Supporting Information

Substrate-constituted three-liquid-phase system: a green, highly efficient and recoverable platform for interfacial enzymatic reaction

Zhigang Li,[a] Huayong Chen,[a] Weifei Wang,[b] Man Qu,[a] Qingyun Tang,[b] Bo Yang*[a] and Yonghua Wang*[b]

[a]*School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, P. R. China*

[b]*School of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510641, P. R. China*

*Corresponding author: E-mail yangbo@scut.edu.cn; yonghw@scut.edu.cn

Experimental

Materials

All experiments were performed with deionized water. Olive oil was supplied by Sinopharm Chemical Reagent Co. (Shanghai, China). Lipozyme TL-100L (*Thermomyces lanuginosus* lipase) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Lipase AYS and lipase AY30 (*Candida rugosa* lipases) were obtained from Amano (Nagoya, Japan). Lipase T1 (*Geobacillus sp* T1 lipase) was produced in our laboratory by expressing its gene (GenBank accession number: AY260764, from Geobacillus zalihae) in recombinant *Escherichia coli*. Acylglycerol standards of

trioleoylglycerol, dioleoylglycerol (15% of 1,2-dioleoylglycerol and 85% of 1,3-dioleoylglycerol, CAS no. 25637-84-7), monooleoylglycerol were obtained from Sigma-Aldrich (China). Isopropanol and *n*-hexane were of HPLC grade from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). BCA kit was purchased from Nanjing KeyGen Biotech. Co. Ltd. Ion liquids, including 1-butyl-3-methylimidazolium bromide ([BMIM]Br), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4), 1-octyl-3- methylimidazolium chloride ([OMIM]Cl), 1, 3-dimethylimidazolium chloride ([DMIM]Cl), 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), 1-ethyl-3-methylimidazolium bromide ([EMIM]Br), 1-alkyl-3-methylimidazolium chloride ([AMIM]Cl) and 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM]$EtSO_4$)) was obtained from Lanzhou Institute of chemical physics. All the other chemicals were of analytical grade.

Partition behavior of lipase in various systems

Lipase were dissolved with 50 volumes of water (solvent to sample ratio, w/v) at room temperature. The experiment of partition behavior of lipase in various systems was first initiated adding polar solvents and solid salt into the lipase solution to form a biphase system consisting of 15-30% (wt.%) salt ([BMIM]Br/salt system: 20% of Na_2SO_4, 30% of $(NH_4)_2SO_4$; the other systems: 15% of Na_2SO_4, 20% of $(NH_4)_2SO_4$) and 20-30% (wt.%) ([EMIM]$EtSO_4$ and [OMIM]Cl: 30%, the others: 20%) polar phase-forming component at a 2 g scale, then 0.4 g of olive oil was added into the biphase system. The mixture was vortexed for 10 s and then centrifuged at 3000 rpm

for 5 min. (*ca.* 20 ºC). This experiment was conducted with different salts and with different polar phase-forming component using separate samples of the solution of different lipases. The hydrolytic activities of lipase in the middle and bottom phases were analyzed by colorimetric techniques, using *p*-nitrophenyl esters as the substrate.[1] The partition coefficient (K) of lipase was defined as the ratio of the activities of lipase in the middle phase to that in the bottom phase. The recovery (Y) was the ratio of lipase activity partitioned in the middle phase to the total amount activities of lipase. It was determined by the partition coefficient of lipase and the volume ratio of the middle phase to bottom phase via the equation,

$$Y(\%) = 100\% \times \frac{RK}{(RK+1)} \qquad (1)$$

where R is the ratio of the volume of the middle phase to that of the bottom phase. The purification factor (PF) of lipase is defined as the ratio of specific activity of lipase in middle phase to that of crude extract and can be calculated as per the following equation:

$$PF = \frac{A_m}{A_c} \times \frac{P_c}{P_m} \qquad (2)$$

where A_m, A_c and P_m, P_c are the activities of lipase (in Uml^{-1}) and protein concentration (in $mgml^{-1}$) in the middle phases and the crude lipase solution, respectively. The selective coefficient of lipase to glycerol was calculated as the ratio of the partition coefficient of lipase to that of glycerol.

