Wageningen Academic Publishers

Magnetic immobilisation of phospholipase C and its hydrolysis of phospholipids in crude soybean oil

D. Yu[1], C. Yu[1], Y. Jiang[1], X. Zhang[1], T. Yuan[1], L. Wang[2*], W. Elfalleh[3*] and L. Jiang[1*]

[1]School of Food Science, Northeast Agricultural University, Harbin 150030, China P.R.; [2]School of Computer and Information Engineering, Harbin University of Commerce, Harbin 150028, China P.R.; [3]Laboratoire Energie, Eau, Environnement et Procèdes (LEEEP), LR18ES35, Ecole Nationale d'Ingénieurs de Gabès, Université de Gabès, 6072 Gabès, Tunisia; hsdwlq@163.com; walid.elfalleh@fst.rnu.tn; jlzname@163.com

Received: 25 April 2018 / Accepted: 25 October 2018

RESEARCH ARTICLE

Abstract

In this study, free phospholipase C (PLC) was immobilised onto magnetic carriers, including sodium alginate (MPCSA), magnetic chitosan microparticles (MCM), Fe_3O_4/P (glycidyl methacrylate (GMA)-EDGM-St) and Fe_3O_4/SiOx-g-P (GMA). The magnetic immobilised PLC had a broader pH activity and temperature tolerance, which could be separated quickly and reused. The highest enzyme loading and enzymatic activity (135.64 mg/g and 8,560 U/g, respectively) were obtained using the magnetic immobilised PLC-Fe_3O_4/SiOx-g-P (GMA) system. Its optimum pH range was 5.5-7.0, and activity remained stable between 50 and 70 °C. After 6 cycles in soybean lecithin emulsion, the magnetic immobilised enzyme retained more than 80% of its initial activity. Enzymatic degumming with PLC-Fe_3O_4/SiOx-g-P (GMA) was carried out 2.0 h at 60 °C, resulting in 1,2-diacylglycerol (1,2-DAG) content of 1.07%, which improving the refining rate of soybean oil. Moreover, PLC-Fe_3O_4/SiOx-g-P (GMA) still possessed more than 80% of its initial activity after 5 cycles of degumming in crude soybean oil. The results show that magnetic immobilised PLC has a good industrial application.

Keywords: phospholipase C, magnetic immobilisation, stability, oil degumming, 1,2-DAG

1. Introduction

Crude soybean oil contains approximately 3% of phospholipids, which leads to dark-colouring and off-flavours during refining and storage; it therefore needs to be removed (Li *et al.,* 2016; Zamora *et al.*, 2004). Traditional degumming processes include acid treatment and water degumming, which remove most of the phospholipids but produce a large amount of waste water polluting the environment, and also cause the loss of neutral oil, reducing the soybean oil refining rate (Dayton *et al.,* 2013; Mei *et al.,* 2013). The new degumming technologies include membrane degumming, adsorption degumming and enzymatic degumming (Laatikainen *et al.,* 2015; Subrahmanyam *et al.,* 2006). Enzymatic degumming is widely used because of its mild reactive conditions, wide scope of application, high oil yield and high quality of oil. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA) are the main components of phospholipids in crude soybean oil (Lamas *et al.,* 2014, 2016; Yang *et al.,* 2014). Phospholipase C (PLC) hydrolyses the sn-3 phosphate ester bond on the glycerol side, producing a 1,2-diacylglycerol (1,2-DAG) and a free head group (Palvannan and Boopathy, 2005; Piel *et al.,* 2017). This process removes PC, PE and increases the recovery of oil. Since the generated 1,2-DAGs remain in the oil during refining, they contribute to the oil yield. In addition, the smaller quantity of triacylglycerols are trapped due to the reduction of the emulsion produced by the gum (Dijkstra, 2010; Elena *et al.,* 2016). Phospholipase A_1 (PLA_1) cleaves the sn-1 ester bond, yielding a free fatty acid and an sn-2-lysophospholipid that easily form sn-1-lysophosphatide. PLA_1 continues to hydrolyse sn-1-lysophospholipid resulting in the reduction of hydrolysis efficiency. Phospholipase A_2 (PLA_2) targets the sn-2 ester, but its enzyme activity is lower than PLA_1. PLA can

significantly reduce the levels of phospholipids in crude soybean oil, but the acid value of soybean oil could increase, and the degumming time takes longer. Some researchers used PLC and PLA to reduce the phosphorus content of crude soybean oil, but PLC and PLA have their own suitable temperature and pH. If they hydrolyse phospholipids under the same conditions, the effect of degumming will be reduced. At the same time, the free phospholipases are very sensitive to pH and temperature and cannot be easily reused. So, the immobilisation of phospholipases is of great significance for improving their reusability.

Enzymes are immobilised on different materials by physical or chemical methods. Enzyme immobilisation methods usually involve entrapping, covalent bonding and cross-linking (Garcia-Galan *et al.,* 2011; Xie and Wang, 2012). The immobilised materials are generally natural polymer materials and nanomaterials. The nanoparticle can provide a larger surface area for enzyme attachment, leading to higher enzyme loading per unit mass of particles. Previously we successfully immobilised PLA on calcium alginate, calcium alginate-chitosan and calcium alginate-gelatine, and applied it to the degumming of crude soybean oil. Since the viscosity of heavy phase produced during degumming is high, it is difficult to separate the immobilised enzyme (Yu *et al.,* 2012, 2014). Recently, some studies focused on immobilised phospholipase and lipase on a magnetic carrier to separate enzyme from substrate (Liu *et al.,* 2013; Qu *et al.,* 2016). Yu *et al.* (2013) immobilised PLA_1 on Fe_3O_4/SiOx-g-P (glycidyl methacrylate (GMA)); it had a broader pH-activity profile of pH 4.5-6.5, was remarkably stable at 45-55 °C for 7 h and was easily separated from the substrate. It still possessed more than 80% of its initial activity after 6 cycles of the degumming process. Therefore, the magnetic immobilised enzyme is not only separated from the substrate, but it can also be reused in a magnetic field.

In this work, free PLC was immobilised onto magnetic nanoparticle carriers. The effect of enzyme loading, enzymatic activity and enzymatic properties of four types of magnetic immobilised PLC were investigated. The effects of the hydrolysis temperature and time on the residual phosphorus levels in crude soybean oil were also studied. The reusability of magnetic immobilised PLC and content changes of crude soybean oil compositions during hydrolytic process were also evaluated. This research provides a theoretical basis for the application of magnetic immobilised PLC to continuous degumming in magnetic fluidised bed.

