Enzymatic production of biodiesel: strategies to overcome methanol inactivation

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Keywords: biodiesel, immobilization, lipase, methanol, sustainability

Abstract

Lipase-catalyzed transesterification of triglycerides and alcohols to obtain biodiesel is an environmentally friendly and sustainable route for fuels production since, besides proceeding in mild reaction conditions, it allows for the use of low-cost feedstocks that contain water and free fatty acids, for example non-edible oils and waste oils. In this contribution, we report on recent advances in the field and focus in particular on a major issue in the enzymatic process, the inactivation of most lipases caused by methanol, the preferred acyl acceptor used for alcoholysis. We describe recent results about immobilization of enzymes on nano-materials and the use of whole-cell biocatalysts, as well as the use of cell-surface display technologies and metabolic engineering strategies for microbial production of biodiesel. We discuss also insight into the effects of methanol on lipases obtained by modelling approaches and report on studies aimed at mining novel alcohol stable enzymes or at improving robustness in existing ones by protein engineering.

1 Introduction

The current trend towards sustainable and eco-friendly products and processes is very relevant in the field of biofuels, including biodiesel, a mixture of fatty acid alkyl esters (FAAE) obtained through esterification of fatty acids or transesterification of triglycerides with short-chain alcohols (**Figure 1**). Sustainability addresses both the raw materials used and the technology of production. As for feedstocks, it implies a shift from food oils and lipids to non-edible oils, waste oils, or to lipids from oleaginous microorganisms and algae. Currently, the predominant technology of production is chemical transesterification by alkali. However, the relevance of bioprocesses catalyzed by lipases is steadily growing, driven by the unique ability of lipases to allow for the use of a variety of feedstocks not suited for alkali catalysis because they contain water and free fatty acids, for example waste oil and non-edible oils [1-3].

Despite the two fold or seven fold higher costs of immobilized or soluble enzymes, respectively, than of the alkali catalyst [4], enzymatic industrial plants are operative, for example Lvming Co. LTD (Shangai, China), Piedmont Biofuel (North Carolina, USA), Hainabaichuan Co. LTD (Hunan, China), Purolite (Bala Cynwyd, PA), Transbiodiesel (Shfar-Am, Israel) and Sunho Biodiesel Corporation (Taipei, Taiwan) [5,6]. Overall, the potential of enzymatic biodiesel is enormous, and its economical attractiveness depends on both the possibility to use cheap raw materials that are not suitable for the chemical reaction and on the refinement of several steps of the production processes where there is still room for optimization [5, 6]. Worth to be mentioned is also the observation that transesterification with triglycerides releases as a by-product free glycerol that, only in the case of enzymatic reaction, is endowed with enough purity to be used for the production of renewable fuels and chemicals [7].

In this contribution, we report on the advancements obtained in the field in the last three years, the time elapsed from the publication of a previous review article [8] and focus in particular on a major issue in the enzymatic process, the inactivation of most lipases caused by methanol, the preferred acyl acceptor used to date. As a matter of fact, only very few lipases are endowed with enough stability towards

methanol to tolerate the methanol:oil ratios optimal for the process (higher than 3:1). Stepwise addition of alcohol during the reaction is generally used to circumvent this limitation. Nonetheless, in order to make the enzymatic route competitive, further developments are still necessary, both in terms of overall process design and intensification, upstream processing (selection of the raw materials) and, of paramount relevance, biocatalyst design (availability of robust catalysts). This review follows this conceptual flow and aims at providing an update of the latest advancements in enzymatic biodiesel process engineering, with emphasis on the biocatalyst stability. Section 2 summarizes studies in process design and optimization suited to make the reaction faster and more efficient, thus also reducing exposition of the enzyme to alcohols. Immobilization on nano-materials and use of whole-cell biocatalysts are reviewed. This section also includes alternative strategies such as the use of cell-surface display technologies or metabolic engineering strategies for the microbial production of biodiesel. Insight into the effects of methanol on lipases obtained by modelling approaches is discussed in section 3 in the frame of a more general view about enzyme robustness to polar organic solvents. Finally, section 4 reports on studies aimed at mining novel alcohol stable enzymes or at improving robustness in existing ones.

2 Implementation of the bio-process: design, whole cell biocatalysis and metabolic engineering

Enzyme pre- and post-reaction treatments have been proven to be a promising tool in relieving inhibitory effects caused by reaction components, included those exerted by methanol. Beneficial pre-treatments of lipase before the start of enzymatic reaction include immersion, incubation, or washing of lipases with substrates, organic solvents, salts, or enzyme lycoprotectants [5, 9]. Post-treatments of immobilized lipases, for example washing after each reaction cycle with solvents (i.e. hexane) were shown to help avoiding the adsorption to the support of glycerol and oil/ FAMEs mixtures which could block lipase activity [5,10,11].

