REVIEW

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A Review of the Current State of Biodiesel Production Using Enzymatic Transesterification

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ABSTRACT: Enzymatic biodiesel production has been investigated intensively, but is presently employed industrially only in a 20,000 tons/year pilot plant in China (Du et al. [2008] Appl Microbiol Technol 79(3):331–337). This review presents a critical analysis of the current status of research in this area and accentuates the main obstacles to the widespread use of enzymes for commercial biodiesel transesterification. Improved results for enzymatic catalysis are seen with respect to increased yield, reaction time and stability, but the performance and price of the enzymes need further advances for them to become attractive industrially for biodiesel production. Critical aspects such as mass transfer limitations, use of solvents and water activity are discussed together with process considerations and evaluation of possible reactor configurations, if industrial production with enzymes is to be carried out. Results of published studies on the productivity of enzymes are also presented and compared to the use of chemical catalysts.

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KEYWORDS: biodiesel; enzyme; lipase; transesterification; productivity

Introduction

There are several reasons for the introduction of biodiesel as an alternative to conventional fossil based diesel. These include decreasing dependency on foreign energy supply from declining fossil fuel resources; helping to reduce global warming by using renewable biofuels for the transport sector; and lowering emissions of particles, sulfur, carbon monoxide and hydrocarbons (Demirbas, 2007; Meher et al., 2006; Mittelbach et al., 1983; Sheehan et al., 1998).

Biodiesel can be produced from fat, lard, tallow, and vegetable oils. These mixtures of fatty acids (FFA) and triglycerides (TAG) need to be chemically altered to fatty acid alkyl esters (FAAE) to be useful as biodiesel fuel for currently used diesel engines (Ma and Hanna, 1999; Meher et al., 2006; Mittelbach et al., 1983; Pryde, 1983; Srivastava and Prasad, 2000).

Catalysts investigated for transesterification are either acids, bases, both liquid and heterogeneous, as well as free or immobilized (imm.) enzymes (Haas et al., 2006; Kaieda et al., 1999; Komers et al., 2001; Ma and Hanna, 1999; Meher et al., 2006; Suppes et al., 2001, 2004).

Most often used industrially today is alkaline transesterification (Kaieda et al., 1999; Meher et al., 2006; Srivastava and Prasad, 2000; Zhang et al., 2003), where raw material with a high water or free fatty acid (FFA) content needs pretreatment with an acidic catalyst in order to esterify FFA (Freedman et al., 1984; Kaieda et al., 1999; Zhang et al., 2003), illustrated in Figure 1. Pretreatment is necessary to reduce soap formation during the reaction and ease the extensive handling for separation of biodiesel and glycerol together with removal of catalyst and alkaline wastewater (Meher et al., 2006; Mittelbach, 1990). The amount of wastewater from a traditional biodiesel plant is around 0.2 ton per ton biodiesel produced (Suehara et al., 2005). Therefore the wastewater treatment and eventual need for water reuse is a severe problem both from an energy consuming and environmental point of view.

Contrary to alkaline catalysts, enzymes do not form soaps and can esterify both FFA and TAG in one step without the need of a subsequent washing step. Thus enzymes are an interesting prospect for industrial-scale production for reduction of production costs. This is especially the case when using feeds high in FFA such as rice bran oil (Lai et al., 2005), inedible *Madhuca indica* oil (Kumari et al., 2007) or second-generation raw materials like spent oils, animal fat and similar waste fractions, with high FFA and water content and large variation in raw material quality. Besides a reduction in the cost of biodiesel as spent oils are less expensive than virgin oils (Hsu et al., 2001; Kulkarni and Dalai, 2006; Srivastava and Prasad, 2000), the use of waste

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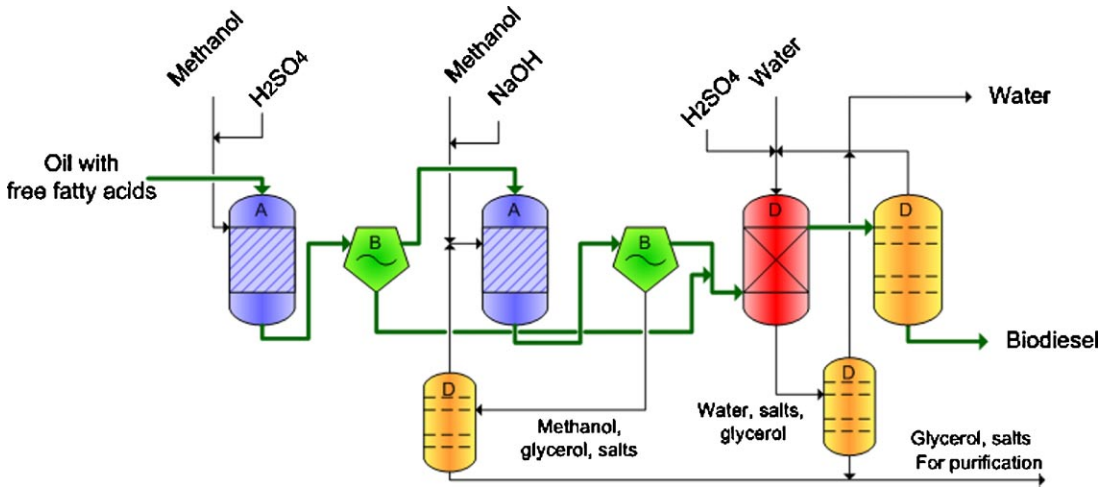

Figure 1. Traditional biodiesel process with an acidic pretreatment step followed by alkaline catalysis. (A) Reactor; (B) Separation (centrifuge or decanter); (D) Product purification and alcohol recovery.

oils etc. is also commendable as waste is turned into a resource reducing the pressure on farm land otherwise used for food production. Unfortunately, waste oils are much more complicated and expensive to transform into biodiesel with chemical catalysts (Freedman et al., 1984; Zhang et al., 2003), though Daka Biodiesel A/S (Løsning, Denmark) produces 2nd generation biodiesel from animal fat waste with a capacity of 55,000 m^3 biodiesel per year using this process.

Enzymes are potentially useful compared to alkaline or acid catalyst, because they are:

- more compatible with variations in the quality of the raw material and reusable;
- able to produce biodiesel in fewer process steps using less energy and with drastically reduced amount of wastewater;
- able to improve product separation and to yield a higher quality of glycerol (Fukuda et al., 2001; Kaieda et al., 1999; Kumari et al., 2007; Meher et al., 2006).

Drawbacks for the use of enzymes are:

- low reaction rate (Zhang et al., 2003);
- their cost (Fukuda et al., 2001; Jaeger and Eggert, 2002; Ma and Hanna, 1999; Meher et al., 2006; Shimada et al., 1999) for industrial-scale use 1,000 US$ per kg compared to 0.62 US$ (Haas et al., 2006) for sodium hydroxide;
- loss of activity, typically within 100 days of operation.

These are the key issues to be addressed for industrial use of lipases in biodiesel production to be viable.

This article presents a detailed review of the use of enzymes, free or imm., for biodiesel production. Reaction mechanisms and the reported productivity of lipases for transesterification are discussed. Lipases used together with different kinds of biomass (oils and fats) for biodiesel production, reaction conditions, and reactor configurations, together with stability/inactivation of the lipases when used for multiple cycles, are also included in the discussion.

Transesterification With Enzymes

Choice of Enzymes

Lipases for biodiesel production from TAG should be non-stereospecific, so all tri-, di and monoglycerides can be converted to FAAE. At the same time, they should also catalyze the esterification of FFA. A wide range of lipases has been used for enzymatic transesterification and esterification. Other aspects are low product inhibition with high FAAE yield, low reaction time, possible reuse of the enzyme, temperature and alcohol resistance and ease of lipase production. Table I gives an overview of the most promising results for enzymatic biodiesel production to date.

Lipases from bacteria and fungi are the most commonly used for transesterification, and optimal parameters for the use of a specific lipase depend on the origin as well as the formulation of the lipase. In general, the best enzymes are able to reach conversions above 90%, while reaction temperatures vary between 30 and 50°C. Reaction time also vary greatly from a low of 8 h for immobilized *Pseudomonas cepacia* lipases transesterifying *jatropha* oil with ethanol to a high of 90 h for the same free enzyme transesterifying soybean oil with methanol. Thus not only the origin of the lipase, but also optimal water activities, reaction temperature, if the enzyme is immobilized or not, choice of alcohol and alcohol to oil molar ratios influence the maximum biodiesel yield, reaction time and enzyme life time. In order to elucidate these points, a closer look at the reaction mechanism and kinetics is necessary.

