Enzyme Technology for the Lipids Industry: An Engineering Overview

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Enzymes useful in the lipids industry, i.e. lipases, phospholipases and lipoxygenases, are surveyed as to source, pH optimum, specificity and so on. Some useful biochemical reactions catalyzed by these enzymes are discussed: hydrolysis of fats and oils by lipases, transesterifications (acidolysis, alcoholysis, interesterification and aminolysis) of fats and oils by lipases, hydrolysis of lecithin by phospholipase A_2 and transphosphatidylation of phospholipids by phospholipase D. Research and development activities in these fields in the academic and industrial sectors of Japan are discussed.

With reference to the lipolytic enzymes' applications, forms or states with which enzymes and microorganisms are used in microaqueous solvent systems, i.e. in low water-activity media or in nearly anhydrous solvents, are summarized. Some configurations of reactors for the microaqueous biosystems are shown, and some engineering problems involved in the systems are identified. The importance of optimal moisture content control is emphasized.

In this review, lipids cover not only edible fats and oils but also natural phospholipids and glycolipids. Among interesting and promising aspects of old and new biotechnologies relevant to the lipids industry, this overview will be confined to enzyme (isolated and whole cells as an container) technology and engineering.

Many enzymes are involved in the catabolic and anabolic metabolisms of lipids but there are not many enzymes and the types of reactions that are or will be considered to apply to lipids industry. They are mostly lipolytic enzymes such as lipases and phospholipases. This should not be surprising in consideration of the present state of the art of enzyme technology and engineering. How many enzymes have been adopted in industry? Only some hydrolases, isomerases and lyases are sharing success stories among thousands of enzymes that are known to exist in life forms. The lipolytic enzymes (lipases and phospholipases), an oxidase (lipoxygenase) and some engineering aspects in their industrial applications are discussed here. Recent trends in biochemical and biotechnological studies on these enzymes and R & D in both academic and industrial sectors of Japan also will be introduced briefly.

LIPASES AND ESTERASES

Variety of lipases. Generally, the properties of enzymes, even though they catalyze the same type of reaction, differ with their origins. Nature is surprisingly profound and of great variety in this respect—far beyond man's imagination. This is valid completely with lipase, which is a single enzyme numbered as triacylglycerol acylhydrolase E.C. 3.1.1.3. The enzyme has a very wide range of properties, depending on its source, with respect to positional specificity, fatty acid specificity, pH optimum, thermostability. This suggests that one can find a suitable lipase (or lipase-producing organism) from nature that fits a given application. It often is stated that the lipases can be placed into two groups according to their positional specificity, 1,3-specific and nonspecific. However, the author agrees with H. Machida's comment that the positional specificity of lipases is not divided clearly into two but it continuously changes from very distinctly specific to very weakly specific or completely nonspecific (1). The situation is made more complicated due to nonenzymatic acyl migration from β- to α-position in glycerides (2).

According to the endoplasmicreticulum (EC of IUB, there is another enzyme, monoacylglycerol lipase (E.C. 3.1.1.23, glycerol-monoester acylhydrolyase), which is different from genuine lipase. This often is found in animal tissues and also is produced by a microorganism. It is difficult to differentiate this enzyme from what is called "esterase."

Fatty acid specificity of lipases is ambiguious. The substrate specificity of a lipase usually is reported as the relative hydrolysis rate of a single triglyceride vs the number of carbons in the fatty acid. The spectrum diverges significantly, which indicates that lipases do not have strict substrate specificity. The substrate specificity is made more vague by the fact that the rate of hydrolysis is affected not only by the substrate but also by physical factors (solid or liquid, particle or emulsion size, degree of turbulence, solubility in water in case of low carbon-numbered triglyceride).

As with other enzymes, each lipases has its own optimal pH, ranging from acid to neutral to alkaline. Development of alkaliphilic lipase aims at two applications: its addition in laundry detergents to enhance cleaning and a substitute for pancreatic lipase in digestive medicine.

