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Author: Efstratios Nikolaivits Giannis-Florjan Norra Epaminondas Voutsas Evangelos Topakas

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Cutinase from *Fusarium oxysporum* catalyzes the acylation of tyrosol in an aqueous medium: optimization and thermodynamic study of the reaction

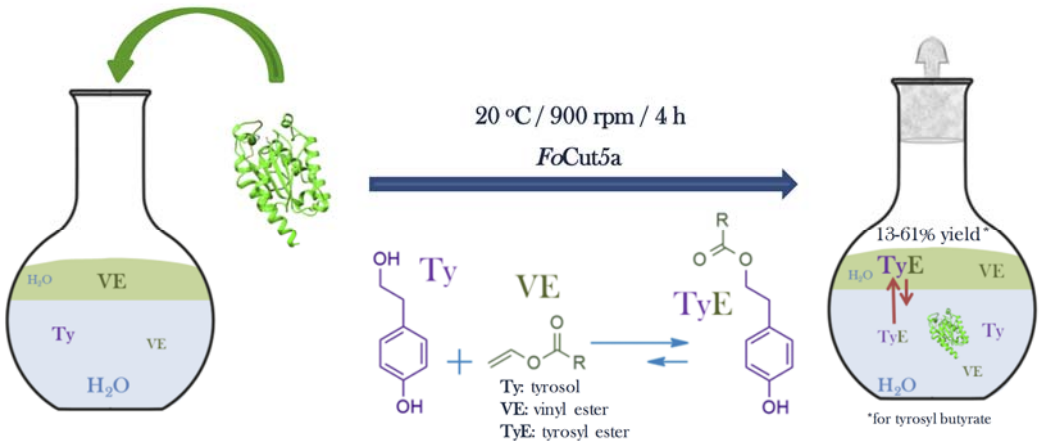
Efstratios Nikolaivits[1], Giannis-Florjan Norra[1], Epaminondas Voutsas[2] and Evangelos Topakas[1]*

[1]Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 9 Heroon Polytechniou Str., Zographou Campus, 15780 Athens, Greece

[2]Laboratory of Thermodynamics and Transport Phenomena, School of Chemical Engineering, National Technical University of Athens, 9 Heroon Polytechniou Str., Zographou Campus, 15780 Athens, Greece

*Correspondence to: E. Topakas. Tel: +30-210-7723264; fax: +30-210-7723163; e-mail: vtopakas@chemeng.ntua.gr

Graphical abstract



Highlights

- *Fo*Cut5a cutinase is capable of synthesizing tyrosyl esters in an aqueous medium
- A biphasic reaction system was optimized for optimal product yield
- Maximum conversion yield of 61% was achieved for the production of tyrosyl butyrate
- Optimal reaction conditions were pH 7, 12.5mM tyrosol, 5 μg_{enz} mL^{-1} for 4 h at 20°C
- Reactant/product solubility and distribution in the biphasic system was predicted

Abstract

Recently, tyrosol has gained attention as a result of its many pharmacological properties and due to the fact that it can be isolated from cheap and abundant resources. Lipophilic tyrosyl esters, which are scarce in nature, have proven in certain cases to acquire

improved biological activity compared to tyrosol itself, increasing their potential use in the food and cosmeceutical industries. The enzymatic approach for the synthesis of such esters has prevailed, as it is "green", compared to chemical practices. We hereby report the enzymatic synthesis of tyrosyl esters of various aliphatic fatty acids performed by a recombinant cutinase from *Fusarium oxysporum* (*Fo*Cut5a). The reaction system used consists of an aqueous phase saturated with the corresponding fatty-acid vinyl ester, which played the role of the acyl donor. We also proceeded to the study of several parameters on the yield of the tyrosyl butyrate ester synthesis. The maximum yield achieved was 60.7% after 4 h at 20 ºC, in pH 7.0, with initial tyrosol concentration of 12.5 mM and using 5 μg *Fo*Cut5a mL^{-1} reaction as catalyst. The optimum reaction conditions can be considered mild, highlighting the environmentally friendly nature of this reaction, along with the fact that there are not any harmful reagents involved. Additionally, the use of two thermodynamic models, Conductor-like Screening Model for Real Solvents (COSMO-RS) and UNIquac Functional-group Activity Coefficients (UNIFAC), were employed for the prediction of reactants' and products' solubilities and their distribution in the reaction biphasic system, aiming to correlate the reaction yields with these important thermodynamic quantities and understand the ability of this enzymatic reaction in synthesizing tyrosyl esters.

Keywords: Fusarium oxysporum; cutinase; tyrosyl esters; COSMO-RS; UNIFAC

1. Introduction

Tyrosol is a phenolic compound primarily occurring in olive oil [1], as well as olive leaf extracts [2] and olive-mill wastewaters [3]. During the past decade many pharmacological properties of tyrosol have been unveiled. Being an antioxidant [4], tyrosol is considered to be cardioprotective, inhibiting LDL-oxidation, thus preventing coronary heart disease [5], inducing survival and longevity proteins, which act as myocardial protection against ischemia related stress [6] and showing antithrombotic activity during platelet aggregation [7,8]. Some researchers have further proved that tyrosol acts as a neuroprotective agent, preventing Alzheimer's [9] and Parkinson's diseases [10] and also as a preventing agent of tumoral diseases [4,11]. Lipophilization of natural antioxidants is a familiar practice (see review from Figueroa-Espinoza et al. [12]) aiming to broaden their applications in oil-based food processing and cosmetics. Acylating tyrosol with a lipophilic moiety makes it an amphiphilic molecule, which could accumulate at oil-water or oil-air interfaces where oxidation is considered to occur, increasing the oil protection. Synthesis of phenolic derivatives can be achieved either chemically or enzymatically. Chemical procedures involve reagents, such as *N*,*N*'-dicyclohexylcarbodiimide, tetrahydrofuran and dichloromethane [13], which may prove harmful for the human health and are not environmentally friendly. Using enzymes as catalysts has been demonstrated as a "green", safe and efficient alternative for food, pharmaceutical and cosmeceutical applications.