Table I. Biodiesel production with various lipases.

Lipase	Oil/fat	Alcohol	Yield	Form	Conditions and observations	References
Pseudomonas fluorescens[a]	Soybean oil	Methanol	90%	Free	35°C, 3:1 molar alcohol added in three steps, 90 h, 150 rpm	Kaieda et al. (2001)
Pseudomonas cepacia[b]			>80%	Free		
Candida rugosa			90%	Free		
Pseudomonas fluorescens[a]	Sunflower oil	Methanol	>95% (24 h, molar ratio 4.5:1)	Imm.	40°C, 200 rpm, 3:1 molar alcohol added in three steps, 10 wt% enzyme based on oil weight, 30 h	Soumanou and Bornscheuer (2003)
Rhizomucor miehei[c]			>80%	Imm.		
Thermomyces lanuginosa[d]			>60%	Imm.		
Candida antarctica[e]	Sunflower oil	Methanol	93.2% (1-propanol)	Imm.	40°C, 3:1 molar ratio of alcohol added in four steps, 10 wt% enzyme based on oil weight, 24 h	Deng et al. (2005)
Rhizomucor miehei[c]		Ethanol	79.1% (96% EtOH)	Imm.		
Thermomyces lanuginosa[d]		1-Propanol	89.8% (methanol)	Imm.		
Thermomyces lanuginosa[f]		2-Propanol	72.8% (1-propanol)	Imm.		
Pseudomonas cepacia[g]		1-Butanol	88.4% (96% EtOH)	Imm.		
Pseudomonas fluorescens[a]		Isobutanol	45.3% (96% EtOH)	Imm.		
Pseudomonas cepacia[b]	Mahua oil	Ethanol	96% (6 h)	Imm.	40°C, 200 rpm, 4:1 molar ratio of alcohol to oil, 10 wt% enzyme based on oil weight; lipases were pH-tuned	Kumari et al. (2007)
			92% (2.5 h)	CLEA[m]		
			99% (2.5 h)	PCMC[n]		
Porcine pancreatic lipase[h]	Babassu oil	Butanol	95%	Imm.	40–50°C, 150 rpm, 10:1 molar alcohol to oil, 20 wt% of total substrate enzyme	Paula et al. (2007)
Pseudomonas cepacia[i]	Jatropha oil	Ethanol	98%	Imm.	50°C, 200 rpm, 4:1 molar ratio of alcohol to oil, 5 wt% water based on enzyme weight, 10 wt% enzyme based on oil weight, 8 h	Shah and Gupta (2007)
Candida antarctica[j]	Tallow	Methanol	74%	Imm.	30°C, 200 rpm, 3-step addition of 3:1 molar alcohol to tallow, 10 wt% enzyme based on oil weight, 72 h	Lee et al. (2002)
Candida sp. 99-125	Rapeseed oil	Methanol	83% (36 h, 5 wt% enzyme, BSTR)	Imm.	40°C, 180 rpm, alcohol molar ratio 3:1 added in three steps. Solvent: *n*-Hexane for salad oil, otherwise petroleum ether	Deng et al. (2003); Nie et al. (2006); Tan et al. (2006)
	Salad oil		95% (30 h, 20 wt% enzyme, BSTR)	Imm.		
	Waste oil		92% (22 h, three PBRs in series)	Imm.		
	Vegetable oil, unspecified		96% (30 h, 15 wt% enzyme, BSTR)	Imm.		
Pseudomonas cepacia[k] mixed with *Candida antarctica*[e]	Restaurant grease	Ethanol 95%	>96%	Imm.	35°C, 200 rpm, 4:1 molar ratio of alcohol, 5 wt% enzyme based on oil weight, respectively; addition of CA after 1 h, 24 h	Wu et al. (1999)
Rhizopus oryzae mixed with *Candida rugosa*[l]	Soybean oil	Methanol	>99%	Imm.	45°C, 200 rpm, 4.5:1 molar ratio of alcohol added in 10 steps, 30 wt% enzyme based on substrate, 10 wt% water, imm. on silica gel, 1 wt% RO and 1 wt% CR, 21 h	Lee et al. (2006)
Thermomyces lanuginosa (TL)[d] mixed with *Candida antarctica* (CA)[e]	Rapeseed and waste oil	Methanol	95%	Imm.	35°C, 130 rpm, 4:1 molar ratio alcohol to oil, *tert*-butanol to oil volume ratio 1:1, 3 wt% TL and 1 wt % CA, 12 h	Li et al. (2006)

Rhizopus oryzae– whole-cell biocatalyst	Soybean oil	Methanol	80% (Cao et al., 2007) 71% (165 h)	Free Imm.	37°C, 150 rpm, 3:1 molar ratio of alcohol added in three steps, air-dried cells	Matsumoto et al. (2001)
Rhizopus oryzae biomass particles supported by polyurethane foam	Soybean oil	Methanol	83%		35°C, 150 rpm, 3:1 molar alcohol added 6 times, 72 h	Ban et al. (2002)

Solvent free if nothing else is stated.
Imm., immobilized; PBR: packed-bed reactor; BSTR, batch-stirred reactor.
[a]Lipase AK from Amano Enzyme Inc.
[b]Lipase PS from Amano Enzyme Inc.
[c]Lipozyme RM IM from Novozymes.
[d]Lipozyme TL IM from Novozymes.
[e]Novozym 435 from Novozymes.
[f]Lipase LA201 from Novozymes.
[g]Lipase PS-C from Amano Enzyme Inc.
[h]CLEA: Cross-linked enzyme aggregates.
[i]PCMC: Protein-coated microcrystals.
[j]PPL type II from Sigma.
[k]Lipase PS-D from Amano Enzyme Inc.
[l]Chirazyme L-2 from Amano Enzyme Inc.
[m]Lipase PS-30 from Amano Enzyme Inc.
[n]Lipase OF from Meito Sangyo Co.

Enzyme Kinetics and Reaction Mechanism for the Transesterification

Lipase transesterification of triglycerides with an alcohol (alcoholysis) involves a two-step mechanism when looking at a single ester bond. The first step is hydrolysis of the ester bond and release of the alcohol moiety followed by an esterification with the second substrate (Kaieda et al., 1999; Miller et al., 1988; Posorske et al., 1988; Xu, 2000). The two steps are represented in Equations (1) and (2) (Paiva et al., 2000)

$$E + \mathrm{Es_s} \rightleftarrows E \cdot \mathrm{Es_s} \rightleftarrows F \cdot B_\mathrm{p} \rightleftarrows F + B_\mathrm{p} \tag{1}$$

followed by

$$F + A_\mathrm{s} \rightleftarrows F \cdot A_\mathrm{s} \rightleftarrows E \cdot \mathrm{Es_p} \rightleftarrows E + \mathrm{Es_p} \tag{2}$$

Subscripts s and p indicate substrate and product, respectively. For biodiesel, A_s = alcohol substrate (i.e., methanol or ethanol), B_p = product with alcohol moiety (di- or monoglyceride or glycerol), E = free enzyme, $\mathrm{Es_s}$ = ester substrate (tri-, di- or monoglyceride), $\mathrm{Es_p}$ = FAAE, F = fatty acid.

This mechanism conforms to a ping-pong bi bi mechanism as each product is released between addition of the substrates (Biselli et al., 2002) and is the widely accepted mechanism for alcoholysis of triglycerides (Dossat et al., 2002; Paiva et al., 2000), although simplifications such as Michaelis–Menten kinetics are applied when fitting to experimental results (Hari Krishna and Karanth, 2001; Paiva et al., 2000). An example of an initial rate equation for a ping-pong bi bi mechanism can be seen in Equation (3) and has been used by, among others, Dossat et al. (2002) and Xu et al. (2005)

$$v_i = \frac{V_\mathrm{max}[\mathrm{TG}][\mathrm{A}]}{K_\mathrm{m,TG}[\mathrm{A}](1 + [\mathrm{A}]/K_{i,\mathrm{A}}) + K_\mathrm{m,A}[\mathrm{TG}] + [\mathrm{TG}][\mathrm{A}]} \tag{3}$$

where v_i = initial rate; V_max, $K_\mathrm{m,TG}$, $K_{i,\mathrm{A}}$, and $K_\mathrm{m,A}$ = kinetic constants; and [TG] and [A] = concentrations of triglycerides and acyl acceptor, respectively.