Thermostability of lipases also varies considerably with their origins. Animal and plant lipases usually are less thermostable than microbial extracellular lipases. Relatively thermostable lipases produced by *Pseudomonas* species (3,4) and by *Humicola* sp. (5) have been reported in Japan.

Microbial lipases produced industrially in Japan and their properties are summarized in Table 1. Other lipases would be available on request but their scale of production may be small.

Biochemical reactions catalyzed by lipases. Lipases and esterases catalyze three types of reaction (Fig. 1). The catalytic action of lipases is reversible. It catalyzes ester synthesis in a microaqueous system (see Section 5.1). However, in view of biotransformation in oleochemical industry yielding value-added products,

TABLE 1

Microbial Lipases Produced Industrially in Japan

Strain	Manufacturer	Thermostability (remaining activity)	Optimal pH	Positional specificity	Molecular weight
Candida cylindracea (Candida rugosa)	Meito Sangyo	40% (50 C, 10 min)	7.0	α, β, α'	55,000
Aspergillus niger	Amano Pharmaceutical	50% (60 C, 15 min)	5.6	α, α'	38,000
Rhizopus niveus	Amano Pharmaceutical	60% (50 C, 30 min)	7.0	α, α'	–
Pseudomonas fluorescens	Amano Pharmaceutical	60% (60 C, 30 min)	7.0	α, α'	31,000
Rhizopus japonicus	Osaka Saikin Kenkyusho	50% (55 C, 30 min)	5.0	α, α'	30,000

In addition to these, Sapporo Breweries, Ltd. is producing *Pseudomonas fragi* lipase and Toyo Jozo Co., Ltd. is producing *Chromobacterium viscosum* lipase.

transesterification action seems more worthwhile than hydrolysis and ester synthesis. The difference in free energy involved in triglyceride hydrolysis is quite small and the net free energy of transesterification is zero. Consequently, transesterification reactions take place easily. Transesterification is categorized into four subdivisions according to the chemical species with which the ester reacts (Fig. 1). Some researchers designate these four types of reaction by "interesterification" but the author prefers transesterification to interesterification as the technical term covering all four types of reaction because in biochemistry transfer of a group from one chemical species to another is called "trans", such as transglycosylation, transpeptidylation, transphophatidylation. Therefore, the author confines the term interesterification only to the type 3 reaction (ester exchange).

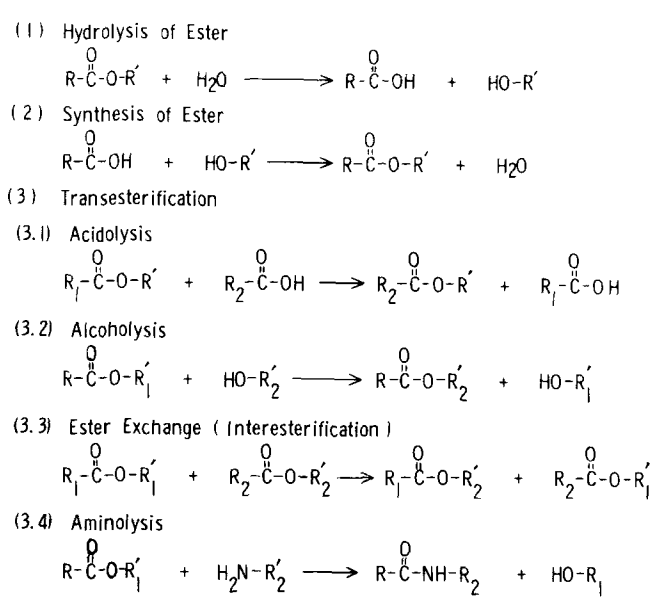

FIG. 1. Types of reaction catalyzed by lipase.

