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Review

Production of triterpenoids with cell and tissue cultures*

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Triterpenes are group of biologically active compounds which can be found in higher plants. Their main source are plants bark, leaves, twigs, fruits, resins or oils. The biological activity of triterpens is very diversified and many studies have already confirmed the following therapeutic effects: anti-inflammatory, antimicrobial, antiviral, antifungial, immunomodulatory, and hepatoprotective. Synthesis of triterpenes derivates can be performed by chemical or enzymatic reactions, however biotransformation is more specific and eliminates the side products and the molecule alterations. These processes use isolated enzymes or microorganisms. Cell culture *in vitro* eliminates problems like extract variability as well as instability of the compounds being obtained during the extraction process. What is more, it ensures high reproducibility and optimal regio- and enantioselectivity. The most widely used technique is a classical screening of a series of microbial strains. Studies on triterpene biotransformation give a lot of information about new biologically active compounds and let predict the metabolism of biological compounds. This review presents most important advancements in the metabolic engineering of microorganisms for the production of triterpenoids. Moreover, the review highlights general strategies to obtain rich biochemical diversity of plants by employing the biocatalysts produced by microorganisms or tissue cultures.

Key words: biotransformation, tritepenoids, cell cultures, microorganisms, *in vitro*

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INTRODUCTION

Triterpenes are a group of biologically active compounds which are present in plant tissues in almost every geographical region in the world. The group consist of approximately 30 000 identified compounds (Dzubak *et al.*, 2006; Mufflera *et al.*, 2011). The number of terpenoids that are produced by plants is probably larger than that of any other group of natural compounds. Plants have the ability to produce thousands of chemical variations of a single isoprenoid unit. This group includes essential oils and resins, cytokinins, gibberellins, strigolactones, dolichols, various steroids and carotenoids (Lohr *et al.,* 2012).

In most of these compounds, two or more isoprene units are joined together in a head-to-tail configuration of the carbon atoms, either in open chain or in cyclic systems containing one or more rings (Fig. 1). The main exception to the head-to-tail arrangement involves the formation of artemisia ketone (and related monoterpenoids), squalene, gossypol, and the carotenoids (Waller, 1970; Chatterjee *et al.*, 2000; Parra *et al.*, 2009; Marienhagen & Bott, 2013).

The major isoprenoids having important function in plant cells are sterols which control fluidity of the plasma membranes, the long-chain polyprenyl dolichol as sugar-carrier-lipid used in protein glycosylation in eukaryotic endoplasmic reticulum. The primary metabolites of eukaryotic organisms includes also phytol, an isoprenoid whis is part of chlorophylls, tocopherols and phylloquinones, the various carotenoids, that are essential as functional and structural components of the photosynthetic routes. What is more, the medium-chain polyprenyl plastoquinone plays an important role in electron transfer during this process. Moreover, isoprenoids serve as precursors of components of various phytohormones (Lohr *et al.*, 2012).

The biological activity of triterpens is very diverse. Many studies have already confirmed the following therapeutic effects: anti-inflammatory, analgesic, antimicrobial, antiviral, antimycotic, immunomodulatory, and hepatoprotective (Dzubak *et al.,* 2006). What is more, modification of the triterpenes structure to enhance their pharmaceutical application can be efficiently carried out using bioprocesses. As previously mentioned, many triterpenes exhibit significant biological activity, but several triterpenoids show hemolytic and cytostatic properties that can restrict their pharmaceutical use. To overcome these limitations and to expand the range of usable triterpenes, a transformation of the compound by means of chemical or biotechnological techniques is possible. However, biotransformation is used to achieve compounds with an optimal regio- and enantioselectivity (Mufflera *et al.,* 2011). Microbial transformations of triterpenoids have been developed primarily in the last 10 or 15 years to produce new and useful compounds (Parra *et al.*, 2009). Biotransformation methods are used as an alternative route to the traditional chemical synthesis in search of new production routes for chemical, pharmaceutical, and agrochemical compounds (Fu *et al.,* 2013). Advantages of these processes are also associated with the environmentally friendly reaction conditions. Moreover, microbial transformation is often the only way to obtain desired product (Zhanga *et al.,* 2005). Recently, several studies have shown that microbial transformation is a useful tool to improve the structural diversity of naturally occurring triterpenoids (Cheng *et al.,* 2004; Qian *et al.,* 2009). Finding out an optimal organism is the most

