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Enzymatic Esterification in Aqueous Miniemulsions

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Abstract: Monoesters of various linear carboxylic acids (C7–C12) with ω-phenyl-labeled primary alcohols (C1–C5) were synthesized in aqueous miniemulsions with various lipases as biocatalysts. The reactants were dispersed in an aqueous solution of a nonionic surfactant to form long-term stable miniemulsions. The esterification of all of the systems could be catalyzed by the applied enzyme and yielded significant conversions of about 90%. The hydrophilicity of the reactants and the specificity of the enzyme toward the substrates determine the reaction velocity and the final conversion. As a model system the reaction of nonanoic acid and 3-phenylpropanol was extensively studied. Among various lipases, Lipase PS was determined to be the most effective, and for this reaction the parameters were optimized. A maximum conversion of 80% could be obtained in less than one hour of reaction time. In comparison with an acid-catalyzed esterification performed in miniemulsion with the same reaction parameters, the enzyme-catalyzed reaction showed a significantly faster conversion. The reactions proved that the application of the miniemulsion technique enables efficient direct enzyme-catalyzed esterification reactions from carboxylic acids and alcohols in the presence of large amounts of water.

Keywords: enzyme catalysis · esterification · lipases · miniemulsion

Introduction

Dehydration reactions, for example, esterification reactions, are of key interest to the synthesis of basic organic chemicals as well as the production of polymers.[1–3] The water generated during the reaction has to be removed from the locus of the reaction to shift the equilibrium to the product side. In the presence of water, the equilibrium is displaced to the reactant side and hydrolysis of the desired products takes place. Dehydrative esterification from a carboxylic acid and an alcohol needs to be catalyzed to perform the reaction in reasonable time under ambient conditions. Acid catalyst systems or specialized enzymes can be employed for these reactions. The generated water can be removed by evaporation, by conducting the reaction in vacuum,[4–7] or by adding water-binding agents, such as zeolites or salts.[8,9] Still, considering economic and environmental issues, the employment of water as a solvent for organic reactions would be desirable. Water is nontoxic, cheap, and environmentally benign. Additionally, the reactants do not have to be dried before the reaction, thus drying agents, time, and energy can be saved. These reasons, only briefly mentioned, are reviewed extensively elsewhere.[10–12]

Despite the chemical obstacles, several groups have already shown that dehydrative esterification reactions, for the synthesis of esters and polyesters, can be conducted in water. It acts as a continuous phase of an emulsion containing hydrophobic droplets and not as a solvent.[13–15] Thus, several requirements have to be met by the reactants: For the formation of an emulsion without the addition of further organic solvents, the starting materials have to be sufficiently immiscible with water, and liquid at the temperature at which the experiment is performed. For the stabilization of the emulsion, a surfactant is required; for the reaction the catalyst. As the catalyst, being a Lewis or Brønsted acid[11,16,17] or an enzyme,[18] is generally water-soluble, the reaction takes place at the interface between the hydrophobic droplets and the aqueous phase.[19] Thus, it is convenient to combine the stabilizing and catalyzing properties in one molecule ("CATASURF").

Regarding enzymes, two types are capable of catalyzing hydrolysis and esterification reactions: esterases and lipas-

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es.[20] Esterases usually act on water-soluble reactants, whereas lipases are specialized toward hydrophobic substrates, present as droplets in an aqueous phase. Thus, most lipases exhibit their catalytic abilities at the interface between the hydrophobic substrates and the aqueous continuous phase.[21–23] Because the catalysis takes place at the interface of the emulsion droplets, a large interfacial area is desirable for a fast conversion. Obviously, the interfacial area has to be present during the whole course of the reaction, thus requiring a highly stable emulsion. Therefore, dehydrative esterification reactions catalyzed by an interfacially active lipase benefit from a large interfacial area and long-term stable emulsions.

By decreasing the droplet size from, for example, 5 μm in a conventional emulsion to 500 nm, the interfacial area increases by a factor of 100. Droplets in the submicron range can be found in systems called "miniemulsions". Direct (oil in water) miniemulsions are generated by dispersing organic liquids in water supported by the application of high-shear forces as ultrasound.[24] The droplets are of a uniform size and are stabilized against coagulation by the presence of an ionic or nonionic surfactant. Diffusional degradation (Ostwald ripening) of the droplets can be suppressed by the addition of a costabilizing, extremely hydrophobic agent.[25] Thus, the size and the composition of the droplets remain constant, even when chemical reactions are performed in the miniemulsion droplets.[25]

So far, only dehydrative polycondensations have been conducted in a miniemulsion. Barrère and Landfester investigated dodecylbenzenesulfonic acid (DBSA)-catalyzed polycondensation reactions of several diols and diacids of different hydrophobicity in miniemulsions.[26] The polymerization yield was found to be dependent on the hydrophobicity of the monomers. With the most hydrophobic monomers, yields of nearly 90% were achieved, despite the presence of an aqueous continuous phase. Moreover, enzymatic ring-opening polymerization of lactones, which can be described as a series of transesterification reactions, conducted in a miniemulsion was reported by Taden et al.[27] The authors noted a significant decrease in the reaction time relative to enzymatic synthesis in organic solvents from several days[12,28–31] to 12 h and less. Herein, the principle of dehydrative polyesterification reactions is extended from polymers to low-molecular-weight systems. Based on other earlier work,[17,26,27] we can assume that the mechanism for the esterification in miniemulsions could run as outlined in Figure 1. The reactants, and eventually the product, form a miniemulsion droplet of about 500 nm stabilized by the surfactant. The enzyme located at the interface of the droplet and the aqueous continuous phase catalyzes the esterification. The water generated during the reaction is expelled from the hydrophobic droplet and is transported to the aqueous continuous phase of the miniemulsion (Figure 1).

To study the dehydrative esterification reaction in the presence of water as a continuous medium, we chose linear carboxylic acids of different chain lengths with 7–12 carbon atoms and linear phenyl alkanoles with 1–5 carbon atoms in the alkyl chain as alcohol substrates (Scheme 1). The influence of the different chain lengths on the reaction velocity and the final conversion has been studied. The phenyl label offers the opportunity for easy detection with a UV detector in an HPLC setup. All of the experiments were preformed with enzymatic catalysis, thus ensuring high conversions with short reaction times at low temperatures. Besides the application of various substrates, the activities of several nonimmobilized enzymes, the reaction conditions, the temperature, and the pH value of the continuous phase were evaluated. The hydrolysis of 3-phenylpropyl nonanoate in a miniemulsion was performed to determine the position of the equilibrium. The efficiency of the enzyme-catalyzed reaction in a miniemulsion was shown by the comparison with the same enzyme-catalyzed esterification in a macroemulsion and a homophase and an acid-catalyzed esterification in a miniemulsion.