R & D activities on lipase applications in Japan. Research on microbiology, biochemistry and biotechnology of lipases and on their industrial applications are quite active in Japan. Division of Biochemistry, Osaka Municipal Technical Research Institute has a long history of research on microbial lipases. They currently are interested in gene cloning of *Geotrichum candidum* lipase and its crystalographic structure. Extensive microbiological and biochemical studies on *Saccharomycopsis lipolytica* (*Candida paralipolytica*) have been carried out by Y. Otha et al. (6). Development of polyethylene glycol (PEG)-modified lipases (organic solvent-soluble lipases) and their application to a number of biochemical reaction in organic solvent media have been reported by Y. Inada and his associates (7). The use of hydrophobic gels for the immobilization of lipase was reported by S. Fukui, A. Tanaka and coworkers (8). Lipase-catalyzed synthesis of macrocyclic lactone (cyclopentadecanolide) was reported by Y. Yamada et al. (9). This will be useful in the production of synthetic musks. Hydrolysis of fats and oils dissolved in isooctane with aqueous lipase solution was studied by J. Takahashi and coworkers (10). Esterification of high acid-value rice bran oil have been studied with a thermostable lipase immobilized in cationic resin beads by Y. Kosugi et al. (11). Enzymic sugar ester syntheses were attempted by H. Seino (12) and by S. Nagai (13), both in collaboration with Dai-ichi Kogyo Seiyaku Co. Ltd. The author and coworkers have studied lipase-catalyzed glycerolysis of fats and oils (14).

The chemical process of fat-splitting currently being performed in industry is very efficient in energy recycling, and it is unlikely that enzymatic fat-splitting will compete economically with the conventional chemical process in the near future. The interest of people in the enzymatic hydrolysis is shifting from bulky fatty acid products to high value-added products or to unstable fatty acids that may be decomposed through

the superheated steam splitting procedure. Industrial preparations of optical active alcohols related to synthetic pyrethroids insecticides with an *Arthrobacter* lipase has been realized by H. Hirohara and S. Mitsuda working with Sumitomo Chemical Co. Ltd. (15). Enrichment or isolation of some long-chain polyunsaturated fatty acids from refined fish oil through lipase action are getting much attention in Japanese fats and oils companies because of their potential for reducing the incidence of thrombosis and related diseases.

Production of cocoa butter substitutes through acidolysis of inexpensive fats by lipase are under active R & D in several Japanese companies (Fuji Oil, Ashai Denka Kogyo, Kanegafuchi Chemical Industry). Isolated lipase immobilized in an appropriate carrier or dry microbial cells having lipase are used in packed bed bioreactors. Moisture content control is the key factor of the acidolysis reaction.

Characterization and application of *Pseudomonas fragi* lipase are being investigated by T. Nishio working with Sapporo Breweries Ltd. (16). A unique monoacylglycerol lipase produced by *Penicillium* sp. has been commercialized recently from Amano Pharmaceutical Co., Ltd., with the aim of monoglyceride synthesis from free fatty acid and glycerol (17). Enzymatic production of monoglyceride still is too costly to compete with a chemical process (a glycerolysis reaction).

Phospholipases. Five kinds of phospholipases exist in nature according to their sites of attack of ester bonds of phospholipids. Only a phospholipase A_2 ("Lecitase" from Novo Industrie A/S) manufactured from porcine pancreatic glands and a phospholipase D (PLD) excreted from *Streptomyces chromofuscus* (Toyo Jozo Co., Ltd.) are available commercially on large scale. The class of phospholipids, substrates for phospholipases, is synonymis with lecithin, and is isolated in various grades from soybean and egg yoke. They are essential constituents of biomembrane and have surface active properties. Lecithins have been utilized as one of five emulsifiers permitted in the food industry. Patents claim that when soybean lecithin is partially hydrolyzed by phospholipase A_2, the resulting phospholipids (containing lyzolecithin) increase in hydrophilicity and have an enhanced power of o/w type emulsification (18). The modified lecithin is on sale now though the production scale is unclear. Phospholipids also find application in medical and pharmaceutical fields. Liposomes, made of various kinds of phospholipids, are expected to be good drug delivery systems.

