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Entrapment in polymeric material of resting cells of *Aspergillus flavus* with lipase activity. Application to the synthesis of ethyl laurate

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The lipase activity of resting cells of *Aspergillus flavus* was improved by entrapment in various polymeric acrylates (PAAFs). Commercially available ethyl acrylate, butyl acrylate and ethyl methacrylate, and acrylates synthesized from 1,3-dichloropropan-2-ol were used as monomers for the *in situ* polymerization. The cells were physically entrapped *via* free-radical-polymerization in aqueous medium or by bulk polymerization. The percentage of resting cells immobilized in the polymers was assessed by analysing the ergosterol content. Bulk polymerization with ethyl methacrylate allowed the greatest incorporation ratio, with 85% of entrapped biocatalyst. Entrapped resting cells were used to prepare ethyl laurate, achieving yields of up to 98%. The specific activity (*r*) of PAAFs was determined using a batch reactor and a packed-bed reactor (PBR). The highest *r* was observed for the resting cells entrapped in poly(ethyl methacrylate) using PBR. These results demonstrate that entrapped resting cells of *A. flavus* can be used to prepare commercial products.

1. Introduction

Biocatalysts (enzymes, resting cells, and whole cells) are crucial for industrial processes as they allow for the efficient production of many valuable compounds. However, the use of these catalysts is limited because of their often considerable prices and their unstable nature. Among biocatalysts, purified enzymes or cells of bacteria and yeasts are commonly immobilized in various supports and matrices, but little attention has been devoted to the immobilization of filamentous fungi.[1] The morphology of fungi is highly complex. Depending on the substrate and solvents used, the cells tend to agglomerate, thereby diminishing catalytic activity and hampering the cells recovery. One approach to maintain the morphological structure of these biocatalysts is through their immobilization on polymeric material. The stability, desired catalytic activity, and recovery capacity of immobilized cells can be preserved, thus allowing their application in the laboratory and industry.[2] Immobilization offers many advantages for biotransformations such as convenient separation by filtration or centrifugation for reuse after reaction, application in continuous processes, straightforward product separation and recovery, better reaction control and increased product purity and yield.[3] Immobilization can also facilitate the use of biocatalysts in a variety of solvents, at extremes of pH, and at exceptionally high temperature and substrate concentrations. At the same time, substrate specificity, enantioselectivity, and reactivity can be modified.[4]

To date, many immobilization methods have been developed. These techniques are divided into two main categories.[5] The first involves immobilization by attachment, whereby macroscopic insoluble aggregates are formed by either linking the biocatalysts (carrierless crosslinking) or by binding them to the surface of an organic or inorganic support (adsorption onto a carrier, covalent binding to a carrier). The second category is entrapment, which comprises encapsulation in semi-permeable membranes and embedding in a matrix. Given the reduced contact with the carrier in the entrapment approach compared to immobilization by attachment, the residual mobility and flexibility of the biocatalysts is much higher.[6] Among these methods, physical entrapment in a matrix is relatively simple and inexpensive, and it causes a relatively small perturbation on the native enzyme structure and function.[7] Hence, this method is regularly used in large-scale processes, particularly when the enzyme of interest is inexpensive. For this purpose, enzymes have been commonly incorporated directly into the polymer matrix during free radical polymerization.[8]

Whole mycelia of filamentous fungi have been extensively used in biocatalytic redox reactions.[9] Recently, the use of whole fungal cells as lipases is receiving increasing attention. In particular, fungal cells with lipase activity grown in porous biomass support particles (BSPs) have been used in diverse

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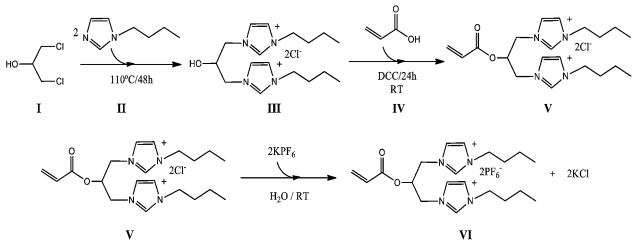

Scheme 1 Synthesis of 1,1′-(2-(acryloyloxy)propane-1,3-diyl)-bis(3-butylimidazol-1-ium) hexafluorophosphate (IL-HFP).

hydrolytic reactions.[10–13] Although the resting cells of several fungi have been identified as robust candidates for cell biocatalysis, research has focused on *R. oryzae* strains.[14] We consider it pertinent to study other microbial sources as potential producers of intracellular lipases of commercial significance. In this context, our research group isolated a fungal strain of *Aspergillus flavus* that showed lipase-like activity and described its usefulness as a biocatalyst in hydrolytic[15] and synthetic reactions.[16–18]

