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Supplementary information

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

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MATERIALS and METHODS

Chemicals

All reagents used were analytical grade. D-glucose was obtained from Amresco (Ohio, USA). Peptone and agar were obtained from Oxoid (Hampshire, UK). $MgSO_4.7H_2O$ and were obtained from Biopack (Buenos Aires, Argentina). Yeast extract was obtained from BD Biosciences (California, USA). KH_2PO_4, H_2SO_4, NaH_2PO_4 and $CoSO_4.7H_2O$ were obtained from Emsure (Massachusetts, USA). K_2HPO_4 was obtained from Dorwil (Buenos Aires, Argentina). Pure glycerol, $CaCl_2.2H_2O$ and Tris were obtained from Carlo Erba (Barcelona, Spain). Crude glycerol, product of the high-pressure splitting process (water <12%, ash <7%, glycerol 70-80%, lipids <1%, methanol <5%, chlorides <3%) was donated by Alcoholes del Uruguay (ALUR). Sodium alginate and serinol standard were obtained from Sigma-Aldrich (Missouri, USA). DHA standard and HEPES were obtained from Merck (Darmstadt, Germany). Pyruvate was obtained from Aldrich Chemistry (Missouri, USA). 2-phenylethylamine (FEA) was obtained from Acros Organics (Pennsylvania, USA). Agarose-IDA (Chelating Sepharose Fast Flow) was obtained from GE Healthcare (Illinois, USA). L-alanine and PLP were obtained from Sigma (Missouri USA).

Bacterial strains

Gluconobacter oxydans NBRC 14819 was obtained from the National Institute of Technology and Evaluation (NITE) (Tokyo, Japan). *E. coli* BL21 and *E. coli* DH5α were obtained from Novagen (NJ, USA).

G. oxydans growth conditions

Pre-cultures from isolated colonies of *G. oxydans* were prepared in 3 mL of medium containing glucose (peptone 5 g/L, yeast extract 5 g/L, D-glucose 5 g/L, $MgSO_4.7H_2O$ 1 g/L, pH 6,5) and incubated 16 h at 30°C and 210 rpm. Successively, 1 L flasks containing 250 mL of medium with pure glycerol (glycerol 100 g/L, peptone 9 g/L, yeast extract 1 g/L, KH_2PO_4 0,9 g/L, K_2HPO_4 0,1 g/L, $MgSO_4.7H_2O$ 1 g/L, pH 6,0) were inoculated with the aforementioned pre-cultures. The cultures were incubated at 30 °C and 180 rpm until they reached an OD_{600nm} value of 1. After spinning down the 250 mL of culture at 4211g for 15 minutes, 20 mg dry weight of cells were obtained. The bacterial pellet was washed with 30 mM phosphate buffer (NaH_2PO_4 4.14 g/L, K_2HPO_4 5.23 g/L, pH 7.0) in order to remove the growth medium and was subsequently centrifuged at 4211g for 15 minutes, discarding the supernatant.

Production of Pf-ATA

ω-Transaminase from *Pseudomonas fluorescens* was cloned and overexpressed in competent *E. coli* BL21 cells transformed with the respective plasmid as previously reported[26]. Briefly, 5 mL of an overnight culture of *E. coli* BL21 (DE3) harbouring the plasmid was used to inoculate 250 mL of Luria-Bertani (LB) medium containing kanamycin (final concentration 50 $\mu g mL^{-1}$). This was performed by octuplicate. Then, the resulting cultures were aerobically incubated at 37 °C with orbital shaking at 230 rpm until the OD_{600nm} reached 0.6. Afterwards, the cultures were induced with 1 mM IPTG. The enzyme production was induced for 18 h at 21 °C. After the induction time, 100 mL aliquots were centrifuged at 4211g for 30 min at 4 °C to harvest cells. Supernatant was discarded and the pellet was resuspended in 10 mL of 25 mM HEPES buffer at pH 8.0 containing 1 mM PLP. Cells were broken by sonication using a Sonics Vibracell VCX 130 at 30% amplitude (10 s ON/30 s OFF) for 5 min at 4 °C. The suspension was then centrifuged at 10,528g for 30 min at 4 °C and the pellet was discarded. The

supernatant containing cell extracts with the his-tagged protein was collected and employed for further immobilisation.

