Synthesis Optimization of Isoamyl Butyrate by Newly Microbial Lipases in solvent-free system.

Gabriela Alves Macedo[1], Gláucia Maria Pastore[2] , Maria Isabel Rodrigues [3]
[1,2,3] Universidade Estadual de Campinas, Faculdade de Engenharia de Alimentos,Campinas, SP, CP 6121; CEP : 13081-970, Brazil

Introduction

Esters from isoamyl alcohol, such as isoamyl butyrate, have economic value as chemicals for flavor and fragrance manufacturing, particularly if the ester may be considered a natural product (1). The synthesis of isoamyl esters by lipases in organic solvents has been studied (2). Enzymatic synthesis offers many advantages over traditional extraction of botanical materials for the production of flavors (3). These include highly specific end product generation , high yields, and purity (4). An optimized process for high yield enzymatic synthesis of isoamyl esters would benefit food manufacturers and be more appealing to consumers, yielding natural instead of artificial or synthetic flavors (5).The purpose of this work was to develop a lipase-mediated process in a solvent-free reaction system for the conversion of isoamyl alcohol into isoamyl butyrate using a natural substrate, which is a by-product of industrial production of ethyl alcohol from the fermentation process of sugar cane in Brazil and is generally discarded. The reactions were catalyzed by newly microbial lipases. A mathematical model using response surface methodology (RSM), was employed to optimize the synthesis process (6).

Results and Discussion

The set of first experiments was carried out in order to compare the lipases of *Geotrichum* sp. and *Rhizopus* sp. as catalysts for the synthesis of isoamyl butyrate. The maximum yield of isoamyl butyrate obtained with *Geotrichum* sp. lipase was 68.5% after 72 hours of reaction at Molar Ratio = 1:2; Temperature = 60 ^{o}C, and lipase amount = 10 % (Table 1). Higher reaction temperature did not increase the molar conversion, probably due to lipase denaturation. Therefore, it is possible to fix the process temperature at 40 ^{o} C leading to a less expensive process. When lipase from *Rhizopus* sp. was used, the variables Molar Ratio and Lipase Amount were the most significant in the process (Table 2). The lipase from *Rhizopus* sp. acted more quickly and gave a more stable product than the lipase from *Geotrichum* sp. in the proposed medium (Tables 1 and 2). After 48 hours of reaction with *Rhizopus* sp. lipase, the yield curve tended to stabilize. The productivity (Yield / h) of the lipase from *Rhizopus* sp. was higher than that obtained using the lipase from *Geotrichum* sp. The maximum yield obtained by the lipase from *Geotrichum* sp. after 48 hours of reaction was 30.8 %, as compared to 76 % of the lipase of *Rhizopus* sp. Thus, lipase from *Rhizopus* sp. was chosen for further study. A five level, two variable central composite rotatable design (CCRD) adopted in this second study required 11 experiments, which included 4 factorial points and 3 central points. The estimated effects for each variable, as well as the interactions between them, were determined. Table 3 shows the analysis of variance (ANOVA) for yield. The pure error was very low, indicating good reproducibility of the data. The coefficient of determination (R^2) 0.92, and *F*-test (4.2 times higher than $F_{4,6} = 4.53$) were adequate to obtain a model to represent the actual relationship between the response (percent molar conversion) and the experimental variables (equation 1). The R-squared value is also called the coefficient of determination and values above 0.90 are considered very good in statistical methods.

$$Y = 74.34 + 15.28\mathrm{MR} + 16.60\mathrm{L} - 26.05(\mathrm{MR})^2 - 13.38(\mathrm{L})^2 \quad \text{(Eq. 1)}$$

The optimum conditions for high yield of isoamyl butyrate were projected to be 1.8:1 for MR and 6.14 % for Lipase amount, giving a predicted value at the stationary point of about 82%. The best experimental yield (Y around 76%) was obtained at: molar ratio =1.5:1; amount of lipase = 5.5%; temperature = 40^{o}C; reaction time of 48 hours. Under these conditions, the yield predicted by Equation 1, would be 74%. The observed yield was 76% validating Equation 1 with a 95% confidence interval. Similarly, synthesis of isoamyl butyrate using a nylon-immobilized lipase from *Candida cylindracea* has been carried out with and without hexane in a reaction system described by Carta *et al*, 1992 (7). In presence of hexane, the isoamyl alcohol was added in stoichiometric proportions; when hexane were not used, the acid was dissolved in isoamyl alcohol. The excess of isoamyl alcohol diminished the lipase activity reduction caused by the acid in the reaction mixture. We observed that the water formed during the esterification reaction appeared to dissolve and did not have a significant effect on the initial rate of the esterification reaction. These observations, also registered by Carta *et al* (7), could explain why high yields were obtained without control of water formed during the reaction.

Table 1 Experimental factorial design arrangement and responses for *Geotrichum* sp. lipase.

Variable levels [a]				Responses (Y%) [b]			
Run	MR[c]	T, °C	L, %	6hrs	24hrs	48hrs	72hrs
1	(1:2)	40	1	5.5	3.0	5.0	7.4
2	(4:1)	40	1	5.5	5.5	5.5	7.4
3	(1:2)	60	1	2.6	30.2	18.7	30.8
4	(4:1)	60	1	5.5	8.8	19.8	27.5
5	(1:2)	40	10	15.4	28.6	27.5	52.8
6	(4:1)	40	10	16.5	22.0	30.8	53.6
7	(1:2)	60	10	7.7	11.0	22.0	68.5
8	(4:1)	60	10	2.2	2.2	6.0	5.5

a) Independent variables;b) Response after 6, 24, 48 and 72 hours ; Y% = % of molar conversion;c) Molar ratio of isoamyl alcohol to butyric acid; d) Loading of lipase, in wt% of total reaction mixture

Table 2. Experimental factorial design arrangement and response for *Rhizopus* sp. lipase.

Variable levels [a] [b]				Responses (Y%)			
Run	MR[c]	T, °C	L, %[d]	6hrs	24hrs	48hrs	72hrs
1	(1:2)	40	1	5.5	15.4	15.4	12.1
2	(4:1)	40	1	22.0	18.7	18.7	33.0
3	(1:2)	60	1	33.0	16.5	29.7	45.1
4	(4:1)	60	1	9.7	25.3	22.0	29.3
5	(1:2)	40	10	4.4	35.2	76.0	46.0
6	(4:1)	40	10	15.4	38.0	38.5	34.1
7	(1:2)	60	10	51.7	60.5	66.0	84.2
8	(4:1)	60	10	15.7	28.6	34.1	26.4

a) Independent variables;b) Responses after 6, 24, 48 and 72 hours ; Y% = percent of molar conversion;c) Molar ratio of isoamyl alcohol to butyric acid;d) Loading of lipase enzyme, in wt % of total reaction mixture

Table 3 Analysis of variance for synthetic variables, molar conversion *Rhizopus* sp. Lipase:40 °C, 48 hrs.

Source of variation	Sum of squares	Degrees of freedom	Mean squares	F Test[a]
Regression	8069.7	4	2017.4	18.8
Residual	643.7	6	107.2	
Lack of fit	635	4	158.1	
Pure error	8.6	2	4.3	
Total	8713.4	10		

Coefficient of determination: R^2= 0.92; $^{a}F_{(0.95;\,4;6)}$ = 4.53

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