Adv Biochem Eng Biotechnol
DOI 10.1007/10_2014_303

Isoprene

Claudia E. Vickers and Suriana Sabri

Abstract Isoprene is a volatile C_5 hydrocarbon. It is produced by a wide variety of organisms and has been shown to play a role in protection of plants under abiotic stress conditions. It also has many different uses as an industrial chemical: most notably as a precursor for synthetic rubbers, but also for production of elastomers, copolymers, adhesives, and specialised chemicals. Modifying and/or engineering isoprene production in plants has the potential to contribute to engineered stress resistance. Moreover, as petrochemical sources of isoprene increase in price and become more scarce, bioproduction routes through microbial processes are becoming more attractive. Here we examine biotechnological aspects of isoprene production and review the current state of the art for both microbial-based industrial bioprocesses and plant engineering.

Keywords Isoprene · Metabolic engineering · Abiotic stress · Rubber · Industrial biotechnology · Microbial fermentation · Synthetic biology

Abbreviations

Ac	Acetyl
DMAPP	Dimethylallyl pyrophosphate
DX	Deoxyxylulose
DXP	Deoxyxylulose 5-phosphate
DXR	Deoxyxylulose reductase
DXS	Deoxyxylulose synthase
FPP	Farnesyl pyrophosphate

C.E. Vickers (✉)
Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, QLD 4072, Australia
e-mail: c.vickers@uq.edu.au

S. Sabri
Enzyme and Microbial Technology Research Center, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

GC	Gas chromotography
GA3P	Glyceraldehyde-3-phosphate
HDR	Hydroxymethylbutenyl diphosphate reductase
HDS	Hydroxymethylbutenyl diphosphate synthase
HMBPP	Hydroxymethylbutenyl diphosphate
HMGR	Hydroxymethylglutaryl-CoA reductase
MK	Mevalonate kinase
IDI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl pyrophosphate
IspS	Isoprene synthase
MEP	Methylerythritol phosphate
MVA	Mevalonate
MVD	Diphosphomevalonate decarboxylase
PGL	Phosphogluconolactonase
PMK	Phosphomevalonate kinase
s	Second(s)
ROS	Reactive oxygen species
Tg	Teragram

