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Biotransformations using plant cells, organ cultures and enzyme systems: current trends and future prospects

Archana Giri[a], Vikas Dhingra[a], C.C. Giri[b,1], Ajay Singh[c], Owen P. Ward[c], M. Lakshmi Narasu[a,*]

[a]*Centre for Biotechnology, Institute of PG Studies and Research, Jawaharlal Nehru Technological University, Mahaveer Marg, Hyderabad 500 028, India*

[b]*Centre for Plant Molecular Biology, Department of Genetics, Osmania University, Hyderabad 500 007, India*

[c]*Department of Biology, University of Waterloo, Waterloo, ON, Canada N2L 3G1*

Abstract

Plants are valuable sources of a variety of chemicals including drugs, flavours, pigments and agrochemicals. Some of the biochemical reactions occurring in plant cells are complex and cannot be achieved by synthetic routes. In vitro plant cell and organ cultures and plant enzymes act as suitable biocatalysts to perform these complex reactions. A wide variety of chemical compounds including aromatics, steroids, alkaloids, coumarins and terpenoids can undergo biotransformations using plant cells, organ cultures and enzymes. The biocatalyst-mediated reactions are regiospecific and stereospecific. Reaction types include oxidations, reductions, hydroxylations, methylations, acetylations, isomerizations, glycosylations and esterfications. Genetic manipulation approaches to biotransformation offer great potential to express heterologous genes and to clone and overexpress genes for key enzymes. Biotransformation efficiencies can further be improved using molecular techniques involving site-directed mutagenesis and gene manipulation for substrate specificity.

Keywords: Biotransformation; Biocatalysts; Plant cell cultures; Hairy root cultures; Plant enzymes; Immobilized cells and enzymes

* Corresponding author. Tel.: +91-40-339-1442; fax: +91-40-339-7648.

E-mail addresses: giricc@hotmail.com (C.C. Giri), mln28@usa.net (M.L. Narasu).

[1] Fax: +91-40-701-9020.

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1. Introduction

Biotransformations are chemical reactions catalyzed by cells, organs or enzymes. Biotransformations explore the unique properties of biocatalysts, namely their stereo-and regiospecificity and their ability to carry out reactions at nonextreme pH values and temperatures. Biotransformations may be used to carry out specific conversions of complex substrates using plant, animal or microbial cells or purified enzymes as catalyst. Biotransformations are different from biosynthesis where complex products are assembled from simple substrates by whole cells, organs or organisms. They are also different from biodegradations in which complex substances are broken down to simple ones. Biotransformations have great potential to generate novel products or to produce known products more efficiently.

Plant cell cultures exhibit a vast biochemical potential for production of specific secondary metabolites. Formation and accumulation of some important secondary metabolites do not occur in plant cell cultures. However, such cultures may retain an ability to transform exogenous substrates into products of interest. The chemical compounds, which can undergo biotransformations mediated by plant enzymes, are varied in nature (Franssen and Walton, 1999). They include aromatic, steroid, alkaloid, coumarin, terpenoid, lignan and other molecular species. It is not necessary for the compounds to be natural intermediates in plant metabolism. The substrates can also be of synthetic origin (Pras et al., 1995a). Plant cell cultures and enzymes have the potential to transform cheap and plentiful substances, such as industrial byproducts, into rare and expensive products. Plant bioconversion systems may be used alone to produce novel chemicals or in combination with organic synthesis. Multistep processes catalyzed by cell or organ culture often generate intermediary metabolites which help to establish biosynthetic pathways (Berlin et al., 1989).

Biotransformation capabilities of microorganisms and their enzymes for the production of a wide variety of fine chemicals are well known (Nikolova and Ward, 1993; Schulze and Wubbolts, 1999; Ward and Singh, 2000). Microbial systems are advantageous in that biomass doubling times are short and hence, production of biomass can be achieved quickly (Ward, 1991). In addition, methods for genetic manipulations of microbes are well established (Singh, 1999). Plant systems, on the other hand, produce a more limited range of enzymes and undifferentiated plant cells have longer doubling times than microbial cells. In addition, the desired enzymes are often produced in minute quantities. Despite these drawbacks, the plant kingdom contains some unique enzymes, which produce a variety of chemicals. Chemical synthesis of some of these compounds is extremely complicated and costly. Hence, biotransformations using plant cells and isolated enzymes have immense potential for production of pharmaceuticals, their disadvantages notwithstanding. Plant enzyme biocatalysts may be applied to the production of totally new drugs and also may be used to modify existing drugs by improving their bioactivity spectrum. Biological availability of pharmaceuticals can be enhanced by introduction of hydrophilic moieties in the substrate and the therapeutic action can be prolonged by introduction of protecting groups. Side effects of drugs may be reduced and drug stability may be increased by modification of the parent molecule. For example, podophyllotoxin and related lignans (starting compounds for the semisynthesis of antitumour drugs), catechols, including the anti-Parkinson drug L-DOPA,

and new dopaminergics can all be modified to improve bioactivity-related properties (Pras et al., 1995b).

2. Biotransformations using plant cells and organ cultures

The bioconversion rates by plant cells and organs will depend on a variety of factors including the solubility of precursors, the amount of enzyme activity present, localization of enzymes, presence of side reactions producing undesired byproduct and presence of enzymes degrading the desired product (Fowler and Stafford, 1992). Elicitation, permeabilization, pH variation and osmotic effects can also influence bioconversion capacity of cells (Xiong Tang and Suga, 1994). Dimethylsulphoxide and organic alcohols may be used for permeabilization to promote substrate uptake and product release (Berlin et al., 1989). Biotransformation reactions performed by plant cell and organ cultures are shown in Table 1.

Cyclodextrin-facilitated biotransformation of water-insoluble precursors has also been investigated. Some precursors are either insoluble or very poorly soluble in the aqueous phase, resulting in very low bioconversion rates. Cyclodextrins are cyclic oligosaccharides that are able to form inclusion complexes with a variety of apolar ligands (Szejtli, 1990). Since tolerance of plant cell cultures to organic phases is low (Woerdenbag et al., 1990), cyclodextrin-complexed precursors could be used to facilitate bioconversion of water-insoluble precursors in a more compatible aqueous environment (Van Uden et al., 1994).

Peganum harmala cell culture converted geranyl acetate to geraniol and linalyl acetate to linalool and α-terpineol (Zhu and Lockwood, 2000). Controlled-release polymer discs made from poly-2-hydroxyethyl methacrylate containing geranyl acetate or linalyl acetate produced higher concentrations of their biotransformation products.