Enzymatic activity measurement

Enzyme activity was spectrophotometrically measured in UV transparent 96-well microplates, employing a Tecan Infinite M200Pro plate reader, with the Software Magellan V 7.2 (Männedorf, Switzerland). Transaminase activity was determined by recording the increase in the absorbance at 245 nm of 200 µL of a reaction mixture containing FEA 2 mM, pyruvate 2 mM, PLP (0.1 mM) in HEPES buffer 100 mM pH 8.0 at 30 °C. The reaction was initiated by adding 10 µL of the enzymatic solution or suspension properly diluted to the reaction mixture. One unit (U) of TA activity was defined as the amount of enzyme required to produce one µmol of acetophenone per minute at the assessed conditions.

Bacterial immobilisation and glycerol conversion to DHA

20 mg of washed *G. oxydans* cells were mixed with 3 mL of 3% alginate solution. The homogenous mixture was added dropwise to a 50 mM $CaCl_2$ solution (crosslinking solution). The resulting beads were incubated in the crosslinking solution for 30 minutes, harvested using a strainer and subsequently washed with distilled water.

Conversions of glycerol by the immobilised resting cells were carried out in 250 mL flasks with 30 mL volume of reaction medium supplemented with pure glycerol (glycerol 50 g/L, KH_2PO_4 0.9 g/L, K_2HPO_4 0.1 g/L, $MgSO_4.7H_2O$ 1 g/L, pH 6). The reactions were carried out at 30 °C and 180 rpm. Conversion kinetics were elucidated by taking samples at different times during the reaction and analysing them by HPLC.

Enzyme immobilisation

Immobilised Pf-ATA on AG-Co^{2+} (Pf-ATA@AG-Co^{2+}) was prepared by mixing 20 mL or 40 mL of crude extract containing the His-tagged protein (~10 U/mL) with 1 gram of AG-Co^{2+} microbeads and incubated under orbital shaking for 1 h at 25 °C. The suspension was then filtered and the microbeads containing the enzyme were washed with 50 mL of 25 mM HEPES buffer at pH 8.0 containing PLP 0.1 mM.

Preparation of co-immobilised bacteria-enzyme system

To obtain the co-immobilised preparations, 2 mg dry cell weight of *G. oxydans* and 500 mg of immobilised Pf-ATA (Pf-ATA@AG-Co^{2+}) were mixed with 50 µL of distilled water. 300 µL of 3% alginate was then added to the mixture. The homogenous mixture was added dropwise to a 50 mM $CaCl_2$ solution (cross-linking solution). The resulting beads were incubated in the cross-linking solution for 30 minutes, then harvested using a strainer and finally washed with distilled water.

HPLC analysis

At different reaction times, samples were withdrawn and centrifuged at 18,000g for 15 minutes. Subsequently, the supernatant of each sample was filtered with a 0.22 µm filter treated with 2% polyvinylpyrrolidone (PVP) and analysed by HPLC. DHA, glycerol and pyruvate were quantified using a Shimadzu Nexera X2 HPLC (Kyoto, Japan) with a diode array detector, equipped with an Aminex® HPX-87C 300 x 7.8 mm from Bio-Rad (California, USA) and a SecurityGuard ™ 4 x 3.0 mm cartridge for Carbo-H columns from Phenomenex (California, USA). The mobile phase was 5 mM H_2SO_4. Detection was carried out at 70 °C at 271 nm for DHA and 190 nm for glycerol and pyruvate, with a flow of 0.6 mL/min for 19 minutes. The retention times recorded were: DHA (16.7 min), glycerol (14.7 min) and pyruvate (9.1 min). Each injection was of a volume of 20 µl. Calibration curves were constructed for DHA (0.15 –5 g/L), glycerol (0.15 – 20 g/L) and pyruvate (0.06 – 2.4 g / L). The samples were analysed using the LabSolutions software from Shimadzu (Kyoto, Japan).