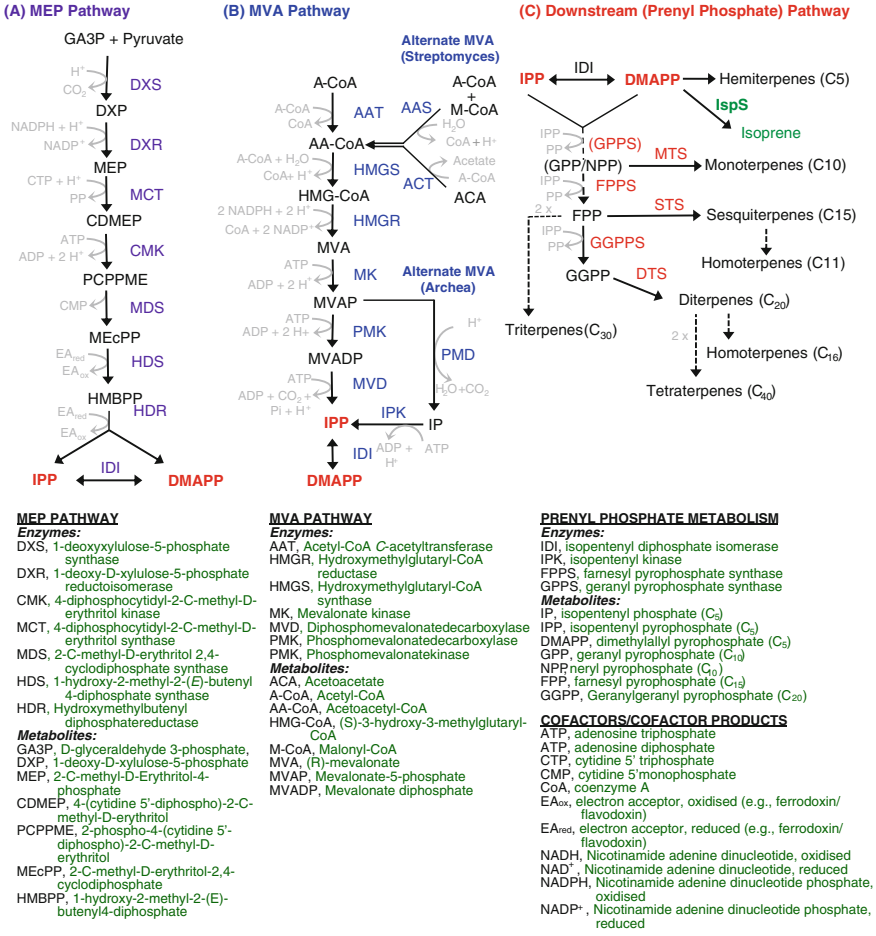
Contents

1 Introduction
2 Isoprene Synthase
3 Engineering Isoprene Production in Microbes
3.1 Engineering Isoprene Synthase for Microbial Expression
3.2 Engineering Precursor Availability
3.3 Engineering Isoprene Production in *E. coli*
3.4 Novel Isoprene Production Pathways
3.5 Engineering Isoprene Production in Other Organisms
3.6 Bioprocess Considerations
4 Engineering Isoprene Production in Plants
5 Summary, Conclusions, Outlook
References

1 Introduction

Williams [146] first identified the volatile C_5 hydrocarbon hemiterpene isoprene (2-methyl-1,3-butadiene) as a product of destructive distillation (thermal decomposition) of gutta percha (natural latex) and caoutchouc (natural rubber). It was recognised that a five-carbon 'isoprene unit' forms a building block for the vast array of different natural products in the terpene (isoprenoid) family; this is known as the 'isoprene rule' [102]. Synthesis of these products does not proceed via

isoprene itself, but through the universal C_5 prenyl phosphate precursors, dimethylallyl pyrophosphate (DMAPP) and its isomer isopentenyl pyrophosphate (IPP). These are both produced by the mevalonate (MVA) and methylerythritol phosphate (MEP) isoprenoid pathways (Fig. 1). Isoprene was identified as a leaf emission in the mid-1900s [63, 95, 106, 107]. Subsequently, many isoprene-producing plants,



MEP PATHWAY
Enzymes:
DXS, 1-deoxyxylulose-5-phosphate synthase
DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase
CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
MCT, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase
MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase
HDR, Hydroxymethylbutenyl diphosphatereductase
Metabolites:
GA3P, D-glyceraldehyde 3-phosphate,
DXP, 1-deoxy-D-xylulose-5-phosphate
MEP, 2-C-methyl-D-Erythritol-4-phosphate
CDMEP, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
PCPPME, 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
MEcPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate
HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl4-diphosphate

MVA PATHWAY
Enzymes:
AAT, Acetyl-CoA *C*-acetyltransferase
HMGR, Hydroxymethylglutaryl-CoA reductase
HMGS, Hydroxymethylglutaryl-CoA synthase
MK, Mevalonate kinase
MVD, Diphosphomevalonatedecarboxylase
PMK, Phosphomevalonatedecarboxylase
PMK, Phosphomevalonatekinase
Metabolites:
ACA, Acetoacetate
A-CoA, Acetyl-CoA
AA-CoA, Acetoacetyl-CoA
HMG-CoA, (S)-3-hydroxy-3-methylglutaryl-CoA
M-CoA, Malonyl-CoA
MVA, (R)-mevalonate
MVAP, Mevalonate-5-phosphate
MVADP, Mevalonate diphosphate

PRENYL PHOSPHATE METABOLISM
Enzymes:
IDI, isopentenyl diphosphate isomerase
IPK, isopentenyl kinase
FPPS, farnesyl pyrophosphate synthase
GPPS, geranyl pyrophosphate synthase
Metabolites:
IP, isopentenyl phosphate (C_5)
IPP, isopentenyl pyrophosphate (C_5)
DMAPP, dimethylallyl pyrophosphate (C_5)
GPP, geranyl pyrophosphate (C_{10})
NPP, neryl pyrophosphate (C_{10})
FPP, farnesyl pyrophosphate (C_{15})
GGPP, Geranylgeranyl pyrophosphate (C_{20})

COFACTORS/COFACTOR PRODUCTS
ATP, adenosine triphosphate
ATP, adenosine diphosphate
CTP, cytidine 5' triphosphate
CMP, cytidine 5'monophosphate
CoA, coenzyme A
EA_{ox}, electron acceptor, oxidised (e.g., ferrodoxin/flavodoxin)
EA_{red}, electron acceptor, reduced (e.g., ferrodoxin/flavodoxin)
NADH, Nicotinamide adenine dinucleotide, oxidised
NAD^+, Nicotinamide adenine dinucleotide, reduced
NADPH, Nicotinamide adenine dinucleotide phosphate, oxidised
$NADP^+$, Nicotinamide adenine dinucleotide phosphate, reduced