In the present study we report the entrapment of resting cells of *A. flavus via* the free-radical polymerization of six monomers. To the best of our knowledge, the direct entrapment in a polymeric material of wild-type resting fungal cells with esterase activity is unprecedented. Three commercial (ethyl acrylate, butyl acrylate and ethyl methacrylate) and two previously described acrylates (allyl acrylate and dichloropropyl acrylates)[18,19] were used as monomers for the production of PAAFs. Moreover, a polymerizable ionic liquid 1,1′-(2-(acryloyloxy) propane-1,3-diyl)bis(3-butylimidazol-1-ium) hexafluorophosphate (IL-HFP) was also used (Scheme 1). Specific activity was determined using either a batch reactor or a packed-bed reactor (PBR). Moreover, the catalytic activity of the PAAFs and of free mycelia of *A. flavus* was assessed using an esterification reaction yielding ethyl laurate. Ethyl esters of short- and medium-chain fatty acids are particularly important in the food, beverage, cosmetic, and pharmaceutical industries. Furthermore, fatty acid ethyl esters synthesized by biocatalysts using natural raw materials can be considered of natural origin, thus adding value to the product obtained.

2. Results and discussion

The esterification of lauric acid with ethanol was used to evaluate the activity of each biocatalyst. The results were compared with those obtained using free *A. flavus* resting cells. Reactions were carried out in a batch reactor or a PBR. The former was used to select the optimum reaction conditions and polymeric material for comparison of the two reaction modes.

2.1. Entrapment of *A. flavus* in polymeric materials (PAAFs)

Allyl acrylate, ethyl acrylate, butyl acrylate, dichloropropyl acrylate, ethyl methacrylate, and polymerizable ionic liquid (IL-HFP) were used as monomers. The synthesis of IL-Cl from dichloropropanol is the first step to obtain the last polymerizable monomer (IL-HFP). Scheme 1 shows the three-step synthesis performed to prepare the IL-HFP: (a) synthesis of compound **III** by substitution of both chlorides present in 1,3-dichloro-2-propanol (**I**) with 1-butylimidazole (**II**); (b) esterification of the product (**III**) with acrylic acid (**IV**); (c) substitution of the counter ion Cl^- with hexafluorophosphate (PF_6^-) anion. The IL-HFP monomer is an ionic liquid, and the resulting polymer is a solid salt that had the capacity to establish new interactions with the resting cells, substrates, and solvents.

Water-dispersive polymerization was used to obtain the corresponding PAAFs as described in the Materials and methods section. Polymerization was carried out for 18 h to increase the yield of the polymer and achieve a greater incorporation of the biocatalyst. A turbid stiff gel was formed after a polymerization reaction, possibly due to the presence of unreacted monomer materials. The PAAFs were recovered by filtration, washed with deionized water and hexane, and then dried under vacuum. The polymeric materials containing *A. flavus* were ground to a powder. The percentage of recovered mass for each polymer was as follows: poly(ionic liquid) (Af-Pol IL-HFP) 60%; poly(allyl acrylate) (Af-Pol AA) 50%; poly(ethyl acrylate) (Af-Pol EA) 52%; poly(butyl acrylate) (Af-Pol BA) 52%; poly(dichloropropyl acrylate) (Af-Pol DCPA) 55%; and poly(ethyl methacrylate) (Af-Pol EM-D) 70%.

In an attempt to improve the efficiency of the polymerization, we carried out a bulk polymerization using ethyl methacrylate as monomer. This compound was selected because it showed the highest mass recovery in the water-dispersive polymerization. Nevertheless, in the new polymeric material (Af-Pol EM-B) the bulk process allowed the recovery of 47% of the whole starting mass (monomer plus resting cells). Similarly, this polymer was dried and ground to a powder (Fig. 1). Morphology and particle size are key factors to considerer when assessing the catalytic performance of an immobilized biocatalyst, as these factors will allow the substrate diffusion to the enzyme containing polymeric material.[20] In this case, the granules obtained were hard, showed minimum-swelling characteristics, and were mechanically stable and therefore suitable for applications in PBRs as well as stirred batch operations.