All substances produced in plant cells are not necessarily the result of enzyme-catalyzed reactions. The alkaloid nitraramine, which contains seven stereogenic centers, is present in *Nitraria schokeri* as a racemate. Isolation of a chiral metabolite might be due to spontaneous nonenzymatic reactions starting from an achiral precursor followed by enzyme-catalyzed metabolism of one of the enantiomers (Wanner and Koomen, 1995).

Catharanthus roseus suspension cell cultures can oxidize the phenylsulphonyl group from completely synthetic molecules to phenylsulphonyl derivatives (Bourgogne et al., 1989; Dantas Barros et al., 1992). Incubation of dibenzylbutanolides with cell-free extracts of *C. roseus* yielded an enzyme-catalyzed oxidative coupling of these compounds to picropodophyllotoxin analogues (Kutney et al., 1992).

Podophyllotoxin, a precursor of the semisynthetic anticancer drug, is generally extracted from its source plants *Podophyllum hexandrum* and *Po. peltatum* (Broomhead et al., 1991; Giri and Lakshmi Narasu, 2000a). Kutney (1993) demonstrated that a cell line of *P. peltatum*, active in biosynthesis of podophyllotoxin, was able to maintain repeated biotransformation of butanolide to the podophyllotoxin analogue (50% yield) by oxidative coupling in a bioreactor for more than 15 cycles of 24 h. Biotransformation of dehydroabietic acid into isotriptophenolide by *Tripterygium wilfordii* has also been reported (Kutney et al., 1997). Cell suspension cultures of *Linum album* accumulate around 0.2% of the cell dry weight of podophyllotoxin and therefore may serve as an alternative source for this important aryltetralin lactone lignan

Table 1
Biotransformation reactions performed by in vitro plant cell and organ cultures

Plant	Precursor	Product	Reference
Acer nikoense	(*RS*)-Rhododendrol	(*RS*)-Rhododendrol 2-*O*-β-D-glucopyranoside and ®-rhododendrol 2-*O*-β-D-xylopyranosyl -(1β6)-β-D-glucopyranoside	Fujita et al. (1995)
Artemesia annua (cell cultures)	Artemisinic acid	Artemisinic acid β-D-glucopyranosyl ester, 9-β-hydroxyartemisinic acid β-D-glucopyranosyl ester and 3β-hydroxyartemisinic acid β-D-glucopyranosyl ester	Kawamoto et al. (1998)
Astasia longa	(*R*)- and (*S*)-Carvone	Dihydrocarvone and isodihydrocarveol	Shimoda and Hirata (2000)
Capsicum frutescens (immobilized cell cultures)	Ferulic acid and vanillylamine	Capsaicin, vanillin	Johnson et al. (1996)
C. frutescens (immobilized cell cultures)	Protocatechuic aldehyde and Caffeic acid	Vanillin, capsaicin	Ramachandra Rao and Ravishankar (2000)
Catharanthus roseus (cell suspension cultures)	Vinblastine	Vincristine	Hamada and Nakazawa (1991)
C. roseus (cell suspension cultures)	Tabersonine	Lochnerinine, lochnericine	Furuya et al. (1992)
C. roseus (cell suspension cultures)	Hydroquinone	Arbutin	Inomata et al. (1991)
C. roseus (cell suspension cultures)	Glychyrrhizin	Glycyrrhetinic acid	Hamada and Nakata (1992)
C. roseus (cell suspension cultures)	Geraniol, nerol, (+) and (−) carvone	5β-hydroxyneo-dihydroxycarveol	Hamada et al. (1997)
C. roseus (immobilized cell cultures)	4-Pyridyl-1-ethanol	(*R*)-4-pyridyl -1-ethanol	Takemoto and Achiwa (1998)
C. roseus (hairy root cultures)	2,4,6-Trinitrotoluene (TNT)	Aminodinitrotoluens (ADNTs)	Hughes et al. (1997)
Centella asiatica	Thiocolchicine	2-*O*- and 3-*O*-monoglucosyl derivatives	Solet et al. (1993)
C. asiatica (suspension cultures)	3-Demethylthio-colchicine	Thiocolchicinoside	Bouhouche et al. (1998)
Coffea arabica	Theobromine	Caffeine	Furuya et al. (1991)
C. arabica	Vanillin	Vanillin-β-D-glucoside	Kometani et al. (1993)
Crocus sativus (cell culture)	Crocetin	Crocetin di-neapolitanosyl	Dufresne et al. (1999)
Curcuma zedoaria (suspension cultures)	Germacrone	Guaiane type sesquiterpene	Sakui et al. (1992)
Datura innoxia (cell suspension culture)	2,4,6-Trinitrotoluene (TNT)	Aminodinitrotoluens (ADNTs)	Lucero et al. (1999)
Daucus carota (immobi-lized plant cells)	Codeinone	Codeine	Jones and Veliky (1981)

Table 1 (*continued*)

