



## Strategies to inhibit the lipid oxidation in the enzymatic synthesis of monoglycerides by glycerolysis of Babassu oil

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**ABSTRACT.** Different strategies to avoid the lipid feedstock oxidation in the enzymatic synthesis of monoglycerides (MAG) from glycerolysis of babassu oil were tested. The reactions were catalyzed by *Burkholderia cepacia* lipase immobilized on SiO<sub>2</sub>-PVA and the tests carried out in batchwise. The best strategy was tested in a continuous packed-bed reactor. Different antioxidants and emulsifiers were used, including: Butyl-hydroxy-toluene (BHT), tocopherol, soy lecithin and Triton X-100. The influence of inert atmosphere (N<sub>2</sub>) on the MAG production was also investigated. Results were compared with those attaining in the control reaction. The best performance was obtained using N<sub>2</sub> in the reaction medium, preventing the oxidation of babassu oil. MAG concentrations were 60 and 24% in batch and continuous mode, respectively. Among the tested antioxidant and emulsifying agents, only soy lecithin was found to be efficient but its application showed limit performance to be used in continuous runs.

**Keywords:** lipase, monoacylglycerol, antioxidant.

## Estratégias para inibir a oxidação lipídica na síntese enzimática de monoglicerídeos via glicerólise do óleo de Babaçu

**RESUMO.** Diferentes estratégias foram testadas para inibir a oxidação da matéria-prima lipídica na síntese enzimática de monoglicerídeos (MAGs) via glicerólise do óleo de babaçu. As reações foram catalisadas pela lipase de *Burkholderia cepacia* imobilizada em sílica-álcool polivinílico (SiO<sub>2</sub>-PVA) e os testes realizados em reator batelada, visando aplicar a melhor condição em reator de leito fixo operando continuamente. Foram testados diferentes agentes antioxidantes e emulsificantes como: Butil-hidroxi-tolueno (BHT), tocoferol, lecitina de soja e Triton 100X; sendo também estudada a influência da atmosfera inerte (N<sub>2</sub>) na produção de MAGs. Os resultados obtidos foram comparados com a reação controle. Nos sistemas testados, o melhor desempenho foi alcançado pelo emprego do N<sub>2</sub> no meio reacional, retardando a oxidação do óleo de babaçu, obtendo-se concentrações de 60 e 24% de MAGs no processo batelada e contínuo, respectivamente. Entre os agentes antioxidantes e emulsificantes testados, apenas lecitina de soja foi eficaz, entretanto apresentou limitações para uso em regime contínuo.

**Palavras-chave:** lipase, monoglicerol, antioxidante.

### Introduction

Monoglycerides (MAG) are nonionic surfactants, widely used in pharmaceutical, food, and cosmetic industries, for not having side effects when ingested, or skin irritation, unlike ionic surfactants. MAG can be synthesized by different enzymatic pathways: selective hydrolysis of triglycerides, glycerolysis of triglycerides and direct esterification of glycerol with different fatty acids (FREITAS et al., 2008). However, due to its high yield and productivity, glycerolysis reactions seem to be more advantageous than the other reactions. Vegetable oils are considered to be readily available raw material and low cost source for several

important fatty acids (DAMSTRUP et al., 2006). In addition, glycerolysis route has additional relevance in the present worldwide context, due to the sharp increase on the glycerol supplied in the market as a primary byproduct from biodiesel plants. Therefore, converting glycerol into value-added products provides an alternative for glycerol disposal and for its surplus problems (FREITAS et al., 2008; SILVA et al., 2009).

