Minireview

Current state and perspectives of producing biodiesel-like compounds by biotechnology

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Summary

The global demand for crude oil is expected to continue to rise in future while simultaneously oil production is currently reaching its peak. Subsequently, rising oil prices and their negative impacts on economy, together with an increased environmental awareness of our society, directed the focus also on the biotechnological production of fuels. Although a wide variety of such fuels has been suggested, only the production of ethanol and biodiesel has reached a certain economic feasibility and volume, yet. This review focuses on the current state and perspectives of biotechnological production of biodiesel-like compounds. At present by far most of the produced biodiesel is obtained by chemical transesterification reactions, which cannot meet the demands of a totally 'green' fuel production. Therefore, also several biotechnological biodiesel production processes are currently being developed. Biotechnological production can be achieved by purified enzymes in the soluble state, which requires cost-intensive protein preparation. Alternatively, enzymes could be immobilized on an appropriate matrix, enabling a reuse of the enzyme, although the formation of by-products may provide difficulties to maintain the enzyme activity. Processes in presence of organic solvents like *t*-butanol have been developed, which enhance by-product solubility and therefore prevent loss of enzyme activity. As another approach the application of whole-cell catalysis for the production of fatty acid ethyl esters, which is also referred to as 'microdiesel', by recombinant microorganisms has recently been suggested.

Introduction

In recent years, the growing global demand for commodities based on the growing economic and personal prosperity of many developing countries' societies like in Brazil, Russia, India or China, and a stable high demand of industrialized countries has led to a steep increase in most commodity prices. Among others, the most public attention has been paid to the sharp increases in energy costs, especially of crude oil prices. As petroleum has driven the economy and mobility of our modern society for more than 100 years now, and as no suitable replacement for fossil energy sources, especially for oil, has been found so far, reports of continuously rising oil demand but decreasing numbers of newly discovered oil resources have stoked fears of oil scarcity, which could shake the modern world's economy and mobility to its core (Grant, 2005). Additionally, our society has an increased awareness that the combustion of fossil carbon was associated with an increase of the carbon dioxide concentration in the atmosphere, which is one reason for a drastic global warming causing massive environmental, economical and social problems. The combination of the aforementioned economical pressure by likely continuously rising prices, and the increase of environmental awareness are driving the development towards new bio-based, renewable and therefore environmental-friendly substitutes for petroleum, like bioplastics or biofuels.

Theoretically, many biotechnological products could have an application as fuels (Wackett, 2008), but the use of most of them is restricted by transportation or storage difficulties (e.g. for hydrogen, methane or propane), by production problems (e.g. for methanol; Wood, 2002) or by unfavourable chemical properties (e.g. the water solubility of methyl-*t*-butyl ether; Suffet 2007). Furthermore, in spite of being subject of intensive research, the biotechnological production of such promising molecules like *n*-butanol is still suffering from high costs and low yields respectively (Huang *et al.*, 2004). On a medium term, the biotechnological production of alkanes, which provide an even higher energy to mass ratio than *n*-butanol and therefore make them an ideal alternative for petroleum-based fuels, may become economically feasible. Several

start-up biofuel companies are planning to start biofuel production based on microbial alkanes in the next years (Keasling and Hu, 2007; Renninger and Newman, 2007; Friedman and Da Costa, 2008; Renninger and Ryder, 2008). However, at present only two biotechnologically produced fuels are on the market to a significant extent beside biogas as a different microbially produced energy source: ethanol and biodiesel.

Ethanol has been reported as the fermentation product of different partially genetically engineered prokaryotic microorganisms like *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca* (Ingram *et al.*, 1987; Dien *et al.*, 2003) as well as of *Saccharomyces* and various other yeast strains, which are the most commonly used microorganisms for ethanol fermentation since thousands of years. Biomass is used as feedstock for the respective fermentation processes. In North America and Europe mostly starch from corn provides the glucose for fermentation, whereas ethanol production in Brazil and other tropical countries is based on sugarcane. In 2008 nearly 20.4 billion gallons of ethanol have been globally produced of which the United States and Brazil accounted for about 78% or 15.8 billion gallons followed by the European Union, China and India. About 77% of the produced ethanol was used as fuel (Lichts, 2008).

Methyl and ethyl esters of fatty acids are commonly referred to as 'biodiesel'. Biodiesel can be obtained by (i) chemical or (ii) enzymatic transesterification of the fatty acids of vegetable oils, animal fats as well as of waste oils and fats to mostly methanol or (iii) by esterification of fatty acids to an alcohol. As in previous years the European Union remained also in 2007, the world's largest biodiesel producer with an output of more than 6.4 billion litres representing 61% of the world's biodiesel production. In the US, Indonesia and Brazil, which are the next largest biodiesel producers, additional 3.9 billion litres were produced in 2007 (ENERS Energy Concept, 2008).

This review focuses on the current developments in this vigorously studied field with a focus on the enzymatic synthesis of microbial biodiesel-like compounds and on its future perspectives.

Chemical production of biodiesel

Biodiesel is mostly if not almost exclusively obtained chemically by alkali- or acid-catalysed transesterification of plant oils and waste fat with ethanol or methanol as the most frequently used acyl acceptors or by esterification of fatty acids (Fig. 1). The used feedstock oils and alcohols as well as the resulting products are biodegradable, non-toxic, nearly free of sulfur, contain no aromatic compounds and provide a high energy to mass ratio. Additionally, biodiesel is not corrosive and can in principle substitute common diesel without major modifications of the combustion engine, whereas engines operated with the more corrosive ethanol as fuel require several modifications regarding for example fuel lines and sealings.

Due to their chemical properties and environmental friendliness a wide variety of renewable oils is used for transesterification. In general, feedstock oils should have a high oleic acid content, which increases the stability of the alkyl ester product towards temperature and other storage conditions, and their production should be as cheap as possible (Pinto *et al.*, 2005). Therefore, the source of the oil depends much on the regional situation and on its availability. In temperate zones mostly oils from
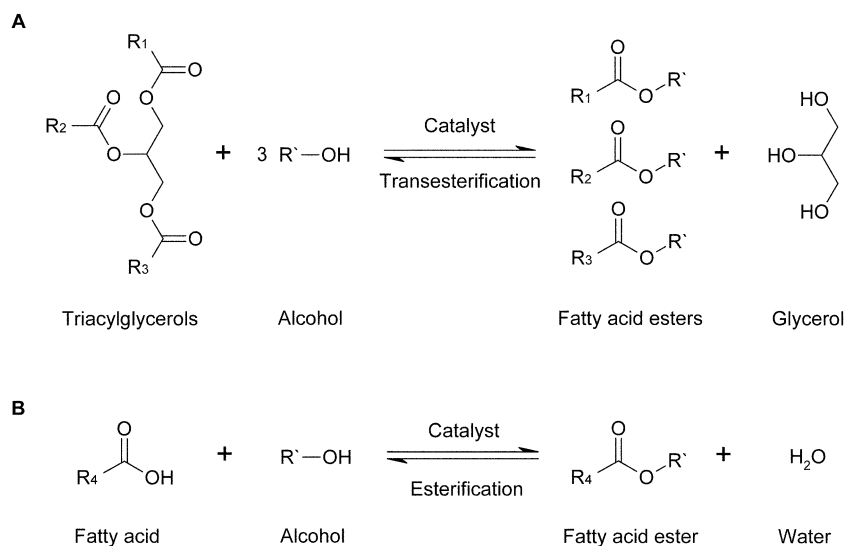

