Co-immobilization of P450 BM3 and Glucose Dehydrogenase on different supports for application as a self-sufficient oxidative biocatalyst

Short title: Co-immobilization of P450 BM3 and Glucose dehydrogenase on different supports

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Abstract

BACKGROUND. The oxy-functionalization of non-activated carbon bonds by the bacterial cytochrome P450 BM3 from *Bacillus Megaterium*, presents a promising field in biosynthesis and it has gained much interest in recent decades. Nevertheless, the need for the expensive cofactor NADPH, together with low operational stability of the enzyme have made the implementation of this biocatalyst unfeasible in most cases for industry.

RESULTS. P450 BM3 and glucose dehydrogenase (GDH), as a cofactor regeneration enzyme, were successfully co-immobilized obtaining a bi-functional self-sufficient oxidative biocatalyst. Firstly, a broad screening on 13 different supports was carried out. Afterwards, five selected agaroses with three different functionalities (epoxy, amine and aldehyde) were studied and their immobilization processes optimized. Finally, P450 BM3 and GDH, were co-immobilized on those supports showing the best performance for P450 BM3 immobilization: Epoxy-agarose (Epoxy-agarose-UAB) presenting 83% and 20% retained activities respectively; AMINO-agarose presenting 28% and 25% and Lentikats® with which both enzymes retained 100% of the initial activity. Furthermore, the re-utilization of the self-sufficient immobilized derivatives was tested in 5 repeated cycles.

CONCLUSIONS. P450 BM3 and GDH have been successfully immobilized on three supports and their re-usability has been tested in a model reaction. It represents a step-forward for future P450 BM3 industrial implementations.

Introduction

Cytochromes P450 (CYPs) are versatile monooxygenases able to hydroxylate non-activated carbon bonds with the only requirement of molecular oxygen and an electron donor. CYPs are found as: (i) double domain proteins, presenting a heme-containing oxidative part and a FAD-/FMN-containing reductase; as (ii) triple domain enzymes with a third Ferredoxin subunit and (iii) as fusion proteins with just one enzymatic unit [1]. These enzymes have been in the focus of research in recent decades due to interest in their application as catalysts for the efficient production of fine chemicals, polymers, active pharmaceutical ingredients or nutritional supplements. They display a broad substrate range as well as the capability to catalyse a variety of oxidations including epoxidations, hydroxylation of aromatics, N-oxidation, deamination, dehalogenation and others [2].

P450 BM3 (EC 1.14.14.1) from *Bacillus megaterium* was discovered in 1986 and excels among CYPs because it presents the highest turnover numbers (17000 min^{-1} for arachidonic acid) [3], [4]. In contrast with eukaryotic CYPs, P450 BM3 is a self-sufficient soluble protein that contains both oxidative and reductase domains in the same polypeptide chain. Its natural substrates are medium to long chain fatty acids, however, as with other CYPs, P450 BM3 is a promiscuous enzyme. It accepts fatty amides and alcohols, hydroxylated fatty acids and ω-oxo fatty acids. It also displays, not only hydroxylating activity, but olefin epoxidation, ring expansion, heteroatom oxidation and dealkylation, and dehydrogenation across C-O, C-N and C-C bonds, as well as carbon-carbon bond formation and cleavage [5].

There are some limitations in the large-scale application of P450 BM3 such as, low operational stability, a dependence on the expensive NADPH electron donor and low retained activity in organic media [6]. Thus, there have been few cases where it has been attempt to be used industrially [7], [8]. In order to tackle these bottlenecks, protein engineering, cofactor recycling systems, surface modifications, reaction media engineering or immobilization are the main strategies usually followed in biocatalytic processes.

Regarding the low operational stability, immobilization has proven to be an efficient methodology for a broad type of oxidoreductases, not only for improving their stability but also for enhancing process metrics by biocatalyst recycling [9], [10]. In addition, immobilization can confer operational advantages such as the possibility to operate in continuous mode, reduction of foam formation, increased stability in organic solvents or simplified product purification [11], [12]. Immobilization of P450 BM3 has been pursued by other authors. For example, Maurer et. al. tried to immobilize it on a variety of commercially available supports [13]. They succeeded using the positively charged resins DEAE and SuperQ; and a Sol-Gel, but only the last one was found suitable for bioconversions. Axarli et. al immobilized a triple mutant onto Epoxy-sepharose, achieving retained activities of 81% and improved stabilities at 37°C [14]. Weber et. al. entrapped the heme-domain of the P450 BM3 into mesoporous molecular sieves (MCM-41 and SBA-15) and found a correlation between the activity observed from the derivative and pore diameter [15]. Furthermore, Zhao et. al. immobilized a mutant on DEAE-650S, further entrapped into k-carrageenan together with catalase and zinc dust (Zn/Co(III)sep) which served as electron source. They could operate the reactor during 10 batch cycles with continued conversions above 80% [16]. Other successful and more recent examples involved the fusion of the enzyme to linkers that enabled immobilization on solid surfaces [17]–[19]. From all the works present in the literature, few are the cases where P450 BM3 has been successfully immobilized covalently for bioconversion purposes. Many of the

above works focused on bio-sensing, in which low amounts of enzyme are required, or on the electrochemical study of the enzyme [20], [21]. For large scale applications, the attachment of large quantities of the enzyme to solid matrices by covalent bonds is a robust method that often confers novel properties to the enzyme and it does not present leaching.

With regard to electron donors, there have been attempts to substitute the expensive NADPH co-substrate for less costly products, *e.g.* NADH or H_2O_2 by protein engineering [22]–[24]. Strategies for regenerating the expensive NADPH has been also studied both in enzymatic and non-enzymatic manners [25]–[27]. The use of whole cell biocatalyst expressing P450 has also been considered as a suitable and much cheaper strategy by taking advantage of the cellular metabolism for NADPH regeneration [28], [29]. However, the use of whole cells entails several drawbacks such as secondary undesired reactions or low reaction rates due to the substrate or product mass transfer limitation. Among the different strategies for cofactor regeneration, the utilization of secondary enzymes that make use of sacrificial substrates is one of the most attractive systems. The use of enzymatic NADPH regeneration systems in P450-catalyzed reactions has been reported by other authors. Different enzymes such as glucose dehydrogenase, formate dehydrogenase or phosphite dehydrogenase proved to be efficient and suitable biocatalysts for this purpose [13], [30], [31], [32].

In this work, a bi-functional self-sufficient oxidative biocatalyst is obtained by the successful co-immobilization of P450 BM3 and a GDH from *Thermoplasma acidophilum* as cofactor-regenerating enzyme. Firstly, P450 immobilization was analysed prior to the selection of the best co-immobilization strategy since P450 is a less robust enzyme compared to GDH. Thus, deeper understanding of P450 BM3 immobilization by two different enzyme-support interactions (adsorption or covalent attachment) is presented. In this sense, different supports presenting a broad range of features were tested to get insight in the optimal immobilization procedure that should be followed. Finally, co-immobilization of P450 BM3 and GDH was

115 performed by applying the best methodologies resulting for the P450 BM3. The encapsulation
116 in polyvinyl alcohol particles (Lentikats®) was also studied for the confinement of both P450
117 BM3 and GDH as an alternative to adsorption and covalent attachment strategies.

118 The resulting-self-sufficient biocatalysts were tested in terms of operational stability in the
119 hydroxylation of sodium laureate as a model reaction for proof-of-concept.

120

121 Experimental

122 Materials and supports

123 Nicotinamide adenine dinucleotide phosphate sodium salts in its oxidized and reduced form
124 (NADP+ disodium salt and NADPH tetrasodium salt) was purchased from BONTAC
125 Bioengineering (Shenzhen, Guandong, China). All the other reagents were purchased from
126 Sigma Aldrich® (St. Louis, MO, USA) and were of analytical grade if not stated elsewhere.

127 The supports used in the screening section were donated by Purolite® Life Sciences (Bala
128 Cynwyd, PA, USA) and their features are summarized in Table 2.

129 The Epoxy-agarose-SIGMA was purchased from Sigma-Aldrich®. According to the supplier's
130 specifications it presented spherical shape, it had an activation degree of ≥ 20μmol/mL and 12
131 atoms spacer.

