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Biocatalysis Made to Order

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ABSTRACT

Recombinant DNA technology is now being explored to engineer enzyme molecules. It has many far-reaching applications in biocatalytic processes of enzyme engineering. The facts have pursued certain important industrial, biomedical, and environmental problems. These current excitements are mainly focused on the basis of gene cloning and in vitro mutagenesis for overproduction and redesigning of enzymes, as well as their probable implications in industry, antibiotic research, and waste degradation.

Index Entries: Gene cloning; site-directed mutagenesis; enzyme overproduction; enzyme redesigning; antibiotic synthesis; catabolic genes; ribozymes; abzymes.

INTRODUCTION

Independent and enormous advancements in molecular biology and enzyme chemistry developed the concepts of recombinant DNA technology and enzyme engineering. In the last decade enzyme technology acquired more and more potential by genetic engineering techniques. Experiments based on cloning and selection of specific genes coupled with in vitro mutagenesis opened novel avenues for finer tunings in protein engineering. Automatically, the questions what, when, and how enzyme technology has to learn from genetic engineering, puzzled many scientists.

Enzyme technology fundamentally is based on immobilization of enzymes as practical biocatalysts for their fruitful and wider applications in terms of biocatalytic manipulations for industrial, analytical, biomedical, and environmental purposes. Gene cloning essentially follows the princi-

ple of insertion of a desired gene into the transport vehicles (plasmids, cosmids, and bacteriophages) for its subsequent introduction into bacterial systems. The host has the capacity to transcribe and translate the cloned gene up to several generations within biological economy and molecular accuracy. Then the foreign protein product is isolated in sufficient quantity for its characterization and application. The in vitro mutagenesis can be used as a powerful tool to change specific nucleotide(s) of the cloned gene to substitute desired amino acid(s) in the native protein molecule for biocatalytic modifications. The field is very young, and undoubtedly there will be massive contributions from directed mutagenesis to protein chemistry, folding, and catalysis, by continued tinkering in and around the active sites of enzymes (*1*).

The most optimistic idea is to reconstruct the structure of the gene

1. For more synthesis of an enzyme,
2. For synthesis of a physically or catalytically superior enzyme; or
3. Not to synthesize any or to synthesize a defective enzyme to stop catalysis partially or fully.

The third possibility is a very difficult task. Still, our bioengineers have proved their creativity in redesigning the basic architecture as well as the temporal sculpture of enzyme molecules in terms of biocatalysis. Everybody will be afraid if somebody comes up with a complete alternative to the enzyme molecule.

OVERPRODUCTION

A large quantity of an enzyme can be produced by cloning its gene in an appropriate host. It includes isolation of the gene, cloning it into restriction site(s) of a suitable vector (commonly, plasmid), and allowing it to express in the host. The gene of interest should either contain the complete genetic information or external control elements along with the original coding sequences. Following transfection by the vector, DNA containing the foreign gene, the host cell is transformed. There is multiplication and amplification of the vector with the growth and division of the host cell. These recombinant clones of the host cell synthesize abundant amounts of the foreign gene product. This results in the overproduction of the enzyme. The efficient and sustained expression of a foreign gene is greatly dependent on the host cell physiology, plasmid stability, and its copy number. Transcriptional, translational, and posttranslational events also largely influence the expression of a cloned gene. An accurate gene expression requires both transcriptional start signals (promoters) and transcriptional stop signals (terminators) (*2*). The promoter contains the TATA box and an enhancer sequence, which are needed for maximal transcription initiation (*3*). Translational signal is also one of the key factors

affecting the expression of a cloned gene, and differences in the different translational initiation sequences determine the variation in the efficiency of mRNA. In several cases the processing of translational product by cleavage of leader sequence is necessary to form an active protein molecule. Activation of certain proteins can only be performed by their glycosylation in glycosylation-proficient (fungal or mammalian) systems, but not in bacterial systems. Such posttranslational modifications occur in the yeast system possessing genes for some bacterial, plant, and mammalian proteins (*4*).

Genes for different enzymes have been cloned in a number of prokaryotic and eukaryotic systems (*4–6*). Expression of the genes causes an increased enzyme production, sometimes at very high levels, when introduced into bacterial and yeast systems (*5*). The plasmid, RSF1010 *trp* hybrid, carrying *trp* operon of *Escherichia coli* has been constructed that is capable of producing over 200-fold more *E. coli*-type tryptophan synthetase in *Pseudomonas aeruginosa* bacterium (*7*). A plasmid vector pBR322 bearing *E. coli* tyrosyl tRNA synthetase (TyrTS) gene expresses TyrTS up to 10% of the total soluble proteins in the *E. coli* cell (*8*), whereas TyrTS expresses 50% of soluble proteins when its gene from *Bacillus stearothermophilus* is cloned into the M13mp93 vector and expressed in *E. coli* (*9*). Cloning of *E. coli* citrate synthase gene into pWK452 and pWK401 plasmids shows 2–2.5-fold and 50-fold production of the enzyme, respectively, in the mutant bacteria over its wild type strain (*10*). The *Aspergillus awamori* species secretes two glycosylated forms of glucoamylase (GAI and GAII), and both forms are encoded by a single gene. A vector (pACl) comprising yeast regulatory signals is employed for the expression of *Aspergillus* glucoamylase in *Saccharomyces cerevisiae.* Gene expression in the yeast cells indicates 25–30% of the total supernatant proteins as glucoamylase (*11*). Similarly, the glycosylated secreted (S) and nonglycosylated cytoplasmic (C) forms of invertase are encoded by separate transcripts from a common *SUC* gene in *S. cerevisiae.* Its signal coding sequence lies within the *SUC* 2 gene, which can be altered by generating deletion mutations in plasmid pAB202. Such deletion mutations have been constructed, and some of them are reported to be the overproducing mutants (pAB273, D71), which can overproduce both forms (S and C) of invertase (*12*). It has also been indicated that the pAB8 transformants of the yeast exclusively synthesize C invertase polypeptide, and that D27 and D36 deletions produce about twice as much total enzyme as the wild-type sequence (*12*).

The transposable element generates an efficient site-specific recombination and encodes resolvase protein that regulates the expression of resolvase gene (*tnp* R) itself. When this *tnp* R gene is closed into the vector pλ8, it expresses 2–3% of total cell protein as γδ resolvase in bacteria (*13*). The dihydrofolate reductase (DHFR) enzyme extends a considerable interest to drug designers and pharmaceutical chemists owing to its assumed clinical importance. Recombinant plasmids (pCV29 and pCV32) containing the *E. coli* DHFR gene code for 6% of total soluble bacterial protein

(*14*). Thymidine kinase (TK) participates in pyrimidine biosynthesis, and the TK gene of herpes simplex virus (HSV) has a widespread use in gene cloning as a selectable marker. A hybrid adenovirus carrying HSV type I TK gene when transfected to human or monkey cell lines produces an enzyme that is nearly 1% of the total cell protein (*15*). Recently, it has been reported that certain polynucleotides able to form Z-DNA structure (Z conformers) can be used to stimulate cloned gene expression. It is demonstrated by cotransfection of mouse LTK$^-$ (murine L) cells with some potential Z conformers and the plasmid pSV2cat containing bacterial chloramphenicol acetyltransferase (CAT) gene. The result indicates 2–10-fold stimulation of CAT gene expression in the mouse cells when compared to that of the plasmid transfection alone (*16*).

