# Kinetically Controlled Synthesis of Cefaclor with Immobilized Penicillin Acylase in the Presence of Organic Cosolvents

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Enzymatic syntheses of cefaclor with immobilized penicillin acylase in organic cosolvents under kinetic control were carried out. KcPGA from *Kluyvera citrophila* was selected as the best catalyst among the three species of immobilized penicillin acylase. Ethylene glycol, glycerol, methanol, ethyl acetate and polyethyleneglycol (PEG) were selected accordingly and cefaclor syntheses were preformed respectively. Best results in terms of yield were obtained in ethylene glycol, with which further studies were investigated and the maximum yield was $Y = 93.5$ %. The optimal conditions were pH 6.5, temperature $\theta = 5$ °C, 3 mol D-phenylglycine methyl ester (PGME) per mol 7-amino-desacetoxymethyl-3-chlorocephalosporin acid (7ACCA) and $x = 30$ % ethylene glycol fraction. Under above mentioned conditions, the yield was $Y = 91.1$ %.

*Key words:*
3-chloro-7-D-(2-phenylglycinamide)-3-cephem-4-carboxylic acid, organic cosolvents, penicillin acylase, enzymatic synthesis

## Introduction

Cefaclor, 3-chloro-7-D-(2-phenylglycinamide)--3-cephem-4-carboxylic acid, a semisynthetic $\beta$-lactam antibiotic with an annual production over 350 tons[1] has a broad spectrum of antibiotic activity for oral treatment. It is still manufactured through chemical routes in the pharmaceutical companies worldwide although the industrial synthesis of $\beta$-lactam antibiotics is experiencing a transfer from chemical processes to biocatalytic ones. In the chemical synthesis, the protection of the $\alpha$-amino group of phenylglycine (PG), the use of highly reactive derivatives of PG, low temperature, anhydrous conditions, and the use of highly toxic compounds (pyridine, dimethylaniline and dichloromethane) are all required. Accordingly, enzymatic synthesis approach becomes an attractive alternative to the production of cefaclor for its moderate operational conditions.

Kinetic control strategy is more interesting because the non-equilibrium concentration can be obtained in kinetically controlled synthesis than equilibrium controlled or thermodynamic controlled process.[2] Enzymatic synthesis of cefaclor under kinetic control has been achieved by penicillin acylase (PA, EC 3.5.1.11; also known as penicillin amidase), with PGME as the activated acyl donor and 7ACCA as acyl acceptor.[3] It is known that the yield is determined by the balance between synthetase, amidase and esterase activities of PA, so improving synthesis/hydrolysis capacity of enzyme is very important. To achieve high yield, *in situ* product removal (ISPR) was used to decrease the hydrolysis of product with complexing of cefaclor to form insoluble compounds,[4,5] and feeding acyl donor was used to depress the acyl donor hydrolysis.[6] In our previous study, the factors such as the ratio of acyl donor to nucleus, the substrate concentration,[7] the operation temperature[8] were optimized to obtain high yield. However, the complextant, naphthol and its derivatives, are deleterious to the human immune system, blood circulation system and kidney. In the strategies used in the synthesis of $\beta$-lactam antibiotics, organic solvents have often been added to aqueous medium to reduce the enzymatic hydrolysis reactions. Water-miscible organic cosolvents have also been studied as media for enzymatic syntheses where more hydrophobic solvents are inapplicable.[9] Adding organic cosolvent reduces the rate of hydrolytic reactions by lowering the water activity of the reaction solution and changes the pK values of the substrates. It is possi-

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ble to improve the product yield of a reversible enzyme reaction by adding organic cosolvents,[10] especially the production of penicillins and cephalosporins in the presence of organic cosolvents under thermodynamically[9,11] and kinetically controlled synthesis.[12,13] Although conditions that favor equilibrium towards synthesis are hardly compatible with high PA activity,[12] higher yields and productivity can be expected because synthesis can be performed under milder conditions, more favorable for PA activity and stability.

In the present work, enzymatic syntheses of cefaclor under kinetic control catalyzed by immobilized PA with organic cosolvents were performed, the yield was evaluated to optimize the reaction conditions. The results showed that the yield can be improved significantly in the presence of organic cosolvents, especially ethylene glycol. Furthermore, the effect of ethylene glycol on the synthesis of cefaclor was also studied.

