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MINI-REVIEW

A literature update elucidating production of *Panax* ginsenosides with a special focus on strategies enriching the anti-neoplastic minor ginsenosides in ginseng preparations

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Abstract Ginseng, an oriental gift to the world of healthcare and preventive medicine, is among the top ten medicinal herbs globally. The constitutive triterpene saponins, ginsenosides, or panaxosides are attributed to ginseng's miraculous efficacy towards anti-aging, rejuvenating, and immune-potentiating benefits. The major ginsenosides such as Rb1, Rb2, Rc, Rd., Re, and Rg1, formed after extensive glycosylations of the aglycone "dammaranediol," dominate the chemical profile of this genus in vivo and in vitro. Elicitations have successfully led to appreciable enhancements in the production of these major ginsenosides. However, current research on ginseng biotechnology has been focusing on the enrichment or production of the minor ginsenosides (the less glycosylated precursors of the major ginsenosides) in ginseng preparations, which are either absent or are produced in very low amounts in nature or via cell cultures. The minor ginsenosides under current scientific scrutiny include diol ginsenosides such as Rg3, Rh2, compound K, and triol ginsenosides Rg2 and Rh1, which are being touted as the next "anti-neoplastic pharmacophores," with better bioavailability and potency as compared to the major ginsenosides. This review aims at describing the strategies for ginsenoside production with special attention towards production of the minor ginsenosides from the major ginsenosides via microbial biotransformation, elicitations, and from heterologous expression systems.

Keywords Ginseng · Minor ginsenosides · Microbial biotransformation · Heterologous co-expression · Elicitation

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Introduction

Plant species have long been investigated as a prospective sink of phyto-molecules which are extremely valued because of their pharmacological significance. Discovered in the mountains of Manchuria, China, ginseng enjoys a prime position in the medicinal herb market. Ginseng roots are esteemed as a panacea, "the kingly herb" in the Chinese community. Native to the Asian continent, classified under the genus "*Panax*" (*Panax ginseng* or Korean ginseng), it shares its popularity with two other members, i.e., *Panax quinquefolius* (American ginseng) and *Panax notoginseng* (Sanchi ginseng).

True ginseng, i.e., *P. ginseng*, is a small, shade loving, perennial herb, belonging to the ivy family "Araliaceae." The generic name, i.e., "*Panax*," literally means "cure all," with a very curious feature of the subterranean parts of the plant, specially the roots which strongly resemble the human form. As was the popular belief with naturopathy practices, the probable potency of a plant would be indicated by nature itself, with clues in its color, shape, or characters. Considering the anthropomorphic appearance of the roots, ginseng was more than naturally accepted as a tonic for overall good health, as a cure all with specific value towards treatment of impotence and lack of sexual vigor.

Used since centuries in traditional Chinese medicines, as a general tonic, aphrodisiac, and to promote mental and physical health rejuvenation, current medicinal research has also found ginseng or its derived extracts extremely beneficial as anti-aging, neuroprotective, cerebral maintenance, immune-modulators, and for enhancing cardiac health (Christensen 2009). The triterpene saponins, collectively known as ginsenosides/panaxosides, are reported to be exerting these miraculous effects of ginseng. More than 150 types of ginsenosides are known and new subtypes are being constantly added to the existing database. Predominantly found in the

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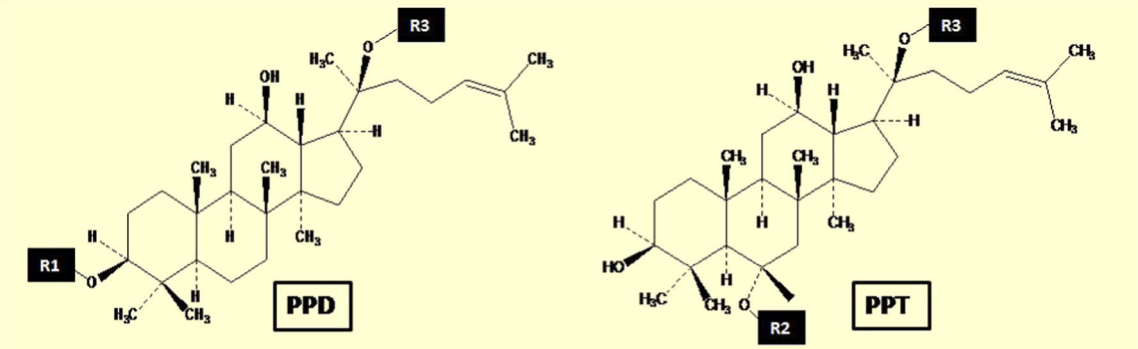
roots, although its presence is also observed in leaves and berries, they are subdivided into two classes (Fig. 1) based on their terpenoid backbone, i.e., 20S protopanaxadiol (Rb1, Rb2, Rc, Rd.) and 20S protopanaxatriol (Re and Rg1). These ginsenosides are the major components, contributing extensively to the chemical profile of a ginseng preparation in vivo/in vitro. The relative proportions of the individual ginsenosides and their ratio, i.e., Rb/Rg, vary extensively among the three commercial species. Rb1, Rg1, and Rb2 are among the major ginsenosides in *P. ginseng* and *P. notoginseng* and Rb1, Re, and Rd. are among the major ginsenosides present in *P. quinquefolius* (Kim 2012a). These major ginsenosides are reported to improve memory function, exert anti-aging, anti-diabetic activities and also offer neuroregulation among a host of other bioactivities reported in vitro (Christensen 2009; Kim et al. 2013b).

However, the current attention of ginseng biotechnologists has been redirected towards a group of relatively less glycosylated, minor ginsenosides, which are not detected in nature as frequently or are completely absent from natural sources. These include Rg2, Rg3, Rh1, Rh2, compound K, among a few others, which are reported to exert anti-neoplastic and specifically anti-metastatic properties (Park et al. 2010). These minor ginsenosides have been implicated in attenuation of tumor angiogenesis, prevention of cell proliferation, and apoptosis induction and are gaining scientific popularity for their anti-cancer potential. This review will summarize the different strategies exploited such as biotransformations, microbial fermentations, and heterelogous expression since the last decade, to enrich ginseng extracts with these minor ginsenosides that are implicated to be comparatively more potent, and harboring potential anti-cancer activities.

The ginsenosides: biosynthesis pathway and chemistry

Even though the pharmacological significance of ginsenosides has largely been recognized, the biosynthesis routes leading to the cytoplasmic manufacturing of these triterpene ginsenosides remains to be elucidated in detail. Belonging to the class of "triterpene saponins," they are predominantly synthesized from mevalonic acid in the cytoplasm (MVA pathway, Fig. 2). An alternate pathway has also been suggested from methylerythritol phosphate (MEP pathway) from the plastids; however, significant questions still need to be answered about any form of cross-talk



PROTOPANAXADIOLS (PPD)			
GINSENOSIDE	R1	R2	R3
Rb1	-Glc-Glc-	--	-Glc-Glc-
Rb2	-Glc-Glc-	--	-Glc-Arab-
Rc	-Glc-Glc	--	-Glc-Araf-
Rd	-Glc-Glc	--	-Glc-
PROTOPANAXATRIOLS (PPT)			
Re	--	-Glc-Rham-	-Glc-
Rf	--	-Glc-Glc-	-Glc-
Rg1	--	-Glc-Rham-	-H-

Fig. 1 The two main classes of the major ginsenosides and their chemical structures. (*Glc*, glucose; *Arab*, arabinopyranose; *Araf*, arabinofuranose; *Rham*, rhamnose; R1= C3; R2= C6; R3= C20 carbon positions of the aglycone)

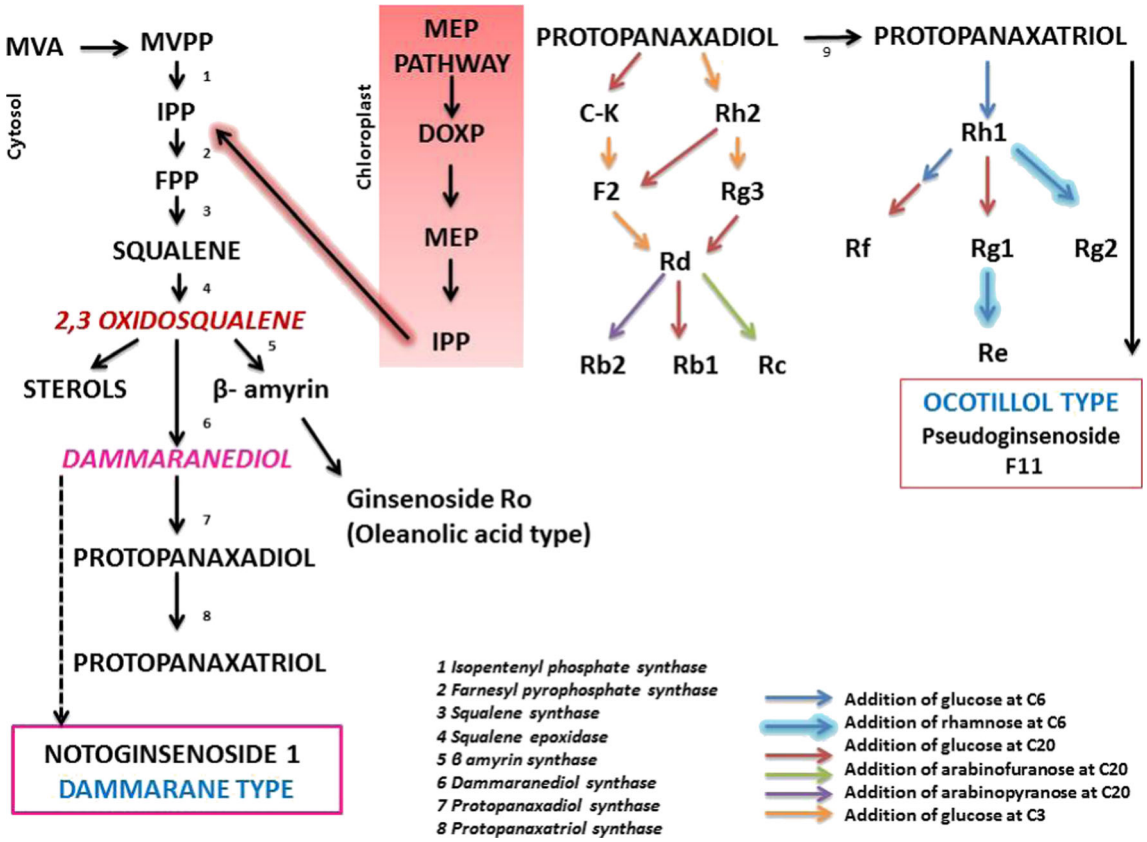


Fig. 2 Putative ginsenoside biosynthesis pathway (*MEP*, 2-C-methyl-D-erythritol 4-phosphate; *DOXP*, 1-deoxy-D-xylulose-5-phosphate; *IPP*, isopentenyl phosphate; *MVA*, mevalonic acid; *MVPP*, diphosphomevalonate; *FPP*, farnesyl diphosphate)

and regulation involving these two independent biosynthesis routes (Zhao et al. 2014).

