**ABSTRACT**
The potential application of rice husk ash (RH26) as support for the immobilization of a recombinant *Rhizopus oryzae* (rROL) lipase as biocatalyst in the enzymatic biodiesel production using alperujo oil and the comparison with commercial hydrophobic support OD403 (RelOD) has been made. Although the specific activity (UA mg support⁻¹) was around one-half lower in RH26 than in RelOD when they were used as biocatalyst in biodiesel reaction, the normalized initial rate was similar, between 1.6 and 2.4 μmol FAME mL⁻¹ min⁻¹ UA⁻¹. Thus in terms of biocatalysis performance, rice husk as is an alternative to commercial supports. However, the main problem is the more complex recovery of RH26 for the reutilization compared with commercial ones.

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**Introduction**

Rice husk ash (RHA) from rice husk (RH) biomass power plant is abundantly available in rice producing countries as a waste, recycling mainly for low-value applications. For instance, in Asian countries it is used in RH gasification power generation, obtaining RHA as a waste product under a control of the temperature in the burning process (Pode 2016). RHA is mainly composed by SiO₂ – about 95% – and other trace elements like potassium, calcium, magnesium, iron, copper, manganese and zinc (Della et al. 2002). This composition of RHA makes it a candidate in industrial applications, with the advantage of its low cost (Turmanova et al. 2012).

Three different types of RHA from the incarceration of RH depending of the temperature can be obtained: amorphous, partially crystalline and crystalline, the first one is considered the most active (Vinh 2012), with pore distribution as mesoporous (Srivastava and Mall 2005).

There is a large development in the uses of RHA with high potential future applications due to its hydrophobicity (Andreola et al. 2013; Pode 2016). Concretely, applications in the basic catalysis of biodiesel production to its use as natural adsorbent for improving the quality of waste frying oil by removing its free fatty acids (FFA) are related (Ismail and Ali 2015).

Also monoglyceride removal in crude biodiesel production is done by coating aluminium oxide on extracted silica from RHA (Saengprachum and Pengprecha 2016), with the support vanadium pentoxide for transesterification of different triglyceride sources as acyl-acceptor group ethanol (Almeida et al. 2016).

Recently, RHA has been used as a carrier, using cross-linking and adsorption methods, for the recovery of activity of *Candida antarctica* lipase B (CALB) with good results, also increasing stability, reusability and application adaptability of the biocatalyst (Ulker et al. 2016a) and applied in the biosynthesis of poly(ε-caprolactone) (Ulker et al. 2016b).

Although the biodiesel production through basic chemical transesterification is mainly used, enzymatic transesterification using lipases has become a real alternative to chemical process in the last years. This is due to its advantage as a green bioprocess using less energy, biodiesel recovery is minimized since no emulsions are presented, with the implementation of less unit operations (Rodrigues et al. 2016) and with the attractive to work directly with substrates containing high quantity of FFA (Juan et al. 2011; Fan et al. 2012; Gog et al. 2012; Rodrigues et al. 2016). However, its industrial implementation is limited by a longer reaction time, the well-known lipase denaturation and inhibition by methanol (ethanol), the cost of the
biocatalyst (You et al. 2013; Canet et al. 2014; Duarte et al. 2015; Kuo et al. 2015; Lotti et al. 2015) and the problems associated to the presence of glycerol. This by-product adsorbs onto carriers when lipase is immobilized provoking lipase deactivation and diminishing the efficiency of the process (Hama et al. 2011).

To avoid part of these problems, some recombinant 1–3 positional specific lipases have been used, avoiding the presence of glycerol as by-product, obtaining as final product 2-monoglycerides (Calero et al. 2014) and minimized the cost of the enzyme due to the production in cell factories (Arnau et al. 2010).

On the other hand, lipases are majority applied in biodiesel synthesis immobilized on commercial supports. Thus, the use of cheaper supports is a clear strategy to diminish the cost of this bioprocess.

Recently, RHA has been used as support for the immobilization of recombinant \textit{Rhizopus oryzae} (rROL) lipase demonstrating the viability of this support as alternative to commercial lipases (Martin et al. 2013). The performance of rROL immobilized on commercial supports under different strategies in biodiesel production has been deeply studied (Canet et al. 2014, 2016; Bonet-Ragel et al. 2015; Canet et al. 2017).