Plant	Precursor	Product	Reference
D. carota, *N. tabacum* and *Gardenia jasminoides* (Immobilized cell cultures)	Acetophenone	(*R*)- and (*S*)-β-Phenethyl alcohol	Akakabe and Naoshima (1994)
D. lanata (immobilized plant cells)	β-Methyldigitoxin	β-Methyldigoxin	Alfermann et al. (1980)
D. lanata (shoot cultures)	Deacetyllanatoside	21′-Di-dehydro-deacetyl-lanatoside C	Rhenius et al. (1997)
Eucalyptus perriniana (cell suspension cultures)	Isoeugenol and eugenol	Eugenyl β-rutinoside, isoeugenyl β-gentiobioside	Orihara et al. (1992)
E. perriniana (suspension cultures)	(+)- and (−)-Fenchone	(1*R*,4*R*,5*S*)-5 hydroxy fenchone-2-one 5-*O*-β-D-glucopyranoside and 5-*O*-β-gentiobioside	Orihara and Furuya (1994)
E. perriniana (suspension cultures)	(+)-Camphor	(1*S*,4*R*,6*S*)-6 Hydroxy bornan-2-one 6-*O*-β-D-glucopyranoside	Orihara et al. (1994)
E. perriniana (suspension cultures)	Caryophyllene oxide	(1*R*,3*Z*,5*R*,8*S*,9*S*)-Caryophylla-3-ene-5,14-diol-*O*-β-gentobioside	Orihara et al. (1994)
E. perriniana (suspension cultures)	p-Aminobenzoic acid	*p*-Aminobenzoyl β-D-glucopyranoside, *p*-(*N*-β-D-glucopyranol amino) benzoy β-D-glucopyranoside	Furuya et al. (1998)
E. perriniana (cell suspension culture)	(−)-Borneol	(−)-Borneol 2-*O*-β-gentiobioside, (−)-borneol 2-*O*-β-sophoroside	Orihara and Furuya (1993)
Glycyrrhiza glabra (cell suspension culture)	Papaverine	Papaverinol	Dorisse et al. (1988)
G. glabra (cell suspensions)	18β-glycyrrhetinic acid	3-*O*-β-D-glucuronopyranosyl 24-hydroxy-18β-glycyrrhetinic acid	Hayashi et al. (1992)
Heteroscyphus planus (cultured cells)	Cubenene	7-Hydroxycalamene	Hashimoto et al. (1999)
Ilex paraguariensis (cell suspension culture)	Ethanol, methanol	1-*O*-Ethyl-β-glucopyranoside, 1-*O*-methyl-β-glucopyranoside	Kraemer et al. (1999)
Lobelia sessilifolia (hairy root cultures)	(−)-Epicatechin or protocatechuic acid	(−)-Epiafzelechin 7-*O*-β-D-glucopyranoside, protocatechuic acid 3-*O*-β-D-glucopyranoside	Yamanaka et al. (1995)
Linum flavum (cell suspension cultures)	Deoxypodophyllotoxin	5-Methoxypodophyllotoxin β-D-glucoside	Van Uden et al. (1997)
Lonicera japonica (suspension cultures)	Cardione	(2*S*)-2-Hydroxy curdione, (2*R*)-2-hydroxy curdione, (8*S*)-6-hydroxycurdione,	Horiike et al. (1997)

(continued on next page)

Table 1 (*continued*)

Plant	Precursor	Product	Reference
		(2*R*,8*S*)-8-hydro-2-hydroxy curdione, (1*S*,10*S*)-1, 10-epoxycurdione	
L. japonica, *Bupleurum falcatum*, *Polygonum tinctorium* and *Solidago altissima* (cell suspension cultures)	Germecrone	Guaiane, eudesmane and secoguaiane	Sakamoto et al. (1994)
L. japonica (cell suspension cultures)	Loganin and 7-deoxyloganin	Secologanin	Yamamoto et al. (1999)
Mentha sp. (immobilized cells)	(−)-Menthone	(+)-Neomenthol	Galun et al. (1985)
Mucuna pruriens (immobilized cells)	L-Tyrosine	L-DOPA	Wichers et al. (1983)
Myriophyllum sp.	2,4,6-Trinitrotoluene (TNT)	Aminodinitrotoluens (ADNTs)	Hughes et al. (1997)
Myrtillocatus geometricus (callus tissues)	Δ_2-Carene	Carenols, arenols	Gil et al. (1995)
Nicotiana plumbaginifolia (cell suspension cultures)	Butyric acid	6-*O*-Butyryl-D-glucose	Kamel et al. (1992)
N. tabacum (immobilized cells)	α- and β-Ionones	Corresponding saturated ketones and alcohols	Xiong Tang and Suga (1994)
N. tabacum and *C. roseus*	3-Carene (1*S*,5*R*)-2-Pinene	(1*S*,3*S*,4*R*,6*R*)-3,4-epoxycarene (1*S*,2*S*,3*R*,5*S*)-2,3-epoxy pinane	Hirata et al. (1994)
Peganum harmala (cell suspension culture)	Geranyl acetate, linalyl acetate	Geraniol, linalool, α-terpineol	Zhu and Lockwood (2000)
P. harmala (cell suspension culture)	Benzyl acetate, neryl acetate *m*-, *p*- and *o*-anisaldehyde, β-ionone, α-pinene, β-pinene, furfural, menthone, citronellal	Benzyl alcohol, nerol, geraniol *m*-, *p*- and *o*-anisyl alcohol β-ionol, 7-hydro-β-ionone verbenol, verbenone myrtenol, myrtenal furfuryl alcohol menthol, piperitol citronellol	Zhu et al. (2000)
Panax ginseng (cell suspension cultures)	Digitoxigenin	Digitoxigenin β-D-glucoside malonyl ester	Kawaguchi et al. (1996)
Papaver somniferum (immobilized cells)	Codeinone	Codeine	Furuya et al. (1984), Corchete and Yeoman (1989)
P. somniferum (immobilized cells)	Silybin	Silybin-7-*O*-β-D-glucopyranoside	Keran et al. (1998)
P. somniferum and *Mahonia* (cell suspension cultures)	Thebaine	($N_{14}CH_3$)-Thebaine	Wilhelm and Zenk (1997)
Pinus radiata (cell suspension culture)	6-*n*-pentyl-2*H*-pyran-2-one	5-(2-pyron-6-yl)pentan-5-ol	Cooney et al. (2000)

Table 1 (*continued*)

Plant	Precursor	Product	Reference
Rauwolfia serpentina (cell suspensions)	Hydroquinone	Arbutin	Lutterbach and Stockight (1992)
Rhodiola rosea (cell culture)	*Trans*-cinnamyl alcohol	Rosavin	Furmanowa et al. (1999)
Solanum amosum (suspension cultures)	o- and *p*-Amino benzoic acid and *N*-acetyl *p*-aminobenzoic acid	*p*-Amino benzoic acid, *N*-acetyl *P*-aminobenzoic acid, 7-*O*-β-D-glucopyranosyl ester	Syahrani et al. (1999)
S. aviculare and *Dioscorea deltoidea* (cell cultures, immobilized cells)	(*S*)-(−)- and (*R*)-(+)-Limonene	*Cis*- and *trans*-carveol carvone	Vanek et al. (1999a,b)
Spirodela punctata (cell suspension culture)	3-Alkyl substituted derivatives of citronellol and citronellic acid	Derivatives with a hydroxy group on C-6 or C-7 and the double bond at C-7, C-8 and C-5	Pawlowicz et al. (1992)
Strophanthus hybrid cells	Digitoxigenin	17β H-periplogenic β-D-glucoside	Kawaguchi et al. (1998)

(Smollny et al., 1998; Empt et al., 2000). In dark and light grown cultures, maximal product yields of lignans up to 0.2% and 0.5% of the dry weight respectively, were achieved, mainly consisting of podophyllotoxin (Smollny et al., 1998). Callus cultures of *Juniperus chinensis* produced low amounts (0.005% of dry weight) of podophyllotoxin (Muranaka et al., 1998). The production of podophyllotoxin could be increased by 11-fold and 15-fold by addition of phenylalanine, a biogenic precursor of podophyllotoxin and chito-oligosaccharides, an elicitor to calli, respectively.

