catalysts

MDPI

*Article*

# Agarose vs. Methacrylate as Material Supports for Enzyme Immobilization and Continuous Processing

**Ana I. Benítez-Mateos [1] and Martina L. Contente [2,3,*]**

[1] Department of Chemistry, Biochemistry and Pharmaceutical Sciences, University of Bern, Freiestrasse 3, 3012 Bern, Switzerland; ana.benitez@dcb.unibe.ch
[2] School of Chemistry, University of Nottingham, University Park, Nottingham NG7 2RD, UK
[3] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Via Celoria 2, 20133 Milan, Italy
* Correspondence: martina.contente@unimi.it

**Abstract:** Enzyme immobilization has become a key strategy to improve the stability and recycling of biocatalysts, resulting in greener and more cost-efficient processes. The design of the immobilized catalysts is often focused only on the immobilization strategy, the binding chemistry between the enzyme and the support, while less attention has been paid to the physico-chemical properties of material supports. Selecting the best carrier for a specific application may greatly influence the performance of the biocatalytic reaction. Herein, we present a comparative study between the two most used material supports for protein immobilization, agarose and methacrylate. Hydrophilic agarose microbeads ensure higher retained enzymatic activity and better catalyst performance when hydrophobic compounds are involved in the biotransformation. Due to the high stickiness, lipophilic molecules represent a major limitation for methacrylate carriers. $O_2$-dependent reactions, in contrast, must be carried out by immobilized enzymes on methacrylate supports due to the low mechanical stability of agarose under dehydration conditions. All these parameters were tested with a special focus on continuous-flow applications.

**Keywords:** enzyme immobilization; flow biocatalysis; agarose beads; methacrylate resins



**Citation:** Benítez-Mateos, A.I.; Contente, M.L. Agarose vs. Methacrylate as Material Supports for Enzyme Immobilization and Continuous Processing. *Catalysts* **2021**, *11*, 814. https://doi.org/10.3390/catal11070814

Academic Editor: Aniello Costantini

Received: 27 May 2021
Accepted: 1 July 2021
Published: 2 July 2021



## 1. Introduction

Forty years ago, protein immobilization emerged as a technique to both increase the stability of enzymes and allow for their reuse and recycling. Since then, the incorporation of enzymes into solid matrices has been applied for different purposes: several types of biomaterials have been assembled by using proteins and different carriers in the field of light energy conversion (e.g., photovoltaics), biosensing (e.g., detection of pesticides or heavy metals), integrated optical devices (e.g., optical switches, micro-imaging systems, telecommunications technologies) as well as chemical reactions [1].

In particular, efficient immobilization strategies have allowed for the development of heterogeneous biocatalysts with retained activity for longer periods, their easy separation from the reaction media once the reaction is completed, and even incorporation into continuous-flow reactors for process intensification [2–4].

These advances have contributed to the integration of enzymes in chemical manufacturing processes, operating under harsh reaction conditions that are often very far from their native environments [5–8].

The development of a "perfect biocatalyst" requires the combination of different areas of expertise such as molecular biology, protein engineering, material science, biophysics, biocatalysis and chemical engineering. From the material perspective, enzymes can be attached to solid supports, entrapped into gels or encapsulated in vesicles [4,9]. Enzyme immobilization can also be performed by crosslinking the protein molecules, creating aggregates (CLEAs; crosslinked enzyme aggregates) that do not require the presence of any

support. However, their use in industrial processes is not widely adopted due to the lower activity and stability of CLEAs compared to material-based immobilization strategies [10].

Among the immobilization strategies involving a material, the attachment of enzymes to a prefabricated support is noteworthy for the easy preparation of the immobilized biocatalyst [8]. Different methodologies involving both physical and chemical approaches have been reported. Among the chemical ones, particular attention has been paid to a covalent bond between the matrix and the catalyst, especially to increase the enzyme operational stability, the easy incorporation in flow chemistry reactors and to avoid catalyst leaching when high flow rates are necessary for fast reactions [11]. Moreover, the prefabricated supports are frequently commercially available in a ready-to-use format. Typically, different material supports (agarose, methacrylate, silica, zeolites, chitosan, etc.) with various particle sizes, porosity and functional groups are tested for the immobilization of an enzyme of interest [4]. However, most of the process optimization for enzyme immobilization has been carried out by trial-and-error approaches to date, while rational immobilization procedures or predictive tools are still scarcely applied.

In this work, we present a comparative study between the most widely used prefabricated material supports for purified enzyme immobilization, agarose and methacrylate microbeads. The advantages and drawbacks of each material have been analyzed and discussed, based also on previous works. The key parameters for the assessment of immobilized biocatalysts, such as retained enzyme activity after immobilization, stability in the presence of co-solvents, stability against temperature, cross-reactivity with substrates/products, and performance in packed-bed flow reactors (PBRs), are the criteria used to evaluate each material support.

