Microbial Conversion with Cofactor Regeneration using Genetically Engineered Bacteria

Tetsuo Endo,* Satoshi Koizumi

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., 3-6-6, Asahi-machi, Machida-shi, Tokyo 194–8533, Japan
Fax: (+81) 427–26–8330, e-mail: endo.tetsuo@kyowa.co.jp

Received May 31, 2001; Accepted July 6, 2001

Abstract: In the synthesis of fine chemicals, biotransformations have been recognized as useful methods and applied for large-scale manufacturing. Especially, the recent development of recombinant DNA technology has greatly expanded the whole microbial cell processes for manufacturing fine chemicals. The whole-cell approaches have been applied not only to single conversion process but also to biotransformations requiring cofactor regeneration, such as reductions with NADH or NADPH and phosphorylations with ATP. The whole microbial cell conversion process with cofactor regeneration enables the production of complicated compounds, such as sugar nucleotides and oligosaccharides, on an industrial scale.

Keywords: biotransformation; cofactor regeneration; genetic engineering

Abbreviations: Gal: galactose; Glc: glucose; GlcNAc: *N*-acetylglucosamine; LacNAc: *N*-acetyllactosamine; Man: mannose; NeuAc: *N*-acetylneuraminic acid; NTP: nucleoside 5'-triphosphate; ORT: orotic acid; PEP: phosphoenol pyruvate; Pyr: pyruvate

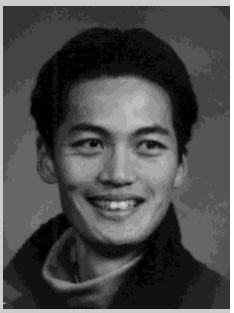
1 Introduction

In the synthesis of fine chemicals, biotransformations have been recognized as useful methods and applied for large-scale manufacturing during the past century, because enzymes are excellent catalysts with rigid regio- and stereospecificities under mild conditions. Many enzymes are used for the syntheses of various compounds, and some of them are immobilized and used repeatedly without decreasing the activities.[1] As isolations of enzymes are laborious procedures, whole-cell reactions without isolating enzymes have been developed since the 1950's.[2] Baker's yeast is the most popular microorganism for biocatalysis.[3] Other microorganisms such as *Rhodobacter sphaeroides*,[4] *Rhodococcus rhodocurous*,[5] *Fusarium oxysporum*,[6] *Psudomonas putida*[7] were also utilized. However, bioconversions using whole cells as 'bags of enzymes' have some drawbacks, 1) weakness of the enzymatic activities, 2) permeability of the substrate or the product, 3) inhibition of the cell growth by the substrate or the product, 4) degradation of the substrate or product by cellular activity, 5) difficulties of isolation and purification from the reaction mixture. Recently, many approaches for the whole-cell conversion have been investigated, and some of them are applied to industrial production.[8]

Among the enzymatic conversions, there are the reactions requiring cofactors such as reductions with NADH or NADPH and phosphorylations with ATP. For the efficient utilization of the cofactors, various cofactor regeneration systems have been developed.[1,9,10,11,12] The most popular regeneration of NADH was carried out by dehydrogenases, and these include the use of baker's yeast (*Saccharomyces cerevisiae*) as a catalyst. Although *S. cerevisiae* has been widely used for the supply of reducing power, it is rather difficult to freely combine it with the desired reactions. Recent genetic engineering technologies have made it possible to construct a strain with the capability for cofactor regeneration by introducing heterologous genes.[13]

In this review, we focus on bioconversion processes with cofactor regeneration, especially those using whole-cells of genetically engineered bacteria.

  1615-4150/01/34306–7-521–526 $ 17.50-.50/0



Tetsuo Endo received his B.Agr and M.Agr. in Agricultural Biological Chemistry from the University of Tokyo in 1993 and 1995, respectively. He joined Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd. in 1995 as a researcher. His research has focused on the large-scale production of sugar nucleotides and oligosaccharides, and he received his Ph.D. from the University of Tokyo in 2000.

Satoshi Koizumi received his B.Agr. and M.Agr. in agricultural biological chemistry from the University of Tokyo in 1984 and 1986, respectively. Since 1986 he has been working on the microbial production of vitamins, nucleotides, and oligosaccharides for Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd. He got his Ph.D in agriculture and life sciences at the University of Tokyo. He is currently the head of the section of applied microbiology.