McLaughlin Gromley (Minnesota, USA) patented a process for conversion of mevalonic acid or isopentenyl pyrophosphate to pyrethrins using cell-free homogenates of *Chrysanthemum* and *Tagetes* species and cofactors (Jovetic and De Gooijer, 1995). By hydrolyzing chrysanthemyl alcohol phosphate, the process can also be used to produce chrysanthemyl alcohol (Hitmi et al., 2000).

Biotransformation by cell cultures and hairy root cultures serves as an important tool in the structural modification of compounds possessing useful therapeutic activity. However, a major drawback of suspension cultures is the phenomenon of somaclonal variation, which may lead to unstable biochemical behaviour (Casas et al., 1998). Since variation and instability are the main problems associated with cell cultures, continuous screening is required to maintain highly productive lines (Doran, 1997). This problem can also be circumvented by the use of organized tissues such as shoot and root cultures. Hairy roots, obtained by transformation of plant cells with the bacterial soil pathogen *Agrobacterium rhizogenes*, grow at high rates as compared to plant cell suspension cultures. Differentiation, which enhances genetic stability, is the advantage in organ cultures and can be produced by genetic transformation (Giri and Lakshmi Narasu, 2000b). Cells can also be genetically engineered or genes encoding for relevant enzymes can be introduced into the host cells.

Hairy root cultures of *Brugmansia candida* were investigated for the biotransformation of hydroquinone into arbutin (Casas et al., 1998). Sugars (sucrose, glucose, mannitol and sorbitol) at a concentration of 30–120 g/L enhanced bioconversion yields. *Panax ginseng* hairy root cultures biotransformed 18-β-glycyrrhetinic acid to 30-*O*-β-D-glucopyranosyl-(1–2)-β-D-glucopyranosyl 18-β-glycyrrhetinic acid (Asada et al., 1993).

2.1. Pathway biotransformation

Pathway biotransformations exploit a characteristic biosynthetic pathway of the plant or use a natural intermediate of the normal biosynthetic pathway. While there are many examples of pathway biotransformations, only a few examples are mentioned here.

Kreis and Reinhard (1990) studied the biotransformation of digitoxin and digitoxigenin in cultures of *Digitalis purpurea*. *Digitalis* sp. produces digitoxin and its 12-hydroxy derivative digoxin, both of which are important cardiovascular drugs. Digoxin is more important for pharmaceutical applications, but it is present in plants in lower concentrations than digitoxin. Approximately 80% of the digoxin leaches into the medium. Digitoxin 12β-hydroxylase, a cytochrome *P*450 monoxygenase, plays a vital role in this biotransformation by *Digitalis.* Digitoxigenin was converted to digitoxigen-3-one, 3-epidigitoxigenin and digoxigenin by *D. lantana* shoot cultures. In addition, various cardiac glycosides were formed including monoglycosides with glucose, glucomethylose, fucose and digitalose (Theurer et al., 1998).

Furuya et al. (1984) studied biotransformation of benzylisoquinoline alkaloids by cell cultures of *Papaver somniferum.* In *Rauwolfia serpentina* cell suspension cultures, biotransformation was accomplished by borohydride reduction of ajmaline to dihydrochanoajmaline followed by a flavin-mediated photooxidation to raumcline (Obitz et al., 1995). Peroxidase-catalyzed reactions, particularly radical coupling reactions, are prominent in secondary product biosynthesis, e.g. in formation of lignins, lignans and suberin. These reactions can be utilized to perform in vivo biotransformations of biotechnological interest.

2.2. Nonspecific biotransformations

Plant cell cultures have the capacity to biochemically transform exogenously added foreign substrates. Biocatalyst types and substrates are chosen to exploit the desired regio-, stereo- and enantioselectivity of the reaction as well as substrate specificity. The reaction type and stereochemistry depend upon functional groups in the substrate and the structural moieties in the vicinity of functional group. Therefore, biotransformations by plant cell cultures are considered to serve as important tools for structural modification of molecules to produce compounds possessing useful properties. Some of the more important biotransformation types are described in the following sections.

2.3. Hydroxylations

Plant cell cultures may be used to transform exogenous substrates by introduction of oxygenated functions regio- and stereoselectively at various positions in the molecules. The

cells possess the ability to hydroxylate with regio- and stereospecificity the C–C double bonds in the allylic position. The cells can also differentiate between different enantiomers of substrates and hydroxylating one enantiomer selectively (Suga and Hirata, 1990).

Hydroxylation of warferin to the corresponding alcohol by *C. roseus* has been reported (Hamada et al., 1993). Cell suspension cultures of *C. roseus* hydroxylated geraniol, nerol, (+) and (−) carvone to 5β-hydroxyneodihydroxycarveol (Hamada et al., 1997). *Pinus radiata* cell cultures transformed the antifungal metabolite 6-*n*-pentyl-2*H*-pyran-2-one via hydroxylation of the pentyl side chain into a series of monohydroxylated isomers (Cooney et al., 2000).

2.4. Glucosylation

Glucosylation reactions are of special interest because they facilitate the conversion of water-insoluble compounds to water-soluble compounds. Plant cell cultures play an important role in this regard since it is difficult to perform this reaction by microorganisms or by chemical synthesis. Plant cell cultures are capable of glucosylation of a variety of exogenously added compounds, e.g. phenols, phenylpropanoid acid and their analogues.

Butyric acid is a potent inhibitor of tumor cell proliferation in vitro and it is effective against acute leukemia. However, its application is limited because it has a short half-life in mammalian systems. Glucosylation of butyric acid has been achieved by cell suspension cultures of *Nicotiana plumbaginifolia* to obtain 6-*O*-butyryl-D-glucose, which extends its half-life and prolongs its bioactivity (Kamel et al., 1992). Ushiyama et al. (1989) studied glucosylation of phenylcarboxylic acids by cells of *Glycyrrhiza echinata*, *Aconitum japonicum*, *Dioscoreophyllum cumminssi* and *N. tabacum*. Van Uden et al. (1993a,b) reported glucosylation of podophyllotoxin by *L. flavum* cells.