However, lipid feedstock for lipases very often have unsaturated fatty acyl groups in their molecules and these non- conjugate all-*cis* olefin structures are extremely vulnerable to highly oxidative conditions provide by active oxygen species such as superoxide

anion, hydroxyl radical, hydroperoxy radical and radical initiating metal ions (ALUYOR; ORI-JESU, 2008). As a result, autoxidation of the unsaturated lipids easily occur affording hydroperoxides and decomposition products giving rise to the undesirable odors and flavors characteristic of rancid fats (RAMALHO; JORGE, 2006). In addition, fat peroxides are considered as harmful substances which may cause cancer or arteriosclerosis or both or accelerate aging (OHTA et al., 1989). In this way, chemical compounds known as antioxidants are used to interrupt the oxidation process by preferentially reacting with the fat radical to form a stable radical which does not quickly react with oxygen (RAMALHO; JORGE, 2006). These compounds are normally used to increase shelf life of manufactured products for which there is available a vast literature and information (ALUYOR; ORI-JESU, 2008; RAMALHO; JORGE, 2006). On the other hand, little quantitative information has been available concerning the effects of lipid peroxide on the reaction rate and on enzyme inactivation in the course of enzymatic reaction. Specifically in relation to the enzymatic glycerolysis, emphasis can be given to the results reported by Ohta et al. (1989), which investigated the inhibition and inactivation of lipase by fat peroxide in the course of batch and continuous glycerolysis. Another strategy was tested by Valério et al. (2010) using food grade surfactants in the synthesis of monoglycerides by glycerolysis of olive oil, such as soy lecithin and Triton X-100.

In this context, this study aimed to test different strategies to inhibit the oxidation of babassu oil in the enzymatic synthesis of monoglycerides, carried out in batch wise to apply the best condition in a continuous operating system. For this purpose, different antioxidants and emulsifiers were used, such as Buthyl-hydroxy-toluene (BHT), tocopherol, soy lecithin and Triton X-100. The influence of inert atmosphere, by sparging N<sub>2</sub> in the reactor vessel, on the production of MAG was also investigated. The monoglyceride syntheses were catalyzed by the *Burkholderia cepacia* lipase immobilized on SiO<sub>2</sub>-PVA hybrid particles. This lipase preparation was chosen due to promising results obtained using this biocatalyst as previously described (DA RÓS et al., 2010; FREITAS et al., 2009, 2010). The reason for using babassu oil is because of its low cost, composition rich in saturated fatty acids (about 90%), and not compete directly with the food chain (SUAREZ et al., 2009).

## Material and methods

### Material

All experiments were carried out with a commercial lipase preparation from *Burkholderia cepacia* (Lipase PS – Batch number: 01022TD) purchased from Amano Pharmaceuticals (Nagoya, Japan) and used as received without further purification. Glycerol (99.5%, propane-1,2,3-triol) was purchased from Merck (Darmstadt, Germany). Tetraethylorthosilicate-TEOS (98%, Aldrich®), polyvinyl alcohol (88%, Across Organics), HCl (minimum 36%, Isolar), epichlorohydrin (99%, Aldrich®) and polyethylene glycol (MM 1500, Reagen) were used in the support synthesis. Refined, bleached and deodorized babassu oil was kindly provided by Pulcra Chemicals (Jacarei, São Paulo State, Brazil) with the following properties: acid number: 0.65 mg KOH g<sup>-1</sup>; peroxide number: 1.82 mEq kg<sup>-1</sup>; iodine number: 25 g I<sub>2</sub> g<sup>-1</sup>; saponification number: 238 mg KOH g<sup>-1</sup> and composition of fatty acids (% wt): octanoic (3.5), decanoic (4.5), lauric (44.7), myristic (17.5), palmitic (9.7), stearic (3.1), oleic (15.2), linoleic (1.8), and average molecular weight of 709.90 g mol<sup>-1</sup>. BHT (butylated-hydroxytoluene) from SAFC Supply Solutions, tocopherol and Triton X-100 (Sigma-Aldrich), soy lecithin (Coleman) were used as antioxidant and emulsifying agents. All the other reagents were of analytical grade.

