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Studies on the specificity of *Candida rugosa* lipase catalyzed esterification reactions in organic media

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Abstract: In this study, the feasibility of the synthesis of various flavor esters catalyzed by a commercial lipase from *Candida rugosa* was investigated and the process parameters were optimized. Lipase from *C. rugosa* successfully catalyzed the synthesis of 19 esters. The highest yields, of more than 90 % after 20 h, were observed in the synthesis of short-chain esters, pentyl propanoate, isopentyl butanoate, and butyl butanoate. Increasing the number of carbon atoms of both substrates above 8 caused a significant decrease of the initial reaction rates and the final yields. The enzyme showed surprisingly low affinity towards pentanoic acid and hexanoic acid, compared with the higher homologues, octanoic acid and decanoic acid. In addition to the number of carbon atoms, the structure of the substrates had a significant influence on the enzyme activity. Namely, the activity of the enzyme towards isopropanol was significantly lower compared with *n*-propanol. Additionally, *cis*-9-octadecenoic acid was a better substrate than octadecanoic acid, its saturated analogue.

Keywords: lipase, *Candida rugosa*, esterification, specificity.

INTRODUCTION

Flavor esters of short-chain or medium-chain fatty acids and alcohols are important aroma compounds used as flavor enhancers in the food industry and fragrances in the cosmetic industry.[1] Esters are usually manufactured by extraction from natural materials or by chemical means, which includes the use of homogenous acids as catalysts. The use of such aggressive catalysts leads to numerous problems, such as the corrosion of equipment, hazards of handling corrosive acids which are not reused, low yields and lack of selectivity, which necessitates extensive refining in order to remove off-colors and odors. On the other hand, esters extracted from plant materials are often too scarce or expensive for commercial use.[2]

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The drawbacks of commercially used methods, accompanied with the growing awareness of environmental protection and the importance of safety in food technology, induced numerous investigations of enzymatic procedures for the synthesis of flavor esters. For this purpose, lipases of microbial and animal origins were used. Since substantial yields of the products in lipase-catalyzed esterification can be achieved only in low-water environments, these reactions are usually performed in organic solvents or solvent-free systems.[3–5] A broad range of esters has been synthesized by various lipases and the observed yields of esters and initial rates of reaction differed strongly. The mechanism of enzymatic esterification was investigated in several studies and it was unambiguously determined that acylation, which results in the formation of an acyl-enzyme complex, is the first step of the reaction.[2,4,6] In order to maximize the yield of esters, serious attention was given to the optimization of the process parameters (such as enzyme concentration, molar ratio of substrates, and temperature), the development of appropriate kinetic models of esterification, and the monitoring and control of the water concentration in the reaction mixture.[1–7] The enantioselective catalytic properties of lipases make them very attractive for the synthesis of pharmaceutical products of enhanced activity.[8,9] Additionally, numerous attempts have been made in the field of immobilization of lipases in order to obtain highly active biocatalysts for multiple application.[1,10,11]

Studies of the influence of the alcohol and acid class on the yield of esters and initial rate of synthesis are scarce, and the obtained results differed strongly depending on the origin of enzyme and the reaction conditions. In a study by Okumura *et al.*, four microbial lipases exhibited strong affinity towards medium-chain, and particularly long-chain fatty acids.[12] In a study conducted with lipase from *Candida rugosa* in organic media, the initial rates of ester formation were significantly higher for tetradecanoic acid and octadecanoic acid compared with short-chain acids (C_2–C_5).[13] Additionally, the rate of formation of esters of *cis*-9-octadecenoic acid was five-times higher than the rate of formation of esters of the analogous saturated acid (octadecanoic acid), which indicates that lipase from *C. rugosa* has stronger affinity for unsaturated acids. On the other hand, Janssen *et al*. reported that, in experiments with the enzyme of same origin, the dependence of the specificity constant (V_m/K_M) on the fatty acid chain length was a bell-shaped function with a maximum at C_{10}.[14] In a study of the specificity of goat pregastric lipase, C_4–C_{16} fatty acids and C_2–C_{10} alcohols were used as substrates.[15] Increasing the number of carbon atoms in the fatty acid was found to lead to a continuous decrease of initial rate of ester formation. Alcohols showed a somewhat different influence in the examined range of number of carbon atoms. The maximum activity was reached with butanol, thereafter increasing the number of C-atoms in alcohols lowered the activity of the enzyme. The decrease of enzyme activity was even sharper when ethanol was applied, probably due to denaturation of the enzyme by ethanol.