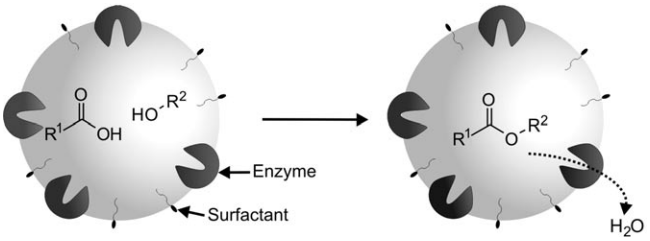
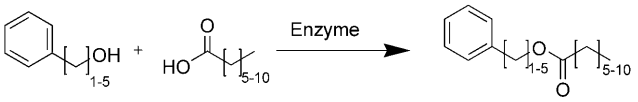


Figure 1. Schematic diagram of an esterification in the miniemulsion.



Scheme 1. General representation of the enzyme-catalyzed esterification reaction.

Results and Discussion

Carbon-chain length: For the formation of a miniemulsion, two phases have to be sufficiently insoluble in each other. To ensure that no phase separation occurs during the reaction, hydrophobic linear carboxylic acids with seven and more carbon atoms were selected for the reaction with 3-phenylpropanol. Although the solubility of the reactants in the aqueous phase is quite high (e.g., heptanoic acid: ca. 2×10^{-3} mol L^{-1}) effective and quick reactions with high conversions were observed. For all of the investigated systems, the pH was 3.8 before, during, and after the reaction. The miniemulsions used for the reactions comprised a 17% hydrophobic phase, stabilized by a nonionic surfactant. The nonionic surfactant Lutensol AT 50 was applied at 10 wt% with respect to the dispersed, hydrophobic phase. Although lipase-catalyzed reactions could be performed in the presence of an ionic surfactant, such as sodium di(2-ethylhexyl)

sulfosuccinate (AOT) or hexadecyltrimethylammonium bromide (CTAB),[32] it is known[33] that ionic surfactants can interfere with enzyme activity. Some anionic surfactants, for example, sodium dodecylsulfate (SDS), are even used to denature and thus deactivate enzymes.[27] Polysorbates (e.g., Tween80) could not be used because they are esters themselves and thus prone to lipase-catalyzed hydrolysis.

Figure 2a shows the reaction profiles of the lipase-catalyzed reaction of long-chain, linear carboxylic acids with 3-phenylpropanol at 40 °C in a miniemulsion. All of the examined systems show a significant conversion of at least 60% after 24 h. The reaction profile with the fastest conversion (rate = 0.32 min^{-1} for nonanoic acid (C9) with 3-phenylpropanol) reached its maximum yield of 78% in 6 h. The profiles of the reactions of decanoic acid (C10) and dodecanoic acid (C12) have slightly lower values (C10: 0.22; C12: 0.21 min^{-1}) and reached their maximum yield of 80% after about 7–8 h. The maximum conversion (80%) of undecanoic acid (C11) was reached after 47 h, whereas the reaction of the two shortest chain acids, heptanoic (C7) and octanoic acid (C8) yielded a maximum conversion of 70% after 60 hours. It is evident from the reaction profiles that the reaction of nonanoic acid with 3-phenylpropanol is the fastest.

From the initial slopes (first 2 h) and the molar masses of the acid substrates, it is possible to calculate the activities of the enzyme towards these substrates (see Figure 2b). Enzyme activities are expressed in U mg^{-1}, with U ("unit") defined as conversion in μmol min^{-1}. The activity of Lipase PS is highest for the reaction of nonanoic acid with 3-phenylpropanol with about 2.7 U mg^{-1}, followed by decanoic and dodecanoic acid with both little less than 2 U mg^{-1}. Surprisingly, the lowest activity was exhibited in the reaction of undecanoic acid with 3-phenylpropanol. As enzymes are highly specialized catalysts, the results obtained from the experiments summarized in Figure 2 can be explained so that Lipase PS shows distinct substrate specificity for nonanoic acid. Still, as observed in nonenzyme-catalyzed systems, the solubility of the substrates in the aqueous continuous phase (their hydrophilicity) might also influence the reaction rate and final conversion[17,26,27] as a concentration gradient of the reactants in the droplets might occur. The more hydrophilic substrates will accumulate at the interface between the droplet and aqueous phase, whereas the hydrophobic reactants will "retreat" to the center of the droplet. Moreover, water can diffuse into the more hydrophilic regions of the droplets, thus favoring hydrolysis reactions, and hydrophilic substrates can diffuse into the continuous phase and are no longer available for esterfication. As the generated ester is the most hydrophobic component of the system, it will accumulate more in the center of the droplet and will not interfere with the enzyme action at the droplet interface.

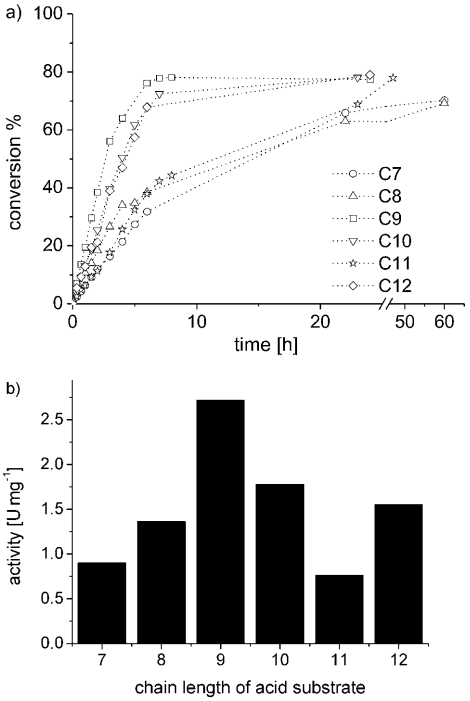


Figure 2. a) Conversion versus time of the reaction of 3-phenylpropanol with linear carboxylic acids with chain lengths of C7–C12 in the presence of Lipase PS. The conversion data was obtained by HPLC analysis and confirmed with NMR spectroscopic analysis. The conversion rates were calculated from the initial slopes: C7: 0.10; C8: 0.15; C9: 0.32; C10: 0.22; C11: 0.10; C12: 0.21 min^{-1}. The dotted lines are only a guide for the eye. b) Enzyme activity of the reactions of 3-phenylpropanol with carboxylic acid substrates with increasing carbon-chain length. The values are calculated from the initial slopes obtained from the profiles in graph (a).

