Revision

Effects of methanol on lipases: molecular, kinetic and process issues in the production of biodiesel

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Abbreviations:
a: thermodynamic activity; a_w:water activity; BGL: *Burkholderia glumae* lipase; CALB: *Candida antarctica* lipase; FAME: fatty acid methyl ester

Abstract

The biotechnological production of biodiesel is based on transesterification/esterification reactions between a source of fatty acids and a short-chain alcohol, usually methanol, catalysed by enzymes of the class of lipases. Several industrial lipases, though stable to other organic solvents, are inactivated by methanol at the concentration required in the process or even lower, what makes it necessary to use stepwise methanol feeding or pre-treatment of the lipases. In this review, we focus on the knowledge available about the basis of methanol-induced lipase inactivation, which is not shared by all lipase enzymes, in order to pave the way for the possible implementation of the biocatalytic process. We suggest that different mechanisms, either substrate inhibition or protein unfolding, can lead to inactivation of different lipases. Attempts to improve the performance of methanol-sensitive lipases by mutagenesis as well as process engineering approaches, such as adding methanol stepwise, using organic solvents, or performing the reaction in salt-based solutions, supercritical carbon dioxide or ionic liquids are also summarized. Available information provides evidence that the mechanism of methanol-mediated inactivation is lipase-specific and call for enzyme-specific strategies of stabilisation.

Introduction

Enzymes are powerful tools for performing industrially relevant reactions. Advantages of the biocatalytic routes are related to the possibility of introducing environmentally compatible and sustainable processes, whereas the main drawback is still the high cost of enzymes. In this context, the stability of enzymes towards the reaction conditions, for example temperature, presence of contaminants or solvents, repeated cycles of catalysis, is of paramount importance for the economical competitiveness of the whole process.

In this review article we focus on the effects exerted by methanol on enzymes of the family of lipases, the catalysts of choice for the biotechnological production of biodiesel [1-5]. In this reaction, fatty acids from triglycerides are esterified to alcohols and fatty acid alkyesters (biodiesel) are produced. Raw materials used can be fats, vegetable oils, waste oils, as well as lipids from microorganisms and algae. Though this last issue is out of the aim of this review, it is very relevant for application since availability of cheap sources of triglycerides contributes to lower the overall costs. Moreover, raw materials differ from each other in the quantity of water and free fatty acids they contain. For example, differently from vegetable oils that are made up mainly by triglycerides, waste oils contain water and free fatty acids. Free fatty acids can be converted by lipases (but not through chemical catalysis) to alkylesters, while the amount of water in this reaction can be either detrimental or beneficial for the conversion, depending on the specific enzyme and the conditions applied. The choice of the alcohol is also crucial because of the associated costs, the properties of the biodiesel obtained, and its effect on the catalyst itself [6]. Short-chain alcohols, in particular methanol and ethanol are widely used, with preference for methanol that is cheaper. Methanol however, is reported to hamper the activity of several lipases when used at the concentrations that would be optimal for the alcoholysis reaction. While several approaches are exploited to overcome this problem, as summarized later in this paper, its origin is still poorly understood, as one can guess also from the non-specificity of the terms used, alternatively inactivation, deactivation, inhibition, denaturation.

Understanding in depth the effects on methanol on enzymes is of broader scope since alcoholysis with short chain alcohols is used, besides for the production of biodiesel, in a variety of reactions, for example in preparing food emulsifiers, personal care and cosmetic products [7], flavours and pharmaceuticals [8].

Enzymatic production of alkylesters: enzymes, alcohols and water

In low water systems lipases (E.E.C.3.1.1.3) catalyse transesterification and esterification reactions involving long-chain fatty acids, while in water medium hydrolysis predominates. Lipases are very popular biocatalysts and are easily obtained from a variety of biological sources that provide enzymes endowed with different substrate specificities, catalytic properties and robustness towards organic solvents. Several purified and recombinant lipases, either free or immobilized, are commercially available. Moreover, academic and industrial laboratories worldwide pursue the search for new enzymes and the modification of existing ones to improve their properties.

Both specific and non specific lipases have been employed for the synthesis of alkylesters [1-4], though enzymes non-specific for the position of fatty acids on the glycerol backbone and for the chemical structure of fatty acids appear to be more flexible in the use of substrates that might contain triglycerides of different structure and composition. In addition to well known lipases, cold-active enzymes are being exploited to reduce the energy costs of the process [9, 10] and lipases stable to temperature and pH are considered of interest because of their overall robustness [11]. Recently, approaches of mutagenesis have been applied to improve the resistance of lipases to the reaction conditions, in particular to the presence of methanol [12-14].

It is generally accepted that transesterification follows a Ping Pong Bi Bi mechanism in which the enzyme reacts first with triglycerides (or free fatty acids) giving a covalently modified enzyme (acyl-enzyme), and then with the alcohol to release the akyl ester [15-17]. The amount of water in the reaction mix seems to impact in opposite ways on the yield of processes catalyzed by different lipases. For example, the synthesis of alkylesters by *Candida antarctica* lipase B (CALB) decreases as water increases [18], whereas water improves the yields of reactions catalysed by *Rhizopus oryzae, Candida* sp 99-125 and *Pseudomonas cepacia* [6, 19, 20].