The biosynthesis pathway initiates with mevalonic acid, sequentially leading to the production of isopentenyl phosphate (IPP). IPP may also be produced via the alternate MEP pathway operative in the plastids (Fig. 2). The MEP pathway originates from condensation of glyceraldehyde-3-phosphate and a 2C pyruvate unit to form DXP (1-deoxy-D-xylulose-5-phosphate). DXP is the source of MEP via reduction and rearrangement to yield IPP and dimethylallyl-diphosphate (DMAPP). Cytosolically, two IPP molecules combine with a DMAPP, leading to biosynthesis of FPP (farnesyl diphosphate), which further assembles head-to-head, with another of itself, to yield the C30 molecule, squalene. Following, cyclization, squalene is converted to 2,3-oxidosqualene, mainly by a class of oxidosqualene cyclases (OSC) (Phillips et al. 2006). Key oxidations of the 2,3-oxidosqualene ring to yield the "dammaranediol" structure are considered to be the committed step towards ginsenoside biosynthesis. Other anabolic branch points commencing from 2,3-oxidosqualene may be either towards sterols—another vital plant requirement—or may be diverted towards the oleanolic acid flux.

The dammaranediol ring structure may undergo further oxidations to yield the protopanaxadiol (PPD) aglycone backbone. This aglycone is further converted to the protopanaxatriol (PPT) aglycone backbone. Protopanaxadiol synthase and protopanaxatriol synthase (PPDS, PPTS) belonging to a family of cytochrome P450-dependent monoxygenases catalyze these key conversions towards synthesis of the two aglycones from dammaranediol. The different ginsenosides are then synthesized from these PPD and PPT aglycones via subsequent glycosylations at C3/C20 positions for the protopanaxadiols and C6/C20 positions for the protopanaxatriols. Among the protopanaxadiols, glycosylation at either C3 or C20 leads to synthesis of ginsenoside Rh2 or compound K, respectively. Ginsenoside Rd. may be synthesized from either of these two "precursors" (via formation of compound F2 from C-K or ginsenopside Rg3 from Rh2; Fig. 2). Ginsenosides Rb1, Rb2, and Rc are further derived from Rd. via addition of glucose (Rb1), arabinopyranose (Rb2), and arabinofuranose (Rc) at the C20 position. Similarly, among the protopanaxatriols, glycosylation at the C6 position yields ginsenoside Rh1, followed by addition of glucose to yield Rf or rhamnose to yield Rg2 at the C6 position. Alternatively, addition of glucose moiety at the C20 position leads to formation of ginsenoside Rg1, which may further accept a rhamnose moiety at the C6 position to yield ginsenoside Re (Fig. 2).

The glycosylation of the aglycone backbone is facilitated by a UGT, i.e., UDP-glycosyltransferase family of enzymes. A vital enzymatic group for ginsenoside biosynthesis, they exhibit

stereospecificity towards stepwise addition of monosaccharide groups at either the C3, C6, or C20 positions of the respective aglycone, for both the ginsenoside classes. Differences in the glycosylation patterns may be the reason for the diversity in the biological activities reported to be exerted by the different classes of ginsenosides, either individually or in combinations. Several of the UGTs have been identified in the ginseng system, especially for the protopanaxadiol group; however, their biochemical functions need further elaboration (Luo et al. 2011; Jung et al. 2014; Xiang et al. 2012, Yan et al. 2014). A *UGRdGT* and a *UGRh2GT* gene have been identified in *P. ginseng* system, responsible for biosynthesis of Rb1 from Rd. with the former and Rh2 to Rg3 with the latter (He and Yue 2010; Yue and Zhong 2005). Some UGTs may exhibit specificity towards their substrates, while some may accept a broad range of chemistry at their active sites. Yan and co-workers recently identified a*UGTPg1*, from *P. ginseng*, which was observed to be involved in the glycosylation of many intermediates like dammarane, PPD, and Rh2 (Yan et al. 2014). Only a few UGTs functional at different levels of ginsenoside biosynthesis are well characterized. This class of enzymes is an attractive target for further research and for envisaging the biosynthetic pathway in detail, which may help in modulating their production patterns through biotechnological interventions.

Although these minor ginsenosides such as Rg3, Rh2, Rh1, and C-K are produced within the cell as part of the ginsenoside biosynthesis pathway, still their presence in nature is negligible. Possibly their intra-cellular accumulation may be extremely low with rapid flux diversion towards the biosynthesis of the major ginsenosides, which may be the reason for their rare occurrence *in planta*. They may be merely acting as "inter mediates" enroute the ginsenoside biosynthesis. However, since not much is known about the ginsenoside biosynthesis and factors regulating their stepwise production, exact reasons as to the rare accumulation of these minor ginsenosides *in planta* still needs to be investigated.

Modulating the ginsenoside biosynthetic pathway via tissue culture interventions

Traditional ginseng cultivation is riddled with many complications. It requires a well prepared and enriched soil bed for sowing the ginseng seeds, and has a long gestation period of about 2 years for the seedling to emerge. The emergent seedling requires another 3–4 years for developing into a mature plant. A stringent and tedious downstream processing of the root is mandatory. Further, the plants are prone to insect/pest attack and the optimum requirements such as weather and soil characteristics are extremely typical (Wu and Zhong 1999).

Consequently, ginseng is an ideal candidate for alternate secondary metabolite production via cell/tissue cultures. Ginsenoside production via callus, cell suspensions, and adventitious/hairy roots have been reported frequently (Asaka et al. 1994; Liu and Zhong 1996; Mathur et al. 1994, 1999, 2003, 2010a, b; Kochan et al. 2012; Biswas et al. 2015, 2016). Many studies exist in the last two to three decades for rapid and mass production of ginsenosides (as reviewed by Wu and Zhong 1999; Murthy et al. 2014; Kim et al. 2015) as summarized in Table 1.

Callus and cell suspension cultures of *P. ginseng* have been the most widely studied and are comparatively simpler systems for in vitro ginsenoside production (Table 1). The ginsenoside profile of ginseng preparations either in vitro or in vivo mostly comprises of the major ginsenosides which include protopanaxadiols Rb1, Rb2, Rc, Rd., and protopanaxatriols such as Re, Rf, and Rg1. However, the ratio between protopanaxadiols to protopanaxatriols may differ between in vivo and in vitro conditions, with mostly the diols predominating in the former whereas triols dominating in the latter. In terms of cell cultures, a combinatorial influence of many factors operative at the physical and chemical environment of the cells seems to decide the ginsenoside production pattern. The medium composition, specifically phytohormonal additives, is among one of the key factors in deciding the net output of ginsenosides and even the ratio of triols to diols. Another most important component is the carbon source, i.e., sucrose. Doubling the sugar concentration is found to have a doubling effect on the total ginsenosides in *P. quinquefolius* and *P. notoginseng* (Mathur et al. 2000; Zhang et al. 1996b). Inorganic factors such as increase in phosphate or nitrate content is also observed to have a significant effect on the ginsenoside production (Liu and Zhong 1998; Zhong and Zhu 1995). Other physical parameters such as temperature and light intensity also reportedly influence in vitro ginsenoside production. In a report by Yu and co-workers (Yu et al. 2005), the highest hairy root biomass was obtained with the cultures incubated at day/night temperature 20/13 °C. However, total ginsenosides was optimum (10.5 mg g^{-1} of DW) in the cultures incubated at 25/25 °C (day/night temperature). They also reported that hairy root growth was stimulated by red-light-incubated cultures when compared to dark-incubated cultures. They also noticed a differential accumulation of the Rb and Rg groups of ginsenosides in dark- and light-grown cultures with Rb-group ginsenosides highest in the dark-grown cultures (4.5 mg g^{-1} of DW), and the Rg group ginsenosides were optimal in the light-grown cultures (5.3 mg g^{-1} of DW). Callus and cell suspensions cultures of *P. quinquefolius* have also been extensively explored for their ginsenoside content (Mathur et al. 1994, 1999, 2003; Kochan and Chmiel 2011). Recently, the adventitious root system, especially in *P. notoginseng*, is gaining popularity as an excellent system for in vitro ginsenoside production (Murthy et al. 2014; Thanh et al. 2014).

Adventitious root cultures are generally considered more robust systems that can tolerate physical conditions during upscaling in bioreactors, with the ease of adaptation into the different types and forms of bioreactors (Paek et al. 2009).

Table 1 Representative examples of in vitro production of ginsenosides

Species	Culture system	Introduced gene/chemical modification	Reported content of the major ginsenosides[a]	Reference
P. ginseng	CC	None	1.46 mg g^{-1} DW	Asaka et al. (1994)
	CC	60 mm K+	15.4 mg L^{-1} day^{-1}	Liu and Zhong (1996)
	CS	0.42 mM phosphate	643 mg L^{-1}	Liu and Zhong (1998)
	CS	6% sucrose	275 mg L^{-1}	Akalezi et al. (1999)
	CS	0.2 M sorbitol + 0.5 gL^{-1} CH	1130 mg L^{-1}	Wu et al. (2005)
	HR	None	2.8 mg g^{-1} DW	Jeong et al. (2005)
	HR	Bioreactor upscaling (5 L)	145.6 mg g^{-1} DW	Palazón et al. (2003)
	AR	Mutation induced by gamma irradiation	82 mg g^{-1} DW	Kim et al. (2013a)
	HR	Transgene (Pg FPS) introduced	30–35 mg g^{-1} DW	Kim et al. (2014c)
	AR	Transgene (Pg HMGR1) introduced	10–13 mg g^{-1} DW	Kim et al. (2014b)
	Tobacco	Transgene (Pq DDS) introduced	5.2 mg L^{-1}	Han et al. (2014)
P. quinquefolius	CC, CS	None	(0.56–1.2% FW)	Mathur et al. (1994, 1999, 2003)
	CS	12.5 μM IBA	33.9 mg L^{-1} day^{-1}	Zhong and Wang (1998)
	CS	1.25 mM phosphate	960 mg L^{-1}	Liu and Zhong (1998)
	CS	None	197 mg L^{-1}	Kochan and Chmiel (2011)
	HR	None	0.3 g g^{-1} DW	Mathur et al. (2010a)
	HR	Bioreactor upscaling (10 L)	6 mg g^{-1} DW	Kochan et al. (2012)
	HR	Bioreactor upscaling	9 mg g^{-1} DW	Kochan et al. (2014)
	HR	Transgene(Pq DDS) introduced	5.2 mg L^{-1}	Wang et al. (2014b)
	CS	None	3.3 mg g^{-1} DW	Biswas et al. (2015)
P. notoginseng	CS	1.25 Mm phosphate	980 mg L^{-1}	Zhong and Zhu (1995)
	CS	4% sucrose	177 mg L^{-1}	Zhang et al. (1996b)
	CS	NO_3^-/NH_{4+} (60:0)	850 mg L^{-1}	Zhang et al. (1996a)
	CS	High density cell culture using centrifugal impeller bioreactor	2.1 g L^{-1}	Zhang and Zhong (2004)
	AR	None	120 mg L^{-1}	Gao et al. (2005)
P. sikkimensis	C, CS	5 μM NAA	77 mg L^{-1} ginsenoside; 199 mg L^{-1} anthocyanin	Biswas et al. (2015)

CC callus culture, *CS* cell suspension, *AR* adventitious root, *HR* hairy root, *YE* yeast extract, *CH* casein hydrolysate

[a] Major ginsenosides: Rb1, Rb2, Rc, Rd., Re, Rg1

Bioreactor upscaling of adventitious roots has also been successfully implemented (Paek et al. 2009; Murthy et al. 2014), and the ginsenoside accumulation has also been reported to improve by medium replenishment strategy (Sivakumar et al. 2005; Jeong et al. 2008). Hairy root culture systems are also another preferred system of choice obtained by infecting explants with *Agrobacterium rhizogenes*. They are favored due to their quick growth and flexibility towards upscaling in bioreactors (Mallol et al. 2001; Palazón et al. 2003; Yu et al. 2005; Mathur et al. 2010a). Hairy root clones differ significantly in their morphology, growth pattern, and ginsenoside accumulation, generally favoring Rg group production. Bioreactor upscaling of hairy roots has been successfully attempted in *P. ginseng* and *P. quinquefolius* (Palazón et al. 2003; Kochan et al. 2012, 2014). Trangenic hairy root lines have also been reported with an incorporated DDS (dammaranediol synthase) gene in *P. quinquefolius* (Wang et al. 2014b) and an FPS gene (farnesyl phosphate synthase) in *P. ginseng* (Kim et al. 2014c), with markedly improved ginsenoside accumulation.