Steady-state kinetics such as Michaelis–Menten can possibly describe the enzymatic conversion satisfyingly with appropriate fitting to a long range of models of varying complexity, but the accuracy of this can be questioned. Fitting of data to models cannot by itself elucidate intermediates and confirm a mechanism without further evidence, that is, spectroscopy (Voet and Voet, 2004). Furthermore, none of the fitted models used to date to the authors' knowledge include the formation and conversion of the mono- and diglycerides, the temperature's influence on enzyme deactivation or the equilibrium limitation for conversion. This could be included by using a simple expansion of Equation (3) substituting the triglyceride concentrations with mono- and diglyceride concentrations respectively creating a rake mechanism as known from solid

catalyzed kinetics (Bourdart and Djega-Mariadassou, 1984). Equilibrium limitations could be included simply using the reversibility term as suggested by Bourdart and Djega-Mariadassou (1984), while temperature deactivation could be formulated by a simple first order enzyme deactivation kinetics. This leads to an expanded form of the kinetic equation, see Equation (4)

$$v_j = \frac{V_{\max}(1 - e^{-k_d})[jG][A]}{K_{m,j}[A](1 + [A]/K_{i,A}) + K_{m,A}[jG] + [jG][A]} \times \left(1 - \frac{[B_P][Es_P]/[jG][A]}{K_{eq,j}}\right) \quad (4)$$

where j relates to specific mono-, di- or triglycerides, k_d is the deactivation constant, typically following an Arrhenius behavior on temperature and $K_{eq,j}$ is the equilibrium constant for each individual step.

For a full picture to be accomplished, much work therefore needs to be done and for that purpose, kinetics cannot be used on its own.

To add to the complexity when investigating the literature regarding kinetics of alcoholysis of triglycerides, it is important to compare similar systems regarding amounts of reactants and products, molecule size, and whether solvents and immobilization are used or not. This is important as traditional enzyme kinetics regards the reaction mixture as one homogenous phase, where all reactants and enzymes are considered as soluble in the solvent. This is not the case when using imm. enzymes or solvent-free systems, where multiple phases can be present and their nature changes during the reaction. This must be taken into account when evaluating imm. enzymes in solvent-free systems and components solubility in the different phases becomes important when trying to evaluate or determine kinetics in such a system. In addition, mass transfer limitations for imm. enzymes must be examined in regard to the molecular size of substrates and products.

The Influence of Water Activity on Transesterification in Solvent-Free Systems

Protection of the water surrounding the lipases is important for optimal conformation of the enzyme, and removal of the water can lead to both reversible, but in particular irreversible, changes in the protein structure (Miller et al., 1988; Yamane, 1987). Optimal water activity for the enzymatic transesterification reaction system including substrates, enzymes, solvent and possibly also carrier material is specific for a given lipase, as seen from the results of Kaieda et al. (2001), Linko et al. (1995), Tan et al. (2006), Zhang et al. (2002, 2005), and Lu et al. (2008). Kaieda et al. (2001) tested free lipases from *C. rugosa*, *P. cepacia*, and *P. fluorescens*. If the system was water free, no reaction took place while the rate of reaction increased with increased water content (1–20 wt% water).

Contrary to this *C. antarctica* (Novozym 435) displays the highest activity with low availability of water (Watanabe et al., 2005; Deng et al., 2005) while other lipases such as *P. cepacia* lipase (Deng et al., 2005) show higher activity with higher water activities. But in the latter case, too high a water activity can decrease the lifetime of the enzyme, so the optimal water content must be evaluated for each given lipase with regard to optimal yield as well as stability.

An important factor when deciding on the water activity therefore is if the enzyme is already stabilized in its active conformation due to immobilization or water is needed to stabilize the enzyme.

The Combined Influence of Water Activity and Solvents on Transesterification

The use of organic solvents for transesterifications can serve more than one purpose: It can be used to ensure a homogeneous reaction mixture alleviating the problems with having the reactants in two phases; it reduces the viscosity of the reaction mixture increasing the diffusion rate reducing mass transfer problems around the enzyme; for immobilized enzymes non-polar solvents might force the residue water to stay around the enzyme increasing the water activity locally and solvents might help stabilizing enzymes.

Park et al. (2008) and Kojima et al. (2004) found that conventional diesel could be used instead of *n*-hexane as a solvent for *C. rugosa* lipase. *tert*-Butanol has been shown to stabilize the activity of Novozym 435 (Chen and Wu, 2003; Li et al., 2006; Royon et al., 2007). This forms the basis for the pilot plant production being tested in China (Du et al., 2008).

Lu et al. (2008) tested a long range of organic solvents for the transesterification of glycerol triolate with methanol using *Candida* sp. 99-125 immobilized on textile fibers. They also tested the influence of the water added to the solution in the range 0–10%. Some general trends can be observed though only the conversion of TAG and yield of FAME after 24 h is stated making it difficult to ascertain when deactivation, change in equilibrium conditions or kinetics are the main cause for the observed differences in yield and conversion. The general trend is that there is no correlation between yield or conversion with the solvents' polarity P, Hildebrand parameters or dielectricity constants. On the other hand, Lu et al.'s results show for polar solvents adding water decrease both conversion and product selectivity (lower FAME yield), while for non-polar solvents the opposite is true. The only exceptions are *tert*-butanol keeping a high conversion of around 96% and DMSO keeping a constant low conversion around 40% independent of the water content. This correlates well with the usefulness of *tert*-butanol as a solvent, since it is only moderately polar and its stabilizing effect on the enzyme, if any, seems not to be overly influenced by the polarity of other solvents like water and therefore not by polar reactants like methanol or products like glycerol. To some extent, these results might be explained by mass transfer, changes in equilibrium

conditions and perhaps an increase in enzyme stability and underlines the fact that for multiphase systems a whole system thermodynamic approach combined with thorough biochemical and kinetic knowledge is necessary in order to predict reaction rate behavior.

Apart from situations where biodiesel is to be used blended with fossil diesel, in which case fossil diesel can be used as a solvent, solvent-based enzymatic production of biodiesel does not comply well with large scale industrial productions. Plant safety is often compromised as organic solvents in general are volatile and potentially hazardous. Furthermore, reactor volumes have to be larger to accommodate both solvents and reactants and, except for fossil diesel, the solvents have to be recovered adding extra investment and running costs for distillation equipment. Therefore solvent-free use of enzymes must be the goal for a future industrial production, if the application of enzymes should be environmentally, economically and energetically superior to traditional catalysts (Kaieda et al., 1999). Unfortunately although similar yields have been obtained with and without solvent (Kumari et al., 2007; Soumanou and Bornscheuer, 2003), solvent-free enzymatic biodiesel production is characterized by lower reaction rate than when using solvents (Mittelbach, 1990), and that needs to be improved for the process to be viable.

Temperature

Optimal temperature is discussed by Balcão et al. (1996). In general, the optimal temperature can be expected to increase when immobilizing an enzyme (Balcão et al., 1996; Montero et al., 1993), because binding to the carrier material gives stability to the enzyme and therefore decreases the effect of thermal deactivation compared to the free enzyme.

There can be distinctions regarding reaction temperature and enzyme preparation to give the optimum yield and thermal stability.

As the heat of reaction for transesterification in general is small (estimated to -18.5 kJ/mol FAME at 25°C based on Poling et al. (2002)), the equilibrium conversion cannot be expected to be influenced much in the temperature range available for enzymatic biodiesel production, that is, 20–70°C. Thus long time batch experiments showing that an enzyme might lead to the same yield at different temperatures or that different enzymes have the same yield at different temperatures in itself does not give any information except that the enzymes were not totally deactivated. Only batch experiments showing yield as function of time combined with long-term experiments in continuous production fermentors such as CSTRs and PBRs can give a useful picture. The first set of experiments will yield the optimal short-term reaction temperature for the individual enzyme while the second will give the long-term effects of prolonged production.

Unfortunately such data are often lacking. A few conclusions though can be drawn based on the results shown in Table II. Firstly, immobilized lipases show more temperature resistance than free lipases. Also Lipozymes TL and RM are being partly deactivated at 60°C within the first 24 h, while Novozym 435 is not similarly affected for this short reaction time neither with methanol or ethanol. Thirdly, the initial rate of reaction does increase with reaction temperature.