Enzyme overproduction can also be achieved by the gene dosage effect that is caused by a high copy number of the vector per host cell. Dramatic overproduction depends upon increasing gene dosage and efficient transcription. Cloning of *E. coli* DNA ligase gene into a recombinant λ phage results 500-fold overproduction of the enzyme representing 5% of the total cellular protein of *E. coli* (*17*). When the plasmid pKC16 containing *E. coli* exonuclease III gene is exposed to higher temperature, induction of the replication leads to an increase in the copy number of the gene. As a result, 125-fold exonuclease III is produced in the bacterial cells (*18*). Likewise, the plasmid pKN410 containing *E. coli* β-lactamase gene also shows temperature induced replication forming multiple copies of the gene. It leads to 400-fold overproduction of β-lactamase in *E. coli* (*19*). These temperature-induced enzyme overproductions are a result of "runaway replication." The positive regulator gene (*pho* B) for alkaline phosphatase of *E. coli*, when cloned into a pBR322 vector, shows multicopies of the *pho* B gene in host cells, and produces alkaline phosphatase in a large quantity (*20*). Three *nif* genes (H, D, K) of *Klebsiella pneumoniae* encoding nitrogenase, which contributes to biological nitrogen fixation, can be obtained in multiple copies when cloned into *Rhodopseudomonas capsulata*. The extra copies of *nif* HDK genes can be activated for the high level nitrogenase production (*21*).

Similarly, a number of host–vector systems have been constructed to increase the production of several enzymes (Table 1). In addition to other vectors, the transposons also act as a broad host range vector that can be inserted into plasmid or bacterial genomes randomly. This insertion is independent of the host cell recombination system. The transposon vector provides an extra advantage over the other vector by permitting a stable insertion of the cloned gene into the DNA in question. This type of vector may be useful for industrial applications because it doesn't provide an extra-genomic load on the host cell. In addition, the gene cloning technique involves some other possible applications of transposons that, in the near future, may become powerful tools for genetic manipulations (*22*). Thus, the application of gene cloning with plasmid construction,

Table 1
Overproduction of Certain Enzymes

Gene	Vector	Host	Level of expression, % cell protein or fold	Reference
Tryptophan synthetase	RSF1010	*Pseudomonas aeruginosa*	200-fold	*5*
Tyrosyl tRNA synthetase	pBR322	*Escherchia coli*	10%	*6*
	m13mp93	*E. coli*	50%	*7*
Citrate synthase	pWK452	*E. coli*	2–2.5-fold	*8*
	pWK401	*E. coli*	50-fold	*8*
Glucoamylase	pAC1	*Saccharomyces cerevisiae*	25–30%	*9*
Invertase	pAB8	*S. cerevisiae*	2-fold	*10*
Resolvase	pλ8	Bacterial cell	2–3%	*11*
Dihydroiolate reductase	pCV29 pCV32	*E. coli*	6%	*12*
Thymidine kinase	HSVtype-I-adenovirus (Hybrid virus)[a]	CV-1 monkey cell	1%	*13*
Chloramphenicol acetyl-transferase	pSV2cat	Mouse LTK$^-$cell	2–1-0-fold	*14*
DNA ligase	λ phase	*E. coli*	500-fold	*15*
Exonuclease III	pKC16	*E. coli*	125-fold	*16*
β-lactamase	pKN410	*E. coli*	400-fold	*17*

[a]HSV = Herpes simplex virus.

transfection, cotransfection, mutation, and gene dosage amplification in enzyme engineering for enzyme overproduction has a great commercial value.

MOLECULAR REDESIGNING

Knowledge of gene cloning, coupled with in vitro mutagenesis, have provided the means to put deletion, insertion, and point mutations at any desired site of a gene. Directed mutagenesis is a promising technique to generate any point mutation. It can be performed either with sodium bisulfite or with the help of an oligonucleotide. Generally, oligonucleotide-directed mutagenesis is used to redesign enzyme molecules. It comprises enzymic extension of an oligonucleotide primer hybridized to a single-stranded circular template by *E. coli* DNA polymerase, ligation of the newly synthesized strand with T4DNA ligase forming a closed circular double-stranded DNA (CC-DNA), transformation of cells with CC-DNA, and screening for the mutants (*23*). The oligonucleotide has complementarity to a region of the template, except for the mismatch that directs the mutation in question. In addition, site-saturation and spontaneous mutation can also be employed to redesign and dissect the structural and functional properties of an enzyme molecule. These techniques involve nucleotide substitution at a specific-site(s) in the gene of interest, which in turn causes the amino acid substitution(s) in the primary structure of the enzyme leading to its physical and/or catalytic modification.

Only a very small number of enzymes could yet be redesigned, such as, tyrosyl-tRNA synthetase (Tyr TS), which has been well documented (*9,24,25*). Two mutant forms of Tyr TS have been constructed by site-directed mutagenesis, causing nucleotide substitution for Ala and Pro at 51 codon site containing nucleotides for Thr in wild-type template. Although both mutant enzymes (Thr51→Ala51, Thr51→Pro51) have increased activity, but the mutant Pro51 has 25-fold increased activity that is mainly owing to lower Km for ATP. The engineering of this particular single point mutant (Thr51→Pro51) in TyrTS improves its affinity (Km) by a factor of 100 for ATP (*25*). It provides an excellent example for catalytic improvement (a large increase in enzyme-substrate affinity) by protein engineering. The catalytic modification of β-lactamase has been performed by site-saturation method. It is a mutagenic technique that can saturate a particular site in a protein molecule with all possible amino acid substitutions. Replacement of Thr71 by Cys71 or Ser71 in β-lactamase results in the higher catalytic activities of its mutant forms (*26*). Conversion of wild-type codons, GGC216→GCC216 and GGT226→GCT226, replaces Gly216→Ala216 and Gly226→Ala226 in the trypsin molecule, respectively. The mutant enzymes show a reduced catalytic rate but an enhanced substrate specificity. This kinetic modification is carried out by changes in the catalytic constant (Kcat) not the Km. Thus, the change in specificity (kcat/Km)

is caused by the differential effects on Kcat but not on Km (*27*). Heat-stable variants can be generated by spontaneous mutations through introducing a gene for a given enzyme from a mesophilic into a thermophilic organism. The strategy has been applied to kanamycin nucleotidyltransferase (KNTase) and heat-stable variants have been produced. The mutation involves the conversion of GAT (Asp80)→TAT (Tyr80) and ACG (Thr130)→AAG (Lys130). It leads to the formation of a thermostable KNTase without any significant change in kinetic properties (Kcat, Km, Vmax) of enzyme (*28*). One or other ways these enzymes (Tyr TS, β-lactamase, Trypsin, KNTase) are superior to their natural (wild-type) forms, representing either catalytic improvement, substrate-specificity enhancement, or thermal-stability increment.