Fig. 1 Isoprenoid pathways **a** MEP pathway, found in plant chloroplasts, most bacteria, and some eukaryotic parasites [45, 98, 100, 154]. **b** Mevalonate (MVA) pathway, with various modifications, found in most eukaryotes, archaea, and some bacteria [41, 79, 90]. Both pathways produce the five-carbon intermediates isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). **c** downstream prenyl phosphate metabolism continues from DMAPP and IPP, with the availability of prenyltransferase enzymes catalysing condensation of prenyl phosphate precursors determining which prenyl phosphates are available to the organism. Isoprene, a hemiterpene, is produced by the action of isoprene synthase (IspS) on DMAPP. Figure modified from [133, 135]

primarily in the C3 group, have been identified [60, 94]. Isoprene is produced in large amounts by phytoplankton in the oceans [84], and animals and bacteria can also emit isoprene [70, 114].

Isoprene produced by plants contributes an enormous amount of carbon—some 440–660 Tg (million tons) per year—to the atmosphere [53]. This isoprene has a significant impact on atmospheric chemistry. It is highly reactive and affects the oxidative capacity of the troposphere [92, 129]. Models indicate that it extends the residence time of greenhouse gases [113], thereby having a climate forcing effect. Isoprene also contributes to local pollution: it reacts with free radicals and ozone, the products of which contribute to formation of secondary organic aerosol particles, ozone, and carbon monoxide; and in the presence of high levels of nitric oxides (typically from industrial activities) it also reacts to produce tropospheric ozone [35, 39, 132]. These pollutants can have severe negative effects both on human health and on plant/animal agriculture. Moreover, because isoprene production is a function of temperature and light, it plays a role in biosphere–climate chemistry feedback [113]. The biological role of isoprene production in plants appears to be the protection of photosynthetic and other processes under stress conditions [80, 118, 136].

Williams [146] observed that the liquid isoprene he distilled reacted with atmospheric ozone over time to become ozonised, and that on distilling the ozonised liquid, a 'violent reaction' took place, whereby all unaltered hydrocarbon volatilised and the remaining product solidified suddenly to produce a 'pure, white, amorphous mass', which was unique and had the chemical formulation $C_{10}H_8O$, the first observation of (presumably) naphthol. Isoprene polymerises with itself and various different partners to form polymers and copolymers with many different properties. It can therefore be used to make a wide variety of useful products. These include synthetic rubbers, elastomers, thermoplastic elastomer block copolymers (e.g., styrene–isoprene–styrene, SIS), adhesives, and specialized chemicals (e.g., vitamins, pharmaceuticals, flavourings and perfumes, and epoxy hardeners) [16, 25, 105, 126, 142]. The primary isoprene product is synthetic rubber (*cis*-1,4-polyisoprene); this is used to make vehicle tires, surgical gloves, golf balls, and adhesives among others. Approximately 1 million tons of petrochemical-derived isoprene are made per annum [142]. Market growth per annum is estimated at 1–2 %, and there is a potential market of 5 billion kg/year through product expansion [83].

Industrial isoprene is currently produced mainly from petroleum as a by-product of thermal cracking of naphtha or gas oil [105, 142]. This petroleum-derived isoprene is available in limited supply, occasionally precipitating global shortages; it therefore suffers from price volatility due to fluctuating petrochemical prices. In addition, it has a high carbon footprint [126, 142]. The production process is relatively expensive and energy-intensive, and yields may be insufficient for future demand [16]. As fossil resources become more expensive to extract and purify, the price of isoprene (and other petrochemical by-products) will also increase. A cost-effective and renewable alternative source of isoprene is therefore attractive.

Although some plants produce enormous amounts of isoprene (Hewitt et al. 2004), harvesting it from this source is not feasible. However, microbes which

normally cannot produce isoprene can be engineered to produce it at significant levels. Production of biochemicals in microbial systems as alternatives to petrochemical routes is a rapidly growing industry, and microbial bioprocesses can be significantly less expensive and more straightforward compared with other methods [125]. The California company Genencor (now owned by DuPont), in collaboration with The Goodyear Tire & Rubber Company, has pioneered development of microbe-based isoprene bioprocesses [54, 142], and several other research groups/companies are also working in the field. Moreover, the biological role of isoprene lends itself to the possibility of engineering for improved stress resistance in crop plants. Herein, we examine the biotechnological aspects of isoprene and review the current state of the art for both microbial-based industrial bioprocesses and plant engineering.

