Electronic Supplementary Material (ESI) for Green Chemistry.

Tuneable 3D printed enzymatic reactors for continuous-flow biotransformations

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1 Fabrication of 3D printed reactor device

This platform has a minimum layer thickness 75 microns to 500 microns when coupled with a 0.5mm extruder nozzle. The minimum build layer thickness, although beneficial in creating near net-shaped parts and finer surface roughness, is largely dependent on processing conditions as well as utilised materials. Building with a controlled heated bed enhances the control of warping as well as layer of glue stick to ensure bonding with build bed.

The reactor device for the immobilisation of enzymes was fabricated on an extrusion based 3D printing platform, Lulzbot Taz 5, using off the shelf, Nylon 618 filament. Fabricating on such a platform allows the process parameters, such as the nozzle temperature, build plate temperature, layer height and the deposition patterns to be easily configured. It is evident from previous studies that a canvas processing parameter cannot be applicable for all geometries even when employing similar materials. The geometry and size of the build as well as the material play an important role in choosing the processing parameters, hence an iterative approach was used in the fabrication of the bioreactor devices. Producing materials having a high melt temperature is challenging, as there is a larger temperature difference between the extrudate and the already cooled deposited polymer. The pulling effect towards the warmer region of the build coupled with shrinkages cause the parts to warp. A glue stick was used to ensure the Nylon filament adhered to the build bed.

As fabricating on the selected platform was an inexpensive process, an iterative approach was used to optimise the fabricated geometry close to the intended design. The main parameters modified during the iterative process are highlighted in Table S1, while the intended reactor to be fabricated is shown in Figure S 1. The reactor device was designed featuring a 1.5mm channel diameter with a volume of 0.8ml and an all-round dimension of 110 x 60 x 11 mm.

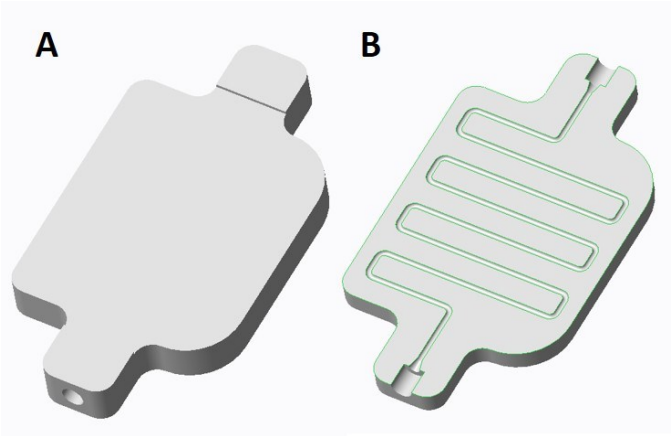
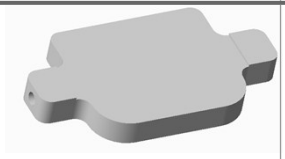
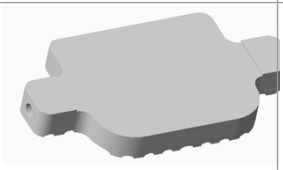
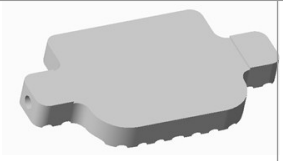
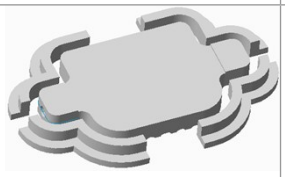
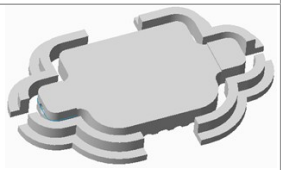


Figure S 1. (A) CAD model of 3D printed bioreactor (B) Section of the bioreactor device, showing the inner channel.

Table S 1 Optimisation of printing parameters for reactor manufacturing.

