# The continuous oxidation of HMF to FDCA and the immobilisation and stabilisation of periplasmic aldehyde oxidase (PaoABC)

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## Electronic Supporting Information

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## 1.0 General experimental information and materials

Chemically competent *E. coli* BL21 Star™ (DE3) cells for expression of GOase variants $M_{3-5}$ were purchased from Invitrogen and transformed according to the manufacturer's protocol. The empty vector pET-30a used for cloning of GOase enzymes originates from Novagen. The *E. coli* TP1000 mutant strain (kindly provided by Professor Palmer of Dundee University, UK) used for PaoABC expression is a derivative of MC4100 with a kanamycin cassette inserted in the mobAB gene region. Horseradish peroxidase (HRP), catalase and chemicals were sourced from Sigma-Aldrich unless otherwise stated. Cell lysis was performed by sonication using a Soniprep 150 (MSE UK Ltd.)

## 2.0 Preparation of biocatalysts

### 2.1 Galactose oxidase enzyme variants $M_3$ and $M_{3-5}$

GOase mutants[1] were transformed into *E. coli* BL21 Star™ (DE3) cells (Invitrogen) according to manufacturer's specifications. A single colony was picked from an overnight LB plate containing 30 µg/mL kanamycin and used to inoculate 5 mL LB medium supplemented with 5 µL kanamycin and grown overnight at 37°C and 250 rpm. 500 µL of the overnight culture was used to inoculate 250 mL of an autoinduction medium (8ZY-4LAC) as described by Deacon and McPherson[2] and supplemented with 250 µL of kanamycin in a 2-L-baffled Erlenmeyer flask. The cells were grown at 26°C and 250 rpm for 60 h. Cells were harvested by centrifugation at 6000 rpm and 4°C for 20 min and subsequently prepared for protein purification.

### 2.2 *E. coli* Perisplasmic Aldehyde Oxidase (PaoABC)

For PaoABC expression,[3] the plasmid pMN100 derived from pTrcHisA (Invitrogen), containing the PaoABC genes with a His6 tag fused to the N-terminus of PaoA, was used. For heterologous expression in *E. coli*, pMN100 was transformed into *E. coli* TP1000 cells, containing a deletion in the mobAB genes responsible for Moco dinucleotide formation. One liter of LB supplemented with 1 mM sodium molybdate and 20 mM isopropyl thio-β-D- galactoside and 150 mg/mL ampicillin was inoculated with 2 mL of an overnight culture and incubated for 24 h at 37 °C and 130 r.p.m. The cells were harvested by centrifugation at 4000 g at 4°C for 20 min.

### 2.3 Purification of GOase enzyme variants $M_3$ and $M_{3-5}$

The cell pellet from a 250-mL-culture was resuspended in 30 mL lysis buffer containing 50 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), 25 % sucrose (w/v), 1 mg/mL lysozyme, 5 mM $MnCl_2$ and 1 % Triton X-100 (v/v). The suspension was gently shaken at 4°C for 20 min. Afterwards, cells were mechanically disrupted *via* ultrasonication (30 s on, 30 s off; 20 cycles) followed by ultracentrifugation (20000 x *g*, 30 min, 4°C). The cleared crude extract was transferred into a flexible tubing (30 kDa cut-off), dialysed into buffer C (50 mM NaPi buffer, 300 mM NaCl, pH 8.0) for 12 h at 4°C and subsequently passed through a syringe filter with a 0.22 µm pore size. Protein purification was accomplished with a peristaltic tubing pump (Thermo Scientific) equipped with a 5-mL-Strep-Tag®-II column (GE Healthcare) pre-equilibrated with buffer C. After loading with crude extract, the column was washed with 5 column volumes of buffer C followed by protein elution with 70 mL of buffer D (50 mM NaPi buffer, 300 mM

NaCl, 5 mM desthiobiotin, pH 8.0). For copper-loading, GOase-containing fractions were pooled, concentrated and subsequently transferred into flexible dialysis tubing (30 kDa cut-off) and dialysed for 12 h into buffer E (50 mM NaPi buffer saturated with $CuSO_4$, pH 7.4) at 4°C. Removal of excess $CuSO_4$ was attained by two cycles of dialysis into buffer E (without $CuSO_4$) for 12 h at 4 °C and protein samples concentrated to approximately 3 mg/mL using a Sartorius Vivaspin 6 spin column (30 kDa mass cut-off). The protein samples were aliquoted and aliquots were frozen in liquid nitrogen prior to storage at -80°C.

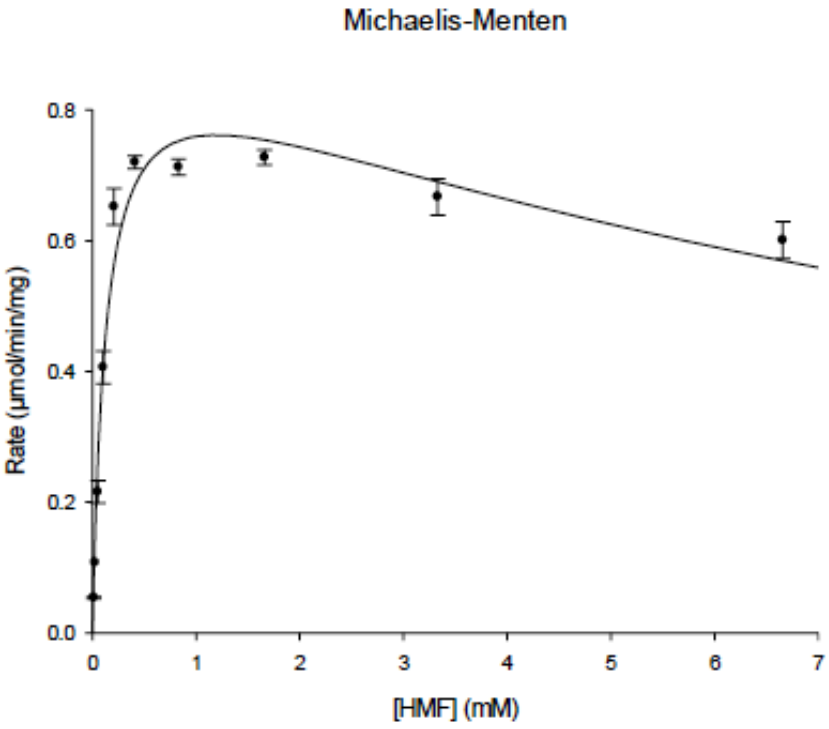
**2.4 Purification of PaoABC**

The cell pellet was resuspended in 8 volumes of 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, 10 mM Imidazole and cell lysis was achieved by sonication (MSE Soniprep) with cooling on ice (20 bursts of 20s on/off at 14u). After addition of DNase I, the lysate was incubated for 30 min at 30$^{o}$C with shaking. After centrifugation at 17000 x *g* for 25 min at 4$^{o}$C, supernatant was filtered though 0.45 µM and 0.2 µM sterile filters before loading onto $Ni_2$-nitrilotriacetic agarose HiTrap 1mL column (GE Healthcare). The column was washed with 2 column volumes of 10 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, followed by a wash with 10 column volumes of the same buffer with 20 mM imidazole. His-tagged PaoABC was eluted with 20 mL of 100 mM imidazole in 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Fractions containing PaoABC were buffer exchanged using a PD-10 column into 50 mM Tris, 1 mM EDTA, pH 7.5. The yield of protein was about 12.5 mg/L of *E. coli* culture.

**2.5 Enzyme Kinetics PaoABC.**

The initial rates were followed on a TECAN Infinite M200 spectrophotometer at 25 °C and 510 nM. A 1:1 ratio was assumed in the oxidation of substrate : production of hydrogen peroxide. The rate of production of hydrogen peroxide was detected by HRP (Type VI, Sigma) and 4-amino antipyrine/2,4,6-tribromo-3-hydroxybenzoic acid dye (ε = 29400 L mol-1 cm-1). The dye was made up by adding 100 µl 4-amino antipyrine (100 mg / mL) and 30 µL (20 mg / mL) 2,4,6-tribromo-3-hydroxybenzoic acid to 10 mL of pH 7.0 potassium phosphate buffer. Substrates were dissolved in DMF to 1 M then diluted in buffer. Typically 10 different substrate concentrations were examined. HRP was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, to give 0.2 mg / mL concentration.

To a 96 well plate, 75 µL substrate, 75 µL HRP and 75 µL dye were added. The assay was started by adding 50 µL purified enzyme (0.5 mg/mL). Rate was plotted against substrate concentration and Vmax and Km values extracted using non-linear regression analysis with a fit to the equation Vmax.[S]/Km+[S]. Note that kinetic parameters reported are apparent since a detailed kinetic analysis of each intermediate and dissolved oxygen was beyond the scope of this work and will be part of a forthcoming study to be reported separately.




Vmax = 0.9273
Km = 0.1296
Ki = 10.9

05/04/2016 11:37:20
Substrate Inhibition (Uncompetitive)
Number of Replicates: 3

**Parameters**

| | Value | ±Std. Error | 95% Conf. Interval | | |
|---|---|---|---|---|---|
| Vmax | 0.9273 | 0.0445 | 0.8360 | to | 1.0186 |
| Km | 0.1296 | 1.732e-2 | 9.407e-2 | to | 0.1651 |
| Ki | 10.9270 | 2.3252 | 6.1559 | to | 15.6980 |

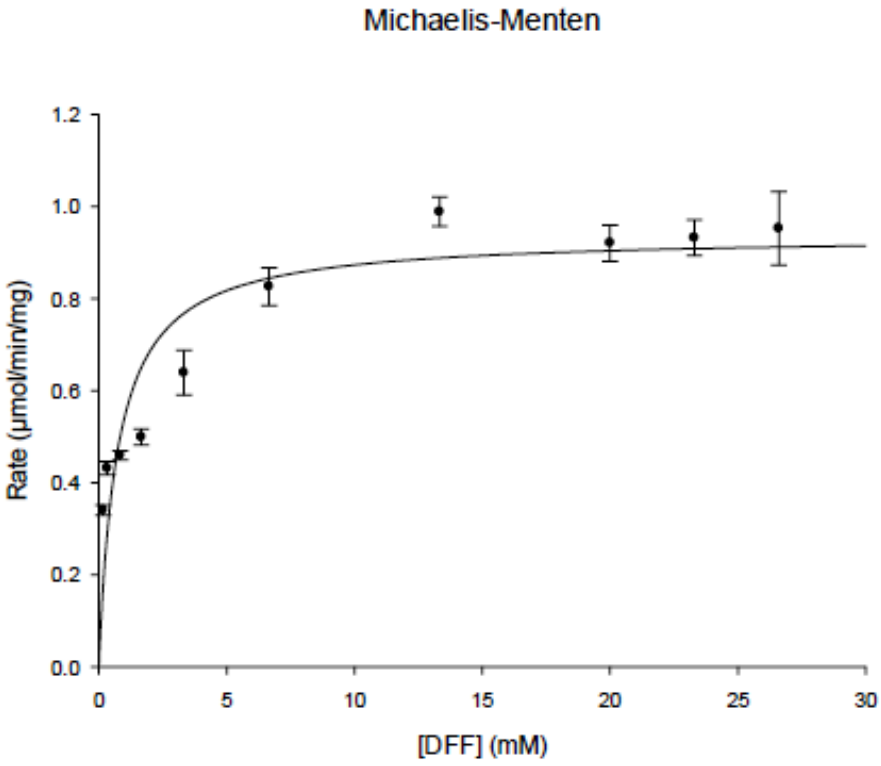
**Goodness of Fit**

| | |
|---|---|
| Degrees of Freedom | 27 |
| AICc | -169.388 |
| $R^2$ | 0.961 |
| Sum of Squares | 7.692e-2 |
| Sy.x | 5.337e-2 |
| Runs Test p Value | 0.034 |

**Data**

| | |
|---|---|
| Number of x values | 10 |
| Number of replicates | 3 |
| Total number of values | 30 |
| Number of missing values | 0 |

**Figure S1** Enzyme Kinetics of PaoABC with HMF




Vmax = 0.9368
Km = 0.7326
Ki = 8.262e+5

Substrate Inhibition (Uncompetitive)
Number of Replicates: 3

**Parameters**

| | Value | ±Std. Error | 95% Conf. Interval | | |
|---|---|---|---|---|---|
| Vmax | 0.9368 | 9.291e-2 | 0.7462 | to | 1.1275 |
| Km | 0.7326 | 0.2250 | 0.2708 | to | 1.1944 |
| Ki | 8.262e+5 | 3.281e+9 | -6.731e+9 | to | 6.733e+9 |

**Goodness of Fit**

| | |
|---|---|
| Degrees of Freedom | 27 |
| AICc | -120.309 |
| $R^2$ | 0.780 |
| Sum of Squares | 0.395 |
| Sy.x | 0.121 |
| Runs Test p Value | 0.132 |

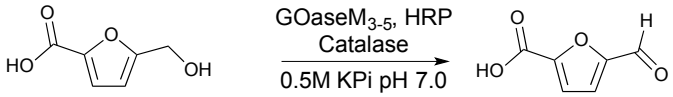
**Data**

| | |
|---|---|
| Number of x values | 10 |
| Number of replicates | 3 |
| Total number of values | 30 |
| Number of missing values | 0 |

**Figure S2** Enzyme Kinetics of PaoABC with DFF

## 3.0 Developing a continuous oxidation of HMF to FDCA

### 3.1 HMFCA oxidation by GOase$M_{3-5}$




HMFCA **3** FFCA **4**

**Table S1**

| Entry[a] | [HMFCA] (mM) | GOase$M_{3-5}$ (3.0mg/mL) | Catalase (3.3mg/mL) | HRP (1mg/mL) | Conversion (%)[b] |
|---|---|---|---|---|---|
| 1 | 50 | 103 µL | 33 µL | - | 100 |
| 2 | " | " | '' | 70 µL | 100 |

[a]Reaction conditions: Final volume 0.3 mL, KPi pH 7.0 0.2 M, 37°C, 1 hr. [b]Conversion calculated by RP-HPLC and adjusted with a 1:1:1 standard of HMF:DFF:FFCA *HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.