### Synthesis of the support and lipase immobilization

The hybrid composite of silica-polyvinyl alcohol (SiO<sub>2</sub>-PVA) was prepared and activated with epichlorohydrin and used to immobilize the *Burkholderia cepacia* lipase according to Da Rós et al. (2010). The support and resulting biocatalyst were characterized for their textural properties, using the BET method (Table 1). The immobilized systems had an average hydrolytic activity of 1,600 ± 120 U g<sup>-1</sup> and water level lower than 10%. The specific mass of the solid determined according to procedure described by Freitas et al. (2010) was 1.865 g mL<sup>-1</sup>.

**Table 1.** Textural properties of the support and lipase immobilized on SiO<sub>2</sub>-PVA.

Material	Specific surface area (m <sup>2</sup> g <sup>-1</sup> )	Specific pore volume (cm <sup>3</sup> g <sup>-1</sup> )	Average pore diameter (Å)
Support	461	0.28	22.91
Immobilized derivative	337	0.25	29.42

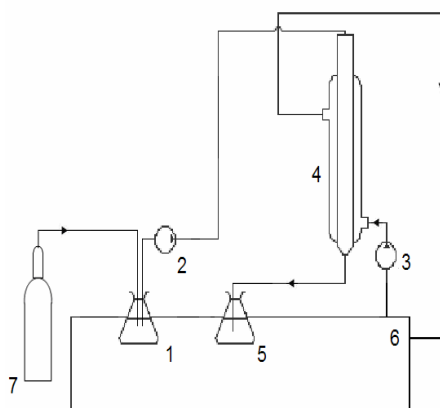
### General procedure of glycerolysis reactions in batch reactor

Reactions were carried out in cylindrical glass reactors (50 mL) containing glycerol and babassu oil, at molar ratio glycerol/babassu oil = 15, and the

mixtures incubated with immobilized lipase on  $\text{SiO}_2$ -PVA at proportion of 10% in relation to the total mass of reagents in the reaction medium (FREITAS et al., 2009). The antioxidant agents (BHT and tocopherol) and emulsifiers (soy lecithin and Triton X-100) were added in adequate amounts (30-100 ppm in relation to the total mass of substrate) at the beginning of the reactions. The syntheses were performed under magnetic stirring (150 rpm) at 50°C for a maximum period of 12h. Inert atmosphere was obtained by sparging  $\text{N}_2$  in the reactor vessel, avoiding in this way exposure of the reaction medium to oxygen. Samples were collected and storage at -2°C for chromatographic analyses. Results were expressed in molar fraction of monoglycerides, diglycerides and triglycerides.

#### General procedure of glycerolysis reactions in continuous mode

The Figure 1 shows the experimental setup of the reaction system, consisting of a jacketed glass column with 15 mm internal diameter, 55 mm length, and 10 mL volume. The reaction mixture made up by babassu oil and glycerol (molar ratio glycerol/oil = 15) was kept in the feeding reservoir, under magnetic stirring at 50°C and pumped through the fixed bed reactor in a downflow at 0.018 mL h<sup>-1</sup>.



**Figure 1.** Experimental scheme of the fixed bed reactor: 1 - feeding reservoir; 2, 3 - peristaltic pumps; 4 - glass column; 5 - output of the product; 6 - bath for temperature control; 7 - nitrogen cylinder.

The column was packed with a suspension of immobilized lipase (6.70 g) in the reaction medium and stabilized by the recirculation of the substrate for 4h, which also allowed the elimination of air bubbles formed during the column packaging. The soy lecithin was added in adequate amounts in the feeding medium. The inert atmosphere when used was ensured by inserting a point of  $\text{N}_2$  in the feeding

reservoir, protecting the substrate from air exposure. Samples were taken and stored at -2°C for chromatographic analyses. The spatial time was calculated according to Levenspiel (1972).

