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Studies on the synthesis of short-chain geranyl esters catalysed by *Fusarium oxysporum* esterase in organic solvents

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Abstract

A novel esterase isolated from *Fusarium oxysporum* was investigated for the synthesis of short-chain esters of geraniol by alcoholysis and direct esterification reactions in organic solvents. The enzyme was used as a dried powder (i.e., not immobilized). The reaction parameters affecting the enzyme behavior such as the nature of organic solvent and acyl donor, the concentration of substrates and the water activity of the system were studied. High yields (80–90%) were obtained by both approaches (alcoholysis and direct esterification) at low values of water activity ($a_w = 0.11$) in *n*-hexane. The enzyme retain its catalytic activity even after fifth reuse in *n*-hexane at $a_w = 0.11$, demonstrating its stability and efficiency under the conditions of this study.

Keywords: Enzymatic synthesis; Geranyl ester; Organic solvent; *Fusarium oxysporum* esterase

1. Introduction

Terpene esters of short-chain fatty acids are essential oils that present a great interest in the food, cosmetic and pharmaceutical industries as flavor and fragrance compounds [1]. Among them, the acetates, propionates and butyrates of acyclic terpene alcohols (geraniol and citronellol), are the most important. Traditionally, these esters are obtained by various methods such as chemical synthesis, extraction from natural products and by fermentation [1,2]. However, these methods exhibit a low yield of the desired product and are often expensive for commercial production [3]. With the great interest given to natural products, the flavor industry is interested in the use of biotechnology to produce the natural aromas, in particular by enzymatic methods [2,3].

On the other hand the use of enzymes as industrial catalysts in organic media offers numerous potential advantages compared to aqueous media from a biotechnological perspective [4]. In such system, hydrolytic enzymes (lipases, esterases and proteases), can be employed to usefully carry out synthetic reactions since the equilibrium position of the reaction is shifted sufficiently to give a high yield of the synthesis product [5,6]. Lipase-catalyzed synthesis of terpene esters by direct esterification [7–10] and transesterification [9,11–13] in media with low water content have been described. However,

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the yield of short-chain terpene esters, such as terpenyl acetates, synthesized by direct esterification was found in most cases to be very low [8,9,13]. According to these researchers, acetic acid is a potent inhibitor of lipase activity. Therefore, an efficient biocatalyst with a high affinity for short-chain acyl donors (especially acetic acid) and terpenols need to be identified.

In this work, we report the use of a novel, partially purified, esterase from *Fusarium oxysporum* (laboratory strain), to obtain geranyl esters by two different approaches: a) alcoholysis of different short chains acyl donors by geraniol and b) direct esterification of geraniol with acetic and butyric acid. Our objective was to investigate the role of various physicochemical parameters, such as the water activity (a_w), the nature of acetyl donor, the polarity of organic solvent and the concentration of reactants on enzyme catalytic behavior.

2. Materials and methods

2.1. Materials

Geraniol, geranyl acetate, various acetates and butyrates and short-chain acids were purchased from Sigma. All organic solvents, ethylene glycol diacetate and isopropylacetate were purchased from Merck. Salts used for water activity fixing were analytical grade. Substrates and organic solvents were dried over molecular sieves (4 Å) before use. The laboratory strain F3 of *F. oxysporum*, isolated from cumin [14], was used in the present investigation. The stock culture was maintained on potato-dextrose agar (PDA).

2.2. Growth conditions

The composition ($g\,l^{-1}$) of the mineral medium was: KH_2PO_4, I; $CaCl_2$ 0.3; $MgSO_4 \cdot 7H_2O$, 0.3; supplemented with 1% (w/v) ammonium phosphate as nitrogen source. The pH of the medium was adjusted to 7 by addition of NaOH.

Liquid state fermentation in the above described mineral medium, prepared as follows: different concentrations of tomato skin (carbon source) suspended in 100 ml of mineral medium, placed in 250 ml Erlenmeyer flasks and sterilized at 121°C for 30 min. The cultures were incubated on rotary shaker (250 rpm) at 30°C.