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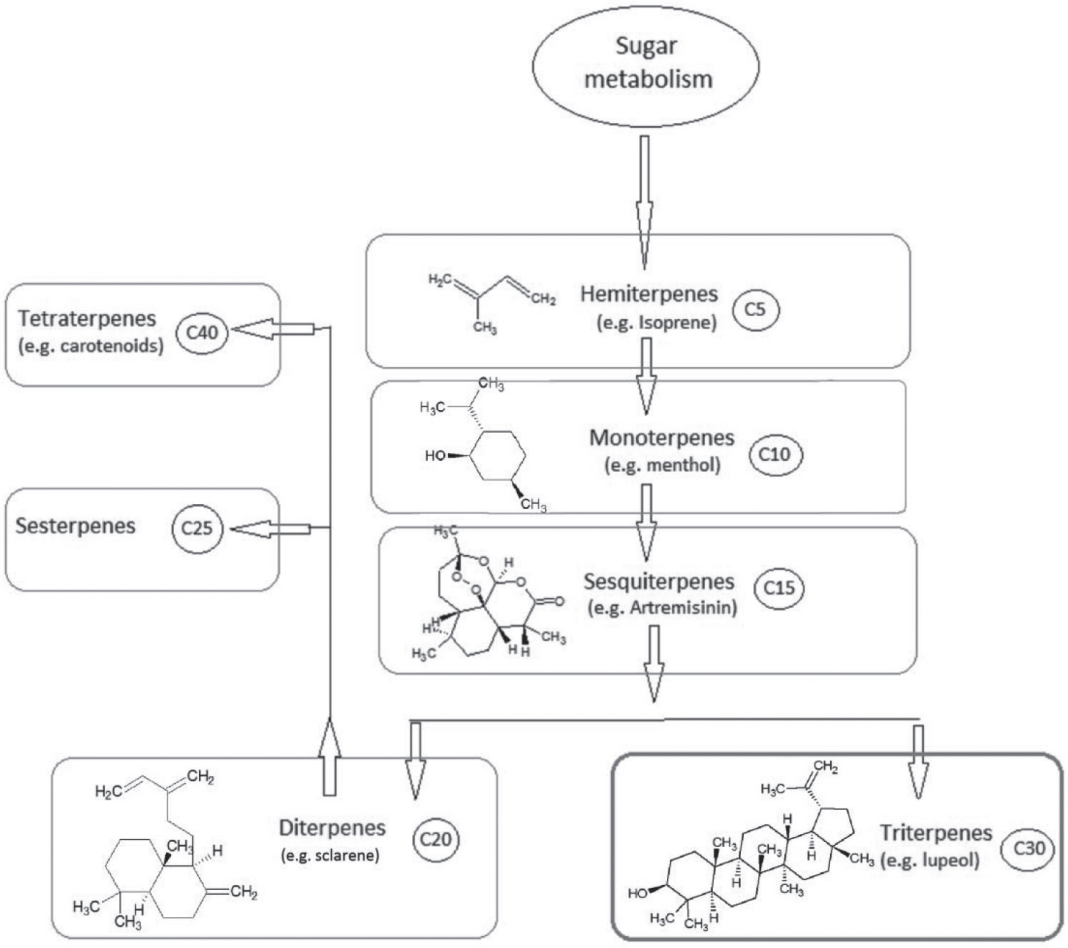

Figure 1. Schematic overview of plant biosynthetic routes of the terpenes (Waller, 1970)

important step in mentioned technology. Comprehensive review on microbial transformation of triterpenoids has been already published (Parra *et al.,* 2009). Much of the rich chemical diversity of natural compounds arises from a limited number of chemical scaffolds (e.g. terpene and polyketide structures), which are modified by specific types of chemical substitutions (hydroxylation, glycosylation, acylation, *O*-methylation) brought about by substrate- and/or proper enzymes. The way these substances are formed is explained by concerted molecular, genomic and genetic approaches (Dixon, 2005). Such approach provides sufficient quantities of the desired plant natural products from inexpensive renewable resources. Because biological raw materials must be derived from well-known plant sources with reproducible content levels of the drug or its precursors, domestic cultivation of the plant sources has several advantages compared to harvesting from the wild. These advantages minimize the risk of misidentification, genetic and phenotypic variability, extract variability and instability, toxic compounds, and contaminants (Canter *et al.,* 2005). This review, presents a general information concerning processes used for the production of pharmaceutically relevant triterpenes. Moreover, examples of triterpene production by cell and tissue biotransformation and the corresponding analytical techniques, to obtain bioactive compounds from precursor molecules are covered.