#### Separation of the products formed during glycerolysis reactions

Samples from the reaction medium ( $\approx$  1gram) were subjected to a step of separation of fractions (mono, di and triglycerides), by adding a mixture of 9 mL chloroform and 2.5 mL glacial acetic acid, with mixing for 10 min. under constant stirring. Then the content was placed in a 100 mL-volumetric flask and the volume was completed with distilled water. Afterwards, the volumetric flask content was transferred to a separating funnel and left to settle for phase separation (AOCS, 2004). The aqueous phase (also containing glycerol) was discarded and the organic phase was concentrated in rotaevaporator (Fisaton 801).

#### Chromatographic analysis

Mono-di and triacylglycerols were analyzed by gas chromatograph (GC) using a Varian 3800 model (Varian, Inc. Corporate Headquarters, Palo Alto, CA, USA) equipped with flame-ionization detector and a 10 m X 0.25 mm X 0.12  $\mu\text{m}$  CPSil 5CB capillary column. Nitrogen was used as the carrier gas at flow rate of 2 mL min<sup>-1</sup>. The detector and injector temperatures were 300°C. The column temperature was set to 80°C for 1 min. and then programmed at 20°C min<sup>-1</sup> to 300°C which was maintained constant for 2 min. Other conditions were split ratio of 1:20 and attenuation equal to 1. An organic phase was dissolved in hexane/ethyl acetate (proportion of 1:1) which contained tetradecane as internal standard, and the injection was carried out into the GC (FREITAS et al., 2009). Data collection and analyses were performed using the software Galaxie Chromatography Data System version 1.9.

#### Calculation of selectivity for forming monoglycerides

The selectivity values obtained in the glycerolysis of the babassu oil were determined using the Equation 1.

$$\% \text{ Selectivity} = \frac{\% \text{ MAG}}{\% \text{ MAG} + \% \text{ DAG} + \% \text{ TAG}} \quad (1)$$

where:

% MAG = is the amount of monoglycerides formed in the reaction;

% DAG = is the amount of diglycerides formed in the reaction;

% TAG is the residual level of triglycerides.

## Results and discussion

### Glycerolysis reactions in batch reactor

Different glycerolysis reactions were performed, such as: i) without any device to prevent the oxidation of the feedstock (Control); ii) with inert atmosphere; and iii) using different antioxidants and emulsifying agents, aiming to study their influence on the monoglycerides synthesis from babassu oil. The progress of these syntheses is shown in Figure 2a to f.

Figure 2a illustrates the formation of mono- and diglycerides profile in the control reaction, in which 49% of MAG was obtained in 12h, with no reaction reversibility. The products obtained in the reaction carried out under inert atmosphere, as shown in Figure 2b, indicated that this strategy was highly efficient, increasing the MAG formation to about 57% in 12h-reaction.

These data corroborate to the reported results (AGUEDO et al., 2008, 2009; BALCÃO; MALCATA, 1997), being this strategy the most used to prevent the oxidation of oils and fats, warranting the enzymatic synthesis to occur without interference or inhibition by changes in the reaction conditions.

