Application of Lipases from *Candida rugosa* in the Enantioselective Esterification of (*R,S*)-*Ibuprofen*

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Abstract: Three commercially available lipases from *Candida rugosa* (OF and MY from Meito Sangyo Co., and CRL from Sigma-Aldrich Co.) were used for the enantioselective esterification reaction of (*R,S*)-ibuprofen with 1-propanol and 2-propanol in saturated cyclohexane as reaction medium. All tested lipases preferentially catalysed the esterification of the *S*-enantiomer of ibuprofen. However, each one of the analysed lipases demonstrated differences in the catalytic activity. Lipase OF showed the highest conversion degree, and the best enantioselectivity was observed for MY and CRL lipases. The influence of temperature, reaction time and addition of *N,N'*-dicyclohexylcarbodiimide (DCC) on the enantioselectivity and on the conversion degree in the enzymatic esterification was studied and the optimal condition for enantioselective esterification was evaluated. Moreover, the application of new commercial cellulose-based tris(3,5-dimethylphenylcarbamate) HPLC chiral column was demonstrated for effective separation, qualification and quantification of both substrates and products within one chromatographic analysis.

Keywords: Esterification, biocatalytic reaction, resolution, (*R,S*)-ibuprofen, *Candida rugosa* lipase.

INTRODUCTION

Lipases are very suitable enzymes for organic synthesis thanks to their capacity of catalysing different reactions such as asymmetric esterification, asymmetric hydrolysis and asymmetric transesterifiaction [1,2]. These enzymes have been applied in the resolution of racemic mixture for the preparation of optically pure compounds. The active centre of these enzymes builds a specific environment, that is able to distinguish between the enantiomers [3]. It is demonstrated, that lipases can exist in two different forms, closed and open. The first one is inactive form, where by a polypeptide chain called 'lid' the active centre of the lipase is secluded from the reaction medium. The second one is considered to be active, because the 'lid' is displaced and the active centre exposed to the reaction medium [4]. Over the application of these enzymes, it has been proved that lipases act at the interface between hydrophobic and hydrophilic regions and water content is one of the most important factors affecting the enantioselectivity of lipases. The small amount of water is needed to retain their active three-dimentional conformation state, stability and active site polarity [5]. Furthermore, it should be considered, that lipases tolerate a great number of non-natural substrates, are stable and active in organic solvents and necessitate no cofactors. Based on the previous reports, it seems to be obvious, that use of these enzymes as biocatalysts in the industry will be continuously increasing due to their high activity and low price [6-9].

2-Arylopropionic acids (profens) are known as major nonsteroidal anti-inflammatory drugs (NSAID) used in the treatment of headache, rheumatoid arthritis, cephalgia, muscular strain [10-12]. All those profen drugs have the chiral carbon atom within the propionic acid moiety. One of the most frequently used drugs within this therapeutic group is ibuprofen [13]. The pharmacological activity of ibuprofen results mainly from the (*S*)-enantiomer, which is 160 times more active than its antipode in the *in vitro* inhibiting prostaglandin synthesis [14,15]. What is more, from the pharmacological studies results, that the accumulation of *R*-ibuprofen will cause serious side effects on the human organism such as gastrointestinal pain [16,17], as well as production of 'hybrid' triglycerides between (*R*)- ibuprofen and Coenzyme A, which disturb normal membrane function and lipid metabolism. Thus, an important effort has been done to synthesise optically pure (*S*)-enantiomer [18]. Facing these facts, the two enantiomers of ibuprofen can be regarded as two different drugs thanks to the difference in their pharmacological properties [19]. Moreover, the application of the pure (*S*)-enantiomer instead of racemic ibuprofen allows to reduce the amount of total drug prescribed to achieve the expected therapeutic effect [20].

Nowadays, immobilization methods of lipases on different supports are developed, due to the possibility to reuse them since they can be easily recovered from the reaction medium. Additionally, immobilization of lipases improves their stability and facilitates to control water activity, which is very important factor in activity of these enzymes. From economical point of view the use of immobilized lipases as biocatalyst is favorable, because it allows to reduce cost of the enzymatic reactions [21-23]. However, the application of crude lipases is better in the preliminary research of the enzymatic activity in various reaction medium, because it gives a possibility to select enzymes with high catalytic activity, which can be immobilized on different supports. Therefore, this step is very important in the development new procedures with the use of immobilized lipases as biocatalysts.