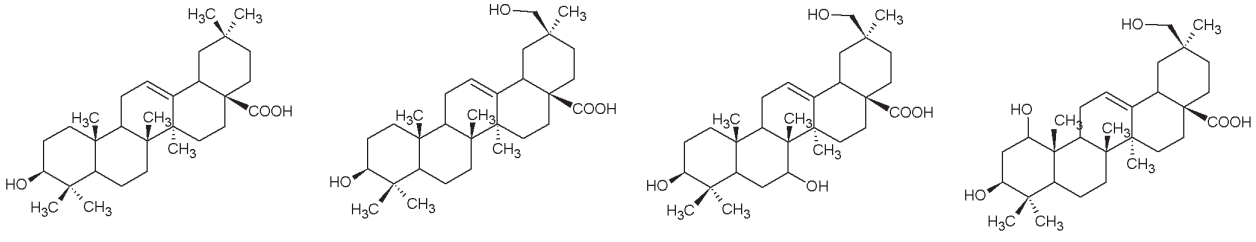

Figure 2. Metabolites from biotransformation of oleanolic acid with *Rhizomucor miehei*

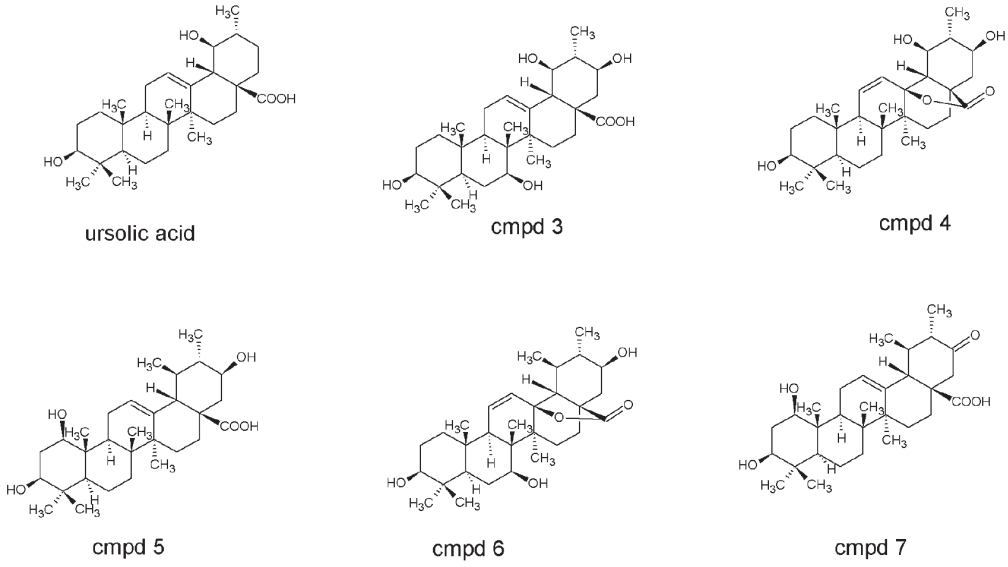

Figure 3. Biotransformation of ursolic acid by *Syncephalastrum racemosum*

MATERIALS AND METHODS OF TRITERPENOIDS BIOTRANSFORMATION

The most widely used technique is still classical screening of a series of microbial strains. The proper cell suspension is prepared in sterile conditions (Chatterjee *et al*., 2000; Parra *et al.,* 2009; Fu *et al*., 2013; Mariengagen & Bott, 2013). The substrate can be micro-emulsified in mixtures of detergents and organic solvents. Proper cells are cultivated in flasks containing a culture medium (i.e. D-(−)-glucose, malt and yeast extract) (Cheng *et al*., 2004; Canter *et al*., 2005; Zhanga *et al*. 2005; Chen *et al.,* 2009; Qian *et al*., 2009). When the culture medium is separated, proper triterpene dissolved in a nontoxic solvent is added to the flask (Carvalho *et al*., 2010; Leipolda *et al*., 2010; Parshikov *et al*., 2012; Baratto *et al*., 2013). After few days the broth is filtered and extracted. The medium culture is then sonicated (Bastos *et al*., 2007; Gallo *et al*., 2009). The organic fractions are combined, dried and analyzed by Thin Layer Chromatography (TLC). The crude extracts are then fractionated using column, flash or circular chromatography (Charlwood & Rhodes, 1990; Dornenburg, 2004; Lee *et al*., 2004; Vanisree *et al.,* 2004; Pavlov *et al*., 2007). The most common analytical methods used for triterpens identification are TLC (Thin Layer Chromatography), NMR (Nuclear Magnetic Resonance) and IR (Infra Red spectroscopy), HR-MS (High Resolution Mass Spectra) and Polarimetry (Parra *et al.,* 2009).

