



## Lipase-catalyzed synthesis of isoamyl acetate in free-solvent media– optimization using a central composite design

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### Abstract

The catalytic action of two non commercial lipases (E.C. 3.1.1.3) in the synthesis of isoamyl acetate in a solvent-free system, were tested using an experimental design in order to better understand the relationship between the reaction variables. Yields ranging from 67 to 77 % molar conversion were achieved. The synthesis of isoamyl acetate catalyzed by the chosen lipase (*Rhizopus sp.*) was optimized, in order to maximize the molar conversion (yield, Y) in a solvent-free system. Response surface methodology involving the substrate molar ratio (RM) and amount of lipase (L) was used, and a second order model was employed to generate response surfaces. The optimum conditions to obtain Y max = 80% were: RM= (2:1); L= 8.7% ( w/w), 40°C, and 48 hours of reaction time.

**Key words:** Flavour esters, esterification, response surface methodology, lipase.

### Introduction

Natural flavour esters extracted from plant materials are often either too scarce or expensive for commercial use. Esters produced from natural substrates by biocatalysts (considered “natural” by some regulatory agencies) may satisfy the increasing commercial demand<sup>1</sup>. Biotechnology offers many advantages over the traditional extraction of botanical materials for the production of flavours. These include highly specific end product generation (e.g. optically-active compounds)<sup>2,3</sup>, high yields and purity, along with guaranteed supply<sup>4</sup>. Commercial exploitation of biotechnology in this area relies not only on technical advances but also on satisfying certain regulatory considerations<sup>5</sup>. An optimised process for the high yield enzymatic synthesis of isoamyl ester would benefit food manufacturers and be more appealing to consumers<sup>6</sup>, since it would yield natural instead of artificial or synthetic flavours, especially if it used a solvent-free system. Ester formation by lipases is a well known reaction<sup>7,9</sup>. The synthesis of short chain esters catalyzed by lipases has been studied for years. Already in 1990, crude preparations of lipases in flavour ester synthesis were reported:<sup>10,13</sup> for ethyl butyrate, esters derived from geranyl, citronellyl alcohol, with butyric, acetic, propionic and capric acids. Different reaction systems were used. Some researchers used organic solvents in order to control the activity of the water formed during the esterification reaction<sup>14</sup>. Since these esters are extensively used in foods and beverages to create various flavours, it is desirable to eliminate the use of organic solvents. Elimination of organic solvents not only leads to “natural” food products, but also simplifies downstream processing<sup>15</sup> and makes the process economically exploitable by reducing production cost. Other studies report on aqueous systems for synthesis, with the addition of buffer solutions for protection of the lipase activity<sup>16</sup>. Response surface methodology (RSM) is an effective tool for optimizing the process<sup>17</sup>. If the proposed model is adequate, as revealed by the diagnostic checking provided by an analysis of variance (ANOVA) and residual plots, contour plots

can be usefully employed to study the response surface and locate the optimum operational conditions (Y max). The experiments were planned using a factorial experimental design<sup>17</sup>. The purpose of the work described here was to develop a simple system for synthesis, without addition of organic solvents, using natural substrates comparing two microbial lipases never tested in that type of synthesis before. The synthesis of isoamyl acetate was accomplished with 99% isoamyl alcohol (non commercial, a by product obtained from the industrial production of ethyl alcohol by sugar cane fermentation process, in Brazil).

### Material and Methods

**Lipase preparation:** *Geotrichum sp.* was grown for 48 hours in a liquid medium<sup>18</sup> at 30°C, with shaking at 150 rpm. *Rhizopus sp.* was grown in a solid medium composed of wheat flour (40% water w/w) for 72 hours at 30°C. Cells were removed by centrifugation and the supernatants treated with ammonium sulphate (80% saturation). The precipitates were dialyzed in phosphate buffer pH 7.0 and used in powder form as crude lipase preparations. For both microorganisms, lipase is the dominant protein in the supernatant. The lipase activities of the two precipitates were quantified by triolein<sup>18</sup>. The method was calibrated with a commercial lipase from *Candida rugosa* (Meito Sangyo Company). One unit (U) is defined as one mmole of oleic acid released per minute. *Geotrichum sp.* lipase with 3.6 U/ mg and *Rhizopus sp.* lipase with 4.16 U/mg were used.