GC-FID analysis

At different reaction times, samples were withdrawn and centrifuged at 18,000g for 15 minutes. The supernatant of the samples were derivatised before analysis as follows: 30 µL of sample were mixed with 30 µL of N-methylimidazole (NMIM). Then, 225 µL of acetic anhydride was added and the sample was incubated for 5 minutes at 25°C. Finally, 300 µL of mQ water were added and the resulting aqueous solution was extracted with 300 µL of dichloromethane. The aqueous phase was discarded, and the organic phase was dried with 30-40 mg of anhydrous $MgSO_4$ and kept for GC analysis. The derivatized and extracted samples were analysed using a Hewlett Packard 7890 series II gas

chromatograph (California, USA) using a 5.5% phenyl silicone column (Zebron ZB-5HT Inferno 30 m × 0.25 mm × 0.25 µm), helium as mobile phase and a flame ionization detector (FID). The injector temperature was 210°C and the detector temperature was 250°C. During separation, an initial temperature of 140°C was held for 2 minutes, then the temperature was raised to 240°C at 20°C/min and finally held for 2 minutes. A calibration curve was created for serinol, using a pure serinol standard in the concentration range of 0.07 - 4.5 g/L. Serinol retention time was 5.6 min under the above describe analysis conditions. Under this analysis conditions, glycerol and DHA could also be detected with retention times of 4.2 and 3.3 respectively. The conversion of serinol was determined as

GC-MS analysis

The GC-MS separation conditions were the same as those described in the GC-FID section, changing the detector for a mass spectrometer. The mass spectrometer was a Bruker MicroTof-Q (Massachusetts, USA). The analyses were performed with a 3-minute delay. Serinol retention time was 5.3 min.

Confocal microscopy

PLP cofactor distribution was analysed in a spectral ZEISS LSM880 confocal microscope (Oberkochen, Germany). A 10X and 40X objective was used while the sample was excited with a Diode excitation laser λ_{ex} = 405 nm. The emission wavelength was at 418 nm. The images were processed with ImageJ software developed by the National Institutes of Health (Maryland, USA).

Serinol synthesis from commercial DHA

100 mg of Pf-ATA@AG-Co^{2+} where mixed with 300 µL of a reaction mixture containing DHA (20, 100, 500 and 1000 mM), 500 mM L-Alanine, 1mM PLP in HEPES buffer 100 mM pH 8. Reactions were incubated 24 h at 30 °C with gentle agitation. Afterwards, reactions where filtered, derivatised and analysed by GC-FID.

Serinol synthesis from pure glycerol

Two-pot reaction. First, 543 mM of glycerol was transformed into DHA by 20 mg of resting cells of *G. oxydans* in the same conditions as described above (Bacterial

immobilisation and glycerol conversion to DHA). Then, the reaction crude was diluted in 25 mM HEPES buffer, pH 8.0, 1 mM PLP, 0.5 M L-alanine to a concentration of 20 mM of DHA. 600 µL of this solution was mixed with 200 mg of Pf-ATA@AG-Co^{2+} and incubated at 180 rpm and 30 °C for 24 hours.

One-pot reaction. The material resulted from co-entrapping 2 mg of *G. oxydans* resting cells and 500 mg of Pf-ATA@AG-Co^{2+} (37.2 U) into alginate 2,6 % was incubated with 3 mL of 25 mM HEPES buffer, pH 8.0, 1 mM PLP, 0.5 M L-alanine and 0.543 M pure or crude glycerol were incubated with the beads at 30°C and 180 rpm, for 23 or 44 hours. Samples were taken and analysed by HPLC and GC. The reusability of this hybrid biocatalysts was assessed in successive 23-hour cycles. After each cycle, the immobilised preparations were separated from the reaction crude with a strainer and washed with distilled water. Subsequently they were mixed with fresh reaction media and incubated again at 180 rpm and 30 °C.