132 Non-functionalized agarose (spherical beads Ø 50-150μm) purchased from Agarose Bead
133 Technologies® (ABT®, Madrid, Spain) was used for obtaining Epoxy-agarose-UAB by support
134 functionalization carried out as described by Axarli et. al. [14]. The quantification of the epoxy
135 groups present on the supports was done following the method described by Gupta [33].

Amino functionalized agarose (AMINO-agarose) presenting amino ethyl groups and aldehyde functionalized agarose (GLYOXYL-agarose) with an extent of labelling of 40-60µmol/mL both were also purchased from ABT®. All this information was always according to the supplier's specifications.

Lentikats® polymer was purchased from GeniaLab® (Braunschweig, Germany) and its composition consists on a mixture of polyvinyl alcohol (10%), polyethylene glycol (6%) and demineralized water (84%).

Recombinant expression of P450 BM3 and Glucose dehydrogenase in *E. coli*

P450 BM3 (CYP102A1 from *Bacillus megaterium* BM3) was recombinantly produced in *Escherichia coli* in a 20 L scale batch fermentation employing an *E. coli* K12 derivative and a pBAD/myc-HisC based expression vector. Glucose Dehydrogenase (GDH) from *Thermoplasma acidophilum* (GDH-Tac) was co-expressed from the same vector in a poly-cistronic arrangement of the GDH gene downstream of the P450 BM3 gene. A 500 ml pre-culture were used to inoculate 20 kg main culture medium with 100 µg/ml neomycin. The pre-culture was prepared in standard Luria-Bertani (LB) medium supplemented with 100 µg/ml neomycin. The fermentation was performed using terrific broth (TB) medium with glycerol. 2.5 h after inoculation of the fermenter as inducer, pre-sterilized L-arabinose was added to the fermenter to final concentration 0.02 % (w/v). 24 hours after inoculation of the fermenter, the cell material was harvested by centrifugation. Cell free extract was prepared by adding 2 weight equivalents of 100 mM potassium phosphate (KP_i) buffer (pH 7.0) to 1 weight equivalent of *E. coli* wet cells and sonication of this cell suspension with an ultrasound probe for 20 min with cooling on ice and centrifugation to remove the cell-debris.

Total protein and enzyme content

The cell lysate was pre-clarified by centrifugation (3220g for 15min.) and analysed accounting for total protein content by means of Bradford Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA) using bovine serum albumin as standard [34].

Enzyme content was assessed using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (NuPage 12%, Invitrogen, USA) run in a Mini-PROTEAN II apparatus (BioRad, USA) following the protocol of Laemmli *et. al.* [35]. Low range protein markers were used for molecular weight determination. Gels were stained using Comassie G250 colloidal stain solution [34% (v/v) ethanol, 2% (v/v) H_3PO_4, 17% (w/v) NH_4SO_4 and 0.066% Comassie G250] and the Image LAB^{TM} software (BioRad, USA) was used for image processing.

Active P450 BM3 content determination

P450 BM3 active form concentration was determined using the CO-differential spectra analysis described by Omura and Sato, using $\varepsilon = 91\ mM^{-1}\ cm^{-1}$ [36], [37].

P450 BM3 activity measurement

The activity of the P450 BM3 was measured spectrophotometrically (λ=340nm) following the consumption of NADPH ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$, 200µM), using sodium dodecanoate (sodium laurate) as substrate (1,3 mM dissolved in sodium phosphate buffer 50mM pH=7,5) and the correspondent amount of sample [1/20 of the total volume (V_T)]. The basal consumption of NADPH by unspecific enzymes present in the lysate was determined by this same test but avoiding the addition of the substrate and adding buffer instead. This consumption rate is subtracted from the measurement with sodium dodecanoate. One unit of activity (UA) is defined as the enzyme required to convert 1µmol of NADPH per minute at those given conditions (30°C and pH = 7.5). The absorbance was recorded using a spectrophotometer Cary 50 Bio UV-visible (Palo Alto, USA). The equipment allows temperature control and stirring. Plastic micro cuvettes BRAND® UV (Sigma Aldrich®) were used for soluble enzyme

characterization and 3.5mL quartz cuvettes HELLMA® 100-QS (Hellma Analytics, Müllheim, Germany) with magnetic stirring, were used for the immobilized derivatives (suspension). In the first case a total volume of 500µL was reached while in the second it was 2mL.

Glucose dehydrogenase activity measurement

GDH activity is measured spectrophotometrically (λ=340nm) following the formation of NADPH. Certain amount of sample (1/20 of the V_T) was added to a solution containing D-Glucose (200mM dissolved in sodium phosphate buffer 100mM pH=8) and $NADP^+$ (400µM). The same procedure explained above was applied for measuring the non-GDH formation of NADPH and the same consideration is taken to obtain the UAs. The absorbance was also recorded using the aforementioned equipment and conditions.

Immobilization metrics

In every immobilization, a characterization was pursued in order to obtain the retained activity (equation 1) and immobilization yield (equation 2). Supernatant and suspension activities were analysed over time until a steady state was reached and in all cases the activity of a blank (no support) was also monitored to ensure that the enzyme activity was not affected by protocol's conditions. A 10% w/v relation between the support and the total volume was always stablished and small amounts of enzyme were loaded (4-5UA of P450 BM3/g of support and 5-6UA of GDH/g of support) so that diffusional limitations were minimized.

$$Retained\ activity\ (\%) = \frac{Suspension\ activity\left(\frac{UA}{g}\right) - Supernatant\ activity\left(\frac{UA}{g}\right)}{Initial\ offered\ activity\left(\frac{UA}{g}\right)}\ x\ 100 \quad \textit{(Equation 1)}$$

$$Immobilization\ yield\ (\%) = \left(1 - \frac{Supernatant\ activity\left(\frac{UA}{g}\right)}{Initial\ offered\ activity\left(\frac{UA}{g}\right)}\right)\ x\ 100 \quad \textit{(Equation 2)}$$

For the assessment of the loading capacity the supports were saturated with enzyme and the activity was calculated taking into account that diffusional limitations do not allow the actual visualization of the activity in the support (equation 3).

$$Loading\ capacity\ \left(\frac{UA}{g}\right) = \left(Offered\ initial\ activity\ \left(\frac{UA}{g}\right) - Supernatant\ activity\ \left(\frac{UA}{g}\right)\right) x\ \frac{Retained\ activity\ (\%)}{Immobilization\ yield\ (\%)} \quad \textit{(Equation 3)}$$

Moreover, the amount of protein per gram of support was also calculated, analysing the initial protein content and subtracting from it, the concentration still remaining in the supernatant at the end of the process. In all cases protein concentration was determined by means of Bradford Protein Assay Kit, as already described.

Immobilization of P450 BM3 onto Purolite® resins

From Purolite®, a set of commercially available resins presenting a variety of matrices, pore sizes, linker lengths, and functional groups (Table 2) were tested for the immobilization of P450 BM3. The conditions in which immobilizations were carried out in each case were those specified by the supplier's protocols. In all cases, the relation of 10% w/v between support and total volume was maintained. The immobilizations were done at 25°C and mild agitation. The incubation times ranged from 0.5 to 5 hours depending on the speed at which the enzymes were attached. The epoxy containing methacrylates (ECR8204F, ECR8215F and ECR8285) were used in 50mM sodium phosphate buffer pH 8 containing 0.5M NaCl. The amino functionalized resins (ECR8309F, ECR8315F, ECR8409F and ECR8415F) were mixed with the enzyme in 50mM sodium phosphate buffer pH 6. The supports made of a mixture of styrene and methacrylate (ECR1061M and ECR1030M) were utilized in 50mM sodium phosphate buffer pH 7.5; finally, immobilizations using the epoxy agaroses (Praesto 45, 65 and 90) were performed in 1M potassium phosphate buffer pH 8.

Immobilization of P450 BM3 onto Epoxy-agarose-SIGMA and Epoxy-agarose-UAB supports

For the other epoxy-agaroses (Epoxy-agarose-SIGMA and Epoxy-agarose-UAB), the enzyme was dissolved in 1M (pH 8) potassium phosphate buffer, mixed with the resin (10% w/v) and left at 25°C with mild agitation. The Epoxy-agarose-UAB was incubated for 4 hours and Epoxy-agarose-SIGMA was incubated for 0.5h. At the end of the process, in order to eliminate the unreacted epoxy groups, the samples were incubated with 0.2M β-mercaptoethanol for at least 2h at 25°C.

