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Highly efficient enzymatic preparation for dimethyl carbonate catalyzed by lipase from *Penicillium expansum* immobilized on CMC–PVA film☆

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ABSTRACT

Herein, we demonstrated a environmental-friendly biocatalytic route for the synthesis of dimethyl carbonate (DMC), which is the first example to use enzyme in the process. Moreover, immobilization of *Penicillium expansum* lipase (PEL) using environmentally benign and biodegradable CMC–PVA polymer has significantly enhanced the catalytic activity thus making them an eligible biocatalyst for synthesis of DMC. The biocatalyst revealed high catalytic performance even under ambient pressure and low temperature (conversion of EC to 94%, yield of DMC to 93% and selectivity of DMC to 99%). The immobilized lipase was effectively recycled for four consecutive cycles providing good yields of the desired product. Accompanying with the intense requirement for the green chemicals and process, our work can provide a useful idea for "green and clean" of harmful chemical reaction.

1. Introduction

In past decades, the synthesis of DMC has inspired intense interests from scientific and industrial fields, due to its low toxicity. It can be effectively used as an environmentally benign substitute for highly toxic phosgene and dimethyl sulfate in carbonylation and methylation reaction, as monomer for several types of polymers and intermediate in the synthesis of pharmaceutical and agricultural chemicals. Nowadays, three preparation routes have been commercialized to produce DMC: the phosgene process, the oxidative carbonylation of methanol [1], and the two-step transesterification process utilizing CO_2 as a raw material (Scheme 1). Among these processes, the transesterification route has been the most advantageous and intensely investigated, since it uses no harmful reagents, and can be operated under ambient pressure. The production of five-membered cyclic carbonates from CO_2 has been industrialized since the 1950s [2–4]. Ethylene glycol (EG) can be obtained as co-product, which is an important and basic organic material and mainly used in the production of polyester fiber, antifreezing agent, unsaturated polyester resin, lubricant and plasticizer. Therefore, the transesterification is an excellent green chemical process from an economic point of view.

Many homogeneous and heterogeneous catalytic systems have been reported for the transesterification of cyclic carbonates with methanol with high selectivity of DMC [5]. While homogeneous catalyst systems have their own drawbacks of catalyst separation after the reaction for reuse, most of the reported heterogeneous catalysts need severe experimental conditions like high temperature and/or rigorous pressure [6–12].

However, only a few solid heterogeneous catalysts were reported so far for the transesterification of ethylene carbonate (EC) with methanol [13] and most of these catalysts contained metal. Nowadays, as the universal awareness of environmental protection, eliminating metal component involvement for catalyst design and abating volatile organic solvent usage in the process of chemical synthesis are more pressing and promising for our future. Hence, there is a need to identify "green" catalyst systems for the transesterification reactions which are "environmental-friendly" and obtaining high DMC yield at ambient conditions.

One of the most promising strategies to achieve these goals is the utilization of enzymes which exhibit a number of features that make their use advantageous as compared to conventional

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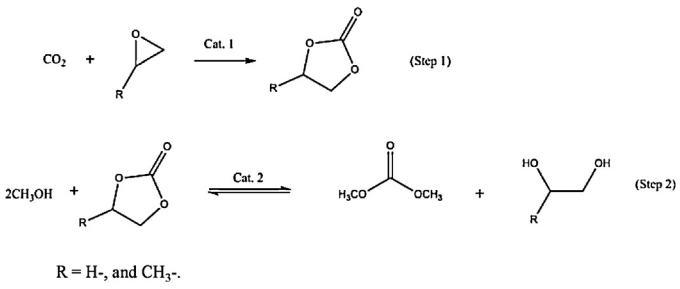


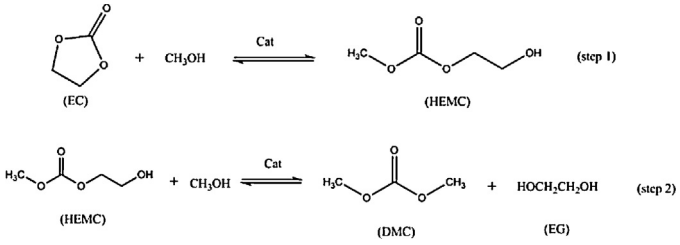
Scheme 1. Two-step process for DMC production utilizing CO_2 as a raw material.

chemical catalysts. Except for a high level of catalytic efficiency, lipases generally operate at mild conditions of temperature, pressure and pH with reaction rates of the order of those achieved by chemical catalysts at more extreme conditions. Lipase, as the potential candidates for "environmental-hazardous" reactions [14], has been applicated in various synthesis routes such as transesterification [15–18], hydrolysis [19], esterification [20], amidation [21,22], polyester synthesis [23–25], oxidation–reduction reaction and methyl like group transfer reaction [26–28].