In this work, we compared catalytic activity of 3 *Candida rugosa* lipases, used to obtain the *S*-enantiomer of ibuprofen. We reported on the enantioselective HPLC sampling of lipase-catalysed enantioselective access to enantiomerically pure ibuprofen. We showed the ability of these lipases to resolve (*R,S*)-ibuprofen enantiomers by esterification reaction with primary and secondary alcohols. Additionally, we presented the influence of temperature, reac-

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tion time and adding the DCC, on the conversion degree and on the enantioselectivity in the enzymatic esterification reaction of (*R,S*)-ibuprofen. The study also involves the optimization of enantioselective chromatographic process resolution of (*R,S*)-ibuprofen with the use of new commercial cellulose-based tris(3,5-dimethylphenylcarbamate) HPLC column. The optimization of chromatographic conditions such as suitable selection of flow rate and mobile phase, concentration and volume of the injected analytes allowed for a good resolution of both substrates and products during one chromatographic analysis.

MATERIALS AND METHODS

Enzymes

Lipase CRL type VII from *Candida rugosa* (activity ≥700 units/mg solid) was from Sigma-Aldrich Co. (Poland). Lipase OF from *Candida rugosa* (activity 380 000 units/g solid) and Lipase MY from *Candida rugosa* (activity 32 000 units/g solid) were a gift from Meito Sangyo Co., LTD. (Japan).

Chemicals

Racemic (*R*,S)- ibuprofen and pure *S*(+)- enantiomer were purchased from Sigma-Aldrich Co. (Poland). 2-propanol, 1-propanol, cyclohexane, *n*-hexane, acetic acid and water were purchased from POCH S.A. (Poland).

Instrumentation

The Shimadzu HPLC system (Japan) equipped with two solvent pumps model LC-20AD, UV-VIS detector model SPD-20A, degasser model DGU-20A_5, an autosampler model SIL-20AC_{HT} and a column oven model CTO-10AS_{VP}. Chiral Lux Cellulose-1 (4.6mm x 250 mm x 5µm) column with tris(3,5-dimethylphenylcarbamate) stationary phase and Guard Cardridge System model KJO-4282 were purchased from Phenomenex Co.

Analysis of Ibuprofen

The enantiomeric excess of the substrate (ee_s) and the product (ee_p) as well as the conversion (c) and enantioselectivity (E) were calculated as below [24,25]:

Enantioselectivity (E):

$$E = \frac{\ln[(1- c)(1- ee_s)]}{\ln[(1- c)(1+ ee_s)]} \quad (1)$$

The ee_s and ee_p values:

$$ee_s = \frac{R - S}{R + S} \quad (2)$$

$$ee_p = \frac{R - S}{R + S} \quad (3)$$

For R>S

where S and R represent the chromatographic peak areas of the *S*- and *R*- enantiomers, respectively. The amount of ibuprofen and ibuprofen esters was expressed by the value of the chromatographic peak areas.

The conversion (c):

$$c = \frac{ee_s}{ee_s + ee_p} \quad (4)$$

Additionally, in the presented results the ee_s and ee_p values are expressed in percentage using equations:

$$ee_s = \frac{R - S}{R + S} \times 100 \quad (5)$$

$$ee_p = \frac{R - S}{R + S} \times 100 \quad (6)$$

The concentration (c) is also expressed in percentage, using (5) and (6) for calculation of conversion equations.