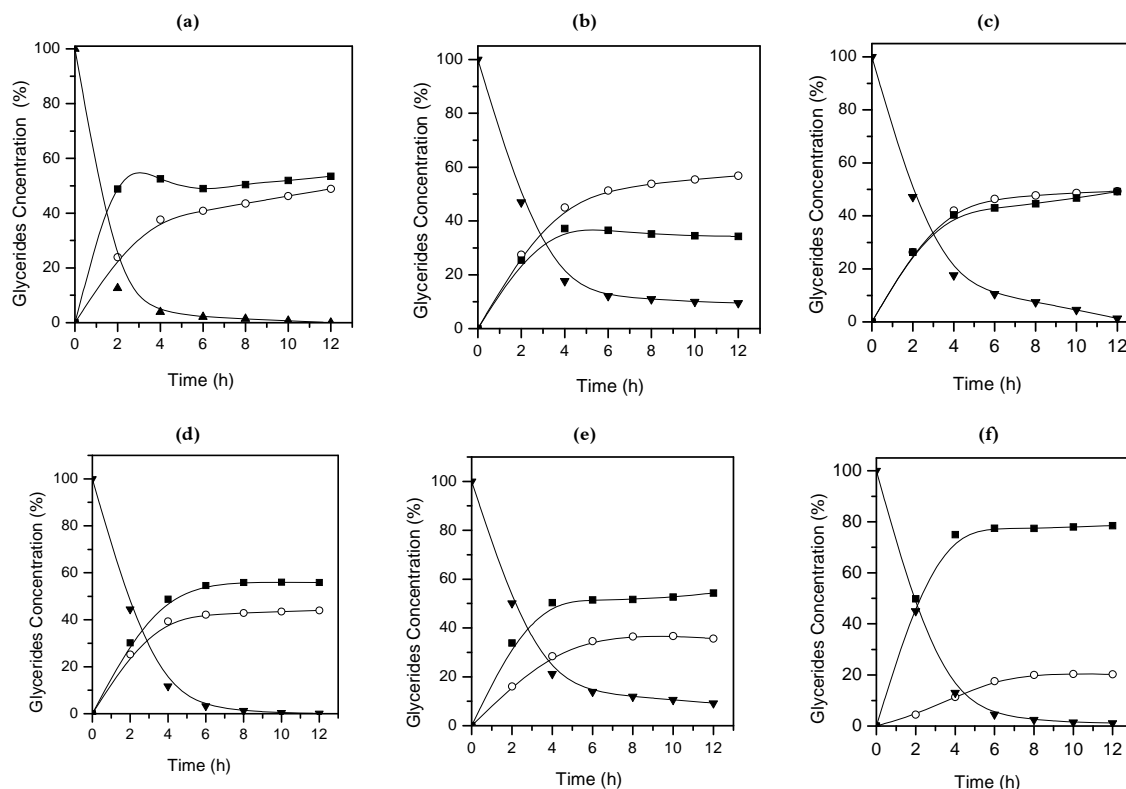
The lecithin also showed good oxidative capacity for the studied reaction system, allowing obtaining 49% of MAG in 12h. In addition higher reaction rate

was observed than the reaction control, resulting in the formation of 42% MAG in only 4h-reaction (Figure 2c).

This may be associated with the antioxidant and emulsifying properties of soy lecithin, widely used in pharmaceutical or food industries (chocolate, margarine). Besides, some studies (KURASHIGE et al., 1993; SOARES et al., 2003) have demonstrated a good compatible of this surfactant with enzymes and successfully employed as stabilizing agent of lipase during immobilization on solid supports.

The antioxidant activities of BHT (Figure 2d) and tocopherol (Figure 2e) were found to be unsatisfactory, since lower MAG concentrations (37-44% MAG) than the one obtained in the reaction control were quantified in 12h reaction. Moreover, in both reactions, a remarkable reaction reversibility was observed reducing MAG concentration after 12h reaction (results not shown).

The BHT is a polyphenol, synthetic antioxidant, very efficient in controlling the oxidation of animal fats and short chain fatty acids, as those in coconut and palm oil (RAMALHO; JORGE, 2006), and the tocopherol is a fat-soluble vitamin E, natural antioxidant, used to inhibit the oxidation of edible oils and fats, preventing oxidation of unsaturated fatty acids (RAMALHO; JORGE, 2006).



**Figure 2.** Profile of monoglycerides (○) and diglycerides (■) formation and consumption of triglycerides (▲) in glycerolysis reactions of babassu oil under different conditions: (a) Control; (b) inert atmosphere, (c) lecithin, (d) BHT, (e) tocopherol and (f) Triton X-100.

Furthermore, the profile of mono- and diglycerides indicated a change in the enzyme activity, with preferential formation of diglycerides, probably associated with some inhibition produced by these two compounds (BHT and tocopherol) on the lipase activity.

Triton X-100 (surfactant) was tested because the literature cites it as the most adequate for reactions mediated by lipases (VALÉRIO et al., 2010). Triton X-100 is a nonionic surfactant, a viscous liquid at room temperature, with clear yellow appearance. It has antimicrobial properties, and frequently used in biochemical applications to solubilize proteins.