2. Materials and methods

Materials

PLC, with a phospholipase activity of 17,000 U/g, was purchased from Dutch State Mines (DSM) nutritional products Ltd (Heerlen, the Netherlands). Crude soybean oil was provided by Jiusan Group (Harbin, China P.R.) with an original phosphorus content of 800 mg/kg. Sodium alginate (MPCSA), magnetic chitosan microparticles (MCM), Fe_3O_4/P (GMA-EDGM-St) and Fe_3O_4/SiOx-g-P (GMA) were prepared by our own laboratory. PC, PE, PI, PA, 1,2-DAG and p-nitrophenylphosphorylcholine with purities greater than 98% were purchased from Sigma Chemical Ltd. (St. Louis, MO, USA). All other analytical grade reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China P.R.).

Production of magnetic immobilised PLC

The free PLC was cross-linked with MCM and MPCSA by glutaraldehyde (Qu *et al.,* 2016). Epoxy groups on the surface of Fe_3O_4/P (GMA-EDGM-St) and Fe_3O_4/SiOx-g-P (GMA) form covalent bonds with amino groups on the surface of PLC to complete the immobilisation under mild conditions (Lin and Doong, 2011; Milosavić *et al.,* 2007; Xu *et al.,* 2005). The preparation of PLC-MCM and PLC-Fe_3O_4/P (GMA-EDGM-St) was performed according to the method described by Qu *et al.* (2016). At first, 1.0 g of MPCSA was soaked in phosphate buffer (0.1 mol/l, pH=7) for 24 h. After separation with a magnet, the supports were transferred into a PLC solution (0.02 g/ml). The mixture was shaken for 5 h at 55 °C and subsequently mixed with 3.0 ml of glutaraldehyde solution. This mixture was then kept for 5 h at 55 °C. Next, the magnetic nanoparticles containing immobilised PLC were separated with a magnet. Following separation, the supernatant was decanted, and the particles were washed at least three times with phosphate buffer to obtain PLC-MPCSA. PLC-MPCSA was preserved at 4 °C until use. 1.0 g Fe_3O_4/SiOx-g-P (GMA) were immersed in phosphate buffer (0.1 mol/l, pH=7) for 24 h. After magnetic separation, the magnetic carriers were transferred into a PLC solution (0.02 g/ml) and stirred at 55 °C for 5 h. The magnetic immobilised PLC was separated and washed with phosphate buffer three times to prepare PLC-Fe_3O_4/SiOx-g-P (GMA). PLC-Fe_3O_4/SiOx-g-P (GMA) was preserved at 4 °C (Qu *et al.,* 2016). The enzyme loading of magnetic immobilised PLC was detected as described by the Bradford method (Bradford, 1976). The activity of magnetic immobilised PLC was determined by the method of Jiang *et al.* (2015a). The secondary structure of PLC after immobilisation was determined by circular dichroism spectra.

Enzymatic characteristics of magnetic immobilised PLC

Several samples (2 g/100 g soybean lecithin emulsions) were prepared in phosphate buffer (0.1 mol/l) with different pH values. The optimum temperature was 55 °C, and the additive contents of free and magnetic immobilised PLC were 2.7 mg/kg and 110 mg/kg, respectively. The effect of pH (in the range 4.0-7.5) on the relative activity of free and magnetic immobilised PLC was studied. The effect of temperature on the relative activity of free PLC and magnetic immobilised PLC was determined using a 2 g/100 g lecithin emulsion at temperatures ranging from 40 °C to 75 °C at the previously determined optimum pH. The highest activity measured over the range of pH and temperatures was designated as 100%, and the activities at other pH and temperatures were calculated as proportions of the highest activity.

Reusability of magnetic immobilised PLC

The magnetic immobilised PLC was added to substrate and hydrolysed for 2 h at optimum pH and temperature conditions. The magnetic immobilised PLC was separated by magnets and then washed with 1 mol/l phosphate buffer to its optimum pH. The residual enzyme activity after each cycle was defined as the value proportional to the original activity (100%).

Effect of magnetic immobilised PLC on hydrolysis of phospholipids in crude soybean oil

Crude soybean oil (100 g) was heated to 70 °C in a water bath, and 0.13 ml of a 45% citric acid solution was added under high shear rate (29 g) for 20 min. The temperature was then decreased to 45-65 °C, and a 4% (w/w) NaOH solution was added to adjust the mixture pH. Magnetic immobilised PL was then added to the oil at a certain dosage rate. The mixture was incubated with continuous stirring at 5×*g* for various times (0-2.5 h). After the reaction, the oil mixture was quickly centrifuged at 117×*g* for 10 min. The residual phosphorus content in the oil phase was determined according to the AOCS method 12-55 (Brühl, 1997). Reusability of magnetic immobilised PLC in the process of crude soybean oil degumming was also studied. Magnetic immobilised PLC, which has better characteristics than other magnetic immobilised PLC, was used to degum in crude soybean oil. The mixture was dried with a vacuum of 0.096 MPA and at a temperature of 80 °C for 4 h in a vacuum dryer, and then homogenised after reaction. The content changes of crude soybean oil compositions during hydrolytic process were studied, and the method of determination referenced to Ravasi *et al.* (2015). The formula for determining the oil yield after degumming is as follows:

$$\text{Oil yield (\%)} = M_1 / M_2 \times 100$$

Where, M_1 = the quantity of oil after degumming (g); M_2 = the quantity of crude soybean oil (g).

Statistical analysis

All experiments were carried out in triplicate to allow for the calculation of means. Statistical analysis was performed with Origin 8.5 software (OriginLab Ltd., Northampton, MA, USA). One-way ANOVA was performed using SPSS 17 Statistical software (SPSS Inc., Chicago, IL, USA). According to Duncan's Multiple Range Test, differences were considered to be significant at $P \leq 0.05$.

3. Results and discussion

Magnetic immobilised PLC

The enzyme loadings and activity

As shown in Figure 1A, the enzyme loadings of MPCSA, MCM, Fe_3O_4/P (GMA-EDGM-St) and Fe_3O_4/SiOx-g-P (GMA) were 61.62, 68.95, 90.63 and 135.64 mg/g, respectively. The enzyme loading of Fe_3O_4/SiOx-g-P (GMA), compared to the other three types of magnetic immobilised PLC, was highest. The results were close to Lin's, that was 98.4 mg/g protein Fe_3O_4/SiOx-g-P (GMA) (Lin and Doong, 2011). The enzyme activities of PLC-MPCSA, PLC-MCM, PLC-Fe_3O_4/P (GMA-EDGM-St) and PLC-Fe_3O_4/SiOx-g-P (GMA) were 6,912, 7,106, 7,890, 8,560 U/g, respectively (Figure 1B). The free PLC is suitable for immobilising on the four magnetic carriers due to the enzyme loading, and the enzyme activities of magnetic immobilised PLC were higher.

