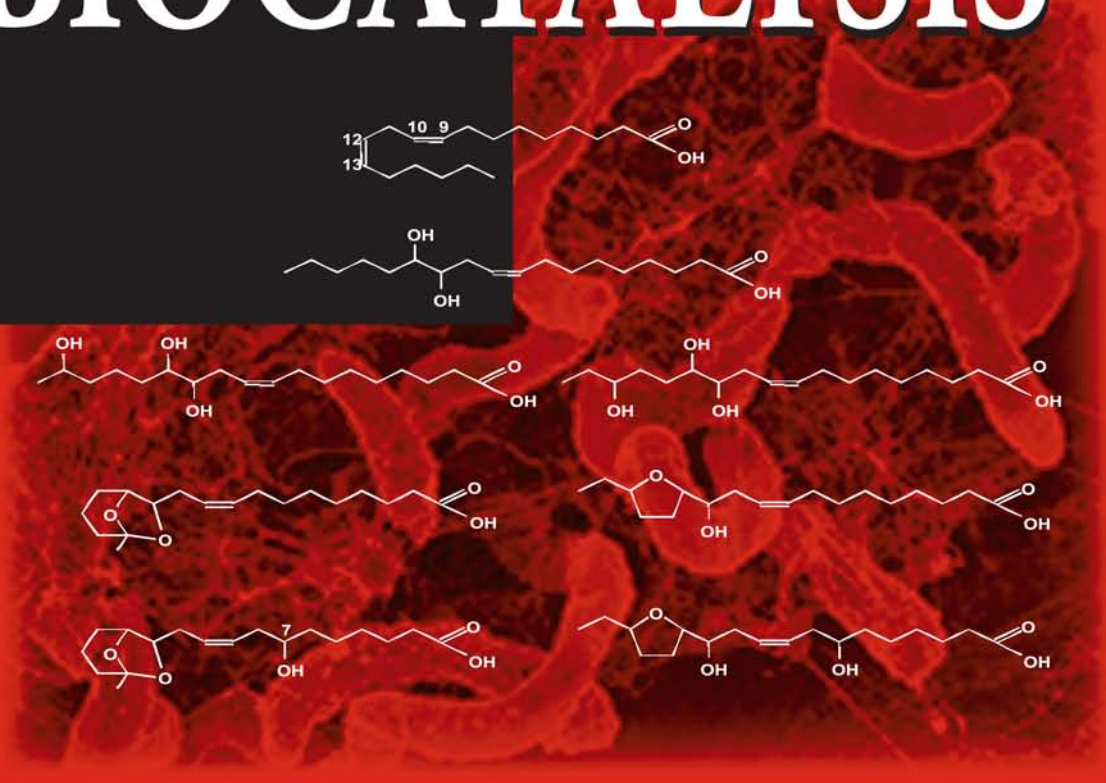
Handbook of

INDUSTRIAL BIOCATALYSIS



edited by

Ching T. Hou

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Preface

Pasteur initiated the scientific study of fermentation. When it was appreciated that microbes catalyzed the chemical reactions used in the production of wine, cheese, and other foods, it became reasonable to expect that they could be put to work in the manufacture of chemicals for industry. Beginning in the late 1960s, both chemical industry and government agencies moved away from petroleum-based nonrenewable feedstocks for production of commodity and specialty chemicals to emphasize the use of renewable resources such as carbohydrates, oils, and fats. In addition, in recent years, the oil and fat industry has started to emphasize quality rather than quantity of oil and fat for human consumption. The definition of biocatalysis includes enzyme catalysis, biotransformation, bioconversion, fermentation, and biotechnology. It deals not only with one-step catalytic reaction, but also includes many sequential reaction steps to produce a product. Biocatalysis is a bioprocess including molecular manipulation of enzymes, the reaction itself, and product recovery.

This handbook was assembled with the intent of bringing together all types of industrial biocatalysis. It consists of 29 chapters whose authors are the world's most famous and most active researchers in this field. The basic information and theoretical considerations for a specific topic area or a specific biotechnological application are provided, and every effort has been made to include the current information. This is the most up-to-date handbook on industrial applications of biosciences and biotechnology.

The book is divided into three sections. The first describes the world's newest biotechnology, including bioprocesses on producing potential industrial products from hydrophobic substrates such as oils and fats. The products include healthy food, nutritional supplements, neutraceuticals, chiral synthons, specialty chemicals, surfactants, biopolymers, and antimicrobial and physiologically active agents. Metabolic pathways and function of polyunsaturated fatty acids (PUFAs) in mammals as well as transgenic production of long-chain PUFA-enriched oils are presented by Vic Huang of Abbott Labs. J. Ogawa and S. Shimizu of Kyoto University, Japan introduce examples of bioprocess development that started from process design stemming from the discovery of the unique metabolites hydrantoin, cyclic imide, microbial nucleosides, and conjugated fatty acids. Tsuneo Yamane of Nagoya University, Japan describes biocatalysis in microaqueous organic media including lipase, esterase, protease, and so on. Rolf Schmid of the University of Stuttgart, Germany contributes a chapter on biocatalysts for the epoxidation and hydroxylation of fatty acid alcohols including fermentation/bioreactor process using oxygenases. Kumar Mukherjee of Munster, Germany uses lipase specificities toward fatty acids and their derivatives—alcohols, alkanethiols, and sterols—to enrich n-3 and n-6 PUFAs, very long-chain monounsaturated fatty acids, other acids, and alcohols. K. Lee and J. Shaw of the Academia Sinica, Taiwan describe a successful story about recombinant *Candida rugosa* lipase with improved catalytic properties and stabilities. Ching Hou of NCAUR, USDA shares his discovery of novel oxygenated fatty acids and their potential industrial application from vegetable oils. Yuji Shimada of Osaka Municipal Technical Research Institute (OMTRI) describes many examples of the application of lipase to industrial-scale purification of oil- and fat-related compounds including production of PUFA-enriched oil, conversion of waste edible oil to biodiesel fuel, and purification of tocopherols, sterols, and steryl esters.

Casmir Akoh's group at University of Georgia give a thorough overview on lipase modification of lipids. Shuji Adachi of Kyoto University describes lipase-catalyzed condensation in an organic solvent including substrate selectivity of lipase for various carboxylic acids and continuous production of esters such as acyl ascobates. Naoto Yamada of Kao Corporation, Japan presents enzymatic production of diacyl glycerol and its beneficial physiological function. Diacyl glycerol functions like oil for all food preparation including frying, yet prevents the accumulation of body fat. Satoshi Negishi of Nisshin OilliO, Japan Ltd., describes the use of nonimmobilized lipase for industrial esterification of food oils. M. Hosokawa and K. Takahashi of Hokkaido University, Japan describe their design for industrial production of polyunsaturated phospholipids and their biological functions (health application). Dan Solaiman's group at ERRC, USDA presents the production of biosurfactants by fermentation of fats, oils, and their coproducts including microbial glycolipids, sophorolipids, and rhamnolipids. Tsunehiro Aki of Hiroshima University, Japan describes current metabolic engineering on development and industrialization of transgenic oils. Tom Foglia's group at ERRC, USDA presents lipase-catalyzed production of structured lipids as low-calorie fats. Toro Nakahara of National Institute of Advanced Industrial Science and Technology, Japan describes microbial polyunsaturated fatty acid production including lipids from bacteria, yeasts, and fungi. Gudmundur Haraldsson of University of Iceland describes lipase-catalyzed production of EPA or DHA containing triacylglycerols derived from fish oil. Rich Ashby et al. of ERRC, USDA describe biopolyesters derived from the fermentation of renewable resources including polylactic acid, polytrimethylene terephthalate, and polyhydroxyalkanoates.

The second section of the handbook deals with producing value-added products from carbohydrate substrates. The scope includes ethanol production, oligosaccharides and glycosides, utilization of hemicelluloses, and carbohydrate-active enzymes. Hajime Taniguchi of Chubu University, Nagoya, Japan presents carbohydrate-active enzymes for the production of oligosaccharides including enzymes for most of the oligosaccharides, such as isomalto-, nigero-, gentio-, fructo-, galacto-, chitosan-, and xylo-oligosaccharides, trehalose, palatinose, trehalulose, and lactosucrose. Peter Biely and Gregory Cote of NCAUR, USDA describe a special group of carboxylic acid esters that operate on highly hydrated substrates such as partially acylated polysaccharides. H. Nakano and S. Kitahata of OMTRI, Osaka, Japan describe industrial-scale production of various cyclodextrins from starch by cyclodextrin glucotransferase. Bruce Dien of NCAUR, USDA contributes a review on converting herbaceous energy crops to bioethanol with emphasis on pretreatment processes. Badal Saha of NCAUR, USDA describes enzymes as biocatalysts for conversion of lignocellulosic biomass to fermentable sugars. Its substates include various agricultural residues such as corn fiber, corn stover, wheat straw and rice straw.

The third section deals with other potential industrial bioprocesses. Gregory Zeikus of Michigan State University describes applications of bioelectrocatalysis for synthesis of chemicals, fuels, and drugs. Ramesh Patel of Bristol-Myers Squibb, New Jersey uses bioprocesses to synthesize chiral intermediates for drug development including anticancer drugs (paclitaxel, orally active taxane, deoxyspergualin and antileukemic agent), antiviral drugs (BMS-186318, HIV protease inhibitor, Atzanavir, crixivan), reverse transcriptase inhibitor (Abacavir, Lobucavir), antihypertensive drugs (angiotensin converting enzyme inhibitor, captopril, monopril), neutral endopeptidase inhibitors, squalene synthase inhibitors, thromboxane A2 antagonist, calcium channel blockers, potassium channel blockers, β-3-receptor agonists, melatonin receptor agonists, anti-Alzheimers drugs, anti-infective drugs, respiratory and allergic diseases, acyloin condensation, enantioselective and enzymatic deprotection. R. Sakaguchi and L. Junejia of Taiyo Kagaku Company, Japan present a novel nutrition delivery system that also preserves the stability of food components and flavor. Sima Sariaslani et al. of Dupont Central Research & Development, Experimental

Station, Wilmington, Delaware describe pathway engineering for production of trans-para-hydroxycinnamic acid from renewable resources. Finally, Chiara Schiraldi and Mario De Rosa of Second University of Naples, Italy describe industrial applications of extremophiles.

The *Handbook of Industrial Biocatalysis* is intended for teachers, postdoctorate and graduate students, and industrial scientists who conduct research in biosciences and biotechnology. It is therefore expected that it will serve as a valuable reference for researchers in the field and as a complementary text for graduate-level reading and teaching.

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Contents

1

Enzymes for the Transgenic Production of Long-Chain Polyunsaturated Fatty Acid-Enriched Oils

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Amanda E. Leonard

1.1 Introduction

Polyunsaturated fatty acids (PUFAs) are fatty acids of 18 carbons or more in length with two or more methylene-interrupted double bonds in the *cis* position. Depending on the position of the first double bond proximate to the methyl end of fatty acids, PUFAs can be designated by the omega (ω-) or (n-) number, and classified into two major groups: ω6 (or n-6) and ω3 (or n-3) families. For example, linoleic acid (LA) in the n-6 family is designated as C18:2n-6 to indicate that this fatty acid contains 18 carbons and two double bonds, with the first double bond at the sixth carbon from the methyl end. Similarly, α-linolenic acid (C18:3n-3) in the n-3 family has 18 carbons and three double bonds, with the first double bond located at the third carbon from the methyl end (Figure 1.1).

1.1.1 Metabolism of Linoleic Acid and α-Linolenic Acid

Animals are incapable of synthesizing both linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3) due to lack of the Δ12 and Δ15-desaturases. However, animals can metabolize these two fatty acids obtained from the diet to form longer and more unsaturated PUFAs to meet the metabolic needs. Since these two fatty acids must be obtained from the diet, they are considered to be essential fatty acids.

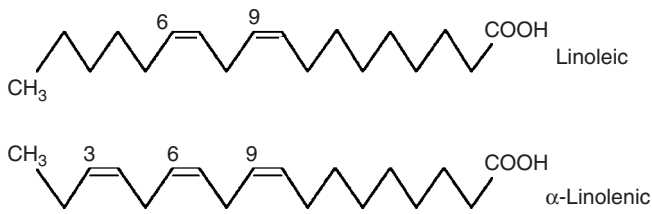

FIGURE 1.1 Nomenclature of polyunsaturated fatty acids

In most eukaryotes, the biosynthesis of PUFAs involves a complex series of desaturation and elongation steps (Figure 1.2).[1] For example, eicosapentaenoic acid (EPA, C20:5n-3) is synthesized from ALA by the addition of a double bond by a Δ6-desaturase to form stearidonic acid (SDA, C18:4n-3); the elongation of SDA to form ω3-eicosatetraenoic acid (ω3-ETA, C20:4n-3); and the addition of another double bond by a Δ5-desaturase to form EPA.[2] The formation of DHA from EPA occurs via different mechanisms in eukaryotes. In higher eukaryotes like mammals, EPA is elongated to ω3-docosapentaenoic acid (ω3-DPA, C22:5n-3), which is further elongated to ω3-tetracosapentaenoic acid (ω3-TPA, C24:5n-3). ω3-TPA is then desaturated by a Δ6-desaturase to generate ω3-tetracosahexaenoic acid (THA, C24:6n-3) in the microsomes. The THA is then transported to the peroxisomes, where it is β-oxidized to form DHA.[3,4] However, in lower eukaryotes like the *thraustochytrid* sp., EPA is elongated to ω3-DPA followed by the addition of a double bond directly to ω3-DPA, by Δ4-desaturase, to generate DHA.[5] The synthesis of long-chain n-6 PUFAs from LA occurs via similar alternating desaturation and elongation steps (Figure 1.2).