So far, there has been a plethora of reports for synthesis reactions performed by hydrolytic enzymes. The water content in such kind of reactions is a very important parameter. On one hand, water molecules bound on the enzyme ensure its catalytic properties [14], while the use of miscible organic solvents causes the loss of the enzyme's essential surface water molecules affecting its catalytic activity [15]. On the

other hand, the excess of water triggers the enzyme's hydrolytic activity at the expense of its synthetic activity [16]. This type of reaction has also been carried out in supercritical carbon dioxide [17–19] and in micro- [20] and mini- [21] emulsion systems with 2% (v/v) and 50 or 80% (w/w) water content respectively. Furthermore, some reactions of this sort have been reported to occur in diphasic systems: water-heptane [22] or water-oil [23].

Cutinases are small members of the serine-hydrolase family that have shown to hydrolyze esters, preferably with short-length chains (C_2 or C_4 being the optimum for them) [24]. Their active site is accessible to the solvent, in contrary to the one of lipases [25], therefore there is no need for interfacial activation. Cutinases, except from the wide range of substrates that can hydrolyze, they are also capable of catalyzing esterification and transesterification reactions. *Fusarium solani pisi* and *Burkholderia cepacia* cutinases have been shown to synthesize flavor compounds such as short-chain alkyl esters [21,26,27] in isooctane or miniemulsion systems. The esterification of various natural phenolic acids with aliphatic alcohols has been performed by a commercial *F. solani* cutinase, but with low conversion yields [28]. Furthermore, esterification and transesterification of various phenolic acids or phenolic acid esters with *n*-butanol or *n*-propanol, have been carried out in hexane-miniemulsion systems, by different feruloyl esterases, with good yields [29–31].

In this study, the enzymatic synthesis of alkyl esters of tyrosol was carried out in an aqueous system under mild reaction conditions, increasing the lipophilicity of tyrosol for its potential use in cosmeceutical or oil-based foods. Their synthesis, so far, has been performed with either chemical synthesis [32] or enzymatically with the use of lipases [33]. In the present reaction system, the aqueous phase is saturated with the acyl donor (vinyl ester) and the transesterification reaction is catalyzed by *Fo*Cut5a, a

cutinase from *Fusarium oxysporum*. The synthesis of five tyrosyl esters was performed and the effect of several parameters including temperature, pH, enzyme loading, and substrate concentration on the transesterification reaction of tyrosol with vinyl butyrate was studied. To increase the understanding of the reaction system, the use of activity coefficient models was carried out, such as the quantum chemistry based Conductor-like Screening Model for Real Solvents (COSMO-RS) that is the thermodynamic tool for the prediction of reagents/products solubility or the group contribution UNIquac Functional-group Activity Coefficients (UNIFAC) model.

2. Materials and methods

2.1 Enzyme and chemicals

The recombinant cutinase from *F. oxysporum* was heterologously produced from *Escherichia coli* BL21 (DE3) and purified, as previously described [25]. Enzyme concentration was calculated by UV spectrometry at 280 nm [34] using molar extinction coefficient of 16180 M^{-1} cm^{-1} [25]. Vinyl esters (acetate, propionate, butyrate, decanoate and laurate) and tyrosol were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals used were of HPLC grade.

2.2 Acylation reactions and product analysis

Vinyl esters with different carbon-chain lengths (acetate-C_2, propionate-C_3, butyrate-C_4, decanoate-C_{10} and laurate-C_{12}) were tested as acyl donors in order to determine the specificity of *Fo*Cut5a in the transesterification reaction of tyrosol.

A typical tyrosyl ester synthesis reaction was carried out in a final volume of 0.5 mL, containing 3.5 mg tyrosol diluted in 0.375 mL phosphate-citrate buffer pH 7.0 (0.1

M), 0.1 mL vinyl ester and 25 μg *Fo*Cut5a diluted in 20 mM Tris-HCl pH 8.0 buffer. Reactions were typically performed in 30 ^{o}C in an Eppendorf Thermomixer® Comfort (Eppendorf, Germany) operated at 900 rpm.

Aliquots of reaction mixtures (10 μL) were analyzed by thin layer chromatography (TLC) on aluminum sheets coated with Silica gel 60 F_{254} (Merck, Germany). An elution system consisted of petroleum ether (40-60^{o}C):ethyl acetate (4:1 v/v) was used for the resolution of the acylated product. Tyrosol and its derivatives were developed through evaporated iodine or detected under UV light (254 nm).

For the quantification of the reaction products, an HPLC method was constructed and followed. Reaction aliquots (0.5 mL) were extracted with 1 mL (0.5-0.25-0.25 mL gradually) of ethyl acetate prior to analysis. 0.2 mL of the extract was dried and then diluted in 0.5 mL methanol (50% v/v) prior to analysis on a SHIMADZU LC-20AD HPLC equipped with a SIL-20A autosampler. The column used is a C-18 reverse-phase NUCLEOSIL® 100-5 (Macherey-Nagel, Germany) at a flow rate of 0.8 mL min^{-1}. Analysis of the samples was executed with a linear gradient method using double distilled water (A) versus acetonitrile (B) as eluents. The total running time for samples containing esters C_2, C_3 and C_4 was 15 min during which the following proportions of the solvent B were used: 0-5 min 10-30%, 5-8 min 30-90%, 8-13 min 90%, 13-15 min 90-10%. For medium-chain tyrosol derivatives (C_{10} and C_{12}) the isocratic step (90% B) lasted 20 min instead of 5 min. Detection took place with the photodiode array detector Varian ProStar and the wavelength at which the absorption was recorded was 280 nm. The conversion yield for the synthesis of tyrosyl esters was calculated from the amount of tyrosol having reacted compared to its initial quantity, using a reference curve constructed by standard solutions of tyrosol.

