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Optimizing the Synthesis of Citronellyl Valerate Using Lipase from *Rhizopus* sp

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Abstract Citronellyl valerate was synthesized by a lipase from a *Rhizopus* sp strain isolated and the lipase produced, at UNICAMP, Brazil. Direct esterification was performed in a solvent-free medium to produce the flavor ester. Response surface methodology was used to optimize the process with respect to the substrate molar ratio and lipase concentration. The results show that the synthesis of citronellyl valerate can be carried out in a solvent-free medium, the maximum ester conversion rate achieved being 91.5% after 48 hours of reaction time.

Keywords: enzymatic synthesis, esters, *Rhizopus*, lipase

Introduction

Since the end of the last century people have become more concerned about their health. Thus natural foods are a very common requirement nowadays, including mainly natural ingredients. This means that, due to the great demand, it is no longer possible to obtain enough natural flavor compounds only by extraction from plants, it being necessary not only to create natural flavors but also to develop new technologies (1). The food legislation of many countries recognizes that natural flavors can be obtained by biotechnological methods (2). The three principal techniques of biotransformation can be distinguished in the following way: use of enzymes, use of microorganisms, use plant cells and tissue cultures. The biotransformation technique most used is that of enzyme application (3).

The use of lipases for direct esterification reactions in solvent-free media is a good alternative for the production of flavor compounds for the food industry. Since the esters are extensively used in foodstuffs and beverages, it is desirable to eliminate the use of organic solvents. Several studies have shown a high potential for the industrial use of modified terpenes (4). Esters of the acyclic terpene alcohol citronellol with short chain fatty acids are very important flavor and fragrance substances (5).

In this research, the ability of the lipase from a *Rhizopus* sp to catalyze the formation of citronellyl valerate by a direct esterification reaction in solvent-free media was studied. Additionally, response surface methodology (RSM), based on a five-level, two-variable central composite rotatable design (CCRD), was used to optimize the process with respect to the following important reaction variables – substrate molar ratio and lipase concentration.

Material and Methods

Lipase Lipase from the *Rhizopus* sp was produced in a solid medium (60% wheat bran and 40% water) at 30°C. After 120 hours, water was added to the solid medium, followed by homogenization, 1 hour of incubation and finally simple filtration. The supernatants were treated with ammonium sulfate (80% saturation) and the precipitates dialyzed against water and freeze dried for use as the crude lipase preparation in powder form.

Experimental design A five level, two variable central composite rotatable design was adopted to optimize the enzymatic reaction, requiring 11 experiments. The variables studied in the process of citronellyl valerate synthesis were molar ratio of citronellol to valeric acid (3:1 to 1:3) and the amount of enzyme (1-7% by weight of the total system). The independent variables and their levels and real values are presented in Table 1.

Esterification method Citronellyl valerate synthesis was carried out in screw-capped test tubes. DL-citronellol and valeric acid were added at different molar ratios followed by different amounts of lipase, according to table 2. The mixture was stirred in an orbital shaker water bath (200 rpm) at 40°C, and samples were taken for analysis after 24 and 48 hours of reaction time. The residual enzyme was removed from the samples by passing through a column containing anhydrous sodium sulfate. The samples were then frozen and analyzed by gas chromatography.

Analysis Twenty μL of supernatant was added to 980 μL of isooctane. The analysis was performed by injecting a 1 μL aliquot in the split (1:100) mode into a CHROMPACK CP 9001 gas chromatograph equipped

Table 1. Variables and levels for the central composite design

Variables	Coded variable levels				
	-1.41	-1	0	1	1.41
Molar ratio (alcohol:acid)	3:1	2.41:1	1:1	1:2.41	1:3
Lipase amount (%w/w)	1	1.88	4	6.12	7

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Table 2. Central composite design and responses for citronellyl valerate synthesis

Run	Variable levels[1]		Responses (Y%)[2]	
	Molar ratio	Lipase amount	24 hours	48 hours
1	-1	-1	19.7	58.8
2	+1	-1	30.3	11.6
3	-1	+1	91.5	75.8
4	+1	+1	12.7	52.9
5	-1.41	0	75.1	50.9
6	+1.41	0	4.77	21.9
7	0	-1.41	0.297	4.15
8	0	+1.41	66.7	81.5
9	0	0	28.0	65.9
10	0	0	37.5	72.7
11	0	0	28.6	51.1

[1]Coded variables
[2]Response after 24 and 48 hours of reaction time; Y%, % of molar conversion (yield)

