1
Applications of Biocatalysis to Biotechnology

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Introduction

Enzymes are protein catalysts synthesized by living systems. The major emphasis in this review will be on the activities of single enzymes, either purified or confined within cells, living and dead. There will be less consideration of the biocatalytic activity of whole, living cells.

Before proceeding to technical detail, it is appropriate to indicate what is to be covered and what is not to be covered in this chapter.

This is not an enzyme textbook: therefore, only brief attention will be given to enzyme structure, properties and kinetics. General enzyme classification will be outlined to indicate the considerable breadth of biocatalytic reactions, and a few specific examples, subjectively selected, will be given.

Enzymes are important as synthetic and degradative catalysts. This variety of effects will become clear during the discussion of present applications of enzymes in the food, chemical, medical, detergent and textile industries.

Enzyme technology has several new techniques under development that are

Table 1. Some key references to biocatalysis in biotechnology.

Topic	Reference
Industrial enzymes—broad coverage	Aunstrup (1978), Dunnill (1980), Fogarty (1983a), Godfrey and Reichelt (1983a), Katchalski-Katzir and Freeman (1982), Rose (1980), Schmid (1979a), Scott (1980), Yamamoto (1978).
Enzymes in the food industry	Birch, Blakebrough and Parker (1981), Ruttloff (1982), Schwimmer (1981), Vanbelle, Meurens and Crichton (1982).
Enzyme production	Lambert and Meers (1983).
Enzyme purification	Bruton (1983).
Enzyme immobilization	Keyes (1980), Klibanov (1983), Trevan (1980).
Enzyme stabilization	Barker (1983), Klibanov (1979).
Pharmaceutical enzymes	Cooney, Stergis and Jayaram (1980), Ruyssen and Lauwers (1978).
Enzyme structure/chemistry	Dixon and Webb (1979), Scrimgeour (1977), Walsh (1979).
Enzymes in organic synthesis	Jones (1980), Kieslich (1976, 1982), Sebek and Kieslich (1977), Sih, Abushanab and Jones (1977).

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Table 2. Enzyme classification.

Class	General description	Selected examples	EC numbers
1. Oxidoreductases	Enzymes of this group catalyse oxidation–reduction reactions involving oxygenation or overall removal or addition of hydrogen atom equivalents.	Alcohol dehydrogenase	1.1.1.2
		Glucose oxidase	1.1.3.4
		Amino acid oxidase	1.4.3.2
		Cytochrome oxidase	1.9.3.1
		Catalase	1.11.1.6
		Peroxidase	1.11.1.7
		Steroid 11β-monooxygenase	1.14.15.4
2. Transferases	These enzymes mediate the transfer of a group, such as aldehydic or ketonic, acyl, sugar, phosphoryl, methyl or a sulphur-containing one, from one molecule to another.	Homocysteine methyltransferase	2.1.1.10
		Hexokinase	2.7.1.1.
		Aryl sulphotransferase	2.8.2.1
		Transketolase	2.2.1.1
		Transaldolase	2.2.1.2
		Alanine aminotransferase	2.6.1.2
3. Hydrolases	The range of functional groups hydrolysed by such enzymes is very broad. It includes esters, anhydrides peptides and others. C–O, C–N and C–C bonds may be cleared as well as some others.	Triacylglycerol lipase	3.1.1.3
		Pectinesterase	3.1.1.11
		Alkaline phosphatase	3.1.3.1
		Ribonuclease II	3.1.13.1
		Deoxyribonuclease I	3.1.21.1
		α-Amylase	3.2.1.1
		Cellulase	3.2.1.4
		α-D-Glucosidase	3.2.1.20
		Aminopeptidase	3.4.11.11
		Chymosin (rennin)	3.4.23.4
		Trypsin	3.4.21.4
		Papain	3.4.22.2

4. Lyases	The types of reactions catalysed are additions to, or formation of, double bonds such as C=C, C=O, C=N.	Pyruvate decarboxylase	4.1.1.1
		Citrate (*pro*-3*S*)-lyase	4.1.3.6
		Carbonate dehydratase	4.2.1.1
		Enolase	4.2.1.11
		Phenylalanine ammonia-lyase	4.3.1.5
5. Isomerases	A variety of isomerizations, including racemization, can be effected	Methionine racemase	5.1.1.2
		Glutamate racemase	5.1.1.3
		Glucosaminephosphate isomerase (glutamine-forming)	5.3.1.19
		Xylose(glucose) isomerase	5.3.1.5
		Alanine racemase	5.1.1.1
		UDPglucose 4-epimerase	5.1.3.2
		S-Methylmalonyl-CoA mutase	5.4.99.2
6. Ligases	These are often called synthetases, and catalyse the formation of C—O, C—S, C—N and C—C bonds with accompanying adenosine triphosphate (ATP) or other nucleoside triphosphate cleavage.	Glutathione synthetase	6.3.2.3
		D-Alanylalanine synthetase	6.3.2.4
		Arginyl-tRNA synthetase	6.1.1.19
		5,10-Methenyltetrahydrofolate synthetase	6.3.3.2
		Carbamoyl-phosphate synthetase (ammonia)	6.3.4.16
		Pyruvate carboxylase	6.4.1.1

certain to change the face of applied biocatalysis. Some of these will be discussed to indicate new directions that are imminent: enzyme reactions in solvents, metal exchange at the active site of the enzyme, and chemical and genetic modification of enzyme structure and activity, for example.

Finally, consideration will be given to deficiencies in existent biocatalysts in the sweetener, detergent, diagnostic, food and other industries and what may be done to improve their suboptimal performance.

Many useful books and reviews are available in the literature that cover in detail many topics of direct or indirect relevance to this review. A selection of these is shown in *Table 1*.

General properties of enzymes

For nearly every chemically catalysed reaction, there is an enzyme-catalysed equivalent (Sih, Abushanab and Jones, 1977). The question that must be asked, then, is: For any given catalytic application, which—the chemical or the biochemical catalyst—solves the problem most economically and acceptably? For any given case, there may be one or more reasons why an enzyme might offer an effective solution. Some of the general properties of enzymes that can contribute are:

1. High catalytic power, up to 10^9–10^{12} the rate of the non-enzymatic reaction, is provided.
2. A broad range of reactions can be catalysed.
3. Reactions can be run under mild conditions of temperature, pH and pressure.
4. Often, high specificity is offered with regard to regioselectivity and stereospecificity.
5. A chiral centre may be created at a prochiral centre.
6. Nature provides a vast reservoir of available enzyme variants for special requirements.

Enzyme classification

At present, more than 2100 enzymes have been recognized by the International Union of Biochemistry (International Union of Biochemistry, 1979; Lowe, 1983). It has been speculated that 25000 natural enzymes exist (Kindel, 1981). If this is true, about 90% of the vast reservoir of biocatalysts still remains to be discovered and characterized. As will be discussed below, through methods of chemical modification, random mutation, and genetic and protein engineering, a large number of modified protein catalysts will be available for industrial consideration as well.

Table 2 provides a glimpse at enzyme classification (International Union of Biochemistry, 1979). Six classes of enzymes have been designated by official taxonomic dictum. *Table 3* indicates the number of representatives in each of the six enzyme classes (Schmid, 1979b), while *Table 4* clearly indicates which of these enzyme classes is the major source of industrial enzymes (Godfrey and Reichelt, 1973a): about 85% are hydrolases, with the remaining 15% divided

Table 3. Number of enzymes in each class.

Enzyme class	Number in class
Oxidoreductases	537
Transferases	559
Hydrolases	490
Lyases	231
Ligases	83
Isomerases	98
Total	1998

Data from Schmid (1979b)

Table 4. Industrial use of enzyme classes

Class	Major enzymes used
Oxidoreductases	Glucose oxidase†
	Catalase†
	Peroxidase†
	Steroid hydroxylases, e.g. EC 1.14.15.4, EC 1.14.99.9, EC 1.14.99.10
Transferases	—
Hydrolases	Protease (acid, neutral, alkaline)
	α-Amylase†
	β-Amylase, EC 3.2.1.2
	Cellulase†
	Amyloglucosidase, EC 3.2.1.3
	Invertase (β-D-Fructofuranosidase), EC 3.2.1.26
	Pectinase (polygalacturonase), EC 3.2.1.15
	Lactase (β-D-Galactosidase), EC 3.2.1.23
	Naringinase
	Anthocyanase
	Lipase (triacylglycerol lipase)†
	Aminoacylase, EC 3.5.1.14
	Penicillinase, EC 3.5.2.6
Lyases	—
Ligases	—
Isomerases	Xylose (glucose) isomerase†

† For EC numbers see Table 2

among oxidoreductases and isomerases. Of the hydrolases, some 70% hydrolyse proteins, 26% hydrolyse carbohydrates and 4% hydrolyse lipids. Relatively few oxidoreductases and no transferases are commercially important, despite their large numbers.

The fact that such an overwhelming percentage of industrial enzymes are hydrolytic in nature is of particular interest in that many of these same hydrolytic reactions are reversible under certain reaction conditions discussed below. The synthetic use of hydrolytic enzymes will be a new direction for industrial enzymology.