Enhancement of in vitro secondary metabolite production has been frequently attempted using elicitation strategies. An elicitor may be defined as a substance which when introduced into a living system initiates or improves biosynthesis of a certain target set of specific compounds, often produced in response to stress or threat (Radman et al. 2003). Numerous examples pertaining to enhanced metabolite production in *Panax* spp. via elicitation are cited in literature and many are being added consistently (Table 2). The major species that has been investigated frequently is cell cultures of *P. ginseng* and

Table 2 Representative examples of in vitro elicitation studies in *Panax* species

Species	Cell system	Elicitor used	Fold enhancement	References
P. ginseng	AR	JA	2.5	Yu et al. (2002)
	HR		2	Yu et al. (2000)
	AR	MeJa	Rb/Rg = 11	Kim et al. (2004)
	HR		5.5–9(Rb);1.85–3.8(Rg)	Kim et al. (2009b)
	AR		4.76	Wang et al. (2013)
	CS		2.9	Thanh et al. (2005)
	AR		3–4	Paek et al. (2009)
	CC	Yeast extract	2.0	Lu et al. (2001)
	CS	Hydrogen peroxide	2.3	Hu et al. (2003a)
	AR	Ethephon + MeJa	2.17 more than MeJa treated	Bae et al. (2006b)
	HR	Tween 80	3	Liang et al. (2014)
	CC	Vanadate	4.4	Huang and Zhong (2013)
	CC	DCCD	3	Huang et al. (2013)
	AR	SNP + MeJA	2.6	Rahimi et al. (2015)
	AR	IBA + MeJa	1.4-fold better than MeJa treated	Kim et al. (2007b)
	AR	Linolenic acid	3.6 (Rb); 2.1(Rg)	Dewir et al. (2010)
	AR	α Linolenic acid	2.5	Wu et al. (2009)
	CC	Salicylic acid	4	Tewari and Paek (2011)
	CC	OGA	2–3	Hu et al. (2003b)
	CC	SNP	1.8	Hu et al. (2003b)
	CC	Fungal elicitor	3	Xu et al. (2005)
P. notoginseng	CC	Phenobarbital	Rb/Rg ratio improved by a factor of 2	Yue and Zhong (2008)
	CC	MeJa	3.5	Wang et al. (2005)
	CC		2.16	Zhong and Zhang (2005)
	CC	MeJa + hydroxyethyl jasmonate	3.1–4.4	Hu and Zhong (2008)
	CC	Hydroxyethyl jasmonate	4.45	Hu and Zhong (2007)
P. quinquefolius	CC	Lactalbumin hydrolysate + MeJa	1.46-fold better than MeJa treated	Wang et al. (2011)
	CS	*Trichoderma harzianum* CF	3.2	Biswas et al. (2016)

CC callus culture, *CS* cell suspension, *AR* adventitious root, *HR* hairy root, *JA* jasmonic acid, *MeJa* methyl jasmonate; *DCCD* N,N′--dicyclohexylcarbodiimide, *SNP* sodium nitroprusside, *IBA* indole butyric acid, *OGA* oligogalacturonic acid, CF culture filtrate

the most common and widely exploited elicitor is methyl jasmonate (MeJa) with more than 90% of elicitation studies in ginseng involving MeJa. Certainly, this elicitor has proved extremely effective in enhancing ginsenoside biosynthesis at all levels of cellular system such as cell suspensions, hairy and adventitious roots. However, the last decade has witnessed an urge to search for other promising elicitors for improved biomass and metabolite retrieval from cell cultures.

Methyl jasmonate has been frequently used in combination with other elicitors to enhance ginsenoside production. Methyl jasmonate is reported to function via the JA signaling pathway. That has been found to enhance the transcriptional activity of squalene synthase and squalene epoxidase upstream genes in elicited ginseng cultures (Rahimi et al. 2015). Enhancements in Rb-group ginsenosides have been frequently observed with jasmonate elicitation (Kim et al. 2004, 2009b). Even adventitious root systems are observed to be influenced by MeJa treatments. Wang and co-workers observed a 4.76-fold enhancement in total ginsenoside count with 200 μM MeJa treatment (Wang et al. 2013). Elicitors such as ethephon, sodium nitroprusside (SNP) and even hormones such as IBA have been reported to enhance yields (1.4–4.4-fold) in cell cultures of *P. ginseng* (Bae et al. 2006b; Rahimi et al. 2016). Recently, "unconventional" elicitors such as vanadate and DCCD have been observed to enhance ginsenoside productivity with concomitant increase in the transcriptional activity of the upstream biosynthesis genes (Huang and Zhong 2013; Huang et al. 2013). Sodium nitroprusside has also gained considerable interest as a potential elicitor. Being a "NO" donor, it is reported to facilitate elicitation signaling through the NO signaling pathway. Even OGAs and fungal

elicitors are reported to function via a similar pathway (Hu et al. 2003a, b; Xu et al. 2005; Rahimi et al. 2016). Salicylic acid is another exogenous elicitor which has been reported to be effective at inducing NO accumulation along with enhanced ginsenoside production (Tewari and Paek 2011). *P. notoginseng* cell cultures have also been subjected to MeJa elicitation and successfully upscaled in bioreactors (Hu and Zhong 2008; Zhong and Zhang 2005). Another jasmonic acid derivative, HeJa, α hydroxyethyljasmonate has been reported to lead to a 4.1–4.4-fold enhancement in cell cultures of *P. notoginseng* (Hu and Zhong 2007, 2008). Elicitations reported in *P. quinquefolius* cell cultures are sporadic. Wang and team have explored the potential of lactalbumin hydrolysate on MeJa-treated cell culture of *P. quinquefolius*. Enhancement was observed to the tune of 1.4-fold over MeJa-treated cultures (Wang et al. 2011). We have previously reported significant ginsenoside elicitation (3.2-fold) with *Trichoderma harzianum*-derived biotic elicitor in *P. quinquefolius* cell suspensions (Biswas et al. 2016). We have also reported a 2.1-fold increase in total ginsenoside yield with a bacterium (*Pseudomonas monteili*)-derived biotic elicitor in *P. quinquefolius* cell suspensions (Biswas et al. 2016).

However, it can be observed that in spite of abundance of reports related to phenomenal enhancements in in vitro ginsenoside production, it is the production of the major ginsenosides which are the most affected. Reports pertaining to biosynthesis of the minor ginsenosides from in vitro cultures, without any biotechnological manipulation, are negligible. It may be assumed that accumulation of the minor ginsenosides within the cell may be hindered under in vivo or in vitro conditions, which may explain their rare occurrence in nature or in cell cultures. These minor ginsenosides are rapidly fed into the metabolic flux to biosynthesize the major ginsenosides via glycosylations (Fig. 2), thereby accounting for the observation that ginseng tissues substantially accumulate major ginsenosides in comparison to the minor ginsenosides either in vivo or in vitro conditions.

The minor ginsenosides: products of intestinal degradation of the major ginsenosides

In general, ginseng is an orally ingested health supplement in the form of capsules or as traditional ginseng root concoctions. The evidence from in vitro and in vivo studies always led biotechnologists to naturally conclude that the major ginsenosides such as Rb1, Rb2, Rc, Re, and Rg1 are responsible for the medicinal efficacy of ginseng on the human body. However, a hypothesis proposed by Kobashi et al. (1992) suggested that plant glycoside by itself may not be the active principle, but it could be a form of "prodrug" that could be metabolically "activated" by intestinal bacterial degradation.

Studies, tracking fate of these intact major ginsenosides after oral ingestion, and detecting their presence in the plasma and urine, have provided ample evidence of the fact that these major ginsenosides exhibit poor absorption from the gastrointestinal (GI) tract. Cui et al. (1997) reported a miniscule 1.2% of the intact triol and less than 0.2% of the diols recovered in human urine. Ginsenoside Rb1 and Rb2 have demonstrated almost negligible absorption from the GI tract, corresponding to their absence in plasma and urine samples (Tawab et al. 2003; Lee et al. 2009). This could be due to the hindrance posed by the bulky sugar side chains, which may retard their cross membrane transport across the intestinal membranes into the bloodstream. Surprisingly, these studies also revealed the presence of simple less glycosylated ginsenosides such as compound K and G-Rh1/G –F1, in blood plasma/urine after a few hours of ginseng ingestion. Under controlled experimental conditions, the only plausible explanation for their detection was that these chemical species were the major intestinal metabolites of the major ginsenosides which were ingested. Structurally, these simple compounds were observed to be similar to the major ginsenosides, in terms of the aglycone backbone but with comparatively fewer sugar groups attached to the aglycone. Stepwise removal of the monosaccharides from the major ginsenosides, by the intestinal microflora, leads to production of different intestinal metabolites which often culminate in accumulation of the minor ginsenosides such as Rg3, Rh2, Rh1, compound K, etc. as shown in Fig. 3. These minor ginsenosides may crossover to the systemic circulation, which explains the presence of "simple" ginsenosides a few hours after ginseng ingestion. Compound K was the principle metabolite reported in such pharmacokinetics studies after protopanaxadiol metabolism and G-Rh1/G-F1 was the principle metabolite detected after protopanaxatriol metabolism (Lee et al. 2009).

These "simple" ginsenosides reported enhanced absorption from the GI tract and were reported to manifest the pharmacological activity of the ginsenoside. Essentially, the major ginsenosides were observed to be manifesting their bioactivities by "reverting" back to its precursor, i.e., the minor ginsenosides. The difference lies in the fact that these major ginsenosides, once ingested, were intestinally degraded by gut microflora to deglycosylated ginsenosides with improved bio-absorption by the intestinal walls. Their absorption could be attributed to greater water solubility of the monoglucosylated ginsenosides and absorption by the intestinal sodium-dependent glucose transporter (Wolffram et al. 2002). Recent research on pharmacokinetics and bioactivities of Rg3, Rh2, compound K, and Rh1 have implicated them to be manifesting the effects of ginseng. These minor ginsenosides have been implicated in many studies to exert strong anti-proliferative effects on cancer cell lines and oxidative stress-mediated cellular damage (Park et al. 2010). Touted as the "new" anti-neoplastic pharmacophores, owing to their overwhelming response in cancer prevention, these minor ginsenosides are now the current focus of ginseng biotechnology.