Serinol synthesis from crude glycerol

One-pot reaction. The material resulted from co-entrapping 2 mg of *G. oxydans* resting cells and 500 mg of Pf-ATA@AG-Co^{2+} (19.6 U) into alginate 2,6 % was incubated with 3 mL of 25 mM HEPES buffer, pH 8.0, 1 mM PLP, 0.5 M L-alanine and 0.5 M crude glycerol were incubated with the beads at 30°C and 180 rpm, for 23 or 44 hours. For comparison, we incubated the same biocatalytic composite with 0.5 M pure glycerol under exactly the same conditions above described. In both cases, samples were taken and analysed by HPLC and GC.

Green metrics

The green metrics calculated for the determination of the sustainability of this work and their comparison among selected references were calculated as follows[1]:

Product mass: Product obtained at the end of the reaction(g).

Waste mass: Is the product mass subtracted from the total mass in the bulk accounting for the reagents, the solvent (including water) and the catalyst.

$$\boldsymbol{Waste\ mass} = (Total\ reagents\ mass\ (g) + totals\ solvent\ mass(g) + total\ biocatalyst\ mass\ (g)) - Product\ mass\ (g) \quad \text{Eq. 3}$$

$$\boldsymbol{E\ factor} = {}^{Waste\ mass}\!\big/_{Product\ mass} \quad \text{Eq. 4}$$

$$\boldsymbol{Efactor_{reagents}} = {}^{Total\ reagents\ mass\ (g)}\!\big/_{Product\ mass\ (g)} \quad \text{Eq. 5}$$

$$\boldsymbol{Efactor_{solvents}} = {}^{Total\ solvents\ mass\ (g)}\!\big/_{Product\ mass\ (g)} \quad \text{Eq. 6}$$

$$\boldsymbol{Efactor_{water}} = {}^{Total\ water\ mass\ (g)}\!\big/_{Product\ mass\ (g)} \quad \text{Eq. 7}$$

$$\boldsymbol{Efactor_{catalysts}} = {}^{Total\ catalyst\ mass\ (g)}\!\big/_{Product\ mass\ (g)} \quad \text{Eq. 8}$$

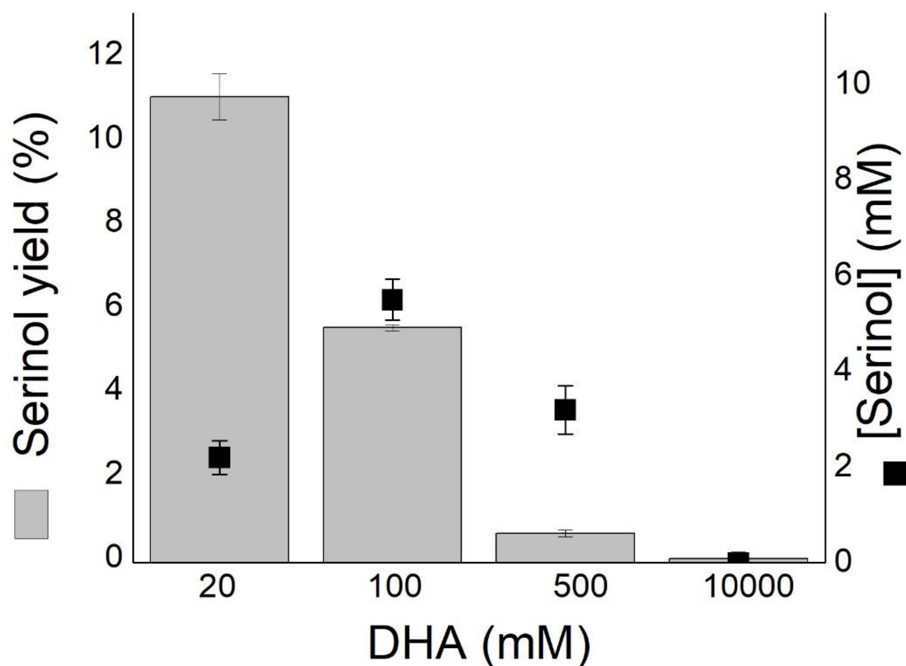


Figure S1. Conversion of DHA and serinol titers catalysed by Pf-ATA@AG-Co^{2+} using different concentrations of DHA. L-Ala concentration was 500 mM in all cases.