Circular dichroism analysis

The secondary structures of free PLC and four magnetic immobilised PLC were analysed by circular dichroism. The results are shown in Figure 2. All five PLCs had positive peaks near 190 nm and negative peaks at 222 nm, indicating the existence of α-helix; positive peaks at 195-198 nm and negative peaks near 218 nm, indicating the existence of β-sheet; and absorption peaks at 212 nm, indicating the existence of random coil. The ratio of secondary structure is shown in Table 1. After immobilisation, the secondary structure of PLC changed, the content of α-helix decreased, and the content of β-sheet, β-turns and random coil increased in varying degrees. The change in alpha helix of PLC-Fe_3O_4/SiOx-g-P (GMA) was relatively small, and the content of random curl increased slightly, so the effect of immobilisation on its secondary structure was relatively small.

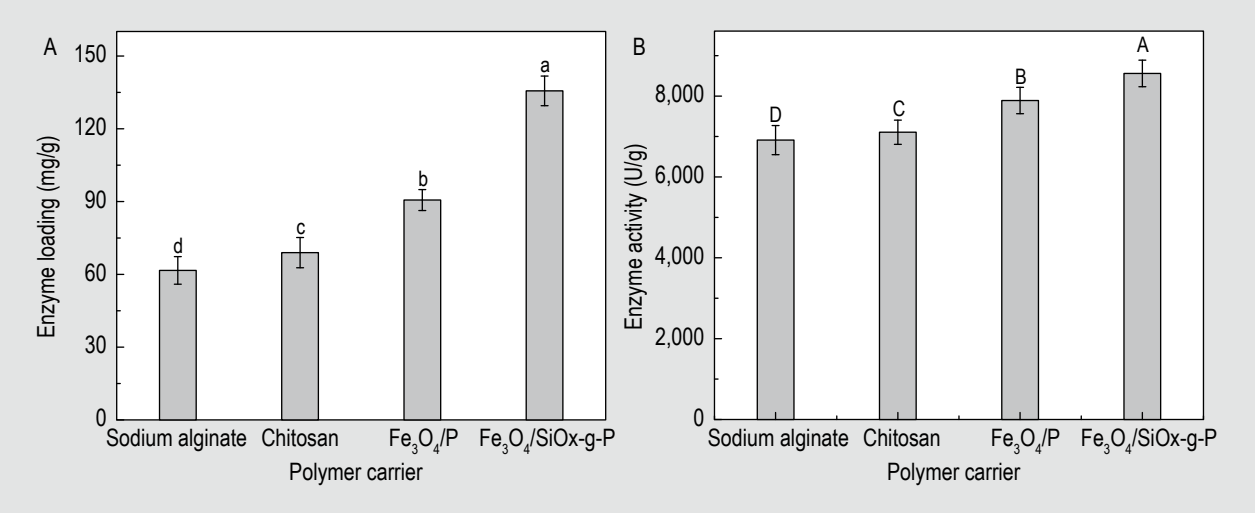

Figure 1. Enzyme load of magnetic carriers (A) and enzyme activity of the four types of magnetic immobilised phospholipase C (PLC) (B). Superscript letters indicate homogenous sub-classes as resulted from ANOVA (P<0.05, Duncan's new multiple range test).

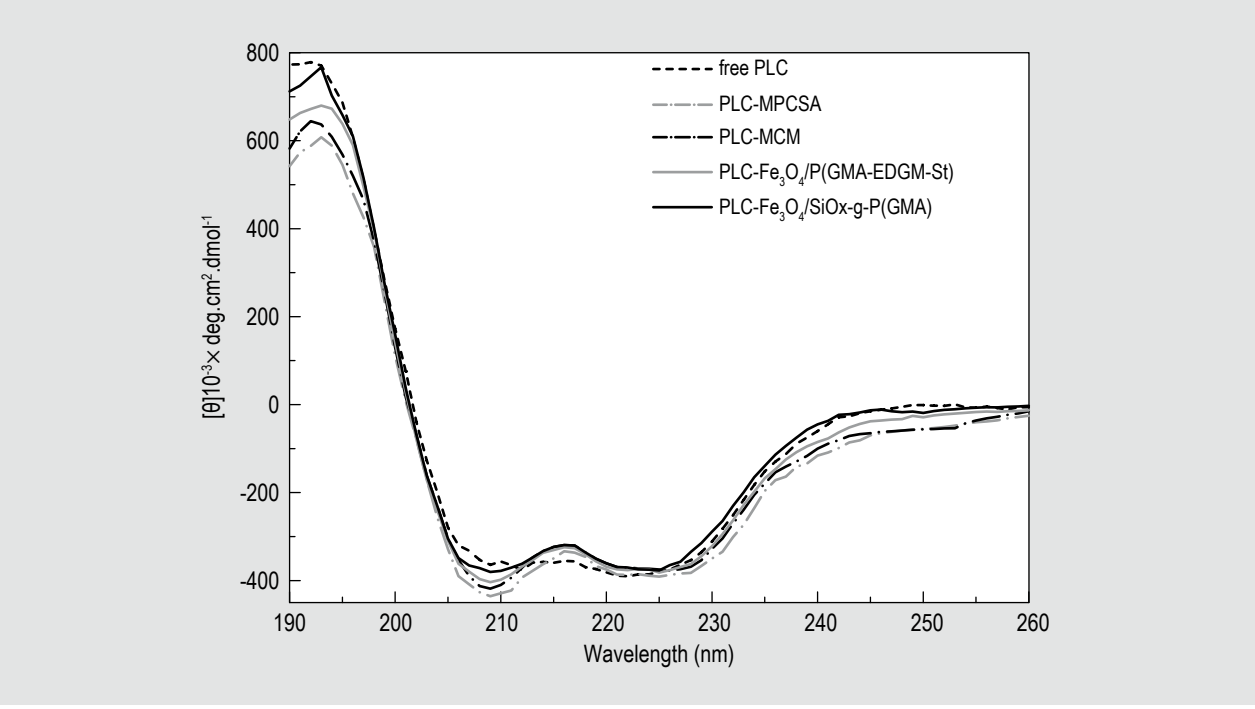

Figure 2. Circular dichroism spectra of free and magnetic immobilisation phospholipase C (PLC).

Table 1. Secondary structure composition of free phospholipase C (PLC) and magnetic immobilised PLC (%).[1]

Enzyme	α-helix (%)	β-sheet (%)	β-turns (%)	random coil (%)
Free PLC	11.2	26.6	22.9	39.3
PLC-MPCSA	6.8	27.4	23.3	42.5
PLC-MCM	7.3	27.2	23.3	42.2
PLC-Fe_3O_4/P (GMA-EDGM-St)	8.7	27.0	23.1	41.2
PLC-Fe_3O_4/SiOx-g-P (GMA)	9.7	26.9	23.0	40.4

[1] MCM = magnetic chitosan microparticles; MPCSA = sodium alginate; GMA = glycidyl methacrylate.