**Experimental design – preliminary study:** A two level factorial design was adopted in the preliminary study to define the best lipase to synthesize isoamyl acetate. This first part of the study required 16 experiments (8 trials for each lipase source). The variables studied in isoamyl acetate synthesis were the temperature (40-60°C); molar ratio (MR) of isoamyl to acetic acid (1:2 - 4:1); amount of enzyme (1-10 % by weight of total system) and type of lipase used (*Geotrichum sp.* or *Rhizopus sp.*). These parameters were

chosen based on our earlier studies.<sup>19</sup> The time of reaction was not considered as a variable in the experimental design because the experiments were accomplished in four different times in order to study the reaction kinetics. The independent variables (MR; T; L) and their levels are presented in Table 1 and the experimental designs are shown in Tables 3 and 4 for the *Geotrichum sp.* and *Rhizopus sp.* lipases, respectively. Following the first factorial design mentioned previously, a second experimental design was used to optimize the synthesis of isoamyl acetate. The study was conducted using the lipase from *Rhizopus sp.*, selected as the more productive according to the results. The reaction parameters involved were the amount of lipase (L) and molecular ratio (MR) between the substrates. The temperature was fixed at 40°C, leading to a less expensive process. The independent variables, their levels and real values are presented in Table 2. A five level, two variable CCRD (central composite rotatable design) was adopted in this study and required 11 experiments, which included 4 factorial points, 3 central points to provide information about the interior of the experimental region, allowing to check for curvature. The central points also provided additional degrees of freedom for error, which resulted in greater power when testing the significance of the effects. To fit a second order model<sup>20</sup>, 4 extra points at a distance  $\pm 1.41$  from the central point, were added to the matrix for this design, as shown in Table 2.

**Ester production and analysis:** Esterification method: isoamyl acetate synthesis was carried out in screw-capped test tubes (3 x 10 cm). Isoamyl alcohol and acetic acid were added in different molar ratios followed by different amounts of lipase, at different temperatures, according to Tables 3, 4 and 5. The mixture was stirred in an orbital shaken water bath (130 rpm) and samples were taken for analysis after 6, 24, 48 and 72 hours of reaction time. Samples were taken and residual enzyme removed by centrifugation at 4°C and 10,000 rpm for 5 min. The samples were frozen and analyzed by gas chromatography within 24 hours.

**Analysis:** 150  $\mu$ L of supernatant was added to 750 mL of n-hexane plus 150 mL of hexanol as internal standard. The analysis was performed by injecting a 1 mL aliquot in a split (1:100) mode into a CHROMPACK CP 9001 gas chromatograph equipped with a flame-ionization detector. A CP WAX 52 CB fused silica capillary column (30 x 0.32 mm i.d.; film thickness 0.2 mm) was used. Injector and detector temperatures were set at 150°C and 200°C respectively. The oven temperature was held at 35°C for 2 minutes, before being raised to 90°C at 5°C/min and held for 2 minutes. The carrier gas was hydrogen. The percentage yield (% molar conversion) was defined according to equation 1.

$$Y(\%) = 1 - \frac{[ALC]_f \times 100}{[ALC]_i} \quad (1)$$

**Table 1.** Variables and their levels for the First Factorial Design.

Variable	Symbol	Coded variable levels		
		-1	0	1
Molar Ratio (alcohol:acid)	MR	(1:2)	(2.25:1)	(4:1)
Temperature, (°C)	T	40	50	60
Lipase amount, (% w/w)	L	1	5.5	10

