Melamine-based Covalent Organic Polymers (MCOPs) as Lipase Nanocarrier for Recyclable Esters Hydrolysis and Transesterification

Zhihao Wang†, Ying Chen†, Jiahe Zhao, Guoliang Gao, Worawan Panpipat, Ling-Zhi Cheong*, and Cai Shen

1 Department of Food Science and Engineering, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832, CHINA
2 Institute of Materials Technology & Engineering, Chinese Academy of Sciences, 1219 Zhongguan Road, Ningbo, 315201, CHINA
3 Food Technology and Innovation Research Center of Excellence, Department of Agro-Industry, School of Agricultural Technology, Walailak University, Thasala, Nakhon Si Thammarat 80161, THAILAND
† These authors contributed equally to the work

Abstract: Present study has successfully synthesized melamine-based covalent organic polymers (MCOPs) and applied it as lipase carrier for recyclable esters hydrolysis and transesterification. The synthesized MCOPs are composed of dense nanosheet structures having a thickness of 3.5 nm. Three immobilization methods namely physical adsorption, cross-linking and carrier activation were employed to prepare the MCOPs-immobilized CRL. Cross-linked MCOPs-immobilized CRL (41.30 mg protein/g MCOPs) and carrier activated MCOPs-immobilized CRL (33.20 mg protein/g MCOPs) had higher enzyme loading as compared to physical absorb MCOPs-immobilized CRL (29.30 mg protein/g MCOPs). Nevertheless, physical absorb MCOPs-immobilized CRL demonstrated the highest esters hydrolysis (49.85 U) and transesterification (1.04 U) activities. Despite having the highest enzymatic activity, physical absorb MCOPs-immobilized CRL were not able to maintain its catalytic stability with more than 30% decreased in enzymatic activity during consecutive hydrolysis and transesterification activities. Meanwhile, cross-linked MCOPs-immobilized CRL demonstrated highest catalytic stability with highest enzymatic activities at the end of consecutive reactions. All the MCOPs-immobilized CRL can be easily recovered and reused through centrifugation with more than 85% of recovery rate.

Key words: melamine-based covalent organic polymers, Candida rugosa lipase, immobilization, hydrolysis, transesterification

1 Introduction
Green chemistry refers to production of chemical compounds based on green processes, emphasizes the use of renewable resources and environmentally friendly processes[1]. Enzymes which can be obtained from renewable sources and capable of catalyzing various chemical reactions under mild conditions are an important part of green chemistry. Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3), which catalyze hydrolysis and synthesis of long-chain acylglycerols and many other esters in aqueous/organic biphasic media, are one of the most widely used biocatalysts in organic chemistry[2]. They are able to preserve their catalytic activity in organic solvents, biphasic systems and micellar solutions[3]. Candida rugosa lipase (CRL, 6.46 × 6.31 × 4.77 nm) is one of the most commonly used lipases and has been widely used for biosynthesis and biotransformations such as resolution of racemic acids, high enantioselective esterification and selective hydrolysis of saturated fatty acids. CRL has great application potentials in chemical and biological industries[4–7]. However, high cost, marginal stability in harsh reaction conditions and difficulties in recovery and reusability has limit its industrial application[8].

Enzyme immobilization refers to confinement or localization of enzymes within a defined space which allows the enzymes to be separated physically from substrate and product for reuse[9]. Enzyme immobilization is also able to increase enzyme catalytic stability in harsh environment[10].

*Correspondence to: Ling-Zhi Cheong, Department of Food Science and Engineering, College of Food and Pharmaceutical Sciences, Ningbo University, Ningbo 315832, CHINA
E-mail: cheonglingzhi@nbu.edu.cn
Accepted February 23, 2020 (received for review February 5, 2020)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
Lipases had been immobilized on various inorganic (hydro-
talcite, GO/ZnO composite, metals, controlled pore metal
oxides) and organic (agarose, chitin, collagen, nylon, cellu-
lose) carriers using either non-covalent physical adsorption,
encapsulation, covalent bonding and cross-linking
methods. Some of these carriers, however, may cause
enzyme conformational transition and decrease mass
transfer resulting in low biocatalytic activity.