Fig. 1. Transesterification (A) and esterification (B) reactions carried out during biodiesel production from triacylglycerols or free fatty acids, respectively, in presence of a chemical or biological catalyst and a short-chain alcohol. R_1 to R_4 represent the fatty acid side-chains, whereas R′ indicates the alcohol side-chains.

rapeseed, soybean and sunflower might be suitable, while in warmer regions also palm kernel, peanut, coconut, olive or cottonseed oils might be good lipid sources for biodiesel production (Akoh *et al.*, 2007). As most of these possible feedstock oils are also valuable edibles, which have to be grown on agricultural acreage, current processes of biodiesel production compete with food production (Cassman and Liska, 2007) and additionally may boost environmental damage (e.g. for palm oil). Therefore, alternatives to edible oils might emerge from the biotechnological processing of jatropha (Berchmans and Hirata, 2008) or algal and microbial oils (Li *et al.*, 2008; Xiong *et al.*, 2008).

Although waste oils and fats are useable sources of biodiesel, too, their high contents of free fatty acid and polymerization products as well as their high viscosity may require a cost-intensive treatment to clean such oils inevitable prior to transesterification (Akoh *et al.*, 2007).

The conventional large-scale chemical synthesis of biodiesel is an alkali-catalysed transesterification (Fig. 1A) that is carried out with a reaction temperature at the alcohol's boiling point and at a 6:1 molar ratio of alcohol to oil (Al-Zuhair, 2007). Although this process is characterized by high reaction rates making this process therefore preferential for biodiesel production, it has a few limitations. The alkali-catalysed process is sensitive towards the presence of water and free fatty acids in the oil. Both can cause saponification of the ester, which comes along with the consumption of the catalyst (Basu and Norris, 1996). The resulting soaps may form emulsions creating difficulties in downstream processing (Ma and Hanna, 1999). Therefore, a pretreatment of the reaction educts to remove water and free fatty acids may be required, resulting in higher cost (Jeromin and Peukert, 1987).

In contrast to the alkali-catalysed process, the acid-catalysed process is not affected by the content of free fatty acids, making it a suitable alternative to alkali-catalysed processes for transesterification of waste oils and fats. Although different studies reported the utilization and optimization of the acid-catalysed transesterification process (Canakci and van Gerpen, 2001; Ataya *et al.*, 2007; Guan *et al.*, 2009), the relatively slow reaction rate has so far prevented its commercialization.

Enzymes used for transesterifications

In comparison with the described alkali- and acid-catalysed chemical transesterification processes, enzymatic approaches may offer many advantages, and have therefore received much attention recently. Enzymatic approaches for biodiesel production can generally be classified into whole cell- and lipase-mediated catalysis,

which again can be subdivided into alcoholysis processes mediated by soluble or by immobilized lipases.

In several studies during the last years, the use of enzymes for the transesterification process has been proposed to overcome most of the drawbacks of the conventional chemical biodiesel production processes. Enzymatic production of biodiesel can be carried out at moderate reaction conditions and at a lower alcohol to oil ratio. These reactions are not interfered by free fatty acids and water, and the products are easier recovered and need not to be purified from catalyst residues (Du *et al.*, 2008). Pretreatment of the feedstock as well as treatment of the wastewaters is not necessary, because free fatty acids contained in the feedstock can completely be converted to biodiesel. Furthermore, glycerol, the most important by-product of biodiesel production, is easily recovered as it is not miscible with fatty acid acyl esters (Janssen *et al.*, 1996).

Lipases play an important role in the metabolism of all living organisms. They can roughly be divided into intracellular and extracellular lipases and are easily obtained biotechnologically in high yields by fermentation and purification. As lipases are widely used in industry as well as in all-day applications like in detergents, they belong to the bulk enzymes and are therefore easily available and cheap. It is thus not surprising that the vast majority of the enzymes studied for transesterification reactions are lipases. Most of the used lipases are of microbial or fungal origin and have mostly been described in eukaryotic microorganisms such as *Candida antarctica*, *Candida rugosa*, *Cryptococcus* ssp. S-2, *Rhizopus oryzae*, *Rhizomucor miehei* or *Thermomyces lanuginosus*. Only a few have been purified from prokaryotic microorganisms, like *Burkholderia cepacia*, *Enterobacter aerogenes*, *Pseudomonas cepacia* and *Pseudomonas fluorescens* (Table 1).

Contrary to all these lipases, only one other enzyme has been reported so far as a catalyst for the production of biodiesel-like compounds. The wax ester synthase/diacylglycerol acyltransferase of *Acinetobacter baylyi* ADP1 has been identified and described as a promiscous acyltransferase involved in wax ester and triacylglycerol synthesis (Kalscheuer and Steinbüchel, 2003; Stöveken *et al.*, 2005).

Soluble lipases

A relatively simple approach for enzymatic production of biodiesel-like compounds employing lipases is the usage of lipases in the soluble state. On one hand, the use of soluble lipases has its advantages in the easy preparation procedure, and therefore low preparation costs. But on the other hand, the enzyme can often only be used once as it is inactivated (i) during the catalysis process due to

Table 1. Biodiesel production by utilization of different purified lipases.