Figure 3 describes in detail the putative degradation of the major ginsenosides by the gut microflora after oral ingestion of the major ginsenosides. The different pathways were hypothesized based on numerous metabolites detected in the

DIOL	C3	C20
Rb1	0-Glc-Glc	0-Glc-Glc
Rb2	0-Glc-Glc	0-Glc-Arap
Rc	0-Glc-Glc	0-Glc-Araf
Rd (M10)	0-Glc-Glc	0-Glc
Rg3	0-Glc-Glc	OH
Rh2	O-Glc	OH
M9(GYP-XVII)	0-Glc-	0-Glc-Glc
M7(Mb)	0-Glc-	0-Glc-Araf
M6	0-Glc	0-Glc-Arap
M 13 (GYP-LXXV)	OH	0-Glc-Glc
M5 (F2)	0-Glc-	0-Glc-
M3(Mc)	OH	0-Glc-Araf
M2(C-Y)	OH	O-Glc-Arap
M1(C-K)	OH	O-Glc
M 12 (APPD)	OH	OH

TRIOL	C6	C20
Re	O-Glc-Rha	O-Glc
Rg1	O-Glc	O-Glc
Rg2	O-Glc-Rha	OH
Rh1 (M8)	O-Glc	OH
M11 (F1)	OH	O-Glc
M4 (APPT)	OH	OH

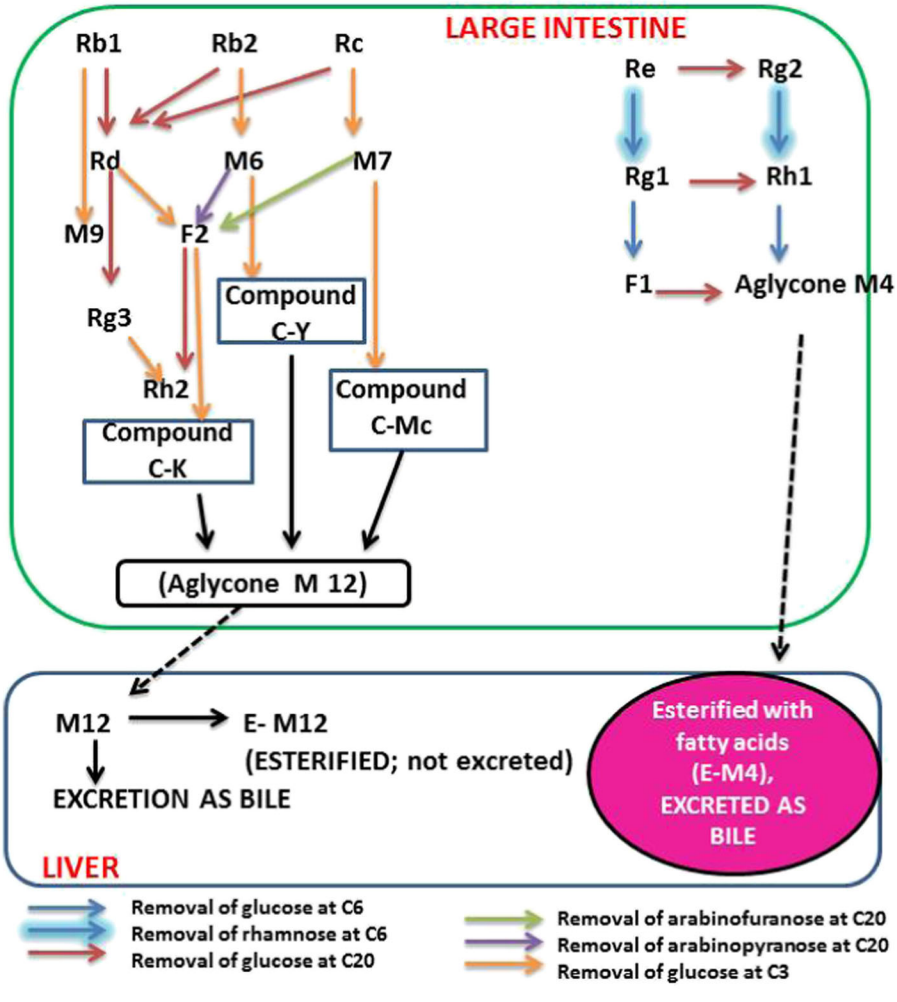


Fig. 3 A schematic representation of the possible metabolic pathways of the major ginsenosides after ingestion in the human alimentary canal. (*Glc*, glucose; *Rham*, rhamnose; *Arap*, arabinopyranose; *Araf*, arabinofuranose; *APPD*, aglycone protopanaxadiol; *APPT*, aglycone protopanaxatriol)

blood plasma and urine in pharmacokinetics studies. A few hours after ingestion of ginseng, gut microbiome active in the large intestine breaks down the monosaccharides one at a time, attached to the C3/C6 and C20 positions of the aglycone moiety. Since the sugar may be removed from either of the carbon positions, multiple degradation pathways arise for a single major ginsenoside as shown in Fig. 3. Ginsenoside Rb1 has "-Glu-Glu" sugar linkage to both the C3 and C20 positions of its protopanaxadiolaglycone. Based on whether the glucose sugar is removed from C3 initially or C20, the first step of ginsenoside Rb1 degradation leads to formation of ginsenoside Rd. (-Glu- removal from C20) or compound M9 also known as gypsenoside XVII (-Glu- removal from C3). Similarly ginsenoside Rd. may again lose a glucose moiety from either of the carbon positions to yield compound M5 also known as compound F2 (-Glu- removal from C3) or ginsenoside Rg3 (-Glu- removed from C20). Compound M9, may alternatively lose glucose from C20 position to yield compound M5. Compound M5 and ginsenoside Rg3 are again subjected to de-glycosylation at C3 to yield compound C-K and ginsenoside Rh2, respectively. In the case of ginsenosides Rb2 and Rc, they possess a terminal arabinopyranose and an arabinofuranose monosaccharide unit at the C20 position of the aglycone. During intestinal degradation, these ginsenosides are almost always observed to lose the glucose from the C3 position initially, to form compound M6 from ginsenoside Rb2 and compound M7 from ginsenoside Rc. Possibly, it may be sterically difficult to remove the "bulky" arabinopyranose and arabinofuranose from the C20 positions of the Rb2 and Rc ginsenosides. Compounds M6 and M7 may further lose another glucose sugar from the C3 position to yield compound C-Y (M2) and compound Mc (M3), respectively. Alternatively in some cases, compound C-Y and Mc may lose their arabinopyranose/arabinofuranose units to yield C-K. Compound C-K, compound C-Y, and compound Mc are the principle intestinal metabolites observed after degradation of the major protopanaxadiols, Rb1, Rb2, and Rc. These

metabolites are reported to exhibit better absorption, bioavailability, and extended half lives in the bloodstream and reportedly manifest the pharmacological effects of ginseng with strong potencies (Park et al. 2010). The fate of these intestinal metabolites is not known in exhaustive detail. It has been hypothesized that they may lose the single glucose unit which they possess to yield compound M12, i.e., the aglycone protopanaxadiol backbone, which may be excreted into bile in the liver.

As compared to the protopanaxadiols, degradation of the protopanaxatriols Re and Rg1 follows a similar but comparatively simpler route. Ginsenoside Re has a –Glu-Rham- sugar linkage to the C6 position and a –Glu- linkage to the C20 of its protopanaxatriol backbone. Alternatively, it may lose glucose from the C20 to yield ginsenoside Rg2 or may lose the terminal rhamnose sugar at the C6 to yield ginsenoside Rg1. The fate of the degradation pathway depends largely on the quality of the gut microflora and their de-glycosylation specificities, steric permissibility, and catalytic efficiency. Ginsenoside Rg1 may again lose a glucose unit from the C6 which may lead to formation of compound F1 (M11) or from C20 to yield ginsenoside Rh1. On the other hand, ginsenoside Rg2 may now lose the terminal rhamnose at its C6 position to lead ginsenoside Rh1 formation. Compound F1 and ginsenoside Rh1 are the principle metabolites obtained from the major protopanaxatriols. They are reportedly excreted from the human body as esters of fatty acids in the bile (Hasegawa 2004).

But the question still remains as to if major ginsenosides can be conveniently broken down to minor ginsenosides, why is there a need to enrich ginseng preparations with the latter. Interestingly, the efficacy of ginseng has been observed to vary among populations and different ethnicities. The answers to the above problems could be found in the metabolism pattern of the ginsenosides. The production of these minor ginsenosides heavily relies on the de-glycosylation efficacy (β-glycosidase/rhamnosidase/arabinase activity) of the intestinal microflora, which further depends on the health of the intestinal microbiome. Intestinal bacteria mainly reported to be involved in such extensive de-glycosylations include *Eubacterium*, *Prevotella*, *Streptococcus*, and *Bifidobacterium* (Park et al. 2010). Based on the different environmental conditions and lifestyle habits, it is common for two unrelated individuals to have a different gut microflora profile. Hence, the pharmacokinetics of the major ginsenosides will vary among the two individuals, in terms of extent of the metabolism, the time lag for the production of these minor ginsenosides, and the subsequent absorption from the intestinal membranes, leading to differences in manifestation of the pharmacological activities. This is one of the major complexities observed with treatment involving ginseng, as this inconsistency can completely defeat the purpose for a certain class of the population.

However, if ginseng extracts are fortified or are enriched with the minor ginsenosides itself, the efficacy will no longer be dependent on the gut microflora to produce these ginsenosides and to exert their effect, and will aid in ensuring uniform medical manifestations within a population. Consequently, ginseng biotechnologists are now concentrating research on these minor ginsenosides with various strategies to supplement ginseng extracts with them, for which enhancing their production in nature is an important pre-requisite.

Biotechnological interventions for production of the minor ginsenosides

Since the minor ginsenosides are obtained after intestinal degradation of the major ginsenosides itself, it was logical to devise strategies that can successfully mimic this biological phenomenon, in a laboratory-controlled environment. Biotechnological techniques that may facilitate stepwise de-glycosylations from the major ginsenosides for production of the desired minor ginsenosides are detailed in the following sections.

Steaming as an age-old traditional strategy to improve ginseng potency

Raw ginseng roots have always undergone a tedious downstream processing before it can be consumed in various forms such as ginseng tea, soups, and other traditional oriental formulations. The raw roots are air-dried (white ginseng), alternatively the roots may be steamed at 90–100 °C for 2–3 h prior to air-drying. Now known as Red ginseng, it has always been considered more potent and pharmaceutically active than white ginseng (Ha et al. 2007). Studies analyzing the changes related to the chemical profile of ginseng after steaming led to detection of less polar ginsenosides such as Rg3, Rh2, Rg5, Rk1, and Rh6 among others, obtained after thermal degradation of the major ginsenosides. These minor ginsenosides were exclusively detected in the red ginseng (Yun et al. 2001; Ha et al. 2007), possibly due to the spontaneous removal of the sugar groups from the major ginsenosides.

