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COMMUNICATION

One-pot biotransformation of glycerol into serinol catalysed by biocatalytic composites made of whole cells and immobilised enzymes

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Biocatalytic cascades afford the development of economically sustainable and green processes. Herein, we examined the unprecedented coupling of co-immobilised *Gluconobacter oxydans* and an isolated transaminase to synthesise serinol from glycerol. Through this approach, we manufactured up to 36 mM serinol, the highest titer ever reported for a non-fermentative biosynthesis. More importantly, similar productivities are obtained starting from the industrial by-product crude glycerol, demostrating the possibilities of this hybrid heterogenenous biocatalyst for valorising bio-based raw materials.

Introduction

The climate emergency demands more sustainable chemical manufacturing processes that ultimately transform renewable chemical sources or industrial by-products into commodities and fine chemicals[1]. Applied biocatalysis and industrial microbiology emerge as enabling disciplines to enhance the sustainability of these processes[2,3]. Nowadays, microbial fermentations dominate the valorisation of bio-based feedstocks[4–6]. However, microbes are limited to physiological conditions and tightened to genomic regulations that may jeopardise the process productivity and product purity. Therefore, the designed biosynthetic pathways are often re-wired due to microbial host metabolic burdens that impose constraints on the microbe productivity[7].

Furthermore, microbial fermentations are mainly limited to natural synthetic schemes under physiological conditions, which are often far from the industrial demands[8]. Whole-cell biotransformations using resting cells harbouring the synthetic enzymes are preferred when non-conventional media (i.e organic solvents, high temperatures and pressures, and substrate saturating conditions) are required to transform raw materials into the target products[9,10]. Resting cells act as enzyme reservoirs where the biotransformations are independent of the cell viability. When using cofactor-dependent enzymes, the metabolic background of resting cells regenerates the pool of cofactors, avoiding their exogenous supply and reducing the process costs. Whole-cells bioreduction of ketones is one of the most illustrative examples of resting cells that self-recycle redox cofactors[11].

For certain biotransformations, biosynthetic routes hardly perform out of their natural hosts, not even in engineered and domesticated chassis such as *E. coli*. The dihydroxyacetone (DHA) production from glycerol is one example where its natural producer *Gluconobacter oxydans* (*G. oxydans*) is highly efficient and copes with the global industrial production of this chemical[5]. Re-wiring the metabolism of this host to upgrade DHA to other chemicals presents some difficulties and has started to be explored in the last decade[12].

Although the metabolism of resting cells hibernates due to the lack of main carbon and nitrogen sources, sometimes compounds derail their planned synthetic schemes, giving rise to unwanted products that damage the process selectivity. As an alternative to resting cells, cell-free enzyme systems ameliorate these selectivity issues since one strictly uses the enzymes participating in the synthetic route[13]. However, working with isolated enzymes entail some

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Electronic Supplementary Information (ESI) available: Materials and methods and additional experimental data including HPLC and GC chromatograms and mass spectrum. Moreover, we add the experimental data using crude glycerol from biodiesel synthesis. See DOI: 10.1039/x0xx00000x

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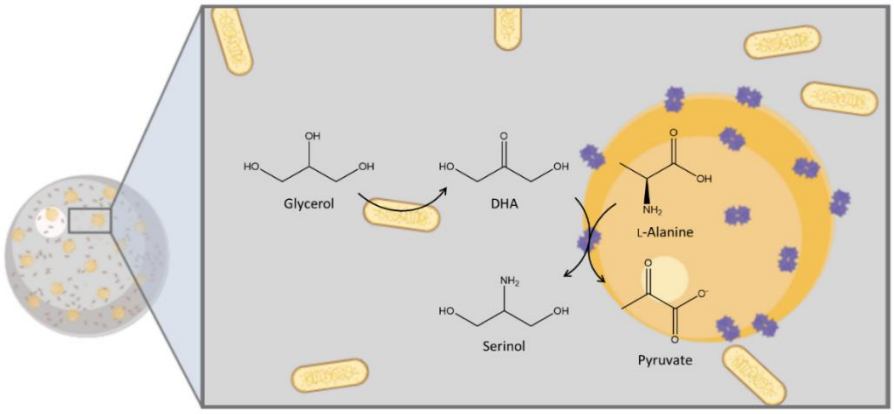