Chromatographic Conditions

The effect of different compositions of mobile phase consisted with three compounds: *n*-hexane, 2-propanol and acetic acid on the separation selectivity of both substrates and products was investigated. Finally, the most appropriate chromatographic conditions were optimised with *n*-hexane/2-propanol/ acetic acid (99.6/0.4/0.15 v/v/v) or (99.9/0.1/0.15 v/v/v) mobile phase with flow rate of 1 mL/min for 1-propanol and 2-propanol as acyl acceptors, respectively. In order to obtain optimum HPLC conditions for separation of (*R*,*S*)-ibuprofen and its esters, three types of chiral chromatographic columns were investigated, including Lux Cellulose-1, Lux Cellulose-2 and Lux Cellulose-3 based on the cellulose tris(3,5-dimethylphenylcarbamate), cellulose tris(3-chloro-4-methylphenylcarbamate) and cellulose tris(4-methylbenzoate), respectively. Lux Cellulose-2 and Lux Cellulose-3 have not proved to be suitable for this purpose, because of the insufficient chromatographic resolution of enantiomers. The use of Lux Cellulose-1 (4.6mm x 250 mm x 5µm) HPLC column gave enhanced resolution for a studied compounds. The chromatographic process was operated at 30°C, due to the better mass transfer and lower viscosity of the eluent. The detection UV wavelength was 254 nm. The representative chromatogram of sufficient separation of *R*- and *S*-enantiomer of ester and *R*- and *S*-ibuprofen in a single run is demonstrated in Fig. (**1**).

Lipase-Catalysed Esterification of (*R,S*)-*Ibuprofen*

The reaction mixture was composed of saturated cyclohexane (625 µL), racemic ibuprofen (2.58 mg, 12.49 µM) and 1-propanol (5.64 µL, 75.02 µM) or 2-propanol (5.77 µL, 74.99 µM) as acyl acceptors. The reaction was started by adding crude (free) lipase (10mg/mL) to the solution. The suspension was incubated at 25°C, 37°C, 45°C and 55°C, shaken (600rpm) in a Thermomixer (Thermomixer comfort from Eppendorf Co.) for a 96 hours. The samples (50uL) were withdrawn at equal time intervals (every 24h), and were than centrifugated (centrifuge minispin plus from Eppendorf Co.). The collected supernatant was removed by evaporation at room temperature and the residue was dissolved in 0.7 mL mobile phase and injected into HPLC. The esterification reaction of racemic ibuprofen with 1-propanol or 2-propanol is shown in Scheme **1**.

RESULTS AND DISCUSSION

Influence of Time Reaction

Table **1** shows the enantiomeric excess of the substrate (ee_s), the conversion (c) and the enantioselectivity (E-value) as a function of

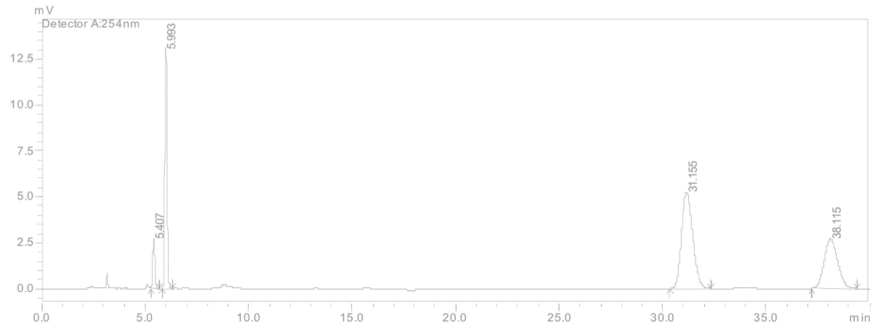
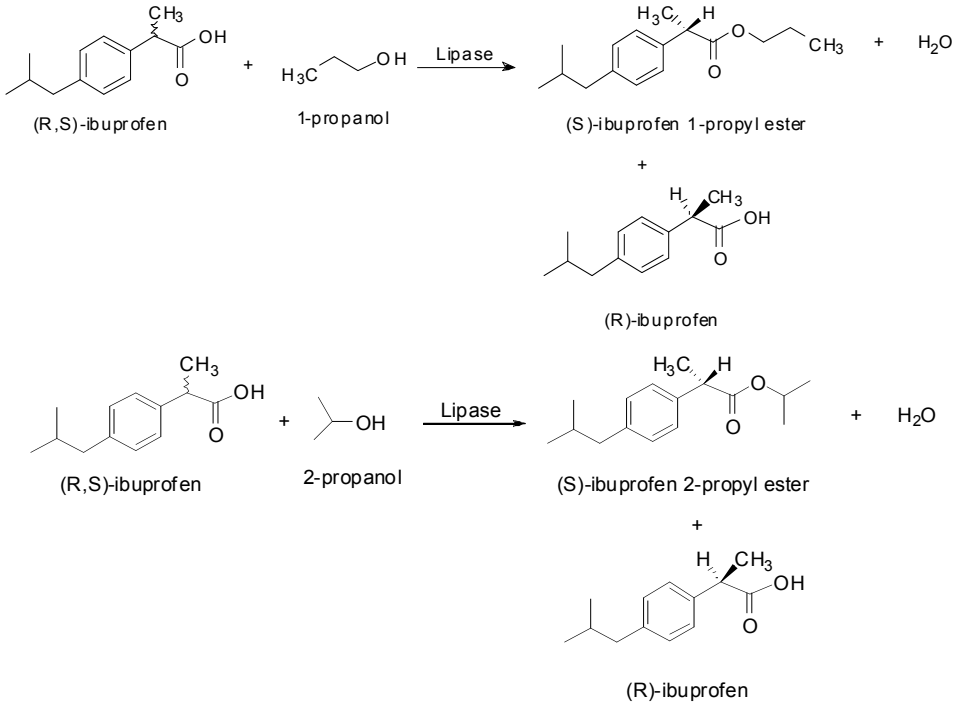