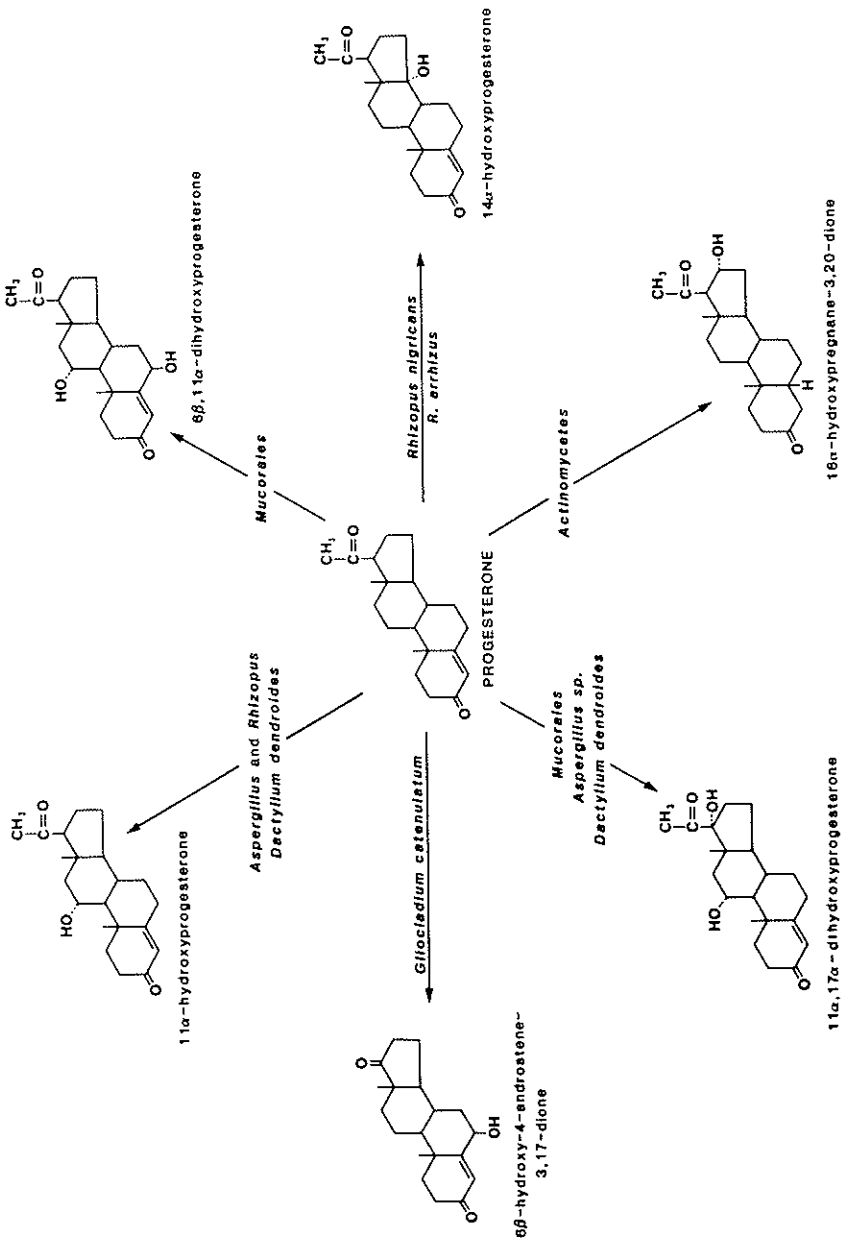


Figure 1. Selected specific steroid hydroxylations.

Many of the specific industrial applications of these and other enzymes will be discussed below. Preceding this, a brief illustration of enzyme talent will be given in the form of some personal favourites.

Some examples of enzyme activity

The use of microbial enzymes to catalyse specific and useful reactions in academic and commercial organic chemistry was stimulated by the success in steroid transformations (Charney and Herzog, 1967; Kieslich, 1980). *Figure* 1 offers just a superficial view of the variety of micro-organisms and hydroxylations that have been studied. In many of these cases, chemical catalysis would be difficult, if not impossible.

It is often the case that an enzyme recognizes only a small region of a complex molecule and can also interact with a less complex molecule having the same or a similar region. A practical outcome of this regiospecificity is that a broad structural range of substrates is often accessible using a very limited number of enzymes. For example, CH(OH)→C=O oxidations can be effected on substrates ranging in complexity from the simple aliphatic alcohols to complex polycyclic alcohols, using only three alcohol dehydrogenases with overlapping specificities. This is shown in *Figure 2* (Jones, 1980).

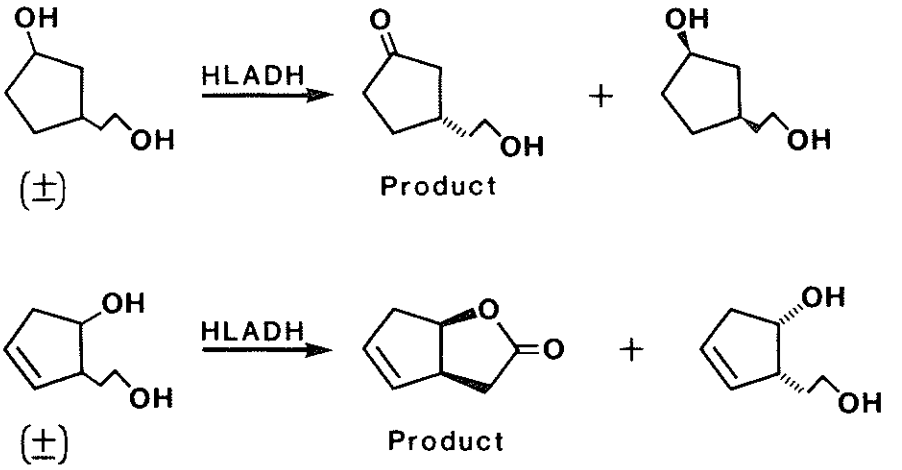


Figure 3. Enantiomeric selectivity and regiospecificity. HLADH, horse liver alcohol dehydrogenase.

In *Figure 3*, it is indicated that an enzyme may be able to achieve enantiomeric selectivity as well as regiospecificity as in a single oxidative reaction catalysed by horse liver dehydrogenase (Jones, 1980). *Figure 4* shows how this exquisite specificity is carried further, by illustrating enantiomeric selectivity combined with enantioface specificity in reduction reactions catalysed by horse liver alcohol dehydrogenase (Jones, 1980).

Enzymes have very subtle stereochemical distinctions that can be applied to particular synthetic problems. This is indicated in *Figure 5*, where three different

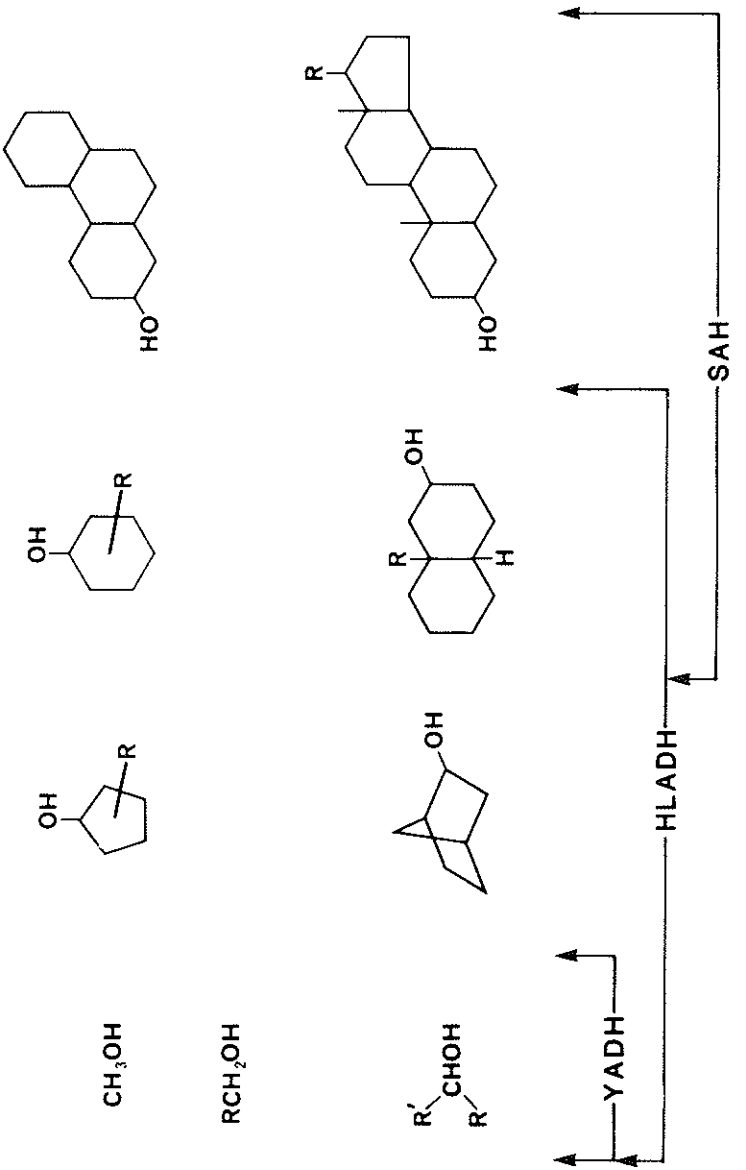


Figure 2. Specificity overlap of alcohol substrates. YADH, yeast alcohol dehydrogenase; HLADH, horse liver alcohol dehydrogenase; SAH, steroid alcohol dehydrogenase.

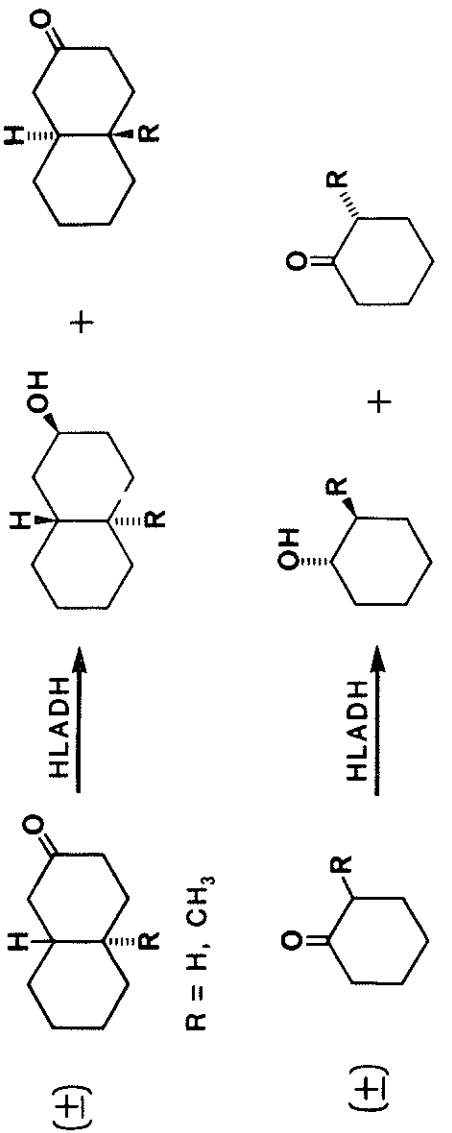


Figure 4. Enantioface specificity and enantiomeric selectivity. HLADH, horse liver alcohol dehydrogenase.