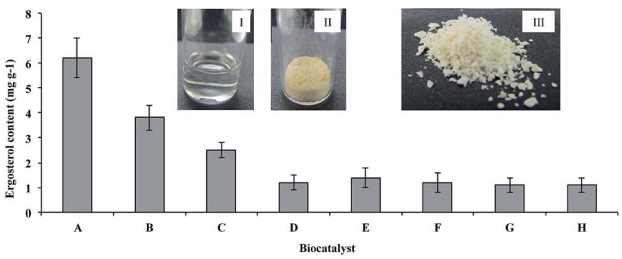

Fig. 1 Content of ergosterol (mg ergosterol g^{-1} of sample) in resting cells of *A. flavus* (A) and in the PAAF: (B) Af-Pol EM-B; (C) Af-Pol EM-D; (D) Af-Pol BA; (E) Af-Pol DCPA; (F) Af-Pol AA; (G) Af-Pol EA; and (H) Af-Pol IL-HFP. Pictures: (I) Ethyl methacrylate monomer; (II) free resting cells of *A. flavus*; (III) *A. flavus* resting cells entrapped in poly(ethyl methacrylate) (Af-Pol EM-B (B)).

2.2. Determination of the resting cells of *A. flavus* entrapped in the polymeric materials

Ergosterol is specific to the fungal kingdom and is found mainly as a membrane constituent. Moreover, membrane area is assumed to be comparable to the total biomass of microbial cells.[21,22] Thus, Zill *et al.*,[23] showed a correlation between ergosterol production, mycelial wet weight, and mycelial protein in *Fusarium graminearum*. Consequently, we used ergosterol content to determine the amount of *A. flavus* entrapped in the polymeric materials tested.

Ergosterol content was 6.2 mg g^{-1} on average in free fungal mycelia (Fig. 1). Considering this value and the ergosterol content in each PAAF, the highest amount of *A. flavus*, 2.5 mg g^{-1} (40%), was found in Af-Pol EM-D, followed by Af-Pol DCPA with 1.4 mg g^{-1} (23%). The other polymers incorporated about 1.2 mg g^{-1} (19%). In contrast, Af-Pol EM-B showed the highest concentration of resting cells (3.8 mg g^{-1}, 60%), although the whole mass recovery was only 47%. This bulk polymerization was markedly faster than the polymerization carried out by suspension and less biocatalyst was lost (85% of the resting cells entrapped).

The differences in the percentage of resting cells incorporated into the supports vary between 19 and 60%. These high differences could be explained considering the heterogeneity of the monomers used and the polymeric processes performed as has already described for other biocatalysts.[22–26]

2.3. Effect of water activity (a_w) on enzymatic activity

The influence of water activity on the esterification reaction catalysed by *A. flavus* resting cells and PAAFs was studied at 5 levels of a_w. Fig. 2 shows the effect of a_w on the yield of ethyl laurate using a batch reactor. The biocatalysts showed very similar behaviour in terms of a_w. The highest yield of ethyl laurate was reached at $a_w = 0.54$ for all the biocatalysts. The highest aw tested ($a_w = 0.75$) caused an evident decrease in the activity of all the biocatalyst. Consequently, all the following experiments were performed using $a_w = 0.54$.

These results are very similar to those reported for most esterification reactions catalysed by lipases, which show the highest catalytic activity with a_w around 0.60.[27] Water activity is one of the main factors affecting enzyme activity. In addition to favouring hydrolysis over condensation reactions, water excess can affect reaction rate, yield and enzyme stability.[18] Solvent can also influence the amount of water bound to the enzyme, but this effect can largely be avoided by the use of fixed water activity.

2.4. Evaluation of the biocatalytic activity of PAAFs in a batch reactor

The enzymatic activity of polymers containing *A. flavus* resting cells was determined by the esterification of lauric acid with ethanol in a batch reactor. Fig. 3 shows the specific activity (r = U g^{-1} of resting cells) of each PAAF and of the resting cells at $a_w = 0.54$. The *A. flavus* resting cells entrapped in poly(ethyl methacrylate), polymerized by bulk polymerization (B), showed the highest r followed by the free resting cells of *A. flavus* (A) and the poly(ethyl methacrylate) polymerized by water-dispersive polymerization (C). The other PAAFs showed r lower than 1.3 U g^{-1}. Kanwar *et al.*[28] had prepared ethyl laurate using purified lipase of *Bacillus coagulans* MTCC-6375 immobilized onto a synthetic hydrophobic poly (MAc-*co*-DMA-cl-MBAm)-hydrogel and a nonane solution of lauric acid and ethanol. The specific activity of the biocatalyst was 18 U mg^{-1} protein and the conversion was 66%, which is clearly lower than the one attained using Af-Pol EM-B at $a_w = 0.54$.