Biotransformation of tyrosol by *Rhodiola sachalinensis* cell cultures into salidroside was studied by Xu et al. (1998). The glucosylation reaction in *R. sachalinensis* is catalyzed by tyrosol glucosyltransferase. Addition of 1 mmol/L tyrosol to the suspension culture converted 95% of the tyrosol to salidroside in 24 h. By repeated addition of 3 mmol/L tyrosol at 24-h intervals over 72 h, a high salidroside yield of 516 μmol/g was obtained.

Crocus sativus cell suspension culture converted crocetin into several glycosyl esters when the culture was fed with the encapsulated substrates (Dufresne et al., 1999). The major pigment identified was crocetin di-neapolitanosyl ester. The other pigments were mixed forms of neapolitanosyl, gentiobiosyl and glucosyl esters. A method of producing the biologically active compound rosavin and other cinnamoylglycosides via glucosylation of *trans*-cinnamyl alcohol by *R. rosea* (roseroot) cell cultures has been reported (Furmanowa et al., 1999).

2.5. Oxido-reductions between alcohols and ketones

Alcohols may be converted to the corresponding ketones by the plant cell cultures. Some enantioselective oxidations that are carried out by cultured cells are useful for the preparation of chiral compounds. Cell cultures of *N. tabacum* converted mono and bicyclic monoterpene alcohols enantioselectively. The cultured cells discriminated between enantiomers of

methane-2-ol, bicyclic heptan-2-ol and bicyclo heptane-3-ol derivatives and oxidized hydroxyl group enantioselectively.

Gil et al. (1995) studied biotransformation of Δ_2-carene by callus cultures of *Myrtillocactus geomtrizans* and *N. tabacum*. Both the cultures biotransformed Δ_2-carene into diastereomeric alcohols and the cultures of *Myrtillocactus* oxidized these alcohols to the corresponding ketones.

2.6. Hydrolysis

Enantioselective hydrolysis is useful for the optical resolution of racemic acetates and has been observed in biotransformation of (*RS*)-1-phenylethyl acetate and its derivatives with cultured cells of *Spirodela oligorrhiza*, in which biotransformation gave (*R*)-alcohols (Pawlowicz and Siewinski, 1987). The hydrolysis of acetates such as (±)-1-phenylethyl ((±)-1), (±)-1-(1-naphthyl)ethyl ((±)-2), (±)-1-(2-naphthyl)ethyl ((±)-3) and (±)-menthyl ((±)-4) using potato and artichoke tubers for the synthesis of alcohols was investigated (Mironowicz, 1998).

2.7. Epoxidation

Epoxidation is very useful for the modification of cytotoxic sesquiterpenes. Sakui et al. (1992) described epoxidation of germacrone by cell suspension cultures of *Curcuma zedoaria*. The biotransformation of (−)-(4*R*)-isopiperitinone by *Mentha piperita* cell suspension culture yielded three hydroxylated derivatives and two epoxidized derivatives including (−)-7-hydroxyisopiperitonone and its glucosides (Park and Kim, 1998). Epoxidation of fatty acids with oat (*Avena sativa*) peroxygenase, immobilized on synthetic membranes, was evaluated in aqueous and heptane media (Piazza et al., 2000). Oleic acid was a preferred substrate compared to its *trans* analogue, elaidic acid.

2.8. Reductions of carbonyl groups

There are many reports of the reduction of ketones and aldehydes to the corresponding alcohols with plant cell cultures. The hydrogen attack in the reduction takes place preferentially from the re-face of the carbonyl group to give the hydroxy compounds with the *S*-chirality at the position bearing the hydroxyl group. Whole cells, cell-free extracts or culture broth from cell suspension cultures of *N. sylvestris* or *C. roseus* can implement reactions of this type. This was attributed to extracellular secretion of peroxidases into the culture medium. Under appropriate conditions, an 87% conversion of substrate was achieved in a 40-min reaction period (Botta et al., 1996).

2.9. Reduction of C–C double bond

Cultured cell lines of *Astasia longa* produced two different enone reductases, which reduced the C–C double bond of carvone (Shimoda and Hirata, 2000). The enzymes have been isolated and the stereochemistry of the enone reduction reactions has been characterized.

2.10. Nitroreduction

Uptake and nitroreduction of 2,4,6-trinitrotoluene (TNT) has been demonstrated in plant cell and organ cultures. Biotransformation of TNT into 2,4,6-aminodinitrotoluene (ADNT) has been investigated. Plant cell cultures of *Datura innoxia*, *C. roseus* and *Myrophyllum* plants transformed TNT into ADNT via nitroreduction (Hughes et al., 1997; Lucero et al., 1999).

3. Biotransformations using immobilized cell culture

Whole cells offer the opportunity to implement multistep biotransformations and to utilize and recycle essential cofactors and co-enzymes. Isolated enzymes may be sensitive to denaturing conditions including pH extremes, heat and specific organic solvents. In order to be useful in biotransformation reactions, biocatalysts need to be stable and reusable. Use of whole cell immobilized system may help overcome some stability problems. Immobilized plant cells have some additional advantages over freely suspended cells. They are more resistant to shear damage and can be used repeatedly over a prolonged period (Panda et al., 1989). Whole cell immobilization may also create nongrowth conditions under which production of secondary metabolites may be improved (Rosevear and Lambe, 1985). Immobilization or entrapment of cells may produce a microenvironment that resembles the organized tissue in the intact plant, causing differentiation and production of secondary metabolites (Williams and Mavituna, 1992).

General methods for immobilization of plant cells are gel entrapment by ion exchange, precipitation, polymerization and in preformed structures (Hulst and Tramper, 1989). For adsorption of plant cells, solid surfaces can be used. Enzymes may be adsorbed to insoluble supports by hydrogen bonding, dipole–dipole interactions and hydrophobic interactions. Commonly used supports are polypropene (e.g. Acceurel TM) and diatomaceous earth (Celite). When the optimum pH of an enzyme is not close to its isoelectric point, enzymes may be immobilized by ion exchange. Enzymes may also be linked covalently to a solid support to overcome leaching. Polyacrylamide is a commonly used matrix for enzyme immobilization. While a high degree of cross-linking prevents leakage and loss of the biocatalyst diffusion, problems may arise with larger substrates. Microencapsulation, forming a microsphere of polymeric membranes around the enzyme in solution, is another elegant method for immobilization of enzymes.

Increased biotransformation yields of capsaicin and dihydrocapsaicin, major pungent principles of chilli pepper fruit, were obtained when immobilized placental tissues of *Capsicum frutescens* were fed with intermediate metabolites of the capsaicinoid pathway, i.e. L-phenylalanine, *p*-coumaric acid, cinnamic acid, caffeic acid, ferulic acid and vanillylamine in combination with L-valine (Johnson and Ravishankar, 1998). A productivity of 3.07 mg capsaicin/g dry weight/day was obtained with a precursor combination of *p*-coumaric acid and L-valine.