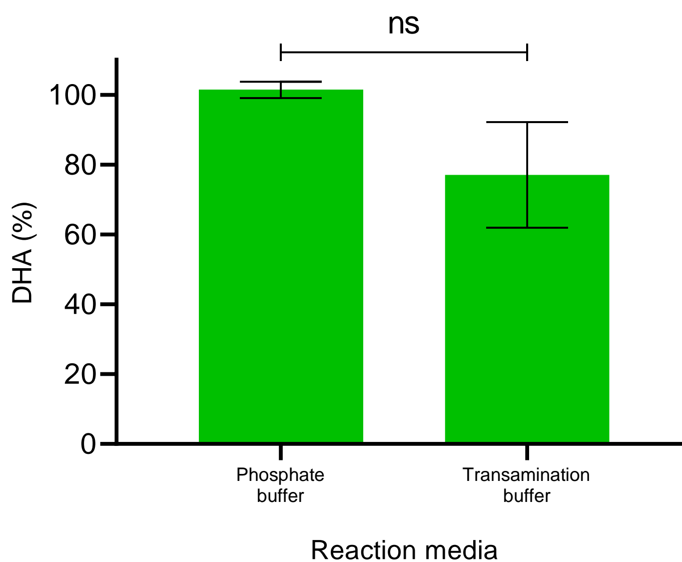
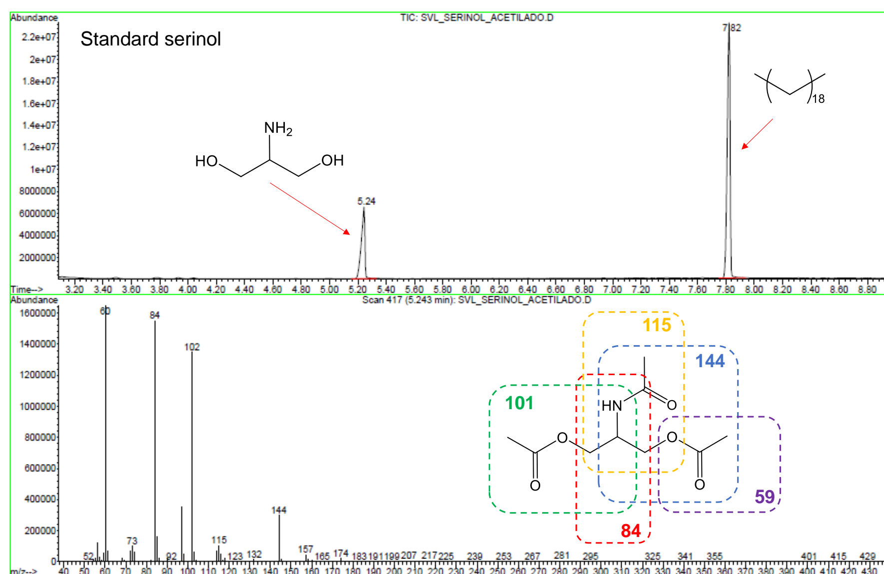


Figure S2. DHA production from 543 mM of glycerol after 24 hours. The reaction was carried out at 30 °C and 180 rpm in phosphate buffer (KH_2PO_4 0.9 g/L, K_2HPO_4 0.1 g/L, $MgSO_4.7H_20$ 1 g/L, pH 6) and transamination media (HEPES 25 mM pH 8.0, PLP 1 mM, L-Alanine 500 mM).