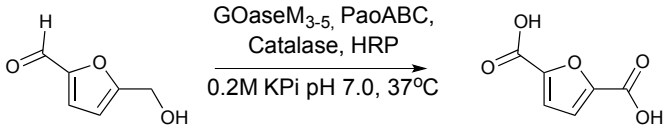
### 3.2 Screening of additional GOase Variants for the oxidation of HMF and HMFCA

**Table S2**

| Entry[a] | Enzyme | Substrate | HRP (1mg/mL) | Catalase (3.3mg/mL) | Conversion to DFF/FFCA [b] (%) |
|---|---|---|---|---|---|
| 1 | $M_3$ | HMF | 70 µL | 33 µL | 50.5 |
| 2 | $M_{3-5}$ | " | " | " | 71 |
| 3 | $M_3$ | HMFCA | " | " | 81 |
| 4 | $M_{3-5}$ | " | " | " | 91 |

[a]Reaction conditions: Final volume 0.3 mL, KPi pH 7.0 0.2M, 50 mM Substrate, 37°C, 30min, [b] Conversion calculated by RP-HPLC and adjusted with a 1:1:1:1:1 standard of HMF:DFF:FFCA:HMFCA:FDCA. *HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.

### 3.3 Continuous oxidation of HMF to FDCA.




HMF **1** FDCA **5**

### HPLC Timecourses for Table 2 in Manuscript

*HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6mL/min

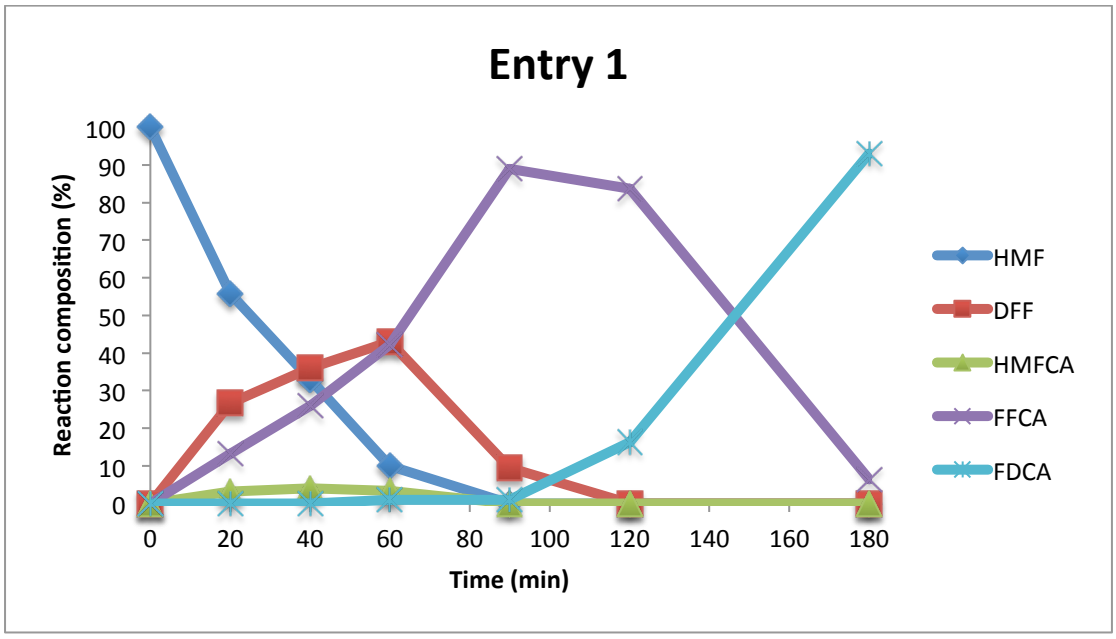



**Figure S3** Time course analysis of entry 1

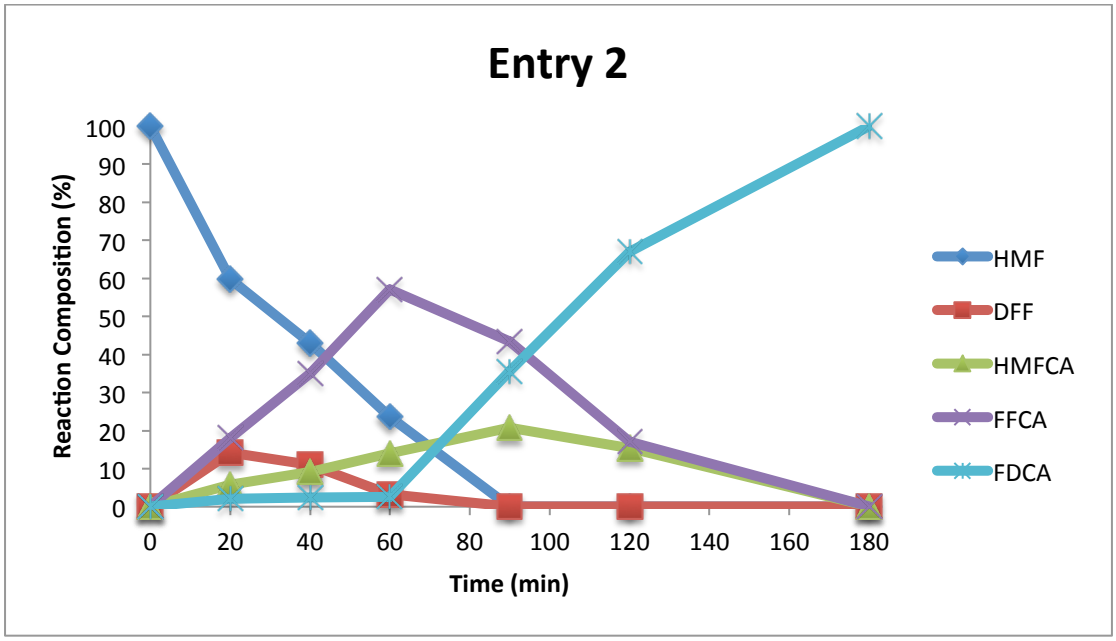



**Figure S4** Time course analysis of entry 2

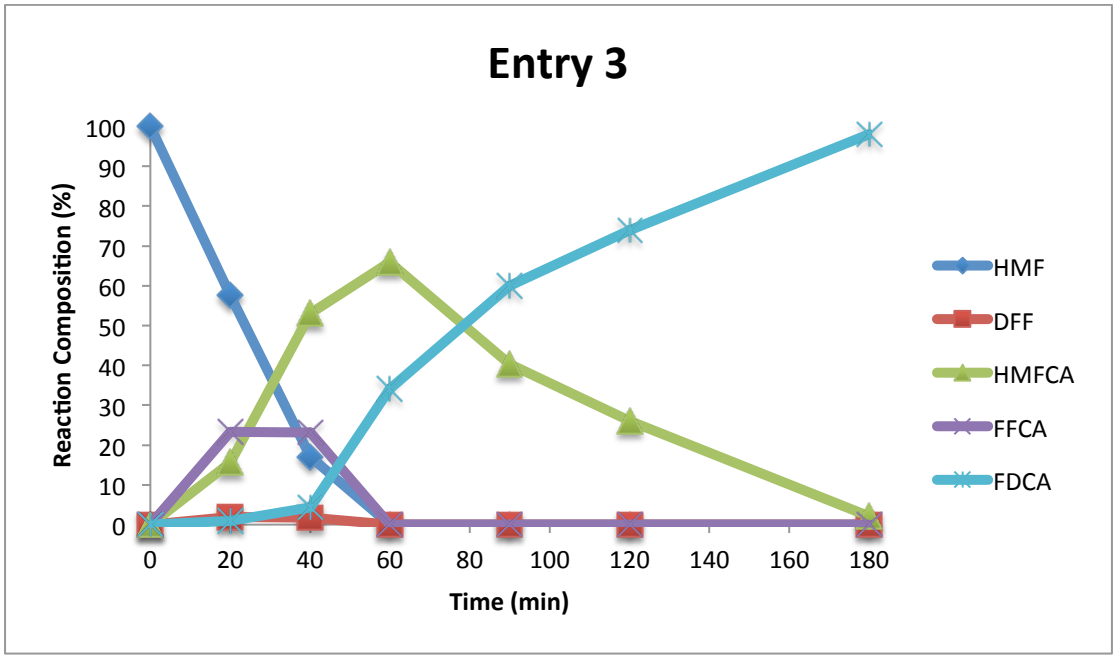



**Figure S5** Time course analysis of entry 3

**3.4 Experimental for the 100mM continuous oxidation of HMF to FDCA using PaoABC, GOase$M_{3-5}$, Catalase and HRP.**

To 69 µL of 0.2M KPi pH 7.0 was added 33 µL catalase (3.3 mg/mL), 1 µL PaoABC (28.9 mg/mL), 70 µL HRP (1 mg/mL). 130 µL GOase$M_{3-5}$ (3.0 mg/mL) was then added to a final volume 300 µL. All enzymes were prepared in 0.2 M KPi, pH 7.0. HMF (37 mg) was added and the solution left in a shaking incubator at 37$^{o}$C for 6hr. During this time the pH was kept to 7.0 by titrating with 2 M NaOH. For analysis, 5 µL of the reaction mixture was extracted, diluted with 80 µL water and quenched with 15 µL 1 M HCl. The aliquots were analysed by RP HPLC. After the reaction was complete, the Eppendorf was heated at 80$^{o}$C and centrifuged for 10min. The reaction was placed in ice and 3 drops of conc HCl was added to precipitate the FDCA. The reaction was centrifuged again and the supernatant discarded. FDCA was washed twice with 0.5 M HCl and heat dried yielding an off white powder in 85% yield.

*HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.

## 4.0 Immobilisation of PaoABC

### 4.1 Carrier-Free immobilisation procedure (CLEA)

PaoABC Aggregation studies - effect of aggregation on PaoABC activity

90µl of the cooled aggregating agent (see Figure S6) was added to 10 µL PaoABC (28 mg/mL) and left in ice for 30mins. The resulting solution was centrifuged at 10,000 rpm at 4°C for 10 mins to aid aggregation. The supernatant was then removed. In a separate eppendorf, 3 µL of benzaldehyde (1 M in MeCN), 33 µL catalase (3.3 mg/mL) and 264 µL 50 mM potassium phosphate buffer pH 7.5 was prepared and then subsequently added to the aggregated enzyme and vigorously shaken. The reaction was left for 15 mins before being quenched by 50 µL 1M HCl, filtered and analysed by RP-HPLC. *HPLC conditions* = ODS-Hypersil C-18 column, flow rate = 1mL/min, 70% water + 0.1% TFA, 30% MeCN.

Catalase Aggregation Studies - effect of catalase aggregation on ability to protect PaoABC activity

90 µL of the cooled aggregating agent (IPA, DIOX, t-But, MeCN, Acetone) was added to 10 µL catalase (9.9 mg/mL) and left in ice for 30mins. The resulting solution was centrifuged at 10,000rpm at 4°C for 10 mins to aid aggregation. The supernatant was removed. 3 µL of benzaldehyde (1 M in MeCN) and 295 µL 50mM potassium phosphate buffer pH 7 was added to the aggregated enzyme and vigorously shaken. 2 µL of PaoABC (28.9 mg/mL) was then added and the reaction left for 60mins before being quenched by 50 µL.1 M HCl, filtered and analysed by RP-HPLC. *HPLC conditions* = ODS-Hypersil C-18 column, flow rate = 1mL/min, 70% water + 0.1% TFA, 30% MeCN.