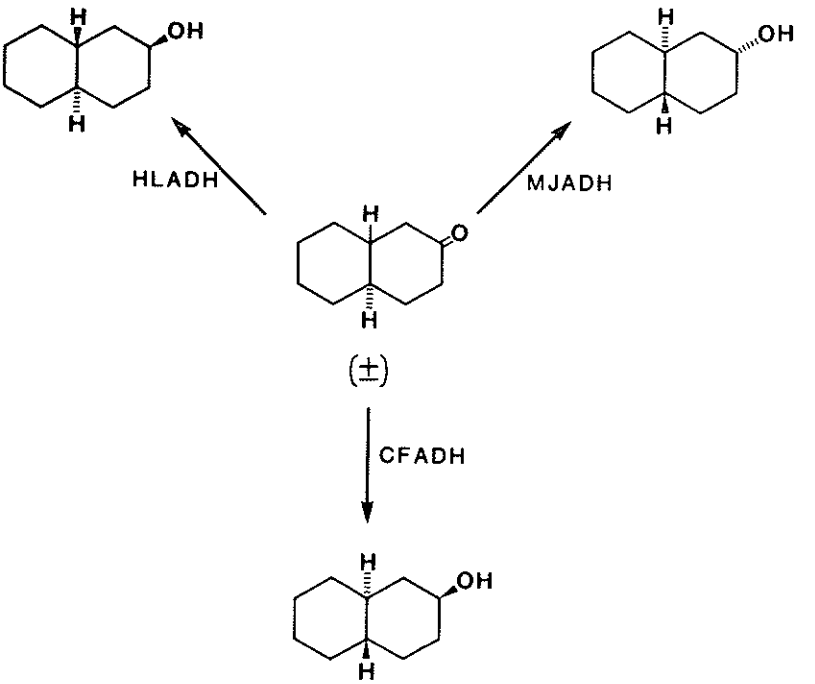


Figure 5. Diastereoisomer formation with different enzymes. HLADH, horse liver alcohol dehydrogenase; MJADH, *Mucor javanicus* alcohol dehydrogenase; CFADH, *Curvularia falcata* alcohol dehydrogenase.

diastereoisomers are formed, depending upon which alcohol dehydrogenase is used (Jones, 1980).

Figure 6 illustrates the extreme specificity of enzymatic oxidations in the carbohydrate field. With two enzymes (D-glucose-1-oxidase, EC 1.1.3.4, and pyranose-2-oxidase, EC 1.1.3.10) alone and in sequence, three useful derivatives of D-glucose can be prepared in excellent yields: (1) D-glucosone, which can be chemically reduced to D-fructose (Geigert, Neidleman and Hirano, 1983); (2) D-glucono-1,5-lactone, which converts to D-gluconic acid in water; and (3) 2-D-ketogluconic acid (Geigert *et al.*, 1983d). Distinguishing C1 and C2 of D-glucose to this degree using chemical catalysis is not possible.

A prime example of selective catalyst design in nature is that of enzymes that catalyse halogen incorporation into a wide spectrum of substrates. Among the known enzymes that catalyse halogenation are those indicated in *Table 5* (Neidleman and Geigert, 1983). They are widespread in nature, and they are all haem-containing peroxidases that vary in their ability to activate particular halide ions. None of these enzymes are able to activate F^- (Morrison and Schonbaum, 1976), and all the marine haloperoxidases that have been carefully studied are bromoperoxidases; that is, they cannot activate Cl^- (Hager, 1982). These facts have interesting consequences, as discussed below.

These enzymes can cause halide incorporation into sulphur- and nitrogen-containing compounds, as well as β-diketones and β-ketoacids (Neidleman,

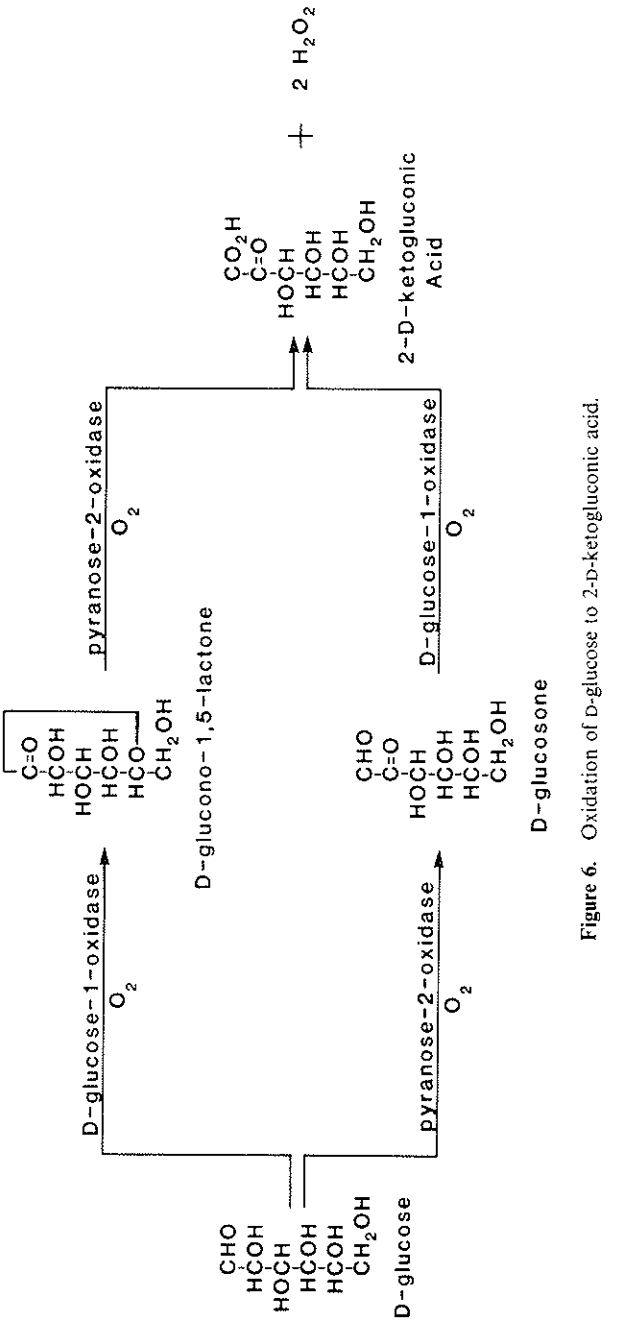


Figure 6. Oxidation of D-glucose to 2-D-ketogluconic acid.

Table 5. Haloperoxidase variants

Enzyme	Source	Approximate optimum pH	Halide activated
Chloroperoxidase (CPO) (chloride peroxidase), EC 1.11.1.10	*Caldariomyces fumago*	3	Cl^- Br^- I^-
Myeloperoxidase (MPO), EC 1.11.1.7	Leucocytes	6–7	Cl^- Br^- I^-
Lactoperoxidase (LPO), EC 1.11.1.7	Milk	6–7	Br^- I^-
Bromoperoxidase (BPO)	Seaweed	6–7	Br^- I^-
Thyroid (iodide) peroxidase (TPO), EC 1.11.1.8	Thyroid	6–7	I^-
Horseradish peroxidase (HRPO), EC 1.11.1.7	Horseradish	6–7	I^-

1975). In addition, recent work has shown that various alkenes, alkynes and cyclopropanes are good substrates for halogenation (Geigert, Neidleman and Dalietos, 1983; Geigert *et al.*, 1983b, c, e). *Figure 7* shows a variety of products that can be synthesized from allyl alcohol in the presence of chloroperoxidase (CPO) (Geigert *et al.*, 1983a; Neidleman and Geigert, 1983). It should be noted that the nature of the halogenated product is dictated by the concentration of the halide ion and by whether one or two different halides are present. This effect will be discussed in a later section. It is also of interest that, in the absence of halide ion, CPO oxidizes allyl alcohol to acrolein.

Figure 8 illustrates the catalytic incorporation of Cl^- into the alkyne, methyl acetylene, and the cyclopropane, methylcyclopropane (Geigert, Neidleman and Dalietos, 1983). The products obtained with methyl acetylene are very responsive

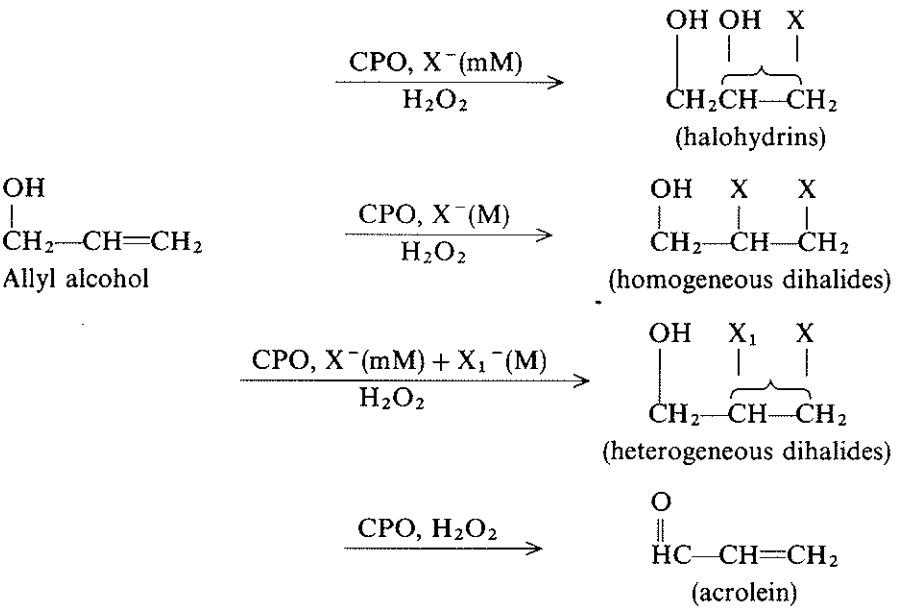


Figure 7. Various reactions between allyl alcohol and chloroperoxidase (CPO), EC 1.11.1.10.