1.1.2 Function of PUFAs

In mammals, PUFAs are important structural components that modulate membrane fluidity and permeability.[6] For example, docosahexaenoic acid (DHA, C22:6n-3), a long-chain n-3 PUFA, and arachidonic

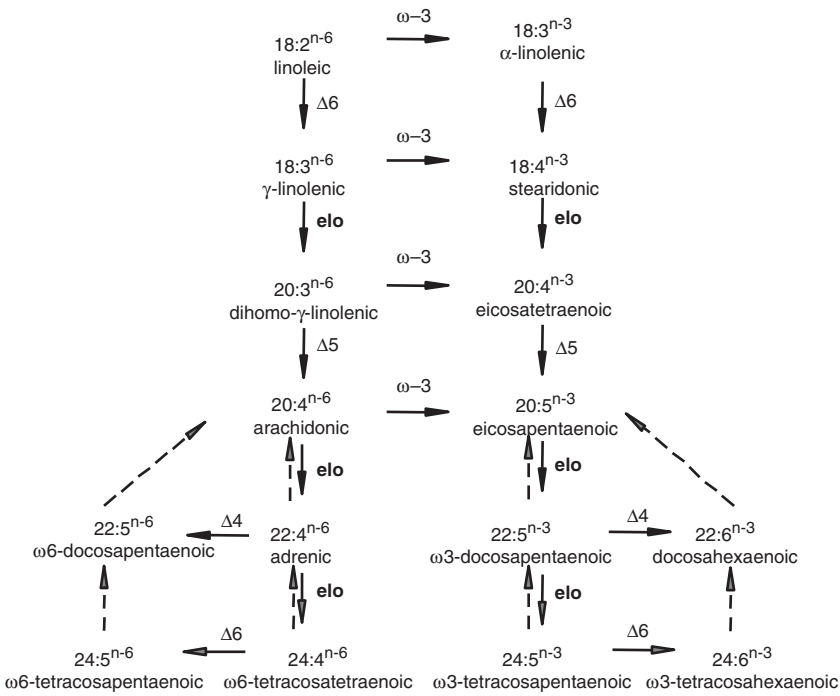

FIGURE 1.2 Metabolic pathway of linoleic and α–linolenic acids

acid (AA, C20:4n-6), a long-chain n-6 PUFA, are found in high proportions in neuronal tissues such as brain and retina, and testis.[7,8] PUFAs also serve as precursors for a number of biologically active molecules, such as eicosanoids, growth regulators, and hormones.[9] In mammals, eicosanoids such as prostaglandins, leukotrienes, and thromboxanes act locally on various signaling mechanisms that have effects on numerous cellular functions including chemotaxis, vascular permeability, inflammation, vasoconstriction, etc.[9] Thus, PUFAs have profound effects on various physiological processes, such as cognitive function, and immunosuppressive and anti-inflammatory actions.[10]

1.1.3 PUFA Production and Chronic Diseases

The availability of long-chain PUFAs depends on the diet providing the precursors, such as LA and ALA, and the activity of enzymes involved in the biosynthesis.[11] Generally, only a small proportion of dietary linoleate and α-linolenate (3.0% and 1.5%, respectively) can be converted to longer PUFAs, with the rest getting β-oxidized to provide energy.[12] The slow formation of PUFAs can be further compromised by various nutritional and hormonal factors.[13] For example, the activity of the Δ6-desaturase *in vivo* is regulated by certain dietary components, age, and hormones.[14] In addition, in chronic diseases like cancer and diabetes, altered expression levels of this enzyme in different tissues have been observed.[15–21] The activity of the Δ5-desaturase is also regulated by diet,[22] and altered expression levels of this enzyme have been associated with various disease conditions, including eye disorders, Alzheimer's disease, and diabetes.[23–25] Thus, low levels of long-chain PUFAs have been associated with disorders of the neurovisual development and other complications of premature birth[26–29] as well as implicated with incidence of chronic diseases, such as diabetes, hypercholesterolemia, rheumatoid arthritis, autoimmune disorders, Crohn's disease, and cancer.[13,30,31]

Clinical evidence has shown that dietary supplementation of PUFAs, such as γ-linolenic acid (GLA, C18:3n-6) and EPA/DHA, can exert the anti-inflammatory, antithrombotic, and antiarrhythmic activities, and provide beneficial effects on glucose and lipid metabolism.[32–38] These findings have received much attention from food manufacturers and pharmaceutical companies, as well as the general public. As a result, sales of these long-chain PUFAs as supplements and fortified foods, such as "DHA plus" eggs, and DHA- and AA-fortified infant formulas have drastically increased in the past few years.

1.2 Commercial Sources of PUFAs

1.2.1 Fish Oil

Currently, the richest sources of EPA and DHA are derived from fish oils obtained from mackerel, herring, salmon, and sardines. Fish obtain these long-chain PUFAs (LC-PUFAs) from the LC-PUFA-rich microalage and phytoplankton they consume. Commercially, fish oils are available in the form of gelatin capsules or oily preparations. Fish oils obtained from fish liver (e.g., cod liver oil) are rich in vitamin A and D, and contain lower amounts (13% to 22%) of EPA/DHA. In contrast, fish oils obtained from fish bodies (e.g., salmon oil) contain 20% to 30% EPA and DHA and are low in cholesterol and vitamin A and D(reviewed in reference 39). These fish oils are used to enrich food products, animal feeds, and aquaculture feeds, in addition to their use for direct human consumption. These oils are not very economical due to the high costs involved in processing, refining, and stabilizing the oils. In addition, the effects of overfishing and the vulnerability of global fisheries to environmental and climatic changes have resulted in decreased yields, which has further driven up the cost of fish oils.[40]

1.2.2 Plant Oils

Plants do not produce EPA or DHA. However, certain plants can produce oils rich in GLA or ALA, and serve as the current commercial sources of these PUFAs. Plant oils derived from borage, evening primrose, and black currant are found to be rich in GLA (reviewed in reference 41). Borage oil is derived from the seed of *Borago officinalis* and contains ~23% GLA. Evening primrose oil obtained from the seed of

Oenothera biennis contains ~9% GLA. Black currant oil derived from the seeds of *Ribes nigrum* is attractive in that it contains 12% ALA in addition to 16% GLA. However, these oils are expensive due to high costs of cultivation, seed harvesting, and oil extraction. Linseed oil (flax) is the richest source of ALA (57% of total fatty acids). Dietary ALA can also be obtained from oils of canola, soybean, wheat germ, and walnut.

1.2.3 Microbial Oils

LC-PUFAs can also be extracted from single cell organisms like microalgae and fungi that can be commercially cultivated in fermentors (heterotrophic producers), or in photoautotrophic cultivation systems.[42,43] Oleagenous fungi such as *Mortierella alpina* accumulate up to 40% (by wt.) oil, of which up to 40% represents AA.[44] Thus, this organism is commercially used in production of AA.[45] Diatoms such as *Nitzschia*, a good producer of EPA, and dinoflagellates such as *Crypthecodinium cohnii* that produce large amounts of DHA, are currently commercially utilized for the production of EPA- and DHA-enriched oils.[42] Currently, marine protists such as the *Thraustochytrids* that make large amounts of DHA are being explored for their potential to make DHA-rich oils for human consumption.[42,46,47] The costs of production of these oils are still considerably high, and further work involving strain-improvement and cultivation-optimization need to be carried out to make these oils more economical.

1.3 Transgenic Production of PUFAs

Although LC-PUFAs-enriched oils are commercially available as discussed in Section 1.2, the cost of production of these oils is generally very high, and the sources of supplies are often unreliable or nonrenewable. The increase in demand for these PUFAs has raised interest in obtaining these from alternate sources that are more economical and sustainable. One attractive option is the production of LC-PUFA-enriched vegetable oils in oilseed crops like soybean, canola, and others. Since these plants can only synthesize 18-carbon (C_{18}) PUFAs such as LA and ALA, it is necessary to genetically manipulate their lipid biosynthetic pathways in order to produce long-chain PUFAs such as GLA, EPA, and DHA. For this, genes encoding the enzymes involved in LC-PUFA biosynthesis need to be isolated from LC-PUFA-rich organisms, and transgenically expressed in oilseed crops. These include the various desaturases and elongases outlined in Figure 1.3.

Desaturases are enzymes that catalyze the addition of a double bond (unsaturation) in a fatty acyl chain (reviewed in reference 48). These enzymes are specific to the location, number, and stereochemistry of double bonds already present in fatty acids.[49] In addition, they have specificity for their substrate carriers, which can be CoA-linked substrates, acyl carrier protein (ACP)-linked substrates, or glycerolipid-linked substrates.

Elongases are enzymes that are responsible for the addition of two-carbon units to the carboxyl end of a fatty acid chain. In both plants and animals, the elongase system is composed of four enzymes: a condensing enzyme, β-ketoacyl CoA synthase (also referred to as elongase), β-ketoacyl CoA reductase, β-hydroxyacyl CoA dehydrase, and *trans*-2-enoyl CoA reductase. Fatty acid elongation is initiated by the condensation of malonyl-CoA with a long chain acyl-CoA, yielding a β-ketoacyl-CoA in which the acyl moiety has been elongated by two carbon atoms. This reaction is catalyzed by the condensing enzyme β-ketoacyl CoA synthase (also referred to as "elongase"). β-ketoacyl-CoA is then reduced, dehydrated, and further reduced by the remaining enzymes in the system to yield the elongated acyl-CoA.[50] The condensing enzyme (elongase) is known to be the rate-limiting enzyme,[50–52] which regulates the systems specificity for the fatty acid substrate in term of chain length and degree of unsaturation. The elongases involved in the elongation of LC-PUFAs are distinct from the plant and yeast elongases that are involved in the elongation of saturated or monounsaturated fatty acids (reviewed in reference 53).

The following sections will focus on the characterization and transgenic expression of nonmammalian sources of LC-PUFA biosynthetic genes, with applications for the production of PUFA-enriched transgenic oils. Enzymes known to exist in all plants, such as the Δ9- and the Δ12-desaturase that are involved in LA production (Figure 1.3), will not be described here since these enzymes are highly active in native

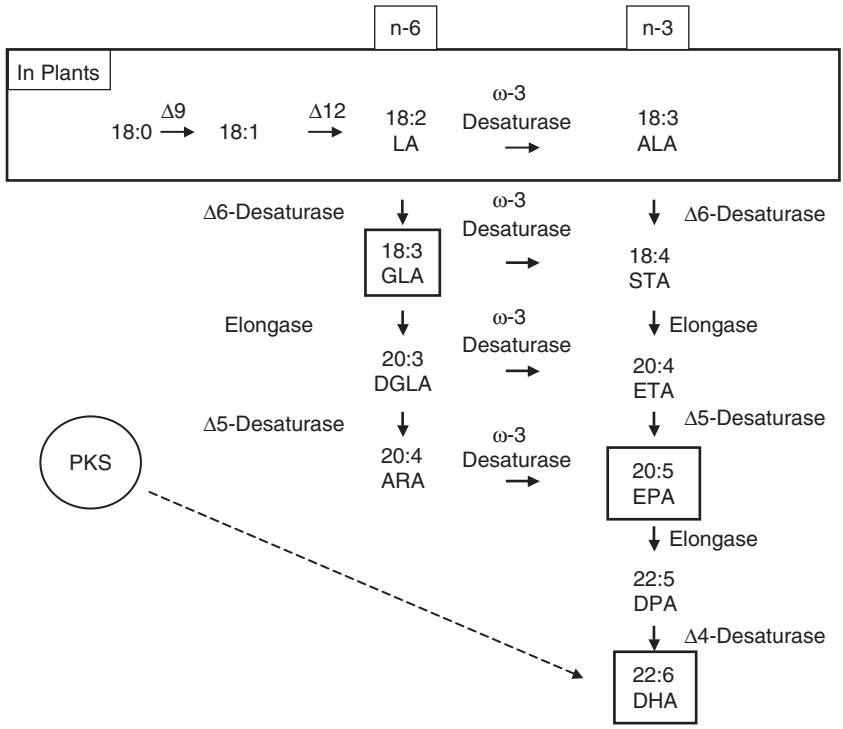

FIGURE 1.3 Pathway and transgenic production of LC–PUFAs

oil-seed crops. Although the discussion will focus on production of transgenic plant oils, it should be noted that these enzymes also have applications for the production of transgenic oils in other oleaginous organisms such *Yarrowia* and *Rhodotorula*.

1.3.1 Enzymes Required for Transgenic GLA Production

Commercially available sources of GLA-enriched oils from borage, evening primrose, and black currant[54] are not economical for large-scale production. Hence efforts are ongoing to transgenically produce GLA in an oil-seed crop. The key step in GLA production involves the insertion of a double bond between carbon #6 and #7 of LA to generate GLA (Figure 1.3). This reaction is mediated by the Δ6-desaturase, a membrane-bound enzyme located in the endoplasmic reticulum. This enzyme is classified as a "front-end" desaturase because it is capable of introducing a double bond between a preexisting double bond and the "front" (carboxyl end) of the fatty acid. It also contains a fused cytochrome b_5 domain at the N-terminus, which plays a role as an electron donor during desaturation, and this domain is essential for activity.[55]

Δ6-desaturases has been isolated from several fungal, plant, microbial, and mammalian sources that produce GLA.[48] Some of these include *M. alpina,*[56] *Mucor rouxii,*[57] *Phytium irregulare,*[58] *Physcomitrella patens,*[59] borage,[60] *Echium* plant sp.,[61] *Primula* sp.,[62] and *Synechcocystis.*[60,63] Most of these Δ6-desaturases are thought to act exclusively on the phospholipid-linked LA substrate.[44,64] In contrast, the Δ6-desaturases from mammalian sources are thought to recognize CoA-linked LA substrates.[65]

Most of the Δ6-desaturases have been functionally expressed in yeast, and many have also been tested in plants.[56,58,60,61,66] In addition, some Δ6-desaturases have also been expressed in oil-seed crops, resulting in production of GLA in seeds. In the early study conducted in our laboratory, expression of the *M. alpina* Δ6-desaturase in a low-linolenic acid variety of *Brassica napus* resulted in the generation of low amounts (~13%) of GLA in addition to the production of an uncommon fatty acid, Δ6,9-18:2.[67,68] This uncommon

fatty acid was derived by the desaturation of oleic acid (OA, 18:1) by the Δ6-desaturase. This problem was resolved by coexpressing the *M. alpina* Δ6-desaturase with its Δ12-desaturase, an enzyme that converts oleic acid to LA (Figure 1.3). This resulted in the accumulation of >40% GLA in the transgenic canola oil, with no detectable Δ6,9-18:2.[67,68] Subsequent studies have been carried out using the *Phytium irregulare* Δ6-desaturase in *Brassica juncea*, and have also resulted in successfully generation of 25% to 40% GLA in the transgenic seed.[58] This oil, however, was also found to contain 2% to 10% stearidonic acid (SDA, 18:4n-3) in addition to the uncommon fatty acid Δ6,9-18:2.[58]

1.3.2 Enzymes Required for Transgenic ARA and EPA Production

The production of the C_{20}-PUFA arachidonic acid (ARA, 20:4n-6) from LA involves the desaturation of LA to GLA (see Section 1.3.1.), followed by the elongation of GLA to dihomo-γ-linolenic acid (DGLA, 20:3n-6), and a subsequent desaturation of DGLA to ARA (Figure 1.3). Thus three major enzymes are involved in this process: a Δ6-desaturase, a C_{18}-PUFA-specific elongase, and a Δ5-desaturase. These same enzymes also function on the n-3 pathway intermediates and are thus also involved in the biosynthesis of EPA (20:5n-3) (Figure 1.3). Since Δ6-desaturase has just been discussed in the previous section (Section 1.3.1), this section will focus only on the C_{18}-PUFA-specific elongase and the Δ5-desaturase.

The first C_{18}-PUFA-specific elongase to be isolated was identified in our laboratory from the ARA-rich fungus, *Mortierella alpina.*[67] This enzyme when tested in baker's yeast specifically recognized and elongated the n-6 and n-3 C_{18}-PUFA substrates, GLA and stearidonic acid (SDA, 18:4n-3), respectively, whereas it demonstrated no activity on monounsaturated or saturated fatty acid substrates.[69–72] Enzymes with similar elongating activity have been isolated from *Caenorhabditis elegans,*[73] *Physcomitrella patens,*[74] and from the marine protist, *Thraustochytrium* sp.[75] In addition, several PUFA-specific elongases have been isolated from mammalian sources.[53] All these PUFA-specific elongases contain five hydrophobic regions predicted to be membrane-spanning regions. In addition, they contain a highly conserved histidine-box motif composed of three histidine residues (HXXHH) embedded in the fourth membrane spanning region.[76–78] These features distinguish them from the plant elongases that are involved in elongation of very long chain saturated and monounsaturated fatty acids, but not PUFAs.[79,80] In addition, the C_{18}-PUFA-specific elongases are thought to recognize CoA-linked substrates[64] as opposed to the plant elongases that recognize acyl carrier protein (ACP)-linked substrates.