Lipase source organism	Condition of used lipase	Substrate	Acyl acceptor	Solvent	Conversion rate (%)	Time (h)	Temperature (°C)	Reference
Fungi:								
Aspergillus oryzae	Soluble	Oleic acid	Methanol	None	~95	8	35	Chen *et al.* (2008)
Mucor miehei	Adsorption	Sunflower oil	Ethanol	Petroleum ether	82	5	45	Mittelbach (1990)
M. miehei	Soluble	Palm oil	Methanol	*n*-Hexane	45.4	1	40	Al-Zuhair *et al.* (2006)
Rhizopus oryzae	Soluble	Soybean oil	Methanol	None	90	70	35	Kaieda *et al.* (1999)
Thermomyces lanuginosus	Adsorption	Waste cooking oil	Methanol	None	92.8	10	45	Yagiz *et al.* (2007)
T. lanuginosus	Soluble	Soybean oil	Ethanol	None	96	7	31.5	Costa Rodrigues *et al.* (2008)
Yeasts:								
Candida antarctica	Adsorption	Palm kernel oil	Ethanol	Supercritical CO_2	63	4	40	Oliveira and Oliveira (2001)
C. antarctica	Adsorption	Soybean oil	Methyl acetate	None	92	14	40	Du *et al.* (2004)
C. antarctica	Adsorption	Soybean oil	Methanol	Ionic liquid	80	12	50	Sung *et al.* (2007)
C. antarctica	Adsorption	Cotton seed oil	Methanol	*t*-Butanol	97	24	50	Royon *et al.* (2007)
C. antarctica	Adsorption	Jatropha oil	Ethyl acetate	None	91.3	12	50	Modi *et al.* (2007)
C. antarctica	Adsorption	Soybean oil	Methyl acetate	None	92	14	40	Xu *et al.* (2003)
C. antarctica	Adsorption	Rape seed oil	Methanol	*t*-Butanol	95	12	35	Li *et al.* (2006)
C. antarctica	Adsorption	Jatropha crude oil	Methanol	2-Propanol	92.8	8	50	Modi *et al.* (2006)
C. antarctica	Adsorption	Soybean oil deodorizer distillate	Methanol	*t*-Butanol	94	25	40	Wang *et al.* (2006)
C. cylindracea	Soluble	Palm oil	Methanol	*n*-Hexane	78	24	37	Lara and Park (2004)
Candida sp. 99–125	Adsorption	Salad oil	Methanol	Hexane	96	30	40	Lu *et al.* (2006)
Candida sp. 99–125	Adsorption	Soybean oil	Methanol	Hexane	98.8	28	40	Yang *et al.* (2006)
Cryptococcus spp. S-2	Soluble	Rice bran oil	Methanol	None	80.2	120	30	Kamini and Iefuji (2001)
Bacteria:								
Burkholderia cepacia	Encapsulation	Sunflower oil	Methyl acetate	Isooctane	64	30	NA	Orcaire *et al.* (2006)
Chromobacterium viscosum	Adsorption	Jatropha oil	Ethanol	None	92	10	40	Shah *et al.* (2004)
Enterobacter aerogenes	Cross-linking	Jatropha oil	Methanol	*t*-Butanol	94	48	55	Kumari *et al.* (2009)
Pseudomonas cepacia	Cross-linking	Madhuca oil	Ethanol	None	92	2.5	40	Kumari *et al.* (2007)
P. cepacia	Adsorption	Jatropha oil	Ethanol	None	98	12	50	Shah and Gupta (2006)
P. cepacia	Adsorption	Sunflower oil	2-Butanol	None	100	6	40	Salis *et al.* (2005)
P. cepacia	Entrapment	Soybean oil	Methanol	None	56	30	35	Noureddini *et al.* (2005)
P. cepacia	Entrapment	Tallow and grease	Ethanol	None	94	20	50	Hsu *et al.* (2001)
P. cepacia	Soluble	Palm kernel oil	Ethanol	None	72	8	40	Abigor *et al.* (2000)
P. fluorescens	Adsorption	Sunflower oil	1-Propanol	None	91	20	60	Iso *et al.* (2001)
P. fluorescens	Adsorption	Sunflower oil	Methanol	Hexane	91	48	40	Soumanou and Bornscheuer (2003)
P. fluorescens	Soluble in organic solvents	Soybean	Ethanol	*iso*-Octane	71	24	70	Zhao *et al.* (2007)

the inevitable use of organic solvents forming a virtually homogenic reaction system (Knezevic *et al.*, 1998; Al-Zuhair *et al.*, 2003) or (ii) during the downstream processing following synthesis, which makes the use of soluble lipases of course cost intensive. These facts may limit the use of soluble lipases for biodiesel production in the future, and therefore studies on such a production system are relatively rare. Kaieda and colleagues (2001) studied the biodiesel production of different soluble lipases. It was found that the use of *P. cepacia* lipase yielded the highest methyl ester production rate of around 80% (w/v), although the reaction time was very long (Table 1). Abigor and colleagues (2000) reported a maximum biodiesel production when using the soluble PS30-lipase of *B. cepacia* (Amano Enzyme, Lombard, IL, USA) for transesterification of palm kernel oil with ethanol or iso-butanol, yielding a conversion rate of 72% and 62%, respectively, as the most productive reactions. In another study several factors facilitating an as high as possible esterification rate by using an *Aspergillus oryzae* soluble lipase together with oleic acid and methanol were determined. It was pointed out that among others the highest methyl ester production rates were obtained with water contents of 10% (w/v) of the oleic acid amount, at methanol to oil ratios of 3.5:1, at temperatures of 30°C and at high stirring rates yielded (Chen *et al.*, 2008, Table 1).

Zhao and colleagues (2007) suggested the use of a lipase from *P. fluorescens* modified by addition of acyl side-chains that is soluble in organic liquids. The authors demonstrated that this lipase was active towards soy bean oil in the absence of water. Thus, further purification of the biodiesel product might not be necessary. Although the application of such lipases for biodiesel production might be an alternative to two-phase processes in future, the commercial use of the latter as well as of other soluble lipases for transesterification processes will not be promising as long as the costs for the preparation of lipases cannot be reduced significantly. For example, a doubling of the enzyme yield per preparation would lead to a 50% reduction of preparation costs and is therefore a significant cost-saving potential. In general, this interesting field needs more research.

Immobilized lipases

As already mentioned above, lipases dissolved in aqueous solutions are not sufficiently stable and are inactivated during the reaction process. Hence, it is not surprising that many studies focus on overcoming this problem. By far most of the studies in the field of enzymatic biodiesel production propose the use of immobilized lipases. If the used enzyme could be immobilized on an appropriate matrix or carrier material, it could be easily removed from the reaction mixture after catalysis, which would ease downstream processing significantly. Additionally, an immobilized enzyme is supported by its carrier material, which may enhance the enzyme's stability and temperature optimum, and may therefore allow to increase the process temperature resulting in a higher conversion rate of the enzyme and a shorter reaction time respectively (Knezevic *et al.*, 1998; Al-Zuhair *et al.*, 2003). Furthermore, higher enzyme stability would limit inactivation and may therefore allow a repeated reuse of the enzyme, also resulting in a significant reduction of the enzyme preparation costs of 50% if the number of reuses could be doubled. Another positive effect of carrier materials on immobilized lipases has been reported by Du and colleagues (2005) who demonstrated that carrier materials might influence the acyl availability for the enzyme because it was found that 1,3-regiospecific lipases, which should theoretically reach a conversion rate of only 66%, converted more than 90% of their substrate. In general, different immobilization techniques can be applied that can be divided into adsorption, entrapment, encapsulation and cross-linking techniques.