To evaluate the influence of substrate polarity, competitive reactions were performed. With a given alcohol substrate, two or three acids of different chain lengths were combined in one miniemulsion droplet. The acid with the shorter chain, which is more soluble in the aqueous phase, is expected to accumulate at the interface between the organic and aqueous phases. As the concentration of the longer carboxylic acid will be less at the interface, the reaction rate of the more hydrophobic, longer chain acid is expected to be less than 50% relative to the noncompetitive reactions. Equal interfacial concentrations would result in 50% of the conversion rate observed in the reaction with the individual substrates. The acid substrates with the highest reaction rate (i.e., nonanoic acid), the lowest reaction rate and simultaneously the most hydrophilic (i.e., heptanoic acid) and most hydrophobic acid (i.e., dodecanoic acid) were chosen for the competitive esterification reactions with 3-phenylpropanol.

Nonanoic acid and heptanoic acid, with the highest and the lowest reaction rates in the single-acid experiments, were combined and esterified with 3-phenylpropanol. The conversions of the individual acids and the alcohol, which represents the total conversion are shown in Figure 3. It is clearly visible that the reaction rate of nonanoic acid with 3-phenylpropanol is higher than the one of heptanoic acid with 3-phenylpropanol (0.12 and 0.08 min^{-1}, respectively). As expected, the conversion rates are lower than the values of the reactions with individual substrates (C9: 0.32; C7: 0.10 min^{-1}). The conversion rate of the reaction with nonanoic acid is only one third of the individual reaction, whereas the reaction rate of heptanoic acid is only slightly lower than without the addition of a second substrate to the reac-

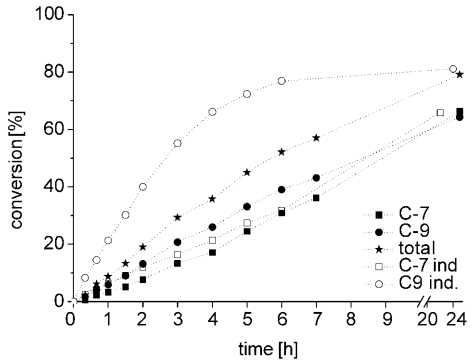


Figure 3. Conversion of acids and total conversion of both acids versus time for the competitive enzymatic esterification of heptanoic and nonanoic acid with 3-phenylpropanol (filled symbols), all catalyzed by Lipase PS. Conversion rates from the initial slopes: $C7_{ind}$: 0.10; $C9_{ind}$: 0.32; C7: 0.08; C9: 0.12 min^{-1}. The dotted lines are only a guide for the eye.

tion mixture. This outcome indicates a higher concentration of the more hydrophilic heptanoic acid at the oil/water interface and thus displacement of the more hydrophobic nonanoic acid to the core of the droplet. Nevertheless, nonanoic acid is converted faster than heptanoic acid, thus indicating a higher specificity of the applied enzyme for nonanoic acid. The total reaction rate is lower, although the total conversion still reaches 80% after 24 h.

The competitive esterification of dodecanoic acid and heptanoic acid with 3-phenylpropanol (Figure 4) shows the same trends as observed in the previous experiment. Both conversion rates are lower than in the experiments with the individually applied acid. Whereas the initial conversion rate of the esterification of dodecanoic acid is only 34% of the rate of the individual reaction, heptanoic acid is converted nearly as quickly (81%) as without the presence of dodecanoic acid. The explanation for this behavior is the same as for the previous experiment: the concentration of the heptanoic acid seems to be higher at the interface than the concentration of the dodecanoic acid. The total conversion

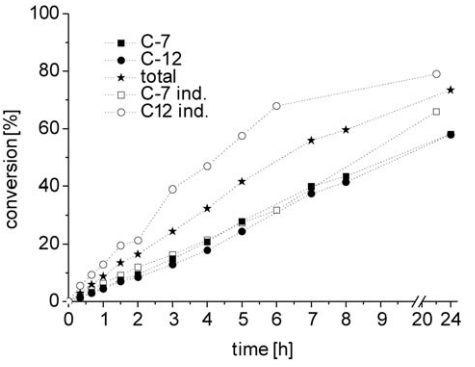


Figure 4. Conversion of acids and total conversion of both acids versus time for the competitive enzymatic esterification of combined heptanoic and dodecanoic acid with 3-phenylpropanol (filled symbols), all catalyzed by Lipase PS. Conversion rates from the initial slopes: $C7_{ind}$: 0.10; $C12_{ind}$: 0.21; C7: 0.08; C12: 0.07 min^{-1}. The dotted lines are only a guide for the eye.

is about 75%, which is slightly below the value observed for the individual reaction.

Figure 5 shows the reaction profiles of the competitive esterification of nonanoic and dodecanoic acid with 3-phenylpropanol. The reaction profile for the esterification of

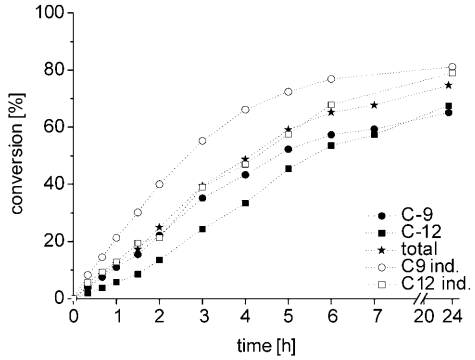


Figure 5. Conversion of acids and total conversion of both acids versus time for the competitive enzymatic esterification of combined nonanoic and dodecanoic acid with 3-phenylpropanol (filled symbols), all catalyzed by Lipase PS. Conversion rates from the initial slopes: $C9_{ind}$: 0.32; $C12_{ind}$: 0.21; C9: 0.19; C12: 0.11 min^{-1}). The dotted lines are only a guide for the eye.

nonanoic acid with 3-phenylpropanol indicates a faster conversion than the profile for dodecanoic acid (0.19 and 0.11 min^{-1}, respectively). Because the slopes of both curves are approximately half of the value obtained from the reaction profiles of the individual esterification reactions, comparable concentrations of both acids can be assumed at the interface between the organic and the aqueous phase. This finding is a strong indication for a preferred catalysis of the esterification of nonanoic acid with 3-phenylpropanol. The total conversion is about 75% after 24 h.

Finally, all of the previously used acids (i.e., heptanoic, nonanoic, and dodecanoic acid) were combined in the droplet phase and esterified with 3-phenylpropanol (Figure 6). As already observed in the previously described competitive reactions, the conversion profile of the esterification of nonanoic acid with 3-phenylpropanol shows the fastest conversion. The curves of the reactions of dodecanoic and heptanoic acid have almost the same shape. With three (hypothetically equally active) acid substrates present, a conversion rate of one third of the reactions of the individual acids can be assumed. Indeed, the conversion rate of the reaction of nonanoic acid shows 34% of the value of the reaction of the individual acid. Regarding the other acids, deviations from the hypothetical value can be found. Whereas the conversion rate of heptanoic acid is 67% of the value for the individual reaction, the conversion rate for dodecanoic acid can be calculated as only 27% of the value for the individual reaction.