2.1 Nanomaterials, new supports for immobilization

Although the number of studies reporting the use of free lipases in biodiesel synthesis is still limited, this strategy has recently called significant interest due to the lower preparation costs, especially when raw cell extracts are used instead of purified enzymes [12]. For instance, the thermostability and methanol/ethanol tolerance of a recombinant *Rhizomucor miehei* lipase used in the methanolysis of microalgae oil with high yields (91%) was increased by two folds engineering the sequence of the propeptide [13]. Ethanolysis of rapeseed oil using the commercial lipase Callera Trans L, a liquid formulation of *Thermomyces lanuginosus* lipase, reached yields higher than 95% [14,15] specifically in the Novozyme BioFAME process [16]. Although data about reusability are not presented in these articles, the economic feasibility of the process surmises that the catalyst should be active over several cycles.

Nevertheless, immobilization is still the method of choice for improving lipase stability [6]. The most relevant innovation in recent years is the use of nanomaterials (NMs) such as nanoparticles (NPs), nanotubes (Nts) and nanofibrous membranes (NFMs) for immobilization. Because of the difference in size, nanomaterials may have distinct influence on the performance of immobilized lipases and therefore other parameters such as activity retention, protein loading, production cost, aggregation potential, non-uniformity and lack of knowledge on immobilized enzymes aggregates have to be also considered [17]. Just to quote a few examples, nanoporous carbon (NPC) derived from metal-organic framework has been used as the support for the immobilization of *Burkholderia cepacia* lipase, improving the enzyme loading efficiency as well as the catalytic performances [18]. Magnetic nanoparticles are drawing special attention due to their high surface area and proper physical properties [19]. For instance, CALB (*Candida antarctica* lipase B) covalently immobilized on functionalized magnetic nanoparticles performed high conversion rates in the sinthesys of biodiesel and retained 100% activity after 6 cycles of reaction [20].

Lipases with different specificity and alcohol tolerance (from *B. cepacia, R. miehei* and *C. rugosa)* covalently immobilized on dendrimer functionalized magnetic carbon nanotubes after 10 cycles of biodiesel synthesis reached a biodiesel yield of 89.4%, 80.5% and 58.3% [21]. Fluidized bed bioreactors assisted by

electromagnetic field have been developed using *Pseudomonas fluorescens* lipase immobilized on chitosan with magnetic properties [22].

2.2 Whole- cell lipases

Whole cell biocatalysis with bacteria, yeasts and fungi is also exploited, since it reduces the cost of enzyme isolation, purification and immobilization [23,24]. Nowadays three types of whole cells are applied: wild type lipase-producing cells, recombinant lipase-producing cells, and recombinant yeast cells displaying lipases on their surface [25]. Whole cell catalysts containing lipolytic activities from *Rhizopus oryzae*, *R. chinensis*, *R. miehei*, *Thermomyces lanuginosus*, *Geobacillus thermocatenulatus*, *P. fluorescens*, *Fusarium heterosporum*, lipase 2 from *Yarrowia lipolytica* and Lipase B from *C. antarctica* have been obtained either by cultivating the original (native) lipase-producing strains or by expressing their lipase-encoding genes in hosts such as *Aspergillus oryzae*, *Escherichia coli*, *Saccharomyces cerivisiae*, and *Pichia pastoris* [6,24]. An interesting application was the use of *Aspergillus niger* whole cells producing a recombinant *F. heterosporum* lipase in the production of FAMEs using partial soybean oil hydrolysate with high free fatty acid contents as starting raw material. In this reaction, such a catalyst performed better than both *A. niger* whole cells expressing CALB and the commercial Novozym 435. The reason for this limitation could be related to the activity of rCALB which is better suited for esterification, and to the low water tolerance of Novozyme 435. [26]. In another study, native *A. niger* lipase-producing whole cells showed conversion higher than 90% using microalgae (*Scenedesmus obliquus*) as feedstock. In this case the biocatalyst was reused for three batches with a loss of conversion efficiency lower than 15% [27]. Lipase-producing *R. oryzae* NBRC 4697 whole cells immobilized onto polyurethane foam coated with activated carbon were successfully tested in biodiesel synthesis using soybean oil [28]. Biodiesel production from waste cooking oil was studied in a magnetic fluidized bed reactor using *P. mendocina* cells immobilized on magnetic microspheres with high yields and good reusability [29]. **Table 1** reports an update of the results described in previous reviews [6,23-25] using whole cells in biodiesel synthesis.