## Materials and methods

### Enzyme

The immobilized penicillin acylase, IPA750 from *Escherichia coli* and PCA from *Bacillus megaterium* were purchased from Hunan Flag Bio-Tech Co. Ltd. (Changsha, China) and Zhejiang Shunfeng Haider Co., Ltd (Dongyang, China), respectively. The immobilized penicillin acylase from *Kluyvera citrophila* (KcPGA) was donated by Shijiazhuang Pharmaceutical Co. Ltd. (Shijiazhuang, China). A unit of penicillin acylase was defined as the amount of enzyme required to produce 1 μmol of 6-aminopenicillanic acid (6APA) per minute in $\gamma$ = 40 kg m$^{-3}$ solution of penicillin G at pH 7.8 at $\theta$ = 37 °C. The enzyme activity was determined by a spectrophotometric assay with *p*-dimethylamino benzaldehyde (PDAB) as a colorimetric substrate.[14]

### Chemicals

PGME and D-(–)-phenylglycine (PG) were purchased from Shanghai Qiude Biochemical Engineering Co. Ltd., China. PDAB was from Sinopharm Chemical Reagent Co. Ltd. Penicillin G potassium salt, cefaclor and 7ACCA were donated by Shijiazhuang Pharmaceutical Co. Ltd. All other reagents used were of analytic grade.

### Enzyme stability assays

The stability of immobilized PA was determined in monophasic water/organic cosolvent mixtures. The enzyme was suspended in 20 mL buffer containing cosolvents at the selected conditions. The residual activity of immobilized PA was measured after per 24 hours incubation.

### Enzymatic synthesis of cefaclor

Enzymatic syntheses of cefaclor were performed in a stirred bioreactor with jackets for water circulation to keep the temperature constant. A pH controller was used to monitor pH values during the reaction process. The initial reaction volume was 100 mL. The enzyme load was 8 IU mL$^{-1}$ and the stirring rate was 120 rpm. The ratio of produced cefaclor and PG (synthesis/hydrolysis) of PA and the conversion of 7ACCA ($X$/mol mol$^{-1}$) in the process was determined by HPLC.

### Analytical methods

Each reactant and prodcuct was identified and analyzed by HPLC with Agilent G1311A pump and Agilent G1315B DAD detector. An Agilent XDB C-18 column (250 mm length and 4.6 mm internal diameter, 5 μm particle diameter and 8 nm pore diameter) was used. Samples of 100 μL were taken from the reaction mixture, added to the 900 μL of eluent in order to dilute the sample, then subjected to HPLC analysis, eluted at 30 °C with 85 % phosphate sodium buffer (25 mmol L$^{-1}$, pH 6.5) and 15 % acetonitrile at $Q$ = 1mL min$^{-1}$, and monitored at 254 nm (7ACCA and cefaclor) and 214 nm (PGME and PG).

## Results and discussion

### Selection of cosolvents

In the synthesis of cefazolin by *E. coli* PA, the yield increased by 65 % and 56 % in the presence of 30 % ethyl acetate and 30 % carbon tetrachloride, respectively.[15] Illanes and Fajardo[16] synthesized ampicillin by *E. coli* PA in the presence of organic cosolvents, and the best results were achieved with ethylene glycol. Fernandez-Lafuente *et al.*[13] found that the presence of methanol exerts a strong and complex modulation on the different antibiotics according to the acyl donor and nucleus. Organic cosolvents were screened in light of the published literatures[11,16–18] and according to our preliminary work on the synthesis of ampicillin in the presence of ethylene glycol.[19]

Therefore, ethylene glycol, glycerol, methanol, ethyl acetate and PEG 6 000, PEG 10 000 were selected as the organic cosolvents in the synthesis of cefaclor. The stability of PA in different cosolvents was presented in Table 1; the enzyme activity did not decrease after 24 hours incubation in ethylene glycol, glycol, PEG 6 000 and PEG 10 000. The stability in polyols was better and this has been

Table 1 – *Effects of organic cosolvents on the synthesis of cefaclor*

| Cosolvents (20 %) | log(*P*) | % of initial activity | Conversion ($X/X_0$, %) |
|---|---|---|---|
| glycerol | –3.04 | 100 | 106 |
| ethylene glycol | –1.8 | 100 | 118 |
| methanol | –0.77 | 48 | 105 |
| PEG 10 000 | N/A[a] | 98 | 65 |
| PEG 6 000 | N/A | 97 | 66 |
| isopropanol | 0.14 | 34 | 75 |

N/A[a]: not available.