Enzymes can also be redesigned to have a lower catalytic activity than the wild-types. The mutation of Cys (TGC) to Ser (AGC) at position 35 in the amino acid sequence of TyrTS creates a less active enzyme than the wild one. After mutation, Km of the enzyme for ATP in aminoacylation reaction is increased, whereas Vmax is decreased (*9*). The enzyme aspartate transcarbamoylase (ATCase) comprises catalytic (C) and regulatory (r) chains. Replacement of tyr165 by Ser165 causes a large decrease in catalytic activity of the holoenzyme as well as its catalytic (C) trimer. The mutant C-trimer shows a 12-fold higher Km value and four-fold reduced Vmax than the wild-type enzyme. It reflects a great decrease in the affinity of the enzyme for aspartate (*29*). Conversion of Glu165 to Asp165 in triosephosphate isomerase (TIM) using site-selective mutagenesis indicates reduced catalysis. The Kcat of TIM with glyceraldehyde 3-phosphate (GAP) substrate is 1/1500th of that of the wild-type enzyme and its Km is reduced 3.6 times. Whereas, with dihydroxyacetone phosphate (DHAP), the Kcat is 1/240th of that of the wild-type TIM, and Km is increased by a factor of 2. In this case, the catalytic steps of the mutant enzyme are quite slower than the binding steps. Since the Glu165 shuttles protons between C-1 and C-2 of triosephosphates, and since altered properties of the mutant TIM involves an increase in the transition state free energies for the protonation and deprotonation steps, it is explained that the substitution of Asp165 for Glu165 principally affects the free energy of transition state(s) for the catalytic reaction (*30*). Substitution of Asn27 for Asp27 in dihydrofolate reductase (DHFR) demonstrates a considerable reduction in the specific activity of the enzyme representing about 0.1% of the wild-type specific activity (*31*). But the Asp27 is not the main residue responsible for the stabilization of the DHFR molecule in its protonated transition state. The decrease in Kcat and affinity for nucleotide substrates as well as increase in thermosensitivity and susceptibility to proteolytic digestion of the mutant adenylate kinase result from the substitution of Ser87 for pro87 in the wild-type enzyme (*32*). The former changes in kinetic properties are likely because of the destabilization of the protein molecule, whereas the latter may be a result of modifications in the secondary and tertiary structures of the enzyme, which occur in response to higher tem-

perature (*32*). Conversion of Asn155 by Leu155 in subtilisin (a bacterial serine protease) leads to a 200–300-fold decrease in its Kcat without changing the Km for sucinyl-L-Ala-L-Ala-L-pro-L-Phe-*p*-nitroanilide peptide as the substrate (*33*). It is apparent that certain structural modifications in enzyme molecules can decrease their catalytic efficiency more than the wild-type enzymes, emphasizing worsening in biocatalysis.

In addition, it is also possible to generate an inactive enzyme molecule through site-selective mutagenesis. Replacement of Lys84 by Gln84 or Arg84 in ATCase (*34*), and Gly95 by Ala95 in DHFR (*32*) causes catalytic inactivation of the mutant enzymes because of their altered conformations. On the other hand, some molecular modifications have no effect on catalytic properties of enzymes. It is evident from the substitution of Cys39 for Pro39 in DHFR (*31*), and Ser172 or Cys172 for Met172 in cytochrome C peroxidase (CCP) (*35*). Thus, depending on need, one may either abolish or deprove the catalytic quality of a desired enzyme, or otherwise can create catalytically and/or physically different enzymes superior to their native forms (Table 2). Such genetically engineered enzymes can be of valuable importance in a number of industrial processes.

ANTIBIOTIC RESEARCH

A wide variety of antibiotics are used as chemotherapeutic agents. Its common and sustained utilization generates resistance in organisms and simultaneously imposes the problem of unabated consumption. Hence, the reduction or elimination of resistance and large-scale production of antibiotics seem to be reasonably warrantable. The recombinant DNA technology is trying to tackle the problem. Consequently, the genes imparting antibiotic resistance can be directed not to synthesize the enzymes that confer resistance to the cell against antibiotics. The thiol-β-lactamase confers ampicillin resistance in *E. coli*. By site-directed mutagenesis the codon AGC for the active site Ser70 of pBR322 β-lactamase can be converted to TGC for Cys70. This mutant enzyme confers a reduced ampicillin resistance in *E. coli* (*36*). It occurs because of reduced β-lactamase activity than the wild-type, whereas, the replacement of Ser70 by Thr70 results in the formation of an inactive β-lactamase (*37*), indicating lack of resistance to ampicillin. Similarly, using the site-saturation technique, a number of mutants can be produced by conversion of Thr71, in class A β-lactamase, which lies adjacent to the active site Ser70. The mutants having Tyr, Trp, Asp. Lys, or Arg at amino acid residue 71 instead of Thr do not show ampicillin resistance in *E. coli* (*26*). Since antibiotic resistance in microorganisms occurs because of transposition of mobile genetic elements (transposons), it is possible to eliminate certain antibiotic resistance by manipulating the transposons. The enzymes transposase and resolvase are coded by *tnp*A and *tnp*R genes of Tn3 (in bacteria) and $\gamma\delta$ (in bacteriophage) transposable elements, respectively. Any alteration in the nucleotide sequence of these

Table 2
Structural and Functional Redesigning of Enzyme Molecules[a]

Category	Property	Enzyme	Amino acid conversion	Catalysis	Reference
Superior	Catalytic improvement	Tyrosyl tRNA synthetase (TyrTS)	Thr51→Ala51 Thr51→Pro51	↑	23
		β-lactamas	Thr71→Cys71 Thr71→Ser71	↑	24
	Substrate-specificity enhancement	Trypsin	Gly216→Ala216 Gly226→Ala226	↓	25
	Thermal-stability increment	Kanamycin nucleotidyl-transferase (KNTase)	Asp80→Tyr80	⇌	26
Modified	Catalytic deprovement	TyrTS	Cys35→Ser35	↓	7
		Aspartate transcarbamolyse (ATCase)	Tyr165→Ser165	↓	27
		Triosephosphate isomerase (TIM)	Glu165→Asp165	↓	28
		Dihydrofolate reductase (DHFR)	Asp27→Asn27	↓	29
		Adenylate kinase	Pro87→Ser87	↓	30
		Subtilisin	Asn155→Leu155	↓	31
Inactive	Inactivation	DHFR	Gly95→Ala95	—	29
		ATCase	Lys84→Gln84 Lys84→Arg84	—	32
Unmodified	Catalytic unalteration	DHFR	Pro39→Cys39	⇌	29
		Cytochrome C peroxidase (CCP)	Met172→Ser172 Met172→Cys172	⇌	33

[a] → Amino acid replacement, ↑ increased, ↓ decreased, ⇌ unchanged, — abolished.

genes causes formation of a mutated transposon and interferes in the synthesis of such enzymes, which are essential for the normal transposition process. Consequently, it may lead to the elimination of antibiotic resistance. In *Drosophila* the oligonucleotide site-directed mutagenesis has been used to generate a mutated transposon (*38*). So an approach to create mutation in transposon to synthesize catalytically inactive enzyme may prove to be rewarding in eliminating antiobiotic resistance.