Scheme 1: Biocatalytic cascade for the synthesis of serinol from glycerol, concurrently catalysed by *G. oxydans* resting cells (yellow rods) and an amine transaminase (in purple) immobilized on agarose microbeads (orange sphere). Both resting cells and the immobilised enzymes are co-entrapped into alginate pearl (gray sphere).

stability burdens due to the fragility of soluble enzymes under operational conditions. To overcome the low stability of isolated enzymes, biotechnologists have exploited immobilisation techniques to stabilise them, resulting in more robust heterogeneous biocatalysts[14]. Now, these solid biocatalysts are easily separated from the reaction media and recycled, which positively impacts the downstream, logistics and economy of the bioprocess. In particular, the co-immobilisation of resting cells and isolated enzymes within the same solid carrier is an interesting approach that has been scarcely explored to concurrently valorise and up-grade bio-based raw materials. Creating artificial microenvironments where the microbes synergistically work with isolated enzymes may improve the exchange of intermediates between biocatalysts.

In this work, we approach the biosynthesis of serinol from glycerol through a two-step sequential process combining *G. oxydans* resting cells and an immobilised amine transaminase from *Pseudomonas fluorescens* (Pf-ATA). Serinol is an important building block in synthetic chemistry that is found in many bioactive compounds (sphingosines, ceramides) and pharmaceuticals[13–15]. Despite its interest, serinol biosynthesis is underexplored in applied biocatalysis and the few works on this topic rely on fermentative processes using glycerol and glucose as starting materials. Glycerol is the archetype of an industrial by-product with ample potential for valorisation through biotransformations. As it is generated in a 10:1 ratio during biodiesel production, large amounts of heavily contaminated glycerol flood the markets reducing its value and generating by-product disposal issues[16]. Therefore, the conversion of this by-product into new value-added compounds like serinol would have a positive impact in the economic equation of biodiesel industry.

Here, we merged the well-established process of glycerol oxidation catalysed by *G. oxydans* to produce dihydroxyacetone (DHA)[17], with an immobilised transaminase that concurrently aminates that DHA to yield serinol *via* a transamination reaction using L-Alanine as amine donor (Scheme 1). We evaluated the most synergetic combination between resting cells and the immobilised enzyme to maximise the serinol titers. Through a rational design, we fabricated and characterised a hybrid heterogeneous biocatalyst capable to produce one of the highest titers of serinol ever reported starting from bio-based glycerol and L-Alanine.

Results and discussion

Recently, we have assembled cell-free multienzyme systems into porous materials to transform diols into aminoacohols[18]. This strategy failed to transform glycerol into serinol because the employed alcohol dehydrogenase from *Bacillus stearothermophilus* was inactive towards glycerol. To address the synthesis of serinol through a biocatalytic oxidation/transamination sequential reaction, we exploited *G. oxydans* resting cells as biocatalyst. These resting cells display a PQQ-glycerol dehydrogenase (GlyDH) anchored to the microbial membrane able to oxidise glycerol efficiently, selectively, and robustly to DHA.

To accomplish this synthetic scheme using fully bio-based materials, we needed an amine transaminase (ATA) capable to transfer the amine group from a bio-based molecule (i.e L-alanine) to DHA to form serinol. According to previous studies, we demonstrated that Pf-ATA efficiently transfers the amine group from L-alanine to several aldehydes to yield the corresponding aminoalcohol[18]. In that work, a His-tagged variant of Pf-ATA was site-selectively immobilised on porous agarose microbeads functionalised with cobalt-chelate groups (Pf-ATA@AG-Co^{2+}). However, this heterogeneous biocatalyst was not previously challenged with ketones. For this purpose, we used a colorimetric assay to test DHA as an amine acceptor using *rac*-phenyl-ethylamine as an amine donor. According to this assay, we found that the transamination activity of Pf-ATA@AG-Co^{2+} using DHA as acceptor was 0.048 ± 0.004 U mL^{-1}, whereas the one measured using pyruvate was 1.310 ± 0.050 U mL^{-1} under the same reaction conditions (Table S1).

