Electronic Supporting Information for

Enzyme-catalyzed selective oxidation of 5-hydroxymethylfurfural (HMF) and separation of HMF and 2,5-diformylfuran using deep eutectic solvents

Ye-Zhi Qin, Yan-Mei Li, Min-Hua Zong,* Hong Wu, Ning Li*

State Key Laboratory of Pulp and Paper Engineering, College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510640, China

* Corresponding authors

Prof. M. H. Zong, Tel: +86 20 8711 1452; Fax: +86 20 2223 6669; Email: btmhzong@scut.edu.cn

Dr. N. Li, Tel/Fax: +86 20 2223 6669; Email: lining@scut.edu.cn

Materials

Alcohol oxidases from *Candida boidinii*, *Hansenula* sp. and *Pichia pastoris* (One unit will oxidize 1.0 μmole of methanol to formaldehyde per min at pH 7.5 at 25 ℃), galactose oxidase from *Dactylium dendroides* (One unit will produce a ΔA_{425} of 1.0 per min at pH 6.0 at 25 ℃, in a peroxidase and *o*-tolidine system. Reaction volume = 3.4 mL. Light path = 1 cm), laccase from *Trametes versicolor*, catalase from bovine liver (2000-5000 U/mg protein,one unit will decompose 1.0 μmole of H_2O_2 per min at pH 7.0 at 25 °C), xanthine oxidase from *Escherichia coli* (One unit will convert 1.0 μmole of xanthine to uric acid per min at pH 7.5 at 25 ℃), 2,2'-azinobis(3-ethylbenzylthiozoline-6-sulfonate) (ABTS), HMF (99%) and TEMPO (98%) were bought from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (>200U/mg protein, one pyrogallol unit will form 1.0 mg purpurogallin from pyrogallolin 20 sec at pH 6.0 at 20 ℃) was purchasedfrom Aladdin Industrial Inc. (Shanghai, China). Immobilized lipase B from *Candida Antarctica* (Novozym 435) was from NovozymesCo., Ltd. (China). Laccases from *Panus conchatus* and *Flammulina velutipes* were provided by Prof. Shiyu Fu. DFF (>98%) was from TCI (Japan). FFCA (98%) and FDCA (97%) were from J&K Scientific Ltd. (Guangzhou, China). HMFCA (98%) was obtained from Adamas Reagent, Ltd (China). All the materials were used as received.

General procedure for enzymatic oxidation of HMF

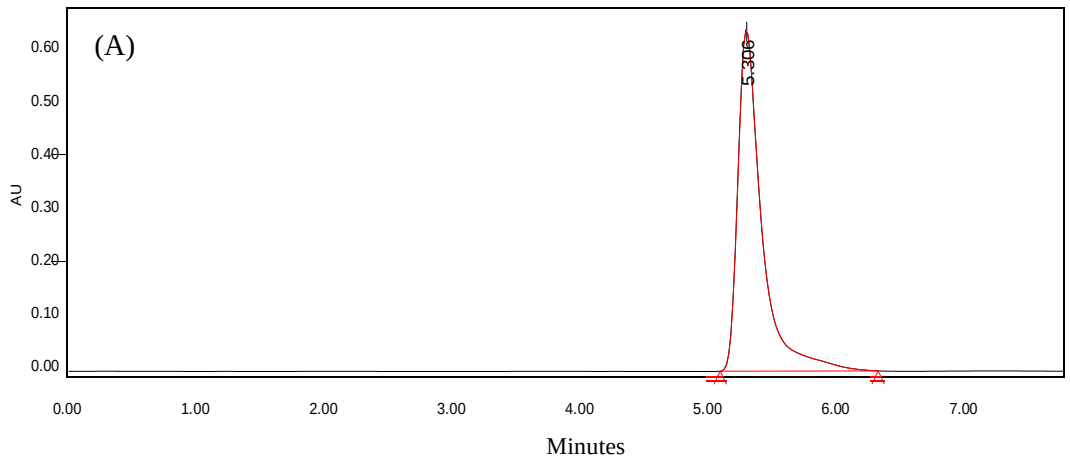
In a typical experiment, 2 mL buffer or deionized water containing 30 HMF and enzymes was incubated in a 15 ml Erlenmeyer shaking flask capped with a septum at 150 rpm. The detailed reaction conditions for each experiment were described in the captions of figures and tables. Aliquots were withdrawn at specified time intervals from the reaction mixture. The samples were treated at 100 °C for 5 min to denature the enzyme, and then diluted by 91 times with the corresponding mobile phase prior to HPLC analysis. All the experiments were conducted in duplicate, and all the data were the averages of experimental results. The yields were determined by HPLC, based on the corresponding calibration curves.