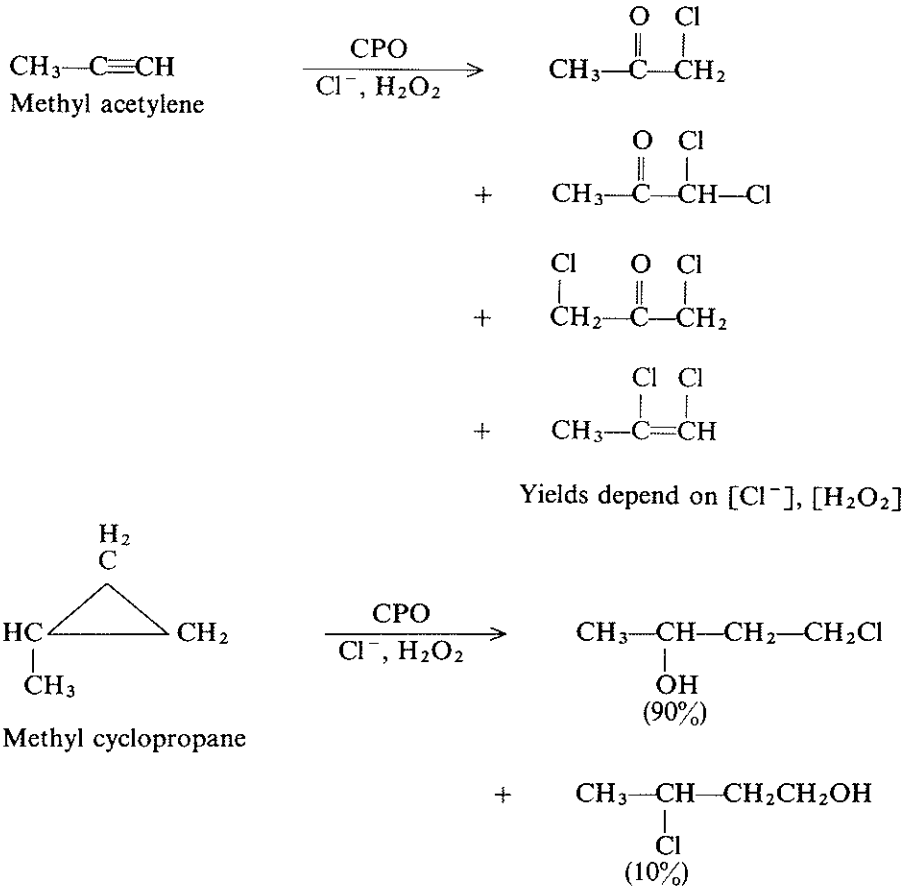


Figure 8. Reactions of chloroperoxidase (CPO) on methyl acetylene and methyl cyclopropane.

to the concentrations of halide ion and hydrogen peroxide—this would be equally true in nature as it is in the laboratory.

Another useful reaction in the synthesis of chemicals is the specific insertion of double bonds into lipids. Depending upon the properties of the desaturating enzyme, double bonds can be placed at different positions within the lipid chain (Fulco, 1977; Weete, 1980). Selectively unsaturated esters and glycerides are of value as flavours, fragrances and as components of various food oils and baked products (Heath, 1981; Macrae, 1983a, b; Strobel, 1983; Strobel *et al.*, 1983). As an example of the production of unsaturated wax esters (monoesters), *Table 6* shows the capacity of *Acinetobacter* sp. HO1-N to form such compounds from ethanol and *n*-eicosane (C_{20}-*n*-alkane) (DeWitt *et al.*, 1982; Ervin *et al.*, 1983). The wax esters contain 0, 1 or 2 double bonds. In the case of a monoenic wax ester, the unsaturation may be in either the acyl or alkoxy moiety of the ester. In the case of a dienic wax ester, one double bond is in the acyl moiety, and the other is in the alkoxy moiety. The ability to control the level of unsaturation in these wax esters with temperature will be illustrated below.

Table 6. Formation of wax esters by *Acinetobacter* sp. HO1-N from *n*-eicosane and ethanol

Substrate ⟶	Fatty acids RCOOH	+ Fatty alcohols + R'OH	⟶ Wax esters ⟶ RC(=O)—OR'		
n-eicosane	16:0, 16:1*	20:0, 20:1	36:0	38:0	40:0
	18:0, 18:1		36:1	38:1	40:1
	20:0, 20:1		36:2	38:2	40:2
Ethanol	16:0, 16:1	16:0, 16:1	32:0	34:0	36:0
	18:0, 18:1	18:0, 18:1	32:1	34:1	36:1
			32:2	34:2	36:2

* 16, 18, 20, 32, 34, 36, 38, 40 = number of carbon atoms: 0, 1, 2 = number of double bonds, located at Δ^9 or Δ^{11} in either the acyl or alkoxy fragment of the wax ester.

Industrial applications

FOODS

The use of biocatalysts in the food industry involves a wide range of effects, including the production of food components such as flavours and fragrances, and the control of colour, texture, appearance and nutritive value. In many cases, the subtleties and nuances of these effects require exquisite control bordering more on art than science. *Table 7* shows various enzymes and their application to particular aspects of the food industry (Shahani *et al.*, 1976; Crouzet, 1977; Yamada, 1977; Aunstrup, 1978; Yamamoto, 1978; Schmid, 1979b; Ter Haseborg, 1981; Marschall *et al.*, 1982; Norman, 1982; Pilnik, 1982; Schindler and Schmid, 1982; Stewart, 1982; Vanbelle, Meurens and Crichton, 1982; Atkinson, 1983; Felix and Villetaz, 1983; Godfrey, 1983a, c; Hollo, Laszlo and Hoschke, 1983; Janda, 1983).

DETERGENTS

One of the major industrial applications of enzymes is in the detergent area. More than 95% of the enzymes sold for laundry detergents are alkaline serine proteases from *Bacillus* sp., especially the *Bacillus subtilis* group. The enzymes must have the following important properties to be acceptable:

1. Stable and active at pH 9·0–10·5.
2. Thermotolerant in the range 55–100°C.
3. Compatibility with perborate, surfactants, chelators, etc.

There is a real possibility that future detergents will contain enzymes other than alkaline serine proteases: lipases for oily or fatty soiling, amylases for starch soiling, and perhaps hydrolases or oxidases (Starace & Barfoed, 1980; Barfoed, 1983; Starace, 1983).

Table 7. Applications of enzymes in the food industry.

Enzymes	Sources	Substrates	Applications
α-Amylase†	Fungal, bacterial	Starch	Liquefaction to dextrins, alcohol production, proper volume in baked goods
β-Amylase‡	Plant	Starch	Maltose production, proper volume of baked goods
Anthocyanase	Fungal	Anthocyanine Glycoside	Decolourization of juice/wine
Catalase†	Fungal, mammals	Hydrogen peroxide	Milk sterilization, cheese-making
Cellobiase (β-D-glucosidase), EC 3.2.1.21	Fungal	Cellobiose	Ethanol production
Cellulase†	Fungal	Cellulose	Ethanol production
Glucoamylase (exo-1,4-α-D-glucosidase), EC 3.2.1.3	Fungal	Dextrins	Dextrin degradation to glucose
Glucose isomerase†	Bacterial	Glucose	High fructose syrup
Glucose oxidase† (± catalase)	Fungal	Glucose, oxygen	Flavour and colour preservation
Hemicellulase*	Fungal, bacterial	Hemicellulose	Clarification of plant extracts
Hesperidinase	Fungal	Hesperidin glycoside	Clarification of juice
Invertase‡	Yeast	Sucrose	Production of invert sugar, sugar confectionery
Lactase‡	Fungal, yeast	Lactose	Lactose hydrolysis in cheese whey
Lipase†	Fungal, bacterial, goat, calf, lamb throat	Lipid	Cheese ripening
	Fungal, bacterial	Lipid	Modify milk fat for butter
	Bacterial	Lipid	Sausage curing
Lipoxidase (lipoxygenase), EC 1.13.11.12	Plant	Carotene	Bleaching agent in baking
Melibiase (α-D-galactosidase), EC 3.2.1.22	Fungal	Raffinose	Improve sucrose production from sugar beets
Naringinase	Fungal	Naringin glycoside	Debittering of juice
Pectinase (polygalacturonase), EC 3.2.1.15	Fungal	Pectin	Wine/fruit juice clarification, viscosity reduction in fruit processing
Proteases, e.g. EC 3.4.22.2, 3.4.22.4, 3.4.23.4	Fungal, plant (papain, bromelain)	Protein	Meat tenderizer
	Fungal	Protein	Condensed fish solids
	Fungal, bacterial, calf stomach (rennin)	Casein	Cheese production
	Fungal	Gluten	Dough conditioner
	Bacterial	Protein	Sausage curing
	Plant (papain)	Protein	Beer haze removal
	Pancreas	Protein	Peptone manufacture
	Bacterial	Protein	Soy sauce preparation
Pullulanase, EC 3.2.1.41	Bacterial	Amylopectin	Beer production, improve glucose and maltose processes

* Mixture † For EC numbers see Table 2 ‡ For EC numbers see Table 4

Table 8. Application of enzymes in the medical field.