2.3. Crude enzyme production

Mycelium was separated from the culture fluid by centrifugation ($7000 \times g$). The supernatant from centrifugation was filtered (milipore 0.45 μ). The permeate was contained esterase activity and was concentrated by ammonium sulfate precipitation (80%, w/v). The precipitate dissolved in 100 mM citrate–phosphate pH 7. The esterase activity was 0.24 $U\,mg^{-1}$ of protein.

2.4. Enzyme assay

Esterase activity was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNB) (Sigma) as substrate [15]. The assay mixture (1 ml) contained 0.5 mM PNB in 50 mM phosphate buffer pH 6.0. From 25 to 50 μl of culture fluid was added, and the change in absorbance at 405 nm was monitored for 1 min during which active preparations showed a linear increase in A_{405} nm at 35°C. The rate of reaction was determined by reference to a standard curve prepared by adding various amounts of *p*-nitrophenol to the assay mixture.

2.5. Enzymatic synthesis

In a typical synthesis of geranyl esters, 0.1 M geraniol and 0.1 M acyl-donor and 60 mg of lyophilized esterase preparation were added to 3 ml of organic solvent. The reaction mixture was incubated at 45°C and magnetically stirred. Samples were withdrawn at various times to determine the concentration of substrate and product by gas chromatography. Control experiments were conducted without enzyme or with

inactivated enzyme by incubation at 100°C for one hour. The enzyme and the reaction media (substrate + organic solvent) were separately preincubated in closed vessels containing saturated salt solutions for 48 h, to obtain various thermodynamic water activities [16]: LiCl ($a_w = 0.11$), $MgCl_2$ ($a_w = 0.33$), $Mg(NO_3)_2$ ($a_w = 0.54$), NaCl ($a_w = 0.75$) and K_2SO_4 ($a_w = 0.97$).

2.6. Analytical methods

The reactions were monitoring by measuring the geraniol and geranyl esters concentrations by gas chromatography, using a Perkin Elmer 8500 chromatographer, equipped with 3 ft glass packed column, loaded with 1%-Dexsil 300 (Supelco) and a FID) detector. Nitrogen was used as the carrier gas at a flow rate of 12 $ml\,min^{-1}$, with detector port temperature at 250°C. The oven temperature was kept constant for 1 min at 80°C, linearly increased ($10°C\,min^{-1}$) up to 170°C.

Water concentrations in the liquid organic phase were measured by coulometric Karl–Fischer titration.

3. Results and discussion

3.1. Synthesis of geranyl esters by alcoholysis

In order to investigate the effect of the nature of the acyl donor on *F. oxysporum* esterase activity, six different acyl donors have been tested for the alcoholysis reaction in *n*-hexane. The acylation of geraniol was carried out at 45°C since at higher temperatures the reaction rate decreased (data not shown). Under the present reaction conditions, among the acyl donors investigated ethylenglycol diacetate and tributyrin appeared to be the most suitable for the synthesis of geranyl acetate and butyrate respectively (Table 1). Conversions as high as 70–80% were observed after 96 h of incubation with both substrates. Conversion yields observed in this work are two to three times higher than that observed for the synthesis of geranyl and citronellyl acetates catalyzed by lipozyme in *n*-heptane [13] or by *Candida cylindracea* and *Pseudomonas sp.* lipases in *n*-hexane [11]. Immobilized lipase from *Candida antarctica* (SP435) on the other hand gave similar or better yields in *n*-hexane depending on the acyl donor used [11]. The reaction progress for the acylation of geraniol with ethylenglycol diacetate and tributyrin respectively, catalyzed by esterase in hexane, are shown in Fig. 1. The geranyl esters concentration increased while the geraniol concentration decreased, as expected, reaching a

Table 1
Effect of the acyl donor (300 mM) on acylation of geraniol (300 mM) catalyzed by *F. oxysporum* esterase preparation (20 mg/ml), in *n*-hexane, at 45°C $a_w = 0.33$

Acyl donor	Reaction rate (mM/h)
Ethyl acetate	2.7
Isopropyl acetate	0
Ethylenglycol diacetate[a]	3.8
Triacetin[b]	3.0
Ethyl butyrate	3.0
Tributyrin[b]	4.3

[a] 150 mM.
[b] 100 mM.