The most abundantly applied immobilization method is the adsorption technique of lipases by van der Waals or other weak forces to a special carrier material, because it is an easy way with low preparation cost and it does not need toxic chemicals. In addition, enzyme activity can be easily retained and regenerated after the end of the transesterification process (Guit *et al.*, 1991). In various studies a wide variety of carrier materials has been considered for the adsorption of a lipase such as acrylic resins, which have most often been used, macro- and microporous resins, silica gels, hydrotalcite, celite or similar materials or even textile membranes (Du *et al.*, 2004; Orcaire *et al.*, 2006; Shah and Gupta, 2006; Lu *et al.*, 2006; Mukesh *et al.*, 2007; Yagiz *et al.*, 2007; Dizge *et al.*, 2008, Table 1). The overall conversion rates of various vegetable oils when using lipases immobilized by adsorption ranged from 63% to 100%, with most conversion rates being above 90%. The highest yields were obtained by Salis and colleagues (2005) using a lipase of *P. cepacia* adsorbed to diatomaceous earth converting sunflower oil with 2-butanol. It is remarkably that a conversion rate of 100% was obtained after only 6 h of reaction and with a relatively low amount of lipase. The lowest yields of 63% and 80%, respectively, were obtained by Oliveira and Oliveira (2001) and Ha and colleagues (2007) using immobilized *C. antarctica* lipase; however, it has to be mentioned that these authors used supercritical CO_2 or ionic liquids, respectively, as rather unusual solvents during reaction. Although the adsorption method has been chosen by the majority of researchers studying immobilized lipases, this immobilization technique is by

far not suitable for industrial applications as enzymes immobilized by the relatively weak adsorption forces tend to be stripped of from their supporting material by shearing forces imposed by the stirrer. This will result in a loss of activity, thereby limiting the reuse of the enzyme (Yadav and Jadhav, 2005). If release of the enzyme from its support could be minimized, adsorption immobilization might have a promising future in biotechnological biodiesel production.

Contrary to lipases that are adsorbed to the surface of a carrier material, lipases that are entrapped or encapsulated within a carrier matrix are much more stable than their absorbed counterparts as they are not directly exposed to shearing forces. Thus, this exclusion avoids leaching from the carrier material, and it may therefore help to increase the number of reuses of the enzyme. Although uses of entrapped or encapsulated lipases have been reported frequently, there are only few studies that investigated the use of lipases immobilized in this way for production of biodiesel-like compounds.

In different studies successful entrapments of *P. cepacia* and *T. lanuginosus* lipase within a phyllosilicate sol-gel matrix have been reported (Hsu *et al.*, 2001; 2004). The transesterification of grease and tallow with methanol, 95% ethanol or n-butanol to different alkyl esters catalysed by this *P. cepacia* lipase yielded conversion rates of up to 94%, whereas *T. lanuginosus* lipase converted up to 100% of the substrate. Problems with low activities occurred and were due to low diffusion into the matrix and erosion of lipase from the surface of the matrix during processing (Noureddini *et al.*, 2005). Orcaire and colleagues (2006) investigated lipases encapsulated in a silica aerogel and the authors reported production of biodiesel and repeated recycling of the immobilized lipase. Although leaching was not the problem, these studies revealed that protein contaminations within the used enzyme caused clogging of the matrix pores. The latter limited diffusion and thereby yields were low. The aforementioned problems might be solved by the use of highly purified enzymes, which could increase the yield of biodiesel production but also comes along with higher costs for enzyme preparation.

Also cross-linking of enzymes is a suitable method for their immobilization. By this method intermolecular cross-links are formed by the reaction of multifunctional chemicals like glutaraldehyde or hexamethylene diisocyanate with enzyme molecules, yielding small aggregates that provide higher stability to the enzyme. Kumari and colleagues (2007) reported on the application of cross-linked *P. cepacia* lipase for transesterification of *Madhuca indica* oil with ethanol, resulting in a yield of 92%, whereas in another study the recently described use of cross-linked *E. aerogenes* extracellular lipase for biodiesel production from *Jatropha* oil and methanol occurred at a conversion

rate of 94% (Table 1). The latter lipase could be repeatedly used without significant loss of enzyme activity (Kumari *et al.*, 2009).

As cross-linked lipases form matrix-free aggregates of particle sizes of usually less than 10 µM (Jegannathan and Abang, 2008), their separation from the product may be difficult if these lipases are used in heterogeneous reaction systems (Cao *et al.*, 2003). Therefore, their application for biodiesel production may remain limited to a small number of processes.

Another possible way to immobilize lipases may be the use of protein coated microcrystals as reported by Kumari and colleagues (2007). Such crystals showed lower mass transfer limitations in comparison with cross-linked enzymes and high conversion rates of 99% in non-aqueous reaction systems.

In few studies, authors tried to overcome problems arising from the use of only one single immobilization method by combining different immobilization techniques. In 2005, Yadav and Jadhav described the application of *C. antarctica* lipase B absorbed to hexagonal mesoporous silica, which was encapsulated by calcium alginate for the transesterification of *p*-chlorobenzyl alcohol with vinyl acetate. For this immobilized hybrid enzyme system, conversion rates of 68%, an activity depletion of only 4% and excellent reusability were reported. This system combines the advantages of proteins absorbed to a carrier matrix with those of the encapsulation technique, as it provides a stable cage for the enzyme-limiting enzyme leaching. Thus, it might arise as an alternative to single immobilization techniques, although the practicability for biodiesel production remains to be demonstrated, yet.

Effects of acyl acceptor, solvent and water content on enzyme-catalysed biodiesel production

Besides the lipases applied and the used oil substrate, whose chemical and physical properties influence the temperature of the transesterification process, the alcohol–oil ratio and the purity of substrates and enzymes, the choice of acyl acceptors, solvents and the water content of the reaction mixture are the most important parameters that affect the effectiveness of the transesterification reaction.

In general, one has to state that different lipases have different preferences towards primary and secondary alcohols. Therefore, the best combination of lipase, alcohol and oil has to be evaluated for every reaction process (Nelson *et al.*, 1996). The most commonly used alcohols for transesterification processes remain methanol and ethanol (Table 1). Methanol is cheap, easily available, highly reactive and more volatile than ethanol, but it is toxic, and its production is non-sustainable as it is synthesized from natural gas, and 'green' production tech-

niques are still not economically viable. Thus, ethanol should be the preferred alcohol for biodiesel production, as it can be produced in sustainable processes via fermentation of sugars or perhaps in the near future directly from cellulosic materials. Therefore, the possibility to produce totally 'green' biodiesel would support ethanol as the acyl acceptor for biodiesel production.