The Δ5-desaturase catalyzes the final step in the production of the C_{20}-PUFAs, ARA and EPA. This enzyme is so called because it introduces a double bond at the Δ5-position of the fatty acid. This desaturase is also considered a "front-end" desaturase and shares all the conserved structural characteristics displayed by other front-end desaturases such as the Δ6-desaturase. Δ5-desaturase genes have been identified from fungi and algae such as *Mortierella alpina.*[81,82] *Thraustochytrium* sp.,5 and *Phaeodactylum tricornutum,*[83] and these have been functionally characterized in yeast. Additional Δ5-desaturases have been identified from *C. elegans,*[84] human,[85] and rat.[86] All the Δ5-desaturases identified so far are capable of desaturating both the n-6 and n-3 PUFA substrates, DGLA and eicosatetraenoic acid (ETA, 20:4n-3), respectively. Coexpression of the *M. alpina* Δ5-desaturase along with its C_{18}-PUFA-specific elongase in yeast, in the presence of exogenously supplied free fatty acid substrate, resulted in the production of significant amount of ARA or EPA.[69] When introduced into a low linolenic variety of *Brassica napus*, the *M. alpina* Δ5-desaturase was capable of desaturating oleic acid (OA, 18:1n-9) to taxoleic acid (Δ5,9-18:2), and LA to pinolenic acid (Δ5,9,12-18:3).[81] This demonstrates its functionality in desaturating fatty acids at the Δ5-position in higher plants, even in the absence of its preferred PUFA substrates.

Since plant oils often contain LA and ALA, the transgenic expression of the Δ6-desaturase, C_{18} PUFA-specific elongase, and Δ5-desaturase would result in ARA and EPA. For production of an EPA-enriched transgenic oil that does not contain ARA, it is necessary to shunt the n-6 PUFA metabolites to their n-3 counterparts. This reaction is catalyzed by a group of enzymes designated the ω3-desaturases. These enzymes are absent from mammals, but can be found in some plants, lower eukaryotes, and cyanobacteria.[87] These enzymes are so called because they introduce a double bond at carbon atom #3 when counted from the methyl- (ω-) end of the fatty acyl chain. ω3-desaturases share all the conserved features

present in other membrane-bound desaturases. These include the presence of two long stretches of hydrophobic residues that traverse the lipid bilayer, and three histidine-rich motifs proposed to be involved in the ligation of iron atoms within the active site domain of these enzymes.[88] This protein also contains the C-terminal motif, KAKSD, proposed to be a retention signal for many transmembrane proteins in the ER.[89] Unlike the front-end desaturases, the ω3-desaturases do not contain a fused cytochrome b_5 domain at their N-terminus, and are thus assumed to interact with a separate cytochrome b_5 for their activity.

All plant and cyanobacterial ω3-desaturases act exclusively on the C_{18}-PUFA substrate, LA, converting it to ALA (Figure 1.3). Although many oilseed crops do contain endogenous ω3-desaturases, these enzymes do not efficiently convert LA to ALA as evidenced by a high LA-to-ALA ratio in their total lipids.[54] Thus for transgenic EPA production, it might be necessary to transgenically express ω3-desaturases with high enzymatic activity, in order to increase the shunt through the n-3 PUFA pathway. A novel ω3-desaturase was identified from *C. elegans* that was capable of recognizing multiple n-6 PUFA substrates, which included the C_{18}-PUFAs, LA and GLA, as well as the C_{20}- PUFA, DGLA.[90,91] This enzyme was found to be functional in plants[90] and thus has potential applications for transgenic EPA production. In addition, a novel fungal ω3-desaturase was recently identified in our laboratory that could specifically convert ARA to EPA, and this enzyme was found to be functional in an oilseed crop.[92] This enzyme has applications for the removal of ARA from EPA- and DHA-enriched transgenic oils, by converting ARA to EPA. This is especially necessary if the transgenic oils are targeted for adult nutrition, since ARA is a precursor for synthesis of proinflammatory eicosanoids that are implicated in inflammatory and cardiovascular disease development.

Thus the transgenic production of EPA will be contingent on the success in coexpressing at least four different enzymes in a single system. Coexpression of three of the PUFA biosynthetic enzymes, the Δ6-desaturase, the PUFA-specific elongase, and the Δ5-desaturase, has been successfully demonstrated in reconstituted baker's yeast, resulting in ARA and EPA production when their respective substrate, LA or ALA, was supplied exogenously.[73,83] However, the yields of ARA or EPA obtained in these studies were poor. Similar results were reported by Domergue et al.[64] in their attempt to coexpress the Δ6- and Δ5-desaturase from *P. tricornutum* along with the C_{18}-PUFA elongase from *P. patens* in transgenic linseed. From these experiments, it appears that there is an accumulation of the Δ6-desaturated fatty acids in the membrane fractions and almost none in the acyl-CoA pool. It is thought that the Δ6-desaturase from most fungi and algae function on phospholipid-linked (mainly phosphatidylcholine-linked) LA or ALA substrates. However, the consecutive step is catalyzed by a PUFA-specific elongase that requires its substrates to be present in the acyl-CoA pool. Thus it appears that a bottleneck in the pathway is created due to the inefficient transfer of the Δ6-desaturated products from the phospholipids to the acyl-CoA pool.[64] To overcome this bottleneck, it might be necessary to identify and coexpress additional enzymes that are involved in the transfer of phospholipid-linked PUFAs to the acyl CoA pools.

1.3.3 Enzymes Required for Transgenic DHA Production

The pathway for the biosynthesis of DHA varies among different groups of organisms. In lower eukaryotes such as DHA-rich algae and fungi, it is thought that DHA is synthesized from EPA in a two-step process (Figure 1.3): a) An initial elongation step that is catalyzed by a C_{20}-PUFA recognizing elongase, wherein EPA is elongated to ω3-DPA; b) The desaturation of ω3-DPA, catalyzed by a Δ4-desaturase, resulting in the generation of DHA. Thus the production of transgenic DHA would involve coexpressing these two new genes in addition to the four previously described genes (Section 1.3.2.) needed for EPA production.

The only enzymes identified to date that can recognize and elongate C_{20}-PUFAs are from mammals.[53] Expression of these genes in baker's yeast revealed that some of them could elongate C_{18}-PUFAs in addition to C_{20}- and C_{22}-PUFAs, whereas others had a specificity for C_{20}- and C_{22}-PUFA substrates. None of these enzymes acted on monounsaturated fatty acids or saturated fatty acid substrates.[53] Attempts are currently under way to identify similar C_{20}-PUFA recognizing elongases from lower eukaryotes, which can then be used for the production of transgenic oils.

The first Δ4-desaturase to be described was identified from a marine protist, *Thraustochytrium*, which produces copious amounts of DHA.[5] Like the Δ5- and Δ6-desaturase, the Δ4-desaturase is also a front-end desaturating enzyme capable of introducing a double bond at carbon # 4 of ω3-DPA. In addition, this enzyme can also desaturate the n-6 substrate adrenic acid (ADA, C22:4n-6) to generate ω6-DPA (C22:5n-6) (Figure 1.3). Expression of the Δ4-desaturase gene in a oilseed crop, *Brassica juncea*, in the presence of exogenously supplied ω3-DPA substrate resulted in the production of 3–6% DHA in the leaves, stems, and roots of the transgenic *Brassica*.[5] A new Δ4-desaturase was recently described from *Euglena gracilis*, and this enzyme was found to desaturate C_{16}-fatty acids in addition to C_{22}-PUFAs.[93]

1.3.4 Alternate Enzymes for Transgenic EPA/DHA Production: The PKS System

LC-PUFA biosynthesis in bacteria such as *Shewanella* and *Vibrio* occurs via a novel polyketide synthase (PKS) pathway.[94–96] This pathway is thought to be analogous to the fatty acid synthase (FAS) pathway involved in the synthesis of short-chain fatty acids.[97] This system was also identified to be involved in DHA production in the marine eukaryote, *Schizochytrium*.[98] Here, PUFA production is thought to be initiated by the condensation between a short-chain starter unit like acetyl CoA, and an extender unit like malonyl CoA. The four-carbon acyl chain formed is covalently attached to an acyl carrier protein (ACP) domain of the PKS complex, and goes through successive rounds of reduction, dehydration, reduction, and condensation, with the acyl chain growing by two carbon units with each round. A novel dehydratase/isomerase has been proposed to exist in this PKS complex that can catalyze trans- to cis-conversion of the double bonds, thus generating double bonds in the correct position of EPA and DHA.[98] Genes involved in this PKS system exist sequentially on long (20–30 Kb) open reading frames (ORFs), and the identity of every region within these ORFs are still unknown.[95,96,98] Expression of the *Shewanella* PKS system in an *E coli* or *Synechococcus* expression system resulted in EPA production, although the levels of EPA produced were low.[94,98] Although none of these PUFA-PKS genes have been expressed in plants as yet, this system offers an attractive alternative to the desaturase/elongase system for the production of EPA/DHA-enriched transgenic oils.

1.4 Conclusion and Future Perspectives

Fatty acids are critical for the normal development and function of all organisms, and in particular, very long chain PUFAs are necessary for the health and maintenance of higher organism such as mammals. Although the biosynthetic pathway of long-chain PUFAs has been studied for a while, detailed biochemical analysis of the enzymatic machinery has been especially hard. This is because the extreme hydrophobicity of the desaturases and elongases creates difficulties during purification of large amounts of these enzymes that are required for biochemical characterization. However, much progress has been made over the last few years in the cloning and identification of genes encoding the PUFA biosynthetic enzymes from different organisms. These findings have important biotechnological applications. For example, these genes can be used in the production of PUFA-rich transgenic oils to meet the increasing demands of the chemical, pharmaceutical, and nutraceutical industry for therapeutic and prophylactic use. Advances in understanding gene regulation in PUFA biosynthesis will also impact the single-cell oil industry. This in turn will affect the marine fish-farming industry, which depends on PUFAs generated by microalgae and fungi for enhancing the levels of PUFAs in fish.

However, some challenges still need to be overcome with respect to transgenic production of PUFAs. Although the overall scheme of PUFA biosynthesis appears to be common for most organisms, enzymes from different organisms may not necessarily be compatible. This may result in unanticipated bottlenecks in the pathway leading to lower yields of LC-PUFAs. This had already been observed during preliminary studies on transgenic EPA production (Section 1.3.2). In addition, many unknowns still need to be addressed with respect to coexpressing several genes from multiple sources simultaneously. It is still early to predict if the transgenic oils thus generated will have a fatty acid profile reflective of natural fish

oil, without the accumulation of unwanted fatty acid byproducts. In addition, it is not known if the transgenically produced LC-PUFAs will indeed accumulate in the triacylglycerol (TAG) fraction. Once these challenges have been overcome however, these transgenic PUFA-enriched oils will afford the public an economical source of desirable PUFAs that will greatly impact general health and nutrition in the future.

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2

Screening for Unique Microbial Reactions Useful for Industrial Applications

Jun Ogawa

Sakayu Shimizu

2.1 Introduction

In the coming postpetrochemical period, production processes will be required to save energy and to reduce environmental damage. In this sense, biological reactions are now widely recognized as practical alternatives to conventional chemical reactions.[1,2] On the other hand, novel catalytic procedures are necessary to produce the emerging classes of organic compounds that are becoming the targets of molecular and biomedical research. Therefore, screening for novel biocatalysts that are capable of catalyzing new reactions is constantly needed.

A bioprocess is sometimes designed from an organic chemistry standpoint, regardless of whether or not a suitable biocatalyst has already been found. This forces screening from a certain level to ascertain the existence of a desirable biocatalyst. Thus, it is also important to increase the catalog of biocatalysts to maintain the motivation for such a steady search. One of the most efficient and successful means of finding new biocatalysts is to screen large numbers of microorganisms, because of their characteristic diversity and versatility.[3–6] The keys for increasing the probability of discovery in the screening are to examine as many samples as possible and to maintain a thoughtful insight.

Usually, screening is simply focused on a one-step target reaction. In such screening, the cells of microorganisms are incubated with target substrates under various reaction conditions and their transformation is monitored. Successful examples of such screening are the finding of novel carbonyl reductases and lactonohydrolases. You can refer to the recent reviews presenting the details of these enzymes.[7,8] Also, information obtained on detailed analysis of a microbial metabolic process leads to unexpected new reactions and substrates. Examples of the latter, i.e., unique reactions found in the microbial metabolism of cyclic amides, nucleosides, and fatty acids, are described together with their applications in this chapter.

2.2 Analysis and Application of Microbial Cyclic Amide Metabolism

2.2.1 Overview of Microbial Cyclic Amide Metabolism

The chemical structure of cyclic amides can be found in many natural and unnatural compounds. The transformation of naturally occurring cyclic amides of pyrimidines and purines plays an important role in nucleobase metabolism. These metabolic activities comprise those of various cyclic amide hydrolases (EC 3.5.2.-), such as dihydropyrimidinase in reductive pyrimidine metabolism,[9] barbiturase in oxidative pyrimidine metabolism,[10] dihydroorotase in pyrimidine biosynthesis,[11] and allantoinase in purine metabolism.[12] In the 1970s, studies on rat liver dihydropyrimidinase showed that the enzyme hydrolyzed 5-monosubstituted hydantoin to *N*-carbamoyl amino acid and that the reaction proceeded D-stereospecifically.[13,14] Later on, Yamada and coworkers showed that microbial cells are good catalysts for this reaction.[15] Based on these observations, hydantoin metabolism in microorganisms was intensively studied and applied to optically active amino acid production.[16,17] *Blastobacter* sp. A17p-4 was screened from soil as a hydantoin-assimilating bacterium for the purpose of D-amino acid production from DL-5-monosubstituted hydantoins.[18] During the course of studies on hydantoin metabolism in this bacterium, it was found that it showed not only hydantoin- but also cyclic imide-metabolizing activity.[19] A recent study revealed that the strain has not only hydantoin-metabolizing enzymes but also enzymes specific to cyclic imide derivatives. Since then, cyclic imide metabolism and specific enzymes have been widely found and studied in bacteria, yeasts, and molds.