In this study, we extended the scope of catalyst in the synthesis of DMC (Scheme 2). The main advantage of the developed synthesis method is that the strategy follows an eco-friendly and non-toxic route. Immobilization technique [29] offers a range of advantages, like increased synthetic activity, ease of separation from a reaction mixture, and improved stability by preventing enzyme from thermal or solvent denaturation during repeated use in continuous processes. A variety of natural supports, such as magnetic nanoparticles [30], titania submicrospheres [31], polymers [32], silica [33], hydroquinone [34] and toyonite [35], have been used for enzyme immobilization.

However, despite of many reported methods, the immobilization procedure still needs to be developed to enhance the synthetic activity of lipases so as to make them the most versatile biocatalyst. In context, entrapment of lipases into a film proves to be a simple and efficient method of immobilization as they provide a high surface area for interaction of enzyme with substrate followed by ease of separation and greater enzyme stability with fewer chances of leaching. Dhake et al. [36] reported immobilization of commercially *Rhizopus oryzae* lipase on a film using a blend of hydroxypropyl methyl cellulose (HPMC) and PVA.

Considering the above issues, in the present paper, we report immobilization of PEL on a film prepared using a blend of CMC–PVA as the biocatalyst for the synthesis of DMC from EC with methanol. The main ideas behind using CMC–PVA as immobilization support were (1) biodegradable CMC–PVA evades the use of harmful chemical reagents, on the other hand, this method may have a higher commercial potential because this immobilization procedure is simple and both CMC and PVA are very cheap; (2) immobilization of PEL using CMC–PVA polymer has significantly enhanced the catalytic activity thus making them an eligible biocatalyst for synthesis of DMC; it leads to an effective synthesis process for DMC



Scheme 2. The preparation of DMC by the transesterification of EC with methanol.

production (conversion of EC to 94%, yield of DMC to 93% and selectivity of DMC to 99%). (3) The enzyme stability and recyclability under operation conditions have been proved for four reaction cycles; the chemical reaction was carried out under mild conditions (60 °C and atmospheric pressure) with a convenient atomic economy.

2. Experimental

2.1. Chemical reagents

Crude lipase from *Penicillium expansum* (PEL) (5000 U mg^{-1} solid), *Penicillium neutral expansum* (PNEL) (10,000 U mg^{-1} solid), *Aspergillus niger* (ANL) (120,000 U mg^{-1} solid), *Rhizopus chinensis satio* (RCSL) (10,000 U mg^{-1} solid), *Aspergillus oryzae* (AOL) (30,000 U mg^{-1} solid), *Porcine pancreatic* (PPL) (135 U mg^{-1} solid), neural proteolytic enzyme (50,000 U mg^{-1} solid), acidic proteolytic enzyme (60,000 U mg^{-1} solid), alkaline proteolytic enzyme (40,000 U mg^{-1} solid) was kindly donated by Shenzhen Leveking Bio-engineering Co. Ltd., China. These enzymes were produced by spraying the concentrated supernatant from fermentation with addition of a certain amount of starch as a thickening agent. Immobilized (Novozyme435) *Candida antarctica* lipase B (EC 3.1.1.3) was donated from Novozymes (China) Investment Co. Ltd. EC, HPMC, CMC, PVA and all other chemicals were purchased from Aladdin Reagent Company, China. Methanol (99.5%) was purified using magnesium. Other reagents were of analytical grade and were used without further purification.

2.2. Immobilization of lipase

The immobilization of PEL was carried out as described elsewhere [35]. The 500 mg of HPMC–PVA (1:1), CMC–PVA (1:1), CMC and PVA blend were dissolved in 25 mL of deionized water at room temperature with continuous stirring until complete dissolution. Lipase 100 mg was dissolved in 1–2 mL deionized water and was added to the solution of polymer formed. The solution was gently stirred for about 60 min and then was slowly poured into a teflon petridish and was allowed to dry at 45–47 °C for 24 h, which was then cut off into several small sections of 2–3 mm^2. The protein content of immobilized lipase was determined in triplicate by Lowry method using bovine serum albumin as a standard [37].