2.3 Microbial production of biodiesel

As an alternative to the use of lipase-displaying microbial cells several efforts focused on the microbial production of biodiesel. In particular, bacteria (*E. coli*) and yeast (*S. cerevisiae, Y. lipolytica*) have been metabolically engineered to produce fatty acid ethyl esters (FAEEs) directly from renewable feedstocks such as carbohydrates [30,31]. These compounds are potentially attractive diesel fuel replacements due to their high energy density and low toxicity [32]. FAEEs can be synthetized by condensation of acyl-CoAs and ethanol by using a wax ester synthase/acyl-CoA diacylglycerol acyltransferase (WS/DGAT). Early attempts at FAEE biosynthesis in *S. cerevisiae* using a wS/DGHAT from *Acitenobacter calcoaceticus* ADP1 resulted in very low yields [33]. Similar attempts were carried out in metabolically engineeried *E. coli* coexpressing the *Zymomonas mobilis* pyruvate decarboxylase and alcohol dehydrogenase and the unspecific acyltransferase from *Acinetobacter baylyi* ADP1 [34]. Also, the US company LS9 used *E. coli* as cell factory platform to produce FAEEs, reporting a FAEE production of 674 mg/L, ca. 9.4% of the theoretical yield on glucose [30]. However, to our knowledge, this company only managed to produce biodiesel at low volumes in a demonstration facility, and never reached commercial scale [35].

Further metabolic engineering strategies for improved production of FAEEs in *S. cerevisiae* have been pursued [36,31], including overexpression of different wax ester synthases encoding genes (and increasing the copy number of such genes), disruption of genes encoding for pathways competing for acyl-CoA (i.e formation of triacylglycerols and esteryl esters, and the β-oxidation pathways), overexpression of genes encoding for the pathways synthetizing the precursors of acyl-CoA such as *ACC*1 encoding acetyl coenzyme A carboxylase and cofactor NADPH, or introducing deregulated Acc1 mutants. Titers ranging from few miligram per liter to about 0.5 g/L have been achieved in this cell factory. Also, targeting to the endoplasmatic reticulum of an *Acinetobacter bayli* ADP1 wax-ester synthase, or a gene cluster expressing a fatty acyl-CoA reductase from *A. baylyi* ADP1 and an aldehyde deformylating oxygenase from *Prochlorococcus marinus*)

produced 130 mg/L FAEE in the yeast *Y. lipolytica* [37]. Although several of these strategies have been combined using stable chromosomal integration in *S. cerevisiae* [38], yields are still very low and, therefore, such strategies still need further engineering to reach industrial feasibility.

2.4 Operational strategies

The goal of reducing the time of contact of the lipase with methanol and, consequently, keeping inactivation by methanol as limited as possible, is pursed by new process strategies that increase bioprocess reactions rates. A summary of recent different bioreactor configurations applied in biodiesel production over the last two years is presented in **Table 2** and the most relevant achievements are summarised here.

A rotary packed bed reactor (RPBR) has been tested successfully using *Candida sp* 99-125 lipase immobilized on diatomite in the alcoholysis of soybean oil. This reactor configuration improves mass transfer efficiency and better micro-mixing environment compared with continuous stirred tank reactors (CSTR), being a possible candidate for industrial application [39].

Another configuration recently tested is a biodiesel reactor that integrates liquid-liquid reactions and a subsequent phase separation by means of a centrifugal contactor separator (CCCS) using the commercial enzyme TranZyme, showing promising results compared to a conventional CSTR [40]. In order to avoid problems associated to glycerol accumulation, a two-stage packed bed reactor with glycerol extraction column was used in a process based on *B. cepacia* lipase immobilized on SiO_2-PVA, increasing the productivity of the system [41].

In order to overcome major problems of the conventional biodiesel reactors, microreactors are considered as a potential alternative. Microreactors increase the dispersion of two phases, providing much higher interface area and avoiding mass transfer hindrance. As a result, shorter reaction time are achieved [42]. The cost analysis demonstrated that this green chemistry innovation alone is not enough and process intensification is needed to make this approach competitive in the future [43].

Enzyme-catalyzed transesterification by means of ultrasonic techniques is an emergent new reaction system. Ultrasounds contribute to a more homogeneous reaction mixture, facilitating dispersion of the lipase in the substrate media and reducing agglomeration, enhancing mass transfer and hence the rate of transesterification [44,45]. Ultrasounds have been recently applied to biodiesel synthesis using a wild type biocatalyst and an engineered *Proteus vulgaris* lipase immobilized on polysulfone [46] or Novozyme CAL-B immobilized on celite [47], increasing more than 10 fold the reaction rates in all cases. Also, an ultrasound two compartment reactor has been developed for the production of FAMEs from rapessed oil using Callera Trans L™, reaching a yield higher than 90% and increasing the reaction rate two fold [48]. Although the application of ultrasound improves both reaction rate and mass transfer, thermal denaturation caused by ultrasound waves in free and immobilized lipases still has to be studied to exclude effects hampering the reutilization of the biocatalyst.