The objective of this study is the comparison of the performance of rROL immobilized by adsorption on commercial hydrophobic support OD403 (RelOD) and rice husk ash (RH26) in the enzymatic biodiesel production using alperujo oil, a waste oil rich in FFA using methanol as acyl-acceptor in terms of immobilization performance, initial FAMES production reaction rate.

**Materials and methods**

**Materials**

Recombinant \textit{Rhizopus oryzae} lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group from Universitat Autónoma de Barcelona (UAB) following production methods referenced in the previous works (Arnau et al. 2010). Olive waste oil (alperujo) was kindly donated from Sierra Mágina olive oil extraction mill (Jaén, Spain).

Heptane, methanol and acetone were purchased from Panreac (Barcelona, Spain). Buffer constituents tris-(hydroxymethyl)-aminomethane, di-sodium hydrogen phosphate anhydrous and hydrochloric acid (37\% purity) were obtained from Scharlab (Barcelona, Spain). Oleic acid and calibration standards of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate were obtained from Sigma-Aldrich (St. Louis, MO). Colorimetric kit for enzymatic assay (11821729) was acquired from Roche (Mannheim, Deuschland). RH26 support was kindly donated by Dr. María de los Ángeles Martín Luengo from ICMM-CSIC (Madrid, Spain). Relizyme OD403 carrier was purchased from Resindion (Milano, Italy).

**Enzyme recovery, lyophilization and storage**

Once performed \textit{Pichia pastoris} fermentation, the recombinant enzyme obtained should be recovered and purified. Since \textit{Rhizopus oryzae} is mostly extracellular, the medium is firstly centrifuged at 10,000 rpm during 10 minutes. Supernatant is then vacuum filtered in order to remove remaining cells and other solids in suspension. Ultrafiltration is needed afterwards, concentrating 10 times the initial volume. This is achieved with the equipment Centrasette\textsuperscript{®} Pall Filtron (East Hills, NY), using a 10 kDa-cut off membrane.

The remaining fluid contains a high amount of salts. In order to reduce this concentration, it is diluted to meet the initial volume using Tris buffer 10 mM, and after diafiltered by membrane until the volume is reduced again 10 times. The resulting quantity is then frozen at –20°C. Lyophilization is finally performed at –50°C and 100 Torr during 48–72 hours, after which the resulting enzyme is recovered and stored at –20°C (Guillén 2012).

**Lipolytic activity**

Lipolytic activity of enzyme solutions is obtained following the modified method described by Roche, enabling to use their colorimetric kit assay. The procedure is always performed in triplicates, where 500 µL of sample are mixed with 500 µL of Tris–HCl buffer (pH 7.25) and 300 µL of Roche reagent in 1.5 mL cuvettes.

They are then introduced in the Cary Varian 300 spectrophotometer (Palo Alto, CA) at 30°C and 580 nm measuring their absorbance for seven minutes (Resina et al. 2004).

**Lipase immobilization**

In order to immobilize rROL with the support ReLOD/RH26, 5 mM phosphate buffer at pH 7.5 is prepared. The appropriate amount of buffer is mixed with lyophilized lipase in order to get solutions ranging its activity between 90 and 1800 UA/mL. The resulted mixture is kept under magnetic stirrer for 30 minutes at 4°C until rROL is totally dissolved.

On the other hand, support must be treated before immobilization. The total weight must be put in contact with 100 mL of water–acetone solution (50/50, v/v) and let it under low stirring for 30 minutes
at room temperature. After that, solution and support are separated by vacuum filtration, washing with distilled water for complete acetone removal.

Pretreated RelOD/RH26 is mixed with phosphate–lipase solution and kept under low stirring for seven hours either at 4 °C or 25 °C, depending on the experiment. When finished, the enzyme immobilized is separated from solution by vacuum filtration washing with phosphate buffer, and let it dry with silica gel, at room temperature, in a desiccator until its weight reached a constant value (1–2 days). Enzyme lyophilized is stored at −20 °C.