We propose that the interactions between the biocatalyst and the hydrophobic support stabilize the structure with the highest activity.[29] This is the case of lipases containing a lid (part of the enzyme molecule) that encloses the catalytic site. These lipases can take two forms: a closed form (inactive) where the catalytic

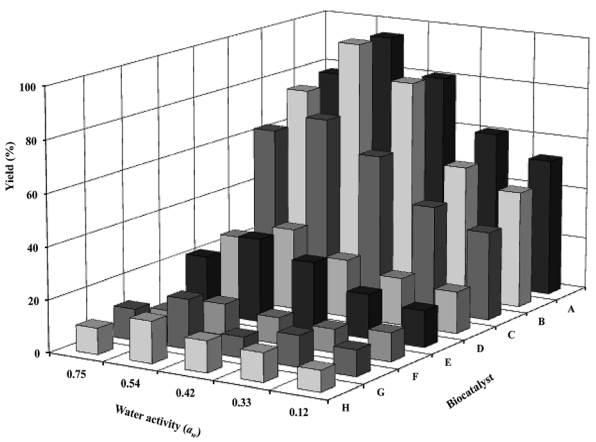

Fig. 2 Effect of a_w on the yield of ethyl laurate using a batch reactor. Reaction conditions: reaction time 24 h, 20 mg of each biocatalyst, and a_w: LiCl (0.12), $MgCl_2$ (0.33), K_2CO_3 (0.42), $Mg(NO_3)_2$ (0.54) and NaCl (0.75). (A) free *A. flavus* resting cells; and *A. flavus* entrapped in different polymeric materials (B) Af-Pol EM-B; (C) Af-Pol EM-D; (D) Af-Pol BA; (E) Af-Pol DCPA; (F) Af-Pol AA; (G) Af-Pol EA; and (H) Af-Pol IL-HFP.

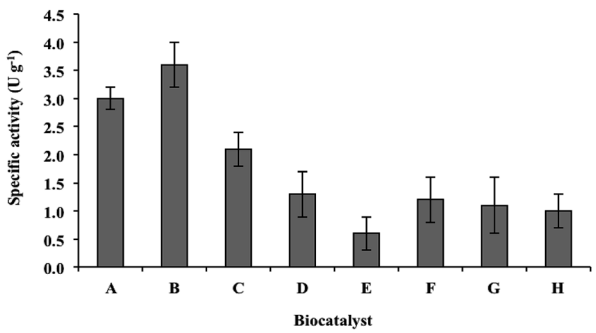

Fig. 3 Specific activity of free *A. flavus* resting cells (A) and *A. flavus* resting cells incorporated in various polymeric materials (B) Af-Pol EM-B; (C) Af-Pol EM-D; (D) Af-Pol BA; (E) Af-Pol DCPA; (F) Af-Pol AA; (G) Af-Pol EA; and (H) Af-Pol IL-HFP. The activity was assayed using a molar ratio of 1 : 2 of lauric acid/ethanol in hexane. The reactions were carried out in triplicate in a batch reactor at $a_w = 0.54$ and 40 °C for 24 h.

site is not accessible because it is sealed by the lid, and an open form (active) in which the catalytic site is accessible to the substrate as a result of lid displacement. In the presence of low amounts of water-insoluble substrates, the lipases are activated by hydrophobic interfaces ("interfacial activation") that bring about a conformational change by opening the lid of the lipase.[29] Accordingly, the immobilization of lipases on hydrophobic supports may be a suitable and simple method for lipase hyper-activation by interfacial activation.[30] This effect might explain the results described above when entrapping *A. flavus* resting cells in poly(ethyl methacrylate) polymerized by bulk polymerization. However, the different behaviours of the PAAFs could be attributed to diffusional limitation within the polymer material. In general, polymer particles produced with highly concentrated cross-linking are rigid, thus providing smaller pores or cavities and therefore restricting diffusion of the substrate, which in turn leads to a reduction in the reaction rate.[31] However, these supports are mechanically resistant and allow the reuse of the biocatalyst. Moreover, transport capacity of substrates through the polymeric matrix could differ depending on the functional groups present, thus increasing or diminishing the concentration of substrate available for the biocatalyst.

2.5. Stability of resting cells and PAAFs in a batch reactor

Next, the stability of the biocatalysts that show the higher specific activity was studied. *A. flavus* resting cells, Af-Pol EM-B and Af-Pol EM-D were used to test the catalytic activity along six cycles of lauric acid esterification using the same conditions described above. Fig. 4 shows that Af-Pol EM-B maintained constant the activity during six cycles, while free *A. flavus* resting cells and Af-Pol EM-D showed a decrease in the catalytic activity along cycles. The free cells *A. flavus* exhibited very poor stability after their repeated use for ethyl laurate synthesis. Fungal agglomeration, diffusional limitations and deactivation could be responsible for the decrease in catalytic activity. The agglomeration may be caused by the water produced as a by-product during the reaction.[32] Furthermore, the use of cells as catalysts has some drawbacks, such as low specific activity and mass transfers limitations of substrates (or products) through the cell envelope.[33] However, the biocatalyst Af-Pol EM-B does not seem to show strong diffusional limitations in the polymeric material.