On the other hand, much effort is being made to study the genetic and biochemical basis of antibiotic synthesis for the develoment of more competent and new antibiotics in large quantity. Mainly, the remarkable advancements in antibiotic syntheses have contributed to the antibacterial and antitumor antibiotics. Enzymatic and chemico-enzymatic syntheses of various medically important antibiotics are being explored for cheaper and higher productions and novel qualities and varieties. Pencillin acylase has a broad substrate specificity and catalyzes hydrolytic deacylation of natural pencillins, which results in the formatiion of 6-amino-penicillanic acid (6-APA) and acyl side chain compounds. Its reverse reaction synthesizes natural penicillins. The enzyme penicillin acylase is used to synthesize various types of penicillins and cephalosporins with the help of certain microorganisms having the ability to produce sufficient quantities of penicillin acylase. The microorganism *Kluyvera citrophila* has the capability to synthesize ampicillin (α-amino penicillin) using the reverse reaction of penicillin acylase, however, *Pseudomonas melanogenum* has the ability to synthesize a high level of ampicillin and cephadroxil but does not have the capacity to synthesize penicillin G or V from 6-APA (*39*). The bacterial penicillinase (β-lactamase) may degrade ampicillin, hence, mutant strains deficient in β-lactamase (KY8540 and KY8541) are used in the synthesis of some β-lactam antibiotics using their penicillin acylase activities. The β-lactam compounds can also be enzymically *N*-acylated using esters and amides to produce their derivatives. In the last 15 years many interesting strides have been made in antibacterial antibiotic research, particularly that related to β-lactams and aminoglycosides. Consequently, the application of chemico-enzymatic syntheses became an important approach for the production of β-lactam compounds and their analogs, as well as for other useful antibiotics (*40*). Cell fusion and microbial transformation techniques are being employed for the production of new aminoglycosidases and new aminoglycosides (such as, combimicins, fortimicins, and istamycin B) with improved antibiotic activities. It is believed that a wide range of distribution of a gene or a gene set among microorganisms is responsible for the synthesis of the common structural parts of antibiotics. There are plasmids (pSNS1, pSPUI, pSCY1, pSHY1) that can produce antibiotics (neomycin, viomycin, cycloheximide, and geldanamycin, respectively), and in many other cases construction of appropriate plasmids can increase antibiotic production.

In addition to site-directed mutagenesis, certain recently emerged techniques are being applied to engineer enzymes involved in antibiotic

research. The site-saturation is used to substitute Thr71 by Cys71 or Ser71 in the amino acid sequence of β-lactamase. Both mutant enzymes have high catalytic activity toward benzylpenicillin and ampicillin (*26*), although its catalytic activity confers resistance to penam and cephem antibiotics by hydrolyzing the β-lactam ring. The improved β-lactam activity in conjunction with some possible chemical and/or enzymic reactions may be proved of worth in producing new classes of β-lactam derivatives. Similarly, the spontaneous mutation technique has been used to replace Asp80 by Tyr80, and Thr130 by Lys130 in the primary sequence of kanamycin nucleotidyltransferase. It results in the formation of the mutant enzyme, which is thermally more stable (*28*). This may have probable importance in certain reactions operating at higher temperatures in industries involved with antibiotics. Attention is also being paid to apply newer techniques for the overproduction of such enzymes that may be useful to chemical factories engaged in antibiotic syntheses. Recently, an enhanced production of chloramphenicol acetyl-transferase has been obtained by the cotransfection of the plasmid containing the gene for this enzyme with potential Z conformers (synthetic polynucleotides) (*16*). A general survey indicates that newer methods are being introduced regularly in antibiotic research to modify the genes and in turn to produce mutant enzymes. These enzymes may be physically and/or catalytically inferior or superior to natural ones. Whatever may be the case, both are equally valuable for biomedical and industrial applications (Table 3). With the increasing advancements in antibiotic research one day it may be possible to create new genes for novel enzymes and establish new methods for the development of additional useful compounds (*40*).

WASTE DEGRADATION

Since last two decades the rapid progress of industries has led to the production of different varieties of chemical compounds that are harmful otherwise. Most of them are nonbiodegradable and persist in the environment as industrial products and wastes. The long-term fate and toxicological properties of these compounds cause risks and hazards to humans and animals. Several synthetic organic compounds are common environmental pollutants. Many of these are hydrocarbons or halogenated compounds. They are widely encountered in the forms of solvents, refrigerants, fire retardants, varnishes, and biocides. Some toxicants enter into biological systems and generate toxicity in the organisms. Ultimately, the problems of environmental protection and biological safety emerge as a challenging question to bioengineers. Engineers are trying to detoxify the environment and biological systems through enzymic degradation or modification of toxicants to nontoxic compounds. During the last eight years some useful approaches have been proposed by various laboratories, which are based on genetic engineering of enzymes.

Table 3
Application of Genetically Engineered Enzymes in Antibiotic Research[a]

Gene	Technique	Structural modification	Resulting changes	Probable application	Reference
β-lactamase	Site-saturation	Thr71 → Tyr71, Trp71, Lys71, Asp71, Arg71	Lack of ampicillin resistance	Biomedical	*24*
	Site-directed mutagenesis	Ser70 → Cys70	Reduced ampicillin resistance		*34*
	— do —	Ser70 → Thr70	Lack of ampicillin resistance		*35*
Chloramphenicol acetyltransferase	Cotransfection with Z-conformer	Formation of Z-DNA structure	Overproduction		*14*
β-lactamase	Site-saturation	Thr71 → Cys71, Ser71	Catalytic improvement	Industrial	*24*
Kanamycin nucleotidyl-transferase	Spontaneous mutution	Asp80 → Tyr80 Thr130 → Lys130	Enhanced thermal stability		*26*

[a] → Amino acid replacement.

As a general rule, the accumulation of unwanted chemicals in the environment changes the selective force of nature, which has to be adapted by the evolution of new enzyme systems capable of degrading the wastes. Certain microorganisms have adapted to the chemicals that came into existence some time ago. The enzymic machinery dealing with the catabolic pathways of several microorganisms are not sufficiently and widely adapted to a number of new chemicals constantly introduced, but they possess the potential to degrade certain unnatural synthetic compounds. Hence, a rapid evolution of catabolic pathways in microorganisms in relation to the toxic compounds as substrates is eagerly desired to prevent them from accumulating. This evolution may be achieved in two ways (*41*):

1. Enzyme overproduction through a gene dosage effect, inactivation or modification in the stringent control of the regulatory genes involved, or enzyme production having altered specificity by mutational divergence; or
2. Derivation of novel enzyme activities from heterologous or preexisting genes for related enzymes by gene recruitment, genetic rearrangment, and so on.

The former may be applicable if wastes are to some extent chemically analogous to the natural substrates, whereas the latter is relevant to the nonanalogous wastes as substrates, which has been performed in the laboratory through designing the microevolutionary processes with the help of genetic engineering techniques.

