



ELSEVIER

Contents lists available at ScienceDirect

Comptes Rendus Chimie

www.sciencedirect.com



Full paper/Mémoire

## Entirely solvent-free biocatalytic synthesis of solketal fatty esters from soybean seeds



*Synthèse biocatalytique entièrement sans solvants d'esters gras du solcétal à partir de fèves de soja*

Rubén Torregrosa<sup>a</sup>, Edinson Yara-Varón<sup>a</sup>, Mercè Balcells<sup>a</sup>, Mercè Torres<sup>b</sup>,  
Ramon Canela-Garayo<sup>a,\*</sup>

<sup>a</sup> Department of Chemistry, University of Lleida, ETSEA, Av. Rovira Roure 191, 25198 Lleida, Spain

<sup>b</sup> Department of Food Science and Technology, University of Lleida, ETSEA, Av. Rovira Roure 191, 25198 Lleida, Spain

### ARTICLE INFO

#### Article history:

Received 31 July 2015

Accepted 15 December 2015

Available online 7 April 2016

#### Keywords:

Biocatalysis

Biomass

2,2-Diméthyl-4-hydroxyméthyl-1,3-

dioxolane

Resting cell

*Rhizopus oryzae*

#### Mots-clés:

Biocatalyse

Biomasse

2,2-Diméthyl-4-hydroxyméthyl-1,3-

dioxolane

Cellules au repos

*Rhizopus oryzae*

### ABSTRACT

Solvent-free reactions are the systems of choice in green chemistry. In addition to contributing to lowering the environmental impact of chemical processes, solvent-free systems can reduce production costs, reaction times, and the dimensions of reactors, thereby decreasing investment costs. An improved procedure to prepare 2,2-dimethyl-4-hydroxymethyl-1,3-dioxolane (solketal) fatty esters from soybean seeds has been developed. Yields higher than 90% were achieved by combining 15 h of hydrolysis with 6 h of esterification with a stepwise addition of solketal. The synthesis was performed in a solvent-free medium, and the final extraction was accomplished using supercritical CO<sub>2</sub>. Hence, we have successfully prepared these esters from soybean beans without using organic solvents. In addition, given the non-toxicity of *Rhizopus oryzae* and the composition of the remaining solid, it might be used as a raw material for feedstock production.

© 2016 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

### RÉSUMÉ

Les réactions sans solvants sont des systèmes de choix en matière de chimie verte. En plus de contribuer à réduire l'impact environnemental des procédés chimiques, les systèmes sans solvants peuvent réduire les coûts de production, les temps de réaction et les dimensions du réacteur, ce qui va diminuer les coûts d'investissement. Nous avons développé un procédé amélioré pour préparer des esters gras du 2,2-diméthyl-4-hydroxyméthyl-1,3-dioxolane (solcétal) à partir de graines de soja. Des rendements supérieurs à 90% ont été obtenus en combinant 15 h d'hydrolyse à 6 h d'estérification avec addition par étapes de solcétal. La synthèse a été réalisée dans un milieu exempt de solvants, et l'extraction finale a été effectuée en utilisant du CO<sub>2</sub> supercritique. Par conséquent, nous avons préparé avec succès ces esters à partir de fèves de soja sans utiliser de solvants organiques. En outre, compte tenu de la non-toxicité de *R. oryzae* et de la composition du solide restant, ce dernier peut être utilisé comme matière première pour la production d'autres matières premières.

© 2016 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

\* Corresponding author.

E-mail address: [canela@quimica.udl.cat](mailto:canela@quimica.udl.cat) (R. Canela-Garayo).

## 1. Introduction

Solvent-free reactions are the systems of choice in green chemistry [1, 2]. In addition to contributing to lowering the environmental impact of chemical processes, solvent-free systems can reduce production costs, reaction times, and the dimensions of reactors, thereby decreasing investment costs [3]. Such systems are particularly relevant in the food industry, a sector governed by strict legislation regarding the use of organic solvents.

