Research Article

Highly active biocatalyst for transesterification: Cross linked enzyme aggregates of *Thermomyces lanuginosus* and *Candida antarctica* B

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The preparation of cross-linked enzyme aggregates (CLEAs) with albumin as an additive is demonstrated successfully with lipase *Thermomyces lanuginosus* (TL) and lipase B of *Candida antarctica* (CalB). Investigations regarding the albumin admixture and the temperature optimum are employed. Compared to non-cross-linked lipase, CalB delivers the best results at a mass ratio of 1:16 lipase/albumin, which leads to a 24-fold increase of activity; TL at a mass ratio of 1:12, which leads to a eightfold increased activity. A transesterification yield of 61% m/m ethyl ester can be reached with 2.5% m/m CLEAs of lipase TL applied on rapeseed oil within 1.5 h reaction time. A catalyst mass of 10% m/m yields 94% m/m ethyl ester in the same time. Within six cycles of reuse, the catalyst remains stable. CLEAs of CalB and Novozym 435 (a commercial immobilized lipase B of *C. antarctica*) show comparable rates in ethanolysis and glycerolysis reaction.

Practical applications: CLEAs have become of increasing interest in terms of synthesis of organic compounds via bio-catalytic (green) approaches. The findings presented here are targeting at transesterification of fats and oils into corresponding alkyl esters applicable, e.g., as alternative fuel substitutes. High conversion rates coupled with hyperactivation effects as well as highly appealing reusability behavior indicate the practical benefits even for technical applications. Conversion of lipids into derivatives by CLEAs as demonstrated here, might extend utilization and conversion possibilities of biomass.

Keywords: Cross-linked enzyme aggregates / Glycerolysis / Immobilization / Reusability / Transesterification

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1 Introduction

Enzyme supported substrate conversion especially in non-aqueous media is mostly linked to immobilized catalysts. Traditionally such enzymes are used as lyophilized powders and/or fixed on a non-catalytic carrier. Both are affected with some specific drawbacks. Lyophilization might lead to structural perturbations, resulting in significant deactivation of the active center. On the other hand, the utilization of ineffective carriers which represent a large portion compared to the active catalyst, leads to dilution of activity [1]. Especially at high loadings more than 50% loss of activity are reported [2, 3]. As alternative, carrier-free immobilized enzymes like cross-linked enzyme crystals (CLECs) or cross-linked enzyme aggregates (CLEAs) can be used [4–6]. These aggregates or crystals show high catalytic enzyme activity, high stability, and reduced production costs due to not requiring additional expensive carriers.

The preparation of CLEAs and combi-CLEAs combines purification (precipitation) and immobilization via single step followed by cross-linking mostly by utilization of glutaraldehyde [7, 8]. Additionally, in order to stabilize the enzyme activity as well as provide additional amino groups for crosslinking, e.g., bovine serum albumin or polyethylenimine can be used as proteic feeder [9–11]. However, for successful industrial scale application still further research will be necessary. Properties like mechanical strength, filterability, and especially reusability/re-activation procedures have to be investigated. Furthermore, it has to be pointed out, that each enzyme type needs its specific operating condition and adopted cross-linking procedure. Therefore, no all-encompassing "one size fits all" solution will exist [1].

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Abbreviations: CalB, Candida antartica lipase B; **CLEA,** cross-inked enzyme aggregate; **CLEC,** cross-linked enzyme crystal; **FAEE,** fatty acid ethyl esters; **IU,** International Units; **TAG,** triacylglycerol; **TL,** *Thermomyces lanuginosus*

Transesterification of fats and oils into corresponding esters is a classical example of how conversions can be supported or even catalyzed by lipases. Especially since fatty acid methyl esters (FAME) have become of increasing interest as sustainable fuel substitute, the applicability of different enzymes to catalyze the transesterification has been widely investigated [12–15]. The aspired goal is to replace classical catalysts like sodium or potassium hydroxide and reduce the enclosed by-product (glycerol, fertilizer) purification steps paired with more favorable overall production costs [16]. However, such enzymes are still not as cost competitive than expected. Therefore, the need for cheaper enzymes on the one hand linked with higher efficiency (=reduced reaction times and lower catalyst amount) as well as optimized re-usability on the other hand are of highest interest. Only if these criteria are fulfilled industrial scale biofuel production via enzyme catalyzed transesterification can be accomplished economically efficient.

One approach to enhance the performance of lipases in terms of transesterification performance is, to immobilize them as CLEAs. Investigations have demonstrated that such aggregates showed improved esterification activity, higher organic solvent and temperature stability as well as conversion rates than compared to the free enzyme [17, 18]. The work presented here demonstrates the improved activity of CLEA's from *Thermomyces lanuginosus* (TL) and *Candida antartica* B on the ethanolysis of rapeseed oil and fish oil as well as on the glycerolysis of the corresponding fatty acid ethyl esters. Furthermore, reusability studies of the aggregates underline their stability.