The application of supercritical carbon dioxide (SC-CO_2) and ionic-liquids technologies are presented as alternatives to classical technologies. Lipase-catalysed biodiesel synthesis using ionic-liquids solvent systems have generally given excellent results. Furthermore, since diesel oils are hydrophobic compounds, organic solvent free separation form the reaction mixture has sometimes been carried out [49]. Earlier studies of enzymatic alcoholysis of natural lipid source in SC-CO_2 are reviewed in [24]. Recently, SC-CO_2 has been used as green solvent in the lipase catalysed ethanolysis of fish oil by Lipozyme RM IM. In this study, no ethanol inhibition was observed at high concentrations of this compound [50]. Overall, these studies point at the application of supercritical carbon dioxide technology as the most promising strategy which specifically minimizes the alcohol inhibition.

A further relevant innovation in the field is the use of deep eutectic solvents (DESs). These can be seen as a new generation of ionic liquids and are formed by mixtures of ammonium salts and a hydrogen bonds donor, such as urea, glycerol, or ethylene glycol. DESs have a low melting point, and they are cheap and biodegradable. DESs potential role in both the chemical and the enzymatic production of biodiesel is multifaceted, as catalysts (acid or basic), as cosolvents, and in the purification of the

produced biodiesel [51, 52]. While DESs have been tested in enzymatic reactions with different enzymes, mainly hydrolases, specific information in the enzymatic production of biodiesel is still preliminary, though promising. DESs with different compositions were tested on a set of lipases in the transesterification of seed oil [53]. The best performing combination was CALB Novozyme 435 in 2:1 Choline acetate/glycerol solvent with a 55% yield after 48 hours. CALB Novozyme 435 was also employed in the methanolysis of a mixture of triglycerides (C8+C10) with 97% conversion after 3 hours reaction with 20% methanol [54].

3 Inactivation by organic solvents

Enzyme activity is mediated by organic solvents via three major mechanisms. Solvents and co-solvents modify the solubility of substrates and products and thus change the Michaelis constant K_m and substrate or product inhibition constants K_i. Unspecific binding of solvent molecules to the protein might promote unfolding or aggregation which lead to irreversible inactivation. Solvent molecules might also bind non-covalently to the substrate binding site or the substrate entrance channel of the enzyme which results in competitive inhibition. The knowledge of the mechanism is the prerequisite for designing engineering strategies to overcome these limitations.

3.1 Solubility effects

Macrokinetic models of the Michaelis-Menten type are widely applied to analyze enzyme kinetics, and the substrate-concentration dependency of the initial reaction rate v_0 is determined to derive the parameters K_m and K_i. In non-ideal mixtures, binding affinities depend not only on the enzyme-substrate interaction, but also on the type of solvent and on the concentration of the substrate. Therefore, binding affinities have to be expressed as thermodynamic activity rather than concentration to obtain a molecular interpretation of enzyme-substrate interactions [55]. Thus, the increase of the Michaelis-Menten constant of a-chymotrypsin upon addition of methanol as co-solvent was quantitatively

explainable by the alteration of the activity coefficient of the substrate methylhydrocinnamate [56]

Expressing K_m in terms of thermodynamic activity has a major advantage: its value is independent of the solvent, in contrast to K_m expressed as concentration [57,58]. By correcting for the different solubilities of short and long chain fatty acids, the chain-length profile of lipases upon alcoholysis reactions in different organic solvents became independent from the solvent [59]. Short chain alcohols also promoted the formation of microaggregates resulting in the activation of lipases [60].

3.2 Irreversible inactivation

Apart from their effect to substrates and products, organic solvents have a major impact on the structure and dynamics of the enzyme. Aggregation, misfolding and unfolding have been attributed to the observed decrease in catalytic activity upon transfer of an enzyme to organic solvent mixtures. In general, unfolding and aggregation are slow processes, therefore irreversible inactivation mostly occur on a time scale of minutes to hours. In molecular simulations of *C. antarctica* lipase B in binary water-methanol mixtures, the affinity of the protein surface towards methanol was considerably higher than towards water, resulting in a gradual replacement of loosely bound water molecules by tightly bound methanol molecules as the methanol concentration increased [61]. These observations are consistent with simulation results on organic solvents interacting with surface loops that led to the opening of pathways for solvent molecules to the protein core, which resulted in a collapse of the secondary and tertiary structure [62,63].

