B. cenocepacia* with solid-state fermentation (SSF) using sugarcane bagasse and sunflower seed cake as substrates. The maximal olive oil-hydrolyzing activity was 72.3 units per gram of dried solid, which was more than two times over the values reported in literature,[66] at 96 h through adding 8 % (v/w) olive oil as inducer during the SSF. After lyophilization and delipidatation, the whole fermented solid was directly used to catalyze the ethanolysis of soybean oil for biodiesel production in *tert*-butanol system. The highest biodiesel yield was about 93 % after 96 h. The kinetic model was firstly proposed as the function of ethanol concentration with competitive inhibition and the regression coefficients of the kinetics model were determined from experimental data. Furthermore, the rate-limiting reaction step from diglyceride to monoglyceride was firstly illustrated for the ethanolysis of biodiesel production directly by fermented solid in *tert*-butanol. Therefore, ethanolysis of biodiesel production directly catalyzed by a low cost fermented solid from *B. cenocepacia* has been considered a promising option in the future (unpublished data).

Therefore, from the above studies, it can be seen that the enzymatic catalyzed biodiesel technologies here can achieve advantage over chemical technologies and traditional enzymatic catalysis technology, and can substantially reduce the production cost of biodiesel production.

4. Biofining byproduct glycerin into high-valued product

Since the price of by-product glycerol of biodiesel has steeply dropped from US $1/lb to US $0.34/lb. So, biodiesel manufacturers are losing benifit in glycerol purification. How to increase the value of glycerin-containing waste has been an important issue. If glycerol can be converted into other valuable chemicals, it will greatly improve the economic competitiveness and environmental sustainability of biodiesel industry. Nowadays, the high-valued products based on glycerol are mainly 1, 3-propanediol, bioethanol, critic acid and polyunsaturated fatty acids.

1, 3-propanediol is an important precursor for manufacturing high-valued polymers and platform chemicals. 1, 3-propanediol fermentation using glycerol as substrate is drawing great attention. Liu et al. described the first one pot microbial and chemo-catalytic conversion of waste glycerol to 1, 3-propanediol coupled with amination mediated by hydrogen transfer catalysis.[67] *C. butyricum* DSM10703 was chosen as the biocatalyst to ferment waste glycerol from biodiesel production. Finally, direct conversion of crude glycerol to valuable secondary amines in a biphasic system without intermediate separation of 1, 3-propanediol was demonstrated[67]. A newly isolated strain

Kluyvera cryocrescens produced 27 g/L of ethanol from crude glycerol with high molar yield of 80% and productivity of 0.61 g/L/h. *K. cryocrescens* also metabolized glycerol to produce ethanol in the presence of limited oxygen, but high concentration of oxygen would lead to low ethanol yields as most of the carbon converted into carbon dioxide.[68]

Citric acid, representing more than 70% (*w/w*) of all food acidulates and having various usages, with an annual demand of more than 1,200 million kg, is one of the most widespread metabolites produced via biotechnological methods on an industrial scale.[69] *Y. lipolytica* ACA-DC 50109 was employed successfully to convert crude glycerol into single-cell oils (SCO) or citric acid. This production could lead to synthesis of specific high-added value lipid.[70]

The fungus *Pythium irregular* was explored to produce eicosapentaenoic acid using crude glycerol from biodiesel industry.[71] The optimal condition of *P. irregulare* growth contains 30 g/L crude glycerol and 10 g/L yeast extract, with 90 mg/L of EPA yield and 14.9 mg/L of productivity. Pure vegetable oil addition might enhance long chain fatty acid production. Removing major impurities such as soap and methanol can help boost fungal growth.

The feasibility of producing docosahexaenoic acid (DHA, 22:6 n-3) through fermentation of the algae *Schizochytrium limacinum* on crude glycerol has been proved. The maximum specific growth rate was determined as 0.692 d^{-1}. The cells had a true growth yield of 0.283 g/g but with a relatively high maintenance coefficient (0.2216 d^{-1}). The highest biomass productivity of 3.88 g/L-day was obtained at dilution rate (D) = 0.3 d^{-1} and crude glycerol concentration (S_0) = 60 g/L, while the highest DHA productivity (0.52 g/L-day) was obtained at D= 0.3 d^{-1} and S_0 = 90 g/L due to the higher DHA content at S_0 = 90 g/L. The EPA-fortified product has great potential serving as animal feed.[72]

Conclusion

The lipase production cost is the main obstacle to commercialization of enzymatic catalyzed biodiesel preparation. Thus, attempts have been made to develop cost-effective lipases, including screening new gene resources of lipases, constructing genetically engineering strain for over-expressing lipases, improving the characterization of lipases by immobilization and physical-chemical modification. Meanwhile, another effective way is to develop new technologies for enzymatic catalyzing biodiesel production through medium engineering, utilizing synergetic combined lipases. In addition, conversion of byproduct glycerol into high-valued products is a further effective strategy to improve commercial efficiency and enhance biodiesel market competition. Therefore, this strategy is a potential way of future development of biodiesel production.

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