2 Materials and methods

2.1 Chemicals

The lipases were supplied as solutions. TL lipase was purchased from Sigma–Aldrich (Germany). *C. antarctica* lipase B (CalB) was kindly provided from VTU Holding (Austria). CalB immobilized on acrylic resin (Novozym 435), glutardialdehyde (25% v/v in water, grade II), and bovine serum albumin (lyophilized powder, 96%) were obtained from Sigma–Aldrich. Manufacturer of fish oil was Reutter S.A. (Chile) and of rapeseed oil Vereinigte Fettwarenindustrie (Austria). Fish oil ethyl ester, technical grade, was kindly provided from BioEnergy International (Austria). Acetone, ethanol and *n*-hexane were of technical grade. All chemicals for the synthetic activity assay were of analytical or HPLC grade.

2.2 Protein determination

Protein concentration was determined colorimetrically using bicinchoninic acid (BCA) as reducing agent [19]. "Thermo Scientific Pierce BCA Protein Assay" – test kit was applied using bovine serum albumin as standard protein.

2.3 CLEA synthesis

The respective lipase and bovine serum albumin were dissolved in phosphate buffer (pH 7, $I = 65$ mM). Acetone (RT, 94 mL) was added dropwise to the chilled protein solution (4°C, 6 mL, 10 mg/mL) under continuous stirring. The suspension was stirred for further 15 min (500 rpm, 4°C) for completing the precipitation of proteins and glutardialdehyde (pH 10, 63 mM) was added dropwise. Cross-linking was carried out for 1 h under continuous stirring (500 rpm, 4°C). The resulting CLEAs were filtered (14–16 μm) supported by a vacuum pump and washed twice by re-suspending the CLEAs in acetone (20 mL) followed by filtration. Afterwards, CLEAs were dried for 3 h at RT.

2.4 Acetone powder

The respective lipase was diluted with phosphate buffer (pH 7, $I = 65$ mM) 1:1 v/v. Acetone (RT, 30 mL) was added dropwise to the chilled protein solution (4°C, 6 mL, 10 mg/mL) under continuous stirring. For a complete precipitation, the suspension has been kept stirring for further 15 min at 500 rpm and 4°C. The resulting enzyme aggregates were centrifuged (15 min, 4000 rpm, 4 °C) and washed by re-suspending in acetone once (~10 mL) followed by centrifugation, before drying the aggregates under a constant stream of nitrogen.

2.5 Synthetic activity assay

Transesterification in organic media catalyzed by (i) free lipase and (ii) CLEAs was carried out with 1-hexanol and tributyrin (500 mM each) in isooctane [20]. To start the reaction 1 mL of pre-warmed substrate was added to the catalyst (5–15 mg) and incubated at 40°C (500 rpm). Hexanol content was analyzed by GC analysis using lauric acid methyl ester as internal standard during the transesterification. The amount of product (hexyl butyl ester) is directly proportional to the decrease of hexanol. Activity of investigated biocatalysts is expressed in International Units (IU) which corresponds to μmol product produced per minute and gram of catalyst [μmol/(min g)]. Absolute activity is related to the total mass of inserted catalyst. Specific activity corresponds to the mass of inserted protein of enzyme.

2.6 Transesterification reactions

2.6.1 Ethanolysis

Ethanolysis was carried out with 1 g of fish and rapeseed oil, each. Reaction took place in closed 5 mL vessels (glass, polyethylene) thermostated in a water bath. A layer of oil was put on the catalyst (2.5, 5, 10% related to oil weight) and EtOH (4:1 mol/mol) was added. The reaction mixture was incubated at 40°C (100 rpm during the first 30 min to avoid

enzyme inhibition by EtOH, 500 rpm for the remaining reaction time). HPLC analysis was done for a time-dependent investigation of the reaction.

2.6.2 Glycerolysis

Glycerolysis was carried out with fish oil ethyl esters (11 g). Glycerol (1:3.4 mol/mol) and catalyst (1.8% related to ester weight) were added. Reaction took place in a suction filter under continuous stirring (500 rpm). Temperature was held at 80°C while injecting a constant stream of nitrogen to the mixture. HPLC analysis was done for a time-dependent investigation of the reaction.

2.6.3 Ethanolysis in presence of different reactor surfaces

A notable influence of the reactor material could be observed. Especially (CalB, Novozym 435, and Combi-CLEA) is sensitive to the kind of reactor's surface. It tends to adsorb on glass. Thus, when used on surfaces of polyethylene within a reaction time of 24 h up to 20% more product can be reached. CLEAs containing TL showed no significant difference in reaction rate due to the influence of plastic or glass.