Pretreatment of Lipase

Pretreatment of imm. lipases as seen in Table III, in either ester, oil, or *tert*-butanol appears to be a way to improve the apparent performance of the lipases, making them more economically attractive for industrial biodiesel production. It is not clear in what way the pretreatment works, but it is likely to intrude in the imm. enzymes and possibly increase mass transfer by surface layer or intra-particle phase change as well as shielding the enzyme vicinity from inhibiting alcohols and glycerol. This area needs to be investigated further to elucidate interaction between carrier, enzymes and oil, but the apparent impact of pretreatment must be held up against multiple reuses of enzymes for industrial production. The impact of a pretreatment may show significant effect in batch reactors, but probably has no

Table II. Influence by temperature on enzymatic biodiesel production.

Temperature	Lipase oil/fat alcohol	Conditions and observations	References
50–60°C	*P. fluorescens*, oleic acid, propanol + butanol	Optimum for as well free as enzymes imm. on kaolinite	Iso et al. (2001)
70°C	*P. fluorescens*, oleic acid, propanol + butanol	Higher thermal stability for enzyme imm. on kaolinite than free enzyme	Iso et al. (2001)
Range 20–60°C	Novozym 435, soybean and rapeseed oil mixture, methanol	Increasing conversion after 6 h with increasing temperature, but comparable conversion after 24 h at all temperatures	Shimada et al. (1999)
Range 25–60°C	Novozym 435, Lipozyme TL IM, Lipozyme RM IM, Soybean oil, ethanol	60°C is optimal for all enzymes for highest conversion after 1 h, while for highest conversion after 24 h optimal temperature is 25°C for RM and TL, but 60°C for Novozym 435	Hernández-Martín and Otero (2008)

Table III. Pretreatment of lipases.

Pretreatment (Pt) and Pt length	Lipase oil/fat alcohol	Conditions and observations	References
tert-Butanol for 1 h + soybean oil for 1 h	Novozym 435, soybean oil, methanol	No solvent. 24.5 wt% yield after 30 min of reaction (2,5 wt% yield without pretreatment) Decrease in methanol inhibition	Chen and Wu (2003)
tert-Butanol for 1 h + soybean oil for 1 h	Novozym 435, soybean oil, methanol	In CSTR, a yield >70 wt% for 70 days was obtained with a feed of 20 mL/min and 3:1 molar alcohol to oil with three regenerations of lipase with *tert*-butanol	Chen and Wu (2003)
tert-Butanol for 1 h	Novozym 435, rice bran oil, methanol	Yield after 7 h reaction time increased from 48% without to 98% with Pt of the lipase	Lai et al. (2005)
Methyl oleate for 30 min + soybean oil for 12 h	Novozym 435, soybean oil, methanol	At 30°C and no solvent, yield was 98.7% after 3.5 h. Pt reduces effect from variations in water activity and methanol inhibition	Samukawa et al. (2000)
Crude soybean oil for 5–120 h	Novozym 435, soybean oil, methanol	Increased Pt length gave increased reaction rate. Pt with crude oil gave comparable yield (94%) and rate for refined and crude soybean oil as substrate	Wei et al. (2004)

considerable effect on long time use in continuous reactors as CSTRs or PBRs. In this case, regeneration, if carried out, must be done either by using a solvent, that is, *tert*-butanol or regenerating the enzymes by other means from time to time off-line.

Pretreatment and regeneration seem to be important aspects in achieving high productivities in enzymatic biodiesel production. However, the effect of the treatments and how treatments are to be used efficiently in full-scale operations still need to be determined.

The Influence of Internal and External Mass Transfer Limitations When Using a Carrier

Immobilization of an enzyme on a carrier increases the enzyme stability towards temperature, chemical as well as shear denaturation and ease handling and recovery of the enzyme. It does however pose a problem, because large molecules (TAG, FAME) have to diffuse through small pores to reach the enzymes while only sparingly soluble reactants (MeOH) have to travel through oil filled channels. If the carrier becomes too large, internal transport limitations might occur leading to a decrease in enzyme efficiency. Equally external mass transport limitations might arise, should a filmlayer form around the carrier pellet.

Internal mass transport limitations have been reported by Yong and Al-Duri (1996) for esterification of oleic acid and octanol and by Park et al. (2006) investigating the influence of pellet size and specific surface area on reaction rate using Lipase QLM immobilized on silica for FAME production. Park et al. though attribute their findings to uneven enzyme distribution in their carriers.

External mass transfer limitations due to formation of an external filmlayer have often been reported in literature and can in most cases be alleviated by increased stirring in batch reactors and CSTRs or increased flow in PBRs.

In the case of biodiesel production, an extra complicating factor might influence the filmlayer formation. Glycerol has been reported to decrease enzyme activity (imm.) (Belafibako et al., 2002; Dossat et al., 1999) as well as increase their stability (Watanabe et al., 2006), while no product inhibition due to fatty acid acyl ester has been reported. The glycerol effect is more likely due to mass transfer limitation in imm. enzymes than enzyme inhibition in the strict sense (see Table IV).

Stevenson et al. (1994) increased ester yield in butanolysis of mutton tallow using a lipase imm. on an anion exchange resin with addition of silica gel to adsorb glycerol, but the evidence for that conclusion has not been supported by extensive experiments. Later by Belafibako et al. (2002), product inhibition from methyl esters towards Novozym 435 (acrylic resin) was not observed, but yield decreased with addition of glycerol when tested for biodiesel production. It was suggested that glycerol should be removed during the reaction with a hydrophilic ultrafiltration membrane in order to diminish the influence of produced glycerol.

Further investigations with imm. enzymes have been carried out by Dossat et al. (1999). The larger the fatty acids (more hydrophobic), the larger the decrease in reaction rate with adsorbed glycerol compared to the situation when no adsorbed glycerol was present. This suggests formation of a hydrophilic layer of water and glycerol on the carrier, which decreases reaction rates due to lowered mass transfer of hydrophobic substrates (Dossat et al., 1999).

The contradicting results by Watanabe et al. (2006) compared to the other references mentioned in this section can be explained by pretreatment of the lipase in a mixture of oil, methyl oleate, and methanol. The pretreatment solution can be expected to intrude the carrier and take up place where otherwise the glycerol would be able to intrude if the biocatalyst was not pretreated. This effect might also explain why increased water content in non-polar solvent increase the TAG conversion and FAME yield

Table IV. Mass transfer limitations by glycerol.

Action	Lipase oil/fat alcohol	Conditions and observations	References
Addition of silica gel to absorption of glycerol	Lipozyme RM IM, mutton tallow, butanol	Increased yield from 68% to 98% when adding silica at 50°C, no solvent and a molar ratio of 3.1:1 alcohol to tallow	Stevenson et al. (1994)
Determination of theoretical yield of glycerol compared to glycerol adsorbed to carrier	Lipozyme RM IM, high oleic sunflower oil, butanol	The theoretical yield of glycerol could be found adsorbed to enzyme carrier after a decrease in reaction yield from 95% to 10% after 14 h in plug flow reactor at 40°C using hexane as solvent	Dossat et al. (1999)
Initial reaction rate followed by addition of glycerol and water	Lipozyme RM IM, oleic acid, ethanol	Water alone decreased initial rate with increasing concentration, but not to the same extent as when glycerol was present. Washing with butanol removed glycerol, and initial conversion of 95% was recovered	Dossat et al. (1999)
Comparison of batch experiments without and with addition of glycerol	Novozym 435, sunflower oil, methanol	For transesterification of 3 g imm. lipase, 44 g sunflower oil and 6.4 g methanol after 15 h at 50°C, a yield of 20% and 12% was achieved without and with addition of 5g glycerol, respectively	Belafibako et al. (2002)

while the opposite is seen in non-polar solvents. Adding water to a non-polar solvent will attract glycerol away from the carrier surface to the water phase leading to faster reactions while adding water to an already polar solvent might just decrease the methanol concentration in the oil filled pores.

Based on this, a hydrophobic carrier therefore is expected to give less external and perhaps internal mass transfer limitations than a hydrophilic carrier. This aspect needs further investigation.