Short chain alcohols are one of the reaction substrates, what in principle should suggest to increase their concentration to drive product formation. Accordingly, while the theoretical optimal methanol:triglycerides molar ratio is 3:1, several authors found highest yield at higher alcohol concentrations, typically up to 6:1 [19, 21-23]. In several cases however, high methanol concentration is rather detrimental and even the stoichiometric condition is not affordable. This is the case of reactions catalysed by CALB, that is rapidly inactivated by methanol at concentrations exceeding 1:1, by far lower than the molar ratio optimal for the conversion [24]. The example of CALB is of interest, since this enzyme is stable in a broad range of other organic solvents [25].

Analyses about the response to methanol of a number of free and immobilised lipases are abundant and, though not directly comparable for they are performed in different systems, depict a clear picture allowing to classify lipases on the basis of their sensitivity/robustness [6, 19]. Among the best characterised lipases, those from the *Pseudomonas/Burkholderia* genus appear to be highly

tolerant to methanol while CALB, *Candida rugosa* lipase and several others are methanol sensitive (**Fig. 1**).

Though even methanol-sensitive lipases have been successfully employed in the synthesis of biodiesel using appropriate experimental procedures (see below), the availability of robust biocatalysts could be of advantage for easier experimental set-ups. In this scenario, understanding the molecular detail of the mechanisms by which methanol inactivates lipases, may pave the way for the implementation of experimental strategies of stabilisation.

Solvent effects: thermodynamic issues

Since the first systematic studies on the catalytic activity of enzymes in organic solvents and the relevance of low amounts of water [26], the relative contribution of the organic solvent and the water content to biochemical properties such as catalytic activity was under discussion. It was observed that catalytic activity and selectivity of many enzymes depends sensitively on the water content. In addition, the effect of the choice of organic solvent to enzymatic activity was studied, and a good correlation between the logP (logarithm of the partition coefficient between 1-octanol and water) of the solvent and the enzyme activity was found [27]. However, many experimental results published so far are difficult to compare because frequently the details of the mixtures are not rigorously controlled.

Two major effects of the organic solvent to the catalytic activity of an enzyme were discussed. It was suggested that solvents of different logP values result in different amounts of enzyme-bound water and thus mediate catalytic activity [28]. Since then, extensive studies were performed to quantify the binding of water to enzymes and to relate water binding to catalytic activity. Systematic quantitative investigations of water binding to proteins were performed in a gas/solid reactor [29, 30]. Up to a water activity of 0.5, the number of protein-bound water molecules increased linearly with water activity, for higher water activity an exponential increase was observed. To our knowledge, there are no experimental studies on water binding in organic solvents at low water content. X-ray studies on water binding in organic solvents always referred to aqueous mixtures [31, 32] and thus are restricted to thermodynamic activities of almost 1.

A second effect of organic solvents is their interaction with the substrates, thus modifying the chemical potential of the substrates [33]. The contribution of substrate-solvent interactions to enzyme kinetics was accounted for by replacing substrate concentrations in the model of enzyme kinetics by thermodynamic activities [34]. Using this approach, the kinetics of *Pseudomonas cepacia* lipase in an isooctane-aqueous biphasic system could be adequately described with the rate equation for a ping-pong mechanism [35]. This approach was later confirmed to analyse enzyme

kinetics in solvent-free systems [36]. For lipase-catalyzed ester alcoholysis, a model was established based on a ping-pong bi-bi mechanism with competitive inhibition by the alcohol substrate [36] (**Fig. 2**). The initial rate v of lipase-catalyzed methanolysis of an ester depends on the thermodynamic activities a_{MeOH} ·and a_{est} of methanol and ester, respectively (eq 1).

$$v = v_{max} \cdot a_{MeOH} \cdot a_{est} / (K_{M,est} \cdot a_{MeOH} + K_{M,MeOH} \cdot a_{est} + a_{MeOH} \cdot a_{est} + K_{M,est} \cdot a_{MeOH}^2 / K_{i,MeOH}) \quad \text{(eq 1)}$$

with four kinetic parameters, the maximum reaction velocity v_{max} , the Michaelis constants $K_{M,est}$ and $K_{M,MeOH}$ of ester and methanol, respectively, and the inhibition constant $K_{i,MeOH}$ of methanol.
The thermodynamic activities a_{MeOH} ·and a_{est} were calculated from the respective mole fractions χ_{MeOH} and χ_{est} by

$$a_{MeOH} = \gamma_{MeOH} \cdot \chi_{MeOH}, \qquad a_{est} = \gamma_{est} \cdot \chi_{est} \qquad \text{(eq 2)}$$

The activity coefficients γ_{MeOH} and γ_{est} were estimated using the UNIFAC group contribution method [37].
This assumption has three major consequences: (1) Even if all components are completely miscible, the thermodynamic activity coefficient deviates from unity. Thus, the thermodynamic activity of each component depends not only on its concentration but may change of the concentrations of the other components change. In a previous study, the thermodynamic activity of an ester substrate at constant concentration varied by 35% upon variation of the methanol concentration [38]. (2) At a given water concentration, solvents with different logP result in a considerable change of the thermodynamic activity of water which is much higher for non-polar solvents with high logP value than for polar solvents, thus leading to the observed correlation of catalytic activity with logP of the solvent [27]. However, additional effects of the solvent have been suggested [39]. (3) The kinetic model applies exclusively to miscible components. However, if substrates and solvent are immiscible, a further deviation from the expected concentration dependence is expected due to partial phase separation.
The importance of developing thermodynamic strategies for controlling both the reaction kinetics and equilibrium of lipase-catalyzed reactions is further emphasized in a recent review [40], and thermodynamical methods for reaction optimization are recommended. However, because many papers still report on concentrations rather than thermodynamic activities [41], results from different research groups obtained under different experimental conditions are generally difficult to compare.