2 NAD(P)H Regeneration

There are many enzymatic reactions including hydrolysis (ester, amide, epoxide, nitrile), esterification, reduction, oxidation, carbon-carbon bond formation, and so on.[3] Reactions catalyzed by oxido-reductases requiring a nicotinamide coenzyme NAD(P)H have been used in various fields, and to circumvent the high cost and instability of the coenzyme, the recycling of NAD(P)H has been intensively investigated and well developed. Formate dehydrogenase, as the NADH regeneration system, has been used for the production of (*R*)-2-pentanol,[14] deoxyfructose 6-phosphate,[15] alcohols,[16,17] D-amino acids,[18] and L-amino acids.[19,20,21] To retain the cofactor in the reaction mixture, a representative method was developed using polymer-bound NADH, which had two advantages, the increase in molecular size and the improvement of stability.[13,22] Sorbitol[23] and xylitol[24] were produced by using negatively charged membranes, which were able to retain NAD(P)H in the reaction mixture. An alternative approach such as microcapsules was also investigated.[25]

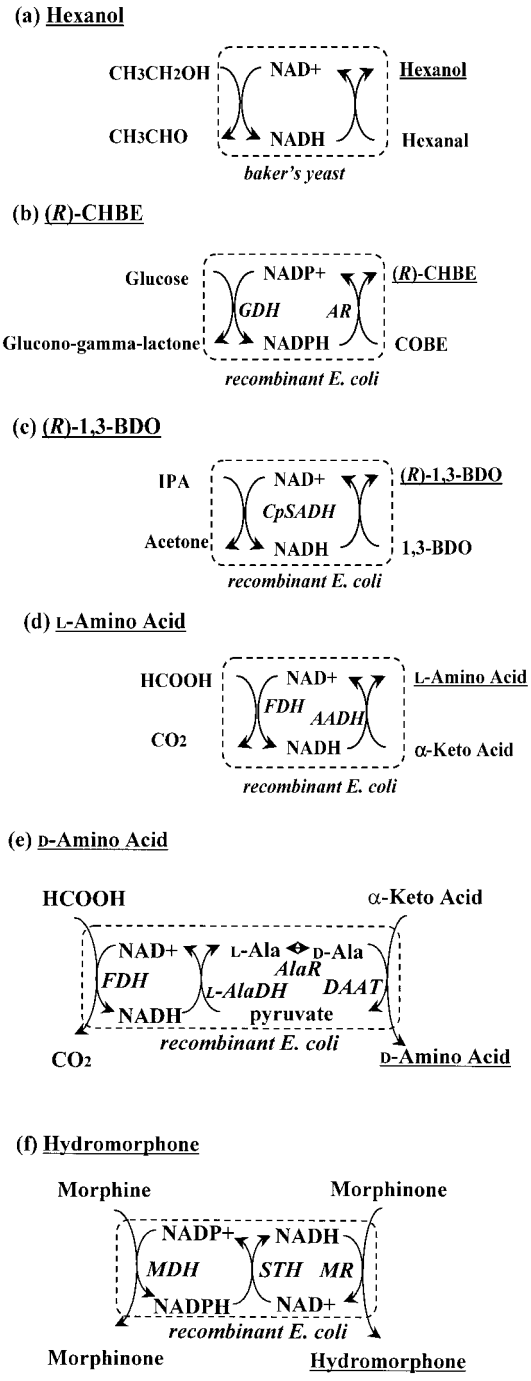


Figure 1. Microbial conversions with cofactor regeneration. (**a**) Hexanol production with baker's yeast. (**b**) (*R*)-CHBE production with recombinant *E. coli*; GDH: glucose dehydrogenase, AR: aldehyde reductase. (**c**) (*R*)-1,3-Butanediol production with recombinant *E. coli*; CpSADH: secondary alcohol dehydrogenase. (**d**) L-Amino acid production with recombinant *E. coli*; FDH: formate dehydrogenase, AADH: L-amino acid dehydrogenase. (**e**) D-Amino acid production with recombinant *E. coli*; L-AlaDH: L-alanine dehydrogenase, AlaR: alanine racemase, DAAT: D-amino acid aminotransferase. (**f**) Hydromorphone production with recombinant *E. coli*; MDH: morphine dehydrogenase, STH: soluble pyridine nucleotide transhydrogenase, MR: morphinone reductase.