Several aerobic and anaerobic saprophytic microorganisms are distributed in different environmental conditions, out of which bacteria and fungi are of great importance in the dissimilation of a number of toxic compounds. The biodegradative capabilities of some bacterial species have been screened, manipulated, and tested for the waste degradation. Among them, *Pseudomonas* is well explored, and the plasmid pWWO (a TOL plasmid) of *P. Putida* is the most extensively studied. It carries the genes that encode catabolic enzymes for degradation of toluene, *m*-xylene, *p*-xylene, 3-ethyltoluene, and 1,2,4-trimethylbenzene, as well as their alcohol, aldehyde, and carboxylic acid derivatives (*42*). There are two clusters of genes on the plasmid pWWO (117 Kb). One comprises the genes *xyl*A, *xyl*B, and *xyl*C, coding xylene oxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase, respectively, which convert the hydrocarbon into carboxylic acid, whereas the other cluster includes *xyl*D, *xyl*E, and *xyl*F genes, which encode toluate or benzoate oxygenase, catechol 1,2,3-oxygenase, and hydroxymuconic semia ldehyde hydrolase, respectively, and are known as "meta-cleavage" pathway enzymes. There are two regulatory genes, *xyl*R and *xyl*S, which are believed to control the catabolic pathway. The plasmid pKT530 has been constructed by cloning of the genes involved in the meta-cleavage pathway (*43*). This recombinant plasmid also carries the *xyl*S gene, which in conjunction

with carboxyic acid substrates, stimulates gene expression for the meta-cleavage pathway (*43*). When the genes present on the plasmid pKT530 coding and degradative enzymes are placed under the control of an *E. coli* promoter, a high level of expression occurs (*44*), leading to the overproduction of the degradative enzymes. Certain *Pseudomonas* strains have an alternative pathway for benzoate degradation that is known to be chromosomally encoded. All TOL plasmids have the common meta-cleavage pathway that is also involved in degradation of phenol and cresol compounds by *P. putida* and *P. aeruginosa* bacteria. Phenol and cresol degradation are also chromosomally encoded, and the chromosomal pathway can be more efficient when compared to that of the plasmid encoded pathway. It is possible that the catabolic genes for the entire pathway are carried on a large transposable element that may explain the widespread occurrence of such pathways in a variety of genera (*42*).

However, the microbial degradation of halogenated compounds seems to be more critical because only a few microorganisms are available in nature that can degrade the halogen-substituted compounds. The reason behind it is insufficient adaptation in enzymic machinery caused by a limited number of naturally occurring halogenated compounds that are also newly introduced. Certain bacterial strains have been experimentally designed to degrade a number of such compounds that came into existence after the industrial establishments. They are 3-, 4-, and 3,5-dichlorobenzoates (*45*); chlorophenoxyacetic acids (*45*); fluoro-, chloro-, and bromoacetate, propionate and butanoate, chloroethane, chloroethanol, and chloromethane (*47*); 3-chlorobenbenzoic acid (*48*), and so on. All these are amenable to enzymic degradation by a number of different plasmids. The biochemical and genetic basis of degradation of certain chlorobenzoic acids and chlorinated phenoxyacetic acids has been analyzed in detail. The degradative pathway of benzoate is chromosomally coded, however, the pathway involved in the degradation of 3-chlorobenzoate and 2,4-dichlorophenoxyacetic acid are borne on pAC27 and pJP4 plasmids, respectively. It has been proposed that any chlorobenzoate or chlorophenoxyacetate can be degraded, but there is a great deal of specificity inherent in such degradations (*41*). The plasmids encoding a complete degradative pathway of 3-chlorobenzoate cannot utilize 4-chlorobenzoate. It can only be utilized or catabolized if the host cell also contains a TOL plasmid (pWWO) that encodes the degradative pathway for toluene and xylene. It is because the TOL plasmid encodes benzoate oxygenase having broader substrate specificity and can catalyze the first degradative step. In this way one can combine different plasmids for still broader substrate specificities, thereby extending the range of substrate utilization by bacterial systems (*49*). Enzymic degradation of 3-chlorobenzoic acid (3 Cba) by *Pseudomonas* sp. (strain B13) (*50,51*) shows the involvement of benzoate oxygenase and dihydrodiol benzoate dehydrogenase in the conversion of chlorobenzoate to chlorocatechol. The chlorocatechol is oxidized to chloromuconic acid by pyrocatechase II (a ring-cleaving enzyme), and the reaction is further

carried out by cycloisomerase II (a lactonizing enzyme), causing dehalogenation of chloromuconic acid into chlorolactone. The chlorolactone undergoes spontaneous conversion and subsequently forms maleylacetic acid using hydrolase II. It is followed by the formation of succinic acid. Only pyrocatechase II, cycloisomerase II, hydrolase II, and the enzyme(s) involved in maleylacetic acid conversion are present on the plasmid pWR1 in *Pseudomonas* B13 (*46*) or the plasmid pAC25 in a strain (3 Cba^+) of *P. putida* (*48*). Both plasmids are mainly homologous, and a deletion in pAC25 (117 Kb) results in the formation of pAC27 (110 Kb), which has genes of pAC25 for 3 Cba degradation (*52*). The plasmid pJP4 (85 Kb) in *Alcaligenes eutrophus* JMP134 encodes for the gradation of 3 Cba as well as for 2,4-dichlorophenoxyacetic acid (2,4-D) (*41,53*). The 2,4-D and 2,4,5-trichlorophenoxyacetic acids (2,4,5-T) are the two phenoxy alkanoic acids, and are widely used as herbicides (the compound 2,4,5-T is highly toxic and has been used in the Vietnam war as a main component of "agent orange"). When *P. cepacia* strains are selected on new substrate(s) enriched in chemostate, the plasmids undergo considerable genetic rearrangements and consume the substrate as a major source of carbon and energy. A novel plasmid is formed as a result of selection of *P. cepacia* strains on new substrates. It is composed of a fragment of the TOL plasmid comprising the replication, incompatibility, and the copy number genes as well as a duplicated segment of the plasmid pAC25 required for the degradation of chlorobenzoic acid (*54*). This way, *P. cepacia* AC1100 can develop the capacity to use 2,4,5-T (*49*), and its resting cells have been reported to oxidize or dehalogenate pentachlorophenol; pentafluorophenol; tetra-, tri-, and dichlorophenols; and 2,4-D (*55*).

Various kinds of hydrocarbons can be used by bateria, yeasts, and filamentous fungi as a sole source of carbon and energy. Mainly monooxygenase enzyme systems are involved in oxidation of straight-chain alkanes into their corresponding alcohols. A complex multicomponent enzyme system requires molecular oxygen for reaction. One such system is composed of cytochrome P-450, with an iron protein and a flavoprotein, whereas the other comprises a nonheme iron protein and rubredoxin as an electron carrier. The former occurs in *n*-octane utilizing *Corynebacterium* sp., whereas the latter occurs in *Pseudomonas* sp. Its catabolic information is believed to be present on a transmissable plasmid. Unlike straight-chain, the branched-chain alkanes cannot be completely degraded by microbial catabolism, and even their β-oxidation enzymes are incompetent toward such alkanes. A synthetic polyester is the branched-chain alkane that can be broken down into 2-ethylhexanol with the help of a freshwater microbial attack using lipase activity. Some plasmids have been recognized for degradation of simple hydrocarbons, short-chain alkanes, and monocyclic as well as dicyclic compounds. The degradation of more complex hydrocarbons is known to be chromosomally coded, and mobilization of the chromosomal genes may be helpful for a wider application of genetically manipulated microorganisms (*56*). Similarly, nylon-6 is an unnatural syn-

thetic compound and about 35 years ago, before industrial establishments, it was not present in the environment. A nylon oligomer consists of more than 100 residues of 6-aminohexanoic acid. The nylon-6 compound is generated from the ring-cleavage polymerization of ϵ-caprolactam, in which head-to-tail condensation of some molecules leads to the formation of cyclic oligomers. The remaining molecules do not polymerize at oligomeric stage, and when they are discharged into the vicinity of *Flavobacterium* sp. as byproducts with the drainage of waste water from the nylon factories, the bacterium is alarmed. Since this process has continued for the last three decades, the *Flavobacterium* sp. K172 has evolved the degradative capability to hydrolyze 6-aminohexanoic acid cyclic dimers. Its degradation pathway involves two hydrolases (EI, 6-aminohexanoic acid cyclic dimer hydrolase; and EII, 6-aminohexanoic acid linear oligomer hydrolase). The degradative pathway is known to be borne on the plasmid pOAD2 in *Flavobacterium* sp. K172 (*57*).

