2. Rao, K. P. C., Verchot, L. V. and Laarman, J., Adaptation to climate change through sustainable management and development of agroforestry systems. *SAT eJournal*, 2007, **4**, 1–30.
3. Sheikh, A. Q., Skinder, B. M., Pandit, A. K. and Ganai, B. A., Terrestrial carbon sequestration as a climate change mitigation activity. *J. Pollut. Effects Control*, 2014, **2**, 110; doi:10.4172/jpe.1000110.
4. Calfapietra, C., Gielen, B., Karnosky, D., Ceulemans, R. and Mugnozza, G. S., Response and potential of agroforestry crops under global change. *Environ. Pollut.*, 2010, **158**, 1095–1104.
5. Wani, N. R. and Qaisar, K. N., Carbon per cent in different components of tree species and soil organic carbon pool under these tree species in Kashmir valley. *Curr. World Environ.*, 2014, **9**(1), 174–181; http://dx.doi.org/10.12944/CWE.9.1.24.
6. Wani, N. R., Qaisar, K. N. and Khan, P. A., Growth performance, biomass production and carbon stocks of 19 year old Fraxinus floribunda (ash tree) plantations in Kashmir valley. *Agric. Forest.*, 2014, **60**(1), 125–143.
7. Wani, N. R., Qaisar, K. N. and Khan, P. A., Biomass, carbon stocks and carbon dioxide mitigation potential of *Cedrus deodara* under temperate conditions of Kashmir. *Can. J. Pure Appl. Sci.*, 2014, **8**(1), 2677–2684.
8. Nabuurs, G. J. and Schelhaas, M. J., Carbon profile of typical forest types across Europe assessed with CO2FIX. *Ecol. Indicators*, 2002, **1**, 213–233.
9. Masera, O. *et al.*, Modelling carbon sequestration in afforestation, agroforestry and forest management projects: the CO2FIX V.2 approach. *Ecol. Model*, 2003, **164**, 177–199.
10. Schelhaas, M. J. *et al.*, CO2FIX V 3.1 – a modelling framework for quantifying carbon sequestration in forest ecosystems. ALTERRA Report 1068, Wageningen, The Netherlands, 2004.
11. Gaboury, S., Boucher, J. F., Villeneuve, C., Lord, D. and Gagnon, R., Estimating the net carbon balance boreal open woodland afforestation: a case study in Quebec's closed crown boreal forest. *Forest Ecol. Manage*., 2009, **257**, 483–494.
12. Kaul, M., Mohren, G. M. J. and Dadhwal, V. K., Carbon storage and sequestration potential of selected tree species in India. *Mitig. Adapt. Strateg. Global Change*, 2010, **15**, 489–510.
13. Ajit, *et al*., Modelling analysis of potential carbon sequestration under existing agroforestry systems in three districts of Indo-Gangetic plains in India. *Agroforest. Syst*., 2013, **87**, 1129–1146.
14. Ajit, *et al*., Estimating carbon sequestration potential of existing agroforestry systems in India. *Agroforest Syst.*, 2016, 1–20; doi: 10.1007/s10457-016-9986-z (published online 12 August 2016).
15. Liski, J., Palosuo, T., Peltoniemi, M. and Sievanen, R., Carbon and decomposition model YASOO for forest soils. *Ecol. Model*, 2005, **189**, 168–182.
16. Dar, J. A. and Sundarapandian, S., Variation of biomass and carbon pools with forest type in temperate forests of Kashmir Himalaya, India. *Environ. Monitor. Assess.*, 2015, **87**(2), 55; doi: 10.1007/s10661-015-4299-7.
17. Jana, B. K., Biswas, S., Majumder, M., Roy, P. K. and Mazumdar, A., Carbon sequestration rate and above ground biomass carbon potential of four young species. *J. Ecol. Natural Environ*., 2009, **1**, 15–24.
18. Yadava, A. K., Biomass production and carbon sequestration in different agroforestry systems in Tarai region of central Himalaya. *Indian Forester*, 2010, **136**, 234–244.