2.2.2 Analysis and Application of Microbial Hydantoin Metabolism

2.2.2.1 Diversity of Hydantoin-Metabolizing Enzymes

Hydantoin is metabolized to an amino acid through two-step hydrolysis via an *N*-carbamoyl amino acid. The enzyme catalyzing the first step, hydrolysis of hydantoin to *N*-carbamoyl amino acid, is called hydantoinase. Three typical hydantoinases with stereospecificity to D-, L-, and DL-5-monosubstituted hydantoin are named D-hydantoinase, L-hydantoinase, and DL-hydantoinase, respectively (Figure 2.1).[17,20–22] D-Hydantoinases have been found in various genera of bacteria such as *Pseudomonas*, *Bacillus*, *Blastobacter*, and *Arthrobacter*, and most of them show dihydropyrimidinase activity. The existence of L- and DL-hydantoinases might be rarer in nature than that of D-hydantoinase. These enzymes can be divided into two groups: one needing ATP for activity and the other not. The ATP-requiring enzyme from *Pseudomonas putida* 77, which functions in creatinine metabolism, showed L-hydantoinase activity.[23]

The second step of hydantoin metabolism, *N*-carbamoyl amino acid hydrolysis to amino acid, ammonia, and carbon dioxide, is catalyzed by carbamoylase. A variety of carbamoylases have been reported (Figure 2.2). Two typical carbamoylases with stereospecificity to *N*-carbamoyl D- and L-amino acids are named D-carbamoylase and L-carbamoylase, respectively.[17] D-Carbamoylase generally shows wide substrate specificity to both aromatic and aliphatic *N*-carbamoyl-D-amino acids.[24] L-Carbamoylase shows rather limited specificity to aromatic or aliphatic *N*-carbamoyl-L-amino acids. An L-carbamoylase with relatively broad substrate specificity has been found in *Alcaligenes xylosoxidans*.[25] β-Ureidopropionase from

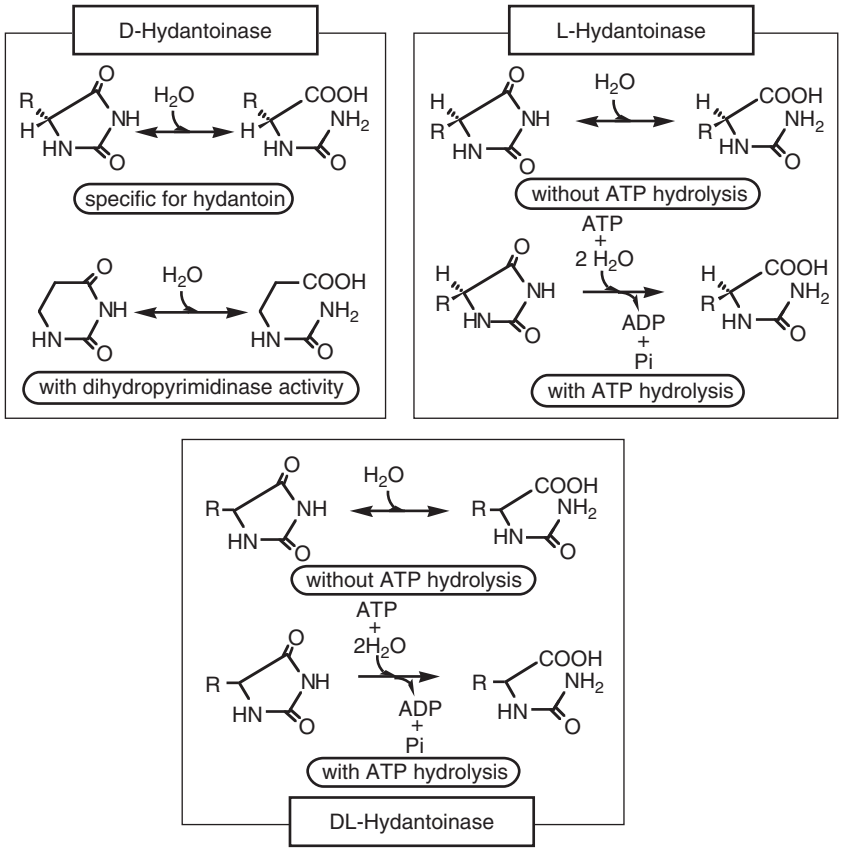

FIGURE 2.1 Reactions catalyzed by typical hydantoinases.

P. putida IFO 12996, which functions in the pyrimidine degradation during *N*-carbamoyl-β-alanine hydrolysis, showed broad substrate specificity not only toward *N*-carbamoyl-β-amino acids, but also toward *N*-carbamoyl-γ-amino acids and several *N*-carbamoyl-α-amino acids.[26] The hydrolysis of *N*-carbamoyl-α-amino acids is strictly L-stereospecific (Figure 2.2).

2.2.2.2 Optically Active Amino Acid Production by Hydantoin-Metabolizing Enzymes

Different combinations of hydantoin-metabolizing enzymes, i.e., hydantoinases and carbamoylases, provide a variety of processes for the production of optically pure α-amino acids (Figure 2.3).[17] The broad substrate range of the processes is valuable, especially for the production of D-amino acids and unnatural L-amino acids.[27,28] Some enzymes recognize multichiral centers other than the α-carbons of amino acids, so they enable simultaneous resolution of multichiral amino acids such as β-methylphenylalanine.[29] Recent research on hydantoin-metabolizing enzymes has been concentrated on newly isolated or improved enzymes, and has included directed evolution techniques, structure elucidation, studies on fusion proteins, and the use of specially designed whole cell biocatalysts.[30]

A practical representative is the production of D-*p*-hydroxyphenylglycine, a building block for semisynthetic penicillins and cephalosporins. The process involves one chemical step and two enzymatic steps (Figure 2.4). The substrate, DL-5-(*p*-hydroxyphenyl)hydantoin, is synthesized by an efficient chemical method involving the amidoalkylation reaction of phenol with glyoxylic acid and urea under acidic conditions. Then, the D-5-(*p*-hydroxyphenyl)hydantoin is hydrolyzed enzymatically to *N*-carbamoyl-D-*p*-hydroxyphenylglycine with a thermostable immobilized D-hydantoinase under alkaline conditions.

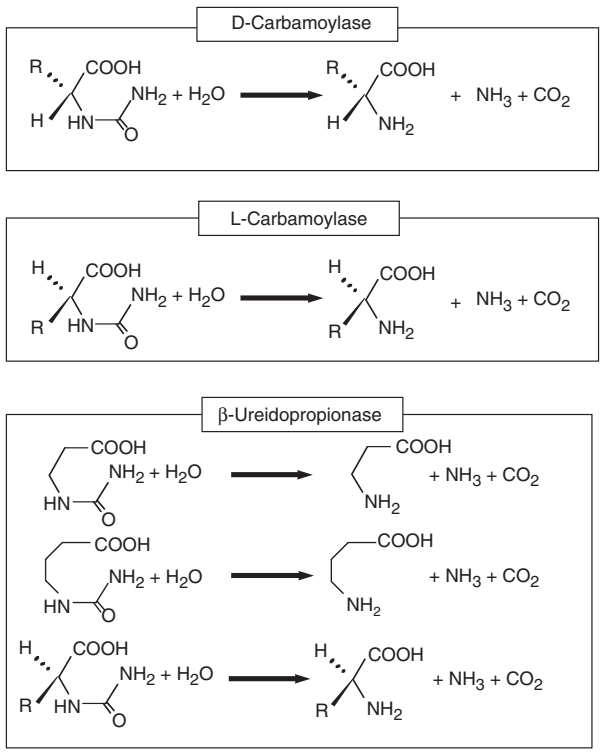

FIGURE 2.2 Reactions catalyzed by typical carbamoylases.

Under these conditions, the L-isomer of the remaining 5-(*p*-hydroxyphenyl)hydantoin is racemized through base catalysis. Therefore, racemic hydantoins can be converted quantitatively into *N*-carbamoyl-D-*p*-hydroxyphenylglycine through this step. Decarbamoylation to D-*p*-hydroxyphenylglycine is performed with an immobilized mutant D-carbamoylase. D-Carbamoylase from *Agrobacterium* sp. KNK712 has been improved into a mutant enzyme showing 20°C-higher thermal stability.[31,32] This process involving immobilized hydantoinase and carbamoylase has been used for the commercial production of D-*p*-hydroxyphenylglycine since 1995.

2.2.3 Analysis and Application of Microbial Cyclic Imide Metabolism

2.2.3.1 Analysis of Microbial Cyclic Imide Metabolism

Based on the finding of cyclic imide-hydrolyzing activity in *Blastobacter* sp.,[19] the metabolism of various cyclic imides by microorganisms was systematically investigated. The fact that *Blastobacter* sp. grows well in a synthetic minimum medium containing succinimide as the sole carbon source indicates that the bacterium has a metabolic system for the assimilation of cyclic imides as energy sources and nutrients.[33] The bacterium can metabolize various cyclic imides with structures similar to that of succinimide such as maleimide, 2-methylsuccinimide and glutarimide, and sulfur-containing cyclic imides such as 2,4-thiazolidinedione and rhodanine. Further investigation of the metabolic fate of these cyclic imides showed that they were metabolized through a novel metabolic pathway (Figure 2.5). This pathway comprises in turn the hydrolytic ring-opening of cyclic imides into half-amides, hydrolytic deamidation of the half-amides to dicarboxylates, and dicarboxylate transformation similar to that in the tricarboxylic acid (TCA) cycle. Two novel enzymes, imidase and half-amidase, and D-hydantoinase were found to function in this pathway.

The nature of imidase, which hydrolyzes a cyclic imide to a half-amide, was further investigated in detail. Three types of enzymes with different substrate specificities were found (Figure 2.6). An imidase

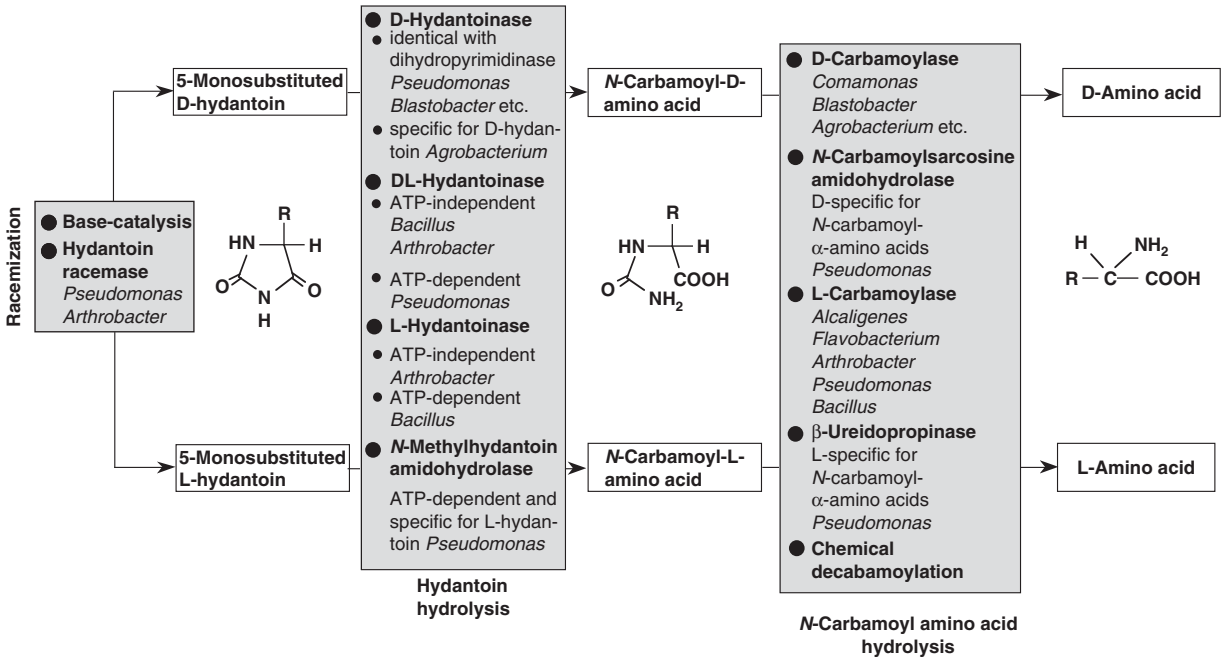

FIGURE 2.3 Processes for optically active α-amino acid production with combinations of various hydantoin–metabolizing enzymes.

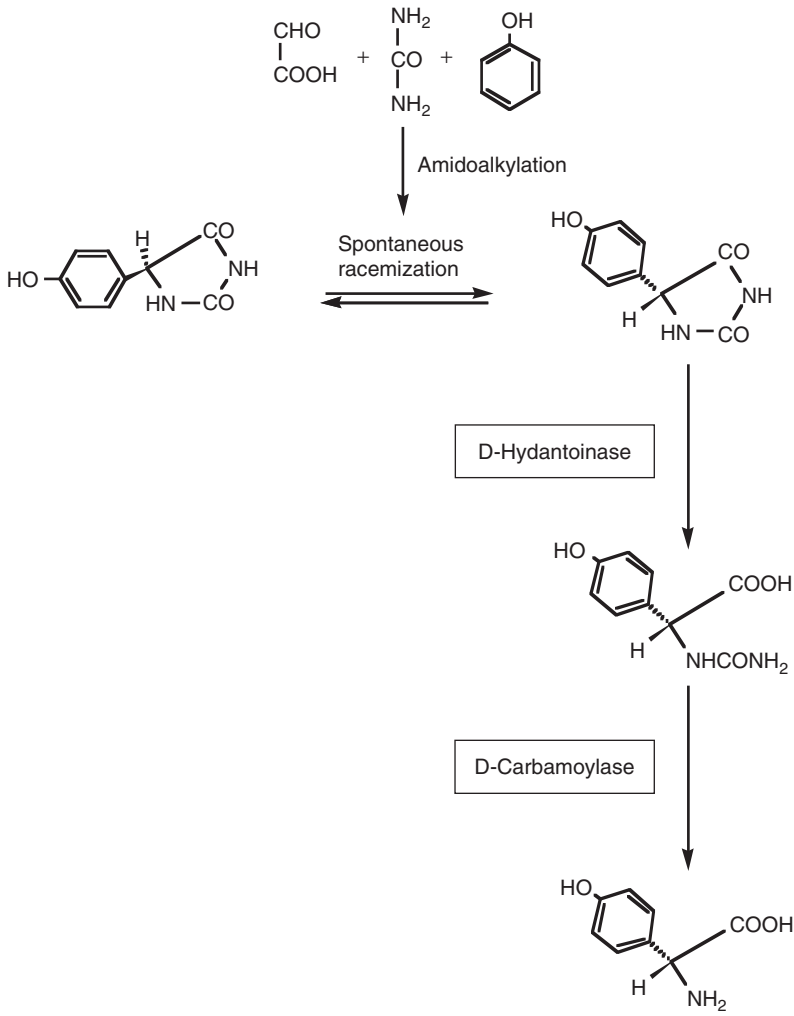

FIGURE 2.4 Industrial process for D-*p*-hydroxyphenylglycine production with hydantoin-metabolizing enzymes.

with specificity toward simple cyclic imides was purified from *Blastobacter* sp.[34] This enzyme is also active toward sulfur-containing cyclic imides such as 2,4-thiazolidinedione and rhodanine. However, bulky cyclic imides or monosubstituted cyclic ureides are not hydrolyzed. Bulky cyclic imides are hydrolyzed by the D-hydantoinase of *Blastobacter* sp. and mammalian dihydropyrimidinases.[35] Another imidase, phthalimidase, with specificity toward phthalimide derivatives was found in *Alcaligenes ureafaciens*.[36] Half-amides, the products of imidase, were further metabolized to dicarboxylates by half-imidase. The enzyme was purified from *Blastobacter* sp. and found to be specific toward half-amides.[37] These enzyme activities were widely distributed among bacteria, yeast, and molds.[38] Cyclic imide metabolism and the enzymes involved have practical potential for the production of high-value organic acids such as pyruvate from cyclic imides or their metabolites, and also the stereo- and regiospecific production of half-amides and dicarboxylates.

