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ULTRASOUND EFFECTS ON THE ACTIVITY AND ENANTIOSELECTIVITY OF CANDIDA RUGOSA LIPASE IN ORGANIC SOLVENTS

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ABSTRACT

This work reports a study on the effect of ultrasound on the behavior of Candida rugosa lipase in the enantioselective transesterfication of some racemic secondary alcohols (1-phenylethanol, 2-pentanol, menthol) with different acyl donors (vinyl acetate, vinyl propionate, vinyl butyrate) in the presence of organic solvents. The experiments were conducted with two different approaches: ultrasonic irradiation without stirring and stirring after ultrasonic pre-irradiation of lipase. The same experiments were conducted under conventional stirring to evaluate the effect of the ultrasound. The influence of pre-irradiation time, organic solvents, acyl donors and temperature were investigated. With the two approaches, the ultrasound has advantage over the conventional stirring. The best results were, however, obtained when the reactions were conducted under ultrasound without stirring. In the resolution of the menthol by vinyl acetate in hexane, the conversion increased 3-fold and the enantioselectivity 5-fold.

Keywords: *Candida rugosa lipase; ultrasound; secondary alcohols; enantioselectivity; transesterification;*

1. INTRODUCTION

Lipases as biocatalysts for the production of enantiomerically pure compounds have great interest because they can catalyze variety of reactions such as: esterification, hydrolysis, aminolysis and transesterification, under milder and simpler process conditions, and with ability to recognize chirality [1-4]. However, their activity and enantioselectivity for various synthetic substrates are not always sufficient [5]. Various methods, such as solvent engineering, enzyme immobilization, lyophilization of the enzyme, and application of mechanical waves, have been done in attempts to enhance activity and to improve enantioselectivity [6-9].

Currently, ultrasound irradiation has been introduced into organic chemistry and biotechnology as an efficient way to accelerate chemical transformations [10, 11]. The effect of ultrasound is based on the cavitation phenomenon: a liquid subjected to ultrasonic field expands and creates bubbles that grow and then implode. The energy generated by this implosion can enhance mass transfer and thus increase the reaction rate and also help to increase the catalytic activity [12, 13]. Ultrasound is also a useful tool in enzymatic reactions. It can enhance substrate dissolution and improve mass transfer

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within and outside the active site of the enzyme [14, 15], it can also affect weak interactions in protein and induce its conformational change, which may improve some enzymatic reactions. Many studies available in the literature reports that the low frequency ultrasound (around 40 kHz) is not able to inactivate enzymes [16-18], and with the use of low frequency and optimum power input, ultrasound can be effective for the biocatalyst. The lipase from *Candida rugosa* is a relatively cheap commercial enzyme widely used in hydrolysis and synthesis of esters, and it is well known for its remarkable stability inorganic solvents [8, 19-22]. The present work focuses on the possibility to increase the activity and enantioselectivity of *Candida rugosa* lipase by using ultrasound irradiation. We studied a transesterification of three racemic secondary alcohols, 1-phenylethanol, 2-pentanol and menthol, by vinyl esters as acyl donors. Starting from reaction parameters, temperature, substrate molar ratio, and enzyme loading optimized previously, the experiments were conducted in an ultrasonic water bath and/or magnetic stirrer with three different approaches: ultrasonic irradiation without stirring, stirring after ultrasonic pre-irradiation of lipase, and under conventional stirring. The effect of the time of ultrasonic pre-irradiation of lipase, organic solvents, acyl donors, and temperature were investigated.