Immobilization of P450 BM3 onto GLYOXYL-agarose support

For the immobilization using GLYOXYL-agarose different pH's were tested (6.5 – 8.5) using 50mM sodium phosphate buffers. The immobilizations lasted 6 hours and were done at 25°C and mild agitation. For the reduction of the Schiff base, two different reducing agents were studied: sodium borohydride (1mg/mL) added at the end of the immobilization and sodium cyanoborohydride (0.05mg/mL for characterization and 0.4mg/mL for high loads of enzyme) added at the beginning of the immobilization.

Immobilization of P450 BM3 onto AMINO-agarose support

For AMINO-agarose 50mM sodium phosphate buffer (pH 6) was used to dissolve the enzyme and mix it with the support. Coming up next, the enzyme was left to ionically adsorb onto the support for 0.5h-1h. After that, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) was added and incubated for 30min. at different concentrations to activate the carboxylic groups and promote the covalent binding. The concentration of EDC was optimized in the characterization phase (1, 2, 3, 4, 5, 10, 15, 20mM) and also when using high loads of enzyme (3, 6, 12, 30, 60mM). Finally, 0.5M NaCl was introduced to desorb all the protein attached non-covalently. The immobilization was carried out at 25°C and mild agitation.

Co-immobilization of P450 BM3 and GDH in AMINO-agarose and Epoxy-agarose-UAB

The conditions in which immobilizations were carried out were exactly the same as previously described for P450 BM3 for AMINO-agarose and Epoxy-agarose-UAB. The activity of the GDH present in the lysate was also monitored in this stage together with the P450 BM3. Retained activities and immobilization yields were obtained for GDH and P450 BM3.

Co-Immobilization of P450 and GDH into polyvinyl alcohol lenses (Lentikats®)

Co-immobilization of GDH and P450 BM3 by entrapment was carried out using an organic polymer commercialized as Lentikats®. The solid mixture was heated up to 95°C until it turned into a transparent liquid. Then it was cooled down to 40°C and 8mL of the polymer were mixed with 2mL of a suspension containing the desired amount of biocatalyst. Afterwards the mixture was poured on a petri dish and the lenses were printed using the LentiPrinter®. The resulting plates were left to dry at 25°C for 1h until they lost 80% of its weight approximately. Once printed, the support adopted a lentil-like shape of 1-2mm diameter and 200-300µm wide where the proteins were entrapped inside.

Re-usability of the biocatalyst for the hydroxylation of sodium laureate

The utilization of the immobilized enzymes in repeated reaction cycles were studied following the consumption of sodium laurate. The reactor (V_T=5mL) contained 1.53mM sodium laurate, 0.2mM $NADP^+$ and 12.5mM D-glucose. All dissolved in 50mM sodium phosphate buffer pH 7.5. The enzyme added was the minimum necessary to convert at least 90% of the initial substrate in 1hour. The reactors used were purchased from MultiSynTech (Witten, Germany) and consisted of 10mL plastic vessels with a porous plate at the bottom. It allowed the extraction of the whole reactor liquid content by filtering with this same porous plate without losing any fraction of the immobilized derivative. The reaction took place at 25°C and 1000 rpm using a Multi-Therm H5000-HC-E thermo-shaker (Benchmark Scientific Inc., Sayreville, NJ, USA). After

each cycle, the immobilized enzymes were washed twice with sodium phosphate buffer 50mM pH 7.5 then new reaction medium was added and substrate conversion was quantified. Experiments were carried out in duplicate.

GC-FID analysis of sodium laurate

Samples containing sodium laurate and its products were analysed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped with a HP-INNOWAX 19095N-123 column (30m, 0.53mm, 1µm, Agilent Technologies). Reaction samples were filtered (Ø 0.22µm) and 50µL of hexanoic acid (2.8mg/mL), that served as internal standard, were added to 300µL of sample. The column temperature started at 150 °C, increased to 240 °C at 24 °C/min. and it was held at final temperature for 11 minutes. The injector temperature was kept at 300 °C; for the flame ionization detector, the temperature was 320 °C. Helium was used as a carrier gas at a flow rate of 8 mL/min. All analyses were carried out in duplicate.

Results and discussion

Characterization of the *E. coli* lysate containing P450 BM3 and GDH

As already mentioned, P450s are enzymes that require a cofactor to perform the redox reactions they catalyse. One of the strategies widely used for overcoming the disadvantages of co-factor dependency is the use of an enzyme-coupled reaction for the regeneration of the expensive NADPH. In this work, GDH (EC 1.1.1.47) from the archaea *Thermoplasma acidophilum* was used as NADPH-recycling enzyme using glucose as sacrificial substrate. This robust and stable tetrameric protein is reported to be an efficient enzyme for NADPH regeneration [31], [32], [38].

In the present work, GDH was co-expressed with P450 BM3 in *E. coli* and both enzymes were present in the cell lysates utilized.

The characterization of the lysate was performed regarding protein content, enzyme content (SDS-PAGE), activity and P450 BM3 active form concentration and the results are shown in Table 1.

The P450 BM3 was expressed at a higher level than the GDH, however, under the conditions in which the activity tests were performed, GDH gave higher specific activity. Table 1, also shows the correlation between Units activity (UAs) and concentration (nmols) of active enzyme; for the P450 BM3 case, 2.85 UA/nmol.

Screening of supports for the immobilization of P450 BM3 – Purolite®

The main objective of the present work was to obtain a bi-functional self-sufficient oxidative biocatalyst by co-immobilization of P450 BM3 and GDH. To accomplish this, supports and immobilization conditions that were found to be most suitable for the P450 BM3 enzyme were prioritised, since cytochrome is the key enzyme performing the target reaction and it has been described as an unfavourable enzyme for immobilizing. GDH, on the contrary, has been described as a robust and more stable enzyme so difficulties in its immobilisation were not anticipated [38]. In fact, successful immobilization of GDH on different kinds of support has been reported by several authors [39]. The results obtained for P450 BM3 immobilization, the materials tested and its features are summarized in Table 2. The supports purchased in the screening kits encompass a range of different particle sizes (45µm to 710µm), matrices (methacrylate, styrene and agarose), functional groups (amino, epoxy and aldehyde), enzyme-support interaction (covalent binding or hydrophobic adsorption) and linker lengths (2 to 18 carbon atoms). All of these properties have been identified as critical for successful enzyme immobilization [12].

As shown in Table 2, the immobilization yields obtained were higher than 90% in all cases, except for the supports that interact with the enzyme by hydrophobic adsorption: ECR1061M and ECR1030M. In both cases the immobilization yields only reached 21% and 33% respectively. On the other hand, no retained activities or very low values (<20%) were observed in all tested supports, except for Praesto 45, 65 and 90 (Entries X to X; all epoxy-agarose supports). According to the titration performed, Praesto 45, 65 and 90 all contained an epoxide concentration of 9, 6 and 8µmol per gram of support respectively and resulted in retained activities of 60-68% after 4h incubation. The maximum loading capacities of these epoxy-agarose resins ranged from 18 to 20UA/g and 7 to 8mg protein/g of support. The immobilization profiles were very similar among the three supports and they did not show any significant correlation with the particle size or the initial epoxide concentration (Fig. 1A).

Regarding the methacrylate and styrene matrices, even though different ionic strengths and pH values were assessed, none of the tested supports showed retained activities above 20%. P450 BM3 had adhered to the matrices in most cases, but did not remain active. Two methacrylate-based materials harbouring amino groups on six carbon chain linkers (ECR8409F and the ECR8415F) gave retained activities of 24% and 22% respectively when the enzyme was adsorbed onto the resins solely by ionic interactions, however once carbodiimide was added, to promote covalent bond formation, the activity was reduced to 6% and 18% respectively. Furthermore, following the supplier's protocol, the four amino methacrylate based supports were further functionalized with glutaraldehyde prior to enzyme binding, but none resulted in successful immobilization either.

It should be also mentioned that methacrylic matrices with the same functional groups than the epoxy-agarose support (ECR8204F, ECR8215F and ECR8285) did not lead to actively immobilized enzyme, as can be seen by the low retained activities obtained (0%, 2% and 3% respectively). These results could indicate that for P450 immobilization the hydrophilicity of

the matrix is a key factor. The higher hydrophobicity of the methacrylate/styrene matrices, together with the flexible nature of the enzyme and the presence of two active sites, are probably the causes behind the deactivation of the biocatalyst [40]. This hypothesis would be also in accordance with the low immobilization yields and retained activities obtained with the styrene matrices (ECR1061M and ECR1030M) where, because the immobilization mechanism is also based on hydrophobic adsorption to the highly hydrophobic nature of the matrix, both the immobilisation yield and retained activity are low. Other authors have also reported unsuccessful attempts to immobilize P450s by hydrophobic adsorption [13].