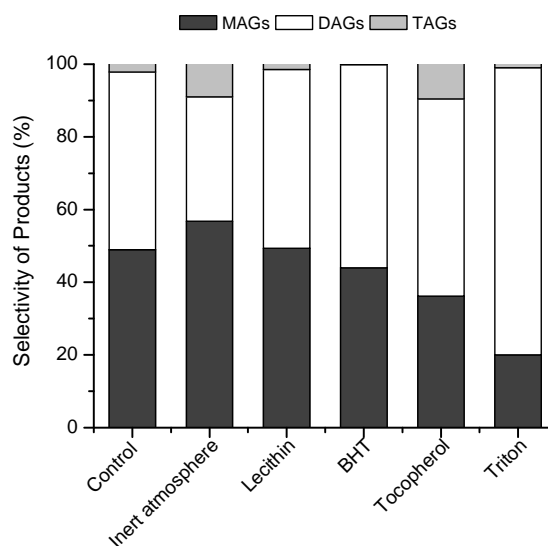
In Figure 2f, one can see that the use of Triton X-100 in the reaction medium was not effective and promoted a sharp reduction in MAG formation (maximum value 20%) in relation to the Control (Figure 2a) and the reaction carried out with soy lecithin (Figure 2c), which has emulsifying properties, as already described. This result also differed from those reported by Valério et al. (2010), but it should be considered that these authors had used the commercial lipase preparation Novozym® 435 as catalyst for the glycerolysis reaction of olive oil. The negative influence of Triton X-100 on the glycerolysis reaction of babassu oil catalyzed by PS lipase immobilized on SiO<sub>2</sub>-PVA may be better understood based on the complex mechanism of interaction between surfactant and immobilized system. The Triton X-100, by being a surfactant, has both hydrophilic (head) and hydrophobic (tail) fractions, and when in contact with lipase it is open arranged, exposing its active site. According to Fernandez-Lorente et al. (2007), the hydrophobic portion of Triton binds to the active site of lipase and may cause a distortion of the enzyme or inhibition of its catalytic activity (competitive inhibition), a competition between Triton and substrate, hindering the access of substrate to the active site of lipase and limiting the product formation. Nevertheless, this behavior depends on the type of support used to prepare the immobilized system, as described by Cabrera et al. (2009). In that study, the authors described a comparative analysis of the Triton effects on the immobilized derivatives of the *Candida antarctica* lipase in different supports (butyl-agarose, octadecyl-agarose, and octadecyl-Sepabeads) and Novozym® 435. Among all tested immobilized derivatives, only Novozym® 435 suffered no distortion or adsorption as a function of the high hydrophobicity of the support employed in the manufacture of this enzyme preparation. Therefore, the use of Triton as emulsifier of the reaction medium should be considered carefully,

and probably recommended only for reactions catalyzed by Novozym® 435.

Of all tested agents, only soy lecithin had no interference on the enzyme activity, attaining similar profile for mono- and diglycerides formation to the control, with additional advantage of increasing the reaction rate. These results may be related to the emulsifying properties of the lecithin.

The use of emulsifiers or surfactants in the reaction medium may provide higher homogeneity to the system oil/glycerol/enzyme, increasing the surface area, and promoting increase in reaction rate, as well as the biocatalysts efficiency, especially for lipases (enzymes that act on an interface). The characteristic of a surfactant is the formation of a micellar system that increases the enzymatic stability in terms of process conditions, such as temperature and pH, besides increasing the substrate solubility, reducing mass transfer limitations, promoting thus an increased conversion (VALÉRIO et al., 2010).

The Figure 3 shows the different strategies tested in terms of selectivity for forming each product (MAG and DAG) in 12h of reaction. Evidently the use of inert atmosphere was the strategy with the best performance for forming MAG, reaching the highest value of selectivity for this glyceride (57%), probably because the N<sub>2</sub> inhibited the oxidation of the oil feedstock without affecting the lipase activity.