Studies in the past decade have further substantiated the fact that steaming process increases the anti-cancer potential of ginseng extracts (Yun et al. 2001; Sun et al. 2011; Wong et al. 2015). Wang and co-workers reported better anti-proliferative activity of heat-processed *P. quinquefolius* roots (when steamed at 120 °C) with decreased Rb1 and Rd. contents and a concomitant Rg3 increase after heat treatment (Wang et al. 2007). Red ginseng has been reported to constitute a higher proportion of Rg3, Rh2, and Rk group of ginsenosides (Ha et al. 2007; Kim et al. 2007a). In another study, it was observed that steamed (at 120 °C for 2 h) *P. notoginseng* roots had a richer Rg3, Rh1, and Rh2 ginsenoside profile as compared to Rb1, Rb2, and Rd. The Rg3 content increased drastically if the treatment was prolonged for another 2 h. This heat-treated red notoginseng extract could effectively reduce

the proliferation of human colorectal cancer cell lines (Sun et al. 2011). Similar observation was also reported for steamed ginseng berries with enhanced Rg3 production under similar steaming protocol which effectively exhibited anti-proliferative activity on human colorectal cancer cell lines (Wang et al. 2006). A majority of the studies concluded that steaming of ginseng roots at 120 °C, as opposed to 100 °C was more effective at enriching Rg3 and Rh2 concentrations with a consequent decrease in Rb1 and Rd., which could be further improved if the duration of steaming was increased. In another interesting study, Choi and his team elucidated a microwave-assisted production of Rg3, Rh2, and Rk group of ginsenosides with improved in vitro anti-cancer activities (Choi et al. 2015). Red ginseng is consistently being reported for its prospective anti-neoplastic behavior (Park et al. 2009, 2011; Lee et al. 2011) due to the enriched presence of the minor ginsenosides. These minor ginsenosides in red ginseng are also reported to exhibit anti-diabetic (Vuksan et al. 2008; Kim et al. 2011a) and anti-hyperlipidemic activity (Kwak et al. 2010), antioxidant properties (Bae et al. 2006a; Kang et al. 2009; Kim et al. 2010, 2011a; Hong and Lyu 2011), pro-apoptotic and induction of a hyper immune response (Park et al. 2011; Lee et al. 2014b), amelioration of cisplatin induced damage (Im et al. 2010; Kim et al. 2014a), alleviation of atopic dermatitis and skin aging (Kim et al. 2009c, 2011b), anti-obesity (Kim et al. 2009a; Lee et al. 2013b), protection from lethal infection by H5N1 influenza virus (Park et al. 2014a), etc. Clearly, this class of compounds holds immense potential as "medicinal miracles," further necessitating exhaustive research into their commercial biosynthesis, in vivo bioactivities, and plausible incorporation into market grade drugs.

However, production of minor ginsenosides via as simple a technique such as steam processing has its own limitations, because of which its commercialization is speculative, in spite of being an extremely easy, less labor, and cost-intensive procedure. The conversion yields are usually low, with the red ginseng being a mixture of major and minor ginsenosides, which might need to undergo extensive quality checks, if commercially prepared, to ensure uniformity in ginsenoside composition. Maintaining the composition could be a limiting factor, as the extent of transformation is extremely sensitive to the steaming temperature and the duration of steam treatment. Moreover, it is also not safe from the phenomena of side reactions, which only adds to the complexity of the procedure (Park et al. 2010).

The search for an active β-glycosidase from microbial systems: biotransformation strategies to convert major ginsenosides to minor ginsenosides by removal of monosaccharides from C3/C6 and C20 positions

As detailed in the previous sections, it is clear that the conversion from major ginsenosides to minor ginsenosides involves subtle "chemical trimming," primarily removal of sugar groups mostly glucose, at the C3 and C20 position of the protopanaxadiols and at C6 and C20 of the protopanaxatriols. Chemically, such de-glycosylations can be facilitated by mild acidic hydrolysis. Indeed, major ginsenosides were observed to be rapidly transforming to Rg3 via mild acid hydrolysis to compound K and the aglycon PPD (Han et al. 1982). However, this methodology could not gain popularity, as the conversion yields were low, resulting in a mixture of major and minor ginsenosides, from which the desired ginsenoside must be purified. These types of reactions are usually poorly selective and result in undesirable side reactions such as epimerization and hydroxylation. Consequently, enzyme-based biocatalytic systems were natural targets for ginseng biotechnologists to facilitate such a transformation. Biological systems offer numerous advantages over chemical procedures, the most important being biological enzyme systems being extremely specific for its substrates, in terms of even their stereochemistry, which leads to higher yields and almost no undesirable side reactions that require elimination from the final product.

A host of microorganisms have been studied for their β-glycosidase enzyme activity and their specificity towards the major ginsenosides in facilitating the desired transformation. The human intestinal microbiome has always garnered significant interest, as primarily it is these microbes which are producing minor ginsenosides from the ingested major ginsenosides in the human intestinal tract. *Bacteroides*, *Bifidobacterium* sp., and *Lactobacillus delbrukeii* (Bae et al. 2004; Chi and Ji 2005; Chi et al. 2005, 2006) are among some common intestinal species investigated for their biotransformation potential. Chi et al. (2005) investigated the efficacy of cell-free preparations of various *Bifidobacterium* and *Leuconostoc* strains in their ability to biotransform Rb1 and Re. They reported a faster degradation rate of Rb1 to compound F2, as compared to bioconversion of ginsenoside Re to Rh1. It required fewer enzymes from cell-free extracts of *Bifidobacterium* sp. Int57, *Bifidobacterium* sp. SJ32, *Aspergillus niger*, and *Aspergillus usamii*. *Lactobacillus delbrueckii* and *Leuconostoc paramesenteroides* transformed Rb1 into Rh2 via Rd. and F2. In another study, they reported that *Bifidobacterium* sp. SH5 transformed Rb2 and Rc into F2, *Aspergillus niger* transformed Rb2 into compound K via compound O and compound Y and ginsenoside Rc into compound K via Mc (Chi et al. 2006). However, as rightly pointed out in an excellent review by Park and team, these microorganisms might not be commercially lucrative, as their cultivation requires expensive medium, which may increase their cost of production (Park et al. 2010). Poor yields and molar conversion ratios leading to poor productivity is another crucial factor deterring their commercial exploitation.

The past decade has witnessed extensive investigation with a host of bacterial and fungal species for their biotransformation capabilities (Table 3). However, it is always preferred if

Table 3 Reported microbial system-based biotransformations (GRAS + non-GRAS) of protopanaxadiols and protopanaxatriols for production of minor ginsenosides

Microorganism	Substrate	Product	Putative enzyme	Conversion efficiency (% molar conversion)	Reference
Protopanaxadiols					
GRAS microorganisms					
Leuconostoc mesenteroides (*KFRI690*)	Rb1	C-K	WC	97.8	Park et al. (2012)
Lactobacillus paralimentarius LH4	Rb1	C-K	Cell-free extract	88	Quan et al. (2013)
Lactobacillus pentosus strain 6105	Rb1	C-K	Purified β-glycosidase	NR	Kim et al. (2012b)
Lactobacillus pentosus DC 101	Rd	C-K	Cell-free extract	87	Quan et al. (2010)
Aspergillus niger	Rg3	APPD via Rh2	β-glycosidase (crude enzyme extract)	100	Liu et al. (2010a)
Aspergillus niger g.848	Ginseng root extract (Rb1, Rb2, Rc, Rd)	C-K	Crude enzyme preparation	69.5	Liu et al. (2015a)
		C-Y		42.4	
		Mc		43.7	
		C-K		69.5	
	Rb1, Rb2, Rc	C-K	Purified β-glycosidase	48.2	Liu et al. (2015b)
	Rb3	Mx		41.2	
Lactococcus lactis (*NZ 9000*)	Rb1	F2	Recombinant β-glycosidase from *Paenibacillus mucilaginosus*	74	Li et al. (2015)
Saccharomyces cerevisiae HJ014	Red ginseng ethanol extract	C-K	Purified β-glycosidase	NR	Choi et al. (2014a)
Non-GRAS microorganisms					
Paecilomyces bainier sp. *229*	Rb1, Rb2, and Rc	C-K	WC	82.6	Zhou et al. (2008)
	Rb1	C-K	Isolated β-glycosidase	NR	Yan et al. (2010)
Microbacterium esteraromaticum (*KCTC 12040BP*)	Rb1	Rg3	Recombinant β-glycosidase expressed in *E. coli* BL21	71	Quan et al. (2012d)
Microbacterium esteraromaticum (*KACC 16318*)	Rb1	C-K	Recombinant β-glycosidase	77	Quan et al. (2012b)
	Rb2	C-K	Recombinant β-glycosidase	NR	Quan et al. (2012a)
Caulobacter leidyia Gp45	Rb1	Compound F2, C-K	WC	91	Cheng et al. (2006)
Acremonium strictum (*AS 3.2058*)	Rb1	C-K	WC	40	Chen et al. (2008)
Achatina fulica China jade snail	Rb1	C-K	β-Glycosidase	NR	Hu et al. (2007)
Microbacterium GS514	Rb1	Rg3	WC	41.4	Cheng et al. (2008)
Terrabacter ginsenosidimutans sp. *Gsoil 3082*	Rb1	Gyp XV11 Gyp LXXV C-K	Recombinant β-glycosidase expressed in *E. coli EPI300-T1*	NR	An et al. (2010)
Penicillium oxalicum sp. *68*	Rb1	C-K	β-Glycosidase	87.7	Gao et al. (2013)
Leuconostoc and *F. johnsoniae*	Rc, Rb1	Rg3	Combination of recombinant α arabinofuranosidase and β-glycosidase	88.3	Kim et al. (2013d)
Pyrococcus furiosus (*DSMZ3638*)	Rb1, Rb2, Rc, Rd	APPD via C-K	Recombinant β-glycosidase expressed in *E. coli ER2566*	82.5	Yoo et al. (2011)
Esteya vermicola (*CN120806*)	Rg3	Rh2	Cell-free extract	90.7	Hou et al. (2012)
Sulfolobus acidocaldarius	Rb1	C-K	Thermostable β-glycosidase	94	Noh and Oh (2009)
	Rb2	C-Y		80	
	Rc	Mc		100	
Sulfolobus sulfotaricus	Ginseng extract	C-K	Recombinant β-glycosidase expressed in *E. coli ER2566*	80	Noh et al. (2009)

Table 3 (continued)