**Transesterification reactions**

Transesterification reactions are carried out in 10 mL closed vials placed in an incubator (IKA KS 400, Staufen, Germany) at 30 °C under continuous orbital stirring (350 rpm). Solvent and water-free reactions are performed stepwise adding each time 0.16 mL of methanol to the 8 g of *alperujo* substrate (previously dewaxed by centrifugation) and 150 mg of selected biocatalyst. Biocatalyst is easily recovered after each reaction. Its deposition allows removing the reaction medium, washing it with heptane–acetone–heptane and the biocatalyst is kept stored afterwards at 4 °C until it is reused in order to perform cycles.

**FAME and oleic acid analysis**

Fatty acid methyl esters and total oleic acid amount were analysed by gas chromatography (GC) following the referenced work (Canet et al. 2014).

**Results and discussion**

**Immobilization of rROL on RH26 and RelOD: comparison**

Two immobilization processes were performed. In one hand, rROL was adsorbed on RelOD after its acetone pretreatment during seven hours (Canet et al. 2014). This is consistent with the fact that multipoint interaction between the non-complementary enzyme and the surface is a low and time-dependent process that requires correct alignment of groups (Mateo et al. 2007). By the other hand, rROL was also immobilized by hydrophobic adsorption on rice husk ash support RH26 using the same method (Canet et al. 2014), which has an amorphous surface due to its silica composition (Martin et al. 2013).

A range of lipase solutions from 50 to 1800 UA/mL was performed in order to evaluate the optimal lipase load in the immobilization process for each support.

| Time (min) | Lipolytic activity (4 °C) | Lipolytic activity (25 °C) |
|------------|--------------------------|---------------------------|
| 0          | 106.74 ± 2.56            | 106.97 ± 0.11             |
| 60         | 104.01 ± 3.45            | 102.30 ± 2.70             |
| 120        | 100.52 ± 3.83            | 108.92 ± 1.68             |
| 360        | 98.40 ± 2.76             | 110.24 ± 2.22             |

Whereas enzyme performance was known to be better at lower temperature, moderately higher ones may favour the vibration of enzyme and support, increasing the possibilities of getting more enzyme—support linkages (Mateo et al. 2007). However, temperature should not be too high since this may cause enzyme denaturation. Thus, for commercial support two temperatures were tested.

In order to ensure if temperature had a significant effect on the lipase stability during the immobilization, two rROL solutions with similar initial activity were prepared. They were left at room temperature (25 °C) and 4 °C. Lipolytic activity (expressed in UA/mL) was followed during the next four hours that corresponds to the immobilization time described in section “Materials and Methods”. As shown in Table 1, activity remained the same along the time regardless of the temperature.

As shown in Figure 1, it can be noted that increasing the initial lipolytic activity leads to an equal increasing in the lipase immobilized on the support and to a proportional decrease of immobilization yield. However, specific lipolytic activity remains almost constant (saturation) when higher values than 800 UA/mL were offered.

As it was expected, immobilization higher loads of lipase activity at room temperature showed more adsorption capacity than at 4 °C, without enzyme denaturation.

In the case of alternative support RH26, shown in Figure 2, the immobilization capacity at room temperature showed the same profile as the commercial one. Nevertheless, it seemed that immobilization capacity decreased when higher loads than 800 UA/mL were offered (1000 UA/mL). However, support was able to adsorb more enzyme as loading values increased up to 1800 UA/mL, leading to believe in a possible double-layer effect since lipases linked in the carrier surface were able to act as an adsorption sites for other lipases, conforming a second layer of enzyme (Masel 1996).

**Biodiesel initial rates**

In order to test if the obtained biocatalysts showed mass transfer problems, enzymatic biodiesel reactions
were performed. As shown in section “Materials and Methods”, alperujo oil was used as a substrate. This waste oil showed a high efficiency in biodiesel synthesis due to its FFA content giving a high stability to the biocatalyst and enhancing the initial rate (Bonet-Ragel et al. 2015).