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Fusion of ginseng farnesyl diphosphate synthase and *Centella asciatica* squalene synthase involved in triterpenoid biosynthesis

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Farnesyl diphosphate synthase (FPS) is a key enzyme in isoprenoid biosynthesis, generating farnesyl diphosphate as the central precursor for the broad classes of sesquiterpenoids and triterpenoids. On the one hand, cyclization of farnesyl diphosphate catalysed by various sesquiterpene synthases leads to structurally diverse sesquiterpenoids, while on the other, dimerization catalysed by squalene synthase (SQS) yields squalene as the first intermediate in the production of triterpenoids. To optimize triterpenoid production, the activities of an FPS generating farnesyl diphosphate and an SQS converting it should be coupled. Here, we constructed a fusion protein combining a ginseng FPS and a *Centella asiatica* SQS via a short peptide (Gly–Ser–Gly) linker. Heterologous expression in *Escherichia coli* resulted in a soluble fusion protein detected by SDS–PAGE. The fusion protein had both FPS and SQS activities, at approximately 94% and 71% of the single enzyme levels respectively. This novel fusion protein will serve as a valuable tool for genetic engineering of triterpenoid compounds, including saponins.

Keywords: Farnesyl diphosphate synthase, fusion protein, squalene synthase, triterpenoids.

THE isoprenoid biosynthetic pathways produce a number of important primary as well as secondary metabolites in many plants[1–3]. Sesquiterpenoids and triterpenoids are two major groups of isoprenoid compounds with important biological functions and medicinal properties. In particular, triterpenoid saponins have a variety of interesting pharmaceutical activities, including anti-inflammatory, anti-cancerogenic and anti-bacterial effects, depending on their chemical structures[4].

Sesquiterpenoid and triterpenoid biosynthesis starts by the conversion of three units of acetyl-coenzyme A (CoA) to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG–CoA),

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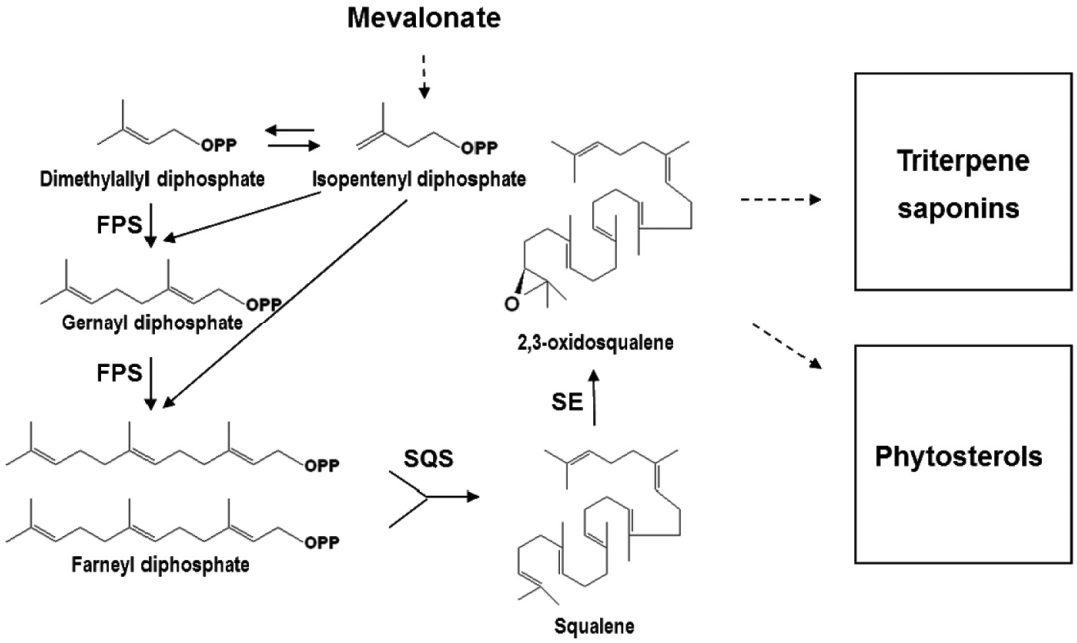

Figure 1. Mevalonate pathway in plants. FPS, Farnesyl diphosphate synthase; SQS, Squalene synthase; SE, Squalene epoxidase.