Laccase activity assay

Laccase activity was determined by oxidation of ABTS, according to a previous method with minor modifications.[1] The assay mixture contained 0.25 mM ABTS in 0.1 M sodium acetate (pH 5.0), and a suitable amount of enzyme. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\varepsilon_{420}=3.6\times10^4\ M^{-1}\ cm^{-1}$) in 3 min. Enzyme activity was expressed in units (U= 1 μmol of ABTS oxidized per minute). The specific activities of laccases from *T. versicolor*, *P. conchatus* and *F. velutipes* were 5.2, 2.0 and 0.8 U/mg, respectively.

Separation of HMF and DFF using DES

After the mixture of HMF and DFF was dissolved in 2 mL ethyl acetate, DES was added and stirred violently for efficient extraction. Then, the mixture stood a while, for creating a biphasic system. The amounts of HMF and DFF in the upper and lower phaseswere determined by HPLC. If necessary, repeated extraction by DES was conducted.



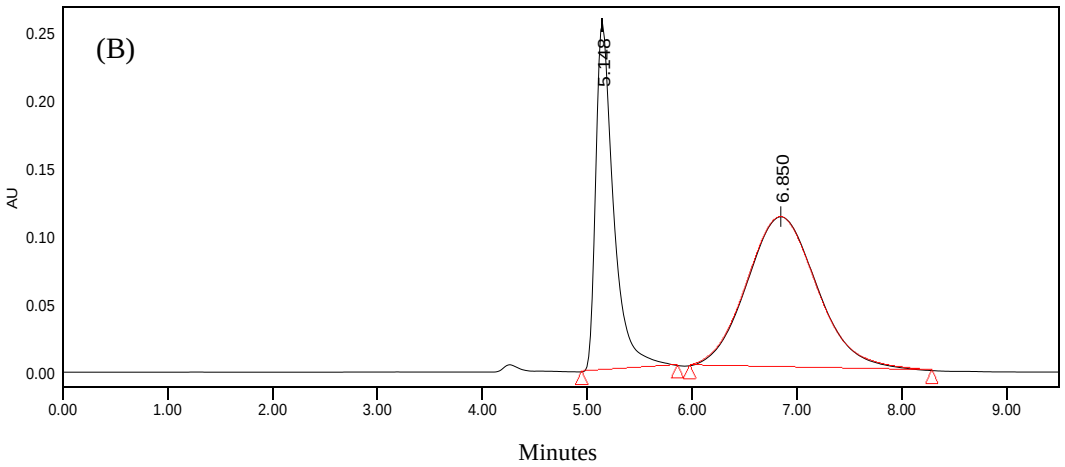


Fig. S1 HPLC analysis of the reaction mixture in the oxidation of HMF to DFF by GO and AO. A: before the reaction; B: after the reaction

The retention times of HMF and DFF were 5.1 and 6.9 min, respectively.

Analytic conditions: The reaction mixture was analyzed on a Zorbax SB C-18 column (250 mm×4.6 mm, 5 μm, Agilent, USA) by a RP-HPLC with a Waters 1525 pump and a Waters 2489 UV detector. The mobile phase consisted of acetonitrile/0.1% trifluoroacetic acid (TFA) aqueous solution (15:85, v/v) with a flow rate of 0.8 mL/min. The UV absorption wavelength and column temperature were 284 nm and 35°C, respectively.