The whole cells of baker's yeast, *Saccharomyces cerevisiae*, have been used as a cofactor regeneration system,[13] and it was demonstrated that even in the solid/gas system, acetaldehyde and hexanol were produced in the gas phase (Figure 1, reaction **a**).[26]

Recently, *Escherichia coli* cells expressing the heterologous NAD^+-dependent dehydrogenase were constructed and used for biotransformations with NAD(P)H regeneration. Ethyl (*R*)-4-chloro-3-hydroxybutanoate [(*R*)-CHBE] is a promising chiral building block for the chemical synthesis of various useful compounds. The asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) to (*R*)-CHBE was achieved by using recombinant *E. coli* overexpressing the aldehyde reductase gene from *Sporobolomyces salmonicolor* and the glucose dehydrogenase (GDH) gene from *Bacillus megaterium* as a catalyst (Figure 1, reaction **b**).[27] (*R*)-CHBE was formed at 268 g/L (94.1% yield; 91.7% ee) in an organic solvent-water two-phase system. To produce (*R*)-1,3-butanediol, which is also an important chiral synthon for the synthesis of various optically active compounds, the (*S*)-1,3-BDO dehydrogenase gene (CpSADH) was cloned from *Candida parapsilosis* and overexpressed in *E. coli* (Figure 1, reaction **c**).[28] (*R*)-1,3-BDO was produced at an amount of 47.4 g/L (94.7% yield; 93.5% ee). This recombinant *E. coli* expressing CpSADH also produced ethyl (*R*)-4-chloro-3-hydroxybutanoate from ethyl 4-chloro-3-oxobutanoate with isopropanol (36.6 g/L, 99% ee).

The synthesis of optically active amino acids has been extensively studied. L-Amino acids (L-leucine, L-valine, L-methionine, L-alanine, L-phenylalanine, L-tyrosine, etc.) were produced from α-keto acids with *E. coli* cells overexpressing heterologous genes of thermostable L-amino acid dehydrogenases (AADH), such as leucine dehydrogenase (LeuDH) from *Bacillus sphaericus*, alanine dehydrogenase (AlaDH) from *Bacillus stearothermophilus*, and formate dehydrogenase (FDH) from *Candida boidinii* with only an intracellular pool of NAD^+ for the regeneration of NADH (Figure 1, reaction **d**).[29] D-Amino acids (D-glutamate, D-leucine, D-methionine, D-valine, etc.) were also produced from α-keto acids with *E. coli* cells overexpressing thermostable heterologous genes of D-amino acid aminotransferase (DAAT) such as alanine racemase (AlaR), L-alanine dehydrogenase, and FDH (Figure 1, reaction **e**).[29] The soluble pyridine nucleotide transhydrogenase (STH) of *Pseudomonas fluorescenes* was overexpressed in *E. coli* and applied for regeneration of both NADH and NADP in the production of the important semisynthetic opiate drug hydromorphone (Figure 1, reaction **f**).[30]

3 ATP Regeneration

The development of an efficient and economical ATP regeneration system is also indispensable for the synthesis of many bioactive compounds. Several ATP regeneration systems using isolated enzymes or whole cells have been published as shown in Figure 2. The reaction catalyzed by acetate kinase requires acetyl phosphate as the phosphorylating agent, which is very easily prepared and stable in solution (Figure 2, reaction **a**).[1,31] Pyruvate kinase, a key enzyme of the glycolytic pathway of bacteria, catalyzes pyruvate release from phosphoenol pyruvate (PEP), along with its major synthetic product ATP (Figure 2, reaction **b**).[32] Polyphosphate kinase catalyzes the phosphorylation of not only of ATP but also other nucleoside diphosphates (NDPs) using polyphosphate [$(P)_n$] as a phosphoryl donor, yielding nucleoside triphosphates (NTPs) (Figure 2, reaction **c**).[33]

On the other hand, a whole-cell ATP regeneration process has been reported by using yeast[34] and *Corynebacterium ammoniagenes* (Figure 2, reactions **d** and **e**).[35] In yeast, several treatments (freeze-drying, addition of solvent of surfactant, etc.) are required to permeabilize the cells. The yeast system was well studied and many high-energy compounds, such as ATP (GTP, CTP, UTP), deoxy-ATP (dGTP, dCTP, dTTP), UDP-Glc, UDP-Gal, UDP-GlcNAc, GDP-Man, CDP-choline, glucosamine 6-phosphate, were successfully produced using glucose as an energy source.[34]