which is then reduced to mevalonate (by 3-hydroxy-3-methylglutaryl-coenzyme A reductase) and further converted into isopentenyl diphosphate (IPP). Isomerization of IPP yields dimethylallyl diphosphate (DMAPP), and sequential condensation of IPP and DMAPP by farnesyl diphosphate synthase (FPS, EC2.5.1.1/EC2.5.1.10) leads to geranyl diphosphate (GPP) and farnesyl diphosphate (FPP). FPP is a branch-point intermediate for biosynthesis of either sesquiterpenoids or triterpenoids, and thus both fluxes share FPP, and relative activities of enzymes utilizing it as substrate will determine the amounts of sesquiterpenoids or triterpenoids formed. FPP has been localized in the cytosol, mitochondria, peroxisomes and chloroplasts[5–8]. However, sesquiterpenoid and triterpenoid biosynthesis is generally thought to occur largely, if not completely, in the endoplasmic reticulum (ER), and thus likely relies on the cytosolic FPP substrate pool.

Sesquiterpenoids are formed directly through cyclization of FPP, catalysed by specific sesquiterpene synthases. In contrast, triterpenoid biosynthesis requires the combination of two FPP molecules in squalene (SQ), by an ER-bound squalene synthase (SQS, EC.2.5.1.21). SQ is then (in seed plants) activated into epoxysqualene, which serves as the common precursor for sterols and triterpenoids (Figure 1). They are formed by highly stereo- and regioselective cyclization of epoxysqualene, catalysed by various triterpene synthases[9].

It has been demonstrated that *in planta* overexpression of FPS leads to significant increase in sterol as well as carotenoid concentration[10]. Similarly, increased SQS activities also result in the accumulation of sterol and triterpene products[11,12]. Taken together, the two enzymes, FPS and SQS, thus play an important role in controlling biosynthetic rates of plant sterols and triterpenoids. Conversely, flux towards sesquiterpenoid products largely depends on the activities of FPS and sesquiterpene synthases, and several studies have shown that sesquiterpenes such as linalool, *epi*-aristolochene and α-farnesene may be formed effectively by fusion proteins of FPS and sesquiterpene synthase enzymes[13–15]. However, similar experiments attempting to fuse FPS and SQS proteins for enhancing triterpenoid biosynthesis have not been reported to date.

In the present study, we have constructed a vector fusing the previously characterized FPS and SQS genes from ginseng and *Centella asiatica* respectively. Heterologous expression in *Escherichia coli* cells was used to produce the FPS/SQS fusion protein for biochemical characterization, in particular, to compare its FPS and SQS activities with those of the two separate proteins.

The genes of *C. asiatica* SQS (GenBank accession AY787628) and ginseng FPS (GenBank accession DQ087959) were PCR-amplified using primer sets CSSn_N and CSSc_X as well as PFSn_N and PFSc_X respectively (Table 1). A hydrophobic sequence (87 bp) at the C-terminus of the SQS gene was eliminated during amplification, as described by Kim *et al.*[16]. All PCR reactions were carried out by heating to 94°C for 5 min, followed by 30 reaction cycles (94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec) and a final extension step (72°C for 10 min). The reaction products were inserted into the multi-cloning site of the pET32a vector; the resulting construct was sequenced for confirmation, and then transformed into *E. coli* BL21 (DE3) pLysS.

Table 1. Primers used for cloning genes

Genes	Primers	Sequence (5′–3′)
PgFPS	PFSn_N	CCACCATGGGCGATCTGAAGA (*Nco*I)
	PFSc_X	CTCCTCGAGTTACTTTTGCCGCTTA (*Xho*I)
	PFSc_B:	AATGGATCCCTTTTGCCGCTTATA (*Bam*HI)
CaSQS	CSSn_N	TAACCATGGGAAGTTTAGGGG (*Nco*I)
	CSSc_X	CAACTCGAGTCATCCCGAGTCATTCTCAAG(*Xho*I)
	CSSn_B	AACGGATCCGGAATGGGAAGTTTAGGGG (*Bam*HI)

Underlined sequences are recognition sites of the restriction enzymes.