In order to fulfill green chemistry principles, besides green synthesis reaction design, extraction procedures must be taken into account since it is known that they require at least 50% of the energy of industrial processes [4]. In this context, some techniques of green extraction such as microwave irradiation, instantaneous controlled pressure drop, supercritical fluid extraction or pressing have been intensely explored in order to modernize conventional processes, making them greener, cleaner, safer and easier to perform [4–6].

Solketal (4-hydroxymethyl-2,2-dimethylursor-1,3-dioxolane, 1,2-acetonide glycerol or 1,2-*O*-isopropylidene glycerol) is an important chiral synthon in the synthesis of diglycerides, glyceryl phosphates, tetraoxaspiroundecanes, and many biologically active compounds, such as glycerophospholipids,  $\beta$ -blockers (aryloxypropanolamines such as (*S*)-propranolol), prostaglandins and leucotrienes [7, 8]. The enantioselective hydrolysis of racemic solketal esters using lipases has been extensively studied, as has the synthesis of these esters, usually in a solvent medium [9–11]. However, there are fewer reports about their preparation in a solvent-free medium starting from either a carboxylic acid or an acylglyceride [12, 13].

Lipases (triacylglycerol acyl hydrolases EC 3.1.1.3) are valuable catalysts in organic synthesis and are widely used in several industries, such as food, dairy, oil and fat, pharmaceutical, detergent, textile, pulp and paper, animal feed, leather, and cosmetics sectors [14–19]. These enzymes are a part of a family with hydrolytic activity that catalyzes reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis [20]. Lipases do not require cofactors, and many enzymes are commercially available in free and immobilized forms. Recent years have brought about considerable interest in the direct use of intracellular lipases as whole cell biocatalysts. The application of such biocatalysts in organic synthesis offers many advantages, one being the preparation of the biocatalyst in a straightforward and inexpensive manner [21].

In previous studies, we described solvent-free processes to prepare biodiesel directly from oilseeds and coffee grounds using whole cell biocatalysts. Yields ranged from 24 to 94% depending on the material and biocatalyst used [22, 23]. The procedure has also been applied to prepare solketal esters, although in this case with moderate yields (60%) [22]. Nevertheless, although ester synthesis was carried out directly from the seeds in a solvent-free medium, the final step involved organic solvent extraction to recover the final esters.

Here we studied the synthesis of solketal fatty esters from soybean seeds using *Rhizopus oryzae* resting cells as the biocatalyst (Scheme 1). The synthesis was performed in a solvent-free medium, and the final extraction was accomplished using supercritical CO<sub>2</sub>. We thus prepared these esters without using organic solvents in the whole process. We determined the effect of pre-mixing, temperature, reaction time and the addition of the alcohol in the transformation of the acylglycerides present in the soybean beans.

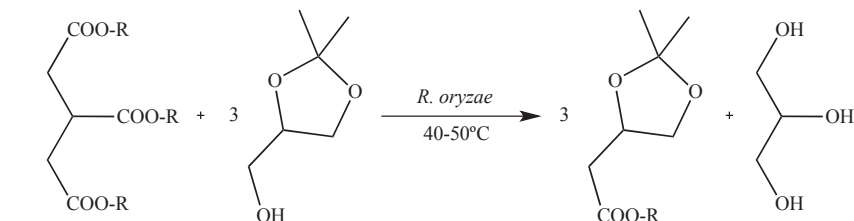
## 2. Experimental

### 2.1. Materials

Solketal (2,2-dimethyl-4-hydroxymethyl-1,3-dioxolane) (98%) was purchased from Fluka Chemicals. CO<sub>2</sub> was obtained from Messer Iberica de Gases S.A (Tarragona, Spain). All the reagents were used as received. Soybean seeds were purchased from a retail store in Lleida, Spain. The seeds were ground in a standard coffee grinder before use. The moisture content of the material was determined by drying 5 g of the ground solid at 130 °C for 1 h. The <sup>1</sup>H NMR spectrum was recorded on a Varian AS400 spectrometer operating at 400 MHz and using CDCl<sub>3</sub> as the solvent.