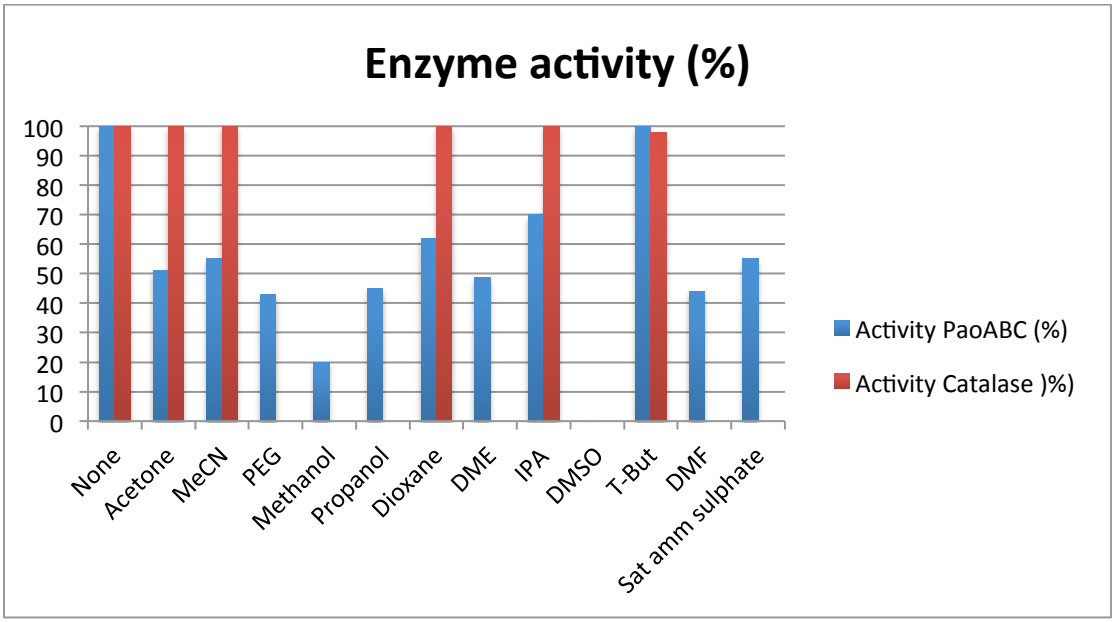


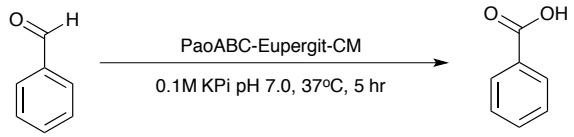

**Figure S6**: Enzyme activity of PaoABC and catalase after aggregation

## 4.2 Immobilisation of PaoABC on epoxy based resins.

### 4.2.1 Immobilisation of PaoABC on Eupergit CM

1 mg of PaoABC was dissolved in 1 mL 1M KPi buffer pH X. The concentration before immobilisation was recorded by UV detection. 10 mg of Eupergit CM was added and the sample was left in a shaking incubator at 150 rpm, 25°C for 24hr. The amount of enzyme absorbed onto the resin was determined by UV spectroscopy (428 nm). Blocking buffer (0.2 M ethanolamine in 0.1 M KPi pH 7) was added and left to shake for 30 minutes. The immobilised enzyme was filtered through a sinter funnel and washed with KPi Buffer pH 7 and, KPi buffer pH 7 with 1 M NaCl. The immobilised beads were tested for activity.

The amount of Eupergit CM required to give a 0.1 mg PaoABC concentration was resuspended in 500 µL 0.1M KPi pH X and 100 mM benzaldehyde was added and left in a shaking incubator for 5 hr at 37°C. The reaction was then acidified and analysed by RP-HPLC. *HPLC conditions* = ODS-Hypersil C-18 column, flow rate = 1 mL/min, 70% water + 0.1% TFA, 30% MeCN.




**6** **7**

**Table S6** pH screen for optimal enzyme bonding to Eupergit CM.

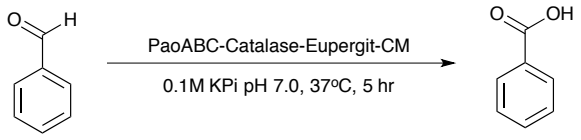
| Entry[a] | pH | PaoABC (mg/mL) | PaoABC Immobilized (mg) | Conversion (%)[b] |
|---|---|---|---|---|
| 1 | 6 | 0.85 | 0.7 | 44 |
| 2 | 7 | 1 | 0.97 | 48 |
| 3 | 8 | 0.94 | 0.8 | 60 |

[a]Reaction conditions: 0.1 mg PaoABC (On resin), 2.5 µL benzaldehyde, 500 µL 100mM KPi pH X, 37°C, 5Hr [b] Conversion calculated by RP-HPLC. *HPLC conditions* = ODS-Hypersil C-18 column, flow rate = 1 mL/min, 70% water + 0.1% TFA, 30% MeCN.

### 4.2.2 Co-immobilisation of PaoABC (1mg) and catalase (2mg) on Eupergit-CM

1 mg of PaoABC + 2 mg catalase was dissolved in 1 mL 1M KPi buffer pH X. The concentration before immobilisation was recorded by UV detection. 10 mg of Eupergit EC was added and left in a shaking incubator at 150 rpm, 25°C for 24 hr. The amount of enzyme absorbed onto the resin was determined by UV spectroscopy (428nm). Blocking buffer (0.2 M ethanolamine in 0.1 M KPi pH 7) was added and left to shake for 30 min. The immobilised enzyme was filtered through a sinter funnel and washed with KPi Buffer pH 7 and, KPi buffer pH 7 with 1 M NaCl. The immobilised beads were tested for activity.

The amount of Eupergit CM required to give a 0.1 mg PaoABC concentration was resuspended in 500 µL 0.1 M KPi pH 7.0 and 100 mM benzaldehyde was added and left in a shaking incubator for 5 hr at 37°C. The reaction was then acidified and analysed by RP-HPLC. *HPLC conditions* = ODS-Hypersil C-18 column, flow rate = 1 mL/min, 70% water + 0.1% TFA, 30% MeCN.




**6** **7**

**Table S7** Screening of co-immobilised catalase and PaoABC on eupergit CM.

| Entry[a] | Carrier | PaoABC (mg/mL) | PaoABC immobilized (mg) | Conversion (%)[b] |
|---|---|---|---|---|
| 1 | Eupergit CM pH 6 | 0.85 | 0.7 | 80 |
| 2 | Eupergit CM pH 7 | 1 | 0.97 | 82 |
| 3 | Eupergit CM pH 8 | 0.94 | 0.8 | 71 |

[a]Reaction conditions: 0.1 mg PaoABC (On resin), 2.5 µL benzaldehyde, 500 µL 100 mM KPi pH 7.0, 37°C. [b] Conversion calculated by RP-HPLC. *HPLC conditions* = ODS-Hypersil C-18 column, flow rate = 1 mL/min, 70% water + 0.1% TFA, 30% MeCN.

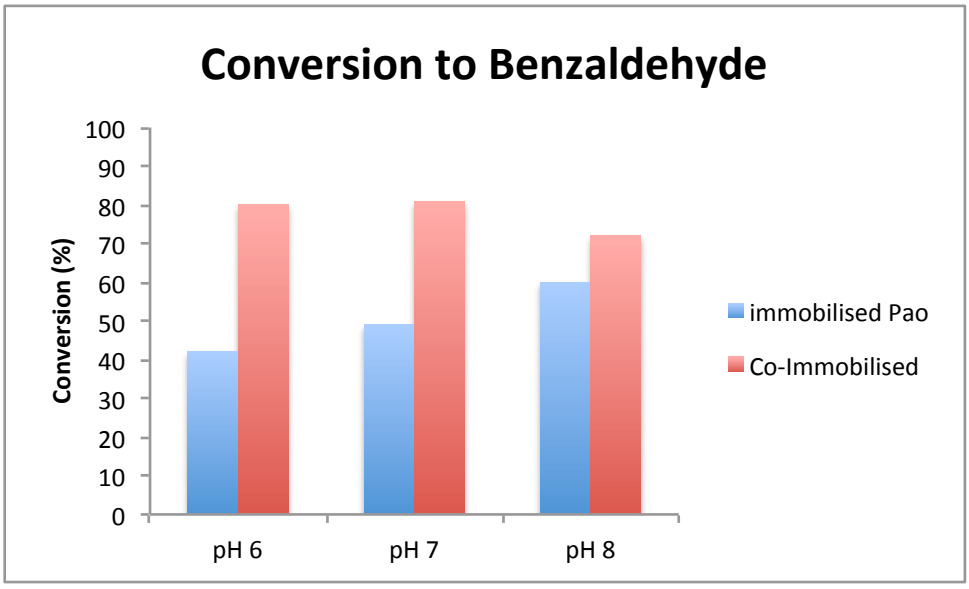
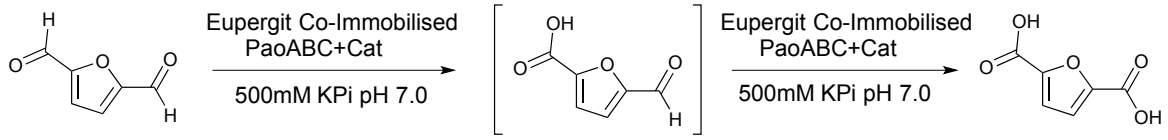



**Figure S7** Immobilised PaoABC vs Co-immobilised PaoABC + Catalase for the oxidation of benzaldehyde to benzoic acid.

### 4.2.3 PaoABC + Catalase co-immobilized on Eupergit CM

10 mg of co-immobilised PaoABC + catalase-Eupergit CM was added to 500 µL 0.5M KPi pH 7.0. DFF was then added to a final concentration of 50mM. The reaction was then placed in a shaking incubator at 37°C and left for 3 hours. After this time, a 10 µL aliquot of the reaction was extracted, diluted with 80 µL water and acidified with 15 µL 1M HCl. The aliquot was centrifuged and analysed by RP HPLC. To recycle the catalyst, the reaction was centrifuged at 100 rpm and the supernatant removed. The pelleted beads were washed twice with water before being being subjected to reaction conditions *HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6mL/min.




DFF **2** FFCA **4** FDCA **5**

**Table S8** Recyclability of co immobilised PaoABC + Catalase.

| Entry[a] | Recycle number. | DFF (%) | FFCA (%) | FDCA (%) |
|---|---|---|---|---|
| 1 | 1 | 0 | 0 | 100 |
| 2 | 2 | 20.2 | 77.7 | 2 |
| 3[c] | 2 | 0 | 0 | 100 |
| 4[c] | 3 | 0 | 52 | 47 |
| 5[c] | 4 | 0 | 100 | 0 |

[a] Reaction conditions: 10 mg Eupergit CM co-immobilized PaoABC + Catalase, 500 µL 500mM KPi pH 7, 37°C, 50mM DFF, 7hr [b] Conversion calculated by RP-HPLC and adjusted with a 1:1:1:1:1 standard of HMF:DFF:FFCA:HMFCA:FDCA *HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6mL/min [c] additional portion of catalase (30 µL of 3.3 mg/ml solution) added.

- PaoABC + catalase immobilised on the resin is not thermostable as the enzyme loses activity after being left overnight at 37 degrees
- Catalase co-immobilised on the resin loses activity after one reaction (Entry 2)
- Additional portion of catalase is required after every reaction (Entry 3)
- PaoABC may be deactivated as catalase loses activity

### 4.2.4 Optimised Immobilisation of PaoABC on Eupergit-CM and additional epoxy supports EC-HFA/S and EC-HFA/M via multipoint attachment and recyclability.

1 mg of PaoABC was dissolved in 10 mL of 1 M potassium phosphate buffer pH 7.0. 100 mg of Eupergit CM was then added and left in a shaking incubator at 25°C for 16 hours. The pH was then raised to pH 8.5 by the addition of 2 M NaOH to facilitate multipoint attachment to the resin. This was left for a further 5 hours. After this time the reaction was filtered and washed with 0.1M KPI pH 7.0 and 0.1M KPi pH 7.0 with 1M NaCl to remove any absorbed protein. After the resin was dry it was blocked with 3M glycine for 16hr hours. The resin was again filtered and washed with deionized water and stored in the fridge.