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Table 1. Glycerol conversion and Serinol yield and titer obtained with different immobilized hybrid biocatalysts

Entry	*G. oxydans*	Pf-ATA	Mode		Initial substrate concentration (mM)	Glycerol conversion[e] (%)	Serinol yield[f] (%)	Serinol titer[h] (mM)
1	Resting	-	Batch[a]		Glycerol (543)	94±9[e]	0	0
2	-	@AG-Co^{2+}	Batch[b]		DHA (500)	-	0.64±0.1[f]	3.2 ± 0.4
3	Resting	@AG-Co^{2+}	Two-pot[c]	1st Pot	Glycerol (543)	96±11[e]	-	0
				2nd Pot	DHA (20)	-	15±5[f]	3 ± 1
4	Co-entrapped into alginate	Co-entrapped into alginate	One-pot[d]		Glycerol (543)	10±2[e]	3.9±0.4[g]	21 ± 2

[a] 543 mM Glycerol was converted to DHA in a 30 mL reaction volume by *G. oxydans* resting cells (20 mg) in 24 h.

[b] Reactions were carried out mixing 100 mg (2.3 U) of Pf-ATA@AG-Co^{2+} with 0.3 mL of reaction mixture containing 500 mM DHA, 500 mM L-Alanine and 1 mM PLP in HEPES buffer 25 mM pH 8 and incubating for 24 h.

[c] In the first pot, 543 mM glycerol was converted to DHA by *G. oxydans* resting cells (20 mg) in 30 mL reaction volume in 24 h. For the second pot, the reaction crude was diluted to 20 mM DHA and mixed with 4.2 U of immobilised Pf-ATA in a 0.6 mL volume for 24 h.

[d] The one pot-system consisted of *G. oxydans* resting cells (2 mg) and 37.2 U Pf-ATA@AG-Co^{2+}co-entrapped in 3% alginate and incubated with 3 mL of 543 mM glycerol for 20 h.

[e] Glycerol conversion is defined as 100 x ($[Glycerol]_{t=24h}$ / $[Glycerol]_{t=0h}$).

[f] Serinol yield starting from DHA is defined as 100 x ($[Serinol]_{t=24h}$ / $[DHA]_{initial}$).

[g] Serinol yield starting from Glycerol is defined as 100 x ($[Serinol]_{t=24h}$ / $[Glycerol]_{Initial}$).

[h] Serinol titer was determined by GC-FID

Previous studies from our groups showed that resting cells of *G. oxydans* quantitively convert 543 mM glycerol into 510 ± 49 mM DHA in 24 hours (Table 1, entry 1)[19]. Encouraged by this result, we incubated Pf-ATA@AG-Co^{2+} with 500 mM DHA and 500 mM L-Ala. We obtained an extremely low product yield (<1%), producing only 3.2 ± 0.4 mM of serinol (Table 1, entry 2). At L-Ala:DHA molar ratio of 1, the amination reaction seems to be very inefficient. Under this condition, we suggest that the transaminase misses the excess of the amine donor needed to shift the reaction equilibrium towards the amination reaction[18]. As result, the productivity of resting *G.oxydans* for DHA (50 g L^{-1} day^{-1}) is 167 times that the productivity of Pf-ATA@AG-Co^{2+} for serinol (0.3 g L^{-1} day^{-1}).