## 2. Results and Discussion

### *2.1. Comparison of Enzyme Activity upon Immobilization*

The retained activity of 11 enzymes after immobilization on either agarose or methacrylate supports is compared in Table 1, also including recently reported studies. Noteworthy, the immobilization procedures were carried out using the same binding chemistry on both carriers for the generation of a covalent bond between the biocatalysts and the support. Gs-Lys6DH (lysine-6-dehydrogenase from *Geobacilus stearothermophilus*), He-P5C (pyrrolidine-5-carboxylate reductase from *Halomonas elongata*), HeWT (*S*-selective ω-transaminase from *Halomonas elongata*), He-AlaDH (alanine dehydrogenase from *Halomonas elongata*), and Ts-RTA (*R*-selective transaminase from *Thermomyces stellatus*) were immobilized, employing the epoxy/$Co^{2+}$-strategy, while HRP (horseradish peroxidase), Tt-NOX (NADPH-oxidase from *Thermus thermophilus*), MsAcT (acyl transfersase from *Mycobacterium smegmatis*), HOR (β-glycosidase from *Halothermothrix orenii*), GalOx (galactose oxidase from *Dactylium dendroides*) and KRED1-Pglu (NADPH-dependent benzyl reductase from *Pichia glucozyme*) were immobilized on glyoxyl supports. Independently on the enzyme and the binding protocol, the biocatalysts immobilized on agarose showed a higher retained activity in all the cases (Table 1). Whereas the particle size (50–150 μm for agarose beads; 100–300 μm for methacrylate beads) and the porosity (10–200 nm) of both materials are similar, the chemical nature differs. Agarose is made of sugars and is therefore more hydrophilic compared to methacrylate, which is formed by acrylic polymers. These different physicochemical properties are plausibly the reason why immobilized enzymes on agarose show an increased retained activity [12,13]. During the enzyme immobilization on methacrylate supports, the biocatalysts are first attracted to the carrier by hydrophobic interactions, which can provoke unfavorable rearrangements of the protein structure. Moreover, the hydrophilic nature of agarose may enhance the intraparticle mass transport of substrates and products, improving the overall performance of the immobilized enzyme [12,14].

**Table 1.** Enzyme recovered activities after immobilization.

| Enzyme | Material Support | Recovered Activity [1] (%) | Results Source |
|---|---|---|---|
| Gs-Lys6DH | Agarose<br>Methacrylate | 91<br>63 | Ref. [15] |
| He-P5C | Agarose<br>Methacrylate | 10<br><5 | Ref. [15] |
| HeWT | Agarose<br>Methacrylate | 60<br>30 | This work<br>Ref. [16] |
| He-AlaDH | Agarose<br>Methacrylate | 42<br>19 | Ref. [17] |
| TsRTA | Agarose<br>Methacrylate | 95<br>67 | This work |
| HRP | Agarose<br>Methacrylate | 48<br>27 | This work |
| Tt-NOX | Agarose<br>Methacrylate | 55<br>24 | Ref. [12] |
| MsAcT | Agarose<br>Methacrylate | 78<br>35 | This work |
| HOR | Agarose<br>Methacrylate | 62<br>30 | This work |
| GalOx | Agarose<br>Methacrylate | 100<br>98 | This work |
| KRED1-Pglu | Agarose<br>Methacrylate | 35<br>16 | This work |

[1] Recovered activity = (specific activity of immobilized enzyme (U/mg)/specific activity of free enzyme (U/mg)) × 100. The protein loading was the same for the enzyme regardless of the material support: Gs-Lys6DH (2 mg/g), He-P5C (2 mg/g), HeWT (5 mg/g), He-AlaDH (0.5 mg/g), Ts-RTA (5 mg/g), HRP (1 mg/g), Tt-NOX (1 mg/g), MsAcT (1 mg/g), HOR (1 mg/g), GalOx (0.1 mg/g), KRED1-Pglu (10 mg/g).

Noteworthy, a tendency was observed between the recovered activities after immobilization on agarose and methacrylate. Enzymes immobilized on agarose supports were on average 2-fold more active than when immobilized on methacrylate materials. Such a difference should be taken into consideration when high enzyme activities are required for the biocatalyst application. As an exception, GalOx showed similar recovered activities regardless of the material support. Due to the low protein loading (0.1 $mg/g_{support}$) and the high activity of its free counterpart (500–1500 $U/mg_{enzyme}$ as reported by the supplier), the difference in the chemical nature of the material may not have an influence at such a scale [12].

In addition, the cost of these commercial supports should be considered for the development of efficient and cost-effective immobilized biocatalysts. For example, the price of methacrylate materials is between 250 and 1800 EUR/Kg, while the cost of agarose microbeads is around 1000–2000 EUR/Kg.

### *2.2. Stablity of the Immobilized Enzymes in Non-Natural Conditions*

In order to incorporate enzymes into industrial processes, the biocatalyst preparation has to be resistant to harsh conditions, which typically include high temperatures, the presence of co-solvents to enhance the substrate solubility in aqueous media, among others. Enzyme immobilization has proven to be an excellent strategy to increase the protein stability under such conditions [8,18,19].

Temperature is a key parameter for any bioprocess, but is especially relevant for biocatalytic reactions, as enzymes are complex catalysts whose industrial potential relies on their operational stability. Many studies have been performed to decrease the temperature-induced inhibition of enzymes by immobilization, thus comparing the immobilized catalyst

with its free counterpart [19]. In terms of binding chemistry between the enzyme and the support, a multipoint covalent attachment is the best strategy to promote the stability of the biocatalyst at moderately high temperatures due to the induced rigidification of the protein structure [20]. Herein, we have tested the stability at 30 °C, 37 °C and 45 °C of the immobilized transaminase (imm-HeWT) either on agarose (HeWT-agarose) or a methacrylate support (HeWT-methacrylate) to decipher the effect of the material on the thermal stability of the immobilized enzyme. As reported in the thermal inactivation curves (Figure 1A), no significant difference has been observed between the half-life of HeWT-agarose and HeWT-methacrylate even at the highest tested temperature (45 °C). These results indicate that the thermal stability is an intrinsic parameter, directly related to the enzyme and the binding chemistry rather than the material support.

