TWO-STEP ENZYMATIC PRODUCTION OF BIOLUBRICANTS: COMBINING THE REUSE OF FREE AND IMMOBILIZED LIPASES

José R. Guimarães[1,2], Kaíque S. G. C. Oliveira[3], Letícia P. Miranda[2], Renato F. C. Bento[2], Roberto Fernandez-Lafuente[4*] and Paulo W. Tardioli[2,*]

[1]*Graduate in Bioprocess Engineering, Institute of Natural Resources, Federal University of Itajubá, Itajubá, MG, Brazil*
[2]*Graduate Program in Chemical Engineering, Department of Chemical Engineering, Federal University of São Carlos, São Carlos, SP, Brazil*
[3]*Graduate Program in Chemical Engineering, Federal Institute of Education, Science and Technology of the South of Minas Gerais, Pouso Alegre, MG, Brazil*
[4]*Departamento de Biocatálisis, ICP-CSIC, Madrid, Spain*
** Corresponding author's email address: jrenatoguimaraes@unifei.edi.br*

ABSTRACT

Enzymatic synthesis of octyl alcohol-based biolubricants was carried out in two catalytic steps. In the hydrolysis stage of degummed soybean oil, several lipases were evaluated as catalysts. Under optimal conditions, lipase from Pseudomonas fluorescens (PFL) yielded 97% conversion after 23 h using 50 U/g oil and DSO:water mass ratio of 1:0.5. Sequential batch strategies have been developed for the recovery and reuse of free enzyme in further hydrolysis cycles. When hexane was used in the separation between the FFA-rich phase and the phase composed of water, enzyme and glycerol, there was better PFL recovery and FFA-conversion of 65% was still achieved after five 24-hour hydrolytic cycles. In the esterification step, the potential of four lipases immobilized on Purolite C18 were evaluated in the production of octyl esters in a solvent-free medium (FFA/octanol molar ratio of 1:2.5) using screw-capped glass bottles as reactor. Immobilized Eversa was used in a vortex flow reactor and a yield of around 94 wt.% was reached after 3 h of reaction using a biocatalyst load of 1% (w/v) and FFA/octanol molar ratio of 1:2.5. The immobilized lipase could be reused for five consecutive 3 h-batches of esterification, maintaining the octyl esters yield of the first batch.

Keywords: degummed soybean oil, recovery and reuse of free lipase, lipase catalyzed hydrolysis and esterification, octyl esters, vortex flow reactor.

1 INTRODUCTION

Fatty acid alkyl esters (FAAE) having from 22 to 26 carbon atoms are promising substitutes for mineral oil lubricants. FAAE can be obtained by direct esterification of fatty acids, transesterification between fatty acid monoalkyl esters or glycerides and the desired alcohol, or hydroesterification, a sequential process of oil/fat hydrolysis followed by purification of the fatty acids and their esterification (Monteiro et al., 2023). This eliminates glycerin as a possible competitor in the esterification reaction and a likely inactivator or inhibitor of the enzyme when using immobilized lipase forms (Pourzolfaghar et al., 2016). The use of biocatalysts (mainly lipases) in these reactions are alternatives to chemical catalysts. In the biocatalytic hydroesterification reaction, the feedstocks do not require high purity, in addition, the glycerol produced in the enzymatic hydrolysis step is a by-product with pharmaceutical quality that can be used in the production of other interesting products (Pourzolfaghar et al., 2016).

In the triglyceride enzymatic hydrolysis step, there are reports showing the use of lipases in their soluble or immobilized formulation for the synthesis of FAAE. As the cost of immobilization has been reported to be competitive with the cost of the enzyme itself, the use of free lipases can maximize the technical and economic viability of the process, even more so if it may be coupled to the recovery and reuse of the free enzyme during the product downstream (Su et al., 2018). Although the use of immobilized enzymes may also have some advantages in this hydrolysis process, we have decided to try free lipases in this approach, developing strategies that may permit their recovery and reuse for several reaction batches.

In the esterification step, lipases immobilized on a hydrophobic support (Purolite Lifetech™ ECR8806F) were used. This immobilization strategy is a simple immobilization procedure and the open and adsorbed form of lipases on hydrophobic supports is very stable, even more stable than multipoint covalently immobilized lipases, and more resistant to changes in reaction conditions (e.g., changes in ionic strength), increasing the range where the biocatalysts can be used. As the enzyme is just physically immobilized, the support can be recovered and reused after enzyme inactivation. Similar supports were used by Tacias-Pascacio et al. (Tacias-Pascacio et al., 2016) with very good results to produce biocatalysts useful for biodiesel production. These highly hydrophobic supports can mitigate the problem of water accumulation inside the biocatalyst particles.