PLD not only hydrolyzes phospholipids but also catalyzes a transphosphatidylation reaction in which exchange of base takes place as indicated in Figure 2. Attempts are being made by Yakurt Central Institute to convert soybean lecithin to phosphatidylglycerol with the transphosphatidylation action of PLD. Phosphatidylglycerol is claimed to be an effective emulsifier even in the presence of calcium ion. The author and others are studying this reaction with the gaol of producing each component of phospholipid in pure state (19). Kinetic estimation of transphosphatidylation activities of several PLD from different sources reveals that the activity varies considerably with the enzyme sources (unpublished data).

Lipoxygenase. The major or sole source of lipoxygenase now utilized is soybean. However, lipoxygenases from *Fusalium oxysporum* were discovered and investigated enzymatically by K. Arima et al. (20). Chemical modification of lipoxygenase has been attempted by H. Hirata et al. of the National Chemical Laboratory for Industry, MITI, as a national project of the Research Association for Biotechnology. If the products, fatty acid hydroperoxides or their reduced derivatives (hydroxyconjugated fatty acid), find useful industrial application, lipoxygenase-catalyzed oxidation of unsaturated fatty acids will be realized commercially.

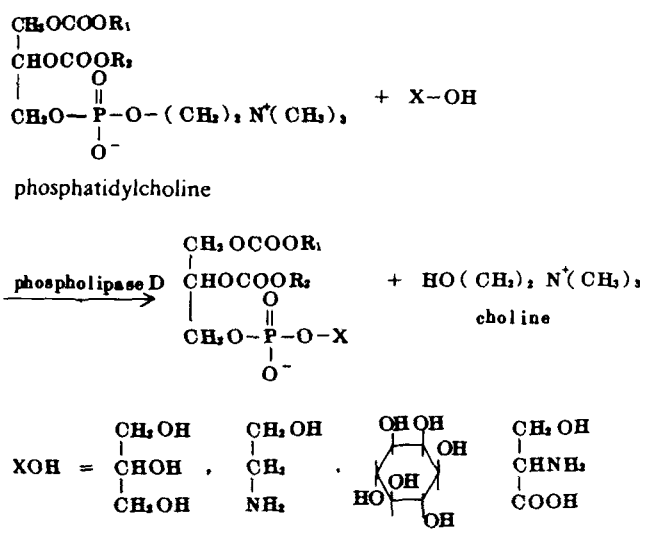

FIG. 2. Transphosphatidylation reaction by phospholipase D.

GENETIC AND PROTEIN ENGINEERING

The production costs of industrial lypolytic enzymes are high as compared with amylases and proteases. The cost seems to hamper wider industrial application of the enzymes; it depends heavily on the degree of purity. There is a hope that the cost can be reduced by gene technology such as gene amplification in addition to a traditional random mutation. The first and essential step of genetic manipulation is cloning of genes involved in the enzyme's biosynthesis. Recently, genes of bacterial lipases (21,22), a mammalian phospholipase A_2 (23) and a bacterial sphingomyelinase (24) have been cloned. A number of genes of lypolytic enzymes will be cloned rapidly in the coming years. Many useful reactions catalyzed by the enzymes in oleochemistry are conducted in microaqueous organic media (see the next section). In this respect, protein engineering approaches will help the elucidation of mechanism of solvent-denaturation and will create novel enzyme proteins that are more resistant to the organic solvents.

BIOCATALYSIS IN MICROAQUEOUS ORGANIC SOLVENT SYSTEM

What is a microaqueous biosystem? As mentioned above, many industrially intriguing reactions catalyzed by the lipolytic enzymes are carried out in organic media. However, one should realize, that the organic media are

not completely anhydrous. Recent research at many laboratories in the world have shown that a complete depletion of water from the system results in no biochemical reaction. Water is essential for the enzyme to display its full catalytic activity because it is a protein. With reference to biocatalysts' application in organic media, some technical phrases have been used: "in low water-activity media," "in nearly anhydrous solvent," "in an organic solvent containing a little amount of water," etc., to show that the reaction systems are not absolutely anhydrous. To emphasize the importance of water for biocatalysis in organic solvents and to cover all possible forms or states of biocatalysts utilization in organic media, would like to propose a shorter but clearer term "microaqueous," which means that the system is not aqueous, nonaqueous or anhydrous. "Microaqueous biosystem" is defined roughly as a biochemical or biological system in which moisture content is low as compared with a conventional one. That is, microaqueous biochemical systems are made up of water-soluble organic substances or water-insoluble organic solvents.