2.3 Optimization of tyrosyl butyrate (TyC_4) synthesis

The effect of several parameters was investigated in order to maximize the conversion yield of tyrosol to its lipophilic derivative. Tyrosol was diluted in different pH, using the following buffers (0.1 M salt concentration): phosphate-citrate for pH 3-7, Tris-HCl for pH 8-9 and glycine-NaOH for pH 9-10. The effect of the enzyme loading (1, 2.4, 5, 25 μg mL^{-1}) and tyrosol concentration (5, 10, 12.5, 25, 50, 75 mM), as well as temperature conditions (15, 20, 25, 30, 35 ^oC) was also investigated. Control reactions in the absence of enzyme, were also realized, during all reaction sets.

2.4 Preparative-scale production and purification of tyrosyl butyrate

Tyrosol (69.1 mg) was mixed with 8 mL MOPS buffer pH 6.0 (0.1 M) saturated with 2 mL vinyl butyrate. Reaction was initiated by addition of 0.5 mg enzyme and incubated for 24 h at 30 ^oC under shaking. At the end of the reaction, the mixture was gradually extracted 3 times with 10 mL ethyl acetate and the organic phases were pooled. The product was purified by adsorption chromatography using a 500 mm×25 mm ID glass column packed with silica gel 60 (0.040–0.063 mm) in the elution solvent (petroleum ether 40-60^oC:ethyl acetate 4:1 v/v). The occurrence of ester was systematically checked on each elution fraction by TLC.

The identification of TyC_4 was done by ^1H and ^{13}C NMR spectroscopy, which was performed in CD_3OD, with a Bruker DRX spectrometer, equipped with broad band probe at 400 MHz. ^1H NMR ($CDCl_3$), δ, ppm: 7.10 (2H, *d*, J = 8.1 Hz, H-1, H-5); 6.80 (2H, *d*, J = 8.3 Hz, H-2, H-6); 4.27 (2H, *t*, J = 7.1 Hz, H-8); 2.88 (2H, *t*, J = 7.0 Hz, H-7); 2.29 (2H, *t*, J = 7.4 Hz, H-11); 1.65 (2H, *m*, H-12); 0.95 (3H, *t*, J = 7.4 Hz, H-13). ^{13}C NMR, δ, ppm: 173.9 (C-10); 154.5 (C-4); 130.0 (C-3); 129.7 (C-1, C-5); 115.4 (C-

2, C-6); 65.0 (C-8); 36.2 (C-7); 34.3 (C-11); 18.4 (C-12); 13.6 (C-13). Carbon numbering corresponds to the product of acylated tyrosol shown in Figure 1.

2.5 Solubility and partition coefficient predictions with the COSMO-RS model

The COSMO-RS developed by Klamt and co-workers [35–37] is an efficient method for the a priory prediction of thermophysical data of liquid mixtures on the basis of unimolecular quantum chemical calculations for the individual molecules that provide the necessary information for the evaluation of molecular interactions in liquids. In the COSMO-RS model, a liquid is considered to be an ensemble of almost closely packed ideally screened molecules. Each piece of surface is characterized by its value of screening charge density (σ_i). The interaction energy of the ensemble is then obtained by a statistically correct consideration of all possible pairs of pieces of surface. The composition of the ensemble that is needed to apply this procedure is delivered by the distribution function $p(\sigma)$. This function that is called as the σ profile, describes the amount of surface in the ensemble having a screening charge density between σ and $\sigma+d\sigma$. The representative σ profile of a mixture is the concentration weighted average of the pure σ profiles. The σ profile of a component needs to be calculated only once. Aside from the electrostatic interactions, other intermolecular forces such as dispersive and repulsive interactions and hydrogen bonds also occur in fluid mixtures. They are merged in an energy concept within the COSMO-RS model. As a result, a COSMO-RS calculation provides the chemical potential or the activity coefficient of component *i* in the mixture. For further details, see the publications of Klamt and co-workers.

2.6 Computational details

The standard procedure that is also described in more details in [38,39] was applied for COSMO-RS calculations. Quantum chemical COSMO calculations were performed for all esters, except from vinyl acetate, which were not included in the database from COSMOlogic GmbH & Co KG, Leverkusen, Germany. Vinyl acetate and water conformers were obtained from the COSMOlogic database. The structures of the various esters were designed using HyperChem Professional (Version 8.0) and were initially optimized using the Geometry Optimization tool of the same program. The quantum chemical COSMO calculations were then performed on the density functional theory (DFT) level, utilizing the BP functional [40–42] with RI (resolution of identity) approximation and a triple-ζ valence polarized basis set (TZVP) [43,44]. All structures were fully optimized using the TURBOMOLE program package. Solubilities were predicted at 30 ºC with the COSMOtherm program (Version C2.1 Release 01.11).

Partition coefficients (K) of tyrosyl esters in the biphasic system of water and vinyl ester were predicted with the following equation:

$$K = x_p^w / x_p^E \quad (1)$$

where x_p is the molar fraction of the product in aqueous phase (x_p^w) and in its corresponding vinyl ester phase (x_p^E). Using the standard thermodynamic equation of liquid-liquid equilibrium, and since the solubility of water in the vinyl ester phase and vice versa is very low, the distribution ratios were approached by the ratio of the activity coefficients at infinite dilution, which were calculated with COSMOtherm, according to the following equation:

$$x_p^w \gamma_p^w = x_p^E \gamma_p^E \Rightarrow K = \frac{x_p^w}{x_p^E} = \frac{\gamma_p^E}{\gamma_p^w} \approx \frac{\gamma_p^{E,\infty}}{\gamma_p^{w,\infty}} \quad (2)$$

where γ_p is the activity coefficient of the product in water (γ_p^w) and its corresponding vinyl ester (γ_p^E) and γ_p^∞ is the activity coefficient of the product at infinite dilution in water ($\gamma_p^{w,\infty}$) and the corresponding vinyl ester ($\gamma_p^{E,\infty}$). Initially, a liquid-liquid equilibrium (LLE) calculation was performed for each water/acyl donor binary mixture at 30 ^oC using COSMOtherm

2.7 Solubility and partition coefficient predictions with UNIFAC

For comparison purposes, the group contribution UNIFAC model [45] was also applied in the prediction of solubilities and partition coefficients. The basis of UNIFAC method is to consider molecules as an aggregation of functional groups. This approach offers the great advantage of being able to describe a virtually infinite number of different compounds with a small number of parameters. UNIFAC has two important drawbacks. The first is that UNIFAC predictions are independent of the location of the functional groups and cannot account for the effect that one group has to another if they are close in the molecule (proximity effect). As a result, the identification of chemical groups is independent of the intramolecular environment. Moreover, any kind of contact between two groups in UNIFAC is associated with the same energy, independent of their individual locations and directions. Despite these drawbacks UNIFAC is widely applied due to its broad parameterization and its high calculation speed.