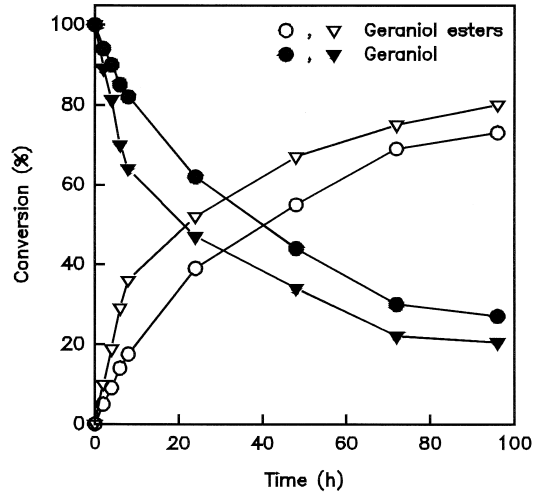

Fig. 1. Typical time course for the acylation of geraniol by ethylenglycol diacetate (●) and tributyrine (♦) in *n*-hexane. Reaction conditions as described in Table 1.

constant value after 96 h. The effect of branching of the acetyl donor carbon chain (isopropyl acetate) had a dramatic effect on the transesterification progress. Similar inhibitory effect, due to branching of the acetyl donor, (isopropyl acetate, *tert*-butyl acetate or isoamyl acetate), have been observed for the synthesis of geranyl and citronellyl esters by various lipases in hexane [11,12].

3.2. Effect of enzyme concentration

The reaction rate for the alcoholysis of ethylenglycol diacetate by geraniol catalyzed by *F. oxysporum* esterase in hexane as a function of amount of enzymatic preparation (7.5–50 $mg\,ml^{-1}$) is shown in Fig. 2. As it can be seen, the reaction rate increased as well as the conversion yield of the reaction as the enzyme concentration increases reaching a maximum (73%) when the amount of enzyme preparation is higher than 15 $mg\,ml^{-1}$ (data not shown). This behavior is similar to that observed for the synthesis of geranyl esters catalyzed by esterase from *Mucor miehei* and lipase from *C. antarctica* respectively [10,17].

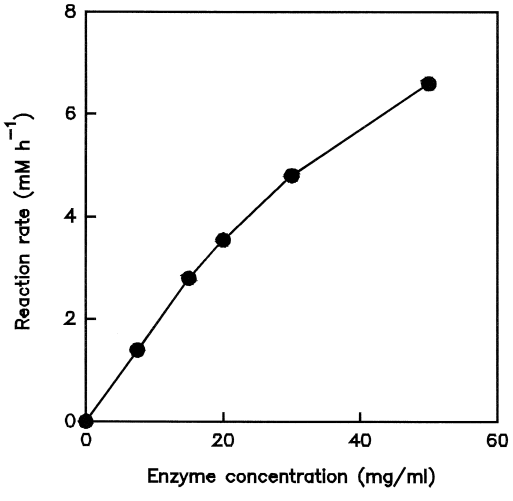

Fig. 2. Effect of *F. oxysporum* esterase concentration on the initial rate of acetylation of geraniol by ethylenglycol diacetate in *n*-hexane. Reaction conditions as described in Table 1.