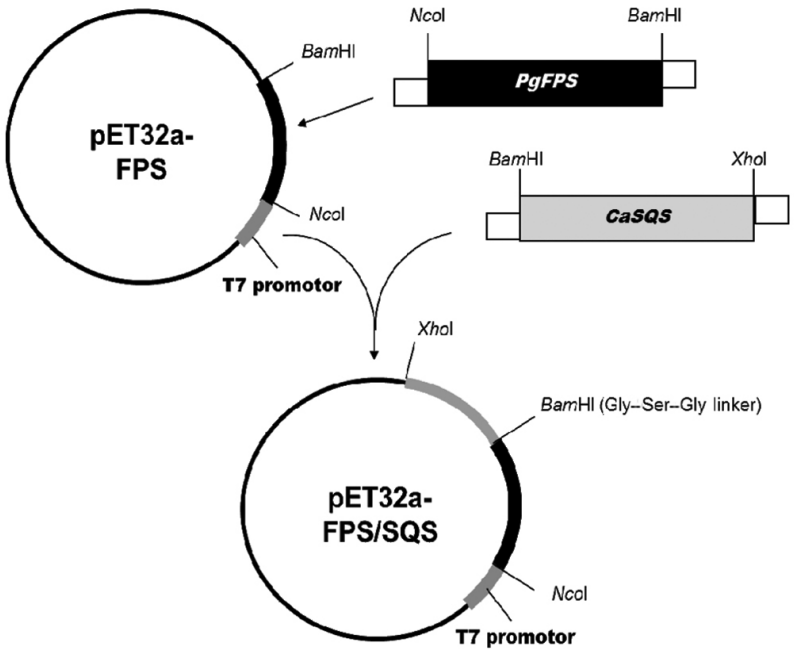

Figure 2. Schematic outline of *Escherichia coli* expression vector construct for the fusion protein combining *Panax ginseng* FPS and *Centella asiatica* SQS.

To construct the FPS/SQS fusion protein expression vector (Figure 2), the *PgFPS* gene in pET32a-FPS was PCR-amplified with the PFSn_N and PFSc_B primers (Table 1) also encoding *Nco*I and *Bam*HI recognition sites respectively. The PCR product was digested with the two restriction enzymes and then ligated into the pET32a vector, resulting in a pET32a–FPS intermediate construct. A truncated SQS gene of *C. asiatica* was amplified with CSSn_B and CSSc_X primers, including *Bam*HI and *Xho*I recognition sites, and the PCR product was digested with both restriction enzymes and ligated into the pET32a–FPS vector, resulting in pET32a–FPS/SQS. To select positive clones, colony PCR was performed with PFSn_N and CSSc_X primers. All the PCR reactions involved were carried out as described above. Finally, presence of the insert was confirmed by DNA sequencing analysis, and the construct transformed into *E. coli* BL21 (DE3) pLysS.

A colony of BL21 (DE3) pLysS cells harbouring the desired expression vector was added to 10 ml of LB medium containing 50 μg/l ampicillin and grown at 37°C to an optical density of 0.5 at 600 nm. Then isopropyl thio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for 3 h. Finally, the cells were collected via centrifugation, resuspended in 4 ml of extraction buffer (50 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$ and 20% glycerol) and sonicated three times for 30 sec. The resulting suspension was centrifuged for 10 min at 14,000 *g*, and a 10 μl aliquot of the supernatant was separated on an 10% SDS-polyacrylamide gel and stained with Coomassie blue for protein detection.

FPS activity assays were conducted as described by Brodelius *et al.*[14]. Cell-free proteins (100 μg) were assayed in 50 mM Tris-HCl (pH 8.0), containing 15 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20% glycerol, 55 μM GPP and 50 μM [1–^{14}C] IPP (55 mCi/mmol), in a total volume of 50 μl. Concentration of cell-free proteins was determined by the Bradford method[17]. The mixtures were incubated for 10 min at 30°C; then 6 M HCl was added to stop the reaction, and incubation was continued for a

further 10 min to hydrolyse the FPP product into extractable farnesol. The reaction mixture was neutralized with 6 M NaOH and then extracted with hexane. The resulting hexane solution was subsequently extracted with water, and an aliquot from it was utilized to measure radioactivity using a scintillation counter.