Entry	Print Conditions	CAD model	Fabricated part	Comments
1	Layer height: 0.3mm Nozzle temp: 235°C Build temp: 100°C			The designed reactor device was fabricated on the platform as designed without. The build warped and went off the build bed half way before completing. (Blocked reactor)
2	Layer height: 0.3mm Nozzle temp: 230oC Build temp: 100°C			'Track' were then added to the device to device, which aimed to break up the bottom layers and reduce warp. The build completed, however it still had a high degree of warpage causing the channels to block. (Blocked reactor)
3	Layer height: 0.3mm Nozzle temp: 230°C Build temp: 100°C			Increased the extraction flow rate of the fume cupboard to minimise build-up of temperature. This minimised warp and reduced the blistering effect see in the layers. (Blocked reactor)
4	Layer height: 0.3mm Nozzle temp: 230°C – 215°C Build temp: 100°C			Although the heated platform at 100 °C assisted adhesion of the build, this led to an over-heated bottom layers of the part noticed in previous builds. Two tactics were employed: (a) Edge temperature controllers around the part (b) A dynamic deposition temperature reduction from bottom layers to the top to compensate for the temperature of the build bed. (230-215 °C) (No blockage)
5	Layer height: 0.3mm Nozzle temp: 230°C – 215°C Build temp: 100 °C			Further increased the extraction flow rate of the fume cupboard with the parameters in test 4. Three further reactor devices were fabricated for experimentation using this condition without a blockages.

The final geometry obtained from the iterative process, Test 5, was then repeated three times. The devices were found to be leak free and similar. The track on the bottom of the print allowed for adhesion to the build bed, while the wall, designed around the build in test 4 & 5, assisted the retention of heat. This lead to the fabrication of a device with minimal warp.

The selected conditions were a layer height of 0.3mm, build and nozzle temperature of 100 °C and 230 – 215 °C respectively. The reactor was designed to a total volume of 0.8mL and 1.5mm diameter with a single inlet-outlet configuration, see Figure 4A. The volume after fabrication was 0.5mL, due to the reduction in diameter as a result of larger XY resolution on the platform.

2 Experimental methods

2.1 Enzyme expression and purification

ω-transaminases ATA117 ((*R*)-selective) and ATA256 ((*S*)-selective) were purchased from Codexis, CA. The (*S*)-selective ω-transaminase from *Silicibacter pomeroyi,* referred here by its PDB code 3HMU was expressed in *Escherichia coli* as described by Steffen-Munsberg *et al*.[1] The cell pellet was lysed by sonication on ice for 8 cycles of 15 s on, 30 s off in 50 mM Tris buffer pH 9.0, 0.3 M NaCl and 0.1 mM PLP, centrifuged for 30 min at 3 220 g at 4 °C and filtered through 0.45 µm filters and loaded onto Ni-NTA superflow (IBA, Germany) and purified by gravity flow. The column was washed with wash buffer (100 mM Tris buffer pH 9.0, 0.3 M NaCl, 0.1 mM PLP, 12.5 mM imidazole) and the enzyme was eluted with elution buffer (100 mM Tris buffer pH 9.0, 0.3 M NaCl, 0.1 mM PLP, 250 mM imidazole). The buffer was exchanged to 100 mM Tris buffer pH 9.0, 0.1 mM PLP by the use of Vivaspin columns (GE Healthcare, Sweden) with 10 kDA molecular weight cut off.

Protein concentration was measured using the Bradford method and purity was assessed by SDS-PAGE, where a single band of the expected size of 50 kDa was visible.

2.2 Chemical modification of Nylon and enzyme immobilisation

The protocol described in Table S2 was used to functionalise the small pieces of Nylon (1cm x 0.6cm x 0.2cm) and the sets of wells. The pieces of Nylon were submerged in 5 mL of the solutions described and washed using tweezers (to catch the pieces) and pipettes. The wells were filled with these solutions and also filled with the washing solutions. In the case of the wells, the modification was stopped depending on the row.