To further study the effect of L-Ala:DHA molar ratio on serinol yield, we firstly performed a study challenging Pf-ATA@AG-Co^{2+} with different DHA concentrations maintaining the L-Ala at 500 mM (Figure S1). After 24 hours using a 0.5 L-Ala:DHA molar ratio (1000 mM DHA), serinol was not detected. Contrariwise, 2.2 and 5.5 mM serinol, corresponding to 11% and 5.5% conversion of DHA were obtained using 20 and 100 mM of DHA, respectively. We observed that upon raising the L-Ala:DHA molar ratio to 25, Pf-ATA transfers more efficiently the amine from L-Ala to DHA to form serinol. This experiment suggests that we need to maintain an optimal donor:acceptor molar ratio that facilitates the reaction shifting towards the amination during the whole biotransformation.

In the light of these results, we performed a two-pot reaction where we first obtained a quantitative conversion of glycerol into 523 ± 60 mM of DHA using *G. oxydans* after 24 h (Table 1, entry 3). In order to maintain the aforementioned L-Ala:DHA ratio and as it was unfeasible to add 13 M of L-Ala due to solubility issues, the resting cells were withdrawn and the reaction crude of this first pot was diluted with the transamination buffer (1 mM PLP and 500 mM of L-Alanine in 25 mM HEPES buffer at pH 8) to a final concentration of 20 mM DHA. In the second pot, the diluted reaction crude was mixed with the Pf-ATA@AG-Co^{2+} biocatalyst and after 24 hours, a serinol titer of 3 mM was detected, representing a 15% conversion of DHA (Table 1, entry 3). The overall serinol productivity of the two-pot system was 0.13 g L^{-1} day^{-1} accounting for the 48 h needed to complete the two sequential, but not concurrently reactions.

Ideally, a one-pot system where both glycerol oxidation and DHA amination reactions are orchestrated should avoid the transfer and dilution steps from one reactor to the next, saving time and infrastructure resources. To that aim, we performed the glycerol oxidation reaction catalysed by *G. oxydans* resting cells in the transamination media (Figure S2). The reaction reached similar glycerol conversion in both phosphate and HEPES buffer supplemented with 1 mM PLP and 500 mM L-Ala, after 24 hours reaction at 30°C. Thus, the whole-cell biocatalyst is negligibly affected by the transamination reaction media. We then fabricated a hybrid heterogeneous biocatalyst that entrapped both *G. oxydans* resting cells and the transaminase within alginate particles. Since the entrapment of the free enzyme might cause the protein diffusion to the bulk owing to the large pores of the hydrogel macroparticles, we co-entrapped *G. oxydans* resting cells and Pf-ATA@AG-Co^{2+} into alginate pearls to avoid the enzyme lixiviation. A similar approach was used by Wilson *et al.* to entrap cross-linked enzyme aggregates into alginate gel particles for the simultaneous synthesis and purification of galacto-oligosaccharides from lactose[20].

First, we evaluated the effect of this biopolymer on the oxidation activity of the entrapped *G. oxydans* resting cells (Figure 1). Stable alginate macroparticles were formed after calcium-driven cross-linking and the entrapped cells were viable (Figure 1A). Despite their viability, the resting cells entrapped into alginate achieved 5-fold lower glycerol conversion than the free cells (Figure 1B). The time-course of glycerol biotransformation showed that the alginate-