Hydrolysis of olive oil in various systems

Olive oil hydrolyses by lipase in various systems were investigated. Batch reactions

were carried out in 10 ml conical flasks containing 2.4 g of SC-TLPS as described in the experiment for studying the partition behavior of lipase in various systems. The control was also carried out by adding 0.4 g of olive oil into 2 g of lipase solution containing the maximal quantity lipase with the SC-TLPS. The various systems were placed in a conical flask equilibrating the desired reaction temperature for 3 h (24 h for lipase T1). The reaction systems were agitated by shaking bath at 200 rpm. Finally, the reaction mixture was withdrawn periodically and centrifuged at 3000 rpm for 5 min (control is 10000 rpm for 10 min). The sample of the top phase preparation was analyzed by HPLC. All the reactions were performed at atmospheric pressure, and all experiments were carried out in triplicate.

Effects of PEG-400 and Na_2SO_4 concentrations on hydrolysis of olive oil in SC-TLPS

The effects of PEG-400 and Na_2SO_4 were investigated by adding solid Na_2SO_4 and PEG-400 into the lipase solution (5%, wt.%) to form a biphase system consisting of 5-34% (wt.%) PEG-400 and 6-23% (wt.%) Na_2SO_4 at a 2 g scale, and then 0.4 g of olive oil was added into the bi-phase system. The control was also carried out by adding 0.4 g of olive oil into 2 g of lipase solution contain the maximal quantity lipase with the SC-TLPS. The reaction systems were agitated by shaking bath at 200 rpm. Then, the reaction mixture was withdrawn periodically and centrifuged at 3000 rpm for 3 min. Finally, the reaction mixture was withdrawn periodically and centrifuged at 3000rpm for 5 min. Furthermore, the various agitation speeds also were investigated in the optimum PEG-400 and Na_2SO_4 concentration.

Reuse of middle lipase-rich phase

A SC-TLPS was first formed by adding 3 g of solid Na_2SO_4, 3 g of PEG-400, and 4 g of olive oil into 14 g of lipase solution (1200 Uml^{-1}). The SC-TLPS were agitated in 37 ºC for 25 min. The reaction mixture was withdrawn periodically and centrifuged at 3000 rpm for 5 min; then, the bottom phase was replaced by equal volume of the bottom (raffinate) phase. The SC-TLPS was agitated in the above conditions for 25 min. The middle phase was collected by centrifugation and recycled for next reaction. The experiment of middle phase reuse was studied by adding an original top and bottom phase into the reused middle phase to form a SC-TLPS, which is the same as the initial one.

Analytical methods

Confocal microscopy. Fluorescent images of the SC-TLPS microstructure were obtained on Laser scanning confocal microscopy (LSCM, Leica, Germany). For fluorescent imaging, the oil-phase marker (Nile Red) was used together with the middle phase marker (fluorescein), the former was excited by the 553nm line and the latter by the 460nm line. A SC-TLPS and a control (described in the section of the reuse of middle lipase-rich phase, except no lipase was contained) were agitated for at least 15 min just before the observations. A drop of the stained sample was placed on a concave confocal microscope slide about 1.2-1.3 mm thick.

Particle size analysis. Particle size analysis was investigated by using a Mastersizer 2000 laser diffractometer with the Hydrosizer 2000S module (Malvern Instruments, UK). The olive oil was extemporaneously dispersed in purified water, middle phase

and bottom phase of SC-TLPS at 2250 rpm until an obscuration rate of 10-20% was obtained, respectively. Then, the droplet size distributions ($d_{3,2}$ and $d_{4,3}$) were investigated at 30 min after. Each sample was measured in triplicate.