Beside of these alcohols, few other alcohols, such as 1-propanol (Iso *et al.*, 2001) and 2-butanol (Salis *et al.*, 2005), have been investigated, and conversion rates of up to 100% were obtained. In several studies also methyl or ethyl acetate were used as the acyl donor for transesterification of oils by immobilized lipases (Xu *et al.*, 2003; Du *et al.*, 2004; Orcaire *et al.*, 2006; Mukesh *et al.*, 2007; Huang and Yan, 2008). On the one hand, such esters remove the hydrophilic, and therefore not oil-soluble by-product glycerol from the reaction mixture by forming triacetylglycerol. This prevents negative effects on enzyme activity and operational stability caused by adsorption of glycerol onto the surface of the immobilized lipase (Soumanou and Bornscheuer, 2003). Thus, enzyme stability and product yield can be increased (Xu *et al.*, 2003) without the costly removal of the produced glycerol by its absorption on silica gels (Stevenson *et al.*, 1994) or by periodically rinsing the lipase by organic solvents (Dossat *et al.*, 1999). On the other hand, methyl or ethyl acetate do not lead to the formation of alcohol droplets in the oil, which occur if the solubility limit of the alcohol in the oil has been exceeded, as the alcohol molecules are slowly released from their acyl esters. The formation of these droplets is not favourable, because it has been demonstrated in different studies that the contact with insoluble linear short-chain alcohols such as methanol and ethanol easily leads to the inactivation of immobilized lipases. Chen and Wu (2003) reported that the degree of inactivation is inversely proportional to the number of carbon atoms of the alcohol.

To avoid an alcohol-caused inactivation, the stepwise addition of the alcohol to the reaction mixture has been proposed, which would constantly keep the alcohol concentration below its solubility limit (Shimada *et al.*, 1999), thus preventing the formation of alcohol droplets. Another approach might be the use of lipases that are stable in presence of short-chain alcohols such as lipases from *Pseudomonas* strains or the use of long-chain alcohols that do not affect the stability of the used lipase (Al-Zuhair, 2007). However, the products of the latter approach might have unfavourable chemical properties, making the acyl esters unsuitable for fuel purposes. The aforementioned alcohol solubility problems and enzyme blocking by glycerol seem to be the greatest drawbacks for the use of immobilized lipases in solvent-free reaction systems so far.

Thus, the use of organic solvents to dissolve alcohols and glycerol has been investigated by several authors.

However, it must be considered that additional solvents might require the costly addition of purification steps at the end of the production process. Soumanou and Bornscheuer (2003) demonstrated that the enzymes show higher activity in relative hydrophobic solvents. With *n*-hexane or petroleum ether for lipase-catalysed biodiesel production conversion rates of up to 80% were obtained, but the overall yield of biodiesel production in an solvent-free reaction system remained higher than with a solvent (Soumanou and Bornscheuer, 2003; Lara and Park, 2004). Glycerol is also insoluble in *n*-hexane, and it was found that it still remains in the reactor where it adsorbs to the immobilized lipase, and therefore causes the same problems as in solvent-free reaction systems (Dossat *et al.*, 1999). Therefore, the use of hydrophobic organic solvents cannot be recommended for use in lipase-catalysed biodiesel production as efforts for enzyme regeneration would not be reduced while additional costs for hydrophobic organic solvents would occur.

Another approach that recently was successfully adopted for commercial use is the addition of a hydrophilic organic solvent. Such a solvent is *tert*-butanol, which has been developed as a novel reaction medium for lipase-mediated transesterification (Li *et al.*, 2006; Wang *et al.*, 2006; Du *et al.*, 2007a). *t*-Butanol is relatively hydrophilic and is able to solve glycerol as well as the used alcohol, which is in most processes methanol. By using *t*-butanol the negative effects of short-chain alcohols and glycerol on enzyme activity could be totally eliminated. Li and colleagues (2006) were able to obtain biodiesel yields of 95% when using a combination of Lipozyme TL IM and Novozyme 435 in a *t*-butanol containing system. Furthermore, the lipase could be reused for more than 200 cycles without a significant loss of enzyme activity. Additionally, Royon and colleagues (2007) reported a 97% conversion rate for the methanolysis of cottonseed oil with *t*-butanol as solvent during a continuous reaction over 500 h without enzyme activity loss.

Although *t*-butanol possesses a Log*P*-value of only 0.35, which normally lowers catalytic activities of enzymes (Soumanou and Bornscheuer, 2003), enzymes can exhibit rather high activity and stability, which could be explained by the equation developed by Du and colleagues (2007b) describing a Log*P*-value, which takes into account the situation of the whole environment of the used lipase in this system. Furthermore, Türkan and Kalay (2008) suggested that *t*-butanol induces a conformational change of the enzyme.

As a result of the experiences made with *t*-butanol as solvent for transesterification, the world's first industrial-scale plant for lipase-catalysed production of biodiesel with a capacity of 20 000 tons year^{-1} has been put into operation in Hunan, China, in late 2006 emphasizing the great prospects of this technology (Du *et al.*, 2008). In

deed, *t*-butanol could solve the problems emerging from enzyme inactivation or blocking by short-chain alcohols or glycerol respectively.

As water is a basic component of all physiological activities, it is not astonishing that enzymes need a minimum concentration of water to retain their activity. For many lipases it has been clearly demonstrated that enzyme activities are low in the absence of water, and that with increasing water contents a significant increase in enzyme activity could be detected. Nevertheless, some authors reported decreasing enzyme activities with increasing water content (Table 1). It was therefore speculated that the water content leading to inhibition of enzyme activity depends on the source of the used lipase. Al-Zuhair and colleagues (2006) and Tan and colleagues (2006) reported that the *R. miehei* and *Candida* sp. 99–125 lipases maintained their activity up to 20% of water while Shimada and colleagues (1999) reported an inhibition of the Novozyme 435 activity at water contents of lower than 0.1%.

Lipases act on interfaces between an organic and an aqueous phase. Their activation by water involves unmasking and restructuring of the active site by conformational changes of the enzyme, which is dependent on the availability of an oil–water interface (Panalotov and Verger, 2000). Therefore, yields of transesterification processes depend on the size of the interfacial area, which can be increased by addition of certain amounts of water, as this facilitates the formation of oil–water droplets. However, excess water will stimulate the reverse and competing hydrolysis reaction, as lipases usually act as hydrolysing enzymes in aqueous environments, causing the decrease of transesterification yields in biodiesel production processes. Thus, the optimum water content always is a compromise between maximizing enzyme activity by maximizing the interfacial area and concomitant minimization of the hydrolysis reaction.