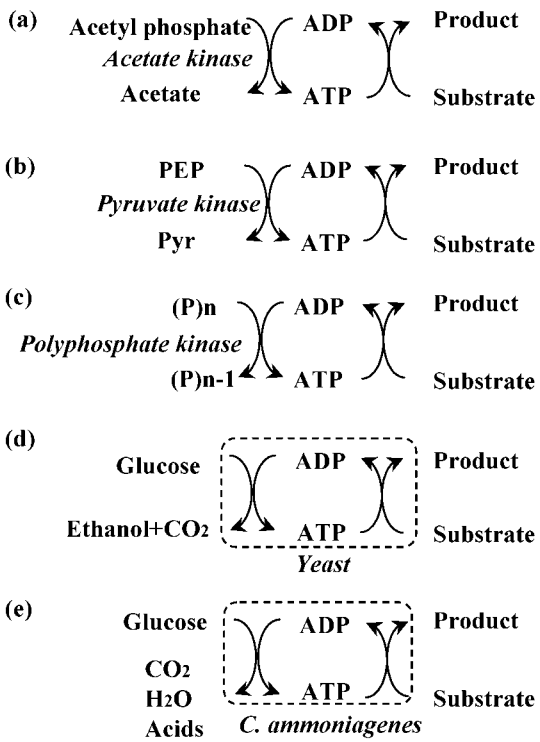


Figure 2. ATP regeneration processes.

Another system using *C. ammoniagenes* has been also studied and applied for large-scale production. Some examples of the production system with *C. ammoniagenes* and recombinant *E. coli* are shown in Figure 3. Reaction **a** of Figure 3 is the basic ATP production scheme with *C. ammoniagenes*.[35] In this reaction, adenine is a precursor of AMP and glucose is used both as a starting material for phosphoribosyl pyrophosphate (PRPP) and an energy source. A solvent and surfactant are added for permeabilizing the cells. ATP formation lasted for 13 h and 37 g/L of ATP were produced (yield of adenine to ATP was approximately 82%).[35]. Reactions **b – e** in Figure 3 show examples of the reactions with the combination of *C. ammoniagene* cells and recombinant *E. coli* cells. In these examples, *C. ammoniagene*s cells contribute to ATP regeneration, and recombinant *E. coli* cells contribute to the catalysis. GMP synthetase, encoded by *guaA* in *E. coli*, catalyzes the glutamine- or NH_3-dependent synthesis of 5'-guanylic acid (GMP) and it requires ATP as a cofactor. GMP was produced by the coupling of *C. ammoniagenes* cells and recombinant *E. coli* cells overexpressing the *guaA* gene. Both cells are treated with organic solvent and surfactant to permeabilize the cells. After 23 h, 70 g/L (85% yield) of GMP were produced from 5'-xanthylic acid (XMP) (Figure 3, reaction **b**).[36] When the gene for inosine kinase instead of GMP synthetase was expressed in *E. coli*, IMP was produced from inosine and glucose (Figure 3, reaction **c**).[37]

