Slovak Society of Chemical Engineering
Institute of Chemical and Environmental Engineering
Slovak University of Technology in Bratislava

PROCEEDINGS

39$^{\mathrm{th}}$ International Conference of Slovak Society of Chemical Engineering

Hotel Hutník
Tatranské Matliare, Slovakia
May 21–25, 2012

Editor: prof. J. Markoš

ISBN: 978-80-89475-04-9, EAN: 9788089475049

Knezevic-Jugovic, Z., Jakovetic, S., Jugovic, B., Gvozdenovic, M., Grbavčić, S., Bezbradica, D., Antov, M.: Enzymatic synthesis of aliphatic esters of phenolic acids and evaluation of their antioxidant properties, Editor: Markoš, J., In *Proceedings of the 39th International Conference of Slovak Society of Chemical Engineering*, Tatranské Matliare, Slovakia, 1426–1432, 2012.

Enzymatic Synthesis of Aliphatic Esters of Phenolic Acids and Evaluation of Their Antioxidant Properties

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Key words: phenolic acid esters; enzymatic esterification; ethyl cinnamate; *Candida antarctica* lipase; radical-scavenging activity; antioxidant activity; cyclic voltammetry

1. Introduction

Cinnamic acid, along with other phenolic compounds, is common component of our daily diet due to its presence in plants. Phenolic acids and their esters are components of soybeans, cotton seeds, peanuts, coffee and they are proven to be one of the reasons for antioxidative potential of oilseeds [1,2].

Natural antioxidative properties of phenolic compounds make them interesting for many usages not only to prevent lipid oxidation in traditional food but also for functional food production to prevent free-radical-induced diseases such as cancer and atherosclerosis or aging caused by oxidative tissue degeneration. Further, phenolic comounds have been proven to have antimicrobial activity and they are widely used as antimicrobial preservatives in pharmaceuticals, cosmetics, foods, beverages [3]. Consequently, these phenolic acids are very interesting substrates for the production of a variety flavor and fragrance compounds such as ethyl cinnamate or butyl cinnamate.

There is growing interest in phenolic acid esters synthesis using free or immobilized lipase in non-aqueous solvents [4]. Although their synthesis can also be chemically catalyzed by acids or bases, enzymatic esterification offers environmental advantages and a reduction in energy consumption. Furthermore, the enzyme synthesis is strongly preferred when product quality is a main issue, as is the case for food production.

Guyot and coworkers were first to report enzymatic esterification of phenolic acids and fatty alcohols catalyzed with lipase B from *Candida antarctica* [5]. Soon afterwards, Stamatis and his research team described enzymatic esterification of cinnamic acid with aliphatic alcohols using lipase from *Rhizomucor miehei* as the catalyst [1], however data related to enzyme catalyzed esterification of cinnamic acid and its esters are very incomplete and contradictory, making them an interesting topic for research.

Focus of this work was to examine opportunities for lipase catalyzed esterification of cinnamic acid and its derivatives with different aliphatic alcohols and to optimize ester production using ethyl cinnamate as a model reaction. This research was mostly done using commercial enzyme preparation of lipase B from *C. antarctica* immobilized on macroporous acrylic resin, but it also included examining the possibility of using lipases from other sources. Esterifications were

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carried out in different solvents as well as in solvent-free systems, where excess of alcohol replaced solvent and provided efficient stirring of reaction mixture and proper dispersal of insoluble matters. This work also describes attempt to examine antioxidant properties of cinnamic acid and its derivatives, thus looking into influence of esterification on antioxidant properties of cinnamic acid.

2. Experimental

2.1. Materials

Lipase B from *Candida antarctica*, expressed in *Aspergillus oryzae* immobilized on macro porous acrylic resin (Novozyme 435) and used in syntheses was obtained from Sigma Aldrich (St Louis, MO, USA). The enzyme activity is ≥ 10,000 propyl laurate units per g of solid enzyme, where PLU is defined as the amount of propyl laurate formed per minute, catalyzed by one unit of enzyme. The microorganism used *Pseudomonas putida* B-21 (NRRL culture colection) and *R. oryzae* NRRL 1526 were donated by Agricultural Research Centre (USA) to the Microbiological Laboratory of the Faculty of Technology and Metallurgy, Belgrade, Serbia. Cinnamic acid, *p*-coumaric acid, *p*-methoxycinnamic acid and alcohols, all of analytical grade, were purchased from Sigma Aldrich (St Louis, MO, USA), while 3 Å molecular sieves, isooctane and other solvents were purchased from Fluka (StQuentin-Fallavier, Switzerland). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) used for radical scavenging test was also purchased from Sigma Aldrich (St Louis, MO, USA).