Taking into account the obtained results in the screening which showed a strong effect of the hydrophilicity of the matrix on P450 immobilization, agarose was chosen to perform further studies.

Immobilization of P450 BM3 onto different functionalized agaroses

In addition to the epoxy resins already tested (Praesto 45, 65 and 90), two other agarose matrices with free epoxy groups and different expoxide concentrations were studied: a commercial lyophilized epoxy-agarose resin from Sigma-Aldrich® (Epoxy-agarose-SIGMA) and an in-house functionalized agarose resin (Epoxy-agarose-UAB) prepared as shown in the experimental section. In addition an agarose based matrices containing aldehyde (GLYOXYL-agarose) and primary amine (AMINO-agarose) functional groups were tested.

The Epoxy-agarose-SIGMA resin, according to the titration performed, contained an epoxide concentration of 110µmol/g and gave an immobilization yield of greater than 95% after 0.5h (Figure 1B). However, a rapid decrease in activity of the suspended biocatalyst was noted during the immobilization procedure, indicating a strong deactivation of the enzyme after immobilization. Thus, after just half an hour, low retained activities (25%) were observed from this support. These results could indicate that the utilization of an epoxy-agarose with high

368 epoxide concentration, such as the Epoxy-agarose-SIGMA, results in a progressive loss of
369 activity over time due to the multipoint attachment of the enzyme to the support [41]. The
370 explanation can be found in the reactivity of the epoxy groups which differs over time: firstly,
371 reacting with the amines present on the surface of the enzyme and secondly reacting with the
372 carboxyl and thiol groups and [42]. At the end, if the incubation time is long enough, the
373 protein can be attached by too many points and lose its whole catalytic activity. This issue can
374 be stopped adding a blocking agent that removes the unreacted epoxy groups once the
375 protein is immobilized. In Figure 1B, β-mercaptoethanol (0.2M final concentration) was added
376 after 0.5h and the suspension was incubated for 3.5h at 25°C. No significant loss of activity was
377 observed during this period which validates the hypothesis of the multipoint attachment as
378 the main reason for enzyme deactivation. However, due to its elevated cost (118€/g) and low
379 retained activities, compared to the Praesto supports, the Epoxy-agarose-SIGMA was
380 discarded from further studies.

381 On the other hand, the Epoxy-agarose-UAB displayed an epoxide concentration of 30μmol/g
382 and gave more than a 95% immobilization yield and 83% retained activity after 0.5h (Figure
383 1C), with a maximum loading capacity of 30UA/g (7.5mg protein/g support). A decrease in
384 retained activity can be also seen, reaching 50% after 4h of incubation probably due to the
385 excessive number of covalent bindings. In this case, as well as for Praesto supports (Figure 1A),
386 the agarose contains a lower epoxide concentration compared to Epoxy-agarose-SIGMA.
387 Therefore, the activity loss due to the multipoint attachment is lower for the Epoxy-agarose-
388 UAB or even negligible for the Praesto compared to the Epoxy-agarose-SIGMA. Thus, when
389 choosing the right support, a consensus should be found. Higher epoxide concentration may
390 imply higher loading capacity as is seen when comparing Praesto (18 to 20 UAs/g) and Epoxy-
391 agarose-UAB (30UAs/g); however, at the same time, higher epoxide concentration entails
392 higher deactivation of the enzyme over time as it is the case comparing all three epoxy-

agaroses: Praesto (32-40% activity lost), Epoxy-agarose-SIGMA (75% activity lost) and Epoxy-agarose-UAB (50% activity lost). In this sense, due to the loading capacity, the high retained activities reached and the low cost (2.5€/g), the Epoxy-agarose-UAB was considered the best candidate for the immobilization of P450 BM3 among epoxy-agaroses. This study represents a deeper insight into the work of Axarli et. al. [14] in the understanding of the immobilization of P450 BM3 on epoxy-agaroses and sets the bases for an optimal process.

As mentioned before, other agarose-based supports with different functional groups were also tested such as GLYOXYL-agarose and AMINO-agarose. The GLYOXYL-agarose (aldehyde activated) immobilization must be pursued in alkali media [43], however, P450 BM3 is not stable above pH 8.5 (data not shown). After screening a range of immobilization pH's (6.5 – 8.5), 7.5 was the best performing one resulting in 50% retained activity after 6h incubation (data not shown). For the reduction of the Schiff base formed between the aldehyde of the resin and the amine of the enzyme, two reducing agents were tested: i) sodium borohydride (1mg/mL) which is added at the end of the incubation and ii) sodium cyanoborohydride (0.05mg/mL) which has slower kinetics and can be added at the beginning of the immobilization. Sodium borohydride resulted in a complete deactivation of the enzyme whilst the sodium cyanoborohydride strategy resulted in 36% of retained activity. Thus, sodium cyanoborohydride was selected as the most suitable reducing agent. Regarding the studies on maximum loading capacity, the values previously obtained in the characterization (36% retained activity) at low enzyme loadings were not extrapolated and a major inactivation occurred. This support has proved suitable for the immobilization of P450 BM3, nevertheless, since the covalent immobilization using high loading was not successful, it was discarded from further studies.

Regarding AMINO-agarose, as for the other tested supports functionalized by amino groups (i.e. ECR8309F, ECR8315F, ECR8409F and ECR8415F) the immobilization is based on a three-

418 stage process. In the first stage, the enzyme is adsorbed onto the support surface by ionic
419 interaction of the amino groups of the support and the carboxyl groups of the enzyme, thus an
420 ionic adsorption occurs. In the second stage, carbodiimide is added to the medium aiming to
421 activate the carboxyl groups present on the enzyme making covalent bonding possible.
422 Solutions with high ionic strength such as sodium chloride (0.5M) can be finally added to
423 desorb all the protein attached non-covalently (third stage). Ionic adsorption on AMINO-
424 agarose was performed at pH 6 and gave a 58% retained activity (Figure 1D phase I). This value
425 was 2.8-fold higher than the retained activities obtained during the ionic adsorption for the
426 methacrylate based materials ECR8409F and ECR8415F harbouring the same functional group
427 (21%), as previously discussed. The maximum loading capacity achieved was 112UA/g (50mg
428 protein/mL) which represents the highest value among all the resins tested. Covalent binding
429 of the amine and the carboxyl groups was carried out by adding EDC following ionic adsorption
430 phase (Figure 1D phase II). Since carbodiimide can lead to enzyme deactivation, different
431 concentrations and incubation times were studied to determine the optimal conditions for the
432 covalent binding phase. These studies showed optimal values of 3mM EDC and 30min reaction
433 time respectively. Following covalent attachment, the biocatalyst was incubated with 0.5M
434 NaCl for 2h (Figure 1D, phase III) in order to increase the ionic strength of the media and
435 release all the protein not adhered covalently. 28% of the initial activity offered was covalently
436 bound to the agarose, which represents 30% less activity than the value obtained for the ionic
437 adsorption phase (Figure 1D, phase I). On the other hand, compared to the methacrylate-
438 based supports, the retained activity was 4.7-fold and 1.6-fold higher than the obtained with
439 ECR8409F and ECR8415F respectively. Regarding the maximum load, 53UA/g was reached (47
440 mg protein/mL), representing a 52% decrease in immobilized activity compare to the obtained
441 by ionic adsorption, likely due to enzyme deactivation caused by the carbodiimide. When
442 testing for the maximum loading capacity the concentration of EDC was further optimized

since more enzyme was added (Figure 2). It resulted in an optimal concentration between 6 and 12mM. This second value was chosen due to the higher immobilization yield that it entailed. This support represents a step forward in comparison with the other tested agaroses and it is the first work reporting a successful covalent immobilization of P450 BM3 in an amino-based matrix.

Co-immobilization of P450 BM3 and Glucose dehydrogenase

As it has been already mentioned, for co-immobilization of both GDH and P450 BM3, it was considered to prioritize the supports and immobilization conditions that were found to be suitable for P450 BM3, i.e. Epoxy-agarose-UAB and AMINO-agarose.