**Table 2.** Variables and levels for Central Composite Design

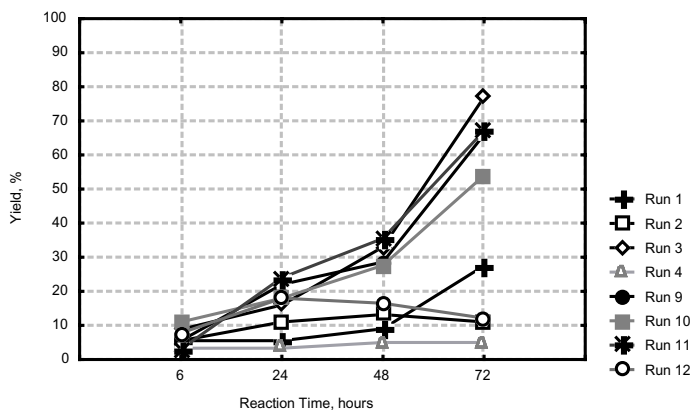
Variable	Symbol	Coded variable levels				
		-1.41	-1	0	1	1.41
Molar Ratio (alcohol:acid)	MR	(0.09:1)	(1:2)	(1.5:1)	(2.5:1)	(2.91:1)
Lipase amount (% w/w)	L	1	2.3	5.5	8.7	10

**Table 3.** Experimental factorial design arrangement and responses for *Geotrichum sp.* lipase.

Run	Variable levels <sup>a</sup>			Responses (Y%) <sup>b</sup>			
	MR	T	L	6 h	24 h	48 h	72 h
1	-1	-1	-1	5.5	5.5	9.0	27.5
2	+1	-1	-1	5.5	11.0	13.2	11.0
3	-1	+1	-1	8.8	16.0	33.0	77.0
4	+1	+1	-1	3.3	3.3	5.0	5.0
9	-1	-1	+1	5.5	22.0	28.6	66.0
10	+1	-1	+1	11.0	18.0	27.6	54.0
11	-1	+1	+1	3.0	24.0	35.5	67.1
12	+1	+1	+1	7.7	18.0	16.5	12.1

<sup>a</sup>)Coded variables

<sup>b</sup>)Response after 6, 24, 48 and 72 hours of reaction time; Y% = % of molar conversion (Yield)



**Figure 1.** Time course synthesis reaction of isoamyl acetate for *Geotrichum sp.*

Where :

[ALC]<sub>f</sub> = final concentration of isoamyl alcohol

[ALC]<sub>i</sub> = initial concentration of isoamyl alcohol

### Results and Discussion

The first experimental design was carried out in order to compare the lipases of *Geotrichum sp.* and *Rhizopus sp.* as possible catalysts for the synthesis of isoamyl acetate. The results obtained are shown separately for each lipase. Table 3 shows the results obtained for the *Geotrichum sp.* lipase in the synthesis of isoamyl acetate. The maximum yield observed was 77% after 72 hours of reaction time

with an MR of (1:2); temperature of 60°C and 1% lipase. In general it can be observed that 1% lipase with an MR of (4:1) were not the best conditions for synthesis, decreasing the molar conversion by up to 38.7%. Increasing the temperature from 40°C to 60°C did not cause statistically significant effects on the molar conversion. On the other hand, increasing the lipase concentration from 1 to 10 % resulted in a 19.7% increase in process yield. The experimental data obtained with the *Rhizopus sp.* lipase in the synthesis of isoamyl acetate are shown in Table 4. It can be seen that the effect of temperature can be considered significant for the process; which was not necessarily true for the *Geotrichum sp.* lipase. Therefore, it was possible to fix the process temperature at 40°C for the next experimental design (aimed at making the process more economic). The variable MR was the most significant for the process. It can also be observed that a change in MR from 1:2 to 4:1 decreased the yield by 14.4%, as observed with the *Geotrichum sp.* lipase. The difference in the type of catalysis performed by the two lipases, observed in the statistical analysis, became evident when the kinetic data were compared during the time course of the reaction as shown in Figures 1 and 2. The *Rhizopus sp.* lipase is more active and stable than *Geotrichum sp.* lipase in the reaction medium used. After 48 hours of reaction, the conversion values observed repaired similar to those obtained after 72 hours. The *Geotrichum sp.* lipase showed lower molar conversion values than the *Rhizopus sp.* lipase, and only after 72 hours of reaction were the conversion values significant. Comparing the kinetic data of the two lipases, the productivity (Y/h) of the *Rhizopus sp.* lipase was definitely higher than that of *Geotrichum sp.* lipase. For example, it can be seen that