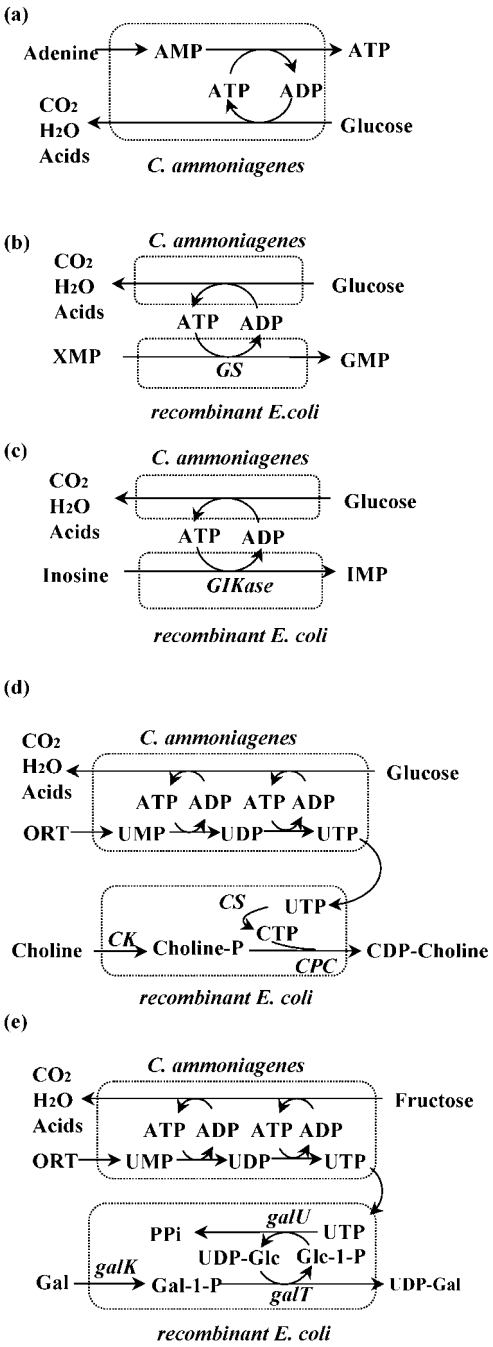


Figure 3. Whole-cell processes with ATP regeneration. (**a**) ATP production from adenine by *C. ammoniagenes* cells. (**b**) GMP production from XMP; GS: GMP synthetase. (**c**) IMP production from inosine; GIKase: inosine kinase. (**d**) CDP-choline production from orotic acid and choline; CS: CTP synthetase, CK: choline kinase, CPC: choline phosphate cytidylyltransferase. (**e**) UDP-Gal production from orotic acid and galactose; galK: galactokinase, galU: glucose 1-phosphate uridyltransferase, galT: galactose 1-phosphate uridyltransferase.

Cytidine diphosphate choline (CDP-choline) is an important intermediate in the biosynthesis of phospholipids such as lecithin, and is used as a drug for brain injuries. Through the combination of *C. ammoniagenes* and recombinant *E. coli* overexpressing CDP-choline biosynthetic genes (CTP synthetase, choline kinase, and choline phosphate cytidylyltransferase), CDP-choline was efficiently produced from orotic acid, choline, and glucose (11 g/L, 23 h) (Figure 3, reaction **d**).[38] In the same manner, UDP-Gal, which is the substrate of galactosyltransferases, was efficiently produced from galactose and orotic acid by using *C. ammoniagenes* and recombinant *E. coli* overexpressing UDP-Gal biosynthetic genes (Figure 3, reaction **e**). In this case, 44 g/L of UDP-Gal accumulated after a 21 h reaction.[39] Similarly, 17 g/L of CMP-NeuAc were produced after a 27 h reaction starting with orotic acid and NeuAc through the coupling of recombinant *E. coli* cells overexpressing the genes of CMP-NeuAc synthetase and CTP synthetase, and *C. ammoniagenes*.[40] 18 g/L of GDP-Fuc were produced after a 22 h reaction starting with GMP and mannose through the coupling of recombinant *E. coli* cells overexpressing the GDP-Fuc biosynthetic genes.[41]

4 NTP Regeneration System

Recently, applications of oligosaccharides as pharmaceuticals in the fields of the prevention of infections by pathogens or viruses, neutralization of toxins, and

immunotherapy for cancer have been widely recognized.[42] Therefore, numerous methods for oligosaccharide synthesis have been developed.[43] Among these methods, glycosyltransferases are recognized as good tools for oligosaccharides synthesis because of their rigid regio- and stereospecificities. The glycosyltransferase requires a sugar nucleotide as substrate, and an excellent sugar nucleotide recycling system with several isolated enzymes, which is promoted by an ATP regeneration system, has been reported.[44] A similar concept was also applied for the production of 3'-phosphoadenosine 5'-phosphosulfate (PAPS).[45]

Recently, many approaches have been conducted to produce oligosaccharides through whole-cell processes.[46] Especially, by the combination of recombinant *E. coli* and *C. ammoniagenes*, a system for oligosaccharides production that could be applied to the industrial manufacture was successfully constructed. By coupling recombinant *E. coli* overexpressing the α1→4 galactosyltransferase gene from *Neisseria gonorrhoeae* and the UDP-Gal production system, globotriose (Galα1→4Galβ1→4Glc) accumulated to 188 g/L for 36 h from orotic acid, galactose and lactose (Figure 4, reaction **a**).[39] Globotriose was produced seven times more efficiently than UDP-Gal and it was supposed that UDP-Gal was recycled within the system via UTP and UDP. In the same manner, by the combination of CMP-NeuAc production system and *E. coli* overexpressing the α2→3 sialyltransferase of *N. gonorrhoeae*, 33 g/L of 3'-sialyllactose (NeuAcα2→3Galβ1→4Glc) were produced after 11 h starting with orotic acid, NeuAc, and lactose (Figure 4, reaction **b**).[40] Through coupling of the GDP-Fuc production system and *E. coli* overexpressing the α1→3 fucosyltransferase of *Helicobacter pylori*, 21 g/L of Lewis X [Galβ1→4(Fucα1→3)GlcNAc] were produced after 11 h starting with GMP, mannose, and *N*-acetyllactosamine (Figure 4, reaction **c**).[41]