Enzyme	Source	Substrate	Effect
Amylase, EC 3.2.1.1, 3.2.1.2	Fungal, bacterial	Starch	Digestive aid
L-Asparaginase, EC 3.5.1.1	Bacteria	L-Asparagine	Antitumour
Bromelain, EC 3.4.22.4	Pineapple	Protein	Digestive aid, reduction of oedema
Cellulase, EC 3.2.1.4	Fungal	Cellulose	Digestive aid
Chymopapain, EC 3.4.22.6	Papaya	Protein	Treatment of herniated discs
Chymotrypsin, EC 3.4.21.1	Pancreas	Protein	Treatment of inflammatory conditions
Diphtheria toxin	Bacterial	Elongation factor 2	Antitumour
Ficin, EC 3.4.22.3	Fig	Protein	Digestive aid
Hyaluronidase, EC 3.2.1.35 and EC 3.2.1.36	Beef seminal vesicles	Hyaluronic acid	In conjunction with anaesthetics (facilitates absorption)
Kallikrein, EC 3.4.21.8	Pancreas	Protein	Reduce blood pressure
Lactase (β-D-galactosidase)	Yeast, fungal	Lactose	Increase milk digestibility
Lipase (triacylglycerol lipase), EC 3.1.1.3	Fungal, pancreas	Lipids	Digestive aid
Lysozyme, EC 3.2.1.17	Chicken egg white	Mucopolysaccharide	Antiviral, antibacterial
Pancreatin (mixture)	Pancreas	Starch, lipid, protein	Digestive aid
Papain, EC 3.4.22.2	Papaya	Protein	Digestive aid, reduction of oedema, treatment of herniated discs
Pepsin, EC 3.4.23.1	Mammalian stomach	Protein	Digestive aid
Plasma, EC 3.4.21.7	Human blood	Protein	Dissolve blood clots
Protease	Bacterial	Protein	Digestive aid
Streptodornase (deoxyribonuclease I), EC 3.1.21.1	Bacterial	DNA	Wound débridement
Streptokinase (plasmin, EC 3.4.21.7)	Human urine	Protein	Dissolve blood clots
Trypsin, EC 3.4.21.4	Pancreas	Protein	Digestive aid, treat athletic injuries
Urokinase, EC 3.4.21.31	Human urine	Protein	Dissolve blood clots

MEDICAL FIELD

Table 8 indicates that the major use of enzymes in the medical field is in digestive aids. However, it is also clear that a range of other applications is existent: use as antitumour or antimicrobial agents, and in the treatment of blood clots and herniated discs, for example. There is considerable research in this area at present, and there is no doubt that more sophisticated applications, such as the treatment of enzyme deficiencies, will be forthcoming (Ruyssen and Lauwers, 1978; Cooney, Stergis and Jayaram, 1980; Anonymous, 1982).

ANALYTICAL APPLICATIONS

The use of enzymes in various aspects of chemical analysis is a rapidly growing area of interest. One of the major applications is in the diagnostic field, as indicated in *Table 9*. These uses have been aided by the development of highly sophisticated solid-support systems and instrumentation. The most common

Table 9. Analytical applications of enzymes.

Enzyme	Source	Substance analysed
Alcohol dehydrogenase, EC 1.1.1.1	Yeast, horse liver	Ethanol
Cholesterol oxidase, EC 1.1.3.6	Bacterial	Cholesterol
Creatinase, EC 3.5.2.10	Bacterial	Creatinine
Galactose oxidase, EC 1.1.3.9	Fungal	Galactose
Glucose oxidase, EC 1.1.3.4	Fungal	Glucose
Glycerol kinase, EC 2.7.1.30	Bacterial, yeast	Triglycerides
L-Glycerol-3-phosphate dehydrogenase, EC 1.1.99.5	Rabbit	Triglycerides
Lactate dehydrogenase, EC 1.1.1.27	Mammalian	Triglycerides, transaminase
Lipase (triacylglycerol lipase), EC 3.1.1.3	Fungal, yeast, wheat germ, pig	Triglycerides
Luciferase (luciferin sulphotransferase) EC 2.8.2.10	Firefly	ATP
Peroxidase, EC 1.11.1.7	Horseradish	Hydrogen peroxide from various reactions
Urease, EC 3.5.1.5	Jack bean	Urea
Uricase (urate oxidase), EC 1.7.3.3	Yeast, pig	Uric acid

tests are carried out on human serum, a liquid of reasonably consistent characteristics. This fact has allowed for the development of regularized, miniaturized and highly automated analytical systems. Other industrial applications are primarily aimed at monitoring of various industrial processes for such compounds as acetic acid, citric acid, galactose and glucose. Another use is in the analysis of pesticides: organophosphates and carbamates (Keyes, 1980; Langley, 1983).

PRODUCTION OF CHEMICALS

The production of the chemicals listed in *Table 10* is usually carried out with enzymes immobilized within living or dead cells, or by fermentation with live cells. The wide diversity of compounds that have been produced is obvious.

Table 10. Chemicals

Compound	Producing organism
I. Organic Acids, Alcohols, Aldehydes	
Acetic acid	*Clostridium* sp., *Gluconobacter* sp.
Acetone	*Clostridium* sp.
Butanol	*Clostridium* sp.
Citric acid	*Aspergillus niger, Candida lipolytica*
Erythorbic acid	*Penicillium notatum*
Ethanol	Various yeasts
Fumaric acid	*Rhizopus delemar*
Gluconic acid	*Aspergillus niger*
Itaconic acid	*Aspergillus terreus*
2-Ketogluconic acid	*Pseudomonas meldenbergii*
5-Ketogluconic acid	*Gluconobacter suboxydans*
α-Ketoglutaric acid	*C. hydrocarbofumarica*
Kojic acid	*Aspergillus oryzae*
Lactic acid	*Lactobacillus delbrukii*
Malic acid	*Lactobacillus brevis*
Propionic acid	*Propionibacterium shermanii*
Succinic acid	*Bacillus succinicum*
II. Amino Acids	
L-Alanine	*Pseudomonas dacunhae*
DL-Alanine	*Corynebacterium gelatinosum*
L-Arginine	*Brevibacterium flavum*
L-Aspartic acid	Aspartase from *Escherichia coli* and *Erwinia herbicola*
L-Citrulline	*Brevibacterium flavum, Pseudomonas putida*
L-Glutamic acid	*Corynebacterium glutamicum, Brevibacterium flavum*
L-Histidine	*Brevibacterium flavum*
L-Isoleucine	*Brevibacterium flavum*
L-Leucine	*Bacillus lactofermentum*
L-Lysine	*Corynebacterium glutamicum, Brevibacterium flavum,* H-enzyme (*Cryptococcus laurentii*) plus R-enzyme (*Achromobacter obae*)
L-Ornithine	*Corynebacterium glutamicum*
L-Phenylalanine	*Bacillus subtilis, Brevibacterium flavum*
L-Proline	*Brevibacterium flavum*
L-Serine	*Corynebacterium glycinophilum*
L-Threonine	*Brevibacterium flavum*
L-Tryptophan	*Bacillus subtilis, Brevibacterium flavum*, tryptophanase from *Proteus rettgeri*
L-Tyrosine	L-Tyrosinase from *Erwinia herbicola*
L-Valine	*Bacillus lactofermentum*
III. Miscellaneous Chemicals	
Adenosine	*Bacillus* sp.
Carotenoids	Algae, fungi
Gibberellins	*Gibberella fujikuroi*
Guanosine	*Bacillus subtilis*
Inosine	*Bacillus subtilis*
Inosine-5′-phosphate	*Brevibacterium ammoniagenes*
Riboflavin	*Clostridium* sp., *Ascomycetes* sp., *Candida* sp.
Vitamin B_{12}	*Propionobacter shermanii, Bacillus* sp., *Pseudomonas* sp.
Xanthan gum	*Xanthomonas campestris*

IV. Possible Chemicals of the Future	
Acrylic acid	Propylene glycol
Adipic acid	Phthalic anhydride
Ethylene oxide	Tartaric acid
Ethylene glycol	Propylene oxide
Glycerol	

V. Chemicals Not Detailed	
Antibiotics	Steroid derivatives
Enzyme inhibitors	Flavours
Alkaloids	Fragrances
Lipid derivatives	Surfactants

The list is not exhaustive, and not all examples are currently commercially significant. Usually, the use of purified enzymes is precluded by the economic disadvantage of the high cost of preparing the enzyme. In other cases, the starting material is D-glucose and its conversion to the final product requires multiple enzymatic activities with associated cofactors; this is most feasible with whole cells. There is considerable research and progress, however, in the application of coenzyme-dependent enzymes in industry (Lowe, 1983). Many references may be consulted for further information on production of chemicals (Kieslich, 1976; Arima, 1977; Perlman, 1977; Sebek and Kieslich, 1977; Yamada and Kumagai, 1978; Gray and Tribe, 1979; Tribe and Gray, 1979; Janshekar and Fiechter, 1982; Rosazza, 1982; Tong, 1982; Chibata, Tosa and Sato, 1983; Linden and Moreira, 1983).

WASTE TREATMENT

The use of enzymes for waste treatment is widespread. Among the enzymes that are used are amylase, amyloglucosidase, cellulase, glucoamylase, lipase, pectinase and protease. They are used to digest their normal substrates, the carbohydrates, proteins, fats and oils. Often, the goal is to 'recycle' the waste for reuse: for example, to convert starch to sugar, to convert whey to various useful products, and for the recovery of additional oil from oil-seeds (Callely, Forster and Stafford, 1976; Godfrey, 1983b; Moo-Young, 1983). A novel procedure for the removal of phenols and aromatic amines, which occur widely in industrial waste waters, has been reported (Klibanov, 1982; Klibanov, Tu and Scott, 1983) and utilizes horseradish peroxidase. The recovery and treatment of lignocellulosic substances is one of the greatest challenges of waste—or, rather, resource—recycling. Economic processes for cellulose and lignin upgrading await improvements in the enzymes involved in their degradation.

MISCELLANEOUS APPLICATIONS

An effort has been made in this review to give a broad view of the use of enzymes in industry. However, the field is so complex and diverse that only partial coverage could be given. *Table 11* gives additional examples of areas where biocatalysis has been applied, which did not fall gracefully into other classifications. Not all are in present use, but the possibility of commercial application exists.

New techniques and concepts

The purpose of this section is to illustrate in several ways that the application of biocatalysis to industrial problems is approaching an explosion of sophistication at least equal to that of genetic engineering. Among the elements in this surge of biocatalysis will be the following:

1. Enzyme reactions in organic solvents.
2. Metal exchange at the active site of enzymes.

Table 11. Miscellaneous applications of enzymes

Enzyme	Substrate	Use	Reference
Aminoacylase EC 3.5.1.14	L-Amino acids	Production of L-amino acids	Chibata, Tosa and Sato (1983)
α-Amylase, EC 3.2.1.1	Starch	Textile desizing, dental hygiene	Godfrey and Reichelt, 1983c, d
Dextranase, EC 3.2.1.11	Dextran	Dental hygiene	Godfrey and Reichelt, 1983d
Glucose oxidase, EC 1.1.3.4	Glucose	Dental hygiene	Godfrey and Reichelt, 1983d; Schmid, 1979b
Papain, EC 3.4.22.2	Protein	Dental hygiene	Godfrey and Reichelt, 1983d
Pectinase, EC 3.2.1.15	Pectins	Wood preservation, retting of textile fibres	Fogarty and Kelly, 1983
Pencillin amidase, EC 3.5.1.11	Penicillins	Synthesis of antibiotics	Arima, 1977
Protease	Protein	Dental hygiene	Godfrey and Reichelt, 1983d
		Leather tanning, photographic silver recovery	Godfrey and Reichelt, 1983; Ward, 1983; Cowan, 1983

3. Chemical modification of enzymes.
4. Enzyme variants from nature, mutation, and genetic (protein) engineering.
5. Effects of temperature on lipid unsaturation.
6. Effects of reactant concentration on reaction product chemistry.
7. Effects of enzyme immobilization.