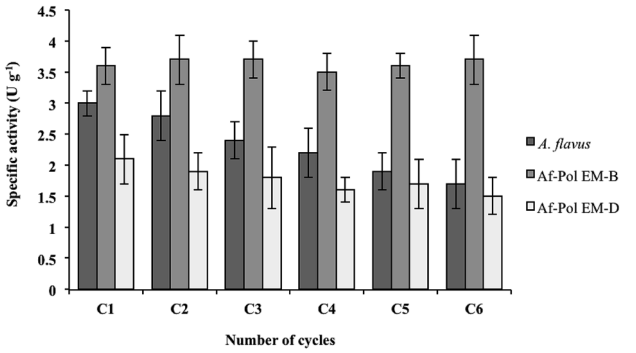

Fig. 4 Biocatalyst stability over repeated batches of esterification reaction using free resting cells of *A. flavus* and *A. flavus* resting cells entrapped in poly(methyl methacrylate) prepared by bulk polymerization (Af-Pol EM-D) and water-dispersive polymerization (Af-Pol EM-D). The stability was assayed using a molar ratio of 1 : 2 of lauric acid/ethanol in hexane. Six cycles of 24 h reactions were carried out in triplicate in a batch reactor at 40 °C and $a_w = 0.54$.

2.6. Enzymatic esterification in a packed-bed reactor (PBR)

A new set of experiments to study the esterification of lauric acid with ethanol was performed in a PBR system using Af-Pol EM-B or resting cells of *A. flavus*. The esterification reaction was carried out using a 1 : 2 mole ratio of carboxylic acid/alcohol, and the minimum flow rate allowed for our system (10 μL min^{-1}). The residence time (R_t) was calculated for Af-Pol EM-B and resting cells of *A. flavus* biocatalyst with the ε/q expression (ε is the catalyst bed porosity, determined experimentally, and q is the flow rate).[34] The R_t for the free *A. flavus* resting cells was 28 min, a value lower than that of Af-Pol EM-B ($R_t = 32$). Fig. 5 shows that the yield of ethyl laurate synthesized using Af-Pol EM-B and free mycelia increased constantly for the first 24 h. Once the steady state was achieved, the catalytic activity of the immobilized cells was constant until the end of the experiment (144 h). In contrast, the biocatalytic activity for free mycelia decreased beyond 24 h. These results similar to the ones obtained in a batch reactor confirm an acceptable operational stability for the Af-Pol EM-B biocatalyst, extending its usability in the continuous operation system. These behaviours could be explained as indicated above by the mass transfer limitations of substrates (or products) through the resting cells and the agglomeration of resting cells inside the PBR. Mass transfer is a crucial aspect when using a PBR. This parameter is much more relevant for biocatalyst bed reactors than for batch reactors because of the greater biocatalyst-to-substrate ratio in the former. In fact, the immobilization of fungal cells growth in a suitable support matrix improves features such as catalytic efficiency and stability and decreases biocatalytic inhibition.[13,35,36] Furthermore, cell immobilization can also enhance mass transfer and minimal clogging in continuous-flow systems.[37] Indeed, the agglomeration of the biocatalyst was not observed during the whole reaction period when the PBR was filled with Af-Pol EM-B. This might be due to the enhanced

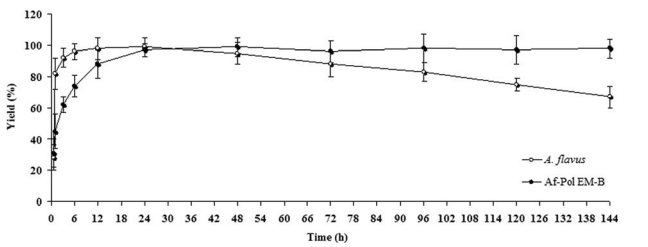

Fig. 5 Yield of esterification reactions using free *A. flavus* resting cells and *A. flavus* resting cells entrapped in poly(ethyl methacrylate) (Af-Pol EM-B). Experiments were carried out in triplicate using the same biocatalyst for each run. The biocatalyst was thoroughly washed between runs.