2. MATERIALS AND METHODS

2.1. Materials and reagents

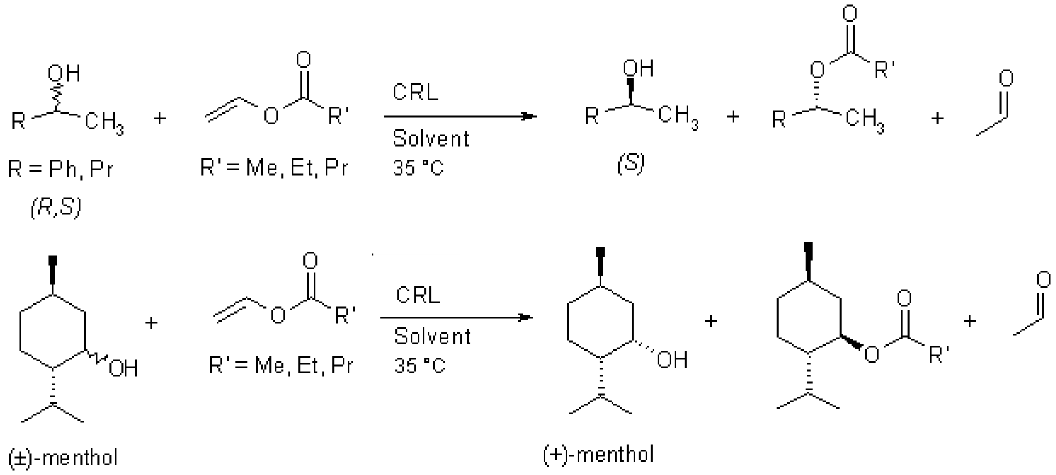
Lipase from *Candida rugosa* (CRL, Type VII, 760 U/mg), (*R,S*)-1-phenylethanol, (*R*)-1-phenylethanol, (*R,S*)-2-pentanol, (*S*)-2-pentanol, (±)-Menthol, (−)-menthol, vinyl acetate, vinyl propionate, vinyl butyrate, were purchased from Sigma-Aldrich. All other chemicals were purchased from local sources and were of the highest purity commercially available. The ultrasonic bath, Branson 1510E-MTH (Branson Ultrasonics Corporation., USA), was basically a rectangular container (14.0 cm×15.0 cm×10.0 cm). The maximum rating power was 143 W, frequency 42 kHz. The temperature of water in bath was controlled with accuracy of ±1°C.

2.2. Ultrasonic pre-irradiation of lipase in organic solvents

The lipase from *Candida rugosa* (50 mg) in 4 mL of organic solvent was ultrasonicated for different time periods (i.e. 0.5, 1, 2, 3 and 4 h). The temperature of the sample could be kept constant at 35 ± 1°C throughout. Secondary alcohol (5 mM) and acyl donor (10 mM) were added to the ultrasonically pre-irradiated lipase, and incubated at 35°C with constant shaking at 200 rpm for different times according to alcohol and acyl donor.

2.3. Transesterification of secondary alcohols

Racemic secondary alcohol (5 mM) and acyl donor (10 mM) were solubilized in 4 ml of organic solvent and then the reaction was initiated by the addition of 50 mg of lipase for different times according to alcohol and acyl donor (Scheme 1). Shaking experiments with lipase or pre-irradiated lipase were carried out over an oil bath under magnetic stirring (200 rpm). Experiments under ultrasound irradiations were carried out by placing the reaction mixture at the center of the ultrasonic water bath. In all cases, the temperature of the reaction mixture was maintained at 35°C.



Scheme.1: Enzymatic transesterifiction of secondary alcohols with different acyl donors.

2.4. Sample analysis

The mixture was filtered at the end reaction and analyzed using a gas chromatography (GC- 17, SHIMADZU) equipped with a hydrogen flame ionization detector (FID) and chiral capillary column Beta-dex^{TM} 325 (30m x 0.25mm x 0.25µm). The temperatures of the injector and the detector were 220 and 250°C, respectively. Nitrogen was used as carrier gas. The temperature programming was performed between 120 and 220°C with the increment of 6°C/min. The following retention times were observed (table 1):

Table 1. Retention times for substrates and products in chiral GC.