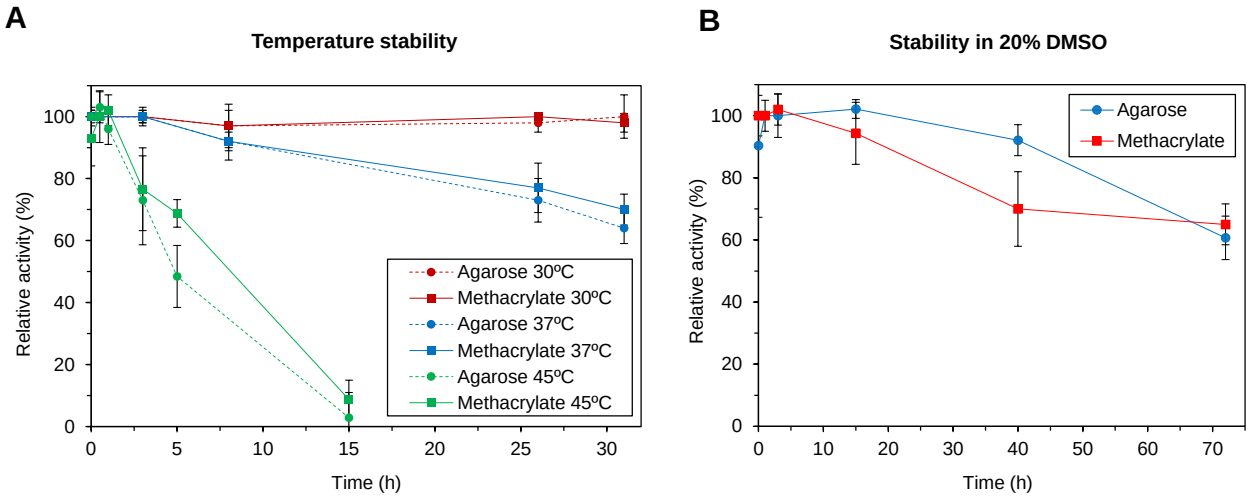


**Figure 1.** Stability of imm-HeWT at (**A**) different temperatures and (**B**) 20% $v/v$ DMSO. The relative activity (%) was calculated by comparison with the immobilized activity (U/g) at the starting point. The 100% activity of the transaminase immobilized on agarose corresponds to 5.7 U/g, while the one immobilized on methacrylate was 3.0 U/g. The comparison of the stability of the imm-HeWT and the free HeWT in 20% DMSO was previously reported [16].

Dimethyl sulfoxide (DMSO) is one of the most commonly used organic co-solvents in water-based biocatalytic reactions. Studies concerning the imm-HeWT in the presence of different concentrations (10–20% $v/v$) of various water-miscible solvents (DMSO, ethanol, 1-propanol, 2-propanol, acetonitrile, and methanol) have demonstrated a better enzymatic stability and performance using DMSO [16], making it the selected co-solvent in all the reported experiments [15–17,21–25]. For this reason, the potential effect of the two matrices on the imm-HeWT stability in the presence of 20% DMSO was compared (Figure 1B). The differences in the residual activity between the biocatalyst immobilized either on agarose or methacrylate were barely noticeable over a period of 70 h, although HeWT-agarose maintained more than 90% of its initial activity during the first 40 h. In a previous study, a higher co-solvent resistance of imm-HeWT versus the free biocatalyst has already been demonstrated [16].

As reported for the temperature effects, the solvent resistance also relies mainly on the enzymatic architecture and the binding chemistry rather than the carrier. Remarkably, the residual activity of HeWT-agarose after 72 h in 20% $v/v$ DMSO was 3.5 U/g, while HeWT-methacrylate preserved 2 U/g. These differences can be the consequence of a higher initial recovered activity of the enzyme immobilized on agarose support.

### *2.3. Stickiness of the Susbtrate/Product to the More Hydrophobic Support*

Methacrylate microbeads are more hydrophobic when compared to agarose. This physico-chemical difference may affect the inertness of the carrier, especially if in close contact with apolar substrates/products. Indeed, we have experienced strong hydrophobic

interactions between aromatic compounds and the methacrylate support, even in flow conditions where no substrate/product accumulation phenomena should be observed due to the optimization of the residence time [23]. In this experiment, HeWT-agarose or HeWT-methacrylate was integrated in a packed-bed reactor (PBR) to perform a model deamination reaction by using pyruvate and *S*-methylbenzylamine (*S*-MBA) as starting materials. The reactor was operated as previously described [16]. The consumption of *S*-MBA as well as the formation of acetophenone were monitored by HPLC. In both the bioreactors, full conversion of the substrate was achieved, since no *S*-MBA was detected in the flow-through. However, less than 50% of the product (acetophenone) was collected in the case of HeWT-methacrylate after 10 column volumes, while 100% of product formation was detected when operating with HeWT-agarose (Figure 2).