Effect of pH on the relative activity of free and magnetic immobilised PLC

The effect of pH values on the relative activity of free and magnetic immobilised PLC to hydrolyse soybean lecithin emulsions was determined at 55 °C for 2 h, and the results are shown in Figure 3. The maximum activity of free PLC was obtained at pH 5.5. The optimum pH was 6.0 for PLC-MPCSA and PLC-MCM and 6.5 for PLC-Fe_3O_4/P (GMA-EDGM-St) and PLC-Fe_3O_4/SiOx-g-P (GMA). The optimum pH of magnetic immobilised PLC is obviously higher than free PLC, and it shifts from 5.5 to neutrality. Magnetic carriers attach to negative charges since a large number of carboxyl groups exist on the surface of MPCSA, Fe_3O_4/P (GMA-EDGM-St), Fe_3O_4/SiOx-g-P (GMA); the pH of magnetic immobilised PLC shifts toward the alkaline side (Liu *et al.,* 2013). Amino/amine groups of MCM carrier also cause the shift of pH to the alkaline side (Chang and Juang, 2005). The conformation of free PLC was changed when it was immobilised, and destruction of the catalytic centre of the magnetic immobilised PLC was reduced by the change in solution pH (Qu *et al.,* 2016). Thus, magnetic immobilisation improves the optimum pH of PLC.

Effect of temperature on the relative activity of free and magnetic immobilised PLC

The effect of temperature on the relative activity of free and magnetic immobilised PLC was determined using soybean lecithin emulsions as substrate at optimum pH for 2 h (Figure 4). In the graph, the optimum temperature for free PLC was found to be 55 °C. The optimum temperature

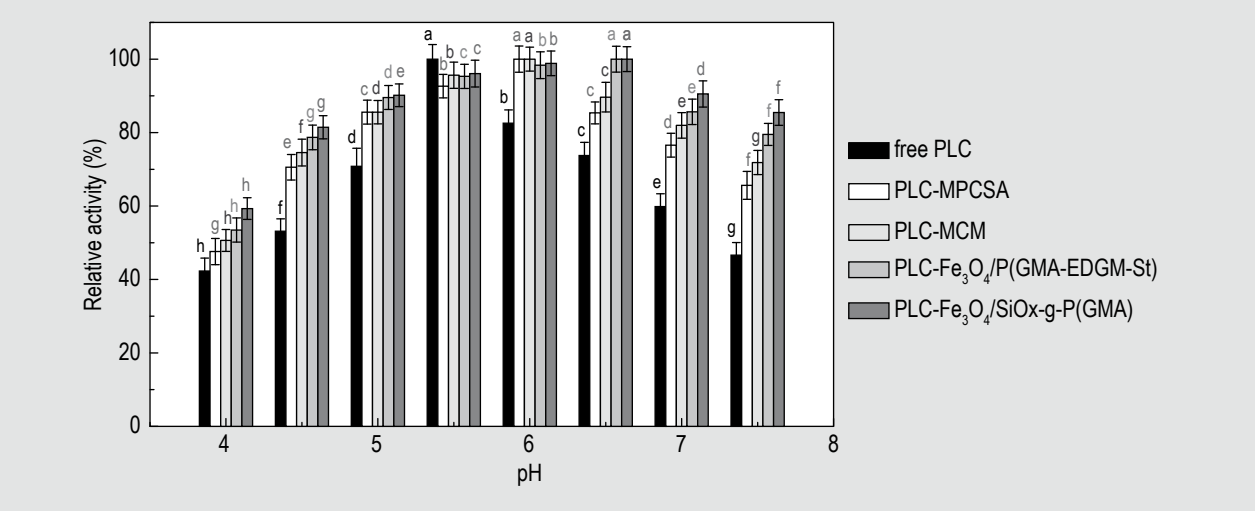

Figure 3. Effect of pH on the relative activity of free and magnetic immobilised phospholipase C (PLC). Superscript letters indicate homogenous sub-classes as resulted from ANOVA (*P*<0.05, Duncan's new multiple range test).

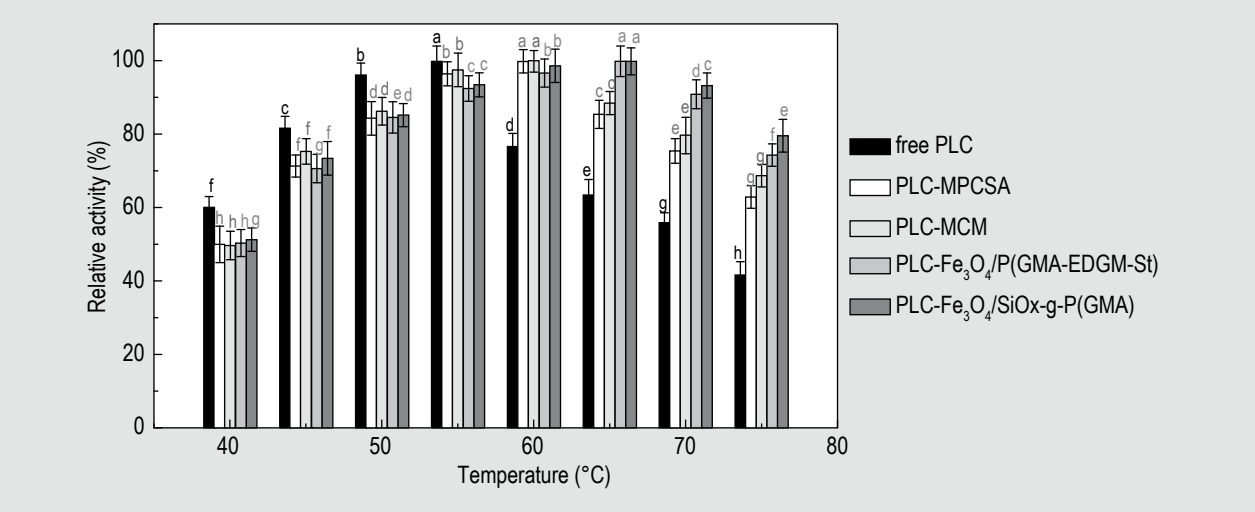

Figure 4. Effect of temperature on the relative activity of free and magnetic immobilised phospholipase C (PLC). Superscript letters indicate homogenous sub-classes as resulted from ANOVA (*P*<0.05, Duncan's new multiple range test).

for PLC-MPCSA and PLC-MCM was 60 °C. The optimum temperature for PLC-Fe_3O_4/P (GMA-EDGM-St) and PLC-Fe_3O_4/SiOx-g-P (GMA) was 65 °C. These results indicate that the optimum temperature for magnetic immobilised PLC is higher than free PLC, and that PLC-Fe_3O_4/SiOx-g-P (GMA) is 10 °C higher than for free PLC. The carriers can protect the enzyme proteins against changes in the enzyme conformation after immobilising, which in turn protects magnetic immobilised PLC against thermal denaturation particularly at high temperatures (Ibrahim *et al.,* 2013). Lima *et al.* (2017) immobilised cellulase on magnetic nanoparticles encapsulated in polymeric nanospheres, and its thermal stability increased by 10 °C. Therefore, the results confirm that the immobilisation process influenced the optimum temperatures of PLC.