**Table 4.** Experimental factorial design arrangement and response for *Rhizopus sp.* lipase.

Run	Variable levels <sup>a</sup>			Responses (Y%) <sup>b</sup>			
	MR	T	L	6 hrs	24 hrs	48 hrs	72 hrs
5	-1	-1	-1	49.5	44.0	45.1	55.0
6	+1	-1	-1	5.5	8.8	14.3	23.1
7	-1	+1	-1	22.0	36.0	37.4	24.2
8	+1	+1	-1	49.0	55.0	56.2	56.5
13	-1	-1	+1	49.0	47.0	57.5	67.1
14	+1	-1	+1	21.0	23.0	20.2	25.3
15	-1	+1	+1	28.2	44.0	57.2	37.7
16	+1	+1	+1	5.5	22.0	22.4	21.5

a) Coded variables; b) Responses after 6, 24, 48 and 72 hours of reaction time; Y% = % of molar conversion (Yield)

**Table 5.** Central composite design arrangement and responses for isoamyl acetate synthesis.

Run	Variable Levels <sup>a</sup>		Responses (Y%) <sup>b</sup>			
	MR	L	6 hrs	24 hrs	48 hrs	72 hrs
1	-1	-1	50	55	70	73
2	+1	-1	13	15	26	28
3	-1	+1	55	60	80	80
4	+1	+1	25	27	39	40
5	-1.41	0	30	32	48	45
6	+1.41	0	22	25	40	43
7	0	-1.41	30	32	51	50
8	0	+1.41	20	23	46	45
9	0	0	43	56	74	76
10	0	0	45	55	75	75
11	0	0	48	53	72	71

a) Coded variables; b) Response after 6, 24, 48 and 72 hours of reaction time, Y% = % of molar conversion (Yield)

**Table 6.** Analysis of variance for synthetic variables pertaining to response % molar conversion after 48 hours-isoamyl acetate.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F Test <sup>a</sup>
Regression	2380.5	4	595.1	3.9
Residual	912.1	6	152	
Lack of fit	907.5	4	226.8	
Pure error	4.6	2	2.3	
Total	3292.7	10		

