Conversions of Lipophilic Substances by Encapsulated Biocatalysts

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It is a great honor and pleasure to be selected as the laureate of the prestigious Normann Medal of the Deutsche Gessellschaft für Fettwissenschaft (DGF) in 1987. I am deeply grateful to Karl Gander, DGF president, Bruno Werdelmann, former DGF president, the members of the selecting committee, and all the members of DGF.

This paper is comprised of two parts. The first deals with the metabolism of alkanes and fatty acids in yeast cells by the action of the enzyme system localized in the organelles, particularly the function of peroxisomes and, namely, bioconversions of these lipophilic compounds in vivo by the enzymes biologically encapsulated. The second part is concerned with bioconversions of lipophilic and slightly water-soluble substances in organic solvent media by using artificially encapsulated biocatalysts.

These works were carried out in my laboratory in collaboration with A. Tanaka, my successor, K. Sonomoto, and my former coworkers Numa and Mishina, and others in the Medical Chemistry Department of Kyoto University and Professor Osumi, Japan Women's University, Tokyo, and some Japanese industries.

METABOLISM OF ALKANES AND FATTY ACIDS BY YEAST (1,2,3)

One specific feature of alkane-utilizing yeasts and higher fatty acid-utilizing yeasts is conspicuous appearance of peroxisomes in the cells. The subtle diversity in the metabolism of alkanes and fatty acids is mediated by subcellular localization of enzymes. My paper describes the metabolism of alkanes and fatty