Table S1 Comparison of enzyme performances in various buffers for oxidation of HMF

Entry	Enzymes in various buffers	Conditions[a]	Enzyme dosage (U)	Time (h)	DFF yield (%)
1	GO + HRP in phosphate buffer (50 mM, pH 7.0)	A	8	72	39
2	GO + HRP in sodium acetate buffer (50 mM, pH 7.0)	A	8	72	50
3	GO + HRP in deionized water	A	8	72	47
4	GO+catalase +HRP in phosphate buffer (50 mM, pH 7.0)	B	8	72	34
5	GO+catalase +HRP in sodium acetate buffer (50 mM, pH 7.0)	B	8	96	60

[a]: Conditions A: 2 mL phosphate buffer (50 mM, pH 7.0), 30 mM HMF, 8 U GO, 1.3 mg HRP, air bubbling for 5 min each day, 25ºC, 150 rpm. Conditions B: 2 mL phosphate buffer (50 mM, pH 7.0), 30 mM HMF, 8 U GO, 1.1 mg catalase, 1.3 mg HRP, air bubbling for 5 min each day, 25ºC, 150 rpm.

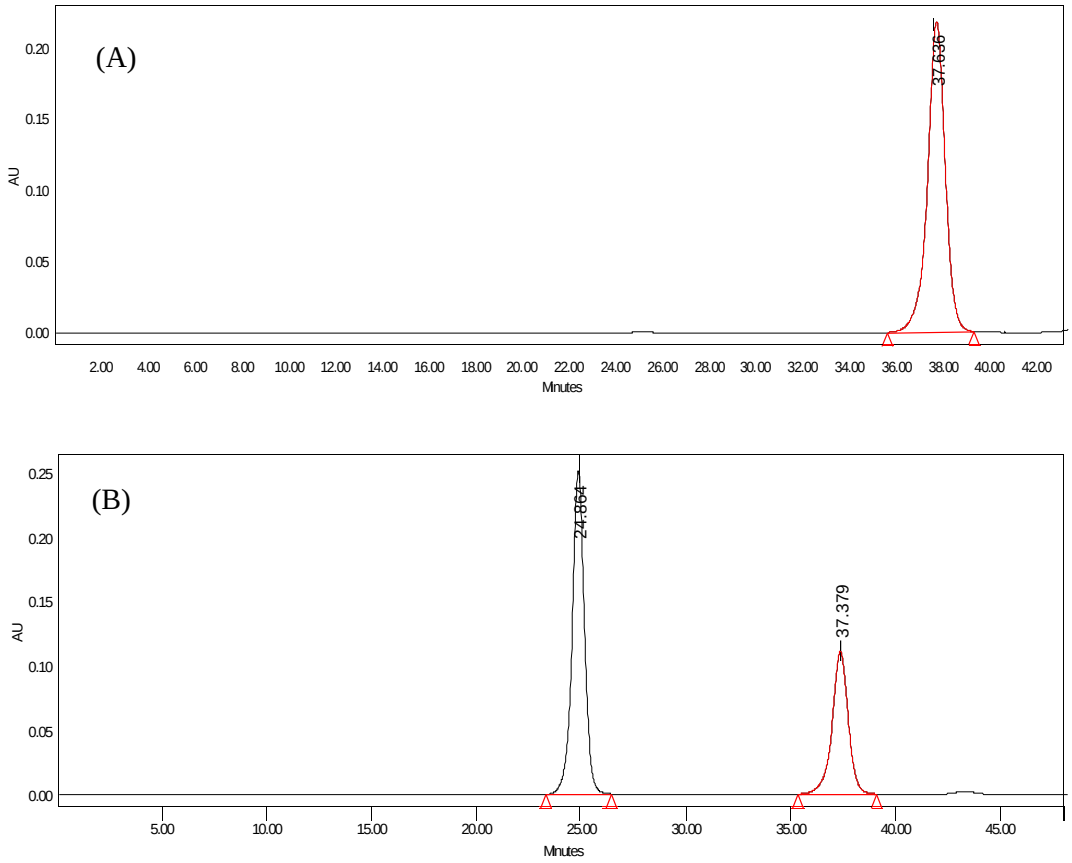
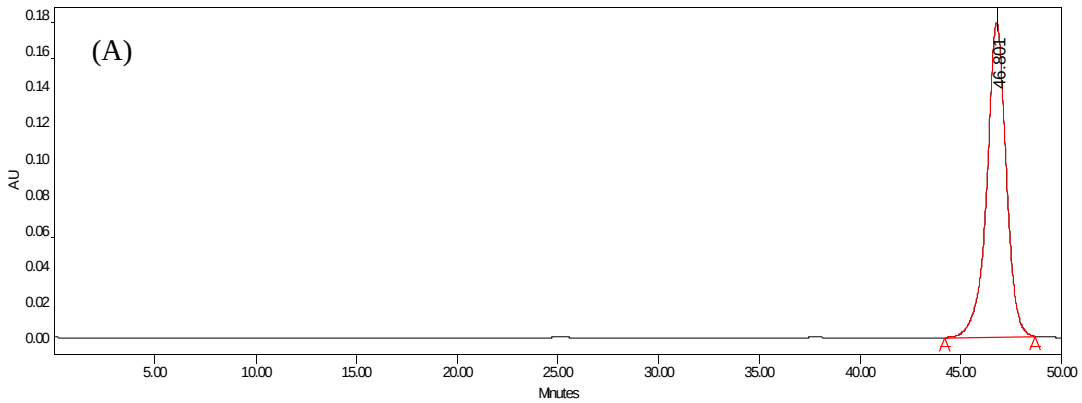


