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SCIENTIFIC PAPER

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LIPASE–CATALYZED ESTERIFICATION IN A REVERSED MICELLAR REACTION SYSTEM

In this work, the lipase–catalyzed synthesis of i–amyl oleate was performed in a reversed micellar system of a cationic surfactant, CPC. The influence of the RM–system constituents on the biocatalysis characteristics (initial esterification rate and conversion extent) was studied and discussed in terms of the RM–structure. The initial water content in the RM–system (the water to surfactant mole ratio, Wo), affected both reaction parameters in a bell–shaped manner, with the maximum depending on the alcohol amount. In general the optimal Wo–values remained between 34 ÷40 for a wide range of the initial substrate ratio. The polarity (logP) and the structure of the organic solvent used as the RM–continuum strongly affected the kinetic parameters. A linear function of the initial rate on arg(logP) was established for the alkanes up to n–C10. The highest rates were measured using a medium of cyclo– or branched alkanes. The esterification under inhibiting concentrations of the substrates could be intensified by a small change in the initial Wo, thus improving the enzyme RM–accommodation and manipulating the effective concentrations of both substrates around the lipase. In the studied RM–system of 0.115 mol dm^{-3} CPC, the maximal initial rate of the lipase–catalyzed i–amyl oleate synthesis was found to be limited at ~340 μmol min^{-1} g^{-1}.

Key words: Lipase–catalyzed esterification, i–amyl oleate, Reversed micelles, Cationic surfactant, Organic solvent.

Reversed micellar systems (RMS) imply their application as a macro–homogeneous medium in which heterogeneous biocatalysis could be performed [1–5]. This appears possible mainly due to the structure of the reversed micelle aggregates. Their nano–sized structure has been associated with the idea of a micro–reactor where the problem of solubility of the substances with different polarity and their contact with the enzymatic catalyst could be solved on a molecular scale [6,7]. Concerning lipases in a reversed micellar environment, the lipase is solubilized in its natural aqueous environment (inside the aqueous core of the reversed micelle) or within the surfactant shell, thus, being protected from the adverse effects of the organic solvent, and also, assuming an easy way to run lipase–catalyzed reactions in a dominant–organic solvent phase (Figure 1).

On the other hand, both the apolar substrates/products dissolved in the organic continuum and the polar substances included inside the dispersed reversed micelles are distributed uniformly in one phase, which is thermodynamically stable and isotropic. Besides, RMS offer other biocatalytic advantages such as:

- High interfacial contact area (10–100 m^2 cm^{-3}) [8].
- Fast exchange processes, intermicellar, and between each micelle and the bulk organic solvent.
- No agitation or weak system mixing is needed.
- Enzyme aggregation is avoided.

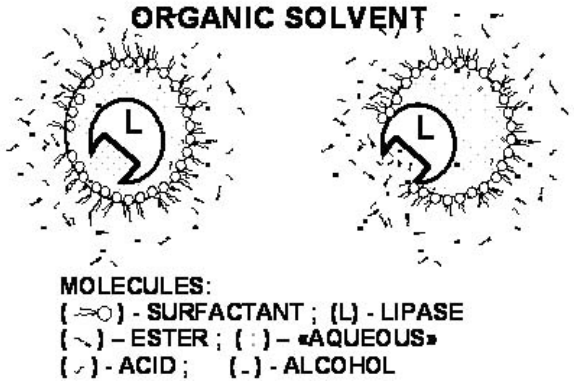

Figure 1. Schematic representation of a reversed micellar esterification system. Two possible schemes of lipase location against the micellar interfaces

- Strict control of the amount of the water present, which is a crucial factor affecting the thermodynamic equilibrium of the reversible reaction synthesis ⇆ hydrolysis.

The main disadvantage refers to product recovery and enzyme re–use which still remain difficult.

The current work in volues lipase–catalyzed esterification in a RMS of a cationic surfactant. This type of RMS has been poorly investigated as a potential medium for synthetic reactions in the case of lipases. The studied process involves the use of a crude enzyme preparation in the manufacturing of a natural biolubricant which has recently gained much attention [9,10].