The effectiveness of such microorganisms in the environment has also been explained. So far, microbial degradation of hydrocarbons occurs during weathering, which is a process used for the removal of contaminating oil spill. Since the growth of microorganisms at their higher cell concentrations is associated with the available surface area of liquid hydrocarbons, the availability of oil to microbial attack (surface area) is one of the major limiting factors involved in the degradation of the weathered oil. The indigenous microflora have an increased competition toward hydrocarbon degradation in the previously contaminated environment and show a greater degradation potential than in the pristine environment. The catalytic properties of hydrocarbon-utilizing strains of microorganisms have also become relevant to industrial interest for production of peterochemicals. It has been suggested that the likelihood of industrial processes arising for petrochemical production based on biocatalysis has increased substantially, particularly in view of the prospect of applying recombinant DNA technology to relevant organisms (*56*). The genetically manipulated microorganisms may also be applied at waste dump sites for removal of toxic chemicals even when present in their high concentrations. It has been shown that a *Flavobacterium* sp. decontaminates 10 ppb-100 ppm concentrations of pentachlorophenol from river, lake, and ground waters to its undectable level within 2–3 d (*58*). Further, it has been demonstrated that the weekly applications of *P. cepacia* strain AC1100, carrying degradative capabilities for 2,4,5-T, can remove more than 90% of 10,000–20,000 ppm concentrations from soil within six w (*59*). The major exhaustion of 2,4,5-T from soil causes death of AC1100 cells. Subsequently, it has been pointed out that the treatment of contaminated soil with such laboratory-developed microorganisms may permit almost complete restoration of the soil with little threat of potential ecological disaster (*59*).

The application of genetically improved microorganisms in the open environment may be of importance to metabolize a number of toxic compounds, including poisonous spills on the sea surface. It may also prove

useful for oil recovery, plant growth promotion, and prevention of ice nucleation in agriculture (*41*), as well as for the production of petrochemicals in industries (*56*). But before such applications, one must be sufficiently careful about its adverse environmental consequences. At this point it is relevant to cite the example of the field application of *P. syringae* (used for the prevention of frost nucleation), which has been proposed in US courts and forbidden (*60*). It has been suggested that the construction of appropriate microbial strains for the enhanced degradation of high-priority toxic chemicals, and the development of guidelines to govern their applications will be key ingredients of the pollution problems associated with toxic chemical compounds (*41*). Consequently, in December 1986, the Recombinant DNA Advisory Committee (RAC) of the US National Institutes of Health discussed the release of recombinant organisms into the environment for field testing experiments and considered relaxing its regulatory reins.

The effective and stepwise consequences of environmental chemical pollution are the generation of toxicity in organisms, which leads to serious biochemical and genetic abnormalities and sometimes to death. The Bhopal, India gas leak in December 1984 is an unforgettable consequence of methyl isocyanate as a potential and fast poison. That menace cost thousands of lives and left behind many diseased, disabled, and destituted through no fault of their own. A major accident in November 1986, in Basel, Switzerland, caused by a fire at a plant containing 1250 tons of fungicides, pesticides, and other agricultural chemicals, caused a spill of 30 tons of these toxic chemicals into the river Rhine. As a result, more than half a million fish and eels are believed to have died, and a 25-mile-long chemical slick moved down the river, demanding a serious ecological damage. The repair, as scientists estimated, will take 10 years. No doubt there are a number of toxic compounds that disturb the molecular physiology, and, in turn, cause either temporary and/or permanent defects in biochemical machinery of the cell, including DNA damage. Some of the common xenobiotics responsible for generating toxicity in various organisms are DDT, BCB, phenobarbital, dioxins, and petroleum-derived polycyclic aromatic hydrocarbons. All of these are synthetic products that induce the activities of mono-oxygenase (MO) enzyme system temporarily. MO induction can cause the production of some metabolites that bind covalently to DNA (*61*). Binding of such metabolites with DNA may be a related cause of DDT-induced mutagenicity and carcinogenicity. Several alkylating agents are also known to induce toxic, mutagenic, teratogenic, and carcinogenic effects in organisms, thought to be a result of the interaction of the alkylating agents with DNA-evoking DNA damage (*62,63*). Similarly, a large body of information is available, but the trickle of papers on in vivo detoxification seems to be of particular importance. The toxicant aflatoxin B_1 is first converted into 2-3-epoxide in vivo and then the product induces hepatic carcinoma. This derivative can be degraded by epoxide hydrase and glutathione-5-epoxide transferase (*64*).

An alkylating agent *N*-methyl-*N*-nitrosourea, alkylates guanine residues at 0^6 position and forms O^6-alkylguanine, which can be repaired (*65*). It is also known that the alkylating agent can react with many biomolecules, including proteins (*66*). Hence, the O^6-alkylguanine ($O^{6\text{-}AG}$) in the DNA molecule can be repaired by such proteins (repair proteins) (*67*). The gene coding a repair protein can be cloned and expressed in repair-deficient cells as well as in the animal itself. It has been demonstrated that the cloned O^6-AG alkyltransferase (ATase) gene of *E. coli* encodes a 37-KDa protein that displays activity on both O^6-AG and alkylphosphotriesters (AP) in DNA (*67,68*). A Chimera plasmid, pJCB06C, has been constructed carrying the entire coding sequence of *E. coli* O^6-AG ATase gene and transfected into the wild-type Chinese hamster cell line RJK0 (*69*). The repair analysis indicates that the resulting 37-KDa ATase protein rapidly repairs O^6-methylguanine of the host genome. The mammalian cells harboring *E. coli* ATase gene show reduced toxicity as well as mutagenicity against alkylating agents (*69*).

Therefore, the genetic engineering of enzymes can be proved to be a useful and rewarding approach to degrade a number of xenobiotics and pollutants in the environment as well as employed to restore or rectify the defects in biological machinery. Such environmental and biological detoxification by manipulating genes involved in detoxification (detoxifying genes) can provide environmental protection as well as biological safety (Table 4) and extend positive interaction for a long-term benefit to organisms.