2.3. Characterization of free and immobilized lipase

The scanning electron microscopy (SEM) analysis (FEI, Quanta 200) was carried out to study the surface morphology. The representative film samples were placed on carbon stub and the images were recorded at 5–15 kV using LFD detector under low vacuum. The thermo gravimetric analysis (TGA) was carried out using Qseries 600 analyzer. About 8–10 mg of samples were placed in a ceramic crucible and the analysis was programmed from 30 to 600 °C with 10 °C min^{-1} rise in temperature, under 99.99% pure nitrogen atmosphere with flow of 100 mL min^{-1}. The reference run was carried out with an empty sample crucible pan and the results were recorded accordingly. The various immobilized film biocatalysts and free lipase were investigated for their native confirmation using FT-IR analysis (Bruker, Vertex 70). The water content of 100 mg immobilized biocatalyst films was determined by Karl Fischer titration analysis (METTLER TOLEDO Titrators).

2.4. Procedure for DMC synthesis from EC and methanol

Enzymatic synthesis of DMC has been performed in a 25 mL conical flask. Given amounts of EC (2.2 g, 25 mmol) and MeOH at different MeOH/EC molar ratios (4:1, 8:1, 16:1 and 24:1) were

Table 1
Screening the lipase sources for DMC synthesis. Conditions: 25 mmol EC; MeOH:EC molar ratio = 16:1; 4.45% (w/w) lipase; temperature 60 °C; 48 h incubation time.

Entry	Cat.	Conv. (%)	Yield (%)		
			DMC	EG	HEMC
1	No catalyst	24	13	14	11
2	*Penicillium expansum*	78	64	63	14
3	*Penicillium neutral expansum*	48	46	47	2
4	*Aspergillus niger*	30	24	23	6
5	*Aspergillus oryzae*	11	0	0	11
6	*Rhizopus chinensis satio*	3	0	0	3
7	*Porcine pancreatic*	14	0	0	14
8	*Novozym 435*	5	0	0	5
9	Neural proteolytic enzyme	4	0	0	4
10	Acidic proteolytic enzyme	7	4	5	3
11	Alkaline proteolytic enzyme	9	3	3	6

Table 2
Screening the support film of PEL for DMC synthesis. Conditions: 25 mmol EC; MeOH:EC molar ratio = 16:1; 6.82% (w/w) catalyst; temperature 60 °C; 48 h incubation time. The composition of the films: CMC–PVA–Lip (CMC:PVA:Lip = 5:5:2 m/m/m), HPMC–PVA–Lip (HPMC:PVA:Lip = 5:5:2 m/m/m), CMC:Lip (CMC:Lip = 5:1 m/m), PVA–Lip (PVA:Lip = 5:1 m/m), CMC–PVA–control (CMC:PVA = 1:1 m/m).

Entry	Cat.	Conv. (%)	Yield (%)		
			DMC	EG	HEMC
1	PEL	78	64	63	14
2	CMC–PVA–Lip	94	93	93	1
3	HPMC–PVA–Lip	82	80	79	2
4	CMC–Lip	85	76	77	9
5	PVA–Lip	75	68	68	7
6	CMC–PVA–control	38	27	26	11

mixed together with the lipase catalyst (2.27, 4.55, 6.82, 9.09%, on the base of EC). The mixtures were incubated for a maximum of 72 h under stirring at temperatures in the range 50–80 °C with agitation speed of 180 rpm. After the reaction was completed, the vessel was then cooled to room temperature. The products were centrifugated to separate the catalyst, and then the supernatant was analyzed by gas chromatography (sp-6890) equipped with a flame ionization detecter (FID) and a capillary column (SE-54, 30 m × 0.25 mm × 0.25 μm). The column temperature was kept at 100 °C for 6 min and then raised to 200 °C for 10 min with a rise of 40 °C min^{-1}. The temperature of the injector and detector was maintained at 200 and 260 °C, respectively. The products were further identified by GC (varian 3900)–MS (varian saturn 2100 T) by comparing retention times and fragmentation patterns with authentic samples. The catalyst enzyme was recovered by centrifugation of the resulted suspension and washed using acetone. The residue obtained was dried at 45 °C under reduced pressure overnight (at 1 Torr for 24 h) and was then used for the next generation. The MeOH/DMC mixture (weight composition 16.5% of DMC, 83.5% of methanol) was distilled from the mixture at 80 °C, and then, DMC was separated from the MeOH/DMC mixture by means of high pressure distillation or extractive distillation [38].

