Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 30 (2004) 167–172
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Efficient immobilization of whole cells of *Methylomonas* sp. strain GYJ3 by sol–gel entrapment

Jianbo Chen [a,*], Yi Xu [b], Jiaying Xin [c], Shuben Li [c], Chungu Xia [c], Junru Cui [c]

[a] *College of Life and Environment Science, Shanghai Teachers University, Shanghai 200234, PR China*
[b] *Laboratory of Biocatalytic Engineering, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200231, PR China*
[c] *State Key Laboratory of Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, PR China*

Received 7 February 2004; received in revised form 20 May 2004; accepted 20 May 2004

Available online 26 June 2004

Abstract

The whole cells of Methanotrophic bacteria *Methylomonas* sp. strain GYJ3 immobilized by the sol–gel technique was investigated in the present work. After the cells were immobilized by entrapment in sodium silicate sol–gel matrix, a higher activity for propylene epoxidation were observed at a common loading amount. In a batch reaction system, the immobilized cells can be repeatedly used for more than 25 times and no significant loss of the activity was found. The activity was well preserved when the immobilized cells were stored at 4 °C for 45 days. Determination of optimal reaction conditions showed that the immobilized cells took on increased activity at a higher pH and temperature. However, the cells entrapped in methyltrimethoxysilane (MTMS) gel matrix gave a lower activity as compared to that of the free cells. Our experimental results indicate that the sol–gel entrapment based on silica matrixes was a simple, efficient and cost-efficient method for the immobilization the whole cells of *Methylomonas* sp. strain GYJ3 cells.

Keywords: Methanotrophic bacteria; Whole cells; Sol–gel entrapment; Immobilization; Propylene epoxidation

1. Introduction

The small molecular weight gaseous alkene is difficult to utilize for the low solubility of propylene in aqueous media and the associated mass transfer resistances. Methane monooxygenase (MMO) is a type of enzyme that catalyzes NADH-dependent insertion of an oxygen atom into C–H bond of methane as well as other substrates, such as propylene [1–3]. The reaction of propylene epoxidation is of importance since propylene oxide is used widely as a kind of significant refined chemical. The biocatalytic reaction is mild and thus harmless to environment as compared with the chemical process, so the potential utilization of this reaction has received considerable attention in recent years. A thorough understanding of the mechanism of alkene epoxidation with methane monooxygenase is being intensively studied at the same time [4–7]. However, the low activity and instability of purified enzyme made it impossible to utilize this enzyme in practical application. Furthermore, the coenzyme NADH is expensive and cannot be regenerated in the enzymatic reaction. From the view point of industrial application, whole cells will be more stable and suitable than the isolated enzyme.

To obtain whole cells with high activities and excellent stabilities, various methods including adsorption, covalent binding and gel entrapment, have been used for immobilization of whole cells of MMO [8–10]. Wingard et al. [8] reported that, under certain conditions the activity of immobilized cells by adsorption was three times that of the free ones. However, the cells by entrapment in calcium alginate showed about the same propylene oxide activity and stability as the free cells. Though the cells by adsorption took on higher activity compared with the ones by entrapment, desorption will also easily take place due to the weak interac-

* Corresponding author. Tel.: +86 21 6432 2933; fax: +86 21 6432 2933.
E-mail address: xuyiecust@yahoo.com.cn (J. Chen).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.05.005

tion between the cells and supports [11]. And this drawback can be avoided by entrapment technique.

In recent years, sol–gel technique has gradually became the focus of interest and been widely used for the immobilization of many biomolecules including enzymes, catalytic antibodies, antigens, fungi, plant and animal cells [12–15]. The process of immobilization involves no high temperature and harsh chemical reaction, and it has the ability to immobilize biomolecules without modifying their structures and functions greatly. Moreover, the porosity can be controlled to an appropriate degree by the judicious selection of precursors, modifiers, polymerization conditions and so on [16]. Sol–gel entrapment technique often uses orthosilicate such as tetramethyl orthosilicate (TMOS), tetraethyl orthosilicate (TEOS) or methyltrimethoxysilane (MTMS) as the precursors [17,18]. In an aqueous media, the hydrolysis of precursors results in the formation of oligomer. Then, the oligomer is further hydrolyzed to form an aqueous sol. The occurrence of gelation process leads to the development of a three-dimensional network and the biomolecules were finally trapped into the cages of network [16,19]. However, it is usually considered that the relatively high concentration of methanol or ethanol produced in the immobilizing process is harmful to the activity of enzymes or cells. As in the case of lipase, only 5% activity can be retained after entrapment in orthosilicate-based matrix [20]. An exception is that Reetz et al. [17] obtained excellent activity on the immobilization of lipase by utilizing alkoxysilane derivatives as gel precursors. Therefore, a new route for synthesis of alcohol-free sol–gel matrix should be developed. Recently, sol–gel process based on sodium silicate silica was frequently used to immobilize biomolecules [19,21]. This approach can avoid the production of alcohol completely. Thus, the denaturation of the biomolecules due to the presence of alcohol can be eliminated. On the other hand, the advantages of sol–gel technique can be reserved.