In this work the UNIFAC-LLE model [46] has been used since it has been especially developed for liquid-liquid equilibrium predictions. Solubility and partition coefficient predictions with UNIFAC were performed in the same way as for COSMO-RS.

2.8 Evaluation of the thermodynamic models by experimental data

For the evaluation of the predictions emanated from the thermodynamic models, distribution experiments were performed for the binary (water-vinyl propionate, water-vinyl butyrate) and tertiary systems (water-vinyl propionate-TyC_3, water-vinyl butyrate-TyC_4). The amount of vinyl and tyrosyl esters was calculated by HPLC, using reference curves constructed by standard concentrations of these esters. The amount of water in the two vinyl esters was measured by the Karl-Fischer titration method, using a TitroLine KF titrator.

3. Results

3.1 Effect of acyl donor chain length on the reaction yield

Except from acylating tyrosol with vinyl butyrate (C_4), other vinyl esters were also used as acyl donors in order to study the effect of the chain length on the reaction yield. As shown in Figure 1, tyrosol was acylated with various fatty-acid vinyl esters in order to determine the specificity of *Fo*Cut5a for the different acyl donors. The conversion of short-chain vinyl esters (C_2, C_3, C_4) is relatively high (over 30%) in contrast to the medium-chain esters (C_{10}, C_{12}), which resulted to c.a. 1% tyrosyl ester production. The highest yield was achieved using vinyl butyrate as acyl donor (41.8%), followed closely by vinyl propionate (38.6%) (Table 1). To our knowledge, this is the first report so far for the enzymatic synthesis of TyC_4 that was preferentially synthesized by *Fo*Cut5a, therefore we proceeded to the optimization of this esterification reaction.

3.2 Study of various parameters in the yield of TyC_4 synthesis

3.2.1 Effect of enzyme concentration

In most industrial applications of biocatalysis, the enzymes prove to be a limiting factor. Increasing the amount of enzyme means increase of initial rate and completion of the reaction in a shorter period, while rising of the process cost. In the transesterification reaction for the production of TyC_4, while the initial reaction rate (mM h^{-1}) increases with the addition of more enzyme (data not shown), the initial rate per enzyme amount (mM h^{-1} mg_{enzyme}^{-1}) reaches a peak for 2.4 μg enzyme mL^{-1} and is low for 25 μg mL^{-1} (Fig. 2). In addition, the conversion yield for 5 μg enzyme mL^{-1} is maximum (29.5% after 4 h), while the specific initial rate is not the highest. Considering the conversion yield as the most crucial parameter, 5 μg *Fo*Cut5a mL^{-1} of reaction was used for further experiments.

3.2.2 Effect of reaction pH

Typically, transesterification reactions take place in organic solvents and are performed by immobilized enzymes. Since this synthetic reaction is catalyzed by a free enzyme in an aqueous medium, it is necessary to investigate the effect of pH on the acylation yield. Optimum reaction environment proved to be phosphate-citrate pH 7.0 buffer (Fig. 3A). The reaction seemed to be favored in alkaline pH, in which the control reactions were also high. Cutinase could act in a wide range of pH (6-10) with a good yield (more than 75% of the optimum) as seen in Fig. 3.

3.2.3 Effect of tyrosol concentration

Under standard reaction conditions (30 ºC; pH 7.0; 5 μg enzyme mL^{-1}) the effect of tyrosol concentration on the initial reaction rate and the TyC_4 formation was studied.

The results (Fig. 4) were fitted to the Michaelis-Menten model using the GraphPad Prism 5 software (R^2=0.9989). The kinetic constants were calculated as follows: K_m=119.2±12.6 mM, k_{cat}=134.6±9.8 s^{-1} and k_{cat}/K_m =1128.9±144.9 s^{-1} M^{-1} respectively. The maximum conversion yield (40.6%) is achieved with 12.5 mM initial tyrosol concentration.

3.2.4 Effect of reaction temperature

*Fo*Cut5a exhibits highest hydrolytic activity at 40 ºC, however at that temperature, intense enzyme deactivation from the first minutes takes place [25]. Under this view, we carried out transesterification reactions at different temperatures in order to optimize the tyrosol conversion yield. This set of reactions was performed under optimal conditions (pH 7, 12.5 mM Ty concentration, 5 μg mL^{-1} enzyme loading). As seen in Fig. 3B, the initial reaction rates at temperatures 15 ºC and 20 ºC are almost identical and very low compared to higher temperatures. There is a great leap between temperatures 20 ºC and 25 ºC, after which the initial reaction rate increases in a nearly linear fashion. The maximum yield is observed at 20 ºC after 4 h (60.7%), which is very close to the yield achieved at 15 ºC after 5 h (58.4%).

3.3 Prediction of mutual solubilities of vinyl esters and water

The solubility of the acyl donor in the water phase and vice versa may play a very crucial part in the reaction yield, as the enzyme's accessibility at this reactant may depend on it. In Table 1, the solubilities of the acyl donors in water and vice versa, as predicted from the COSMO-RS and UNIFAC models are presented. It can be observed that increase of the vinyl ester's carbon-chain length decreases its solubility in water with medium-chain vinyl esters (decanoate and laurate) showing very low solubility, in

the order of 10^{-8} or 10^{-9}. Similarly, increase of the vinyl ester's carbon-chain length decreases the solubility of water in it. However, this effect is much weaker than the one observed for the solubility of the vinyl esters in water (Table 1). COSMO-RS and UNIFAC yield similar and satisfactory mutual solubility predictions, with UNIFAC to be slightly better than COSMO-RS.