The conversion of EC, the yield in EC and the selectivity to DMC were calculated using Eqs. (1)–(3), where the number of moles was determined by External Standard Method from the chromatographic analysis.

$$\text{EC conversion}\ (\%) = \frac{\text{sum of the moles of DMC products} + \text{sum of the moles of HEMC products}}{\text{moles of EC introduced in the reaction}} \times 100 \quad (1)$$

$$\text{DMC yield}\ (\%) = \frac{\text{sum of the moles of DMC products}}{\text{moles of EC introduced in the reaction}} \times 100 \quad (2)$$

$$\text{DMC selectivity}\ (\%) = \frac{\text{sum of the moles of DMC products}}{\text{sum of the moles of DMC products} + \text{sum of the moles of HEMC products}} \times 100 \quad (3)$$

3. Results and discussion

3.1. Screening of biocatalyst for DMC synthesis

The catalytic activity of lipase from various natural sources has been directly evaluated in the transesterification of EC with methanol. All the available lipases were screened based on their activities for the production of DMC. As shown in Table 1, PEL showed high catalytic performance for the production of DMC, while other lipases revealed little activity and almost no DMC can be found. (More details about free PEL catalyst for DMC synthesis will be presented in supporting information). It is interesting to find that the condition with no catalyst showed higher conversion than those with some other lipases including Novozyme 435, which means that these lipases could not activate this reaction. The decreasing DMC yield was probably ascribed to those lipases, inhibiting intermolecular interactions of substrates. Based on these results, we selected PEL as the lipase for the immobilization. A dependence on the support was also observed in Table 2. When PEL was immobilized in CMC/PVA blends, the DMC were obtained in 93% yield. This value is higher than those using the same lipase in a free form, where the yield was 64%. It seems that after immobilization, there was an enhancement of the enzyme activity and stability, as shown by Dalla-Vecchia in esterification reactions of carboxylic acids with n-pentanol in organic media by *R. oryzae* lipase [39]. Therefore, considering the data shown in Table 2, CMC/PVA blends were selected as the immobilized film in the following studies.

3.2. Characterization of immobilized lipase films

The surface morphology of immobilized lipase was determined using SEM analysis as shown in Fig. 1. The SEM images reveal the even distribution of PEL as a small globule in the film (Fig. 1a–d), whereas the image of supports without lipase shows absence of globular structures with plane background (Fig. 1e). The obtained images were compared with an SEM image of a commercially available lipase (Fig. 1f) under same instrumental conditions signifying the entrapment of lipase into the film.

The FT-IR (Fig. 2) of free lipase containing a broad envelope at 3421 cm^{-1} was due to the overlap of —NH stretching of amide group of proteins. The FT-IR absorption spectrum of lipase generally shows three major bands caused by peptide group vibrations in the range of 1800–1300 cm^{-1} [40]. The FT-IR spectrum of free lipase (Fig. 2g) in the present study shows that the amide I band at 1645 cm^{-1} is mainly due to the C—O stretching vibrations, free lipase illustrates a characteristic band of amide II with the maximum of 1601 cm^{-1} due to N—H bending with contribution of C—N stretching vibrations. The presence of amide III band present at maximum of 1420 cm^{-1} is due to N—H bending with C—Cα and C—N stretching vibrations. These significant bands were observed in the free lipase as well as immobilized lipase emphasizing presence of lipase in its native conformation. The amide I and II bands are the most sensitive to the secondary structure of the protein and if these bands are disturbed in the FT-IR spectrum of a protein, then