Nanosheets materials have recently been used for
enzyme immobilization. Covalently-linked porous
organic polymers (COPs), is a novel nanosheets material
constructed through covalent bonds. It possess unique
properties including high porosity, high surface area, low
density, good thermal and solvent stability. COPs have
been applied in gas storage/separation, catalysis, metal
adsorption, concentration of phenolic antioxidants and
sensing. Melamine (MA), 66% N by mass, is a cheap
and abundant triazine monomer used extensively in plastic,
medicinal, decorative, and paper industries. One of the
unique properties of MA include its molecular recogni-
tion ability through donation and acceptance of hydrogen
bonds, metal chelation, and \( \pi-\pi \) interactions. Recently, MA
has been used as monomers for synthesis of MA-based
COPs (MCOPs) with high porosity, special surface morphol-
ogy and amorphous nanostructures which are applied for
adsorption of heavy metal ions. The aforementioned
unique characteristics of MCOPs has deemed it a promising
novel candidate for enzyme immobilization. Nevertheless,
limited studies have been conducted to date to investigate
the use of this unique materials for enzyme immobilization.

Present study aimed to synthesize MCOPs and investi-
gate its application as carrier for CRL (Fig. 1). CRL was
immobilized on the MCOPs using three methods namely
physical adsorption, cross-linking and carrier activation.
Catalytic activity of the MCOPs-immobilized CRLs was
evaluated in terms of esters hydrolysis and transesterifica-
tion activities. In addition, recovery and reusability of the
MCOPs-immobilized CRLs were also investigated through
consecutive hydrolysis and transesterification reactions.
Findings from present study will guide development of a
metal-free organic polymer materials for lipase immobiliza-
tion.

2 Experimental Procedures

2.1 Materials

_Candida rugosa_ lipase (CRL) was obtained from Amano,
JAPAN. \( p \)-Nitrophenyl palmitate (\( p \)-NPP) and Bicinchonic
acid assay (BCA) were purchased from Sigma Aldrich.

![Fig. 1](image-url) a) Experimental flow diagram. Hydrolysis b) and transesterification c) reactions catalyzed by MCOPs-immobilized CRL.
2.2 Apparatus
Fourier-transform infrared spectroscopy (Nicolet 6700, Thermo-fisher, USA), Bruker’s Dimension icon Atomic Force Microscope, Hitachi Scanning Electron Microscope (SEM, Hitachi TM3030).

2.3 Measurements
2.3.1 Synthesis of MCOPs
Synthetic paths in references\(^{31}\). Firstly, 0.939 g of melamine was added to a beaker containing 50 mL of dimethyl sulfoxide. The mixture was magnetically stirred for 10 min to form a homogeneous solution. Following that, 1.36 mL of 1,4-dibromobutane was added to the mixture and stirred 30 min. The resultant mixture was then heated at 100°C for 2 h. Acetone was then added to the mixture to obtain a yellowish product. The yellowish product was purified by centrifugation and washed 6 times with a mixture of acetone and methanol (volume ratio of 1:1) to remove residual DMSO. The purified product was dissolved in absolute ethanol and ballmilled (600 r/min) for 3 h to obtain MCOPs. Finally, the obtained MCOPs was vacuum dried at 80°C for 10 h.