In order to confirm the chemical nature of the FPS reaction products (of single FPS enzyme as well as FPS/SQS fusion protein), neutralization was performed with 6 M NaOH. The mixture was extracted with hexane, and the hexane phase was subsequently extracted with water. An aliquot of the extracted hexane phase was then analysed using a scintillation counter. In order to analyse the radioactive reaction products, enzymatic reactions were conducted in accordance with the method described by Mekkriengkrai *et al.*[18]. The prenyl diphosphate products were extracted with 1-butanol saturated with water and then treated with potato acid phosphatase at 37°C overnight to hydrolyse the diphosphate moiety. The products were analysed by reverse phase TLC (LKC-18, Whatman, Maidstone, England) developed in acetone : water (9 : 1, v/v). The radioactive products were then identified in comparison with an authentic standard visualized by iodine vapour, and each of the radioactive spots was analysed with a Bio-image Analyzer BAS1500 (Fuji, Tokyo, Japan).

SQS activity was determined as described by Nakashima *et al.*[9], using reaction mixtures of 50 mM Tris-HCl (pH 7.6), 10 mM KF, 0.1 mM $MgCl_2$, 3 mM NADPH, 20 μM [1–^{3}H]FPP (16 Ci/mmol^{-1}; Amersham-Pharmacia Biotech) and 30 μg proteins, and incubating for 1 h at 30°C. The reaction was stopped by placing the tube on ice and adding 100 μl of *n*-hexane, mixing and then centrifuging. Aliquots of the *n*-hexane phase were separated by silica gel TLC (Merck) with ethylacetate : benzene (1 : 4, v/v). After development, the plates were sprayed with I_2, and the spot containing squalene was scraped-off and extracted with chloroform. The resulting solution was subjected to radio-counting in a scintillation counter.

The goal of the present study was to fuse a FPS and a SQS, and then compare the enzymatic activities of both moieties constituting the fusion protein with those of the native, single enzymes. To this end, cDNAs encoding the *Panax ginseng* FPS and the *C. asiatica* SQS enzymes were inserted into the pET32a expression vector, either separately or together in a single reading frame connecting both coding sequences with a three-amino acid linker. In previous studies, heterologous expression in *E. coli* had been used to characterize the FPS and SQS enzymes[16,19]. Therefore, similar protocols were employed here for comparative investigation of the fusion protein. In particular, SQS has a highly hydrophobic sequence at the C-terminal region that might help anchor the enzyme to the endoplasmic reticulum membrane[20]. We should eliminate the sequence at the C-terminal region by PCR amplification to transfer hydrophobic properties of SQS into the soluble protein. Various sizes of linkers have been used in other studies to fuse two proteins, and long linkers were found unsuitable because they tended to be sensitive to proteolytic attack and also because the distance between the two active sites may have impeded substrate channelling between protein domains[21,22]. In case of no linker between two genes, production of the sesquiterpenoid was less effective in *E. coli* cells[15]. It is likely that a fuse protein without a linker peptide caused steric hindrance which affected both structural configurations and enzyme activities. To avoid these negative effects, a relatively short Gly–Ser–Gly linker was used here for construction of the FPS/SQS fusion. We successfully generated the fuse protein by the linker between two genes and confirmed it by PCR analysis with primers of FPS (a forward) and SQS (a reverse).

The expected molecular weights of the FPS, SQS and FPS/SQS fusion proteins, including N-terminal Trx/His/S-tags were 55.911, 62.532 and 102.148 kDa respectively. SDS–PAGE analysis revealed that the extract of bacterial cells over expressing either FPS or SQS contained prominent proteins not found in the empty-vector control, with molecular weights of about 56 and 63 kDa respectively (Figure 3, lanes 2 and 3). The cell-free extract from *E. coli* cultures expressing the protein fusion construct pET32–FPS/SQS instead contained a protein of approximately 100 kDa (Figure 3, lane 4).

To assess the FPS activities of the separate FPS enzyme and FPS/SQS fusion protein, we assayed the incorporation of GPP and [1–^{14}C]IPP into FPP. The reaction products were dephosphorylated and analysed by reversed-phase TLC (Figure 4), showing that single FPS and fused FPS with SQS resulted in farnesol (FOH). In