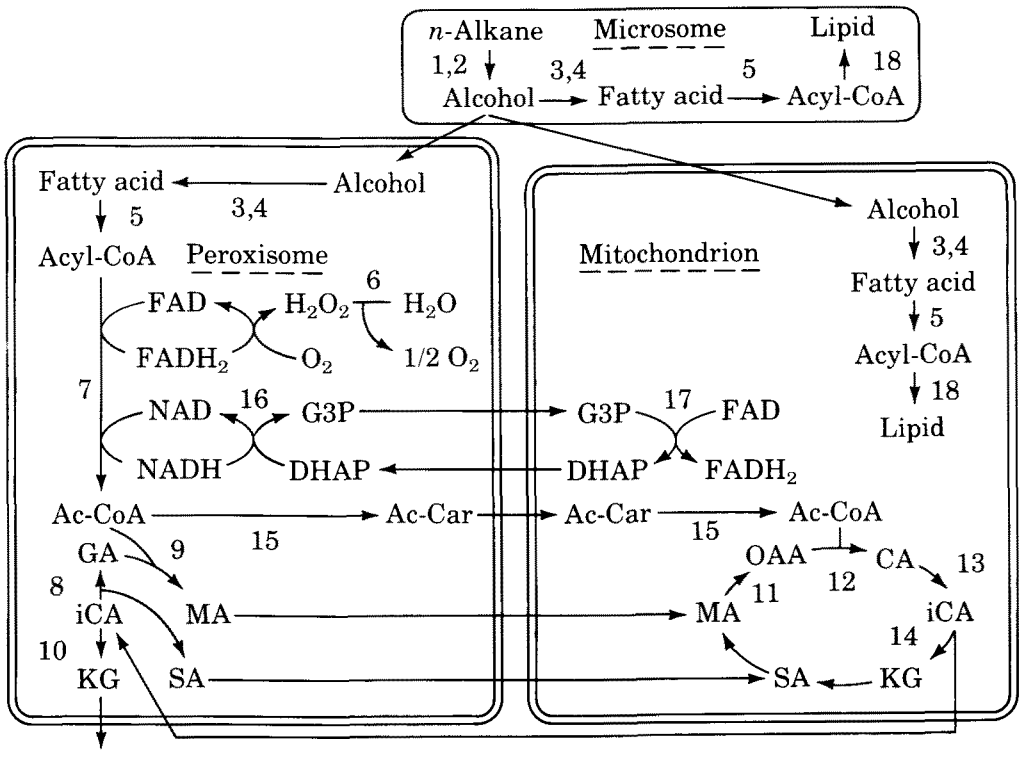


FIG. 1. Presumptive roles of peroxisomes, mitochondria and microsomes in alkane-assimilating yeasts. Enzymes: 1, cytochrome P-450; 2, NADPH-cytochrome P-450 (cytochrome c) reductase; 3, long-chain alcohol dehydrogenase; 4, long-chain aldehyde dehydrogenase; 5, acyl-CoA synthetase; 6, catalase; 7, β-oxidation system; 8, isocitrate lyase; 9, malate synthase; 10, NADP-linked isocitrate dehydrogenase; 11, malate dehydrogenase; 12, citrate synthase; 13, aconitase; 14, NAD-linked isocitrate dehydrogenase; 15, carnitine acetyl transferase; 16, NAD-linked glycerol-3-phosphate dehydrogenase; 17, FAD-linked glycerol-3-phosphate dehydrogenase; 18, glycerol-3-phosphate acyl transferase.
Abbreviations: Ac-Car, acetyl carnitine; Ac-CoA, acetyl-CoA; CA, citrate; DHAP, dihydroxyacetone-phosphate; GA, glyoxylate; G3P, glycerol-3-phosphate; iCA, isocitrate; KG, α-keto-glutarate; MA, malate; OAA, oxalacetate; SA, succinate.

acids in yeasts with special emphasis on the physiological function of peroxisomes.

In microsomes, alkanes are hydroxylated to the corresponding fatty alcohols which are further oxidized to fatty acids via aldehydes in microsomes, mitochondria and peroxisomes, respectively. Degradation of fatty acids to acetyl-CoA via β-oxidation pathways is carried out exclusively in peroxisomes while fatty acids formed in microsomes and mitochondria are incorporated into cellular lipids, each after being activated to acyl-CoAs. Acetyl-CoA produced in peroxisomes is converted to C_4-compounds by the cooperative action of peroxisomes and mitochondria. The existence of acyl-CoA synthetases of different subcellular localization has been demonstrated. The distinctly localized acyl-CoA synthetases play an important role to supply acyl-CoAs, which will be utilized for chain elongation and intact incorporation, or for degradation yielding acetyl-CoA, the substrates for the de novo synthesis system of cellular components. Figure 1 shows the presumable roles of peroxisomes, mitochondria and microsomes in alkane (or fatty acid)-assimilating yeasts. Figure 2 depicts fatty acid metabolism in alkane (or fatty acid)-utilizing yeasts with special reference of the distinct functions of acyl-CoA synthetase I and acyl-CoA II, which are localized exclusively in peroxisomes. The mutants lacking acyl-CoA synthetase I and those lacking acyl-CoA synthetase II were obtained by Numa and his group. When mutants lacking acyl-CoA synthetase I were cultivated on alkanes of odd-chain carbon skeletons, the proportion of odd-chain fatty acids to total cellular fatty acids was completely different from that of the wild strains (Table 1).

As mentioned above, peroxisomes of alkane- and fatty acid-grown yeast cells contain various enzymes,

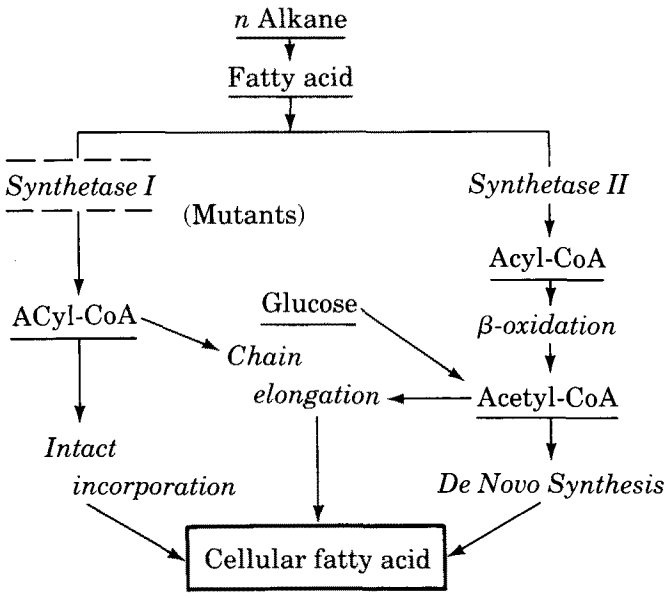


FIG. 2. Proposed scheme of fatty acid metabolism in alkane (fatty acid)-utilizing yeasts. If the mutant of *Candida lipolytica* lacking acyl-CoA synthetase I is grown on alkanes or fatty acids, only the pathway in the peroxisomes is operative, showing the different pattern of the cellular fatty acid from that of the wild strain (Table 1).