2.3.2 Physical characterization of the MCOPs
MCOPs was characterized using Fourier-transform infrared analysis (FTIR), atomic force microscope (AFM) and scanning electron microscopy (SEM). FTIR analysis was conducted using a Fourier-transform infrared spectroscopy. The incident light was oriented at the Brewster angle of silicon of 74°, and spectra between 650 and 4000 cm\(^{-1}\) were acquired over 100 scans with a resolution of 8 cm\(^{-1}\). AFM analysis was conducted using Bruker’s Dimension icon under ambient condition with a silicon nitride cantilever (SNL-10) using a measured spring constant of 0.4 N/m. All AFM images were acquired in ScanAsyst mode with an image resolution of 256 × 256 pixels. Structural morphology of the MCOPs was observed and imaged using a focused ion beam scanning electron microscope (Carl Zeiss) with a working potential of 3 kV under magnifications ranging from 7.35 K to 44.87 K.

2.3.3 Immobilization of CRL on MCOPs
CRL was immobilized on MCOPs using three different methods namely physical adsorption, cross-linking and carrier activation according to previously published method with slight modifications\(^{20}\).

2.3.3.1 Physical adsorption method
Twenty five milligram of MCOPs was mixed with 2.5 mL of CRL solution [10 mg CRL/mL of phosphate buffer (pH 7.0)]. The resulting mixture was shake in incubator (300 rpm) for 15 min. Reaction was terminated by adding 4 mL of 0.5 M Na\(_2\)CO\(_3\) followed by centrifugation at 6000 rpm for 10 min. The supernatant (0.5 mL) was diluted 10-fold with deionized water and 4-Nitrophenol (p-NP) liberated was extracted by aqueous alkaline phase, and detected at 410 nm against a blank without enzyme using UV-vis spectrophotometer.

2.3.3.2 Cross-linking method
Twenty five milligram of MCOPs was mixed with 2.5 mL of CRL solution [10 mg CRL/mL of phosphate buffer (pH 7.0)]. The resulting mixture was centrifuged (280 rpm) for 8 h at 40°C. Following that, the mixture was centrifuged (6000 rpm/min) for 10 min and precipitates were collected and immersed in 5 mL of 25 % glutaraldehyde (5 min).

2.3.3.3 Carrier activation method
MCOPs was activated by immersing in 5 mL of 25% glutaraldehyde solution for 5 min. Following that, MCOPs were separated by centrifugation at 6000 rpm/min for 10 min. Activated MCOPs was mixed with 2.5 mL CRL solution [10 mg CRL/mL phosphate buffer (pH 7.0)] and shake in incubator (280 rpm/min) for 8 h at 40°C. At the end of the immobilization process, the mixture was centrifuged (6000 rpm/min) for 10 min and precipitates were collected as MCOPs-immobilized CRL (PA).

2.3.4 Catalytic activity of the MCOPs-immobilized CRL
Esters hydrolysis activity was determined according to a previously reported method with slight modifications\(^{32}\). Four milliliters of ethanol containing p-NPP (15 mM) and phosphate buffer solution (4 mL of 50 mM, pH 7.5) were mixed in a screw-capped 15 mL vial. Twenty five milligram of MCOPs-immobilized CRL was then added to initiate the hydrolysis reaction. The mixture was incubated at 37°C in incubator shaker (300 rpm) for 15 min. Reaction was terminated by adding 4 mL of 0.5 M Na\(_2\)CO\(_3\) followed by centrifugation at 6000 rpm for 10 min. The supernatant (0.5 mL) was diluted 10-fold with deionized water and 4-Nitrophenol (p-NP) liberated was extracted by aqueous alkaline phase, and detected at 410 nm against a blank without enzyme using UV-vis spectrophotometer. The amount of liberated p-NP was determined according to standard curve prepared by varying amounts of p-NP. Enzyme activity (U) was defined as amounts of p-NP liberated per min by per gram of MCOPs-immobilized CRL. All reactions were performed in triplicate.
Esters of transesterification activity was conducted using method by Teng et al.\textsuperscript{24}. MCOPs-immobilized CRL (25 mg) was added to screw-capped vials containing 10 mM \textit{p}-NP and ethanol (300 μL) and \textit{n}-hexane (5 mL). Transesterification was performed at 40°C in a shaking incubator (200 rpm) for 45 min. At the end of the reaction, Na\textsubscript{2}CO\textsubscript{3} solution (1 mL) was added to terminate the reaction. The reaction mixture was centrifuged at 6000 rpm for 10 min and lower aqueous phase was diluted 5 times and 4-Nitrophenol (\textit{p}-NP) liberated was quantified at 410 nm. Enzyme activity (U) was defined as amounts of \textit{p}-NP liberated per min by per gram of MCOPs-immobilized CRL. All reactions were performed in triplicate.