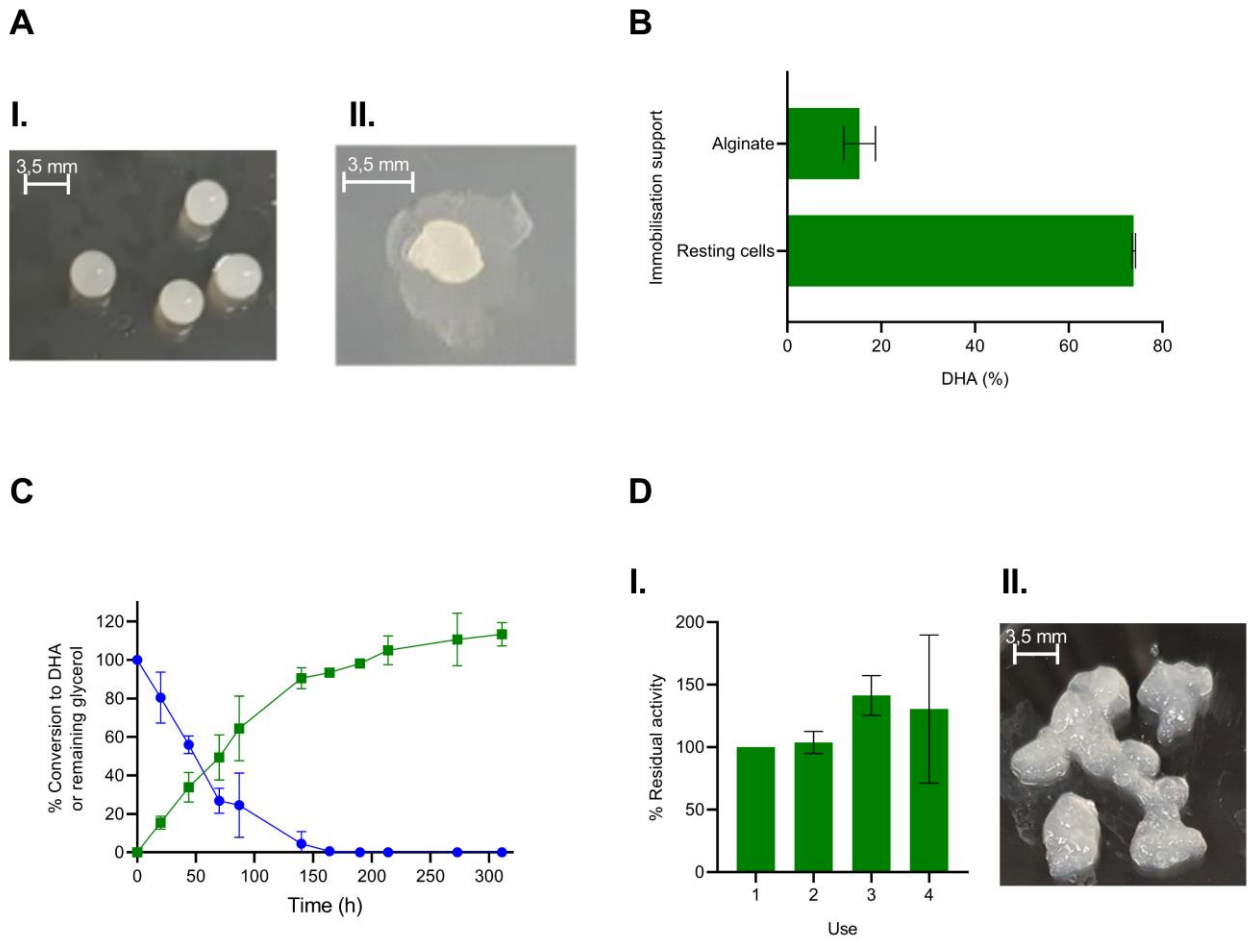

Figure 1: A) I. Immobilised preparations of *G. oxydans* in alginate. II. Viability assay of G. *oxydans* cells immobilised in alginate. B) DHA production from 543 mM of glycerol using free *G. oxydans* and *G. oxydans* entrapped in alginate, after 20 hours. C) DHA production kinetics starting from 543 mM of pure glycerol by *G. oxydans* entrapped into alginate. DHA (green squares), Glycerol (blue circles). D) I. Residual activity of reused *G. oxydans* entrapped into alginate. II. G. oxydans immobilised preparations after 4 consecutive uses.

entrapped *G. oxydans* cells needed up to 200 hours to quantitatively transform 543 mM glycerol into DHA (Figure 1C). This time-course indicates that immobilised cells were 8 times less productive than the free ones. Remarkably, the entrapped resting cells maintained the same exquisite regioselectivity as the free cells, exclusively yielding DHA as product. Furthermore, entrapped resting cells could be re-used up to four operational cycles without losing efficiency, although the particles suffered mechanical stability issues after the fourth cycle (Figure 1D).