### 2.2. Preparation of standards

Triacylglycerides (TAGs) were used as the standards for the HPLC analysis and were obtained from the soybean seeds using a previously reported method (Min, 1994) [24]. Free fatty acids (FFAs) were prepared from vegetable oil using a standard method (Liu, 1994) [25]. Stock solutions of TAGs and FFAs (10 mg/mL) in an acetone:acetonitrile mixture (1:1, v/v) were prepared and standard 1, 0.5, 0.2, and 0.1 mg/mL solutions were then obtained from the same. These solutions were used to plot the corresponding standard curves.



Soybean acylglycerides

**Scheme 1.** Hydrolysis and esterification reactions of soybean acylglycerides to yield solketal fatty esters.

### 2.3. HPLC analysis

HPLC analysis was carried out using a Waters Series 600 pumping system, a Waters 710 autosampler, and a Waters 2690 UV detector operating at  $\lambda = 210$  nm (Waters Cromatografía SA, Spain). Ten microliters of each sample were injected into a Waters Cromatografía SA Symmetry C18 5- $\mu\text{m}$  (150  $\times$  3.9 mm) reverse-phase column operating at 40 °C. The following solvents were used: acetonitrile; 0.1% TFA aqueous solution (95:5 v/v) (solvent A); and acetone (solvent B). The gradient program was started with 100% of solvent A at a flow rate of 0.7 mL/min. After 5 min, the flow rate was increased to 1 mL/min, and the gradient was increased to 50% of solvent B at 5 mL/min. Finally, the flow rate was increased to 1.5 mL/min and held for 15 min. The chromatographic peaks were recorded and integrated using the Millennium 32 computer software package (Waters Cromatografía SA, Spain).

### 2.4. Preparation of resting cells

*R. oryzae* was grown in a synthetic liquid medium containing 2 g of asparagine, 1 g of  $\text{K}_2\text{HPO}_4$ , 0.5 g of  $\text{MgSO}_4$ , 5 mg of thiamine hydrochloride, 1.45 mg of  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ , 0.88 mg of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.235 mg of  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  per liter of distilled water. The initial pH of the medium was adjusted to pH 6.0. Next, 250-mL aliquots of the medium were sterilized at 121 °C for 15 min, and 1% (v/v) of refined sunflower oil was added aseptically. The medium was inoculated with 2.5 mL of a *R. oryzae* spore suspension ( $1\text{--}4 \times 10^6$  spores/mL) and then incubated at 28 °C for 5 d using an orbital shaker at 200 rpm. Mycelium was harvested from the culture medium using a Buchner funnel and washed with distilled water followed by acetone. The mycelium was then dried under vacuum for 18 h and ground to a powder [26]. The *R. oryzae* (CECT20476) strain is housed in the Spanish Type Culture Collection (CECT).

### 2.5. Preliminary hydrolytic studies

Mixtures of 0.9 g of milled soybean seeds and 0.09 g of resting cells were added to a 3-mL Eppendorf vial in order to prepare 3 vials. One was either pre-mixed for 1 min in a vortex mixer or immersed in an ultrasonic bath for 20 min. Subsequently, sets of 3 vials were shaken at 1400 rpm in a Thermomixer at various temperatures and periods. Finally, the resulting mixture was suspended in 8 mL of hexane, agitated using an orbital shaker (Thermomixer) at 1400 rpm, and maintained at 50 °C for 20 min. The resulting organic solution was recovered by filtration, and the solid was re-extracted as before. The two organic solutions were pooled and evaporated. Aliquots of the crude extract were dissolved and analyzed using the method described above. Control experiments were performed to determine the effect of the extraction path. Experiments were performed in triplicate.