Alternative Immobilization Methods

A relatively new area of promising immobilization methods is cross-linked enzyme aggregates (CLEAs) and protein-coated microcrystals (PCMCs). These have been tested with success for production of biodiesel with a *P. cepacia* lipase in solvent-free conditions by Kumari et al. (2007). The traditional immobilization on an inert carrier dilutes the enzyme activity giving lower volumetric and space–time productivity as well as increases enzyme cost (Sheldon et al., 2005). CLEAs of lipases have been prepared for synthesis in organic media with increased activity compared to commercially available imm. enzymes (Novozym 435). Several lipases have been stabilized with this technique successfully (Kumari et al., 2007; Wilson et al., 2006). Also immobilization of lipases as CLEAs in cellulose and PTFE membranes for esterification of oleic acid and *n*-butanol in an organic solvent has been done with promising results (Hilal et al., 2004). Further studies regarding CLEAs and PCMCs and their usage for biodiesel production without solvents are needed for better evaluation of the potential of these techniques.

Raw Materials

Oils and Fats

A comprehensive review was published in 1996 (Krawczyk, 1996) discussing the future of biodiesel. At that time, the cost of biodiesel compared with petroleum-based products was an issue, just as it is now, since oil prices reached an all time high in early 2008 (FAO, 2008). Other important issues are the quality of the biodiesel, that is, residual salts and water content, which can be affected by production methods as well as the choice of raw materials with respect to sustainability. Due to the applicability of enzymes for biodiesel production regardless of large variations in quality of the raw material, enzymes can have an industrial potential, which is worth further elaboration, because of the advantages named in the introduction compared to the traditional two-step process with chemical catalysts. An ideal process design for enzymes could be as simple as Figure 2.

Industrial production today is based on vegetable oils such as palm, rapeseed, soybean, castor and *Jatropha curcas* oil as well as with various waste products such as grease and animal fat, but currently only one plant is using enzymes (Novozym 435) for their production. Sunflower oil was first used by Mittelbach (1990) and up until now a wide range of edible and nonedible oils has been investigated for their prospective use in biodiesel production. In Appendix 1 (part A), the virgin oils reported to have been tested with lipases can be seen.

Few of these crops have the potential of going from mere research into commercial scale biodiesel production due to many obstacles as reported by Venendaal et al. (1997) for European energy crops. Even fewer has potential of being

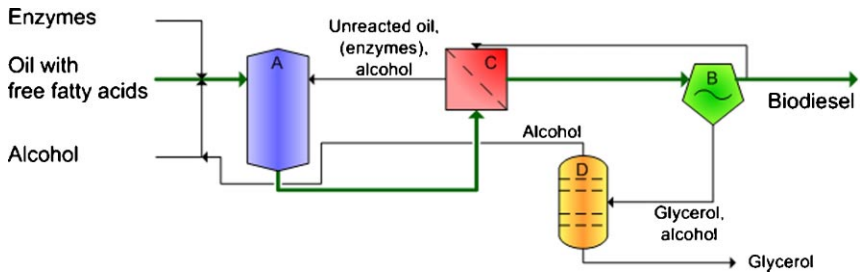

Figure 2. Ideal process design for enzymatic biodiesel production. (A) Reactor; (B) Separation (centrifuge or decanter); (C) Filter.

used in enzymatic industrial biodiesel production due to their low content of FFAs, unless drastic improvements occur with enzyme performance or price of usage.

A way to decrease the cost of biodiesel is to use waste products instead of virgin oils. These often also have a high content of FFAs, which is favorable to the use of enzymes. A variety of oil and fat waste products has been used with enzymes for biodiesel production and can be seen in Appendix 1 (part B). Enzymes, unlike alkaline catalysts, are very capable of esterifying FFA with methanol, and this favors the use of enzymes for catalysis for substrates rich in FFA. Table V shows how variations in FFA and

Table V. Oil, fats, and quality influence on yield.

Triglyceride substrate	Lipase acyl acceptor	Conditions and observations	References
Soybean oil deodorizer distillate (SODD) with 28% FFA	Novozym 435, methanol	Higher enzyme tolerance toward MeOH and higher reaction rates when using SODD compared to refined soybean oil. MeOH inhibition is linearly minimized with increasing FFA concentration. Higher molar ratios of MeOH to oil of up to 40:1 for 100% FFA (oleic acid) are needed in order to observe MeOH inhibition compared to the observed inhibition for 5% FFA at a molar ratio of 4:1	Du et al. (2007)
Acid oil with 77.9 wt% FFA	Novozym 435, methanol	Optimum at higher molar ratio 5–8:1 MeOH to oil than what is seen for low acid oil	Watanabe et al. (1999, 2005, 2006)
Oleic acid	Novozym 435, ethanol	High reaction rate compared to triglyceride substrates. In the range of 0.5:1–10:1 mole alcohol to acid, yield increase with increasing molar ratio	Trubiano et al. (2007)
Soybean oil and olive oil	Lipozyme, lauric acid	Decrease in acidolysis activity is faster when using food-grade soybean oil compared to highly purified olive oil	Posorske et al. (1988)
Refined, degummed and crude soybean oil	Novozym 435, methanol	Yield was respectively 32.4%, 30.9%, and 0.5% methyl ester after 10 cycles with repeated reuse of lipase and three step additions of 1:1 methanol to oil in each cycle. Conversion decreased with increase in added amounts of phospholipids and use of not degummed oil	Watanabe et al. (2002)
Refined, degummed/dewaxed and crude rice bran oil	Novozym 435, methanol	Increased reaction time and decreased yield with decrease in oil quality. Ester yield decreased 10% when adding 2 wt% soybean phospholipids (based on oil weight) to refined oil before methanolysis, but not when adding 2 wt% rice bran wax	Lai et al. (2005)
Refined and crude soybean oil	Novozym 435, methanol	Decreasing yields from usage of refined soybean oil to refined soybean oil added phospholipids and to crude soybean oil. Lipase pretreatment in crude oil for 120 h increased yield and rate when using crude oil as to being with refined oils	Wei et al. (2004)

phospholipid content can affect yield, rate and enzyme performance.

Enzymes show higher yield and have longer lifetime in substrates rich in FFA than in triglyceride rich substrates, but phospholipids in crude oil are found to inhibit lipases during biodiesel production (Lai et al., 2005; Watanabe et al., 2002; Wei et al., 2004). The phospholipids are the main components removed in degumming and were identified in extracts from immobilized lipases used for methanolysis of the crude oil (Watanabe et al., 2002). These reports indicate the importance of using refined oils with respect to phospholipids, when the lipases are less robust toward substrate variations than would be optimal. An option is to perform simultaneous enzymatic degumming and transesterification as a one step process, but this area needs further elaboration.

Acyl Acceptors

An extensive selection of alcohols as well as a few esters has been tested for enzymatic biodiesel production, see Table VI. The choice of alcohol has some influence on the properties of the biodiesel produced, that is, cold flow properties (Lee et al., 1995; Wang et al., 2005) and lubricity (Drown et al., 2001), though this does not draw as much attention as the price of the alcohol to be used for biodiesel production.

The alcohols used for enzymatic as for traditional chemical industrial biodiesel production must be cheap as is methanol and ethanol, for optimization of production costs and supply of raw material.

Methanol is widely used, but severe methanol inhibition, compared to other alcohols, typically lower enzyme

Table VI. Results with various acyl acceptors and lipases.