MICROBIAL TRANSFORMATION OF TRITERPENOIDS

Studies on triterpene biotransformation give various information on newly syntethised biologically active compounds. Moreover, they let predict the metabolism of biological compounds. Oleanolic acid, (Fig. 2)

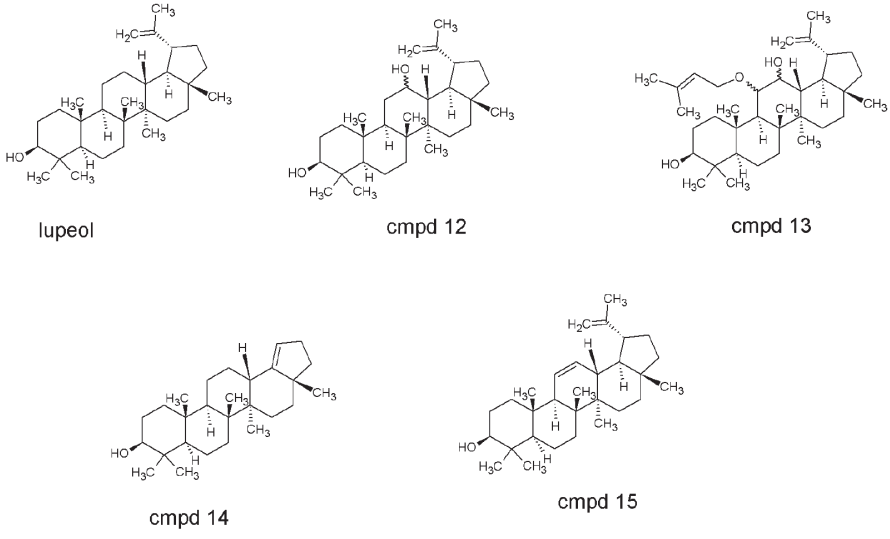

Figure 4. Biotransformation products of lupeol, caused by *A. ochraceus* and *M. rouxii*

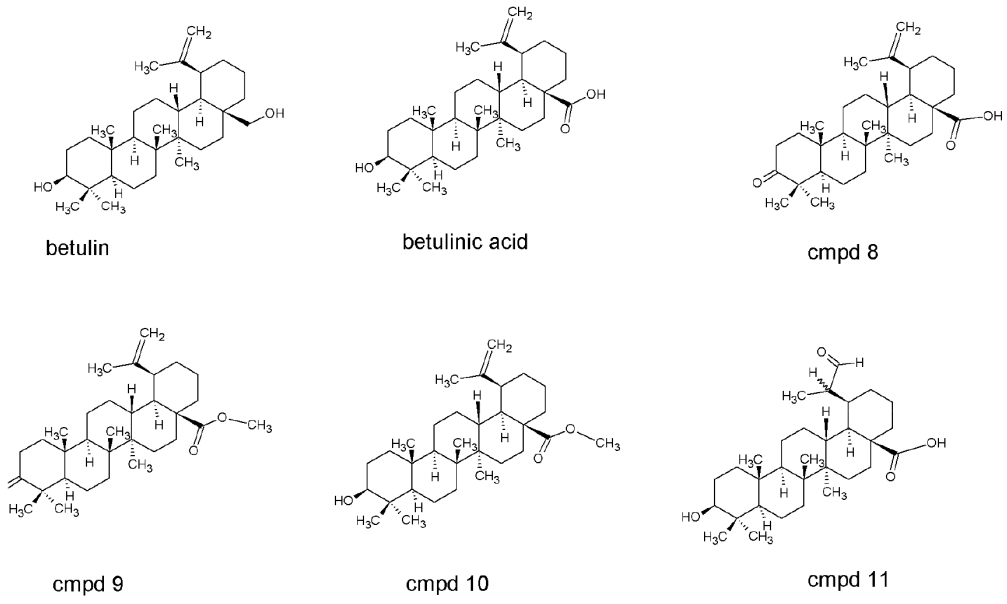

Figure 5. Biotransformation of betulin and betulinic acid

is a natural pentacyclic triterpenoid compound. It is present in olive-pomace oil, being the main components of the protective wax-like coating of the olive skin. Oleanolic acid displays remarkable pharmacological role due to its antitumor, antibacterial, anti-HIV, anti-inflammatory, antioxidant, and hepatoprotective activity. Biotransformation of oleanolic acid with *Rhizomucor miehei* yielded a mixture of three metabolites. Chromatography of this mixture on a silica-gel column resulted in recovery of 64% of the substrate and isolation of the following compounds: queretaroic acid, compound 1 and compound 2 (Fig. 2) (Leipolda *et al*., 2010).

