Biotransformation Enzymes

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Abstract

Biocatalysis, which was an area of least concern during the past years, has now a remarkable space in the field of chemistry. Biotransformation is the process through which the functional groups of organic compounds are modified by living cells to a chemically different product. The process explores the specific properties of biological catalysts, which include stereospecificity and region specificity and their capability to withstand reactions at no extreme temperatures and pH values. It may involve the use of plant cells, animal cells or microbial cells or purified enzymes as catalysts to bring about specified transformations of complex substrates. Biotransformation enzymes help to produce "nature-like" biodegradable compounds, and are gaining importance in this aspect. This chapter focuses on biotransformation enzymes, which catalyse highly reaction-specific and stereo-specific reactions to synthesize compounds that cannot be produced by chemical means. Moreover, these enzymes help meet the increasing demand of society for eco-friendly compounds by producing the bulk of biodegradable 'green' products. This chapter discusses the recent advances and applications in biotransformation.

Keywords

Biotransformation • Enzymes • Biocatalysis • Natural products • Xenobiotics

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5.1 Introduction

Biotransformation or biocatalysis entails the use of the catalytic part of the biological systems such as plant cells, animal cells or microbial cells or purified enzymes for the biosynthesis of novel compounds. Biotransformation has a high prospective to bring about novel products and to improve known products more effectively. It differs from biosynthesis and biodegradation in that it involves interconversion of molecules by living systems, whereas biosynthesis is the combining of simpler substrates to form complex products and biodegradation is the decomposition of complex substances to simpler ones.

Biotransformation commonly occurs in human, plant and microbial cells as part of metabolizing xenobiotics. Also, it serves as an efficient, specific, and eco-friendly process for the production of industrial products, thereby replacing the toxic chemical transformation reactions. In many instances, chemical modification of compounds by biotransformation alters their biological effects, either making them useful to the organism or ending up with toxic effects. But in most cases, biotransformation terminated with the production of propitious products.

Recent reviews suggest that biocatalysis also forms a sustainable way in environmental fields to mop up environmental pollutants. It helps harness the catabolic activity of living systems to degrade and transform a wide range of compounds to chirally pure compounds, thereby offering several advantages over the use of chemicals and microorganisms. Thus, biotransforming enzymes are gaining importance.

Properties of biotransforming enzymes are as follows:

- Biocatalysis, which is normally performed in an aqueous environment but can, in many cases, also be conducted in solvent mixtures, liquid–liquid two-phase systems, and even in pure organic solvents. A relevant practical example is the use of esterases and lipases to catalyse esterifications in organic solvents such as vinyl acetate.
- They require mild reaction conditions. Therefore, biocatalysis offers great chances and advantages for successful applications (also in cases where either the substrates or the products of the reaction are chemically labile).
- There is no, or only limited, use of protecting groups, for example, for the chemoenzymatic synthesis of complex carbohydrates and glycoconjugates.
- They have high chemo-, regio- and stereoselectivities (Oreste Ghisalba et al. 2010).

A wide variety of biotransforming enzymes exist and are highly substrate specific. Some of the biotransforming enzymes are similar in plants, animals and in humans but there are a few species specific ones. In this context, we can classify the biotransformation enzymes into three as occurring in humans, plants and microbial cells.

5.2 Biotransforming Enzymes in Humans

In the case of humans, all those molecules which the body does not recognize as its own are considered as ‘foreign’ or *xenobiotics*. This section explains the biotransformation of xenobiotics by respective enzymes, thus lessening their toxic effects and easily removing them from the body.

Today all living beings of the earth are constantly and unavoidably exposed to xenobiotics, which include both manufactured and naturally occurring chemicals such as industrial chemicals, pesticides, drugs, pollutants, alkaloids, secondary plant metabolites, and toxic substances. Xenobiotics are readily absorbed by the human body because of their lipophilic property. It is due to this physical property that the homeostasis becomes altered. Consequently, there comes the role of biotransformation enzymes, which aid in the elimination of xenobiotics by converting them to hydrophilic compounds. The process of biotransformation ends up with the change in properties of foreign molecules, i.e. from those preferring absorption (lipophilicity) to those which are easily eliminated either through urine or faeces (hydrophilicity).

Having a broad substrate specificity, the biotransforming enzymes metabolize a wide range of endogenous compounds such as acetone, ethanol, steroid hormones, bilirubin, bile acids, fatty acids, eicosanoids, vitamins A and D, etc. Some of these enzymes are either expressed constitutively or are induced by the xenobiotic itself. The rates of xenobiotic biotransformation among individuals may vary depending upon the amino acid sequence, i.e. the structure of a given biotransforming enzyme. In general, a variant form of a xenobiotic biotransforming enzyme (*allelic variant* or an *allelozyme*) has diminished enzymatic activity compared with that of the wild-type enzyme, although this is not always the case. However, the impact of amino acid substitution on the catalytic activity of a xenobiotic biotransforming enzyme is usually substrate-dependent (Parkinson et al. 2013). The reactions catalysed by xenobiotic biotransforming enzymes are generally divided into two groups: Phase I and Phase II (Table 5.1).

5.2.1 Phase I Reactions

Phase I reactions mainly involve three reactions – oxidation, reduction and hydrolysis. These reactions introduce a functional group (–OH, –SH, $-NH_2$ or –COOH), and usually end up in the formation of slightly hydrophilic compounds. The functional groups added during phase I biotransformation form the site for phase II biotransformation.

Oxidative Reactions Oxidation is the most prevalent and an important way of metabolizing xenobiotics. It includes withdrawal of an electron, followed by addition of oxygen into the molecule. Molecular oxygen most often forms the source and in some cases the oxygen is obtained from water. Most organic compounds undergo single-electron or double-electron redox reactions. Some undergo a 4e-

Table 5.1 Xenobiotic metabolizing enzymes

Reaction	Enzyme	Localization
Phase I		
Oxidation	Cytochrome P450	Microsomes
	Flavin monooxygenases	Microsomes
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Cytosol, mitochondria
	Xanthine oxidase	Cytosol
Hydrolysis	Carboxyl esterase	Cytosol, microsomes, lysosomes, blood
	Peptidase	Lysosomes, blood
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microsomes, cytosol, microflora
	Carbonyl reduction	Microsomes, cytosol, blood
	Quinone reduction	Microsomes, cytosol
Phase II		
	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Glutathione conjugation	Microsomes, cytosol
	Amino acid conjugation	Microsomes, mitochondria
	Acylation	Mitochondria, cytosol
	Methylation	Microsomes, cytosol, blood

oxidation. The most common electron acceptor is molecular oxygen. This can undergo a 2e- reduction to yield H_2O_2 or a 4e- reduction to generate water.

Common oxidative reaction includes

- Dehydrogenation (involves a hydride abstraction)
- Hydroxylation of aromatic carbons
- Hydroxylation of aliphatic carbons
- Oxidations involving carbon-heteroatom systems (Amin M. Kamel 2007).