Reaction conditions: PCA 432 IU, 20 °C, pH 6.5, 7ACCA 50 mmol $L^{-1}$, PGME 100 mmol $L^{-1}$. The residual activity was the value after 24 hours incubation.

proved in the stabilization of PA by Arroyo *et al.*[20] It maybe that the polyols prevent the unfolding of protein by strengthening the hydrogen bonds in the hydrophilic interactions;[21] polyols and glycols are the best cosolvents to maintain both the solvophobic interactions essential for the native structure and the water shell around the protein molecules. The effects exerted by these cosolvents are attributed to a positive change in the standard free energy denaturation.[22] The residual activity of PA in methanol and isopropanol were 25 % and 34 % of the initial activity. Yields were only 65 % and 66 % in presence of PEG 6 000 and PEG 10 000, respectively. And ethylene glycol, glycerol and methanol were found to enhance the yield of cefaclor compared to those in the buffer; especially in ethylene glycol and glycerol the yield were improved to 118 % and 106 %, respectively. Thus, ethylene glycol was selected as the best cosolvents for further study.

## Effects of ethylene glycol content on the yield of cefaclor

The effects of substrate concentration and pH on the yield of cefaclor were studied (data were not shown) with PCA from *B. megaterium* and the results were similar to that in the cefaclor synthesis with ISPR.[7] Substrates concentrations: 7ACCA $c$ = 50 mmol $L^{-1}$; PGME $c$ = 100 mmol $L^{-1}$; and pH 6.5 were chosen. The time course of the cefaclor yield was studied with different concentration of ethylene glycol and immobilized enzyme from different microoganisms. As shown in Fig. 1, with ethylene glycol concentration increasing, the reaction rates decreased because the viscosity of the reaction medium increased. However, the dynamic viscosity of the reaction medium increased to up to $\eta$ = 26.5 mPas did not affect the synthesis yield of cepha-

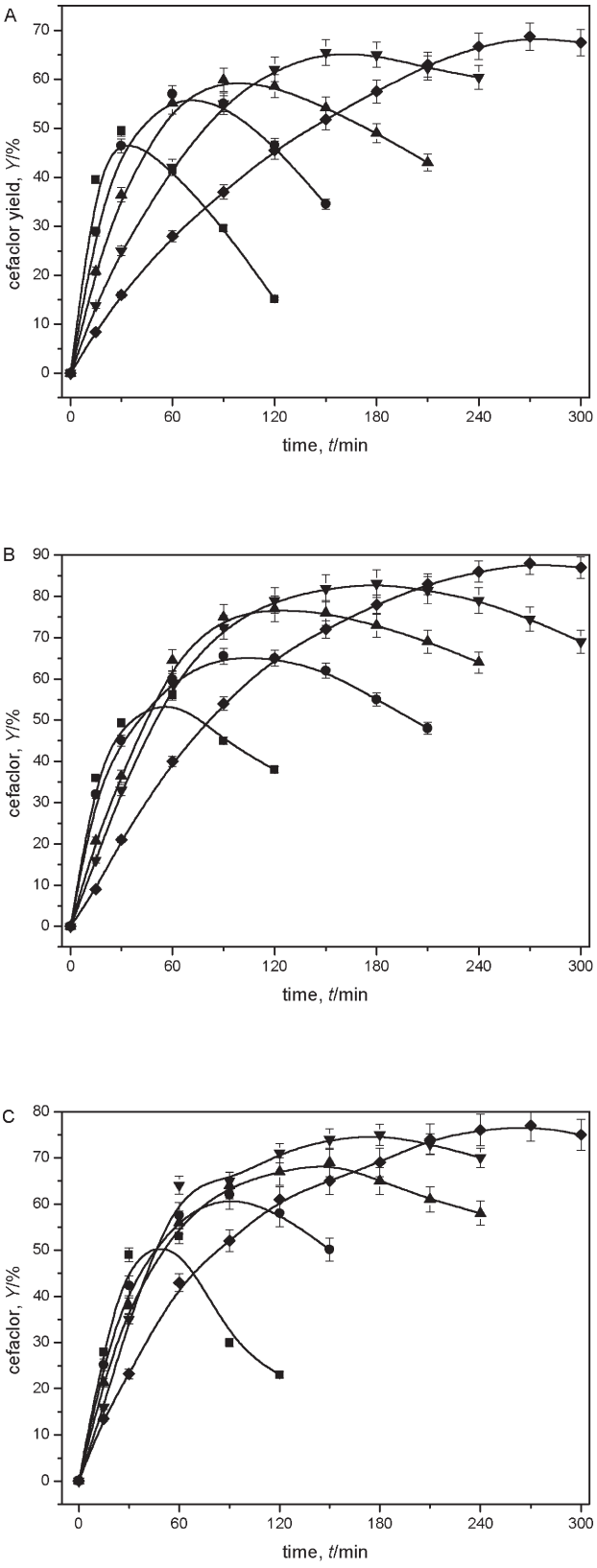
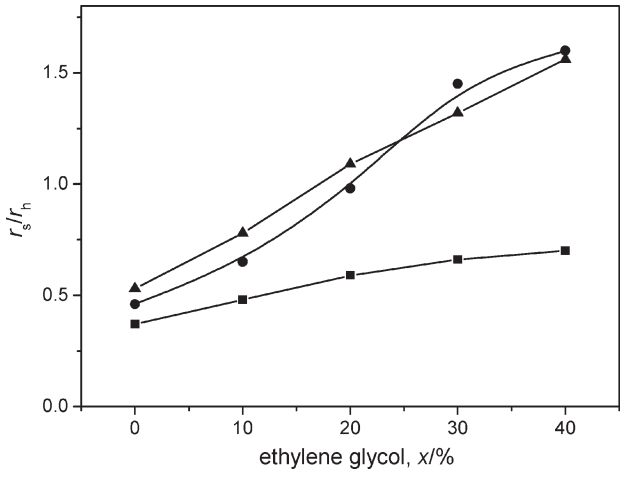