High-performance liquid chromatography analysis (HPLC). The compositions of the major compounds from the hydrolysates were analyzed by HPLC (Waters 2695) with refractive index detector (HPLC-RID). The components were separated on a Phenomenex Luna silica column (250 mm × 4.6 mm i.d., 5 μm particle size) at a column temperature of 35 °C. The mobile phase was a mixture of *n*-hexane, 2-propanol and methanoic acid (15:1:0.003, by volume) and the flow rate was 1.0 $mLmin^{-1}$. Sample injection volume was 10 μL. Peaks in HPLC were evaluated by comparison of their retention times with reference standards. Each acylglycerol species and FA was expressed as weight percentage. Acquisition and processing of data were collected and processed using the instrument integrated soft-ware (Waters 2695). The hydrolytic ratios of olive oil was the ratio of the total amount of free fatty acids generated ($[FFA]_t$) to the theoretical amount of free fatty acids produced.[2] The theoretical amount of free fatty acids produced was assumed to be equal to three times the amount of triglycerides (TAG), ($3\times[TAG]_0$). The conversion of oil into free fatty acids is calculated as in the following equation.

$$R_{FFA} = \frac{[FFA]_t}{3[\mathrm{TAG}]_0} \times 100\% \qquad (3)$$

Glycerol concentration was determined by an enzymatic kit.[3] The concentration of protein was determined by BCA kit (Sangon, Shanghai, China) and standard curve was made by different concentrations of BSA standard protein.[4]

Table S1 Number of phases in different systems.

	PEG-400	PEG-600	[BMIM]Br	[BMIM]BF_4	[EMIM]$EtSO_4$	methanol	ethanol	isopropanol	isoamyl alcohol
$(NH_4)_2SO_4$	III	III	III	III	III	II	III	III	II
Na_2SO_4	III	III	III	III	III	II	III	III	II
NaCl	II	II	III	II	II	II	II	III	II

Table S2 Relative activities of lipases treated by different PEGs. Lipases were incubated in the different polymer solution (50%, wt.%) for 24 h, respectively.

	PEG-400	PEG-600	PEG-1000	PEG-2000	Na_2SO_4	$(NH_4)_2SO_4$
R_{CRL} (%)	173.53	195.06	206.21	-	39.72	206.15
$R_{T\text{-}1}$ (%)	140.20	131.22	141.54	170.07	128.91	114.11
R_{CALB} (%)	167.63	168.69	141.23	162.47	35.45	49.20
$R_{P20000L}$ (%)	137.67	142.06	123.90	120.53	77.41	56.87
$R_{TL\text{-}100L}$ (%)	104.83	118.76	60.02	93.19	71.86	70.92

Table S3 Relative activities of lipases treated by different ionic liquids. Lipases were incubated in the different ionic liquid solution (50%, wt.%) for 24 h, respectively.

	[EMIM]Cl	[BMIM]Cl	[BMIM]Br	[BMIM]BF_4	[OMIM]Cl	[DMIM]Cl	[EMIM]Br	[AMIM]Cl	[EMIM]$EtSO_4$
R_{CRL} (%)	144.44	145.01	136.95	142.36	1.13	9.38	5.04	44.08	16.87
$R_{T\text{-}1}$ (%)	103.42	222.99	166.06	53.41	347.05	38.97	91.04	112.16	-
R_{CALB} (%)	166.77	153.82	128.03	50.77	58.91	110.49	133.04	104.42	151.50
$R_{P20000L}$ (%)	31.63	16.06	16.50	17.18	45.74	95.59	33.93	17.97	122.73
$R_{TL\text{-}100L}$ (%)	104.76	71.12	58.54	3.90	86.66	143.12	77.18	22.47	92.52

Table S4 Partition coefficients of lipases in different SC-TLPSs.