2.2. Lipase catalyzed phenolic acid esters production

All esterification reactions were performed batchwise under temperature control at 55 ºC and 250 rpm in reaction flasks, using phenolic acids as limiting substrate. Also, the reactions were carried out in the presence of 3 Å molecular sieves to remove water produced as the by-product and depress water dependent reversible reactions. Typically, reaction mixture consisted of 0.846 mmol of phenolic acid (cinnamic, *p*-methoxycinnamic and *p*-coumaric acid) and 15 equivalents of alcohol (ethanol, butanol) and was filled with isooctane to reach a total volume of 5 mL. Esterification was started by adding lipase (75 mg of *C. antarctica* or corresponding activity of other two lipases) into the reaction flask (100 mL). Samples were taken each 12 hours and analyzed for phenolic acids and/or esters content. Control reactions, without enzyme, were performed simultaneously under the same conditions. Each reaction was done in duplicate and standard deviations were less than 5%. The second-order kinetic model, the first order to phenolic acid and first-order to alcohol concentration, was used to describe time course of the reactions:

$$r = \frac{-dc_{\mathrm{Ac}}}{dt} = k_1 c_{\mathrm{Ac}} c_{\mathrm{Al}} \qquad (1)$$

where c_{Ac} and c_{Al} are concentrations of phenolic acid and alcohol, respectively, and t is reaction time. The conversion yield, Y, can be then expressed as a function of the initial acid, $C_{0\mathrm{Ac}}$, and alcohol, $C_{0\mathrm{Al}}$, substrate concentrations at any reaction time, t, as:

$$Y = \frac{1 - M}{\frac{C_{0Ac}}{C_{0Al}} - M} \qquad (2)$$

where M is a constant defined as:

$$M = e^{k_1 t (C_{0Al} - C_{0Ac})} \qquad (3)$$

In the proposed kinetic model, adjustable parameter is the rate constant, k_1, which was determined by least-squares fits to the experimental data.

2.3. Optimization of parameters for ethyl cinnamate production

In order to maximize production of ethyl cinnamate, series of experiments were performed, in which type of organic solvent and substarte initial molar ratios (1:1, 1:3, 1:5, 1:7, 1:9, 1:12, 1:15,

and 1:20) were alternatively varied. The effect of solvent polarity on product formation was examined using solvents with different log*P* values.

2.4. Analysis and monitoring of reaction mixtures

Ester formation was monitored by titration of residual cinnamic acid with NaOH (0.05M) in presence of phenolphthalein as indicator. Conversion yields were calculated simply with respect to the limiting reagent (cinnamic acid or its derivatives) by dividing amount of the substrate that was consumed in reaction with the initial amount of substrate. Results were also confirmed by HPLC analysis by measuring the ester concentration on a C18 Spherisorb column (particle size was 5 μm; length 250 mm; diameter 4.6 mm).

2.5. Antioxidant activity measured by DPPH assay

Antioxidative activity of cinnamic acid and its esters was measured by their ability to scavenge DPPH radical, which was monitored by decrease of absorbance on 517 nm, as described elsewhere [6]. A volume of 200 μL of metanolic ester solution was mixed, in spectrophotometric cuvet, with 1800 μL of metanolic DPPH solution (0.1 mM), vortexed and left in dark and after 30 min absorbance was measured on 517 nm. Appropriate ethyl cinnamate dilutions in methanol were experimentally found in order to satisfy the linear dependence of ester concentration vs absorbance. Calculations were done as follow:

$$\boldsymbol{RSA}(\%) = \left[1 - \frac{(\boldsymbol{A}_s - \boldsymbol{A}_0)}{\boldsymbol{A}_b}\right] \qquad (4)$$

where A_s is the absorbance of the tested antioxidant, A_0 is the absorbance of the antioxidant in methanol, and A_b is the absorbance of the DPPH solution without the sample.

2.6. Antioxidant activity measured by cyclic voltammetry

Cyclic voltammetry experiments were performed in a three electrode cell, at ambient temperature. Glassy carbon electrode was used as working, Pt wire as counter, while saturated calomel electrode (SCE) served as reference electrode. The cyclic voltammograms were recorded at scan rate of 50 mV s^{-1}, starting at open circuit potential to potential of 2.0 V (for cinnamic acid) and 1.2 V (for *p*-coumaric acid and ethyl cinnamate). The experiments were performed using SP-200 (BioLogic Science Instruments) potentiostat/galvanostat.