Substrate	**Retention time (min)**							
	For alcohol		For Ester					
			Alkyl acetate		Alkyl propionate		Alkyl butyrate	
	R	*S*	*R*	*S*	*R*	*S*	*R*	*S*
(*R,S*)-1-phenylethanol	10.15	10.31	11.40	11.64	13.15	13.44	14.96	15.39
(*R,S*)-2-pentanol	5.07	5.29	7.02	7.24	8.80	9.15	10.92	11.16
	−	+	−	+	−	+	−	+
(±)-menthol	12.92	13.23	14.50	14.74	16.62	16.87	18.48	18.73

The enantioselectivity (*E*) and conversion (*c*, %) were calculated from the enantiomeric excess of the substrate (ee_s, %) and product (ee_p, %) based on the following equations or relations :

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

Where

$$c = \frac{ee_s}{ee_s + ee_p} \qquad (2)$$

$$ee_s = \frac{|A_R - A_s|}{A_R + A_s} \qquad (3)$$

$$ee_p = \frac{|B_R - B_s|}{B_R + B_s} \qquad (4)$$

Where A_R and A_S represent the concentrations of the (*R*)-enantiomers and (*S*)-enantiomers of secondary alcohol, B_R and B_S represent the concentration of the (*R*)-enantiomers and (*S*)-enantiomers of ester corresponding.

3. RESULTS AND DISCUSSION

Here, we evaluated the effect of ultrasound by two approaches: ultrasound throughout the reaction (US) and the enzymatic pre-irradiation (P-I). The same reactions were performed under conventional stirring (Conv) in order to compare the activity and enantioselectivity displayed by *Candida rugosa* lipase for the transesterifiction of secondary alcohols. Various organic solvents and acyl donor were investigated to find the most appropriate reaction system.

3.1. Effect of ultrasonic pre-irradiation time

It was reported that when using enzyme powders to catalyze reactions in organic solvent, the enzymatic pre-irradiation by ultrasound can reduce the size of particles and consequently enhance the surface area between the enzyme and the substrate, which can contribute to reduce mass transport limitations and improve catalytic activity of enzyme [14, 23]. The effects of ultrasonic pre-irradiation time with the three alcohols were investigated. The results presented in figures 1-3 show that the conversion increase with increasing ultrasonic pre-irradiation time from 0.5 to1 h in the case of 1-phenylethanol and 2-pentanol, and from 0.5 to 2 h in the case of menthol, but a decrease of conversion was observed beyond these times. The enantioselectivity had also similar changes with ultrasonic pre-irradiation time where *E* value was increased from 0.5 to 1 h, and decreased when the time exceeded 1 h with the three alcohols. Considering the best conversion and *E* value obtained, the ultrasonic pre-irradiation time of 1 h was selected for the approach of the enzymatic pre-treatment reaction in the subsequent experiments.

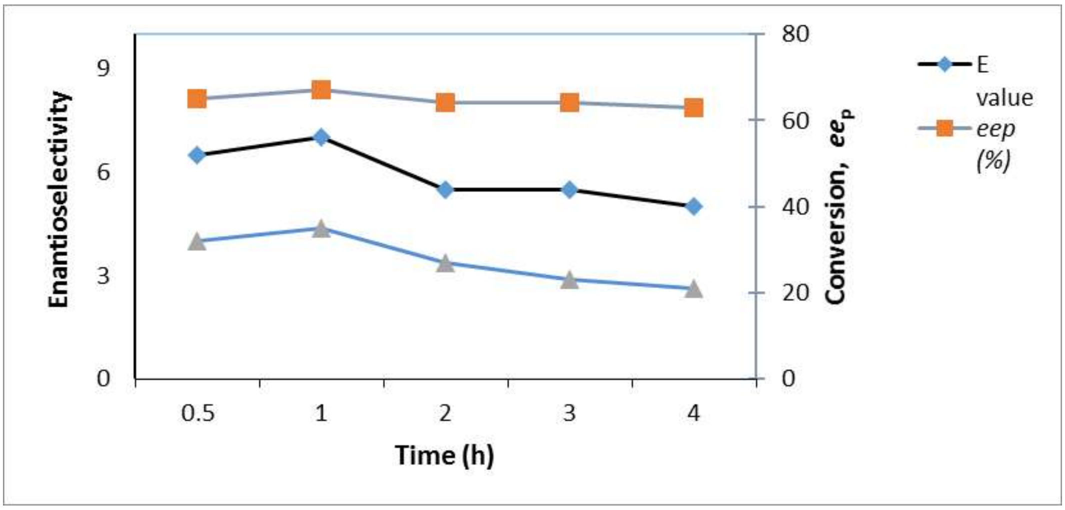


Figure.1. Effet of ultrasounic pre-irradiation time of on the enzymatic transesterifiction of (*R, S*)-1-phenylethanol with vinyl acetate in hexane.