Knowledge of the substrate specificity is necessary to develop an efficient lipase-catalyzed process. It is well known that the substrate specificity is largely dependent on the particular type of lipase, the nature of the reaction system (aqueous, organic solvent or two-phase system) and the process conditions. Although several authors have studied the specificity of the most cost effective lipase from *Candida rugosa*, full details on the specificity of lipase catalyzed esterification reactions in organic media are not available. The aim of this study was to determine the fatty acid and alcohol chain length specificity of the lipase from *Candida rugosa* in organic media. Various reaction parameters affecting the catalytic behavior of the enzyme, such as the structural characteristics of the substrate, the initial water concentration, temperature and the enzyme concentration have been examined.

EXPERIMENTAL

Materials

Candida rugosa lipase (triacylglycerol hydrolase, EC 3.1.1.3) was provided by Sigma (St. Louis, USA). The activity of enzyme was 860 U mg^{-1}, determined by the Sigma method, as previously described.[16] 2,2,4-trimethylpentane, *n*-propanol, isopropanol, *n*-butanol, 3-methyl-1-butanol, heptanol, octanol, dodecanol, propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, octanoic acid, and decanoic acid were provided by Fluka (Buchs, Switzerland). *n*-Pentanol and 2-methylpropanoic acid were provided by Farmitaliana Carlo Erba (Milano, Italy). Tetradecanoic acid, octadecanoic acid, and *cis*-9-octadecenoic acid were obtained from Sigma (St. Louis, USA). All chemicals were of 99 % or higher purity.

Esterification

Ester synthesis was carried out in stoppered flasks (100 ml) in 2,2,4-trimethylpentane. The reaction mixture containing enzyme and 0.25 M of both substrates was diluted up to a volume of 10 ml with 2,2,4-trimethylpentane and was incubated on a shaker at 150 rpm and at 45 ºC unless otherwise specified. All the experiments were carried out in duplicate.

Analyses

The progress of the esterification was monitored by the determination of the residual acid content by titration against sodium hydroxide using phenolphthalein as the indicator and a mixture of ethanol and diethyl ether (1:1) as the quenching agent. The ester formed was calculated as being equivalent to the acid consumed. The initial reaction rates were determined from the slope of the initial linear portions of the plots of ester concentration *vs*. time. The yield was calculated as the quotient of the ester concentration and the initial acid concentration.

RESULTS

The effects of the most important reaction parameters were studied on the synthesis of pentyl 2-methylpropanoate. Subsequently, the enzyme specificity was investigated at the optimum values of the examined parameters. The initial concentrations of both substrates (pentanol and 2-methylpropanoic acid) were 0.25 M in all experiments.

Effect of water

The effect of the water concentration on the lipase activity was studied in the range of 0–0.5 % (v/v). The reaction temperature was 45 ºC and the enzyme concentration was 0.5 % (w/v). The reaction curves are presented in Fig. 1. The high-