Fig. 1 – *Effect of ethylene glycol fraction on the yield of cefaclor with different immobilized penicillin acylase*
*Reaction conditions: 20 °C, 7ACCA 50 mmol, PGME 100 mmol, pH 6.5. Square: control; Circle 10 % ethylene glycol; uptriangle 20 % ethylene glycol; downtriangle: 30 % ethylene glycol; diamond: 40 % ethylene glycol. (A) PCA; (B) IPA750; (C) KcPGA*

lexin[23] and ampicillin.[24] In Fig. 1, it is easy to learn that the maximum yield was achieved in 40 % ethylene glycol solution. There were 12 %, 22 % and 24 % improvement than that of control catalyzed by PCA, KcPGA and IPA750, respectively. In the synthesis, 40 % ethylene glycol slowed down the reaction rate and the maximal yields were obtained after 4.5 hours reaction. So the optimal concentration of ethylene glycol was 30 % based on the results. Ethylene glycol can be classified as a soft monophasic solvent to promote a very slight increase in $pK_a$ of acyl donor and the effects only produced on acyl donor substrate.[9] In the synthesis of cephalexin, a 35 % improvement was obtained in 50 % ethylene glycol.[25] It is known that PA can only accept nonionic substrate[26] and the addition of organic solvents such as ethylene glycol may facilitate the stabilization of nonionic substrate. So the effect of ethylene glycol on the enzyme properties: the ratio of synthesize cefaclor and hydrolysis of PGME were studied and the results were shown in Fig. 2. With the content of ethylene glycol increased, all the ratios catalyzed by PCA, KcPGA and IPA750 increased. It may contribute that the addition of ethylene glycol depresses the hydrolysis of PGME, especially in the syntheses catalyzed by KcPGA and PCA.