Molecular and kinetic effects of methanol

While the issue of the deleterious outcome of methanol on most enzymes is well known and broadly discussed, until now research focussed mainly on approaches to circumvent this experimental limitation (see next paragraph), and information available on molecular and kinetic issues is still poor. Major effects can be ascribed to the solvent effects described in the previous paragraph. However, it is clear that methanol can act directly on the catalyst since, as a matter of fact, different lipases display a different behaviour in the presence of methanol (**Fig. 1**).

Short chain alcohols are supposed to lead to deactivation of lipases by two different mechanisms. High concentrations of alcohol might lead to (partial) unfolding of the enzyme followed by irreversible deactivation. In ethanol/water mixtures, the disruption of intra-protein hydrophobic interactions by adsorption of alcohol molecules on hydrophobic sites on the protein surface was modeled by molecular dynamics simulations [42] and experimentally confirmed by static light-scattering measurements [43]. In this case, addition of alcohols resulted in a transition to a more helical state [44]. Moreover, many organic solvent molecules were shown to act as competitive lipase inhibitors [45]. However, both effects are not always clearly separated in publications on the decreased catalytic activity of lipase in methanol-solvent mixtures or solvent-free systems.

Inhibition has been hypothesized since long to explain the low performances of some lipases, for example CALB, in alcoholysis of triglycerides [2, 24]. Recently, the initial rate of CALB catalysed transesterification in dependence of methanol concentration was studied in a strictly controlled model system composed by vinyl acetate, methanol and toluene taking into account also water activity (a_w) and the thermodynamic activity of reagents [38]. It was shown that CALB is inhibited by methanol at thermodynamic activities above 0.2, corresponding to concentrations as low as 1% in toluene (**Fig. 3**) [38], while structural stability is only decreased at much higher concentrations. On the contrary, the lipases from *Burkholderia sp.* are intrinsically stable towards methanol. Accordingly, the yield of transesterification increases with methanol concentration [19, 22, 46]. Among this group of robust enzymes, the *B. glumae* (BGL) lipase was exposed to methanol, specifically for the time (24-48 hours) and the conditions (up to 75% methanol) used in the biodiesel reaction in a reaction mixture composed by triolein at fixed concentration and increasing methanol (molar ration from 1:1 up to 1:6) [22]. BGL produced a final yield of over 90 % at reactants molar ratio (1: 6) that correspond to ~75 % methanol in the aqueous phase (**Fig. 1**). In this case, even exposition to high methanol (over 50%) did not damage the specific activity of the enzyme. Only prolonged incubation with high methanol affected stability and the protein propensity

towards aggregation. Inactivation in this case is therefore driven by conformational damage, rather than by inhibition. Accordingly, the initial rate of reaction of BGL in the system described by Kulschewski and colleagues [38] was not affected by methanol (unpublished results from our laboratories).

Evolving methanol-resistant lipases

Tolerance to methanol is an inherent property of some lipases and, interestingly, it seems to be independent from robustness to other environmental factors, as for example temperature and organic solvents. Though some effort has been devoted to the identification of novel enzymes suitable to be used in one-step biodiesel production processes, in our opinion this approach still did not provide amazing improvements in the catalogue of methanol resistant catalysts available.

Lipases used for biodiesel are very often immobilised, on a wide range of supports and using different techniques, including adsorption, covalent binding, encapsulation and entrapment [4, 47-50]. While it is demonstrated that immobilization improves the catalyst's longevity and reusability and may enhance lipase activity, for example favouring the interaction enzyme/substrate, to the best of our knowledge evidence about a specific role in the methanol tolerance is still lacking.

In recent years, a few novel studies focussed on the development of improved enzymes through protein engineering methods. In this case, the issue is more specific that lipase stabilisation (see for a recent review [51]) and the success is uncertain since, generic robustness towards the presence of methanol is not always coupled with high activity in transesterification.

Rational mutagenesis was applied to build additional hydrogen bonds at the surface of CALB to counteract stripping of water molecules by the solvent. Mutants were found to be more resistant to incubation with high methanol and to maintain increased residual activity in hydrolysis reactions but were not tested in alcoholysis and, therefore, it is not known how they perform at increasing oil:methanol ratios [14].

Dror and colleagues [13] implemented methanol stability in the lipase from *Geobacillus stearothermophilus* T6, an enzyme able to resist high temperature but poorly stable in polar organic solvents. Authors used two different and complementary approaches: a structure guided method based on the identification of specific mutations from sequence alignments, phylogenetic and structural analysis and random mutagenesis by error prone PCR, identifying three amino acids crucial for stability they modified by saturation mutagenesis. The best obtained variant was significantly more stable than the wild type to both methanol and ethanol when assayed in hydrolysis and alcoholysis. Stabilisation however, did not abolish the trend of decrease in the enzyme activity in dependence of methanol characteristic of the wild type protein, since highest yields were obtained at the lower alcohol:oil ratio. Interestingly, one of the stabilised variants was

even less active in methanolysis than the wild type, showing once more that structural stability does not necessarily counteract the impact of methanol.

Korman and colleagues [12] applied directed evolution to a *Proteus* lipase that is relatively tolerant to short chain alcohols but is irreversibly inactivated when incubated at over 50% methanol. A crystal structure of this variant revealed additional hydrogen bonds and salt bridges, suggesting that polar interactions may become particularly stabilizing in the reduced dielectric environment of the oil and methanol mixture. Other advantageous substitutions concerned the calcium binding site of the enzyme, a region known to stabilise the structure of a number of microbial lipases [52].