These results encouraged us to co-entrap both *G. oxydans* resting cells and Pf-ATA@AG-Co^{2+} microbeads into alginate pearls, through dripping an alginate suspension containing cells and agarose microbeads into a calcium chloride solution. Figure 2 shows the presence of agarose microbeads containing transaminases within the alginate pearls. The micrographs taken with a confocal laser scanning microscope demonstrate that the autofluorescence of PLP from the holoenzyme only appears in the microbeads where the Pf-ATA is immobilised. Finally, this hybrid heterogeneous biocatalyst was employed for the one-pot serinol synthesis starting from 543 mM glycerol and supplementing the reaction with 500 mM of L-Ala.

Figure 3 shows the progress of the serinol biosynthesis where 14 % of the initial glycerol was converted into 55 mM of DHA and 21 mM of serinol after 20 hours which translates into a volumetric productivity of 2.30 g L^{-1} day^{-1} (Table 1, entry 4, and Figure 3). The one-pot system exhibits a serinol productivity 18-fold higher than the two-pot system.

Remarkably, the one-pot system continued working to produce up to 36 mM of serinol after 44 hours. This titer means that 6.6 % of the glycerol was transformed into serinol. Under this condition, 1 mol of serinol was produced with 2.8% (in mol) of PLP and a 14-fold molar excess of the starting materials (L-Ala and Glycerol). The resulting serinol was characterized by GC-MS (Figure S3). This effect could be an added advantage of the whole cell-enzyme biocatalytic composite over the resting cells and the immobilised enzyme physically segregated in two different heterogeneous biocatalysts.

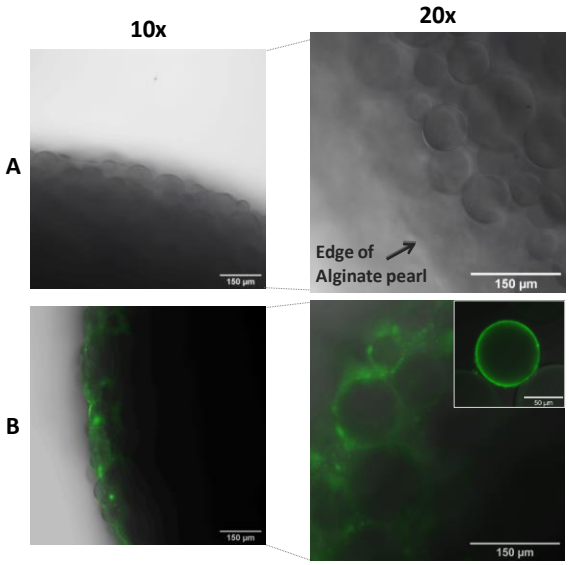

Figure 2: Fluorescence confocal microscopy images of the co-immobilised preparation containing *G. oxydans* and Pf-ATA@AG-Co^{2+} within alginate pearls. Overlay of bright field and PLP auto-fluorescence (green) of alginate beads co-entrapping the bacteria and the agarose microbeads without (A) and with the Pf-ATA (B) at 10X (left panels) and 20X magnification (right panels). The inlet in the right B panel represents the 40X magnification of a single bead of Pf-ATA@AG-Co^{2+} within the microstructure of the alginate bead.

Despite the reaction stoichiometry, we detected almost 7 times less pyruvate than serinol after 20 hours and the pyruvate almost completely disappeared after 44 hours of reaction (Figure 3, Figure S4). This by-product might be depleted by the metabolic background of *G. oxydans* resting cells. In fact, experiments where pyruvate was fed as co-substrate show that the resting cells oxidise glycerol to DHA and consume the pyruvate to unidentified products (Figure S5). Likely, pyruvate enters in the central metabolism of *G. oxydans* cells as carbon source. This unexpected effect also contributed to shift the transamination equilibrium towards the synthesis of serinol as the pyruvate, despite being the preferred amine acceptor of Pf-ATA, was not available to accept the amine group from the aminated product. Thus, the architecture of the heterogenous multi-biocatalyst herein developed, provides a constant supply of substrate without accumulating large amounts of the intermediate, which passively aids the transamination reaction.