2 Isoprene Synthase

The enzyme isoprene synthase (IspS; EC 4.2.3.27; systematic name dimethylallyl-diphosphate diphosphate-lyase [isoprene-forming]) is responsible for the prodigious global production of isoprene. Isoprene synthase catalyses the removal of pyrophosphate from DMAPP to produce isoprene and pyrophosphate (Fig. 2). Mg^{2+} or Mn^{2+} are required as cofactors, with Mg^{2+} being the preferred cofactor [120, 121, 144].

IspS genes and/or proteins have been characterised from several *Populus* sp. (poplar/aspen; [13, 49, 85, 109, 119, 121, 138, 139]), *Quercus ilex* (oak; [13, 73]), *Salix discolour* (pussy willow; [144, 145]), *Salix babylonica* (weeping willow; [57]), *Liquidambar styraciflua* (sweetgum; [57]), *Arachis hypogaea* (peanut; [13]), *Myrtus communis* (myrtle; [57]), *Pueraria montana/Pueraria lobata* (kudzu; [13, 57, 119]), and *Mucuna bracteata* [57]. Although many prokaryotes also produce isoprene [46, 70], a prokaryotic *IspS*/IspS has yet to be identified.

Isoprene synthase belongs to the Tps-b group of angiosperm isoprenoid synthases [52, 115, 119]. The genes have six introns and seven exons [49, 85, 109, 115, 119, 138, 139]. In poplar (and presumably also in other plants), isoprene synthase is found in three to four copies; these genes are differentially regulated [139], but it is thus far

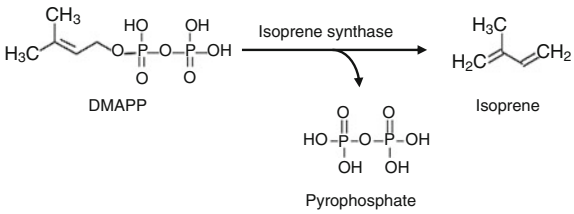


Fig. 2 Synthesis of isoprene from DMAPP as catalysed by isoprene synthase

unclear what the biological significance of this is. In in vitro analyses, isoprene synthase enzymes have a broad pH optimum (usually peaking at ~7–8) and temperature optima between 40–60 °C [47, 69, 73, 120]. They are relatively inefficient enzymes, with a high K_m (~2–20 mM) and variable but generally low k_{cat} values [13, 73, 111, 119, 121, 145, 151]. Substrate availability and thermal influence on enzyme kinetics have a strong controlling effect on isoprene emission [117]. IspS enzymes from different source organisms also have different susceptibilities to substrate-mediated inhibition [13]. The crystal structure of several IspS enzymes has been solved [14, 25, 68].

3 Engineering Isoprene Production in Microbes

Although plants produce large quantities of isoprene, approaches for engineering industrial production (for use as an industrial chemical) have focused on the use of microbial fermentation. Although plants represent an attractive starting point due to the naturally high emission levels of some species and the potential for direct conversion of carbon dioxide into isoprene via photosynthesis, bioprocess requirements are far cheaper and simpler in microorganisms. The challenges of capturing isoprene from a plant-emitting system would add significant cost and complication to the process. Microbial fermentation represents a sustainable alternative to produce isoprene directly from abundant and cost-effective renewable resources, for example, simple sugars sourced from plants, and ultimately, lignocellulosic feedstocks [134].

Isoprene production from bacteria was first identified by Kuzma et al. [70]. Of the bacteria surveyed, *Bacillus subtilis* was the highest producer [46, 70]. However, isoprene production in *B. subtilis* is only 50 μg/L/h^{-1} [64], insufficient for commercial processes. Common industrial microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (yeast) do not naturally produce isoprene. In order to achieve sufficient titres from microbial bioprocesses, substantial engineering is required, starting with introducing an appropriately engineered isoprene synthase gene. Due to the low native flux through isoprenoid pathways in most microbes, it is also necessary to engineer upstream pathways to provide sufficient precursors for industrial-scale isoprenoid production. This includes engineering central carbon metabolism to improve precursor availability or balance subcellular precursor concentrations for isoprenoid pathways, engineering through the core isoprenoid pathways themselves (MEP/MVA), and engineering availability of prenyl phosphate precursors (including controlling competition from downstream prenyltransferases). In the case of isoprene, the synthase uses DMAPP so the latter engineering steps are not required. With respect to engineering isoprene production in microbes, much of the experimental data is found in the patent literature, and we refer to patents frequently in the sections below.