Table 1 Effects of biocatalyst and reactor system on the yield and specific reaction rate (r = U g^{-1}). A 1 : 2 mole ratio lauric acid/ethanol was used in all experiments

	Yield (%)		r (U g^{-1})	
Biocatalyst	Batch[a]	PBR[b]	Batch[a]	PBR[b]
A. flavus	95 ± 10	97 ± 12	3.0 ± 0.2	6.2 ± 0.6
Af-Pol EM-B	96 ± 7	99 ± 13	3.6 ± 0.4	7.4 ± 0.7

[a] Batch reactions at 24 h (40 °C, 1200 rpm, 20 mg of biocatalyst). [b] Flow reactions in the PBR (40 °C, flow = 10 μL min^{-1}, samples taken 24 h after starting, column reactor filled with each biocatalyst).

mechanical stability, size and shape of the particle. In contrast, agglomeration of free resting cells of *A. flavus* was observed inside the PBR. This agglomeration points to the occurrence of mass transfer limitations, thereby producing a decrease in the reaction rate. Indeed, when the biocatalyst was thoroughly washed after each run, it was reverted to the former behaviour, as shown in Fig. 5.

In addition, the specific activities of free *A. flavus* resting cells and Af-Pol EM-B were determined to compare batch and continuous flow reactions. Ester yields at 24 h of reaction were considered for this comparison. The reaction achieved an almost steady state at 24 h. Table 1 shows that yields were similar in the two reactors, while Af-Pol EM-B showed a higher r than free *A. flavus* resting cells. This result is in agreement with that described by Csajagi *et al.*[38] using free and supported lipases.

3. Materials and methods

3.1. Materials and reagents

Lauric acid, ascorbic acid, ethyl dodecanoate, ethyl acrylate, butyl acrylate, ethyl methacrylate, benzoyl peroxide (BPO), poly-(vinylpyrrolidone), and ergosterol were purchased from Sigma-Aldrich (Sigma-Aldrich Quimica, S.A., Madrid, Spain). Hexane, methanol, ethanol, acetone, acetonitrile, ethyl ether, isopropanol, and dichloromethane were supplied by J. T. Baker (Quimega, Lleida, Spain). 1,3-Dichloro-2-propanol, *N*′*N*′-dicyclohexylcarbodiimide (DCC), and potassium hexafluorophosphate (KPF_6) were supplied by Across Organics (Barcelona, Spain). Potassium hydroxide was purchased from Panreac (Barcelona, Spain). Allyl acrylate and dichloropropyl acrylate (regioisomeric mixture of 2-chloro-1-(chloromethyl)-ethyl acrylate and 2,3-dichloro-1-propyl acrylate) were prepared using a previously described method.[18,19]

3.2. Procedure for preparing fungal resting cells

A strain of *A. flavus* isolated from sunflower seeds was deposited in the culture collection "Colección Española de Cultivos Tipo" (Burjassot, Valencia-Spain) under reference number CECT 20475. The microorganism was maintained on potato/dextrose/agar (PDA) at 4 °C. The fungal strain was cultured in a synthetic liquid medium, as previously described.[16] Mycelia were harvested from the whole culture broth by filtration using a Buchner funnel and washed with distilled water followed by acetone. Mycelia were then dried under vacuum for 18 h and ground into a powder. The enzymatic units (U) were determined beforehand on the basis of the enzymatic hydrolysis rate of methyl stearate.[19] The specific activity of resting cells of *A. flavus* was 118 000 U g^{-1}.

3.3. Preparation of 1,1′-(2-(acryloyloxy)propane-1,3-diyl)-bis(3-butylimidazol-1-ium) hexafluorophosphate (IL-HFP) (VI)

3.3.1. Synthesis of 1,1′-(2-hydroxypropan-1,3-diyl)-bis(3-butylimidazol-1-ium) chloride (IL-Cl) (III). A mixture of 1,3-dichloro-2-propanol (**I**) (6.45 g, 50 mmol) and *N*-butylimidazole (**II**) (12.42 g, 100 mmol) was heated at 110 °C for 48 h in a capped reactor under Ar atmosphere. The mixture was cooled to room temperature and acetonitrile was added until no more product precipitated. The white solid formed was recovered by filtration and washed 3 times with cold acetonitrile. After drying 17.36 g of the product **III** (92% yield), a highly hygroscopic compound, were recovered.

3.3.2. Synthesis of 1,1′-(2-(acryloyloxy)propane-1,3-diyl)-bis(3-butylimidazol-1-ium) chloride (V) and hexafluorophosphate (IL-HFP) monomers (VI). The IL-HFP monomer was synthesized following the Steglich esterification method.[39] In this regard, 80 mL of a dichloromethane solution containing 4 g (55.5 mmol) of acrylic acid (**IV**), 4.1 g (10.8 mmol) of **III** and 35.18 mg (0.288 mmol) of 4-dimethylaminopyridine was added to a 250 mL one-necked round flask equipped with a calcium chloride drying tube. The solution was cooled in an ice-bath, and 6.68 g (32.4 mmol) of DCC were added at 0 °C for 5 min under stirring. After a further 5 min at 0 °C, the ice bath was removed and the white-color reaction mixture was stirred for 24 h at room temperature in a water bath. The reaction mixture was filtered and the precipitate was washed with dry dichloromethane (3 × 20 mL). The organic solutions were combined and evaporated under vacuum. The recovered solid (**V**) was dissolved in water (20 mL) and KPF_6 (828.3 mg, 4.5 mmol) was added. The mixture was stirred for 15 min at room temperature. Then 20 mL of dichloromethane were added. The organic layer was then separated and dried over anhydrous $MgSO_4$. The solvent was evaporated under vacuum and the compound (**VI**) was identified by ^{1}H-NMR. ^{19}F and ^{31}P NMR was also carried out to confirm the identity of the anion in the ionic liquid.