Acyl acceptor	Lipase oil/fat	Conditions and observations	References
Methanol, 99% and 96% ethanol, 1- and 2-propanol, 1- and isobutanol	Novozym 435, sunflower oil	Lipase from *Candida antarctica* showed >92% conversion with methanol, 99% EtOH and 1-butanol, while only 45.3% conversion was obtained when using 96% EtOH	Deng et al. (2005)
Methanol, 99% and 96% ethanol, 1- and 2-propanol, 1- and isobutanol	Lipozyme TL IM, Lipozyme RM IM, LA201, PS-C and AK-C, sunflower oil	Lowest yield was seen for 2-propanol, while the highest yield (>60%) for the lipases was with methanol, ethanol (96% and 99%), 1-propanol as well as 1- and isobutanol	Deng et al. (2005)
Methanol, ethanol, propanol, 1-, 2-, and isobutanol, isoamyl alcohol (a mixture of pentanol isomers)	*Pseudomonas cepacia* (imm.), Triolein	Lowest yield seen for methanol, followed by 2-butanol and ethanol, while complete conversion was obtained when using the other alcohols	Salis et al. (2005)
Methanol, ethanol, propanol, butanol, 2-propanol, 2-butanol, and isobutanol	Novozym 435, Soybean oil	Linear alcohols inhibit lipases to a higher degree than branched alcohols do when comparing initial rates	Chen and Wu (2003)
2-Ethyl-1-hexanol	*Candida rugosa*, rapeseed oil	Complete conversion after 1 h with 22wt% lipase and after 5 h with 5 wt% lipase (wt% based on oil weight)	Linko et al. (1995)
Methyl acetate	Novozym 435, soybean oil	Comparable yield observed for transesterification of refined and crude soybean oil with 12:1 molar methyl acetate and oil, while this was not the case when using methanol in a 3:1 molar ratio. Constant activity was observed for 100 cycles' use of methyl acetate	Du et al. (2004)
Ethyl acetate	Novozym 435, Crude *Jatropha curcas*, karanj, and sunflower oil	Conversion above 90% has been obtained for all oils with an ethyl acetate to oil ratio of 11:1, one-step addition of ethyl acetate and 10 wt% enzyme based on oil weight at 50°C for 12 h. The enzyme was reused for 12 cycles without loss of activity. No loss in reactivity was seen with enzyme pretreatment for 72 h in ethyl acetate before an interesterification, compared to untreated enzyme	Modi et al. (2007)
Methanol	Novozym 435, sunflower oil	Yield increases with no. of additions of in total 4:1 mol MeOH to oil. 94% yield with 8 additions and 97% yield with continuous addition during a 16 h reaction	Belafibako et al. (2002)

performance (Chen and Wu, 2003; Salis et al., 2005; Shimada et al., 1999). Inhibition of *C. antarctica* (Novozym 435) by methanol was also reported by Shimada et al. (1999) to give irreversible loss in activity. A solution to minimize alcohol inhibition is a stepwise addition of the alcohols, which was introduced and successfully performed by Shimada et al. (1999) and Watanabe et al. (1999).

When screening available lipases for their ability to be used in biodiesel production, the chosen parameters such as molar ratio alcohol:oil, alcohol addition in multiple steps or, that is, water activity determine the outcome of the screening. The effect of the choice varies from lipase to lipase as reported by Hernández-Martín and Otero (2008) and Watanabe et al. (1999). Using more than 3–4 mole alcohol per mole triglyceride added in one step favors more alcohol tolerant enzymes. Lipases of *Pseudomonas* origin display more resistance towards methanol inhibition and have a higher optimum molar ratio of methanol to oil than do lipases from *Thermomyces lanuginosa* and *Rhizomucor miehei* as reported by Soumanou and Bornscheuer (2003). Also Noureddini et al. (2005) found at 8.2:1 mole alcohol to oil that a *P. cepacia* lipase gave highest ester yield out of nine lipases tested for methanolysis of soybean oil.

Important to underline is that the observation of methanol inhibition can be masked if using very high enzyme loadings, that is, 50 wt% immobilized enzyme based on oil weight. This is illustrated by the results of Noureddini et al. (2005), Kaieda et al. (2001), and Hernández-Martín and Otero (2008).

An acyl acceptor for industrial production must be cheap and available in large quantities; therefore simple alcohols (methanol and ethanol) are the only realistic options as long as no benefits come from using a more expensive alternative. An advantage from not being able to add the alcohols in stoichiometric surplus due to inhibition is reduced energy consumption, equipment size and handling of the unreacted alcohol.

When the main aim of the use of biodiesel is a reduction in green house gas emission and reduced dependency on fossil fuel, it is also important to evaluate the source of the alcohol used. Most methanol today originates from a fossil fuel source (natural gas or coal), while ethanol is widely produced from renewable sources as well first as second generation bioethanol. Biomethanol though is an emerging concept, but yet production is still very energy consuming (Demirbas, 2007).

If renewable alcohols, preferably second generation, is to be used for biodiesel on a worldwide industrial scale, again only ethanol and potentially with time also methanol are realistic choices.

Problems and Perspectives in Industrial Application

Reactor Configurations

Reactor configurations for free and imm. enzymes in industrial applications need to include a retention of the enzymes from the product stream. For free enzymes, this can be achieved with an ultrafiltration or centrifugation unit, while more options are possible for imm. enzymes, because the immobilization can be carried out in several ways.

PBRs are very applicable for continuous production with heterogeneous catalysts, though most existing biodiesel plants are running in batch mode with stirred tank reactors and homogeneous catalysts. In Table VII, reported results with different reaction setups and reactor designs for biodiesel production with enzymes are shown.

The most commonly used reactor type for research in this area is a batch-stirred tank reactor (Balcão et al., 1996; Freedman et al., 1984) and this is still a very widely used option. With a look at the European production of 1.1 billion tonnes of fossil diesel and 31.1 million tonnes of biodiesel in 2005 (IEA, 2008) and with the latter still increasing, biodiesel must be viewed as a commodity chemical and therefore produced in continuous operated plants to reduce operational costs. Possible solutions could be CSTRs, PBRs, fluid beds, expanding bed, recirculation membrane reactors or once through reactors with static mixers. All have been tested in laboratory scale (Dossat et al., 1999; Hsu et al., 2004; Nie et al., 2006; Royon et al., 2007; Shaw et al., 2008; Shibasaki-Kitakawa et al., 2007; Shimada et al., 2002; Dubé et al., 2007; Darnoko and Cheryan, 2000). PBRs have been tested in the laboratory by Shaw et al. (2008) for biodiesel production from soybean oil using *n*-hexane:*tert*-butanol (9:1, v/v) as cosolvent for methanol. With a space time of 46 min, they reached a conversion of around 75% using Novozym 435 which compares favorably with many batch experiments. Nie et al. (2006) reached a conversion of between 30% and 32% with 1:1 molar ratio of oil:methanol, so maximum conversion was 33%. This was with a space time of between 127 and 51 min using *Candida* sp. 99-125 immobilized on textile with petroleum ether and 10% water as co-solvents. Most importantly, they could prove a nearly constant enzyme activity during 500 h of operation. Watanabe et al. (2001) reached a conversion of 90% with a space time of 2.7 h using Novozym 435 in a solvent-free system producing FAME from waste oil. The enzymes kept their activity for 100 days when three PBRs in series where used with intermediate glycerol removal and methanol addition. Based on these experiments, it must be concluded that for continuous production it is possible to achieve long time enzyme stability in PBRs whether using solvents or not. The use of solvents in itself only increases production costs as they have to be removed and purified for recycling so they should not be used. For full scale production in PBRs, the pressure drop caused by the high viscosity of solvent-free systems becomes a problem. PBRs will need to be operated at low flow velocities in order to minimize the pressure drop. Figure 3 shows that the particle diameter must be above 4 mm for the pressure drop to be <1 bar/m reactor at a rapeseed oil flow velocity of 0.01 m/s, but also that this particle diameter is in the range of several commercial imm. lipases. This problem can partly be

Table VII. Choice of reaction setup and reactor design for enzymatic biodiesel production

Reactor	Lipase oil/fat alcohol	Conditions and observations	References
Batch with free and lab scale imm. lipase	*Pseudomonas fluorescens*, triolein/safflower oil, propanol	Complete conversion after 10 h with imm. enzyme with triolein and safflower oil, while free enzyme only converted 90% within 25 h	Iso et al. (2001)
Batch with lipases lab scale immoblized in silica aerogel	*Burkholderia cepacia/Candida Antarctica*, sunflower oil, methyl acetate	Low reaction rate due to internal mass transfer limitations was observed compared to Novozym 435	Orcaire et al. (2006)
Fixed bed reactor	Novozym 435, cottonseed oil, methanol	The reactor is packed with 1.7 wt% Novozym 435 based on oil weight to reach a conversion of 95% at 50°C with 54 vol% oil, 13.5 vol% MeOH, and 32.5 vol% *tert*-butanol after 24 h. The lower the oil flow rate, the higher the yield achieved when running continuously. A yield of 95% could be maintained for 500 h with 32.5 vol% *tert*-butanol and a flow rate of 9.6 mL/h/g enzyme	Royon et al. (2007)
3 packed bed reactors in series	Novozym 435, vegetable, tuna and waste edible oil, methanol	With addition of 1 molar equivalent MeOH before each bed, a yield above 90% for 100 days was obtained. Glycerol was removed in between beds. No solvent was used	Shimada et al. (2002)
Two and three packed bed reactors in series	Novozym 435, vegetable oil, methanol	For two reactors in series with the addition of 1:1 molar alcohol to oil in the first and 2:1 alcohol in the second reactor, the enzymes were inactivated rapidly, while with three reactors in series with the addition of 1:1 molar alcohol to oil in each gave as high a conversion as 93% for 100 days of continuous operation	Watanabe et al. (2000)

alleviated either by increasing the size of the carrier or by adding a solvent that reduces the fluid viscosity. With increasing particle diameter, the pressure drop decreases as can be seen in Figure 3, but internal mass transfer rate in the biocatalyst decreases. The latter can affect the overall reaction rate, if the rate determining step is internal mass transfer.