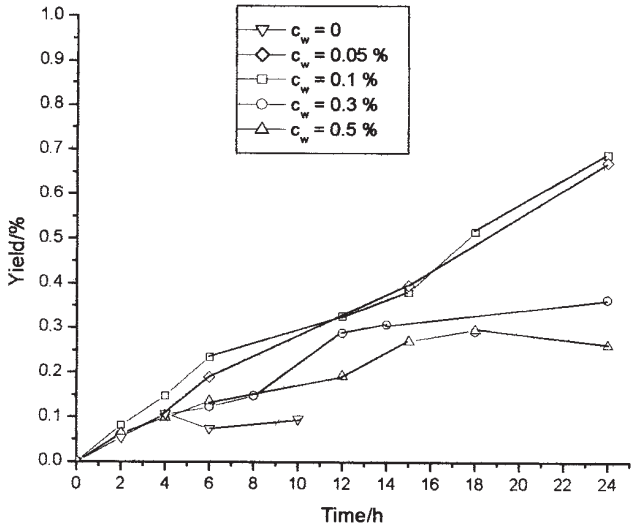

Fig. 1. The effect of the initial water concentration on the synthesis of pentyl 2-methylpropanoate.

est initial rate of synthesis of $9.22 \cdot 10^{-3}$ mol dm^{-3} h^{-1} was determined at a water concentration of 0.1 % (v/v). The initial rates of the ester synthesis at other water concentrations were in a narrow range, between $6.15 \cdot 10^{-3}$ mol dm^{-3} h^{-1} and $6.72 \cdot 10^{-3}$ mol dm^{-3} h^{-1}. The highest yield of esters of 68.8 % after 24 h was observed at a water concentration of 0.1 % (v/v). Therefore, all further experiments were performed at a water concentration of 0.1 % (v/v).

Effect of catalyst concentration

The effect of enzyme concentration was studied in the range of 0.1 – 0.5 % (w/v), at 45 ºC and 0.1 % of water. The results are illustrated in Fig. 2. It can be seen that there is a strong correlation between the concentration of the biocatalyst and

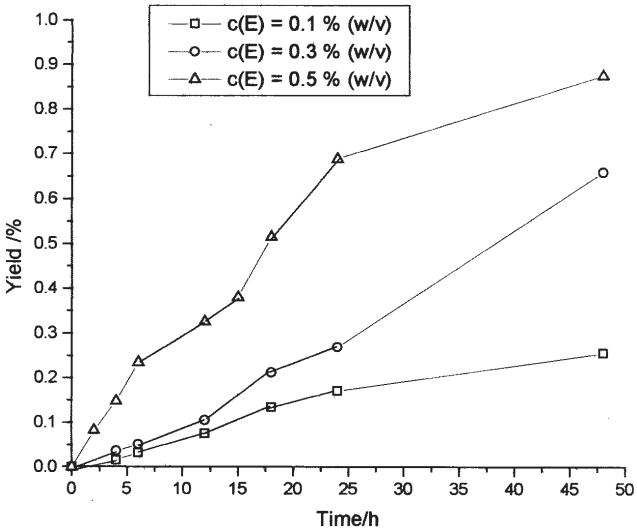

Fig. 2. The effect of the enzyme concentration on the synthesis of pentyl 2-methylpropanoate.

both the initial rate of ester synthesis and the yield of ester after 48 h. At the highest employed concentration of enzyme, the initial rate was $9.22 \cdot 10^{-3}$ mol dm^{-3} h^{-1}. Decreasing the enzyme concentration to 0.3 % and 0.1 % led to a substantial decrease of the initial rates, *i.e.*, to $2.25 \cdot 10^{-3}$ mol dm^{-3} h^{-1} and $9.38 \cdot 10^{-4}$ mol dm^{-3} h^{-1}, respectively. All further experiments were carried out with 0.5 (w/v) of lipase from *C. rugosa*.