Optimal control of the moisture content is the keystone of the microaqueous biocatalysis system as it affects the reaction rate, product yield, product selectivity and operational stability. A rough profile of effects of moisture content on the last two variables is drawn in Figure 3, which suggests that at lower moisture content the yield of the product may be high, but the reaction rate may be lower. While at the greater moisture content the rate becomes higher but the yield drops. In between, there is an optimal moisture content from an engineering viewpoint. The effects of moisture content on the biochemical reactions in organic media are exemplified by glyceride synthesis (25) and glycerolysis reaction (14) both carried out by the author's group using acidolysis (26) and by reaction in reversed micelles (27). These are only several among a number of reactions that follow the scheme shown in Figure 3.

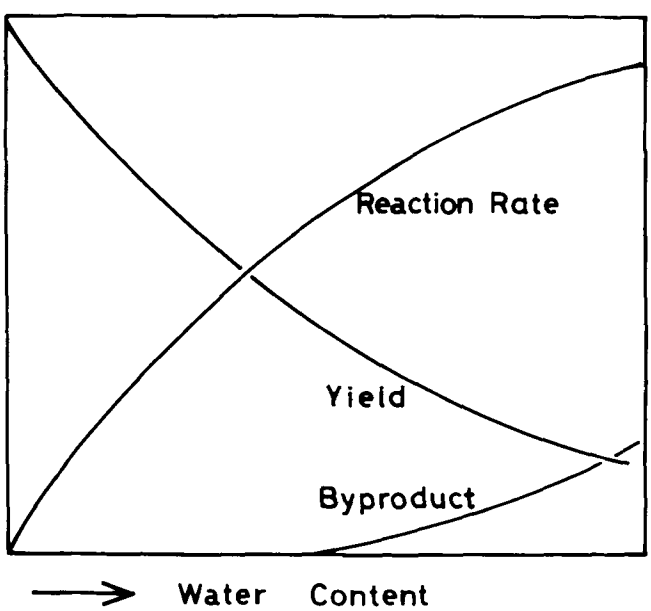

FIG. 3. Effect of moisture content on a reaction conducted in a microaqueous organic medium.

Enzyme forms used in microaqueous solvent systems. In the past several years, a broad range of forms or states have been developed with which enzymes may be used for diversed reactions in microaqueous solvent systems. These can be classified tentatively into six forms:

(1) If a substrate is water-soluble, its highly concentrated aqueous solution becomes microaqueous. Enzymes are dissolved in the concentrated substrate solutions. Carbohydrates like glucose and sucrose are examples. Alternately, enzymes are dissolved in a water-soluble organic solvent that has some amount of water. Methanol, ethanol, ethylene glycol, glycerol, dimethylformamide, dimethylsulfoxide and acetone are some such solvents. In our glyceride synthesis and glycerolysis reaction, lipases are dissolved in microaqueous glycerol (14,25).

(2) Solid enzyme is suspended in a water-immiscible or water-insoluble organic solvent (9,28,29). If the solid enzyme is not suspended easily, it is adsorbed on particles of support like diatomaceous earth prior to its suspension.

(3) An enzyme molecule is confined in a reversed micelle solubilized in a water-insoluble organic solvent (30,31). The formation of reversed micelles using a surfactant, AOT, in aliphatic hydrocarbons such as heptane, octane and isooctane is well-known. Sharp dependence of the activity of enzyme in reversed micelles on the water content as expressed by $w_0 = [H_2O]/[AOT]$ generally is recognized.