Despite the significant differences in the reaction rates and the conversions of the acids, the total conversion of the alcohol substrate almost reaches the values of the reactions of the individual acids after 24 h. The low reaction rate of

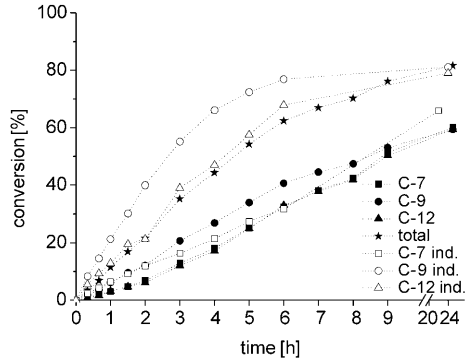


Figure 6. Conversion of acids and total conversion of all acids versus time for the competitive enzymatic esterification of combined heptanoic, nonanoic, and dodecanoic acid with 3-phenylpropanol (filled symbols), all catalyzed by Lipase PS. Conversion rates from the initial slopes: $C7_{ind}$: 0.10; $C9_{ind}$: 0.32; $C12_{ind}$: 0.21; C7: 0.06; C9: 0.11; C12: 0.06 min^{-1}. The dotted lines are only a guide for the eye.

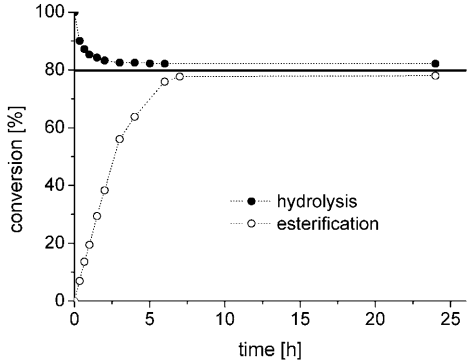


Figure 7. Hydrolysis and esterification of 3-phenylpropyl nonanoate catalyzed by Lipase PS versus time. The dotted lines are only a guide for the eye.

heptanoic acid and the fact that dodecanoic acid is not readily available for reaction at the droplet interface decreases the total yield to 75%. Regarding the reaction profiles, a plateau is not visible, thus indicating that the reaction has not obtained equilibrium state after 24 h of reaction time and that 80% conversion can be obtained.

The interfacial concentration seems to be directly connected to the chain length/hydrophilicity of the acid substrates. Higher hydrophilicity leads to higher interfacial concentration and vice versa. On the other hand, all of the results show a very clear preference for the reaction of nonanoic acid with 3-phenylpropanol over the other acid substrates applied in the experiments. Thus, this system was used for further experiments.

Competitive enzymatic reactions in a homophase were carried out as well to eliminate the interface and the effect of hydrophilicity/hydrophobicity so that the substrate preference of Lipase PS could be investigated. Because the reactions had extremely low conversion rates, no preference could be observed. After five days, the conversion of all three acids (C7, C9, and C12) was about 7% without any significant difference. This observation confirms the advantage of the heterophase for enzymatic ester synthesis.

Equilibrium: To determine the equilibrium of the catalyzed esterification/hydrolysis reaction, pure ester 3-phenylpropyl nonanoate was hydrolyzed enzymatically with Lipase PS. The ester was miniemulsified and hydrolyzed under the same conditions as the corresponding esterification reaction (see Figure 7 for the reaction profile). The hydrolysis profile initially shows a rapid decrease in ester content, and an equilibrium with an ester content of about 80% is reached after 3 h. The esterification reaction reaches a maximum conversion of slightly less than 80% after about six hours (see also Figure 2). This outcome means that the equilibrium is successfully shifted toward the products of the esterification reaction in the presence of large amounts of water. We can expect 80% as the maximum conversion, which can be obtained under the applied conditions with an equimolar ratio of the reactants.

Substrates applied in excess: For an evaluation of potential substrate inhibition, esterification experiments were conducted with an excess (ten- and fivefold) of either nonanoic acid or 3-phenylpropanol under at same conditions as the experiments without substrate excess. The reaction profiles of these experiments are shown in Figure 8 in comparison to the reaction profile of the enzyme-catalyzed esterification of equimolar reactants. The profiles obtained from the reactions with an excess of alcohol substrate show faster conversion than the reactions with equimolar reactants or an excess of acid.

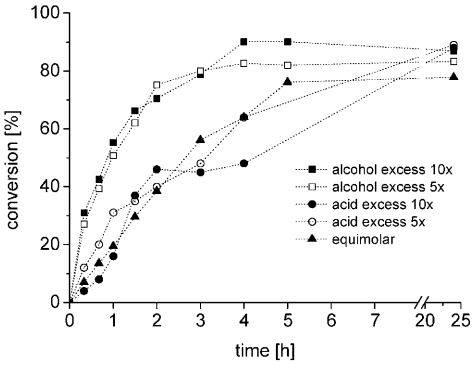


Figure 8. Reaction profiles of the Lipase PS-catalyzed esterification reactions of nonanoic acid with 3-phenylpropanol with an excess of alcohol (circles) or acid (squares) compared to the reaction with an equimolar amount of reactants. The reaction rates: the equimolar reaction: 0.32; excess of alcohol tenfold: 0.70; fivefold: 0.66; excess of acid tenfold: 0.22; fivefold: 0.25 min^{-1}. The dotted lines are only a guide for the eye.

A maximum conversion of slightly more than 80% is obtained after 4 h with a fivefold excess of alcohol, whereas the application of a tenfold excess of 3-phenylpropanol results in yields of nearly 90% after 4 h. Compared to the experiment with equimolar reactants, the values are increased

by 20 and 30% conversion, respectively. The esterification performed with an excess of acid substrate yielded a maximum conversion of nearly 90% after 5 h. The conversion rates are lower (tenfold excess: 0.22 min^{-1}; fivefold excess: 0.25 min^{-1}) than for esterification with an excess of alcohol (tenfold excess: 0.70 min^{-1}, fivefold excess: 0.66 min^{-1}) and slightly lower than for the reaction with an equimolar amount of substrates (0.32 min^{-1}). It can be concluded that an excess of either substrate shifts the equilibrium towards the product side. The maximum conversion increases from slightly less than 80% to nearly 90%. An excess of alcohol significantly increases the reaction velocity as the maximum conversion can be obtained in less than 4 h. The conversion rate of the reactions with an excess of the acid substrate slightly decreases relative to the equimolar application of the reactants.

It seems that excessive acid has a slight inhibitory effect on Lipase PS. Acid inhibition has already been observed by Daneshfar et al.[34] in the lipase-catalyzed reaction (*Candida antarctica*) of 2-ethylhexanol with 2-ethylhexanoic acid in *n*-heptane. As no change in pH value was observed during all the reactions with excessive substrates, the influence of the pH value on the reaction rates could be excluded.