Reusability of magnetic immobilised PLC

To investigate reusability, the immobilised PLC hydrolysed soybean lecithin emulsions at optimum temperature and pH for 2 h. As shown in Figure 5, after 6 recycles, PLC-MPCSA, PLC-MCM, PLC-Fe_3O_4/P (GMA-EDGM-St), PLC-Fe_3O_4/SiOx-g-P (GMA) retained 65.23, 69.03, 73.26 and 80.12% relative activity, respectively. The results show that four magnetic immobilised enzymes have a good reusability. Guo *et al.* (2016) reported that the residual activity of magnetic immobilisation of α-amylase still remained approximately 80% of the initial activity after the 4th reuse, indicating that the immobilised enzyme had good stability and reusability. The loss of enzyme activity was attributed to the denaturation of PLC in the hydrolysis environment, followed by the mechanical agitation and the collision of magnetic immobilised PLC during hydrolysis, which resulted in the PLC shedding off the carrier. If magnetic immobilised PLC is applied in the magnetic fluidised bed, the loss of PLC on the magnetic carrier will be avoided by external force damage, and the reusing effect will be improved.

Effect of magnetic immobilised PLC on hydrolysis of phospholipids in crude soybean oil

Effect of hydrolysis temperature on residual phosphorus levels of degummed crude soybean oil

Effect of the reaction temperature on the residual phosphorus levels of degummed crude soybean oil was presented in Figure 6. The degumming conditions were the enzyme dosage of 110 mg/kg with incubation time of 2.5 h at optimal pH. The residual phosphorus content in crude soybean oil decreased gradually with the enhancement of hydrolysis temperature. There was a significant decrease in phosphorus content of crude soybean oil when the hydrolysis temperature increased from 45 °C to 60 °C. The residual phosphorous content was decreased to 64.82 mg/kg, which was lower than the phosphorous contents obtained with the other magnetic immobilised PLC in this study, when PLC-Fe_3O_4/SiOx-g-P (GMA) was added to crude soybean oil. When the hydrolysis temperature was raised above 60 °C, the residual phosphorous content of crude soybean oil decreased slightly. Temperatures influence the viscosity of the lipid mixture and the activity of enzyme, affecting the removal rate of phospholipids in crude soybean oil (Jiang *et al.,* 2014). These results demonstrate that hydrolysis temperature remarkably affects the degumming of magnetic immobilised PLC.

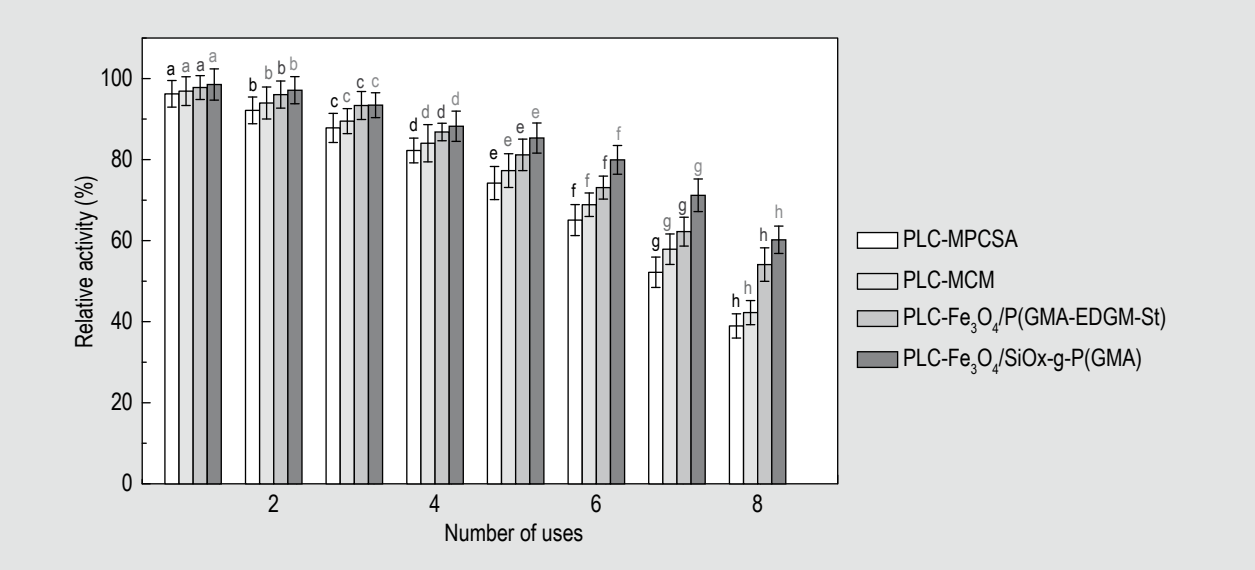

Figure 5. Reusability of magnetic immobilised phospholipase C (PLC). Superscript letters indicate homogenous sub-classes as resulted from ANOVA (*P*<0.05, Duncan's new multiple range test).