3.1 Engineering Isoprene Synthase for Microbial Expression

Despite isoprene being widely produced by different microbes, and despite intensive search efforts [64, 123, 150], no microbial isoprene synthase has been reported to date. Indeed, only plant isoprene synthases have been discovered (see above). Consequently, it is necessary to transfer a plant isoprene synthase into microbes for engineering purposes. To optimise expression and activity, significant engineering is required prior to transfer of plant-encoded enzymes into microbes. Briefly, this involves (1) removal of intron sequences (i.e., use of a cDNA sequence), (2) removal of the chloroplast targeting sequence (if present), (3) codon optimisation of the sequence, and (4) selection of appropriate transcription and translation control sequences (see [135] for details). Further engineering, such as introduction of mutations that improve catalytic activity, thermostability, or other industrially useful traits, might also be performed.

Prior to any engineering, an appropriate *IspS* gene must be selected. Isoprene synthases from different source organisms vary significantly in their performance with respect to isoprene production in microbes [13, 25, 33, 57, 142].

Removal of introns (or selection of a cDNA sequence) and truncation to remove the chloroplast sequence are usually performed first (see below). Modifying isoprene synthase codon usage from plant to *E. coli* improves isoprene production in *E. coli* [25, 31]. Peptide tags, which are often included in cloning constructs and used for isolation of resulting proteins, can interfere with catalytic properties of isoprene synthase [25, 85] and should be avoided for industrial applications when designing constructs.

Isoprene synthase proteins include a chloroplast targeting peptide [85, 108, 119, 139, 140]; removal of this peptide increases isoprene production in microbes [25, 57, 85, 140]. As for other isoprenoid synthase genes [30, 147], selection of the truncation site for removal of the chloroplast leader sequence is critical in isoprene synthase [25]. Mapping of several truncation sites in a *Populus alba* isoprene synthase indicated that, of those tested, removal of 156 nucleotides (a 52-amino–acid deletion) with addition of an ATG (yielding a protein starting with MEARRS…) provided the highest levels of isoprene [25]. This truncation improved the k_{cat} 2-fold, the K_m 1.3-fold, the K_i 1.6-fold, and the specific activity 2-fold.

Somewhat surprisingly, decreasing the copy number using a low copy-number expression vector can improve isoprene production; isoprene synthase apparently forms inclusion bodies when overexpressed [31, 33]. Titrating expression from different promoters, albeit not yet directly examined, will most likely have combinatorial effects with copy number. This should be considered during construct design.

Although there is significant variation in catalytic performance, in general the poor catalytic properties of IspS enzymes (in particular the high $K_{m(DMAPP)}$ and low k_{cat} values) make the wild-type isoprene synthases unattractive as industrial enzymes. IspS proteins also exhibit substrate inhibition at varying levels for different enzymes [13, 14]. An *A. hypogaea* IspS is relatively resistant to substrate inhibition compared to a *P. alba* IspS (which is commonly used for engineering),

as long as high concentrations of the cofactor Mg^{2+} (100 mM) are available. The *A. hypogaea* IspS produced more isoprene in in vivo studies than a *P. alba* IspS, but also has an in vitro K_m of almost an order of magnitude more than the *P. alba* IspS.

A variety of targeted and random approaches was carried out by Bott et al. [25] to identify IspS mutants with improved catalytic activities. A number of mutations that conferred improved activities were identified. Bott et al. [25] also solved the crystal structure of IspS using a number of IspS enzymes and constructs. The structures suggested that several crucial loops forming the active site of isoprene synthase are flexible, and the authors noted the potential to modify metal binding, diphosphate recognition, DMAPP chain binding, and active site; a large number of potentially useful mutations were suggested [25]. A thorough mutation analysis of many different residues was then performed using a truncated (MEARR…) *P. alba* IspS [14]. This revealed a number of mutations that improved activities when nonpolar residues were converted to polar residues, presumably improving solubility (which had been noted to be poor). Many other mutations that improved specific activity in vitro and/or isoprene production in vivo were also identified. A selection of high-performing mutations (numbering based on MEAA… truncation) includes: A453N in a 'flexible loop' of the substrate binding site; L494P/L494C, a hydrophobic residue in a surface-exposed loop, where mutations to hydrophilic residues are thought to affect protein folding, solubility, or activity; and T536I/T536F/T536Y (a 'miscellaneous' mutation that yielded significant increases in activity with several substitutions). Crystallisation confirmed a change in conformation of the loop containing residues 490–497 where the L494P mutation is situated; this mutant provided a twofold higher specific activity, a decreased K_m and an increased k_{cat} in in vivo studies. Both L494P and T536F proved to confer thermostability, with the latter being more effective. It was suggested that the thermostability of T536F was due to replacement of a polar amino acid residue with a large hydrophobic residue (thereby increasing local hydrophobicity). Multiple sequence analysis demonstrated that both proline at 494 and I/V/F (hydrophobic residues) are common in isoprene synthases/terpene synthases (respectively). These data probably provide some clues as to why different IspS proteins from different species perform significantly differently in terms of isoprene production in microbes.