Microorganism	Substrate	Product	Putative enzyme	Conversion efficiency (% molar conversion)	Reference
Sphingopyxis alaskensis	Rb1 Rb2 Rc Rd. Rg3	Gypenoside XVII compound O compound Mc F_2 Rh2	Recombinant β-glycosidase	6.8 gL^{-1} h^{-1} Gyp XVII productivity and Rg3 to Rh2 converted with 89% molar conversion yield	Shin and Oh (2014)
Dictyoglomus turgidum (*DSMZ 6724*)	Rb1 Rb2 Rc	APPD C-Y Mc	Recombinant β-glycosidase expressed in *E. coli* ER2566	100	Lee et al. (2012)
Flavobacterium sp. *BGS36*	Rd	Rg3	Actively growing bacterial culture	NR	Ten et al. (2014b)
Paenibacillus mucilaginosus (*KCTC3870*)	Rb1	F2	Recombinant β-glycosidase expressed in *E. coli* BL21	95.7	Cui et al. (2014)
Caldicellulosiruptor saccharolyticus, *Sulfolobus acidocaldarius*	Rb2 Rc	C-K	Recombinant α-l-arabinofuranosidase and/or β-galactosidase from *C. saccharolyticus* with β-glucosidase from *S. acidocaldarius*	388 and 328 mg L^{-1} h^{-1} (productivity) respectively	Shin et al. (2013)
Fusarium proliferatum (*ECU2042*)	Rg3	Rh2	Purified β-glycosidase	NR	Su et al. (2009)
Leuconostoc sp. *BG78*	Rc	Rg3	β-Glycosidase	70–75	Ten et al. (2014a)
Fusarium sacchari	Rb1 Rb3 Rc	C-K Mx Mc	Actively growing fungal cell broth	82.2 90 57.3	Han et al. (2010)
Thermotoga thermarum (*DSM 5069*)	Rb1	Rg3	Recombinant β-glycosidase expressed in *E. coli*	97.2	Pei et al. (2015)
Cordyceps sinensis (*CICC14017*)	Rb1	C-K	Mycelia mediated	82	Wang et al. (2015b)
Sulfolobus solfataricus (*DSM 1617*), *Caldicellulosiruptor saccharolyticus* (*DSM 8920*)	Red ginseng	C-K	Recombinant β-glycosidase, α-L-arabinofuranosidase expressed in *E. coli* ER2566	100	Shin et al. (2015)
Protopanaxatriol					
Food grade					
Aspergillus niger	Rf	Rh1	Recombinant β-glycosidase expressed in yeast (MGY70)	NR	Ruan et al. (2009)
	Rf	APPT	Crude enzyme extract	90.4	Liu et al. (2010a)
Non food grade					
Microbacterium esteraromaticum (*KACC16318*)	Re Rg1	Rg2 Rh1	Recombinant β-glycosidase expressed in *E. coli* BL21	100 78	Quan et al. (2012c)
Sanguibacter keddieii	Rg1	F1	Recombinant β-glycosidase expressed in *E. coli* BL21	100	Kim et al. (2012b)
Fusarium moniliforme var. *subglutinans* (*KCTC6149*)	Rg1	F1	Purified β-glycosidase	100	Kim et al. (2011c)
Pyrococcus furiosus (*DSMZ 3638*)	Rf Re	Rh1 Rg1	*Recombinant β-glycosidase* expressed	100 55	Oh et al. (2014)

Table 3 (continued)

Microorganism	Substrate	Product	Putative enzyme	Conversion efficiency (% molar conversion)	Reference
	Rg2	Rh1	in *E. coli* ER2566	40	
Pseudonocardia sp. *Gsoil1536*	Re	Rg2	Recombinant β-glycosidase expressed in *E. coli* BL21	87.1	Du et al. (2014)
Dictyoglomus turgidum (*DSM6724*)	Ginseng root extract (R1, Rf, Rg1)	APPT	Recombinant β-glycosidase expressed in *E. coli* ER2566	81.2	Lee et al. (2014a)
Cellulosimicrobium sp. *21*	Re	Rg2	Secreted β-glycosidase	89.8	Gao et al. (2015)
Thermoanaerobacterium thermosaccharolyticum (*DSMZ 571*)	R1, R2	Rg1, Rh1	Purified β-xylosidase	100	Shin et al. (2014)
Protopanaxadiol + protopanaxatriol					
Actinosynnema mirum (*KACC20028*)	Rb1	C-K	Recombinant β-glycosidase expressed in *E. coli* BL21	NR	Cui et al. (2013)
	Re	Rg2			
	Rg1	APPT			
Penicillium aculeatum (*KCTC 6245*)	Rb_2	C-Y	Purified β-glycosidase	98	Lee et al. (2013a)
	Rc,	Mc		100	
	Rd.	C-K		94	
	Rg_3	APPD		46	
	Rg_1	F1		100	
	Rf	APPT		90	
Cladosporium cladosporioides (*GH21*)	Rb1	C-K	WC	74.2	Wu et al. (2012)
	Rg1	F1		89.3	

WC whole cell-based biotransformation, *NR* not reported, *APPD* aglyconeprotopanaxadiol (M12), *APPT* aglyconeprotopanaxatriol (M4)

the microbe under question is "food grade" or is listed under "GRAS" (generally regarded as safe) category, as ultimately, ginseng or its derivatives are orally consumed and their safety towards human consumption is a prime concern. Among the "food grade" microorganisms, *Lactobacillus pentosus* have been found to facilitate conversion of diols to C-K with a high transformation yield of around 87% (Quan et al. 2010; Kim et al. 2012b). Another GRAS classified, *Leuconostoc mesenteroides*, in a whole cell-mediated biotransformation, led to a 97.8% conversion yield of Rb1 to C-K (Park et al. 2012). A purified β-glycosidase from *Aspergillus niger* was reported to facilitate a similar conversion, however, with a lower yield of 41.2 (Liu et al. 2015b). Interestingly, in a previous study with the use of the same microbial species, a glycosidase present in the enzyme extract was reported that could biotransform Rg3 to Rh2 with 100% conversion efficiency (Liu et al. 2010a). Another remarkable observation, encountered while preparing the review, was the fact that microbial biotransformations were mostly accepting the diols as their substrate. The triols were accepted by comparatively limited systems, a majority of which are considered "non-food grade." Consequently, extensive literature exists for biotransformation of the protopanaxadiols as compared to the successful transformation of the triols. The possible reason for such an observation may be differences in the stereochemistry of the diols and the triols, as it is hypothesized that it might be difficult to remove sugar groups at the stereochemically "unfavorable" C6 position of the protopanaxatriols. Successful examples, particularly with the use of "food grade" microbes, are extremely limited. However, an *Aspergillus niger* system has been reported to facilitate Rf conversion to Rh1 (Ruan et al. 2009) and in some studies the de-glycosylation continues till the production of the non-glycosylated protopanaxatriol aglycone (Liu et al. 2010a; Table 3).

On the contrary, "non food grade" classified microorganisms are reported extensively for their protopanaxadiol bioconversion efficiency. Theoretically at least, what is required is an active β-glycosidase enzyme, whether it is present in a cell-free preparation, a purified enzyme system, or may be even as a recombinant enzyme system. In some cases, the enzyme may be localized intracellularly, whereby a whole cell-mediated biotransformation may be proposed. The substrates can be added exogenously to an actively growing culture broth (Cheng et al. 2008; Zhou et al. 2008; Wu et al. 2012). The hypothesis behind this line of experimentation is to facilitate metabolism of the glucose units on the aglycone backbone of the ginsenosides by the actively growing culture as a source of sugar. Zhou and co-workers reported 82.6% conversion yield of Rb1, Rb2, and Rc to C-K, with a *Paecilomyces bainier* culture broth (Zhou et al. 2008). A marginally improved yield of 91% molar conversion efficiency of Rb1 to C-K was reported with *Caulobacter leidyia* culture broth within 48 h of substrate addition (Cheng et al. 2006). However, success with whole cell-mediated biotransformation of protopanaxatriols is still awaited. Cell-free extracts have also been investigated for their putative biotransformation potential. Quan and co-workers reported 87% bioconversion potential of cell-free extract of *Lactobacillus pentosus*, facilitating Rd. to C-K conversion (Quan et al. 2010). An *Esteya vermicola* cell-free extract was also reported to mediate bioconversion of Rg3 to Rh2 with a 90.7% yield at 50 °C, pH 5.0 and a substrate concentration of 3 mg mL^{-1} (Hou et al. 2012).

Whole cell-mediated/cell-free extracts-based biotransformations are advantageous as they are relatively simple and most importantly, do not require specific enzyme purification which is a significant labor and cost incurring process. However, optimized substrate uptake by whole cells for the substrate and the enzyme to encounter each other is a limiting factor which may be one of the reasons for the observed low product yields. Cell-free extracts, on the other hand, solve this problem to a large extent; however, yield may significantly alter with changes in physical conditions like pH and temperature, and maintenance of an active enzyme in cell-free extracts for long durations is technically cumbersome.

Recombinant and purified enzyme systems have been extensively used to biotransform ginsenosides with yields as high as 95–100%. A host of microbial systems have been screened for development of a recombinant β-glycosidase such as *Microbacterium esteraromaticum* (Quan et al. 2012a, b, d), *Penicillium oxalicum* (Gao et al. 2013), *Arthrobacter chlorophenolicus* (Park et al. 2014b), *Sulfolobus* spp. (Noh et al. 2009; Noh and Oh 2009), and *Pyrococcus furiosus* (Yoo et al. 2011) (Table 3). These recombinant enzyme systems are exhaustively characterized for their substrate specificity and optimum physical conditions like pH and temperature of the assay. The optimum pH for the enzyme assays generally range from 5 to 6.5 and the assay temperature rarely exceeding 40 °C (Park et al. 2010).

Another interesting feature of the β-glycosidase enzyme system is the difference in their ease towards removal of sugar groups at three different carbon positions, i.e., C3 or C20 for the diols and C6 or C20 for the triols. Some enzyme systems may initiate de-glycosylation from the C3/C6 position and eventually from C20, whereas for other systems, it might function in reverse. Consequently, multiple pathways may exist for the degradation of a particular ginsenoside based on the stereospecific preference of a particular enzyme system for sugars at the C3/C20 position (for the diols) or C6/C20 position (for the triols). This fact also explains the presence/absence or concomitant presence of two intermediates belonging to two different hydrolytic pathways for a particular ginsenoside, with a single enzyme system.

Similar to ginsenoside metabolism in the human gut, use of enzyme systems also lead to production of minor ginsenosides via a similar pathway. In the case of ginsenoside Rb1, based on the stereochemical freedom of the enzyme system, glucose unit might be removed from either the C3 or C20. Removal of

glucose unit from the C3 position leads to the following degradation pathway: Rb1→Gp-XV11→compound F2→C-K (An et al. 2010; Yan et al. 2010). Alternatively, removal of sugar from C20 position follows a degradation pathway leading to ginsenoside Rd. formation (Chen et al. 2008; Noh et al. 2009; Yoo et al. 2011). Again, based on the removal of glucose from C3/C20 position, the degradation may follow two paths: degradation of ginsenoside Rd. may proceed via compound F2 to C-K (Chen et al. 2008; Noh et al. 2009), or to Rh2 via formation of Rg3 (Quan et al. 2012d; Kim et al. 2013d).

Ginsenoside Rb2 and Rc have an outer arabinopyranose or arabinofuranose unit at the C20 position. In case of whole cell-based biotransformations using active culture, inherent pyranosidase/furanosidase activity may be difficult to obtain; however, Zhou and team have reported the removal of these bulky sugar groups to yield ginsenoside Rd. using whole cell culture of *Paecilomyeces bainier*. The study further reported formation of either C-K/Rh2 from ginsenoside Rd. (Zhou et al. 2008). A recombinant β-glycosidase from *Pyrococcus furiosus* also demonstrated Rb2/Rc conversion to Rd., however with comparatively low efficiencies (Yoo et al. 2011). Consequently, the yield of downstream compound C-K formation also decreases. Recently researchers have investigated the combinatorial use of a recombinant "designed" enzyme system with arabinopyranosidase/furanosidase activity along with β-glycosidase activity, to facilitate the degradation of Rb2 and Rc, comparatively more efficiently than a single enzyme system, with improved yields as high as 100% molar conversion (Kim et al. 2013d; Shin et al. 2013).