A



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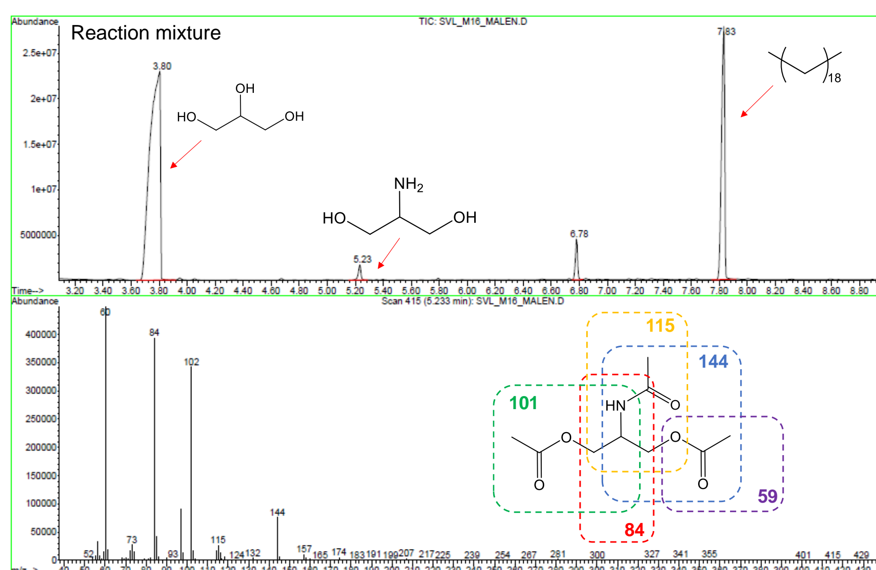

Figure S4. GC-MS analysis of derivatized samples withdrawn from the serinol production. (A) Serinol standard and (B) reaction crude after 44 hours of reaction catalysed by the biocatalytic composite. The GC traces (top panel) and the mass spectrum of the peak at 5.23 min (bottom panel) are shown indicating the chemical structures and the fragmentation pattern.

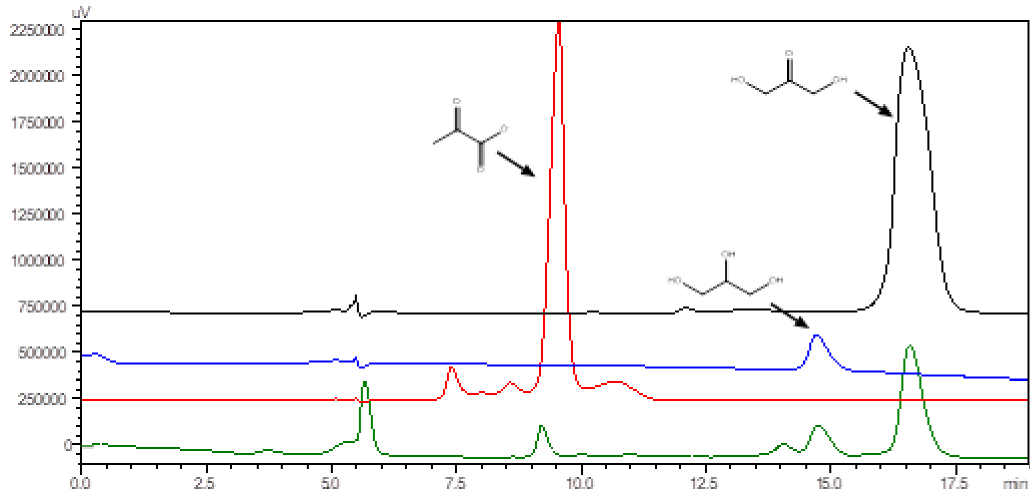

Figure S4. HPLC analysis of serinol production. DHA standard (black). Glycerol standard (blue). Pyruvate standard (red). Media composition of the serinol production reaction with the co-immobilised system after 44 hours (green).