### 2.6. Reactive extraction in a solvent-free system

Nine grams of milled oilseeds and 0.9 g of resting cells were mixed, and aliquots of the solid mixture were added

to 3-mL Eppendorf vials. The vials were then shaken at 250 rpm in a Thermomixer at various temperatures and periods. Solketal was added either in a single addition or stepwise.

Samples were vortex-mixed for 1 min after the addition of the alcohol, and the mixture was shaken under the same conditions during a given time. Finally, all the solids were pooled and extracted using a lab-scale supercritical fluid extractor (Spe-ed SFE-Applied separations). The extraction vessel was packed with 2 g of solids obtained in the reactions in each run. The extraction temperature, pressure,  $\text{CO}_2$  flow rate and time were set at 45 °C, 350 bars, 4 L/min, and 45 min, respectively. The resulting viscous liquid was recovered and analyzed by  $^1\text{H}$  NMR. Experiments were performed in triplicate.

$^1\text{H}$  RMN (400 MHz,  $\text{CDCl}_3$ )  $\delta$  ppm 0.87 (t,  $J = 7.03$  Hz,  $\text{CH}_3$ ), 1.09–1.36 (m,  $\text{CH}_2$ ), 1.36 (s, 3H) 1.43 (s, 3H) 1.52–1.72 (m,  $\text{CH}_2$ ) 2.32 (t,  $J = 7.42$  Hz,  $\alpha\text{CH}_2$ ) 3.73 (dd,  $J = 8.60$ , 6.25 Hz, 1H) 4.04–4.11 (m, 2H) 4.16 (dd,  $J = 11.33$ , 4.69 Hz, 1H) 4.20–4.36 (m, 1H) 5.23–5.40 (m, =CH).

## 3. Results and discussion

Given our previous results show that a preceding hydrolytic step improves the yield of the methyl esters synthesized from oilseeds, we performed a preliminary study to determine the effect of pre-mixing, temperature, and reaction time on the hydrolysis of the TAGs present in the soybeans. No water was added as we considered that the seeds would hold enough moisture to carry out TAG hydrolysis. Most of the conditions tested yielded hydrolysis in excess of 66.66% as would be expected, thereby indicating that the hydrolytic reaction does not stop at the 2-monoacylglyceride (2-MAG) as would be expected considering that the lipases of *R. oryzae* are 1,3-specific (Table 1) [27].

Statistical analysis (ANOVA test and comparisons made by the Student-Newman-Keuls multiple-comparisons test,  $P < 0.05$ ) shows that neither temperature nor reaction time had a significant effect on reaction yield. However, vortex pre-mixing increased the yield compared to ultrasound pre-mixing, but not compared to no pre-mixing.

Given these results and that the pre-mixing time was also shorter for vortex than for ultrasound pre-mixing, the next experiments were performed using the former. Temperature had no evident effect on the final yield, although 50 °C gave the best yield for 15 h when no pre-mixing was used. Consequently, the reaction temperature was fixed at 50 °C for the following experiments.

Table 2 shows the effect of hydrolysis and esterification periods, as well as the addition of alcohol, on the synthesis of solketal fatty esters from soybean seeds. The procedure allows the preparation of this sort of compound using a fully environmentally friendly method as no solvent is used.

Considering the same hydrolysis and esterification periods, the yields resulting from the stepwise addition of the alcohol were consistently higher than those achieved from a single addition. The effect of stepwise addition of alcohols has already been considered in various biocatalytic processes using lipases [28,29]. The stepwise addition of

**Table 1**

Effect of pre-mixing, temperature, and reaction period on the biocatalytic hydrolysis of the triacylglycerides present in soybean seeds ( $n = 3$ ).