(4) An enzyme is solubilized in an aromatic solvent (benzene, toluene, etc.) by binding it with PEG via a triazine ring. It was demonstrated that some amount of water is needed for the PEG-bound enzyme to exhibit its activity (32).

(5) An enzyme is entrapped within a gel whose degree of hydrophobicity can be controlled. The gel-entrapped enzyme is suspended in water-immiscible organic solvent (8).

(6) Porous particles are impregnated with an aqueous solution of the enzyme. The particles are suspended or packed in a water-immiscible organic solvent. The enzyme can be used in free and immobilized states. This is a sort of w/o emulsion but the proportion of water phase to the organic phase is lowered greatly in such a system, and the system is more convenient technologically than the free w/o emulsion (33).

The forms (1), (2), (4) and (5) are microaqueous at molecular level, while the form (6) is microaqueous at the phase level. The form (3) lies between molecular and phase levels. It may be called microaqueous at pseudo-phase level. Thus people have obtained a variety of forms of enzyme workable in organic media. It is difficult to predict which form is most suitable for industrial purposes but economy eventually will decide this.

Microorganism forms used in microaqueous solvent systems. Microbial cells are used in one of the following forms in microaqueous solvent.

(1) Wet or semi-dried cells are suspended in water-immiscible organic solvent (34). Wet microbial cells contain 70–80 wt % of water. If they are dispersed in the solvent, the whole system is microaqueous

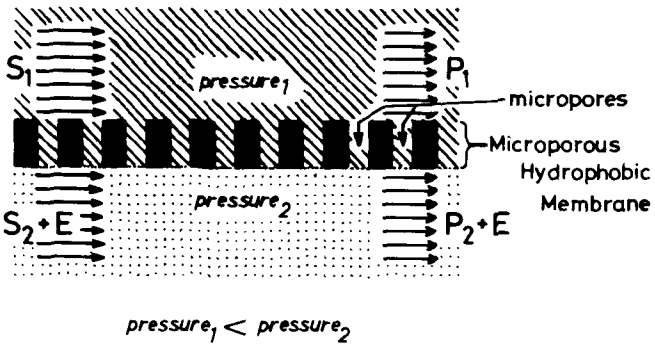

FIG. 4. The neighborhood of the membrane in the microporous hydrohobic membrane bioreactor system performed continuously. The substrates, S_1 and S_2, and the products, P_1 and P_2, for four types of reaction are shown below. Enzyme is dissolved in S_2 phase or adsorbed on the S_2-side surface of the hydrophobic membrane.

	Type of reaction	S_1	S_2	P_1	P_2	Ref.
1.	Hydrolysis	Fat	Water	Fatty acid	Glycerol	37
2.	Glyceride synthesis	Fatty acid	Glycerol	Glyceride	Water	25
3.	Glycerolysis	Fat	Glycerol	Partial glyceride	—	14
4.	Trans-phosphatidylation	Phosphatidyl-choline in ether	Glycerol in water	Phosphatidyl-glycerol	Choline in water	38

because water is confined only within the cells.

(2) Semi-dried mycellium cells packed in a column (35).

(3) Wet or semi-dried cells immobilized by entrapping in gels having an appropriate hydrophobicity-hydrophilicity balance (36) or by holding them in a sponge-like support.

As usual, the use of whole cells having the enzyme under consideration seems more economical than the use of the enzymes isolated from their cultures.

REACTOR SYSTEMS FOR MICROAQUEOUS BIOCATALYST

The reactor configurations for microaqueous biocatalyst may not differ much from those for conventional aqueous media. Batch reactors (stirred tank reactors) and continuous reactors such as packed bed (PBR), stirred tank (CSTR) and fluidized bed (FBR) are conceivable. Membrane reactors with either batch or continuous operation are also a choice. We have developed a novel bioreactor system having a microporous hydrophobic membrane. This is based on the principle of contacting two immiscible liquid phases at the membrane surface so the reactor is suitable for two-liquid phase biocatalysis system as shown by

Substrate 1 (phase 1) + Substrate 2 (phase 2)
biocatalyst
Product 1 (phase 1) + Product 2 (phase 2)

In this bioreactor, reaction and phase separation can be achieved simultaneously. The representations of the membrane in the bioreactor for the four cases are depicted schematically in Figure 4.