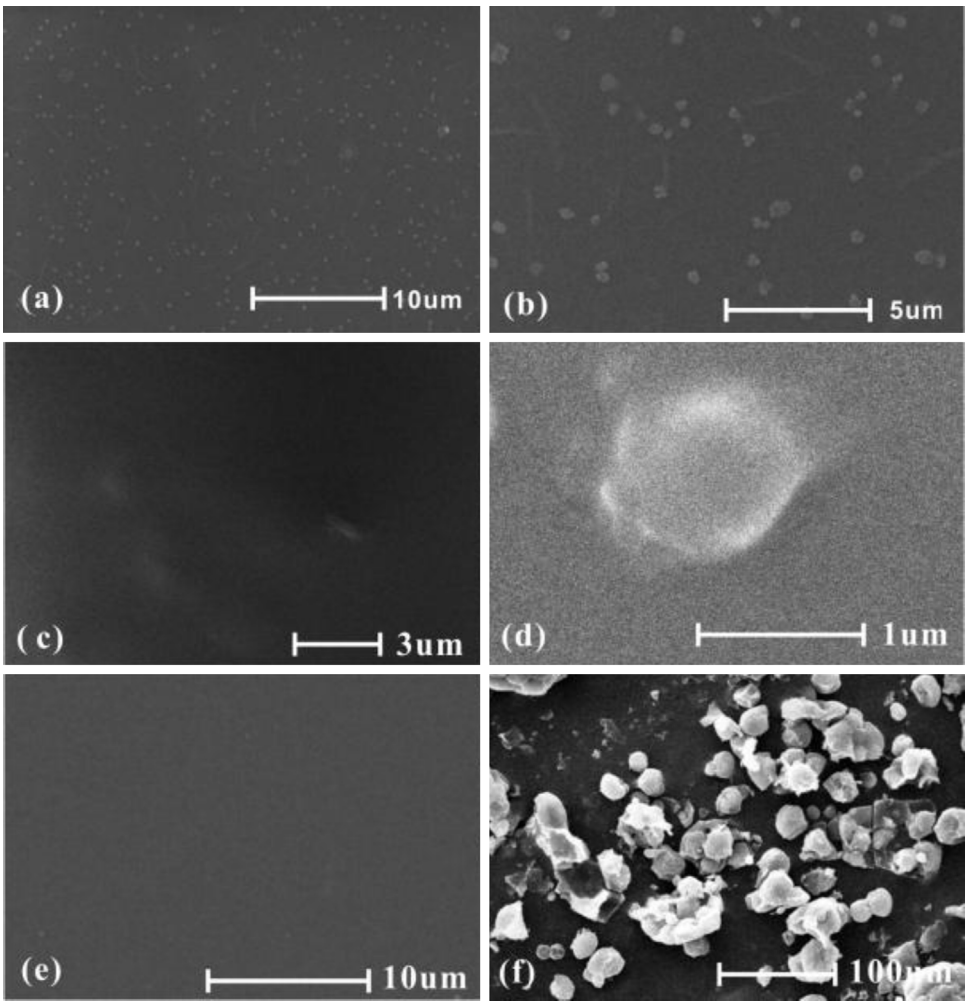


Fig. 1. SEM image of: (a–d) CMC–PVA–Lip, (e) CMC–PVA–control and (f) free PEL.

it can be supposed that the protein has lost its native conformation [41].

The FT-IR spectrum of CMC–PVA–Lip (Fig. 2a) shows that the amide I and II bands of the purified acidic lipase were overlapped with the absorption band of the CMC–PVA (Fig. 2d), and that the intensity of the absorption band of the immobilized support at 1601 cm^{-1} was greater than that of the CMC–PVA. This can be further confirmed by the shift of amide I band from 1645 to 1666 cm^{-1} and C—N stretching of amide III band at 1394 cm^{-1}. Herein, it can be assumed that the intensity of the absorption band of the CMC—PVA was increased with the amide I and II bands. This shows that no major structural change occurred when the enzyme was immobilized into CMC–PVA. These spectral characteristics indicate that the structural conformation of the lipase was retained even after adsorption into the CMC–PVA. Other immobilized enzymes including both HPMC–PVA–Lip (Fig. 2b) and CMC–Lip (Fig. 2c) showed the similar phenomenon compared to their supporting films (HPMC–PVA–control (Fig. 2e), CMC–control (Fig. 2f)).

The TGA showed that temperature above 200 °C is required to remove the tightly bound water from proteins. The obtained results are in agreement with the earlier reports [42] which studied the effect of temperature for which enzymes maintain their catalyst. The similar observation was made for TGA analysis (Fig. 3) of immobilized lipases, where immobilization has also increased the thermal stability of lipase as compared with free lipase.

The amount of residual water was evaluated by using Karl Fischer titration method (Table 3). The water content of free lipase was 6.06% while for the best immobilized lipase HPMC–PVA was 3.52%.

The immobilization efficiency was determined from the protein content by Lowry method [37] and was found to be 85–92% for all the immobilization supports with 89% for the CMC–PVA

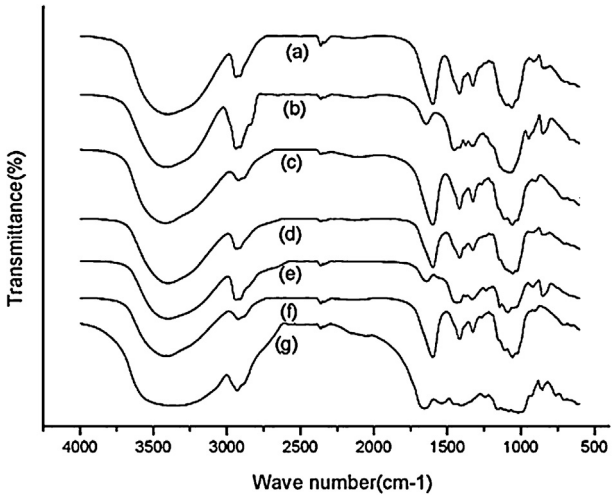


Fig. 2. FT-IR spectrum of (a) CMC–PVA–Lip, (b) HPMC–PVA–Lip, (c) CMC–Lip, (d) CMC–PVA–control, (e) HPMC–PVA–control, (f) CMC–control, and (g) free PEL.