MATERIALS AND METHODS

The studied system was composed of the substrates, *oleic acid* (Merck, Darmstadt, extra pure)

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and *i–amyl alcohol* (Aldrich, ≥98%), in the continuum of an *organic solvent* (under study were *cyclohexane*, *n*–hexane, *n*–heptane, *n*–octane, *n*–decane: all synthesis grade, Merck–Schuchardt; *i*–octane: Sigma–Aldrich Chemie GmbH, Steinheim, p.a.; *n*–dodecane: Acros Organics, Geel, Belgium, 99%) wherein the *lipase enzyme (Candida rugosa*, TypeVII, Sigma) was incorporated inside the reversed micelles of a quaternary ammonium salt, *cetyl pyridinium chloride, CPC* (Sigma Chemical Co., St. Louis, MO USA). The effects of the system constituents on the initial esterification rate and the *i*–amyl oleate yield were examined in a kinetic series following the consumption of the free oleic acid titrimetrically (alcoholic 0.1 mol dm^{-3} KOH/phenolphthalein) [11]. The initial reaction rate was determined from the initial slope of the curve describing the residual free oleic acid in time, which was linear within at least 90 min (with a correlation coefficient of >0.975 based on at least seven sample measurements). In all the studies the constants were: CPC–concentration, 0.115 mol dm^{-3}; aqueous buffer type, phosphate K_2H/KH_2, 0.05 mol dm^{-3}, pH 6.88÷7.09; the system temperature was set at 35°C, and the stirrer speed at ~70 rpm. The analytical and buffer reagents were of the following purity grade: potassium hydroxide, Sigma–Aldrich Inc., St. Louis MO USA, 91%; ethanol absolute, Riedel–de Haën, p.a., ACS; potassium phosphate dibasic anhydrous, Fluka, Buchs, p.a.; potassium dihydrogen phosphate, Aldrich, Steinheim, 98+%.

RESULTS AND DISCUSSION

1. Effect of the initial water content of RMS – Wo

Lipase–catalyzed reactions occur at the oil–water interface. In the case of esterification water plays a versatile role. Its presence is indispensable for the activation of the lipase reactivity, but in contrast, its absence eliminates the competing reaction (hydrolysis), as well as the competing (to the alcohol molecule) nucleophile. Consequently, the initial water dosage strongly affects the rate of reaction as well as the equilibrium position. Figure 2 represents the influence of the initial Wo of the RMS on the esterification kinetics. Within the Wo–range 13÷30 it an acceleration is seen in both the initial rate and conversion after 5 hours. Since an average radius of the aqueous pool of the reversed micelles formed (R_{RM}) can be assumed taking into account the Wo–value [12,13] the results could be discussed in terms of the best fit of one lipase molecule within one reversed micelle. The yeast lipase secreted from *Candida rugosa* has an apparent molecular weight of a single polypeptide chain of M_r ~ 60000Da [14]. The molecular weight of a protein is directly correlated with the characteristic radius of the biomolecule [15] and for lipase, R_L can be calculated to be ≈ 2nm. Within the acceleration–range Wo 13 ÷ 30, R_{RM} increased from ≈