Process strategies to overcome (or minimize) lipase inactivation by methanol

Due to the issues related to the use of short-chain alcohols, different acyl acceptors have been tested, including primary, secondary, straight and branched-chain alcohols, as well as esters such as methyl or ethyl acetate [53]. Chen and Wu [54] demonstrated that inactivation is reduced as the number of carbon atoms of the alcohol increases, with the preference for the type of alcohols depending on each specific lipase [55]. However, as the choice of the alcohol also influences the cold flow properties and lubricity of biodiesel and, therefore, its value and price, as long as no additional benefits derive from using a more expensive alternative, methanol and ethanol seem to be the only realistic options from the point of view of price and availability [2], pointing again to the issue of the enzyme's sensitivity.

One common strategy to protect the enzymes against alcohol inactivation, as well as to improve mutual solubility of hydrophobic triglycerides and hydrophilic alcohols, is the use of organic solvents [53]. Hydrophobic solvents as isooctane, n-heptane, petroleum ether, n-hexane and cyclohexane are the most commonly used in biodiesel synthesis [56]. However, glycerol is insoluble in hydrophobic solvents, and tends to adsorb to the immobilized lipase. The formation of an outer film layer of glycerol decreases reaction rates due to the lowered mass transfer of hydrophobic substrates [2, 57]. Such an effect can be avoided using 1-3 positional specific lipases. By optimizing the reaction time, the migration of 2-acyl groups is minimized, thereby obtaining FAMEs and 2-monoglycerides as the end products of the reaction. Monoglycerides are a potentially interesting product e.g. as emulsifiers, although a purification process would be needed [58-60]. The use of hydrophilic organic solvents is much less useful, because a strong interaction with the essential water layer coating enzymes molecules occurs [61, 62]. However, hydrophilic 1,4-dioxane and tert-butanol favour high transesterification yields by reducing the oil viscosity and dissolving the glycerol by-product [53, 63, 64].

Nevertheless, organic solvent systems are not recommended for the production of biodiesel fuel from waste oil because of the risk of explosion and requirement of solvent removal. For these reasons, solvent-free systems are still the preferred strategy [65, 66]. The main problem to work in a solvent free system is the low solubility of methanol in oils. In several cases, this factor adds to other possible effects on the enzyme molecule, making the use of high methanol concentrations still more difficult. In these cases, methanol effects are overlapping but can be reduced by the same strategy, i.e. its step-wise addition at a concentration below 1/3 molar equivalent [65]. It should be also recalled that the specific problem of solubility is very relevant at the beginning of the reaction in batch processes, when TAGs are the major component; as the reaction progresses and FAMEs are produced, methanol can be increased because its solubility is higher in FAMEs than in TAGs [21, 65].

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Stepwise addition of the alcohol is often exploited in enzymatic bioreactor configurations [2, 21, 24, 67, 68]. Meeting the specifications of biodiesel, namely, very low tolerance to impurities, single reaction stage, without need for separation of glycerol and further processing, is exceptionally difficult [66]. One approach is the use of a two-stage bioreactor with glycerol separation between stages and different biocatalysts in each stage [66]. Alternative novel strategies are, for instance, the use of salt-solution-based reaction systems, which can maintain an acceptable methanol concentration by dissolving methanol in a salt solution [69], the application of supercritical carbon dioxide approaches to reach an efficient mass transfer [70, 71] or the application of ionic liquid technology to lipase-catalyzed methanolysis [72].

All the above considerations must be taken into account in the selection of the bioreactor for biodiesel production. Different batch and continuous set ups, strategies and reactors designs have been proposed. Concerning bioreactor configurations, stirred tank reactor (STR), packed bed reactors (PBR), fluidized-bed reactors (FBR) and membrane reactors (MBR) have been reported as possible options [2, 70]. From the kinetic point of view, PBRs offer better performance than CSTRs, because the high mechanical stress caused by stirring can be avoided, the volumes are reduced and the technology is less expensive [73, 74]. The enzyme can be reused without prior separation and can operate continuously at lower enzyme-substrate ratio compared to a batch process [75]. However, PBRs show operational problems caused by the immiscibility of the substrates, resulting in channeling and lower yields compared to batch processes. This problem could be solved by using structured agents to minimize compacting of the bed, or by using co-solvents. In PBRs flow rates should meet a compromise to avoid mass transfer resistance at liquid film layer at low flow rates and low interaction with the enzyme at high flow rates [75]. Also,

glycerol is difficult to remove due to its high viscosity and hydrophilic nature [76]. To obtain ester purity higher than 96 %, the use of two plug-flow reactors, separated by an operation unit for glycerol removal, has been implemented in the production of butyl ester with Novozyme 435 lipase [77]. A brief summary of different bioreactors applied in biodiesel production and the life-time of the enzymes is presented in **Table 1**. Whole cells instead of enzymes have been also exploited [78].