2.7 Determination of yields by HPLC

Liquid chromatography according to ISO 16931 [21] was performed on an HP 1100 Series HPLC system including a solvent degassing unit, a quaternary pump, an autosampler and a variable wavelength detector. The separations were carried out using two combined columns (Phenogel 100, 500 Å, 300 mm × 7.8 mm) at 40°C and a tetrahydrofuran mobile phase with a flow rate of 1 mL/min. Compounds were detected by a 2301 Knaur refractive index detector. System control and data evaluation were done on the HP Chemstation for LC software package.

2.8 Determination of hexanol by GC

1-Hexanol was determined by GC (Agilent 7890A with a flame ionization detector and a split of 30:1) for the synthetic activity assay. A capillary column (Agilent 19091N-133; 30 m × 250 μm × 0.25 μm) was used for the separation and helium (0.7 mL/min) was used as carrier gas. Injection volume was 1 μL. A temperature gradient from 100 to 220°C (5°C/min) was applied and 220°C were kept for 5 min. Total runtime was 29 min.

2.9 Reusability study

(i) Reusability of CLEAs of TL (6.6 m% based on oil mass) was studied by carrying out the transesterification of rapeseed oil (3 g) with ethanol in a molar ratio of 1:4 (oil/ethanol) at 40°C. Samples were drawn after 1, 2, and 3 h and analyzed by HPLC/SEC. CLEAs were recovered by filtration using a glass frit (14–16 μm). Regeneration was succeeded by washing twice with *n*-hexane, once with acetone, four times with buffer and two times with acetone (amount of solvent of each step: 33 mL/g CLEA). For further cycles adequate amounts of oil and alcohol, related to the regained mass of CLEAs, were added.

(ii) Reusability of CLEAs of CalB (3.9 m% based on ethyl ester mass) was studied by carrying out the transesterification of glycerol with fish oil ethyl ester (11 g) in a molar ratio of 1:3.4 (glycerol/ethyl ester) at 80°C under a constant stream of nitrogen injecting to the reaction mixture. Samples were drawn after 1, 2, 3, and 4 h and analyzed by HPLC. CLEAs were recovered by filtration using a glass frit (14–16 μm). Regeneration was succeeded by washing three times with *n*-hexane, once with acetone, three times with buffer and twice with acetone (amount of solvent of each step: 33 mL/g CLEA). For each further cycle adequate amounts of educt, related to the regained mass of CLEAs, were added.

3 Results and discussion

3.1 BSA amount

We applied the procedure reported by Shah et al. [9] on the two classical lipases TL and CalB examining the influence of the albumin fraction on enzymatic activity. Figure 1a and b demonstrates the activity obtained relative to the activity inserted as a function of the albumin amount. Experimentally determined activities with varying albumin proportions are presented in Table 1.

In case of lipase TL best performance of relative enzyme activity (IU per g inserted enzyme) was reached with a mass ratio enzyme to BSA 1:12, detecting eightfold higher specific activity comparing neat, not cross-linked enzyme. A mass ratio of enzyme to BSA of 1:1 shows the highest absolute activity of 605 IU/g. With increasing albumin content, absolute activity decreases slightly. At a BSA content of 1:6, a lower absolute activity (512 IU/g) was observed in comparison to the reference. Considering the additional quantity of obtained catalyst, a hyperactivation of the enzymes is clear. Relative activities (Table 1a) increase with BSA content up to a mass ratio of 1:12, as indicated in Fig. 1a.

Hyperactivation of CalB was even more successful (Fig. 1b). The maximum of relative enzyme activity was reached with a mass ratio of 1:16 of enzyme to BSA getting 24-fold higher relative activity relating to the reference. Also absolute activity was increased by adding albumin for CalB. Highest absolute activity was detected at a ratio 1:12, detecting 3.8-fold higher activity compared to the reference.