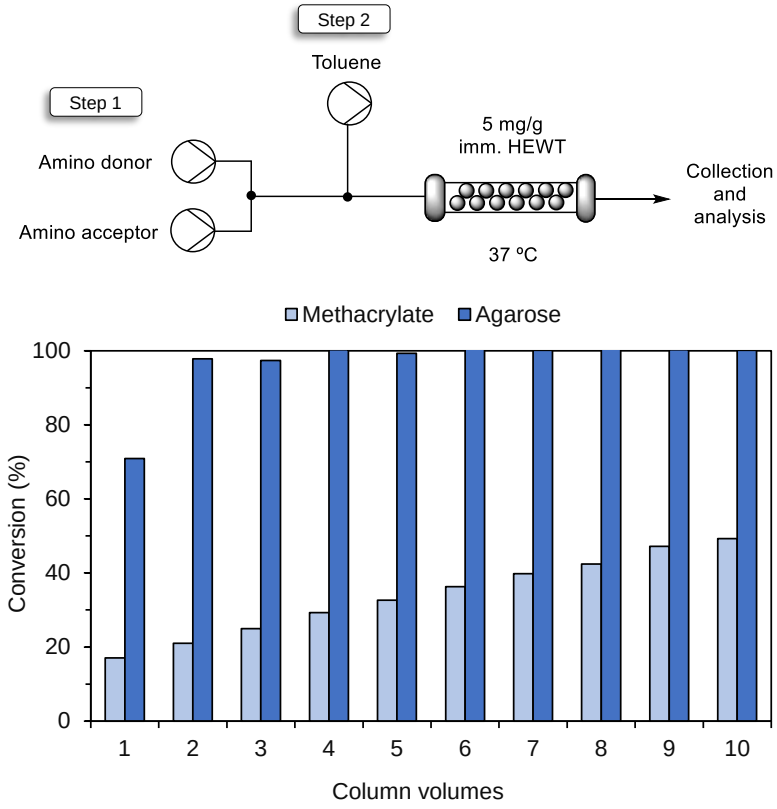


**Figure 2.** Continuous-flow amination by immobilized HeWT on agarose/methacrylate supports. At the top, a scheme of the flow system setup. Step 1: biotransformation reaction; step 2: desorption with toluene. At the bottom, the operational performance of the PBRs. The substrate solution contained 5 mM *S*-MBA, 10 mM pyruvate and 0.1 mM PLP. Flow rate: 1.2 mL/min. Residence time: 1 min.

In order to understand whether acetophenone was stuck to the methacrylate support by hydrophobic interactions, an inlet with an apolar solvent (toluene) was introduced before the reactor to recover the product [22]. After running toluene for 20 min, 67% of the previously stuck acetophenone was recovered (Table 2). However, a considerable part of the product generated by the bioreactor remained trapped in the methacrylate support. The same procedure was applied to the bioreactor with HeWT-agarose to completely remove the traces of acetophenone. These results are of great importance because the conversion of the biocatalyst could be underestimated due to the stickiness of apolar compounds to methacrylate supports. Moreover, it needs to be considered that the use of hydrophobic carriers associated with hydrophobic starting materials could increase time and costs while also reducing the greenness of the process.

**Table 2.** In-flow recovery of acetophenone from the PBR.

| | Material Support | Product | |
|---|---|---|---|
| | | % | mg |
| Trapped on support [1] | Agarose | 4 | 0.2 |
| | Methacrylate | 66 | 4.7 |
| Released with toluene [2] | Agarose | 100 | 0.2 |
| | Methacrylate | 67 | 3.2 |

[1] The acetophenone trapped into the support was calculated over 10 column volumes considering full conversion (59.8 mM acetophenone). [2] The acetophenone released by running toluene was calculated considering the product previously trapped as the 100%.

### *2.4. Integration of $O_2$-Dependent Reactions into Flow Reactors*

The oxygen supply, essential for the oxidation reaction to take place, was ensured by a segmented air–liquid flow stream formed before the column containing the immobilized GalOx (Figure 3). Air was delivered at 20 psi; its flow was measured as previously described [26]. To ensure a constant flow, a BRP (40 psi) was applied before the air tank. An aqueous solution of galactose (50 mM) was pumped, joining the airflow at the T-junction, before entering the column in which the oxidation reaction occurs in approximately 10 min. A BPR (5 psi) ensured a constant and controlled flow of aqueous phase leaving the column. The aqueous stream was collected and the unreacted galactose was measured using a commercially available kit (R-biopharm). Although no difference in the reaction performance could be observed in terms of molar conversion at the beginning of the process (80% m.c. methacrylate support; 76% m.c. agarose carrier), a better operational stability was obtained using the methacrylate material over time. After 48 h of continuous processing, an increase in pressure in addition to dehydration problems was observed in the case of the agarose GalOx, giving rise to lower productivity (60% m.c. after 48 h) and lower enzymatic efficiency. Constant delivery of the desired aldehyde was observed for the methacrylate GalOx (78% mc after 48 h).