One of the advantages of the microaqueous bioreactor system is a lack of microbial contaminations. This should be emphasized in view of the bioreactor commercialization system.

The substrate solution fed to a microaqueous bioreactor system must contain a definite amount of water. If it is anyhdrous, biocatalysts in the reactor would lose moisture gradually, which would result in loss of activity. However, moisture content will be undesirable in terms of yield and selectivity of product. When water is formed through the reaction as in a condensation reaction), it must be removed by an appropriate method such as purging the liquid with bubbles of dry inert gas, reducing the pressure or adsorption with molecular sieves.

For monitoring and automatic control of moisture content, a moisture sensor is required. Several choices are available for this purpose: refractometer, electric conductivity meter, water activity meter, dielectric constant meter and dew point meter (thin aluminum film sensor). The last sensor can detect 0-400 ppm of free water dissolved in hydrocarbon on line. We have found that 0-5% moisture content of glycerol could be detected by an electric conductivity electrode (39). One of these sensors will enable control of the moisture content of an organic medium at an optimal level.

DISCUSSION

Research and development concerning enzyme applications to the lipids industry has not been as active as

those to the areas of carbohydrate, protein and amino acids. This is mainly due to the following facts:

(1) The number of high value-added products in the oleochemical industry is limited.
(2) The enzymes involved in the biotransformations for the lipids industry are costly.
(3) There are many difficulties in solving engineering problems because of heterogeneous and/or micro-aqueous natures of oleochemical bioreactions. Basic quantitative data, especially data on operational stability of enzymes, are scarce.

The future prospects in enzyme technology for the lipids industry will be widened by further endeavor in these three subjects. Fortunately, we are witnessing various and enthusiastic activities of R & D all over the world, and we are optimistic enough to see several new enzyme-catalyzed processes in the lipids industry in the near future.