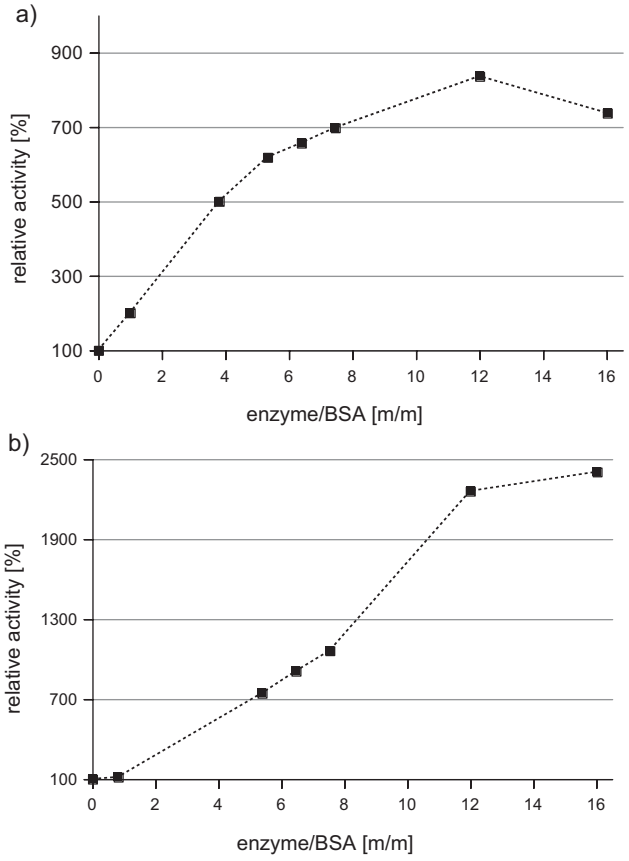

Figure 1. Effect of adding varying amounts of BSA on CLEA activity using (a) *Thermomyces lanuginosus*. lipase and (b) lipase B of *Candida antarctica*. Relative activity [%] = (units obtained in CLEAs/initial units taken) × 100; related to free precipitated enzyme powder.

As demonstrated by Shah et al. [9], bovine serum albumin can be used to avoid enzyme deactivation by cross-linking, achieving hyperactivation (12-fold higher activity compared to the neat enzyme) for lipase *Pseudomonas cepacia*. The results on experiments carried out here on TL and CalB underline these findings. Although CLEAs of TL with BSA 1:12 contained only small amount of enzyme, magnitude of absolute activity (IU per g inserted catalyst) remained comparable to that of enzyme powder. A better hyperactivation could be reached on CalB. This indicates that the structure (in fact the activity) of CalB CLEA showed a greater stability by the provision of additional amino groups coming from BSA. Furthermore, the expected deactivation effects of glutaraldehyde on the lipase were successfully suppressed. In any case, varying BSA amounts lead to significant improvements with respect to the preservation of the two CLEA's investigated here. This means that, within an specific (optimum) BSA range most active CLEAs are obtained. If the BSA amount is too high, cross-linking is already hindered by the high number of free amino groups introduced by BSA which concur with those from the lipase. Generally, CLEAs prepared from TL and especially CalB follow the observations already published by Shah et al. [9] on *P. cepacia*.

3.2 Temperature optimum

To determine the temperature optimum of CLEAs from TL and CalB activity measurements were conducted at various temperatures. The aggregates with the highest specific activities detected at 40°C (CalB 1:16 and TL 1:12) were selected for the synthetic activity assay. For CLEAs of TL the maximum activity was observed between 50 and 55°C (measurements between 40 and 60°C). In this range the aggregates showed three times higher activity comparing to the results at 40°C. CLEAs of CalB and Novozym 435 showed best activity between 75 and 80°C (measurements between 40 and 90°C) also being three times more active than at 40°C (see Fig. 2).

3.3 Transesterification reactions

The productivity of the prepared catalysts was tested by transesterification of fish oil and rapeseed oil. The production of biodiesel in the form of fatty acid ethyl esters and transesterification of fish oil ethyl esters with glycerol to TAGs (e.g., for dietary supplement) were performed. Application of CLEAs of *P. cepacia* for transesterification has been reported before, yielding 90–92% ethyl ester [9, 22].

3.3.1 Ethanolysis of rapeseed oil and fish oil catalyzed by lipase TL comparing free and cross-linked forms

Ethyl esters prepared from rapeseed oil and fish oil catalyzed by cross-linked TL and free enzyme (acetone powder) are compared in Fig. 3. Cross-linked TL (with albumin as an additive in a mass ratio of 1:4) shows 40% more turnover of rapeseed ethyl esters than the not cross-linked form (54 m% vs. 94 m% after 1.5 h reaction time). Inserting CLEAs of TL for ethanolysis of fish oil, 68 m% ethyl esters are produced after 1.5 h. After 6 h, 78 m% fish oil ethyl esters were determined.

3.3.2 Ethanolysis of rapeseed oil by lipases of the organism TL with different amounts of catalyst

Figure 4 shows rapeseed oil ethyl esters produced by CLEAs of TL (1:4) inserting different amounts of catalyst. Thus, with a usage of 2.5 m% catalyst 61 m% ethyl esters were observed after 1.5 h reaction time. A use of 5 m% catalyst leads to 82 m% ethyl esters after 1.5 h and an amount of 10 m% catalyst reaches a turnover of 94 m% ethyl esters. Depending on the amount of catalyst used, the curve begins to flatten after a certain time. With a stake of 2.5 m% it starts after 2 h and at a turnover of approximately 80 m% the reactions stops.

Table 1. Inserted amount of enzyme using (a) 2.5% m/m CLEAs of TL and (b) 10% m/m CLEAs of CalB, considering the BSA amount.