3.4 Prediction of tyrosyl esters' distribution in the reaction medium

The mode of action of *Fo*Cut5a in this particular reaction system may be explained by understanding the behavior of the esters produced in each case. Both thermodynamic models (COSMO-RS and UNIFAC) were used for the prediction of the partition coefficients of all tyrosyl esters produced experimentally. The comparison of the models' predictions with the experimentally measured values for TyC_3 and TyC_4 indicates that COSMO-RS yields satisfactory results, while UNIFAC strongly underestimates the experimental data.

As seen in Table 2, products are distributed in the acyl donor phase in all cases ($K<1$). Additionally, the increase of the tyrosyl ester's chain-length increases its presence in the acyl donor phase, making it scarcer in the aqueous phase. Exception to this is the COSMO-RS prediction for the TyC_3, which shows the highest distribution in the aqueous phase compared to the other tyrosyl esters. UNIFAC model predicts the decrease of the distribution of products (with increasing chain-length) almost linearly.

4. Discussion

Tyrosol is the main phenolic compound found in olive oil, exhibiting biological activity. It is also present in the olive oil production by-products (leaves and mill wastewaters [2,3]) making it a low-cost, easy to access antioxidant, especially in

countries with high olive oil production such as Spain, Italy and Greece (97% of the 2014-2015 EU production according to the International Olive Oil Council-IOOC or 77% of the 1997-2000 world-wide production [47]). It has been suggested that hydrophobic derivatives of natural antioxidants exhibit higher antioxidant activity against LDL oxidation than their respective precursors [20,48]. Tyrosol's monoacetylated derivatives are more effective inhibitors of rabbit platelet aggregation (which causes atherosclerosis), compared to tyrosol itself, rendering them a more potent protection agent against cardiovascular disease [7]. Moreover, although tyrosol does not display any antimicrobial activity, medium-chain tyrosyl esters (TyC_8, TyC_{10}, TyC_{12}) inhibit the growth of several bacterial strains (*Staphyloccocus aureus*, *Staphyloccocus xylosus*, *Bacillus cereus*, *Bacillus flavum*) and two leishmanial strains (*Leishmania. major* and *Leishmania infantum*) [33].

Fatty acid tyrosyl esters have shown to possess sufficient surfactant properties, which adds them to the short list of surfactant-antioxidants [49]. Being such, they can be used in the food industry as antioxidants, in oil-water emulsions or oil matrices. Lipoic acid tyrosyl ester was tested as antioxidant in fish-oil water emulsion system, resulting in 50-60% inhibition of oxidation [50]. On the contrary, tyrosol appeared to be a more adequate antioxidant in olive oil, than its esters that were slightly less effective [51]. This can be explained by the so called "polar paradox", which states that hydrophilic molecules are more effective as antioxidants in the bulk of oil, while lipophilic are in oil-water systems [52].

*Fo*Cut5a was able to catalyze the transesterification of tyrosol with various fatty acid vinyl esters in a water medium saturated with the corresponding vinyl ester. Kremnický et al. (2004) were the ones who discovered a water-based reaction system,

which allowed a crude lyophilized preparation of *Trichoderma reesei*, exhibiting acetyl esterase activity, to catalyze the acetylation of methyl β-D-xylopyranoside, using vinyl acetate as acetylating donor [53]. That also proved to be an equilibrium-controlled reaction. The same reaction system has been then used for acetylation of mono-, di- and oligosaccharides by several purified acetyl esterases [54,55]. To the authors' knowledge, this is the first time where an aqueous biphasic reaction system was employed for the acylation of a biological active compound catalyzed by a cutinase, while describing the thermodynamic distribution of reactants and products.

In our study, we firstly investigated the effect of the acyl donor's (vinyl ester) chain-length on the production yield. The maximum conversion was observed for vinyl butyrate, followed closely by vinyl propionate. Even though cutinases usually show maximum hydrolytic efficiency for the C_2 or C_4 esters [24], little is known about the effect of the carbon-chain length on the synthesis kinetics. *F. solani pisi* cutinase shows to best utilize butyric acid (C_4) for the synthesis of ethyl esters [27] and similarly *B. cepacia* cutinase prefers the C_4 alcohol for the esterification of butyric acid [26]. In both cases substrates with three carbon atoms have not been tested. Concerning the hydrolytic activity, *Fo*Cut5a shows maximum efficiency (k_{cat}/K_m) for the hydrolysis of C_4 esters [25], however, there is no data reported for C_3 substrates. As noted from the experimental data, medium carbon-chain vinyl esters (C_{10} and C_{12}) were difficult for *Fo*Cut5a to utilize in this system. This can be attributed to the fact that cutinases show low efficiency for the medium carbon-chain esters both in hydrolysis [24] and synthesis [26,27]. Moreover, according to the COSMO-RS predictions, vinyl decanoate and laurate have almost zero solubility in water, where the enzyme has access and probably this is an additional reason why the production yields for the respective tyrosyl esters are low.

As previously reported [25], although *Fo*Cut5a shows its highest hydrolytic activity at 40 ºC, the highest yield for the transesterification reaction was achieved at 20 ºC. This could be possibly explained by the low thermal stability of this enzyme at temperatures exceeding 30 ºC. While maximum conversion was obtained at 20 ºC, the highest initial reaction rate was achieved at 35 ºC. The room-temperature esterification reaction mediated by *Fo*Cut5a, results in an environmentally friendly process with minimal requirements in terms of energy consumption. In addition, the optimal enzyme loading was found to be 5 μg mL^{-1}, which is very low, while being very efficient at a low temperature, meaning that the cost of the enzyme does not constitute a limiting factor to the process.