The inactivating effect of methanol on *C. antarctica* lipase B was monitored by CD spectroscopy [64] and was stronger than the effect of ethanol, as shown for *C. antarctica* lipase A [65].

In general, stability toward additives and cosolvents is tightly linked with thermostability [66]. In thermostable proteins, structural fluctuations are restricted by improved packing, hydrogen bonding, and salt bridges, thus

preventing solvent molecules from passing into the core which would result in unfolding [67]. Thus, strategies to increase the thermostability of an enzyme by mutation in general lead to increased stability toward hostile organic solvents, additives, and co-solvents [68].

3.3 Reversible inhibition by competitive binding

There is growing evidence that solvent molecules might also compete with the substrate for binding to the substrate binding site. As a result, the catalytic activity is reduced instantly and will not further decrease on longer time scales. Since long, competitive inhibition by solvent molecules has been reported [69]. Competitive inhibition is mostly expected for enzymes with hydrophobic binding sites such as cytochrome P450 enzymes [70]. Methanol has been demonstrated to act as a competitive inhibitor of some lipases [71-73]. In only few cases, the molecular basis of inhibition has been elucidated by X-ray crystallography [74] or modelling [75]. Water activity-dependent changes in selectivity of a lipase has been attributed to the gradual binding of water to a specific water binding site at increasing water concentration [76].

Depending on the lipase, the substrate, and the (co-)solvent, the experimentally observed inactivation of lipases by methanol could follow one of the three mechanisms. Because the knowledge of the mechanism of inactivation will be the basis of designing an engineering strategy, a careful analysis is highly recommended.

4 Solvent-tolerant lipases: mining biodiversity and protein engineering

Owing to the necessity to reduce costs and complexity of the process, lipases that are tolerant to short chain alcohols are actively investigated. A major goal of this research is to obtain biocatalysts, either as pure proteins or as whole cells, which are able to perform one-shot reactions avoiding the need to add the alcohol stepwise or to re-activate the enzyme upon recycling. The concentrations of

methanol used in transesterification reactions are quite high, ranging between ~30 and 100% v/v of the non-lipid phase with reaction batches ideally containing 1: 3 or even higher triglyceride: alcohol molar ratios since it has been shown that yield of the process is increased at a high methanol (up to 1:5) [3]. Such conditions are unaffordable for methanol sensitive enzymes, as for example CALB, that undergoes inactivation already at 1:1 oil: alcohol molar ratio.

4.1 Naturally tolerant lipases

As a matter of fact, only a very few enzymes are naturally alcohol robust. Among them, best known are the lipases from *Burkholderia* specie that retain high activity after prolonged exposure to high concentrations of short-chain alcohols. Both the *B. glumae* (*Chromobacterium viscosum*) and, to a still higher extent, the *B. cepacia* lipases were shown to support transesterification at 1:5 triglyceride: methanol molar ratio and to be stable in 50% methanol over extended times [77,78]. In the conditions applied, 50% methanol corresponds to a 1:3 molar ratio. *Burkholderia* lipases are also endowed with high stability to heat and organic solvents [79].

During the last two years, further promising biocatalysts were found with long-term robustness toward methanol or ethanol. The metagenomic lipase RK-lip479 retains 70% of its catalytic activity upon incubation for 24 h in 25% methanol. Consistently, the enzyme is active in the methanolysis of waste vegetable oil with highest yields at 1:3 oil to methanol molar ratio [80]. Under the same conditions, a lipase from *Pseudomonas stutzeri* retained 90% of activity. Unfortunately in this case performances in alcoholysis were not assessed [81]. Like many other lipases, the lipase from *Proteus sp.* SW1 was activated during 12 hours of incubation in water-miscible solvents such as ethanol, acetone, isopropanol and acetonitrile, followed by a slow deactivation. After 120 h incubation in 80% ethanol, this enzyme still retained 75% of the catalytic activity. Methanol seems to be more deleterious. Still, a 9 h half-life in 80% methanol was observed [82]. An even higher stability was found for a lipase from *Xanthomonas oryzae* which showed no loss in catalytic activity upon incubation in 20% methanol for 24 h at 70°C [83]. Unfortunately, the molecular basis of methanol stability in these lipases is still

unknown.

4.2 Engineeered lipases

This section provides an overview of a few recent studies performed by protein engineering (**Table 3**) and includes a case study in which the performances of a microbial lipase were improved by combining mutagenesis and immobilization. Methods employed to obtain alcohol resistant mutants are based either on directed evolution or they make use of structural/evolutionary information as the starting point.