Since complete reactions were not needed for these cases, only a pulse of 160 μL of methanol was added to the reaction in order to avoid lipase inactivation and initial rate for each reaction was calculated, corresponding to the maximum conversion of 14%. Figure 3 shows the values obtained for each biodiesel reaction, using both biocatalysts (RelOD and RH26). It can be noted that there is a linear correlation between initial rate and specific activity when lower values of it were used for both types of biocatalysts. In the case of RelOD, it is observed that initial rate remained the same for specific activities up to 46 UA/mg. This led to the conclusion that it was not worth working at values higher than 60–80 UA/mg at these specified transesterification conditions.
Table 2 shows a summary of the most significant results obtained and compared with previous works in the same group. For better comprehension and comparison, initial rate values were divided by total lipolytic activity present in the reaction (biocatalyst weight $\times$ specific activity) obtaining a normalized value for each case. It is clear that maximum initial rates were achieved (2.37 l mol FAME/mL min $\times$ UA) at some particular values of specific activity, for the commercial RelOD (46.21 UA/mg).

| Support | Specific activity (UA/mg support) | Initial rate (µmol FAME/mL-min) | Total UA/reaction | Normalized initial rate (µmol FAME/mL-min-UA) |
|---------|----------------------------------|---------------------------------|-------------------|---------------------------------------------|
| RH26    | 27                               | 4.39                            | 3500              | 1.25                                        |
| RH26    | 48.09                            | 9.97                            | 6250              | 1.59                                        |
| RelOD   | 46.21                            | 16.48                           | 6931              | 2.37                                        |
| RelOD   | 90.29                            | 18.88                           | 13,543            | 1.39                                        |

**Biodiesel production**

Based on previous conclusions, a complete transesterification reaction was performed using the commercial RelOD support. Biodiesel production was carried out with five pulses of methanol – each pulse was added every 45 minutes – aiming to achieve the maximum yield of 66% and to avoid enzyme deactivation (Lotti et al. 2015). This is due to the use of a 1,3-positional specific lipase (see section “Materials and Methods”), which leaves 2nd position of triacylglycerol unreacted. Acyl migration was not considered here, since much longer time would be needed (Kaieda et al. 1999) and no glycerol as a product was detected in medium.

Figure 4 shows complete reaction after 225 minutes, achieving a 64.5% of FAME yield (nearly a 98% of real yield). Through a periodical methanol addition, FAME production increased linearly along the time. Oleic acid was also followed, which is the FFA with major presence in the substrate. In this case, it can be noted the component is decreased during the reaction, indicating that the esterification rate was higher than the hydrolysis one.

**Stability-testing reaction cycles**

It has been tested in past studies that lipases have a noticeable decrease in their activity when put in contact with methanol in the reaction media over long periods of time (Noureddini et al. 2005). Thus, a serial of biodiesel synthesis reaction cycles were performed in order to evaluate its stability.

As presented in Figure 5, a total of seven cycles were carried out which corresponded to 26 hours of reaction. Considering the first one’s yield as the
maximum that could be obtained, it was compared to the successive ones in order to determine the evolution of the biocatalyst deactivation along the reutilizations. Nearly a 64% of the initial yield was maintained after 26 hours of reaction. Despite the long exposure to methanol in the reaction media, these results showed that activity of biocatalyst was well preserved when using alperujo oil as a substrate, although it is not possible to distinguish if the lost activity is due to an inactivation or a possible leaching effect.

**Conclusions**

During the immobilization procedure higher loads of lipase activity were obtained at 25°C comparing with a temperature of 4°C without loss of activity. The UA immobilized per mg of support was twofold higher in commercial support probably due to the highest superficial area exposed to biocatalyst. However, when both biocatalysts were tested in a biodiesel reaction the initial rate expressed as μmol FAME

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**Figure 4.** FAME yield and total oleic acid amount during a complete transesterification using RelOD as a support and alperujo oil as a substrate. Pulses of methanol were added before the very fourth samples.

**Figure 5.** Relative activity for each reaction cycle taking the first reaction yield as the 100% of biocatalyst activity.
mL⁻¹ mL⁻¹ UA⁻¹ were of the same order of magnitude demonstrating RH26 has a similar performance of commercial ones. Thus, RH26 could be considered as a potential carrier alternative to RelOD. However, the main drawback of this cheap support is the not easy recovery for a possible reutilization in successive bio-transformations due to its intrinsic characteristics compared with commercial ones. This point should be improved for a future application as support of lipase in the enzymatic production of biodiesel. In the case of rROL immobilized on commercial support, high yield values were achieved as well as high stability values were obtained after 26 hours of reaction, demonstrating the efficiency of this carrier.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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