RIBOZYMES AND ABZYMES

The need for catalysts in biological systems stems largely from the nature of biological molecules themselves. The molecules that interact in cellular processes are generally quite stable, and without outside intervention, they are poncerously slow to react. Enzymes speed up these interactions and bring them into a time scale compatible with life. Each protein enzyme typically accelerates a biochemical reaction by a factor ranging from a million to a trillion. The discoveries of RNA acting as an enzyme (ribozymes) (*70*) and catalytic antibodies (abzymes) (*71,72*) are startling findings that redefine a biocatalyst. The first example of RNA catalysis was discovered in 1981 and 1982 from the studies of a protozoan *Tetrahymena thermophila.* This single-celled eukaryote, like other eukaryotes, has four ribosomal RNAs, three of which are transcribed as a single unit of RNA. The resulting primary transcript is then cut, spliced, and modified to produce the finished molecules. The *Tetrahymena* pre-rRNA intron is self-splicing, which is only one step in a cascade of reactions that the RNA carries out on its own. Its resemblances to the action of an enzyme are

1. The reaction is accelerated by many orders of magnitude;
2. It is highly specific;

Table 4
Environmental and Biological Detoxification

Plasmid carrying detoxifying genes	Toxic waste	Process	Importance	Reference
pWR1, pAC25, pAC27, pAC29	3,-4-, and 3,5-dichlorobenzoates			*39,44*
pWWO	Toulene, *m*-and *p*-xylene, 3-ethyltoluene and 1,2,4-trimethylbenzene as well as their alcohol, aldehyde and carboxylic acid derivatives			*40*
		Degradative	Environmental protection	
pJP2, pJP4	4-chloro-,2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acid			*45*
pUO1	Chlorethanol, Propionate and butanoate, Chloro-, methane and -ethane, Fluoro-, chloro, -and bromo-acetate			*46*
pAC27, pJP4	3-chlorobenzene			*47*
pKF1	Mono-, and di-chlorobiphenyls			*52*
pOAD2	6-aminohexanoic acid			*56*
pJCB06C	*N*-methyl-*N*-nitrosourea	Repairing	Biological safety	*68*

3. The catalysis depends on the three-dimensional structure of the polynucleotide chain, which is, in turn, decided by the sequence of nucleotides;
4. A common catalytic strategy of enzymes is to bind two substrates (reactants) in close proximity and at a special orientation to each other, thereby facilitating their reaction. The RNA intron employs the same strategy when it binds guanosine and the six pyrimidines (as an attacking group); and
5. The folded structure of the intron activates the phosphate group at each reaction site, bringing it into a state favorable for cleavage. At the splice sites, the folded structure of the intron accelerates the rate of cleavage by a factor of 10 billion, i.e., it reduces the time frame of the reaction from 19,000 years to one minute.

A mitochondrial mRNA intron from the fungus *Neurospora crassa* is also self-splicing. Several other self-splicing mRNA and rRNA introns from yeast mitochondria have also been demonstrated. In all these instances guanosine is required, and the RNA is spliced by the same mechanism as in *Tetrahymena*. An RNA from a bacterial virus (bacteriophage T_4) also splices itself in *E. coli*. The interesting example of ribonuclease P, a tRNA processing enzyme found both in bacteria and in higher cells, proved RNA as a true catalyst. It contains RNA and protein in a single package, but the RNA subunit alone can cut the pre-tRNA molecule at the correct spot, whereas the protein has no such capacity. Even the some RNA subunit from a recombinant DNA template could catalyze the accurate maturation of tRNA precursors. If it can be demonstrated that RNA can catalyze its own replication, the explanation for the first step toward life in a prebiotic environment may be the "self-replicating RNA molecule" itself. In all known examples, the substrate for the RNA enzyme has been RNA (another part of the same molecule, a different RNA polymer, or a single nucleotide).

On the other hand, catalytic antibodies are "specialized protein enzymes." The production of antibodies that can catalyze chemical reactions opens the way to make enzymes with any desired specificity. Antibodies and enzymes share a major point of similarity (both bind their target molecules with high specificity and affinity). Essentially unlimited diversity of antibody molecules may be trapped to produce enzymes with whatever specificities are desired. Enzymes are generally thought to speed up chemical reactions by stabilizing the transition state, the most unstable and therefore the highest energy intermediate formed by the reactants during the conversion to products. Enzymes, by stabilizing the transition state, lower the energy needed for the conversion and, consequently, increase the rate of the reaction. The trick in producing catalytic antibodies lies in obtaining antibody molecules that will stabilize the transition states of the selected chemical reactions. The catalytic antibodies display a number of characteristic enzyme features.

1. They speed up chemical reactions;
2. They show substrate specificity;
3. They can be inhibited; and
4. Their activity has a modest dependence on the pH of the reaction mixture.

Catalytic antibodies cleaving esters (*71*) and catalyzing carbonate hydrolysis (*72*) have been demonstrated. Development of antibodies that can break peptide bonds will be a major futuristic goal (*73*). Catalytic antibodies designed to cut proteins at any desired amino acid sequence could be used for selectively dissecting the proteins, much as restriction enzymes are used for dissecting DNA. The equivalent of restriction enzymes for proteins would be valuable for studying the relation between protein structure and function. Catalytic antibodies also have potential medical applications. Antibodies by themselves do not destroy the target antigens, but essentially serve as signals for triggering the destructive activities of other immume system proteins and cells. Antibodies that cannot only bind to proteins, but also cut them, might be useful for such applications as dissolving blood clots or searching out and destroying tumor cells. Antibody genes are especially subject to mutations, DNA rearrangements, and contribute to the generation of the large diversity of the antigen–antibody sites. It may be possible to apply genetic selection techniques to antibody-producing cells to obtain mutations that lead to the production of catalytic antibodies with desired characteristics. Semisynthetic catalytic atibodies by chemical modification of the antigen-binding site, if possible to synthesize, would result in a combination of the specificity and high binding affinity of an antibody with the activity of a synthetic catalytic compound not normally found in antibodies (*73*). The development of catalytic antibodies opens a way of tapping into the vast repertoire of binding pockets (on antibodies) to do chemical work (*71*).

FUTURISTIC GOAL

The challenges of the modern world are many. Newer discoveries in basic sciences and achievements in technology have added brighter dimensions to the concepts of the applications of science and technology to day-to-day life. Enzymes have been successfully demonstrated to perform miracles in chemical reactions operating in biological systems. Their high catalytic power and precise substrate specificity have been exploited for several industrial, biomedical, and now, environmental applications (Fig. 1). Enzyme engineering is trying to produce enormous amounts of enzymes, to make them more stable in extracellular, unnatural, artificial, and even in unfavorable environments, and to synthesize more efficient enzyme molecules in terms of catalysis, substrate specificity, and the rate of the chemical reactions they catalyze. Attempts are being made to construct enzyme molecules with a higher catalytic rate and wider substrate