Whole cell-mediated biocatalysis of biodiesel production

For the current commercial production of biodiesel, predominantly diverse renewable vegetable oils are used for chemically alkali-catalysed or enzymatically catalysed transesterification reactions (Vasudevan and Briggs, 2008). Advantages of alkali-catalysed transesterification reactions are high conversion rates of triglycerides to their corresponding alkyl esters, and short reaction periods. Disadvantages of the alkaline catalysis are the energy intensiveness, as the alkali-catalysed transesterification needs a high temperature, difficulties in glycerol recovery, removal of the alkaline catalyst from the product and treatment of the highly alkaline waste water (Al-Zuhair, 2007). In contrast, enzymatically catalysed transesterification reactions, especially when using immobilized lipases, are less energy-consuming, and offer a facilitated

separation of glycerol and recycling of the biocatalyst. Nevertheless, disadvantages of the enzymatic catalysis are lower conversion rates comparing with the alkaline catalysis and high costs for isolation, purification and immobilization of the lipase enzyme (Metzger and Bornscheuer, 2006; Al-Zuhair, 2007). Thus, whole-cell biocatalysts, such as filamentous fungi, yeast and bacteria, seem to be promising alternatives to enhance the cost-effectiveness of biotechnological transesterification processes, as at least costs for the isolation and purification of the enzyme could be saved (Fukuda *et al.*, 2008).

Fungi as whole-cell biocatalysts. Filamentous fungi possess a great potential for biotechnological production of biodiesel due to their ability to synthesize intra- and extracellular lipases as well as their robustness. Furthermore, lipase-producing fungi can be immobilized on biomass support particles (BSPs) and used as whole-cell biocatalyst. The intracellular lipase-producing strain *Rhizopus chinensis* spontaneously, which was immobilized on BSPs during the initial cell cultivation, facilitated the later separation of the whole-cell biocatalyst from the reaction mixture as well as its reuse in the transesterification process (Nakashima *et al.*, 1988; 1989). Pretreatment of the mycelium first with yatalase and afterwards with various organic solvents, especially with isooctane, resulted in an enhanced enzyme activity due to partial degradation of fungal cell wall and release of membrane-bound lipase. Highest molar conversion rates of 93% were obtained, when this pretreated and lyophilized cells were employed as catalysts for esterification of ethyl hexanoate in *n*-heptane, using hexanoic acid as substrate oil and ethanol as acyl acceptor (Table 2; Wang *et al.*, 2007). Teng and Xu (2008) improved culture conditions during fermentation of *R. chinensis* in a non-aqueous system containing *n*-heptane, and best lipase activity was obtained using olive oil as substrate oil (Table 2). Usage of lyophilized cells of *R. chinensis* as biocatalyst for transesterification of soybean oil with methanol yielded 86% methyl ester (ME) (Table 2; Qin *et al.*, 2008).

R. oryzae has also been intensively studied for its usability as whole-cell biocatalyst for biodiesel production. Ban and colleagues (2001) used spontaneously immobilized mycelium of *R. oryzae* strain IFO4697 on BSPs made of polyurethane for methanolysis of soybean oil. Different cultivation conditions were tested to enhance methanolytic activity of the immobilized cells, and best results were obtained using olive oil or oleic acid and stepwise addition of methanol to avoid lipase inactivation. In presence of 15% water, the ME content in the reaction mixture reached 90%, which is at the same level as that using extracellular lipase from cells of *R. oryzae* obtained by recovery from culture broth, catalysing methanolysis in presence of 4–30% water (Table 2; Ban *et al.*, 2001).

Table 2. Biodiesel production by utilization of different whole-cell biocatalysts (modified according to Fukuda et al., 2008).

Organism	Condition of organism	Substrate	Acyl acceptor	Solvent	Conversion rate (%)	Time (°C)	Temperature (°C)	Reference
Fungi:								
R. oryzae	Immobilized on Bsps	Soybean oil	Methanol	None	80–90	72	32	Ban et al. (2001)
R. oryzae	Immobilized on Bsps	Soybean oil	Methanol	None	90	48	35	Hama et al. (2006)
R. oryzae	Immobilized on Bsps	Soybean oil	Methanol	t-Butanol	72	NA	35	Wei et al. (2007)
R. oryzae	Immobilized on Bsps	Jatropha oil	Methanol	None	89	60	30	Tamalampudi et al. (2008)
R. oryzae	Immobilized on Bsps	Rape seed oil (refined)	Methanol	t-Butanol	60	24	35	Li et al. (2008)
R. oryzae	Immobilized on Bsps	Rape seed oil (crude)	Methanol	t-Butanol	60	24	35	Li et al. (2008)
R. oryzae	Immobilized on Bsps	Rape seed oil (acidified)	Methanol	t-Butanol	70	24	35	Li et al. (2008)
R. oryzae	Dried	Rape seed oil (crude)	Methanol	None	90	72	25	Jin et al. (2008)
R. oryzae	Dried	Waste vegetable oil	Methanol	None	80	72	25	Jin et al. (2009)
R. oryzae	Dried	Waste grease	Methanol	None	55	72	25	Jin et al. (2009)
R. chinensis	Treated with yatalase and isooctane, lyophilized	Hexanoic acid	Ethanol	Heptane	93	72	30	Wang et al. (2007)
R. chinensis	Lyophilized	Soybean oil	Methanol	None	86	72	30	Qin et al. (2008)
Recombinant A. oryzae	FHL producing, immobilized on Bsps	Soybean oil	Methanol	None	94	72	30	Hama et al. (2008)
Yeasts:								
Recombinant Saccharomyces cerevisiae	Intracellular ROL, permeabilized	Soybean oil	Methanol	None	71	165	37	Matsumoto et al. (2001)
Recombinant S. cerevisiae	ROL at cell surface, lyophilized	Soybean oil	Methanol	None	79	72	37	Matsumoto et al. (2002)
Bacteria:								
Recombinant Escherichia coli	Intracellular WS/DGAT, free cells	Sodium oleate	1-Butanol	None	5	72	37	Kalscheuer et al. (2006a)
Recombinant E. coli	Intracellular WS/DGAT, free cells	Sodium oleate	Ethanol	None	64	72	37	Kalscheuer et al. (2006b)
Recombinant E. coli	Intracellular WS/DGAT, free cells	Sodium oleate	Ethanol	None	75	72	37	Y. Elbahloul and A. Steinbüchel (unpubl data)
Recombinant E. coli	LipK107 producing, permeabilized	Oil	Methanol	None	100	12	15	Gao et al. (2009)
Bacillus subtilis	Immobilized on magnetic particles	Waste-cooking oils	Methanol	None	90	72	35	Ying and Chen (2007)