Ramachandra Rao and Ravishankar (2000) used freely suspended and immobilized cells of *C. frutescens* Mill for the conversion of protocatechuic aldehyde and caffeic acids to vanillin

and capsaicin. The increase in vanillin accumulation was well correlated with an increase in specific activity of caffeic acid *O*-methyltransferase in protocatechuic aldehyde and *S*-adenosyl-L-methionine-treated immobilized *C. frutescens* cell culture. Biotransformation of isoeugenol to vanillin flavour metabolites and capsaicin was more effective in immobilized rather than free cells and this activity was further enhanced by β-cyclodextrin and fungal elicitor (Ramachandra Rao and Ravishankar, 1999).

Vanek et al. (1999a) examined the course of biotransformation of *S*-(−)-limonene into *cis*- and *trans*-carveol and carvone by *Solanum aviculare* and *Dioscora deltoidea* immobilized by entrapment in alginate, carragenan, pectate gels, polyurethane foam and bound to polyphenyleneoxide. Ratio of products was influenced by the immobilization method. Increase of the contact area for mass transfer was an important factor in utilizing an immobilized system to enhance digoxin production by *D. lanata* cell cultures (Hong et al., 1998).

Enzyme and cell biotransformations may also be implemented in membrane reactors. The membrane retains the biocatalyst while allowing substrates, nutrients and products to pass through freely. In comparison to gels, there is better control of fluid dynamics, flow distribution and easier scale up. The membrane system can also facilitate maintenance of sterility of the reactor (Novais, 1988).

4. Genetic engineering approaches towards biotransformation

Bioconversion capacity of cell cultures can be further optimized by cell selection, elicitation, permeabilization, radiation, pH and osmotic shock. A more fundamental approach is the transfer of genes that code for the key enzymes catalyzing the desired biosynthetic reactions into a fungal or bacterial cell because of their ability to produce high amounts of enzymes (Pras et al., 1995b).

Hashimoto et al. (1993a) reported expression of hyoscyamine 6-β-hydroxylase in *Escherichia coli*. This recombinant bacterium was able to convert hyoscyamine to scopolamine. Subsequently, this cloned gene has been transferred to the plant, *Atropa belladonna* and expressed constitutively. Further, transformed hairy roots with increased efficiency of conversion of hyoscyamine to scopolamine have been reported by Hashimoto et al. (1993b). Cloning and expression of bacterial lysine decarboxylase under the control of a 35S promoter fused to the coding sequences of the small subunit of rubisco transit peptide in tobacco root cultures was found to affect two secondary metabolic pathways (Berlin et al., 1998).

Biotransformations using transformed roots of *Nicotiana* sp. have been investigated. When cadaverine (1,5-diaminopentane) was fed to its hairy roots, the formation of nicotine, the usual alkaloid derived from putresine (1,4-diaminobutane), was inhibited and minor alkaloid anabasine formation was markedly stimulated (Walton et al., 1988). This is because of the competition between cadaverine and *N*-methyl putrescine and their metabolites as substrates for the nicotine biosynthetic pathway. Fecker et al. (1993) reported that cadaverine is a product of the decarboxylation of lysine and that its production could be engineered by the introduction and expression of a heterologous gene for lysine decarboxylase (*ldc*).

The *ldc* gene from the bacterium *Hafnia alvei*, expressed in *Nicotiana* hairy root cultures, exhibited a 10-fold increase in cadaverine production and a threefold increase in anabasine.

Herminghaus et al. (1996) reported further enhancement in the expression of the introduced gene by directing the expressed protein to the leucoplast where lysine is synthesized. In this way, the supply of a severely rate-limiting precursor was increased in order to express a latent biosynthetic pathway. Ichinose et al. (1999) reported introduction of a cDNA encoding furostanol glycoside 26-*O*-β-glucosidase of *Costus speciosus* in *N. tabacum* via *A. tumafaciens*-mediated transformation and reported furostanol glycoside 26-*O*-β-glucosidase activity in the transgenic plant.

5. Biotransformations using plant enzymes

A large number of reports on enzymes isolated from plant cell cultures used in bioconversions are depicted in Table 2. While enzyme preparation seems to be most suitable for economical production of pharmaceuticals, enzyme applicability depends upon the balance between activity losses during the isolation procedure and superiority of the bioconversion efficiency of the resulting preparation when compared with cell systems (Pras et al., 1995a). Regioselective hydroxylations and glycosylations offer the best opportunities for the production of improved drugs.

Some examples of important reactions with isolated plant enzymes in free or immobilized state are described in the following sections.

5.1. Papain

High concentrations of the thiol-protease papain are found in the latex of leaves and green fruit of *Carica papaya* (Azarkan et al., 1997). Papain hydrolyzes peptide bonds and, in some cases, ester linkages are also cleaved. This proteolytic enzyme can catalyze both forward and backward reactions. The direction of reaction can be manipulated by changing the water concentration, thereby altering the equilibrium of the reaction. Papain may be used in regioselective hydrolytic reactions. For example, papain regioselectively hydrolyzes dehydroglutamate diester at the 5-ester position only, whereas α-chymotrypsin hydrolyzes only the 1-ester position (Faber, 2000). Papain has specificity for peptide bonds involving Phe, Val or Leu and an unspecified amino acid X (Faber, 2000).

5.2. Oxynitrilases

Oxynitrilases or hydroxynitrile lyases are stereoselective enzymes and produce only one enantiomer (Klempier et al., 1993). They catalyze addition of hydrogen cyanide to aldehydes and have been used for synthesis of chiral cyanohydrins, which are versatile synthetic precursors of α-hydroxy acids and aldehydes, ethanolamines, amino alcohols, pyrethroid insecticides, imidazoles and heterocycles (Wieser and Nagasawa, 2000). On the basis of their enantioselectivity, these enzymes can be separated into (*R*)- and (*S*)-oxynitrilases catalyzing the general reaction indicated in Fig. 1.