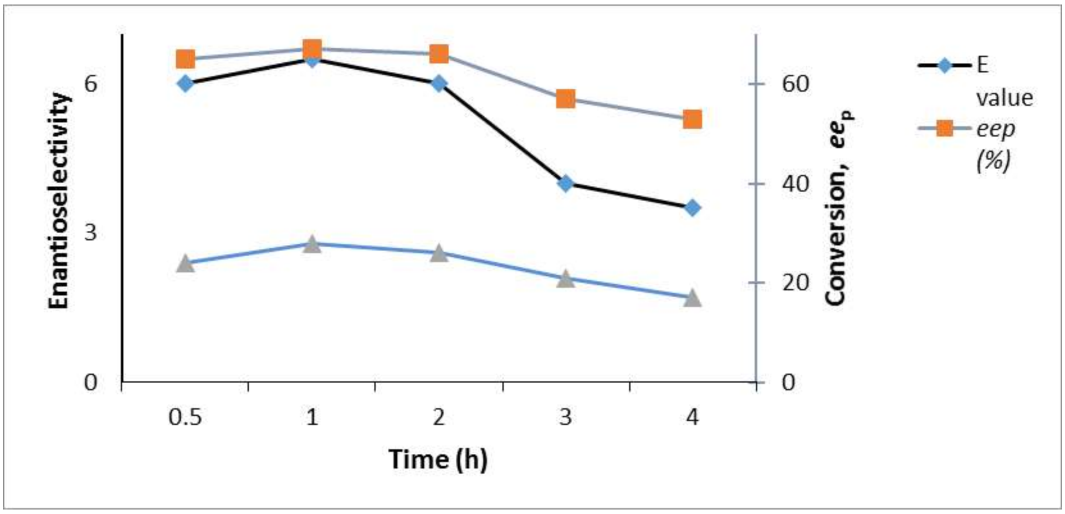


Figure.2. Effet of ultrasounic pre-irradiation time of on the enzymatic transesterifiction of (*R, S*)-2-pentanol with vinyl acetate in hexane.

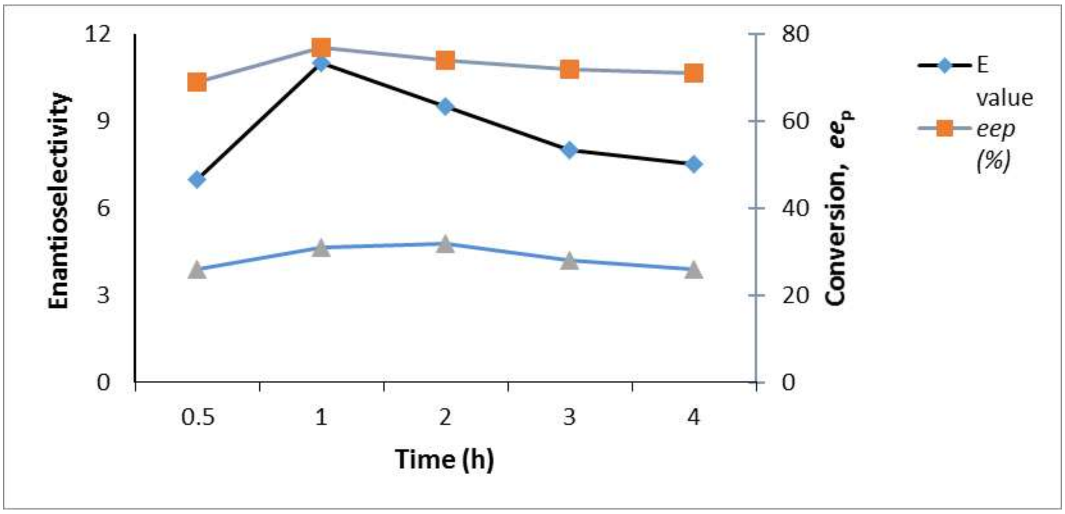


Figure.3. Effet of ultrasounic pre-irradiation time of on the enzymatic transesterifiction of (±)-menthol with vinyl acetate in hexane.