**Figure 3.** Selectivity of the products formed during glycerolysis of the babassu oil catalyzed by the immobilized PS lipase employing different strategies to prevent oxidation and promote better homogenization of the reaction medium.

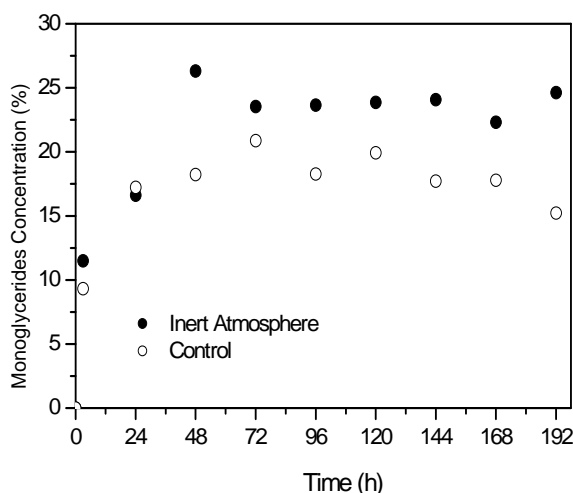
#### Glycerolysis performed in fixed bed reactor

Glycerolysis reactions of the babassu oil in fixed bed reactor were performed employing the strategies

which allowed attaining the highest MAG concentrations in the tests carried out in batch reactor, being: control reaction, under inert atmosphere and employing soy lecithin as oxidant and/or emulsifying agent. The Figure 4 shows the profile of MAG formation for the two first mentioned conditions.

The results obtained in continuous mode are similar to those achieved in the system operating in batch, i.e. when employed inert atmosphere there was a significant increase in the formation of MAG (mean value  $\cong$  24%) in relation to control reaction (mean value  $\cong$  18%), confirming that  $N_2$  inhibited or reduced the oxidation of the vegetal oil in MAG synthesis. Still, regardless the strategy (control or inert atmosphere), the formation of monoglycerides was kept at stable levels with small oscillations, as expected for systems like that, showing a good operational stability of the experimental system and immobilized enzyme.

Nevertheless, when soy lecithin was added to the feeding medium, it was not possible to operate the fixed bed reactor, due to the pore obstruction of immobilized lipase derivative, preventing the substrate migration through the packed bed. This fact may be associated with the increased viscosity of the reaction medium constituted of glycerol and babassu oil (molar ratio = 15) and soy lecithin, a zwitterionic and highly viscous surfactant.



**Figure 4.** Profile of monoglyceride formation in the continuous glycerolysis of babassu oil employing PS lipase immobilized on  $SiO_2$ -PVA.

To overcome the high viscosity of these reaction media, studies carried out by several researchers (DAMSTRUP et al., 2005; XU et al., 2001; YANG et al., 2005) recommend the use of solvents, such as *tert*-butanol, hexane, pentanol, isopropanol, among others. Another possibility would be the variation of

the molar ratio between starting materials, once with reduced amount of glycerol in the reaction medium, this would be less viscous (40.74 cp), due to the high viscosity of glycerol ( $d = 1.22 \text{ g cm}^{-3}$ ).

## Conclusion

Among tested strategies, the most effective was obtained by employing inert atmosphere in the process operated under both discontinuous and continuous modes. Of the antioxidant agents, only the soy lecithin had no influence on enzyme activity and similar MAG was attained in relation to the reaction control performed in discontinuous mode. However, the addition of soy lecithin in the feeding medium to perform the process in a continuous basis was limited due to the pore obstruction of immobilized derivative, causing a pressure drop in the bed and preferential pathways of the substrate.

## Acknowledgements

The authors thank to CNPq and FAPESP by support received to develop this research.

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Received on July 26, 2011.

Accepted on January 16, 2012.

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