Cells of *R. oryzae* were further stabilized and reused for several batches after performing cross-linking treatment with glutaraldehyde (Ban *et al.*, 2002), yielding enzyme activities competitive to the commercial lipase Novozyme 435 (Tamalampudi *et al.*, 2008). Biodiesel-fuel production was optimized for immobilized mycelium of *R. oryzae* on BSPs using a repeated batch-cycle methanolysis reaction in a 20l air-lift bioreactor with soybean oil as substrate, and high flow rates for the reaction mixture due to exfoliation of the immobilized cells from the BSPs (Oda *et al.*, 2005; Hama *et al.*, 2006). Lipases ROL31 and ROL34 from *R. oryzae* responsible for methanolysis were identified by Hama and colleagues (2006). ROL34 was bound to the cell wall, whereas ROL31 was suggested to result from cleavage of an N-terminal residue of ROL34, and was bound to the cell membrane. During cultivation of the cells using olive oil or oleic acid as substrates, the intracellular methanolysis activity strongly correlated with the amount of membrane-bound ROL31, suggesting that the latter has a significant role in methanolysis (Hama *et al.*, 2006). Furthermore, it was demonstrated, that immobilization on BSPs significantly inhibited secretion of membrane-bound lipases and thus, increased intracellular lipase content and activity (Nakashima *et al.*, 1990; Adamczak and Bednarski, 2004; Hama *et al.*, 2006). Wei and colleagues (2007) reused cells of *R. oryzae* in nonaqueous medium containing *t*-butanol as solvent in a repeated process, which resulted in a methyl ester yield of 72% (Table 2). This *t*-butanol system was further optimized by testing different oils as substrate, and it was found that usage of acidified rapeseed oil resulted in a significantly higher reaction rate and final methylester yield in comparison with that from refined and crude rapeseed oil (Table 2; Li *et al.*, 2008). Cells of *R. oryzae* without immobilization were applied for transesterification reactions using crude canola rape seed oil and water-containing waste oils and greases with high free fatty acid contents as substrate oils. A biodiesel yield of 90% was obtained and only 3% residual mono-, di- and triacylglycerols remained (Table 2; Jin *et al.*, 2008; 2009).

Also heterologous expression of lipases in fungi has been investigated (Tamalampudi *et al.*, 2007; Hama *et al.*, 2008). *Aspergillus oryzae* strain niaD300, which was derived from the wild-type strain RIB40, was used as host strain for heterologous expression of lipase B (CALB) from *C. antarctica*. The CALB gene was constructed with and without homologous and heterologous secretion signal peptide and expressed under the control of two different promoters. By the use of antibodies raised against the fused carboxy terminal FLAG tag of CALB, it was demonstrated that cell wall and membrane-bound CALB was responsible for the biocatalytic activity of the whole-cell biocatalyst. Recombinant cells of CALB containing *A. oryzae* were immobilized within BSPs and used

for hydrolysis of *p*-nitrophenol butyrate and for the optical resolution of (RS)-1-phenyl ethanol by enantioselective transesterification with vinyl acetate as acyl donor (Tamalampudi *et al.*, 2007). Hama and colleagues (2008) used recombinant cells of *A. oryzae* containing the lipase-encoding gene *fhl* from *Fusarium heterosporum* as whole-cell biocatalyst for lipase FHL-catalysed methanolysis. It was demonstrated that the recombinant FHL-producing cells of *A. oryzae* attained a higher final methyl ester content and higher lipase stability compared with *R. oryzae* (Table 2). Regiospecificity analyses towards mono- and diacylglycerols revealed that FHL-producing cells of *A. oryzae* contained a lower level of the *sn*-2 isomers than cells of *R. oryzae* (Hama *et al.*, 2008).

Yeasts as whole-cell biocatalysts. Due to their eukaryotic expression mechanisms and bacteria-like growth and handling, yeasts are attractive hosts for heterologous expression of membrane-bound lipases with an enhanced activity on cell surfaces for transesterification processes. A yeast cell surface display system for lipase from *R. oryzae* was developed, which is based on the *FLO1* gene from *Saccharomyces cerevisiae* encoding a lectin-like cell-wall anchor protein (Flo1p). Flo1p is composed of a secretion signal domain, a flocculation functional domain, a glycosylphosphatidylinositol anchor attachment signal domain and a membrane-anchoring domain. Matsumoto and colleagues (2002) fused the amino terminal region of protein ROL, designated as ProROL, to the Flo1p flocculation functional domain, yielding surface-expressed lipase. Usage of lyophilized cells of recombinant *S. cerevisiae* expressing cell surface-bound ROL as biocatalyst for transesterification of soybean oil with methanol yielded 79% methyl esters (Matsumoto *et al.*, 2002), which was a higher value comparing with 71% methyl esters obtained from recombinant *S. cerevisiae* expressing intracellular lipase ROL (Table 2; Matsumoto *et al.*, 2001). Thus, cell surface-expressed lipase offers advantages like an easy access to the substrate during alcoholysis, which renders pretreatment of the catalyst cells unnecessary, and thus decreases production costs. Furthermore, it was demonstrated that these recombinant yeast strains catalyse enantioselective transesterification reactions in various non-aqueous organic solvents, i.e. hexane, heptane, cyclohexane and octane (Matsumoto *et al.*, 2004).

Bacteria as whole-cell biocatalysts. Bacteria are often used as whole-cell biocatalysts in biotechnological production processes, because they can be cultivated to high cell densities and generally offer the possibility of genetic engineering. A well-investigated and -understood bacterium is the Gram-negative bacterium *E. coli*, which does not produce fatty acid methyl esters or fatty acid ethyl esters (FAEE) by its natural metabolism. Ethanol is only

formed anaerobically among other fermentation products during mixed acid fermentation in low levels by this bacterium. To provide significant amounts of ethanol as acyl acceptor for esterification of sodium oleate under aerobic conditions, Kalscheuer and colleagues (2006a) heterologously expressed the pyruvate decarboxylase (PDC, EC 4.1.1.1) and alcohol dehydrogenase B (ADHB, EC 1.1.1.1) from *Z. mobilis* in *E. coli*, catalysing the non-oxidative decarboxylation of pyruvate to produce acetaldehyde and carbon dioxide, and subsequently ethanol by the reduction of acetaldehyde during fermentation, accompanied by the oxidation of NADH to NAD respectively (Wills *et al.*, 1981; Neale *et al.*, 1986). Furthermore, microbial FAEE biosynthesis for microdiesel production was established by heterologous expression of the wax ester synthase/acyl coenzyme A : diacylglycerol acyltransferase (WS/DGAT) from *A. baylyi* ADP1, designated as AtfA. Enzymes of the WS/DGAT class possess an extraordinary low substrate specificity, accept a broad range of various substances as alternative acceptor molecules and allow biosynthesis of compounds structurally as different as triacylglycerols and wax esters (Kalscheuer and Steinbüchel, 2003; Stöveken *et al.*, 2005; Uthoff *et al.*, 2005).