Influence of the enzyme: Besides Lipase PS, several other enzymes were evaluated for their ability to catalyze the reaction of nonanoic acid and 3-phenylpropanol in a miniemulsion. The enzyme preparation was used at a constant concentration of 1 mg mL^{-1} (see Figure 9 for the results of the reactions). With the exception of Esterase 009, only lipases were applied. Besides the enzymes listed in Figure 9, Lipase G and a lipase from hog pancreas were also applied. Both enzymes led to immediate destabilization of the miniemulsion. Thus, no conversion could be recorded. The miniemulsion prepared with lipase *Rhizopus arrhizus* phase separated after about 2 h. The other enzyme preparations did not affect the stability of the miniemulsion during the course of the reaction. Even several weeks after preparation, the emulsions were still stable. There are two reasons for the destabilization of the miniemulsion: As some enzymes can strongly interact with surfactants,[32] the surfactant in such a system is no longer available for the stabilization of the miniemulsion droplets. In addition to the actual enzyme, the preparations consist of different impurities that might also adsorb the surfactant and thus interfere with the stabilization of the dispersed phase.

Figure 9a shows that with the exception of *rhizopus arrhizus* lipase (RAL) all the enzymes yield a conversion of 80% after at least 24 h. Whereas the reaction catalyzed with Lipase PS reaches maximum conversion after 5 h, the Esterase 009-catalyzed reaction shows a conversion of 50% and the Chirazyme L-5-catalyzed reaction only 30%. The maximum conversion of the reaction catalyzed by *rhizopus arrhizus* lipase is only approximately 20%. In the two-phase system formed after the phase separation (2 h) of the miniemulsion, only a slight increase of the conversion can be recorded.

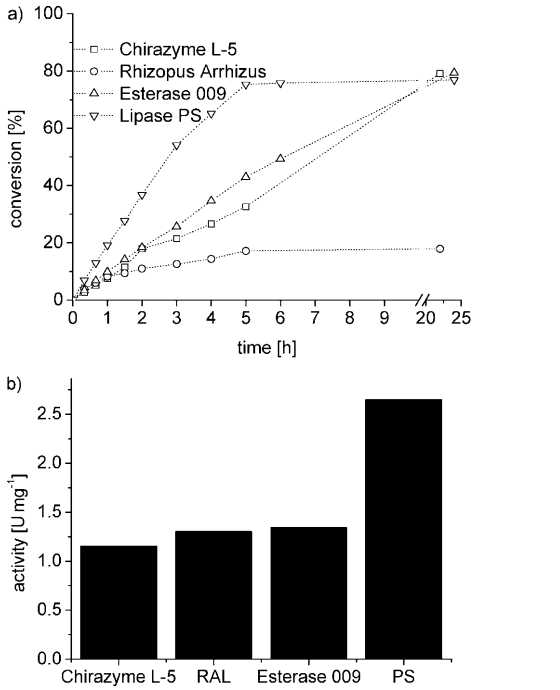


Figure 9. a) Enzyme-catalyzed reaction of nonanoic acid with 3-phenylpropanol with enzymes of different origins. The reaction rates: Chirozyme L-5: 0.14; *Rhizopus arrhizus* lipase: 0.09; Esterase 009: 0.15; Lipase PS: 0.32 min^{-1}. The dotted lines are only a guide for the eye. b) Calculated activities for the different enzymes in the esterification reaction of nonanoic acid with 3-phenylpropanol. RAL = *Rhizopus arrhizus* lipase.

Although all of the activities of the enzymes provided by the suppliers are given as 20–30 U mg^{-1} against olive oil (Lipase PS, *rhizopus arrhizus* lipase, and Esterase 009) or glyceroltributyrin (Chirazyme L-5), significant differences in the activity toward the examined system were found (see Figure 2 for a comparison). With the exception of the reaction catalyzed with *rhizopus arrhizus* lipase, the initial slopes were calculated from the conversion values obtained during the first 2 h of the reaction. Because the miniemulsion with added *rhizopus arrhizus* lipase was destabilized after 2 h, only the conversion values obtained during the first hour were used for the calculation. Lipase PS shows the highest activity for the esterification of nonanoic acid with 3-phenylpropanol, and the activities of the other enzymes are around 1 U mg^{-1}, which is about one third of the activity of Lipase PS (Figure 9b). Lipase PS is obviously the most efficient enzyme of for the esterification of nonanoic acid with 3-phenylpropanol in a miniemulsion at 40°C and pH 3.8, at which the highest reaction rate was observed.

Enzyme activity is determined by several factors, such as pH value and ionic strength of the solvent and temperature of the environment. Figure 10 and Figure 11 show a plot of conversion versus time for the Lipase PS-catalyzed esterification of nonanoic acid with 3-phenylpropanol in a miniemulsion with variation of the reaction temperature (Figure 10) and the pH value of the continuous phase (Figure 11). Three temperatures (i.e., 30, 40, and 50°C) and

two pH values (i.e., pH 3.8 and 6.8) were chosen. Regarding Figure 10, all of the three curves give a maximum conversion of about 80%. Although the differences are not significant, the initial slopes of the reaction profiles change slightly with the reaction temperature. The value calculated for the reactions increases from 30 to 40 and 50°C (0.26, 0.27, and 0.28 min^{-1}, respectively). These results indicate that the activity of the enzyme for the given system is only very slightly dependent on the reaction temperature in the range 30–50°C. As mentioned above, the enzyme activity is generally dependent on the pH value of the reaction environment, which is the continuous phase in the case of the miniemulsion system. Significant amounts of the carboxylic acid will be deprotonated at pH >5. The carboxy anion itself is a surfactant that can stabilize the droplets containing the reactants. It was thus possible to prepare a stable miniemulsion from nonanoic acid and 3-phenylpropanol by using an aqueous phase of pH 6.8 without the addition of further surfactant. To be able to compare the results with those obtained from reactions in miniemulsions stabilized with Lutensol AT50, the esterification reactions at pH 6.8 were performed in miniemulsions with and without the addition of Lutensol AT50.

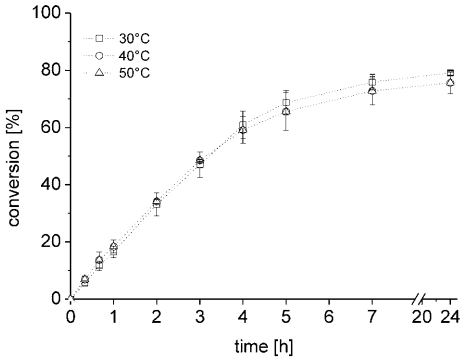


Figure 10. Influence of temperature on the reaction rate of the Lipase PS-catalyzed esterification of nonanoic acid with 3-phenylpropanol. The dotted lines are only a guide for the eye.