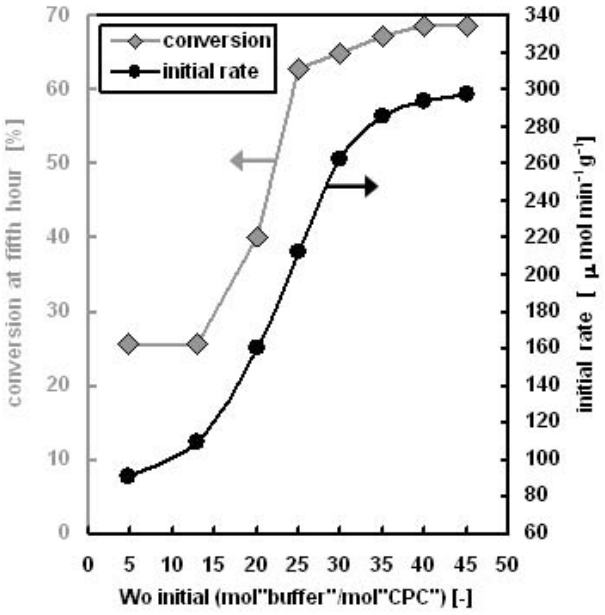

Figure 2. Dependence of the initial esterification rate and the conversion on the initial water content (Wo) of the RMS ([oleic acid]$_{init.}$ = 0.2 mol dm^{-3}, initial molar ratio {i–amyl alcohol}/{oleic acid} = 2.5, [lipase]=3 g {solid} dm^{-3} {RMS}, i–octane)

1.67 to ≈ 3.84nm. It may be concluded that the optimal ratio, $R^* = R_{RM}/R_L$, with respect to the lipase catalytic reactivity is recommended to be $R^* \sim 2$. A similar R*–ratio was reported for the RM–extraction of 9 different proteins with M_r13.7 ÷ 185kDa [16] and recently for the activity of horse radish peroxidase entrapped in reverse micelles of Igepal CO–520/cyclohexane [17].

As represented further (see Figure 6) the optimal R^* can vary with different substrate ratio because R_{RM} also changes with the amounts of the alcohol and the acid both mediating as co–surfactants in the RMS.

2. Effect of the initial molar ratio of the substrates

The initial rates of the esterification obtained at different initial molar ratios of {*i*–amyl alcohol}/{oleic acid} are plotted in Figure 3, while Figure 4 shows the concentration of the ester–product (*i*–amyl oleate) accumulated in the RMS durang a five hour process.

The maximal rates of the synthesis were observed when starting from equal molar concentrations of the substrates in a RMS. At a molar ratio <1 (less alcohol), the initial rate increased with increasing alcohol content. At higher ratios, >1, the reaction rate decreased at an almost equal slope. Inhibition by the acyl–donor could be observed at 0.4 mol dm^{-3} of oleic acid where, also, the ratio–profile was tighter, i.e. the reaction rate was more sensitive to the alcohol content present. The governing role of the acid agreed with the mechanisms which suppose the initial formation of the acyl–enzyme complex [18]. Studies dealing with biosynthese in RMS by various lipase species showed that the esterification

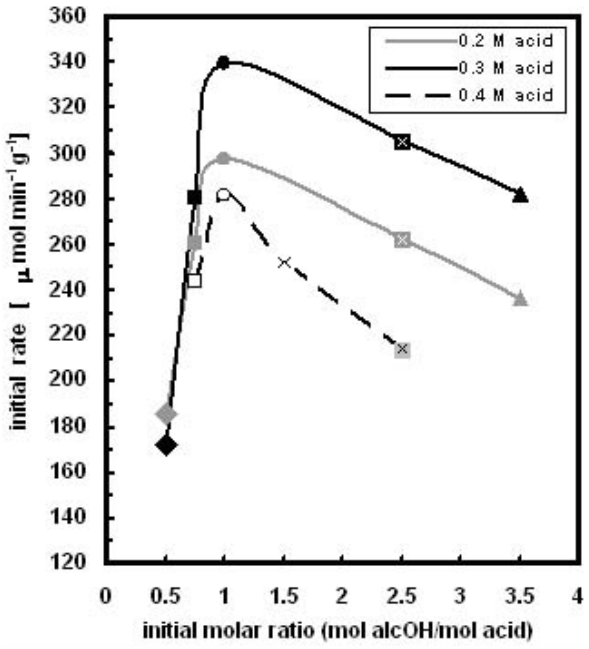

Figure 3. Effect of the initial substrate molar ratio on the initial esterification rate (Wo=30, [lipase] = 3 g dm^{-3})

proceeded according to the Ping–Pong Bi–Bi mechanism [19,20] although this was not the general case [21]. In the current system lipase–catalysis was the fastest at the stoichiometric ratio of the substrates. We have not yet met in the literature such a simple fact regarding non-aqueous enzymology and the kinetics of this reaction deserve further attention. Figure 4 shows that regardless of the slower initial rates at 0.4 mol dm^{-3} of oleic acid, the yields achieved in a 5–hour