3.3. Effect of organic solvent

The polarity of the organic solvent is a fundamental factor that determines the behavior of enzymes. Solvent molecules can associate with the enzyme and influence enzyme hydration. Since hydrophobic solvents do not distort the essential water layer around the enzyme the enzymatic activity should be higher than in hydrophilic ones [18,19]. Moreover, different enzymes are differently sensitive to organic solvents [20]. In this work, the influence of the polarity of the organic solvent on the esterase activity was studied. Table 2, shows the effects of the log *P* value of six selected organic solvent (defined as the ratio between the concentrations of the organic solvent in octanol and in water at equilibrium), on the activity of esterase from *F. oxysporum* for the acetylation of geraniol by ethylenglycol diacetate. As it can be seen, the polarity of the solvent influence the reaction rate of the alcoholysis reaction. The activity of esterase was higher in solvents with log *P* values greater than 2.9. However, the highest conversion yield (73%) and reaction rate was observed in solvents with log *P* values 4.0–4.5, while higher log *P* values does not necessarily sustain a higher enzyme activity. In solvents with log *P* values lower than 2 the activity of esterase decreased drastically. This behavior is similar to that observed for the majority of enzymes in organic media [18,21].

Table 2
Effect of selected organic solvents on reaction rate of the acetylation of geraniol with ethyleneglycol diacetate catalyzed by *F. oxysporum* esterase

Solvent	log *P*	Reaction rate (mM/h)
Dimethyl formamide	−1.0	0
Chloroform	2.0	0.6
Dibutylether	2.9	3.0
n-Hexane	4.0	3.8
Isooctane	4.5	3.5
Dodecane	5.5	2.9

Reaction conditions as described in Table 1.

3.4. Effect of substrate concentration

It has been reported that geraniol act as a dead-end inhibitor for *M. miehei* lipase catalyzed the acetylation of geraniol in organic solvents [12]. Since the effect of substrate concentration depends on the enzyme used, the effect of substrate molar ratio on acetylation of geraniol by ethylenglycol diacetate catalyzed by esterase from *F. oxysporum* was investigated. The reaction was carried out in *n*-hexane at a constant acetyl donor concentration and varying geraniol concentrations. As it can be seen in Fig. 3, no inhibitory effect of geraniol was observed since increasing geraniol concentration led to an increase in esterification rate showing an optimum value at a concentration ratio of 2.

3.5. Effect of water activity

It has been shown that the activity of enzymes used in organic solvents is dependent on the water content, and that only a narrow range gives optimal activity [21,22]. Water is involved in all noncovalent interactions that help to maintain the active enzyme conformation. The water content in the reaction mixture is better expressed by the thermodynamic activity of water a_w [23]. Fig. 4 shows the effect of the a_w on the reaction progress for the acetylation of geraniol with ethylenglycol diacetate in hexane catalyzed by *F. oxysporum* esterase. Table 3 shows the effect of the water activity on the reaction rate of the above reaction.

As it can be seen from Table 3, the transesterification activity of esterase in hexane increased with increasing the water activity reaching a maximum at $a_w = 0.75$. This behavior is also observed for various lipases in similar non-conventional media [16]. Fig. 4 shows that the final yield of the reaction depends also on

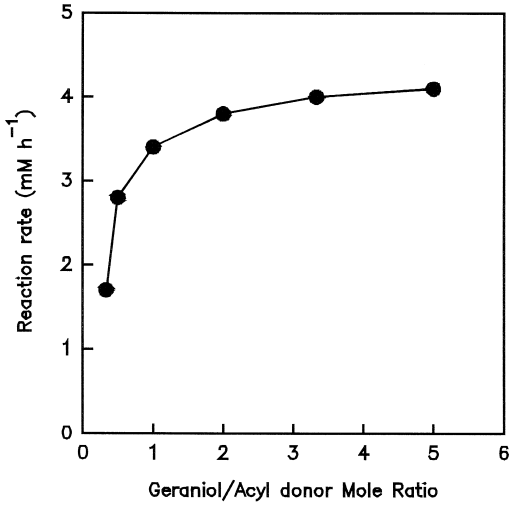

Fig. 3. Effect of geraniol/ethylenglycol diacetate molar ratio on reaction rate of synthesis of geranyl acetate catalyzed by *F. oxysporum* esterase in *n*-hexane. The acyl donor concentration was constant (150 mM). Reaction conditions as described in Table 1.