Aiming to utilise the industrial by-product crude glycerol, we then assayed it as a substrate for serinol production with the hybrid heterogeneous biocatalyst (Figure S6). Even though the starting material contained methanol, ashes and other contaminants, we only observed a 30% reduction in the synthesis rate. The slower production rate was manifested in a 32% lower serinol yield when using crude glycerol compared to the pure one using the same heterogeneous biocatalyst and under the same reaction conditions.

Finally, we studied the reusability of the hybrid heterogenous biocatalysts for the conversion of pure and crude glycerol (Figure S7). For both glycerol qualities, the production of both serinol and DHA diminishes to less than 40% and 10% of its initial capacity after the second and third 23 h cycles, respectively. We suggest that this poor robustness relies on the low stability of Pf-ATA@AG-Co^{2+} as supported by previous results that report the dramatic inactivation of this enzyme during its sequential use for the synthesis of aminoalcohols[20]. Furthermore, we remark that the biocatalyst integrity is affected by the components of crude glycerol, as the alginate matrix completely disintegrates after 3 cycles. Further experiments will be required to stabilise Pf-ATA and improve the physical resistance of the matrix used for the immobilisation.

Previously, Andreeßen and Steinbüchel reported a titer of 36 mM through a fermentative process using *E. coli* cells harbouring the

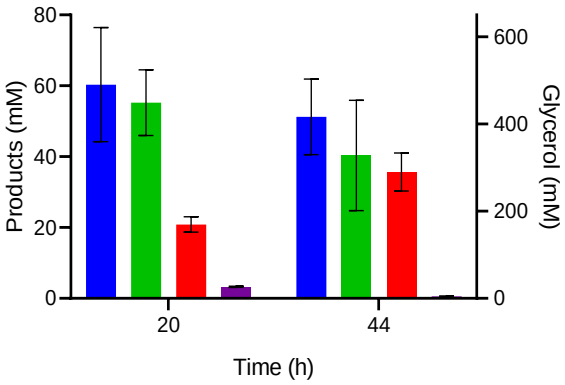

Figure 3: Time-course of serinol production using the co-immobilised system as biocatalyst. The reaction was triggered with 543 mM of glycerol. Substrate: glycerol (blue), and products: DHA (green), serinol (red), pyruvate (purple).

recombinant dihydroxyacetone phosphate aminotransferase/dihydrorhizobitoxine synthase of *Bradyrhizobium elkan*ii USD94[21]. This engineered microbe converts glycerol into dihydroxyacetone-phosphate through its central metabolism, which is then concurrently aminated and dephosphorylated to produce serinol. This process was later intensified in a batch bioreactor fed with glucose and glycerol, achieving up to 77 mM of serinol[22]. When using a more advanced engineered *E. coli* strain and glucose as starting material in a fed-batch reactor, Luo *et al.* recently reported a serinol titer of 160 mM[23]. In contrast, the system we herein report, directly uses glycerol as starting material without any metabolic activation that relies on fermentative processes. Although our process is still far from its optimal version, the serinol titer we produced was similar to that one obtained with a non-optimised fermentative process and the highest one ever reported with a resting biotransformation. The entrapment of both resting cells and immobilised transaminases allows an upgrade of 321 times‡ in the price of pure glycerol (0.15 U$S/g) to serinol (48.14 U$S/g) in a one-pot procedure. If crude glycerol is considered, the valorisation increases three orders of magnitude, as crude glycerol has a price that oscillates around 100 U$S/ton‡.

The high titer of this cell-free process biocatalyst encouraged us to assess its sustainability. For this purpose, we calculated the total E-factor of the process, but also the specific contribution of water, other solvents rather than water, reactants, and catalyst (Figure 4).