2.2.3.2 Application of Microbial Cyclic Imide Metabolism to Pyruvate Production

Cyclic imide metabolism has been applied to the production of a high-value organic acid, pyruvate.[39] The commercial demand for pyruvate has been increasing because of its use as an effective precursor in the synthesis of various drugs and agrochemicals in addition to as a component of mammalian-cell

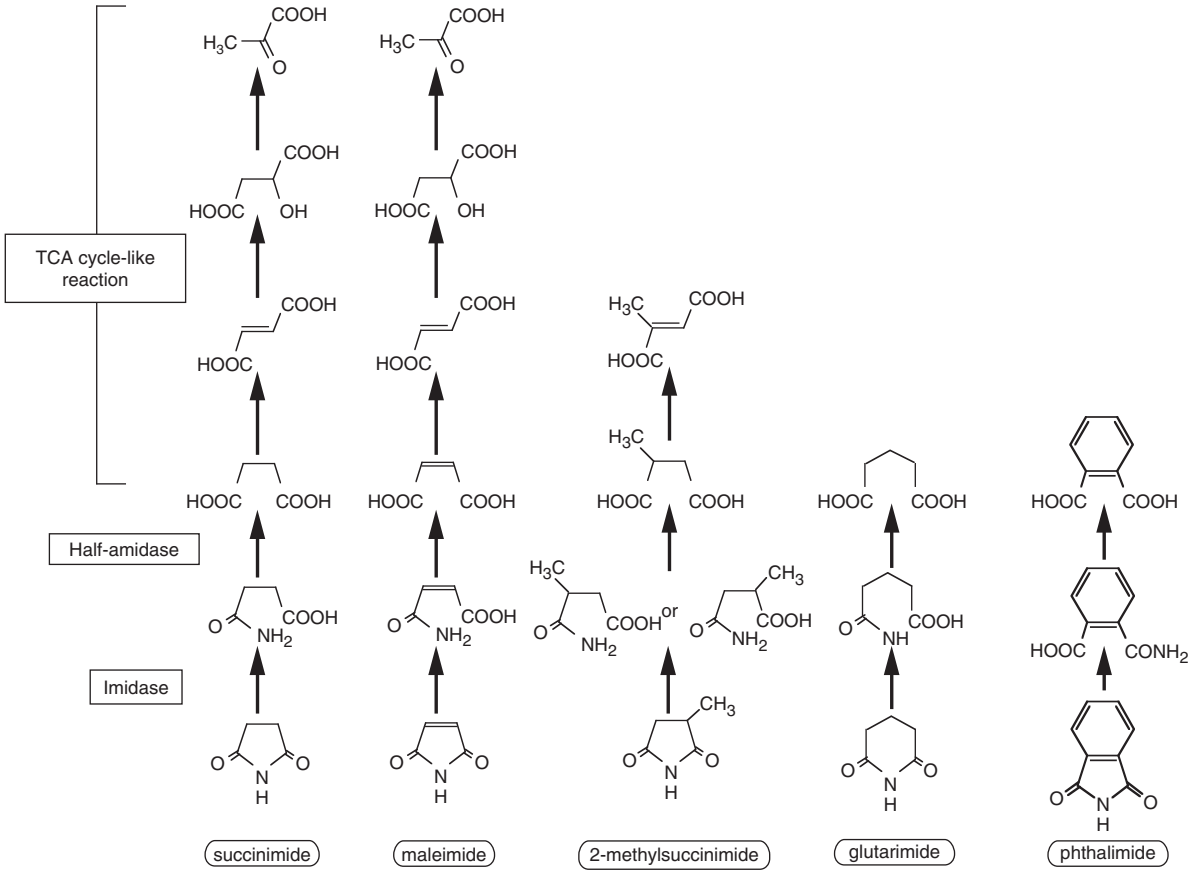

FIGURE 2.5 Pathways of microbial cyclic imide-metabolism.

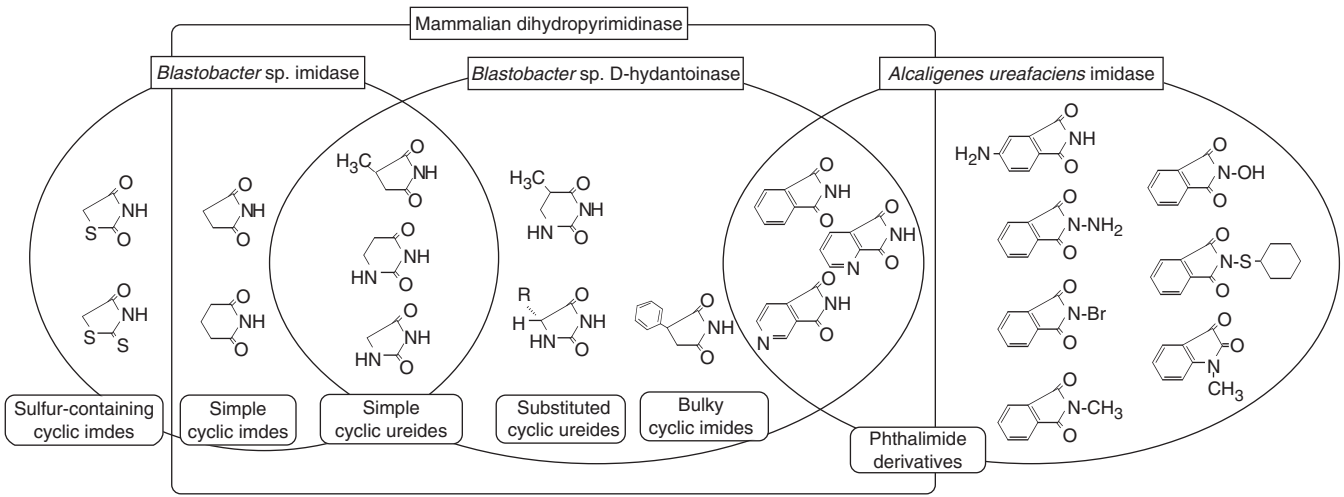

FIGURE 2.6 Substrate spectra of typical imidases.

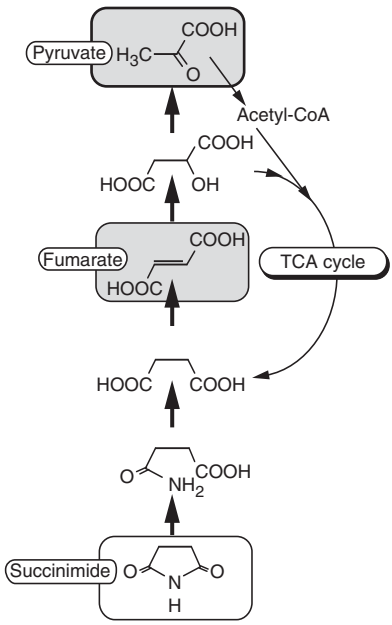

FIGURE 2.7 Pyruvate production from fumarate through microbial cyclic imide metabolism.

culture media. *Pseudomonas putida* s52 isolated with succinimide as the sole carbon source exhibits highly active cyclic imide metabolism. This activity has been used for pyruvate production from fumarate, a cheap cyclic imide metabolism intermediate (Figure 2.7). Using cells cultivated in medium containing 2% (w/v) fumarate as the catalyst, 286 mM pyruvate was produced from 500 mM fumarate in 27 h. Bromopyruvate, a malic enzyme inhibitor, inhibited the pyruvate production and also the growth of *Pseudomonas putida* s52 in the medium with fumarate as the sole carbon source. Bromopyruvate-resistant mutants were derived from *Pseudomonas putida* s52, and their pyruvate production was examined. One of the mutants showed much higher pyruvate production than the parent strain. Using the mutant cells cultivated in medium containing 2% (w/v) fumarate as the catalyst, 770 mM pyruvate was produced from 1000 mM fumarate in 96 h.[40]

2.2.3.3 Application of the Imidase-Catalyzing Reaction to Fine Organic Synthesis

In case of a half-amide, a useful building block for organic synthesis, there is synthetic difficulty in selective amidation at one of two equivalent carboxyl groups. 3-Carbamoyl-α-picolinic acid (α-3CP) is one of the regioisomeric half-amides of 2,3-pyridinedicarboxylic acid (PDC). α-3CP is a promising intermediate for modern insecticide synthesis. Chemical synthesis of α-3CP from PDC via the dimethylester involves troublesome regiospecific diester hydrolysis to the half-ester. Enzymatic regiospecific hydrolysis of 2,3-pyridinedicarboximide (PDI) (Figure 2.8) is one of the attractive methods for overcoming this disadvantage. Some imidases and D-hydantoinases (dihydropyrimidinases) have been found to hydrolyze aryl-substituted cyclic imides such as PDI, 3,4-pyridinedicarboximide and phthalimide.[35,41] Based on these findings, potential imidases that are applicable to the regiospecific hydrolysis of PDI to α-3CP were screened for. Phthalimide-assimilating microorganisms have been isolated as possible catalysts for the regiospecific hydrolysis of PDI to α-3CP. *Arthrobacter ureafacience* O-86 was selected as the best strain and applied to the cyclohexanone-water two-phase reaction system, pH 5.5, where the spontaneous random hydrolysis of PDI was avoided and the enzyme maintained its activity. Under the optimized conditions, with the periodical addition of PDI (in total, 40 mM), 36.6 mM α-3CP accumulated in the water phase with a molar conversion yield of 91.5% and a regioisomeric purity of 94.5% in 2 h.[36]

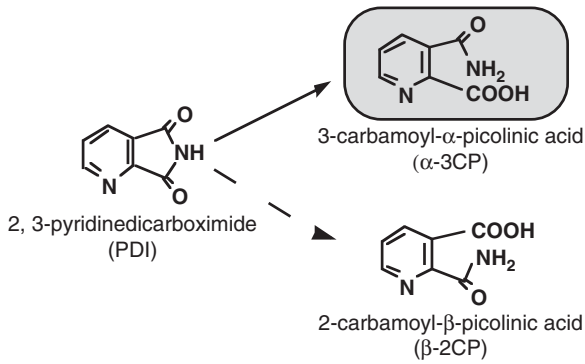

FIGURE 2.8 Imidase-catalyzing regioselective hydrolysis of 2,3-pyridinedicarboximide (PDI) to 3-carbamoyl-α-picolinic acid (α-3CP).

2.3 Analysis and Application of Microbial Nucleoside Metabolism

2.3.1 Overview of Microbial Nucleoside Metabolism

Recently, nucleosides and a variety of chemically synthesized nucleoside analogs have attracted a great deal of interest as they have antibiotic, antiviral, and antitumor effects.[42] In light of this trend, the microbial metabolism of nucleosides was reevaluated in detail, although it had been well studied as an assimilation or salvage pathway.[43] The first reaction in nucleoside metabolism is N-riboside cleavage. Two kinds of enzymes, nucleosidase (nucleoside hydrolase; EC 3.2.2.-) and nucleoside phosphorylase (EC 2.4.2.-), are known to catalyze this reaction (Figure 2.9). Nucleosidase catalyzes the irreversible hydrolysis of nucleosides and participates mainly in the assimilation pathway. A nucleosidase from *Ochrobactrum anthropi*, which specifically catalyzes the N-riboside cleavage of purine nucleosides, has been purified and characterized.[44] The enzyme was revealed to be useful for the decomposition of purine nucleosides in foodstuffs, with these nucleosides causing hyperuricemia, an increasingly common disease in adults.[45] On the other hand, nucleoside phosphorylase catalyzes the phosphorolytic cleavage of nucleosides and shows ribosyl transferase activity.[46] This enzyme functions mainly in the salvage pathway. Nucleoside phosphorylase has been well studied and used as a catalyst for the synthesis of nucleoside analogs through base exchange reactions.[46,47]

In the case of 2'-deoxyribonucleoside degradation, the product of the nucleoside phosphorylase reaction is 2-deoxyribose 1-phosphate. This is further transformed into D-glyceraldehyde 3-phosphate and acetaldehyde via 2-deoxyribose 5-phosphate. These reactions are reversible and successively catalyzed

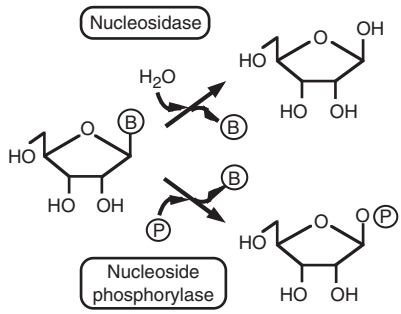

FIGURE 2.9 Reactions catalyzed by nucleosidase and nucleoside phosphorylase.

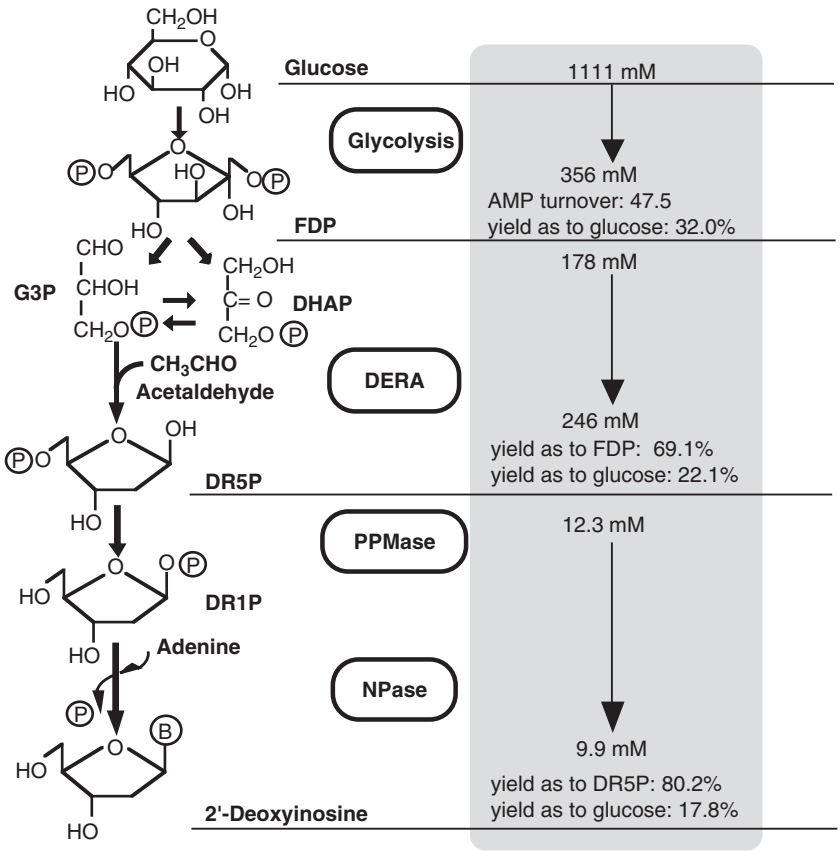

FIGURE 2.10 Biochemical retrosynthesis of 2'-deoxyribonucleoside.

by phosphopentomutase and deoxyriboaldolase, and the products of these reactions, D-glyceraldehyde 3-phosphate and acetaldehyde, finally flow into a central metabolic process such as the glycolytic pathway. Recently, a unique application of these reversible reactions to nucleoside synthesis was investigated, as described below.

2.3.2 Biochemical Retrosynthesis of 2'-Deoxyribonucleoside Through Microbial Nucleoside Metabolism

With the spread of PCR techniques and new antiviral nucleosides, and the advent of antisense drugs for cancer therapy, there will be an urgent need for a DNA building block, 2'-deoxyribonucleoside, on a large scale in the near future. Classical 2'-deoxyribonucleoside sources are hydrolyzed herring and salmon sperm DNA. These sources, however, will not allow us to meet future demands for a stable and economical supply of 2'-deoxyribonucleoside. A possible microbial method for 2'-deoxyribonucleoside production from easily available materials, glucose, acetaldehyde, and a nucleobase, has been examined, that is, the use of reversible reactions involved in nucleoside degradation. In this process, microorganisms possessing glycolytic enzymes, deoxyriboaldolase, phosphopentomutase, and nucleoside phosphorylase, were used as catalysts. The glycolytic enzymes produce D-glyceraldehyde 3-phosphate from glucose. Subsequently, deoxyriboaldolase, phosphopentomutase, and nucleoside phosphorylase cooperatively produce 2'-deoxyribonucleosides from D-glyceraldehyde 3-phosphate, acetaldehyde, and a nucleobase via 2-deoxyribose 5-phosphate (Figure 2.10). A deoxyriboaldolase suitable for 2-deoxyribose 5-phosphate synthesis with tolerance to acetaldehyde has been found on screening.[48] A potential enzyme was found in *Klebsiella pnuemoniae* and transformed into

E coli.[49] Using the *E coli* transformant as a source of glycolytic enzymes and deoxyriboaldolase, 2-deoxyribose 5-phosphate was produced from glucose and acetaldehyde in the presence of ATP, which is required for D-glyceraldehyde 3-phosphate generation from glucose by glycolytic enzymes. The energy derived from ATP was replaced by the energy derived from alcohol fermentation by baker's yeast in the form of fructose 1,6-diphosphate, which was further utilized as a D-glyceraldehyde 3-phosphate precursor by the *E coli* transformant.[50] The 2-deoxyribose 5-phosphate produced was further transformed to 2'-deoxyribonucleoside by *E coli* transformants expressing phosphopentomutase and nucleoside phosphorylase.[51] Typical results of such synthesis are also presented in Figure 2.10. It is noteworthy that the glycolytic pathway supplies the important substrates, fructose 1,6-diphosphate and D-glyceraldehyde 3-phosphate, for 2'-deoxyribonucleoside synthesis via 2-deoxyribose 5-phosphate.