Effect of temperature

The temperature of the ester synthesis was varied in the range of 35 – 55 ºC. The reaction curves are presented in Fig. 3. The highest initial rate ($9.22 \cdot 10^{-3}$ mol dm^{-3} h^{-1}) and yield of ester after 48 h (87.4 %) were observed in experiment performed at 45 ºC. Both a decrease and an increase of temperature by 10 ºC led to a

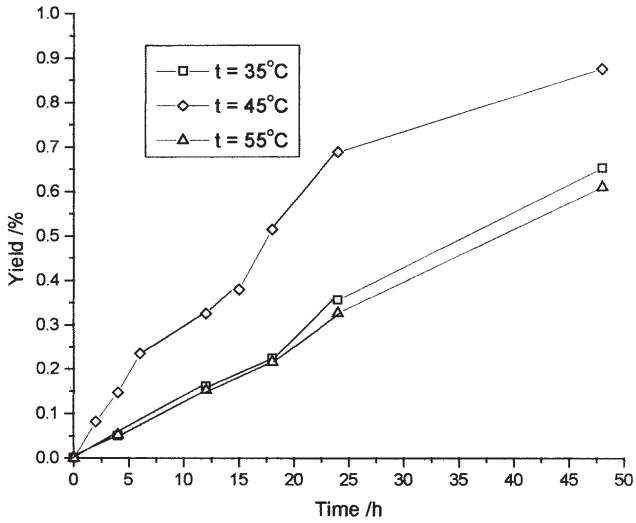

Fig. 3. The effect of temperature on the synthesis of pentyl 2-methylpropanoate.

decrease of the initial rate of ester synthesis ($3.12 \cdot 10^{-3}$ mol dm^{-3} h^{-1} and $3.25 \cdot 10^{-3}$ mol dm^{-3} h^{-1}, respectively) and of the yields of ester (65.2 % and 60.9 %, respectively). Therefore, all further experiments were carried out at 45 ºC.

The effect of fatty acid type

The time courses of several representative ester syntheses ($C_{3:0}$–$C_{6:0}$ acids) are shown in Fig. 4. The initial rates of esterification of straight chain saturated acids ($C_{3:0}$–$C_{18:0}$) and yields after 48 h are illustrated in Fig. 5. High yields of esters (above 90 %) were achieved when the acid donors were propanoic acid, butanoic acid and octanoic acid. Hexanoic acid and octadecanoic acid showed poor acyl donor properties, since yields of just below 15 % of the corresponding ester were reached after 48 h of reaction. A slightly different trend was observed when the initial rates of reaction were compared. The initial rate of the synthesis of propanoic acid ester was among the lowest observed despite the fact that the yield after 48 h

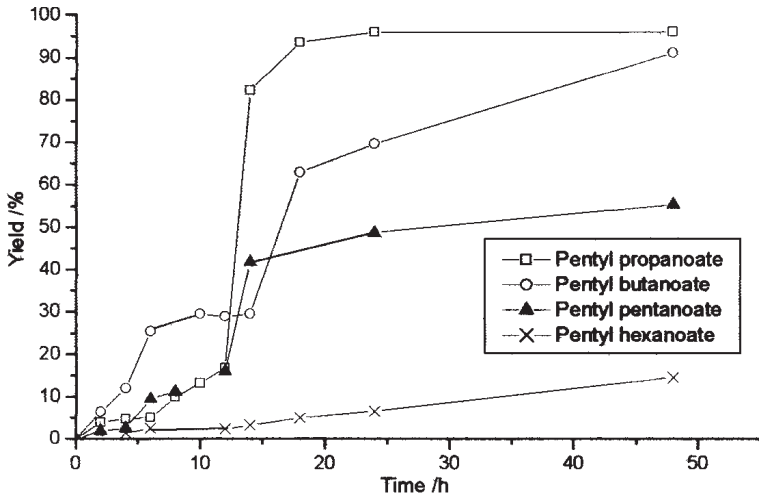

Fig. 4. Time courses of the synthesis of various *n*-pentyl esters.

was, on the contrary, one of the highest. Nevertheless, butanoic acid proved to be a good substrate for ester synthesis with the highest initial rate of ester synthesis of $7.5 \cdot 10^{-3}$ mol dm^{-3} h^{-1}.