The immobilization of GDH using Epoxy-agarose-UAB resulted in poor affinity of the protein for the support with immobilization yields below 20%. However, of the enzyme that did adhere, a slight over-activation was observed compared to the same quantity of free enzyme. At the end of the incubation (4h), retained activity of 20% and a maximum loading capacity of 3UA/g were achieved. Since the conditions were exactly the same as reported above for P450 BM3, the activity for the cytochrome was comparable and resulted in 50% retained activity and 30UA/g (7.5mg protein/g support).

With AMINO-agarose, GDH presented high affinity for the support and it also showed an over-activation effect when adhered to the matrix. It had better tolerance to the presence of EDC compared with P450 BM3, with 10mM being the optimal concentration for this enzyme (57% retained activity) in the characterization phase. Coimmobilisation of both enzymes, initially using a low carbodiimide concentration of 3mM, resulted in a 25% retained activity for GDH and 28% for P450 BM3. Using a higher loading of EDC (12mM) resulted in a loading capacity of 18 UA/g for GDH and 53 UA/g for P450 BM3. In summary, a self-sufficient oxidative biocatalyst was obtained.

Co-immobilization of P450 BM3 and GDH into polyvinyl alcohol particles - Entrapment

Finally, as an alternative to immobilization by adsorption or by covalent attachment, an entrapment strategy was also investigated for co-immobilization. Both P450 BM3 and GDH were successfully embedded into Lentikats® (polyvinyl matrix) as described in the experimental section. The analysis undertaken, showed maximum retained activities of 100% for both P450 BM3 and GDH.

In the sense of the loading capacity employed, the activity and concentration of protein in the polymer was 1.8UA/g of P450 BM3, 0.8UA/g of GDH and 1.1mg protein/g respectively. It was not further increased due to operational issues. The capacity is limited by an aggregation effect observed when charging high concentrations of lysate with which the lenses form a compact mass that cannot be used in our reactions. This entails a serious drawback when looking to industrial feasibility because the enzyme should be previously purified or the specific activity of the support would remain too low [28]. However, since the immobilization had been successful, albeit with low enzyme loading, and it is the first work that reports a successful entrapment of both P450 and GDH in Lentikats ®, it was decided to continue working with this immobilized derivative.

Reusability of the biocatalyst using sodium laurate as substrate

From all the supports tested, AMINO-agarose, Epoxy-agarose-UAB and Lentikats® were considered good candidates to be potential carriers in bio-catalysis applications. It is well-known that uncoupling effects on P450s produce reactive oxygen species that inactivate the enzyme (uncoupling) [44]. This handicap is less relevant when working with native substrates such as sodium laureate and so this substrate was used as a model for studying the reusability of the self-sufficient biocatalyst. Firstly, the coupling efficiency of P450 towards this substrate

was determined and then reusability studies were performed using the self-sufficient P450/GDH biocatalysts obtained with the aforementioned carriers.

Reactions using sodium laureate (1.3mM) and NADPH (0.2mM) were performed, monitoring and comparing substrates consumption by means of GC-FID. A coupling efficiency of 90.4% was observed, which means that, 90.4% of the NADPH consumed was employed for sodium laurate hydroxylation, with the remainder directed towards uncoupled reactions. This substrate was considered adequate to assess the operational stability of the enzymes in repeated reaction cycles.

Total turnover number (mols of substrate consumed/mols of P450 BM3) (TTN) was chosen as the metric to evaluate the yield of the self-sufficient biocatalyst in comparison with the soluble lysate. To assess it, the immobilized P450 BM3/GDH (Epoxy-agarose-UAB, AMINO-agarose and Lentikats®) biocatalysts were tested in sodium laurate reaction cycles of 1 hour each utilizing glucose as the sacrificial substrate and the cofactor in its oxidized form ($NADP^+$) thus forcing the cofactor regeneration to start before the P450-catalyzed reaction. Moreover, $NADP^+$ starting concentration was 6.5-fold lower than the sodium laureate., The amount of P450 BM3 required to convert > 90% of sodium laurate in 1 hour was found to be 0.18-0.2UA/mL (0.063-0.07 nmols/mL). GDH on the other hand, was always ensured to be present in excess so that it did not limit the hydroxylation reaction. The conversions obtained in each cycle for the immobilized systems are presented in Figure 3. The TTN for the soluble reaction was 21,500 mols of sodium laurate consumed/mol of P450 BM3 in 1 hour of reaction. The immobilized derivatives allowed the re-utilisation of the self-sufficient biocatalyst boosting the TTN by 2.31-fold for the Epoxy-agarose-UAB, 2.98-fold for AMINO-agarose and 2.3-fold for the enzymes immobilized into Lentikats®. As shown in Figure 3, the conversion dropped in an exponential manner for epoxy-agarose (Figure 3A). On the contrary, with AMINO-agarose and Lentikats®

514 the decay was more linear (Figure 3B). At the end, all three derivates could be re-used
515 successfully, thus demonstrating a significant operational stability.

516

517 Conclusions

518 P450 BM3 has gained much interest in recent years in biocatalysis for its oxy-functionalization
519 characteristics, although, the implementation of this enzyme in industrial processes has lacked
520 optimal metrics. Operational stability as well as its requirement of NADPH in stoichiometric
521 amounts have been identified as the main bottlenecks. Aiming to overcome these two main
522 drawbacks, P450 BM3 and GDH have been co-immobilized in the present work to obtain a
523 robust bi-functional self-sufficient biocatalyst.

524 P450 BM3 and GDH were covalently co-immobilized on epoxy-functionalized agarose and
525 amino-functionalized agarose. Both enzymes were also successfully co-immobilized by
526 entrapment in a polyvinyl alcohol/polyethylen-glycol matrix named Lentikats®. From the five
527 epoxy-agaroses tested, the optimal support (Epoxy-agarose-UAB) presented an epoxide
528 concentration of 30µmol/g and retained activities of 83% for P450 BM3 and 20% for GDH. On
529 the other hand, AMINO-agarose showed retained activities of 28% for P450 and 25% for GDH.
530 Finally, the entrapment of both enzymes in Lentikats ® lead to P450 BM3 and GDH co-
531 immobilization maintaining 100% of the activity initially loaded.

532 P450 BM3 was also immobilized on aldehyde-functionalized agarose and presented 45%
533 retained activity. However, this support did not allow high enzyme loads required for reaction
534 testing, thus making this support unattractive for further studies.

535 The operational stability of the obtained bi-functional self-sufficient biocatalysts (Epoxy-
536 agarose-UAB, AMINO-agarose and Lentikats®) was analysed in terms of re-usability for sodium

537 laureate oxidation. All three derivatives showed successful reuse in 5 cycles, entailing an
538 increment in the TTN of 2.31-fold the Epoxy-agarose-UAB, 2.98-fold the AMINO-agarose and
539 2.3-fold the Lentikats® compared to the soluble enzymes.

540 The P450 BM3 wild type served as a model in the immobilization studies in this work. On the
541 other hand, mutated P450 BM3s, usually required for oxidative reactions of non-natural
542 substrates which entail high uncoupling effects, can follow the exact same principles reported
543 here since the surface of the enzyme is usually unaffected [1], [5], [6], [45]. In this sense,
544 immobilization can be an added value to the process, for these new enzymes, due to the many
545 advantages that it confers.

546

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557

558 References

559 [1] V. B. Urlacher and M. Girhard, “Cytochrome P450 monooxygenases: An update on

perspectives for synthetic application,” *Trends Biotechnol.*, vol. 30, no. 1, pp. 26–36, 2012.

[2] F. P. Guengerich, “Common and Uncommon Cytochrome P450 Reactions Related to Metabolism and Chemical Toxicity,” *Chem. Res. Toxicol.*, vol. 14, no. 6, pp. 611–650, 2001.

[3] L. O. Narhi and A. J. Fulco, “Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in Bacillus megaterium,” *J. Biol. Chem.*, vol. 261, no. 16, pp. 7160–7169, 1986.

[4] A. J. Warman, O. Roitel, R. Neeli, H. M. Girvan, H. E. Seward, S. A. Murray, K. J. McLean, M. G. Joyce, H. Toogood, R. A. Holt, D. Leys, N. S. Scrutton, and A. W. Munro, “Flavocytochrome P450 BM3: an update on structure and mechanism of a biotechnologically important enzyme,” *Biochem. Soc. Trans.*, vol. 33, no. 4, pp. 747–753, 2005.