The reaction profiles over 24 h are shown in Figure 11. Comparing the reaction performed at pH 3.8 with the esterification reactions performed at pH 6.8, it can be noticed that the reaction profile of the first-mentioned reaction shows a higher conversion rate than the other profiles. Regarding the maximum conversions, the values are 5% (no Lutensol AT50) and 8% (Lutensol AT50) lower for the esterification reactions performed at higher pH values. Among the reactions conducted at pH 6.8, the profile of the reaction performed in droplets stabilized exclusively by the nonanoate species shows the faster conversion.

Usually, the enzyme shows the highest activity at a pH value between 7 and 8 (lipolysis of olive oil). The reaction performed at pH 3.8 has the highest velocity. Regarding the reactions at pH 6.8, the presence of the nonionic surfactant Lutensol AT50 decreases the enzyme activity. The surfactant, which is, like the lipase, located at the interface between the organic hydrophobic droplet and the aqueous continuous phase, might interfere with the active site of the enzyme, block the reactivity, or adsorb on the enzyme, alter the conformation of the active site, and thus decrease the enzyme activity. The system needs to be stabilized at pH 3.8 by the application of a surfactant. The carboxylic acid is protonated at this pH value and cannot act as a surfactant. Thus, no comparison between a Lutensol AT50-stabilized system and a system without an additional surfactant is possible in an acidic medium.

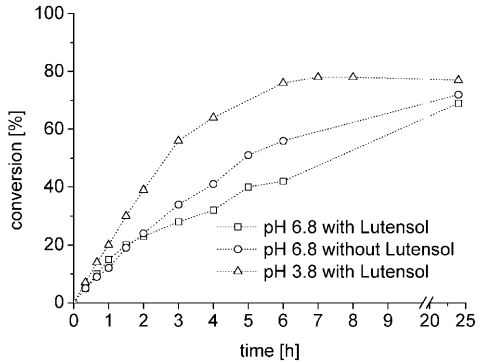


Figure 11. Reaction profiles of the Lipase PS-catalyzed esterification of nonanoic acid with 3-phenylpropanol in miniemulsions with continuous phases of pH 3.8 and 6.8, respectively. Conversion rates at pH 6.8: with Lutensol AT50: 0.16 min^{-1}; without Lutensol AT50: 0.19 min^{-1}; at pH 3.8: 0.32 min^{-1}. The dotted lines are only a guide for the eye.

The miniemulsion droplets provide a large and defined interfacial area. Thus, there must be a certain amount of enzyme that covers the entire interface provided. Until this enzyme concentration is reached, an increase in the conversion rate should be observed. As soon as the entire interface is covered by the enzyme, no further effect should be observable. To determine this critical value, increasing amounts of enzyme were added to the miniemulsion that contained nonanoic acid and 3-phenylpropanol. The reaction profiles and the relative conversions are shown in Figure 12, in which it is clearly visible that the conversion rate is increased after the addition of a higher amount of enzyme that catalyzes the esterification. Whereas the maximum conversion (≈80%) of the reaction catalyzed with 0.5 $mg\,mL^{-1}$ of the enzyme is reached after more than 8 h, it is reached after only 40 min after applying 10 $mg\,mL^{-1}$ of the enzyme. An almost linear relation can be obtained by plotting the relative conversions (min^{-1}) versus the concentration of the enzyme that catalyzes the reaction.

Before the addition of enzyme the z-average size of the droplets is 500 nm. Assuming that this value remains unaltered during the course of the reaction, a total interfacial area of 0.9 m^2 per mL of emulsion can be calculated. With an assumed protein content of 20% of the enzyme preparation and a molar mass of the enzyme of $M = 30000\ g\,mol^{-1}$, the area available for one enzyme molecule can be calculated to be 22 nm^2 for an enzyme content of 10 $mg\,mL^{-1}$. As

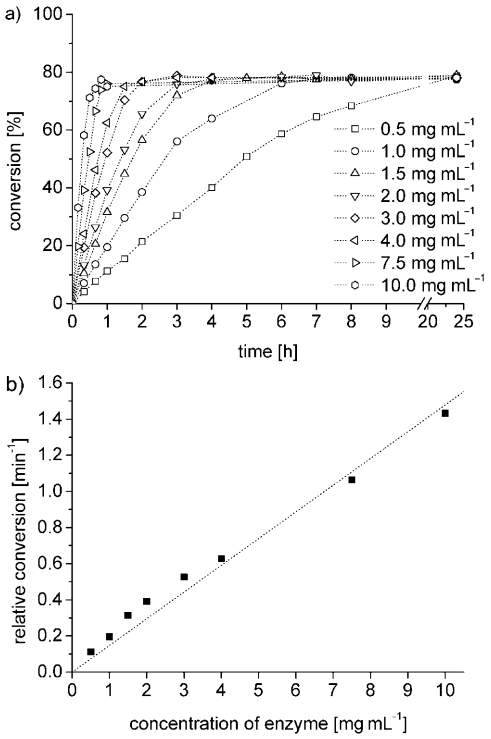


Figure 12. a) Reaction profile of the esterification of nonanoic acid with 3-phenylpropanol with different amounts of Lipase PS (0.5–10.0 mg mL^{-1} with respect to the emulsion). The dotted lines are only a guide for the eye. b) Conversion per minute calculated from the initial slopes of the plot of conversion versus time. The dotted line is the linear fit of the data points.

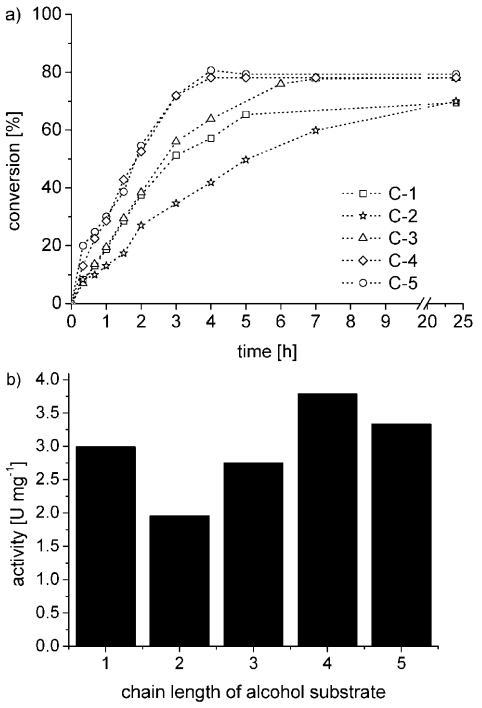


Figure 13. a) Reaction profiles of Lipase PS-catalyzed esterification reactions of nonanoic acid with ω-phenyl-labeled linear alcohols from benzyl alcohol (C1) to 5-phenylpentanol (C5). Reaction rates: C1: 0.31; C2: 0.20; C3: 0.32; C4: 0.42; C5: 0.40 min^{-1}. The dotted lines are only a guide for the eye. b) Enzyme activity of the reactions of nonanoic acid with ω-phenyl alcohol substrates with increasing carbon-chain length. The values are calculated from the initial slopes obtained from the plots shown on the left.

the curve in Figure 12b does not show any sign of saturation, it can be assumed that further enzyme molecules can be brought to the interface for a further acceleration of the conversion. It can be expected that as soon as the complete interfacial area provided by the droplets is completely covered with enzyme no further increase in the reaction velocity will be observed.