Commercial *F. solani* cutinase (30 mg mL^{-1}) has also been used in lipophilization reactions of various phenolic acids, which were esterified with 1-octanol at 45 ºC in *tert*-butanol. At that temperature the initial reaction rate was 15.2 μmol h^{-1} g_{enzyme}^{-1} [28], more than 10^5 times lower compared to the one of *Fo*Cut5a at 20 ºC, which resulted to maximum conversion of 29% after 12 days. The same enzyme expressed in *S. cerevisiae* and lyophilized (1.4 mg mL^{-1}), synthesized ethyl esters at 30 ºC in isooctane. The maximum initial rate observed was 1.15 μmol min^{-1} mg_{enzyme}^{-1} for ethyl butyrate and the maximum conversion was 97% for ethyl valerate after 6 h [27]. The initial rate succeeded in our reaction system under optimal conditions was 28 times higher than the one observed for that enzyme. Wild-type *B. cepacia* cutinase (2.6 mg mL^{-1}) was used for the synthesis of alkyl esters in isooctane. Butyl butyrate synthesis reached the maximum conversion yield (95%) after 12 h at 37 ºC [26].

When it comes to the synthesis of lipophilic tyrosyl esters several studies were previously reported all of them performed in organic solvents, exploiting immobilized

lipases as catalysts. For example, the transesterification of tyrosol with ethyl acetate in hexane using a non-commercial lipase from *Staphylococcus xylosus*, (54 ºC, 500 UI lipase) was employed, which led to 95% yield after 48 h [56]. The direct esterification of tyrosol with several fatty acids has also been investigated, catalyzed by immobilized non-commercial lipases from *Rhizopus oryzae* and *S. xylosus* (1000 UI) and by the commercial lipase Novozyme 435® (5 mg mL^{-1}) [33,57]. The reactions were carried out at 45 ºC in 2-methyl-2-propanol/hexane for 120 h (non-commercial enzymes) or 72 h (Novozyme 435®). *R. oryzae* lipase exhibited maximum conversion yield for the tyrosyl laurate ester (70%), while *S. xylosus* and *C. antarctica* lipases for the tyrosyl acetate (95 and 99.7%, respectively). The novel reaction system proposed in this study does not result in such high production yields, although the employed enzyme amount is much less (1000 times compared to Novozyme 435®) and the reaction is completed in 4 hours. According to our knowledge, tyrosyl butyrate (TyC_4) had not been synthesized enzymatically before.

Concerning the thermodynamic studies, it was observed that the reaction yields can be generally well correlated with the solubilities of the vinyl esters in water, i.e. the decrease in the solubility leads to decrease in the reaction yield, as well as with the distribution ratios of the products (tyrosol esters) in the biphasic reaction medium, i.e. the decrease in the distribution ratio leads also to decrease in the reaction yield. The solubilities of vinyl esters in water can be satisfactorily predicted by both UNIFAC and COSMO-RS, while for prediction of the distribution ratios only COSMO-RS gives satisfactory results.

In the presence of water an amount of the ester produced inevitably gets hydrolyzed. We propose that the reaction takes place in the aqueous phase or the water-

oil interphase, where the enzyme has access. According to our thermodynamic study the majority of the product is transferred in the acyl donor phase (vinyl ester) immediately after its production, due to its low solubility in water, so it gets protected from further hydrolysis. We believe this is the main reason why this reaction keeps moving towards the synthesis, until equilibrium is reached. Reaction mixtures were monitored for 24 h in preliminary experiments (data not shown), but it was observed that the reactants and products reached an equilibrium state after only a few hours (4 h).

Overall, this novel reaction system presented for the production of lipophilic tyrosyl esters, especially tyrosyl butyrate, can prove attractive for industrial utilization, since it is an environmental friendly process compared to the existing synthetic reactions, employing mild conditions in an aqueous medium. In addition, the cost of the enzyme that usually proves to be a limitation in biocatalytic reactions, would be marginal due to low amount needed. Furthermore, no heating or cooling steps are needed prior to the reaction as it is practically performed at room temperature. Another advantage is that the product could be easily collected from the acyl-donor phase free of enzyme. The major shortcoming of this process is the use of activated donors requiring a chemical synthetic step to synthesize the corresponding vinyl esters prior to the enzymatic transesterification reaction. Moreover, the use of an immobilized cutinase may reduce the cost of the enzyme through the recycling of biocatalyst, therefore the immobilization of *Fo*Cut5a will be further explored.

5. Conclusions

The broad applications of tyrosyl esters demand a sustainable and environmentally friendly production that could be provided by Biocatalysis. The discovery of novel

biocatalysts is fundamental, aiming in a decrease of bioconversion cost utilizing mild reaction conditions, whereas diminishing the reaction byproducts and waste. In the present investigation, the potential of *F. oxysporum* cutinase for the transacylation of tyrosol in an aqueous medium was studied, while the reaction conditions were optimized. For the synthesis of short-chain fatty-acid tyrosyl esters, a reaction system primarily consisting of water and a vinyl ester as acyl donor was suggested. The enzymatic-mediated reaction reached a satisfactory yield of 61% in 4 h, employing a miniscule amount of biocatalyst (5 μg *Fo*Cut5a mL^{-1} of reaction) under mild conditions of pH and temperature (7.0 and 20 ^{o}C, respectively). Due to its high water content the reaction system used is very attractive, as the enzyme and the corresponding product are protected in the aqueous and vinyl ester phase, respectively. The reactants' and product's solubilities and their distribution in the reaction biphasic system was investigated by the use of COSMO-RS and UNIFAC thermodynamic models, aiming in understanding the synthetic behavior of this reaction system catalyzed by *Fo*Cut5a cutinase.

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Figure captions

Fig. 1 Schematic depiction of the transesterification reaction of tyrosol with various vinyl esters differing in their carbon-chain length

Fig. 2 Effect of the concentration of added enzyme on conversion yield (●) and initial rate (○). Reactions were performed in phosphate-citrate pH 7 buffer, with 50 mM tyrosol for 4 h at 30 ºC.