Table S 2 Protocol for surface modification and enzymes immobilisation on Nylon

Step	Treatment
1	3D printed nylon was incubated for 15 min. at r.t. in 5M HCl, then washed profusely with H_2O and 0.1M K phosphate buffer pH 8.0
2	Sample 1 was incubated for 2h at r.t. in Glutaraldehyde solution 25%(w/w) in H_2O. Then washed profusely with H_2O and 0.1M K phosphate buffer pH 8.0
3	Sample 2 was incubated for 2h at r.t. in 5%(w/v) polyethyleneimine in 0.1M K phosphate buffer pH 8.0. Then washed profusely with H_2O and 0.1M K phosphate buffer pH 8.0
4	Step 2 was repeated and solution left overnight. Then washed as in previous steps.
5	Sample 3 and/or 4 were incubated in enzyme solution at 4 ºC overnight. The enzyme concentration on 0.1M K phosphate buffer pH 8.0 was be 1 mg/mL. After, the samples were washed with H_2O and pH 8.0 buffer.
6	After enzyme immobilisation, the surfaces were washed for 2h at r.t. in a 0.5M NaCl solution in buffer. This is to eliminate non-covalently bonded enzyme.

The immobilization of the enzyme was performed incubating the polymer S4 with 5 mL solution of the enzyme (1 mg of enzyme/mL) in 0.1M K phosphate buffer at pH=8.0, with 0.1 mM PLP overnight at 4 ^{o}C. Afterwards, the piece was filtered-off and washed with the same phosphate buffer and a 0.5M NaCl solution in the same buffer to eliminate the enzyme immobilised by non-covalent interactions.

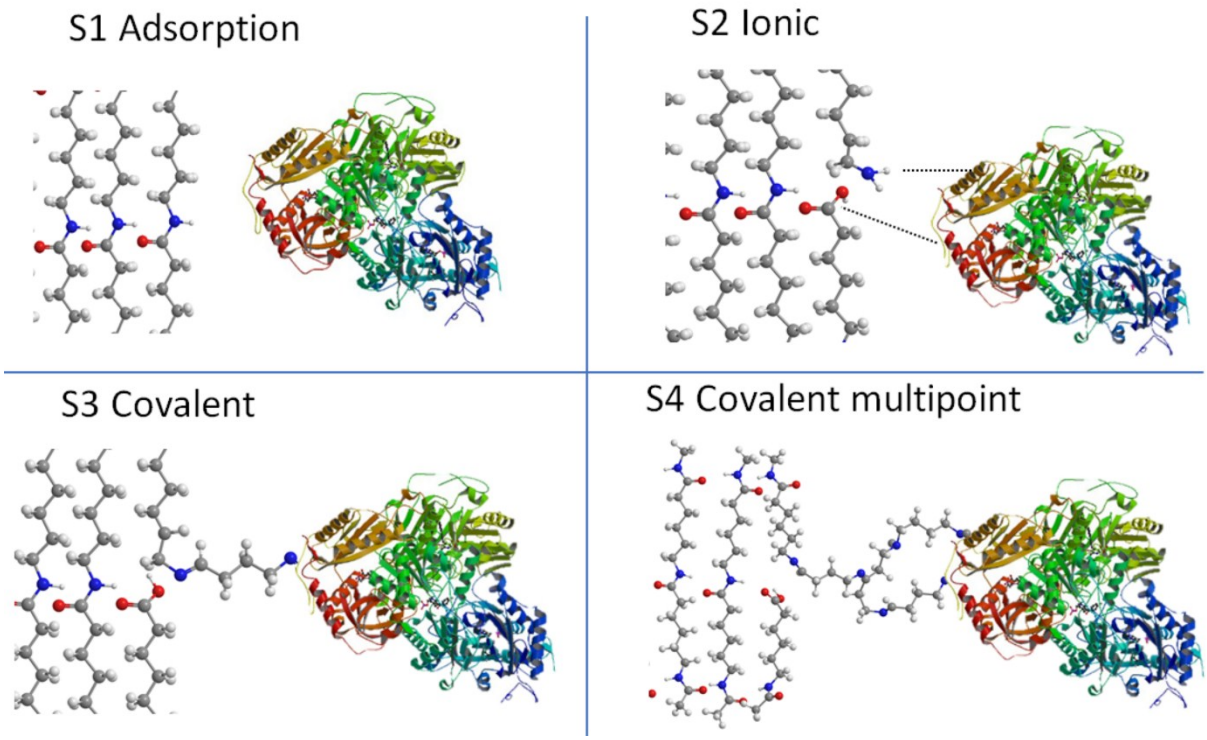
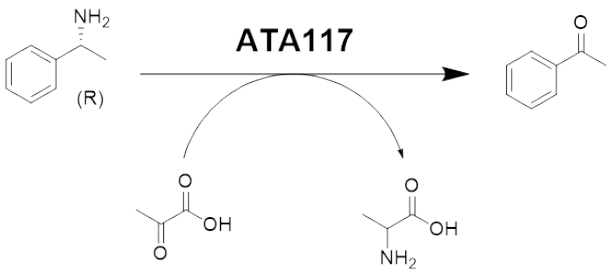