The main enzymes involved in the oxidative reactions include xanthine oxidase (XO), monoamine oxidases (MAOs), diamine oxidases (DAOs), flavin-containing monooxygenases (FMOs), cytochrome P450s (P450s), etc. (Amin M. Kamel 2007).

Cytochrome P450s Cytochrome P450 enzymes (CYPs or P450s) belong to the super family of heme-dependent proteins that are synthesized by different mammalian tissues such as liver, small intestine, lungs, kidneys, brain and placenta. In the present scenario 57 different P450 isoforms have been identified in man. Based on the protein sequence, the enzymes have been classified into 18 families and 43 subfamilies. Of these 18 families, only the first three (CYP1, CYP2, CYP3) are

involved in xenobiotic metabolism and they form approximately 70% of the total CYP content in human liver.

The heme is ferric (Fe^{3+}) containing porphyrin cofactor with cysteine as the fifth ligand and the sixth coordination site to bind and activate molecular oxygen; NADPH is used as electron source and an NADPH reductase system to recycle $NADP^+$ to NADPH.

The liver microsomal P450 enzymes play a very important role in determining the intensity and duration of action of drugs, and they also play a key role in the detoxification of xenobiotics. Cytochrome P450 catalyses the basic reaction of monooxygenation, where one atom of oxygen is incorporated into a substrate (RH), with the subsequent reduction of other to water with reducing equivalents derived from NADPH (Amin M. Kamel 2007).

$$\text{Substrate}\,(\text{RH}) + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Product}\,(\text{ROH}) + \text{H}_2\text{O} + \text{NADP}$$

The reactions catalysed by cytochrome P450 include hydroxylation of an aliphatic or aromatic carbon, an epoxidation of a double bond, heteroatom (S-,N-,I-) oxygenation, an N-hydroxylation, heteroatom (O-,S-,N-) dealkylation, oxidative group transfer, cleavage of esters and dehydrogenation. It can also catalyse the reduction of azo and nitro compounds and cause reductive dehalogenation. Catalysis of these reactions makes cytochrome P450 system a unique one in the overall metabolism of pollutants and the xenobiotics. The protein part of the enzyme determines the substrate specificity. The cytochrome P450 enzymes are now abbreviated in codes as CYP followed by a number to indicate the family and a letter to specify the subfamily and number, which codes further the enzymatic activity of that particular protein. For example, CYP1-3 families have significance in the oxidation of pollutants and drugs (Osmo Hanninen 2009).

Alcohol Dehydrogenases and Aldehyde Dehydrogenases Alcohol dehydrogenases are the zinc-containing cytosolic enzymes present in the highest level in liver and in kidney, lungs, gastric mucosa, etc. Human ADH is a dimeric protein with two subunits of 40 kDa. The subunits may be α, β, γ, π, χ, σ or μ and are encoded by six different gene loci. Based on the different subunits present, ADH may be of various types, given in Table 5.2.

Aldehyde dehydrogenases (ALDH) play a major role in xenobiotic metabolism. They are enzymes which oxidize aldehyde to carboxylic acid using NAD^+ as cofactor. The enzymes also exhibit esterase activity. Twelve ALDH genes were found in humans and they may differ in their primary amino acid sequence and the quarternary structure.

Flavin Monooxygenases FMOs are microsomal enzymes which need NADPH and O_2, and catalyse reactions similar to those catalysed by cytochrome P450. In mammals, the FMO gene family comprises five enzymes, FMO1 to FMO5, with about 550 amino acid residues each. The amino acid sequence shows 50–58% identity across the species lines. The highly conserved glycine-rich region (residues 4 to 32)

Table 5.2 Types of alcohol dehydrogenases (ADH)

Class	Enzyme	Function	Location
Class I	α-ADH	Oxidation of ethanol and other small aliphatic alcohols.	Liver, adrenal glands, lower levels in kidney, lungs, blood vessels.
	β-ADH		
	γ-ADH		
Class II	π-ADH	Oxidation of large aliphatic and aromatic alcohols.	Liver, lower levels in stomach.
Class III	χ-ADH	Oxidation of long-chain alcohols and aromatic alcohols.	All tissues, including brain.
Class IV	σ/μ-ADH	Conversion of ethanol to acetaldehyde and oxidation of retinol.	Stomach and other areas of gastrointestinal tract.

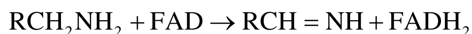
of each FMO enzyme non-covalently binds one mole of FAD present near the active site; adjacent to this is a second highly conserved glycine-rich region (residues 186 to 213) that binds NADPH. FMO is heat-labile and can be deactivated in the absence of NADPH by heating microsomes to a temperature of 50 °C for 1 min in contrast to cytochrome P450, which is deactivated with a non-ionic detergent, such as 1% Emulgen 911. The pH optimum for FMO-catalysed reactions is 8 to 10, which is slightly higher than that for most P450 reactions (pH 7 to 8).

The reactions catalysed by FMO include the oxidation of nucleophilic tertiary amines to *N*-oxides, secondary amines to hydroxylamines and nitrones, and primary amines to hydroxylamines and oximes. It also oxidizes several sulfur-containing xenobiotics (such as thiols, thioethers, thiones, and thiocarbamates) and phosphines to *S*- and *P*-oxides, respectively. With few exceptions, FMO acts as an electrophilic oxygenating catalyst, which distinguishes it from most other flavoprotein oxidases and monooxygenases. During the oxygenation of xenobiotics, the 4a-hydroperoxyflavin is converted to 4a-hydroxyflavin with the transfer of the flavin peroxide oxygen to the substrate. The final rate-limiting step in the catalytic cycle involves dehydration of 4a-hydroxyflavin (which restores FAD to its resting, oxidized state) and release of $NADP^+$. Also this step determines the upper limit of the rate of substrate oxidation. Binding of $NADP^+$ to FMO during catalysis is important because it prevents the reduction of oxygen to H_2O_2. In the absence of bound NADP, FMO would function as an NADPH-oxidase that would consume NADPH and cause oxidative stress through excessive production of H_2O_2.

In humans, FMO plays a major role in the biotransformation of several drugs (e.g. benzydamine, cimetidine, clozapine, guanethidine, methimazole, olanzapine, sulindac sulfide, tamoxifen and various dimethylaminoalkyl phenothiazine derivatives such as chlorpromazine and imipramine), xenobiotics (e.g. cocaine, methamphetamine, nicotine, tyramine) and endogenous substrates (e.g. trimethylamine, cysteamine). The major flavin monooxygenase in human liver microsomes, FMO3, is predominantly involved in the conversion of (*S*) nicotine to (*S*)-nicotine *N*-1-oxide, excreted in the urine of cigarette smokers or individuals wearing a nicotine patch. Therefore, the presence of *trans*-(*S*)-nicotine *N*-1-oxide in urine is clinically used as an in vivo probe of FMO3 activity in humans. FMO3 is also the principal

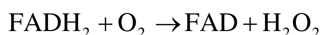
enzyme involved in the *S*-oxygenation of cimetidine, an H_2-antagonist widely used in the treatment of gastric ulcers and other acid-related disorders.