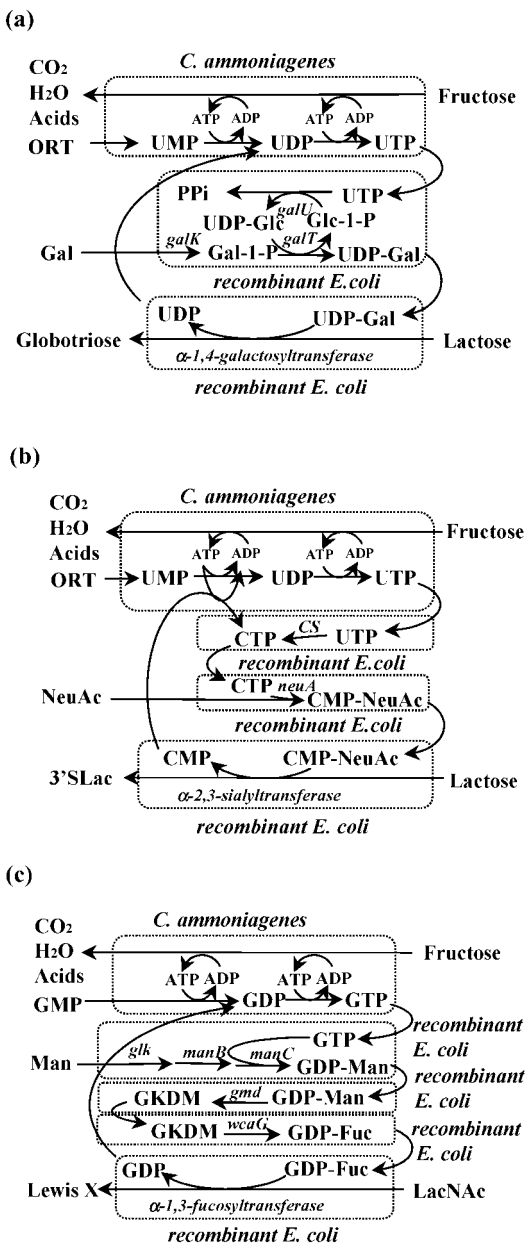


Figure 4. Whole-cell processes with NTP regeneration. (**a**) Globotriose (Galα1→4Galβ1→4Glc) production from orotic acid, galactose, and lactose. (**b**) 3'-Sialyllactose (Neu Acα2→3Galβ1→4Glc) production from orotic acid, *N*-acetylneuraminic acid, and lactose; neuA: CMP-NeuAc synthetase. (**c**) Lewis X [Galβ1→4(Fucα1→3)GlcNAc] production from GMP, mannose, and *N*-acetyllactosamine; glk: glucokinase, manB: phosphomannomutase, manC: mannose 1-phosphate guanylyltransferase, gmd: GDP-mannose dehydratase, wcaG: GDP-4-keto-6-deoxymannose epimerase/reductase.

5 Conclusion

The whole-cell approaches for the production of fine chemicals are attractive methods with high yields, time- and cost-effectiveness, and simple operations. Recent recombinant DNA technologies will also promote the usage of whole-cell approaches. Although several purification steps are required after the whole-cell reaction, improvement of the purification process, such as the utilization of simulating moving bed systems,[47] will contribute to the efficient isolation. Progress in microbial genetics and microbial genome research will make it easier to use bacteria as enzyme sources and make it possible to design new biotransformation processes.[48]

References

[1] (a) A. Liese, K. Seelbach, C. Wandrey, *Industrial Biotransformations*, Wiley-VCH, Weinheim, **2000**; (b) M.-R. Kula, C. Wandrey, *Methods Enzymol.* **1987**, *136*, 9–21.