2.4 Analysis and Application of Microbial Fatty Acid Metabolism

2.4.1 Fatty Acid Desaturation Systems for Polyunsaturated Fatty Acid Production

C20 polyunsaturated fatty acids (PUFAs), such as 5,8,11-*cis*-eicosatrienoic acid (mead acid, MA), dihomo-γ-linolenic acid, arachidonic acid, and 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA), exhibit unique biological activities. Because food sources rich in these PUFAs are limited to a few seed oils and fish oils, the screening for alternative sources of these PUFAs in microorganisms has been conducted, which resulted in the isolation of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4.[52] This fungus produces 30–60 g/l of mycelia (dry weight) containing about 60% lipids. The lipids mainly consist of triacylglycerol, which contains arachidonic acid. The amount of arachidonic acid is 40–70% of the lipids—approximately 13 kg/kl on large-scale fermentation. The fungus operates unique fatty acid desaturation systems involving at least five desaturases with different specificities. EPA has been produced through the ω3 route from α-linolenic acid via successive Δ6 desaturation, elongation and Δ5 desaturation using the same fungus.[53] Further investigation led to the isolation of desaturase-defective mutants and opened routes for the production of other PUFAs (Figure 2.11).[54,55] For example, dihomo-γ-linolenic acid and MA can be produced through the ω6 and ω9 routes using Δ5 and Δ12-desaturase-defective mutants, respectively. A recent review presented the details of this research.[56]

2.4.2 Fatty Acid Metabolism Useful for Conjugated Fatty Acid Production

Conjugated fatty acids have attracted much attention as a novel type of biologically beneficial functional lipids. For example, dietary conjugated linoleic acid (CLA) reduces carcinogenesis, atherosclerosis, and body fat.[57,58] Today, CLA is produced through chemical isomerization of linoleic acid, which results in the by-production of unexpected isomers. Considering the use of CLA for medicinal and nutraceutical purposes, an isomer-selective and safe process is required. A bioprocess is a potential alternative for this purpose.

Dairy products are among the major natural sources of CLA, of which *cis*-9,*trans*-11-octadecadienoic acid is the main isomer.[59] CLA has been shown to be produced from polyunsaturated fatty acids by certain rumen microorganisms such as *Butyrivibrio* species. *cis*-9,*trans*-11-Octadecadienoic acid has been suggested as an intermediate in the biohydrogenation of linoleic acid to octadecaenoic acid by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens*.[60] It has also been reported that *Propionibacterium freudenreichii*, which is commonly used as a dairy starter culture, can produce CLA from free linoleic acid.[61] Recently, the ability to produce CLA from linoleic acid was extensively screened for in lactic acid bacteria.[62] Many strains were found to produce CLA from linoleic acid, and the mechanism of CLA production was investigated with *Lactobacillus acidophilus* AKU 1137 as a representative strain.[63] The

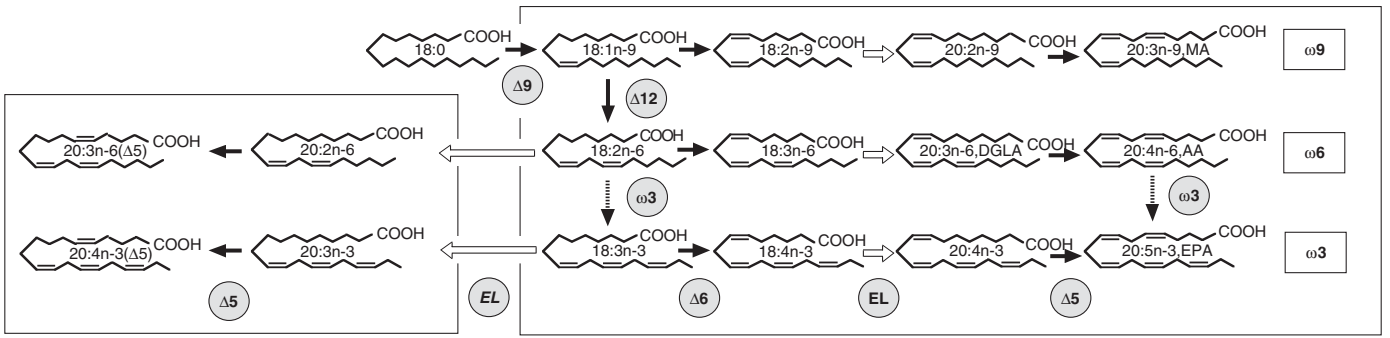

FIGURE 2.11 Polyunsaturated fatty acid–synthesizing systems in *M. alpina* 1S–4 and mutants of it. EL, elongase; AA, arachidonic acid; MA, Mead acid; ALA, α-linolenic acid; EPA, 5,8,11,14,17-*cis*-eicosapentaenoic acid; DGLA, dihomo-γ-linolenic acid.

CLAs produced by *L. acidophilus* were identified as *cis*-9,*trans*-11-octadecadienoic acid (CLA1) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2).[64] Preceding the production of CLA, hydroxy fatty acids identified as 10-hydroxy-*cis*-12-octadecaenoic acid and 10-hydroxy-*trans*-12-octadecaenoic acid were accumulated. The isolated 10-hydroxy-*cis*-12-octadecaenoic acid was transformed to CLA on incubation with washed cells of *L. acidophilus*, suggesting that this hydroxy fatty acid is one of the intermediates of CLA production from linoleic acid (Figure 2.12). Based on these results, the transformation of hydroxy fatty acids by lactic acid bacteria was investigated. Lactic acid bacteria transformed ricinoleic acid (12-hydroxy-*cis*-9-octadecaenoic acid) into CLA (a mixture of CLA1 and CLA2).[65] There are two possible pathways for CLA synthesis from ricinoleic acid by lactic acid bacteria: 1) direct transformation of ricinoleic acid into CLA through dehydration at the Δ11 position, and 2) dehydration of ricinoleic acid at the Δ12 position to linoleic acid, which is a potential substrate for CLA production by lactic acid bacteria (Figure 2.12). In a similar manner on linoleic acid transformation to CLA, lactic acid bacteria transformed α- and γ-linolenic acid into the corresponding conjugated trienoic acids.[66,67] Those produced from α-linolenic acid were identified as *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (18:3) and *trans*-9,*trans*-11,*cis*-15-18:3, and those from γ-linolenic acid as *cis*-6,*cis*-9,*trans*-11-18:3 and *cis*-6,*trans*-9,*trans*-11-18:3 (Figure 2.13). Washed cells of lactic acid bacteria exhibiting high productivity of conjugated fatty acids were obtained by cultivation in medium supplemented with polyunsaturated fatty acids such as linoleic acid and α-linolenic acid, indicating that these enzyme systems are induced by polyunsaturated fatty acids, maybe for their detoxication.[60]

2.4.2.1 Preparative CLA Production from Linoleic Acid by Lactic Acid Bacteria

After screening 14 genera of lactic acid bacteria, *L. plantarum* AKU 1009a was selected as a potential strain for CLA production from linoleic acid.[62] Washed cells of *L. plantarum* exhibiting a high level of CLA production were obtained by cultivation in a nutrient medium containing linoleic acid. Under the optimum reaction conditions with the free form of linoleic acid as the substrate, washed cells of *L. plantarum* produced 40 mg/ml CLA (33% molar yield) from 12% (w/v) linoleic acid in 108 h. The resulting CLA comprised a mixture of CLA1 (38% of total CLA) and CLA2 (62% of total CLA), and accounted for 50% of the total fatty acids obtained. A higher yield (80% molar yield as to linoleic acid) was attained with 2.6% (w/v) linoleic acid as the substrate in 96 h, resulting in CLA production of 20 mg/ml [consisting of CLA1 (2%) and CLA2 (98%)] and accounting for 80% of the total fatty acids obtained. Most of the CLA produced was associated with the washed cells, and mainly as a free form.[62]

2.4.2.2 Preparative CLA Production from Ricinoleic Acid and Castor Oil by Lactic Acid Bacteria

The ability to produce CLA from ricinoleic acid is widely distributed in lactic acid bacteria. Washed cells of *L. plantarum* JCM 1551 were selected as a potential catalyst for CLA production from ricinoleic acid.[68] Cells cultivated in a medium supplemented with a mixture of α-linolenic acid and linoleic acid showed enhanced CLA-productivity. Under the optimum reaction conditions, with the free acid form of ricinoleic acid as the substrate and washed cells of *L. plantarum* as the catalyst, 2.4 mg/ml CLA was produced from 3.4 mg/ml ricinoleic acid in 90 h, the molar yield as to ricinoleic acid being 71%. The CLA produced, which was obtained in the free fatty acid form, consisted of CLA1 (21% of total CLA) and CLA2 (79% of total CLA), and accounted for 72% of the total fatty acids obtained.[68]

Ricinoleic acid is abundant in a plant oil, castor oil. Castor oil is an economical source of ricinoleic acid. About 88% of the total fatty acids in castor oil is ricinoleic acid. Unfortunately, CLA cannot be directly produced from castor oil by lactic acid bacteria. Lactic acid bacteria only use the free form of ricinoleic acid for CLA production, i.e., not its triacylglycerol form, which is mainly found in castor oil. However, in the presence of lipase, castor oil became an effective substrate for CLA production by lactic acid bacteria.[65] The addition of a polyhydroxy-type detergent enhanced the CLA production from castor oil. Under the optimum conditions with castor oil as the substrate and washed cells of *L. plantarum* JCM 1551 as the catalyst, 2.7 mg/ml CLA was produced from 5.0 mg/ml castor oil in 99 h. The CLA produced accounted for 46% of the total fatty acids obtained, and consisted of CLA1 (26%) and CLA2 (74%).[69]

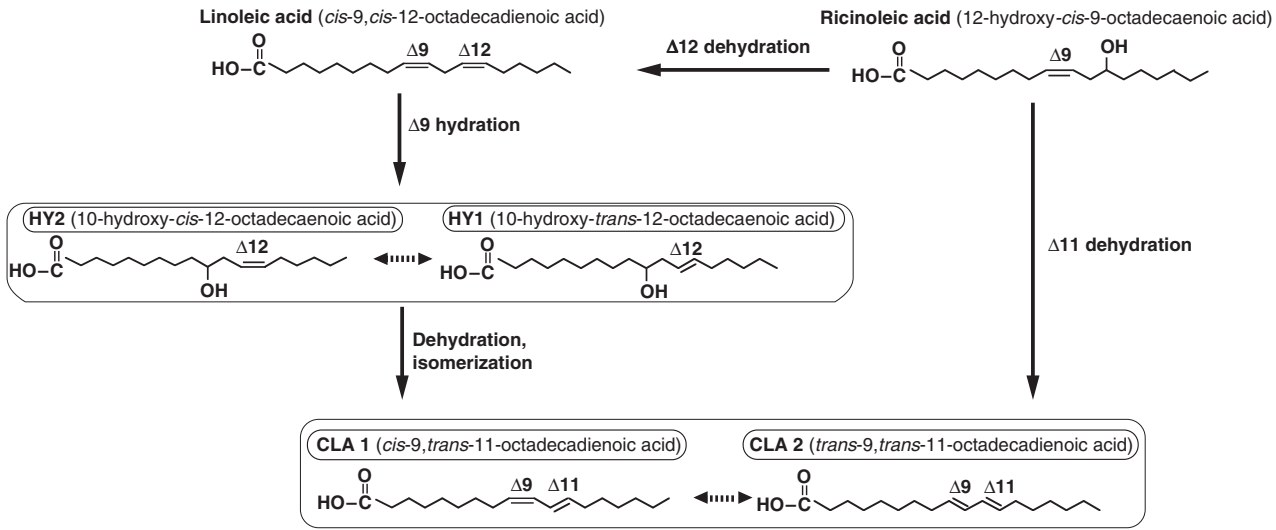

FIGURE 2.12 Conjugated linoleic acid (CLA)–producing systems in lactic acid bacteria.

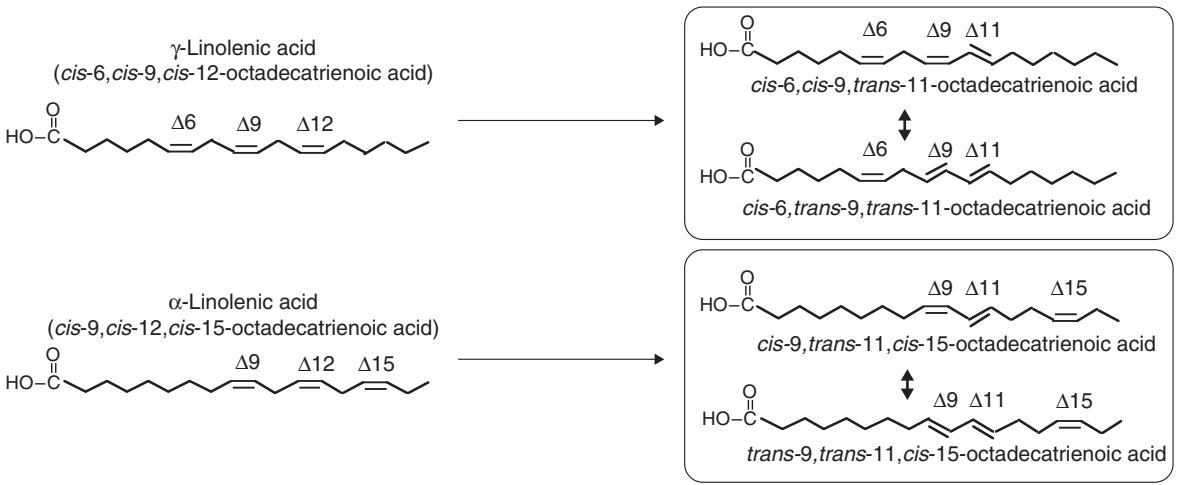

FIGURE 2.13 α-and γ-Linolenic acid transformation into conjugated fatty acids by lactic acid bacteria.

2.5 Conclusions: For Expansion of Biocatalysts for Practical Purposes

In this chapter, the authors introduced several examples of unique microbial reactions useful for practical purposes. The reactions resulted from detailed observation of microbial cyclic amide, nucleoside, and fatty acid metabolisms, and have paved the way to new bioprocesses for the production of optically active amino acids, organic acids, half-amides, 2'-deoxyribonucleosides, polyunsaturated fatty acids, conjugated fatty acids, etc.

Modern society requests the development of processes exhibiting environmental harmonization, economic efficiency, and specificity. This trend is causing the application of biological reactions to a greater variety of industries. Future bioprocesses will generally not be limited by the available technology or the nature of the substrates and products. Instead, the feasibility of new bioprocesses will often be determined by the availability of biocatalysts, the search for which needs patience for steady research but has a deep impression when a new biocatalyst is encountered.