Monoamine Oxidase, Diamine Oxidase and Polyamine Oxidase Monoamine oxidase (MAO), diamine oxidase (DAO) and polyamine oxidase (PAO) – all these three enzymes are principally involved in the oxidative deamination of primary, secondary and tertiary amines. Many of the naturally occurring amines, such as the monoamine serotonin (5-hydroxytryptamine), the diamine putrescine and histamine, and monoacetylated derivatives of the polyamines spermine and spermidine form the substrate for these enzymes. Of the three enzymes, MAO is particularly involved in xenobiotic metabolism. Oxidative deamination of a primary amine produces ammonia and an aldehyde, whereas oxidative deamination of a secondary amine produces a primary amine and an aldehyde. The products of the former reaction, an aldehyde and ammonia, are those produced during the reductive biotransformation of certain oximes by aldehyde oxidase. The two forms of monoamine oxidase are MAO-A and MAO-B. The genetical information suggests that the genes coding MAO-A and -B are of common ancestral origin but encoded by two distinct genes, both localized on the X chromosome and both comprising 15 exons with a similar intron–exon organization. The amino acid sequence of MAO-A (Mr 59.7 kDa) is 70% identical to that of MAO-B (Mr 58.0 kDa).The mechanism of catalysis by monoamine oxidase is illustrated below:



$$RCH_2NH_2 + FAD \rightarrow RCH = NH + FADH_2$$

$$RCH = NH + H_2O \rightarrow RCHO + NH_3$$



$$FADH_2 + O_2 \rightarrow FAD + H_2O_2$$

The substrate is oxidized by the enzyme, which itself is reduced ($FAD \rightarrow FADH_2$) (Parkinson et al. 2013). The oxygen incorporated into the substrate is derived from water, not molecular oxygen; hence the enzyme functions as a true oxidase. The catalytic cycle is completed by reoxidation of the reduced enzyme ($FADH_2$ n FAD) by oxygen, which generates hydrogen peroxide (which may be a cause of oxidative stress). The initial step in the catalytic cycle appears to be abstraction of hydrogen from the α-carbon adjacent to the nitrogen atom; hence, the oxidative deamination of xenobiotics by MAO is generally blocked by substitution of the α-carbon.

Although not present in mitochondria, PAO resembles MAO in its cofactor requirement and basic mechanism of action. Both enzymes use oxygen as an electron acceptor, which results in the production of hydrogen peroxide.

Diamine oxidase is a cytosolic, copper-containing pyridoxalphosphate-dependent enzyme present in liver, kidney, intestine, and placenta. Its substrates include histamine and simple alkyldiamines with a chain length of four (putrescine) or five (cadaverine) carbon atoms. Diamines with more than nine carbon atoms are not substrates for DAO, although they can be oxidized by MAO.

Xanthine Dehydrogenase–Xanthine Oxidase Xanthine dehydrogenase (XD) and xanthine oxidase (XO) are two forms of the same enzyme that differ in the electron acceptor used in the final step of catalysis. In the case of XD, the final electron acceptor is NAD^+ (dehydrogenase activity), whereas in the case of XO it is oxygen (oxidase activity). XD is converted to XO by reversible oxidation of cysteine residues (Cys993 and Cys1326 of the human enzyme) and/or by proteolytic cleavage. Under normal physiologic conditions, XD is the predominant form of the enzyme found in vivo (Parkinson et al. 2013). However, during tissue processing, XO or a combination of XO and XD is involved. The induction of XD and/or the conversion of XD to XO in vivo are thought to play a crucial role in ischemia-reperfusion injury, lip polysaccharide (LPS)-mediated tissue injury, and alcohol-induced hepatotoxicity. During ischemia, XO levels increase because hypoxia induces XD/XO gene transcription and because XD is converted to XO. During reperfusion, XO contributes to oxidative stress and lipid peroxidation because the oxidase activity of XO involves the reduction of molecular oxygen, which results in the generation of reactive oxygen species.

5.2.2 Hydrolytic Enzymes

Primary hydrolytic enzymes are carboxylesterases, peptidases and epoxide hydrolase. Other enzymes are cholinesterases and paraoxonases. Hydrolysis involves cleavage of ester or amide bonds, resulting in carboxylic acids, mainly in the presence of water. Generally, esters are hydrolysed much faster than amides (Fig. 5.1).

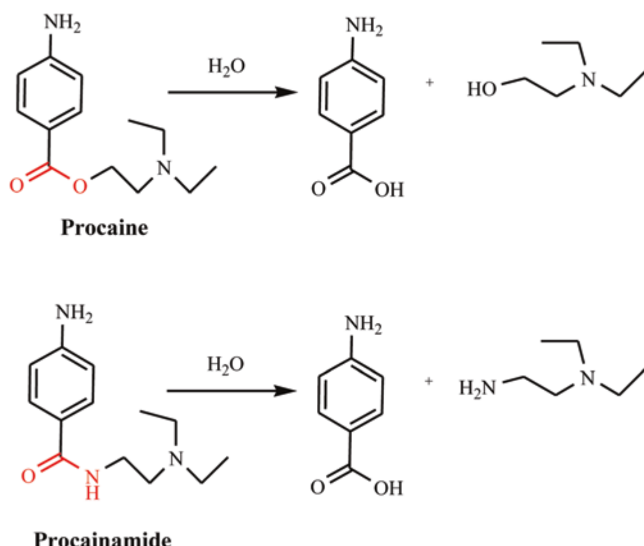


Fig. 5.1 Hydrolysis of esters and amides

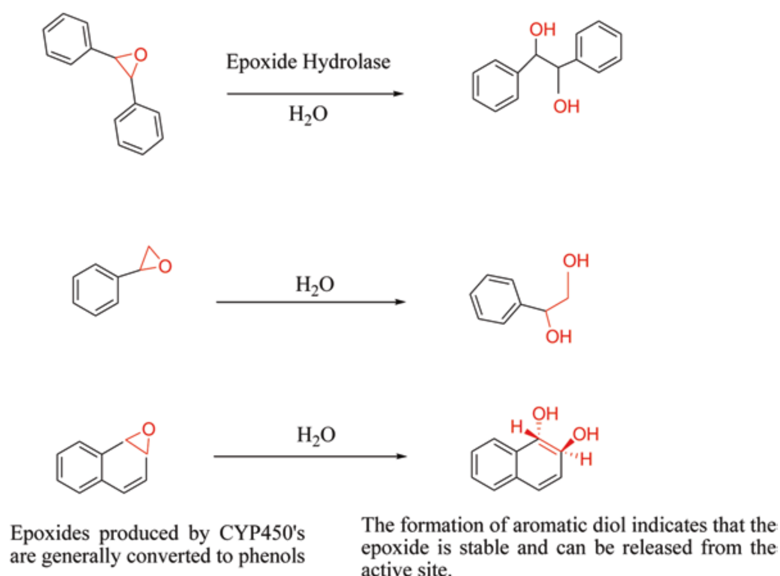


Fig. 5.2 Hydration of epoxides

Hydrolysis of epoxides involves addition of water to alkene epoxides and arene oxide to form the corresponding diols (Fig. 5.2).