Fig. 3 Effect of reaction pH (A) on conversion yield after 24 h and temperature (B) on conversion yield (●) and initial rate (○). Reactions were performed in phosphate-citrate pH 7 buffer, 12.5 mM tyrosol with 5 μg enzyme mL^{-1} for 4 h (optimal conditions)..

Fig. 4 Effect of tyrosol concentration and on the conversion yield (●) and initial rate (○). Reactions were performed in phosphate-citrate pH 7 buffer, with 5 μg enzyme mL^{-1} for 3 h at 30 ºC.

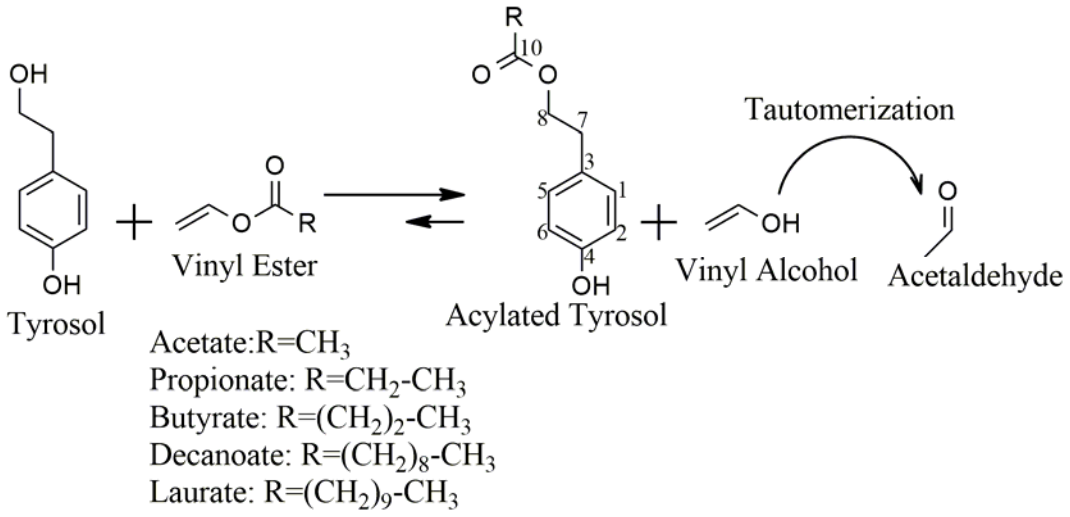

Fig 1

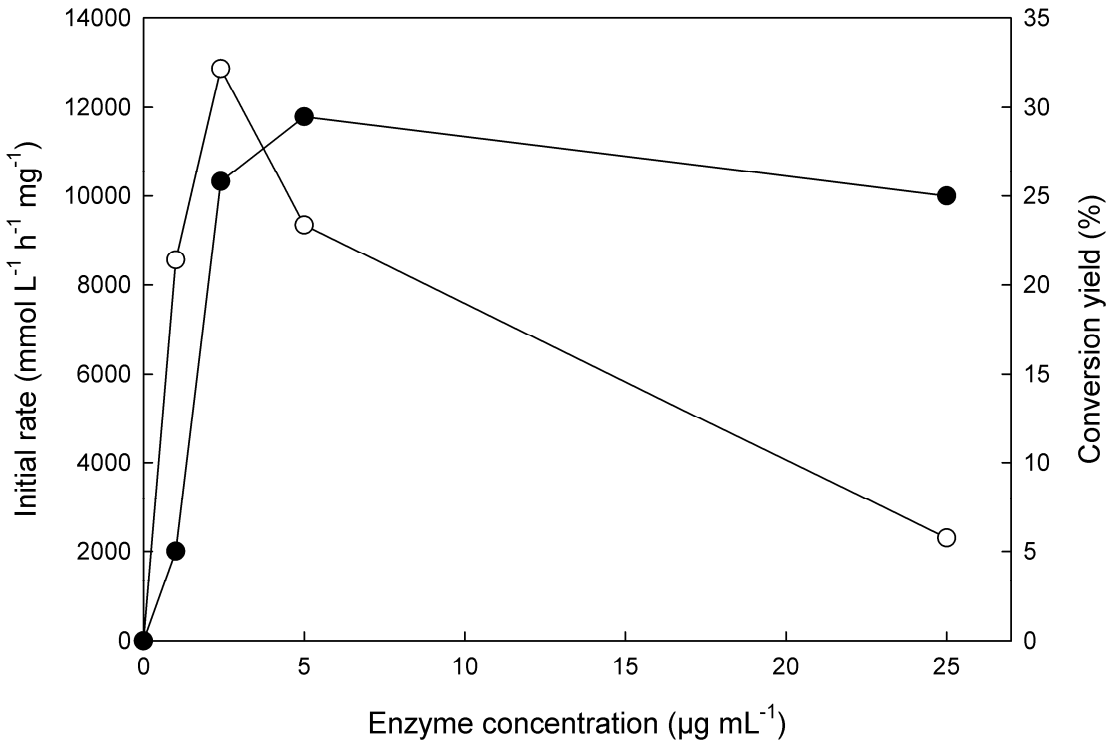

Fig 2

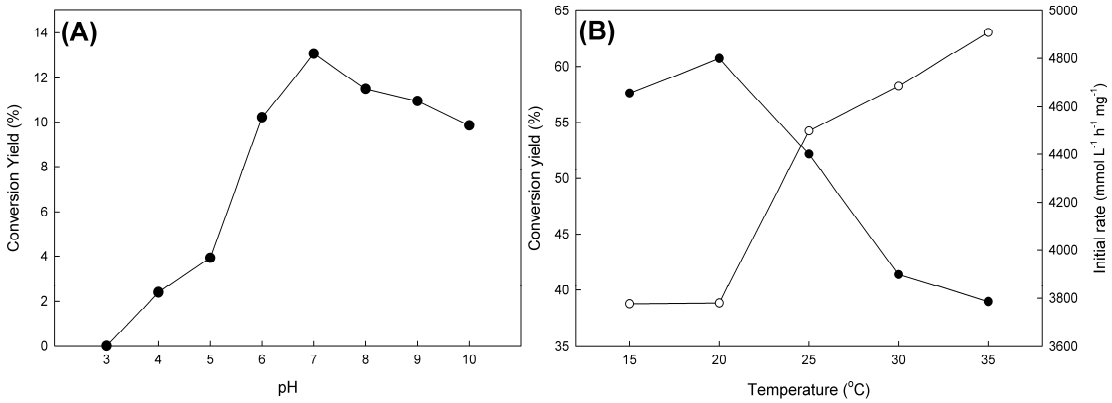

Fig 3

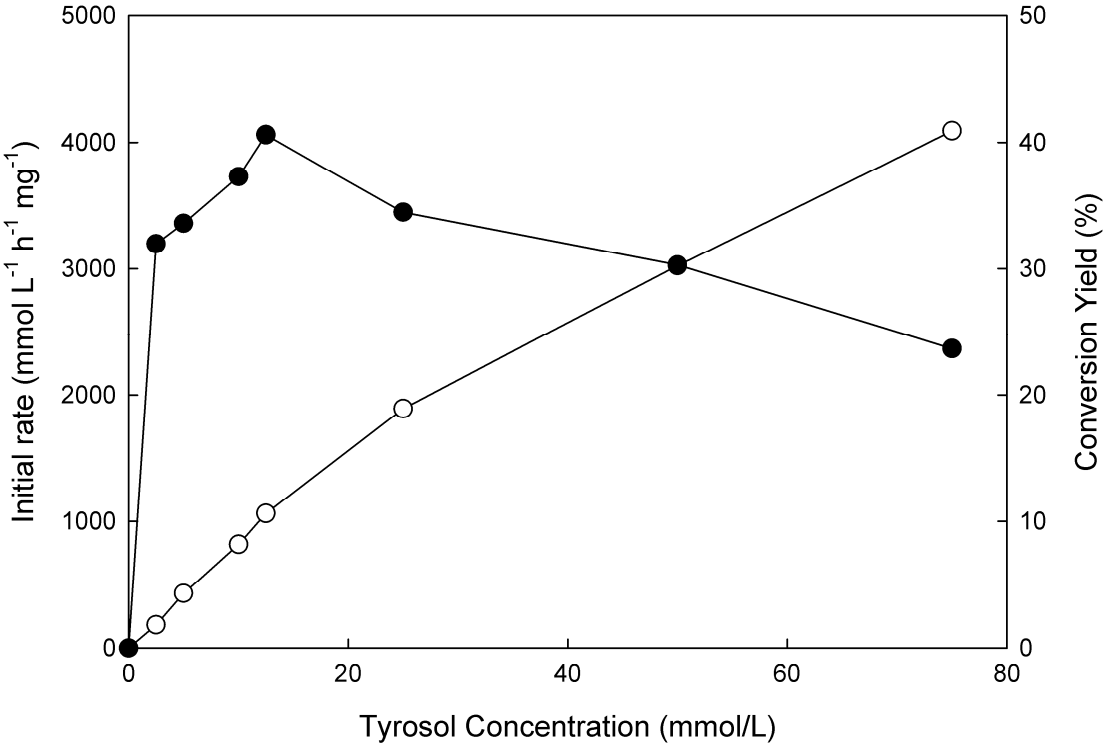

Fig 4

Table 1 Conversion yield for the synthesis of tyrosyl esters (at 30 ºC for 4 h) using different chain-length acyl donors. Experimentally measured and COSMO-RS and UNIFAC predicted mutual solubilities (x), given in mole fractions, of the vinyl ester/water mixtures at 30 ºC are also presented.

Vinyl ester	Yield	Experimental		COSMO-RS		UNIFAC	