1 mg of PaoABC was dissolved in 10 mL 10mM KPi buffer pH 7. The concentration before immobilisation was recorded. 100 mg of EC-HFA/S or EC-HFA/M was added and left in a shaking incubator at 150 rpm, 25°C for 24hr. The amount of enzyme absorbed onto the resin was determined by UV spectroscopy (428 nm). After this time the pH was increased to pH 8.5 to facilitate multipoint attachment to the resin (5 hr). Blocking buffer (3 M Glycine pH 8.5) was added and left shake for 5 hours. The immobilised enzyme was filtered through a filter paper and washed with 0.1 M KPi Buffer pH 7 and, 0.1 M KPi buffer pH 7 with 1M NaCl. The immobilised beads were tested for activity.

The amount of epoxy based support required to give a 0.5 mg PaoABC concentration was resuspended in 470 µL 0.1M KPi pH 7.0, 5 mg CAT-CLEA (see section 4.3 for procedure) and 50 mM DFF was added and left in a shaking incubator for 5 hr at 25°C. After this time, a 10 µL aliquot of the reaction was extracted, diluted with 80 µL water and acidified with 15 mL 1 M HCl. The aliquot was centrifuged and analysed by RP HPLC. To recycle the catalyst, the reaction was centrifuged at 100rpm and the supernatant removed. The pelleted beads were washed twice with water before being being subjected to reaction conditions. *HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.

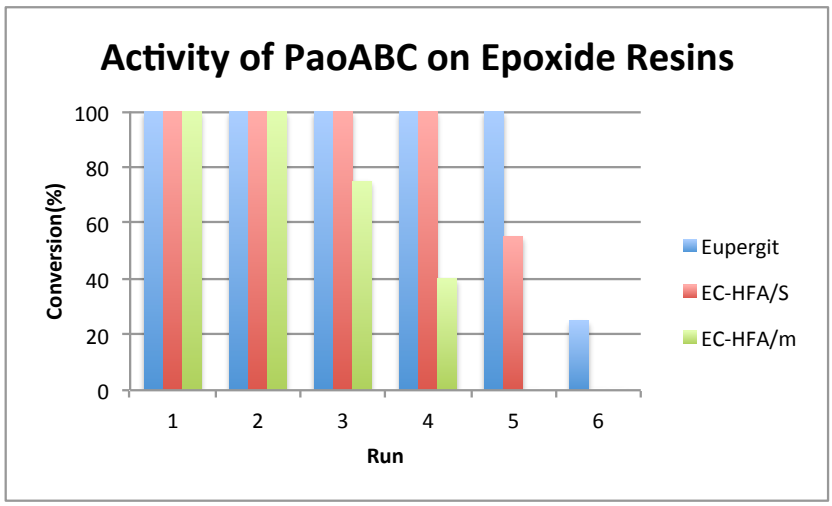



**Figure S8** Recyclability of PaoABC on Eupergit based supports with added CAT-CLEA.

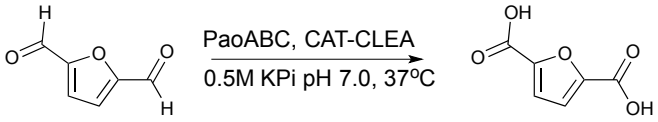
### 4.3 Cross-linked catalase to increase stability in the oxidation of DFF to FDCA

#### 4.3.1 Cross-linked enzyme aggregate (CLEA) of catalase

CAT-CLEAs were prepared by dissolving catalase in potassium phosphate buffer (100 mM, pH 7.0) to a final protein concentration of 40 mg/mL. Protein aggregation was induced by mixing 1 mL of the enzymatic solution and 1 mL of saturated solution of ammonium sulphate. After 1 min, glutaraldehyde was added slowly to the final concentration of 50 mM. After 3 h of cross-linking reaction at 10°C, the suspension was centrifuged at 10,000 g for 10 min at 4 ºC. CLEAs were recovered as pellet and washed with 100 mM potassium phosphate buffer (pH 7.0) three times. After preparation the enzyme was kept in the same buffer (2 mL) at 4 ºC until use.

#### 4.3.2 The recyclability of CAT-CLEA in the oxidation of DFF to FDCA

To 295 µL 0.5 M KPi pH 7.0 was added 5mg of Cat-CLEA and 100 mM DFF. 5 µL of PaoABC (28.9 mg/mL) was added and the reaction was placed in a shaking incubator at 37°C. After 3 hours, a 10 µL aliquot of the reaction was extracted, diluted with 80 µL water and acidified with 15 µL 1 M HCl. The aliquot was centrifuged and analysed by RP HPLC. To recycle the catalyst, the reaction was centrifuged at 100 rpm and the supernatant removed. The pelleted CAT-CLEA were washed twice with water before being being subjected to reaction conditions. *HPLC conditions:* Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.




DFF **2** FDCA **5**

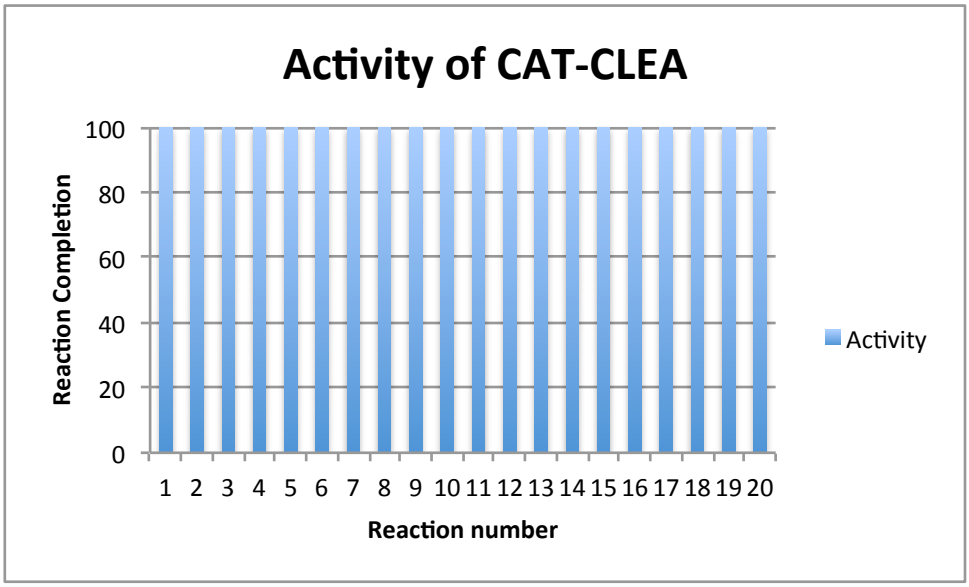


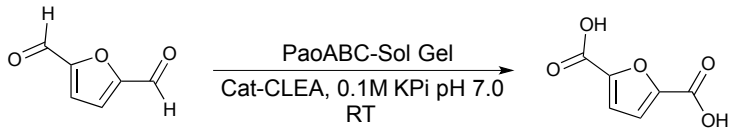

**Figure S9** The recyclability of CAT-CLEA in the oxidation of DFF to FDCA by PaoABC

### 4.4 Entrapment of PaoABC in Silica Hydrogel.

#### 4.4.1 Procedure for the entrapment of PaoABC in hydrogel

Tetramethyl orthosilicate (TMOS) (0.450 g) was placed in a small vial, cooled in an ice bath, and stirred at about 600 rpm. HCl (108 µL, 2.44 mM) was added, and the solution was stirred for 10 min. The solution was adjusted to pH 5.1 by adding 60 µL of 20 mM sodium phosphate buffer (pH 7.4). In a separate small vial, 1 mg of PaoABC was added to 540 µL of 10 mM HEPES (sodium salt) buffer solution (pH 7.5). Then 60 µL of a 20 mg/mL solution of PVI, PEI or PEG were added. The PaoABC solution was added to the TMOS-containing solution, and the resulting mixture was stirred for 1 min. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released, and the gel was rinsed with 2 mL of distilled water three times. The rinsed water was analysed by nanodrop to determine if any PaoABC has not been entrapped. The gel was then soaked in 2 mL of distilled water overnight at 4 °C. The water was decanted off yielding 750 mg of wet hydrogel containing 1mg of PaoABC.

#### 4.4.2 Time course analysis of the oxidation of DFF by different formulations of PaoABC hydrogel.




DFF **2** FDCA **5**

To 300 µL of 0.1 M KPi pH 7.0, was added 5mg of CAT-CLEA and 0.065 mg of PaoABC immobilized in hydrogel (50 mg of gel) in a 1.5 mL eppendorf. The reaction was vigorously shaken and the pH adjusted to pH 7.0. 1.9 mg of DFF (50 mM) was added and the reaction was placed in a shaking incubator at 25ºC. 5 µL of the reaction mixture was extracted and diluted with 80 µL of water and 15 µL of 1 M HCl before being centrifuged for 5mins. The aliquots were analysed by reverse phase HPLC. HPLC-Conditions: Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M H2SO4 at 60°C with flow rate 0.6 mL/min.