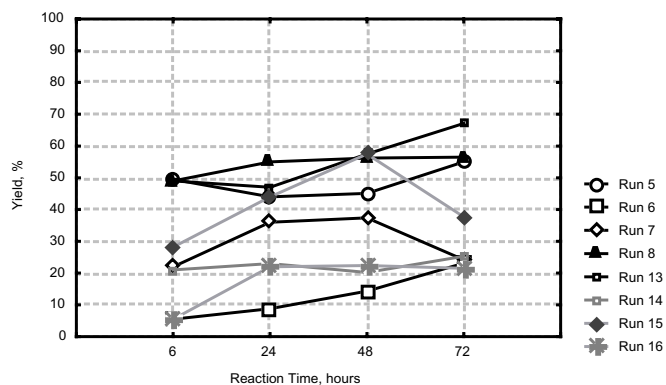
Regression Coefficient: R= 0.851

<sup>a</sup> F<sub>0.75; 4,6</sub> = 1.89

the maximum molar conversion obtained with *Geotrichum* sp. lipase after 48 hours of reaction was 35% as compared to 57.5% with *Rhizopus* sp. lipase. Table 5 shows the experimental conditions and the results for yield according to the second factorial design. After 48 hours the reaction achieved equilibrium under all conditions tested. Thus at this point, the productivity was higher and the process could be considered stable. The yield achieved along the experimental design ranged from 26 to 80%, showing a good distribution of the experimental points. On the other hand, under extreme conditions, axial points (-a,+a), runs 5 to 8, as shown in Table 5, the yield obtained was smaller than in the others. The maximum molar conversion obtained in the synthesis of isoamyl acetate by *Rhizopus* sp. lipase, was 80% (run 3, Table 5), where the variable molar ratio was (1:2) and the amount of lipase 8.7%, after 48 hours at 40°C. However it is important to observe that at the central point, where the molar ratio was 1.5:1 and the amount of lipase 5.5% (w/w), the yield was about 75%, statistically equivalent to the optimum condition of 80 % in run 3. From economic point of view, the central condition might be more interesting since it uses less lipase (5.5% as against 8.7%) and a higher concentration of isoamyl alcohol, a low cost substrate. Table 6 shows the analysis of variance (ANOVA) for yield. The pure error was very low, indicating good reproducibility of the data obtained. The lack of fit was responsible for a poor good correlation coefficient and *F*-test. This could be explained by the aggressive character of acetic acid in the lipase performance, resulting in enzyme inactivation during some of the experiments. However, the correlation coefficient of 0.851 and the *F*-test were enough to obtain an adequate model (equation 2) to represent the real relationship between the response percent molar conversion and the variables.

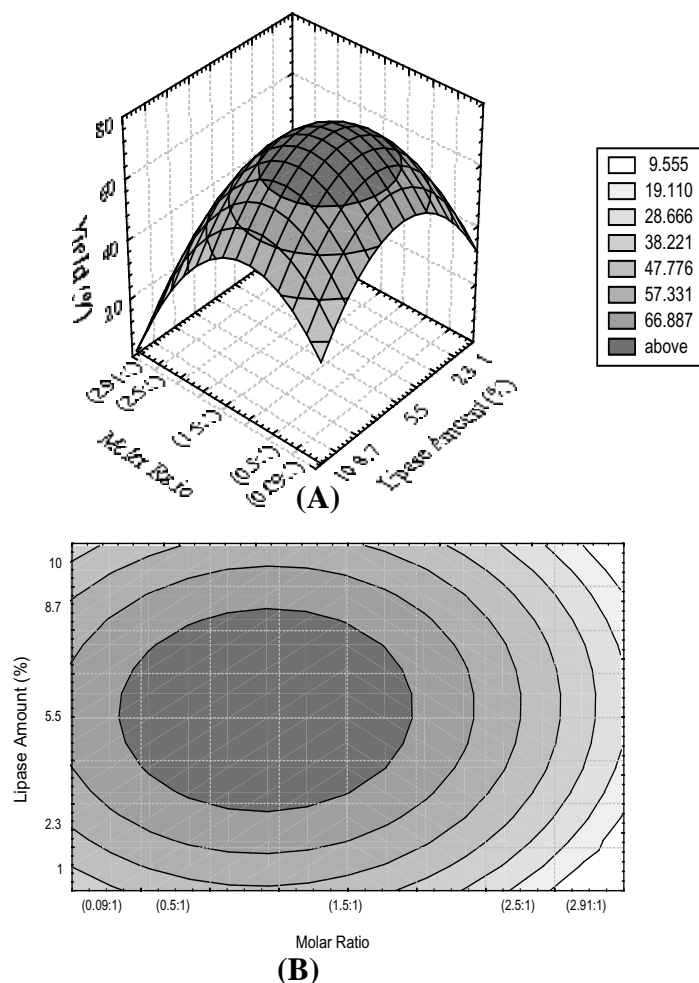
$$Y = 73.63 - 11.90MR + 1.98L - 12.88MR^2 - 10.62L^2 \quad \text{Eq. (2)}$$

Concerning equation 2, the interaction MR versus L was not statistically significant, but both MR and L were shown to be



**Figure 2.** Time course synthesis reaction of isoamyl acetate for *Rhizopus* sp.

significant, mainly the quadratic terms, responsible for the high curvature in the surface plot, as shown in Figure 3.