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Conflict of interest

Authors declare no conflict of interest

Table 1. **Processes for the production of biodiesel in bioreactor**

Lipase	Conversion %	Reutilization	Reactor	Strategy	Reference
C. antarctica Novozyme 435	> 9	52/70 cycles	PBR	Metanol stepwise	[65]
C. antarctica Novozyme 435	99.5	6 cycles	PBR	Batch repeated Metanol stepwise	[79]
T. lanuginosa immobilized on STV-DVB-PGA.	97	10 cycles	Batch reactor	Methanol stepwise	[80]
Candida sp. immobilized on textile cloth with cofixing agents	91	100 hours	3 step PBRs, with glycerol separation	Hexane as cosolvent	[47]
C. antarctica Novozyme 435	80	>120 hours	3 step PBRs,	Tert-butanol as cosolvent	[75]
C. antarctica Novozyme 435	93.2	50 % loss of activity after 24 hours.	RCTA	Methanol stepwise	[81]
C, rugosa AY. P. cepacia S P. fluorescens AK	80	12 days without lost of activity	MBR		[82]
T. lanuginosa Lipozyme TL-IM and different supports	60-100	3 batches of 10 hours	Batch reactor	Different methanol stepwise	[83]
C. antarctica Novozyme 435	96	50 days	PBR 2 PBRS separated by an unit for glycerol removal	Tert-butanol as cosolvent	[77]
P. cepacia immobilized on magnetic Fe_3O_4 nanoparticles	88	55 % loss of conversion after 240 hours	four PBR	n-hexane as cosolvent	[84]
P. fluorescens immobilized on Epoxi-SiO_2-PVA	81.6	10 days	PBR	Tert-butanol as cosolvent	[85]
r-Fusarium heterosporum expressed in *A. oryzae*. Whole cells immobilized on BSP	> 90	5 batches with minimal loss of activity	PBR	Recycling Substrates	[78]
IIT-Sarkzyme	70	20 % loss or conversion after 50 cycles.	Integrated CSTR-PBR	Solvent free system	[86]

Reference List

1. Parawira W: **Biotechnological production of biodiesel fuel using biocatalysed transesterification: A review**. *Crit Rev Biotechnol* 2009, **29:**82-93.

2. Fjerbaek L, Christensen KV, Norddahl B: **A review of the current state of biodiesel production using enzymatic transesterification**. *Biotechnol Bioeng* 2009, **102:**1298-1315.

3. Adamczak M, Bornscheuer UY, Bednarski W: **The application of biotechnological methods for the synthesis of biodiesel**. *Eur J Lipid Sci Technol* 2009, **111:**808-813.

4. Tan T, Lu J, Nie K, Deng L, Wang F: **Biodiesel production with immobilized lipase: A review**. *Biotechnol Adv* 2010, **28:**628-634.

5. Christopher LP, Kumar H, Zambare VP: **Enzymatic biodiesel: Challenges and opportunities.** *Appl Energy* 2014, **119:**497-520.

6. Deng L, Xu X, Haraldsson GG, Tan T, Wang F: **Enzymatic Production of Alkyl Esters Through Alcoholysis: A Critical Evaluation of Lipases and Alcohols**. *JAOCS* 2005, **82:**341-347.

7. Uphues G, Saint Victor M-E, Raths H-C, Biermann M, Behler A, Hill K: **Industrial surfactant syntheses.** In *Reactions and synthesis in surfactant systems*. Edited by Texter J: CRC Press; 2001.

8. Ozyilmaz G, Gezer E: **Production of aroma esteres by immobilized *Candida rugosa* and porcine pancreatic lipase into calcium alginate gel.** *J Mol Catal B-Enzym* 2010, **64:**140-145.

9. Luo Y, Zheng Y, Jiang Z, Ma Y, Wei D: **A novel psychrophilic lipase from Pseudomonas fluorescens with unique property in chiral resolution and biodiesel production via transesterification**. *Appl Microbiol Biotechnol* 2006, **73:**349-355.

10. Yang KS, Sohn JH, Kim HK: **Catalytic properties of a lipase from Photobacterium lipolyticum for biodiesel production containing a high methanol concentration**. *J Biosci Bioeng* 2009, **107:**599-604.

11. Zhang H, Zhang F, Li Z: **Gene analysis, optimized production and property of marine lipase from *Bacillus pumilus* B106 associated with South China Sea *Halichondria rugosa***. *World J Microbiol Biotechnol* 2009, **25:**1267-1274.

12. Korman TP, Sahachartsiri B, Charbonneau DM, Huang GL, Beauregard M, Bowie JU: **Dieselzymes: development of a stable and methanol tolerant lipase for biodiesel production by directed evolution**. *Biotechnol Biofuels* 2013, **6:**70.

13. Dror A, Shemesh E, Dayan N, Fishman A: **Protein engineering by random mutagenesis and structure-guided consensus of Geobacillus stearothermophilus Lipase T6 for enhanced stability in methanol**. *Appl Environ Microbiol* 2014, **80:**1515-1527.

14. Park HJ, Joo JC, Park K, Kim YH, Yoo YJ: **Stabilisation of *Candida antarctica* lipase B in hydrophylic organic solvent by rational design of hydrogen bond**. *Biotechnol Bioprocess Eng* 2012, **17:**722-728.

15. Martinelle M, Hult K: **Kinetics of acyl transfer reactions in organic media catalysed by Candida antarctica lipase B**. *Biochim Biophys Acta* 1995, **1251:**191-197.

16. Bousquet-Dubouch M-P, Graber M, Sousa N, Lamare S, Legoy MD: **Alcoholysis catalyzed by *Candida antarctica lipase B* in a gas/solid system obeys a Ping Pong Bi Bi mechanism with competitive inhibition by the alcohol substrate and water.** *Biochim Biophys Acta* 2011, **550:**90-99.

17. Chulalaksananukul W, Condoret JS, Delorme P, Willemot RM: **Kinetic study of esterification by immobilized lipase in n-hexane**. *FEBS Lett* 1990, **276:**181-184.

18. Samukawa T, Kaieda M, Matsumoto T, Ban K, Kondo A, Shimada Y, Noda H, Fukuda H: **Pretreatment of immobilized Candida antarctica lipase for biodiesel fuel production from plant oil**. *J Biosci Bioeng* 2000, **90:**180-183.