The three respective genes *atfA*, *pdc* and *adhB* were ligated into plasmid pSK⁻ under control of two *lacZ* promoters to ensure effective transcription, yielding plasmid pMicrodiesel that was introduced into *E. coli*. It was demonstrated that the pMicrodiesel-containing strain produced large amounts of ethanol, which was used as an alternative acyl acceptor for esterification of sodium oleate (Fig. 1B), catalysed by the heterologously expressed acyltransferase AtfA, yielding considerable amounts of FAEEs. The FAEE biosynthesis using the engineered *E. coli* strain is strictly dependent on the presence of sodium oleate in the medium, yielding a conversion rate of 64% (Kalscheuer *et al.*, 2006a). Recently, Y. Elbahloul and A. Steinbüchel (unpubl. data) cultivated *E. coli* harbouring pMicrodiesel in a 20 l fed-batch bioreactor, yielding a conversion rate of 75% (Table 2).

Furthermore, Kalscheuer and colleagues (2006b) established heterologous wax ester biosynthesis in a recombinant *E. coli* strain by coexpression of a fatty alcohol-producing bifunctional acyl-coenzyme A reductase from the jojoba plant and AtfA from *A. baylyi* strain ADP1, catalysing the esterification of fatty alcohols and coenzyme A thioesters of fatty acids. In presence of 0.2% oleate in the medium, beside jojoba oil-like wax esters such as palmityl oleate, palmityl palmitoleate and oleyl oleate, also low amounts of fatty acid butyl esters were produced if additionally 1-butanol was present in the medium (Table 2). Although the intention of this study was the synthesis of wax esters, there is also a potential to increase the amount of synthesized butyl esters by applying bacterial strains

able to provide high intracellular concentrations of fatty acid coenzyme A and alcohol (Kalscheuer *et al.*, 2006b).

Gao and colleagues (2009) isolated the lipase-producing bacterium *Proteus* sp. strain K107 and used *E. coli* as host strain for cloning the respective lipase-encoding gene *lipK107*. Recombinant cells of *E. coli* strain BL21 (DE3) containing lipase-encoding gene *lipK107* were used as whole-cell biocatalyst for lipase-catalysed methanolysis. Highest conversion rates of up to 100% were obtained if the cells were permeabilized with 0.3% (w/v) cetyl-trimethylammonium bromide, and transesterification reaction was performed for 12 h at 15°C (Table 2; Gao *et al.*, 2009).

Ying and Chen (2007) used cells of the Gram-positive lipase-producing strain *Bacillus subtilis* immobilized in a hydrophobic magnetic polymicrosphere as a magnetic cell biocatalyst (MCB). Using waste-cooking oils as substrate oil and stepwise addition of methanol, transesterification catalysed by MCB yielded 90% ME after 72 h reaction in a solvent-free system (Table 2). MCB could be recovered by magnetic separation and reused for transesterification (Ying and Chen, 2007), helping to reduce preparation costs for MCBs significantly.

Conclusions and perspectives

The significant increase in biodiesel production over the last years has been possible due to the extension of biodiesel production capacities based on chemically catalysed processes, while biotechnological processes currently play only a minor role in biodiesel industry. Although a small production plant, which uses an enzyme-catalysed production process, has been build in China in 2006, many technical problems prevent a widespread application of such biotechnological transesterification processes. Therefore, many researchers are working on solving these problems sufficiently. Most of the studies aim at achieving higher conversion rates, saving costs for energy, preparation, immobilization and increasing the number of reuses of the enzymes or cells. Preparation costs can be reduced significantly by the use of bulk enzymes from well-known organisms, such as yeasts, which ideally should be reused for several times.

A significant cost-saving potential arises from the reduction of enzyme or cell preparation cost mainly by increasing the number of reuses. This can be achieved by immobilizing such an enzyme or cell, respectively, on an appropriate carrier matrix, easing the separation of the catalysts from the product, whereas interactions with the alcohol educt and/or the by-product glycerol often lead to rapid inactivation of the catalyst and thus decreasing conversion rates. Most studies suggested immobilization by absorption, but during production process erosion of the enzyme from the matrix surface may limit the number of

reuses (Yadav and Jadhav, 2005). Therefore, immobilization by encapsulation or entrapment of the enzyme might offer more stable conditions for catalysts, although this application provokes limitation of diffusion (Hsu *et al.*, 2001; Noureddini *et al.*, 2005). By the use of organic solvents many researchers achieved a significant increase in conversion rates and reusability of the catalysts, as these solvent promote the formation of a homogenous reaction mixture and thus prevent negative effects of the alcohol and by-product on the catalyst. One special hydrophilic organic solvent, *t*-butanol, showed excellent positive effects on enzyme activity, and might have a promising future in transesterification processes, as can be assumed from its use in the aforementioned production plant in China (Wang *et al.*, 2006).

Beside the costs resulting from the process itself, major costs emerge from needed substrates as (i) carbon sources for biomass production of biocatalysts and (ii) substrate oils and alcohols for the alcoholysis reaction. These costs might be reduced by the application of whole-cell catalysts. As renewable resources like cellulosic and hemicellulosic wastes are cheap, occur in large amounts and do not compete with food and feed production, research efforts currently are targeted on screening for suitable strains utilizing the respective renewable carbon sources and synthesize biodiesel-like compounds, or genetically engineering of known whole-cell biocatalysts to obtain recombinant strains possessing these new characteristics. Strobel and colleagues (2008) reported on an endophytic fungus, *Gliocladium roseum* strain NRRL 50072, producing a series of volatile hydrocarbons and hydrocarbon derivatives on a cellulose-based medium. Nevertheless, amounts of these compounds were very low and the production time of 18 days too time-consuming for commercial applications. Further efforts to identify suitable production strains have to be made.

To save costs for substrate oils and alcohols for the alcoholysis reaction, attempts to overproduce fatty acids and alcohols by metabolic engineering seem to be desirable. It was already demonstrated that metabolic engineering to produce ethanol is a promising way to supply sufficient amounts of this acyl acceptor for esterification and production of FAEE (Fig. 1B; Kalscheuer *et al.*, 2006a). Also efforts in producing fatty acids by engineering the pathway of fatty acid biosynthesis and degradation were performed. Lu and colleagues (2008) knocked out the endogenous *fadD* gene in *E. coli* encoding an acyl-CoA synthetase to block fatty acid degradation. An acetyl-CoA carboxylase and a thioesterase were heterologously expressed to increase the supply of malonyl-CoA for fatty acid biosynthesis, and to increase the abundance of shorter chain fatty acids respectively (Lu *et al.*, 2008). Thus, improvement of both fatty acid and alcohol biosynthesis by metabolic engineering of bacteria is possible,

and could be combined to obtain a self-sufficient whole-ell biocatalysts reducing costs for these substrates.

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