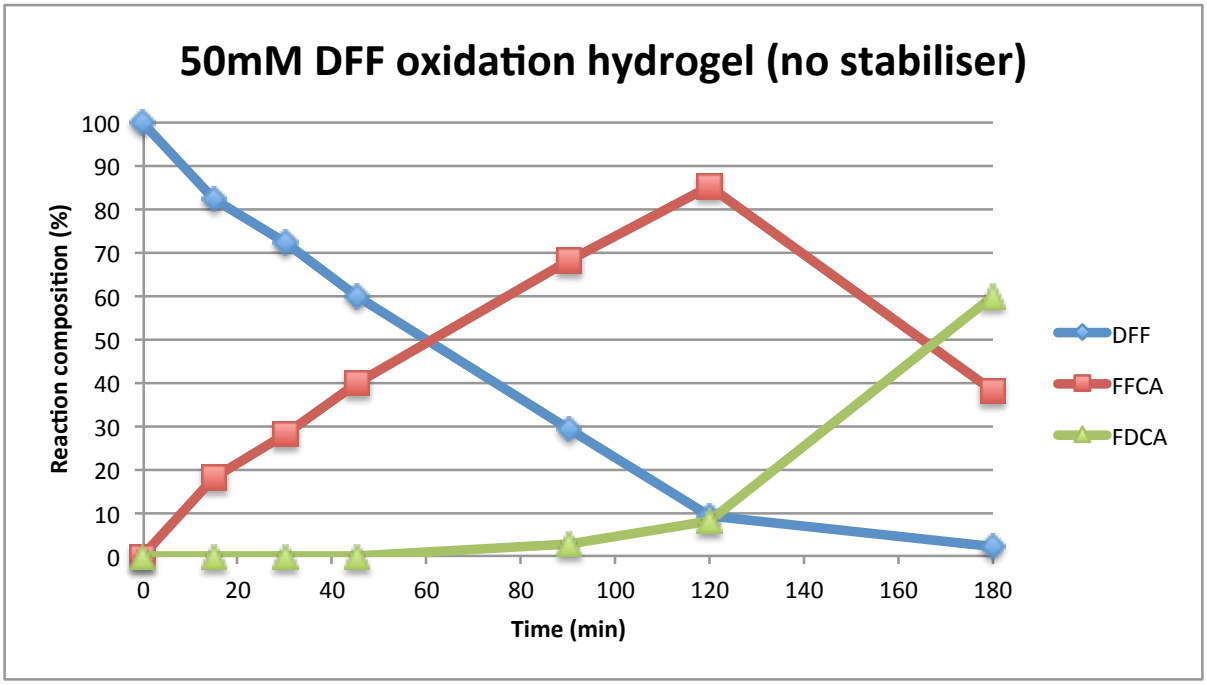



**Figure S10** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel (unstabilised)

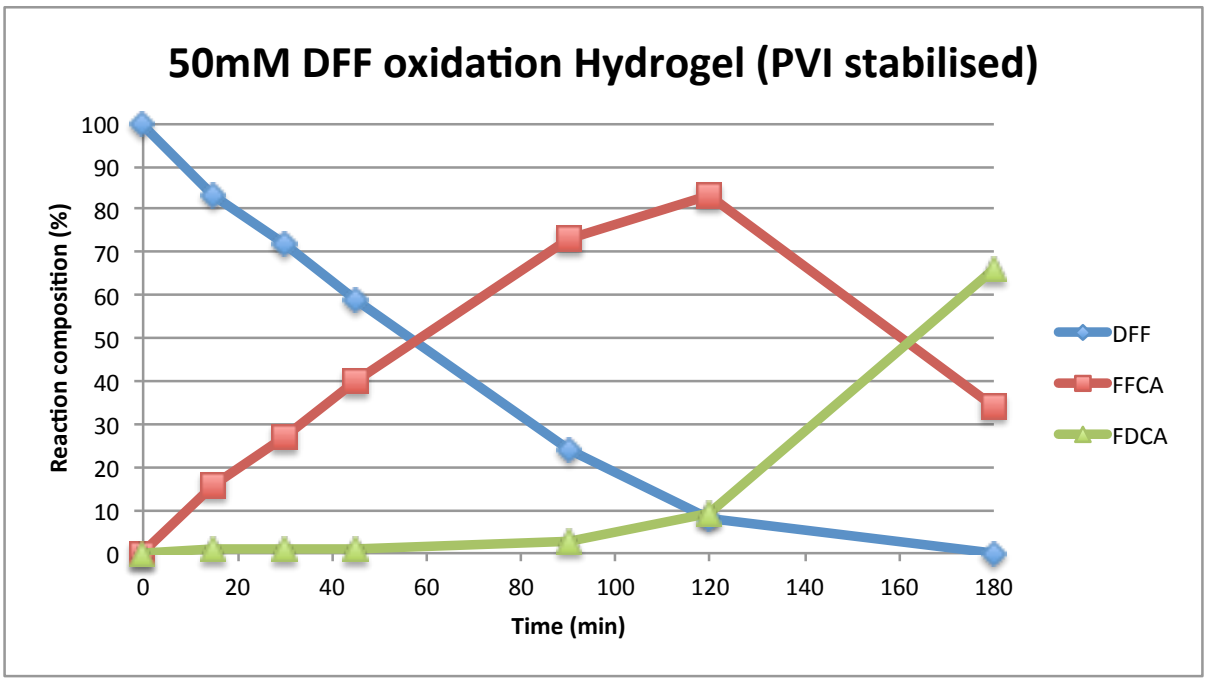



**Figure S11** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel (PVI stabilised)

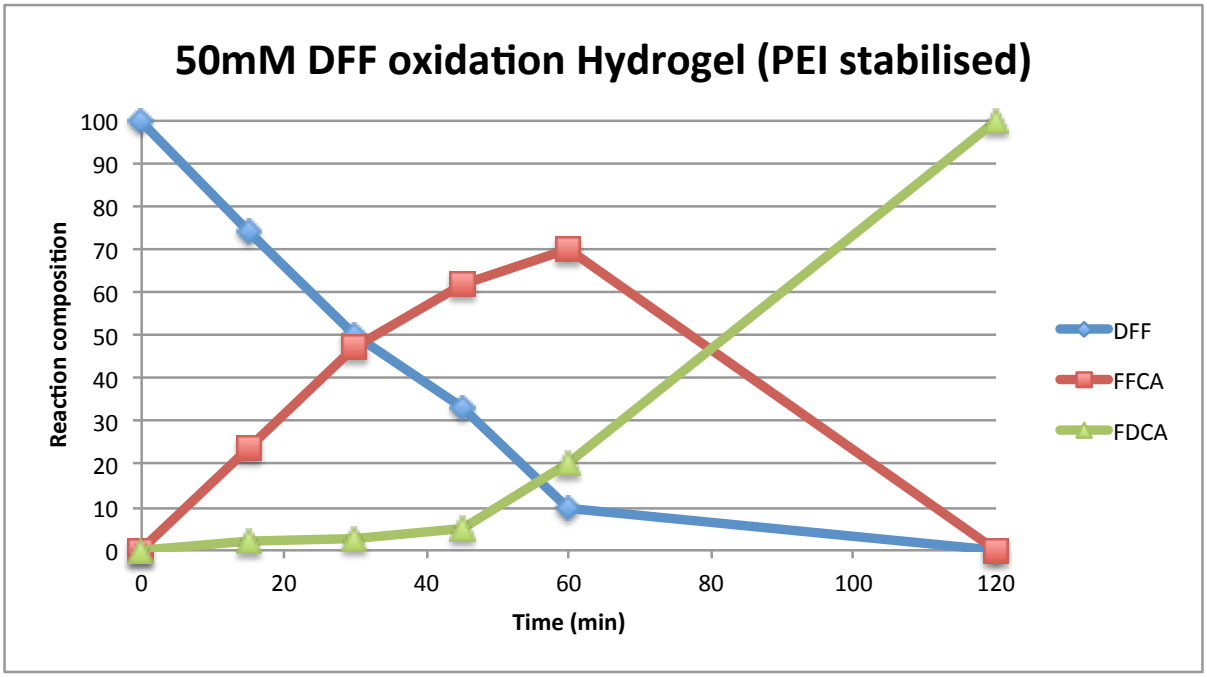



**Figure S12** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel (PEI stabilised)

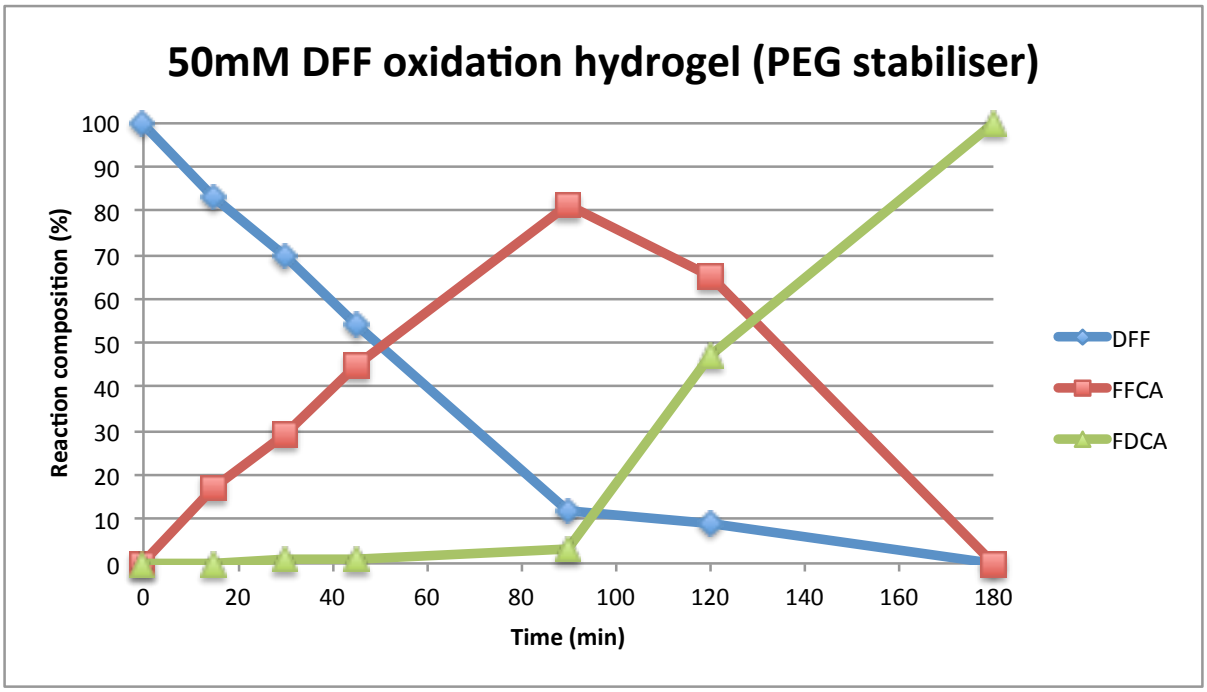



**Figure S13** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel (PEG stabilised)

### 4.4.3 Procedure for the co-entrapment of PaoABC and catalase in hydrogels

Tetramethyl orthosilicate (TMOS) (0.450 g) was placed in a small vial, cooled in an ice bath, and stirred at about 600 rpm. HCl (108 µL, 2.44 mM) was added, and the solution was stirred for 10 min. The solution was adjusted to pH 5.1 by adding 60 µL of 20 mM sodium phosphate buffer (pH 7.4). In a separate small vial, 1 mg of PaoABC and 5mg catalase or 5mg Cat-CLEA was added to 540 µL of 10 mM HEPES (sodium salt) buffer solution (pH 7.5). Then a 60 µL of either a 20 mg/mL solution of PVI, PEI or PEG were mixed. The PaoABC solution was added to the TMOS-containing solution and the resulting mixture was stirred for 1 min. A vacuum was applied to the stirred mixture until a gel formed. The vacuum was released, and the gel was rinsed with 2 mL of distilled water three times. The rinsed water was analysed by nanodrop to determine if any PaoABC has not been entrapped. The gel was then soaked in 2 mL of distilled water overnight at 4 °C. The water was decanted off yielding 750 mg of wet hydrogel containing 1mg of PaoABC with catalase.

### 4.4.4 Activity of the co-immobilised PaoABC + catalase in hydrogels

To 300 µL of 0.3 M KPi pH 7.0, was added 5 mg of CAT-CLEA and 0.065 mg of PaoABC immobilized in hydrogels (50 mg for gel) in a 1.5 mL eppendorf. The reaction was vigorously shaken and the pH adjusted to pH 7.0. 1.9 mg of DFF (50 mM) was added and the reaction was placed in a shaking incubator at 25ºC. 5 µL of the reaction mixture was extracted and diluted with 80 µL of water and 15µL of 1M HCl before being centrifuged for 5 min. The aliquots were analysed by reverse phase HPLC. HPLC-Conditions: Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6mL/min.