19. Kaieda M, Samukawa T, Kondo A, Fukuda H: **Effect of methanol and water contents on production of biodiesel fuel from plant oil catalyzed by various lipases in a solvent-free system**. *J Biosci Bioeng* 2001, **91:**12-15.

20. Tan T, Nie K, Wang F: **Production of biodiesel by immobilized Candida sp. lipase at high water content**. *Appl Biochem Biotechnol* 2006, **128:**109-116.

21. Robles-Medina A, Gonzalez-Moreno PA, Esteban-Cerdan L, Molina-Grima E: **Biocatalysis: towards ever greener biodiesel production**. *Biotechnol Adv* 2009, **27:**398-408.

22. Santambrogio C, Sasso F, Natalello A, Brocca S, Grandori R, Doglia SM, Lotti M: **Effects of methanol on a methanol-tolerant bacterial lipase**. *Appl Microbiol Biotechnol* 2013, **97:**8609-8618.

23. Natalello A, Sasso F, Secundo F: **Enzymatic transesterification monitored by an easy-to-use Fourier transform infrared spectroscopy method**. *Biotechnol J* 2013, **8:**133-138.

24. Shimada Y, Watanabe Y, Samukawa T, Sugihara A, Noda H, Fukuda H, Tominaga Y: **Conversion of vegetable oil to biodiesel using immobilized *Candida Antarctica* lipase**. *JAOCS* 1999, **76:**789-793.

25. Anderson EM, Larsson KM, Kirk O: **One biocatalyst-many applications: the use of *Candida antarctica B* lipase in organic synthesis.** *Biocatal Biotransf* 1998, **16:**181-204.

26. Sym EA: **Action of esterase in the presence of organic solvents**. *Biochem J* 1936, **30:**609-617.

27. Laane C, Boeren S, Vos K, Veeger C: **Rules for optimization of biocatalysis in organic solvents**. *Biotechnol Bioeng* 1987, **30:**81-87.

28. van Erp SH, Kamenskaya EO, Khmelnitsky YL: **The effect of water content and nature of organic solvent on enzyme activity in low-water media. A quantitative description**. *Eur J Biochem* 1991, **202:**379-384.

29. Dimoula K, Pohl M, Buchs J, Spiess AC: **Substrate and water adsorption phenomena in a gas/solid enzymatic reactor**. *Biotechnol J* 2009, **4:**712-721.

30. Branco RJ, Graber M, Denis V, Pleiss J: **Molecular mechanism of the hydration of Candida antarctica lipase B in the gas phase: Water adsorption isotherms and molecular dynamics simulations**. *Chembiochem* 2009, **10:**2913-2919.

31. Deshpande A, Nimsadkar S, Mande SC: **Effect of alcohols on protein hydration: crystallographic analysis of hen egg-white lysozyme in the presence of alcohols**. *Acta Crystallogr D Biol Crystallogr* 2005, **61:**1005-1008.

32. Mattos C, Bellamacina CR, Peisach E, Pereira A, Vitkup D, Petsko GA, Ringe D: **Multiple solvent crystal structures: probing binding sites, plasticity and hydration**. *J Mol Biol* 2006, **357:**1471-1482.

33. Smith RR, Canady WJ: **Solvation effects upon the thermodynamic substrate activity; correlation with the kinetics of enzyme catalyzed reactions. I. Effects of added reagents such as methanol upon alpha-chymotrypsin**. *Biophys Chem* 1992, **43:**173-187.

34. van Tol JB, Stevens RM, Veldhuizen WJ, Jongejan JA, Duine JA: **Do organic solvents affect the catalytic properties of lipase? Intrinsic kinetic parameters of lipases in ester hydrolysis and formation in various organic solvents**. *Biotechnol Bioeng* 1995, **47:**71-81.

35. van Tol JB, Jongejan JA, Duine JA, Kierkels HG, Gelade EF, Mosterd F, van der Tweel WJ, Kamphuis J: **Thermodynamic and kinetic parameters of lipase-catalyzed ester hydrolysis in biphasic systems with varying organic solvents**. *Biotechnol Bioeng* 1995, **48:**179-189.

36. Sandoval G, Condoret JS, Monsan P, Marty A: **Esterification by immobilized lipase in solvent-free media: kinetic and thermodynamic arguments**. *Biotechnol Bioeng* 2002, **78:**313-320.

37. Lee SB: **Enzyme reaction kinetics in organic solvents: A theoretical kinetic model and comparison with experimental observations.** *J Ferment Bioeng* 1995, **79:**479-484.

38. Kulschewski T, Sasso F, Secundo F, Lotti M, Pleiss J: **Molecular mechanism of deactivation of C. antarctica lipase B by methanol**. *J Biotechnol* 2013, **168:**462-469.

39. Bell G, Janssen AEM, Halling PJ: **Water activity fails to predict critical hydration level for enzyme activity in polar organic solvents: Interconversion of water concentrations and activities**. *Enzyme Microb Technol* 1997, **20:**471-477.

40. Castillo E, Torres-Gavilan A, Sandoval G, Marty A: **Thermodynamical methods for the optimization of lipase-catalyzed reactions**. *Methods Mol Biol* 2012, **861:**383-400.

41. Al-Zuhair S, Ling FW, Jun LS: **Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase**. *Process Biochem* 2007, **42:**951-960.

42. Lousa D, Baptista AM, Soares CM: **Analyzing the molecular basis of enzyme stability in ethanol/water mixtures using molecular dynamics simulations**. *J Chem Inf Model* 2012, **52:**465-473.