**Figure 3.** Response surface (A) and contour diagrams (B) of yield as a function of MR.

### Conclusions

The objective of this work was to verify the performance of two microbial lipases in the synthesis of isoamyl acetate, and then optimize the process using a reaction system of interest from the industrial point of view. The proposed system has not been used before with non immobilized lipase because it presents very aggressive characteristics for the enzymes. However, the lipases produced at UNICAMP were able to work in this system, leading to conversion ratios of interest with no water control. One of the main conclusions of this study was revealed by the kinetic behaviour of the lipases in the proposed system. The process using the *Rhizopus* sp. lipase was considered more effective because it

reached higher conversion rates after shorter reaction times. For this reason, the *Rhizopus sp.* lipase was used in the optimization of the synthesis of isoamyl acetate. However it must be noted the *Geotrichum sp.* lipase reached a high conversion ratio value after 72 hours of reaction. In 1994 Razafindralambo et al.<sup>21</sup> chose the synthesis of isoamyl acetate as a model to study enzymatic catalysis, using direct transesterification of acetic acid and isoamyl alcohol, accomplished in 10 mL n-heptane in a shaker at 150 rpm. The molar ratio of the substrates alcohol: acid varied from 1 to 8, the enzyme concentration from 0.1 to 1 g, the temperatures from 32 to 55°C and the amount of water from 0.1-1% (w/v). The researchers obtained 225 grams of isoamyl acetate after 24 hours of reaction or 10.62 g/l hours using immobilized *Mucor miehei* lipase. Comparing the productivity data obtained with *Mucor miehei* lipase with that of *Rhizopus sp.* lipase, the former was only 11% higher than the latter. It should be noted that the reaction systems were not the same in the two studies; Razafindralambo et al added organic solvent and water to the reaction medium, procedures that were not adopted here. Besides, the *Mucor miehei* lipase was a commercial immobilized powder and not a crude laboratory preparation, like the *Rhizopus* and *Geotrichum sp.* lipases used in this study. These considerations show the importance of proceeding with research using new lipases, able to work without the addition of organic solvents (leading to valuable products for use as food additives). Although the mathematical model was only predicted with 75% of confidence, the experimental data, whose behaviour can be evaluated visually from the response surfaces and contour diagrams, showed that the process was viable within the range of variation proposed for the process variables. It must be considered that the proposed reaction system includes no protection for the lipase used, in its crude form; the enzyme being exposed to acetic acid, which has an inhibitory effect on most lipases<sup>10,11,22,23</sup>. Most of the work done on acetate synthesis uses triacetin to solve this problem, this being a less aggressive substrate in a transesterification process, a methodology of indirect synthesis. The process presented in this work is a direct and economic way to produce isoamyl acetate. Since the system was feasible, the lipase could be immobilized to decrease its sensitivity to the acetic acid. Moreover this measure could lead to the reuse of the lipase, making the process even more economical. It seems that the best conditions (Y around 75%) for the direct synthesis of isoamyl acetate *Rhizopus sp.* lipase in an organic solvent-free system were: molar ratio 1.5:1; amount of lipase 5.5%; a temperature of 40°C and 48 hours of reaction time. Under these conditions, 8.6 g of isoamyl acetate were produced per litre of reaction mixture per hour, high productivity when compared to the 10.6 grams obtained by Razafindralambo et al in 1994<sup>21</sup> with *Mucor miehei* lipase, using the transesterification of triacetin and isoamyl alcohol in an organic solvent system.

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