A successful example of engineered methanol tolerance concerns whole cells catalysts producing engineered recombinant *Thermomyces lanuginosus* lipase (TLL) [84,85]. TLL is one of the leading enzymes for biodiesel production because of its high activity in the presence of water [86] and is inactivated by methanol exceeding 1.5:1 molar ratio. Methanol inactivates even the whole cell catalysts, thus requiring its stepwise addition. With the goal of adapting the TTL-based whole cell catalyst to one-shot transesterification with high methanol concentration, 8 surface amino acids with high B-factors were modified by Iterative Saturation Mutagenesis (ISM) [87]. The stabilized enzyme variant was used for one- shot biotransformation of waste grease to FAME with 3: 1 methanol oil molar ratio and produced 81% FAME yields in 8 hours, and 90% after 24 hours, respectively. Under the same conditions *E. coli* (wtTLL) yielded 67% and 82% FAME at 8 and 24 h. Whole cells could be recycled for 4 cycles retaining 92% of their original activity.

4.2.1 From a thermostable but not methanol-stable lipase to an industrial catalyst: a case study

In a successful series of experimental reports, the lipase from *Geobacillus stearothermophilus* T6 was implemented for resistance to methanol and application in FAMEs synthesis [88-90]. This lipase is thermostable yet sensitive to polar organic solvents and 30 minutes incubation in 60% methanol reduces its activity by 70%. The catalyst was targeted by (***a***) a mixed mutagenesis approach, (**b**) rational stabilization of mobile regions, and (**c**) entrapment, as it is briefly summarized in the following as an example of a multi-approach strategy.

(**a**)Mutagenesis. In a first work, the wild type sequence was modified by two complementary methods [88]. A structure-guided consensus approach allowed generating a small library of mutants focused on residues highly conserved in homologous lipases, whereas a second library was produced by error prone PCR. In both cases, lipase variants were significantly more resistant to methanol than the wild type, as it was assessed in reactions of hydrolysis of soluble *p*-nitrophenol esters. Interestingly enough, beneficial substitutions discovered in the two libraries were partly overlapping. The best performing variants carried the amino acid substitutions A269T, H86Y and Q185L, all of them located at the protein surface (**Figure 2**). Mutants were up to 60 fold more stable than the wild type and the H86Y/A269T double variant lost only 25% of its activity after incubation in 70% methanol, whereas the activity of the wild type under the same condition dropped to less than 1%. Tested in the methanolysis of soybean oil the H86Y/A269T lipase variant showed 2-fold improved activity over the wild type with 36.8% yield *vs* ca 16%. Data seem to suggest that stabilization, though significant, still cannot fully withstand the deleterious action of methanol on the protein, consistent with the observation that for all variants the yield of FAMEs decreased with alcohol concentration. Worth of note is the poor performance in transesterification of the methanol stable Q185L lipase variant. Amino acid 185 belongs to the lid structure, thus it is possible that the introduction of the hydrophobic leucine at this position stabilized the close (inactive) conformation of the enzyme via interactions with the hydrophobic residues of the active site.

b) In order to further improve stability, 8 surface charged residues were changed to hydrophobic ones [89], inspired by previous studies showing that the exposure of hydrophobic residues enhances the stability of enzymes to organic solvents. The stabilizing substitution R374W was added to the double variant H86Y/A269T obtaining a lipase endowed with 324 min half-life in 70% methanol that is 87-fold higher than the wild type. 24 hours-methanolysis of soybean oil at 1.5:1 methanol to oil molar yielded 46 % FAME, close to the theoretical highest transformation yield allowed by this methanol/oil ratio (50%). As a further step towards application, the mutant was assayed in the methanolysis of soybean oil and waste chicken oil, two non-expensive substrates that differ in their fatty acids

composition and content of free fatty acids, in the presence of very high methanol concentration (4.5:1 methanol:oil) representative of industrially-relevant conditions. When the performances of the stabilized lipase were compared with those of two industrial lipases (*T. lanuginosus* lipase Lipolase 100L and *C. antarctica* lipase Novozym®435) it turned out to be the best catalyst in the transesterification of chicken waste oil yielding 64 % FAMEs within 24 hours, while Lipolase performed better with soybean oil. Worth of note is the influence of the composition of the substrate (different results with soybean oil and chicken waste oil) that brings under the spotlight not only the relevance of the enzyme FA specificity but also possible inhibitory effects of oil components that can act differently on different lipases.

c) The final step of this work was to implement the stabilized lipase in a catalyst of industrial applicability reducing its costs and improving re-usability through entrapment of crude enzyme preparation in an aromatic sol-gel matrix [90]. The entrapped lipase was stable for at least 16 cycles of esterification. Tested for biodiesel production from chicken waste oil at 4.5:1 methanol/oil molar ratio, it achieved 80% conversion within 24 hours.