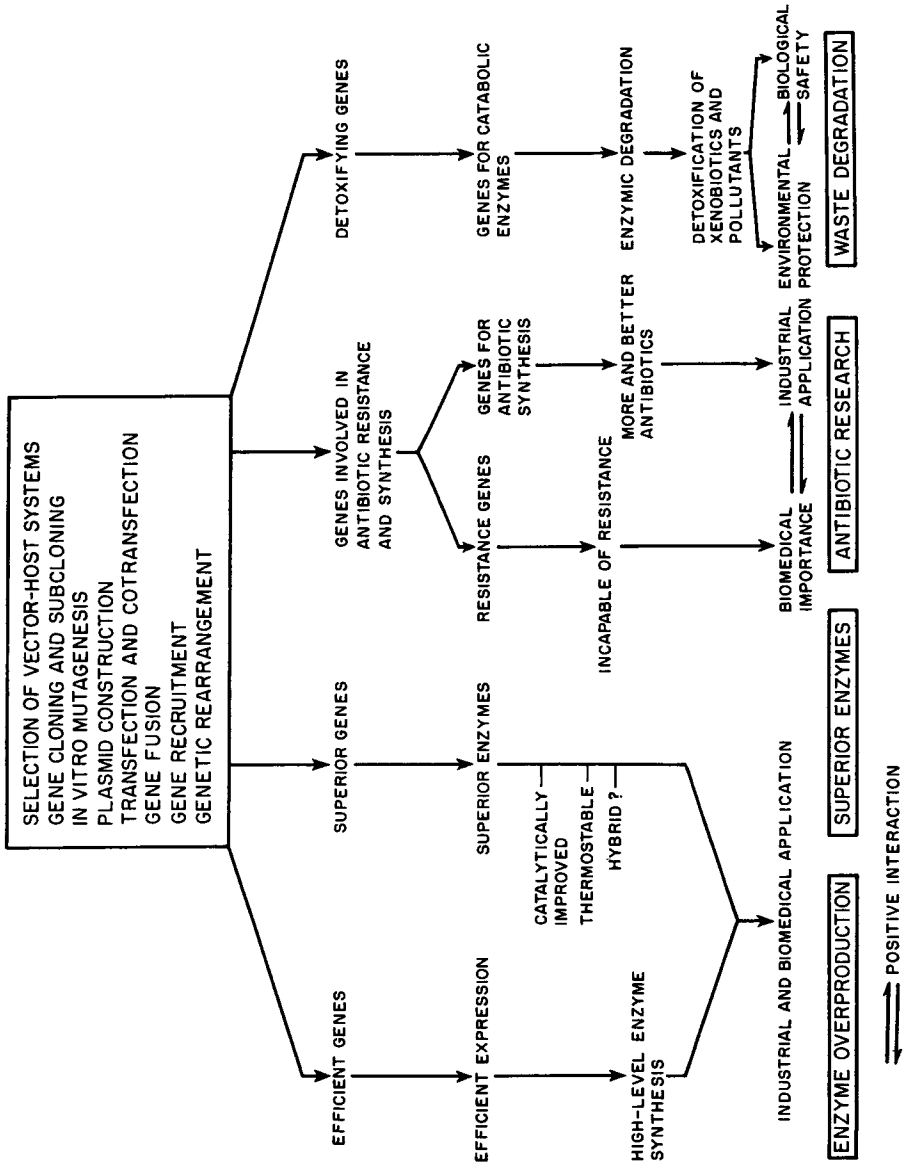
SELECTION OF VECTOR-HOST SYSTEMS
GENE CLONING AND SUBCLONING
IN VITRO MUTAGENESIS
PLASMID CONSTRUCTION
TRANSFECTION AND COTRANSFECTION
GENE FUSION
GENE RECRUITMENT
GENETIC REARRANGEMENT
EFFICIENT GENES
EFFICIENT EXPRESSION
HIGH-LEVEL ENZYME SYNTHESIS
SUPERIOR GENES
SUPERIOR ENZYMES
CATALYTICALLY IMPROVED
THERMOSTABLE
HYBRID ?
GENES INVOLVED IN ANTIBIOTIC RESISTANCE AND SYNTHESIS
RESISTANCE GENES
INCAPABLE OF RESISTANCE
GENES FOR ANTIBIOTIC SYNTHESIS
MORE AND BETTER ANTIBIOTICS
DETOXIFYING GENES
GENES FOR CATABOLIC ENZYMES
ENZYMIC DEGRADATION
DETOXIFICATION OF XENOBIOTICS AND POLLUTANTS
BIOMEDICAL IMPORTANCE
INDUSTRIAL APPLICATION
ENVIRONMENTAL PROTECTION
BIOLOGICAL SAFETY
INDUSTRIAL AND BIOMEDICAL APPLICATION
ENZYME OVERPRODUCTION
SUPERIOR ENZYMES
ANTIBIOTIC RESEARCH
WASTE DEGRADATION
POSITIVE INTERACTION

Fig. 1.

specificity. A true success, in this direction, will be to produce an enzyme with multicatalytic activity (hybrid enzyme). One of the masterplans of modern biology is to approach biocatalysis at the level of the genes. Here, genetic engineering has to shake hands with enzyme engineering. The genes of the enzymes are being manipulated for the effective modulations and modifications of biocatalysis. Transcription, post-transcriptional processing of transcripts, translation, and post-translational modifications are the major events where enzyme molecules may be approached for the desired alterations in their structure and function. The genes of the enzymes can be modified by in vitro mutagenesis to create a biocatalyst, different in its amino acid sequence through the change in the nucleotide sequence of the DNA. This is already in experimental practice for alterations in thermodynamic as well as kinetic components of enzyme molecules to influence catalysis. Transcriptional stimulation is achieved by attaching strong promoters to isolated genes, in order to synthesize more of a message that can be translated into the desired enzyme in a suitable system. Among post-transcriptional modifications, splicing is most important. Tissue-specific splicing of a common message to produce diverse proteins has already been demonstrated in the case of many neuropeptides. It is known that introns in many cases are self-splicing, and some mitochondrial pre-mRNAs splice their own introns through the formation of a lariate, which may or may not be associated with protein factors. If such self-splicing messages of enzymes can be artificially spliced in a tissue-specific manner and then faithfully translated into diverse catalytic subunits capable of performing their jobs independently but accurately, then a situation allegoric to the hybrid enzyme may appear. This may indicate the way to construct a whole metabolic pathway artificially in a cell-free system (cell-free metabolism). Exons of many genes have been successfully attributed to the functional domains of their corresponding proteins. The shuffling of exons takes place during the splicing of pre-mRNA to produce mature mRNA. It gives an insight into how cloning and selection of individual exons of enzyme molecules may lead to a better understanding of the correlation between the structure and function of the domains and their regulation. Structural parts of the enzyme molecules responsible for the catalysis may be better understood through these investigations. Translational efficiency may be enhanced by improving the stability of the messages, which will substantially add to the concept of overproduction of an enzyme. Post-translational modifications of proteins have already been demonstrated for the interconversion of active and inactive forms of enzymes in cellular conditions. Such reactions in extracellular environments may contribute to the flexibility of applications of the enzymes in industries and clinics.

Catalytic antibodies for carbonate hydrolysis and cleaving ester bonds have already been reported. This is a remarkable field, in which genetic engineering can accelerate the development of enzyme engineering through the expertise of molecular immunology. Monoclonal anti-

bodies that can stabilize the transition states of the chemical reactants can act as biocatalysts. The transition state chemicals ought to be antigenic in order to elicit antibody production through hybridomas. We first have to search out compounds analogous to the transition-states of chemical reactants of interest. Once the catalytic antibodies are successfully produced and perform their chemical work, the hybridomas may be maintained to grow continuously and secrete the antibodies into the medium. Genetic selection of such catalytic antibodies to produce desired characteristics of the protein molecules may lead to a situation where programming catalytic properties to antigenic determinants will become possible. Catalytic antibodies against chemically modified antigens may lead to synthetic biocatalysts. If these optimistic speculations stand the test of time, then we will see the start of a new phase in the biocatalytic theater.

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