The immobilization of the whole cells have not been intensively studied, in this article the sodium silicate silica-based sol–gel method was used to immobilize whole cells of *Methylomonas* sp. strain GYJ3. And the technique by using MTMS as a precursor was also checked for a comparison. The effect of entrapment in different sol–gel matrix was evaluated by the determination of activity and stability and the results compared with that of the free cells were discussed.

2. Materials and methods

2.1. General

Methylomonas sp. strain GYJ3, one of the Type I Methanotrophic bacterial, was preserved at the State Key Laboratory of Oxo Synthesis and Selective Oxidation in Lanzhou, China [22]. All the chemicals were obtained commercially and were of analytical grade.

2.2. Cultivation of Methylomonas sp. strain GYJ3

The *Methylomonas* sp. strain GYJ3 was grown in a 15 L jar fermenter with 10 L of the following medium (g/L): NH_4Cl, 0.5; K_2HPO_4, 0.49; $KH_2PO_4{\cdot}7H_2O$, 0.40; $MgSO_4{\cdot}7H_2O$, 0.3; $CaCl_2{\cdot}2H_2O$, 0.02; KNO_3, 1.6; NaCl, 0.3; $FeSO_4{\cdot}7H_2O$, 0.004; $CuSO_4{\cdot}5H_2O$, 0.004; $MnSO_4{\cdot}H_2O$, 0.0004; $ZnSO_4{\cdot}7H_2O$, 0.00034; $Na_2MoO_4{\cdot}2H_2O$, 0.00024; pH 7.0. It was incubated for 96 h at 32 °C with 50% methane–50% air. *Methylomonas* sp. strain GYJ3 cells were harvested by centrifugation at 9000 × *g* and washed three times with 0.05 mol L^{-1} phosphate buffer, pH 7.0. The washed cells were resuspended in the above phosphate buffer and stored at 4 °C.

2.3. Immobilization of Methylomonas sp. strain GYJ3 cells by sodium silicate-based sol–gel method

For the entrapment of cells in sodium silicate-based sol–gel, typically 2 mL of 3.5 mol L^{-1} sodium silicate solution was mixed with appropriate amount of 1.2 mol L^{-1} hydrochloric acid to make the pH of the mixture weak acidic. After the mixture was shaken vigorously, desired amount of phosphate buffer (0.2 mol L^{-1}, pH 7.5) was added to adjust the pH of solution to 7.0. Then, appropriate amount of cell suspension was added, and the mixture was shaken gently until gelation occurred about 10–20 min later at ambient temperature. The immobilized cells were aged at 4 °C for 48 h and dried at ambient temperature for another 72 h before use. Then, the immobilized cell was washed and incubated in a conical flask at 32 °C for 4 days under the same conditions as the incubation of the free cells. Phosphate buffer (0.05 mol L^{-1}, pH 7.0) was used to wash the gel several times prior to propylene epoxidation testing.

2.4. Immobilization of Methylomonas sp. strain GYJ3 cells by MTMS-based sol–gel method

Entrapment of cells in MTMS-based sol–gel technique was similar to Reetz et al.'s procedure [17]. The cell' suspension (0.8 mL) was added to a mixture of aqueous sodium fluoride (0.1 mL, 0.75 mol L^{-1}) and phosphate buffer (0.1 mL, 0.2 mol L^{-1}, pH 7.0). The mixture was shaken and MTMS (1 mL) was added. Then, the reaction mixture was mixed thoroughly until gelation occurred (about 2–3 min later). The following process was the same as that of the sodium silicate-based sol–gel method.