Fig. S2 HPLC analysis of the reaction mixture in the oxidation of HMF to HMFCA by XO. A: before the reaction; B: after the reaction

The retention times of HMFCAand HMF were 24.9 and 37.4 min, respectively.

Analytic conditions: The reaction mixture was analyzed on an Aminex HPX-87H column (300 mm×7.8 mm, Bio-Rad, USA)by a RP-HPLC with a Waters 515 pump and a Waters 996 PDA detector. The mobile phase consisted of 5 mMH_2SO_4 aqueous solution with a flow rate of 0.6 mL/min. The UV absorption wavelength and column temperature were 254 nm and 60°C, respectively.



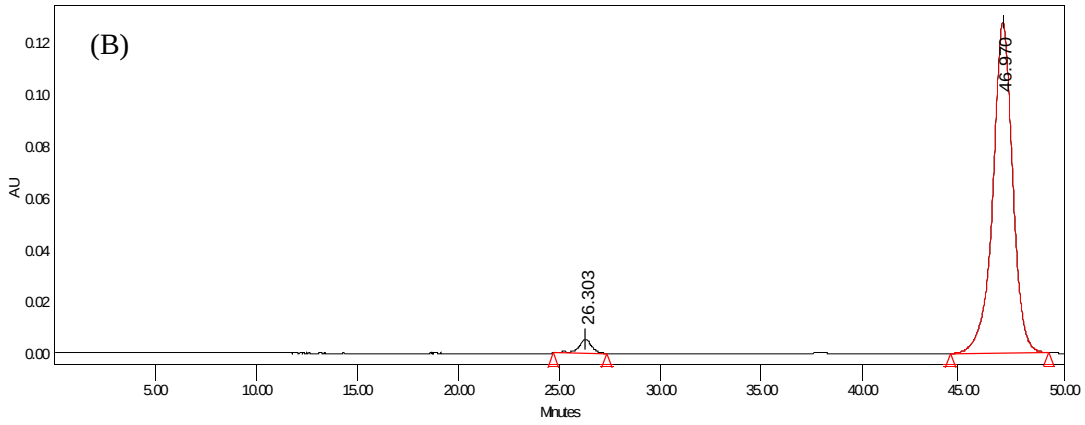


Fig. S3 HPLC analysis of the reaction mixture in the oxidation of DFF to FFCA by XO. A: before the reaction; B: after the reaction

The retention times of FFCA and DFF were 26.3 and46.8 min, respectively. Analytic conditions were the same as those described in Fig. S3