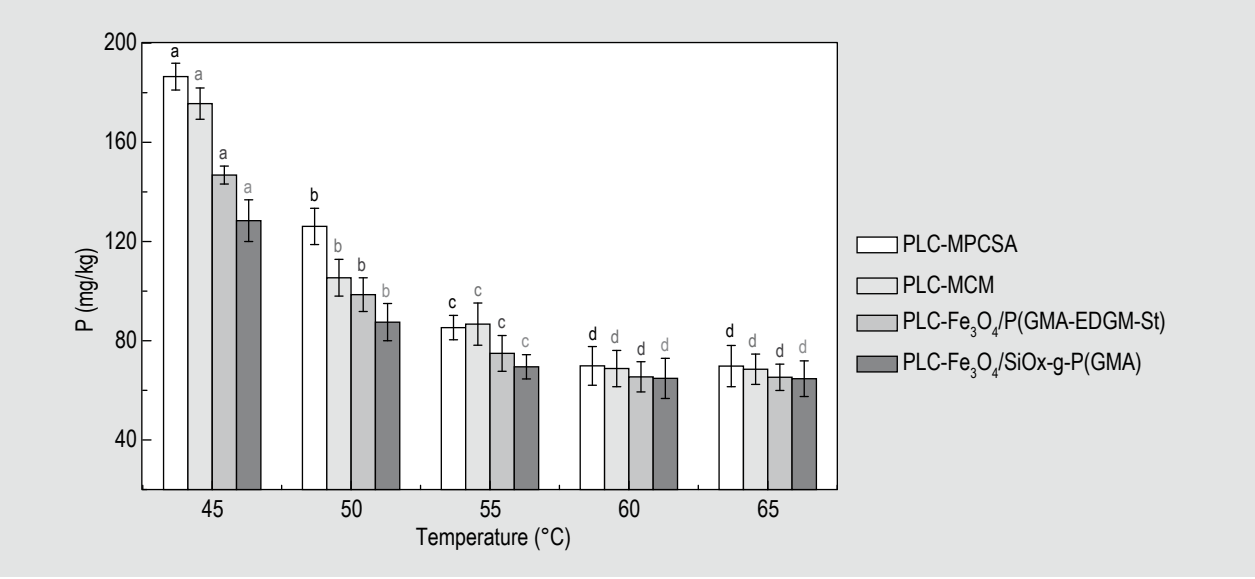

Figure 6. Effect of the hydrolysis temperature on the residual phosphorus levels. Superscript letters indicate homogenous sub-classes as resulted from ANOVA (*P*<0.05, Duncan's new multiple range test).

Effect of hydrolysis time on residual phosphorus levels of degummed crude soybean oil

The degumming of magnetic immobilised PLC in crude soybean oil was conducted with the enzyme dosage of 110 mg/kg and at optimal pH and temperature. Figure 7 shows the effect of hydrolysis time on the residual phosphorus content of degummed crude soybean oil. The residual phosphorous content of crude soybean oil decreased with the prolongation of hydrolysis time. At the beginning of hydrolysis for 1.0 h, there was a rapid decline of residual phosphorus content. When PLC-MCM, PLC-MPCSA, PLC-Fe_3O_4/P (GMA-EDGM-St), PLC-Fe_3O_4/SiOx-g-P (GMA) hydrolysed phospholipids for 2.0 h, the residual phosphorus was 68.71, 69.53, 65.91 and 64.77 mg/kg, respectively. The change in phosphorus content after 2.0-2.5 h treatment was not significant. The reason is that PLC only catalyses PC and PE not PI and PA in phospholipids (Ye *et al.,* 2012). PI and PA were still present in crude soybean oil, so the residual phosphorus content was relatively high. In order to reduce the residual phosphorus content, magnetic immobilised PLC and magnetic immobilised PLA are placed in magnetic fluidised beds at different temperatures and pH for continuous degumming of crude soybean oil.

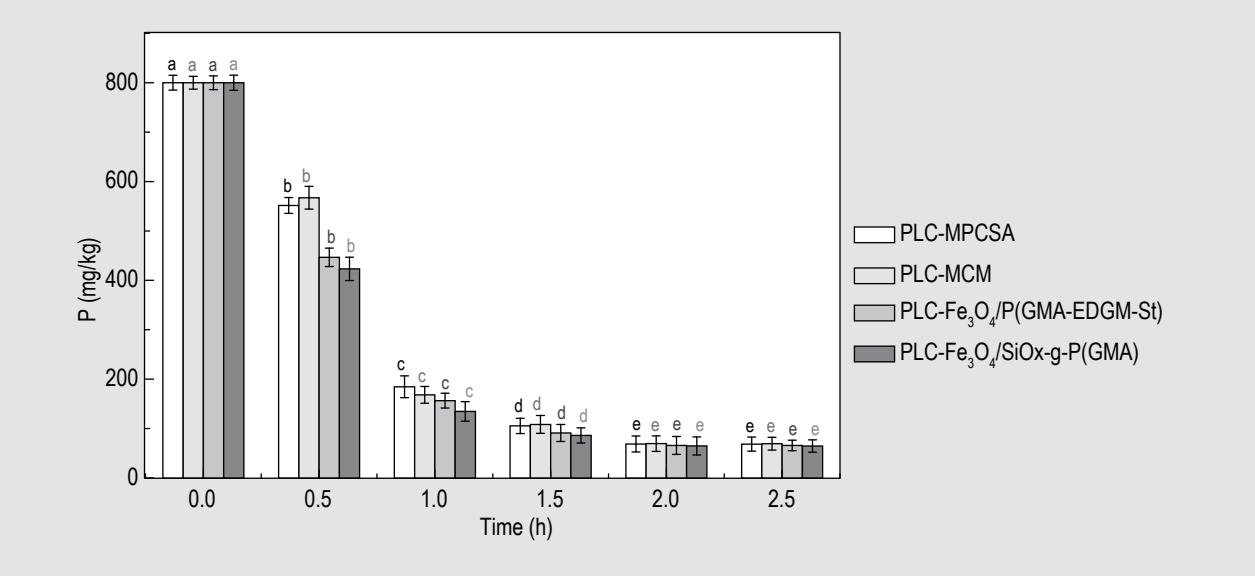

Figure 7. Effect of the hydrolysis time on the residual phosphorus levels. Superscript letters indicate homogenous sub-classes as resulted from ANOVA (*P*<0.05, Duncan's new multiple range test).

Reusability of magnetic immobilised PLC on hydrolysing phospholipids in crude soybean oil

For the magnetic immobilised PLC process, the degumming conditions were 110 mg/kg of enzyme at 60 °C with pH of 6.5 for 2.5 h. The results of immobilised enzyme reusability were shown in Figure 8. After 5 cycles, PLC-MPCSA, PLC-MCM, PLC-Fe_3O_4/P (GMA-EDGM-St), PLC-Fe_3O_4/SiOx-g-P (GMA) retained 72.6, 73.4, 79.5 and 82.5% relative activity, respectively. Their relative activities were 63.3, 66.2, 71.8 and 73.4% after 6 cycles, respectively. The research of Long *et al.* (2014) showed that immobilised pullulanase retained more than 56% of its initial activity after 8 consecutive reuses. This outcome indicates that the reusability of enzymes is improved through magnetic immobilisation.