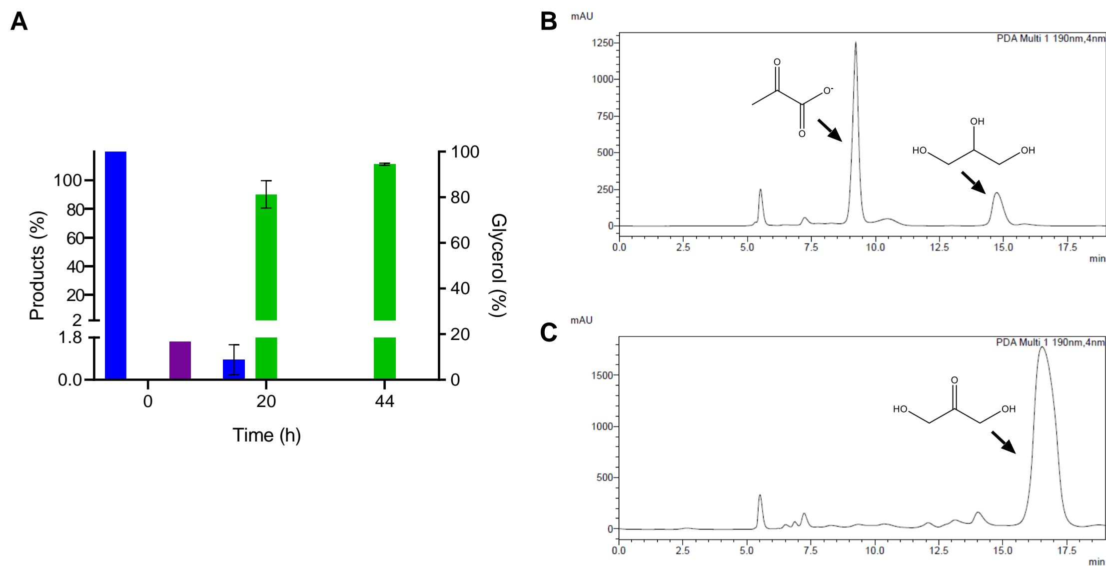

Figure S5. A) Pyruvate consumption during DHA production from 543 mM of glycerol by *G. oxydans* resting cells. Glycerol (blue), pyruvate (purple), DHA (green). The reaction was carried out with 2 mg *G. oxydans* in 3 mL of transamination media (HEPES 25 mM pH 8.0, PLP 1 mM, L-Alanine 500 mM) with the addition of 8 mM pyruvate. The reaction was incubated at 30 °C and 180 rpm for 44 h. B) HPLC analysis of the initial reaction crude. C) HPLC analysis of the final reaction crude.