(a)

Enzyme/BSA (m/m)[a]	Enzyme in CLEA (% m/m)[b]	Enzyme in oil (% m/m)	Ethyl esters after 1 h (% m/m)	Ethyl esters after 6 h (% m/m)	Absolute activity (IU/g)	Relative activity (%)[c]
1:4	18	0.45	52	79	596	501
1:7	11	0.28	43	71	496	698
1:12	6.7	0.17	33	57	363	837
1:16	4.7	0.12	19	52	224	738
1:0	85	–	–	–	551	100

(b)

Enzyme/BSA (m/m)[a]	Enzyme in CLEA (% m/m)[b]	Enzyme in oil (% m/m)	Ethyl esters after 6 h (% m/m)	Ethyl esters after 24 h (% m/m)	Absolute activity (IU/g)	Relative activity (%)[c]
1:4	10	1.0	14	58	804	–
1:7	7.5	0.75	27	66	962	1.06×10^3
1:12	6.3	0.63	36	82	1.72×10^3	2.26×10^3
1:16	4.9	0.49	37	79	1.41×10^3	2.40×10^3
1:0	40	–	–	–	486	100

[a]Cross-linked weight of enzyme/cross-linked weight of BSA.
[b]Cross-linked weight of enzyme/resulting weight of CLEAs (%).
[c]Obtained activity/initial activity (%).

3.3.3 Ethanolysis of fish and rapeseed oil catalyzed by lipase CalB comparing free, cross-linked forms and Novozym 435

In Fig. 5, resulting ethyl esters produced by ethanolysis with CalB free, cross-linked form and Novozym 435, a commercially available immobilized CalB, are shown. Using 10 m% catalyst rapeseed oil and fish oil were transesterified. After 6 h reaction time cross-linked aggregates (with albumin as an additive in a mass ratio of 1:12) expressed six times higher conversions compared to the free enzymes (free: 8 m%; CLEA: 50 m%).

CLEAs showed higher absolute activity than Novozym 435 (Novozym 435: 952 IU/g; CalB 1:12: 1.72×10^3 IU/g). The transesterification with CalB 1:12 compared to Novozym 435 starts a little faster, but the fraction of ethyl esters is

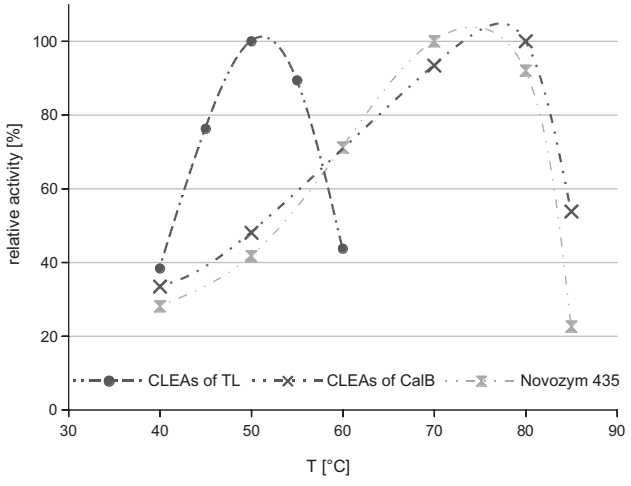

Figure 2. Evaluation of the influence of temperature on activities of CLEAs from TL and CalB.

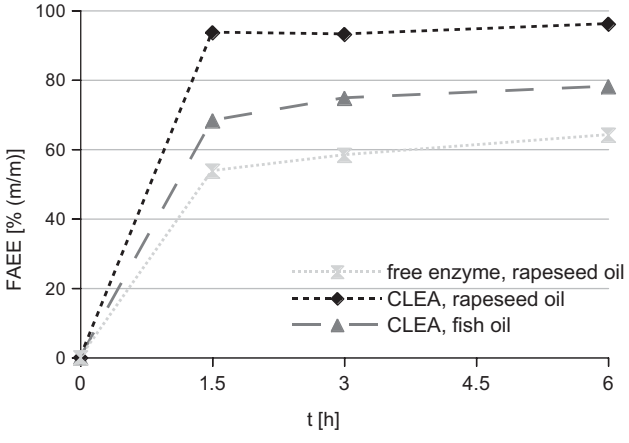

Figure 3. Fatty acid ethyl esters (FAEE) produced by CLEAs of TL (BSA in a mass ratio 1:4) and free enzyme powder in comparison (10% m/m of catalyst related to oil mass, molar ratio of ethanol/oil 4:1).