2.5. Experiments of propylene epoxidation

The activities of free or immobilized cells of *Methylomonas* sp. strain GYJ3 were assayed by the rate of propylene epoxidation to propylene oxide. A conical flask sealed with a screw cap was used to perform the propylene epoxidation experiment. In a 250 mL conical flask, the free or immobilized cells were suspended in 15 mL phosphate buffer

(0.05 mol L^{-1}, pH 7.0). Then, the mixture of propylene and air were injected into the conical flask with a syringe and the proportion of propylene in the mixture was 50%. The reaction was performed on a shaker at 32 °C and 150 rpm.

2.6. Regeneration of the coenzyme NADH

When the epoxidation reaction completed, cells of *Methylomonas* sp. strain GYJ3 were washed and kept at 32 °C for 7–8 h under the incubation conditions of cells. Then, the cells were washed and used for a new batch of reaction.

2.7. Assays for propylene oxide and propylene

The concentration of propylene oxide or propylene were analyzed on a gas chromatography (Tian-mei Co., type 7890-II, Shanghai), equipped with a 25 m × 0.25 mm SE-54 capillary column and a FID detector. The temperatures of oven, injector and detector blocks were set at 60, 180 and 200 °C, respectively.

3. Results and discussion

3.1. Reaction activity of free and immobilized cells of Methylomonas sp. strain GYJ3

The activity of free and immobilized cells of *Methylomonas* sp. strain GYJ3 were determined by the propylene epoxidation experiments under the same conditions. The results were shown in Fig. 1. It was found that the cells entrapped in MTMS sol–gel matrix only took on lower activity than that of the free cells. As for the lower activity of the cells in MTMS sol–gel matrix, many investigators ascribed this case to the denaturation of alcohol on the biomolecules [19,20]. It seemed that some enzymes encapsulated in orthosilicate-based matrix similarly showed no high activities. As for lipase, the activity of lower than 5% relative to the non-immobilized form was ever found when the gel was prepared by acid catalyzed hydrolysis of tetramethoxysilane (TMOS) [21]. However, whole cells entrapped in sodium silicate sol–gel showed a 1.5-fold higher activity than that of the free cells. It was considered that the enhanced activity of the sodium silicate sol–gel entrapped cells may result from a better dispersion of the cells in the gel matrix and the absence of alcohol, which was harmful to the biomolecules. The activity of cells obtained based on MTMS sol–gel process was much lower than that was prepared by sodium silicate sol–gel technique. Then, in the following experiments, only the immobilized cells entrapped in sodium silicate sol–gel were investigated.

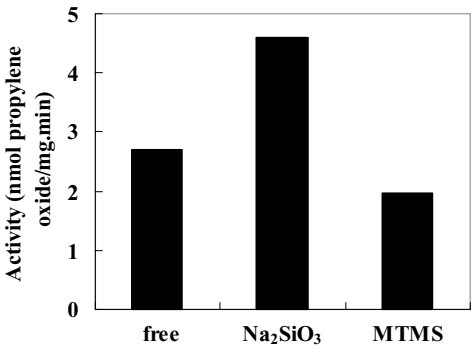

Fig. 1. Activities of free and immobilized cells for propylene epoxidation. Reaction temperature: 32 °C; pH 7.0; reaction time: 6 h; the loading amount was 0.174 g dried cells/g catalyst.

3.2. Effect of loading amount on the activity of cells entrapped in sodium silicate gel

The appropriate amount of cell-loading in the support was an important parameter affecting the activity of immobilized cells. The loading amount varying from 0.029 to 0.435 mg (dry weight) cells/g immobilized catalyst was investigated (Fig. 2). With the activity of free cells as 100%., the relative activity of immobilized cells decreased with the increase in cell-loading. A further increase in cell-loading beyond a certain critical amount resulted in lower catalytic activity. This is the same case as what was found by Reetz et al. [17] where the lipase was entrapped in a hydrophobic sol–gel matrix. The reasons accounting for the result can be rationalized in two aspects. One is the diffusional limitation of the substrate entering into the catalyst particles leads to a decrease in the apparent activity of immobilized cells. Another reason is the aggregation of the cells might occur at relatively high concentrations, resulting in a lower degree of dispersion in the sol–gel matrix [8,17].