		PEG400	PEG600	PEG1000	PEG2000	[BMIM]Br	[BMIM]BF_4	[OMIM]Cl (AY30: [EMIM]$EtSO_4$)
K_{AYS}	$(NH_4)_2SO_4$	6.06	5.78	2.36	∞	∞	7.63	-
	Na_2SO_4	7.36	3.71	0.46	-	5.59	4.63	0.47
K_{T-1}	$(NH_4)_2SO_4$	1.92	2.30	5.50	0.94	5.80	-	-
	Na_2SO_4	3.48	1.43	0.09	1.01	0.24	-	-
K_{AY-30}	$(NH_4)_2SO_4$	4.72	0.66	0.35	0	1.04	1.05	1.27
	Na_2SO_4	1.31	0.45	0.03	-	1.04	2.01	0.98
$K_{TL-100L}$	$(NH_4)_2SO_4$	8.18	4.32	0.93	-	442.46	6.64	∞
	Na_2SO_4	3.30	4.18	-	5.76	55.17	0.27	11.06

Table S5 Diameters and specific surfaces of oil in water, middle phase and bottom phase of SC-TLPS.

	water	middle phase	bottom phase
D-specific surface (μm)	33.7	10.5	25.2
D-volume (μm)	91.5	32.1	68.2
specific surface ($m^2\ g^{-1}$)	0.178	0.571	0.238

Table S6 Comparison of achieved hydrolytic ratios of oils in different enzymatic reaction systems.

Reaction system	lipase origin	catalyst form	Substrate	T (ºC)	Time (h)	Y (%)	catalyst reuse and stability	ref.
SC-TLPS	*Candida rugosa*	free lipase	olive oil	30	1	96.7	more than 90% activity after 8 uses	this paper
O/W system	*Candida rugosa*	free lipase	linseed oil	33	3.3	93.9	-	5
isooctane/water system	*Candida rugosa*	free lipase	cod liver oil	35	1	26.1	-	6
reversed micelles	*Candida rugosa*	free lipase	palm oil	37	2	90	-	7
O/W system	*Candida rugosa*	immobilized lipase	soybean oil	40	5	88	80% activity after 6 uses, 60% activity after 10 uses	8
O/W system	*Thermomyces lanuginosus*; *Rhizomucor miehei*	combined use of two immobilized lipases	soybean oil	30	10	95	20% activity after 10 uses; 90% activity after 10 uses (washed with *n*-hexane)	9
isooctane/water system	*Candida rugosa*	immobilized lipase	canola and olive oil	40	5	<50	26% activity after 10 uses	10
O/W system	*Candida rugosa*	immobilized lipase	olive oil	45	12	<90	11% activity after 3 uses	11
O/W system	*Rhizomucor miehei; C. antarctica*	combined use of two immobilized lipase	soybean oil	53	24	<85	90% activity after 15 uses (washed with n-*h*exane)	12