Fig. (1). Representative HPLC chromatogram of ibuprofen and 1-propyl esters of ibuprofen with retention time of *R*-enantiomer of ester (t_R=5.407), *S*-enantiomer of ester (t_R=5.993), *R*-ibuprofen (t_R=31.155) and *S*-ibuprofen (t_R=38.115); HPLC conditions Lux Cellulose-1 (4.6mm x 250 mm x 5μm) HPLC; mobile phase: *n*-hexane/2-propanol/ acetic acid (99.6/0.4/0.15 v/v/v), F=1mL/min, UV=254 nm.



Scheme 1. Biocatalysed esterification of (*R*,*S*)-ibuprofen with 1-propanol and 2-propanol.

Table 1. Influence of Reaction Time on the Esterification Reaction of (*R*,*S*)-ibuprofen catalysed by *Candida rugosa* lipase

Reaction Time [h]	Lipase OF *C.rugosa*			Lipase MY *C.rugosa*			Lipase CRL *C.rugosa*		
	C(%)	ee_s(%)	E	C(%)	ee_s(%)	E	C(%)	ee_s(%)	E
24	27	26	6.9	2	1	13.5	4	4	13.1
48	51	63	7.3	4	3	11.8	11	11	13.6
72	68	94	8.6	7	6	12.5	19	19	14.2
96	76	98	7	13	13	12.9	28	32	14.4

Reaction conditions: (*R*,*S*)-ibuprofen (12.49μM), 1-propanol (75.02μM), lipase (10mg/mL), cyclohexane saturated (625uL), temp. 45°C ; C-conversion, ee_s - enantiomeric excess, E - enantiomeric ratio.

time reaction. In this esterification reaction conducted in 45°C, there was used lipase from *Candida rugosa* as catalyst in a saturated cyclohexane.

Lipase of from *C. rugosa* has the highest conversion degree (76% after 96h), with enantiomeric excess of substrate equal 98%. It should be emphasised, that despite of high conversion achieved by the use of this lipase, the E-value is not high. Obtained results show, that the highest enantioselectivity has lipase CRL (E =14.4 after 96h), but the conversion degree of this lipase is much lower than of lipase OF.