3.3. Influence of temperature and pH on free and immobilized cells

After entrapment in sol–gel, the optimal reaction conditions of immobilized cells were compared with the free ones. As shown in Fig. 3, the optimum pH and temperature of

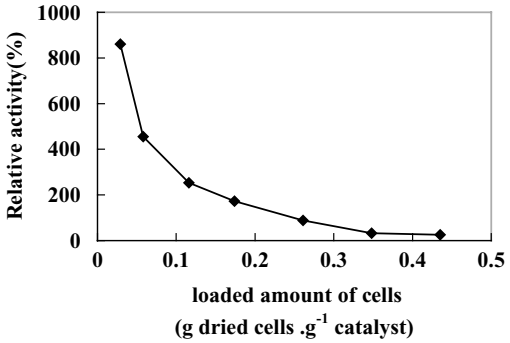

Fig. 2. Relative activity of *Methylomonas* sp. strain GYJ3 cells entrapped in sodium silicate gel at different loading amount of cells on propylene epoxidation. (◆) Relative activity ($v_{rel} = v_{immob}/v_{free} \times 100\%$). The reaction conditions were the same as in Fig. 1, except the loading amount of cells.

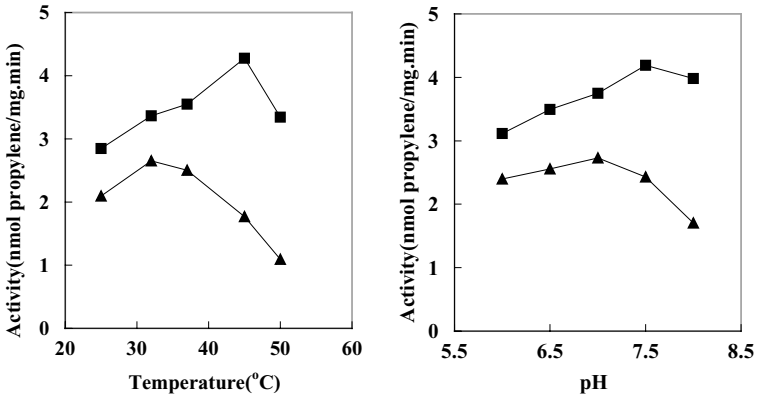

Fig. 3. Effect of temperature and pH on the activity of free and immobilized cells. The reaction conditions were the same as in Fig. 1, except: for temperature test the pH being 7.0 and for pH test the temperature being 32 °C.

cells altered from 7.0 to 7.5 and 35 °C to 40 °C, respectively, after immobilized in sodium silicate gel. This implies that the stability of cells against pH and temperate was increased after the immobilization in the sol–gel.

3.4. Operational and preservational stability of the immobilized cells

The determination of operational stability of the free and immobilized cells was performed in a batch reaction system. Incubation of cells after each batch reaction was performed for the regeneration of coenzyme NADH. As shown in Fig. 4, the immobilized cells of *Methylomonas* sp. strain GYJ3 prepared by entrapment in sodium silicate sol–gel matrix had almost kept the initial activity after 25 batches reaction. However, the free cells of *Methylomonas* sp. strain GYJ3 almost lost its activity after three batches of reaction. Specially, it was found that the activity of cells immobilized in sodium silicate gel used for the second batch was higher than others. We are not sure about this. Perhaps, the amount of coenzyme produced during the regeneration process accounted for this case.

The preservational stability was investigated after the immobilized cells were kept at 4 °C in refrigerator for 45 days. Results showed that the sol–gel entrapped cells of *Methylomonas* sp. strain GYJ3 were highly stable and no significant loss of activity was found after long-term storage. The similar results were found by Liu and Chen [19] for the immobilization of enzyme and protein. It was considered that the complete absence of alcohol and conformation retention of proteins accounted for the high stability.

3.5. Regeneration of the coenzyme NADH

Methane, the substrate of Methanotrophic bacterium, is required for the growth and production of coenzyme NADH [23]. And the process of propylene epoxidation occurred in the presence of NADH. When the immobilized cells were used in the batch reactions, the process of propylene epoxidation can not proceed before the regeneration of the coenzyme.