TABLE 1

Ratios of Odd-Chain Fatty Acids to Total Cellular Fatty Acids in *C. lipolytica* Wild and Mutant Strains

Carbon source	Proportion of odd-chain fatty acids (%)	
	Wild	Mutants
Glucose	3.3	0.7- 0.8
n-Undecane	9.2	1.5- 1.7
n-Tridecane	62.0	2.1- 2.4
n-Pentadecane	97.8	8.9-11.4
n-Heptadecane	98.6	11.7-12.4
Oleic acid	0.8	0.2- 0.3

especially peroxisome-associated enzymes. As shown in Table 2, the levels of these peroxisome-associated enzymes are enhanced markedly when cultivated on these special substrates. Practical applications of these enzymes have been commercialized already.

BIOCONVERSIONS OF LIPOPHILIC AND SLIGHTLY WATER-SOLUBLE COMPOUNDS IN ORGANIC SOLVENT MEDIA BY USING ARTIFICIALLY ENCAPSULATED BIOCATALYSTS (4,5,6)

In the bioconversion of highly lipophilic or slightly water-soluble compounds, it is desirable to carry out the enzymatic reaction in a mixture of water and a suitable organic cosolvent or in an adequate organic solvent that contains a small amount of water. The use of an organic solvent will improve the poor solubility of the substrate and other reaction components that are hydrophobic. Moreover, for the utilization of hydrolytic enzymes for synthetic or group transfer reactions, the water fraction in the reaction mixture should be reduced by replacing water with appropriate organic solvents. Furthermore, along with the recent development of biotechnology much wider applications of biocatalysts are demanded, e.g. bioconversions of xenobiotic compounds such as aliphatic and aromatic compounds, having hydrophobic characters. In such bioprocesses, substrates and products themselves are organic solvents that are unconventional for biocatalysts.

Biocatalysts (enzymes, microbial cells) traditionally have been used in aqueous systems. It generally has been considered that biocatalysts are liable to be denatured in the presence of organic solvents, resulting in the loss of their catalytic abilities.

Attempts to render biocatalysts resistant to organic solvents have been made through different lines of approaches: chemical, biochemical and genetic. Lately, site-specific mutations of enzyme molecules by gene manipulation have attracted worldwide interests. Of these approaches, immobilization of biocatalysts on or in suitable supports seem to be the most general and promising.

We have developed convenient methods for entrapping biocatalysts inside gel matrices formed from

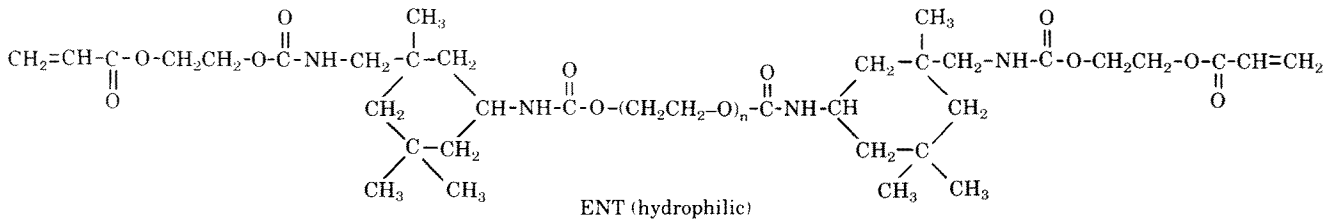
TABLE 2

Levels of Peroxisomal Enzymes in *Candida Tropicalis* pK 233

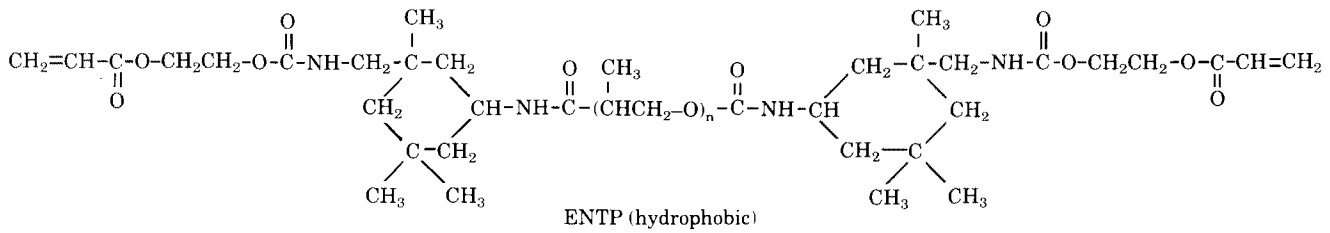
Enzyme activity of cells grown on	Enzyme: Glucose (nmol • min^{-1} • mg protein^{-1})	Enzyme: Alkanes (C_{10}-C_{13}) (nmol • min^{-1} • mg protein^{-1})
Long-chain alcohol dehydrogenase	6	80
Long-chain aldehyde dehydrogenase	9	98
Acyl-CoA synthetase	37	70
β-Oxidation system	*ca.* 0	75
Catalase	206 × 10^3	5,540 × 10^3
Carnitine acetyl transferase	376	7,430
NAD-linked glycerol-3-phosphate dehydrogenase	10	169
Isocitrate lyase	40	81
Malate synthase	33	170
NADP-linked isocitrate dehydrogenase	242	491
Uricase	42	182
d-Amino acid oxidase	112	73