[2] D. H. Peterson, M. C. Murray, *J. Am Chem. Soc.* **1952**, *74*, 1871–1872.
[3] S. M. Roberts, *J. Chem. Soc., Perkin Trans. I* **1999**, 1–21.
[4] M. Abo, A. Okubo, S. Yamazaki, *Tetrahedron: Asymmetry*, **1997**, *8*, 345–348.
[5] H. Yamada, M. Kobayashi, *Biosci. Biotechnol. Biochem.* **1999**, *17*, 13–21.
[6] M. Kataoka, K. Shimizu, K. Sakamoto, H. Yamada, S. Shimizu, *Appl. Microbiol. Biotechnol.* **1995**, *44*, 333–338.
[7] Y. Tamura, M. Nishino, T. Ohmachi, Y. Asada, *Biosci. Biotechnol. Biochem.* **1998**, *62*, 2226–2229.
[8] (a) S. Hashimoto, A. Ozaki, *Curr. Opin. Biotech.* **1999**, *10*, 604–608; (b) M. D. Leonida, *Curr. Med. Chem.* **2001**, *8*, 345–369.
[9] J. Grunwald, T. M. S. Chang, *J. Mol. Catal.* **1981**, *11*, 83–90.
[10] Y. Yamazaki, H. Maeda, *Agric. Biol. Chem.* **1982**, *46*, 1571–1581.
[11] E. Ilan, T. M. S. Chan, *Appl. Biochem. Biotechnol.* **1986**, *13*, 221–230.
[12] (a) R. Wichmann, C. Wandrey, A. F. Buckmann, M.-R. Kula, *Biotechnol. Bioeng.* **1981**, *23*, 2789–2802; (b) T. Ohshima, C. Wandrey, M.-R. Kula, K. Soda, *Biotechnol. Bioeng.* **1985**, *27*, 1616–1618.
[13] (a) J. D. Stewart, *Curr. Opin. Biotechnol.* **2000**, *11*, 363–368; (b) S. Rodriguez, K. T. Schroeder, M. M. Kayser, J. D. Stewart, *J. Org. Chem.* **2000**, *65*, 2586–2587.
[14] R. J. Lamed, E. Keinan, J. G. Zeikus, *Enzyme Microb. Technol.* **1981**, *3*, 144–148.
[15] (a) N. J. Turner, *Curr. Opin. Biotechnol.* **2000**, *11*, 527–531; (b) C. Guerard, V. Alphand, A. Archelas, C. Demuynck, L. Hecquet, R. Furstoss, J. Bolte, *Eur. J. Org. Chem.* **1999**, 3399–3402.
[16] B. Orlich, H. Berger, M. Lade, R. Schomacker, *Biotechnol. Bioeng.* **2000**, *70*, 638–646.
[17] (a) D. Bur, M. A. Luyten, H. Wynn, L. R. Provencher, J. B. Jones, M. Gold, J. D. Friesen, A. R. Clarke, J. J. Holbrook, *Can. J. Chem.* **1989**, *67*, 1065–1070; (b) G. Casy, T. V. Lee, H. Lovell, *Tetrahedron Lett.* **1992**, *33*, 817–820.
[18] A. Galkin, L. Kulakova, H. Yamamoto, K. Tanizawa, H. Tanaka, N. Esaki, K. Soda, *J. Fer. Bioeng.* **1997**, *83*, 299–300.
[19] N. Esaki, H. Shimoi, Y.-S. Yang, H. Tanaka, K. Soda, *Biotechnol. Appl. Biochem.* **1989**, *11*, 312–317.
[20] K. F. Gu, T. M. S. Chang, *Biotechnol. Bioeng.* **1990**, *36*, 263–269.
[21] T. Ohshima, C. Wandrey, D. Conrad, *Biotechnol. Bioeng.* **1989**, *34*, 394–397.
[22] R. Wichmann, C. Wandrey, A. F. Buckmann, M. R. Kula, *Biotechnol. Bioengineer.* **2000**, *67*, 791–804.
[23] (a) M. Ikemi, N. Koizumi, Y. Ishimatsu,, *Biotechnol. Bioeng.* **1990**, *36*, 149–154; (b) M. Ikemi, Y. Ishimatsu, S. Kise, *Biotechnol. Bioeng.* **1990**, *36*, 155–164.
[24] B. Nidetzky, W. Neuhauser, D. Haltrich, K. D. Kulbe, *Biotechnol. Bioeng.* **1996**, *52*, 387–396.
[25] M. Stengelin, R. N. Patel, *Biocatal. Biotransform.* **2000**, *18*, 373–400.
[26] T. Maugard, S. Lamare, M. D. Legoy, *Biotechnol. Bioeng.* **2001**, *73*, 164–168.