Pre-mixing	T (°C)	Reaction time (h)	Yield (%)	Standard error
Vortex pre-mixing	40	15	83.4	2.1
		24	85.6	1.3
		39	85.2	2.2
		48	86.7	1.7
	50	15	76.0	0.9
		24	81.6	0.3
		39	85.1	0.6
		48	88.8	1.6
Ultrasound pre-mixing	40	15	79.3	0.9
		24	80.5	2.7
		39	82.2	0.9
		48	78.7	1.6
	50	15	77.3	0.3
		24	79.9	0.7
		39	77.4	1.8
		48	82.7	0.6
No pre-mixing	40	15	86.0	2.0
		24	80.4	1.3
		39	78.5	1.9
		48	82.1	1.1
	50	15	86.3	2.7
		24	92.3	2.4
		39	86.0	2.7
		48	76.0	2.3

alcohol is frequently carried out in order to overcome lipase inhibition. Kyeong and Yeom, 2014 [30] prepared biodiesel using a packed-bed bioreactor filled with *R. oryzae* NBRC 4697. Those authors stated that a stepwise addition of methanol prevented inhibition of the immobilized cells. Nevertheless, standard errors were also higher for the stepwise addition, thereby indicating that the addition process and the intermedia mixing have a strong influence on the final yields. Yields higher than 90% were reached by combining 15 h of hydrolysis with 6 h of esterification with stepwise addition. Increasing hydrolysis time and esterification period resulted in a 97% yield.

The best yields were similar to those recently described starting from palmitic acid and using a commercial lipase and dichloromethane as solvents [11]. Moreover, the yields were noticeably higher than those achieved using a commercial lipase and either conventional heating or microwave irradiation in solvent-free media [12]. Yields were

**Table 2**

Effect of reaction time and the addition process of the alcohol on the solvent-free biocatalytic synthesis of solketal fatty esters from soybean seeds.

Alcohol addition	Hydrolysis Period (h)	Esterification period (h)	Yield (%)	Standard error
Single addition	0	96	64.4	6.5
	4	92	55.1	4.7
	15	81	75.6	2.0
	24	72	78.9	2.6
Stepwise <sup>a</sup>	0	6	76.8	10.9
	4	6	75.0	7.8
	15	6	90.9	2.5
	24	6	95.0	15.9

<sup>a</sup> Five additions of 4  $\mu$ L each at 2  $\mu$ L/min. Vials were vortex-mixed for 1 min after each addition.

also higher than the one obtained in a preliminary study using sunflower seeds, the same biocatalysts, and a final hexane extraction. The hydrolysis and the esterification periods for this former reaction were 48 h each and the solketal was added stepwise 48 h and 72 h after the process was started.

#### 4. Conclusion

We have developed an improved procedure to prepare solketal fatty esters from soybean seeds. Yields higher than 90% were achieved by combining 15 h of hydrolysis with 6 h of esterification with a stepwise addition of solketal. The synthesis was performed in a solvent-free medium, and the final extraction was accomplished using supercritical CO<sub>2</sub>. Hence, we have successfully prepared and extracted these esters without using organic solvents, making it fully compliant with green chemistry principles. In addition, given the non-toxicity of *R. oryzae* and the composition of the remaining solid, it might be used as a raw material for feedstock production.

#### Acknowledgements

This work was supported in part by a Grant-in-Aid for the Secretaría de Estado de Política Científica y Tecnológica of the Spanish Ministry of Education and Culture (contract grant number: CTQ2009-14699-C02-01) and by a Grant-in-Aid for the Departament d'Universitats, Recerca i Societat de la Informació of the Generalitat de Catalunya (contract grant number: 2014 SGR 1296).