Effect of hydrolysis time on the content of crude soybean oil compositions

It was found that PLC-Fe_3O_4/SiOx-g-P (GMA) was more capable of hydrolysing phospholipids in crude soybean oil, so it was added to crude soybean oil with 110 mg/kg of enzymes, 60 °C of hydrolysis temperature and 6.5 of pH (Table 2). There was 0.37% of PI and 0.16% of PA after 2 h of hydrolysis by PLC-Fe_3O_4/SiOx-g-P (GMA). This is similar to their initial contents in crude soybean oil; meanwhile, crude soybean oil only contained 0.01% PC and 0.18% PE that was converted to 1,2-DAG. It was obvious that the content of 1,2-DAG increased in soybean oil. This result was consistent with Jiang *et al.* (2015b). After degumming with PLC-Fe_3O_4/SiOx-g-P (GMA), the oil yield was 98.1%. The degumming improved refining yield by PLC because PC and PE was hydrolysed to produce 1,2-DAG. However, the residual phosphorus content was high after degumming, so magnetic PLA could be used to reduce the residual

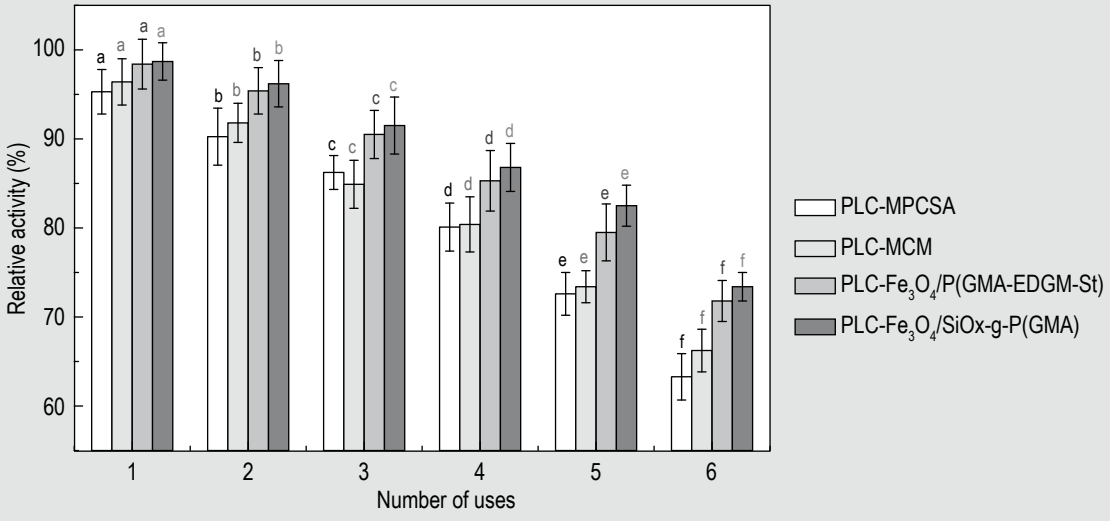

Figure 8. Reusability of magnetic immobilised phospholipase C (PLC) particles in degumming of crude soybean oil. Superscript letters indicate homogenous sub-classes as resulted from ANOVA (P<0.05, Duncan's new multiple range test).

Table 2. Effect of hydrolysis time on the content of crude soybean oil compositions.[1,2]

Time (min)	PC (%)	PE (%)	PI (%)	PA (%)	1,2-DAG (%)
0	0.80±0.09^{a}	0.36±0.01^{a}	0.37±0.02^{a}	0.18±0.03^{a}	0.28±0.03^{e}
30	0.47±0.07^{b}	0.28±0.07^{ab}	0.36±0.03^{a}	0.19±0.02^{a}	0.61±0.04^{d}
60	0.26±0.04^{c}	0.23±0.05^{bc}	0.38±0.01^{a}	0.17±0.03^{a}	0.80±0.05^{c}
90	0.11±0.02^{d}	0.19±0.06^{c}	0.36±0.01^{a}	0.17±0.01^{a}	0.96±0.04^{b}
120	0.01±0.01^{e}	0.18±0.03^{c}	0.37±0.03^{a}	0.16±0.04^{a}	1.07±0.02^{a}
150	0.02±0.02^{de}	0.18±0.02^{c}	0.35±0.04^{a}	0.18±0.02^{a}	1.06±0.02^{a}

[1] DAG = diacylglycerol; PA = phosphatidic acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol.
[2] Different letters represent the differences among contents: the same letter indicates no significant difference (P>0.05).

phosphorus content which was not hydrolysed by PLC in a magnetic fluidised bed.

4. Conclusions

Free PLC was immobilised in magnetic particles, and magnetic immobilised PLC showed better stability over a broad temperature and pH range. The enzyme loading and enzymatic activity of PLC-Fe_3O_4/SiOx-g-P (GMA) was the highest in the four types of magnetic immobilised PLC explored in this study. It hydrolysed PC and PE in crude soybean oil to produce 1,2-DAG, reducing the loss of soybean oil in the refining process, and improved the refining rate of soybean oil. The stability of magnetic immobilised PLC particles was better during the degumming process. Therefore, magnetic immobilised PLC can be applied to industrial crude soybean oil degumming.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China (NSFC): Study on the mechanism of nanomagnetic enzyme hydrolysis of soybean oil by multi-effect orientation and biosynthesis of functional lipids (No: 31571880). This work was supported by a grant from the Province Natural Science Foundation of Heilong Jang: Study on the Mechanism of Continuous Orientation Esterification of Nanometer Magnetic Lipase (No: C2017019). This work was also supported by a grant from Rice bran high-value steady-state processing technology and intelligent equipment research and demonstration (No: 2018YFD0401101). The authors would like to thank the anonymous reviewers and the editor for their comments on an earlier version of this paper.

Conflict of interest

All authors have claimed that there is no conflict of interest.

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Brühl, L., 1997. Official methods and recommended practices of the American Oil Chemist's Society. Physical and chemical characteristics of oils, fats and waxes. Lipid/Fett 99: 197-197.

Chang, M.Y. and Juang, R.S., 2005. Activities, stabilities, and reaction kinetics of three free and chitosan-clay composite immobilised enzymes. Enzyme and Microbial Technology 36: 75-82.

Dayton, C.L., Rosswurm, E.M. and Da Silva Galhardo, F., 2013. Enzymatic degumming utilizing a mixture of PLA and PLC phospholipases with reduced reaction time. U.S. Patent No. 8,460,905. U.S. Patent and Trademark Office, Washington, DC, USA.

Dijkstra, A.J., 2010. Enzymatic degumming. European Journal of Lipid Science and Technology 112: 1178-1189.

Elena, C., Ravasi, P., Cerminati, S., Peiru, S., Castelli, M.E. and Menzella, H.G., 2016. *Pichia pastoris* engineering for the production of a modified phospholipase C. Process Biochemistry 51: 1935-1944.

Garcia-Galan, C., Berenguer-Murcia, Á., Fernandez-Lafuente, R. and Rodrigues, R.C., 2011. Potential of different enzyme immobilisation strategies to improve enzyme performance. Advanced Synthesis and Catalysis 353: 2885-2904.

Guo, H., Tang, Y., Yu, Y., Xue, L. and Qian, J., 2016. Covalent immobilisation of α-amylase on magnetic particles as catalyst for hydrolysis of high-amylose starch. International Journal of Biological Macromolecules 87: 537-544.

Ibrahim, A.S., Al-Salamah, A.A., El-Toni, A.M., El-Tayeb, M.A. and Elbadawi, Y.B., 2013. Immobilisation of cyclodextrin glucanotransferase on aminopropyl-functionalized silica-coated superparamagnetic nanoparticles. Electronic Journal of Biotechnology 16: 10.