Influence of the alcohol substrate: Because the enzyme has specific activities toward the acid substrates, the same behavior can be expected regarding the alcohol component. Further ω-phenyl alcohols with a chain length of C1–C5 (benzyl alcohol to 5-phenylpentanol) were selected for the esterification with nonanoic acid. The reaction profiles are shown in Figure 13.

As observed with the different carboxylic acids, the solubilities of the alcohols in the aqueous continuous medium decreases with increasing carbon chain length, which is expected to increase the stability of the miniemulsion and thus favor an efficient reaction. Even with the very water-soluble benzyl alcohol (0.37 mol L^{-1}), high ester yields can be obtained. This finding underlines the robustness of the applied miniemulsion system.

As shown in Figure 13a, the esterification reaction of nonanoic acid with 2-phenylethanol (C2) has the lowest conversion rate. With the increasing chain length of the alcohol, the reaction profiles indicate a faster reaction. The maximum conversion of the reaction of nonanoic acid with benzyl alcohol and 2-phenylethanol is only 70%. The maximum conversion of the reactions of the other alcohols can be found to be 80%. At first glance, the reaction velocities are directly related to the hydrophobicity of the alcohol substrate and thus to the stability of the miniemulsion. Alcohols with a longer carbon chain are less soluble in the aqueous phase of the miniemulsion, thus there will be a decrease in the amount of diffusion from the particles, which increases the stability of the droplets. Regarding the propanol, butanol, and pentanol derivatives, the statement is valid. According to this hypothesis, benzyl alcohol should be the substrate with the lowest conversion rate because it is the alcohol with the highest solubility in the aqueous phase. In contradiction to this hypothesis, fast conversion and very high activity of the enzyme toward the esterification of benzyl alcohol with nonanoic acid can be found, thus indicating a distinct specificity of Lipase PS toward this alcohol substrate.

Control experiments: acid-catalyzed esterification in a miniemulsion and enzyme-catalyzed reaction in homophase and in a macroemulsion: For comparison, the esterification reactions were conducted with acid catalysis in a miniemulsion and with enzyme catalysis in a homophase (only substrates

without solvent) and a macroemulsion. We applied the same "CATASURF" (i.e., DBSA) for the acid-catalyzed esterification of nonanoic acid and 3-phenylpropanol in a miniemulsion as Manabe et al. used in their macroemulsion system.[17] The plot of conversion versus time was compared with the enzyme-catalyzed reaction of the same educts (Figure 14). Bearing in mind that the acid catalyst was applied as a 2 wt% solution, it can easily be calculated that CATASURF was used at a concentration of about 17 mg mL^{-1}. Relative to the amount of enzyme (1 mg mL^{-1}), this concentration is nearly 20 times the concentration of catalyst in the miniemulsion.

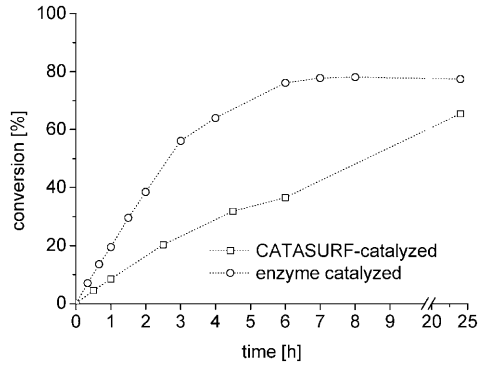


Figure 14. Comparison of conversion rates of DBSA and Lipase PS-catalyzed esterification of nonanoic acid with 3-phenylpropanol. Reaction rates: "CATASURF": 0.14; enzyme catalyzed: 0.32 min^{-1}. The dotted lines are only a guide for the eye.

Regarding the reaction profiles (Figure 14) of the DBSA (CATASURF) and the enzyme-catalyzed reaction, the conversion rate of the enzyme-catalyzed reaction is significantly higher than the rate of the acid-catalyzed reaction. Whereas the enzyme-catalyzed reaction reaches maximum conversion of about 80% after 5 h, the DBSA-catalyzed reaction reaches only about 35% conversion after this time. Even after 24 h, only about 60% conversion can be detected. Still, compared with the conventional emulsion system, employed by the above-mentioned authors, the reaction is faster. Manabe et al. reported that the maximum conversions of most of the esterification reactions were reached after more than 48 h. Because the catalysis takes place at the interface between the organic reactant droplet and the aqueous continuous phase, it can be assumed that the increase in interfacial area from macroemulsion to miniemulsion increases the rate of the catalyzed reaction, which is consistent with the data obtained herein and the data obtained from Manabe et al.[16] Figure 15 shows the reaction profiles of nonanoic acid with 3-phenylpropanol in a macroemulsion in comparison to the same system in a miniemulsion.

The reaction profiles show a slower conversion in macroemulsion (0.18 min^{-1}), which is 56% of the rate of the miniemulsion (0.32 min^{-1}). The final conversion in both reactions is almost 80%. A higher interfacial area offers more space for the adsorption of enzyme, thus generating more reaction

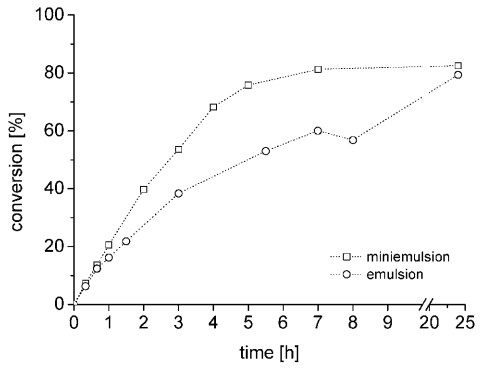


Figure 15. Reaction profiles of Lipase PS-catalyzed esterification of nonanoic acid with 3-phenylpropanol in macroemulsion relative to miniemulsion. The dotted lines are only a guide for the eye.

sites and leading to higher conversion rates. The enzyme-catalyzed reaction in homophase was even slower. Although the maximum conversion of 80% could be obtained, significant amounts of products could only be detected after several days.

The presented data show that an acid-catalyzed esterification is possible in miniemulsion; however, it is obvious from the reaction profiles that the enzyme catalysis is superior to the conventional acid catalysis.