#### References

- [1] M. Escribá, J. Eras, M. Duran, S. Simon, C. Butchosa, G. Villorquina, M. Balcells, R. Canela-Garayoa, *Tetrahedron* 65 (2009) 10370.
- [2] J.O. Metzger, *Angew. Chem., Int. Ed.* 37 (1998) 2975.
- [3] G.D. Yadav, P.A. Thorat, *J. Mol. Catal. B: Enzym.* 83 (2012) 16.
- [4] F. Chemat, M.A. Vian, G. Cravotto, *Int. J. Mol. Sci.* 13 (2012) 8615.
- [5] N. Rombaut, A.S. Tixier, A. Bily, F. Chemat, *Biofuels, Bioprod. Bioref* 8 (2014) 530.
- [6] F. Chemat, A.S. Fabiano-Tixier, M.A. Vian, T. Allaf, E. Vorobiev, *Trends Anal. Chem.* 71 (2015) 157.
- [7] J.S. Clarkson, A.J. Walker, M.A. Wood, *Org. Process. Res. Dev.* 5 (2001) 630.
- [8] F. Molinari, K.S. Cavenago, A. Romano, D. Romano, R. Gandolfi, *Tetrahedron: Asymmetry* 15 (2004) 1945.
- [9] A.C.O. Machado, A.A.T. da Silva, C.P. Borges, A.B.C. Simas, D.M.G. Freire, *J. Mol. Catal. B: Enzym.* 69 (2011) 42.
- [10] I. Itabaiana, F.K. Sutili, S.G.F. Leite, K.M. Gonçalves, Y. Cordeiro, I.C.R. Leal, L.S.M. Miranda, M. Ojeda, R. Luque, R.O.M.A. de Souza, *Green Chem.* 15 (2013) 518.
- [11] X. Wang, Q. Jin, T. Wang, *J. Am. Oil Chem. Soc.* 90 (2013) 1455.
- [12] R. Torregrosa, M. Balcells, M. Torres, R. Canela-Garayoa, *Nat. Prod. Commun.* 9 (2014) 1095.
- [13] K. Araya, A. Ugarte, L. Azocar, O. Valerio, L.Y. Wick, G. Ciudad, *Fuel* 144 (2015) 25.
- [14] K. Faber, *Biotransformations in Organic Chemistry*, 6th ed, Springer, Berlin, Germany, 2011.
- [15] V. Gotor-Fernández, V. Gotor, in: J. Polaina, A.P. MacCabe (Eds.), *Industrial Enzymes*, Springer, Houten, Netherlands, 2007, p. 301.
- [16] K.E. Jaeger, B.W. Dijkstra, M.T. Reetz, *Annu. Rev. Microbiol.* 53 (1999) 315.
- [17] M.T. Reetz, *Curr. Opin. Chem. Biol.* 6 (2002) 145.
- [18] J.B. Jones, *Tetrahedron* 42 (1986) 3351.
- [19] S. Divakar, B. Manohar, in: J. Polaina, A.P. MacCabe (Eds.), *Industrial Enzymes*, Springer, Netherlands, 2007, p. 283.
- [20] A. Houde, A. Kademi, D. Leblanc, *Appl. Biochem. Biotechnol.* 118 (2004) 155.

- [21] G. Grogan, in: *Practical Transformation: A Beginner's Guide*, John Wiley & Sons, Ltd., Chichester, U.K, 2009.
- [22] M. Torres, J.J. Méndez, V. Sanahuja, R. Canela, *Biocat. Biotrans* 21 (2003) 129.
- [23] M. Torres, V. Loscos, V. Sanahuja, R. Canela, *J. Am. Oil Chem. Soc.* 80 (2003) 347.
- [24] D.B. Min, in: S.S. Nielsen (Ed.), *Introduction to the Chemical Analysis of Foods*, Chapman and Hall, New York, 1994, p. 181.
- [25] K.S. Liu, *J. Am. Oil Chem. Soc.* 71 (1994) 1179.
- [26] M. Torres, E. Barbosa, V. Loscos, R. Canela, *Biotechnol. Lett.* 22 (2000) 1265.
- [27] R.N. Farias, M. Torres, R. Canela, *Anal. Biochem.* 252 (1997) 186.
- [28] M. Aarthy, P. Saravanan, M.K. Gowthaman, C. Rose, N.R. Kamini, *Chem. Eng. Res. Des.* 92 (2014) 1591.
- [29] J.M. Cerveró, J.R. Álvarez, S. Luque, *Biomass Bioener* 61 (2014) 131.
- [30] J.S. Kyeong, S.H. Yeom, *Bioprocess Biosyst. Eng.* 37 (2014) 2189.