[5] C. J. C. Whitehouse, S. G. Bell, and L.-L. Wong, “P450 $_{BM3}$ (CYP102A1): connecting the dots,” *Chem. Soc. Rev.*, vol. 41, no. 3, pp. 1218–1260, 2012.

[6] R. Bernhardt and V. B. Urlacher, “Cytochromes P450 as promising catalysts for biotechnological application: Chances and limitations,” *Appl. Microbiol. Biotechnol.*, vol. 98, no. 14, pp. 6185–6203, 2014.

[7] C. J. Paddon, P. J. Westfall, D. J. Pitera, K. Benjamin, K. Fisher, D. McPhee, M. D. Leavell, A. Tai, A. Main, D. Eng, D. R. Polichuk, K. H. Teoh, D. W. Reed, T. Treynor, J. Lenihan, H. Jiang, M. Fleck, S. Bajad, G. Dang, D. Dengrove, D. Diola, G. Dorin, K. W. Ellens, S. Fickes, J. Galazzo, S. P. Gaucher, T. Geistlinger, R. Henry, M. Hepp, T. Horning, T. Iqbal, L. Kizer, B. Lieu, D. Melis, N. Moss, R. Regentin, S. Secrest, H. Tsuruta, R. Vazquez, L. F.

583 Westblade, L. Xu, M. Yu, Y. Zhang, L. Zhao, J. Lievense, P. S. Covello, J. D. Keasling, K. K.
584 Reiling, N. S. Renninger, and J. D. Newman, “High-level semi-synthetic production of the
585 potent antimalarial artemisinin,” *Nature*, vol. 496, no. 7446, pp. 528–532, 2013.

586 [8] I. Kaluzna, T. Schmitges, H. Straatman, D. Van Tegelen, M. Müller, M. Schürmann, and
587 D. Mink, “Enabling Selective and Sustainable P450 Oxygenation Technology. Production
588 of 4-Hydroxy-α-isophorone on Kilogram Scale,” *Org. Process Res. Dev.*, vol. 20, no. 4,
589 pp. 814–819, 2016.

590 [9] U. Guzik, K. Hupert-kocurek, D. Wojcieszyńska “Immobilization as a Strategy for
591 Improving Enzyme Properties-Application to Oxidoreductases,” *Molecules,* vol.19*,* p.
592 8995–9018, 2014.

593 [10] W. Tischer and F. Wedekind, “Immobilized Enzymes: Methods and Applications,”
594 *Biocatal. Discov. to Appl.*, vol. 200, pp. 95–126, 1999.

595 [11] R. DiCosimo, J. McAuliffe, A. J. Poulose, and G. Bohlmann, “Industrial use of
596 immobilized enzymes,” *Chem. Soc. Rev.*, vol. 42, no. 15, p. 6437, 2013.

597 [12] J. C. S. D. Santos, O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R. C. Rodrigues, and R.
598 Fernandez-Lafuente, “Importance of the Support Properties for Immobilization or
599 Purification of Enzymes,” *ChemCatChem*, vol. 7, no. 16, pp. 2413–2432, 2015.

600 [13] S. C. Maurer, H. Schulze, R. D. Schmid, and V. Urlacher, “Immobilisation of P450BM-3
601 and an NADP(+) cofactor recycling system: Towards a technical application of heme-
602 containing monooxygenases in fine chemical synthesis,” *Adv. Synth. Catal.*, vol. 345, no.
603 6–7, pp. 802–810, 2003.

604 [14] I. Axarli, A. Prigipaki, and N. E. Labrou, “Engineering the substrate specificity of
605 cytochrome P450 CYP102A2 by directed evolution : production of an efficient enzyme

606 for bioconversion of fine chemicals,” vol. 22, pp. 81–88, 2005.

607 [15] E. Weber, D. Sirim, T. Schreiber, B. Thomas, J. Pleiss, M. Hunger, R. Gläser, and V. B.
608 Urlacher, “Immobilization of P450 BM-3 monooxygenase on mesoporous molecular
609 sieves with different pore diameters,” *J. Mol. Catal. B Enzym.*, vol. 64, no. 1–2, pp. 29–
610 37, 2010.

611 [16] L. Zhao, G. Güven, Y. Li, and U. Schwaneberg, “First steps towards a Zn/Co(III)sep-driven
612 P450 BM3 reactor,” *Appl. Microbiol. Biotechnol.*, vol. 91, no. 4, pp. 989–999, 2011.

613 [17] J. H. Lee, D. H. Nam, S. H. Lee, J. H. Park, S. J. Park, S. H. Lee, C. B. Park, and K. J. Jeong,
614 “New platform for cytochrome P450 reaction combining in situ immobilization on
615 biopolymer,” *Bioconjug. Chem.*, vol. 25, no. 12, pp. 2101–2104, 2014.

616 [18] S. Zernia, F. Ott, K. Bellmann-Sickert, R. Frank, M. Klenner, H.-G. Jahnke, A. Prager, B.
617 Abel, A. Robitzki, and A. G. Beck-Sickinger, “Peptide-Mediated Specific Immobilization
618 of Catalytically Active Cytochrome P450 BM3 Variant.,” *Bioconjug. Chem.*, vol. 27, no. 4,
619 pp. 1090–7, 2016.

620 [19] C. Y. Tan, H. Hirakawa, R. Suzuki, T. Haga, and F. Iwata, “Immobilization of a Bacterial
621 Cytochrome P450 Monooxygenase System on a Solid Support,” pp. 1–6, 2016.

622 [20] D. Holtmann, K. M. Mangold, and J. Schrader, “Entrapment of cytochrome P450 BM-3
623 in polypyrrole for electrochemically- driven biocatalysis,” *Biotechnol. Lett.*, vol. 31, no.
624 5, pp. 765–770, 2009.

625 [21] V. E. V Ferrero, L. Andolfi, G. Di Nardo, S. J. Sadeghi, A. Fantuzzi, S. Cannistraro, and G.
626 Gilardi, “Protein and electrode engineering for the covalent immobilization of P450
627 BMP on gold,” *Anal. Chem.*, vol. 80, no. 22, pp. 8438–8446, 2008.

628 [22] R. Neeli, O. Roitel, N. S. Scrutton, and A. W. Munro, “Switching pyridine nucleotide

629 specificity in P450 BM3: Mechanistic analysis of the W1046H and W1046A enzymes,” *J.*
630 *Biol. Chem.*, vol. 280, no. 18, pp. 17634–17644, 2005.

631 [23] P. C. Cirino and F. H. Arnold, “A self-sufficient peroxide-driven hydroxylation
632 biocatalyst,” *Angew. Chemie - Int. Ed.*, vol. 42, no. 28, pp. 3299–3301, 2003.

633 [24] A. Vidal-Limón, S. Águila, M. Ayala, C. V. Batista, and R. Vazquez-Duhalt, “Peroxidase
634 activity stabilization of cytochrome P450BM3 by rational analysis of intramolecular
635 electron transfer,” *J. Inorg. Biochem.*, vol. 122, pp. 18–26, 2013.

636 [25] H. K. Chenault, E. S. Simon, and G. M. Whitesides, “Cofactor regeneration for enzyme-
637 catalysed synthesis,” *Biotechnology and Genetic Engineering Reviews*, vol. 6, no. 1. pp.
638 221–270, 1988.

639 [26] F. Hollmann, K. Hofstetter, and A. Schmid, “Non-enzymatic regeneration of
640 nicotinamide and flavin cofactors for monooxygenase catalysis,” *Trends Biotechnol.*,
641 vol. 24, no. 4, pp. 163–171, 2006.

642 [27] W. Liu and P. Wang, “Cofactor regeneration for sustainable enzymatic biosynthesis,”
643 *Biotechnol. Adv.*, vol. 25, no. 4, pp. 369–384, 2007.

644 [28] M. T. Lundemo and J. M. Woodley, “Guidelines for development and implementation of
645 biocatalytic P450 processes,” *Appl. Microbiol. Biotechnol.*, vol. 99, no. 6, pp. 2465–2483,
646 2015.

647 [29] H. Schewe, B. A. Kaup, and J. Schrader, “Improvement of P450BM-3whole-cell
648 biocatalysis by integrating heterologous cofactor regeneration combining glucose
649 facilitator and dehydrogenase in E. coli,” *Appl. Microbiol. Biotechnol.*, vol. 78, no. 1, pp.
650 55–65, 2008.