43. Liu W, Bratko D, Prausnitz JM, Blanch HW: **Effect of alcohols on aqueous lysozyme-lysozyme interactions from static light-scattering measurements**. *Biophys Chem* 2004, **107:**289-298.

44. Yamazaki K, Iwura T, Ishikawa R, Ozaki Y: **Methanol-induced tertiary and secondary structure changes of granulocyte-colony stimulating factor**. *J Biochem* 2006, **140:**49-56.

45. Graber M, Irague R, Rosenfeld E, Lamare S, Franson L, Hult K: **Solvent as a competitive inhibitor for Candida antarctica lipase B**. *Biochim Biophys Acta* 2007, **1774:**1052-1057.

46. Noureddini H, Gao X, Philkana RS: **Immobilized Pseudomonas cepacia lipase for biodiesel fuel production from soybean oil**. *Bioresour Technol* 2005, **96:**769-777.

47. Chen Y, Xiao B, Chang J, Fu Y, Lv P, Wang X: **Synthesis of biodiesel from waste cooking oil using immobilized lipase in fixed bed reactor**. *Energy Convers Manage* 2009, **50:**668-673.

48. Zhang B, Weng Y, Xu H, Mao Z: **Enzyme immobilization for biodiesel production**. *Appl Microbiol Biotechnol* 2012, **93:**61-70.

49. Franssen MC, Steunenberg P, Scott EL, Zuilhof H, Sanders JP: **Immobilised enzymes in biorenewables production**. *Chem Soc Rev* 2013, **42:**6491-6533.

50. Yan Y, Li X, Wang G, Guanlin L, Su F, Wang X, Liu T: **Biotechnological preparation of biodiesel and its high-valued derivatives: A review.** *Appl Energy* 2014, **113:**1614-1631.

51. Hwang HT, Qi F, Yuan C, Zhao X, Ramkrishna D, Liu D, Varma A: **Lipase-catalyzed process for biodiesel production: Protein engineering and lipase production**. *Biotechnol Bioeng* 2014, **111:**639-653.

52. Invernizzi G, Papaleo E, Grandori R, De GL, Lotti M: **Relevance of metal ions for lipase stability: structural rearrangements induced in the Burkholderia glumae lipase by calcium depletion**. *J Struct Biol* 2009, **168:**562-570.

53. Gog A, Roman M, Toça M, Paizs C, Irimie DF: **Biodiesel production using enzymatic transesterification – current state and perspectives**. *Renew Energ* 2012, **39:**10-16.

54. Chen JW, Wu WT: **Regeneration of immobilized Candida antarctica lipase for transesterification**. *J Biosci Bioeng* 2003, **95:**466-469.

55. Nelson L, Foglia T, Marmer W: **Lipase-catalyzed production of biodiesel**. *JAOCS* 1996, **73:**1191-1195.

56. Soumanou MM, Bornscheuer UT: **Improvement in lipase-catalyzed synthesis of fatty acids methyl esters from sunflower oil**. *Enzyme Microb Technol* 2003, **33:**97-103.

57. Dossat V, Combes D, Marty A: **Continuous enzymatic transesterification of high oleic sunflower oil in a packed bed reactor: influence of the glycerol production**. *Enzyme Microb Technol* 1999, **25:**194-200.

58. Caballero V, Bautista FM, Campelo JM, Luna D, Marinas JM, Romero AA, Hidalgo JM, Luque R, Macario A, Giordano G: **Sustainable preparation of a novel glycerol-free biofuel by using pig pancreatic lipase: Partial 1,3-regiospecific alcoholysis of sunflower oil**. *Process Biochem* 2009, **44:**334-342.

59. Freitas L, Paula AV, dos Santos JC, Zanin GM, de Castro HF: **Enzymatic synthesis of monoglycerides by esterification reaction using *Penicillium camembertii* lipase immobilized on epoxy SiO2-PVA composite.** *J Mol Catal B Enzym* 2010, **65:**87-90.

60. Stepan E, Velea S, Tanase C, Raud A, Enascuta CE, Oprescu EE: **Biodiesel and surfactants from fats.** *Rev Chim* 2012, **63:**646-650.

61. Royon D, Daz M, Ellenrieder G, Locatelli S: **Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent**. *Bioresour Technol* 2007, **98:**648-653.

62. Iso M, Chen B, Eguchi M, Kudo T, Shrestha S: **Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase**. *J Mol Catal B Enzym* 2001, **16:**53-58.

63. Du W, Li W, Sun T, Chen X, Liu D: **Perspectives for biotechnological production of biodiesel and impacts**. *Appl Microbiol Biotechnol* 2008, **79:**331-337.

64. Li LL, Du W, Liu DH, Wang L, Li ZB: **Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium.** *Mol Catal B Enzym* 2006, **43:**58-62.

65. Shimada Y, Watanabe Y, Sugihara A, Tominaga Y: **Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing**. *J Mol Catal B Enzym* 2002, **17:**133-142.

66. Xu Y, Nordblad M, Woodley JM: **A two-stage enzymatic ethanol-based biodiesel production in a packed bed reactor**. *J Biotechnol* 2012, **162:**407-414.

67. Watanabe Y, Shimada Y, Sugihara A, Noda H, Fukuda H, Tominaga Y: **Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase**. *JAOCS* 2000, **77:**355-360.

68. Hernandez-Martin E, Otero C: **Different enzyme requirements for the synthesis of biodiesel: Novozym 435 and Lipozyme TL IM**. *Bioresour Technol* 2008, **99:**277-286.