Besides the length of the fatty acid, the effects of branching and the presence of double bonds were investigated. The synthesis of pentyl esters of octadecanoic and *cis*-9-octadecenoic acid were compared for the purpose of determining the effect of double bonds, while butanoic acid and 2-methylpropanoic acid were applied to elucidate the effect of the presence of a methyl group in the β-position of the fatty acids. The obtained results are illustrated in Fig. 6. The obtained results imply that branching in the β-position did not have a significant influence on the ester synthesis, since the outcomes of the reactions were only slightly different. On

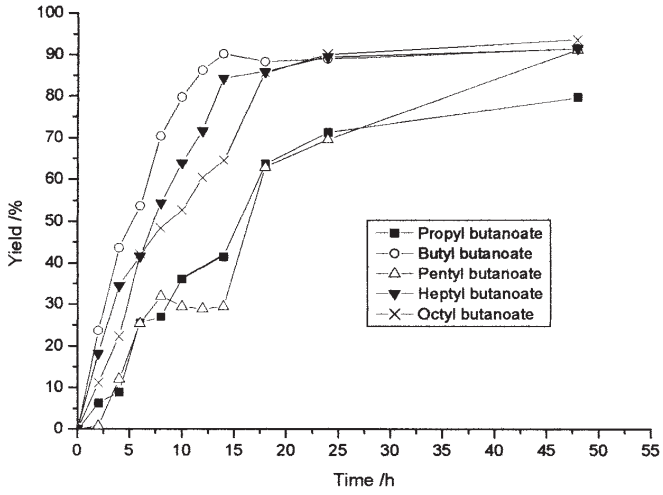

Fig. 5. The influence of the fatty acid chain length on the initial rate and final yield.

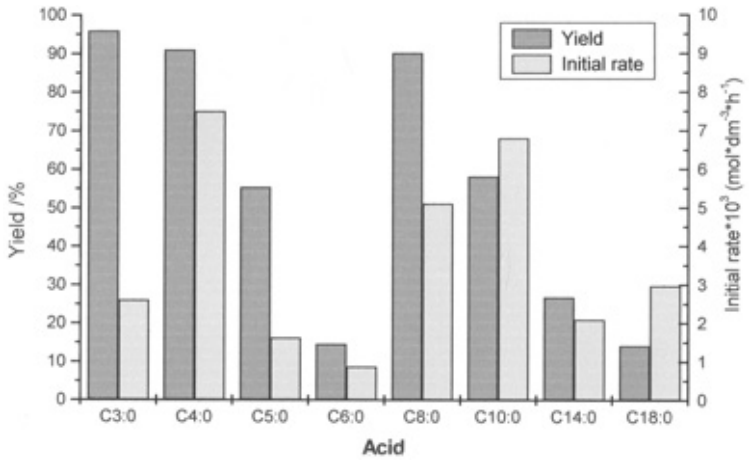

Fig. 6. The influence of the fatty acid structure on the initial rate and final yield.

the other hand, the synthesis of oleic acid ester was twice as fast as that of pentyl octadecanoate, which indicates that unsaturated acids have a stronger affinity towards the ester synthesis.

The effect of the alcohol type

All of the experiments were carried out with butanoic acid as the acyl-donor. The number of carbon atoms was varied in the range of $C_{3:0}$–$C_{12:0}$. The reaction curves of the synthesis of all of the esters of butanoic acid are illustrated in Fig. 7. The initial rates and yields of esters after 48 h are presented in Fig. 8. The time course of the ester synthesis has a similar profile for each of the examined ester, except pentyl butanoate (Fig. 7). The initial rate of this reaction is low, but after 18 hours of reaction a significant acceleration occurred which caused the final yield of ester to be high. High concentrations of esters (around 90 %) were achieved in the reactions with C_4–C_8 alcohols (Fig. 8). On the other hand, dodecanol was a poor substrate for esterification catalyzed by lipase from *C. rugosa*, with a yield of only 40 %. Compared to the yields after 48 h, the initial rates of ester synthesis had greater discrepancies. The highest rate of 29.5 ·

