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A CAPE approach to gamma-Linolenic acid production via lipase-catalyzed enzymatic hydrolysis

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Abstract

In this work, a lipase-catalyzed selective enzymatic hydrolysis of the borage oil was carried out using commercial enzymes and native crude enzymatic extract from Brazil as biocatalysts for the reaction. Two crude enzymatic extracts home-produced and three commercial lipases were screened for concentrating γ-linolenic acid of the triacylglycerols in unhydrolyzed acylglycerols. The kinetics of enzymatic hydrolysis of borage oil using *Geotrichum candidum* lipase in a batch reactor has been investigated. This work also presents a systematic procedure for parameter estimation through of dynamic optimization implemented as a nonlinear programming problem. With the reaction rate, a simulator was developed to explore the possible operational strategies for a large-scale reactor. The proposed computer-aided tool was applied to optimize the parameter of Michaelis-Menten kinetic model.

Keywords: Enzymatic hydrolysis, gamma-linolenic acid, kinetic model, CAPE approach.

1. Introduction

Polyunsaturated fatty acids (PUFA) present important action in physiological functions, therefore intensive effort has been done to identify possible effects of eicosapentaenoic acid (EPA), docosahexaenoic (DHA) and γ-linolenic acid (GLA) in treating some diseases. GLA has been reported to be an effective nutritional supplement to treat premenstrual syndrome [1], cancer [2], certain skin diseases [3], hypertriglycemidemia [4] and hypertension [5].
Borage oil (*Borago officinalis L.*) is rich in polyunsaturated fatty acids (PUFA), which contains a high level of GLA, around 23 %.
Some processes have been developed aiming the enrichment of GLA, as urea complexation [6], low temperature crystallization [7] and supercritical extraction [8], but usually undesired products are obtained. Lipase-catalyzed reactions are an advantageous alternative for this objective since some lipases have high selectivity toward unsaturated fatty acids. PUFA can be concentrated in the remaining acylglycerols, what is preferred considering the nutritional aspect [9].
Some works considered the kinetic parameters of the enzymatic reactions. To verify the kinetics of the enzymatic hydrolysis of palm oil by lipase, Sulaiman et al. [11] proposed a mechanistic model based on change interfacial area lipase-substrate with agitation speed and substrate concentration. Ting et al. [12] used the Michaelis–Menten equation to fit the kinetic parameters and the double reciprocal plot of the enzymatic hydrolysis reaction rate of the soybean oil. The initial weight of oil was used to evaluate Michaelis constant, K_m and the maximum reaction velocity, V_{max}. In this work, lipase-producing microorganisms isolated from natural sources (*Geothicum candidum* and *Aspergillus niger*) were used and compared with commercial lipases from *Candida antartica* B (CALB), *Thermomyces lanuginose* (TL 100L and TLIM) and *Rhizomucor miehei* (RM IM) as biocatalysts. Also, it is presented a systematic procedure for parameter estimation through dynamic optimization implemented as a nonlinear programming problem. The Michaelis-Menten kinetic model was applied in the hydrolysis reaction of borage oil. A mathematical model of the system was developed and the proposed computer-aided tool was used to optimize the parameters.

2. Experimental procedure

The Borage oil was purchased from SP Farma Ltda (São Paulo, Brazil). The employed commercial lipases were *Candida antartica* B (CALB), *Thermomyces lanuginose* (TL 100L and TLIM) and *Rhizomucor miehei* (RM

IM). All commercial lipases were provided by Novozymes SA. The microorganisms used in this study were isolated from soil and fruits samples and collected around Brazil Southwest region.

2.1. Lipase-catalyzed hydrolysis of the borage oil

The conditions used for the enzymatic hydrolysis reaction were: 12g of Borage oil (22.1% of GLA), 28 g of distilled water, 300 U/g of oil of the enzyme, stirring at 500 rpm and temperature of 40º C.

2.2. Separation of the free fatty acids (FFA) and acylglycerols

In the end of each enzymatic reaction, acylglycerols and free fatty acids fractions were separated by the method described by Shimada et al. [13].