651 [30] N. Beyer, J. K. Kulig, A. Bartsch, M. A. Hayes, D. B. Janssen, and M. W. Fraaije, “P450

652 BM3 fused to phosphite dehydrogenase allows phosphite-driven selective oxidations,”
653 *Appl. Microbiol. Biotechnol.*, 2016.

654 [31] Y. Lu and L. Mei, “Co-expression of P450 BM3 and glucose dehydrogenase by
655 recombinant Escherichia coli and its application in an NADPH-dependent indigo
656 production system,” *J. Ind. Microbiol. Biotechnol.*, vol. 34, no. 3, pp. 247–253, 2007.

657 [32] L. D. Smith, N. Budgen, S. J. Bungard, M. J. Danson, and D. W. Hough, “Purification and
658 characterization of glucose dehydrogenase from the thermoacidophilic
659 archaebacterium *Thermoplasma acidophilum*.,” *Biochem. J.*, vol. 261, no. 3, pp. 973–7,
660 1989.

661 [33] N. G. Munishwar, “Methods for Affinity Based Separations of Enzymes and Proteins,” p.
662 120.

663 [34] M. M. Bradford, “A rapid and sensitive method for the quantitation of microgram
664 quantities of protein utilizing the principle of protein-dye binding,” *Anal. Biochem.*, vol.
665 72, no. 1–2, pp. 248–254, 1976.

666 [35] U. K. Laemmli, “Cleavage of structural proteins during the assembly of the head of
667 bacteriophage T4,” *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

668 [36] R. Omura, Tsuneo; Sato, “The Carbon Monoxide-biding pigment of Liver Microsomes,”
669 *J. Biol. Chem.*, vol. 239, no. 7, pp. 2370–2378, 1964.

670 [37] F. P. Guengerich, M. V Martin, C. D. Sohl, and Q. Cheng, “Measurement of cytochrome
671 P450 and NADPH – cytochrome P450 reductase,” vol. 4, no. 9, pp. 1245–1252, 2009.

672 [38] A. Weckbecker and W. Hummel, “Glucose Dehydrogenase for the Regeneration of
673 NADPH and NADH,” *Microb. Enzym. Biotransformations*, vol. 17, no. 8, pp. 225–238,
674 2005.

675 [39] P. K. Srivastava and S. Singh, “Immobilization and Applications of Glucose-6-Phosphate
676 Dehydrogenase: a Review,” *Prep. Biochem. Biotechnol.*, vol. 43, no. 4, pp. 376–384,
677 2013.

678 [40] A. W. Munro, D. G. Leys, K. J. Mclean, K. R. Marshall, T. W. B. Ost, S. Daff, C. S. Miles, S.
679 K. Chapman, D. A. Lysek, C. C. Moser, C. C. Page, L. Dutton, D. G. Leys, K. R. Marshall, C.
680 S. Miles, S. K. Chapman, C. C. Page, and L. Dutton, “P450 BM3 : the very model of a
681 modern flavocytochrome,” vol. 27, no. 5, pp. 250–257, 2002.

682 [41] G. Álvaro, R. Fernández-Lafuente, R. M. Blanco, and J. M. Guisan, “Immobilization-
683 Stabilization of Penicillin G Acylase from Escherichia Coli.” Applied Microbiology and
684 Biotechnology, pp. 181–195, 1989.

685 [42] E. Katchalski-Katzir and D. M. Kraemer, “Eupergit® C, a carrier for immobilization of
686 enzymes of industrial potential,” *J. Mol. Catal. - B Enzym.*, vol. 10, no. 1–3, pp. 157–176,
687 2000.

688 [43] C. Mateo, J. M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. L??pez-Gallego, B. C. C.
689 Pessela, A. Hidalgo, G. Fern??ndez-Lorente, R. Fern??ndez-Lafuente, and J. M. Guis??n,
690 “Glyoxyl agarose: A fully inert and hydrophilic support for immobilization and high
691 stabilization of proteins,” *Enzyme Microb. Technol.*, vol. 39, no. 2, pp. 274–280, 2006.

692 [44] I. I. Karuzina and A. I. Archakov, “The oxidative inactivation of cytochrome P450 in
693 monooxygenase reactions,” *Free Radic. Biol. Med.*, vol. 16, no. 1, pp. 73–97, 1994.

694 [45] V. B. Urlacher and M. Girhard, “Cytochrome P450 monooxygenases: perspectives for
695 synthetic application,” *Trends Biotechnol.*, vol. 24, no. 7, pp. 324–330, 2006.

696 **TABLES**

Table 1. Characterization of the cell free extract containing P450 BM3 and Glucose dehydrogenase.

Enzyme	[Protein] (mg protein/mL lysate)	Specific Activity (U/mg protein)	Enzyme* (mg enz/mg prot)	P450 BM3 active form concentration† (µM)
P450 BM-3	45.4	2.82	0.524	44.9
GDH		3.63	0.146	-

*Data obtained from SDS-PAGE analysis

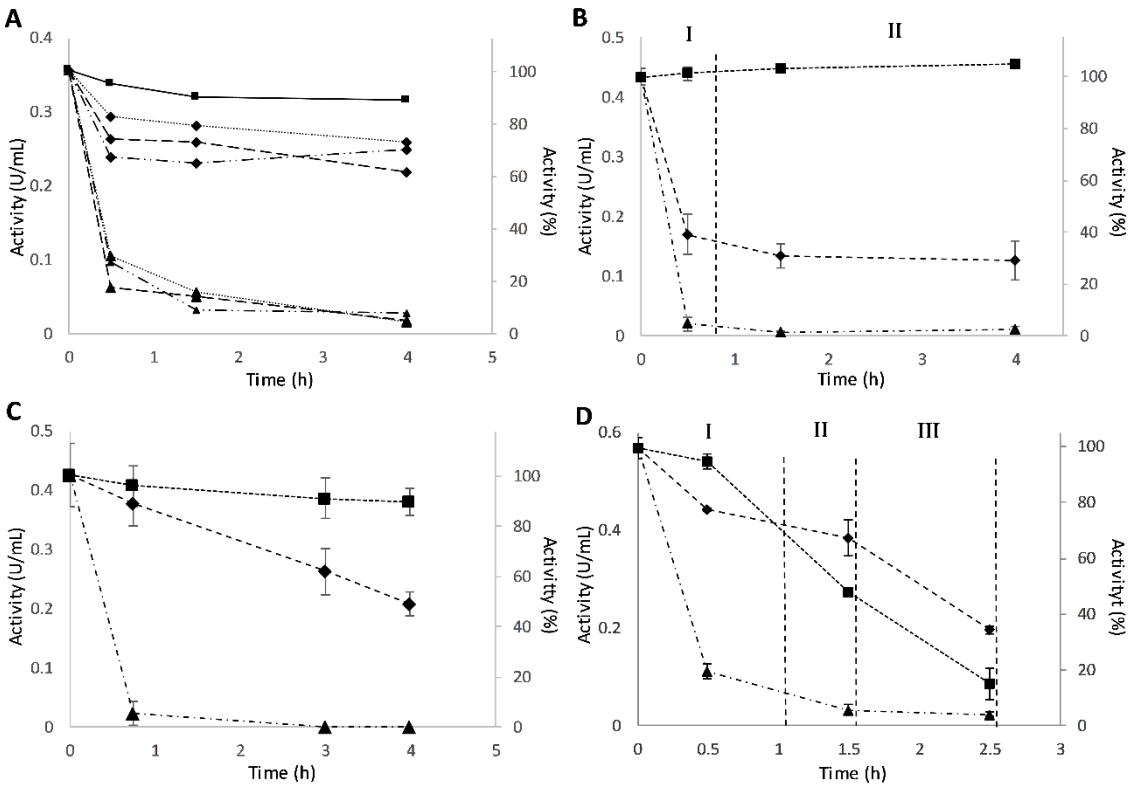
†Data obtained from CO difference spectrum assay

712 **Table 2.** Main characteristics of the matrices tested and their results regarding immobilization and retained activity of P450 BM3. The criterion chosen to

713 select a support for further studies sets a minimum for Immobilization at 90% and 25% for Retained activity.