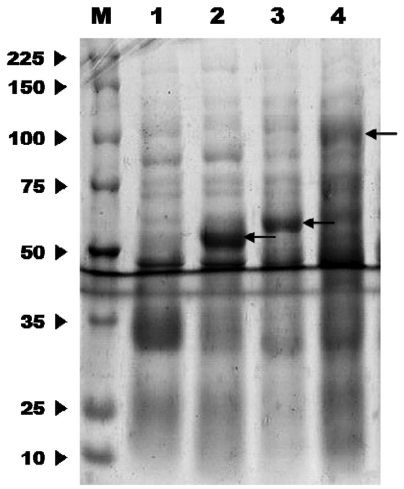

Figure 3. SDS–PAGE analysis of FPS, SQS and FPS/SQS fusion proteins expressed in *E. coli*. Lane M, Molecular weight markers (kDa); lane 1, Proteins extracted from cells transformed with the empty pET32a vector; lanes 2, 3, Proteins extracted from cells transformed with pET32a–FPS and pET32a–SQS respectively and lane 4, Proteins extracted from cells transformed with pET32a–FPS/SQS. Arrows indicate the FPS, SQS and FPS/SQS proteins with calculated M_r of 55.9, 62.5 and 102.1 kDa respectively.

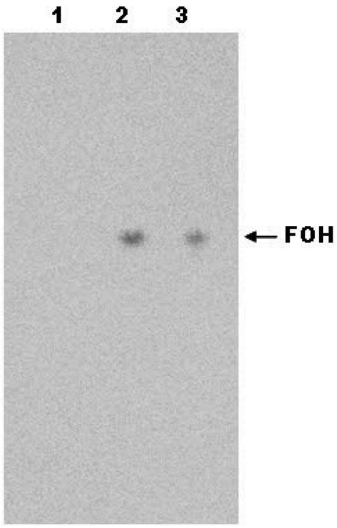

Figure 4. Reversed-phase TLC analysis of FPS enzyme activities. Lane 1, Reaction products of total proteins extracted from *E. coli* cells harbouring empty pET32a vector; lanes 2, 3, Reaction products of total proteins extracted from *E. coli* cells harbouring pET32a–FPS and pET32a–FPS/SQS respectively. FOH indicates the position of farnesol produced from a hydrolysed farnesyl diphosphate.

Table 2. *In vitro* enzyme activities of farnesyl diphosphate synthase (FPS), squalene synthase (SQS) and FPS/SQS fusion proteins produced by heterologous expression in *Escherichia coli*

	Specific activity	
Transformed vectors	FPS[a]	SQS[b]
pET32a (no insert)	40.05 ± 0.12	ND
Single FPS	382.27 ± 16.63	–
Single SQS	–	110.89 ± 8.27
FPS/SQS	359.33 ± 11.54	78.73 ± 6.33

[a,b]Specific FPS and SQS activities of extracts are expressed as nmol of IPP and/or FPP incorporated into FPP and SQ respectively, per min and mg of protein. Each value represents the mean of three independent experiments with standard deviations. ND, Not detected; –, Not measured.

contrast, no FOH was detected in the extracts of bacterial cells harbouring the empty pET32 vector. Further analysis using reversed-phase TLC showed that the radioactive product of the FPS and FPS/SQS assays had retention behaviour identical to an unlabelled standard of FPP, confirming the chemical nature of the product and thus covalent linkage of GPP and IPP into FPP.

The *in vitro* activity of the separate FPS and FPS/SQS fusion proteins was 380 and 295 nmol/min/mg respectively (Table 2). The K_m value of the separate FPS protein for IPP was determined to be 3.4 μM, while the FPS/SQS fusion enzyme had an apparent K_m of 3.2 μM. In comparison, the K_m values for IPP of other FPS enzymes have been reported to range between 3.0 and 4.0 μM (ref. 23). Taken together, our experiments thus demonstrated that the FPS/SQS fusion protein had FPS activity similar to the original stand-alone FPS.

In a separate experiment, SQS activity of the fusion protein was compared with that of a single-enzyme control. The FPS/SQS fusion had SQS activity of about 79 nmol/min/mg, while the separate SQS enzyme had an activity of 110 nmol/min/mg (Table 2).

To improve production of secondary metabolites through substrate channelling between adjacent active sites, fusion of two consecutive enzymes on the respective pathways has been engineered earlier. In particular, protein fusion involving FPS has been reported earlier, albeit in combination with sesquiterpenoid cyclases, aiming to enhance sesquiterpenoid rather than triterpenoid production. It has been reported that a fusion protein combining *Artemisia annua* FPS and tobacco *epi*-aristolochene synthase showed aproximity effect, with increased overall catalytic activity to produce *epi*-aristolochene when expressed in *E. coli*[14]. Wang *et al.*[15] showed that a fusion of FPS and α-farnesene synthase, *in vivo* upon heterologous expression in *E. coli*, produced higher amounts of α-farnesene than using the separate enzymes. Similar to these previous studies, our results showed that FPS can also function in fusion with SQS, with similar FPS and slightly lower SQS activities than the separate enzymes. The latter finding may be explained by the changed SQS domain in the fusion protein, where a hydrophobic sequence at the C-terminus may have caused a slight modification of the protein structure that negatively affected its activity.