Carboxylesterases Carboxylesterases are ~60-kDa glycoproteins that are present in a wide variety of tissues, including serum. Most of the carboxyl esterase activity in liver is associated with the endoplasmic reticulum, although remarkable carboxylesterase activity is present in lysosomes and cytosol (Parkinson et al. 2013). The enzyme plays a significant role in producing pharmacologically active metabolites. For example, the pharmacologically active metabolite lovastatin hydroxy acid, which inhibits HMG-CoA reductase and lowers plasma cholesterol levels, is produced by converting lovastatin to lovastatin hydroxy acid by liver carboxylesterases.

Carboxylesterases in serum and tissues and serum cholinesterase collectively determine the site and duration of action of certain drugs. For example, procaine, a carboxylic acid ester, is rapidly hydrolyzed; hence, this drug is used mainly as a local anesthetic. In contrast, procainamide, the amide analogue of procaine, is hydrolyzed much more slowly, because of which the drug reaches the systematic circulation and is useful in the treatment of cardiac arrhythmia. In general, enzymatic hydrolysis of amides occurs more slowly than that of esters (Parkinson et al. 2013).

Peptidases Peptidases are enzymes which cleave peptides in the blood and tissues. They include amino peptidases, carboxypeptidases and endopeptidases. Amino peptidases hydrolyze amino acids at the *N*-terminus and carboxypeptidases at the *C*-terminus. Endopeptidases hydrolyse peptides at specific internal sites; for exam-

ple, trypsin cleaves peptides on the *C*-terminal side of arginine or lysine residues. Peptidases also function as amidases since they cleave the amide linkage between adjacent amino acids.

Epoxide Hydrolase The *trans* addition of water to alkene epoxides and arene oxides (oxiranes) is catalysed by epoxide hydrolase. Epoxide hydrolase is present in almost all tissues, including the liver, lung, kidney, skin, intestine, colon, testis, ovary, spleen, thymus, brain and heart. The five distinct forms of epoxide hydrolase present in mammals include microsomal epoxide hydrolase (mEH), soluble epoxide hydrolase (sEH), cholesterol epoxide hydrolase, LTA4 hydrolase and hepoxilin hydrolase. As their names imply, cholesterol epoxide hydrolase, LTA4 hydrolase, and hepoxilin hydrolase exclusively hydrolyze endogenous epoxides, but have no particular role in the detoxification of xenobiotic oxides. LTA4 hydrolase is distinct from the other epoxide hydrolases in that it is a bifunctional zinc metalloenzyme that has both epoxide hydrolase and peptidase activities as well as because of the two hydroxyl groups introduced during the conversion of LTA4 to LTB4. Many epoxides and oxides are intermediates formed during the cytochrome P450-dependent oxidation of aromatic and unsaturated aliphatic xenobiotics. These metabolites which may otherwise bind to proteins and nucleic acids and cause cellular toxicity and genetic mutations are removed by epoxide hydrolases, particularly mEH and sEH. They rapidly convert the potentially toxic metabolites to the corresponding dihydrodiols, which are less reactive and easier to excrete. Because of these activities epoxide hydrolases are widely considered as a group of detoxification enzymes (Parkinson et al. 2013).

5.2.3 Reductive Reactions

Certain metals (e.g. pentavalent arsenic) and xenobiotics with an aldehyde, ketone, disulfide, sulfoxide, quinone, *N*-oxide, alkene, azo or nitro group often undergo reduction reaction, although it is sometimes difficult to ascertain whether the reaction proceeds enzymatically or non-enzymatically by interaction with reducing agents (such as the reduced forms of glutathione, FAD, FMN and NAD(P)) (Parkinson et al. 2013).

Azo- and Nitro-Reductions During azo-reduction, the nitrogen–nitrogen double bond is sequentially reduced and cleaved to produce two primary amines, using four reducing equivalents. Nitro-reduction requires six reducing equivalents, which are consumed in three sequential reactions, for the transformation of nitrobenzene to aniline. Azo- and nitro-reductions can be catalysed by enzymes of intestinal flora (Fig. 5.3), two liver enzymes cytochrome P450 (has the capacity to reduce xenobiotics under low oxygen or anaerobic conditions) and NAD(P)H-quinone oxidoreductase (a cytosolic flavoprotein, also known as DT-diaphorase), and interactions with reducing agents (reduced forms of glutathione, NADP, etc.). Under certain circumstances, a third liver enzyme, aldehyde oxidase, may also catalyse azo- and nitro-reduction reactions (Parkinson et al. 2013).