Figure S 2 Scheme of the different types of possible unions of the enzyme in the Nylon surface.

Covalent immobilization (S3) when the chemical modification was stopped after step 2 (Table S2). Step 6 (Table S2) was performed to eliminate S2 type unions.

2.3 Catalytic test with modified nylon part

The enzyme ATA117 was supported on a small Nylon piece as described on Table S2. After that, this piece was tested on the transformation of R-MBA in acetophenone to check if the enzyme still has activity once supported in Nylon.



Scheme S 1 Benchmark reaction employed to test the biocatalytic activity of immobilised w-TA on 3D printed parts

Table S 3 Results of the catalytical test with ATA117 immobilised on Nylon

Enzyme	Conversion (%)[e]			
state	Free[a]	Immobilised[b]	Residual[c]	Washing[d]
ATA117	100	22.6	65.5	-

The reactions were performed in batch. Conditions: 5mM R-MBA, 5mM Pyruvic acid, 0.1mM PLP, 30ºC, stirring, 17h. a) Free: the reaction was performed in 1ml of a 1mg/ml solution of fresh enzyme (the same concentration used for the immobilisation in step5 Table S.I.X). b) Immobilised: the treated piece of Nylon was submerged in 1ml of 0.1M K phosphate buffer pH 8.0. c) Residual: this was performed in 1 ml of the residual enzyme solution that remains after the immobilisation (step5 in Table S2.). This solution was initially 1mg/ml. d) Washing: this was performed in 1 ml of the NaCl 0.5M washing solution used in step6 Table S2 e) The samples were treated and analysed by GC as described in Analytical methods.

2.4 Analytical methods

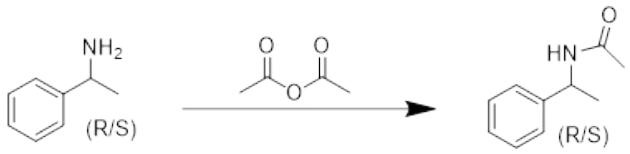
2.4.1 Nylon surface modifications

The surface modifications were followed by IR Alpha IR with a resolution of 5 cm^{-1}.

2.4.2 Catalytical test with pieces. Conditions and enzymes screening in wells.

The reactions were analysed by GC-FID Trace 1310 (Thermo Scientific) with CP Chiralsil Dex CB (25 m x 0.25 mm x 0.25 mm) column from Agilent). The samples were analysed after the following treatment: 100 µL of NaOH (till pH 12) and 750 µL of AcOEt were added to 1 mL of sample. Then it was centrifuged at 15000 g for 2 min. 600 µL of the organic phase were transferred to a MS vial. At this point, 10 µL of acetic anhydride and 10 µL of triethylamine were added to derivatise the MBA.