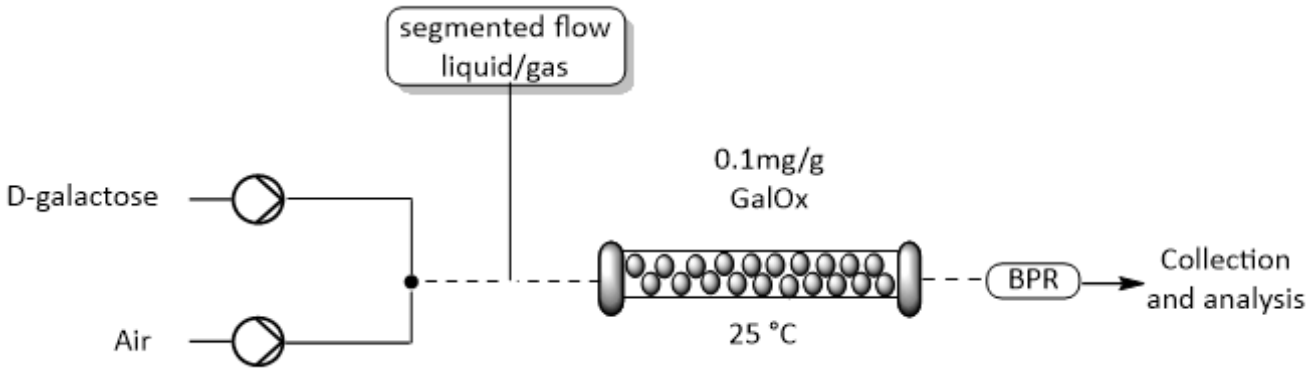


**Figure 3.** Continuous-flow scheme of the D-galactose oxidation through immobilized GalOx on either agarose or methacrylate supports.

## 3. Materials and Methods

### *3.1. Materials*

Galactose oxidase from *D. dendroides* (GalOx), peroxidase from horseradish (HRP), AzBTS-$(NH_4)_2$, *S*-Methylbenzylamine, *R*-Methylbenzylamine, pyruvate, pyridoxal-5′-phosphate, D-galactose, *p*-nitrophenyl acetate, *p*-nitrophenyl-β-D-glucopyranoside, Benzil, NADPH were purchased from Sigma Aldrich Gillingham, U.K. Plain agarose (6BCL) was purchased from Agarose Bead Technologies (ABT), Madrid, Spain. The methacrylate supports were donated by Resindion S.R.L. and Purolite®. All other reagents were of analytical grade.

### *3.2. Protein Expression and Purification*

Protein expression and purification were carried out as previously described: MsAcT [27]; HOR [28]; TsRTA [29]; HeWT [30]; KRED1-Pglu [22]. HeWT, TsRTA, MsAcT as pure proteins were stored at 4 °C, HOR at room temperature and KRED1-Pglu at −20 °C. Protein quantification was performed by Bradford assay [13].

### *3.3. Enzymatic Activity Assays*

Activity assays were carried out in 96-well plates for both the free and immobilized enzymes.

An adapted version of previous protocols [29,30] was used for TsRTA and HeWT. Briefly, 200 µL of a reaction mixture containing 2.5 mM *R*- or *S*-MBA, 2.5 mM pyruvate and in 50 mM phosphate buffer at pH 8.0 with 2.5% DMSO was prepared. The reaction was triggered by adding 5 µL of the free enzyme (0.5 mg/mL) or immobilized one (suspension of 1:5 *w*/*v*). The increase in UV absorbance was monitored at 245 nm for 5 min at 30 °C. Free TsRTA specific activity 2.6 U/mg, free HeWT 1.9 U/mg.

HRP activity: 200 µL of a reaction mixture containing 2.5 mM D-galactose, 2.5 mM AZBTS, 0.02 mg/mL GalOx in 50 mM phosphate buffer at pH 7.5 was prepared. The reaction was triggered by adding 5 µL of free HRP or immobilized HRP (suspension of 1:5 *w*/*v*). The increase in absorbance was monitored at 420 nm for 10 min at 25 °C. Free enzyme specific activity: 150 U/mg.

An adapted version of previous protocols [27,28] was used for MsAcT and HOR. Briefly, 200 µL of a reaction mixture containing 0.56 mM *para*-nitrophenyl acetate, 0.1% *v*/*v* EtOH, or 10 mM *para*-nitrophenyl-β-D-glucopyranoside (*p*NPG) in 100 mM phosphate buffer at pH 8.0 or 50 mM HEPES buffer at pH 7.4 was prepared. The reaction was triggered by adding 2 µL of free MsAcT dil 1:100 (0.0005 mg/mL) or immobilized (suspension of 1:10 *w*/*v*) or 2 µL of free HOR dil 1:10 (0.003 mg/mL) or immobilized suspension (1:5 *w*/*v*). The increase in absorbance was monitored at 400 nm for 5 min at 25 °C. Free MsAcT specific activity: 180 U/mg, free HOR: 7 U/mg

GalOx activity: 200 µL of a reaction mixture containing 2.5 mM D-galactose, 2.5 mM AZBTS, 0.0025 mg/mL HRP in 50 mM phosphate buffer at pH 7.5 was prepared. The reaction was triggered by adding 5 µL of free GalOx or immobilized GalOx (suspension of 1:5 *w*/*v*). The increase in absorbance was monitored at 420 nm for 10 min at 25 °C. Free enzyme specific activity: 700 U/mg.

KRED1-Pglu activity: An adapted version of a previous protocol was followed [22]. A total of 200 µL of a reaction mixture containing 0.25 mM NADPH, 0.5 mM Benzil, 0.1% *v*/*v* DMSO, 0.7 mg/mL KRED1-Pglu in 50 mM Tris-HCl buffer at pH 8.0 was prepared. The reaction was triggered by adding 100 µL of free or immobilized KRED1-Pglu (suspension of 1:5 *w*/*v*). The decrease in absorbance was monitored at 340 nm for 5 min at 25 °C. Free enzyme specific activity: 20 mU/mg.