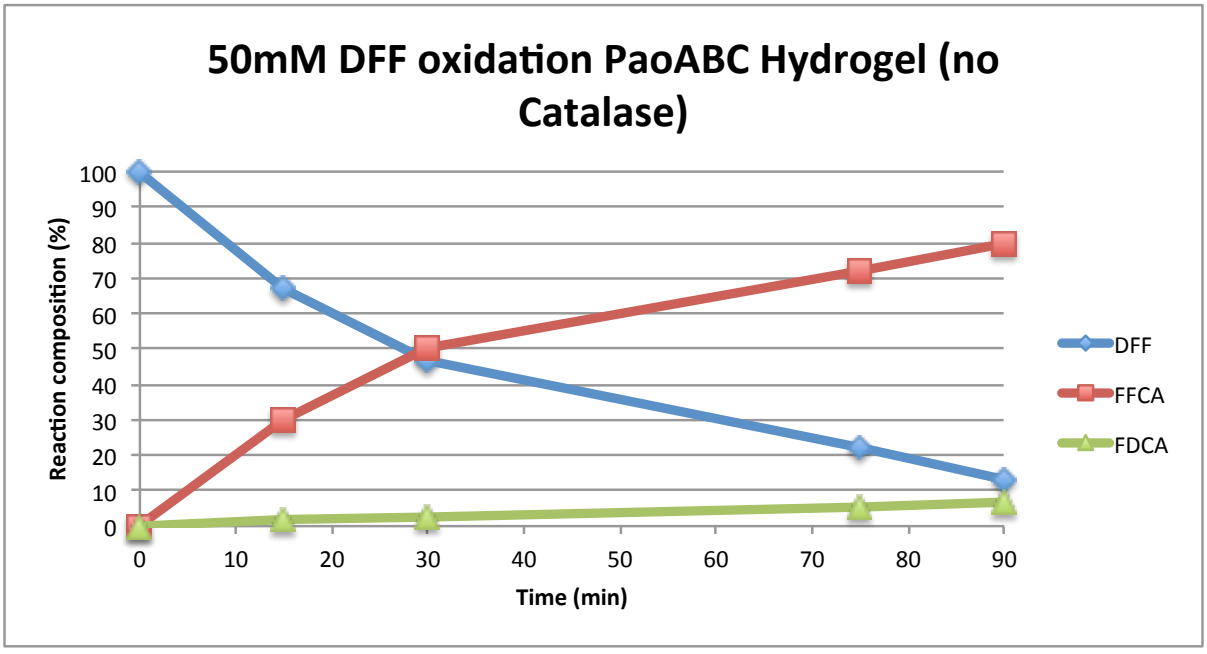



**Figure S14** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel without catalase

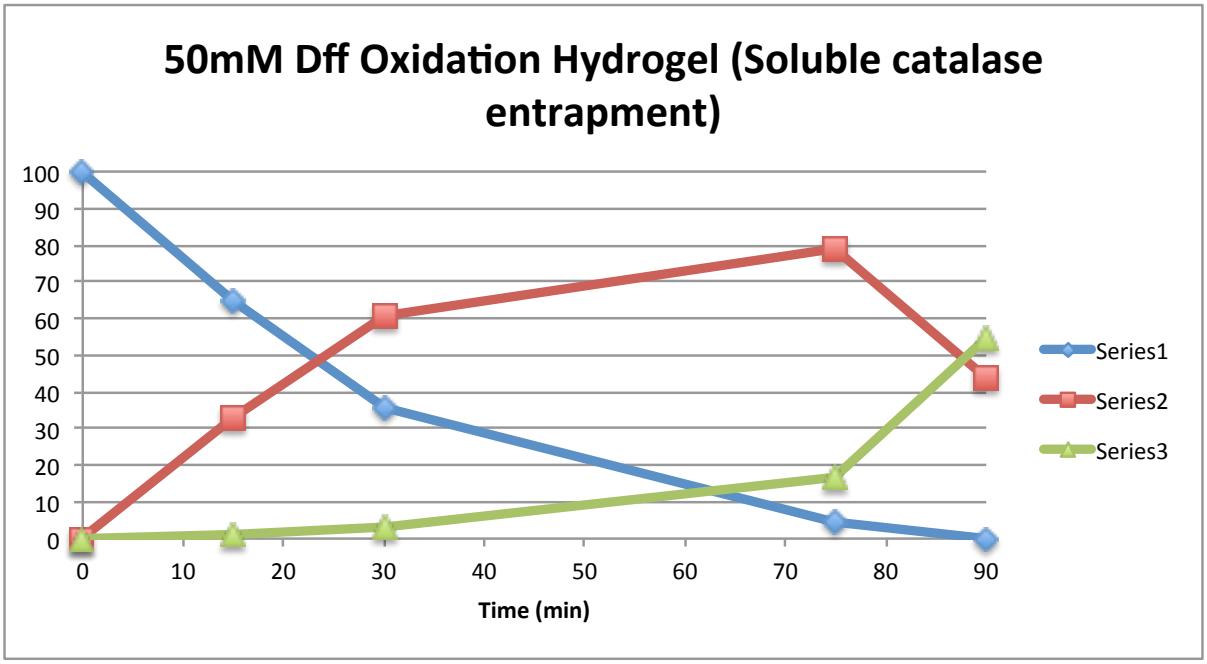



**Figure S15** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel coencapsulated with soluble catalase

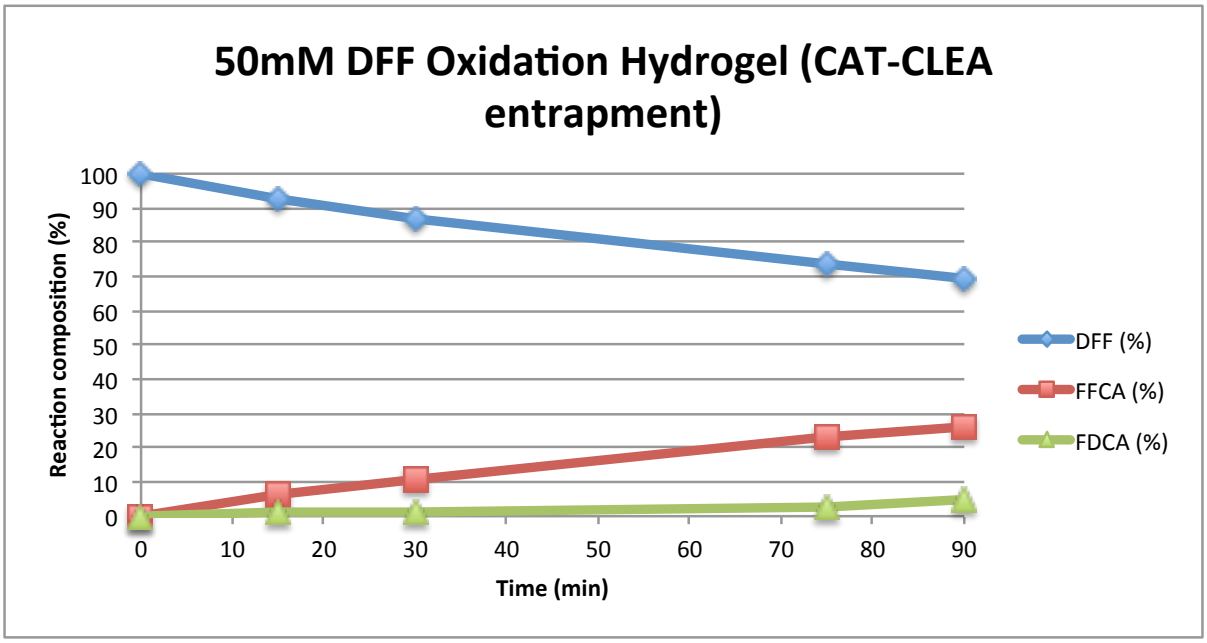



**Figure S16** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel coencapsulated with CAT-CLEA.

### 4.4.5 Time course analysis of the oxidation of DFF to FDCA by soluble PaoABC and PaoABC-Gel at increasing substrate concentrations.

To 300 µL of 0.1M KPi pH 7.0, was added 5mg of CAT-CLEA and 0.065 mg of soluble PaoABC or PaoABC immobilized in hydrogels (50 mg for gel) in a 1.5 mL eppendorf. The reaction was vigorously shaking and the pH adjusted to pH 7.0. DFF (X mM) was added and the reaction was placed in a shaking incubator at 25ºC. The pH was maintained by careful addition of a saturated solution of sodium bicarbonate. 5 µL of the reaction mixture was extracted and diluted with 80 µL of water and 15 µL of 1 M HCl before being centrifuged for 5 mins. The aliquots were analysed by reverse phase HPLC. To recycle the catalyst, the reaction was centrifuged at 100rpm and the supernatant removed. The gel was washed twice with water before being being subjected to reaction conditions. HPLC-Conditions: Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.