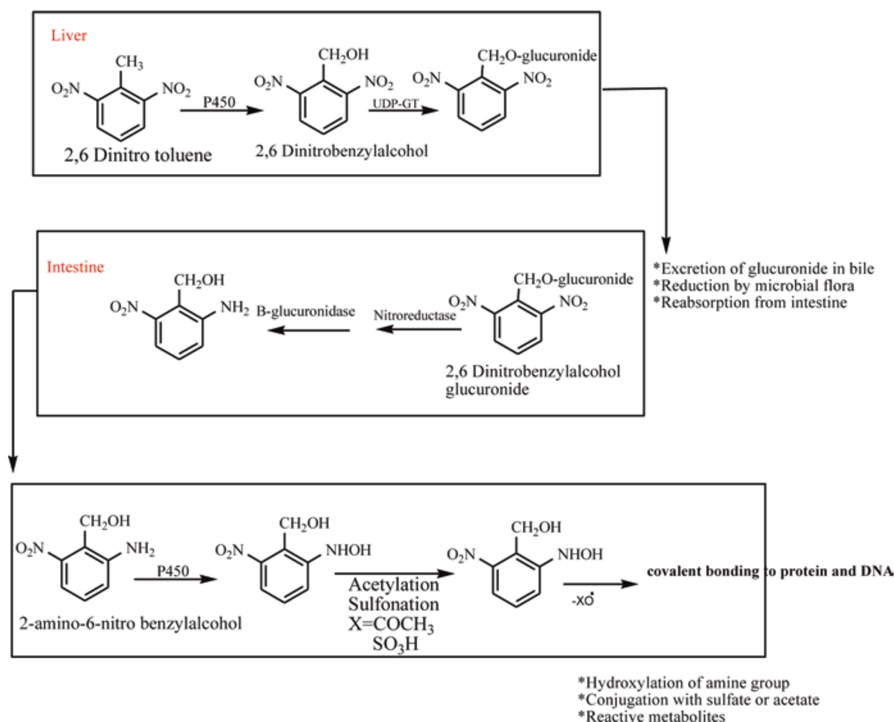


Fig. 5.3 Reduction – role of intestinal microbial flora in biotransformation

Carbonyl Reduction Carbonyl reductases are a class of enzymes which catalyse the reduction of certain aldehydes to primary alcohols and of ketones to secondary alcohols alcohols. These enzymes are monomeric, NADPH-dependent and present in blood and the cytosolic fraction of the liver, kidney, brain and other tissues and structurally belong to the short-chain dehydrogenase/reductase (SDR) super family which includes certain hydroxysteroid dehydrogenases and prostaglandin dehydrogenases. The major circulating metabolite of the antipsychotic drug haloperidol is a secondary alcohol formed by carbonyl reductases in the blood and liver. The enzyme shows a high degree of stereoselectivity in the reduction of ketones to secondary alcohols.

Quinone Reduction The cytosolic flavoprotein NAD(P)H-quinone oxidoreductase, also known as DT-diaphorase, catalyses the two-electron reduction of quinones to stable hydroquinone with stoichiometric oxidation of NAD[P]H without oxygen consumption. The reaction can also be catalysed by carbonyl reductase, especially in humans. Although there are exceptions, this pathway of quinone reduction is essentially nontoxic as it is not associated with oxidative stress, unlike the one-electron reduction of quinones by NADPH-cytochrome P450 reductase (Fig. 5.4). The other substrates for DTdiaphorase include a variety of potentially toxic compounds, including quinone epoxides, quinone imines, azo dyes and *C*-nitroso deriv-

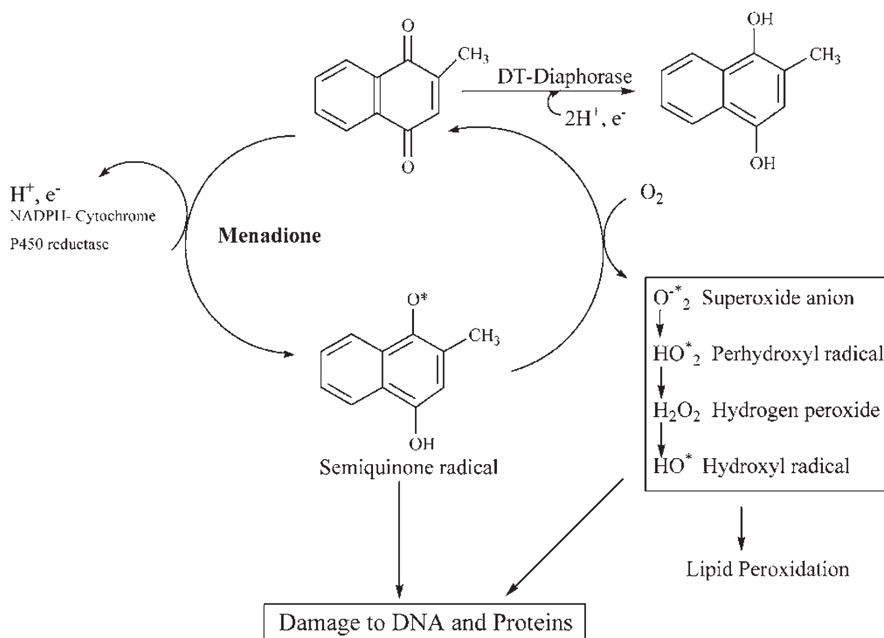


Fig. 5.4 Reduction of quinones

atives of arylamines. The second pathway of quinone reduction is catalysed by NADPH-cytochrome P450 reductase (a microsomal flavoprotein) and results in the formation of a semiquinone free radical by a one-electron reduction of the quinone.

Semiquinones are readily auto-oxidizable, which leads to nonstoichiometric oxidation of NADPH with oxygen consumption. The auto-oxidation of a semiquinone free radical produces superoxide anion, hydrogen peroxide and other active oxygen species, which can be extremely cytotoxic and result in oxidative stress. DT-diaphorase levels are often elevated in tumour cells, since agents that are biotransformed by DT-diaphorase have implications over chemotherapy.

5.2.4 Phase II Reactions

Phase II enzymes are another class of biotransformation enzymes which catalyse biotransformation of endogenous compounds and xenobiotics mainly by conjugating reactions. The products formed thereby are of easily excretable forms as well as act as a metabolic inhibitor of pharmacologically active substances. These include glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. The cofactors involved in these reactions react with functional groups that are either present on the xenobiotics or are introduced during phase I biotransformation. The main consequence of phase II biotransformation

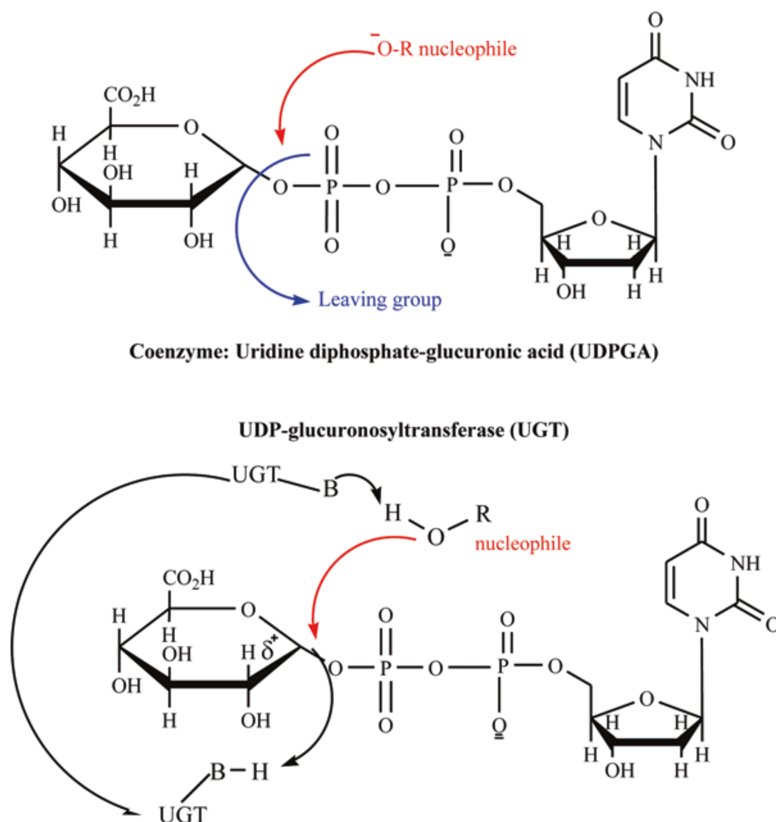


Fig. 5.5 Conjugation with glucuronic acid (glucuronidation)

reactions is to excrete out the foreign chemicals by increasing their hydrophilicity to a larger extent.