Conclusion

To summarize, it was shown that it is very suitable to perform enzyme-catalyzed esterification reactions in an aqueous miniemulsion, as shown for several linear carboxylic acids with chain lengths of 7–12 carbon atoms with different ω-phenylalkanoles with a linear chain of 1–5 carbon atoms. The experimental setup enables the reactions to take place in the presence of large amounts of water, which is not possible with a conventional esterification setup in homogeneous solution. All of the investigated reactions of linear carboxylic acids (C7–C12) esterified with 3-phenylpropanol show more than 60% conversion, in case of excessive acid or alcohol, even 90% conversion after 24 h. Esterification reactions of nonanoic, decanoic, and dodecanoic acid with 3-phenylpropanol reach their maximum conversion after less than 10 h. Competitive esterification reactions of heptanoic, nonanoic, and dodecanoic acid with 3-phenylpropanol indicate that the hydrophilicity of the acid is crucial for its location within the droplets and exhibit a distinct specificity of Lipase PS toward the esterification of nonanoic acid over the other acids investigated.

Although the homophase reaction and the reaction in a macroemulsion of nonanoic acid with 3-phenylpropanol catalyzed by Lipase PS reached the conversion of the reaction conducted in a miniemulsion, the velocities were significantly slower. The same reaction, performed with acid catalysis in a miniemulsion is faster than this reaction performed in

an emulsion but still slower than the enzymatically catalyzed reaction.

High ester yields can be obtained after short reaction times under mild conditions in the presence of large amounts of water with the presented strategy. Additionally, the robustness of the system underlines the high potential of enzyme-catalyzed esterification in a miniemulsion for environmentally friendly organic chemistry.

Experimental Section

Materials: Heptanoic acid (99%), nonanoic acid (>99%), undecanoic acid (>98%), dodecanoic acid (99%), benzyl alcohol (>99%), 2-phenylethanol (99%), and 4-phenylbutanol were purchased from Merck. Octanoic acid (99%) was obtained from Riedel-de Haën and decanoic acid from Acros. 3-Phenylpropanol (98%), 5-phenylpentanol (99%), and hexadecane (HD; 99%) were purchased from Aldrich. 4-Dodecylbenzenesulfonic acid (DBSA; 90%) was purchased from Fluka. Lutensol AT50 (poly(ethyleneoxide)hexadecyl ether) was a gift from BASF. The following enzymes were used: Chirazyme L-5 (*Candida antarctica*) provided by Roche and Lipase G (*Penicillum cammemberti*) and Lipase PS (*Burkholderia cepacia*) provided by Amano. Lipase from hog pancreas and lipase from *Rhizopus arrhizus* were provided by Fluka and Esterase 009 (recombinant *Aspergillus oryzae*) from Jülich Chiral Solutions GmbH.

Acetonitrile (Chromanorm, Prolabo) was obtained from VWR. All the chemicals were used without further purification. Demineralized water was used throughout the experiments.

Enzyme-catalyzed reaction in miniemulsion: An equimolar solution (5 g) of the carboxylic acid and alcohol components with hexadecane (4 wt%) was added to an aqueous solution of Lutensol AT50 (25 g, 2 wt%). This two-phase system was stirred for 1 h. The macroemulsion was subsequently sonicated with a Branson sonifier 450D (1/4-inch tip) for 2 min. Ultrasound was applied in 5-s pulses with 5-s pauses. The vessel was cooled in an ice bath. From the miniemulsion formed, aliquots (20 mL) were transferred to a screwcap glass vial containing the enzyme preparation. The pH value of the reaction mixture was 3.8, except for the reaction without the addition of Lutensol AT50 (see below). If not otherwise stated, a 20-mg aliquot of the enzyme preparation was added after sonication to initiate the reaction. The reaction vessels were placed into a PLS 4×4 thermoshaker (Advanced ChemTech) at a fixed temperature and a speed of 400 rpm for 24 h. The samples were taken after 20 min, 40 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h, and 24 h.

Variation of the pH value of the continuous phase: The miniemulsion was prepared from 0.1 M NaOH solution without Lutensol AT50 by using the generated carboxy anion as a surfactant. Another miniemulsion was prepared using basic Lutensol AT50 solution (2 wt% Lutensol AT50 in 0.1 M NaOH solution). Both miniemulsions were used as described.

Enzyme-catalyzed reaction in a homophase: Lipase PS (30 mg) was added to an equimolar homophasic mixture of 3-phenylpropanol (2.3 g, 17.1 mmol) and nonanoic acid (2.7 g, 17.1 mmol). The mixture was agitated at 40 °C. Samples were withdrawn after selected periods of time.

Acid-catalyzed reaction in a miniemulsion: Nonanoic acid (2.7 g, 17.1 mmol), 3-phenylpropanol (2.3 g, 17.1 mmol), and hexadecane (200 mg) were emulsified as described. Aqueous DBSA (2 wt%) was used as the surfactant. The miniemulsion was agitated in the thermoshaker at 40 °C and 400 rpm. Samples were taken after 30 min, 1 h, 2.5 h, 4 h, 5 h, 6 h, and 24 h.

Hydrolysis of 3-phenylpropyl nonanoate: The miniemulsion of 3-phenylpropyl nonanoate (2.5 g, 8.9 mmol) and hexadecane (100 mg) was prepared with aqueous Lutensol AT50 (12.5 g, 2 wt%) as described. The miniemulsion (10 mL) was poured into a screwcap glass vial containing Lipase PS (10 mg) and put into the thermoshaker at 40 °C and 400 rpm for 24 h.

HPLC analysis: After certain periods of time (see general procedure), a sample of the reaction miniemulsion (10 μL) was injected into acetonitrile/water 90:10 (1 mL). An aliquot (20 μL) was injected into the HPLC setup with an autosampler Ti Series 1050 (Hewlett Packard). The eluent was acetonitrile/water 90:10 with a flow rate of 0.5 mL min^{-1}. The column used was a RP C-18 (nucleosil 120–5 C180) from Macherey–Nagel. The fractions were detected using a UV detector UVD 170U (Dionex) with variable wavelengths. The conversions were calculated from the respective peak areas [see Eq. (1)]. All the calculated reaction data is subject to an error of approximately 5% attributed to weighting and chromatogram-peak integration errors.

NMR spectroscopic analysis: Samples were withdrawn at the same time as for HPLC analysis. A sample of the miniemulsion (30 μL) was diluted with [D$_6$]DMSO (0.5 mL). The measurements were performed on a Bruker DRX 400 NMR spectrometer at 400.13 MHz. The conversions were calculated [see Eq. (1)] from the integrals of the peaks of the hydrogen atoms bound to the α-carbon atom of the acid and the ester at $\delta =$ 2.28 and 2.18 ppm, respectively:

$$\text{Conversion} = [\text{peak area (ester)}]/[(\text{peak area (ester)} + \text{peak area (acid)}] \quad (1)$$

The reaction rates were calculated from the data points acquired during the first 2 h of the experiments, if not otherwise stated in the discussion.

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