Jiang, X., Chang, M., Jin, Q. and Wang, X., 2015a. Application of phospholipase A1 and phospholipase C in the degumming process of different kinds of crude oils. Process Biochemistry 50: 432-437.

Jiang, X., Chang, M., Jin, Q. and Wang, X., 2015b. Optimisation of the degumming process for camellia oil by the use of phospholipase C in pilot-scale system. Journal of Food Science and Technology 52: 3634-3644.

Jiang, X., Chang, M., Wang, X., Jin, Q. and Wang, X., 2014. A comparative study of phospholipase A 1 and phospholipase C on soybean oil degumming. Journal of the American Oil Chemists' Society 91: 2125-2134.

Laatikainen, M., Srithammavut, W., Toukoniitty, B., Turunen, I. and Sainio, T., 2015. Phospholipid adsorption from vegetable oils on acid-activated sepiolite. Adsorption 21: 409-417.

Lamas, D.L., Constenla, D.T. and Raab, D., 2016. Effect of degumming process on physicochemical properties of sunflower oil. Biocatalysis and Agricultural Biotechnology 6: 138-143.

Lamas, D.L., Crapiste, G.H. and Constenla, D.T., 2014. Changes in quality and composition of sunflower oil during enzymatic degumming process. LWT – Food Science and Technology 58: 71-76.

Li, Z., Liu, H., Zhao, G., Wang, P., Wang, L., Wu, H., Fang, X., Sun, X., Wu, X. and Zheng, Z., 2016. Enhancing the performance of a phospholipase A1 for oil degumming by bio-imprinting and immobilisation. Journal of Molecular Catalysis B: Enzymatic 123: 122-131.

Lima, J.S., Araújo, P.H., Sayer, C., Souza, A.A., Viegas, A.C. and De Oliveira, D., 2017. Cellulase immobilisation on magnetic nanoparticles encapsulated in polymer nanospheres. Bioprocess and Biosystems Engineering 40: 511-518.

Lin, F. and Doong, R., 2011. Bifunctional Au-Fe_3O_4 heterostructures for magnetically recyclable catalysis of nitrophenol reduction. Journal of Physical Chemistry C115: 6591-6598.

Liu, Q., Hua, Y., Kong, X., Zhang, C. and Chen, Y., 2013. Covalent immobilisation of hydroperoxide lyase on chitosan hybrid hydrogels and production of C6 aldehydes by immobilised enzyme. Journal of Molecular Catalysis B: Enzymatic 95: 89-98.

Long, J., Jiao, A., Wei, B., Wu, Z., Zhang, Y., Xu, X. and Jin, Z., 2014. A novel method for pullulanase immobilised onto magnetic chitosan/Fe_3O_4 composite nanoparticles by *in situ* preparation and evaluation of the enzyme stability. Journal of Molecular Catalysis B: Enzymatic 109: 53-61.

Mei, L., Wang, L., Li, Q., Yu, J. and Xu, X., 2013. Comparison of acid degumming and enzymatic degumming process for Silybum marianum seed oil. Journal of the Science of Food and Agriculture 93: 2822-2828.

Milosavić, N., Prodanović, R., Jovanović, S. and Vujčić, Z., 2007. Immobilisation of glucoamylase via its carbohydrate moiety on macroporous poly (GMA-co-EGDMA). Enzyme and Microbial Technology 40: 1422-1426.

Palvannan, T. and Boopathy, R., 2005. Phosphatidylinositol-specific phospholipase C production from *Bacillus thuringiensis serovar*. kurstaki using potato-based media. World Journal of Microbiology and Biotechnology 21: 1153-1155.

Piel, M.S., Peters, G.H. and Brask, J., 2017. Chemoenzymatic synthesis of fluorogenic phospholipids and evaluation in assays of phospholipases A, C and D. Chemistry and Physics of Lipids 202: 49-54.

Qu, Y., Sun, L., Li, X., Zhou, S., Zhang, Q., Sun, L., Yu, D., Jiang, L. and Tian, B., 2016. Enzymatic degumming of soybean oil with magnetic immobilised phospholipase A2. LWT – Food Science and Technology 73: 290-295.

Ravasi, P., Braia, M., Eberhardt, F., Elena, C., Cerminati, S., Peirú, S., Castelli, M.E. and Menzella, H.G., 2015. High-level production of *Bacillus cereus* phospholipase C in *Corynebacterium glutamicum*. Journal of Biotechnology 216: 142-148.

Subrahmanyam, C.V., Rao, M.V., Balasubrahmanyam, V. and Bhowmick, D.N., 2006. Membrane degumming of crude rice bran oil: pilot plant study. European Journal of Lipid Science and Technology 108: 746-752.

Xie, W. and Wang, J., 2012. Immobilised lipase on magnetic chitosan microspheres for transesterification of soybean oil. Biomass & Bioenergy 36: 373-380.

Xu, F.J., Cai, Q.J., Li, Y.L., Kang, E.T. and Neoh, K.G., 2005. Covalent immobilisation of glucose oxidase on well-defined poly (glycidyl methacrylate)-Si(111) hybrids from surface-initiated atom-transfer radical polymerisation. Biomacromolecules 6: 1012-1020.

Yang, L., Zou, P., Cao, J., Sun, Y., Han, D., Yang, S., Chen, G., Kong, X. and Yang, J., 2014. Facile synthesis and paramagnetic properties of Fe_3O_4@SiO_2 core-shell nanoparticles. Superlattices and Microstructures 76: 205-212.

Ye, L., Deng, K., Xu, F., Tian, L., Peng, T. and Zan, L., 2012. Increasing visible-light absorption for photocatalysis with black BiOCl. Physical Chemistry Chemical Physics 14: 82-85.

Yu, D., Jiang, L., Li, Z., Shi, J., Xue, J. and Kakuda, Y., 2012. Immobilisation of phospholipase A1 and its application in soybean oil degumming. Journal of the American Oil Chemists' Society 89: 649-656.

Yu, D., Ma, Y., Jiang, L., Walid, E., He, S., He, Y., Xiaoyu, Z., Zhang, J. and Hu, L., 2014. Stability of soybean oil degumming using immobilised phospholipase A2. Journal of Oleo Science 63: 25-30.

Yu, D., Ma, Y., Xue, S.J., Jiang, L. and Shi, J., 2013. Characterisation of immobilised phospholipase A1 on magnetic nanoparticles for oil degumming application. LWT – Food Science and Technology 50: 519-525.

Zamora, R., Olmo, C., Navarro, J.L. and Hidalgo, F.J., 2004. Contribution of phospholipid pyrrolisation to the color reversion produced during deodorisation of poorly degummed vegetable oils. Journal of Agricultural and Food Chemistry 52: 4166-4171.