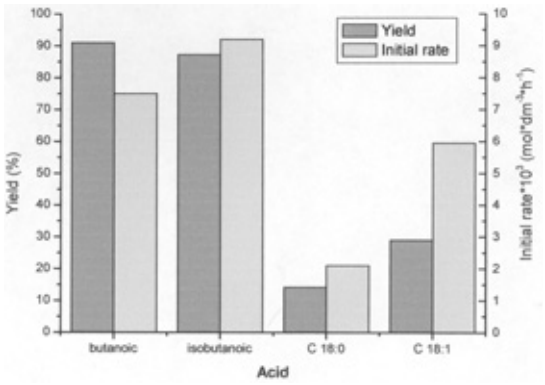

Fig. 7. Time courses of the synthesis of various esters of butanoic acid.

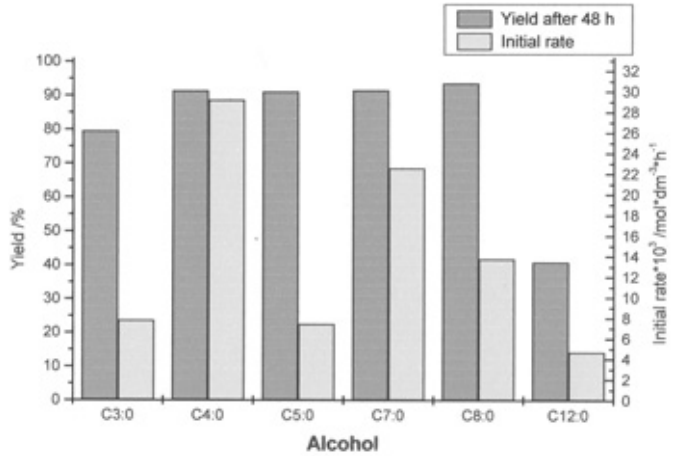

Fig. 8. The influence of the alcohol chain length on the initial rate and final yield.

10^{-3} mol $dm^{-3}h^{-1}$ was observed in the synthesis of butyl butanoate, which is 6-fold greater than the initial rate of synthesis of dodecyl butanoate.

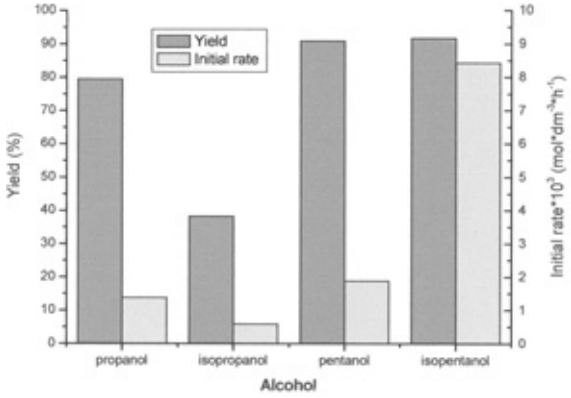

Fig. 9. The influence of the alcohol structure on the initial rate and final yield.

The influence of branching was investigated in two series of experiments: the influence of a methyl group in the α-position was investigated by comparing the syntheses of *n*-propyl butanoate and isopropyl butanoate, while the influence of β-substitution was investigated by comparing the syntheses of pentyl butanoate and isopentyl butanoate. Initial rates and yields (after 48 h) are shown in Fig. 9. Substitution of a hydrogen atom in the α-position led to a significant decrease of both the initial rate of esterification and the final yield (Fig. 9). On the other hand, the influence of β-substitution was more complex: the yield after 48 h was similar to the corresponding straight chain analogue, but the initial rate was 4.5 times higher.