3.2. Effect of organic solvent

The choice of the appropriate organic solvent for a lipase-catalyzed reaction is known to be a crucial factor in determining the activity [24]. Several solvents with different log *P* were investigated for the transesterification of alcohols at 35°C, log *P* defined as the partitioning coefficient of solvent between 1-octanol and water, characterizing the polarity or hydrophobicity of the solvent. The reactions were conducted under three different condtions: ultrasound throughout the reaction (US), stirring using a lipase pre-irradiated for one hour (P-I), and under conventional stirring (Conv) with alcohols. All modes of enzymatic transesterification revealed similar features in terms of changes in log *P*. Except the diethylether (log *P* = 0.89), the results indicate that the conversion and enantioselectivity were favored in solvents with higher log *P* values (table 2). These observations are in accordance with many researchers reported in the literature [4, 25, and 26]. The highest values were obtained when hexane was used as the reaction media and under the effect of ultrasound with the three alcohols. Therefore, hexane was chosen as an appropriate solvent.

Table.2. Effect of solvent on the enzymatic transesterifiction of racemic secondary alcohols with vinyl acetate.

Substrate	**Solvent**	**Log *P***	**Time (h)**	***ee_p* (%)**			***C* (%)**			***E***		
				US	P-I	Conv	US	P-I	Conv	US	P-I	Conv
1-phenylethanol	THF	0.49	4	69	61	60	9	8	4	6	4.5	4
	Diethylether	0.89		71	63	60	33	19	14	8.5	5	4.5
	Dichloroethane	1.25		74	67	65	21	20	13	8	6	5
	Chloroform	1.97		56	50	51	17	15	10	4	3.5	3
	Toluene	2.73		72	66	61	37	35	13	9	5	4.5
	Hexane	3.50		75	69	67	42	35	15	12	8	5.5
2-pentanol	THF	0.49	6	64	56	54	11	7	6	5	3.5	3.5
	Diethylether	0.89		68	62	57	16	12	9	6	4.5	4
	Dichloroethane	1.25		63	57	53	24	22	16	5.5	4.5	3.5
	Chloroform	1.97		60	54	55	13	7	5	4.5	3.5	3.5
	Toluene	2.73		68	59	57	32	29	22	7	5	4.5
	Hexane	3.50		74	67	66	36	28	21	10	6.5	6
menthol	THF	0.49	5	82	73	70	13	8	7	11.5	7	6
	Diethylether	0.89		87	76	69	34	25	20	22.5	9.5	6.5
	Dichloroethane	1.25		91	84	77	26	18	11	29	14	8.5
	Chloroform	1.97		57	55	54	14	12	9	4	4	3.5
	Toluene	2.73		86	71	71	39	26	18	23	7.5	7
	Hexane	3.50		89	77	73	45	31	16	37.5	11	7.5

3.3. Effect of acyl donor

The type of acyl donor can also influence transesterification reactions catalysed by lipases. We studied three different carbon chain lengths (C_2, C_3, C_4) of vinyl esters as acyl donors. The results under the three modes of transesterification with alcohols are presented in table 3. As can be seen in table 3, *Candida rugosa* lipase had a higher activity and enantioselectivity toward: vinyl acetate with 2-pentanol and menthol, and vinyl propionate with 1-phenylethanol in all modes of transesterification. Moreover, the conversion and *E* value decreased with the elongation of chain length of the acyl donors. It might be explained by easier access to the active site of the lipase with a short chain length.