EFFECTS OF ORGANIC SOLVENTS ON ENZYME REACTIONS

Organic solvents can have deleterious effects on enzyme reactions by causing protein denaturation and, therefore, a loss in enzymatic activity. However, as in many other instances, there is another side of the coin: organic solvents can have a positive effect on certain enzyme reactions. Examples are finding their way into the literature with increasing frequency. Several mechanisms appear to be involved in these advantageous cases, including:

1. Increasing substrate solubility in the aqueous phase and therefore increasing substrate availability for enzymatic transformation.
2. Reducing hydrolytic reactions by making water a limiting reactant.
3. Altering the conformation of the active site of the enzyme.

Each of these mechanisms will be illustrated by examples from the literature.

Two basic types of systems have been studied in pursuing enzyme reactions in solvents. One is a monophasic system in which a water-miscible solvent is employed. A second version of a monophasic system is that in which the only solvent is an organic one. In contrast to these monophasic systems, there are biphasic systems in which water and an immiscible organic solvent are used. As not all enzymes can retain activity in the presence of high levels of organic solvent, the biphasic systems have the advantage that the enzyme in the aqueous phase is not necessarily contending with elevated and inhibitory solvent concentrations (Lilly, 1983).

Table 12. Improvement by solvents of cortisone reduction by 20β-hydroxysteroid dehydrogenase (*20β-HSDH*)†

Organic solvent	Activity of 20β-HSDH (% inhibition)	Cortisone solubility in solvent (g/100 ml)	Solvent solubility in water (%; w/v)	Cortisone reduction (%)
n-hexane	0	0·002	0·014	< 5
Carbon tetrachloride	0	0·004	0·08	< 5
Chlorobenzene	0	0·030	0·048	15
Diethyl ether	62	0·017	7·5	10
Butyl acetate	52	0·160	0·5	100
Ethyl acetate	71	0·270	8·6	90

† EC 1.1.1.53.

Some of these principles are illustrated in *Table 12*. In aqueous medium, the percentage conversion of cortisone to preg-4-en-17,20,21-triol-3,11-dione is < 5%. The Table clearly indicates that butyl and ethyl acetate markedly improve the transformation (Antonini, Arrea and Cremonesi, 1981). A number of points should be noted. These solvents do inhibit the activity of 20β-hydroxysteroid dehydrogenase (EC 1.1.1.53). The logical reaction to this inhibitory effect might be to eliminate these solvents as candidates to improve the reaction in favour of a solvent such as chlorobenzene, which does not inhibit activity of the enzyme. However, it is seen that the inhibition by butyl and ethyl acetate is compensated for by the increased solubility of cortisone in the aqueous phase, resulting from a balance of cortisone solubility in the organic solvent and solubility of the organic phase in the aqueous phase. It is necessary to assess carefully the net worth of both positive and negative factors.

Another principle to be illustrated is that certain hydrolytic reactions can be reversed to afford synthetic reactions. Esterases and lipases that normally hydrolyse their ester and glyceride substrates in an aqueous milieu can often synthesize these substances when water is made a limiting reagent. Thus, a reaction which is thermodynamically at a disadvantage in water is thermodynamically favoured in organic solvent. The proportion of organic solvent can approach 100%.

Table 13 shows an example of ester synthesis by α-chymotrypsin in the presence of chloroform (Klibanov *et al.*, 1977). With water as the solvent, the ester

Table 13. Synthesis of esters by 'hydrolytic' enzymes in solvents

Acid	Alcohol		Ester
N-acetyl L-tryptophan	+ ethyl	EC 3.4.21.1 α-chymotrypsin / $CHCl_3$	*N*-acetyl L-tryptophan ethyl ester (100%)
Linoleic	+ cetyl		Cetyl linoleate (~90%)
Palmitic	+ octyl		Octyl palmitate (~90%)
Pentanoic	+ pentyl	Dried mycelia of *Rhizopus arrhizus*	Pentyl pentanoate
Butyric	+ benzyl		Benzyl butyrate
Acetic	+ geranyl		Geranyl acetate (30–70%)

is not synthesized. *Table 13* also illustrates the capacity of one of the reactants, the alcohol, to serve as the solvent for ester synthesis with dried mycelia of *Rhizopus arrhizus* (Bell *et al.*, 1978; Patterson *et al.*, 1979; Strobel, 1983; Strobel *et al.*, 1983). Methods such as these will be employed to produce flavours and fragrances for industrial use.

These techniques can be used to prepare mono-, di- and triglycerides, as well as simple esters. This is shown in *Table 14*. Glycerol and fatty acid are dissolved

Table 14. Synthesis of glycerides in solvent by dried *Rhizopus arrhizus* mycelia

Glycerol (0·2% w/v)	+ Oleic acid (10% w/v)	*R. arrhizus* (dried) → Acetone	1-Monoglyceride 1,2/1,3-Diglycerides 1,2,3-Triglyceride (trace)

70% yield based on conversion of hydroxyl groups to ester.

in a solvent and, in the presence of dried mycelia of *R. arrhizus*, glycerides are synthesized (Bell *et al.*, 1978). A similar process with *Corynebacterium* sp. 5–401, in which glycerol and oleic acid are dissolved in *n*-hexane, yielded triolein, rather than mono- or diolein (Seo, Yamada and Okada, 1982). Methods such as these will synthesize tailor-made glycerides for industrial applications (Tanaka *et al.*, 1981; Fukui and Tanaka, 1982).

It was shown in *Table 12* that the nature of the organic solvent can have a marked influence on the efficiency with which an enzymatic transformation of a steroid can be performed. The same holds true for enzyme-catalysed ester synthesis: *Table 15* illustrates that chloroform is the solvent of choice for ester synthesis with α-chymotrypsin (Martinek and Semenov, 1981; Martinek, Semenov and Berezin, 1981).

Table 15. Synthesis of *N*-benzoyl-L-phenylalanine ethyl ester by α-chymotrypsin in the presence of various solvents

Solvent	Yield (%)
Chloroform	80
Benzene	64
Carbon tetrachloride	63
Diethyl ether	26
Water	0

The process of interesterification is another example of a potential industrial application of biocatalysis in biphasic systems. Typically, lipase-coated inorganic particles are activated with 10% water, and stirred in a reactor with substrates dissolved in a solvent such as petroleum ether (Macrae, 1983a, b). Such a method can use a cheap feedstock such as olive oil, and, under appropriate conditions, a reaction product closely resembling the more valuable cocoa butter can be obtained. Reactions such as these offer a clear indication of the greater specificity that can be achieved with biocatalysis, compared with traditional chemical catalysis.

Table 16. Interesterification of olive and coconut oils (1 : 1; w/w)

Carbon no. in triglyceride fatty acids	Starting mixture (wt %)	Percentage change from starting mixture in interesterified oils treated with:	
		C. cylindracae lipase†	Alkali metal
26–28	29·2	−13·0	−14·0
40–48	12·0	+48·3	+48·9
50–56	58·7	−35·2	−34·2

†Triacylglycerol lipase, EC 3.1.1.3.

Table 16 illustrates that both chemically catalysed and enzyme-catalysed interesterification can result in random acyl migration and exchange (Macrae, 1983a, b). In this case, sodium or sodium alkoxide and a non-specific lipase offer little to choose from with respect to the end product. However, when enzymes with intrinsic catalytic specificity are used, the situation is quite different. For example, in *Table 17* (Macrae, 1983a, b) the use of a 1,3-specific lipase in the presence of olive oil and stearic acid enriches the 1,3-positions of olive oil with stearic acid. In *Table 18* a linoleic-specific lipase results in the enrichment of olive oil with linoleic acid, but not stearic acid (Macrae, 1983a, b). Remarkable reaction specificity such as this can yield novel products with selected properties not achievable through random chemical or enzymatic interesterification.

The final example of organic solvent effects on biocatalysis is the most intriguing because it has basic implications for both industry and nature. It is the regulation of enzyme activity and specificity through solvent-mediated conformational changes in the active site of enzymes. α-Thrombin is an enzyme with both esterase and amidase activity. *Table 19* shows the influence of dimethylsulphoxide (DMSO) on this dual activity: increasing DMSO concentration increases esterase activity and decreases amidase activity (Pal and Gertler, 1983). This is an example wherein the catalytic capacity of the enzyme in an aqueous reaction mixture can be increased by addition of an organic solvent.

The effects of solvents on biocatalysis are complex, but promise exciting developments in industrial applications.

METAL EXCHANGE AT ACTIVE SITES OF ENZYMES

In chemical catalysis, it is legend to believe that to change the catalyst, you change the metal. In biocatalysis, this concept is in its infancy. There are provocative data in the literature to show that this underdeveloped approach to enzyme modification and improvement deserves more attention. Examples will be given to illustrate metal exchange as a means of altering enzyme specificity, stability and inhibition.

Like α-thrombin, carboxypeptidase A is an enzyme with two catalytic activities —esterase and peptidase. In the case of α-thrombin, esterase activity is increased and amidase activity decreased in the presence of increasing concentrations of DMSO. In the case of carboxypeptidase A, esterase activity may be increased, and peptidase activity decreased by metal exchange. *Table 20* shows that when zinc, the natural metal component, is replaced by cobalt, both esterase and

Table 17. Olive oil triglyceride interesterification with a 1,3-specific lipase (EC 3.1.1.3) of *Rhizopus delemar* and stearic acid (5:1, w/w)

	Total triglycerides		Positions 1, 3		Position 2	
Fatty acid	Olive oil (wt %)	Interesterified oil (% change)	Olive oil (wt %)	Interesterified oil (% change)	Olive oil (wt %)	Interesterified oil (% change)
16:0	16·6	−2·9	23·2	−4·3	3·5	−0·3
16:1	1·8	−0·2	2·0	−0·4	1·3	+0·3
18:0	2·0	+13·6	2·5	+20·5	1·0	−0·3
18:1	66·8	−10·2	64·2	−15·4	72·0	+0·2
18·2	12·8	−0·2	8·1	−0·4	22·2	+0·1

Table 18. Olive oil triglyceride interesterification using the linoleic acid specific lipase† of *Geotrichum candidum* with stearic acid, and linoleic acid (1·0:0·15:0·15, w/w/w).