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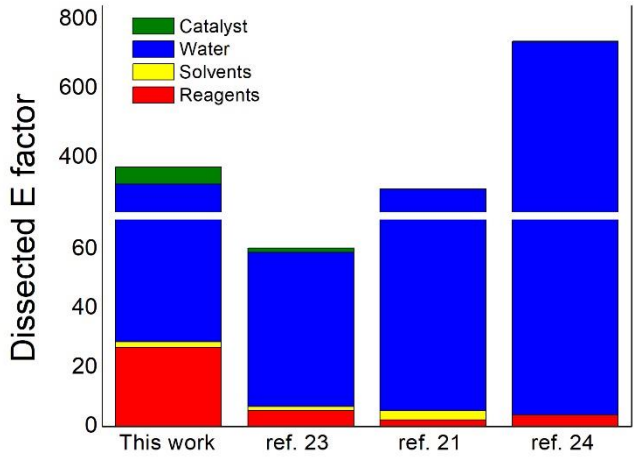

Figure 2: Weighted E factor values of different serinol production processes. Contribution of water (blue), solvents other than water (yellow), reactants (red) and catalyst (green) to the total E factor were plotted. E factor values were calculated from the data reported in this work, Luo et al. (ref 23), Andreen *et al*. (ref. 21), and Bhandare *et al*. (ref. 24).

We also performed the same assessment for the serinol production from fermentative processes previously reported by Luo et al[23] and Andreeßen & Steinbüchel[21] and the chemical synthesis of serinol reported by Bhandare[24]. All biotransformations exhibited a significantly lower E factor value in comparison to the chemical synthesis, although water was the major contributor to the E factor in all cases. The lowest E factor herein calculated was found for the optimised and glucose-driven fermentative processes with an E factor of 60 due to the high titer of serinol. When the fermentation is not optimised and the starting material was glycerol instead of glucose, the E factor increased up to 300, a similar value to the one calculated for the system herein presented and catalysed by the hybrid heterogeneous biocatalyst.

Remarkably, the contribution of the catalyst to the total E factor was notorious in the systems with the *G. oxydans* resting cells and the immobilised transaminase co-entrapped into the alginate pearls. We envision that the weight of the biocatalysts in the total E-factor will be reduced by improving the re-use of this hybrid system.

Conclusions

More sustainable approaches for valorising and up-grading renewable raw materials and wastes contribute to expand the concept of the biorefinery. In particular, glycerol is one of the largest surpluses of the biodiesel industry that need to be transformed into high-added value products. In this work, we have up-graded glycerol to serinol through a hybrid biocatalytic system that co-entraps resting cells and immobilised enzymes into an alginate matrix. The combination of resting cells and isolated enzymes is not trivial, so we herein designed a novel heterogeneous multi-functional biocatalyst able to oxidise glycerol to DHA, and concurrently aminate the intermediate ketone to serinol. This was possible through the spatial confinement of resting *G. oxydans* and an immobilised transaminase inside porous alginate macroparticles that catalyse the concurrent oxidation and transamination reactions in one-pot. Using this hybrid and heterogeneous biocatalyst, we achieved up to 36 mM of serinol, the highest titer ever reported for a non-fermentative serinol biosynthesis. Finally, we analysed the sustainability metrics of the serinol biosynthesis reported in this work and compared it with the fermentative biosynthesis and the chemical synthesis of this molecule. E factor was significantly lower for all biosynthetic processes compared to the chemical one, although water was the major contributor in all cases. The green metrics forecast a decreasing on the E factor of our process if the operational stability of the biocatalyst is maximised according to the weight of the biocatalyst mass in the total value of the E factor.

Conflicts of interest

There are no conflicts to declare.

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‡ Upgrades in price were calculated using the Merck-Sigma-Aldrich catalogue and the market price from crude glycerol provided by Alcoholes del Uruguay (ALUR) (Montevideo, Uruguay).

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