Table.3. Effect of acyl donor on the enzymatic transesterifiction of racemic secondary alcohols in hexane

Substrate	**Acyl donor**	**Time (h)**	***ee_p* (%)**			***C* (%)**			***E***		
			US	P-I	Conv	US	P-I	Conv	US	P-I	Conv
1-phenylethanol	Vinyl acetate	4	75	67	67	42	35	15	12	7	5.5
	Vinyl propionate	6	85	82	75	37	25	18	21	13	8
	Vinyl butyrate	8	78	72	69	22	16	9	10	7	6
2-pentanol	Vinyl acetate	6	74	67	66	36	28	21	10	6.5	6
	Vinyl propionate	8	71	62	60	24	13	7	7.5	4.5	4
	Vinyl butyrate	8	63	58	61	13	9	5	5	4	4
menthol	Vinyl acetate	5	89	77	73	45	31	16	37.5	11	7.5
	Vinyl propionate	7	86	73	68	39	31	17	23	9	6
	Vinyl butyrate	8	80	69	64	27	19	11	12	6.5	5

3.4. Effect of temperature

The effect of reaction temperature on activity and enantioselectivity, in the transesterification of menthol with vinyl acetate as acyl donor, was carried out under ultrasonic irradiation in a range of 25 to 55°C. The results obtained show that the conversion increased with increase in temperature (figure 4). Elevation of temperature may enhance solubility of substrates, and improve mass transfer within and outside of site active. However, the increase of temperature beyond 35°C caused a decrease in enantioselectivity, which might be explained by disruption of the active conformation of enzyme at higher temperatures which leads to loss of enantioselectivity. The optimum temperature selected was 35°C.

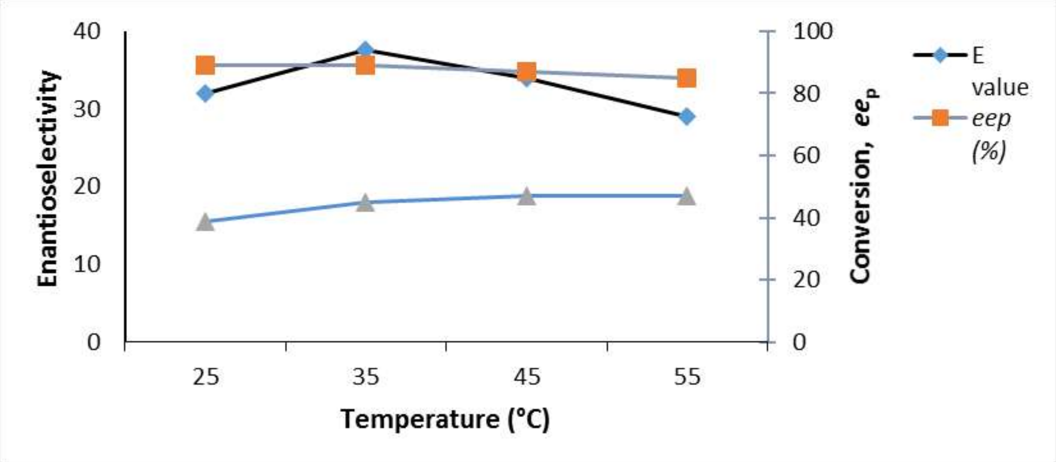


Figure.4. Effect of temperature on the enzymatic transesterifiction of (±)-menthol with vinyl acetate in hexane under ultrasound.

4. CONCLUSIONS

In this work an enantioselective transesterification of some racemic secondary alcohols under ultrasound irradiation using *Candida rugosa* lipase was investigated. Among various alcohols and acyl donors studied, menthol with vinyl acetate as acyl donor and hexane as solvent had the best results regarding conversion degree and enantioselectivity. Our results confirm the improvement of lipases activity and enantioselectivity by ultrasound reported in other researches. Therefore, the ultrasound could be an alternative in ‘‘green’’ enzymatic processes due to its high efficiency, low instrumental requirement and significant enhancement of enzyme catalytic activity compared with conventional techniques.

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