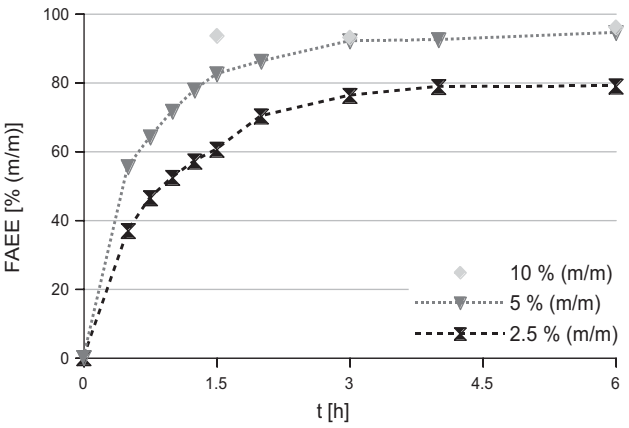

Figure 4. Effect of variation of catalyst amount on rapeseed ethyl esters produced by CLEAs of TL (BSA in a mass ratio 1:4). Molar ratio of ethanol/oil 4:1.

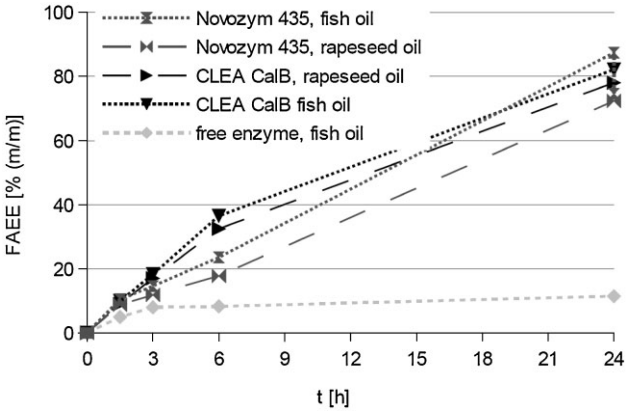

Figure 5. Ethyl esters produced by CLEAs of CalB (BSA in a mass ratio 1:12), free enzyme powder and Novozym 435 in comparison (10% m/m of catalyst related to oil mass, molar ratio of ethanol/oil 4:1).

adapting over time, thus it can be said CalB 1:12 and Novozym 435 catalyzed equally well.

3.3.4 Ethanolysis with CLEAs of different BSA amount

Table 1 shows produced ethyl esters as a function of BSA content in the catalyst. Ethanolysis of fish oil and rapeseed oil were carried out with CLEAs of TL (2.5 m%) and CLEAs of CalB (10 m%). Achieved ester production can be compared to the determined absolute activities. The higher it is, the faster is ester production over time. Observing the specific activities of CalB, productivity increases with decreasing enzyme content. Finally, two common behaviors of TL and CalB were observed: both lipases show best results in relative activity at about 5 m% enzyme in CLEA where the best distribution of enzyme occurs and consequently diffusion of substrate to the active centers and release of products from those is most efficient. Furthermore, both lipases reach for a stake of about 0.5 m% enzyme in oil the highest turnover of ethyl esters within 6 h of reaction time.

When evaluating the overall behavior of different CLEAs in the ethanolysis of rapeseed and fish oil, beneficial effects of the cross-linked forms compared to the neat enzymes were observed. Expected rates of conversion using, e.g., Lipozyme TL IM resulted in ester yields of 25–69 m% found for soybean oil and palm oil conversion [23, 24]. Composition of the oils is different but the conversion rates can be taken as guidance levels as they represent highly unsaturated as well as more "saturated" oils. In the present work, higher conversion rates were reached using cross-linked TL at relatively short reaction times. The difference (94% vs. 68% Fig. 3) between fish oil and rapeseed oil is due to the fatty acid chain length specificity of the lipase, which prefers short-chain and unsaturated fatty acids [25]. Also, the variation (reduction) of catalyst amount from 10% to 2.5% led to promising turnover rates at relatively low catalyst concentrations (Fig. 4). In this case, this is directly related to the improved efficiency of cross-linked forms compared to the neat. However, liberated glycerol inhibited the enzymes by sticking on the surface, even if a lower amount of substrate was used. This is still one of the most hindering effects for scaling up the process, independently if enzymes are used in neat or as cross-linked form. Similar results were obtained when CalB was used for ethanolysis. The catalytic behavior of this enzyme is much slower than compared to TL. A higher conversion rate of fish oil was obtained due to the fatty acid chain length specificity of the lipase, which has higher affinity to long-chain, unsaturated fatty acids [25]. Regarding the activities of TL it has to be noted, that the productivity of aggregates with low relative activity (CLEA 1:4) remained higher than the productivity of aggregates with high relative activity (CLEA 1:16). In case of TL, this seems to be result of the inserted enzyme amount in the CLEAs (18% m/m vs. 4.7% m/m) as shown in Table 1a. However, CalB showed opposite results (Table 1b) but this might be linked to the more linear conversion behavior (Fig. 5). Anyway, a more detailed evaluation in this context is under investigation.