Hou et al. [24] have used methanol, formaldehyde and formate to regenerate NADH. It was found that the cells lost their activities after two or three times regeneration by the

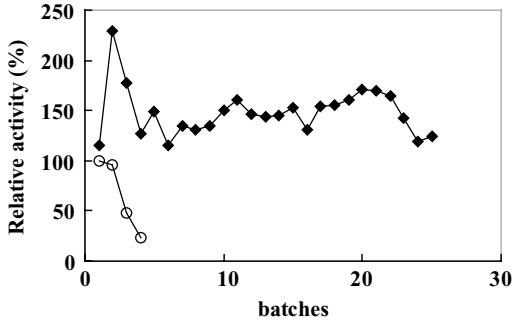

Fig. 4. Operational stability of free and immobilized *Methylomonas* sp. strain GYJ3 cells in repeated reaction of propylene epoxidation. Relative activity = v_{immob}/v_{free}. Symbols: (◆) Whole cells immobilized in sodium silicate gel; (○) free cells. The reaction conditions were the same as in Fig. 1.

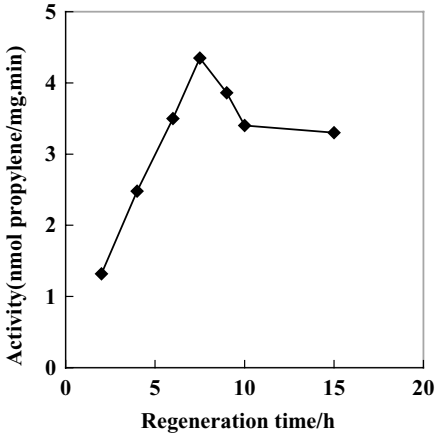

Fig. 5. Relationship between the regeneration time and the activity of cells entrapped in sodium silicate gel matrix.

addition of the three substances. Gao et al. [25] reported that methane was the perfect exterior carbon source and it can be used for several times to reproduce coenzyme. This method was used in our present study. The relation between the regeneration time and activities of the cells was illustrated in Fig. 5. Results indicated that the optimal regeneration time was 7.5 h.

4. Conclusions

For the cells immobilized in sodium silicate gel matrix, a higher activity and excellent stability relative to the free cells were obtained. Though the detailed mechanism study has not been performed on whole cells, we speculated that the microenvironment created by the sol–gel cage seemed to be beneficial for biomolecules to maintain their activity and stability. However, the *Methylomonas* sp. strain GYJ3 cells by entrapment in MTMS-based gel matrix did not show the same properties. It was considered that the presence of alcohol produced in the process of immobilization accounted for the main reason.

The main problem existing in the immobilization of biomolecules is the diffusional limitation. This was further proved by our results. The activity of immobilized cells did not increase with the increase in loading amount. This result suggests that the diffusional resistance might be one of the most significant influencing factors. The activity of immobilized cells by entrapment in sodium silicate gel matrix was about 1.5-folds higher than that of the free cells when the loading amount was 0.174 g dried cells/g catalyst. But this is not the most appropriate loading amount and a higher activity can be obtained at a lower loading amount of cells.

In the following experiment, the optimal reaction conditions including pH, temperature and the optimal regeneration time were determined. Results indicated that the optimal pH value and temperature of immobilized cells was 7.5 and 40 °C, respectively. This suggests that the immobilized cells entrapped in sol–gel matrix can resist against higher pH value and high temperature, as compared with the free cells, whose optimal pH value and temperature was 7.0 and 35 °C, respectively. Results showed that the optimal regeneration time of cells entrapment in sodium silicate gel matrix was 7.5 h.

In summary, the sodium silicate and MTMS-based sol–gel entrapment methods have been used to immobilize the cells of *Methylomonas* sp. strain GYJ3. The immobilized cells entrapped in sodium silicate gel matrix exhibited an excellent activity and stability. The sodium silicate-based sol–gel method appeared to be a feasible and useful method for potential application.

References

[1] H. Dalton, Biological methane activation-lessons for the chemists, Catal. Today 13 (1992) 455–461.

[2] J. Colby, D.I. Stirling, H. Dalton, The solubal methane monooxygenase of *Methylococcus capsulatus* (Bath), Biochem. J. 165 (1977) 395–402.

[3] B.J. Wakkar, J.D. Lipscomb, Dioxygen activation by enzymes containing binuclear non-heme iron clusters, Chem. Rev. 96 (1996) 2625–2657.

[4] S.K. Lee, J.C. Nesheim, J.D. Lipscom, Transient intermediates of methane monooxygenase catalytic cycle, J. Biol. Chem. 268 (1993) 21569–21577.

[5] A.M. Valentine, S.S. Stahl, S.J. Lippard, Methanistic studies of the reaction of reduced methane monooxygenase hydroxylase with dioxygen and substrates, J. Am. Chem. Soc. 121 (1999) 3876–3887.

[6] R. Davydov, A.M. Valentine, S.K. Panicucci, B.M. Hoffman, S.J. Lippard, An ERPstudy of the dinuclear iron site in the soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) reduced by one electron at 77 K: the effects of component and the binding of small molecules to the diiron(III) center, Biochemistry 38 (1999) 4188–4197.

[7] G.T. Gassner, S.J. Lippard, Component interactions in the soluble methane monooxygenase system from *Methylococcus capsulatus* (Bath), Biochemistry 38 (1999) 12768–12785.

[8] L.B. Wingard, R.P. Roach, O. Miyawaki, K.A. Egler, G.E. Klinzing, Epoxidation of propylene utilizing *Nocardia corallina* immobilized by gel entrapment or adsorption, Enzyme Microb. Technol. 7 (1985) 503–509.

[9] G.A. Kovalenko, V.D. Sokolovskii, Epoxidation of propene by microbial cells immobilized on inorganic supports, Biotechnol. Bioeng. 39 (1992) 522–528.

[10] P.K. Mehta, S. Mishra, T.K. Ghose, Methanol biosynthesis by covalently immobilized cells of *Methylosinus trichosporium*: batch and continues studies, Biotechnol. Bioeng. 37 (1991) 551–556.

[11] K. Ryu, J.S. Dordick, How do organic solvents affect peroxidase structure and function? Biochemistry 31 (1992) 2588–2598.

[12] Q. Chen, G.L. Kenausis, A. Heller, Stability of oxidases immobilized in silica gels, J. Am. Chem. Soc. 120 (1998) 4582–4585.

[13] I. Gill, A. Ballesteros, Encapsulation of biologicals within silicate, J. Am. Chem. Soc. 120 (1998) 8587–8598.

[14] J.D. Brennan, Using intrinsic fluorescence to investigate proteins entrapped in sol–gel materials, Appl. Spectrosc. 53 (1999) 106A–121A.

[15] A.K. Williams, J.T. Hupp, Sol–gel encapsulated alcohol dehydrogenase as a versatile, J. Am. Chem. Soc. 120 (1998) 4366–4371.

[16] I. Gill, A. Ballesteros, Bioencapsulation within synthetic polymers (part I): Sol–gel encapsulated biologicals, Trends Biotechnol. 18 (2000) 282–296.

[17] M.T. Reetz, A. Zonta, J. Simpelkamp, Efficient immobilization of lipases by entrapment in hydrophobic sol–gel materials, Biotechnol. Bioeng. 49 (1996) 527–534.

[18] Y. Wei, J. Xu, Q. Feng, H. Dong, M. Lin, Encapsulation of enzymes in mesoporous host materials via the nonsurfactant-templated sol–gel process, Mater. Lett. 44 (2000) 6–11.

[19] D.M. Liu, I.W. Chen, Encapsulation of protein molecules in transparent porous silica matrices via an aqueous colloidal sol–gel process, Acta Mater. 47 (1999) 4535–4544.

[20] R.B. Bhatia, C.J. Brinker, Aqueous sol–gel process for protein encapsulation, Chem. Mater. 12 (2000) 2434–2441.

[21] M.T. Reetz, A. Zonta, J. Simpelkamp, Efficient heterogeneous biocatalysts by entrapment of lipases in hydrophobic sol–gel materials, Angew. Chem. Int. Ed. Engl. 34 (1995) 301–303.

[22] R.N. Shen, C.L. Yu, Q.Q. Ma, S.B. Li, Direct evidence for a soluble methane monooxygenase from Type I methanotrophic bacterial: purification and properties of a soluble methane monooxygenase from

methylomonas sp. GYJ3, Arch. Biochem. Biophys. 345 (1997) 223–229.
[23] H. Dalton, Biological methane activation-lessons for the chemists, Catal. Today 13 (1992) 455–461.
[24] C.T. Hou, R. Patel, A.I. Laskin, et al., Microbial oxidation of gaseous hydrocarbons: epoxidation of C_2 to C_4 *n*-alkenes by Methylotrophic bacteria, Appl. Environ. Microbiol. 38 (1979) 127–134.
[25] C.Z. Gao, S.B. Li, Z.Z. Ning, D.X. Miu, Propylene oxide production from propylene by immobilized living cells and biocatalyst regeneration by the mixture of methane and air, J. Mol. Catal. (China) 4 (1990) 291–297.