synthetic prepolymers. Figures 3 and 4 show the structures of the prepolymers of photo cross-linkable resins (ENT, hydrophilic and ENTP, hydrophobic) and the prepolymers of urethane resin (PU), respectively. Photo cross-linkable resin prepolymers have photosensitive functional groups such as acryloyl groups at both terminals of the linear main chain. The chain length of prepolymers can be adjusted by using poly(ethylene glycol) or poly(propylene glycol) of optional chain length as the starting material for synthesis. Thus, ENT-4000, for instance, means that the prepolymer is formed with poly(ethylene glycol)-4000 (average Mw, ca. 4000; the chain length, ca. 40 nm). When the main skeleton consists of poly(ethylene oxide), the prepolymer and accordingly the gels formed from the prepolymers should have a hydrophilic character. On the other hand, the prepolymers containing poly(propylane oxide) in the main skeleton (ENTP) and the gels formed from ENTP should be hydrophobic. In the case of urethane prepolymers, the hydrophilic or hydrophobic character of prepolymers can be adjusted by changing the ratio of the poly(ethylene oxide) part and the poly(propylene oxide) part in the polyether moiety of the main skeleton. Thus, PU-3 with a high content of poly(propylene oxide) gives more hydrophobic gels while PU-6 and PU-9 with a high content of poly(ethylene oxide) hydrophilic gels.

Gelation of photo cross-linkable resin prepolymers can be completed easily by illuminating the mixture of prepolymer solution, a small amount of photosensitizer, e.g. benzoin ethyl ether or benzoin isobutyl ether, and enzyme solution or microbial cell suspensions by near-ultraviolet irradiation for three to five min. Entrapment of biocatalysts with urethane prepolymers is much



ENT (hydrophilic)



ENTP (hydrophobic)

FIG. 3. Structures of typical photo-crosslinkable resin prepolymers.

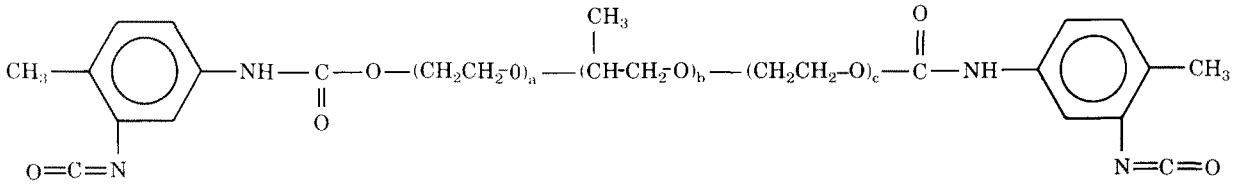


FIG. 4. Structure of polyurethane resin prepolymers (PU).

simpler. When liquid prepolymers are mixed with aqueous solutions of enzymes or aqueous suspensions of microbial cells, prepolymers react each other, being cross-linked by forming urea linkages with liberation of carbon dioxide.

The conformational structure of an immobilized enzyme will be more resistant to the distortion caused by organic solvents as compared with free native enzyme. In the cases of biotransformations of biological substances in vivo, many enzymes, particularly those catalyzing transformations of lipophilic biological compounds, function in membrane-bound states, and the stability of such membrane-associated enzymes is in general greater than that of enzymes released from the membrane.