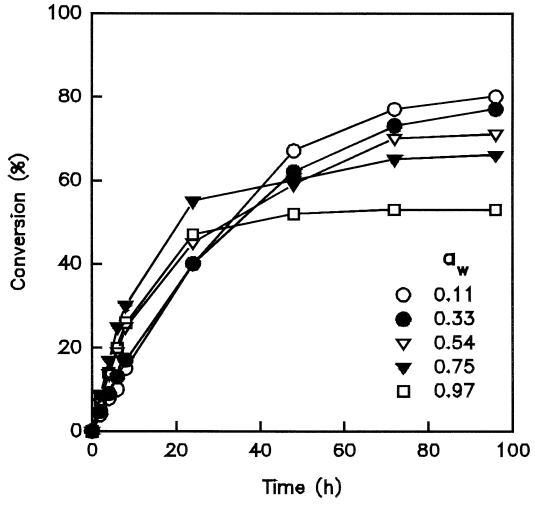

Fig. 4. Effect of water activity (a_w) on reaction progress of acetylation of geraniol with ethylenglycol diacetate catalyzed by *F. oxysporum* esterase in *n*-hexane. Reaction conditions as described in Table 1.

Table 3
Effect of water activity (a_w) on reaction rate of the acetylation of geraniol with ethyleneglycol diacetate catalyzed by *F. oxysporum* esterase

a_w	Reaction rate (mM/h)
0.11	3.1
0.33	3.8
0.54	5.1
0.75	6.2
0.97	5.8

Reaction conditions as described in Table 1.

the water activity of the system. The higher yield (83%) was observed at $a_w = 0.11$ while at higher water activities the final reaction yield progressively decreased to 60% at $a_w = 0.97$. The decrease on the final yield, at high water activity values, could be attributed to the different stability of the enzyme at different hydration states. It was founded that other hydrolytic enzymes such as chymotrypsin and lipase from *Candida rugosa* display a pronounced maximum of catalytic activity at a distinct a_w value at which their stability is decreased as compared to relatively anhydrous conditions [24,25]. Moreover, it was proposed that the thermostable enzymic preparations owe their stability to their low degree of hydration (low water activity values) which would provide a more rigid conformation [25].

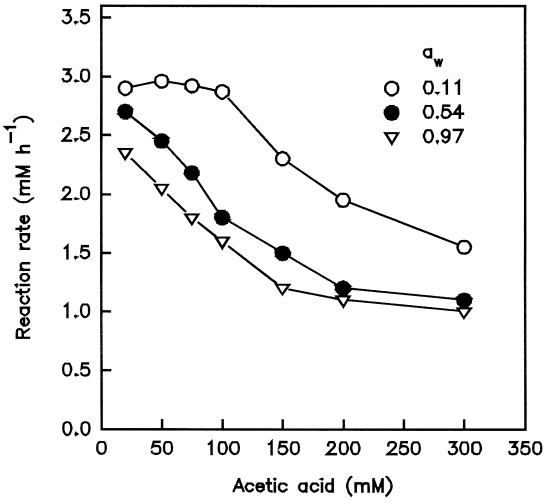

Fig. 5. Effect of acetic acid concentration on reaction rate of the esterification of geraniol (150 mM) at three different water activity values (a_w), catalyzed by *F. oxysporum* esterase in *n*-hexane. Reaction conditions as described in Table 1.

3.6. Synthesis of geranyl acetate by direct esterification

It has been showed that it is more difficult to prepare geranyl acetate by direct esterification, in similar fashion than other geranyl esters [9,26]. Up to now, just a few lipase are able to catalyze the esterification of alcohols with acetic acid [9,27]. Claon and Akoh [27] reported that among four commercial lipases tested, only immobilized lipase from *C. antarctica* gives high yields for the direct esterification of terpenols with acetic acid. It has proposed that the acetic acid can damage the hydration-layer around the protein molecule or can decrease the local pH in the microaqueous enzymatic microenvironment causing reaction inhibition [26,27].