The (*R*)-enzyme is found in rosaceae especially in bitter almonds (*Prunus amygdalus*), but also in *Phlebodium aureum* and *L. usitatissimum* (Brussee et al., 1990). The *Prunus* enzyme

Table 2
Biotransformation reactions performed by enzymes isolated from in vitro plant cell cultures

Plant	Precursor	Product	Enzyme	Reference
Artemisea annua	Arteannuin B	Artemisinin	Oxidoreductase	Dhingra et al. (2000)
Catharanthus roseus (immobilized enzyme from suspension cultures)	Catharanthine and vindoline	3′,4′-Anhydrovinblastine (AVLB) and leurosine	Strictosidine synthase	Kutney et al. (1988)
C. roseus (cell cultures)	Catharanthine and vindoline	Anhydrovinblastine	Peroxidase	Smith et al. (1988)
C. roseus (cell suspension cultures)	AMP	Guanine nucleotides	AMP deaminase	Yakuki and Ashihara (1992)
C. roseus (cell supension cultures)	5-Phosphomevalonate	5-diphosphomevalonate	Phosphomevalonate kinase	Schulte et al. (1999)
Cinchona robusta (suspension cultures)	Secologanin and tryptamine	Stereospecific condensation	Strictosidine synthase	Stevens et al. (1993)
C. robusta, *Morinda citrifolia*, *Rubia inctorum*, *Tabernaemontana divaricata, C. roseus* (cell cultures)	Isopentanyl diphosphate	Dimethyl allyl diphosphate	Isopentanyl diphosphate isomerase	Ramos-Valdivia et al. (1998)
Coleus blumei	Dihydroxyphenyl lactate	Rosmarinic acid	Hydroxyphenyl-pyruvate and rosamarinic acid synthase	Petersen and Alfermann (1988)
Digitalis lanata	β-Methyldigitoxin	β-Methyldigoxin	Digitoxin 12β-hydroxylase	Petersen et al. (1987)
Glycyrrhiza glabra (cell suspensions)	18β-glycyrrhetinic acid	3-*O*-Glycoside	18β-Glycyrrhetinic acid 24-hydroxylase	Hayashi et al. (1990)
Hyoscyamus niger	Hyoscyamine	6β-Hydroxy-hyoscyamine	Hyoscyamine 6β-hydroxylase	Yamada and Hashimoto (1989)
Medicago sativa (suspension cultured cells)	Quercetine	Quercetine-3-*O*-glucoside	*O*-Glucosyl-transferase	Parry and Edwards (1994)
Mucuna pruiens	Monophenols	Catechols	Phenoloxidase	Pras et al. (1990a)
Solanum khasianum (cell cultures)	Acetovanillone	β-hydroxy acetovanillone	Cytochrome *P*450	Muhlenbeck and Barz (1997)
Papaver somniferum (cell culture and differentiated plants)	Salutaridine	(7*S*)-Salutaridinol	Salutaridine NADPH 7-oxidoreductase	Gerardy and Zenk (1993b)
P. somniferum (cell suspension culture)	(*R*)-Reticuline	Salutaridine	Cytochrome *P*450 monooxygenase	Gerardy and Zenk (1993a)
P. somniferum (cell culture and differentiated plants)	Salutaridine	(7*S*)-Salutaridinol	Salutaridine NADPH 7-oxidoreductase	Gerardy and Zenk (1993b)
Rauwolfia serpentina (suspension cultures)	Raucaffricine	Vomilenine	Raucaffricine-*O*-β-D-glucosidase	Warzecha et al. (1999)
Taxus chinensis (cell suspension cultures)	Taxanes possessing an unsubstituted 10-hydroxyl group	2β,5β,10β,14β Tetraacetoxy-4 (20), 11-taxadiene	10-Hydroxytaxane *O*-acetyltransferase	Menhard and Zenk (1999)

Table 2 (*continued*)

Plant	Precursor	Product	Enzyme	Reference
T. cuspidata (cell suspension cultures)	10-Deacetylbaccatin III	Baccatin III	Acetyl Co A:10-Deacetylbaccatin-III-10-*O*-acetyltransferase	Pennington et al. (1998)
Zea mays (cultured cells)	Pyruvate or acetaldehyde	Acetoin (3-hydroxy-2-butanone)	Pyruvate carboligase	Forlani (1999)

can accept aldehyde substrate where R = Ph, 3,4-(CH_2O_2)Ph, *n*-C_3H_7, $CH_3CH{=}CH$ and *c*-$C6H_{11}$, producing products in both high enantiomeric excess (ee) and yields. The (*S*)-enzyme is produced from millet (*Sorghum bicolor*), gum tree (*Hevea brasiliensis*) and *Manihot esculenta*. The *Manihot* enzyme, cloned in *E. coli*, has been used to synthesize a wide range of optically active α-hydroxynitriles in diisopropyl ether organic reaction media (Wajant and Effenberger, 1996). (*S*)-Cyanohydrins with different R substituents have been synthesized with ee of 80–100% using enzyme from *Hevea*, *Sorghum* and *Manihot* as illustrated in Table 3.

Hydroxynitrite lyases operate in plants to release HCN (cyanogenesis) as a defense against herbivores (Stump and Conn, 1981). Natural substrates for cyanogenesis are (*R*)-amygdalin (almond, apple, cherry), (*S*)-dhurrin (millet), linamarin, (*S*)- or (*R*)-lotaustralin (maniok, rubber tree, flax) and prunasin (almond, cherry, apple). Synthetic applications of the enzyme were developed when it was discovered that the hydrolytic conversion is suppressed by organic solvents immiscible in water (Effenberger, 2000).

5.3. Cyclases

Cyclases have broad substrate specificity. They can be used for the smooth cyclization of cyclic dienes and their epoxides (Piet et al., 1995, 1996). A cyclase was detected in chicory which selectively cyclizes the germacrane derivative germanone 4,5-epoxide into neoprocureumenol. The first step is enzyme-mediated protonation of epoxide group followed by ring closure leading to carbonation.

5.4. Phenoloxidases

Phenoloxidases catalyze hydroxylation of monophenols to catechols with regiospecificity. Bioconversions of *para*-substituted (Pras et al., 1988) and bi- and tricyclic mono-

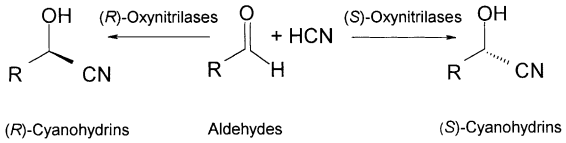

Fig. 1. (*R*)- and (*S*)-hydroxynitrile lyase catalyzed synthesis of (*R*)- and (*S*)-cyanohydrins.