2.3.5 Catalytic stability of the MCOPs-immobilized CRL

Catalytic stability of the MCOPs-immobilized CRL was evaluated by consecutive hydrolysis and transesterification reactions. Following each reaction, MCOPs-immobilized CRL was washed three times with phosphate buffer (5 mL) to remove residual unreacted \textit{p}-NPP from the immobilized enzyme. MCOPs-immobilized CRL was dried under nitrogen stream to constant weight and the recovered immobilized CRL was weighed. Enzyme recovery rate is defined as the ratio of the mass of MCOPs recovered after each reaction to the mass of MCOPs before the start of the reaction. The recovered immobilized CRL was then subjected to subsequent esters hydrolysis or transesterification reactions. Enzyme activity for each cycle of reaction was determined according to aforementioned procedures.

3 Results and Discussion

3.1 Physical characterization of the MCOPs

MCOPs were successfully synthesized and characterized. Figure 2a shows the FTIR spectra of melamine and the synthesized MCOPs. FTIR spectra of the melamine demonstrated the absorption bands near 3469, 3419 cm\textsuperscript{-1} correspond to the anti-symmetric stretching vibration of NH\textsubscript{2} and the absorption bands near 3332, 3129 cm\textsuperscript{-1} correspond to the bending vibration of NH\textsubscript{2}\textsuperscript{25, 26}. Following complete reaction with 1,4 dibromoethane, the reaction product (MCOPs) did not contain any absorption peaks at 3469, 3419, 3332, 3129 cm\textsuperscript{-1} indicating complete reaction of the melamine. Instead, the reaction product (MCOPs) demonstrated new peak at 2917 and 2836 cm\textsuperscript{-1} which was attributed to bending of the CH\textsubscript{2}. Formation of these new peaks indicated that 1,4-dibromoethane has successfully incorporated hydrocarbon groups into the MCOPs. In addition, MCOPs also demonstrated absorption peak at 1650 and 1550 cm\textsuperscript{-1} which are due to bending vibration of NH\textsubscript{2} and stretching vibration of C=N, respectively. A peak at 814 cm\textsuperscript{-1} which represents the bending vibration of triazine can also be observed. Specific details of the FTIR spectra and functional groups of melamine and MCOPs are shown in Table 1.

Figure 2b-c shows morphology of the MCOPs as imaged using SEM and AFM. MCOPs is mainly composed of dense nanosheet structures which are stacked in layers. AFM analysis shows the MCOPs nanosheet has a thickness of 3.5 nm (Fig. 2d).

3.2 Immobilization of the CRL on MCOPs

Three different approaches namely physical adsorption,
cross-linking and carrier activation was used to immobilized the CRL on MCOPs. Among the three methods, cross-linking method demonstrated the highest CRL loading rate of 41.30 mg protein/g MCOPs, followed by carrier activation and physical adsorption methods which have CRL loading rate of 33.20 mg protein/g MCOPs and 29.30 mg protein/g MCOPs, respectively. Higher loading rate of the cross-linking and carrier activation methods can be attributed to the use of glutaraldehyde which introduces reactive functional group forming Schiff base to bind with the CRL enzyme27, 28.