Fatty acid	Fatty acid content of olive oil (%)	Change in interesterified oil product (%)
16:0	11·6	−0·1
16:1	0·8	+0·3
18:0	3·6	+0·9
18:1	72·8	−8·0
18:2	10·6	+7·7
20:1	0·6	−0·2

† Triacylglycerol lipase, EC 3.1.1.3.

Table 19. DMSO effects on the enzyme activity of bovine α-thrombin†

DMSO (% v/v)	Activity (%)	
	Amidase‡	Esterase§
0	100	100
5	60	135
10	30	175
15	30	190
20	9	200

† EC 3.4.21.5.
‡ 0·2 mM *N*-Benzoyl-L-phenylalanyl-L-valyl-arginine-*p*-nitroanilide HCl.
§ 1 mM *p*-Tosyl-L-arginine methyl ester HCl.

Table 20. Effects of metal exchange on the enzyme activity of bovine carboxypeptidase A†

Metal	Relative rates	
	Esterase‡	Peptidase§
Apo (−metal)	0	0
Zinc	100	100
Cobalt	114	200
Nickel	43	47
Manganese	156	27
Cadmium	143	0
Mercury	86	0
Rhodium	71	0
Lead	57	0
Copper	0	0

† EC 3.4.17.1.
‡ 0·01 M-benzoylglycyl-DL-phenyllactate, pH 7·5, 25°C.
§ 0·02 M-benzyloxycarbonylglycyl-L-phenylalanine, pH 7·5, 0°C.

peptidase activities are increased. When zinc is replaced by manganese or cadmium, esterase activity is increased, while peptidase activity is markedly reduced or vanishes completely (Vallee, 1980).

A somewhat more subtle effect of metal exchange is illustrated in *Table 21.* The cases of α-thrombin and carboxypeptidase A involve an alteration in general classes of enzyme activity: esterase, peptidase and amidase. In the case of the aminoacylase, substitution of zinc by cobalt has an effect on substrate specificity in one type of hydrolytic activity, namely deacylation. The data show that

Table 21. Metal exchange effects on substrate specificity of the aminoacylase of *Aspergillus oryzae*

Substrates	Relative activity (%)	
	Co^{2+}	Zn^{2+}
N-Chloro-acetyl-Ala	127	100
N-Chloro-acetyl-Met	163	286
N-Chloro-acetyl-Norleu	191	326
N-Chloro-acetyl-Leu	153	60
N-Chloro-acetyl-Phe	195	218
N-Acetyl-Glu	2·5	1
N-Acetyl-Gln	59	12
N-Acetyl-Ala	39	16·5
N-Acetyl-Lys	6·5	1

substituting cobalt for zinc increases activity against six substrates and decreases activity versus three (Gilles, Loffler and Schneider, 1981); depending on the specific substrate, therefore, either the zinc or the cobalt enzyme might be the choice. Furthermore, the pH optimum of the zinc enzyme towards *N*-chloroacetyl-alanine is 8·5, whereas that of the cobalt enzyme is 7·0.

Table 22. Metal exchange effects in iodosylbenzene-supported oxygenation with cytochrome $P450_{cam}$

Cyt $P450_{cam}$	Reactions	Substrates	Relative activity (%)
Fe(III)	5-hydroxylation	Camphor	100
Fe(III)	5,6-epoxidation	Dehydrocamphor	100
Mn(III)	5-hydroxylation	Camphor	<0·5
Mn(III)	5,6-epoxidation	Dehydrocamphor	53

The examples just discussed are concerned with hydrolytic enzymes. The effects of metal exchange are not limited to this enzyme class. In *Table 22* two different oxidative activities of cytochrome $P450_{cam}$ are considered, namely 5-hydroxylation of camphor and 5,6-epoxidation of dehydrocamphor. Whereas the Fe(III) enzyme (the natural enzyme) carries out both reactions, the Mn(III) enzyme catalyses only the latter (Gelb, Toscano and Sligan, 1982). In a hypothetical case, if one had a substrate that could be hydroxylated and epoxidized but only the epoxide product was desired, the side-reaction (the hydroxylation) could be eliminated by constructing and using the Mn(III) enzyme.

The case of substituting manganese for iron in a superoxide dismutase of *Bacteroides fragilis* (*Table 23*) illustrates a remarkable alteration in enzyme

Table 23. Metal exchange effects on the superoxide dismutase of *Bacteroides fragilis*.

Superoxide dismutase (SOD)	Effect of 5 mM H_2O_2 on half-life (min)	Inhibition by mM NaN_3 (%)		
		0·2	1·0	20·0
Native Fe SOD	4	56	89	—
Reconstituted Fe SOD	5	51	84	—
Reconstituted Mn SOD	>20	—	0	45

stability and inhibition. The natural and reconstituted iron-containing enzymes are considerably more sensitive to sodium azide (NaN_3) inhibition and hydrogen peroxide instability than the reconstituted manganese-containing enzyme. This effect mirrors the difference noted in naturally occurring iron and manganese variants of superoxide dismutase (EC 1.15.1.1): a satisfactory agreement between two 'laboratories', the natural and the academic (Gregory and Dapper, 1983).

It is clear from these dramatic alterations in biocatalytic properties by metal exchange at the active site that more attention should be given to the technique.

EFFECT OF TEMPERATURE ON ENZYMATIC UNSATURATION OF LIPIDS

The production of saturated and unsaturated wax esters by *Acinetobacter* sp. HO1-N was discussed above. The capacity of the micro-organism to vary dramatically the level of unsaturation can be enhanced by imposing a temperature stress on the biosynthetic process. There is in nature a common and inverse relationship between lipid unsaturation and temperature: high temperature leads to low unsaturation; low temperature leads to high unsaturation. The major function of this relationship is to ensure membrane function and fluidity over a range of temperature conditions. *Table 24* illustrates that the wax esters

Table 24. Effect of temperature on unsaturation of wax esters produced from ethanol and *n*-eicosane by *Acinetobacter* sp. HO1-N

Temperature (°C)	Di-ene fractions (Total)	Mono-ene fractions (Total)	Saturated fractions (Total)
Ethanol	(32:2, 34:2, 36:2)	(32:1, 34:1, 36:1)	(32:0, 34:0, 36:0)
17	72%	18%	10%
24	29%	40%	31%
30	9%	25%	66%
n-Eicosane	(36:2, 38:2, 40:2)	(36:1, 38:1, 40:1)	(36:0, 38:0, 40:0)
17	71%	24%	5%
24	41%	37%	22%
30	35%	40%	25%

produced by *Acinetobacter* sp. HO1-N from *n*-eicosane and ethanol at 17°C are much more unsaturated than those produced at 30°C (Ervin *et al.*, 1983). This response may be mediated through temperature-sensitive desaturases, but the mechanism is not yet clarified. However, even in the absence of a biochemical explanation, the technique can, in appropriate situations, give very high levels of specifically unsaturated lipid derivatives.

EFFECTS OF SUBSTRATE (HALIDE ION) CONCENTRATION ON PRODUCT CHEMISTRY

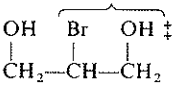
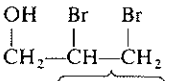
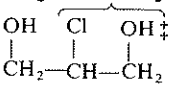
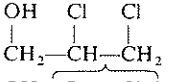
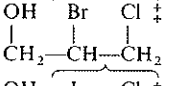
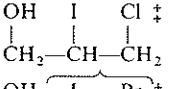
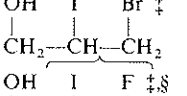
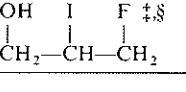
It was noted in *Figures 7* and *8* that the nature of the halogenated products synthesized by haloperoxidases (E.C. 1.11.1.10) from allyl alcohol depends upon halide ion concentration, among other things. At low millimolar levels of activatable halide ion, the major products are halohydrin, whereas at molar levels the product is the homogeneous dihalide. It was further indicated that in the presence of two different halide ions, heterogeneous (mixed) dihalide derivatives could be formed. The requirements for this reaction include the

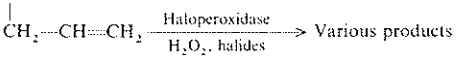
following: (1) at least one of the halide ions must be activatable by the chosen haloperoxidase; (2) the most readily activated halide ion should be present at millimolar concentration with the less or non-activatable halide ion at molar concentrations; and (3) the ratio of the lesser to the more activated halide ion should be > 100. Halide ions can be arranged in the following order according to their ease of activation: $I > Br > Cl \ggg F^-$. In fact, F^- is not activated by any known haloperoxidase.

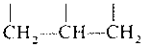
Taking advantage of these various factors, some unusual heterogeneous dihalide derivatives of allyl alcohol can be synthesized, as illustrated in *Table 25*. Some of these compounds are new compositions of matter, showing that biocatalysis can yield truly novel products. The fluorinated derivative represents the first C–F bond synthesized in an enzyme-associated reaction with a purified biocatalyst (Neidleman and Geigert, 1983).

There will be many examples in the future wherein the nature of the end-product of the enzyme-catalysed reaction will depend upon the substrate preference of the enzyme and the relative concentrations of 'competing' substrates.