3.4. Entrapment of *A. flavus* in polymeric materials

3.4.1. Water-dispersive polymerization. A mixture of 620 mg of monomer (ethyl acrylate, butyl acrylate, ethyl methacrylate, allyl acrylate, dichloropropyl acrylate or a polymerizable ionic liquid -IL-HFP-) and 3.41 mg of benzoyl peroxide (BPO) was added under vigorous magnetic stirring to a reaction vial containing 5.6 mL of phosphate buffer (25 mM, pH = 7.0), 310 mg of *A. flavus*, 19.8 mg of poly(vinylpyrrolidone) and 7.89 mg of ascorbic acid at 50 °C (ref. 40) and fitted with a PTFE-lined cap. The reaction mixture was stirred for 18 h at 70 °C. Each PAAF containing resting cells was recovered and washed 3 times with deionized water and hexane. PAAFs were dried under vacuum

until constant weight and then placed in a closed container for water activity equilibration.

3.4.2. Bulk polymerization. Ethyl methacrylate (500 mg) was mixed with 250 mg resting cells of *A. flavus* and 15 mg (2% w/w mixture) of BPO in a 10 mL reaction vial fitted with a PTFE-lined cap. The reaction was performed under a dry and inert atmosphere using nitrogen. Polymerization reactions were carried out in an orbital shaker (Eppendorf1 Thermomixer Comfort, Hamburg, Germany) for 3 h at 70 °C. The solid formed was recovered and washed 3 times with hexane and then dried under vacuum for 18 h. The solid *A. flavus* entrapped in poly-(ethyl methacrylate) obtained by bulk polymerization (Af-Pol EM-B) was ground to powder and dried again under vacuum until constant weight.

3.5. Determination of *A. flavus* content in the polymeric materials

The percentage of *A. flavus* resting cells entrapped in each polymer was assessed by analyzing the ergosterol content on the material. This process was performed following Torres *et al.*[21] with some modifications. For this purpose, 100 mg of each previously prepared PAAF or free resting cells was blended with 6 mL of methanol for 2 min. This mixture was then centrifuged for 5 min at 800 g. The supernatant was poured off and the solid was suspended in 6 mL of methanol, shaken, placed in an ultrasound bath for *ca.* 30 s, and centrifuged again. The two supernatants were combined, mixed with 3 g KOH in 7.5 mL of methanol, and refluxed for 60 min. The reaction mixture was cooled, diluted with 6 mL of distilled water, and extracted three times with 25 mL of hexane. The organic extracts were combined and evaporated to dryness. The residue was dissolved in 1.5 mL of dichloromethane-isopropanol (99 : 1, v/v) and transferred to a vial. The solution was evaporated to dryness. The residue was dissolved in 1 mL of dichloromethane-isopropanol (99 : 1, v/v) and filtered with a HV 45 μm filter (Millipore). The samples were analyzed as described below. All the experiments were carried out in triplicate.

3.6. Equilibration of water activity

The water activity (a_w) was set by equilibrating separately reagents and biocatalysts (PAAFs and *A. flavus*) with aqueous saturated solutions of LiCl ($a_w = 0.12$), $MgCl_2$ ($a_w = 0.33$); K_2CO_3 ($a_w = 0.42$), $Mg(NO_3)_2$ ($a_w = 0.54$) and NaCl ($a_w = 0.75$). for at least 48 h.[19] The water activity of the biocatalyst was measured using an Aqua Lab series 3TE from Decagon Devices Inc. (Pullman, WA, USA).

3.7. Catalytic activity and study of the stability of PAAFs

The catalytic activity of PAAFs was determined using lauric acid and ethanol. For this, 0.5 mL of a solution containing 25 mg mL^{-1} (0.125 mmol mL^{-1}) of lauric acid and 11.52 mg mL^{-1} (0.250 mmol mL^{-1}) of ethanol in hexane were added to a reaction vial (1.5 mL) fitted with a PTFE-lined cap. Then 20 mg of the corresponding biocatalyst was added and the mixture was stirred at 40 °C for 24 h. The resulting solution was analyzed as described below, using a set of ethyl laurate solutions as external standards. Stability studies of the biocatalysts showing the best catalytic activity were performed using each biocatalyst for six consecutive esterification of lauric acid. All the experiments were carried out in triplicate.