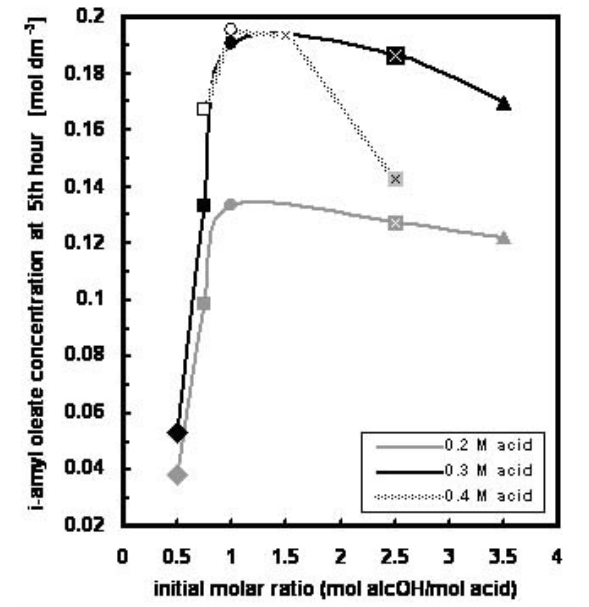

Figure 4. Effect of the initial substrate molar ratio on the i–amyl oleate concentration yielded in five hours (Wo=30, [lipase] = 3 g dm^{-3})

esterification can be as high as those obtained at no acid–level inhibition (0.3 mol dm^{-3}).

3. Effect of the organic solvent used (its polarity and structure)

Several correlations have been established between the solvent properties and the enzyme behavior and stability. Usually, the effect is related to the *logP*–value of the solvent (*logarithm of the partition coefficient of the organic solvent in the system octanol/water*). In RMS there are two aspects of the solvent effect on the reaction rate. The first is the solvent compatibility with the surfactant and biocatalyst (detriment). Another, the physical properties of the solvent determine the aggregation number of the surfactant in RMS [16] and, thus, the effective concentrations of the substrates in the microenvironment that surrounds the enzyme. It has also been shown that the solvent medium affects the orientation of the acyl–substrate with respect to the hydrophobic binding pocket of the lipase active site [22]. As may be seen in Figure 5 when normal alkanes up to *n*–C_{10} were used, the initial esterification rate increased almost linearly with *logP*. This fact might be attributed to the effect of the solvent chain length on the aggregation behavior of the surfactant. R_{RM} was found to increase slightly with carbon number and the effect became negligible in solvents longer than *n*–C_8 [23]. In the can of *n*–hexane, the esterification was not initialized, possibly due to the disposition of the *n*–hexyl–moiety in the lipase hydrophobic groove which blocks the active site [24]. Cyclo– or branched alkanes, in spite of their lower *logP*–values, obviously provided better surfactant packing with respect to the accommodation of both the lipase and the substrates for each other and the highest rates were attained. *i*–Octane and *cyclo*hexane were also reported to be more suitable solvents for the esterification reactions catalyzed by surfactant–coated *Candida rugosa* lipase [25], as well as for lipase–catalyzed hydrolysis in RMS.

4. Optimization of the RMS at high oleic acid concentration

This investigation represents an attempt intensity the reaction rate and improve the ester yield at the acid level, 0.4 mol dm^{-3} considered inhibiting in RMS (Figure 3). The effect of the other system constituents, the substrate initial molar ratio, Wo and lipase concentration in the RMS, was studied. The initial rates of the esterification obtained at variable initial molar ratios of {*i*–amyl alcohol}/{oleic acid} for different Wo are plotted in Figure 6, while Figure 7 shows the concentration of the ester–product (*i*–amyl oleate) accumulated in the RMS in five hours.