Code	Functional group (Linker)	Matrix	Interaction	Pore diameter (Å)	Particle size (µm)	Immobilization Yield (%)	Retained acitivty (%)
ECR8204F	Epoxy	Methacrylate	Covalent	300-600	150-300	92	0
ECR8215F	Epoxy	Methacrylate	Covalent	1200-1800	150-300	99	2
ECR8309F	Amino (C2)	Methacrylate	Ionic/Covalent	600-1200	150-300	95	3
ECR8315F	Amino (C2)	Methacrylate	Ionic/Covalent	1200-1800	150-300	97	4
ECR8409F	Amino (C6)	Methacrylate	Ionic/Covalent	600-1200	150-300	100	6
ECR8415F	Amino (C6)	Methacrylate	Ionic/Covalent	1200-1800	150-300	98	18
ECR8285	Epoxy (C4)	Methacrylate	Ionic/Covalent	400-600	300-710	100	3
ECR8806F	None (C18)	Methacrylate	Hydrophobic	500-700	150-300	93	0
ECR1061M	None	Styrene/Methacrylic	Hydrophobic	600-750	300-710	21	0
ECR1030M	None	Styrene/Methacrylic	Hydrophobic	200-300	300-710	33	7
Praesto 45	Epoxy	Agarose	Covalent	ND	45	92	63
Praesto 65	Epoxy	Agarose	Covalent	ND	65	95	68
Praesto 90	Epoxy	Agarose	Covalent	ND	90	95	60

714 **FIGURES**



715

716 **Figure 1.** P450 BM3 immobilization onto: A) Praesto 45 [discontinued line (-----)], 65 [dotted
717 line (·····)] and 90 [combined dotted and discontinued line (-···-···)] [Sodium Phosphate Buffer
718 1M pH 8] B) Epoxy-agarose-Sigma [Sodium Phosphate Buffer 1M pH 8]; (I) the enzyme is
719 bound to the support (II) β-mercaptoethanol 0.2M final concentration is added to eliminate
720 the unreacted epoxy groups, C) Epoxy-agarose-UAB [Sodium Phosphate Buffer 1M pH 8] and
721 D) AMINO-agarose [Sodium phosphate buffer 0,05M pH 6]; (I) adsorption of the enzyme to the
722 support (II) incubation with certain amount of carbodiimide (EDC) (III) incubation with 0.5M
723 NaCl and desorption of the unattached protein. The activity offered always ranged from 3.5 to
724 6 UA/g of resin. The activities of a blank (squares), the supernatant (rhombus) and the
725 suspension (triangle) were continuously analysed. Error bars correspond to standard deviation
726 (n=2).

727

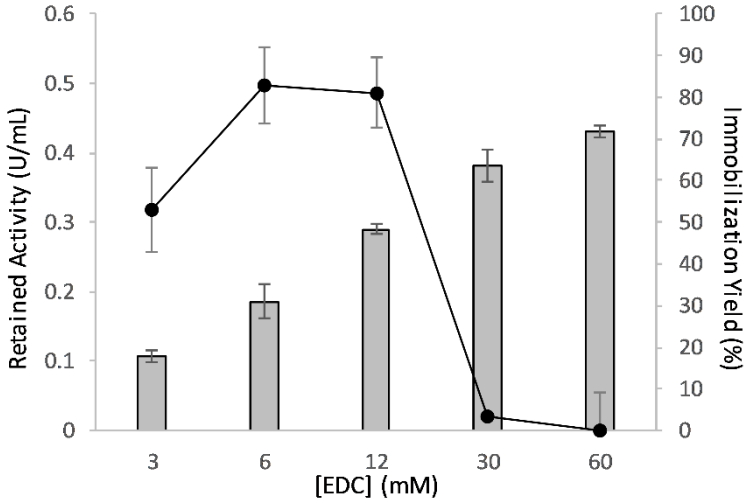


Figure 2. Optimization of the EDC concentration in AMINO-agarose experiments using high loads. The immobilization was carried out using sodium phosphate buffer 50mM pH 6 and offering 92UA/g of resin. Retained activity (black dots) and immobilization yield (grey bars) are represented. Error bars correspond to standard deviation (n=2).

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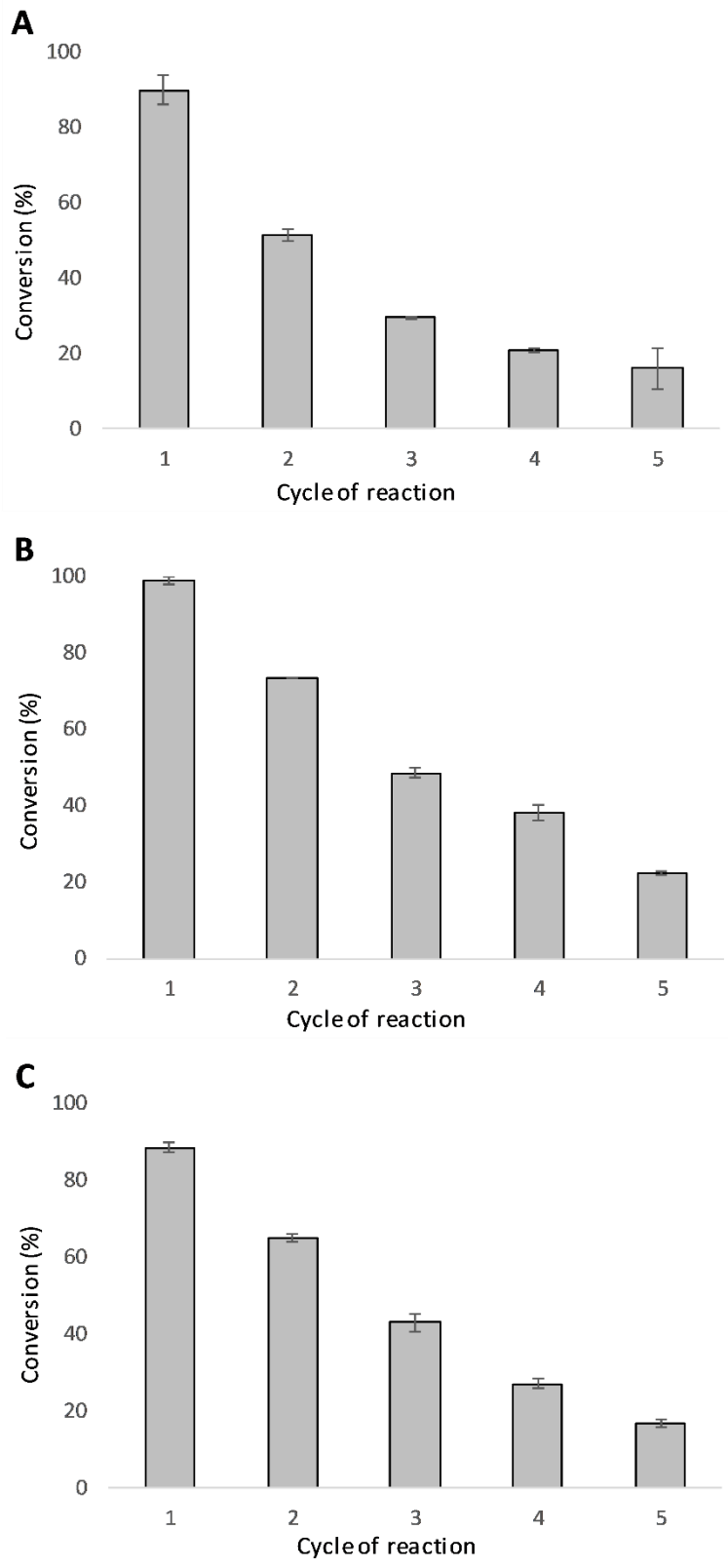

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745 **Figure 3.** Reaction cycles using both P450 BM3 and GDH immobilized onto: A) Epoxy-agarose-
746 UAB (10mg of agarose/mL), B) AMINO-agarose (10mg of agarose/mL) and C) Lentikats® (50mg
747 of Lentikats/mL). Reactions were carried on in 5mL scale adding 0.18-0.2UA of total activity
748 and using sodium phosphate buffer 50mM pH 7.5, 1.3mM sodium laurate, 0.2mM $NADP^+$,
749 12.5mM D-glucose, temperature control at 25°C and constant 1000rpm agitation using a
750 Multi-Therm H5000-HC-E thermos shaker. Error bars correspond to standard deviation (n=2)