### *3.4. Enzyme Immobilization on Epoxy/$Co^{2+}$-Supports*

Epoxy-agarose microbeads were prepared as previously described [31]. Epoxy-methacrylate (EP403/S or ECR8204F) was commercially available. The addition of $Co^{2+}$-chelates to either epoxy-agarose or epoxy-methacrylate was carried out by incubating 1 g of epoxy-support with 2 mL of modification buffer (0.1 M sodium borate, 2 M iminodiacetic acid pH 8.0) for 2 h. After filtration and washing steps, the resin was incubated with 5 mL of Metal Buffer (30 mg/mL of $CoCl_2$). After filtration and washing steps, 5 mL of (6×)Histag-enzyme was added to the resin and the suspension was incubated for 6 h under shaking. The immobilized enzyme was washed with 3 mL of desorption buffer (50 mM EDTA, 0.5 M NaCl in 50 mM phosphate buffer, pH 7.2). Finally, the remaining epoxy groups were blocked by incubation with 4 mL of 3 M glycine pH 8.5 overnight and afterwards the immobilized enzyme was washed with the appropriate buffer for its storage.

### 3.5. Enzyme Immobilization on Glyoxyl Supports

Epoxy-agarose microbeads and epoxy-methacrylate (EP403/S or ECR8204F) were activated with glyoxyl groups following a modified version of a previous protocol [32]. Briefly, 1 g of either epoxy-agarose or epoxy-methacrylate was incubated with 10 mL of 100 mM $H_2SO_4$ overnight under orbital shaking. Then, the support was filtered and washed 10 times with 10 volumes of $H_2O$. The resulting glyceryl support was oxidized with 10 mL of 30 mM $NaIO_4$ for 2 h under orbital shaking. Finally, the glyoxyl support was washed 10 times with $H_2O$ and stored at 4 °C until use.

For the enzyme immobilization, 10 mL of enzyme at the desired concentration in 100 mM sodium bicarbonate buffer at pH 10.0 was mixed with 1 g of glyoxyl support and incubated under orbital shaking for 3 h. Then, the suspension was filtered and the immobilization yield (%) was calculated by measuring the remaining activity in the flow-through. The immobilized enzyme was incubated with 20 mM of piperidine–borane to reduce the imine bonds between the enzyme and the aldehyde groups on the support. The immobilized enzyme was washed with the appropriate buffer for its storage.

### 3.6. Enzyme Stability Test in DMSO

A total of 0.9 mL of 50 mM phosphate buffer at pH 8.0 containing 20% $v/v$ DMSO was added to 0.1 g of immobilized HeWT (5 mg/g) on either agarose or methacrylate. The suspension was incubated at 25 °C under shaking for 72 h. Samples were withdrawn at different time points and the enzyme activity was measured as described in Section 3.3.

### 3.7. Enzyme Stability Test at 45 °C

A total of 0.9 mL of 50 mM phosphate buffer at pH 8.0 was added to 0.1 g of immobilized HeWT (5 mg/g) on either agarose or methacrylate. The suspension was incubated in a water bath at 45 °C with magnetic stirring for 15 h. Samples were withdrawn at different time points and the enzyme activity was measured as described in Section 3.3.

### 3.8. Continuous Flow Reactions

Flow biotransformations were performed using a R2S/R4 Vapourtec flow reactor equipped with a V3 pump and an Omnifit glass column (6.6 mm i.d. × 100 mm length) filled with the immobilized enzyme (1 g) as a packed-bed reactor (PBR). A first equilibration step was performed by running buffer at 1 mL/min for 10 min (50 mM phosphate buffer pH 8.0 for HeWT, pH 7.5 for GalOx). Subsequently, solutions of substrates/cofactors at different concentrations depending on the enzyme were mixed in a T-tube and pumped through the PBR containing the immobilized biocatalyst (5 mM *S*-MBA, 10 mM pyruvate, and 0.1 mM PLP in 50 mM phosphate buffer pH 8.0 with 2.5% $v/v$ DMSO for HeWT, 50 mM D-galactose for GalOx phosphate buffer pH 7.5). In the case of GalOx, a segmented flow air/liquid was formed to provide the $O_2$ necessary for the oxidation reaction. Samples were collected after each column volume and analyzed by HPLC or D-galactose commercial kit by R-biopharm, specific for the detection of D-galactose in complex matrix.

For the recovery of the product stuck to the support, a first washing step was performed by running buffer at 1 mL/min for 10 min. Then, toluene was flushed at 0.59 mL/min for 20 min. Samples were collected after each column volume and analyzed by HPLC.

### 3.9. HPLC Analysis

The products were analyzed by HPLC equipped with a C18 column. The samples were diluted in a solution of 1:1 ($v/v$) 0.1% HCl and acetonitrile. *S*-MBA (Rt. 4.6 min) and acetophenone (Rt. 6 min) were detected using a UV detector at 250 nm after a gradient method 5:95 to 95:5 ($H_2O$:MeCN 0.1%TFA) over 4 min with a flow rate of 0.8 mL/min at 45 °C. Standards of acetophenone were also analyzed for the calibration curve.

## 4. Conclusions

In this study, a comparison between agarose and methacrylate for enzyme immobilization has been carried out. Using the same binding chemistry and enzymatic loading for each enzyme on both of the carriers, we elucidated the role of the material support on typical biotransformations with a special focus on flow processing. Eleven different biocatalysts have been considered, demonstrating that the physico-chemical properties of the matrix may influence the performance of the flow-reaction. While the hydrophilic agarose ensures a 2-fold more active catalyst than the methacrylate counterpart, and a better performance when apolar aromatic compounds are involved in the reaction, more lipophilic methacrylate supports present greater mechanical stability, especially under dehydration conditions. No influence of the carrier properties has been observed in the enzymatic resistance to the reaction parameters, such as temperature and co-solvents, as they are intrinsic characteristics more related to the protein structure. Focusing on the final application, this report can be considered a "first-step-guide" for a more rational selection of the two most employed matrices for protein immobilization, allowing for a fast and cost-efficient optimization of the process parameters (Figure 4).