In summary, we generated a protein fusion of FPS and SQS moieties, which has combined activities leading from IPP and GPP to FPP and on to squalene. It thus catalyses crucial steps en route to triterpenoid formation, effectively by-passing the competition for FPP as a substrate for sesquiterpenoids. This novel fusion protein will therefore serve as a valuable tool for genetic engineering of triterpenoid compounds, including saponins.

Conflict of interest: The authors declare that they have no conflict of interest.

1. Gaffe, J., Bru, J. P., Causse, M., Vidal, A., Stamitti-Bert, L., Carde, J. P. and Gallusci, P., LEFPS1, a tomato farnesyl pyrophosphate gene highly expressed during early fruit development. *Plant Physiol.*, 2000, **123**(4), 1351–1362.
2. Kroymann, J., Natural diversity and adaptation in plant secondary metabolism. *Curr. Opin. Plant Biol.*, 2011, **14**(3), 246–251.
3. Suzuki, M. and Muranaka, T., Molecular genetics of plant sterol backbone synthesis. *Lipids*, 2007, **42**(1), 47–54.
4. Augustin, J. M., Kuzina, V., Andersen, S. B. and Bak, S., Molecular activities, biosynthesis and evolution of triterpenoid sapoinins. *Phytochemistry*, 2011, **72**(6), 435–457.
5. Grunler, J., Ericsson, J. and Dallner, G., Branch-point reactions in the biosynthesis of cholesterol, dolichol, ubquinone and prenylated proteins. *Biochim. Biophys. Acta*, 1994, **1212**(3), 259–277.
6. Cunillera, N., Boronat, A. and Ferrer, A., The *Arabidopsis thaliana FPS1* gene generates a novel mRNA that encodes a mitocondrial farnesyl-diphosphate synthase isoform. *J. Biol. Chem.*, 1997, **27**(24), 15381−15388.