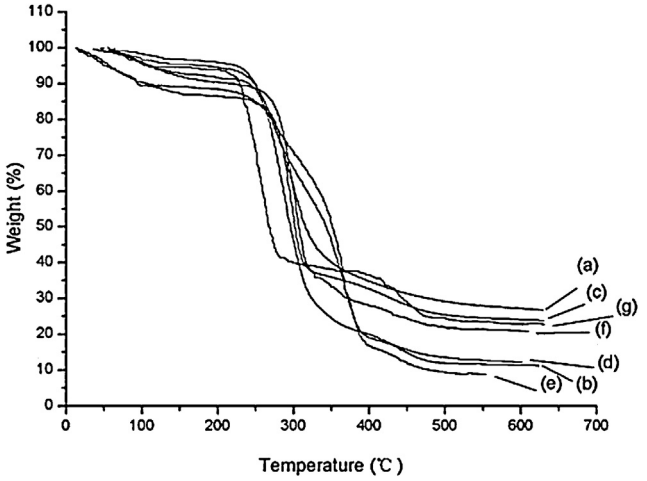


Fig. 3. TGA analysis of (a) CMC–PVA–Lip, (b) HPMC–PVA–Lip, (c) CMC–Lip, (d) CMC–PVA–control, (e) HPMC–PVA–control, (f) CMC–control, and (g) free PEL.

immobilized lipase. The percent immobilization achieved was very high because the method does not follow any washing procedure, which is most commonly followed during the use of cross-linking reagent for immobilization purpose.

3.3. Optimization of the reaction conditions of CMC–PVA–Lip

3.3.1. The effect of molar ratio of MeOH to EC

It is well known that the catalytic performance is closely associated with the added amount of reacted precursors in the reactions. In our present study, the effect of MeOH/EC molar was also investigated. As shown in Fig. 4a, it is apparent that the catalytic performance of lipase on the transesterification reaction was significantly affected by MeOH/EC molar ratio. An increase of MeOH/EC molar ratio from 4:1 to 16:1 generated an increasing DMC yield with an improved product conversion from 51% to 93%. In the chemical kinetical law, the reaction equilibrium is sharply impacted by the reactant concentration, and more reactant can drive the reaction to move in the opposite direction. Therefore, more addition of methanol should produce higher DMC yield due to the reversible nature of the transesterification step [43]. When further increasing MeOH/EC from 16:1 to 24:1, a decrease of the conversion with EC can be observed. It may be attributed that excessive methanol increases the stability of the native conformation of the protein in methanol surroundings and decreases the irreversible inactivation of the lipase [44,45]. It means that MeOH could enhance "apparently" the lipase activity in certain concentration, but exceeding the tolerated limit of MeOH, the enzyme activity is damaged by more and more MeOH. Nevertheless, a suitable value (16:1) was determined in the subsequent investigation in consideration of energy consumption.

Table 3
Determination of water content by Karl Fisher method.

Entry	Sample	Water content (%, w/w)
1	CMC–PVA–Lip	3.52
2	HMPC–PVA–Lip	2.03
3	CMC–Lip	5.83
4	CMC–PVA–control	6.45
5	HPMC–PVA–control	5.39
6	CMC–control	7.54
7	PEL	6.06

3.3.2. The effect of the added amount of lipase

In our reaction process, the amount of lipase not only plays a key role for catalytic performance, but also is a crucial economical factor for successful industrial application due to their expensive value. Here, we selected the amount of enzyme range from 2.27% to 9.09% based on the weight of EC at 60 °C. In Fig. 4b, it is found that both EC conversion and DMC yield exhibited a similar tendency and possessed a turning point with the increase amount of catalyst. The two parameters reached their maximum values corresponding to 94% EC conversion of and 93% DMC yield at 6.82% lipase concentration. It is interesting to find that the highest DMC selectivity occurred at 6.82% rather than 9.09% lipase concentration. It was probably ascribed to the higher enzyme density, causing intermolecular interactions of lipase, in this way blocking the catalytic sites [16].