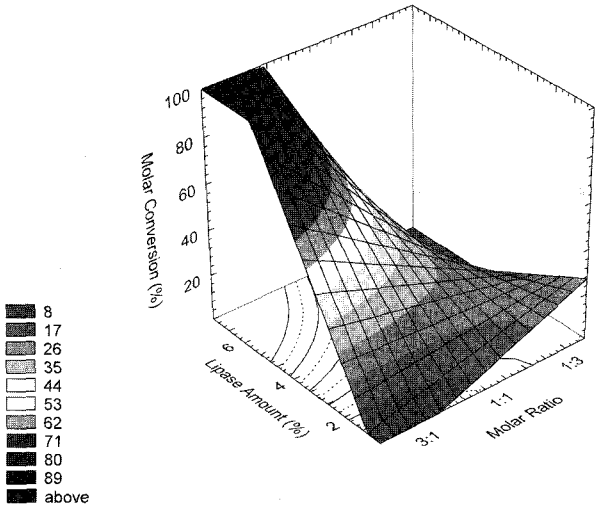

Fig. 1. Response surface of yield as a function of molar ratio and lipase amount for the synthesis of citronellyl valerate.

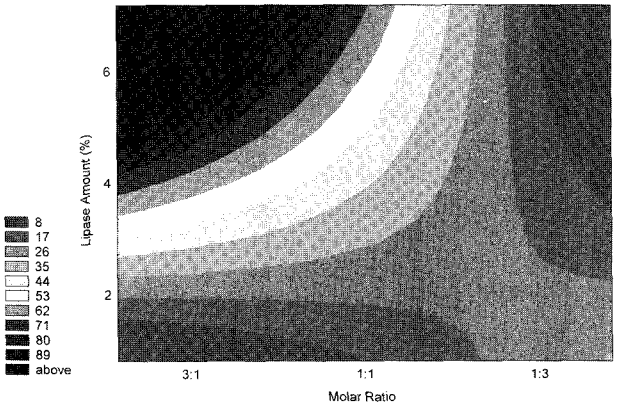

Fig. 2. Contour diagram of yield as a function of molar ratio and lipase amount for the synthesis of citronellyl valerate.

with a flame-ionization detector. A β-DEX™ 225 capillary column was used. The injector and detector temperatures were set at 220 and 300 °C, respectively and the oven temperature was maintained at 95°C. The carrier gas was helium. The extent of synthesis (yield) was calculated based on the consumption of the injected alcohol, and quantified using standard alcohol curves.

Results and Discussion

Table 3 shows the analysis of variance (ANOVA) for the yield after 24 hours. The pure error was low so there was good data reproducibility. The correlation coefficient of 0.94 and *F*-test value (8.7 times higher than $F_{3,7}$=4.35) were good enough to show that the model (equation 1) obtained was representative.

Y = 30.92 – 20.96 (molar ratio) + 18.51 (amount lipase) – 22.35 (molar ratio x amount lipase)

Figures 1 and 2 show the response surface and contour plot, respectively.

Lipase from *Candida antarctica* catalyzed the esterification of citronellol with 74% of conversion yield in a solvent-free medium (6). BOURG-GARROS *et al.* (1998) (7) investigated immobilized lipase from *Candida antarctica* (Novozym 435) for the synthesis of (z)-3-hexen-1-il acetate by direct esterification in a solvent-free medium and achieved a 70% yield. The lipase from *Rhizopus* sp was considered to be very effective during isoamyl butyrate synthesis (8). This enzyme also has the ability to catalyze the formation of citronellyl butyrate by direct esterification, with yields of from 95 to 100% after 24 hours (9).

Table 3. ANOVA for the synthetic variables pertaining to the response % molar conversion after 24 hours – citronellyl valerate

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F_{cal}	F_{tab} (0.95)
Regression	8253.891	3	2751.297	37.78	4.347
Residual	509.6926	7	72.81323		
Lack of fit	453.0859	5			
Pure error	56.60667	2			
Total	8763.584	10			

Regression coefficient: R = 0.94184

In our study, the synthesis of citronellyl valerate exhibited very high yields. The maximum ester conversion rate achieved was 91.5% after 24 hours of reaction time. Figures 1 and 2 show the molar ratio and amount of lipase required to obtain a molar conversion above 89%.

These results indicate that the synthesis of citronellyl valerate can be carried out in solvent-free media, which is very important for its use as a food additive. Furthermore, the synthesis could be manipulated to increase the molar conversion rate as shown by changing the parameters (molar ratio and lipase amount). Therefore more studies are required to obtain the best conditions giving the highest yield at low cost.

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