Alternatively, Rb2/Rc have also been reported to lose glucose units from the C3 position initially, to form compound C-Y or compound Mc, following similar degradation pathways as was detailed in the ginsenoside metabolism section (Noh and Oh 2009; Shin and Oh 2014), which in some cases, may further proceed to lose the arabinopyranose/furanose, to yield compound C-K (Liu et al. 2015b). This particular degradation route facilitated by C-Y/Mc is the more common pathway observed, when Rb2/Rc ginsenosides are subjected to biotransformations, thereby suggesting that it might be "favorable" to remove sugar units at the C3 position, as compared to the C20 position for enzyme systems (Cui et al. 2013; Yoo et al. 2011). Interestingly, Shin and co-workers isolated a β-glycosidase from *Thermus thermophilus* which only exhibited specific hydrolysis of outer sugar at exclusively the C20 position. It converted Rb1 to Rd., GpXV11 to F2, and GpLXXV to C-K with 100% molar conversion. As expected, these products were not hydrolyzed any further (Shin et al. 2014).

Successful microbial-based bioconversions in the case of the protopanaxatriols are comparatively fewer, further limiting the discovery of alternate degradation pathways and intermediates. As compared to the diols, they have fewer sugar groups attached at their C6/C20 position, leading to production of fewer intermediates upon de-glycosylation.

Ginsenosides Re and Rg2 have a "Glu-Rhamnose" sugar linkage at the C6 position. An inherent rhamnosidase activity is essential in the enzyme system used for biotransformation, to completely facilitate its breakdown into its corresponding aglycone. Ginsenoside Re may lose the rhamnose at the C6 position to yield ginsenoside Rg1 (Oh et al. 2014). Ginsenoside Rg1 has a single glucose unit at both C6 and C20 positions. Based on the position of the sugar group removed by the enzyme system, the bioconversion of ginsenoside Rg1 pathway may follow two paths, i.e., either to Rh1 to its PPT aglycone (Quan et al. 2012c; Cui et al. 2013) or via compound F1 to its PPT aglycone (Kim et al. 2011c; Kim et al. 2012a). Alternatively, ginsenoside Re may lose the glucose at the C20 to yield ginsenoside Rg2 (Quan et al. 2012c; Gao et al. 2015). Provision of multiple pathways for bioconversion of ginsenosides by enzyme based systems must be accounting for the significantly high yields that are observed.

A few examples exist for enzyme systems that allow for enough stereochemical flexibility that successfully converts both the diols and triols simultaneously (Table 3). An interesting study by Wu et al. (2012) reported co-transformation of Rb1 and Rg1 when added exogenously to an actively growing *Cladosporium cladosporoides* culture broth with 74.2 and 89.3% conversion yield, respectively. The study also reported that in the absence of Rg1, Rb1 can be metabolized by removal of sugar from either the C3 to yield Gyp XV11 or C20 to yield Rd. However, in the presence of Rg1, only ginsenoside Rd. was detected whereas ginsenoside Rg1 was de-glycosylated at the C6 position to yield compound F1. The enzyme system demonstrated variable affinities with removal of Glu from C20 for the diols and from C6 in the case of triols. Similar efforts by recombinant β-glycosidase (BglAm) from *Actinosynnema mirum* could hydrolyze the outer and inner glucose moieties at the C3 and C20 positions of the protopanaxadiol-type ginsenosides Rb1, Rb2, Rc, Rd., gypenoside XVII to produce protopanaxadiol via gypenoside LXXV, F2, and Rh2 via multiple pathways. Rb2 and Rc were converted to C-Y and Mc, but not further as the enzyme lacked arabinopyranosidase/furanosidase activity. Ginsenoside Re was converted to Rg2 and Rg1 aglycone PPT via Rh1 (Cui et al. 2013).

Microorganisms screened for their β-glycosidase activity are mostly the ginseng rhizosphere-associated microbiome or those isolated from ginseng plantations. Many such novel bacterial strains are being characterized with ginsenoside hydrolyzing potential. These bacterial strains facilitate ginsenoside conversion via different degradation pathways. *Flavobacterium panaciterrae* (Jin et al. 2014) and *Novosphingobium ginsenosidimutans* (Kim et al. 2013e) have been reported to convert Rb1 to F2 via Rd. as an intermediate. Rb1 conversion to GypXV11 was observed with the novel strains *Sphingomonas ginsenosidimutans* (Choi et al. 2010) and *Terrabacter ginsenosidimutans* (An et al. 2010). *Intrasporangium* sp. isolated from soil of ginseng plant was also reported to facilitate such a conversion (Cheng et al.

2007). Rb1 to C-K conversion was observed with a novel *Mucilaginibacter ginsenosidivorax* (Kim et al. 2013c) and *Sphingobacterium ginsenosidimutans* (Son et al. 2013). A comprehensive study based on screening of a host of ginseng soil-associated microbiome led to the conclusion that Rb1-CK conversion is best observed with *Sphingomonas* spp., *Burkholderia* spp., *Luteibacter* spp., and *Streptomyces* sp. (Fu et al. 2014).

Many commercial enzyme sources have also been investigated for their biotransforming potency. Cellulase enzyme commercial preparation (Cellulase 12 T), at a dose of 3.67%, could enrich Rg3 content by 4-fold in white ginseng extract within 72 h (Chang et al. 2009). Choi et al. (2014b) explored a range of enzymes for their ginsenoside converting potential in amylase (Spezyme and Optidex) hydrolyzed ginseng extract. Rapidase, from *Aspergillus niger* and *Trichoderma longibrachiatum*, with pectinase and cellulose activity, was reported to facilitate ginseng saponin conversion to aglycones. In another study, artificial gastric juice was observed to increase de-glycosylations in notoginseng extracts. Around 80 intermediates were detected, with 16 novel dammaranes reported, which exhibited better cytotoxicity towards cancer cells (Wang et al. 2014a).

Another commonly employed strategy is the combinatorial use of steaming and fermentation to improve minor ginsenoside yields. Hong et al. (2011) allowed fermentation of red ginseng extract with a fungus, *Monascus pilosus*. Deglycosylated ginsenosides such as Rh1, Rh2, and Rg3 were comparatively enriched along with the additional synthesis of monakolin K (a fungal derived metabolite with reported cholesterol lowering properties), following the fermentation procedure. Rg3 from black ginseng was converted to aglycone PPD with 100% molar efficiency using a combination of steaming and biotransformation treatments with *Aspergillus niger* (Liu et al. 2010b).

Production of ginsenosides via heterologous expression of the biosynthetic genes

Ginsenoside biosynthesis, particularly the dammaranediol skeleton and the aglycones protopanaxadiol and protopanaxatriols, has been accomplished via synthetic biology. Artificial biosynthetic pathways have been constructed in a variety of organismal systems, with most common being Baker's yeast, i.e., *Saccharomyces cerevisiae*. Table 4 summarizes last 10 years reports that involve production of ginsenosides in heterologous systems.

Bakers' yeast (*Saccharomyces cerevisiae*) has been extensively characterized in terms of genetics and physiology and with the advent of multiple genetic engineering tools, this microorganism is an ideal host for the heterologous production of valuable natural products (Dai et al. 2014). Additionally, this system is exclusively preferred because of its "food grade" applicability as a leavening agent in bread and bakery products, where it converts fermentable sugars that are present in dough into carbon dioxide and ethanol. Since the biosynthesis of 2,3-oxidosqualene is critical for biosynthesis of ginsenosides, often squalene epoxidase and dammaranediol synthase genes, which catalyzes conversion of dammaranediol from 2,3-oxidosqualene, have been frequently expressed in yeast (Dai et al. 2013; Wei et al. 2015; Zhao et al. 2016). Dammaranes have also been reported (8.63 mg L^{-1}) to be expressed in an *Escherichia coli* system whereby the dammarenediol-II biosynthetic pathway was engineered via co-expression of squalene synthase (SS), squalene epoxidase (SE), NADPH-cytochrome P450 reductase (CPR) from *Saccharomyces cerevisiae*, and SE from *Methylococcus capsulatus* (McSE), NADPH-cytochrome P450 reductase (CPR) from *Arabidopsis thaliana* along with dammaranediol synthase from *P. ginseng* (Li et al. 2016). Liu et al. (2015c) have reported production of dammaranediol in *Pischia pastoris*, whereby they modulated the metabolic flux to improve 2,3-oxidosqualene biosynthesis and reducing its competitive requirement for lanosterol synthesis. They facilitated upregulation of *ERG1* gene responsible for 2,3-oxidosqualene biosynthesis, and simultaneously repressing the *ERG7* gene responsible for 2,3-oxidosqualene bioconversion to lanosterol. The yield of dammaranediol was observed to increase by 3.6-fold, which was further improved by precursor feeding in terms of squalene addition to the growth medium.

Dammaranediol have also been reported in transgenic tobacco whereby the dammarenediol-II synthase gene (*PgDDS*) isolated from *P. ginseng* was introduced into the *Nicotiana tobacum* genome under the control of 35S promoter by *Agrobacterium*-mediated transformation. An organ-specific accumulation of dammaranediol was observed with a particular transgenic line 14 (T14) exhibiting a high amount (157.8 μg g^{-1} DW) of dammarenediol-II in the roots. An almost 4-fold increase in dammaranediol biosynthesis was observed from cell suspensions derived from root segments of line T14 (Han et al. 2014). Gwak et al. (2017) have recently reported generation of transgenic tobacco lines expressing CytP450 protopanaxadiol synthase (*CYP716A47*) and UDP-glycosyltransferase (*UGT71A28*) in addition to Pg DDS. Due to the co-expression of the set of genes, dammarane diol was successfully converted to the aglycone protopanaxadiol which was glycosylated at C20 position to yield an important minor ginsenoside, i.e., compound C-K. Interestingly, production of CK in tobacco led to stunted plant growth and abnormal pollen development in transgenic tobacco. Similar deterioration in the health of the host organism due to the presence of ginsenoside precursors was reported in *Saccharomyces cerevisiae* (Zhao et al. 2016) co-expressing *a P. ginseng* dammarenediol-II synthase, a *P. ginseng* cytochrome P450-type protopanaxadiol synthase (*PPDS*), and a *Arabidopsis thaliana* NADPH-cytochrome P450 reductase (*ATR1*). The