3.3.5 Glycerolysis of fish oil ethyl esters

Conversion of glycerol and fish oil ethyl ester to triacylglycerols (TAG) comparing the catalyst Novozym 435 and CLEAs of CalB (1:16) was examined (Fig. 6). After 6 h reaction time CLEAs of CalB achieved a conversion to TAG of 37 m%, after 24 h 87 m% were yielded. Novozym 435 reaches in the same times 34 m% and 75 m% TAG, respectively. Glycerolysis reaction was successful with CLEA's from CalB but unfortunately failed with CLEA's from TL. The catalysts were not keep suspended (even at low

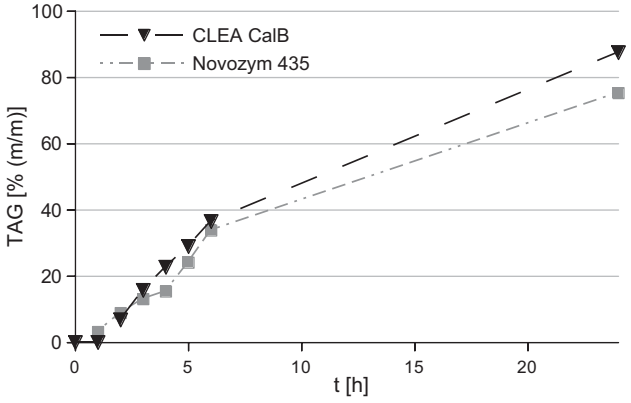

Figure 6. TAG produced by CLEAs of CalB (BSA in a mass ratio 1:12) and Novozyme 435 in comparison. Molar ratio of glycerol/ethylester 1:3.4.

temperature) and built a dark brown aggregate with glycerol, which consequently led to no reaction. The same aggregation was observed during ethanolysis at the time when the reaction started to stagnate due to inhibition by liberated glycerol.

3.4 Reusability

Above-mentioned findings on the preparation of TAG from fish oil ethyl esters show that CLEAs of CalB (as determined for ethanolysis) are reacting as well, or even a little faster compared to the commercially available immobilized Novozym 435. In this context, the possibility of reuse is one of the most important advantages of immobilized biocatalysts. To evaluate the behavior of the CLEAs, reusability trials have been carried out. However, in the case of Novozym 435, inconsistencies in yields of TAG (approximately 10 to −20% of TAG produced comparing to the cycle before) and in regained weight of catalyst were observed. Hence, the chosen washing steps were obviously inappropriate for this catalyst. The method was inefficient to remove residues of reaction adequately, and/or promoted the releasing of the enzyme from the plastic carrier. In particular water, due to its strong negative polarity, is assumed to possibly desorb proteins from the carrier. Nevertheless, Novozym 435 is known as reusable catalyst under proper conditions [26–28] and was not further investigated in the present work.

3.4.1 CLEAs from lipase *Thermomyces lanuginosus* tested on ethanolysis

Rapeseed oil and ethanol were chosen as substrate. Figure 7 shows the yield of ethyl esters by application of CLEAs of TL (1:12) over six cycles of reuse. Full yield of fatty acid ethyl ester was retained the first four cycles, decrease in yield occured in cycle five and six. Washing was done with *n*-hexane to remove oil and ethyl esters, phosphate buffer to

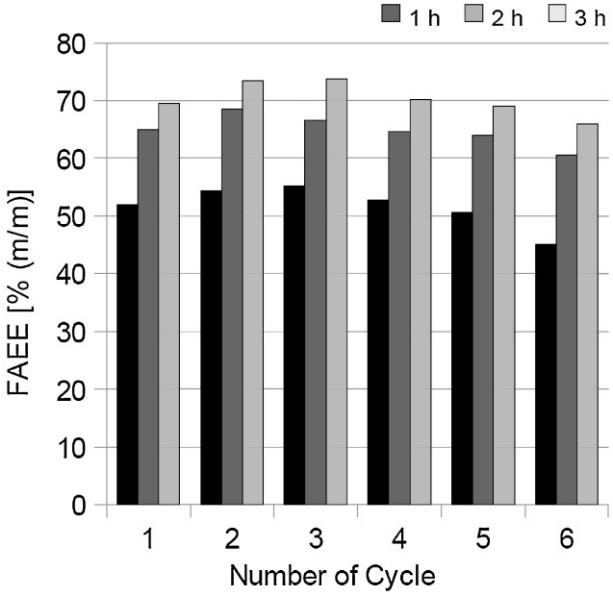

Figure 7. Reusability of CLEAs of TL inserted for ethanolysis.

remove inhibiting effect of glycerol from the active centers and acetone to remove water with final drying on air. An amount of 33 mL per washing step was chosen; preliminary tests with lower amounts of solvent were done (data not shown). Indicators for successful remove of reactants are weight and occurring color of the dry catalyst. Observing 3 h reaction time of six recycling cycles, ethyl ester production remained almost stable. In the 1st cycle an average production of 23 m% FAEE per hour was achieved and during the 6th cycle 22 m% FAEE per hour.