T: temperature; Y: the hydrolysis rate of oil

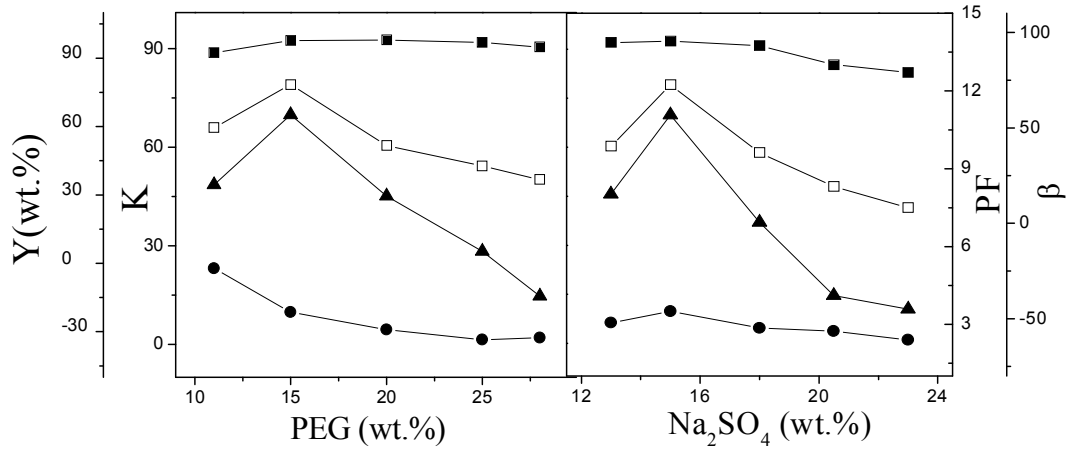

Figure S1 Effects of PEG-400 and sodium sulfate concentrations on the partition coefficients, purification factors and recoveries of lipase as well as the selectivity coefficients of lipase to glycerol in the PEG-400/Na_2SO_4 SC-TLPS. Left: The concentrations of Na_2SO_4 (wt.%) are 15%; Right: The concentrations of PEG-400 (wt.%) are 15%. Symbol: ▲:K, □:β, ●:PF, ■:Y_{lipase}.

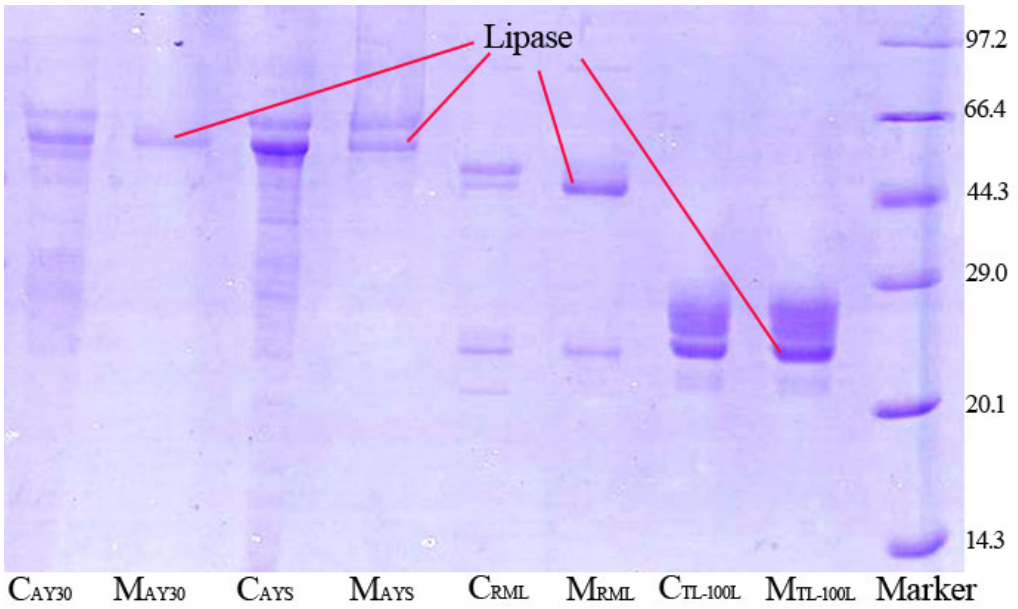

Figure S2 SDS–PAGE analysis of the crude lipases and the purified lipases by SC-TLPS.

C_{RML}: the crude RML lipase; M_{RML}: the purified RML lipase by SC-TLPS;

C_{AY30}: the crude lipase AY30; M_{AY30}: the purified lipase AY30 by SC-TLPS;

C_{AYS}: the crude lipase AYS; M_{AYS}: the purified lipase AYS by SC-TLPS;

$C_{TL\text{-}100L}$: the crude lipase TL-100L; $M_{TL\text{-}100L}$: the purified lipase TL-100L by SC-TLPS.