DISCUSSION

In this study, lipase from *C. rugosa* was shown to have good catalytic properties in the synthesis of esters. The yields of esters and rates of ester formation were in the

proximity of results previously reported in related studies with enzymes of the same origin,[2,17] or even higher.[5] The obtained results showed that high yields of esters, higher than 90 %, could be achieved within the first 20 hours for several of the prepared esters – pentyl propanoate, 3-methylbutyl butanoate, and butyl butanoate.

The obtained optimum temperature and enzyme concentration are within a narrow range of the usual optimum values of these parameters for esterification catalyzed by free lipases.[4] On the other hand, the differences between the optimum water concentrations in various studies are significantly larger, and the water amounts cover an enormous range of values, 0.05 – 5 % (v/v).[2,4,17] A certain amount of water is necessary for keeping the enzyme in its active conformation, but a high water concentration promotes hydrolysis of the formed esters. It can be seen (Fig. 1) that an initial water concentration of 0.1 % (v/v) is optimum concentration during whole time course of the reaction, but also that, in the later stages of the reaction, the yield of ester at a water level of 0.05 % (v/v) is very close to the optimum curve. It is plausible that with an initial concentration of 0.05 %, the water created in the reaction increased the hydration and flexibility of the enzyme, which resulted in enhanced activity in the further course of the reaction.

The most interesting result of the part of study focused on the influence of the chain-length of the fatty acid was the decrease of enzyme activity in the C_5–C_6 region (Fig. 5). In the majority of studies of the lipase specificity in ester synthesis, somewhat simpler trends have been noticed. Pregastric goat lipase[15] showed grater affinity towards short-chain fatty acids, while lipase from *C. rugosa*[5,13] and *Candida* sp.[18] showed greater affinity towards long-chain fatty acids. However, a low activity of lipase from *C. rugosa* towards hexanoic acid was noticed by Janssen *et al*.[14] and Parida and Dordick.[19] Their explanations of this phenomenon differ: Janssen *et al.* ascribed it to the thermodynamics of reaction, *i.e.* to the slow conversion of the acyl-enzyme complex, while Parida and Dordick made the assumption that lipase has two acyl–binding pockets–one for binding fatty acids with a chain length below six, and the other one for binding fatty acids with more than six carbon atoms. The discrepancy between the low initial rate of synthesis and the high yields of propanoic acid esters is probably due to the fact that propanoic acid has hydrophilic properties. In the first stage of the reaction, propanoic acid withdraws water from the enzyme, which consequently, lowers its activity. However with the progress of the reaction, the water concentration increased, due to the fact that water is a reaction product, and ensures the full realization of the catalytic properties of the enzyme.

A higher activity towards *cis*-9-octadecenoic acid, compared with saturated long-chain fatty acids (C_{14}–C_{18}), was noticed in previous studies conducted with lipase from *C. rugosa*[13] and lipase from *Candida* sp.[18] In addition to the difference in the kinetic properties of the substrates, this discrepancy is sometimes attributed to the better solubility of *cis*-9-octadecenoic acid.[13]

The results referring to the synthesis of various esters of butanoic acid showed that lipase from *C. rugosa* has a wide specificity towards alcohols. High activities of the enzyme have been observed with C_4–C_8 alcohols. However, when propanol was used as the nucleophile, the rate of formation of ester was lower, probably due to the destruction of the microaqueous layer around the enzyme by the hydrophilic substrate. This kind of behavior was previously observed with ethanoic acid and ethanol as substrate for lipase-catalyzed esterification.[15,20–21] On the other hand, ester synthesis with dodecanol was even slower than with propanol. The reasons for this phenomenon are probably somewhat different. Namely, since alcohols react with the acyl-enzyme intermediate formed during the acylation process, the rate of synthesis is determined by the diffusion of alcohol molecules into the active site of the enzyme. Since smaller alcohols are able to diffuse into the active site more readily than bulky ones, an increase of the chain length leads to a decrease of the enzyme activity. Such a trend was noticed in previous studies with lipases from *M. miehei*, *Aspergillus*, *C. rugosa*, *Rhizopus arrhizus*,[22] and goat pregastric lipase,[15] but the optimum activity zone was slightly narrower (C_4–C_6).