Table 25. Various halogenated products formed from allyl alcohol† by haloperoxides as a function of halide concentrations and ratios

Enzyme	mM KBr	mM KCl	mM KI	mM KF	Major product
Chloroperoxidase	17	—	—	—	
Chloroperoxidase	3389	—	—	—	
Chloroperoxidase	—	200	—	—	
Chloroperoxidase	—	2000	—	—	
Chloro- or lacto-peroxidase	20	2000	—	—	
Chloroperoxidase	—	2000	20	—	
Horseradish peroxidase	2000	—	20	—	
Horseradish peroxidase	—	—	20	2000	

† 

‡ Positional isomers in 1 : 1 ratio.
§ Actually minor product (10% yield); major product: 

ENZYME VARIANTS FROM NATURE, RANDOM MUTATION, GENETIC ENGINEERING AND CHEMICAL MODIFICATION

The preparation of enzyme variants by metal exchange at the active site has already been discussed. Other ways to isolate or prepare modified enzymes exist, including: (1) screening for enzyme variants in nature; (2) creating new enzymes by random mutation; (3) constructing alternative biocatalysts through genetic engineering and site-specific mutation; and (4) chemically modifying the enzyme.

Nature has an enormous reservoir of variants for many classes of enzymes. *Table 5* shows variant haloperoxidases. *Table 26* illustrates variants of super-

Table 26. Variants of superoxide dismutases.

Metal	Source
Fe	*Rhizobium japonicum*
Mn	Pea leaf
	Escherichia coli
Mn/Fe	*Rhodococcus bronchialis*
Cu/Zn	Vertebrates
	Green pea
	Neurospora crassa
	Saccharomyces cerevisiae
Fe/Zn	*Thermoplasma acidophilum*

$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$.

oxide dismutases (Neidleman, 1983). Other enzymes that show variability in structure and properties include α-amylase (Fogarty, 1983b), glucose isomerase (Bucke, 1983), pectinolytic enzymes (Fogarty and Kelly, 1983), cellulases (Enari, 1983), lipases (Macrae, 1983a, b) and proteinases (Ward, 1983). A particularly interesting example of an enzyme variant is that of a non-haem, manganese-containing catalase of *Lactobacillus plantarum*. Traditional catalase is an iron–haem enzyme. In this case, two markedly different enzymes catalyse the same reaction—the degradation of hydrogen peroxide (Kono and Fridovich, 1983a, b).

In addition to the fact that the search for, and the discovery and characterization of, variant enzymes may offer solutions to problems in industrial biocatalysis, information derived from these studies will supply structure–activity data that will aid in the directed synthesis of tailored enzymes by protein or genetic engineering as well as by chemical modification (Ulmer, 1983). The design of optimized enzymes will include consideration of thermostability, turnover number, substrate specificity, product inhibition, cofactor requirements, pH optimum, size and stability to pH, salts and other enzymes.

Table 27 gives some examples of enzyme alteration by random mutation and site-specific genetic engineering. It should be noted that the effect is not always in a positive direction, but even the negative effects on activity afford useful insights for future studies.

The effects of chemical modification on the catalytic activity of various enzymes are illustrated in *Table 28*. In each of these four cases, a positive change is affected either by increasing the activity of the biocatalyst or by initiating a new catalytic function.

Table 27. Enzyme modifications by mutation and genetic engineering.

Enzyme	Source	Modification†	Effect	Reference
Aspartate transcarbamoylase (aspartate carbamoyltransferase), EC 2.1.3.2	*E. coli* HS513	Change of glycine^{125} to aspartic acid at position other than active site by mutation	Loss of activity, binding of catalytic and regulatory subunits weakened	Kim *et al.*, 1981
β-Lactamase (penicillinase), EC 3.5.2.6	*E. coli* K12	(1) Serine^{70} to cysteine by recombinant DNA	Decreased enzyme activity	Sigal, Harwood and Arentzen, 1982.
		(2) Serine^{70} to threonine and threonine^{71} to serine	Inactive enzyme	Dalbadie-McFarland *et al.*, 1982
Tyrosyl-tRNA-synthetase, EC 6.1.1.1	*Bacillusstearothermophilus* cloned into *E. coli*	Cysteine^{35} to serine	K_m for ATP lowered	Winter *et al.*, 1982.
Xanthine dehydrogenase (purine hydroxylase I), EC 1.2.1.37	*A. nidulans*	Alteration in relative positions of catalytic and orienting sites through mutation	Change in substrate specificity	Scazzocchio and Sealy-Lewis, 1978

† Superscripts indicate the positions of the amino acids in the protein.

Table 28. Effect of chemical modification of enzymes on catalytic activity.

Enzyme	Chemical modification	Effect	Reference
α-Amylase (*B. subtilis*), EC 3.2.1.1	Tryptophan at active site modified	Greater % of maltose and glucose in product	Hollo, Laszlo and Hoschke, 1983
α-Chymotrypsin, EC 3.4.21.1	Pyridoxal added to the reaction mixture	Allows for hydrolysis of D-aromatic amino esters with a free amino group; decreases similar activity against L-enantiomers (the 'normal' substrate)	Kraicsovits, Previero and Otvos, 1981
Papain (pineapple), EC 3.4.22.2	Alkylation of cysteine at active site with 7α-bromoacetyl-10-methylisoalloxazine	Initiates oxidation of dihydronicotinamides	Levine and Kaiser, 1978
Rennet (chymosin) (*Mucor pusillus*), EC 3.4.23.4	Acylation with various anhydrides	>100% increase in milk coagulating activity	Cornelius, Asmus and Sternberg, 1982

The few examples given as illustrations serve to indicate that both the discovery and the synthesis of variant enzymes will be rich sources of novel biocatalysts, differing in properties from the currently available selection of enzymes. One can anticipate that, with an increasing knowledge of structure–activity relationships in enzymes, there will come an era of 'enzymes-to-order', optimized to the needs of specific processes.

Further, it is challenging to realize that 'non-enzymes' can also carry out catalytic reactions under certain conditions. Bovine serum albumin has been shown to cause hydrolysis of *p*-nitrophenyl α-methoxyphenylacetates (Kokubo *et al.*, 1982). The L-enantiomer is hydrolysed three times faster than the D-enantiomer. The level of biocatalytic activity is considerably less than that of hydrolytic enzymes. Another instance of 'non-enzymatic' catalysis is the hydroxylation of aniline by immobilized haemoglobin in the presence of riboflavin and NADH. Activity similar to that with cytochrome P450 was obtained (Guillochon *et al.*, 1982).

Is it possible that chemical or genetic modification of common proteins such as these can produce economically and commercially significant catalysts?

ENZYME IMMOBILIZATION

Discussing the diverse advantages and techniques of immobilizing enzymes or their producing cells on solid supports for industrial applications is beyond the scope of this review. Numerous useful references are available (Keyes, 1980; Trevan, 1980; Klibanov, 1983). Five general methods for immobilization are available: these are covalent attachment, adsorption, encapsulation, entrapment and cross-linking. Each method may have desirable advantages over other procedures, dependent on whether an enzyme or a cell is being immobilized, and on the cost–benefit picture for the process under consideration.

The major advantages that may result from immobilization are: (1) the biocatalytic system may be readily separated from the products and reused; and (2) immobilization often affords improved enzyme stability against temperature, pH and other environmental denaturants. These effects allow for an increase in the useful lifetime for enzymes (*see also* Chapter 5).

Future developments in immobilization will allow for a more interactive role between the enzyme and the immobilization matrix. The chemical and physical nature of the solid matrix will be modified to afford additional stabilization effects, for example, hydrogen peroxide degradation to protect sensitive oxidases, and microenvironments to favour enzyme reactions in solvents. Heterogeneous matrices, allowing a mosaic of hydrophilic and hydrophobic environments, will allow for the simultaneous or sequential immobilization of multi-enzyme systems to produce a variety of chemicals. Sophisticated immobilization methods will serve as an adjunct in optimizing the use of the enzyme variants discussed above.

Desirable enzyme improvements

As nothing is perfect, it is illogical to expect that for each process there exists an enzyme which is ideal in the eyes of those concerned. Some enzymes approach

Table 29. Some suggested enzyme improvements from the literature

Enzyme	Applications	Useful improvement	Reference
α-Amylase	Detergent	Alkali and bleach resistance	Barfoed, 1983.
Amyloglucosidase	High fructose corn syrup	Immobilized, higher productivity	Reichelt, 1983
Esterases, lipases, proteases	Flavour development	More specificity in flavour development	Godfrey, 1983b.
Glucose isomerase	High fructose corn syrup	Increased thermostability, lower pH optimum	Hollo, Laszlo and Hoschke, 1983.
Glucose-1-oxidase and other oxidoreductases	Diagnostic assay, food quality, dentifrice	Increased stability to hydrogen peroxide	Schmid, 1979b; Richter, 1983
Limoninase (limonin-D-ring lactonase), EC 3.1.1.36	Fruit juice debittering	More complete degration of limonin	Janda, 1983.
Protease	Detergent	Bleach resistance	Starace, 1983; Bucke, 1983.
Protease	Chill-proofing beer	More specific enzymes	Godfrey, 1983a.
Pullulanase, debranching enzyme	High fructose corn syrup	Thermostable	Reichelt, 1983.

the ideal more closely than others. *Table 29* offers a selection of desirable enzyme improvements drawn from recent literature. The Table suggests that the methods discussed above for generating enzyme variants can be applied to numerous examples, and that much remains to be accomplished in biocatalysis.

Conclusion

This review has attempted to present an overview of many of the contact points between industry and biocatalysis, and it seems clear that these are both many and varied. The range is from the subtlety of food aroma and texture to the extraordinary specificity of enzymes in the synthesis of complex organic chemicals, and from the much-acclaimed benefits of therapeutic enzymes to the more down-to-earth necessities of waste disposal.

A most significant development, growing more pervasive in biocatalytic research and applications each year, is the realization that enzymes need not be confined to aqueous reaction conditions, room temperature, and atmospheric pressure. They can tolerate more hostile environments: solvents, temperatures greater than 100°C, and pressures far above atmospheric. Note the recent report (Baross and Deming, 1983) of micro-organisms functioning at 250°C, at a pressure of 265 atmospheres (26·5 MPa) and at the salt concentrations of sea water. Enzymes are not as fragile as has been thought, and will often do new 'tricks' in adverse surroundings.

It is also clear that there is a vast reservoir of untapped, undiscovered and unsynthesized enzyme variants with properties to match most process requirements. A search in nature, the use of chemical modification, and the application of the techniques of mutation and genetic and protein engineering will find or construct the answer to a process engineer's dream.

Finally, it is heartening to note that existent enzymes are not perfect, not all problems have been solved, and that an abundance of exciting and profitable research remains to challenge the willing scientist.

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