Another mutagenesis/selection approach was later used to further improve solubility (and improve the in vivo activity) of IspS [97]. To identify improved mutants, challenges with increasing concentrations of DMAPP in both in vitro and in vivo systems were used. The in vivo method involved titration of flux through an engineered MVA pathway, thereby taking advantage of the toxicity of prenyl phosphate precursors (see below) to link improved catalytic properties with growth rate and allow facile selection of desirable mutants. An S288C mutation (numbering based on MEA… truncation) repeatedly emerged from different screens. Other mutations that conferred either improved catalytic properties (K_m, k_{cat}, V_{max}) or improved expression/solubility were also identified. Combining the S288C mutation with an A3T mutation provided a three- to fivefold enhancement.

3.2 Engineering Precursor Availability

One of the largest obstacles to efficient microbial biosynthesis of isoprene is the production of its precursor, DMAPP. In the absence of temperature variation, DMAPP availability is the primary driver for isoprene production in plants [117, 139, 140], and precursor availability in microbes is a key limiting factor for engineering isoprenoid production in microbes [135]. Thus, engineering of the upstream isoprenoid pathway (either MEP or MVA, depending on the production organism) is required. This kind of pathway engineering is necessary for production of any isoprenoid compound in industrial microbes, because isoprenoids share common metabolic precursors. Genetic platforms resulting from work with isoprene can therefore be applied to the biosynthesis of other valuable products (and vice versa).

A problem encountered in isoprenoid pathway engineering is that high levels of prenyl phosphate precursors (DMAPP/IPP/FPP) are apparently toxic to cells [74, 82, 122, 150]. Furthermore, it is known that several prenyl phosphates act as feedback regulators to control flux through isoprenoid pathways [11, 12, 50, 58]. In order to avoid toxicity/feedback from buildup of prenyl phosphates, it is necessary to have a metabolic sink for these precursors in place prior to engineering increased flux. Isoprene is an ideal sink, because it is produced directly from one of the C_5 precursors (DMAPP), thereby providing an immediate drain for the prenyl phosphate pool. The high $K_{m(DMAPP)}$ of IspS also means that it is unlikely to compete effectively with downstream prenyltransferases that use DMAPP as a substrate. This means that potential growth defects caused by depleting the pool of prenyl phosphates required for synthesis of essential isoprenoids (primarily FPP) are avoided. Moreover, being volatile (b.p. = 34 °C), it partitions into the gas phase under normal cultivation conditions and is unlikely to cause toxicity problems in itself.

Broad approaches for engineering improved carbon flux in microbes through both the MVA and the MEP pathways have been reviewed recently [135]; here we focus on published approaches specific to isoprene production. Readers should note that additional approaches used for other isoprenoid products (in particular, for controlling flux upstream of the DMAPP/IPP node and for controlling downstream competition) will most likely also be effective for isoprene production.

3.3 Engineering Isoprene Production in E. coli

E. coli was the first microorganism to be engineered for isoprene production, and the best results by far have been achieved in this organism. The first recombinant isoprene-producing *E. coli* was generated as part of a study to identify the first *IspS* gene [85]. A hybrid poplar (*P. alba* × *P. tremula*, aka *P.* × *canescens*) isoprene synthase cDNA was used; truncation to remove the putative chloroplast targeting peptide resulted in a 1.85-fold increase in isoprene production. However, as later studies also found, simply introducing an isoprene synthase into *E. coli* results in only very low yields; in this case, only 1.85 nmol isoprene/mg cellular protein.