Inclusion of enzymes or microbial cells within suitable gels would give an environment analogous to that in vivo. Multi-point interactions between entrapped enzymes (and microbial cells including enzymes) and gel matrices will give stabilizing effects. If the gels have desired hydrophobic characters and a suitable network structure, the environment around the biocatalysts entrapped in the gels will be more similar to that in vivo. However, the situation of immobilized biocatalysts in vitro is more complicated. For application of such gel-entrapped biocatalysts for conversions of lipophilic compounds in organic solvent systems, affinity of lipophilic substrates for the gels entrapping biocatalysts and diffusion of reactants through gel matrices are important factors. Low affinity of hydrophilic gels for lipophilic substrates will lower the apparent activity of the gel-entrapped biocatalysts. Thus, use of suitably hydrophobic gels with an adequate network structure will be preferable depending on hydrophobicity of substrates and polarity of solvents to be used.

In this article, we would like to report comprehensively on our experimental results using biocatalysts immobilized with prepolymers of photo cross-linkable resins and urethane resins. The biocatalyst-entrapping gels had the desired hydrophobicity-hydrophilicity balances and network structures. They were used for bioconversions of a variety of highly lipophilic or slightly water-soluble substrates carried out in homogeneous reaction systems composed of water-water miscible cosolvents or water-containing organic solvents. The effects of solvents and water content in reaction systems also were investigated.

Bioconversion in water-organic cosolvent systems. Water-water miscible organic cosolvent systems have been employed widely to dissolve water-insoluble, lipophilic compounds to prepare homogeneous reaction systems and to shift reaction equilibrium in a desired direction, especially in the synthetic direction with hydrolyzing enzymes.

We have studied bioconversions extensively by biocatalysts entrapped by our prepolymer methods in appropriate water-water miscible organic solvent systems for carrying out the reactions continuously. Table 3 shows our results on bioconversions of various lipophilic or slightly water-soluble compounds carried out in water-water miscible organic solvent systems by the use of microbial cells entrapped with prepolymers.

Bioconversion in organic solvent systems. Although water-immiscible organic solvents have been used for bioreactions to increase the solubility of substrates and

TABLE 3

Bioconversions in Water-Organic Cosolvent Systems by Microbial Cells Entrapped with Prepolymers

Microorganisms (Condition)	Organic Cosolvent	Application
Arthrobacter simplex (acetone-dried)	10% Methanol	Δ^1-Dehydrogenation of hydrocortisone
Curvularia lunata (living)	2.5% Dimethyl sulphoxide	11β-Hydroxylation of cortexolone
Rhizopus stolonifer (living)	2.5% Dimethyl sulphoxide	11α-Hydroxylation of progesterone
Sepedonium ampullosporum (living)	0.65% Dimethyl formamide	16α-Hydroxylation of estrone
Corynebacterium sp. (living)	15% Dimethyl sulphoxide	9α-Hydroxylation of 4-androstene-3,17-dione
Enterobacter aerogenes (thawed)	40% Dimethyl sulphoxide	Synthesis of adenine arabinoside

TABLE 4

Bioconversions in Organic Solvent Systems by Biocatalysts Entrapped with Prepolymers

Biocatalysts	Organic solvent (water-saturated)	Application
Nocardia rhodocrous (cells)	Benzene-n-Heptane (1:1)[a]	Δ^1-Dehydrogenation of ADD[b]
	Benzene-n-Heptane (4:1)	Δ^1-Dehydrogenation of TS[c]
	Benzene-n-Heptane (1:1)	3β-Hydroxysteroid dehydrogenation
	Benzene-n-Heptane (4:1)	17β-Hydroxysteroid dehydrogenation
	Chloroform-n-Heptane (1:1)	3β-Hydroxysteroid dehydrogenation
Rhodotorula minuta (cells)	n-Heptane	Stereoselective hydrolysis of *dl*-menthyl ester
Candida cyclindracae (lipase)	Cyclohexane or Isooctane	Stereoselective esterification of *dl*-menthol
Rhizopus delemar (lipase)	n-Hexane	Interesterification of triglycerides

[a]Mixed ratio by volume.

[b]4-Androstene-3,17-dione.

[c]Testosterone.

water-organic solvent two-phase systems. Only a limited number of papers have described the use of organic solvents alone (mostly, water-saturated organic solvents) in bioreactions.

We have investigated extensively bioconversions of lipophilic compounds by immobilized biocatalysts in organic solvent systems (Table 4).

The activities of immobilized biocatalysts were found to be affected significantly by the hydrophilicity-hydrophobicity balance of gels, the hydrophobicity of substrates, and the polarity of reaction solvents.

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