The samples were analysed by the following method: injector temperature 230°C, split ratio 1:10, continuous flow 1.7 ml/min; 40 °C for 2 min, 20 °C/min to 150 °C, hold 5 min, then 30 °C/min to 225 °C, then hold for 8 min, FID temperature 250°C. Helium was used as carrier gas.



Scheme S 2 Derivatisation of product for GC analysis

2.5 Continuous-flow set-up

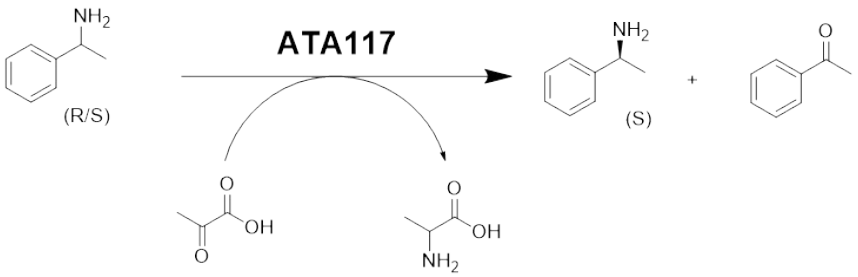
2.5.1 Functionalisation of the Nylon Reactor

i. The reactor was filled manually with HCl 5M using a syringe. The acid solution was left inside for 10 min at r.t.

ii. The HCl 5M solution was flushed out of the reactor also manually using a syringe full of air. Then the reactor was washed also manually with H_2O using a syringe. In this moment the pressure inside increased significantly and it was harder to pass more solvent through the reactor.
iii. The reactor was filled manually with a solution of Glutaraldehyde 25%(w/w) in H_2O using a syringe. This solution was left inside for 16h at r.t.
iv. The Glutaraldehyde solution was flushed out of the reactor also manually using a syringe full of air. Then the channel was washed with 0.1 M K phosphate buffer pH 8.0 using a HPLC pump at flow 0.01 ml/min for 5h.
v. 15 mL of ATA117 enzyme 5mg/ml solution in 0.1 M K phosphate buffer ph 8.0 (with 0.1 mM PLP cofactor) were prepared (75 mg of enzyme were used) and recirculated through the reactor channel at 0.025 ml/min using a HPLC pump at r.t. during 24h.
vi. 0.1 M K phosphate buffer pH 8.0 was passed finally through the channel also at 0.025 ml/min using a HPLC pump at r.t. for 6h in order to flush out the enzyme solution and leave the reactor full of buffer and ready to perform bioreactions inside.

The 3D printed reactor was connected to a pump and filled with HCl 5M and the flow was stopped for 10 min. After this time, deionized water was pumped through to wash out all the acid until the water at the outlet of the reactor gave a neutral pH. Then, the reactor was further washed with a buffer solution 0.1 M of potassium phosphate buffer at pH=8.0. Then, the reactor with a solution of glutaraldehyde 25%(w/w) in H_2O to generate a aldehyde-based reactive reactor surface. Once filled it, the flow was stopped and the modification was allowed for 16h at r.t.. After, the reactor was washed with a buffer solution.

2.5.2 Enzymatic enantiomeric resolution of MBA under continuous flow conditions



Scheme S 3 Benchmark reaction for the kinetic resolution of rac-methylbenzylamine. Reaction mixture: 65 µL of MBA (0.5 mmol), 36 µL of pyruvic acid (0.5 mmol) and 2.5 mg of PLP (0.01 mmol) were solved in 100 mL of 0.1 M K phosphate buffer pH 8.0

Table S 4 Results for the enzymatic enantiomeric resolution of MBA under continuous flow conditions.