[27] (a) M. Kataoka, L. P. S. Rohani, Y. Yamamoto, M. Wada, H. Kawabata, K. Kita, H. Yanase, *Appl. Microbiol. Biotechnol.* **1997**, *48*, 699; (b) M. Kataoka, L. P. S. Rohani, M. Wada, K. Kita, H. Yanase, I. Urabe, S. Shimizu, *Biosci. Biotechnol. Biochem.* **1998**, *62*, 167–169; (c) M. Kataoka, K. Yamamoto, H. Kawabata, M. Wada, K. Kita, H. Yanase, S. Shimizu, *Appl. Microbiol. Biotechnol.* **1999**, *51*, 486–490; (d) Y. Yasohara, N. Kizaki, J. Hasegawa, M. Wada, M. Kataoka, S. Shimizu, *Biosci. Biotechnol. Biochem.* **2000**, *642*, 1430–1436.
[28] A. Matsuyama, H. Yamamoto, N. Kawada, Y. Kobayashi, *J. Mol. Catalysis,* **2001**, *11*, 513–521.
[29] A. Galkin, L. Kulakova, T. Yoshimura, K. Soda, N. Esaki, *Appl. Environ. Microbiol.* **1997**, *63*, 4651–4656.
[30] B. Boonstra, D. A. Rathbone, C. E. French, E. H. Walker, N. C. Bruce, *Appl. Environ. Microbiol.* **2000**, 66, 5161–5166.
[31] H. Nakajima, K. Nagata, H. Kondo, K Imahori, *J. Appl. Biochem.* **1984**, *6*, 19–28.
[32] S. L. Haynie, G. M. Whitesides, *Appl. Biochem. Biotechnol.* **1990**, *23*, 155–170.
[33] K. Murata, T. Uchida, J. Kato, I. Chibata, *Agric. Biol. Chem.* **1988**, *52*, 1471–1477.
[34] T. Tochikura, *Chem. Bio. (Japanese)* **1993**, *31*, 639–649.
[35] (a) T. Fujio, A. Furuya, *J. Ferment. Technol.* **1983**, *61*, 261–267; (b) T. Fujio, A. Furuya. *Appl. Microbiol. Biotechnol.* **1985**, *21*, 143–147.
[36] T. Fujio, T. Nishi, S. Ito, A. Maruyama, *Biosci. Biotech. Biochem.* **1997**, *61*, 840–845.
[37] (a) H. Mori, A. Iida, S. Teshiba, T. Fujio, *J. Bacteriol.* **1995**, *177*, 4921–4926; (b) H. Mori, A. Iida T. Fujio, S. Teshiba, *Appl. Microbiol. Biotechnol.* **1997**, *48*, 693–698.
[38] (a) T. Fujio, A. Maruyama, *Biosci. Biotech. Biochem.* **1997**, *61*, 956–959; (b) T. Fujio, S. Teshiba, A. Maruyama, *Biosci. Biotech. Biochem.* **1997**, *61*, 960–964.
[39] (a) S. Koizumi, T. Endo, K. Tabata, A. Ozaki, *Nat. Biotechnol.* **1998**, *16*, 847–850; (b) T. Endo, S. Koizumi, K. Tabata, S. Kakita, A. Ozaki,. *Carbohydr. Res.* **1999**, *316*, 179–183.
[40] (a) T. Endo, S. Koizumi. K. Tabata, A. Ozaki, *Appl. Microbiol. Biotechnol.* **2000**, *53*, 257–261; (b) T. Endo, S. Koizumi, K. Tabata, S. Kakita, A. Ozaki,. *Carbohydr. Res.* **2001**, *330*, 439–443.
[41] S. Koizumi, T. Endo, K. Tabata, A. Ozaki, *J. Ind. Microbiol. Biotechnol.* **2000**, *25*, 213–217.
[42] R. A. Dwek, *Chem. Rev.* **1993**, *96*, 683–720.
[43] (a) H. M. Flowers, *Methods. Enzymol.* **1978**, *50*, 93–121; (b) M. M. Palcic, *Curr. Opin. Biotechnol.* **1999**, *10*, 616–624.
[44] (a) C.-H. Wong, S. L. Haynie, G. M. Whitesides, *J. Org, Chem.* **1982**, *47*, 5418; (b) Y. Ichikawa, G.-J. Shen, C.-H. Wong, *J. Am. Chem. Soc.* **1991**, *113*, 4698; (c) Y. Ichikawa, G. C. Look, C.-H. Wong, *Anal. Biochem.* **1992**, *202*, 215–238; (d) K. M. Koeller, C.-H. Wong, *Chem. Rev.* **2000**, *100*, 4465–4493; (e) T. Miyazaki, H. Sato, T. Sakakibara, Y. Kajihara, *J. Am. Chem. Soc.* **2000**, *122*, 5678–5694.
[45] (a) C.-H. Lin, G.-J. Shen, E. G. Junceda, C.-H. Wong, *J. Am. Chem. Soc.* **1995**, *117*, 8031–8032; (b) M. D. Burkart, M. Izumi, C.-H. Wong, *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 2747.
[46] T. Endo, S. Koizumi, *Curr. Opin. Struct. Biol.* **2000**, *10*, 536–541.
[47] A. Navarro, H. Caruel, L. Rigal, P. Phemius, *J. Chromatogr.* **1997**, *770*, 39–50.
[48] A. L. Demain, *Trend. Biotechnol.* **2000**, 18, 26–31.