Glucuronidation Glucuronidation plays a major role in the biotransformation of xenobiotics in almost all mammals. Glucuronidation requires the cofactor uridine diphosphate-glucuronic acid (UDP glucuronic acid), and the reaction is catalysed by UDP glucuronosyl transferases (UGTs), localized in the endoplasmic reticulum of the liver and other tissues, such as the kidney, intestine, skin, brain, spleen and nasal mucosa. The site of glucuronidation is generally an electron-rich nucleophilic heteroatom (O, N or S) (Fig. 5.5). Therefore, substrates for glucuronidation contain such functional groups as aliphatic alcohols and phenols (which form *O*-glucuronide ethers), carboxylic acids (which form *O*-glucuronide esters), primary and secondary aromatic and aliphatic amines (which form *N*-glucuronides), and free sulfhydryl groups (which form *S*-glucuronides). Coumarin and certain other carbonyl-containing compounds are glucuronidated to form arylenol-glucuronides. Other than xenobiot-

ics, substrates for glucuronidation include several endogenous compounds, such as bilirubin, steroid hormones and thyroid hormones (Parkinson et al. 2013). Xenobiotics and endogenous compounds on glucuronidation become polar, water-soluble conjugates that are eliminated from the body through urine or bile, which depends on the size of the parent compound or phase I metabolite.

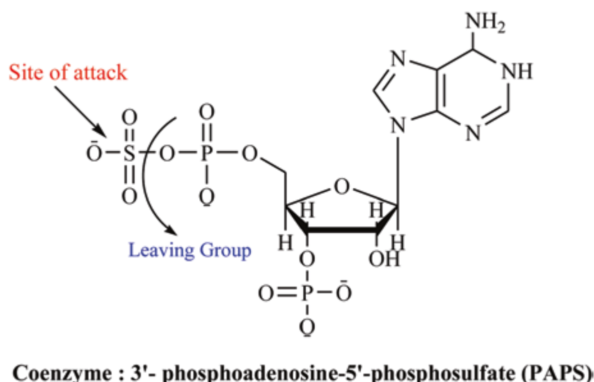
Sulfation Sulfation is a main conjugation pathway for phenols which can also occur for alcohols, arylamines, N-hydroxy compounds and, to some extent, thiols. As with sugar conjugation, the active donor is 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Fig. 5.6). Sulfation results from the interaction of the drug with PAPS in the presence of the cytosolic enzyme sulfotransferase. Sulfate conjugation occurs less frequently than does glucuronidation presumably because PAPS cellular concentration is considerably lower (75 mM) than Uridine 5′- diphosphoglucuronic acid (UDPGA) (350 mM). Hence the capacity of sulfation is low and only fewer numbers of functional groups undergo sulfate conjugation. Functional groups that can be sulfated are phenols: Ar-OH, alcohols: R-OH, arylamines: Ar-NH2, and N-hydroxy compounds: R-NH-OH (Amin M. Kamel 2007).

Methylation Though methylation reaction is common, it is considered as a minor pathway of xenobiotic biotransformation (Fig. 5.7). In this reaction, the methyl group donor SAM (*S*-adenosylmethionine) is converted to *S*-adenosylhomocysteine. Methylation reactions include endogenous compounds such as melatonin, histamine, serotonin, dopamine, etc. Compared to conjugation reactions, methylation reduces the polar and hydrophilic nature of the substrates, thereby deactivating their biological activities.

Acetylation N-Acetylation is a major route of biotransformation for xenobiotics containing an aromatic amine ($R–NH_2$) or a hydrazine group ($R–NH–NH_2$), which are converted to aromatic amides ($R–NH–COCH_3$) and hydrazides ($R–NH–NH–COCH_3$), respectively (Evans 1992). The enzyme which catalyses the *N*-acetylation of xenobiotics is N-acetyltransferases. The enzyme requires acetyl-coenzyme A (acetyl-CoA) as the coenzyme (Fig. 5.8). Except for cystein conjugates, which are transformed to mercapturic acids by N-acetylation, primary aliphatic amines are rarely its substrates. Like methylation, N-acetylated metabolites are less polar than their parent compounds since N-acetylation masks an amine with a nonionizable group.

Conjugation with Amino Acids There are two principal pathways by which xenobiotics are conjugated with amino acids: one which conjugates with the amino group of the amino acid and the other with the carboxylic group. The first pathway involves conjugation of xenobiotics containing a carboxylic acid group with the amino group of amino acids such as glycine, glutamine and taurine. This pathway involves activation of the xenobiotic by conjugation with CoA, which produces an acyl-CoA thioether that reacts with the *amino group* of an amino acid to form an amide linkage. The second pathway involves conjugation of xenobiotics containing an aromatic hydroxylamine (*N*-hydroxy aromatic amine) with the *carboxylic acid group* of such

Fig. 5.6 Sulfation



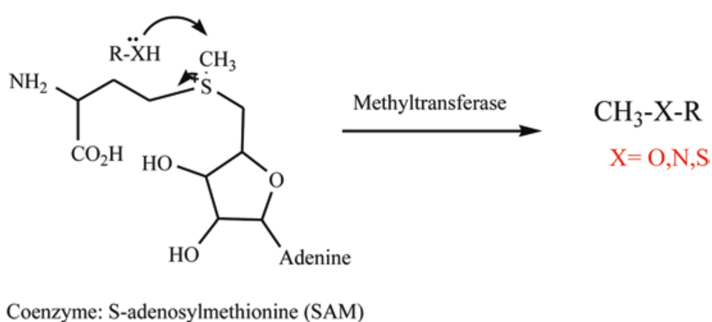


Fig. 5.7 Methylation

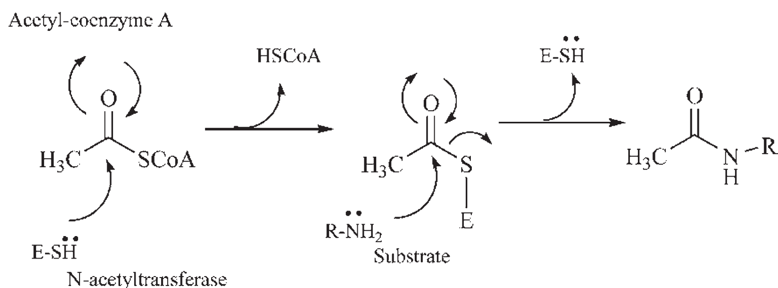


Fig. 5.8 Acetylation

amino acids as serine and proline. This pathway involves activation of an amino acid by aminoacyl-tRNA-synthetase, which reacts with an aromatic hydroxylamine to form a reactive *N*-ester (Kato and Yamazoe 1994).Carboxylic acids, particularly aromatic acids and aryl acetic acids, are conjugated with polar endogenous amino acids. The quantity of amino acid conjugation is minute because of the limited availability of amino acids in the body and competition with glucuronidation for carboxylic acid substrates. Amino acids conjugation of carboxylic acids leads to amide bond forma-