7. Oliver, L. M. and Krisans, S. K., Peroxisomal protein targeting and identification of peroxisomal targeting signals in cholesterol biosynthetic enzymes. *Biochim. Biophys. Acta*, 2000, **1529**(1–3), 89–102.
8. Sanmiya, K., Ueno, O., Matsuoka, M. and Yamamoto, N., Localization of farnesyl diphosphate synthase in chloroplasts. *Plant Cell Physiol.*, 1999, **40**(3), 348–354.
9. Nakashima, T., Inoue, T., Oka, A., Nishino, T., Osumi, T. and Hata, S., Cloning, expression, and characterization of cDNAs encoding *Arabidopsis thaliana* squalene synthase. *Proc. Natl. Acad. Sci. USA*, 1995, **92**(6), 2328–2332.
10. Daudonnet, S., Karst, F. and Tourte, Y., Expression of the farnesyl diphosphate synthase gene of *Saccharomyces cerevisiae* in tobacco. *Mol. Breed.*, 1997, **3**, 137–145.
11. Lee, M. H. *et al.*, Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. *Plant Cell Physiol.*, 2004, **45**(8), 976–984.
12. Seo, J. W. *et al.*, Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. *Phytochemistry*, 2005, **66**(8), 869–877.
13. Deng, Y., Sun, M., Xu, S. and Zhou, J., Enhanced (S)-linalool production by fusion expression of farnesyl diphosphate synthase and linalool synthase in *Saccharomyces cerevisiae*. *J. Appl. Microbiol.*, 2016, **121**(1), 187–195.
14. Brodelius, M., Lundgren, A., Mercke, P. and Brodelius, P. E., Fusion of farnesyldiphosphate synthase and *epi*-aristolochene synthase, a sesquiterpene cyclase involved in capsidiol biosynthesis in *Nicotiana tabacum*. *Eur. J. Biochem.*, 2002, **269**(14), 3570–3577.
15. Wang, C., Yoon, S. H., Jang, H. J., Chung, Y. R., Kim, J. Y., Choi, E. S. and Kim, S. W., Metabolic engineering of *Escherichia coli* for α-farnesene production. *Metab. Eng.*, 2011, **13**(6), 648–655.
16. Kim, O. T., Seong, N. S., Kim, M. Y. and Hwang, B., Isolation and characterization of squalene synthase cDNA from *Centella asiatica* (L.) Urban. *J. Plant Biol.*, 2005, **48**(3), 263–269.
17. Bradford, M. M., A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 1976, **72**, 248–254.
18. Mekkriengkrai, D., Sando, T., Hirooka, K., Sakdapipanich, J., Tanaka, Y., Fukusaki, E. and Kobayashi, A., Cloning and characterization of farnesyl diphosphate synthase from the rubber-producing mushroom *Lactarius chrysorrheus*. *Biosci. Biotechnol. Biochem.*, 2004, **68**(11), 2360–2368.
19. Kim, O. T., Bang, K. H., Jung, S. J., Kim, Y. C., Hyun, D. Y., Kim, S. H. and Cha, S. W., Molecular characterization of ginseng farnesyl diphosphate synthase gene and its up-regulation by methyl jasmonate. *Biol. Plant.*, 2010, **54**(1), 47–53.
20. Tansey, T. R. and Shechter, I., Structure and regulation of mammalian squalene synthase. *Biochim. Biophys. Acta*, 2000, **1529**(1–3), 49–62.
21. Carlsson, H., Ljungcrantz, P., Bülow, L. and Mosach, K., Engineering of lactose metabolism in *E. coli* by introducing β-galactosidase/galactokinase fusion enzymes. *Biotechnol. Lett.*, 1992, **14**(6), 439–444.
22. Carlsson, H., Ljung, S. and Bülow, L., Physical and kinetic effects on induction of various linker regions in beta-galactosidase/galactose dehydrogenase fusion enzymes. *Biochim. Biophys. Acta*, 1996, **1293**(1), 154–160.
23. Ohnuma, S., Hirooka, K., Ohto, C. and Nishino, T., Conversion from archael geranylgeranyl diphosphate synthase to farnesyl diphosphate synthase. *J. Biol. Chem.*, 1997, **272**(8), 5192–5198.

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Optimization of key factors for enhanced fermentative biohydrogen production from water hyacinth by RSM

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This communication discusses the optimization of key factors for the enhanced bio-hydrogen production from water hyacinth. Three critical factors inoculums age (18–24 h), inoculums volume (20–80 ml/l) and concentration of sulphuric acid (0.5–2.0%) were optimized by response surface methodology (RSM) with central composite design (CCD) for better production. RSM analysis showed that all three factors significantly influenced hydrogen production. The optimum hydrogen production was 705 ml/l obtained with 21 h old bacterial culture, 50 ml/l inoculums with 1.25% sulphuric acid pre-treatment. The hydrogen concentration produced by *Clostridium acetobutylicum* NCIM 2877 was enhanced after using RSM. The results obtained indicate that RSM with CCD can be used as a technique to optimize culture conditions for enhancement of hydrogen production by pre-treatment of low-cost organic substrate; water hyacinth using dark fermentation methods may be one of the most promising approaches.

Keywords: Central composite design, *Clostridium acetobutylicum* NCIM 2877, hydrogen production, response surface methodology, water-hyacinth.

BIOHYDROGEN has high energy density (122 kJ/g) and also does not produce any harmful combustion products. These characteristics make it the most promising and advanced biofuel when compared to other biofuels. Processes such as steam reformation and water electrolysis are efficient methods for hydrogen production but require high energy input, whereas biological processes are operated at ambient temperature and mild operational conditions. Certain bacterial species like clostridium and enterobacter are used in the process of dark fermentation for biohydrogen production. However, there are two bottlenecks in the biological hydrogen production process. They are, low hydrogen yield and high-cost substrates. Conventional substrates like glucose, sucrose and starch used for biological hydrogen production are expensive which restricts the application and development of biohydrogen[1].

Water hyacinth (*Eichhornia crassipes*) is a free-floating aquatic plant. In India it was first observed in West Bengal, in the beginning of 1890, and is now found

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