In this work, the ability of *F. oxysporum* esterase to catalyze the direct esterification of geraniol with acetic acid have been studied. Fig. 5 shows the effect of acetic acid concentration on reaction rate for the direct esterification of geraniol in *n*-hexane at three different water activity values (a_w 0.11, 0.54 and 0.97 respectively). As it can be seen, both water activity values and acetic acid concentration affect the reaction rate of the esterification. Increasing a_w values as well as acetic acid concentration, reduce the reaction rate. This behavior is expected in direct esterification reactions studied since low water content favor synthesis over hydrolysis and it was observed by other researchers for similar reaction systems [21]. Moreover, it is expected that the high polar acetic acid, is located in the aqueous microenvironment of the enzyme, affecting their catalytic behavior since damage the hydration layer-protein interaction as described before. Probably the negative influence of acetic acid on enzyme hydration microenvironment and therefore on activity is reduced at low hydration state of enzyme (low a_w values).

The higher reaction rates as well as conversion yields (85–95%), were obtained for acetic acid concentrations < 100 mM and $a_w = 0.11$, while increasing concentrations of acetic acid inhibited esterase activity as illustrated in Fig. 5. The yields and the reaction rates observed in this work, ware significant higher than that observed for the direct esterification of terpenols with acetic acid catalyzed by lipozyme in *n*-heptane [13] or Palatase and *M. miehei* lipases in hexane [27]. It must be noted, that at this

conditions, no inhibition of *F. oxysporum* esterase activity by butyric acid was observed even at high acid concentrations (data not shown). Similar inhibitory effect of high acetic acid concentrations was also observed for the esterification of isoamyl alcohol and citronellol catalyzed by immobilized lipases from *M. miehei* and *C. antarctica* respectively [8,27].

3.7. Effect of enzyme reuse

In this study the ability of esterase from *F. oxysporum* to be reused was investigated. The enzyme preparation was reused 5 times for the acetylation of geraniol by ethylenglycol diacetate in *n*-hexane. Reaction was followed for 24 h incubation at 45°C. After each run the enzyme was washed with dry *t*-butanol and *n*-hexane and the solvent was evaporated. The enzymatic preparation, was adjusted to the water activity value of 0.11, prior to reuse, by procedure described in Section 2. Reaction rates of geranyl acetate formation with repeated use of esterase are shown in Fig. 6. As it can be seen, the reaction rate observed after the fifth reaction cycle (120 h), was reduced only about 10%, demonstrating the relatively high stability of enzymatic activity under the above experimental conditions.

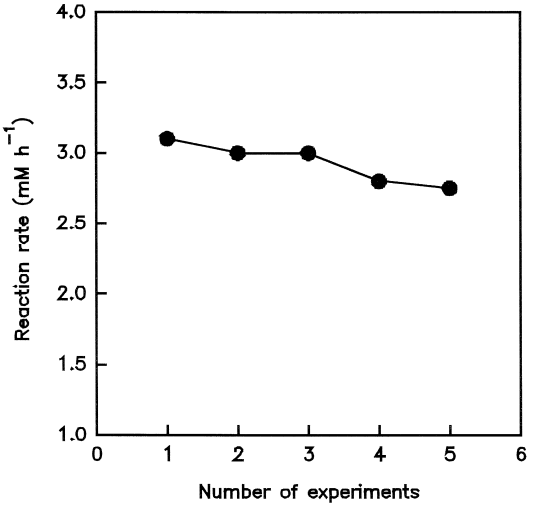

Fig. 6. Reaction rates of the acetylation of geraniol by ethylenglycol diacetate with repeated use of *F. oxysporum* esterase in *n*-hexane at $a_w = 0.11$. Reaction conditions as described in Table 1.

4. Conclusions

Esterase preparation from *F. oxysporum*, is well suited for the production of short-chain geranyl esters by esterification or transesterification in organic solvents. The synthetic action of esterase depends on the nature of organic solvent used, the hydration state of the system and the molar ratio of reactants. Under the conditions of the present study, the enzyme exhibit a high degree of stability and efficiency especially at low water activity values. Indeed, this esterase has the potential of becoming an important enzyme for acetylation reaction in bioorganic synthesis and for production of industrially important short chain flavor esters.

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