As may be seen (Figure 6) a 4–unit increase in Wo, 30 → 34 caused an acceleration in the initial rate and the positive deviance was greater as more alcohol was

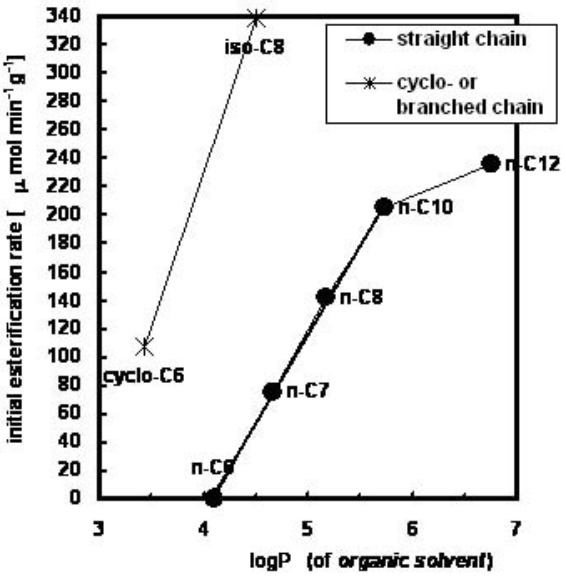

Figure 5. Effect of the organic solvent used on the initial esterification rate. Representation in terms of the logP–value of the organic solvent, carbon chain length and structure (initial ratio {i–amylOH}/{oleic acid} = 1, [oleic acid] = 0.3 mol dm^{-3}, Wo=30, [lipase] = 3 g dm^{-3})

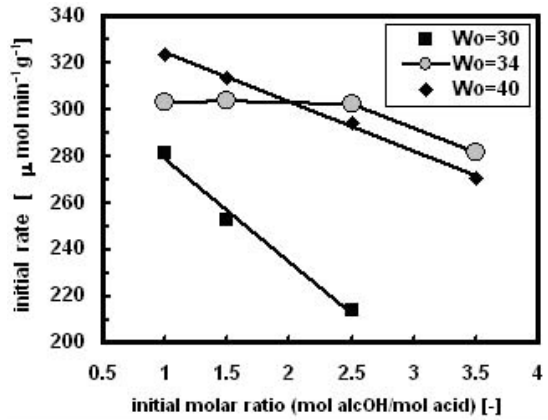

Figure 6. Effect of the initial Wo on the initial rate at different initial substrate molar ratios ([oleic acid] = 0.4 mol dm^{-3}, [lipase] = 3 g dm^{-3}, i–octane)

present in the RMS. Nevertheless, in general, with increasing the initial molar ratio of the substrates above 1, the reaction rate slowed down. Here, except the possible limitations imposed by the esterification reaction specificity ("biocatalytical" point of view), another reason, from the "medium" point of view, might also contribute. It was established that when increasing the co–surfactant (alcohol) concentration in the RMS, smaller micelles were formed [26]. This effect in combination with the reduced Wo probably suppressed the catalytic activity of the incorporated enzyme.

The data for the substrate initial molar ratio 2.5 could be compared with those obtained at a two times lower oleic acid level of 0.2 mol dm^{-3} (Figure 2). The

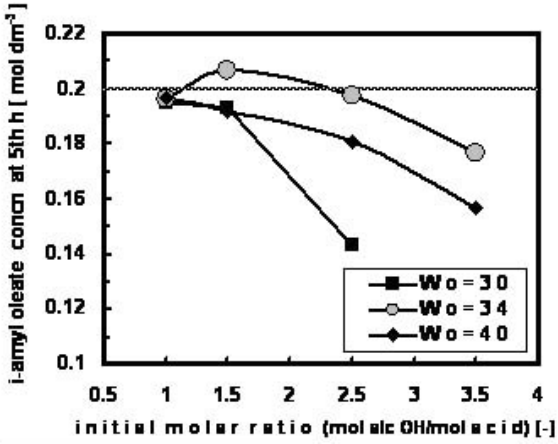

Figure 7. Effect of the initial Wo on the i–amyl oleate concentration yielded in five hours at different substrate ratios ([oleic acid] = 0.4 mol dm^{-3}, [lipase] = 3 g dm^{-3}, i–octane)