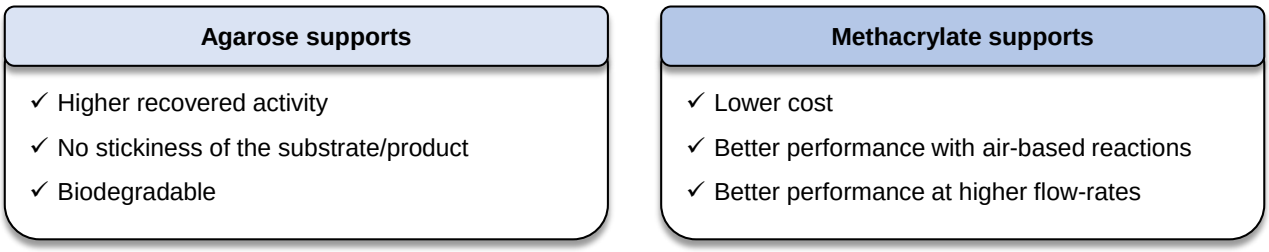


**Figure 4.** Summary of the main advantages of agarose and methacrylate material supports.

**Author Contributions:** Conceptualization, A.I.B.-M. and M.L.C.; methodology, formal analysis, investigation, data curation, and resources, A.I.B.-M. and M.L.C.; writing—original draft preparation, A.I.B.-M.; writing—review and editing, M.L.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the University of Bern "Seal of Excellence Fund", grant number SELF19-03 BIORPHANDRUG (A.I.B.-M); the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant number 792804 AROMAs-FLOW (M.L.C.).

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The authors are grateful to Francesca Paradisi for her support and the insightful discussions. We wish to thank Resindion S.R.L. and Purolite® for donating the methacrylate resins..

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Nagy, L.; Magyar, M.; Szabo, T.; Hajdu, K.; Giotta, L.; Dorogi, M.; Milano, F. Photosynthetic machineries in nano-systems. *Curr. Protein Pept. Sci.* **2014**, *15*, 363–373. [CrossRef] [PubMed]
2. Guisan, J.M.; López-Gallego, F.; Bolivar, J.M.; Rocha-Martín, J.; Fernandez-Lorente, G. The Science of Enzyme Immobilization. In *Methods in Molecular Biology*; Humana Press Inc.: New York, NY, USA, 2020; Volume 2100, pp. 1–26.
3. Bommarius, A.S.; Paye, M.F. Stabilizing biocatalysts. *Chem. Soc. Rev.* **2013**, *42*, 6534–6565. [CrossRef]
4. Sheldon, R.A.; van Pelt, S. Enzyme immobilisation in biocatalysis: Why, what and how. *Chem. Soc. Rev.* **2013**, *42*, 6223–6235. [CrossRef]
5. Sheldon, R.A.; Woodley, J.M. Role of Biocatalysis in Sustainable Chemistry. *Chem. Rev.* **2018**, *118*, 801–838. [CrossRef]
6. Chapman, J.; Ismail, E.A.; Dinu, Z.C. Industrial Applications of Enzymes: Recent Advances, Techniques, and Outlooks. *Catalysts* **2018**, *8*, 238. [CrossRef]
7. Winkler, C.K.; Schrittwieser, J.H.; Kroutil, W. Power of Biocatalysis for Organic Synthesis. *ACS Cent. Sci.* **2021**, *7*, 55–71. [CrossRef] [PubMed]
8. Basso, A.; Serban, S. Industrial applications of immobilized enzymes—A review. *Mol. Catal.* **2019**, *479*, 110607. [CrossRef]