3.3.3. Effect of incubation time

As shown in Fig. 4c, the dependence of DMC yield and EC conversion on reaction time was also investigated. It is apparent that longer reaction time from 0 h to 48 h is more advantageous for the reaction process, yielding more DMC (93%) with the improved selectivity. The increasing DMC selectivity by the time may probably be owing to facile formation of HEMC at the initial stage [11]. The prolonged reaction time is appreciated for further transesterification of HEMC with methanol to DMC formation, accordingly the improved DMC yield and DMC selectivity. When further increasing the reaction from 48 h to 72 h, no apparent change on the DMC yield and EC conversion can be seen, indicating an appropriate reaction time as 48 h.

3.3.4. Effect of temperature

Finally, in our present report, the temperature influence on transesterification process has been also investigated. Seen from Fig. 4d, the temperature increase is favorable for the EC conversion, since the enzyme activity increases with the temperature increase. Both the conversion of EC and the yield to DMC increased gradually with the temperature till a maximum at 60 °C. In addition, when the temperature increases to 70 °C, both the DMC yield and selectivity almost stay the same. However, further increase in temperature (up to 80 °C) led to a slightly decrease of the yield of DMC. Nevertheless, we adopted 60 °C for further experiments in this work, which is in agreement with the earlier reports for the appropriate temperature of this lipase [46].

3.3.5. The recyclability and stability of lipase

To make the process more economical, it is necessary to study the recyclability of immobilized lipase, which is regarded as the most important advantage of an immobilized enzyme compared to the free enzyme. As shown in Fig. 5a, the immobilized lipases show good stability and retain more than 75% of their initial activity after 4 consecutive reuses. The loss of activity is believed owing to deactivation of lipase or possibility of desorption of lipase from the support as cycles are increased. Ozyilmaz et al. [47] carried out enzyme leakage study and discovered that no significant leaching (below 1%) of lipase could be observed from the immobilization support even after a period of 48 h. In addition, it is known that enzymes are naturally insoluble in organic solvents and thus we believe the decrease in lipase activity was because of deactivation due to extended exposure of biocatalyst to the hydrophobic media (e.g., methanol, DMC, etc.) and not due to desorption of lipase from the immobilization support [47–49]. The same observation was made by Lozano et al. for synthesis of citronellol acetate using CaL-B where a continuous decrease in activity was observed as the operation cycles increased [50].

The storage stability study of free lipase and CMC–PVA–Lip was investigated and the immobilized lipase was found to be