4.3 Insight into structural changes in engineered lipases

Structure determination of the triple mutant of *G. stearothermophilus* lipase described in the previous paragraphs revealed the formation of additional, though unexpected hydrogen bonds to structurally conserved water molecules or, upon side chain flipping, to the backbone, resulting in a considerable decrease of the local B-factors [89]. Overall stabilization arose from an enlarged network of hydrogen bonds at the protein surface (directly or through structurally conserved water molecules) and the stabilization of the binding sites for the structural Zn^{2+} ion (H86Y), and Ca^{2+} binding site (R374W).

By directed evolution targeting residues with high B-factor, mutation D27R in *Thermomyces lanuginosus* lipase was identified which resulted in increased specific activity and methanol stability of the enzyme, supposedly by forming a salt bridge on the protein surface [87].

Moreover, one of the most robust lipases, a 13-fold mutant of the *Proteus mirabilis* lipase, revealed additional hydrogen bonds and salt bridges compared to the wild-type enzyme, suggesting that polar interactions might contribute to stabilization in nonpolar media [91].

Overall, structural analysis of the stabilized lipases pinpoints structural stabilization achieved via various mechanisms and mainly localized at the proteins surface: reinforcement of the hydrogen bonding network, increased salt bridges, and stronger interaction with essential water molecules.

The study of methanol tolerance partly overlaps with the more general issue of lipases resistance to organic solvents, in particular to polar organic solvents.

Directed evolution approaches were used in several cases to increase lipases to solvents different from methanol and that we described previously. Overall, beneficial random mutations were mainly located at the enzyme surface and contribute to reinforce protein hydratation. Since a detail analysis of these studies is out of the scope of this review, readers are referred to recent overviews [92,93].

5 Conclusions

Significant innovation is arising in different steps of the enzymatic process for biodiesel production. In particular, we would like to highlight the use of new nanomaterials for enzyme immobilization, the use of whole cells as biocatalysts, the implementation of unconventional techniques such as ultrasounds to accelerate reaction rate and SO-CO2 ionic liquids to enhance biocatalyst stability. In order to get insight in the basis of the observed improvements, it would be useful to take the point of view of the catalyst, to understand, for example, the mechanistic basis of enhanced lipase stability by SO-CO2 and ionic liquids. This information should contributed to enlarge the knowledge necessary for rational approaches to enzyme stability.

Unfortunately, issues of relevance for robustness towards methanol and for the use of lipases in alcoholysis reactions are still elusive.

A first point to consider is that not always methanol-stabilized lipases provide higher yields in transesterification. The reason for this apparent contradiction stems from the experimental approach used to evaluate the evolution of stability

in lipase variants. In most protocols, lipases pre-incubated in methanol are assayed for generic activity in the hydrolysis of soluble substrates. This choice is dictated by the need of performing an easy and high throughput assay to evaluate several mutants at once. However, it does not guarantee highest performances in the synthesis of biodiesel, although we should acknowledge that most stabilized enzymes also better perform in oil alcoholysis.

A second point is that FAME yields featured by engineered enzymes are similar to those of naturally resistant lipases (i.e. *Burkholderia* lipases). The basis of tolerance in these lipases are still unclear, although their 3D structures are known. Thus, as for time being, the production of engineered lipases is of application relevance if they have additional advantages, for example the ease of heterologous expression or specific enzyme features. However, from the point of view of knowledge and ability of control of the processes, the availability of a large body of data on the effects of mutagenesis is the mandatory requirement towards rationalization of the molecular bases of stability towards alcohols and other polar solvents.

We finally wish to pinpoint that for some lipases, as for example the broadly used *C. antarctica* lipase B, a relevant role in methanol driven inactivation is attributed to inhibition by methanol itself [71,72]. While it is reasonable to assume that an approach different from overall structural stabilization should be applied to this catalyst, attempts in this direction are to date not reported. Inhibition was never specifically addressed by mutagenesis and answers cannot be provided by the studies previously described whose experimental design allows revealing structural effects only.

To conclude, in spite of the growing body of information available, the ability to rationally engineer solvent stability seems to be still far of reach and directed evolution remains the technique of choice. However, important hints have emerged allowing more focused (or semi-rational) approaches. Worth of note are recent studies that targets channels or tunnels in the protein molecule to avoid solvent molecules reaching the enzyme active site [94].

Acknowledgement

M.L. acknowledges a grant from the University of Milano-Bicocca (Fondo Ateneo) and partial support by by the BEETOUT (Sugar BEEt biorefinery for the inTegrated prOduction of biofUel and polyesTers) project, funded by Fondazione Cariplo (Bando Ricerca Integrata sulle Biotecnologie Industriali e sulla Bioeconomia 2015-0375). F.V. and P.F. acknowledge support by the project CTQ2016-74959-R (MINECO/FEDER,UE). The group is member of 2014-SGR-452 and the Reference Network in Biotechnology (XRB) (Generalitat de Catalunya). J.P. acknowledges financial support by Deutsche Forschungsgemeinschaft (SFB716, EXC310).