Recently, some rational methods creating new biocatalysts have been rapidly developed. Modern gene technology, crystal structure analysis, and bioinformatics enable the modulation of enzyme function through site-directed mutation, DNA shuffling, etc. One example is modification of the substrate specificity of a monooxygenase, P450 BM-3, from *Bacillus megaterium*. P450 BM-3 is a fatty acid monooxygenase. Its substrate specificity was expanded to aromatic hydrocarbons, and phenolic and arylalkyl compounds by means of crystal structure-based directed mutation, which resulted in the creation of novel catalysts for regiospecific and stereospecific alcohol synthesis.[70–72] However, "rationality" is not the only answer for developing new biocatalysts. Classical screening of microbial diversity and versatility is still important. Such screening is something like a midnight walk without moonlight; however, detailed observation and deep insight with a well-considered strategy will lead to a new biocatalyst. This philosophy has now been succeeded by in vitro random evolution technology.[73]

The industrial success of biocatalysts unfortunately depends on the economics of the specific processes. However, once successful, it provides enormous opportunities. With the introduction of each new process accumulating experience and confidence, it becomes easier to develop and justify the next new bioprocess. Thus, it is important to increase the catalog of biocatalysts waiting to be examined for practical purposes.

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3

Biocatalyses in Microaqueous Organic Media

Tsuneo Yamane

3.1 Introduction

Enzymatic or microbial reactions are generally performed by enzymes or microorganisms that exist in the large excess of water. However, there are cases where yield or rate or productivity increases significantly by reducing water content in the reaction system. Most biocatalytic reactions using organic solvents are involved in this category.

Performing enzymatic or microbial reactions in organic media has several advantages as opposed to using aqueous medium, including the following:

- Shifting of thermodynamic equilibrium to favor synthesis over hydrolysis.
- Reduction in water-dependent side reaction (such as hydrolysis reaction in transfer reactions).
- Immobilization of the enzyme is often unnecessary (even if it is desired, merely physical deposition onto solid surfaces is enough).
- Elimination of microbial contamination (quite critical in view of industrial scale process).
- Increasing solubility of hydrophobic substrates.
- Recovery of product from low boiling-point solvents is easy and also the insoluble biocatalyst is easily separated.

Here organic media as the reaction system are classified into two categories:[1]

1. Solvent systems
2. Solvent-free systems

In the former system one or more substrates are dissolved in an inert organic solvent that does not participate in the reaction in any respect, but to provide an environment for the biocatalyst to exert its action on the dissolved substrate(s).

There are a number of cases where the former is the system of choice: for example, when the substrate is solid at the temperature of the reaction, when high concentration of the substrate is inhibitory for the reaction, when the solvent used yields a better environment (accelerating effect) for the enzyme, and so forth.

In the latter system, no other compounds but substrate(s) and enzyme are present in a reactor. In principle, one substrate can be used in a large excess over another, and if so, it may also act as a solvent for the other reactant. This may be alternatively named "neat" biotransformation.

One of the big attractions of enzymatic solvent-free synthesis is potentially very high volumetric productivity. This, however, does not apply to all reactions, and in many instances it may actually take a longer time to achieve the desired degree of conversion in the absence of added solvent. In this case, volumetric productivity in the reactor [(mass of product formed) (reactor volume)$^{-1}$ (time)$^{-1}$] should be calculated for both solvent-based and solvent-free systems using the same volume of the reaction mixture and the same amount of the enzyme in order to make an economically justified choice between the two. Similarly, no risk of solvent-induced inactivation of the biocatalyst in solvent-free system is another advantage, but the overall loss of the enzyme activity can still be significant if the reaction time is too long. It should be added that avoidance of organic solvents is particularly advantageous to the food industry where stringent legal regulations related to the use of organic solvents are in force. Also, no fireproof and explosion-proof equipment/procedures are necessary for the solvent-free processing and the environment in the factory is less hazardous to the health of workers.

Biocatalytic reactions in organic media are currently being studied very actively as interdisciplinary fields between organic chemistry and enzyme engineering, and between oleochemistry and enzyme engineering to synthesize or convert lipids, saccharides, peptide, chiral compounds, polymers, etc. A comprehensive monograph was published in 1996 reviewing the progress,[2] and methods and protocols related to this topic were summarized in monographs.[3–5]

Types of reactions involve: 1) bond formations of ester, amide, and glycoside by synthetic reaction as reverse ones of hydrolyses or by transfer reactions (transesterification, transpeptidylation, transglycosylation, etc), 2) oxidation and reduction, 3) C-O and C-N bond formation by addition/substitution reactions, 4) C-C bond formation, 5) polymerizations, etc. The use of biocatalysts for these reactions results in the best advantages in their selectivities involving enantio-selectivity, regio-selectivity, functional group selectivity, etc. The process may be made simpler because of these selectivities without having to introduce a protecting group and its deprotection afterward.

3.2 Enzymes

Many enzymes have been used for studying their actions in organic media, including lipases whose origins are bacteria, molds, yeasts, and mammals and plants to lesser extents, esterases, proteases such as thermolysin, -chymotrypsin, and subtilisin, peroxidase, phenol oxidase, alcohol dehydrogenase (yeast), and so on. As a microorganism in organic media, baker's yeast has been most often used.

3.2.1 Techniques Enabling Biocatalyst Highly Active in Organic Media

A number of techniques have been developed that enable biocatalysts active in organic media. These include:

1. Enzyme molecules are dissolved freely (some enzymes are soluble in glycerol or dimethylsulfoxide).
2. Enzyme molecules are derivatized with polyethylene glycol (PEG), or are formed as a complex with surfactant (they are called lipid-coated enzyme or surfactant-modified enzymes, or surfactant-enzyme complexes). All these modified enzymes are soluble in some organic solvents.
3. Enzymes are suspended or dispersed in organic media. If their dispersion is not uniform, it is deposited on the surface of fine solid particles beforehand.
4. The enzymes are confined in a reverse micelle.

5. The enzymes exist in aqueous phase inside micropores of porous solid particles. Enzymes are either free or immobilized.
6. The enzyme or microbial cells are entrapped in hydrophobic gel.
7. Microbial cells (wet or dry) having a particular enzyme are suspended in organic media, or they are first immobilized in macroporous supports followed by suspension or packed in a bioreactor.

Since a number of techniques have been developed as mentioned above, it can be said confidently that any enzyme can make its activity high in organic media. Techniques #2 and #4 exert high activity of the enzyme preparation, but its recovery or continuous usage will be difficult. Technique #3 will give us biocatalysts suitable for industrial usage because of its ease in recovery or of ability of continuous operation, although one must invent a method of pretreatment or addition of activity enhancer to get higher activity.

3.2.2 Reactivation

Powders of some commercial enzyme preparations exhibit low activities when they are suspended in organic media, but they are drastically reactivated when they have been re-lyophilized from their aqueous solutions containing proper amount of sugar alcohols,[6–8] surfactants,[9,10] fatty acids,[11] hydrocarbons,[12] etc. This phenomenon is related to the second technique mentioned above.

3.2.3 Purity

A factor that many researchers have paid little attention to, but that is very important, is the purity of the enzyme preparations they use. As will be mentioned later, a profile of the effect of a trace amount of water depends on the purity of the enzyme preparation, and the enzyme molecules are surrounded by large amounts of impurities remaining around them and influence directly the enzyme catalysis when the crude enzyme preparations are suspended in organic media (Figure 3.1a).[13] Therefore their catalytic activities are affected strongly by the nature and amount of the impurities. Crude enzyme powder exhibit enough activity when it is uniformly dispersed in organic media, but pure enzyme powder is not uniformly dispersed, and shows no activity unless three factors are optimized: water content, proper support material, and the addition of a reactivator (or activity enhancer) (Figure 3.1b).[6] Figure 3.2 indicates that

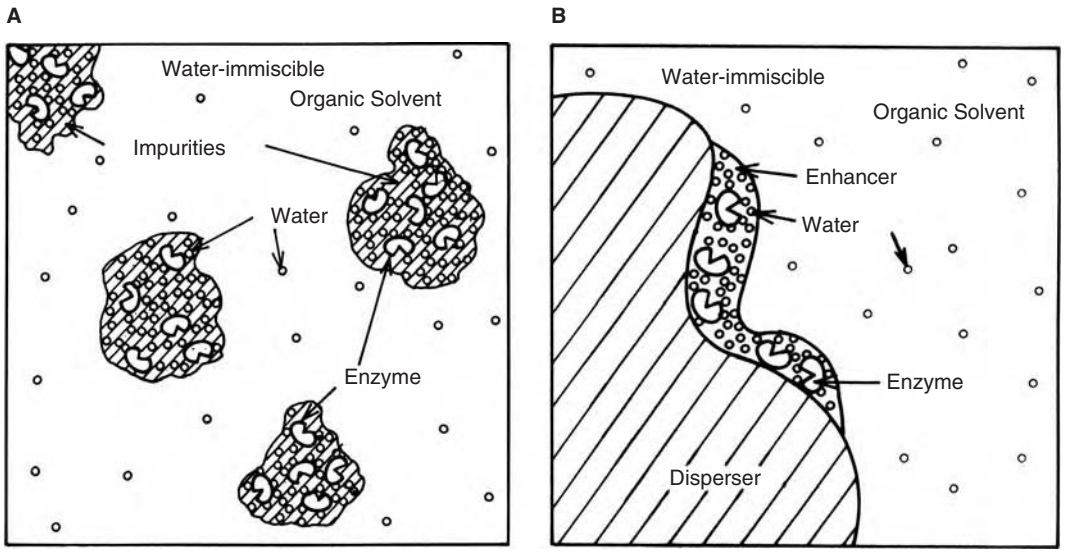

FIGURE 3.1 Schematic pictures of crude enzyme powder particles suspended in microaqueous medium (a), and the system of (pure enzyme + activity enhancer) deposited on a disperser particle which is suspended in microaqueous medium (b).[6]

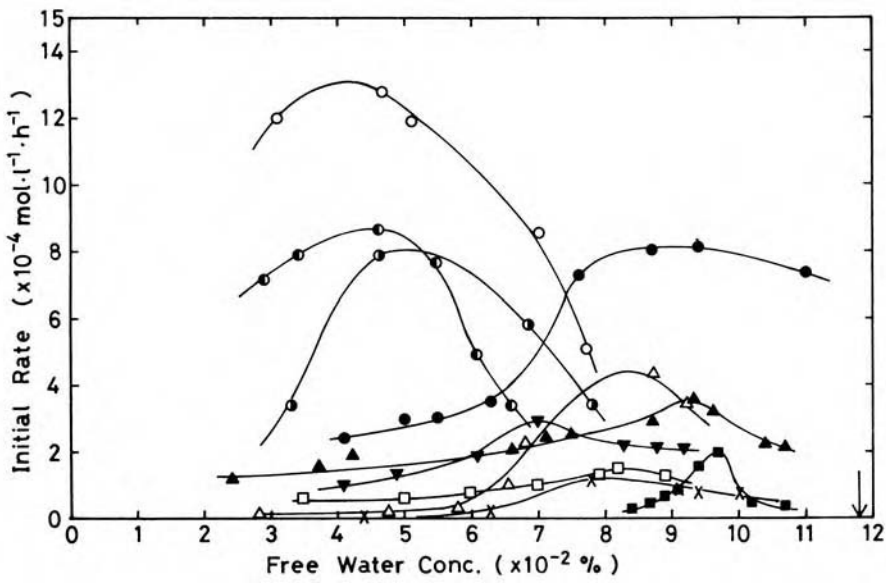

FIGURE 3.2 Effect of additive on the lactonization activity of the pure enzyme at various concentrations of free water. The vertical arrow indicates the solubility of water in benzene at 40°C (0.118%). —Δ—, no addition, i.e., enzyme plus celite; —○—, enzyme plus arabitol plus celite; —◐—, enzyme plus sorbitol plus celite; —◑—; enzyme plus erythritol plus celite; —●—, enzyme plus phosphotidylcholine plus celite; —▲—, enzyme plus lactose plus celite; —×—, enzyme plus BSA plus celite; —■—, enzyme plus casein plus celite; —▽—, enzyme plus PVA plus celite; —❑—, enzyme plus dextran plus celite.

the rate of lactonization by *Pseudomonas fluorescens* lipase is increased significantly when a sugar alcohol such as erythritol, arabitol, or sorbitol is added before freeze-drying together with celite powder.[6] In this case, the pure lipase is not dispersed at all without celite powder, and even if the enzyme is deposited on the celite powder, the catalytic activity is very low.

3.2.4 Properties of Enzymes

Properties of an enzyme change more or less in organic media, including thermal stability and various specificities (selectivities):

1. Thermal stability (half life), $t_{1/2}$—Inactivation of an enzyme is regarded as change of its three-dimensional structure from an active form to an inactive form, and often involves water molecules during this structural change. Such structural changes do not occur in a complete anhydrous state so that its thermostability is enhanced. Therefore, the value of its half life, $t_{1/2}$, becomes much longer in very anhydrous conditions than in an aqueous solution, and approaches to $t_{1/2}$ value of its aqueous solution when the water content increases.[14] Bear in mind, however, that its catalytic activity in nearly complete anhydrous state drops down to a low level, as will be mentioned later. In any cases, $t_{1/2}$ should be evaluated under a strict microaqueous condition, also taking into consideration the nature of the organic solvent.
2. Substrate specificity, k_{cat}/K_m—Substrate specificity of a given enzyme is quantitatively evaluated by the value of k_{cat}/K_m. In nonaqueous enzymology its value changes in various organic solvents.[15] This implies that the specificity of an enzyme can be varied by selecting the kind of organic solvent without applying protein engineering and/or screening another new enzyme from nature.
3. Enantioselectivity, E—Enantioselectivity of a given enzyme is quantitatively evaluated by the value of the so-called enatiomeric ratio, or E value, which is defined as the relative ratio of k_{ca}/K_m values

of *R*-and *S*-isomers.[16] Optical purity of the product, the *ee* value, increases with any increase in the *E* value. It is said that the *E* value should be more than 100 to produce optically pure product industrially. The *E* value of an enzyme changes in various organic solvents. Attempts have been made to correlate the *E* value of enzymes with physicochemical properties of organic solvents, such as dielectric constant, dipole moment, and molar volume.[17,18]

A number of factors affect enzymatic reactions in organic media, among which a trace amount of water and the nature of organic solvents will be discussed briefly.

3.3 Water

Special attention should be paid to the water content when one carries out biocatalytic reactions in organic media because a trace amount of water strongly affects, among other things: 1) reaction rate, 2) yield and selectivity, and 3) operational stability. Numerous studies in many laboratories have shown that a complete depletion of water from the reaction system results in the nonoccurrence of the biochemical reaction. Water seems essential for a biocatalyst to display its full catalytic activity. Enzyme as a protein needs a fluctuation or perturbation to exert its catalytic activity, and water bound to the protein allows its fluctuation. Completely dried enzymes cannot fluctuate.

3.3.1 The "Microaqueous" Concept

In order to emphasize the importance of a trace amount of water for biocatalyst utilization in organic media, the author proposed a novel technical term "microaqueous" to depict the reaction system more accurately.[19–21] This technical term implies that the system is neither aqueous nor nonaqueous/anhydrous. In its strictly scientific sense, nonaqueous/anhydrous implies the complete absence of water. In between these extremities (aqueous and nonaqueous), there is a state where the system has little water. This state could be called "microaqueous." Thus, strictly speaking, an organic solvent in which the biocatalyst works is a microaqueous organic solvent, or more simply a microaqueous solvent. Putting the adjective "microaqueous" in front of (organic) solvent suggests properly that the (organic) solvent contains a trace amount of water.

The effects of a trace amount of water on lipase-catalyzed esterification and transesterification are shown in Figure 3.3a[13] and Figure 3.3b, respectively. In the range of very low water content, the reaction rates are limited by hydration of the enzyme proteins (hydration-limited), whereas in the range of excess water, they are limited by the reverse reaction (reverse reaction-limited). In the transesterification reaction (Figure 3.3b), the hydrolytic side reaction appears at a higher water content, resulting in a decrease of the product yield.