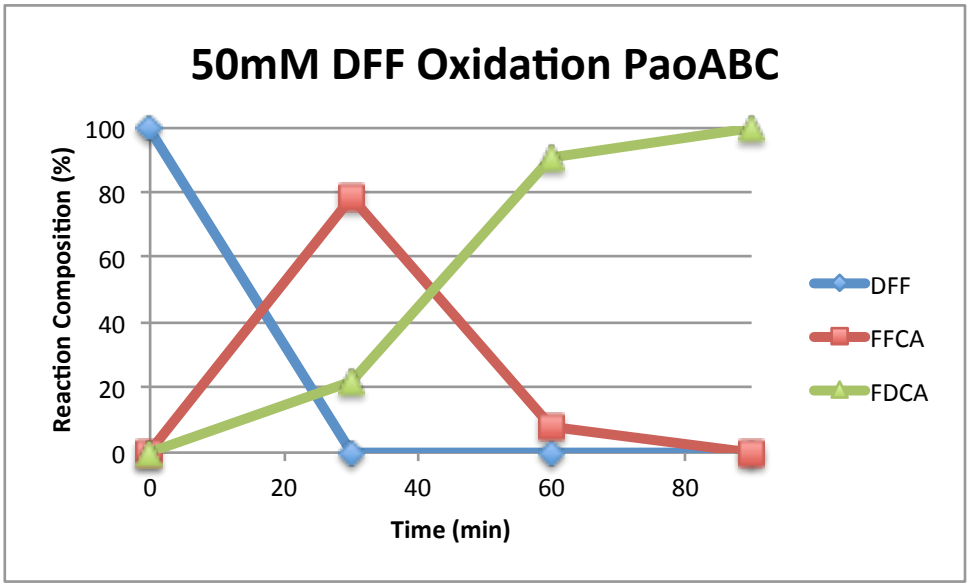



**Figure S17** Time course analysis of the oxidation of 50 mM DFF by soluble PaoABC

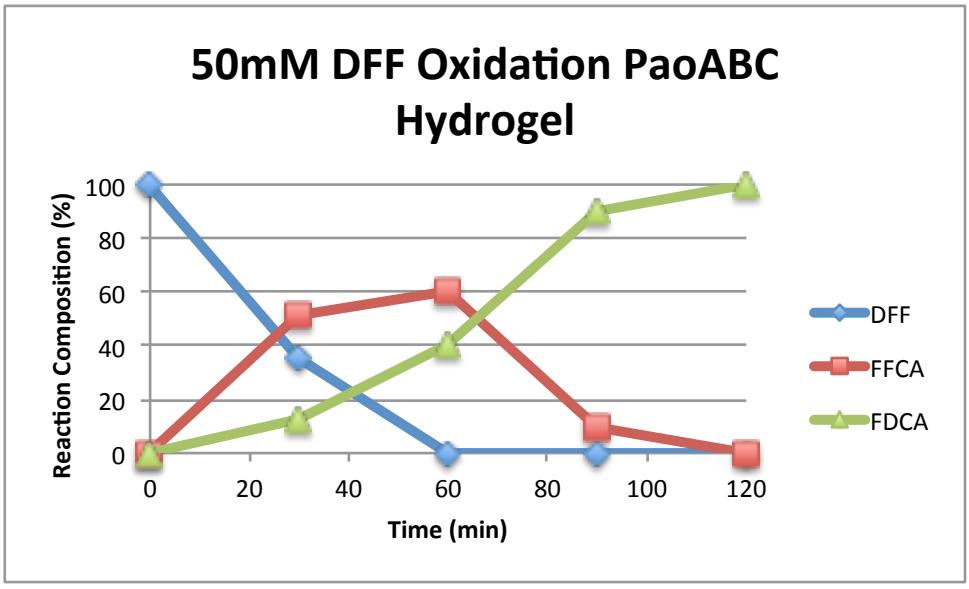



**Figure S18** Time course analysis of the oxidation of 50 mM DFF by PaoABC hydrogel

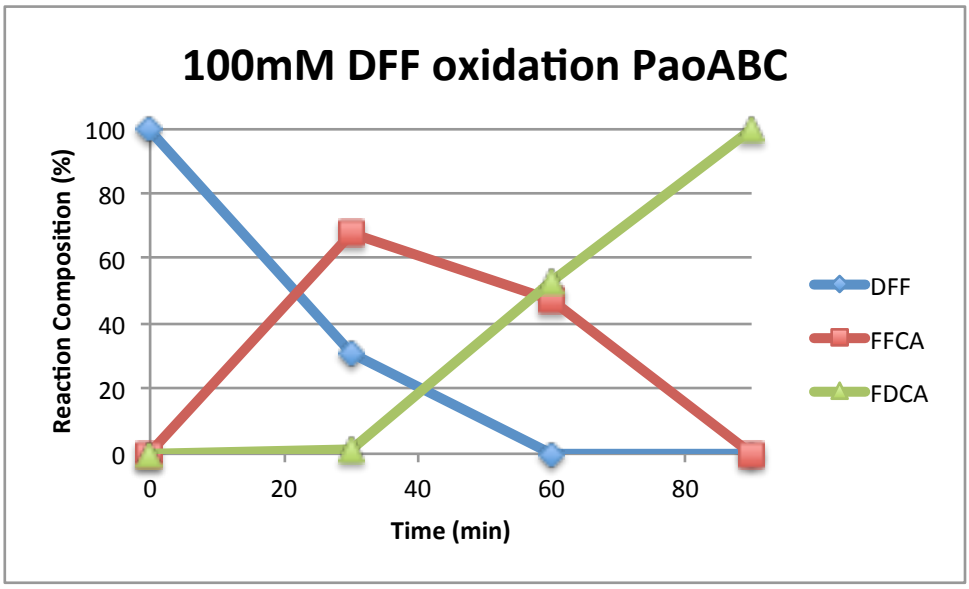



**Figure S19** Time course analysis of the oxidation of 100 mM DFF by soluble PaoABC

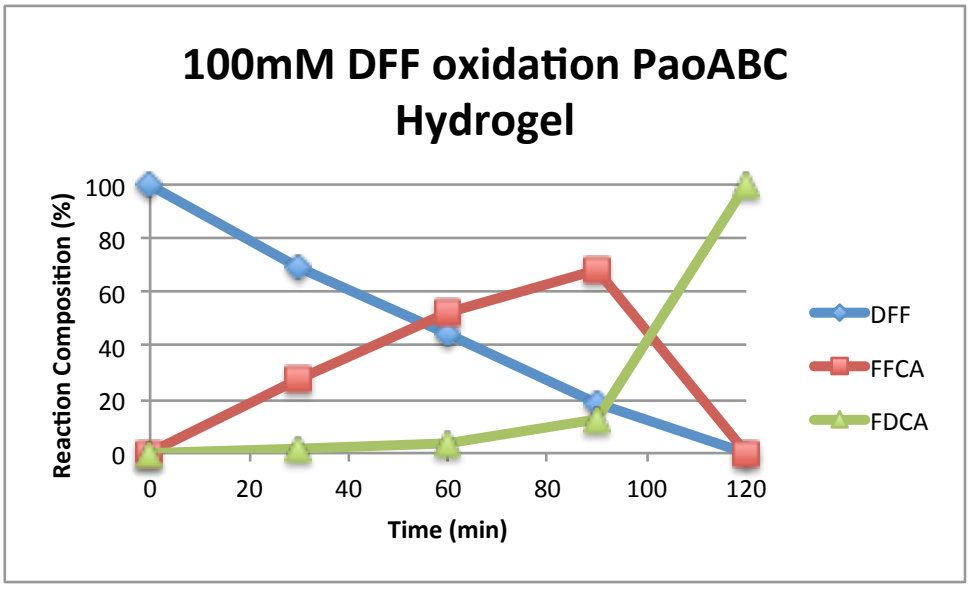



**Figure S20** Time course analysis of the oxidation of 100 mM DFF by PaoABC hydrogel

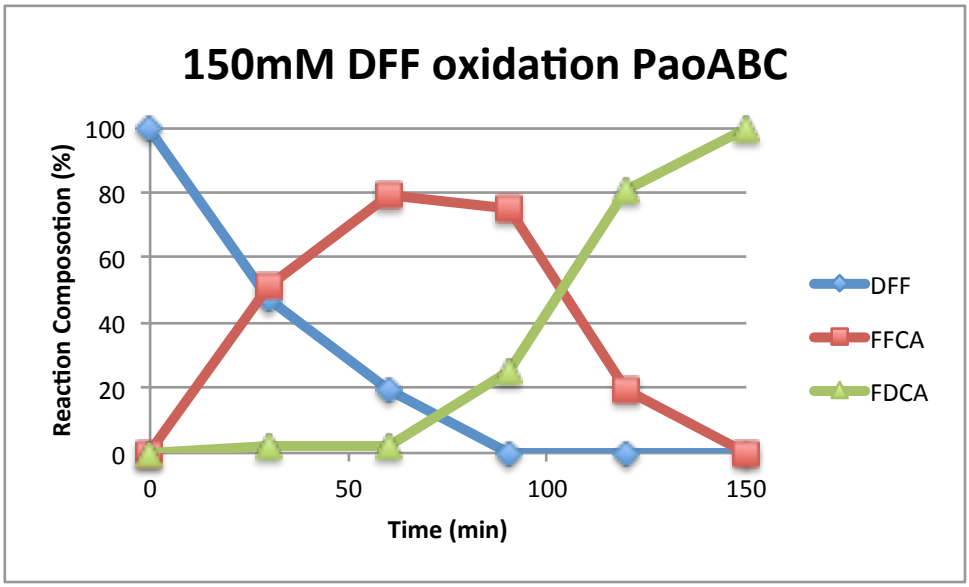



**Figure S21** Time course analysis of the oxidation of 150 mM DFF by soluble PaoABC

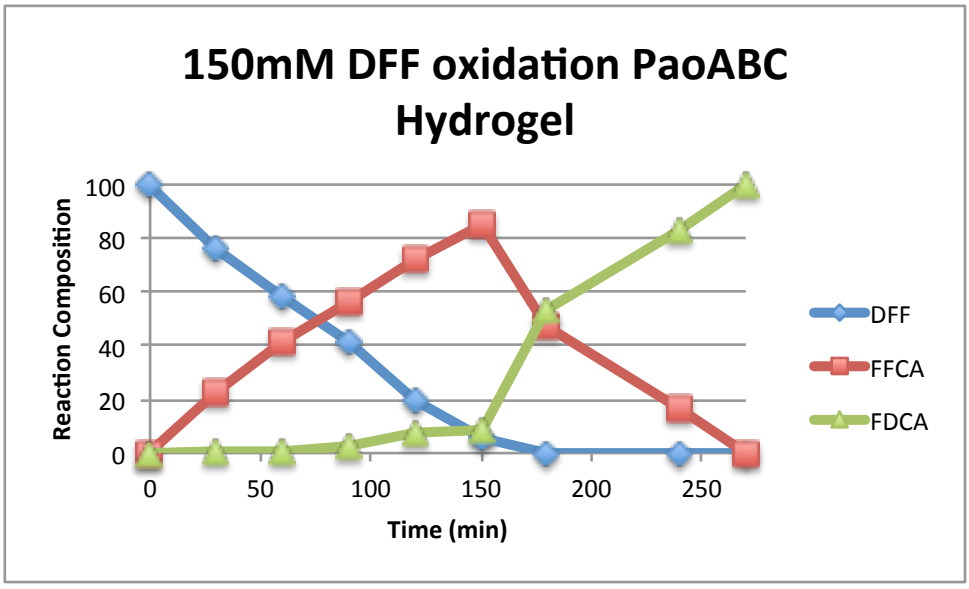



**Figure S22** Time course analysis of the oxidation of 150 mM DFF by PaoABC Hydrogel

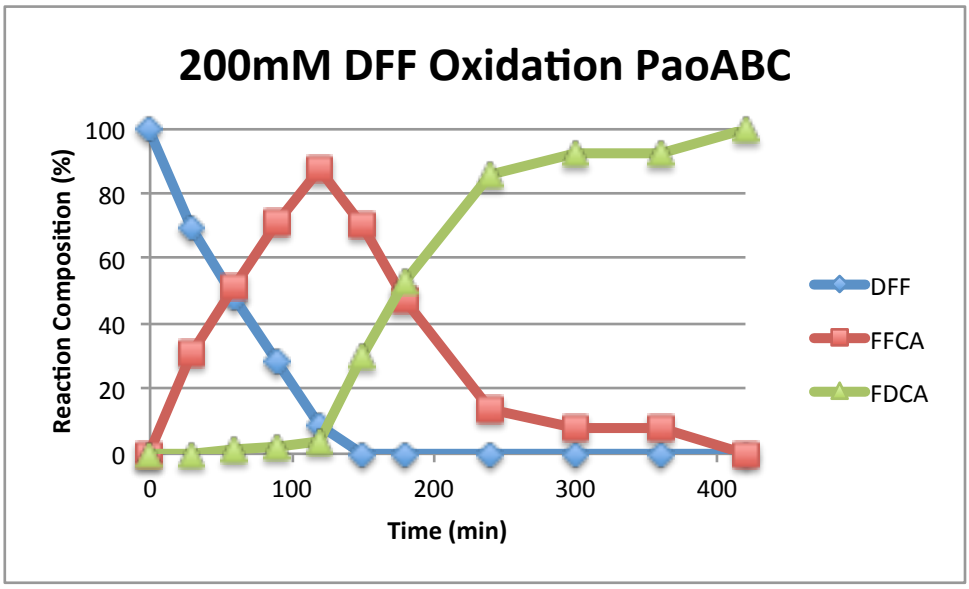



**Figure S23** Time course analysis of the oxidation of 200 mM DFF by soluble PaoABC

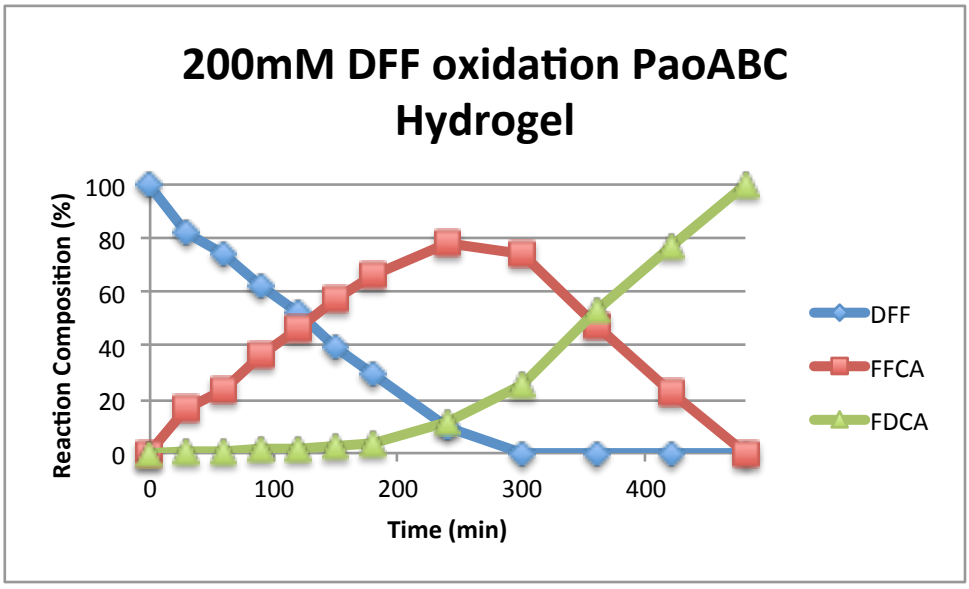



**Figure S24** Time course analysis of the oxidation of 200 mM DFF by PaoABC Hydrogel

### 4.4.6 Time course analysis of the oxidation of DFF to FDCA by soluble PaoABC and PaoABC-Gel at increasing temperature.

To 300 µL of 0.1M KPi pH 7.0, was added 5 mg of CAT-CLEA and 0.065mg of soluble PaoABC or PaoABC immobilized in hydrogels (50 mg for gel) in a 1.5 mL eppendorf. The reaction was vigorously shaking and the pH adjusted to pH 7.0. DFF (200 mM) was added and the reaction was placed in a shaking incubator at XºC. The pH was maintained by careful addition of a saturated solution of sodium bicarbonate. 5 µL of the reaction mixture was extracted and diluted with 80 µL of water and 15 µL of 1 M HCl before being centrifuged for 5 mins. The aliquots were analysed by reverse phase HPLC. HPLC-Conditions: Bio-Rad Aminex HPX-87H, 300 mm × 7.8 mm pre-packed column with 100% 0.005 M $H_2SO_4$ at 60°C with flow rate 0.6 mL/min.