69. Talukder MMR, Beatrice KLM, Song OP, Puah S, Wu JC, Won CJ, Chow Y: **Improved method for efficient production of biodiesel from palm oil.** *Energ Fuel* 2008, **22:**141-144.

70. Hama S, Kondo A: **Enzymatic biodiesel production: an overview of potential feedstocks and process development**. *Bioresour Technol* 2013, **135:**386-395.

71. Lee JH, Kwon CH, Kang JW, Park C, Tae B, Kim SW: **Biodiesel production from various oils under supercritical fluid conditions by Candida antartica lipase B using a stepwise reaction method**. *Appl Biochem Biotechnol* 2009, **156:**24-34.

72. Ha SH, Lan MN, Lee SH, Hwang SM, Koo YM: **Lipase-catalyzed biodiesel production form soybean oil in ionic liquids.** *Enzyme Microb Technol* 2007, **41:**480-483.

73. Rao N, Lutz S, Wurgws K, Minor D: **Continuous biocatalytic processes**. *Org Process Res Dev* 2009, **13:**607-613.

74. Freitas L, dos Santos JC, Zanin GM, de Castro HF: **Packed-bed reactor running on babassu oil and glycerol to produce monoglycerides by enzymatic route using immobilized Burkholderia cepacia lipase**. *Appl Biochem Biotechnol* 2010, **161:**372-381.

75. Halim SF, Kamaruddin AH, Fernando WJ: **Continuous biosynthesis of biodiesel from waste cooking palm oil in a packed bed reactor: optimization using response surface methodology (RSM) and mass transfer studies**. *Bioresour Technol* 2009, **100:**710-716.

76. Severac E, Galy O, Turon F, Pantel CA, Condoret JS, Monsan P, Marty A: **Selection of CalB immobilization method to be used in continuous oil transesterification: analysis of the economical impact**. *Enzyme Microb Technol* 2011, **48:**61-70.

77. Severac E, Galy O, Turon F, Monsan P, Marty A: **Continuous lipase-catalyzed production of esters from crude high-oleic sunflower oil**. *Bioresour Technol* 2011, **102:**4954-4961.

78. Yashida A, Hama S, Tamadani N, Fukuda H, Kondo A: **Improved performance of a packed-bed reactor for biodiesel production through whole-cells biocatalysis employing a high-lipase-expression system**. *Biochem Eng J* 2012, **63:**76-80.

79. Hajar M, Shokrollahzadeh S, Vahabzadeh F, Monozzami A: **Solvent-free methanolysis of canola oil in a packed-bed reactor with use of Novozym 435 plus loofa.** *Enzyme Microb Technol* 2009, **188:**188-194.

80. Dizge N, Keskinler B, Tanriseven A: **Biodiesel production from canola oil by using lipase immobilized onto hydrophobic microporous styrene-divinylbenzene copolymer.** *Biochem Eng J* 2009, **44:**220-225.

81. Sang-Min Y, Yong-Cheol P, Kyungmoon P: **Effect of environmental conditions and methanol feeding strategy on lipase-mediated biodiesel production using soybean oil.** *Biotechnol Bioprocess Eng* 2010, **15:**614-619.

82. Machsun AL, Gozna M, Nasikin M, Setyahadi S, Yoo YJ: **Membrane microreactor in biocatalytic transesterification of triolein for biodiesel production.** *Biotechnol Bioprocess Eng* 2010, **15:**911-916.

83. Rodrigues RC, Pessela BC, Volpato G, Fernández-Lafuente R, Guisan JM, Ayub MAZ: **Two step ethanolysis: A simple and efficient way to improve the enzymatic**

biodiesel synthesis catalyzed by an immobilized-stabilized lipase from *Thermomyces lanuginosus*. *Process Biochem* 2010, **45:**1268-1273.

84. Wang X, Liu X, Zhao C, Ding Y, Xu P: **Biodiesel production in packed-bed reactors using lipase-nanoparticle biocomposite**. *Bioresour Technol* 2011, **102:**6352-6355.

85. Dors G, Freitas L, Mendes AA, Furigo A, de Castro HF: **Transesterification of palm oil catalyzed by *Pseudomonas fluorescens* lipase in a packed-bed reactor.** *Energ Fuel* 2012, **26:**5977-5982.

86. Chattopadhyay S, Sen R: **Development of a novel integrated continuous reactor system for biocatalytic production of biodiesel**. *Bioresour Technol* 2013, **147:**395-400.

Figure Legends

Fig. 1. Effects of methanol in the lipase-mediated alcoholysis of triglycerides might concern the reaction mixture (solubility and miscibility of substrates) and/or the enzyme (denaturation and inhibition). In the middle of the schema: performances of a methanol sensitive (CRL) and two methanol tolerant (BGL and BCL) lipases at growing methanol concentrations. CRL: *Candida rugosa* lipase; BGL: *Burkholderia glumae* lipase; BCL: *Burkholderia cepacia* lipase.

Fig. 2: Ping-pong bi-bi mechanisms of enzyme-catalyzed alcoholysis of ester S-A_1 by alcohol A_2 and substrate inhibition by alcohol A_2. S = acetic acid, A_1 = vinyl alcohol, A_2 = methanol, S-A_1, S-A_2 = esters. Reprinted from [38], with permission from Elsevier.

Fig. 3: Experimentally determined catalytic activity (circles) and predicted modeled activity (dashed line) at a water activity of $a_w = 0.09$. Reprinted from [38], with permission from Elsevier.