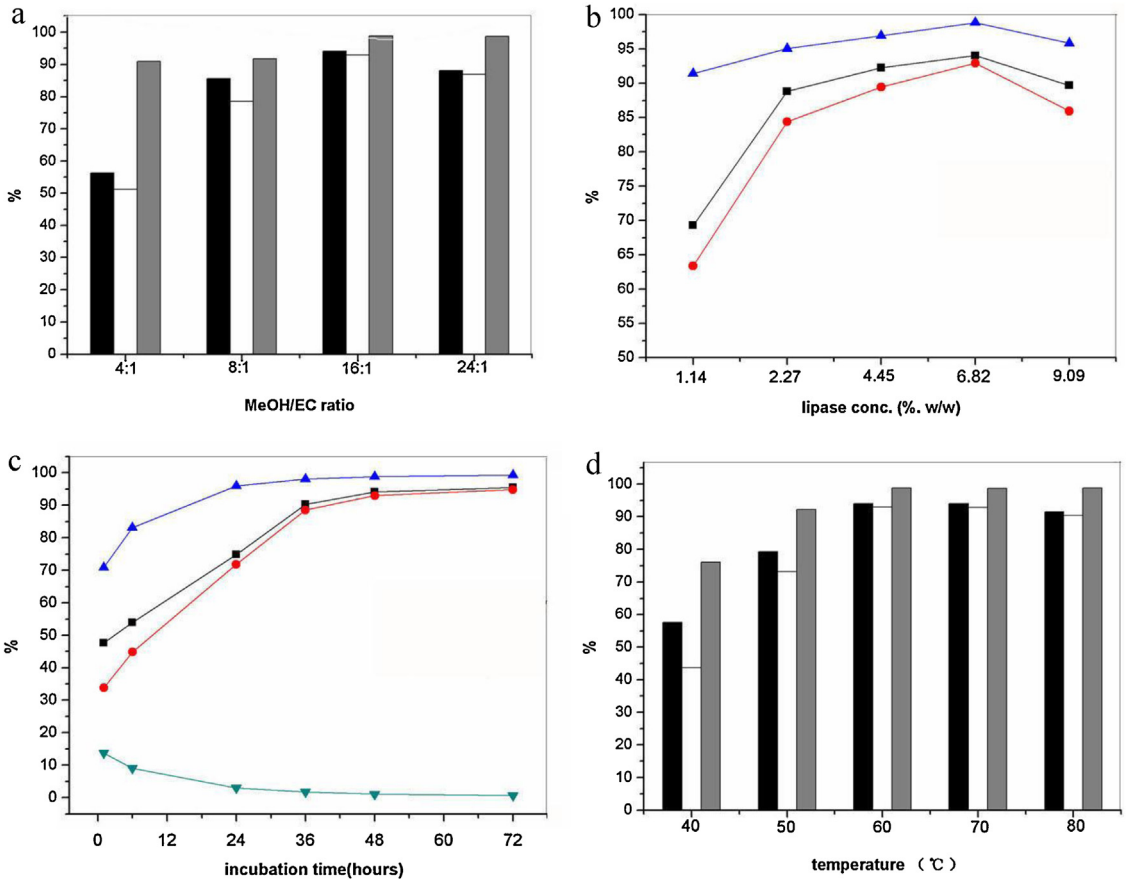


Fig. 4. (a) MeOH/EC molar ratio effect on the DMC synthesis (■, EC conversion; □, yield in DMC; ■, selectivity of DMC). Conditions: 25 mmol EC; 6.82% (w/w) lipase; temperature 60 °C; 48 h incubation time. (b) Influence of the lipase concentration on the DMC synthesis. Conditions: 25 mmol EC; MeOH:EC molar ratio = 16:1; temperature 60 °C; 48 h incubation time. (c) Influence of the incubation time on the DMC synthesis. Conditions: 25 mmol EC; MeOH:EC molar ratio = 16:1; 6.82% (w/w) lipase; temperature 60 °C. (d) Influence of temperature effect on the DMC synthesis (■, EC conversion; □, yield in DMC; ■, selectivity of DMC). Conditions: 25 mmol EC; MeOH:EC molar ratio = 16:1; 6.82% (w/w) lipase; 48 h incubation time.

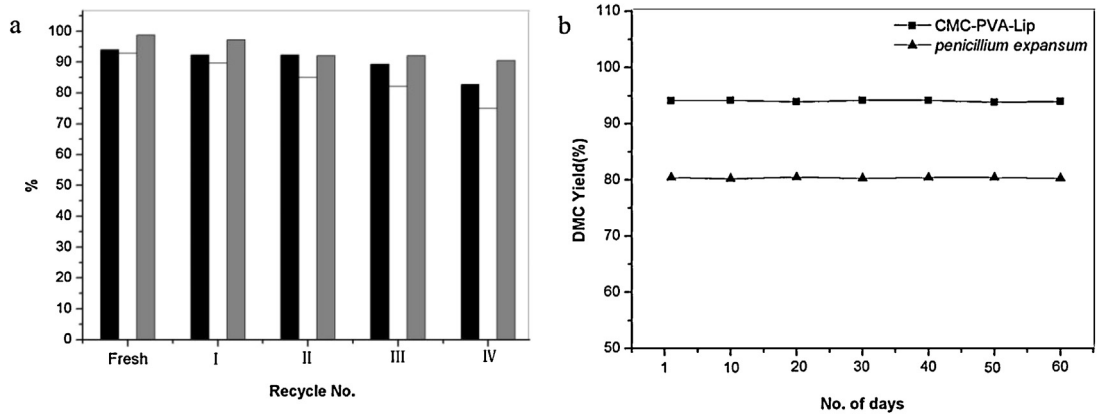


Fig. 5. (a) The reusability of immobilized lipase in DMC synthesis (■, EC conversion; □, yield in DMC; ■, selectivity of DMC). (b) The stability of immobilized lipase in DMC synthesis.

appreciably stable for a period of 60 days retaining its original catalytic activity (Fig. 5b). The immobilized enzyme provided excellent yield up to 94% of desired product whereas free lipase provided 80% yield after a considerable period of 60 days. The same observation was made by Dhake et al. [36] where the original catalytic activity of HPMC–PVA lipase did not decrease after a period of 90 days.

4. Conclusions

In conclusion, we demonstrated an environmental-friendly biocatalytic route for the synthesis of DMC, which is the first example to use enzyme in the process. Moreover, immobilization of PEL using environmentally benign and biodegradable CMC–PVA polymer has significantly enhanced the catalytic activity thus making

them an eligible biocatalyst for synthesis of DMC. The biocatalyst revealed high catalytic performance even under ambient pressure and low temperature (conversion of EC to 94%, yield of DMC to 93% and selectivity of DMC to 99%).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.06.013.

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