Table 4 Ginsenoside production in heterologous systems

Gene	Organism	Ginsenoside type produced	Amount produced	Reference
UDP-glycosyltransferase (PgUGT74AE2 and PgUGT94Q2)	Yeast (*S. cerevisiae*; CEN. PK 2-1D)	Rg3	1.3 mg L^{-1}	Jung et al. (2014)
UDP-glycosyltransferase (UGTPg 45 and UGTPg 29)	Yeast (*S. cerevisiae*)	Rg3 and Rh2	Not available	Wang et al. (2015a)
Dammarenediol-II synthase and *protopanaxadiol synthase* genes of *Panax ginseng* *NADPH-cytochrome P450 reductase* gene of *Arabidopsis thaliana*	Yeast (*S. cerevisiae*)	Aglycone protopanaxadiol	0.05 mg g^{-1} DCW	Dai et al. (2013)
UDP-glycosyltransferase (UGTPg 100 and UGTPg1)	Yeast (*S. cerevisiae*)	F1 and Rh1	42.1 and 92.8 mg L^{-1}, respectively	Wei et al. (2015)
Dammarenediol-II synthase, *cytochrome P450-type protopanaxadiol synthase* (PPDS) from *P. ginseng* and a *Arabidopsis thaliana NADPH-cytochrome P450 reductase* (ATR1)	Yeast (*S. cerevisiae*)	Aglycone protopanaxadiol	1436.6 mg L^{-1}	Zhao et al. (2016)
Oleanolic acid synthase from *Medicago trunculata*, *dammarenediol-II synthase*, *protopanaxadiol synthase*, *protopanaxatriol synthase* from *P. ginseng* and *NADPH-cytochrome P450* from *A. thaliana*	Yeast (*S. cerevisiae*) strain GY-1	Aglycone PPD, PPT, and oleanolic acid	17.2, 15.9, 21.4 mg L^{-1}, respectively	Dai et al. (2014)
Dammaranediol synthase (*PgDDS*) CytP450 protopanaxadiol synthase (*CYP716A47*) UDP-glycosyltransferase (*UGT71A28*)	Tobacco	Compound K	1.55–2.64 μg g^{-1} DW	Gwak et al. (2017)
Dammaranediol synthase (*PgDDS*) CytP450 protopanaxadiol synthase (*CYP716A47*)	Transgenic tobacco cell suspension	Aglycone PPD	125.9 μg g^{-1} DW	Chun et al. (2015)
Dammaranediol synthase (Pg DDS)	Transgenic tobacco cell suspension	Dammaranediol	5.2 mg L^{-1}	Han et al. (2014)
Squalene synthase (SS), *squalene epoxidase* (SE), *NADPH-cytochrome P450 reductase* (CPR) from *Saccharomyces cerevisiae* SE from *Methylococcus capsulatus* (McSE), *NADPH-cytochrome P450 reductase* (CPR) from *Arabidopsis thaliana*; *Dammaranediol synthase* (*Pg DDS*)	*E. coli*	Dammaranediol	8.63 mg L^{-1}	Li et al. (2016)
Dammaranediol synthase (Pg DDS)	*Pischia pastoris*	Dammaranediol	1.073 mg g^{-1} DCW	Liu et al. (2015c)

health of the yeast cells was significantly affected by reactive oxygen species released by the pool coupling between PPDS and ATR1. To improve pathway flux through PPDS, a PPDS-ATR1 fusion construction was attempted which conferred approximately 4.5-fold increase in catalytic activity, and 71.1% increase in protopanaxadiol production compared with PPDS and ATR1 co-expression.

Heterologous expression till the production of aglycone protopanaxadiol has been frequently reported. However, production of the ginsenosides from these aglycones requires presence of UDP-glycosyltransferases that catalyze regioselective transfer of sugar units to these aglycones. UGTs are enzymes that transfer a sugar from UDP-sugar to various metabolites, including hormones and secondary metabolites. UGTs typically act at the final steps of biosynthetic pathways to increase solubility, stability, storability, bioactivity, or bioavailability of metabolites (Jung et al. 2014). However, knowledge of the respective UGT's operative in the biosynthesis of the different ginsenosides is not extensively known. A few of these UGTs have been characterized and co-expressed in yeast systems. Co-expression of PgUGT74AE2 and PgUGT94Q2 has been observed to lead to de novo Rg3 biosynthesis (Jung et al. 2014) and UGTPg 100 and UGTPg1 to ginsenoside F1 and Rh1 biosynthesis (Wei et al. 2015).

Although these systems can be potentially utilized for large-scale production of dammaranediol and aglycone protopanaxadiols, however, these systems will have limited usage for production of specific ginsenosides, at least not until the different UGTs responsible for biosynthesis of the different ginsenosides are not identified and characterized. This strategy is a promising tool for production of minor ginsenosides and efforts oriented towards identification of UGTs and their successful co-expression in heterologous systems without compromising their health require appropriate scientific attention.

Elicitations as a strategy to induce production of the minor ginsenosides

In vitro ginsenoside production has been frequently tampered with a host of elicitors, the most common being methyl jasmonate. However, production of minor ginsenosides such as Rg3 and Rh2, induced by elicitation, is rare. In a study published by Kim et al. (2013f), Rg3 biosynthesis was observed after 7 days treatment of *P. ginseng* hairy roots with methyl jasmonate, to the tune of 0.42mg g^{-1} DW. The cellular accumulation of ginsenoside Rh2 was comparatively less, as majority of the flux was diverted towards production of Rg3, which was stably accumulated within the cell. The proteins isolated from the MJ-treated hairy roots exhibited an enhanced catalytic activity of Rh2 conversion to Rg3, further corroborating the observed results.

Our team has been working in *Panax* biology since the last 15 years, particularly in *P. quinquefolius* and *Panax sikkimensis*, the latter being an important Indian congener of ginseng. The team has reported hairy roots in *P. quinquefolius* (Mathur et al. 2010a) and also elucidated a somatic embyogenesis protocol in *P. sikkimensis* (Mathur et al. 2001a). The host lab has also patented a high ginsenoside yielding cell line of *P. quinquefolius* (US PATENT 6326202; Mathur et al. 2001b) and an anthocyanin yielding cell line of *P. sikkimensis* (US PATENT 6368860; Mathur et al. 2002) and reported ginsenoside and anthocyanin production from their cell suspensions (Biswas et al. 2015, 2016).

Since the last 2 years, we have been involved in upregulating ginsenoside biosynthesis in cell suspensions of these two lines using a host of abiotic and biotic elicitors. We have successfully identified key elicitors that not only increases ginsenoside production, by almost 3-fold, but could also induce production and exudation of minor ginsenosides, i.e., Rg3 and Rh2 (Biswas et al. 2016). Additionally, application of a *Trichoderma harzianum*-derived elicitor led to 8.1 mg L^{-1} production and exudation of Rh1. Rh2 production, to the tune of 4.0 mg L^{-1}, was observed with the use of hydrogen peroxide elicitor. On the other hand, salicylic acid application has been observed to induce around 7.0 mg L^{-1} Rg3 exudation into medium (Biswas 2016).

Conclusion

Even though the database regarding production of minor ginsenosides is huge, and seems to be expanding at a furious pace, unfortunately, biotechnologists are still not even close to defining an optimized system, standardized parameters or technologies that may help in commercializing the enrichment of minor ginsenosides in ginseng samples. This could be due to the tremendous complexity and the cross-linking of many factors that govern ginseng chemistry and pharmacology. The age-old technique of steaming may have been ethnobotanically validated; however, it may not be suitable enough to be conveniently commercialized. It is an extremely "raw" technique, where it is difficult to maintain desirable physical conditions, to prevent the naturally occurring side reactions and maintain the ginsenoside consistency of the end product.

Biotransformations using microbial enzyme systems have shown immense promise; however, most of the microbial strains used are non food grade, which is the biggest hindrance towards development of any resultant technology. Use of GRAS or food grade microbes is highly recommended for biotransformation of ginsenosides which are present in food items, enriching the nutritional content in a simple and cost-effective manner which is also very safe for consumption. But these strains tend to exhibit poor productivity as opposed to non food grade microbes. Whole cell systems are often used

for biotransformation purposes, but the major factor limiting their use is the biomembrane barrier which the substrate needs to cross over, in order to access the intracellular enzymatic machinery. The process can be made for improved and better conversion yields, if the cells are permeabilized and if the enzyme system can be made to leach out extracellularly or be actively transported to the extracellular domain of its outer membrane, whereby it has an easy and unrestricted access to the substrate.

An active β-glycosidase system with flexible stereospecificity for ginsenoside hydrolysis, theoretically at least, is what is required for a successful major to minor conversion. Ginsenosides, however, in addition to glucose, also possess xylose, arabinofuranose, and rhamnose sugars attached to the aglycone structures such as Rb2, Rc, or Re. A combination of β-glucosidase, β-xylosidase, α-L arabinofuranosidase, and α-L rhamnosidase is actually required, if these ginsenosides are also to be converted to their aglycones. It is precisely, because of this reason, that most enzyme systems, comprising of β-D glucosidase only accept the terminal glucose present in the alkyl chains on C3 and C20 positions of ginsenoside Rb1, as the substrate, and exhibit no activity towards Rb2 and Rc. Possibly because of this essential requirement, commercial enzyme systems such as pectinases and cellulases, with bond cleavage specificities, failed to achieve commercial popularity. Purified enzyme systems, even though might be exhibiting high specificity, but face inherent problems such as low enzyme abundance in the cells and difficult purification procedures.

On the other hand, recombinant enzyme systems, offering several advantages over native enzymes such as ease of purification and industrial upscaling and abundant copies of the enzyme, can hydrolyze several ginsenosides simultaneously. They also exhibit simpler purification methodologies and high conversion efficiencies. Targeted research in this direction requires construction of multi enzyme systems translated from multiple gene expression "cassette" consisting of genes encoding for β-glucosidase, β-xylosidase, α-L arabinofuranosidase, and α-L rhamnosidase. They may prove to be extremely beneficial to hydrolyze simultaneously the different classes of ginsenosides. It will be an additional benefit if these gene cassettes may be efficiently expressed in GRAS microbial hosts, in order to eliminate the complexities of toxicity and user safety associated with non-GRAS microbes.

Elicitation strategies are also of potential application, which could be comparatively, one of the cheapest technologies for mass production of these minor ginsenosides as opposed to development of a recombinant enzyme system technology. Presence of minor ginsenosides such as C-K, C-Y, Rg3, Rh2, and Rh1 is rare *in planta*. Not much is known about the factors regulating their biosynthetic pathway and flux transfer. It is however, well established that UGTs or UDP-glycosyltransferases play a key role in inter-conversion from these minor ginsenosides to major ginsenosides via sugar transfer from UDP-glucose. However, the "transient presence" of these minor ginsenosides, during ginsenoside biosynthesis, could probably be due to their being "intermediates," and they are rapidly utilized by glycosyltransferases to synthesize the major ginsenosides. It is a probability that the copy number of these glycosyltransferases may be several times higher than the glycosyltransferases that synthesize the minor ginsenosides from dammaranediol, resulting in a brisk turnover of the minor ginsenosides to the major ginsenosides. Elicitation with biotic and abiotic factors, as was reported in our study (Biswas et al. 2016), might be modulating this delicate balance between the two different glycosyltransferases, favoring the production of the minor classes, in co-ordination with changes in expression pattern of the upstream genes as well. However, it is speculative and definitive evidence is still awaited. However, ginsenosides, which are present in minute amounts in vivo, will also be difficult to induce in vitro in ginseng systems and will require exhaustive elicitation regimes and thorough optimizations regarding dosage and application duration for a better fraction of these minor ginsenosides. However, still much needs to be investigated along the pharmacological front, and only once we have a clearer picture regarding how these ginsenosides, both minor and major, interact with each other, to manifest a particular response, will we be able to effectively intervene to maximize and fine tune the production of the desirable ginsenosides.

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Compliance with ethical standards

Conflict of interest Tanya Biswas declares that she has no conflict of interest.

Ajay Kumar Mathur declares that he has no conflict of interest.

Archana Mathur declares that she has no conflict of interest.

Ethical compliance This article does not contain any studies with human participants or animals performed by any of the authors.

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