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Application of partially concentrated *Candida rugosa* lipase in the enzymatic synthesis of geranyl acetate in organic solvent

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ABSTRACT

Esters of terpinyl alcohols are substances used for adding flavor to several products because of their organoleptic properties. The enzymatic synthesis of these substances is more eco-friendly than conventional chemical synthesis. The aim of this study was to evaluate the concentration of *Candida rugosa* lipase by precipitation with acetone followed by lyophilization and application of the enzyme in the synthesis of geranyl acetate in organic solvent. The steps of enzyme concentration and ester synthesis were performed by applying central composite rotational designs (CCDR) in order to evaluate the process variables. The best condition for the concentration of *Candida rugosa* lipase was at −10 °C and 80% (w/w) acetone saturation, which resulted in 198 U/mL of enzyme activity and 11.7-fold concentration factor (CF). The optimum condition for the synthesis of geranyl acetate was at 45 °C, 12.5% (w/w) (lipase/geraniol mass ratio), 1:1.5 (geraniol: vinyl acetate molar ratio) at 8 h of reaction in organic solvent (n-hexane). Such condition resulted in 79% yield and productivity of 9.88%/h in ester production. The results obtained demonstrate that it is possible to obtain high yields in the synthesis of geranyl acetate even with the use of non-commercial partially concentrated lipase in the free form when using optimized process conditions.

1. Introduction

Esters of terpinyl alcohols are considered natural aromas due to their organoleptic properties and are widely applied in fragrances, foods, cosmetics, among others (Belsito et al., 2008). These esters may be produced by chemical synthesis and enzymatic extraction or catalysis (biocatalysis). Enzymatic synthesis presents numerous advantages for the production of different types of substances due to: greater specificity in the formation of products, better recovery and purification after the synthesis, lower energy consumption and lower by-product formation. Several enzymes can be used in biocatalysis, such as lipases, which are especially prominent due to their great versatility. Lipases can catalyze reactions of triacylglycerol hydrolysis, ester synthesis, alcoholysis, aminolysis, peroxidation, epoxidation, inter and transesterification, etc. Lipases can be applied in both aqueous and organic solvent environments and they also act at the interface between polar and nonpolar solvents (Badgujar and Bhalchandra, 2014; Gupta et al., 2013; Thakar and Madamwar, 2005).

Geraniol-based esters, such as geranyl acetate (3,7-dimethylocta-2,6-dien-1-yl ethanoate), are important components of various essential oils for granting them the scent of roses, and thus are widely used in the manufacture of perfumes. Moreover, geraniol and its geometric isomer (nerol) present different biological activities and are often mentioned due to their high antimicrobial activity (Gupta et al., 2007). Given the importance of geraniol esters and the advantages of employing enzymatic catalysis rather than chemical catalysis, several enzymatic systems have been studied for their production.

The synthesis of geranyl esters can be performed by using different organic solvents. N-hexane is commonly cited in the literature as a solvent for this synthesis. There are also reports of the use of supercritical CO_2, although with less efficiency than n-hexane. N-heptane, isooctane, media with low water activity or with the addition of surfactants are also alternatives for the enzymatic synthesis of geraniol esters (Chulalaksananukul et al., 1993, 1992; Huang et al., 1998;

Abbreviations: Ac, acetone; ANOVA, analysis of variance; CCRD, complete central rotational design; CF, concentration factor; G:A, geraniol:vynil acetate molar ratio; L:G, lipase:geraniol mass ratio; P, productivity; RF, recovery factor; T, temperature; t, reaction time; U, unit of lipase activity (μmol/min); Y, yield

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Stamatis et al., 1998) .On the other hand, solvent-free media can be a promising way of synthesizing ester, since the absence of organic solvents makes these processes more eco-friendly. Ferraz et al. (2015) obtained 53% yield in the synthesis of geranyl propionate using non-commercial immobilized lipase from *Penicillium crustosum* at 60 °C after 50 min of reaction in a solvent-free medium.

In what concerns to the nature of the enzymatic catalyst, a number of strategies can be applied, such as the use of lyophilized cells, free-form enzymes and immobilized enzymes in different types of supports (macroporous anionic resin beads, commercial polymers, polyacrylonitrile nanofiber membranes, physical adsorption and covalent bonding, physical adsorption in nanotubes, sodium alginate, biodegrabable polymer, among others) (Chulalaksananukul et al., 1993, 1992; Huang et al., 1998; Stamatis et al., 1998; Converti et al., 2002; Varma and Madras, 2010; Damnjanović et al., 2012; Gupta et al., 2013; Mohamad et al., 2015; Ferraz et al., 2015).

Processes involving immobilized lipases are the most commonly reported in the literature, mainly due to the possibility of reuse of the enzyme in several reaction cycles. Huang et al. (1998) obtained 84% (in 24 h) and 97% (in 36 h) yield in the synthesis of geranyl acetate using immobilized surfactant-coated lipase from *Candida rugosa* in organic medium (isoocante, 35 °C) and Damnjanović et al. (2012) obtained yield > 99.9% (in 48 h) in batch reaction and 78.9% (in 10 h) in a fluidized bed reactor when employing a commercial immobilized lipase from *Candida rugosa*.