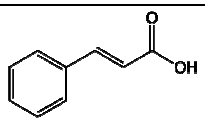
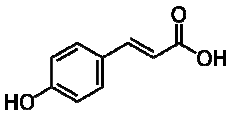
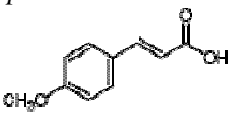
2.7. Evaluation of antimicrobial activity

3. Results and discussion

3.1. Effect of phenolic acid and lipase type on esterification

Esterification reactions of cinnamic acid and its two derivatives, including hydroxylated *p*-coumaric and metoxylated *p*-metoxycinnamic acid, were carried out with ethanol and/or butanol in the isooctane in the conditions described in Section 2.2 and the results are presented in Fig. 1. It appears that esterifications of cinnamic acid and its derivatives were done successfully but in different esterification yields due to differences in acid structure. In order to mathematically describe the reaction kinetics and to compare present processes quantitatively, the second-order kinetic model was adopted to fit the kinetic data. The nonlinear regression fitting of data to the second-order kinetic model equation resulted in the kinetic parameters given in Table 1. In Fig. 1 both experimental and predicted data are presented and apparently correlate well.

Table 1. Kinetic parameters obtained by nonlinear regression fitting of data to the second-order kinetic model and conversion yields for cinnamic acid and its derivatives

Phenolic acid	Alcohol	k/ h^{-1} $(mmol\ dm^{-3})^{-1}$	Max ester yield/ %
 Cinnamic acid	butanol	3.07	50.1±0.4, 96 h
	ethanol	1.95	35.2±1.8, 96 h
 *p*-Coumaric acid	ethanol	0.47	10.4, 96 h
 *p*-Metoxycinnamic acid	butanol	0.32	8.8, 36 h

Esterification yields obtained with cinnamic acid with both ethanol and butanol were greater than those obtained with cinnamic acid derivatives. It appeared that the reaction rate constant for ethyl cinnamate is more than four times higher than for ethyl-*p*-coumarate. This result is in accordance with literature data and it implicates that presence of the substituents on the aromatic ring affects lipase affinity towards acid [1, 5]. Both cinnamic acid derivatives used in this experiment, *p*-coumaric and *p*-methoxycinnamic acid, have electron donating groups, hydroxyl and methoxy, in para position on the ring. Lower affinity towards these acids is explained by electron effect, where electron donating groups on the ring cause deactivation of electrophilic carbon from carboxylic group which is responsible for nucleophilic attack on the alcohol. Also steric effect should not be forgotten, so most authors attribute this low enzyme affinity to combined effect of these factors along with reaction conditions. Higher esterification yield was obtained when cinnamic acid was esterified with *n*-butanol, probably because ethanol in large excess caused denaturation of the enzyme. Later in this work ethyl cinnamate was used as model reaction and all optimizations were done in order to obtain higher ester yields.

Other lipases used in this experiment were not suitable for esterification of cinnamic acid, probably due to its aromatic structure. The esterification reactions with both *R. oryzae* lipase and *P. putida* lipase resulted in low esters yields (<2,5%). Thus, esterification of cinnamic acid with ethanol by using *C. antarctica* lipase has selected as a model reaction for further study.