It was demonstrated, that with increasing the time of esterification reaction, it comes to an increase of conversion and enan -

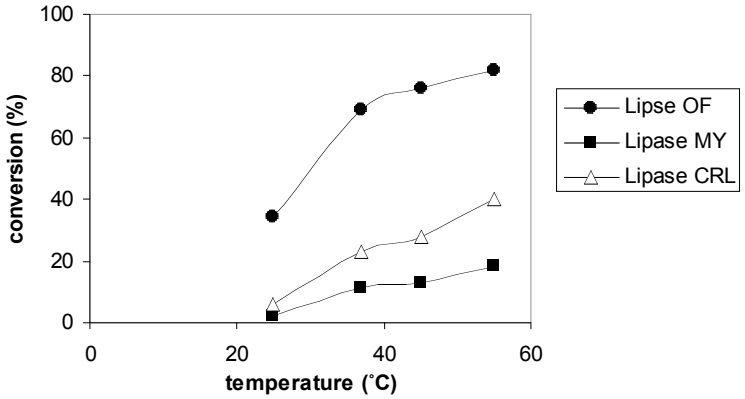


Fig. (2). Influence of temperature on the conversion (*R,S*)-ibuprofen. Reaction conditions: (*R,S*)-ibuprofen (12.49μM), 1-propanol (75.02μM), lipase (10mg/mL), saturated cyclohexane (625 μL), after 96h.

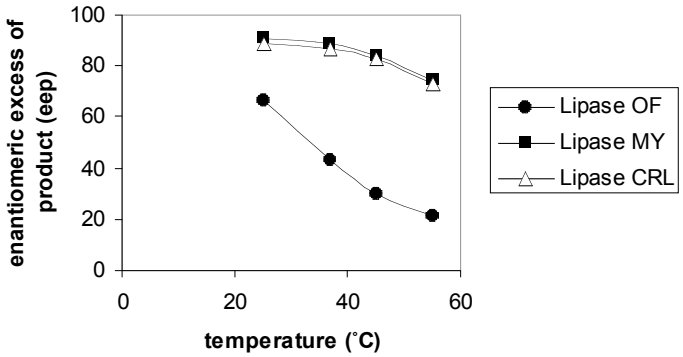


Fig. (3). Effect of temperature on the enantiomeric excess of products. Reaction conditions: (*R,S*)-ibuprofen (12.49μM), 1-propanol (75.02μM), lipase *C.rugosa* (10mg/mL), saturated cyclohexane (625 μL), after 96h.

tiomeric excess of acid, both in various intensity depending on the applied lipase. It was observed as well, that the lipase with the highest conversion degree (OF) showes the lowest enantioselectivity (E=7) in comparison with other analysed lipases.

Influence of Temperature

The influence of temperature on the conversion and enantioselectivity of esterification of ibuprofen was tested at temperature ranging from 25°C to 55°C (Fig. **2**). The study demonstrate, that the conversion of products increases with the increasing reaction temperature. However, increasing the reaction temperature results in a decrease of enzyme enantioselectivity. The reaction temperature of 55°C gave a maximum of conversion, but the enantiomeric ratios are lower than at 37°C (Fig. **3**). Analysing enantiomeric excess of esters, the highest values are observed at 25°C. However, the lower conversion makes this temperature not optimal for an efficient esterification reaction. Therefore, a compromise of conversion and the enzyme enantioselectivity in these reaction conditions is a temperature equal of 37°C.

Effect of Alcohol Moiety

A suitable selection of alcohol moiety has a significant influence on the conversion degree and on the enantioselectivity of esterification reaction. In this experiment (*R,S*)-ibuprofen was esterified by the use of primary and secondary alcohol. Study shows, that 1-propanol (primary alcohol) is a good substrate for the esterification of ibuprofen, due to the high conversion degree and enantiomeric ratio (Figs. **4**, **5**). Application of secondary alcohol (2-propanol) as substrate in esterification reaction gives much lower values of conversion and enantiomeric ratio, in comparison with the primary alcohol. Basing on the results, it is essential to apply the primary alcohol to achieve a high conversion degree and a good enantioselectivity when using the lipase from *Candida rugosa* as catalyst in enantioselective esterification.

Influence of DCC

An influence on the conversion degree and enantioselectivity of esterification reaction was tested by adding a *N,N*'-dicyclohexylcarbodiimide (DCC) into reaction mixture. The results show that conversion degree of estrification reaction when adding the DCC was higher after 8h in comparison to the results achieved after 96h in absence of this compound (Fig. **6**). However, the enantiomeric ratio (E) in the reaction when using DCC was at a very low level ($E \leq 1.2$). Finally, the addition of DCC to enzymatic estrification reaction with the use of tested lipases from *Candida rugosa* as biocatalysts improved the conversion, but reduced the enantioselectivity.