2 MATERIAL & METHODS

All other reagents were of analytical grade.

2.1 Enzyme immobilization, standard activity assay and protein quantification

Enzyme immobilization followed the protocol described by Tacias-Pascacio et al. (Tacias-Pascacio et al., 2016). Standard activity assays and protein quantification followed the procedure described by Beisson et al. (Beisson et al., 2000) and Bradford (Bradford, 1976), respectively.

2.2 DSO enzymatic hydrolysis to produce FFA

Different lipases were evaluated in the hydrolysis of DSO, using an oil/water mass ratio of 1:4 and enzyme load of 25 U/g oil. The reactions were carried out in closed glass bottles at 35 °C and stirred at 250 rpm for 5 min. After selecting the lipase in terms of activity, the effect of several oil/water mass ratios (1:0.06, 1:0.25, 1:0.5, 1:1, 1:2, and 1:4) and enzyme loads (50 and 250 U/g oil) were investigated to maximize the hydrolysis of DSO. In this set of experiments, the conditions used were the same as previously mentioned but time was prolonged to 24 h. The kinetic profiles of the hydrolyses were studied in batch mode using a reactor with mechanical agitation under optimal conditions. For this reaction, the reactor was operated at 35 °C and agitation of 900 rpm. After 24 h of reaction, the reaction medium was submitted to two different processes: 1) it was left to rest for 3.5 h until it formed two phases or 2) hexane was added to the reaction solution (using an FFA/hexane mass ratio of 1:1), followed by a 30 min rest until it formed two phases. The light phase (composed of FFAs and non-hydrolyzed acylglycerols) was removed, while the heavy phase (composed of water, enzyme and glycerol) was used in a new batch of reaction, employing the same oil/water mass ratio than in the first batch. At the end of each reaction batch, aliquots of the light phase were removed and ethanol was added to stop the reaction and FFAs were quantified by titration using KOH.

2.3 Enzymatic esterification to produce fatty octyl esters

FFAs produced in the hydrolysis step were washed twice with distilled water and separated from the glycerol phase by centrifugation at 5,000 rpm for 5 min at 25 °C. Afterwards, they were dried in an oven at 70 °C for 24 h and used in the esterification reaction. Octanol was used as acyl acceptor utilizing a FFA/octanol molar ratio of 1:2.5. EV, CALB and PPL immobilized on Purolite were used as biocatalysts (1%, w/v) and compared with Lypozyme 435. The first lipase evaluation in the esterification reactions was performed in screw-capped glass bottles with 50 mL of capacity containing 5 mL of reaction mixture, at 37 °C, and stirred at 250 rpm for 24 h. The reactions using the optimal biocatalyst were performed at 37 °C in a vortex flow reactor (VFR) at 1700–2000 rpm. Samples were withdrawn to analyze octyl fatty acid esters by gas chromatography.

EV-Purolite biocatalyst was utilized in successive 3 h-batches of esterification of DSO with octyl alcohol at 37 °C in a vortex flow reactor at 1700–2000 rpm stirring. The reaction mixture contained DSO and 1-octanol, and an enzyme load of 1% (w/total volume). After each 3 h-cycle, the biocatalyst was recovered by centrifugation at 5,000 rpm for 5 min. At the end of each batch, octyl esters were analyzed by gas chromatography.

3 RESULTS & DISCUSSION

Among the lipases evaluated in the hydrolysis reaction of degummed soybean oil (DSO), PFL demonstrated the best performance, achieving a conversion 18 times greater than that observed using CALB. The effect of different mass ratios of oil/water in the hydrolysis of DSO was evaluated and the low water mass ratios (0.06 and 0.25), the yields were reduced and increased when the ratio was increased to 0.5. After this water mass ratio, the increase in the yields is not significant (the yields grew from 55 to 65% when increasing the water ratio from 0.5 to 4).

The effect of the enzyme load on the hydrolysis of the DSO as a function of the reaction time showed that there was an increase in the conversion of FFA as the enzyme load was increased from 50 U/g oil (55.47% conversion) to 250 U/g oil (61.24% conversion). However, this small increase did not justify the use of high enzyme loads in this reaction. An appropriate enzyme load is a critical factor in the economic viability of the process. In this sense, an enzyme load of 50 U/g oil was selected to investigate the reaction course of the hydrolysis of DSO catalyzed by PFL.