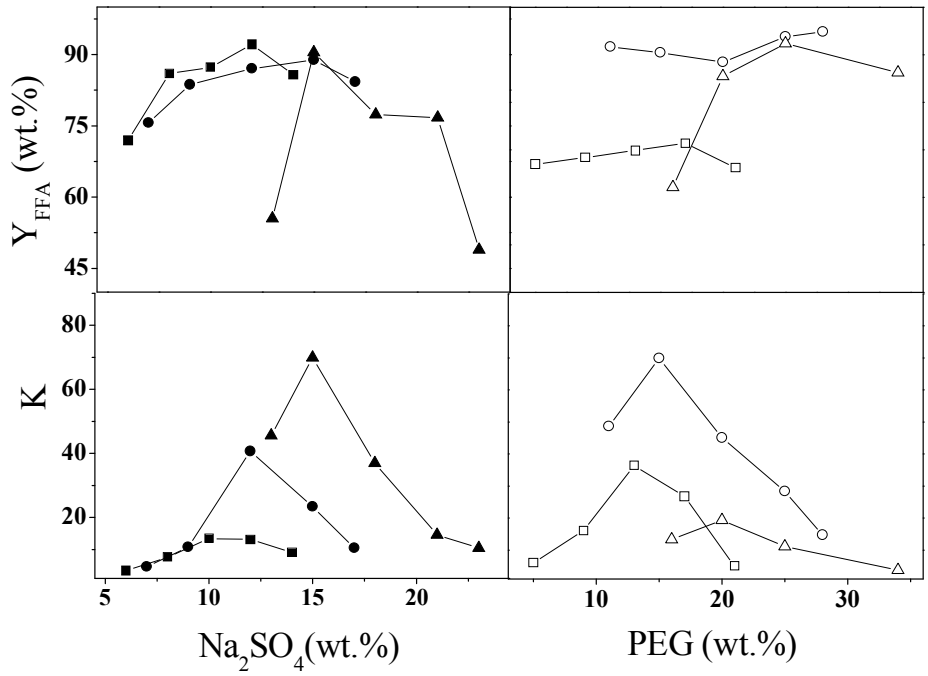

Figure S3 Effects of concentrations of PEG-400 and Na_2SO_4 on partition coefficients of lipase, and hydrolytic ratios of olive oil, with the concentration of PEG-400 (wt.%) being 15% (▲), 25% (●) and 30% (■), the concentrations of Na_2SO_4 being 12% (△), 15% (○) and 20% (□). Top figures: the effects of concentrations of PEG-400 and Na_2SO_4 on the hydrolytic ratios of olive oil; Bottom figures: the effects of concentrations of PEG-400 and Na_2SO_4 on the partition coefficients of lipase.

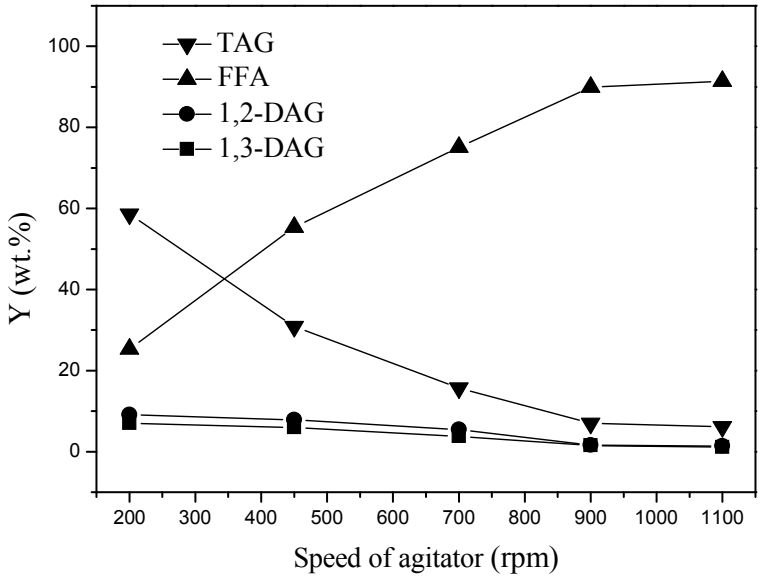

Figure S4 Effects of agitator speeds on the degree of olive oil hydrolysis.