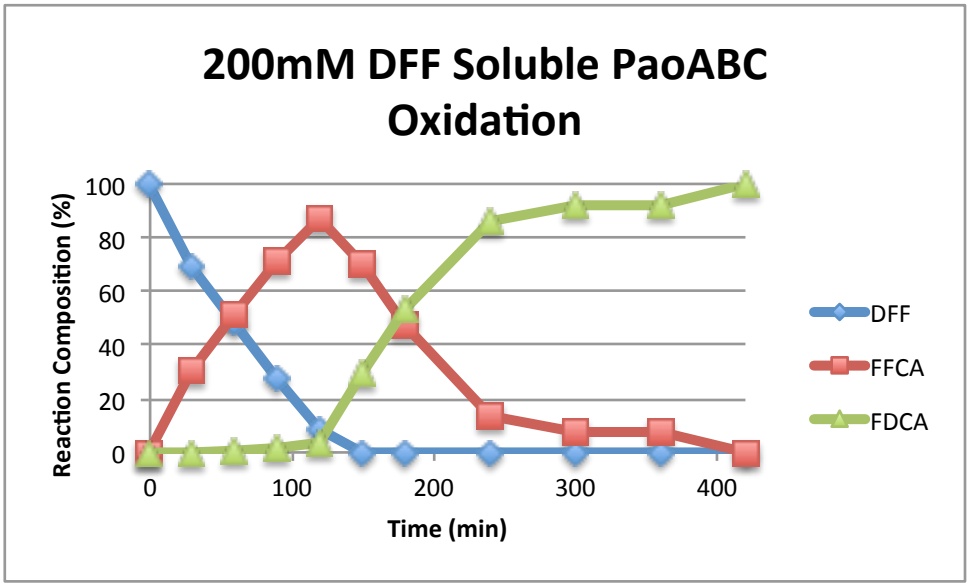



**Figure S25** Time course analysis of the oxidation of 200 mM DFF by soluble PaoABC at 25°C

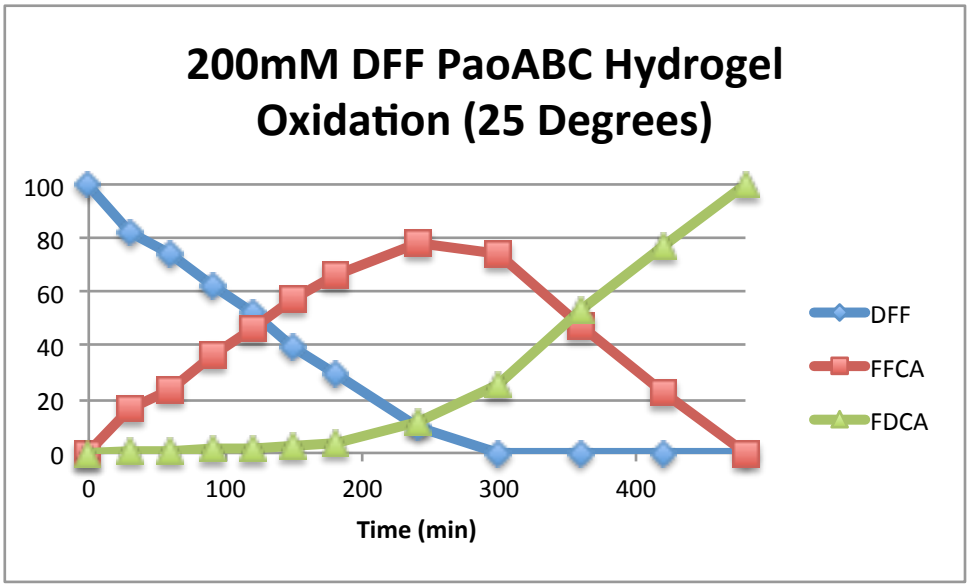



**Figure S26** Time course analysis of the oxidation of 200 mM DFF by PaoABC Hydrogel at 25°C

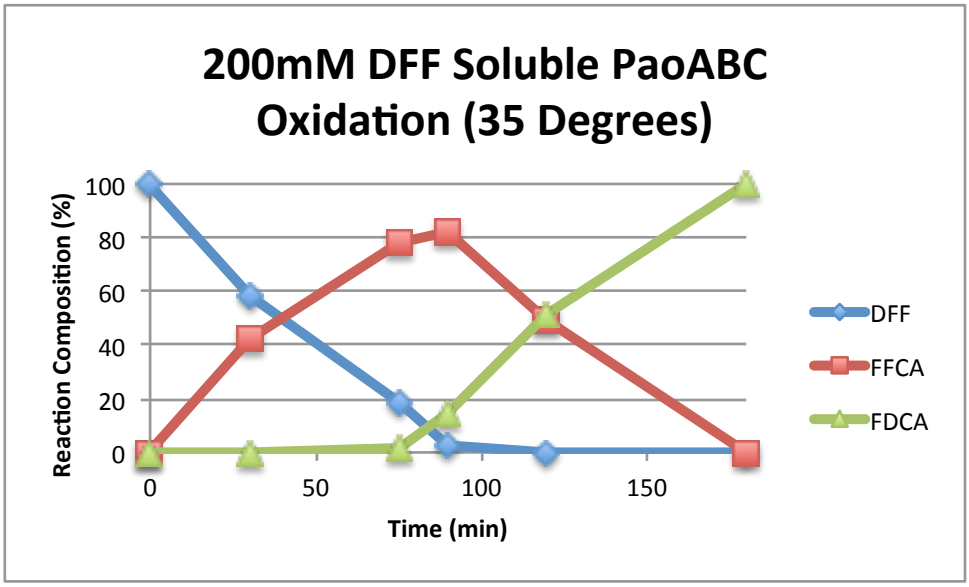



**Figure S27** Time course analysis of the oxidation of 200 mM DFF by soluble PaoABC at 35°C

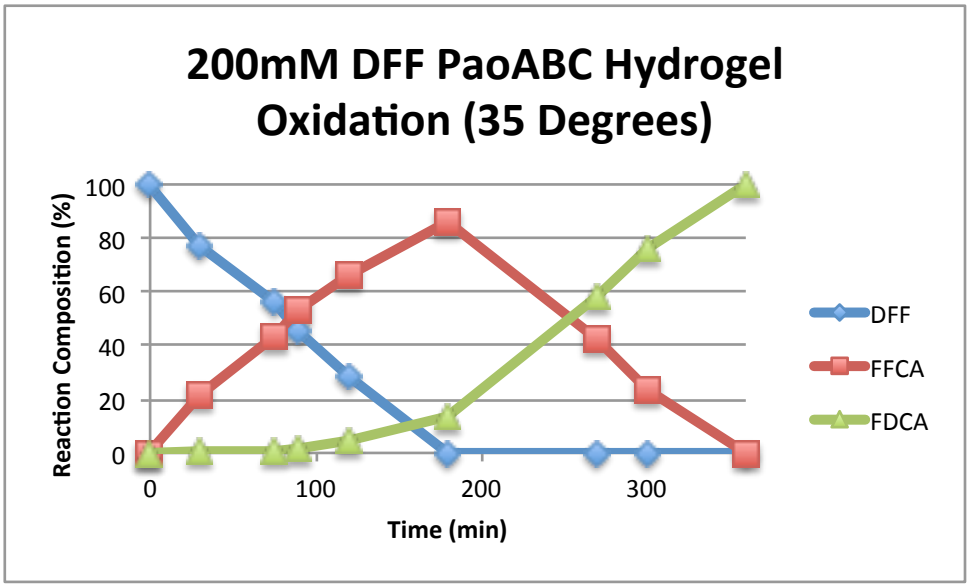



**Figure S28** Time course analysis of the oxidation of 200 mM DFF by PaoABC Hydrogel at 35°C

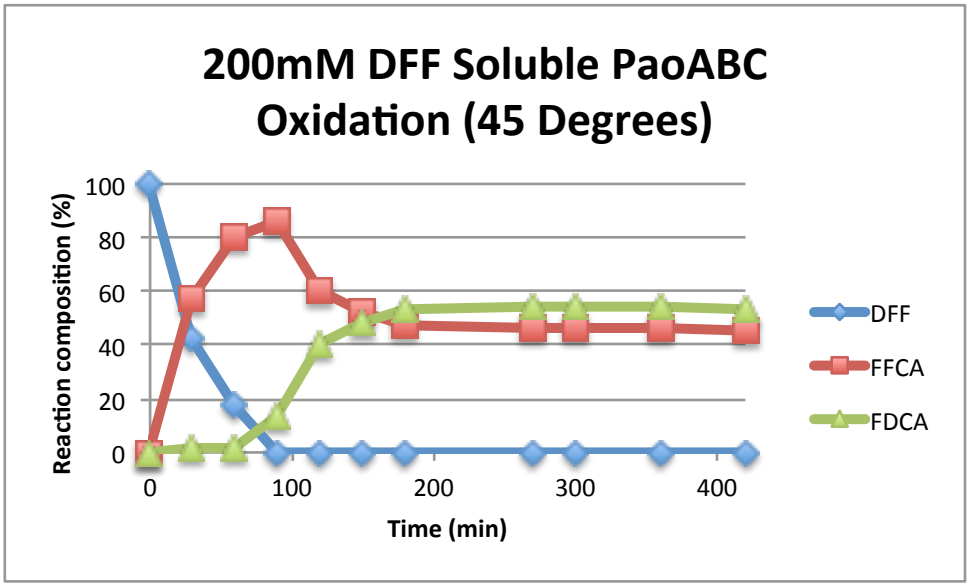



**Figure S29** Time course analysis of the oxidation of 200 mM DFF by soluble PaoABC at 45°C

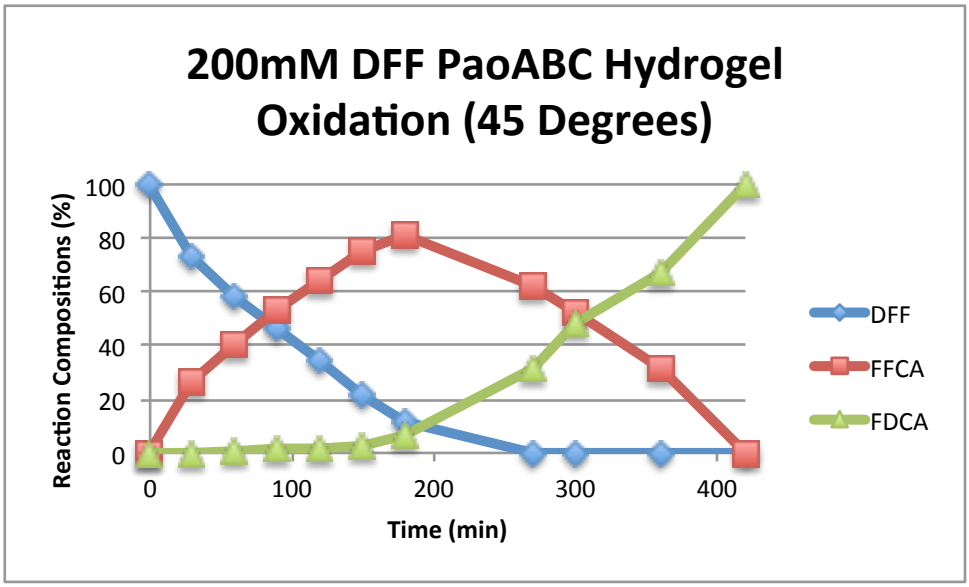



**Figure S30** Time course analysis of the oxidation of 200 mM DFF by PaoABC Hydrogel at 45°C

## 5.0 Standards for Absorbance Calibration.

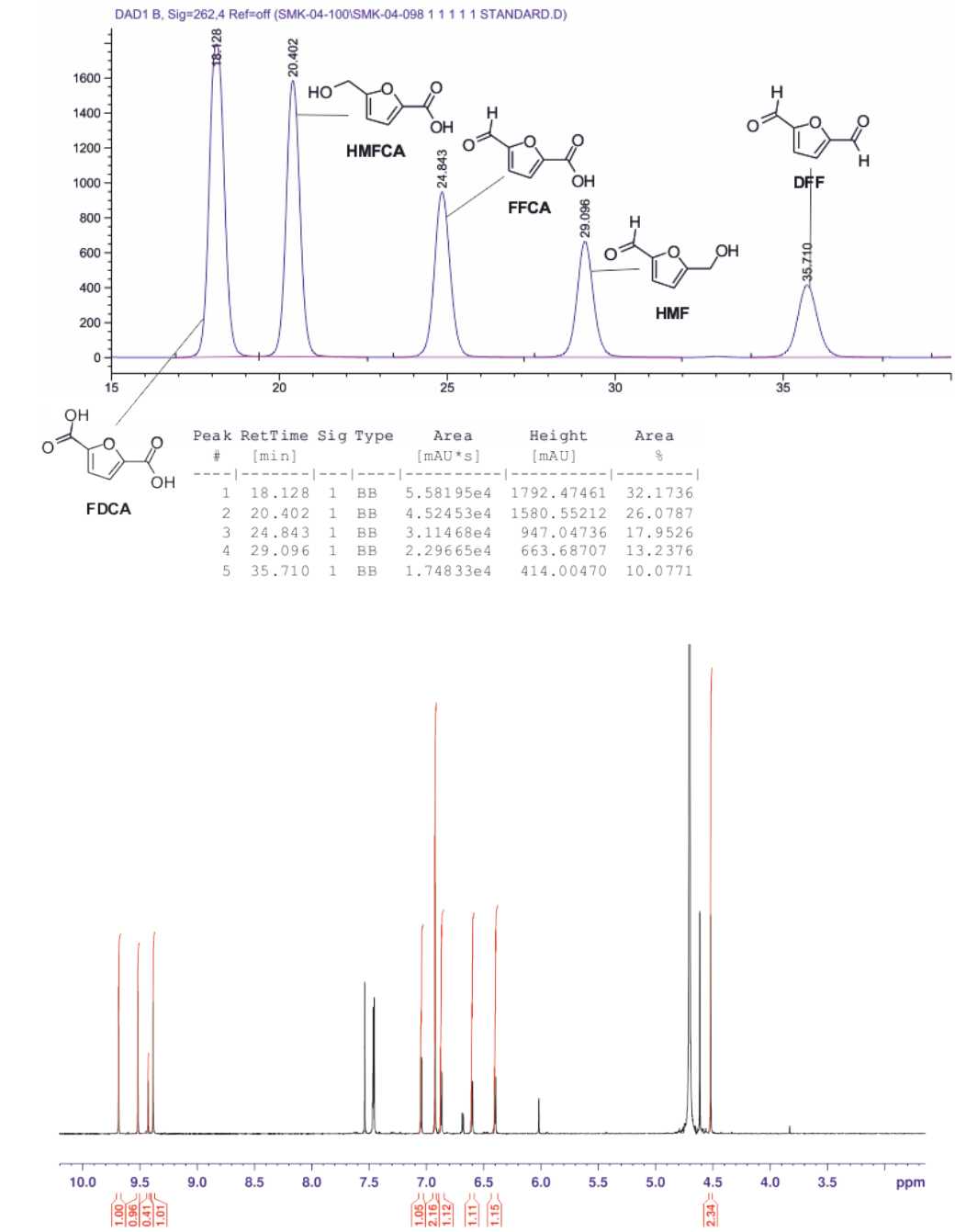



**Figure S33**.0 1:1:1:1 standard of HMF, DFF, HMFCA, FFCA and FDCA used to adjust absorbance in section 7.0. Peaks used in NMR are FFCA = 9.67, DFF = 9.51, DFF Hydrate = 9.421, HMF = 9.38, FDCA = 6.92 and HMFCA = 4.4.

## References

1. Escalettes, F., and Turner, N. J. *ChemBioChem*, 2008, *9*, 857–860.
2. Deacon, S.E. and McPherson, J. *ChemBioChem.*, 2011, *12*, 593-601.
3. Neumann, M., Mittelstädt, G., Iobbi-Nivol, C., Saggu, M., Lendzian, F., Hildebrandt, P., and Leimkühler, S. *FEBS Journal*, 2009, *276*, 2762–2774.