3.3 Catalytic activity of the MCOPs-immobilized CRL

Figure 3 shows the ester hydrolysis and transesterification activities of the MCOPs-immobilized CRL obtained through different immobilization methods. In comparison to free CRL which has a hydrolysis activity of 93 U, all the MCOPs immobilized-CRL showed decreased in hydrolysis activity (physical adsorption: 44.81 U, cross linking: 40.25 U and carrier activation: 49.85 U). Similar trend can be observed for esters transesterification activity. Immobilization resulted in a decrease in transesterification activity from 1.2 U (free CRL) to 1.04 U (physical adsorption), 0.729 U (cross-linking) and 0.878 U (carrier activation), respectively. Decrease in enzyme activity can be attributed to changes in enzyme conformation following binding with the MCOPs carrier. In addition, immobilization may have decrease the mass transfer rate between the immobilized CRL and p-NPP substrate. Interestingly, reduction in hydrolytic activity of the MCOPs-immobilized CRL is more pronounced as compared to the transesterification activity of lipases. As transesterification is performed in an organic solvent system, low reduction of transesterification activity of the MCOPs-immobilized CRL indicated that MCOPs-immobilized CRL may demonstrate good solvent stability.

3.4 Catalytic stability of the MCOPs-immobilized CRL

Figure 4 shows the catalytic stability of the MCOPs-immobilized CRL in consecutive hydrolysis and transesterification reactions.

An interesting phenomenon was observed during the consecutive hydrolysis reactions. Regardless of the immo-

| Group       | Vibration         | Wavelength (cm⁻¹) |
|-------------|-------------------|-------------------|
| N–H         | Stretching        | 3469              |
| N–H         | Stretching        | 3419              |
| N–H         | Bending           | 3332              |
| N–H         | Bending           | 3129              |
| CH₂         | Bending           | 2917              |
| CH₂         | Bending           | 2836              |
| N–H         | Bending           | 1650              |
| C=N         | Stretching        | 1550              |
| N–H         | Ring out of plane deformation | 1024 |
| triazine    | Bending           | 814               |

Table 1 FTIR spectra of melamine and MCOPs.

Fig. 3 a) Esters hydrolysis and b) transesterification activities of free lipase (FL), physical adsorb (PA), cross-linked (CL), and carrier activated (CA) MCOPs-immobilized CRL.
All the MCOPs-immobilized CRL showed a two-fold increase in terms of hydrolytic activity in the second cycle during consecutive hydrolysis reaction. It is possible that the MCOPs-immobilized CRL has its essential water layer surrounding the enzyme molecules stripped off during the immobilization process resulting in lower hydrolytic activity as compared to free CRL. The MCOPs-immobilized CRL then has regained essential water layer to maintain their optimal hydrolytic activity from the first hydrolysis cycle.

Among the three immobilization methods, MCOPs-immobilized CRL obtained through physical adsorption method has the highest enzyme activity during the first few cycles with a hydrolytic activity of 10.93 U during the second cycle. Nevertheless, there is a 31% dropped in hydrolytic activity to 7.54 U during the sixth cycle (Fig. 4a). We postulated that the weak binding force of physical adsorption method has led to lipases leakage and eventually decreased catalytic activity during the recycle test.

MCOPs-immobilized CRL obtained through cross-linking method also demonstrated similar trend with high hydrolytic activity during first few cycles of the hydrolysis reactions. Nevertheless, cross-linked MCOPs-immobilized CRL demonstrated higher catalytic stability as compared to physical adsorb MCOPs-immobilized CRL. Cross-linked MCOPs-immobilized CRL recorded a 19.05% decreased in hydrolytic activity (8.33 U) during the sixth cycle. In the seventh cycle, cross linked MCOPs-immobilized CRL has the highest hydrolytic activity of 5.98 U followed by carrier activated MCOPs-immobilized CRL (3.76 U) and physical adsorb MCOPs-immobilized CRL (3.49 U). In short, physical adsorb MCOPs-immobilized CRL has the highest hydrolytic activity but its catalytic stability is inferior to cross-linked MCOPs-immobilized CRL.