Ursolic acid, a pentacyclic triterpene, exists in many plant species, especially in some medicinal herbs. It has been reported that ursolic acid exhibits a remarkable spectrum of biological activities, such as antiinflammatory, antiallergic, antibacterial, hepatoprotective and antitumor (Fu *et al*., 2013).

The *Syncephalastrum racemosum* converted ursolic acid to five metabolites: compounds 3–7 (Fig. 3) (Fu *et al*., 2013).

Lupeol has been shown to possess a range of proven biological activities in preventing cancer, coronary and hepatic diseases. It can be very efficiently obtained from bark of some trees (Gallo *et al*., 2009). *Mucor rouxii* transformed lupeol to two metabolites, compounds 12 and 13 (Parshikov *et al*., 2012), although *Aspergillus ochraceus* also converted lupeol to two metabolites, compound 14 and 15 (Fig. 4) (Carvalho *et al*., 2010).

Betulinic acid and betulin are bioactive pentacyclic triterpenes. These are the most common triterpenes found in plants together with ursolic and oleanolic acids. They have been isolated from many plants such as birch (*Betula* spp.). Important pharmacological properties have been described for betulin and betulinic acid. Many studies have confirmed their antitumor, anti-HIV, antiinflammatory, antibacterial, antimalarial, antitrypanosomal and analgesic activity (Baratto *et al*., 2013). The biotransformation experiments showed production of compounds 8–11 from betulinic acid (Bastos *et al*., 2007; Baratto *et al*., 2013). Betulin was converted to betulinic acid using *Aspergillus oryzae, Armillaria luteo-virens Sacc, Aspergillus foetidus* (Fig. 5) (Chen *et al*., 2009).

Table 1 shows examples of microbial transformation of triterpenoids and the yields of obtained derivatives which have been already published (Bastos *et al*., 2007; Chen *et al*., 2009; Gallo *et al*., 2009; Carvalho *et al*., 2010; Leipolda *et al*., 2010; Parshikov *et al*., 2012; Baratto *et al*., 2013; Fu *et al*., 2013).

CONCLUSIONS

The use of tissue engineering and cell culture techniques offers the opportunity to optimize the yield of the target compound and to obtain a uniform, high quality product (Mufflera *et al*., 2011). Microbial transformation of triterpenoids has provided new derivatives that are potentially useful for pharmacological studies. In these biotransformation processes, several reactions that are difficult to achieve by chemical methods have been successfully accomplished. These biotransformations can also be used as *in vitro* models to predict the metabolism of biologically active triterpenoids (Parra *et al.,* 2009). Plant *in vitro* production of biologically active metabolites has several advantages because the target substances are produced in cells, tissues or organs that are cultivated in bioreactors under sterile conditions. Moreover, they are completely insulated from adverse environmental factors (Vanisree *et al.,* 2004). Disadvantage of the method is that the amounts of desired metabolites are often lower than the contents in intact plants (Charlwood & Rhodes, 1990). There are more and more strategies to enhance the yields of secondary metabolites in plant *in vitro* cultures like media and hormone optimization and the use of various techniques such as immobilization and genetic modification (Dornenburg, 2004; Lee *et al*., 2004; Pavlov *et al.,* 2007).

Table 1. Cell cultures applied in synthesis of triterpene derivates

Triterpen compound	Cell suspension	Derivate	Yield (%)
Oleanolic acid	*Rhizomucor miehei*	Queretaroic acid	5
		cmpd 1	6
		cmpd 2	4
Ursolic acid	*Syncephalastrum racemosum*	cmpd 3	13
		cmpd 4	3
		cmpd 5	4
		cmpd 6	3
		cmpd 7	12
Lupeol	*Mucor rouxii*	cmpd 12	26
		cmpd 13	16
	Aspergillus ochraceus	cmpd 14	19
		cmpd 15	11
Betulin	*Aspergillus oryzae*	Betulinic acid	1
	Armillaria luteo-virens Sacc QH		2
	Aspergillus foetidus		9
Betulinic acid	*Mycelia sterilia;*	cmpd 8	3
	Penicillium sp.	cmpd 8	2
		cmpd 9	2
	Penicillium citreonigrum	cmpd 10	2
	Daucus carota	cmpd 8	7
		cmpd 11	10

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