Steric effects have a great influence on the activity of lipase in the synthesis of ester, as can be seen in Fig. 9. This influence was the most pronounced when a substrate with a methyl group in the α-position was applied. In this case, both the initial rate and the final yield were significantly decreased compared with the straight chain analogue (*n*-propanol), probably due to significant steric hindrance by the methyl group in the proximity of the hydroxyl group. This kind of behavior has been observed in previous studies focused on the effect of the class of alcohol on ester synthesis with pregastric goat lipase[15] and immobilized commercial lipase (Novo SP 344®).[23] The presence of a methyl group in the β-position had a partial effect, *i.e.,* it led only to an increase of the initial rate of ester synthesis, while the final yield was the same as in the case of the synthesis using amyl ester. Such catalytic properties were observed only in a study of ester synthesis using four different microbial lipases, thus higher yields of 2-methylpropyl *cis*-9-octadecenoate than butyl *cis*-9-octadecenoate were obtained with lipases from *Aspergillus niger* and *Geotrichum candidum*,[12] while pregastric goat lipase showed equal activity towards pentanol and 2-methylbutanol.[15] It can also be noticed that the initial rate of 2-methylbutanol ester synthesis was even slightly higher than that of butyl ester synthesis (Figs. 7 and 9). It is plausible that 2-methylbutanol, being a shorter molecule, can approach more easily the active site of the enzyme than pentanol.

It can be concluded that lipase from *C. rugosa* successfully catalyzed the formation of flavor esters in high yields. The highest enzyme activity was observed in the syntheses of low molecular weight esters, such as pentyl propanoate, isopentyl butanoate, and butyl butanoate. Butanoic acid was the best fatty acid, while butanol and 2-methylbutanol were the best nucleophilic substrates. The presence of a double bond in the fatty acid molecule led to significant increase of the enzyme acitivty.

ИЗВОД

ИСПИТИВАЊЕ СПЕЦИФИЧНОСТИ ЛИПАЗЕ ИЗ *Candida rugosa* У РЕАКЦИЈИ ЕСТЕРИФИКАЦИЈЕ У ОРГАНСКОМ РАСТВАРАЧУ

ДЕЈАН БЕЗБРАДИЦА, ИВАНА КАРАЛАЗИЋ, НЕВЕНА ОГЊАНОВИЋ, ДУШАН МИЈИН, СЛАВИЦА ШИЛЕР-МАРИНКОВИЋ И ЗОРИЦА КНЕЖЕВИЋ

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Циљ овог рада је био испитивање могућности синтезе различитих естара помоћу липазе из *Candida rugosa* као и оптимизација процесних параметара. Показано је да се дата липаза може користити као ефикасан биокатализатор за добијање деветнаест различитих естара. Највећи приноси, већи од 90 %, добијени су у случају синтезе нижих естара као што су пентил-пропаноат, 2-метилбутил-бутаноат и бутил-бутаноат. Почетна брзина синтезе естара и крајњи приноси били су знатно нижи са супстратима који садрже више од 8 угљеникових атома. Ензим је показао изненађујуће мали афинитет према пентанској и хексанској киселини, у поређењу са вишим хомолозима, октанском и деканском. Поред броја угљеникових атома, на активност ензима значајно је утицало присуство двоструке везе у молекулу супстрата и разгранатост супстрата. Наиме, липаза из *C. rugosa* испољила је много мању активност у реакцији са изопропанолом него са пропанолом, док је показала много већи афинитет према 9-*cis*-октадеценској киселини у односу на њен засићени аналог, октадеканску киселину.

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