In terms of transesterification activities, regardless of immobilization methods, there were no significant differences in transesterification activity during the consecutive reactions (Fig. 4b). Similar to hydrolysis reaction, physical adsorb MCOPs-immobilized CRL demonstrated the highest transesterification activity but cross-linked MCOPs-immobilized CRL demonstrated better catalytic stability. After seven consecutive transesterification reactions, cross-linked MCOPs-immobilized CRL was able to maintain 82.5% of its transesterification activity. Meanwhile, physical adsorbed and carrier activated MCOPs-immobilized CRL were only able to maintain 68.7% and 58.26% of its initial activities, respectively.

It is worth noting that both the cross-linked and carrier activated MCOPs-immobilized CRL has lower catalytic activity as compared to physical adsorb MCOPs-immobilized CRL. This may due to changes in the CRL conformation as glutaraldehyde has been reported to reduce substrate affinity.

### 3.5 Recovery of the MCOPs-immobilized CRL

Figure 5 shows the recovery of MCOPs-immobilized CRL in consecutive hydrolysis and transesterification reactions. Regardless of immobilization methods, all the MCOPs-immobilized CRL can be easily recovered through centrifugation process during consecutive hydrolysis and transesterification processes. More than 85% of MCOPs-immobilized CRL can be recovered during the repeated experiments. Both aqueous and organic solvents did not affect the recovery of the MCOPs-immobilized CRL indicating good recyclability of the immobilized CRL.

### 4 Conclusion

MCOPs is successfully synthesized and applied as carrier for CRL for esters hydrolysis and transesterifications. MCOPs-immobilized CRL produced through cross-linking and carrier activation methods had higher enzyme loading as compared to MCOPs-immobilized CRL produced through physical adsorption method. Nevertheless, physi-
cal absorb MCOPs-immobilized CRL demonstrated the highest esters hydrolysis and transesterification activities. Despite having highest enzymatic activity, physical absorb MCOPs-immobilized CRL were not able to maintain its catalytic stability with more than 30% decreased in enzymatic activity during consecutive hydrolysis and transesterification activities. Among all the immobilized CRL, cross-linked MCOPs-immobilized CRL demonstrated the highest catalytic stability with the highest enzymatic activities at the end of the consecutive reactions. All the MCOPs-immobilized CRL can be easily recovered and reused with more than 85% of recovery rate. Findings from present study will guide development of MCOPs for used as a novel carrier for lipase which can be used in wide range of applications involving esters hydrolysis and transesterification reactions.

**Acknowledgment**

This work was sponsored by National Natural Science Foundation of China (21706137) and Ningbo University (421401560). Ling-Zhi Cheong acknowledged funding support from Ningbo "3315" Talent Program.