3.3.2 Existing Places of Water and Equilibrium

In the system of enzyme powder suspended in organic media (state #3 in Section 3.2.1) as the simplest case, a general moisture balance is given by

$$C_{w,\text{total}} = Y_{ap} C_{ap} + Y_{im} C_{im} + C_{w,\text{free}} \tag{3.1}$$

where

$C_{w,\text{total}}$ = total water concentration [g H_2O/mL]
Y_{ap} = amount of water bound to the active protein [g H_2O/g dry protein]
C_{ap} = concentration of the active protein (enzyme)[g dry protein/mL]
Y_{im} = amount of water bound to the inert material [g H_2O/g dry inert material]
C_{im} = concentration of the inert material [g dry material/mL]
$C_{w,\text{free}}$ = concentration of the free water dissolved in organic medium [g H_2O/mL]

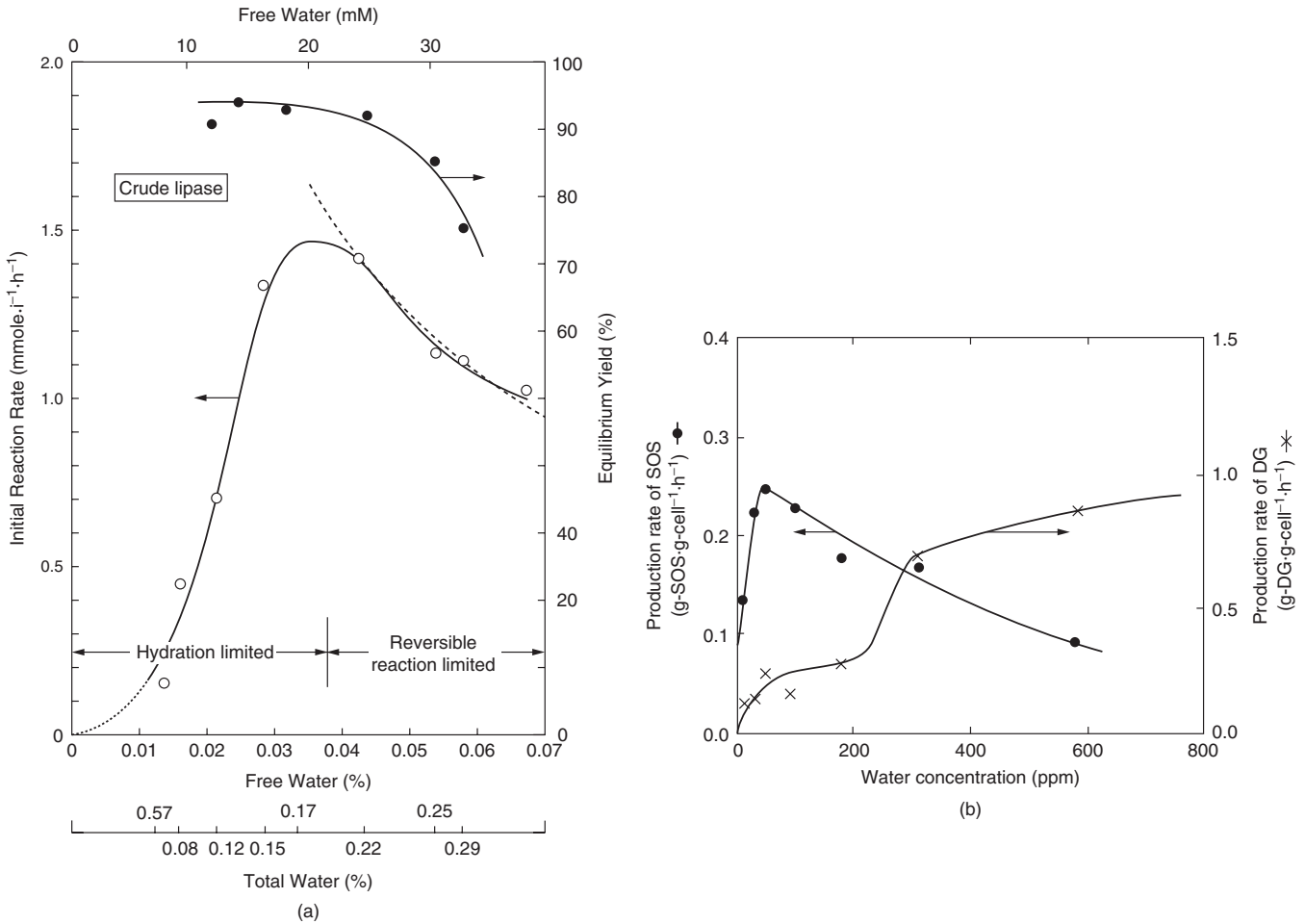

FIGURE 3.3 Effect of trance amount of water on lipase-catalyzed esterification (a)[13] and transesterification (b).[30]

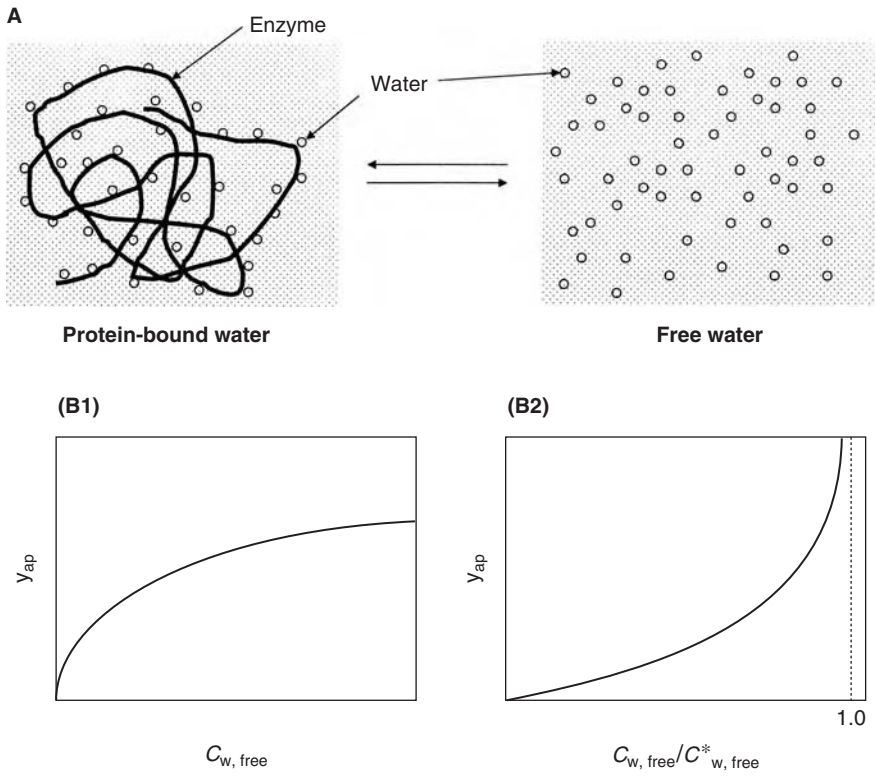

FIGURE 3.4 Dynamic equilibrium between enzyme-bound water and free water in an organic medium (A), and general profiles of the adsorption isotherms of water between the enzyme and water-miscible organic solvent (B1) and between enzyme and water-immiscible organic solvent (B2).[20] $C^{*}_{w,free}$ is water solubility of the organic solvent.

By using Equation 3.1, it is possible to explain the characteristic difference in dependency of the reactivity of variously graded enzyme on the total moisture content. The optimal moisture level will decrease with an increase in the purity of the enzyme preparation, and the optimal total moisture content will increase when the crude enzyme preparation is used. In any case, the optimal total moisture content will increase as the amount of the enzyme added to the reaction mixture increases. Some researchers discuss the effect of water as a function of added water or total water, but they are not scientific variables. It is important to realize that the water only bound to the enzyme molecule, y_{ap}, affects directly its catalytic activity, whereas the water dissolved freely in an organic medium, $C_{w,free}$, only participates in the reaction as a substrate or as a product so that the conversion or yield is determined mostly by the free water concentration $C_{w,free}$.

There is a thermodynamic equilibrium between Y_{ap} and $C_{w,free}$ (Figure 3.4A).[20] Water-miscible organic solvents exhibit Langmuir-type adsorption isotherms having saturation phenomena (Figure 3.4B1):

$$y_{ap} = \frac{y_{ap,max} C_{w,free}}{K + C_{w,free}} \tag{3.2}$$

On the other hand, water-immiscible organic solvents generally show simply rising curves having no saturation (Figure 3.4B2).

They are expressed by BET-type multiple-layer adsorption isotherm:

$$y_{ap} = \frac{y_{ap,max} C_{w,free}/C^*_{w,free}}{(1 - C_{w,free}/C^*_{w,free})(1 - C_{w,free}/C^*_{w,free} + KC_{w,free}/C^*_{w,free})} \tag{3.3}$$

The curves shown in Figure 3.4 suggest that water hydrated on the enzyme, Y_{ap}, can be controlled by changing $C_{w,free}$.

3.3.3 Water Activity, a_w

Effects of water content on the activities of an enzyme in various organic solvents are partly summarized by applying a parameter named water activity, a_w.[22] a_w is a thermodynamic parameter, defined as in an equilibrium the water vapor pressure, of gas phase, p_w, divided by the saturated water vapor pressure of

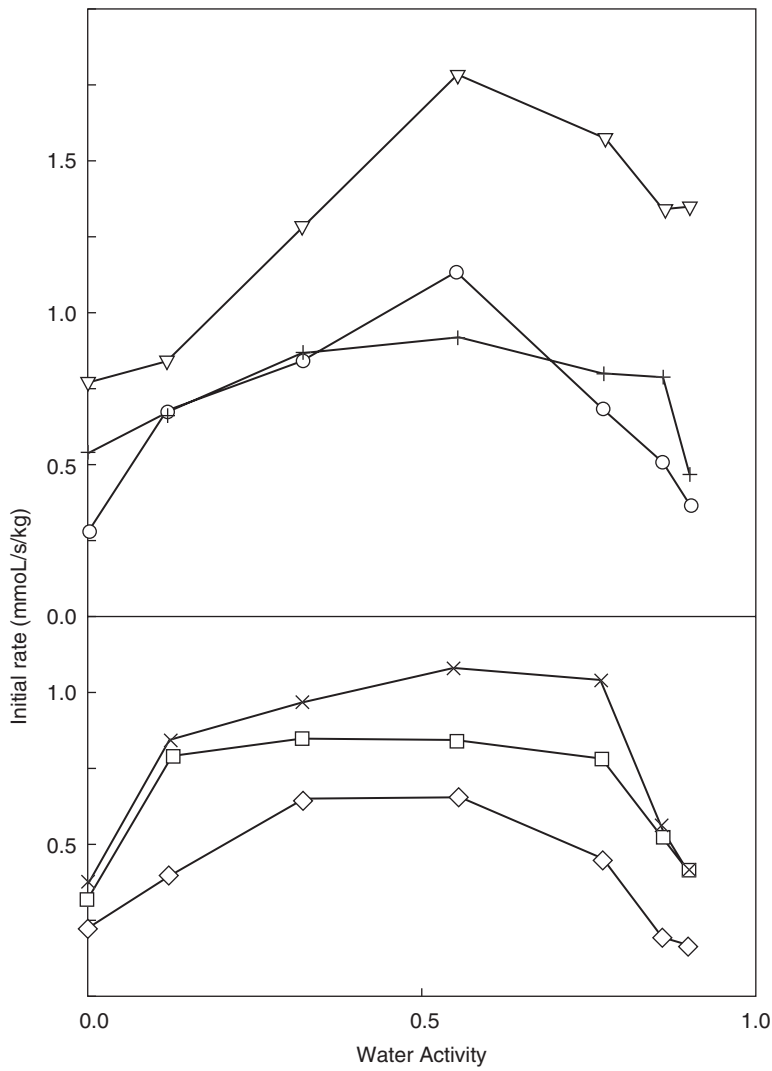

FIGURE 3.5 Activity of Lipozyme catalyst as a function of water activity in a range of solvents.[24] Hexane(∇), toluene(×), trichloroethylene(O), isopropyl ether(□), pentan-3-one(◊), and none, i.e., liquid reactant mixture(+).

the gas phase p_w^*, in a closed vessel of a constantly controlled temperature, in which substances are put for a long time to reach an equilibrium.

$$a_w p_w / p_w^*,\ 0 < a_w < 1 \tag{3.4}$$

The substances, powdered enzyme, organic solvent-containing substrates, and an aqueous saturated solution of a mineral salt are kept separate for the study of biocatalysis in organic solvent. After reaching an equilibrium at which all the components have some value of a_w, the powdered enzyme and the reaction solvent are mixed to measure the initial reaction rate. By changing the kind of the mineral salts, one can change the value of a_w between 0 and 1. A curve is obtained between a_w and $C_{w,free}$ (or more precisely molar fraction of water), which is characteristic for each of various organic solvents.[23]

The reaction rate with suspended enzyme catalyst shows similar dependence on a_w in different organic solvents, as seen in Figure 3.5.[24] However, as also seen in Figure 3.4, not all the data are on a single curve, but the absolute rates are scattering depending on the nature of the organic solvents, indicating that a_w is not almighty. There is a case where a_w fails to predict critical hydration level for enzyme activity in polar organic solvents.[23]

3.4 Organic Solvents

When wanting to apply a solvent system, one must choose a suitable solvent from the vast kinds of organic solvents prior to carrying out an enzymatic reaction in an organic solvent. From active basic research having been carried out in the past two decades, there has been progress in our understanding of properties of enzymes in organic media (mentioned in Section 3.2), and in how organic solvents affect them. Some researchers call the achievement "medium engineering."

Among numerous kinds of organic solvents, those often used for enzymatic reactions are not many, and may be classified into three categories (Table 3.1),[25] in view of the importance of water solubility of the organic solvents concerned:

1. Water-miscible organic solvents—Any cosolvent system having 0–100% ratio of the solvent/water can be prepared from this kind of solvent. Note that some organic solvents having limited solubility

TABLE 3.1 Classification of Solvents Commonly Used for Biocatalytic Reactions in Organic Media

1. Water-miscible organic solvents
   - Methanol, ethanol, ethylene glycol, glycerol, *N*,*N*′-dimethylformamide, dimethylsulfoxide, acetone,
   - formaldehyde, dioxane, etc.
2. Water-immiscible organic solvents (water solubility·g/l at the temperature indicated)
   - alcohols
   - (*n*-, *iso*-) proppyl alcohol, (*n*-, *s*-, *t*-) butyl alcohol, (*n*-, *s*-, *t*-) amyl alcohol, *n*-octanol, etc.
   - esters
   - methyl acetate, ethyl acetate (37.8, 40°C), *n*-butyl acetate, hexyl acetate, etc.
   - alkyl halides
   - methylene chloride (2, 30°C), chloroform, carbon tetrachloride, trichloroethane (0.4, 40°C), chlorobenzene, (*o*-, *m*-, *p*-) dichlorobenzene, etc.
   - ethers
   - diethyl ether (12, 20°C; 14.7, 25°C), dipropyl ether, diisopropyl ether, dibutyl ether, dipentyl ether, tetrahydrofuran, etc.
3. Water-insoluble organic solvents (water solubility·ppm at the temperature indicated)
   - aliphatic hydrocarbons
   - *n*-hexane (320, 40°C), *n*-heptane (310, 30°C), *n*-octane, isooctane (180, 30°C), etc.
   - aromatic hydrocarbons
   - benzene (600, 25°C; 1200, 40°C), toluene (300, 25°C; 880, 30°C), etc.
   - alicyclic hydrocarbons
   - cyclohexane (160, 30°C), etc.