Selection of Lipases

Commercially available lipases from *Candida rugosa* were tested for their catalytic properties of enantioselective esterification of racemic ibuprofen with 1-propyl and 2-propyl alcohols using saturated cyclohexane as a solvent. Performed study proves the ability of these lipases to enantioselective catalysis of (*R,S*)-ibuprofen. All tested lipases preferentially catalysed the esterification of the *S*-enantiomer of ibuprofen, however, each one of the analysed lipases demonstrated differences in the catalytic activity.

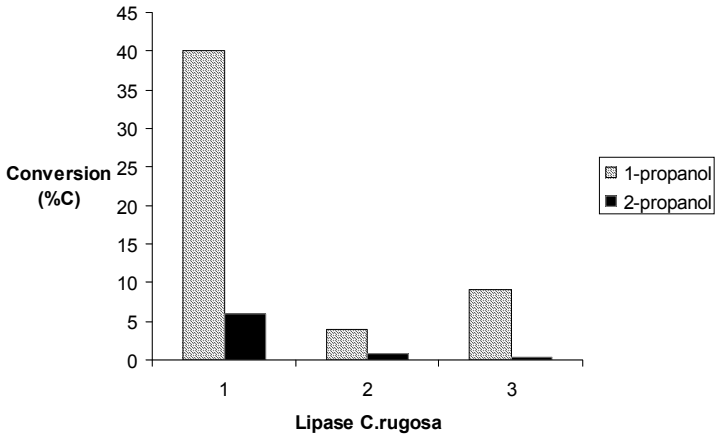


Fig. (4). Conversion degree (%) of the (*R*,*S*)-ibuprofen in dependence of used alcohol moiety. Reaction conditions: (*R*,*S*)-ibuprofen (12.49 μM), 1-propanol (75.02 μM) or 2-propanol (74.99 μM), lipase *C.rugosa* (10mg/mL), cyclohexane saturated (625 μL), after 48h, temp. 37°C.

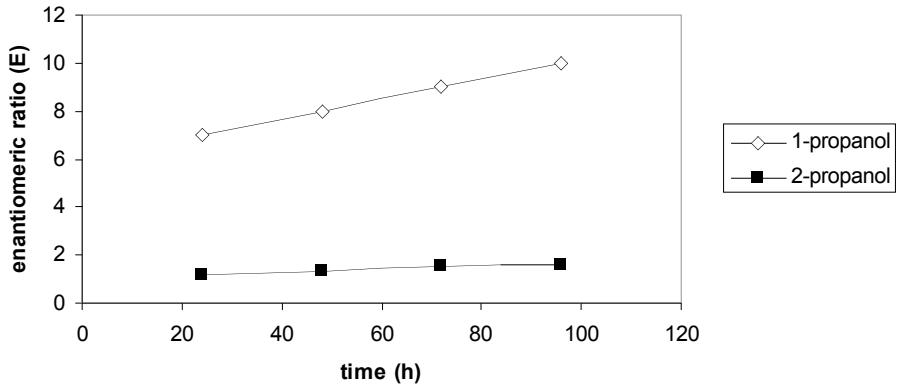


Fig. (5). Enantiomeric ratio of the (*R*,*S*)-ibuprofen in dependence of used alcohol moiety.
Reaction conditions: (*R*,*S*)-ibuprofen (12.49μM), 1-propanol (75.02 μM) or 2-propanol (74.99 μM), lipase *C.rugosa* OF (10mg/mL), saturated cyclohexane (625 μL), temp. 37°C.