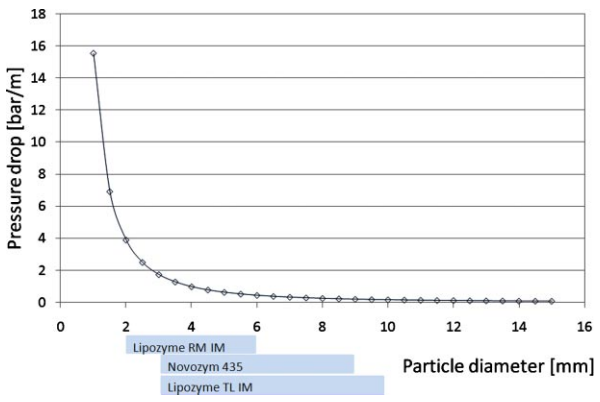

Figure 3. Packed bed reactor pressure drop and velocity for catalyst diameter of 0–16 mm and rapeseed oil at 25°C. Vo = 0.01 m/s.

To clarify this problem, investigation of internal and external heat and mass transfer limitations has been carried out for a solvent-free system in preparation for this review. The kinetic data used is taken from the work of Al-Zuhair (2005) and Soumanou and Bornscheuer (2003), who studied ping-pong bi bi kinetics of enzymatic transesterification of sunflower oil and methanol in *n*-hexane with immobilized enzymes. This study is chosen, even though with solvent, for the kinetic parameters and is combined with the appropriate, that is, diffusion constant and viscosity in solvent-free oil and methanol.

Evaluation of whether heat transfer, as well internal (Aris, 2000) and external (Mears, 1971a), influence the reaction rate based on the kinetic study done by Al-Zuhair (2005) shows that this is not the case, nor does external mass transfer based on Mears criterium (Mears, 1971b).

On the other hand, when evaluating internal mass transfer or the internal catalyst effectiveness factor (Froment and Bischoff, 1990), it can be concluded that internal mass transfer does influence the reaction rate. For an internal catalyst effectiveness factor, η, as defined in Equation (4) equal to 1, surface conditions control reaction rate, while for $\eta < 1$ pore diffusion influences the reaction rate

$$\eta = \frac{\text{rate of reaction with pore diffusion resistance}}{\text{rate of recation with surface conditions}} \quad (4)$$

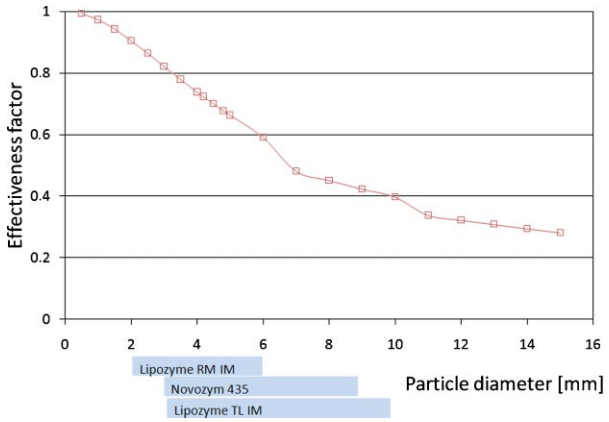

Figure 4. Effectiveness factor as a function of particle diameter. Given is particle diameter of selected commercial lipases.

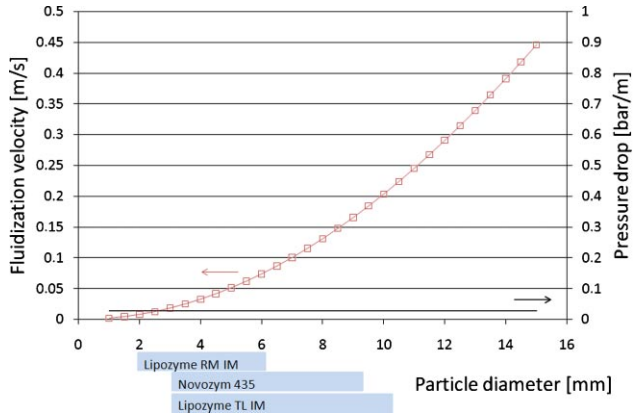

Figure 5. Fluidized bed reactor velocity and pressure drop for catalyst diameter of 0–16 mm and rape seed oil at 25°C.

Assuming pores filled with oil and methanol diffusion through the oil could be limiting, modeling of η as a function of particle diameter for a solvent-free system has been done.

As seen from Figure 4, the effectiveness factor decreases rapidly from 1 towards 0.66 in the range of particle diameters of existing commercial biocatalysts. This is equivalent to a 34% decrease in reaction rate caused by an increased particle diameter and thereby pore diffusion distance. This is in accordance with what has already been suggested from experimental data, and the effect must therefore be included as an important factor when evaluating these systems and the reactor choice.

A reduction in enzyme activity of more than 30% alone caused by internal mass transport limitation is hardly acceptable, so packed bed reactors are not an option for solvent-free enzymatic FAME production. Possible solutions to the problem could be fluidized (expanded) beds or once through reactors using static mixers. In this case, the pressure drop in the reactor is not determined by the friction between pellet and fluid, but by friction between fluid and wall and the force necessary to fluidize the pellets. The pressure drop in fluidized beds at minimum fluidization velocity as a function of pellet diameter is shown in Figure 5. As seen, the pressure drop is at an acceptable level. Experiments using a fluidized bed for biodiesel production have been carried out by Shibazaki-Kitakawa et al. (2007) using an anionic ion-exchanger as catalyst instead of enzymes. The main problem by using fluid bed reactors is that a relatively poor mixing of the fluid phase might be expected and that immobilized enzymes will be carried away by the product stream. The standard solution to the latter problem is to separate the pellets from the fluid phase using hydrocyclones, but this approach in the case of biodiesel production is yet to be tested.

The immiscibility of the lipid and alcohol phase presents a problem in fluid bed reactors, CSTRs and once through reactors and has to be addressed in order to minimize and describe mass transfer limitation together with optimization of yield in biodiesel production (Dubé et al., 2007; Noureddini et al., 1998). This problem exists for enzymatic catalyzed systems in the same way as for chemically catalyzed systems.

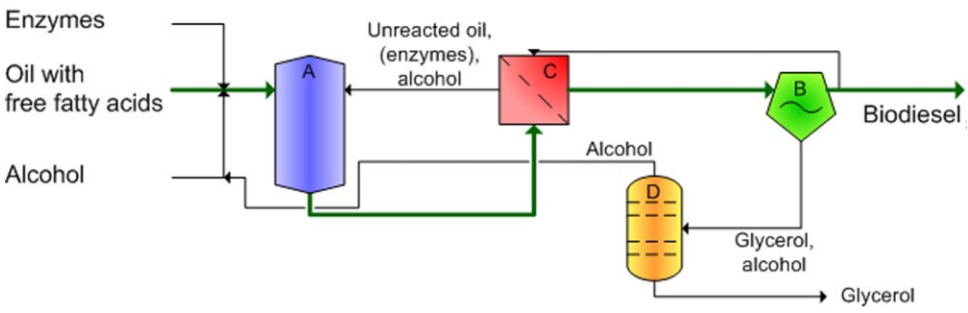

Figure 6. Enzymatic membrane process for biodiesel production. (A) Reactor; (B) Separation (centrifuge or decanter); (C) Filter or membrane; (D) Alcohol recovery.