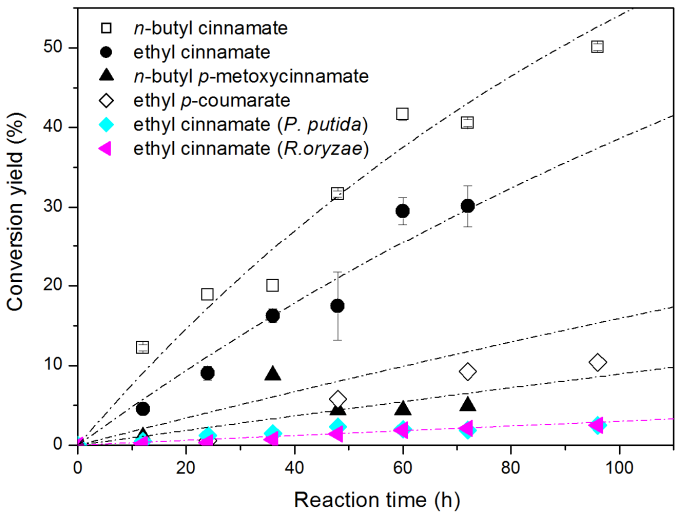


Figure 1. Time course of phenolic acid esters synthesis. Reaction mixture consisted of 0.167 M phenolic acids (cinnamic acid, *p*-methoxycinnamic acid and *p*-coumaric acid) and 2.53 M aliphatic alcohol (ethanol and *n*-butanol). Reactions were carried out at 55 ºC and 250 rpm, with 75mg of immobilized lipase from *C. antarctica* or corresponded amount of *P. putida* or *R. oryzae* lipases.

3.2. Effect of solvent polarity

Enzyme performances are known to be highly dependent on the properties of the organic solvent. Thus, esterification of cinnamic acid with ethanol was conducted in variety of solvents in order to determine optimal solvent for this reaction. Results obtained in this investigation are presented in Table 2.

Isooctane was proven to be the best solvent for esterification of cinnamic acid with ethanol, as can be seen from Table 2. This result agrees with literature, where has been noted that solvents with log*P* > 4 are good for biocatalysis since they do not remove essential water layer around the enzyme [7]. Conversions up to 85% within 5 days were reported for esterification of cinnamic acid with *n*-butanol, when a-polar solvent *n*-pentane was used [8]. In solvents with low log*P* values, that is in polar environments the best results are obtained when *tert*-butanol is used. This result is in accordance with the literature data, where relatively high reaction yields, 52%, have been obtained during esterification of cinnamic acid with *n*-octanol when *tert*-butanol has been used as solvent [1]. This result is probably due to a good solubility of cinnamic acid in polar solvents.

Table 2. Effect of organic solvent on the esterification of cinnamic acid with ethanol catalyzed by immobilized lipase B from *Candida antarctica* at 55 ^{o}C and 250 rpm

Solvent	acetone	*tert-butanol*	chloroform	hexane	heptane	isoocatne	hexadecane
log*P*	-0.23	0.35	2	3.5	4	4.5	8.8
Ester yield, %	traces	20	traces	22	23	31	6.0

3.3. Effect of initial molar ratio on reaction yield

Initial molar ratio of substrates is shown to be one of the most important parameters in enzyme catalyzed ester synthesis. Effect of this parameter on the rate of product formation was examined in isooctane, the solvent that was proven to be the best for synthesis of ethyl cinnamate. Increase in initial molar ratio (acid to alcohol) from 1:3 to 1:5 led to decrease in reaction yield for 45%, indicating that ethanol in great excess indeed deactivated enzyme. Further increase in molar ratio did not have such significant effect on product formation.

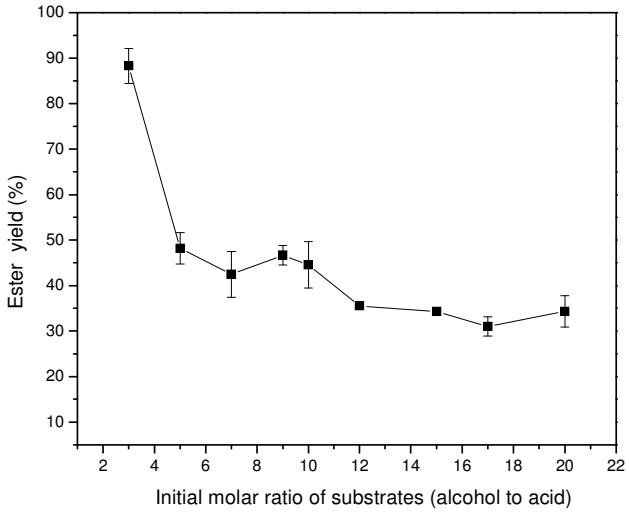


Figure 2. Effect of the initial molar ratio of substrates (alcohol to acid) on ester yield. Reaction mixture consisted of 0.0846 mmol cinnamic acid and alcohol varied according to experimental plan

3.4. DPPH assay and cyclic voltammetry results

Cinnamic acid and ethyl cinnamate were tested for their radical-scavenging activity against DPPH in order to elucidate the influence of esterification on the antioxidant activity. It appeared, as showen in Fig. 3, that ethyl cinnamate has better antioxidant potential (30% for 0.167M methanolic solution) than cinnamic acid itself (4%), though it is still very low comparing to other natural phenolic antioxidants, such as gallic acid.