3.4.2 CLEAs from lipase *Candida antarctica* B tested on glycerolysis

Figure 8 shows the generated conversion to TAG of six cycles of recycling gained with CLEAs of CalB. Observing 4 h reaction time of each cycle, CalB shows an average decrease of the conversion to TAG of 1.9 m% per hour after each cycle (1st cycle: 16 m% TAG per hour, 6th cycle: 6 m% TAG per hour). In the fifth cycle, only less than half of TAG after 4 h reaction time comparing the first cycle were produced (1st cycle: 63 m%; 5th cycle: 28 m%; 6th cycle: 25 m%). The decrease could be attributed to the reaction temperature. Experimentally the optimum of temperature was detected between 75 and 80°C. In the reaction a temperature of 80°C was selected to evaporate ethanol (bp = 78°C) and push the reaction equilibrium to the product side. The temperature is at the edge of the optimum range for the catalyst.

As demonstrated, CLEAs of TL are adequate catalysts for transesterification reactions and results of reusability tests indicated their remaining high activity with high ester yields over six cycles. Ester respectively activity levels are

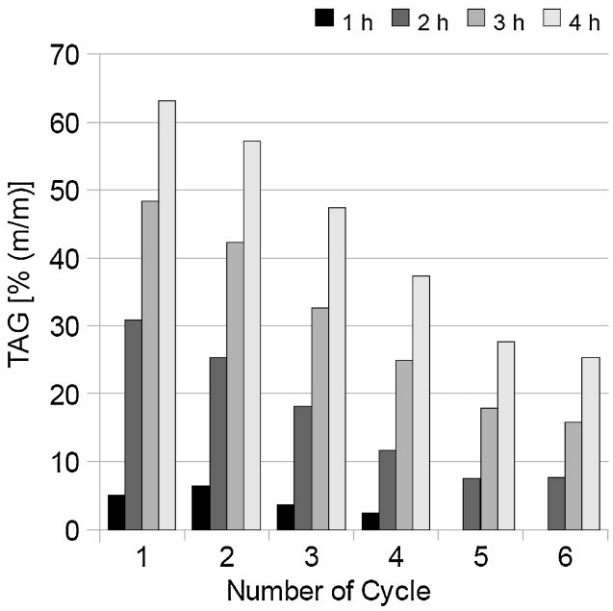

Figure 8. Reusability of CLEAs of CalB inserted for glycerolysis.

comparable to findings of Rodrigues et al. Lipozyme TL IM showed about 80% remaining activity after seven cycles in their experiments [24].

3.5 Shelf-life

Beside the advantage of reusing immobilized biocatalysts, another important factor is durability of catalysts, when stored for an extended periods of time. In order to have a first idea about shelf-life of the produced CLEAs, their behavior was evaluated when stored at 4°C in screw capped jars. This evaluation should just give an indication on further work planned in this context as well as complete the here presented findings.

In order to associate productivity as a function of storage time, (i) CLEAs and (ii) not cross-linked enzyme powder were applied for transesterification. In case of not CLEAs, both lipases showed a decrease of ethyl ester production of 30–40% (using 10 m% catalyst) after eight days of storage compared to the ester yielded after preparation. In contrast, both types of CLEAs (TL and CalB) showed no significant decrease in productivity after a storage period of three months: CLEAs of TL (1:4, 2.5 m%) yielded more than 90% of ester comparing to fresh built aggregates, CLEAs of CalB (1:12, 10 m%) achieved over 85% of ethyl ester compared to aggregates, which were stored for one day only. This was an important observation so that it can be stated that CLEAs are (besides the mentioned improved conversion and reusability behavior) in terms of shelf-life also well suitable to be used over a long period of time.

4 Conclusions

It could be demonstrated, that cross-linking is a proper technique to improve enzyme activities. Both lipases here investigated were hyperactivated (gaining higher activity comparing to the neat enzyme) due to cross-linking with BSA as an additive. These higher activities were demonstrated on ethanolysis and glycerolysis reactions of different oils, and results indicated that high yields in shorter reaction time can be achieved. Varying catalyst amounts as well as BSA proportions during cross-linking led to notable effects on conversion behavior comparable to other lipases already investigated. If scaling up the process is considered, it was shown that especially CLEA's of TL have high potential due to high conversion rates and re-activation possibility.

CLEAs of CalB showed high activity at elevated temperature, which could be utilized, e.g., for glycerolysis or for transesterification of materials with higher melting points such as fats. Furthermore CalB is known as an enzyme with esterase-activity, able to esterify free fatty acids. However, inhibition of enzymes by glycerol, which is formed as by product, is still an unsolved problem especially if a continuous process is desired.

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