However, there have been reports of the use of free-form enzymes with high yields in the formation of geraniol esters under optimized process conditions. Converti et al. (2002) verified the increase of initial speed of the reaction with the increase of lyophilized cells concentration of *Aspergillus oryzae* (from 5 to 30 g/L) and with the increase of temperature (from 30 to 80 °C) without geraniol inhibition up to 75 mM concentration. In other study, Xiong et al. (2014) using lipases from different micro-organisms in free enzyme system obtained a maximum yield of 96.3% (in 3 h) with concentration of geraniol at 100 mM, 200 rpm agitation speed, 35 °C and enzyme concentration of 1% (w/v) for the *Pseudomonas fluorescens* lipase (A = 121.8 U/g). Furthermore, the authors reported 40% yield with *Candida rugosa* lipase (A = 23.2 U/g) under the same conditions, which represents a productivity of 13.33%/h. Stamatis et al. (1998) reported between 70% and 80% yield (after 96 h) in the synthesis of short-chain of geraniol with *Fusarium oxysporum* lipase in organic solvents (hexane).

The literature reports other peculiarities in the synthesis of esters of geraniol. The synthesis of geranyl acetate with immobilized lipase from *Mucor miehei* was five times faster in organic solvent (hexane) than in supercritical CO_2 and there was inhibition of the reaction by the geraniol concentration in the ranging between 30 and 240 mM (Chulalaksananukul et al., 1993, 1992). Varma and Madras (2010) verified that the speed of synthesis of esters of geraniol increased with the increase in the concentration of the lipase Novozyme 435 (*Candida antartica*) in the range between 10% and 15%(w/w), however it did not change above this range. The optimum temperature in the same study varied from 40 to 60 °C as a result of the supercritical solvent used.

Thus, the aims of this study were to evaluate the concentration of *Candida rugosa* lipase and the application of this biocatalyst in the transesterification between geraniol and vinyl acetate for the production of geranyl acetate in organic medium (n-hexane). Factorial designs were applied in order to obtain the optimal process conditions.

2. Material and methods

2.1. Microorganism

The lyophilized microorganism *Candida rugosa* NRRL Y-17506 was kindly donated by the Agricultural Research Service Culture Collection (USA). Microorganism activation was carried out by cultivation in a medium containing 20 g/L agar, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose, which was incubated at 27 °C for 48 h. After cultivation, the stock culture produced was stored at 5 °C.

The fermentation medium used for the production of lipase contained: 3 g/L of yeast extract, 4 g/L of malt extract, 6 g/L of yeast hydrolyzate (Prodex lac®) and 5 g/L of olive oil. The fermentation was carried out in flasks, which were agitated at 180 rpm and 25 °C for 72 h (Ho et al., 2004). After culturing, the fermented medium was centrifuged at 300 rpm for 15 min at 4 °C for cell removal. The supernatant was used for measuring crude lipase activity.

Table 1
Central composite rotational design with 2^2 factorial points + 4 central points for the concentration of *Candida rugosa* lipase at different concentrations of acetone (*Ac*, % v/v) and temperature (*T*, °C).

Run	*Ac* (%v/v)*	*T* (°C)*	Lipase (U/mL)	RF (%)	CF
1	60 (−1)	−10 (−1)	145.42	8.60	8.61
2	80 (+1)	−10 (−1)	198.57	23.50	11.75
3	60 (−1)	0 (+1)	62.87	3.72	3.72
4	80 (+1)	0 (+1)	147.15	39.18	8.71
5	70 (0)	−5 (0)	156.47	46.29	9.26
6	70 (0)	−5 (0)	168.87	44.97	9.99
7	70 (0)	−5 (0)	138.98	41.12	8.22
8	70 (0)	−5 (0)	147.15	43.54	8.71

* Coded values in parentheses.

2.2. Lipase

The concentration of lipase from the cell-free medium (crude lipase) was performed by acetone precipitation. A CCDR consisting of 2^2 factorial points + 4 central points (Table 1) was used for evaluating the independent variables: acetone concentration (*A*, 60–80% v/v) and temperature (T, −10–0 °C). The ranges of variables for concentration of lipase were based on the previous study conducted by Kumarevel et al. (2005). The amount of acetone added in each assay was calculated according to Eq. (1) (Scopes, 1994), in which z = volume of acetone (mL) added to 1000 mL of crude enzyme, x = initial saturation (% v/v), and y = final saturation (% v/v).

$$z = 1000\left(\frac{y-x}{100-y}\right) \quad (1)$$

In all assays, the acetone was added to the crude enzyme broth with a drip speed of 3.5 mL/min under magnetic stirring. After precipitation, the concentrated enzyme was separated from the supernatant by centrifugation (8000 rpm, 15 min, 2 °C) and resuspended in 10 mL of 0.1 mol/L phosphate buffer with pH 7.0. Concentration factors (CF) and recovery factors (RF) were estimated as a function of the initial activity of the crude lipase and of the final activity of the concentrated lipase (Maldonado et al., 2015).

The concentrated enzyme was then distributed into vials with a volume of 3.5 mL, frozen at −25 °C for 24 h, and lyophilized at −55 °C in a benchtop freeze dryer (Terroni®, Interprise) with the aid of an hermetic compressor with forced air ventilation using a vacuum pump with capacity of 5/370 (CFM/W) for 24 h. The obtained lyophilized cake was gently broken to obtain fine powder (Shieh et al., 1996; Maldonado et al., 2015). The enzyme concentrate was placed in hermetically sealed glass vials and stored at room temperature until use in the enzymatic synthesis.

2.3. Geranyl acetate

Geranyl acetate synthesis was carried out in completely sealed 25 mL Erlenmeyer flasks. One hundred (100) mg of geraniol were dissolved in 10 mL of organic solvent (n-hexane) in each flask. The flasks were kept under agitation at 140 rpm in a shaker. The independent variables (factors) assessed were: temperature (*T*, 30–45 °C), enzyme: