Effect of Substrate Concentration on the Synthesis of Cefaclor by Penicillin Acylase with in Situ Product Removal

Ye-Wang Zhang, Deng-Chao Li, Qing-Xun Song, Shu-Lai Liu, and Dong-Zhi Wei*

State Key Laboratory of Bioreactor Engineering,
New World Institute of Biotechnology, East China University
of Science and Technology, Shanghai 200237, P. R. China

Original scientific paper
Received: November 28, 2005
Accepted: March 15, 2006

Enzymatic synthesis of 3-chloro-7-D-(2-phenylglycinamide)-3-cephem-4-carboxylic acid (cefaclor) by penicillin acylase (PA) was carried out with *in situ* product removal (ISPR) under kinetic control. The yield of cefaclor highly depended on substrate concentrations and the ratio of nucleus to acyl donor. Substrate concentrations were optimized as 50 mmol l^{-1} of 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid (7ACCA) and 100 mmol l^{-1} of phenylglycine methyl ester (PGME) at the conditions: temperature 20 °C, pH 6.3; and enzyme load was 8 IU ml^{-1}. It is effective to improve the transfer of acyl donor through controlling the substrate concentration with feeding acyl donor. The conversion of nucleus and acyl donor was improved to 93 % and 62 %, respectively.

Key words:
Acyl donor feeding, 3-chloro-7-D-(2-phenylglycinamide)-3-cephem-4-carboxylic acid, enzymatic synthesis, *in situ* product removal, penicillin acylase, substrate concentration

Introduction

Series of semi-synthetic β-lactam antibiotics showed to be very efficient antibiotics and will represent one of the key groups of anti-infective agents.[1] Now the industrial synthesis of β-lactam antibiotics is experiencing a transfer from chemical procedures to biocatalytic ones. From a viewpoint of environmental acceptability, biocatalytic processes offer obvious benefits: reactions are performed in aqueous system at neutral pH and moderate temperature, without the need for extensive functional group protection and/or activation, and high chem-, region-, and stereo-selectivities to be realized.[2] Penicillin acylase (PA; EC 3.5.1.11), which has a single amino acid as catalytic center,[3] is widely used in the production of 6-aminopenicillanic acid, the precursor for the production of the semi-synthetic β-lactam antibiotics. *Cole* and his coworkers[4] found that semi-synthetic antibiotics could be synthesized by whole microbial cells having PA activity.

Enzymatic condensation processes can be carried out under thermodynamic or kinetic control.[5] In the first case there is no need for activation of acyl donor, but the main drawback of the thermodynamically controlled synthetic strategy is the deleterious effect that the presence of moderate high concentration of organic solvent may exert on the activity and stability of the enzyme.[6–8] To our best knowledge, few β-lactam antibiotics such as cefamandole,[9] amoxicillin,[10] penicillin G,[11] and cephalotin[12] were reported being synthesized successfully under thermodynamic control. The reasons are the thermodynamics of synthesis reactions are generally unfavorable in aqueous medium[12] and PA from some microorganisms can not recognize the protonated form of the acyl donor.[7] In contrast with thermodynamic control, kinetical control is a more interesting strategy used in the enzymatic synthesis, and non-equilibrium concentration of the product can be reached by activation of the acyl donor to an amide, ester or anhydride.[13–16]

Cefaclor, i.e. 3-chloro-7-D-(2-phenylglycinamide)-3-cephem-4-carboxylic acid, a semi-synthetic β-lactam antibiotics with a broad spectrum of antibiotic activity mainly with properties suitable for oral treatment, has been synthesized enzymatically by using PA.[17] As shown in scheme 1, cefaclor was the product of the condensation of 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid (7ACCA) and phenylglycine methyl ester (PGME). In the course of cefaclor synthesis, cefaclor hydrolysis and PGME hydrolysis take place simultaneously. The hydrolysis of cefaclor and PGME results in a low synthetic yield and the trouble practice in the downstream process. To protect cefaclor from hydrolysis, *in situ* product removal (ISPR) can be considered of great interest, because cefaclor can react with naphthol to form insoluble clathrate-type complexes.[18,19]

In the previous study, we have investigated the effect of ISPR[20] and temperature[21] on the synthesis

*Corresponding author: Tel.: + 86-21-64252981; Fax: 86-21-64250068;
E-mail address: dzhwei@ecust.edu.cn

of cefaclor, and the conversion of 7ACCA was improved to 86 % at 5 °C. But the productivity and the yield still need to be improved. In this work, we try to synthesize cefaclor at moderate temperatures, and the effects of substrate concentrations were studied to achieve high conversion of 7ACCA and PGME.

Materials and methods

Enzyme

The soluble PA from *Bacillus megaterium*, provided by the Shanghai Institute of Biochemistry of the Chinese Academy of Science, was immobilized on epoxyacrylic resin as described previously.[20] One unit of PA was defined as the amount of enzyme required to produce $A = 1$ μmol min^{-1} (0.017 μkat) in $w = 4$ % solution of penicillin G at pH 7.8 and 37 °C. The enzyme activity was determined spectrophotometrically using *p*-dimethylaminobenzaldehyde as the substrate.

Chemicals

Penicillin G, D-(-)-phenylglycine (PG) and D-(–)phenylglycine methyl ester (PGME) were purchased from the Shanghai Industrial Chemical Co. Ltd., China. Cefaclor and 7ACCA were donated by Tiantai Pharma Co., Zhejiang, China. Epoxyacrylic resin was supplied by the Institute of Catalysis of East China University of Science and Technology. All other reagents were of analytical grade.

Methods

Cefaclor enzymatic synthesis

Syntheses of cefaclor were performed in two stirred bioreactors with jackets for water circulation. The total reaction volume was 100 ml and the volume flow rate of mixture between two reactors is $Q = 10$ ml min^{-1}. The enzyme load and the stirring speed used in this work ensured the absence of the external diffusional effects. These conditions were investigated in the previous study.[17] The initial rate of synthesis and hydrolysis of PA (Γ_s/Γ_h) in the process was determined by HPLC. Enzymatic synthesis of cefaclor with ISPR was performed as described by *Yang* et al.[17] with slight modification demonstrated in Fig. 2. In the PGME feeding process, PGME solution (1.13 mol l^{-1}) was fed with the speed of 2.5 ml per hour by pumping with a peristaltic pump from a cooler reservoir to the enzyme reactor.

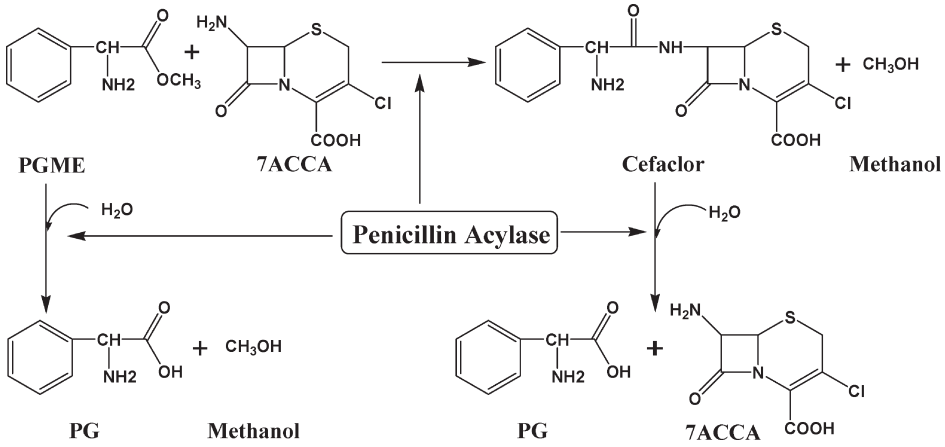
Analysis Methods

Each reactant was identified and analyzed by HPLC (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 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 $\varphi = 85$ % phosphate sodium buffer (25 mmol l^{-1}, pH 6.5) and $\varphi = 15$ % acetonitrile at $Q = 1$ ml min^{-1}, and monitored at 254 nm (7ACCA and cefaclor) and 214 nm (PGME and PG).

Results and discussion

Effects of substrate concentration on the synthesis of cefaclor with ISPR

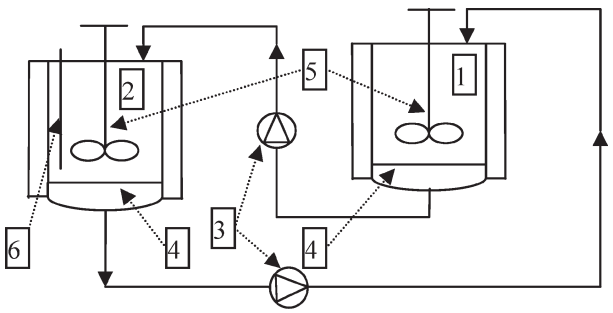
There was about 80 % residual enzyme activity in the presence of 1-naphthol, however after extensive washing more than 95 % activity was regained, this was similar to the reports of *Schroen* at al.[22] The concentration of 7ACCA varied from 25 mmol l^{-1} to 100 mmol l^{-1} and the results are shown in Fig. 1. The yield of cefaclor increased as the in-



F i g . 1 – *The general scheme for penicillin acylase catalyzed synthesis of cefaclor*

crease of the concentration of PGME when the ratio of the concentrations of 7ACCA to PGME was 1 : 2. It is similar to that obtained in the synthesis of cephalexin; *Schroen* et al.[23] reported that high initial acyl donor concentrations are required for a high yield on nucleus in the enzymatic synthesis of cephalexin. The reasons may be that the high concentration of nucleophile showed an inhibititory effect and reduced the rate of antibiotic synthesis, similarly to findings reported by *Ribeiro* et al for the ampicillin synthesis.[24] Since the concentration of 7ACCA above the 50 mmol l^{-1} does not lead to the ideal yield of cefaclor, it appears that the more of 7ACCA is not involved in the reaction at fixed enzyme loading.

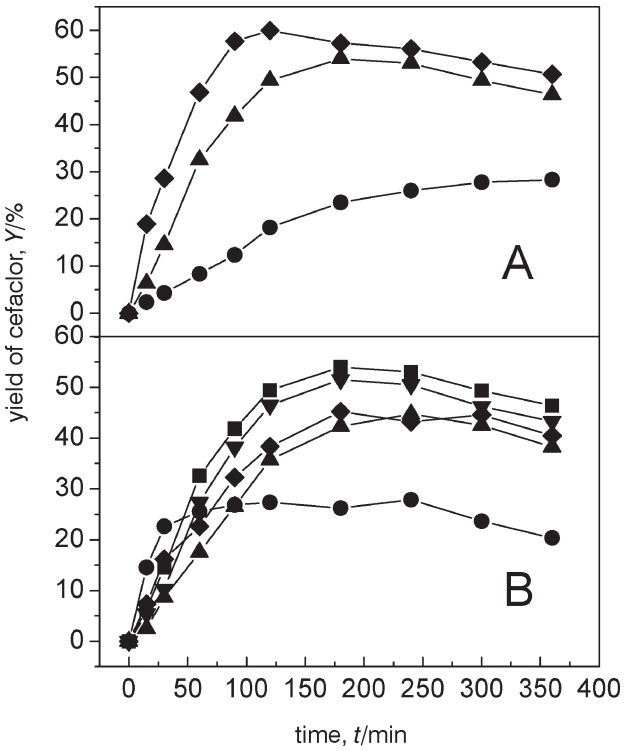
When the concentration of 7ACCA was 50 mmol l^{-1}, the yield of cefaclor increased from 34 % to 53 % with increasing the 7ACCA-PGME mole ratio from 1 : 1 to 1 : 2, but the cefaclor yield increased only 5 % when the mole ratio increased to 1 : 3. Therefore, it can be concluded that high PGME concentration improved the high conversion of 7ACCA. According to the synthesis mechanism of PA,[25] the enzymes bind PGME to form acyl-enzyme intermediate, but the excess of acyl-enzyme complex is attacked by water and gives the unwanted PG, the hydrolysis product of PGME. Thus the large excess of acyl donor led only to a slight increase in the yield of cefaclor. In order to get the optimum substrate concentrations, the effect of concentration on Γ_s/Γ_h was investigated (shown in Fig. 4). When the ratio of 7ACCA to PGME was 1 : 2, the maximum Γ_s/Γ_h value was 2.58 while the concentration of 7ACCA was 50 mmol l^{-1}. When the concentration of 7ACCA was increased up to 50 mmol l^{-1}, the Γ_s/Γ_h began to drop down. Because the ratio of 7ACCA to PGME was fixed at 1 : 2, the mount of PGME was always two times to 7ACCA. Therefore, the hydrolytic speed of PGME increased more quickly than the synthetic rate of cefaclor resulted in the decrease of Γ_s/Γ_h. Thus the 7ACCA concentration was 50 mmol l^{-1} in the following experiments.



F i g . 2 – *Diagram of PA catalyzed synthesis of cefaclor with in situ product removal: 1. Complex reactor, 2. Enzyme reactor, 3. Peristaltic pump, 4. Sintered-glass, 5. Mechanical stirrer, 6 pH controller*

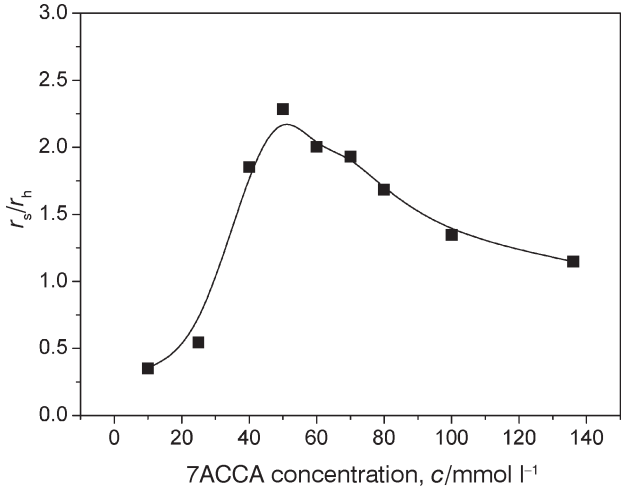
Effects of pH and temperature on the synthesis of cefaclor with ISPR

The reactions catalyzed by PA are reversible in the synthesis of cefaclor. As shown in Fig. 5, the maximum yield of cefaclor was obtained at pH 6.4. The pH of the reaction medium influences the dissociation of the PGME amino group and the hydrolysis of cefaclor. The hydrolysis of product is not serious like the situation without ISPR. Consideration of cefaclor is unstable at pH levels above 6.5 and the solubility of 7ACCA is very low at pH values below 6.5, the optimum pH range was from 6.3 to 6.4. In this pH range, the substrate solution actually was a supersaturation of 7ACCA.[4]

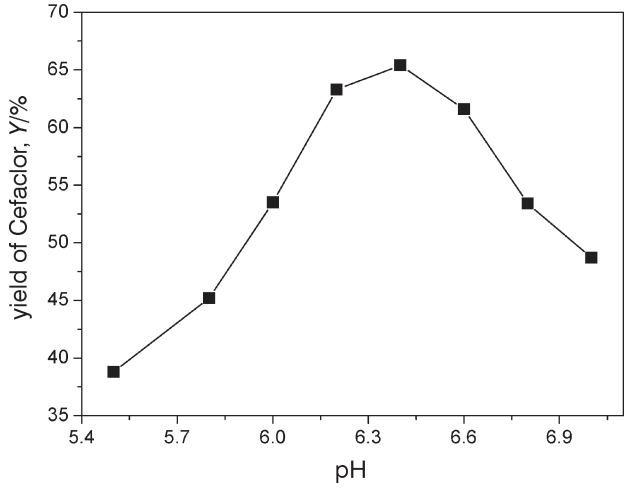


F i g . 3 – *Effects of substrate concentration on the synthesis of cefaclor. Figure A: Dependence of the yield on the different ratio of substrates when the concentration of 7ACCA was 50 mmol l^{-1}, circle 1 : 1, uptriangle 1 : 2, square 1 : 3. Figure B: Dependence of the yield on the concentration of 7ACCA when the ratio of 7ACCA to PGME was fixed at 1 : 2, 7ACCA concentration: circle 25 mmol l^{-1}, diamond 40 mmol l^{-1}, square 50 mmol l^{-1}, uptriangle 75 mmol l^{-1}, downtriangle 100 mmol l^{-1}. Other reaction conditions: pH 6.3, 20 °C, 7.5 mmol naphthol was added to complex reactor and enzyme load was 8 IU ml^{-1}.*

The maximum conversion of 7ACCA with ISPR at different temperatures are presented in Fig. 6. At 5 °C the conversion was 86 %, but the conversion at 30 °C was only 54 %. Therefore, one can conclude that low temperature is favorable to get higher yield of cefaclor. With temperature decrease, the rate of hydrolysis of cefaclor and PGME de-



F i g . 4 – *The effect of the substrate concentration on the* Γ_s/Γ_h. *Reaction conditions: pH 6.3, 20 °C, enzyme load was 8 IU ml^{-1} and the ratio of 7ACCA to PGME is 1 : 2.*



F i g . 5 – *Effects of pH on the synthesis of cefaclor: Reaction conditions: 50 mmol l^{-1} 7ACCA, 100 mmol l^{-1} PGME, 7.5 mmol naphthol was added to complex reactor, 20 °C and enzyme load was 8 IU ml^{-1}.*

creased.Since the concentration of cefaclor in the mixed system also decreased,[21] the conversion of 7ACCA evidently improved. However, the initial synthetic rate was improved 8 times when the reaction temperature was changed from 5 °C to 30 °C, and at 5 °C the productivity was only 5.4 mmol l^{-1} h^{-1}, whereas at 30 °C it was 13.5 mmol l^{-1} h^{-1}. The low productivity at low temperature prevents the enzymatic processes for reaching a commercial acceptance. So the application of higher temperature appears to be necessary to achieve higher productivity in the cefaclor synthesis. At optimal conditions, the conversion of 7ACCA was improved from 69 % to 86 % by using ISPR (data was not shown).

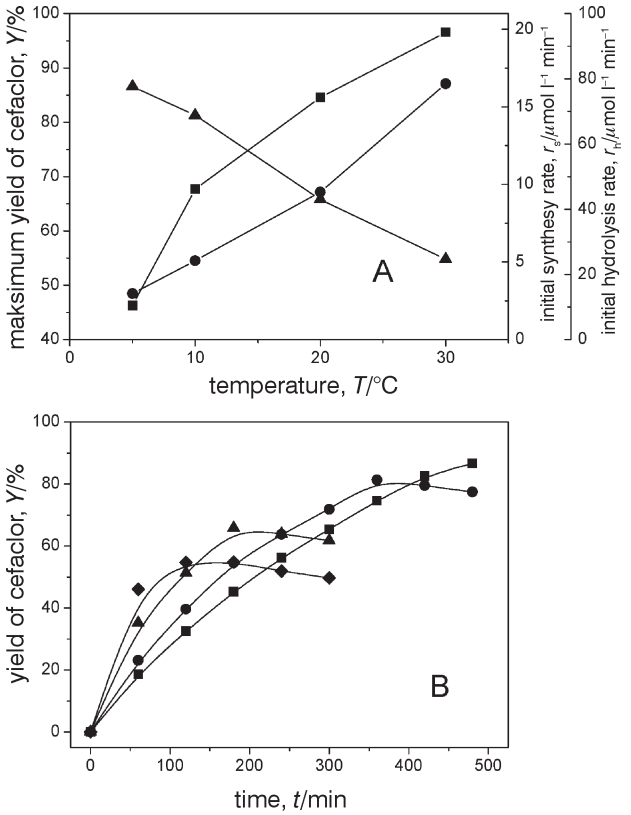
Effects of feeding with PGME on the conversion of 7ACCA with ISPR

In the process of the synthesis of semi-synthetic antibiotics, the conversion of antibiotics nucleus was estimated as primary index because it is more expensive than acyl donor. Therefore, an excess of acyl donor was used in the enzymatic synthesis of semi-synthetic antibiotics. Marladkar, for example, used ten-fold molar excesses of PGME in the synthesis of cephalexin,[26] *Terreni* et al. used three-fold molar excesses of ester in the acylation of 7-aminocephalosprinanic acid (7ACA).[27] However, the yields are still below 90 %, because of the serious hydrolysis of acyl donor at high concentration. In this study, feeding of PGME was used to alleviate the hydrolysis. We found that hydrolysis of PGME could be accelerated at high concentrations of PGME.[21] The results in Fig. 7 showed that the conversion of 7ACCA was improved to 93 %, whereas the conversion of PGME was improved to 62 %. Evidently, the conversion of 7ACCA and the PGME hydrolysis depend not only on the reaction temperature but also on the ratio of 7ACCA to PGME. In addition to the productivity improvement to 7.7 mmol l^{-1} h^{-1}, the reaction time can be shortened by raising the ratio of enzyme to substrate.

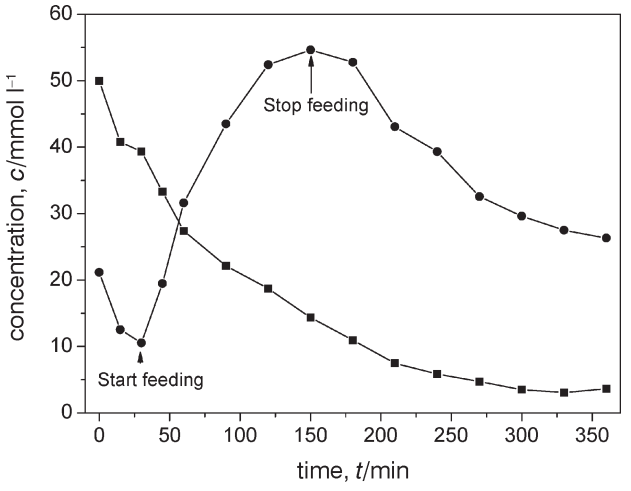
Marcelo et al.[24] put the hypothesis that nucleophile can position the active site of the enzyme. The results in Fig. 7 support conclusions of *Marcelo* et al: the initial high concentration of nucleus induced the high conversion of the 7ACCA when feeding with PGME was applied. Actually, the results demonstrate that the optimization of the reaction conditions and the feeding with acyl donor improve the conversion of substrates to products. *Youshko* et al attribute this to the improving of Γ_S/Γ_H during the process.

Conclusions

The process of the synthesis of 3-chloro-7--D-(2-phenylglycinamide)-3-cephem-4-carboxylic acid (cefaclor) was described by pointing out conditions where PGME hydrolysis was minimized by feeding the reactive PGME and mixture with removing cefaclor *in situ* by complexion with naphthol. Controlling of the initial concentrations of 7ACCA and PGME to 50 mmol l^{-1} and 25 mmol l^{-1}, and a start of PGME feeding 30 min after the beginning of the reaction caused the conversion of 7ACCA and PGME increase to 93 % and 62 %, respectively. The results supported the application of enzymatic synthesis of β-lactam antibiotics as alternative to conventional chemical ones.



F i g . 6 – *Effects of temperature on the synthesis of cefaclor with in ISPR. A: The dependence of maximal yields, maximal rates of cefaclor synthesis and PA activity on the function of absolute temperature. Uptriangle maximum yields of cefaclor, square initial synthetic rate of cefaclor, circle initial hydrolysis rate of PGME. B: Time course of enzymatic synthesis of cefaclor at different temperatures. Square 5 °C, circle 10 °C, uptriangle 20 °C, diamond 30 °C. Reaction conditions: 7ACCA 50 mmol l^{-1}, PGME 100 mmol l^{-1}, 7.5 mmol naphthol was added to complex reactor, pH 6.3 and enzyme load was 8 IU ml^{-1}.*



F i g . 7 – *The time course of synthesis of cefalcor with feeding PGME and ISPR. Square 7ACCA concentration, circle PGME concentration. Reaction conditions: 50 mmol l^{-1} 7ACCA, 25 mmol l^{-1} PGME, 7.5 mmol PGME was feeding at 30 min with peristaltic pump in 2 h, enzyme load was 8 IU ml^{-1}, 7.5 mmol naphthol was added to complex reactor.*

ACKNOWLEDGEMENTS

This work was supported by the Key Disciplinary Foundation of Shanghai

List of abbreviations and symbols

7ACCA – 7-aminodesacetoxymethyl-3-chlorocephalosporanic acid

PGME – phenlglycine methyl ester

Cefaclor – 3-chloro-7-d-(2-phenylglycinamide)-3-cephem-4-carboxylic acid

PG – phenylglycine

ISPR – *in situ* product removal

A – enzyme activity, μkat

c – concentration, mmol l^{-1}

F – mol flow rate, μmol min^{-1}

P – productivity, mmol l^{-1} h^{-1}

Γ_s – rate of synthesis, mmol l^{-1} h^{-1}

Γ_h – rate of hydrolysis, mmol l^{-1} h^{-1}

Q – volume flow rate, ml min^{-1}

t – time, min h

T – temperature, °C

w – mass fraction, %

Y – yield, %

φ – volume fraction

References

1. *Cesar, M., Olga, A., Valeria, G., Gloria, F.-L., Jose, M. P., Manuel, F., Rosa, L. S., Tamara, M., Fernando, L.-G., Lorena, W., Rodrigo, T., Jose, M. G., Rober, F.-L.,* Medicinal Chemistry Reviews-Online. **2** (2005) 207.
2. *Margreth A. Wegman, M. H. A. J., Fred van Rantwijk, Roger A. Sheldon*., Adv. Synth. Catal. **343** (2001) 559.
3. *Duggleby, H. J., Tolley, S. P., Hill, C. P., Dodson, E. J., Dodson, G., Moody, P. C. E.,* Nature. **373** (1995) 264.
4. *Cole, M.,* Biochem. J. **115** (1969) 747.
5. *Giordano, R. C., Ribeiro, M. P., Giordano, R. L.,* Biotechnol. Adv. **24** (2006) 27.
6. *Fernandez-Lafuente, R., Rosell, C. M., Guisan, J. M.,* Enzyme Microb. Technol. **23** (1998) 305.
7. *Schroen, C.G., Nierstrasz, V. A., Kroon, P. J., Bosma, R., Janssen, A. E. M., Beeftink, H. H., Tramper, J.,* Enzyme Microb. Technol. **24** (1999) 498.
8. *Kim, M. G., Lee, S. B.,* J. Mol. Catal. B: Enzym. **1** (1996) 201.
9. *Nierstrasz, V. A., Schroen, C. G. P. H., Bosma, R., Kroon, P. J., Beeftink, H. H., Janssen, A. E. M., Tramper, J.,* Biocatal. Biotransform. **17** (1999) 209.
10. *Diender, M. B., Straathof, A. J. J., Van Der Wielen, L. A. M., Ras, C., Heijnen, J. J.,* J. Mol. Catal. B: Enzym. **5** (1998) 249.
11. *Fernandez-Lafuente, R., Rosell, C. M., Piatkowska, B., Guisan, J. M.,* Enzyme Microb. Technol. **19** (1996) 9.
12. *Svedas, V. K., Margolin, A. L., Berezin, I. V.,* Enzyme Microb. Technol. **2** (1980) 138.
13. *Youshko, M. I., Moody, H. M., Bukhanov, A. L., Boosten, W. H. J., Svedas, V. K.,* Biotechnol. Bioeng. **85** (2004) 323.

14. *Cao, X. J., Zhu, J. H., Wei, D. Z., Hur, B. K.,* J. Microbiol. Biotechnol. **14** (2004) 62.
15. *Fuganti, C., Rosell, C. M., Servi, S., Tagliani, A., Terreni, M.,* Tetrahedron: Asymmetry. **3** (1992) 383.
16. *Shaw, S.-Y., Shyu, J.-C., Hsieh, Y.-W., Yeh, H.-J.,* Enzyme Microb. Technol. **26** (2000) 142.
17. *Cheng, T., Chen, M., Zheng, H., Wang, J., Yang, S., Jiang, W.*, Protein Expr Purif. **46** (2006) 107.
18. *Kemperman, G. J., de Gelder, R., Dommerholt, F. J., Raemakers-Franken, P. C., Klunder, A. J. H., Zwanenburg, B.* Chem.-Eur. J. **5** (1999) 2163.
19. *Yang, Y., Wei, D. Z.,* Biotechnol. Lett. **25** (2003) 1195.
20. *Yang, L., Wei, D. Z., Zhang, Y. W.,* J. Chem. Technol. Biotechnol. **79** (2004) 480.
21. *Wei, D. Z., Yang, L., Song, Q. X.,* J. Mol. Catal. B: Enzym. **26** (2003) 99.
22. *Schroen, C. G., Nierstrasz, V. A., Bosma, R., Kemperman, G. J., Strubel, M., Ooijkaas, L. P., Beeftink, H. H., Tramper, J.,* Enzyme Microb. Technol. **31** (2002) 264.
23. *Schroen, C. G., Nierstrasz, V. A., Moody, H. M., Hoogschagen, M. J., Kroon, P. J., Bosma, R., Beeftink, H. H., Janssen, A. E. M., Tramper, J.,* Biotechnol. Bioeng. **73** (2001) 171.
24. *Ribeiro, M. P. A., Ferreira, A. L. O., Giordano, R. L. C., Giordano, R. C.,* J. Mol. Catal. B: Enzym. **33** (2005) 81.
25. *Youshko, M. I., Svedas, V. K.,* Biochem.-Moscow. **65** (2000) 1367.
26. *Maladkar, N. K.,* Enzyme Microb. Technol. **16** (1994) 715.
27. *Terreni, M., Ubiali, D., Pagani, G., Hernandez-Justiz, O., Fernandez-Lafuente, R., Guisan, J. M.,* Enzyme Microb. Tech. **36** (2005) 672.