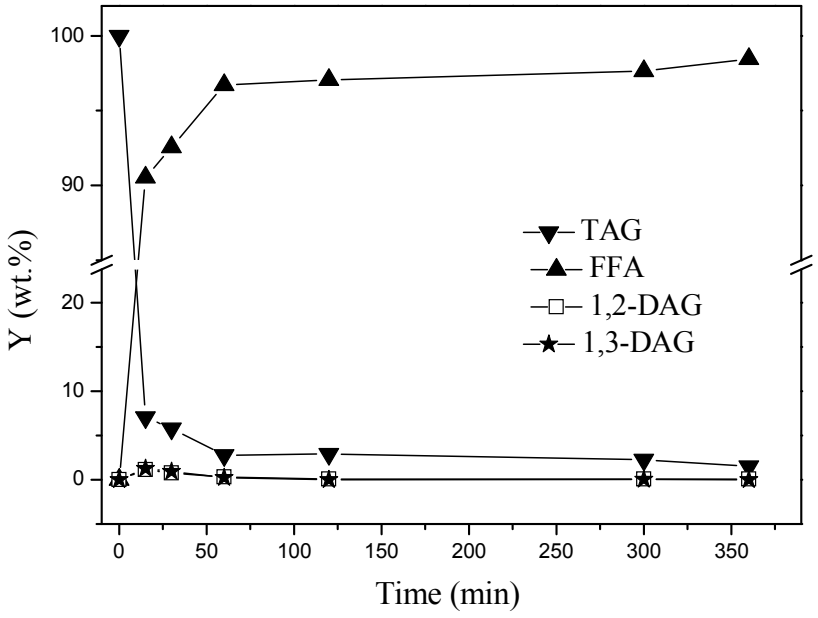

Figure S5 Effects of reaction time on the degree of olive oil hydrolysis.

References

1. A. Shome, S. Roy, and P.K. Das, *Langmuir*, 2007, **23**, 4130.
2. J. Kansedo and K. T. Lee, *Chem. Eng. J.*, 2014, **237**, 1.
3. Z. G. Li, D. J. Zhang and Z. L. Xiu, *Sep. Purif. Technol.*, 2009, **66**, 472.
4. M.M. Lin, D.N. Lu, J.Y. Zhu, C. Yang, Y.F. Zhang and Z. Liu, *Chem. Commun.*, 2012, **48**, 3315.
5. W.W. Chen, S.D. Sun, S.H. Liang, L. Peng, Y.D. Wang, M. Shen, *J. Oleo. Sci.*, 2014, **63**, 619.
6. A. Sharma, S.P. Chaurasia, A.K. Dalai, *Cata. Today.*, 2013,**207**, 93.
7. Z.D. Knezevic, S.S. Siler-Marinkovic, L.V. Mojovic, *Appl. Microbiol. Biotechnol.*, 1998, **49**, 267.
8. W.J. Ting, K.Y. Tung, R. Giridhar, and W.T.Wu, *J. Mol. Catal. B-Enzym.*, 2006, **42**, 32.
9. R. C. Rodrigues, and M.A. Z. Ayub, *Process. Biochem.*, 2011, **46**, 682.
10. M. Yigitoglu, and Z. Temocin, *J. Mol. Catal. B-Enzym.*, 2010, **66**, 130.
11. S. Fadıloglu, and Z. Soylemez., *J. Agric. Food. Chem.*,1998,**46**, 3411.
12. J.S. Alves, N.S. Vieira, A.S. Cunha, A.M. Silva, M.A.Z. Ayub, R. Fernandez-Lafuente, and R.C. Rodrigues, *Rsc. Adv.*, 2014, **4**, 6863.