Table 3
Synthesis of (*S*)-cyanohydrins by (*S*)-hydroxynitrile lyases

	Enzyme source		
R =	*Hevea*	Sorghum	Manihot
Ph	√	√	√
3-C_6H_5O-Ph	√	√	
3-OH-Ph		√	
3-Thienyl	√	√	√
$(CH_3)_2CH$	√		
n-C_3H_7	√		√
i-C_3H_7			√
c-C6H_{11}	√		√

Ph = phenyl.

phenols (Pras et al., 1990a) have been investigated using alginate-entrapped cells of *Mucuna pruriens* and partially purified phenoloxidase isolated from *Mucuna*. Phenoloxidases were isolated from suspension cultures of *M. pruriens*. Continuous production of an important pharmaceutical compound, 7,8-dihydroxy-*N*-di-*n*-propyl-2-aminotetralin, using a phenoloxidase from cell cultures of *M. pruriens* has also been studied by Pras et al. (1990b).

5.5. Haloperoxidases

Haloperoxidases catalyze halogenation of a variety of organic compounds using hydrogen peroxide and halide ions as substrates (Maranon and Van Huystee, 1994). Haloperoxidases are known from various sources including mammals, birds, plants, algae, fungi and bacteria. The enzymes are involved in the biosynthesis of a variety of halogenated natural products (from chlorobromomethane to chiral halogenated hydrocarbons). They are abundant in higher plants where they generally perform one electron oxidation leading to dehydrogenated products or oligomers.

The stereochemistry of the chloroperoxidase-catalyzed epoxidation of indene has been elucidated (Manoj et al., 2000). In aqueous solutions, the epoxide products were unstable and formed *cis–trans* diols. The reactions carried out in the absence of water produced 1*R*,2*S* enantiomers in approximately 30% ee.

An acidic peroxidase, produced by cell suspension culture of *Cassia didymobotrya*, was purified from the culture medium and studied for the biotransformation reactions. The enzyme catalyzed the conversion of 4,3′,4′-trihydroxychalcone and 4,3′,4′-trihydroxy-3-methoxychalcone to the corresponding 3,3′-biflavanones, as mixtures of racemic and *meso* forms (Vitali et al., 1998).

5.6. Lipoxygenases

Lipoxygenase is a nonheme iron-containing enzyme which catalyzes the incorporation of dioxygen into suitable unsaturated substrates. It is a very stereoselective and regioselective enzyme (Deoliveira et al., 1998). Examples of the soybean lipoxygenase-catalyzed oxidation

of various substrates are illustrated in Fig. 2. The natural substrate of the enzyme is linoleic acid, but it can accept a number of substrates provided they contain a *Z*,*Z*-1,4-diene unit with a substituent (R) of 3–10 carbons and a carboxylic acid group (Holland, 2000).

A combination of lipoxygenase and hydroperoxide lyase (soya flour) has been used to convert plant polyunsaturated fatty acids to a mixture of hexenal, hexan-1-ol, *E*-2-hexenal, *E*-2-hexen-1-ol and *Z*-3-hexen-1-ol, the so-called natural 'green note' flavour components present in plants such as mint (*M. arvensis*), which are in high demand by the food industry (Kula and Kragle, 2000).

5.7. Cytochrome P450 monoxygenase

Cytochrome *P*450-dependent oxidations play an important role in plant terpenoid biosynthesis (Bolwell et al., 1994). Because of the broad substrate specificity, the cytochrome *P*450 monoxygenase enzyme offers enormous potential for the catalysis of biotransformation reactions in vitro (Hotze et al., 1995; Kraus and Kutchan, 1995). Digitoxin 12β-hydroxylase, a cytochrome *P*450-dependent monoxygenase present in the microsomes, has been isolated from cell cultures of *D. lanata.* This enzyme catalyzes the hydroxylation of β-methyldigitoxin into β-methyldigoxin (Petersen et al., 1987). Involvement of cytochrome P450 monooxygenases in the biotransformation of (−)-(4*R*)-isopiperitenone into (−)-7-hydroxyisopiperitenone by cell suspension culture of *M. piperita* has been investigated by Park et al. (1999).

5.8. Other enzymes

The synthesis of optically pure 2-hydroxy acids has been achieved on the semipreparative scale by the α-hydoxylation of long-chain carboxylic acids with molecular oxygen, catalyzed by α-oxidase of peas (Adam et al., 1998). The substrate selectivity of the α-oxidation of saturated, unsaturated and heteroatom-containing (oxygen, sulfur) carboxylic acids catalyzed by the enzyme indicated that this biotransformation proceeds with a high degree of enantioselectively.

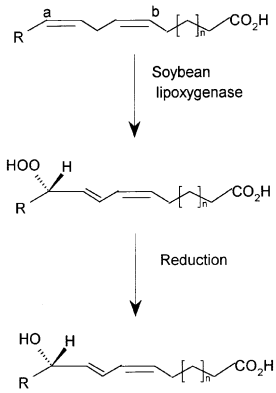

Fig. 2. Soybean lipoxygenase-catalyzed oxidation of 1,4-dienes.

Hyoscyamine 6β-hydroxylase was isolated from cultured roots of *Hyoscyamus niger* (Yamada and Hashimoto, 1989). It is responsible for hydroxylation of hyoscyamine into 6β-hydroxy hyoscyamine and scopolamine. This enzyme acts specifically and only the *S*-derivatives can bind to the active site. Hashimoto et al. (1993b) reported the expression of hyoscyamine 6β-hydroxylase in *E. coli.*

Pfitzner and Zenk (1982) isolated and characterized strictosidine synthase from cell suspension cultures of *C. roseus*. This enzyme forms strictosidine by stereospecific condensation of tryptamine and secologanin. Glycosidases are responsible for selective hydrolysis and the reversibility of the reaction allows these enzymes to be used for the synthesis of glycosides (Kren and Thiem, 1997). Parry and Edwards (1994) characterized *O*-glucosyltransferase from cell suspension cultures of *Medicago sativa.*

6. Conclusion

Progress in the study of biotransformations using plant cells, organs and enzymes in vitro in particular is slow. However, the possibility of success in exploring the biocatalytic capability of plant cells and enzymes is enormous. Specifically, the characterization of secondary metabolic pathway is a multidisciplinary challenge. It includes identification of metabolic intermediates, demonstration of plausible reaction sequences, isolation and characterization of individual enzymes responsible and their tissue and subcellular localization. The limited and fragmented knowledge in this area is a real bottleneck for the exploitation of biotransformations in vitro. A coordinated approach of molecular studies on metabolic pathway engineering to understand the genes and enzymes involved may contribute towards the utilization of biotransformations in vitro for practical applications.

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