REFERENCES

1. Machida, H., *Yukagaku* (in Japanese) *33*:691 (1984).
2. Mattson, F.H., and R.A. Volpenhein, *J. Lipid Res., 3*:281 (1962).
3. Sugiura, M., T. Oikawa, K. Hirano and T. Inukai, *Biochem. Biophys. Acta. 488*:353 (1977).
4. Kosugi, K., H. Suzuki, A. Kanbayashi, T. Funada, M. Akaike and K. Honda, Japanese Patent 57-38239 (1982).
5. Liu, W.-H., T. Beppu and K. Arima, *Agric. Biol. Chem. 37*:2493 (1973).
6. Gomi, K., Y. Ota and Y. Minoda, *Agric. Biol. Chem. 50*:2531 (1986).
7. Yoshimoto, T., K. Takahashi, H. Nishimura, A. Ajima, Y. Tamaura and Y. Inada, *Biotechnol. Letters 6*:337 (1984).
8. Yokozeki, K., S. Yamanaka, K. Takinami, Y. Hirose, A. Tanaka, K. Sonomoto and S. Fukui, *J. Appl. Microbiol. Biotechnol. 14*:1 (1982).
9. Makita, A., T. Nihira and Y. Yamada, *Tetrahedron Letters 28*:805 (1987).
10. Mukataka, S., T. Kobayashi and J. Takahashi, *J. Ferment. Technol. 63*:461 (1985).
11. Kosugi, K., H. Igusa and N. Tomizuka, *Abstract of Annual Meeting of Soc. Agric. Biol. Chem.*, Japan, 1985, p. 657.
12. Seino, H., T. Uchibori, T. Nishitani and S. Inamasu, *J. Am. Oil Chem. Soc. 61*:1761 (1984).
13. Nakata, H., K. Jinno, Y. Chikazawa, I. Morita, N. Nishio, M. Hayashi and S. Nagai, *Abstract of Annual Meeting of Soc. Ferment. Technol.*, Japan, 1986, p. 78.
14. Yamane, T., M.M. Hoq, S. Itoh and S. Shimizu, *J. Jpn. Oil Chem. Soc. 35*:625 (1986).
15. Hirohara, H., S. Mitsuda, E. Ando and R. Komki, *Biocatalysts in Organic Synthesis*, edited by J. Tramper, H.C. van der Plas and P. Linko, Elsevier, Amsterdam, 1985, p. 119.
16. Nishio, T., T. Chikano and M. Kamimura, *Agric. Biol. Chem. 51*:181 (1987).
17. Yamaguchi, S., T. Mase, S. Asada, European Patent 0191217 (1986).
18. Kyowa Hakko Kogyo Co., Ltd., Japanese Patent 58-51853 (1983).
19. Juneja, L.R., N. Hibi, T. Yamane and S. Shimizu, *Appl. Microbiol. Biotechnol.*, in press.
20. Matsuda, Y., T. Beppu and K. Arima, *Agric. Biol. Chem. 43*:1179 (1979).
21. Odera, M., K. Takeuchi and A. Toh-e, *J. Ferment. Technol. 64*:363 (1986).
22. Kugimiya, W., Y. Otani, Y. Hashimoto and Y. Takagi, *BBRC 141*:185 (1986).
23. Seilhamer, J.J., T.L. Randall, M. Yamanaka and L.K. Johnson, *DNA 5*:519 (1986).
24. Tsukagoshi, N., A. Yamada, M. Tomita, H. Ikezawa and S. Udaka, *Abstract of Annual Meeting of Soc. Agric. Biol. Chem.* Japan, 1987, p. 31.
25. Hoq, M.M., T. Yamane, S. Shimizu, T. Funada and S. Ishida, *J. Am. Oil Chem. Soc. 61*:776 (1984).
26. Macrea, A.R., *Biocatalysis in Organic Synthesis*, edited by J. Tramper, H.C. van der Plas and P. Linko, Elsevier Amsterdam, 1985, p. 195.
27. Barbaric, S., and P.L. Luisi, *J. Am. Chem. Soc. 103*:4239 (1981).
28. Klibanov, A.M., *Biocatalysis in Organic Media*, edited by C. Laane, J. Tramper and M.D. Lilly, Elsevier, Amsterdam, 1987, p. 115.
29. Ooshima, H., H. Mori and Y. Harano, *Biotechnol. Letters 7*:789 (1985).
30. Edited by Luisi, P.L. and B.E. Straub, *Reverse Micelle*, Plenum Press, New York (1984).
31. Han, D., and J.S. Rhee *Biotechnol. Bioeng. 28*:1250 (1986).
32. Takahashi, K., H. Nishimura, T. Yoshimoto, M. Okada, A. Ajima, A. Matsushima, Y. Tamaura, Y. Saito and Y. Inada, *Biotechnol. Letters 6*:765 (1984).
33. Martinek, K., and I.V. Berezin, *J. Solid-Phase Biochem. 2*:343 (1977).
34. Buckland, B.C., P. Dunnill and M.D. Lilly, *Biotechnol. Bioeng. 17*:815 (1975).
35. Bell, G., J.R. Todd, J.A. Blain, J.D.E. Patterson and C.E.L. Shaw, *Biotechnol. Bioeng. 23*:1703 (1981).
36. Fukui, S., A. Tanaka and T. Iida, *Biocatalysis in Organic Media*, edited by C. Laane, J. Tramper and M.D. Lilly, Elsevier, Amsterdam, 1986, p. 21.
37. Hoq, M.M., T. Yamane, S. Shimizu, T. Funada and S. Ishada, *JAOCS 62*:1016 (1985).
38. Lee, S.Y., N. Hibi, T. Yamane and S. Shimizu, *J. Ferment. Technol. 63*:37 (1985).
39. Yamane, T., J.S. Rhee, Y. Ohta and S. Shimizu, *J. Jpn. Oil Chem. Soc. 36*, in press.