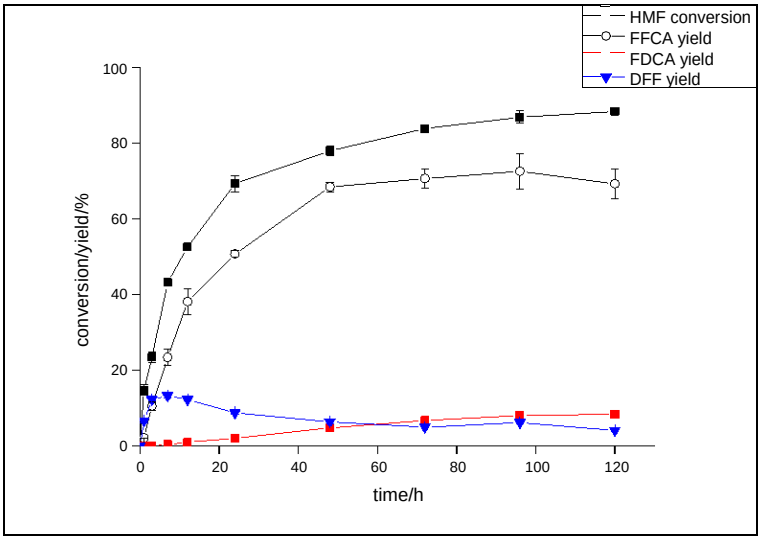
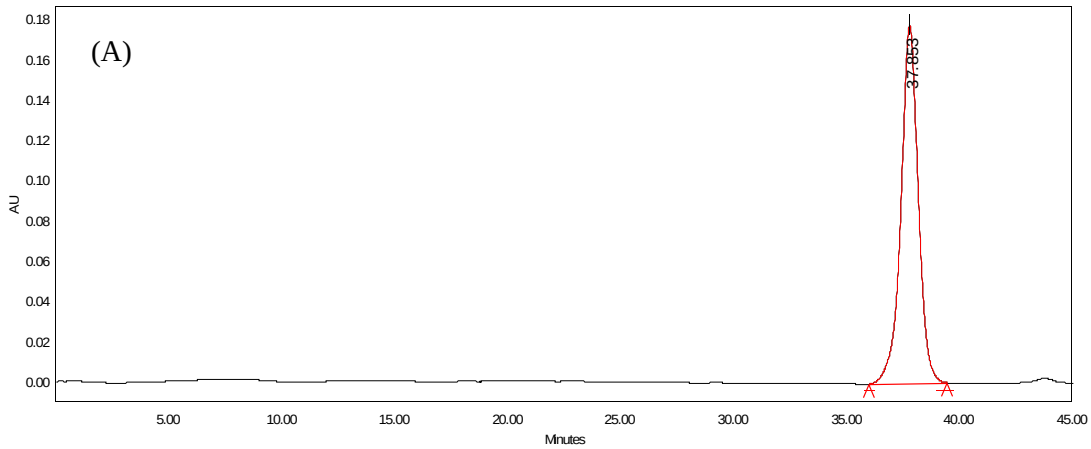