Figure 1a shows the reaction course of the hydrolysis of DSO catalyzed by PFL. The reaction course is quite linear until reaching a value of around 90% after 10-12 h of reaction. After 23 h of reaction, the FFAs yield reached a value of 97%, this slowing down of the reaction may be likely due to some inhibitory effects of the released FFAs and the decrease in the reaction pH. After that time, no significant increase in FFA conversion was observed. These results are promising since the reaction was carried out in a system without emulsifier or buffer, being composed only of DSO, water and enzyme, eliminating the need for FFAs purification steps and reducing process costs. The composition of the FFAs were 10.30 ± 0.17 wt.% of palmitic acid, 6.29 ± 0.03 wt.% of stearic acid, 22.80 ± 0.25 wt.% of oleic acid, 45.25 ± 0.27 wt.% of linoleic acid and 8.85 ± 0.04 wt.% of linolenic acid, which accords with the composition of soybean oil. A reaction time of 24 h was selected to evaluate the reuse of the enzyme, together with the heavy phase (water, glycerol and enzyme).

Figure 1b shows the reuse of PFL in the hydrolysis of DSO. It is observed that it was possible to reuse the heavy phase of the hydrolysis reaction for at least 3 sequential batches, keeping the conversion above 61%, when there was no use of hexane to recover the heavy phase. Using hexane to recover the FFAs (Figure 1b), 65% of conversion in the fifth cycle was obtained, while only 20% was obtained if hexane was not used. The use of solvent proved to be a viable alternative for recovering the heavy phase in the hydrolysis step, considering the possibility of reusing the heavy phase and the hexane in subsequent recycles. In fact, in our experiments, after each batch, hexane was recovered by rota-evaporation and reused in the next cycle. The strategy of reusing the heavy phase can maximize the economic viability of the free fatty acid production process. In addition, it allows the concentration of glycerol with high purity to be used in other process of food, cosmetic and pharmaceutical purposes. 5 cycles were the maximum reuses assayed because the concentration of glycerol made handling this phase more and more difficult. Moreover, less free water will be available for the hydrolytic reactions.

EV, CALB and PPL were immobilized on Purolite for further application in the synthesis of octyl esters in a solvent-free medium. The reaction catalyzed by EV-Purolite shows that the higher the enzyme load, the higher the esterification initial rate (almost 80%

yield using 2% of biocatalysts versus just over 65% using 1% after 30 minutes); however, there is no effect on the final yields of the octyl esters. Using 1% (w/v) of enzyme load, 94.1 ± 0.5 wt% of the octyl esters yield was achieved after 3 h of reaction. After that time, no significant increase was observed in the esters yield. Thus, a time of 3 h was used to analyze the operational stability of EV-Purolite. The yield of octyl esters was fully retained after five 3 h-batches. This result shows that the immobilized EV biocatalyst had high operational stability, indicating that there is no significant enzyme release from the support nor enzyme inactivation. These characteristics are fundamental aspects for the application of immobilized enzymes in industrial processes. Another advantage of using EV immobilized on Purolite Lifetech EC8806F is the possibility of recyclability of the support after enzyme inactivation.