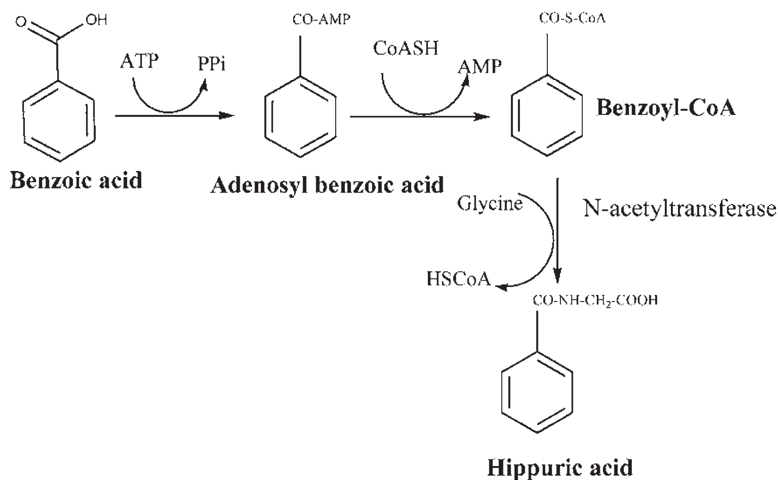
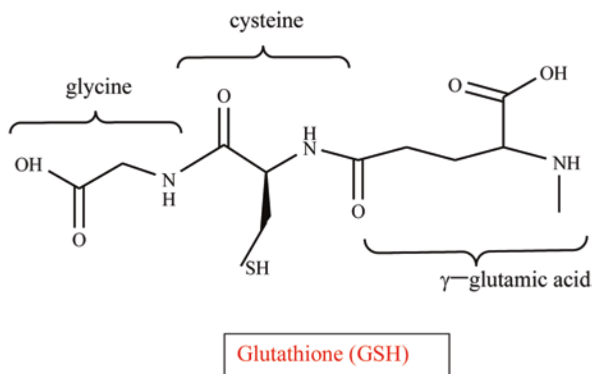


Fig. 5.9 Conjugation with amino acids (glycine conjugation with benzoic acid)

Fig. 5.10 Conjugation with glutathione



tion. Glycine conjugates are the most common amino acid conjugates in animals (Fig. 5.9). Conjugation with L-glutamine is most common in humans and other primates, but it seems to occur to a lesser extent in non-primates. Taurine, arginine, asparagine, histidine, lysine, glutamate, aspartate, alanine and serine are the other amino acid conjugates found in mammals (Parkinson et al. 2013).

Conjugation with Glutathione The tripeptide glutathione (GSH) is found in virtually all mammalian tissues (Fig. 5.10). The presence of a potent nucleophilic thiol group helps glutathione function as a scavenger of harmful electrophilic compounds ingested or produced by metabolism. Xenobiotics conjugated with glutathione are either highly electrophilic as such or are first metabolized to an electrophilic product prior to conjugation. Conjugation with glutathione reduces drug toxicity that results from the reaction of cellular nucleophiles with electrophilic metabolites. The glutathione S-transferases (GSTs) are a family of enzymes that catalyse the conju-

gation of tripeptide glutathione with xenobiotic compounds forming thioether conjugates. To a larger extent, a number of endogenous compounds such as prostaglandins and steroids are also metabolized via glutathione conjugation (Parkinson et al. 2013).

5.3 Biotransforming Enzymes in Plants

Plants are permanently exposed to various xenobiotics and pose potential danger. Like other living beings, plants also include a variety of xenobiotic-metabolizing enzymes for detoxification of compounds (Table 5.3). Though some of them show similarities with those in humans and animals, there are several plant-specific ones. Plant cell suspension cultures serve as tools for the biotransformation of xenobiotics. Our environment is contaminated by unregulated contaminants, which include personal care products, pesticides, herbicides and industrial compounds that are thought to have long-term adverse effects on ecosystems. But according to Powles and Yu (2010), in the case of plants, the increased activity of xenobiotic-metabolizing enzymes results in faster deactivation of xenobiotics, which may be manifested as improved detoxification of environmental contaminants but also as a resistance to herbicides.

Veterinary drugs are used worldwide to treat diseases and protect animal health. However, veterinary drugs are also unwantedly introduced into the environment mostly via animal excrements, which persist in the environment for a long time and may impact on the non-target organisms. Plants are able to uptake the veterinary drugs, transform them to non- or less-toxic compounds and store them in the vacuoles and cell walls. This ability may protect not only the plants themselves but also other organisms, predominantly invertebrates and wild herbivores (Bartikova et al.

Table 5.3 Xenobiotic-metabolizing enzymes in plants

Reaction	Xenobiotic-metabolizing enzymes
Biotransformation phase I	Cytochromes P450
	Peroxides
	Ascorbate peroxidases
	Peroxides III-class
	Alcohol dehydrogenases
	Aldehyde dehydrogenases
	Short-chain dehydrogenases/reductases
	Aldo-ketoreductases
	Carboxylesterases
	Epoxide hydrolases
Biotransformation phase II	Glutathione-S-transferases
	Glycosyltransferases
	Malonyltransferases
	Sulfotransferases
	Methyltransferases

2015). Although antibiotics belong to the most important veterinary drugs, information about their biotransformation in plants is only limited. Migliore and his group (2003) studied phytotoxicity, uptake, and biotransformation of enrofloxacin in crop plants such as *Cucumis sativus*, *Lactuca sativa*, *Phaseolus vulgaris* and *Raphanus sativus*. Their results showed that plants were able to metabolize enrofloxacin into ciprofloxacin via deethylation reaction. The other example includes the biotransformation of the NSAID diclofenac. NSAID diclofenac is found in the environment worldwide due to its intensive use and poor elimination during wastewater treatment processes. In order to test phytoremediation as a tool for the removal of this drug from wastewater, the uptake of the compound into plant tissues and its metabolic pathway was addressed using barley (*Hordeum vulgare*) and a hairy root cell culture of horseradish (*Armoracia rusticana*) as model species. Diclofenac was taken up by plants and underwent rapid biotransformation. A phase I reaction resulted in the hydroxylated metabolite 4OOH-diclofenac, which was conjugated subsequently in phase II to a glucopyranoside, a typical plant-specific metabolite (Huber et al. 2012).

5.4 Biotransforming Enzymes in Microbes

The recognition of microbial biotransformation has created a boom in the chemical and pharmaceutical industries. In recent years, microbial biotransformation has become an inevitable process in green chemistry. The wide variety of microbial strains and enzymes with their selective biotransformation potential has resulted in the bioconversion of a myriad of different substances into desired products.