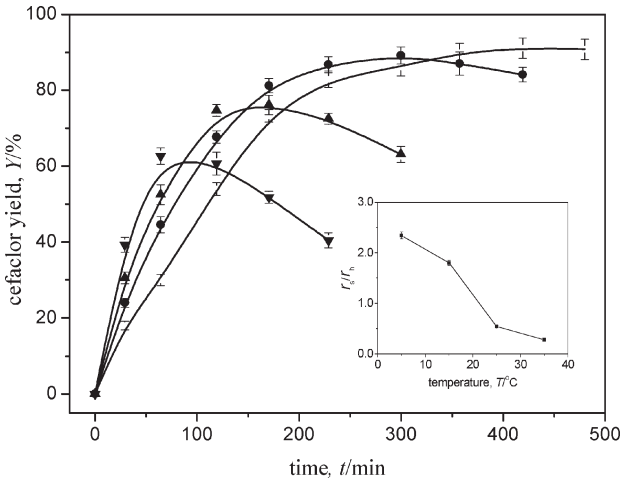



F i g . 2 – *Effect of ethylene glycol fraction on the $r_s/r_h$ of different immobilized penicillin acylase*
*Reaction conditions are the same as Fig. 1; Square: PCA; circle: KcPGA; uptriangle IPA750*

In the synthesis of cefaclor, KcPGA obviously showed have higher yields than IPA750 from *E. coli* and PCA from *B. megaterium* according to Fig. 1 in the range of investigated concentrations of ethylene glycol (10 % – 40 %). It is commonly known that the best PA for one particular synthetic reaction may be unsuitable for another. Hernández *et al.*[27] reported that PA from *A. turbulans* had higher synthetase in the ampicillin synthesis but lower synthetase in the synthesis of cefamondale. Cheng *et al.*[28] compared the PA from *A. faecalis, E. coli, K. cryocrescens* and *P. rettgeri*, and found that PA from *K. cryocrescens* has the highest selectivity in the synthesis of cefalexin. PA from *E. coli* also has higher selectivity in synthesis of cephalexin. However, it has lower yield than KcPGA in the present work (shown in Fig. 2). KcPGA and IPA750 were more suitable for synthesis of cefaclor than PCA. Thus, KcPGA was chosen as the catalyst in the following experiments mainly because the highest yield was obtained at the synthesis conditions of $Y$ = 30 % ethylene glycol.

## Effect of temperature on the synthesis of cefaclor in the presence of ethylene glycol

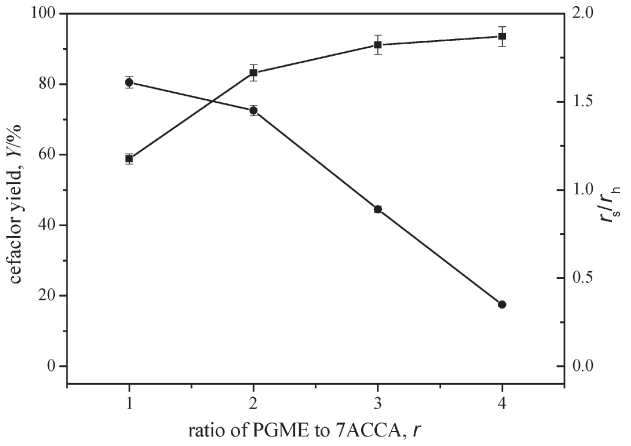
Temperature is the determined factor in the biotransformation because it directly affects the enzyme stability and activity. In our previous study, the effect of temperature on the enzymatic synthesis of cefaclor by PA was described with ISPR.[8] ISPR is beneficial for the synthesis of cefaclor at low temperature, but has little significant increase at high temperatures. It is similar to the results with ISPR in the presence of ethylene glycol (as shown in Fig. 3); the yield of cefaclor was $Y$ = 91.1 % at $\theta$ = 5 °C and $Y$ = 62.7 % at $\theta$ = 35 °C. Low temperature favors the conversion of 7ACCA though the reaction rate is low. The hydrolysis of cefaclor and PGME were abated at low temperatures. These can be reflected by $r_s/r_h$ shown in Fig. 3; it dramatically decreased from 2.34 at $\theta$ = 5 °C to 0.28 at $\theta$ = 35 °C. This is the case in the synthesis of cefalexin at frozen media ($\theta$ = –20 °C) and there was a 58 % improvement than that at $\theta$ = 20 °C.[29]




F i g . 3 – *Effect of temperature on the yield of cefaclor in the presence of ethylene glycol*
*Reaction conditions: KcPGA, 30 % ethylene glycol, 50 mmol $L^{-1}$ 7ACCA, PGME 100 mmol $L^{-1}$, pH 6.5. Square: 5 °C; circle: 15 °C; uptriangle: 25 °C; downtriangle: 35 °C. $r_s/r_h$ at different temperature was present at the inset.*