increase in the initial Wo 30 → 40 (0.4 mol dm^{-3} oleic acid) accelerated the initial esterification rate as well as it was in the respective Wo–transition, 15 → 20 (0.2 mol dm^{-3}, Figure 2). The ester–concentrations achieved in five hours were also doubly reciprocal. It may be concluded that the change in Wo directly affected the effective concentrations of both substrates, which reacted through the lipase enzyme. The values of the initial Wo 34 ÷ 40 appeared optimal in the wide range of the initial substrate ratios, 1.0 ÷ 2.5, where the highest initial rates of the synthesis were obtained, ≥300 μmol min^{-1} g^{-1}. Moreover, at Wo 34 the highest ester yields were accumulated in the RMS (Figure 7).

The increase in the lipase concentration per unit volume of RMS (Wo 34) caused a decrease in the rate at each substrate ratio (data not shown).

CONCLUDING REMARKS

The object of this study was the lipase–catalyzed esterification of a fatty (oleic) acid with a short–chain (*i*–amyl) alcohol and its performance in a reversed micellar system (RMS). The RMS–structure allows a heterogeneous synthesis in one macro–phase wherein the "water/oil" interface is distributed at a nano–dimension. Thus, the inherent b*i*–phasic problems of solubility and the enzyme–substrate contact could be solved on a molecular scale.

The study represented some kinetic investigations of the influence of the RMS–constituents on the process characteristics of *i*–amyl oleate biosynthesis. The following dependences were described and discussed in terms of the RM–structure:

- The initial substrate molar ratio at different levels of the acyl–agent – the inhibiting concentrations were determined.
- The effect of the polarity (*logP*) and the structure of the organic solvent used as the continuum. A linear function of the initial rate on arg(*logP*) was established

for the alkanes up to n–C_{10}. The highest rates were measured in a medium of the cyclo– or branched alkanes.

- The esterification at inhibiting substrate concentrations could be intensified by a small change in the initial water content (W_o), thus improving the enzyme RM–accommodation and manipulating the effective concentrations of both substrates around the lipase.

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IZVOD

ESTERIFIKACIJA KATALIZOVANA LIPAZAMA U REAKCIONOM SISTEMU SA REVERSNIM MICELAMA

(Naučni rad)

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U radu je proučavana sinteza i–amil oleata u sistemu sa reverznim micelama (RM) korišćenjem katjonske površinski aktivne materije (KPAM). Uticaj osnovnih konstituenata RM sistema na biokatalitičku aktivnost lipaze (početna brzina esterifikacije, stepen konverzije) analizirana je i diskutovana u svetlu RM strukture. Početni udeo vode u RM sistemu (odnos vode i površinski aktivne amterije, Wo) utiče na parametre brzine reakcije (princip zaštitnog zvona) sa maksimumom koji zavisi od prisutne količine (udela u reakcionoj smeši) alkohola. Utvrđeno je da je optimalan odnos Wo oko 34 do 40 za sve ispitivane odnose supstrata. Polarnost i struktura organskog rastvarača (log P), koji je kontinualna faza u RM sistemu ima velikog uticaja na kinetičke parametre. Pokazano je da postoji linearna zavisnost između početne brzine i alkana (sve do n–C10). Najveće izmerene brzine su u slučaju primene medijuma zasnovanog na ciklo ili račvastim alkanima. Esterifikacija koja je inhibirana prisustvom supstrata može se ubrzati malim promenama odnosa Wo čime se povećava adaptacija enzima u RM sistemu i stvarno uvećava koncentracija oba supstrata u blizini lipaze. Maksimalna brzina esterifikacije i–amil oleata katalizovane lipazom je ograničena na oko 340 mmol/g.min kada je koncentracija dodate KPAM 0,115 mol/dm^3.

Ključne reči: Esterifikacija, Lipaze, i–amil oleat, Reverzne micele, Katjonska površinski aktivna materija, Organski rastvarač.