Fig. S4 *Trametes versicolor* laccase-TEMPO mediated oxidation of HMF

Reaction conditions: the same as Table 2



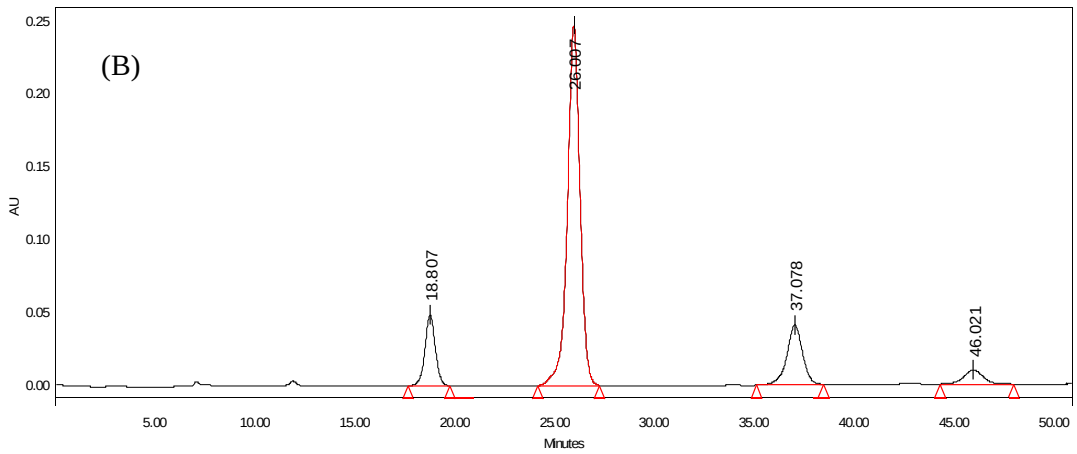


Fig. S5 HPLC analysis of the reaction mixture in the oxidation of HMF to FFCA by laccase. A: before the reaction; B: after the reaction

The retention times of FDCA, FFCA, HMF and DFF were 18.8, 26.0, 37.1 and 46.0 min, respectively. Analytic conditions were the same as those described in Fig. S3.

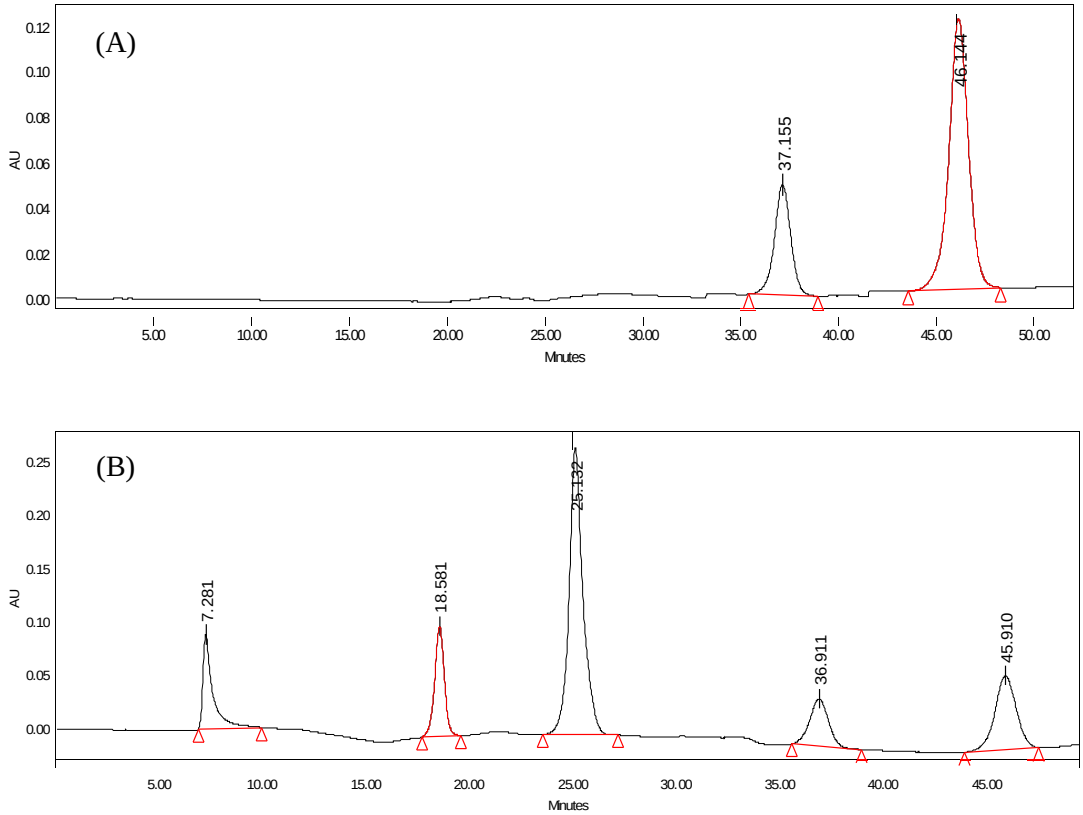


Fig. S6 HPLC analysis of the reaction mixture in the tandem oxidations of HMF to FDCA by GO and lipase.A: after GO-mediated oxidation HMF; B: after lipase-mediated oxidation of the mixture of DFF and HMF