3.8. Esterification in a continuous packed-bed reactor (PBR)

The PBR consisted of a glass column (inner diameter: 3 mm; total length: 100 mm; packed length: 90 mm; inner volume: 0.70 mL) filled with *ca.* 240 mg of *A. flavus* entrapped in poly(ethyl methacrylate) obtained by bulk polymerization (Af-Pol EM-B) or free resting cells of *A. flavus*. The glass column was packed manually with the biocatalyst, which was then fixed in place using glass wool plugs. A solution of lauric acid (25 mg mL^{-1}, 0.125 mmol mL^{-1}) and ethanol (11.52 mg mL^{-1}, 0.250 mmol mL^{-1}) in 85 mL of hexane was pumped through the column reactor at a constant flow rate of 10 μL min^{-1} (FRX System, Syrris Ltd, Royston, UK). The reactor was heated at 40 °C with an FRX Volcano Column Adaptor (Syrris) and a RCT Basic 40 hot-plate (IKA-Werke GmbH & Co., KG, Staufen, Germany) with a digital external control Pt 100 temperature sensor. All the experiments were carried out in triplicate. The biocatalyst was thoroughly washed and dried after each experiment and reused for the next experiment.

3.9. Analytical methods

Progress of the esterification reaction using each biocatalyst was determined by GC using an Agilent HP6890 series gas chromatograph (Barcelona, Spain) coupled to a flame ionization detector (FID). Regarding the parameters for esterification, the analytical column was a 30 m × 0.25 mm fused silica capillary coated with a 0.20 μm film thickness of poly(80% biscyanopropyl 20% cyanopropylphenyl siloxane) (SP-2330; Supelco, Madrid, Spain). The temperature program used was 40 °C for 5 min, followed by an increase of 20 °C min^{-1} until reaching the final temperature of 225 °C, which was then held for 3 min. A 1 : 20 split injection ratio was applied. Hydrogen was used as the carrier gas at a constant pressure of 620 kPa. The injection volume was 1 μL. The injection system was held at 250 °C and the FID at 280 °C.

HPLC analyses of ergosterol were carried out with a Waters series 600 pumping system, a Waters 710 autosampler, and a Waters 2487 UV detector at 282 nm (Waters Cromatografia SA, Spain). Twenty microliters of each sample was injected into a Sunfire C_{18} 5 μm (2.1 mm × 150 mm) (Waters Cromatografia SA) reverse-phase column at 25 °C. The eluent was methanol with a flux of 0.3 mL min^{-1}.

^1H, ^{19}F and ^{31}P NMR spectra of IL-HFP were recorded on a Varian AS400 spectrometer. ^1H NMR (400 MHz, DMSO-d_6): δ: 9.24 (s, 2H), 7.83 (m, 2H), 7.78 (m, 2H), 6.41 (dd, $J = 17.2$, 1.3 Hz, 1H), 6.17 (dd, $J = 17.2$, 10.5 Hz, 1H), 6.02 (dd, $J = 10.5$, 1.3 Hz, 1H), 5.64–5.59 (m, 1H), 4,72–468 (m, 2H), 4.50–4.44 (m, 2H), 4.24 (q, $J = 7.3$ Hz, 4H), 1.90 (t, $J = 7.6$ Hz, 4H), 1.40 (m, 4H), 0.92 (t, $J = 7.4$ Hz, 6H) ^{13}C-NMR (101 MHz, $CDCl_3$) δ: 164.10, 130.4, 120.4, 118.6, 64.8, 53.4, 49.6, 30.1, 18.2, 13.3. ^{19}F (376 MHz, DMSO-d_6) δ: 70.16 (d, J_{FP} = 712,04 Hz) and ^{31}P (162 MHz, DMSO-d_6) δ: 105.77 (Sep, J_{FP} = 712,04 Hz).

4. Conclusions

The catalytic activity of free and entrapped *A. flavus* resting cells in acrylic polymers is reported for the first time. This immobilization increased stability and catalytic efficiency, reduced inhibition and agglomeration of the biocatalyst, thus preventing the loss of biocatalytic activity. Of note, our results highlight the possibility to use *A. flavus* resting cells entrapped in poly-(methyl acrylate) (Af-Pol EM-B) to prepare diverse products such as ethyl laurate. Af-Pol EM-B can be repeatedly used without loss of activity. This biocatalyst showed the highest specific activity in the esterification reaction using both batch and PB reactors.

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