**References**

1) Anastas, P.; Eghbali, N. Green chemistry: principles and practice. Chem. Soc. Rev. 39, 301-312 (2010).
2) Cheong, L.-Z.; Wei, Y.; Wang, H.; Wang, Z.; Su, X.; Shen, C. Facile fabrication of a stable and recyclable lipase@amine-functionalized ZIF-8 nanoparticles for esters hydrolysis and transesterification. J. Nanopart. Res. 19, 280 (2017).
3) Villeneuve, P.; Muderhwa, J.M.; Graille, J.; Haas, M.J. Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. J. Mol. Catal. B 9, 113-148 (2000).
4) Sánchez, A.; RÍO, J.L.D.; Valero, F.; Lafuente, J.; Faus, I.; Solà, C. Continuous enantioselective esterification of trans-2-phenyl-1-cyclohexanol using a new Candida rugosa lipase in a packed bed bioreactor. J. Biotechnol. 84, 1-12 (2000).
5) Dong-Lin, W.; Ahindra, N.; Guan-Chun, L.; Jei-Fu, S. Factors affecting the resolution of dl-menthol by immobilized lipase-catalyzed esterification in organic solvent. J. Agric. Food Chem. 50, 262-265 (2002).
6) Chen, Y.; Cheong, L.-Z.; Zhao, J.; Pampipat, W.; Wang, Z.; Li, Y.; Lu, C.; Zhou, J.; Su, X. Lipase-catalyzed selective enrichment of omega-3 polyunsaturated fatty acids in acylglycerols of cod liver and linseed oils: Modeling the binding affinity of lipases and fatty acids. Int. J. Biol. Macromol. 123, 261-268 (2019).
7) Adlercreutz, P. Immobilisation and application of lipases in organic media. Chem. Soc. Rev. 42, 6406-6436 (2013).
8) Villeneuve, P.; Muderhwa, J.M.; Graille, J.; Haas, M.J. Customizing lipases for biocatalysis: A survey of chemical, physical and molecular biological approaches. J. Mol. Catal. B 9, 113-148 (2000).
9) Brena, B.M.; Batista-Viera, F. Immobilization of enzymes. In Immobilization of enzymes and cells, Springer, pp. 15-30 (2006).
10) Basso, A.; Serban, S. Industrial applications of immobilized enzymes-A review. Mol. Catal. 479, 35-54 (2019).
11) Zou, B.; Hu, Y.; Jiang, L.; Jia, R.; Huang, H. Mesoporous material SBA-15 modified by amino acid ionic liquid to immobilize lipase via ionic bonding and cross-linking method. Ind. Eng. Chem. Res. 52, 2844-2851 (2013).
12) Yilmaz, E.; Can, K.; Sezgin, M.; Yilmaz, M. Immobilization of Candida rugosa lipase on glass beads for enantioselective hydrolysis of racemic Naproxen methyl ester. Bioresour. Technol. 102, 499-506 (2011).
13) Thangaraj, B.; Solomon, P.R. Immobilization of lipases-A review. Part I: Enzyme immobilization. ChemBioEng Reviews 6, 157-166 (2019).
14) Zhang, S.; Shi, J.; Deng, Q.; Zheng, M.; Wan, C.; Zheng, C.; Li, Y.; Huang, F. Preparation of carriers based on ZnO nanoparticles decorated on graphene oxide (GO) nanosheets for efficient immobilization of lipase from Candida rugosa. Molecules 22, 1205 (2017).
Yan, M.; Ge, J.; Liu, Z.; Ouyang, P. Encapsulation of single enzyme in nanogel with enhanced biocatalytic activity and stability. *J. Am. Chem. Soc.* **128**, 11008-11009 (2006).

Li, Y.; Ruan, Z.; Zheng, M.; Deng, Q.; Zhang, S.; Zheng, C.; Tang, H.; Huang, F.; Shi, J. *Candida rugosa* lipase covalently immobilized on facilely-synthesized carbon nitride nanosheets as a novel biocatalyst. *RSC Adv.* **8**, 14229-14236 (2018).

Zhang, S.; Deng, Q.; Shi, J.; Zhang, C.; Tang, H.; Huang, F.; Shi, J. Novel amphiphilic polyvinylpyrrolidone functionalized silicone particles as carrier for low-cost lipase immobilization. *R. Soc. Open Sci.* **5**, 172368 (2018).

Wang, H.; Zhang, H.; Wei, S.; Jia, Q. Preparation of ionic liquid hybrid melamine-based covalent organic polymer functionalized polymer monolithic material for the preconcentration of synthetic phenolic antioxidants. *J. Chromatogr. A* **1566**, 23-31 (2018).

Yang, G.; Han, H.; Du, C.; Luo, Z.; Wang, Y. Facile synthesis of melamine-based porous polymer networks and their application for removal of aqueous mercury ions. *Polymer* **51**, 6193-6202 (2010).

Zhou, Q.Z.K.; Chen, X.D. Immobilization of β-galactosidase on graphite surface by glutaraldehyde. *J. Food Eng.* **48**, 69-74 (2001).

Sahiner, N.; Demirci, S.; Sel, K. Covalent organic framework based on melamine and dibromoalkanes for versatile use. *J. Porous Mater.* **23**, 1025-1035 (2016).