The retention times of an unidentified product, FDCA, FFCA/HMFCA, HMF and DFFwere 7.2, 18.5, 25.1, 36.9, and 45.9 min, respectively. Analytic conditions were the same as those described in Fig. S3

As shown in Fig. S6, the separation of FFCA and HMFCA is not efficient for their quantitative analysis. However, we have found that the peak areas of HMFCA are comparable at 254 nm and at its maximum UV absorption wavelength (based on the whole band scanning by the PDA detector), while the peak area of FFCA at its maximum UV absorption wavelength is 3-fold higher than that at 254 nm. So the qualitative identification of HMFCA and FFCA is feasible based on the changes in the absorbance at 254 nm and the maximum UV absorption wavelength. On the other hand, few HMFCA was formed at the initial reaction (Fig. S7A). During the initial 7 h, no significant difference was found between the absorbance at 254 and the maximum UV absorption wavelength; therefore, the peak at 25.2 min was considered to stem from FFCA instead of HMFCA. The concentration of FFCA could be determined based on the peak area at 25.1 min. After 24 h, FFCA was used up (Fig. S7B). So the concentration of HMFCA could be calculated readily from its peak area after 24 h reaction, based on the corresponding calibration curve.

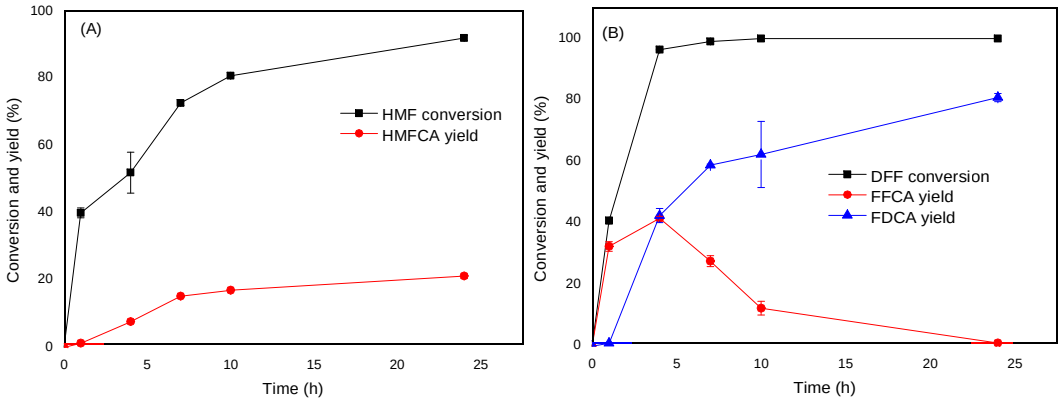


Fig. S7 Lipase catalyzed oxidation of HMF (A) and DFF (B) in the presence of H_2O_2 and EtOAc. Conditions for HMF oxidation: 2 mL EtOAc/*t*-butanol (1:1, v/v), 30 mM HMF, 12 mg Novozym 435, addition of 1.6 equiv. aqueous H_2O_2 (30%, v/v) each hour for 6 times, 30 ºC, 150 rpm. Conditions for DFF oxidation: 2 mL EtOAc/*t*-butanol (1:1, v/v), 10 mM DFF, 12 mg Novozym 435, addition of 1.6 equiv. aqueous H_2O_2 (30%, v/v) each hour for 6 times, 30 ºC, 150 rpm

Table S2 Separation of HMF and DFF with DES

DES	Extraction	HMF extracted (%)	DFF extracted (%)	DFF purity (%)
None	Original	-	-	76%
ChCl:Gly	1st	60	6	88
	2nd	81	17	93
	3rd	95	45	97
ChCl:Ur	1st	48	17	83
	2nd	70	32	87
	3rd	85	41	92

The original concentrations of HMF and DFF in EtOAc are 0.67 and 2.07 mg/mL. DES of the equal volume was added into EtOAc to extract HMF.

References

1. R. Bourbonnais, D. Leech and M. G. Paice, *Biochim. Biophys. Acta*, 1998, **1379**, 381-390.