It should be noted that the choice of strain has a significant effect on initial production of isoprenoids in both *E. coli* and yeast [24, 34, 99, 127]. Strain also affects isoprene production in *E. coli*; for example, *E. coli* BL21(λDE3) produces 10 times higher titres than *E. coli* FM5, and *E. coli* ATCC11303 (an *E. coli* B derivative) performs even better than BL21(λDE3) in fed-batch culture [31]. We have also observed significant interstrain differences, both in isoprene production capability upon introduction of an isoprene synthase and in 'engineerability' (the effect of upstream pathway engineering) in our laboratory (Bongers, Behrendorff, Nielsen and Vickers, unpublished data).

Initial attempts at improving isoprene production focused on engineering through the native MEP pathway. Introduction of an heterologous MVA pathway significantly improves titres. Both pathways can be used in parallel for production, or engineering can be focused on the MVA pathway, which to date has delivered the best yields [17, 37] as discussed below.

3.3.1 Engineering Core MEP Pathway Flux

E. coli uses a MEP pathway for native isoprenoid production. Condensation of the central carbon intermediates pyruvate and glyceraldehyde-3-phosphate (GA3P) by deoxyxylulose synthase (DXS; see Fig. 1) to generate deoxyxylulose 5-phosphate (DXP) represents the primary rate-limiting step in the MEP pathway [3, 55]. Coexpression of an homologous or heterologous deoxyxylulose synthase (*dxs*; see Fig. 1) provides a two- to fourfold increase in isoprene [31, 57, 85].

Conversion of DXP to 2-C-methyl-D-erythritol 4-phosphate (MEP) by deoxylululose reductase (DXR) can also be rate-limiting [3]. A titre of 94 mg/L isoprene with a rate of 2.8 mg/L/h^{-1} was achieved by Zhao et al. [155] in *E. coli* fed-batch cultures when *Populus nigra* (black poplar) *IspS* was overexpressed together with the *E. coli dxs* and *dxr* genes. When heterologous *B. subtilis dxs* and *dxr* were used for overexpression, isoprene production was further enhanced 3.3-fold to 314 mg/L. This suggests that use of heterologous enzymes can provide more efficient catalysis, either from improved native catalytic properties or avoidance of native posttranslational regulatory control. Significant differences between the primary sequences of the homologous and heterologous genes were observed, supporting these possibilities.

In addition to *dxs* and *dxr*, overexpression of isopentenyl diphosphate isomerase (*idi*), which converts IPP to DMAPP and vice versa, improves isoprene production [25, 31, 33]. This presumably allows rapid balancing of the intracellular IPP and DMAPP pools, and may be particularly important given the heavy relative drain on the DMAPP pool exerted by IspS. In the absence of release at the DXS bottleneck by overexpression of *dxs*, overexpression of *idi* has minimal effect [33]. Coexpression of *dxs* and *idi* with engineered kudzu isoprene synthase provides a five- to 12-fold increase in isoprene compared to the IspS alone, allowing titres of up to 300 mg/L broth using *E. coli* strain BL21 [31, 33]. Strains overexpressing *E. coli dxs* and *idi* along with a *P. alba IspS* produced 35–70 mg/L isoprene on rich media in batch culture [76, 93].

The last two steps of the pathway are catalysed by hydroxymethylbutenyl diphosphate synthase (HDS/IspG/GcpE) and hydroxymethylbutenyl diphosphate reductase (HDR/IspH/LytB; see Fig. 1), both of which are $[4Fe–4S]^{2+}$ (iron–sulfur cluster) enzymes [112, 152]. Following upstream release at the DXS node and overexpression of IDI to balance the C_5 prenyl phosphate pools, flux through these two enzymes becomes rate-limiting. Under these conditions, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP, aka cMEPP), the substrate for IspG (GcpE/HDS), accumulates in the cell and is even exported into the medium [156]. It was also observed that a strain expressing kudzu *IspS*, yeast *idi*, and *E. coli dxs* produced more isoprene under noninducing conditions than under inducing conditions, and that the strain also accumulated (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), the substrate for HDR [37]. This suggested that HDR (IspH) activity was limiting.

Overexpression may help in some cases; for example, inclusion of an HDS overexpression, which has been shown to increase isoprenoid production when other bottlenecks are released [156], can further improve isoprene titres twofold in a strain overexpressing *dxs* and *idi* [76]. However, the complex cofactor requirements and biochemistry of the enzymes themselves must also be addressed.