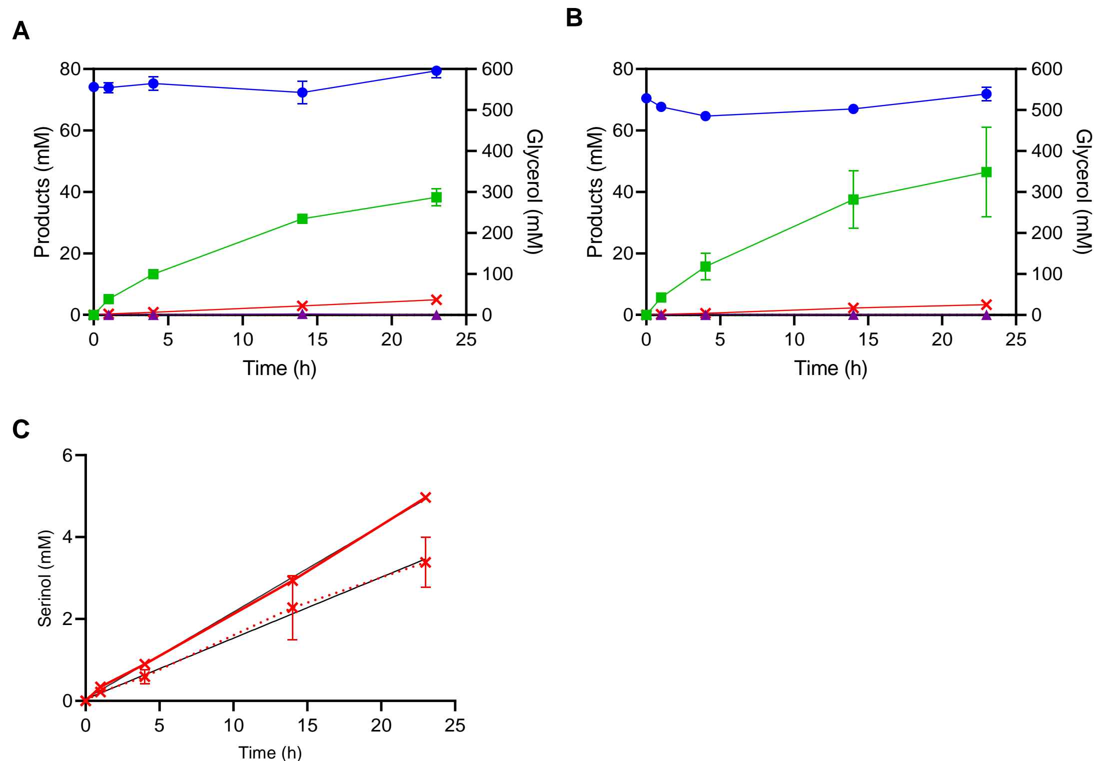

Figure S6. Serinol production kinetics using the co-immobilised hybrid system in one-pot. Both reaction started with 543 mM of either pure or crude glycerol under the reaction conditions described in method section. A) Pure glycerol. B) Crude glycerol. Glycerol (blue circles), DHA (green squares), serinol (red crosses), pyruvate (purple triangles). C) Initial reaction of serinol production using pure glycerol (solid line) and crude glycerol (dash line).

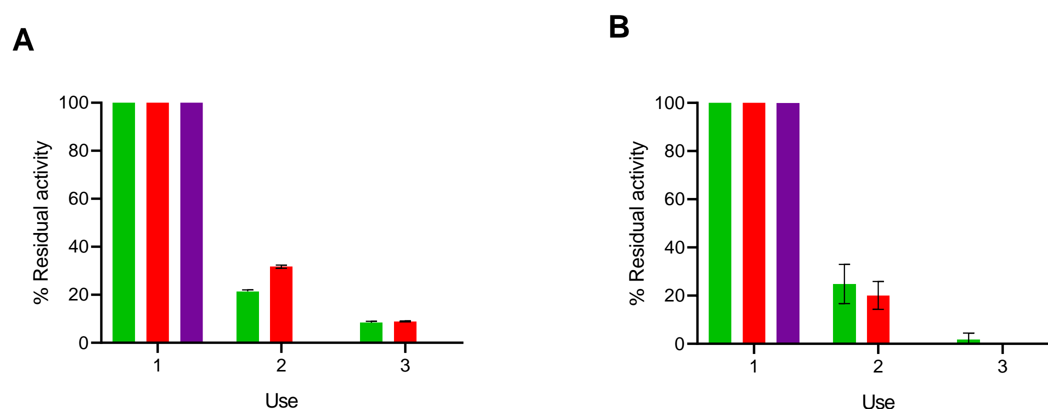

Figure S7. Reuse of the co-immobilised system in consecutive 23-hour cycles, starting from 543 mM of glycerol. DHA (green), serinol (red), pyruvate (purple).

Table S1. Activity of Pf-ATA towards different amino acceptors.

Amino acceptor	Amino donor	Activity (U/mL)	Relative activity (%)
Pyruvate	*rac*-phenyl-ethylamine	1.310 ± 0.050	100
Dihydroxyacetone	*rac*-phenyl-ethylamine	0.048 ± 0.004	4

All reactions were performed with 2 mM of amino acceptor, 2 mM of amino donor, 0.1 mM PLP in HEPES buffer 100 mM pH 8 at 30 °C.

References

1 A. Lapkin and D. J. C. Constable, Eds., *Green Chemistry Metrics*, John Wiley & Sons, Ltd, Chichester, UK, 2008.