Conflict of interest

The authors declare no financial or commercial conflict of interest

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Table 1. Update of whole cell lipases applied in biodiesel synthesis. FBR = Fluidized bed reactor; BSP = Biomass support particle

Microorganism	Substrate	Immobilization	Reactor	Biodiesel yield %	Reusability (cycles)	Reference
Aspergillus oryzae expressing lipase from *F. heterosporum*	Soybean oil partially hydrolysed	BSP	Batch stepwise methanol addition	93	---	26
Aspergillus niger	Microalgae oil	Polystyrene	Batch stepwise methanol addition	90.8	2	27
Rhizopus oryzae	Soybean oil	Polyurethane foams coated with activated carbon	Batch stepwise methanol addition	95.0	--	28
Pseudomonas mendocina	Waste cooking oil	Magnetic microspheres	FBR	91.8	10	29

Table 2. Novel processes for biodiesel production in bioreactor

Lipase	Conversion	Reutilization	Reactor	Strategy	Ref.
Pseudomonas mendocina whole cells immobilized on magnetic microspheres	91,8	10 cycles	Magnetic FBR	Optimized constant methanol feeding rate	[29]
Candida sp. 99-125 immobilized on diatomite	97	5 cycles	Rotating packed bed reactor (RPBR)	Optimized methanol feeding rate	[39]
TransZyme A immobilized on hard shell beads	86	9 hours	CSTR + Centrifugal contactor separator (CCCS)	Optimized methanol feeding rate	[40]
Callera Trans L™	91	---	Two compartment ultrasound reactor	Recirculation	[48]
Burkhoderia cepacia immobilized SiO_2-PVA	96.3	Half time 1512 h.	Two stage packed bed reactor with glycerol extraction column	Optimized constant methanol feeding rate	[41]

Table 3. Protein engineering towards stabilization to methanol

Enzyme	Wild type*	Mutagenesis	Improvement in stability*	Structural changes	Substrate for transesterification	Conversion	Ref
Proteus mirabilis lipase(stabilized with S-S bond)	Inactive after 2 h incubation in 70% methanol	Ep-PCR+SDM	80% residual activity after 16 h incubation in 70% methanol	11 substitutions. Additional polar interactions and salt bridges	Canola oil 5:1 molar ratio	76% in 20 hours (wt 47.7%) Can be recycled (the wt not)	[91]
Geobacillus stearothermophilus Lipase	4 min half-life in 70% methanol	-Consensus-guided -Ep-PCR -Substitution of surface charged residues	324 min half-life in 70% methanol (87x)	H86Y/A269T/R374W. Hydrogen bonds network and structural water molecules	soybean oil 1.5:1 methanol to oil molar ratio + other substrates	46% in 24 hours ** Wt: 8.6% in 24 hours	[88,89]
Thermomyces lanuginosum Lipase	28% residual activity after 1 hour incubation in 75% methanol	Mutagenesis of residues with high B-factor	71% residual activity after 1 hour incubation in 75% methanol	S105C/D27R new hydrogen bond that stabilizes a flexible loop structure	Waste grease	With whole cells S105C/D27R 90% in 24 hours Wt: 82% in 24 hours	[87]

*measured as activity of methanol-incubated biocatalysts in hydrolysis reactions
** highest conversion possible 50%

Figure legends

Figure 1. Reactions for the synthesis of fatty acid alkyl esters (biodiesel) by lipases. (a) Esterification of fatty acids and alcohols. Waste oils are rich in free fatty acids and contain water; (b) Transesterification of triglycerides and alcohols. This reaction produces glycerol that can be used as a platform for the synthesis of chemical building blocks

Figure 2. Crystal structure of wild-type lipase T6 with the mutated residues(H86, A269, R374) shown in orange sticks, catalytic triad residues (Ser114, Asp318, and His359) in green, calcium-binding residues (Glu361,Gly287, Pro367, and Asp366) in magenta, zinc-binding residues (Asp62, His88, Asp239, and His82) in cyan, α-helix lid and α9 in red. and gray spheres, respectively. Reproduced with permission from ref [89].

Graphical abstract

A major issue in the enzymatic production of biodiesel is the sensitivity of most lipases to methanol. Both free and immobilized enzymes can be inactivated by methanol that can induce protein denaturation or act as a competitive inhibitor of the enzyme. Process design and protein engineering are applied to relieve methanol inactivation (courtesy of A. Pischedda)