### Effect of substrate ratio on the yield of cefaclor

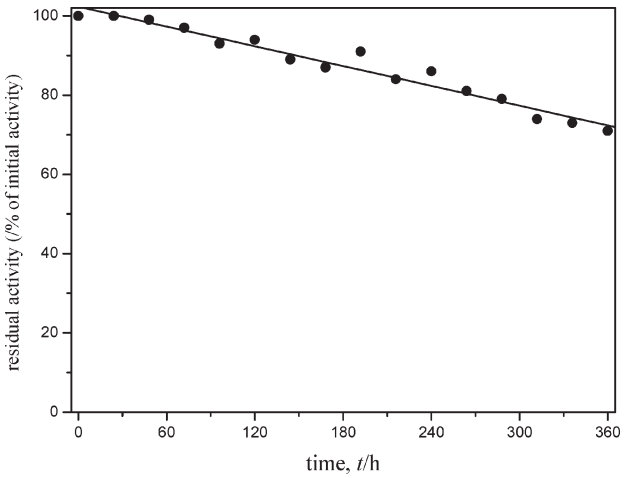
Although the antibiotic nuclei and acyl donor contribute equally to the product, the antibiotic nuclei was chosen as index of yield because nuclei was more expensive than acyl donor. Actually, in the synthesis of $\beta$-lactam antibiotics, excess of acyl donor was often used to obtain high yield. In the synthesis of cephalexin, Maladkar have used tenfold excess of PGME,[30] and Illanes have used threefold excess of PGME.[31] In the synthesis of cefaclor, the yield was improved from $Y$ = 58.8 to 91.1 % when the mole ratio of ($r$) PGME to 7ACCA increased from 1 to 3 (Fig. 4). This yield is higher than that of ISPR at the same temperature.[8] However, the yield was improved only about 2.4 % when the ratio was increased from 3 to 4. This phenomenon indicated that the high concentration of PGME over guideline may lead to the hydrolysis of PGME, and it has been known that the unspecified hydrolysis of PGME was serious at high PGME concentration.[32] Thus, the optimal ratio of PGME to 7ACCA should be 3 in the PA catalyzed synthesis in the presence of ethylene glycol.




F i g . 4 – *Effect of ratio of PGME to 7ACCA on the yield of cefaclor*
*Reaction conditions: KcPGA 30 % ethylene glycol, 5 °C, 7ACCA 50 mmol L$^{-1}$, pH 6.5. Square: cefaclor yield; circle: $r_s/r_h$.*

### Stability of KcPGA at the optimal conditions for cefaclor synthesis

The stability of immobilized biocatalyst was carried out at the selected conditions of synthesis of cefaclor and the data was shown in Fig. 5. After 360 hours incubation, there was still about 71 % of residual activity. A half time of 650 hours was determined, which means that the catalyst is stable under the conditions of cefaclor synthesis.




F i g . 5 – *The stability of KcPGA at selected conditions of cefaclor synthesis*
*Conditions: pH 6.5, 5 °C, 30 % ethylene glycol*

## Conclusions

Effects of cosolvents on the enzymatic synthesis of cefaclor were investigated, and ethylene glycol was selected as the best cosolvent for further studies. Among the three species of immobilized penicillin acylase used, KcPGA from *K. citrophila* was chosen as the best biocatalyst because of its highest yield of cefaclor. The mole fraction of ethylene glycol, reaction temperature, and the mole ratio of PGME to 7ACCA were optimized. Ethylene glycol improved antibiotics synthesis strongly by increasing the ratio of synthesis of cefaclor to hydrolysis of PGME. Consideration of the cost, the optimal reaction conditions were: $x$ = 30 % ethylene glycol, $\theta$ = 5 °C, the ratio of $r_{\mathrm{PGME/7ACCA}}$ = 3:1. The maximum yield obtained was $Y$ = 91.1 % under the optimal conditions.

*ACKNOWLEDGEMENT*

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### List of symbols

$c$ – concentration, mmol L$^{-1}$
$r$ – mole ratio
$X$ – conversion, %
$x$ – mole fraction, %
$Y$ – yield, %
$\gamma$ – mass concentration, mg L$^{-1}$
$\eta$ – dynamic viscosity, mPa s
$\theta$ – temperature, °C
$r_s/r_h$ – ratio of reaction rate

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