2.3. Fatty acids composition analysis

Gas chromatography was used to determine the composition of the GLA in the FFA and acylglycerols fractions. Following, these fractions are converted in methyl esters by the method of Hartman et al. [14]. The analysis were carried out in a Varian chromatograph model STAR 3600CX.

2.4. Acylglycerols, FFA and glycerol analysis

The acylglycerols, FFA and glycerol (GL) components analyses were carried out by High Performance Size Exclusion Chromatography (HPSEC), model 515 HPLC pumps, a refraction index detector model 2410 and a temperature controller (Waters).

3. CAPE tool development

A deterministic mathematical model for the enzymatic reactor was developed and coupled with an optimization procedure. This CAPE tool allows to identify kinetic parameters as well as to explore different operational strategies. Both batch and continuously operated reactor were considered.
For the kinetic parameters estimation, a balanced based model for a batch reactor, represented by Eqs. 1 and 2, was solved using a FORTRAN program with integration by an algorithm based on the fourth-order Runge-Kutta method.

$$\frac{\mathrm{dP}}{\mathrm{d}t} = V_{\max}\frac{S}{K_{\mathrm{m}}+S} = v \qquad (1)$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -v \qquad (2)$$

where S and P are the measured compositions of TG and FFA.
A simultaneous estimation of the parameters (V_{max} and K_m) in the Michaelis-Menten kinetic model was determined by:
Minimizing

$$E(\theta) = \sum_{n=1}^{np}\left[(S_n - Se_n)^2 + (P_n - Pe_n)^2\right] = \sum_{n=1}^{np}\varepsilon_n^2(\theta) \tag{3}$$

Subjet to: $l_p < V_{max} < u_p$
$l_p < K_m < u_p$

A Quasi-Newton algorithm was used to find out the function minimization, but the software allows the use of alternative optimization methods as Levemberg-Marquadt and Genetic Algorithm. The optimization problem is seen a nonlinear programming one (NLP).
In Eq. (3), Se_n and Pe_n are the measured compositions of TG and FFA at the sampling time n. S_n and P_n are the compositions computed by the model at the sampling time n and the term *np* is the number of sampling points. l_p and u_p are specified lower and upper bounds on the parameters, with $l_p \leq u_p$. The $\varepsilon_n(\theta)$ is the error in the output due to the nth sample.

4. Results and Discussion

4.1. Lipase-catalyzed enzymatic hydrolysis

Table 1 presents the activity of the native and commercial lipases. 1 unit of lipase activity (U) is defined as the amount of enzyme that releases 1 μmol of fatty acid from triglyceride (olive oil) per minute of reaction at 37ºC.

Table 1 - Hydrolyze activity of the native crude enzymatic extracts and commercial lipases using olive oil

Native lipases	**Lipase activity (U/mg)**	
	SmF	**SSF**
Aspergillus niger (AN)	5.96	2.84
Geotrichum candidum (GC)	4.32	2.21
Penicillium solitum (PS)	1.34	0.63
Comercial lipases	**Lipase activity (U/mg)**	
Lipozyme RM IM	15.3	
Lipozyme TL IM	56.8	
Lipozyme TL 100L	108	
Candida antartica B (CALB)	1045	

Figure 1 shows the final composition of the triglycerides (TG), diglycerides (DG), monoglycerydes (MG), free fatty acids (FFA) and glycerol (GL) after 24 hours of reaction for each lipase employed in the hydrolysis reaction at 40º C.
It is observed that the lipase *Geotrichum candidum* presented a high potential to hydrolyse the acylglycerols, obtaining a maximum of 88.2% of FFA. The GC

lipase presented higher power of hydrolysis (Figure 1) and higher enrichment of GLA in the acylglycerols fractions (Table 2). This enzyme presented an enrichment of 39.5% in acylglycerols fractions. In fact, this lipase presents an enzymatic activity (U/mg of enzyme) lower than the others employed lipases, but when it is used in high quantities in order to remain the concentration 300 U/g of oil, the GC lipases present higher potential to concentrate GLA. Increasing its concentration to 318 U/g_{oil}, in the same reaction conditions, the enrichment of GLA reaches 41.7%.