It is only after the nineteenth century, targeted application of microbial transformations came into emergence. The fusion of two sciences, namely, organic chemistry and microbiology, has resulted in the tremendous growth of this field. The technology of microbial transformation deals with the isolation of microbial enzymes to catalyse bioconversions of organic compounds.

Microbial transformation offers the advantages of operating at non-extreme pH, near room temperature and reduced levels of toxic waste products with high selectivity. This metabolic flexibility of the microorganisms is exploited in the production of different enzymes for different reactions. However, the syntheses of these enzymes are regulated according to the physiological needs of the cells. The practice of biotransformation with recombinant microbial enzymes is gaining importance; the applications may include the production of hormones, antibiotics and special chemicals. Microbial transformation might be useful in cases where there is no chemical solution for chemical transformations.

Microbial transformations make use of enzyme-catalysed reactions within living cells, typically exploiting single chemical reactions like oxidation, reduction, hydrolysis and degradation, formation of C-C or C-hetero atom bonds (Gopishetty et al. 2007) (Table 5.4).

Economically, microbial biotransformations can be used in the manufacture of alkaloids, antibiotics, vitamins, amino acids, fermented beverages and fermented foods. Another feature of microbial transformation is its ability to imitate

Table 5.4 Reactions catalysed by microorganisms

Microbial strain	Reaction catalysed	Examples
Gliocladium roseum	Hydroxylation	Hydroxylation of unsubstituted coumarin to 7-hydroxy coumarin
	Glycosylation	Glycosylation of 7-hydroxy coumarin to 6,7-dihydroxy-coumarin-6-glycoside
Candida tropicalis	Alkylation	*O*-alkylation of 7-hydroxy coumarin to 7-methoxy coumarin
Streptomyces griseus	Oxidation	*O*-deethylation of 7-ethoxy coumarin to 7-hydroxy coumarin
	O-, *N*-dealkylation	Hydroxylation of 7-hydroxy coumarin to 6,7-dihydroxy coumarin
	Aromatic hydroxylationEpoxidation	Methylation of 6,7-dihydroxy coumarin to 7-hydroxy-6-methyl coumarin
	Carbon-carbon fission	
Aerobacter aerogenes	Microorganisms with mercury-metabolizing capability	
Bacillus megaterium		
Clostridium cochlearium		
Desulfovibrio desulfuricans		
Desulfovibrio vulgaris		
Desulfobulbus propionicus		
Enterobacter aerogenes		
Escherichia coli		

mammalian metabolism of drugs, which enables structure determination of drug metabolites for use in preclinical trials, toxicity studies and regulatory process.

5.5 Industrially Significant Biotransformations

Selected milestones of industrially relevant biotransformation and biocatalytic processes are summarized in Table 5.5.

Table 5.5 Some selected milestones of industrially relevant biotransformation and biocatalytic processes

Year	Process
5000 BC	Vinegar production
800 BC	Casein hydrolysis with chymosin for cheese production
1670	"Orlean" process for the industrial bio-oxidation of ethanol to acetic acid
1680	Anton van Leeuwenhoek first to see microorganisms with his microscope
1897	E. Buchner discovers yeast enzymes converting sugar into alcohol
1934	Regioselective bio-oxidation of sorbite to sorbose for the Reichstein vitamin C synthesis
1940	Sucrose inversion using an invertase
1950	Bioconversion of steroids
1970	Hydrolysis of pencillin to 6-aminopencillanic acid
1973	First successful genetic engineering experiments
1974	Glucose to fructose isomerization with immobilized glucose isomerase
1985	Enzymatic process for the production of acrylamide
1990	Hydrolysis of protease (trypsin) of porcine insulin to human insulin
1995	3000 ton pa plant for the biotransformation of nicotinonitrile to nicotinamide

High Fructose Syrups Production of high-fructose glucose syrups, by glucose isomerase, was one of the earliest biotransformation processes done by immobilized enzyme technology, and it has been operating now since 1972. It is used in the soft drinks and confectionary industries as it has health advantages over the conventional sugar, sucrose, i.e. fructose is twice as sweet, lower in calories and not absorbed into the bloodstream as quickly as sucrose (Collins and Kennedy 1999).

Antibiotics Many antibiotics synthesized from their precursors are produced by the biotransformation process catalysed by the respective enzymes. Some examples include semi-synthetic penicillin produced from its precursor 6-amino-penicillanic acid. This is done by using the immobilized penicillin acylase (*amidase*), which has been operational since 1973.

Vitamins From the economic point of view, microbial biotransformations are widely used to produce vitamin B_{12}, riboflavin, ascorbic acid and p-carotene. For example, oxidation of sorbitol by *Acetobacter suboxydans* or *Acetobacter xylinum* yields ascorbic acid (vitamin C).

$$\text{D-Glucose} \rightarrow \text{D-Sorbitol} \rightarrow \text{L-Sorbose} \rightarrow \text{L-Ascorbic acid (vitamin C).}$$

$$\boxed{\textit{Acetobacter suboxydans}}$$

Flavours and Fragrances The role of biotransformation technologies in the flavours and fragrances industry is becoming more prominent for its ability to produce single enantiomers. For example, the (L) enantiomer of carvone tastes of spearmint, whereas its (D) enantiomer tastes of dill/caraway. Also only (L) form of monoso-

dium glutamate has taste-enhancing properties, and out of eight isomers of menthol only (L)-menthol has the desired combination of mint taste and cooling sensation (Collins and Kennedy 1999).

Food Industries There is an increasing demand by consumers for natural, environmentally friendly and healthy products made from natural, renewable sources, for use in both food ingredients and personal care products. Biotransformation enzymes play a prominent role in the production of various food products like cheese, wine, food additives, etc. The advantages of using biotransformation for these types of preparations are the ability to operate under mild conditions, hence retaining the traditional properties of the food products.

5.6 Conclusions

Biotransformation is a process by which organic compounds are transformed from one form to another, aided by organisms such as bacteria, fungi and enzymes. It is used as a valuable strategy to build molecules similar to parent drugs in the drug discovery programme. It can also be used to synthesize compounds or materials. Microbial biotransformation or microbial biotechnology is extensively used to generate metabolites in bulk amounts. Biotransformation approaches and synthetic methods in tandem provide a source for generating compounds around core structures, which can be screened for various biological activity studies. These studies help in screening and advancing compounds through various stages of the drug discovery programme. Hence, biotransformation experiments can be effectively utilized to synthesize more compounds. Identification of the tentative structure of metabolites helps to design and synthesize new molecules similar to the parent compound. The synthesized metabolites can be a compound or material whose properties might be similar to those of the parent drug, and can serve as an ideal backup compound for parent drug in clinical trials (Ravindran et al. 2012).

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