9. Guisán, J.M.; Bolivar, J.M.; López-Gallego, F.; Rocha-Martín, J. *Immobilization of Enzymes and Cells*; Humana Press Inc.: New York, NY, USA, 2020; Volume 2100.
10. Cao, L.; van Langen, L.; Sheldon, R.A. Immobilised enzymes: Carrier-bound or carrier-free? *Curr. Opin. Biotechnol.* **2003**, *14*, 387–394. [CrossRef]
11. Romero-Fernández, M.; Paradisi, F. Protein immobilization technology for flow biocatalysis. *Curr. Opin. Chem. Biol.* **2020**, *55*, 1–8. [CrossRef]
12. Benítez-Mateos, A.I.; Huber, C.; Nidetzky, B.; Bolivar, J.M.; López-Gallego, F. Design of the Enzyme–Carrier Interface to Overcome the $O_2$ and NADH Mass Transfer Limitations of an Immobilized Flavin Oxidase. *ACS Appl. Mater. Interfaces* **2020**, *12*, 56027–56038. [CrossRef]
13. Liese, A.; Hilterhaus, L. Evaluation of immobilized enzymes for industrial applications. *Chem. Soc. Rev.* **2013**, *42*, 6236–6249. [CrossRef] [PubMed]
14. Rodrigues, R.C.; Ortiz, C.; Berenguer-Murcia, Á.; Torres, R.; Fernández-Lafuente, R. Modifying enzyme activity and selectivity by immobilization. *Chem. Soc. Rev.* **2013**, *42*, 6290–6307. [CrossRef] [PubMed]
15. Roura Padrosa, D.; Benítez-Mateos, A.I.; Calvey, L.; Paradisi, F. Cell-free biocatalytic syntheses of l-pipecolic acid: A dual strategy approach and process intensification in flow. *Green Chem.* **2020**, *22*, 5310–5316. [CrossRef]
16. Planchestainer, M.; Contente, M.L.; Cassidy, J.; Molinari, F.; Tamborini, L.; Paradisi, F. Continuous flow biocatalysis: Production and in-line purification of amines by immobilised transaminase from Halomonas elongata. *Green Chem.* **2017**, *19*, 372–375. [CrossRef]
17. Roura Padrosa, D.; Nissar, Z.; Paradisi, F. Efficient Amino Donor Recycling in Amination Reactions: Development of a New Alanine Dehydrogenase in Continuous Flow and Dialysis Membrane Reactors. *Catalysts* **2021**, *11*, 520. [CrossRef]
18. Stepankova, V.; Bidmanova, S.; Koudelakova, T.; Prokop, Z.; Chaloupkova, R.; Damborsky, J. Strategies for stabilization of enzymes in organic solvents. *ACS Catal.* **2013**, *3*, 2823–2836. [CrossRef]
19. Mateo, C.; Palomo, J.M.; Fernandez-Lorente, G.; Guisan, J.M.; Fernandez-Lafuente, R. Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzym. Microb. Technol.* **2007**, *40*, 1451–1463. [CrossRef]
20. Pedroche, J.; del Mar Yust, M.; Mateo, C.; Fernández-Lafuente, R.; Girón-Calle, J.; Alaiz, M.; Vioque, J.; Guisán, J.M.; Millán, F. Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: Correlation between enzyme-support linkages and thermal stability. *Enzym. Microb. Technol.* **2007**, *40*, 1160–1166. [CrossRef]
21. Benítez-Mateos, A.I.; Contente, M.L.; Velasco-Lozano, S.; Paradisi, F.; López-Gallego, F. Self-Sufficient Flow-Biocatalysis by Coimmobilization of Pyridoxal 5′-Phosphate and ω-Transaminases onto Porous Carriers. *ACS Sustain. Chem. Eng.* **2018**, *6*, 13151–13159. [CrossRef]
22. Contente, M.L.; Paradisi, F. Self-sustaining closed-loop multienzyme-mediated conversion of amines into alcohols in continuous reactions. *Nat. Catal.* **2018**, *1*, 452–459. [CrossRef]
23. Contente, M.L.; Dall'Oglio, F.; Tamborini, L.; Molinari, F.; Paradisi, F. Highly Efficient Oxidation of Amines to Aldehydes with Flow-based Biocatalysis. *ChemCatChem* **2017**, *9*, 3843–3848. [CrossRef]
24. Roura Padrosa, D.; Alaux, R.; Smith, P.; Dreveny, I.; López-Gallego, F.; Paradisi, F. Enhancing PLP-Binding Capacity of Class-III ω-Transaminase by Single Residue Substitution. *Front. Bioeng. Biotechnol.* **2019**, *7*, 282. [CrossRef] [PubMed]
25. Hegarty, E.; Paradisi, F. Implementation of biocatalysis in continuous flow for the synthesis of small cyclic amines. *Chimia* **2020**, *74*, 890–894. [CrossRef] [PubMed]
26. Wang, S.Q.; Wang, Z.W.; Yang, L.C.; Dong, J.L.; Chi, C.Q.; Sui, D.N.; Wang, Y.Z.; Ren, J.G.; Hung, M.Y.; Jiang, Y.Y. A novel efficient route for preparation of chiral β-hydroxycarboxylic acid: Asymmetric hydration of unsaturated carboxylic acids catalyzed by heterobimetallic complex wool-palladium-cobalt. *J. Mol. Catal. A Chem.* **2007**, *264*, 60–65. [CrossRef]
27. Contente, M.L.; Pinto, A.; Molinari, F.; Paradisi, F. Biocatalytic N-Acylation of Amines in Water Using an Acyltransferase from Mycobacterium smegmatis. *Adv. Synth. Catal.* **2018**, *360*, 4814–4819. [CrossRef]
28. Delgado, L.; Parker, M.; Fisk, I.; Paradisi, F. Performance of the extremophilic enzyme BglA in the hydrolysis of two aroma glucosides in a range of model and real wines and juices. *Food Chem.* **2020**, *323*, 126825. [CrossRef]
29. Heckmann, C.M.; Gourlay, L.J.; Dominguez, B.; Paradisi, F. An (R)-Selective Transaminase From Thermomyces stellatus: Stabilizing the Tetrameric Form. *Front. Bioeng. Biotechnol.* **2020**, *8*, 1–13. [CrossRef]
30. Cerioli, L.; Planchestainer, M.; Cassidy, J.; Tessaro, D.; Paradisi, F. Characterization of a novel amine transaminase from Halomonas elongata. *J. Mol. Catal. B Enzym.* **2015**, *120*, 141–150. [CrossRef]
31. Mateo, C.; Fernández-Lorente, G.; Cortés, E.; Garcia, J.L.; Fernández-Lafuente, R.; Guisan, J.M. One-step purification, covalent immobilization, and additional stabilization of poly-His-tagged proteins using novel heterofunctional chelate-epoxy supports. *Biotechnol. Bioeng.* **2001**, *76*, 269–276. [CrossRef]
32. Guisán, J.M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzym. Microb. Technol.* **1988**, *10*, 375–382. [CrossRef]