	%	x (VE in water)	x (water in VE)	x (VE in water)	x (water in VE)	x (VE in water)	x (water in VE)
Acetate (C2)	30.7	-	-	$2.3\ 10^{-3}$	$3.1\ 10^{-02}$	$3.5\ 10^{-03}$	$5.8\ 10^{-02}$
Propionate (C3)	38.6	$2.7\ 10^{-03}$	$9.4\ 10^{-02}$	$5.5\ 10^{-4}$	$2.1\ 10^{-02}$	$6.6\ 10^{-04}$	$3.0\ 10^{-02}$
Butyrate (C4)	41.8	$4.1\ 10^{-04}$	$6.2\ 10^{-02}$	$1.5\ 10^{-4}$	$1.7\ 10^{-02}$	$1.8\ 10^{-04}$	$2.6\ 10^{-02}$
Decanoate (C10)	1.1	-	-	$3.5\ 10^{-8}$	$1.1\ 10^{-02}$	$1.5\ 10^{-07}$	$1.9\ 10^{-02}$
Laurate (C12)	0.9	-	-	$2.2\ 10^{-9}$	$1.0\ 10^{-02}$	$1.6\ 10^{-08}$	$1.8\ 10^{-02}$

Table 2 Prediction of the partition coefficients $K(x_p^w/x_p^E)$, using both thermodynamic models (COSMO-RS and UNIFAC) and the experimental calculations for the two esters tested (propionate and butyrate).

Product	K_{COSMO}	K_{UNIFAC}	K_{exp}
Ty-C_2	$2.3\ 10^{-3}$	$2.5\ 10^{-5}$	-
Ty-C_3	$6.1\ 10^{-3}$	$8.7\ 10^{-6}$	$2.1\ 10^{-2}$
Ty-C_4	$1.8\ 10^{-4}$	$1.3\ 10^{-6}$	$5.2\ 10^{-3}$
Ty-C_{10}	$9.4\ 10^{-6}$	$4.2\ 10^{-9}$	-
Ty-C_{12}	$3.1\ 10^{-8}$	$4.7\ 10^{-10}$	-