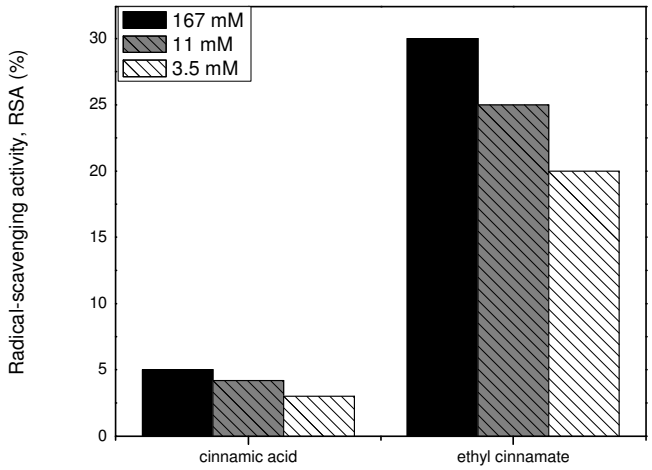
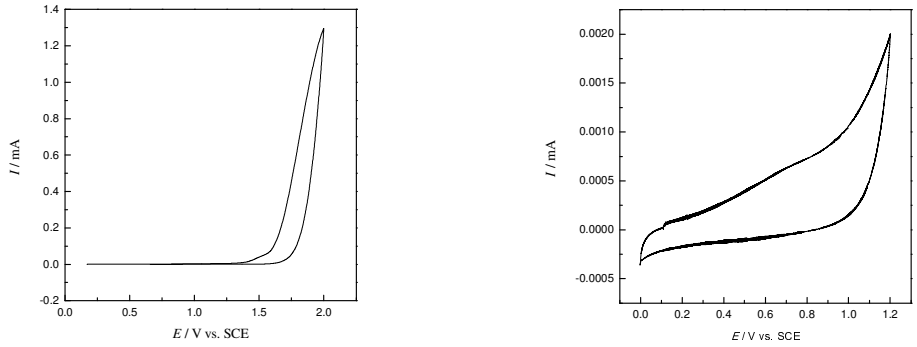


Figure 3. Free radical- scavenging activity of cinnamic acid and ethyl cinnamate

The electrochemical behavior of ethyl cinnamate was investigated by cyclic voltammetry at a scan rate of 50 mV s^{-1} in acetate buffer pH 5.6 on glassy carbon electrode and compared to native cinnamic acid. It seems that the esterification of cinnamic acid increased its antioxidant activity (Fig. 4). It was possible to observe some oxidation process in the cyclic voltammogram of ethyl cinnamate leading to wave broadening compared with that of cinnamic acid as well as appearing of minor anodic peak at E_a 0.7 V.



a) Cinnamic acid (1 mM) in sodium acetate buffer (0.2M)

b) Ethyl cinnamate (1 mM) in sodium acetate buffer (0.2M)

Figure 4. Cyclic voltammetry plots

Conclusion

The esterification activity of the commercial immobilized lipase CALB towards cinnamic acids and its derivative has been studied. Using cinnamic acid as substrate, the reaction rate constants (1.95 h^{-1} mM^{-1} for ethanol and 3.07 h^{-1} mM^{-1} for butanol) were more than four and nine times higher compared to those obtained with *p*-coumaric (0.47 h^{-1} mM^{-1}) and *p*-methoxycinnamic acids (0.32 h^{-1} mM^{-1}), respectively. Thus, esterification of cinnamic acid with ethanol by using *C. antarctica* lipase has selected as a model reaction for further study.

Isooctane is shown to be the best solvent for this reaction even though solubility of cinnamic acid in this a polar solvent is very low. Highest esterification yield of ethyl cinnamate is obtained when initial molar ratio of substrates 1:3 (cinnamic acid is limiting substrate) is used. Esterification of cinnamic acid appeared to result in increasing radical-scavenging ability. The effect of esterification of cinnamic acid was also confirmed by electrochemical method using ethyl cinnamate which appeared to enhance the antioxidant activity. These findings should stimulate the application of such lipase-catalyzed reactions for the preparation of food acceptable esters of cinnamic acid as potential lipophilic antioxidants.

Acknowledgements

This work was supported by Grant numbers E!6750 and III 46010 from the Ministry of the Education and Science, Republic of Serbia.

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