Table 2 – GLA composition in the acylglycerols fraction using different lipases

Lipases	FFA Composition (%)[a] in acylglycerols fraction								
	16:0	**18:0**	**18:1**	**18:2**	**18:3**	**20:0**	**20:1**	**22:0**	**24:0**
Native									
AN	12.3	4.1	16	37.1	21.7	0	3.8	0	1.7
GC	8.1	4.7	11	23.1	39.5	0.2	6.3	0.2	1.9
Commercial									
CALB	10.8	3.5	17	37.2	22.6	0.2	4	0.1	1.6
RMIM	8.4	3.2	15.5	34.4	30.3	0.1	3.3	0.1	1.4
TL 100L	10	3.5	15.9	35.1	26.4	0.2	3.9	0.2	1.5
TLIM	7.9	2.8	15.6	34.4	30.8	0.2	3.5	0.1	1.6

Mass of water and oil = 40g; concentration of lipases of 300 U/g_{oil}.

[a] 16:0 Palmitic acid; 18:0 Stearic acid; 18:1 Oleic acid; 18:2 Linoleic acid; 18:3 γ-Linolenic acid; 20:0 Arachidic acid; 20:1 Eicosenoic acid; 22:0 Behenic acid; 24:0 Lignoceric acid.

4.2. Kinetic parameter estimation

The computed profiles for TG and FFA are shown in Figure 2. It can be seen that the model described well the batch experimental observations, according to reactions represented by equation 4. The obtained values of V_{max} and K_m are shown in Table 3.

$$TG + H_2O \xleftrightarrow{lipase} 3FFA + GL \quad (4)$$

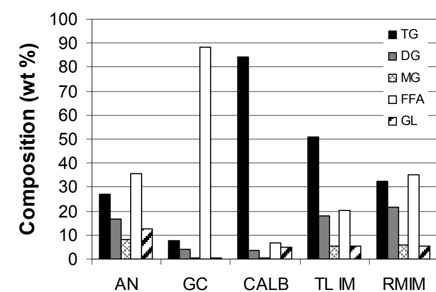

Figure 1 – Composition of the acylglycerols TG, DG, MG, FFA and GL for the different lipases used. 24 hours of hydrolysis reactions at 40ºC and lipase concentration of 300 U/g oil

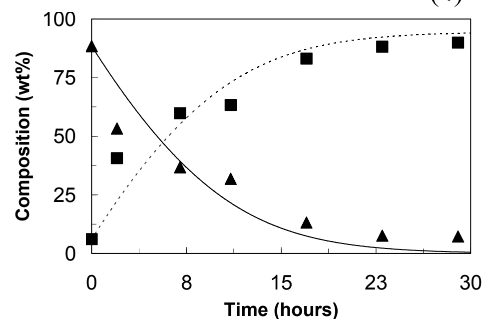

Figure 2 –Experimental and simulated data for batch experiments at 40ºC. The experimental data are for composition of TG (▲) and FFA (■). Simulated results were represented by lines (TG —; FFA ---)

Table 3 – Calculated values of Vmax and Km of the enzymatic hydrolysis using GC lipase

Parameters	Calculated values[a]
V_{max} (mM/min)	0.124
K_m (mM)	10.588

[a] 12 g of borage oil.

5. Conclusions

In this work the production of gamma-linolenic acid (GLA) via lipase-catalysed enzymatic hydrolysis is considered. The native crude enzymatic presented good performance to concentrate the GLA using a free-solvent enzymatic hydrolysis reaction (42 % of the GLA in acylglycerols fraction). To verify the borage oil hydrolysis and the enrichment of GLA, as well as to design a large scale system, a computer-aided procedure is proposed. With such procedure, it was possible to design as well as to define operating strategies a process to have high performance operation (high throughput at reduced residence time with lower enzyme deactivation).

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