Time (h)	Flow (ml/min)	Conversion (%)	%ee
2	0.025	87/44	**77**
5	0.025	96/48	**93**
11	0.01	99.9/49.9	**99.9**
19	0.01	99.9/49.9	**99.9**
25	0.01	99.9/49.9	**99.9**
Stored in buffer at 4 °C for 48 h.			
28	0.01	88/44	**78**
40	0.01	96/48	**92**
48	0.01	99.9/49.9	**99.9**
66	0.01	97/48	**95**
74	0.01	98/49	**96**
90	0.01	98/49	**95**
96	0.01	97/48	**94**

Conditions: 5mM MBA, 5mM pyruvic acid, 0.1mM PLP, r.t., Reactor volume: 0.5ml.

Samples treatment: All the samples were basified with NaOH 2M till pH 12 and then extract with AcOEt (1 ml/ml of sample). The organic phase was dried with $MgSO_4$ anhydrous.

Samples analysis: The samples were analysed by HPLC (Merck HITACHI LaChrom). Conditions: Daicel Chiralcel OD-H, n-hexane/ethanol (99/1), 1ml/min, 210nm, 25ºC, 25µl of sample. Retention times: acetophenone 7.32 min, R-MBA 15.05 min, S-MBA 17.30 min.

2.5.3 HPLC spectra

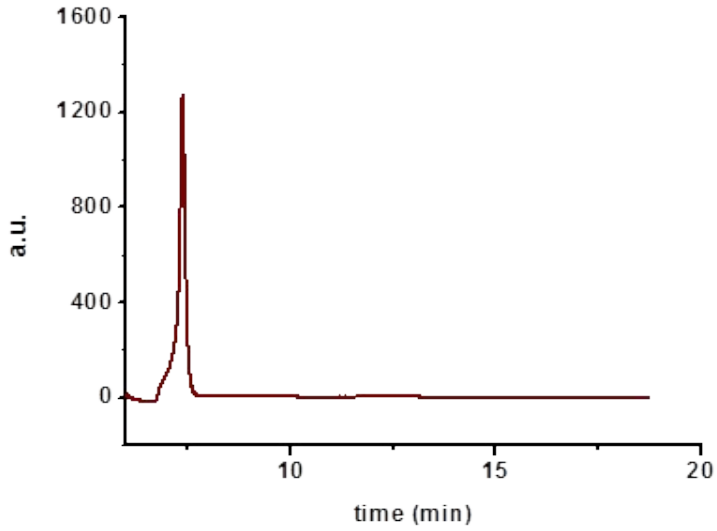


Figure S 3 HPLC spectra of acetophenone 2.5 mM

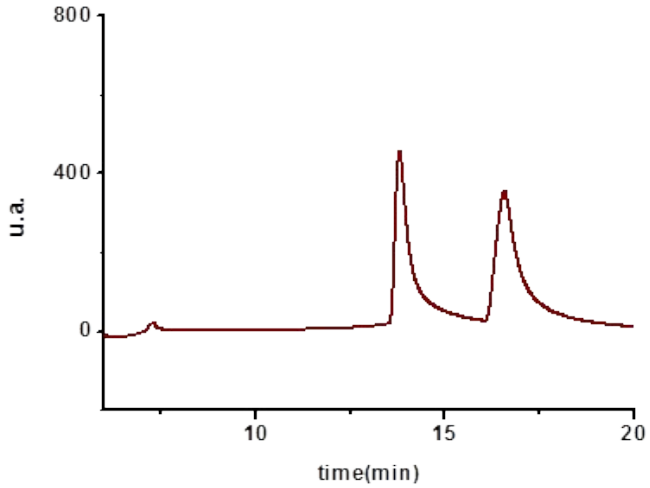


Figure S 4 HPLC spectra of the initial reaction mixture

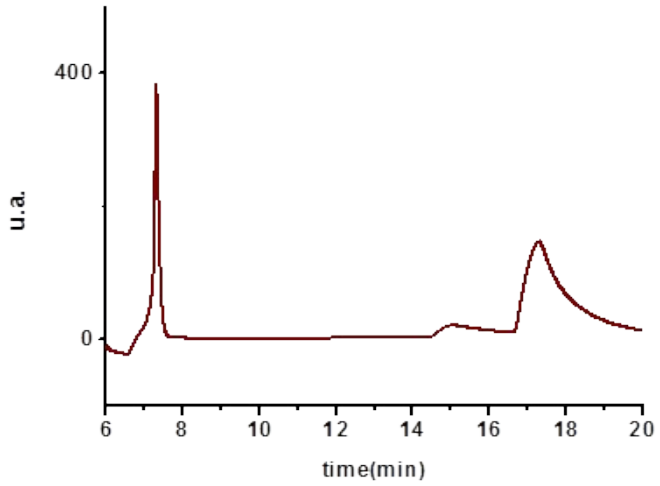


Figure S 5 HPLC spectra of the reaction at flow 0.025 ml/min

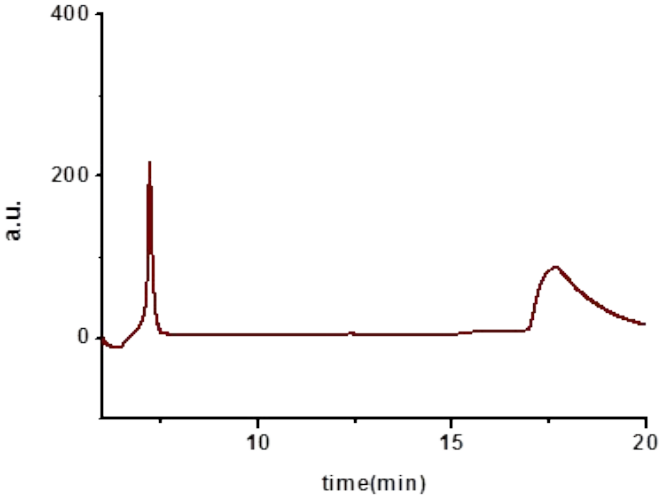


Figure S 6 HPLC spectra of the reaction at flow 0.01 ml/min

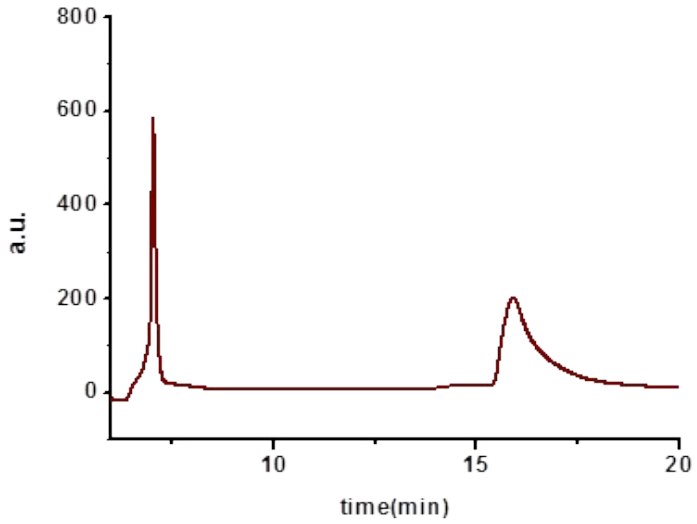


Figure S 7 HPLC spectra of the reaction when reuse the reactor at flow 0.01 ml/min

3 References

[1] F. Steffen-Munsberg, C. Vickers, A. Thontowi, S. Schätzle, T. Tumlirsch, M. Svedendahl Humble, H. Land, P. Berglund, U. T. Bornscheuer, M. Höhne, *ChemCatChem* **2013**, *5*, 150-153.