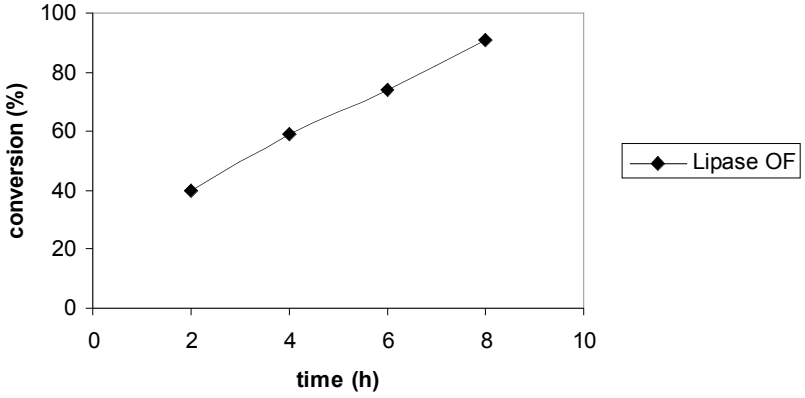


Fig. (6). Effect of DCC on the conversion degree of esterification reaction. Reaction conditions: (*R*,*S*)-ibuprofen (12.49μM), 1-propanol (75.02 μM), lipase *C.rugosa* OF (10mg/mL), saturated cyclohexane (625 μL), DCC, temp. 37°C.

The highest conversion degrees were obtained from lipase OF, the other ones (lipase MY and CRL) exhibited lower performances in the same conditions of reaction (Table **1**). It should be noted, that values of enatiomeric ratios and enantiomeric excess of products were higher in reactions carried out with used lipases MY and CRL, in comparison with OF lipase (Fig. **7**). Therefore, application of the lipases from *Candida rugosa* in enantioselective esterification reactions of racemic ibuprofen require specific optimisation of the reaction conditions, depending on the used lipase.

CONCLUSIONS

In view of the obtained results, there is a significant influence of temperature, reaction time and type of alcohol on the conversion degree and on the enantioselectivity of the studied product. It was

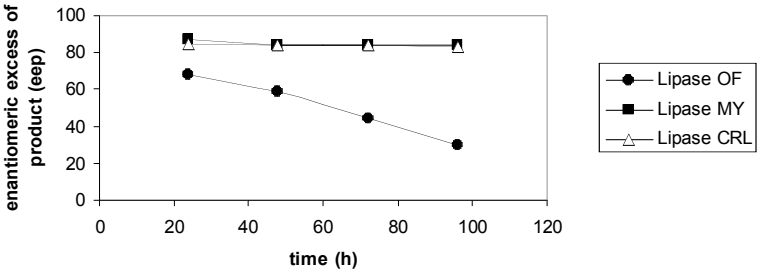


Fig. (7). Enantiomeric excess of products in dependence of used lipases. Reaction conditions: (*R*,*S*)-ibuprofen (12.49 μM), 1-propanol (75.02 μM), lipase *C. rugosa* (10mg/mL), cyclohexane saturated (625 μL), temp. 45°.

demonstrated, that in the examined temperature range, when the temperature increases, the conversion value increases as well, but the enantioselectivity decreases. The performed study confirmed, that the application of primary alcohols gives better results of conversion and enantioselectivity in comparison to secondary alcohols [26]. It was also showed, that the application of DCC in esterification reaction using *Candida rugosa* lipase gives excellent results of conversion within a significantly shorter time, however, the enantioselectivity is on a very low level (E≤ 1,2).

The performed study shows, that there are many factors, which affect the enantioselectivity and conversion of esterification reaction, applying *Candida rugosa* lipase. The obtained results demonstrate, how important is an optimisation of reaction conditions, in relation to each lipase individually. The OF lipase in the applied reaction conditions has the highest conversion among the tested lipases, but the lowest values of enantiomeric excess of products. As opposed to OF, lipases MY and CRL caused a good results of enantiomers excess of products, but low conversion values.

The comparison study of three commercially available *Candida rugosa* lipases showed their biocatalytic activity, indicating the necessity of a specific optimisation of reaction conditions depending on the applied lipase. A appropriate optimisation allows for a simple and efficient enantioselective synthesis of ibuprofen enan – tiomers using biocatalysts with an potential application in the pharmaceutical industry. At the same time, a reliable liquid chromatographic system with the new commercially available cellulose-based stationary phase is proposed for reproducible determination of profens in pharmaceutical analyses.

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