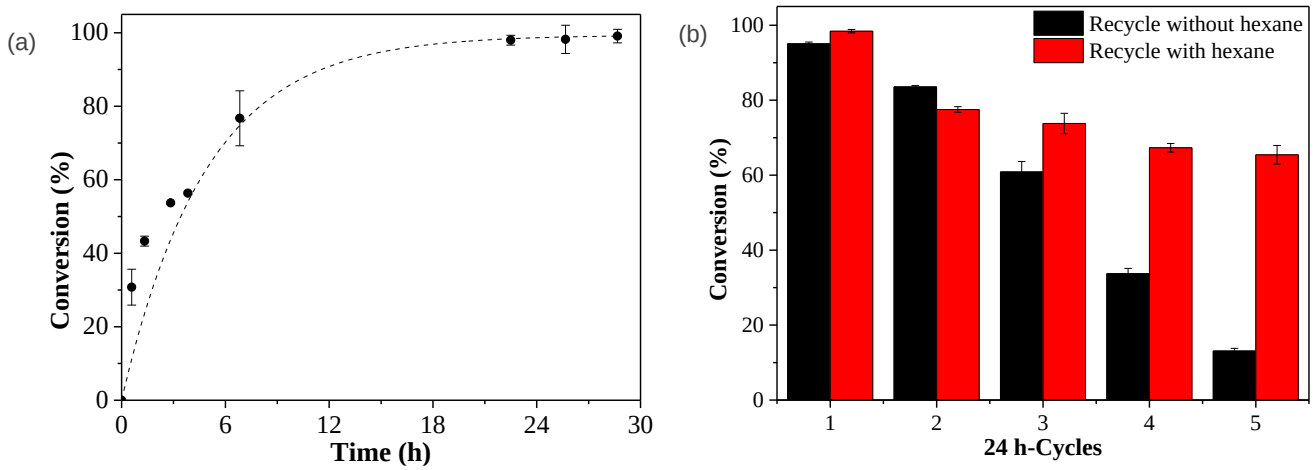

Figure 1. (a) Time course of hydrolysis of DSO catalyzed by PFL. (b) Reuse assay (24 h-cycles) of the PFL in the hydrolysis of DSO. Assay conditions: enzyme load of 50 U/g DSO, oil/water mass ratio of 1:0.5, temperature of 35 °C, 900 rpm stirring in a batch reactor.

4 CONCLUSION

This paper shows the feasibility of the enzymatic approach used for the enzymatic synthesis of octyl alcohol-bases biolubricants carried out in two steps. The use of free PFL in DSO hydrolysis allowed a high FFA yield, performed in an emulsifier-free system without buffer, being only composed of DSO, water and enzyme, thus reducing the need of FFA purification steps and, that way, the process costs. Furthermore, the possibility of reusing the heavy phase of the hydrolysis reaction in sequential batches of DSO hydrolysis was demonstrated. In the esterification step, EV immobilized on Purolite Lifetech EC8806F successfully provided the synthesis of highly yield of octyl esters (around 94 wt.% of ester yield). The immobilized lipase could be reused in five 3 h-batches of esterification, maintaining 100% of the octyl esters yield of the first batch. The nature of the product was confirmed by spectroscopy (ATR-FTIR) and gas chromatography (GC) analyses.

REFERENCES

Beisson, F., Tiss, A., Rivière, C., Verger, R., 2000. Methods for lipase detection and assay: A critical review. Eur. J. Lipid Sci. Technol. 102, 133–153. https://doi.org/10.1002/(SICI)1438-9312(200002)102:2<133::AID-EJLT133>3.0.CO;2-X

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Monteiro, R.R.C., Berenguer-Murcia, Á., Rocha-Martin, J., Vieira, R.S., Fernandez-Lafuente, R., 2023. Biocatalytic production of biolubricants: Strategies, problems and future trends. Biotechnol. Adv. 68, 108215. https://doi.org/10.1016/j.biotechadv.2023.108215

Pourzolfaghar, H., Abnisa, F., Daud, W.M.A.W., Aroua, M.K., 2016. A review of the enzymatic hydroesterification process for biodiesel production. Renew. Sustain. Energy Rev. 61, 245–257. https://doi.org/10.1016/j.rser.2016.03.048

Su, C., Nguyen, H.C., Nguyen, M.L., Tran, P.T., Wang, F., Guan, Y., 2018. Liquid lipase-catalyzed hydrolysis of gac oil for fatty acid production: Optimization using response surface methodology. Biotechnol. Prog. 34, 1129–1136. https://doi.org/10.1002/btpr.2714

Tacias-Pascacio, V.G., Peirce, S., Torrestiana-Sanchez, B., Yates, M., Rosales-Quintero, A., Virgen-Ortíz, J.J., Fernandez-Lafuente, R., 2016. Evaluation of different commercial hydrophobic supports for the immobilization of lipases: Tuning their stability, activity and specificity. RSC Adv. 6, 100281–100294. https://doi.org/10.1039/c6ra21730c

ACKNOWLEDGEMENTS

This work was funded by Federal University of Itajubá (Edital 06/2023 - UNIVERSAL UNIFEI), São Paulo Research Foundation (FAPESP, grant number 2016/10636-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES, Finance Code 001), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, project numbers 405889/2016-0 and 308212/2017-7), and Ministerio de Ciencia e Innovación and Agencia Estatal de Investigación (Spanish Government) (project PID2022-136535OB-I00). Dr A. Berenguer-Murcia´ help and sugestions (University of Alacant) is gratefully recognized.