Table VIII. Productivity for enzymatic biodiesel production

Productivity [kg ester per kg enzyme]	**Enzyme**/Substrate	Time [h]	Enzyme conc. [wt%]	Yield [%]	No. of reuses	Solvent	Source
1,200	**Lipozyme/** Tetradecanoic acid + 1-tetradecanol	20	1	>96	12	No	Miller et al. (1988)
1,200	**Novozym 435/** Soybean and rapeseed oil + methanol	48	4	>96	50	No	Shimada et al. (1999)
470	**Novozym 435 pretreated in methyl oleate and soybean oil /** Soybean oil + methanol	3.5	4	>97	20	No	Samukawa et al. (2000)
2,000	**Novozym 435**/Cottonseed oil + methanol	500	[a]	95	[a]	*tert*-Butanol	Royon et al. (2007)
5,400	**Novozym 435 pretreated with *tert*-butanol**/Soybean oil + methanol	144	[b]	>70	[b]	No	Chen and Wu (2003)
4,250	**Novozym 435 + Lipozyme TL IM**/Rapeseed oil + methanol	12	1 + 3	95	–	*tert*-Butanol	Li et al. (2006)
7,400	**Novozym 435**/Acid byproduct + methanol	24 (not complete)	1	>71	100	No	Watanabe et al. (2006)
1,700	**Novozym 435**/Acid byproduct (from above) + refined rapeseed oil + methanol	48	6	>90	100	No	Watanabe et al. (2006)

[a]Fixed-bed reactor.
[b]Continuous stirred tank reactor.

A way of improving production has been suggested by Dubé et al. (2007) and Cao et al. (2007) for chemical catalysts. For canola oil and methanol transesterification catalyzed by sulfuric acid (Dubé et al., 2007) and NaOH (Cao et al., 2007), a carbon membrane was used in both cases to remove glycerol, methanol, catalyst and biodiesel through the membrane, while canola oil was retained by the membrane. This is a very interesting way of increasing yield by continuous product removal and is a technique that can be used for both imm. and free enzymes in biodiesel production, as the membranes will retain the enzymes on the feed side reducing loss of catalyst. A suggestion for a process design is seen as Figure 6 using a membrane and enzymes, free or imm., simultaneously, where the filter/membrane is selected with respect to the enzyme formulation.

Static mixers are alternatives to stirred tank reactors with lower energy consumption. They can be utilized for both free and immobilized enzymes and give good mixing, which is important to reduce mass transfer limitations when producing biodiesel, though only use with alkaline catalyst has been reported as by Noureddini et al. (1998) in a continuous 2 L reactor system with static mixers in series with a holding tank and a yield above 97%.

As seen, a wide range of configurations are applicable for biodiesel production. Membranes are very promising, but the capital cost and disadvantages due to low mass transfer must be reduced before their use together with enzymes is economically feasible. Known technologies such as stirred tanks for solvent-free systems or PBR systems including solvents are currently superior when it comes to cost and knowledge of operation.

Process Considerations

To the authors' knowledge, only one plant exists that uses enzymatic transesterification of biodiesel. This plant uses transesterification with Novozym 435 with *tert*-butanol as solvent. Though *tert*-butanol is expected to increase enzyme life-time, the experimental data of Watanabe et al. (2001) suggests that Novozym 435 also has long life times in solvent-free systems and a change to a different reactor setup should make solvent-free production a cheaper option than a solvent based production. The key factor here becomes the cost of the enzyme and downstreaming costs.

The cost price on enzymes has to be brought down if enzymes are to compete on the commodity chemical production market. As free enzymes are cheaper than imm. due to the process of immobilization it is worthwhile to investigate whether enzymes for biodiesel production need immobilization, the price of immobilization can be lowered or other techniques than traditional imm. with an inert carrier is applicable. Furthermore, long-term experiments with reuse of enzyme for many cycles are needed in order to make correct evaluations of the industrial potential of a particular process.

The way for enzymes to make up for their higher cost compared with homogeneous, chemical catalysts is reuse (Hsu et al., 2001). The longer the reuse of the same enzyme, the higher the productivity that can be obtained for a given batch of enzyme, thereby lowering the biodiesel production price. Efficient reuse is dependent upon whether the enzymes can obtain and maintain a high initial activity without inactivation or inhibition, where, that is, pretreatment to some extent can improve reuse efficiency. Reuse is made easier with the use of imm. enzymes, which due to their size are more easily separated from product streams than free enzymes. This advantage, however, as well as a change in activity and stability, must outweigh the increased cost due to immobilization.

Productivity calculations of amount of ester produced per amount of enzyme used are important when evaluating the economic potential compared to the state-of-the-art process using alkaline or acidic catalyst. Assuming 1 kg oil yields 1 kg biodiesel, the productivity can be calculated. High productivity depends on yield, numbers of reuse (N) and enzyme concentration as seen in Equation (5) and must compensate for the high price of enzyme, when comparing alkaline to enzymatic catalysts as well as imm. to free enzymes

$$\text{Productivity (kg product/kg enzyme)} = \frac{\text{Yield (\%)} \times N\,\text{(times of reuse)} \times 100\%}{\text{Enzyme conc. (wt\%)}} \quad (5)$$

Productivities are shown in Table VIII. An alkaline catalyst, that is, NaOH used in a concentration of 1 wt% based on oil weight and complete conversion has an approximate productivity of 100 kg ester per kilogram catalyst. According to these calculations, lipases have up to 74 times higher productivity. A price of approx. 1,000 US$ per kg for Novozym 435 compared to approx. 0.62 US$ (Haas et al., 2006) for NaOH together with the productivity leads to an enzyme cost of 0.14 US$ per kg per kg ester compared to 0.006 US$ per kg ester for NaOH. A price reduction of enzyme purchase costs to 44 US$ per kg or an increased enzyme life of around 6 years would make enzymes competitive based on productivity alone. To this must be added increased reactor costs as enzymes lead to longer space times than bases, but reduced separation costs and waste water treatment costs.

Conclusions

Lipases have been used for biodiesel production with promising results. Many types of lipases have been used, giving high yields with a large variety of oil, fats, and acyl acceptors. High yields are obtained with substrates containing large amounts of FFA and water. These two compounds cause problems in the traditional alkaline transesterification. Lipases are, however, inhibited by smaller alcohols, especially methanol, so methanol addition must be done in several steps in less than equimolar amounts. High productivity, involving yield and numbers of reuse, as well as low reaction time, have been achieved, and further improvements such as pretreatment and reactor design can make industrial solvent free enzymatic biodiesel production an option for the future.

The work was supported by The Danish Council for Strategic Research.

Appendix 1

	Reference with reported use for biodiesel
A: Virgin oils used for biodiesel production with enzymes	
Babassu	Merçon et al. (2000)
Borage seed	Stevenson et al. (1994)
Corn	Stevenson et al. (1994)
Cottonseed	Köse et al. (2002)
Jatropha curcas	Shah and Gupta (2007)
Karanj (*Pongamia pinnata*)	Modi et al. (2007)
Mahua (*Madhuca indica*)	Kumari et al. (2007)
Olive	Hoq et al. (1985)
Palm	Knezevic et al. (1998)
Palm kernel	Abigor et al. (2000)
Peanut	Stevenson et al. (1994)
Rapeseed	Linko et al. (1998)
Rice bran	Lai et al. (2005)
Safflower	Iso et al. (2001)
Soybean	Kaieda et al. (1999)
Sunflower	Mittelbach (1990)
Butterfat	Garcia et al. (1992)
Hoki liver oil	Stevenson et al. (1994)
Menhaden oil	Torres et al. (2003)
Tuna oil	Shimada et al. (2002), Torres et al. (2003), Watanabe et al. (1999)

Appendix 1

	Reference with reported use for biodiesel
B: Waste products used for biodiesel production with enzymes	
Olive oil pitch, byproducts from refining olive oil	Torres et al. (2007)
Acid oil byproduct alone or with added rapeseed oil	Watanabe et al. (2006)
Waste oil	Li et al. (2006), Nie et al. (2006), Watanabe et al. (2001)
Soybean oil deodorizer distillate	Du et al. (2007)
Waste activated bleaching earth from refining virgin oils with 35–40 wt% oil	Park et al. (2008)
Waste cooking oil	Kulkarni and Dalai (2006)
Recycled restaurant grease	Wu et al. (1999)
Lard	Lee et al. (2002)
Grease and tallow	Hsu et al. (2001), Nelson et al. (1996)
Beef and mutton tallow	Stevenson et al. (1994)
Cotton oil soapstock	Keskin et al. (2008)

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