The 4Fe–4S cluster is very sensitive to oxidative damage. Oxidative stress causes accumulation of MEcPP in mycobacteria and corynebacteria, most likely due to the interference at the 4Fe–4S group [10]. In *E. coli*, the iron–sulfur cluster (isc) operon enzymes are involved in formation of the 4Fe–4S cluster in Fe–S enzymes [131]. The *isc* enzymes also assist in repair of the 4Fe–4S clusters after oxidative damage [43]. In the presence of appropriate concentrations of flavodoxin and flavodoxin reductase, HDR activity in extracts from *E. coli* overexpressing both *IspH* and the *isc* operon increased 200-fold in vitro [51]. Removal of the *isc* operon repressor (*iscR*) was successfully used to increase specific productivity of isoprene by up to threefold in vivo (though a reduction in growth rate was also observed [37].

Another approach to improve activities of 4Fe–4S enzymes is to improve electron transfer to support redox reactions. Accordingly, overexpression of the *E. coli* electron transferase flavodoxin I (*fldA*) increased isoprene production one- to twofold. Increased expression of IspS did not improve the isoprene titre, suggesting that in this case, IspS activity was not limiting.

After overexpression of *fldA,* the HDS and HDR nodes were further targeted by overexpression of *Thermosynechococcus elongates IspG* (*GcpE*) and *IspH* (*LytB*; [37]). *T. elongates* does not encode an *fldA*; instead it apparently uses a reducing shuttle system consisting of ferredoxin (*petF*) and ferredoxin-NADP(+) oxidoreductase (*petH*; [89]). Expressing *GcpE*, *petF*, and *petH* in strains with a *P. alba* IspS (MEARR… truncation), yeast *idi* and variable levels of *dxs* overexpression increased isoprene production by 10–80 %, with a stronger response observed with increasing promoter *dxr* strength. Both the *dxs*-only and the *dxs/GcpE/petF/petH* strains exhibited accumulation of MEcPP. However, surprisingly, MEcPP was just as high —and in some cases, higher—in the *GcpE/petF/petH* strains than in the strains without *GcpE* overexpressed. Despite this, the HDR (GcpE/IspH) product HMBPP was observed to accumulate in *GcpE* strains, indicating that the overexpression successfully relieved at least a proportion of the flux constraint at that node. Its

accumulation suggested that HDS (IspG) activity was still limiting. Heterologous overexpression of a second *IspG* from a different species and an *IspH* gene from *Anabaena* further improved titres [88]. A strain with several modifications, including overexpressions of *dxs*, *dxr*, *idi*, *fldA-ispG*, *Anabaena IspH-petF-petH*, a lower MVA pathway, a truncated *P. alba IspS*, and a restored chromosomal *pgl* (see the following section) provided titres of 8.4 g/L isoprene in a fed-batch fermentation [88].

3.3.2 Balancing MEP Pathway Precursors

Balancing of GA3P and pyruvate pools to prevent accumulation of either precursor has been shown previously to be important for isoprenoid production via the MEP pathway [4, 5, 48]. Two studies have demonstrated, unsurprisingly, that this is also important for isoprene production. The first examined different pathways for glucose utilisation, namely the Embden–Meyerhof pathway (EMP), Entner–Doudoroff pathway (EDP), pentose phosphate pathway (PPP), and Dahms pathway [76]. Carbon redirection to specific pathways was achieved through various strategies: glucose-6-phosphate isomerase (*pgi*) was disrupted to divert carbon away from the preferred EMP and towards PPP (in this case flux also occurs to a lesser extent via the EDP); a double *pgi* and gluconate-6-phosphate dehydrogenase (*gnd*) knock-out blocks both EMP and PPP, channelling flux towards the EDP; feeding D-xylulose restricts glycolytic carbon flux to the PPP; and finally, carbon from xylose was channelled into a Dahms pathway constructed by introducing a xylonate catabolic pathway, knocking out the PPP by disrupting *xylA*, and introducing D-xylose dehydrogenase to convert D-xylose to D-xylonate. The EDP, which produces equimolar GA3P: pyruvate, provided the highest titres; combining this with an engineered MEP pathway (overexpression of *dxs*, *idi*, and *IspG*) and a modified *P. alba IspS* provided titres of $\sim$225 mg/L isoprene (about threefold higher than the engineered MEP pathway alone).

The second study utilised a novel approach to balance precursor levels on galactose by forcing galactose utilisation via the De Ley–Doudoroff pathway [93]. This involved blocking the Leloir pathway for galactose utilisation and engineering a complete De Ley–Doudoroff pathway by introducing a missing dehydrogenase gene from *Pseudomonas syringae* to catalyse conversion of galactose to galactonase. Equimolar ratios of GA3P and pyruvate were achieved in the resulting strain. Coupling this pathway with overexpression of *E. coli dxs* + *idi* and a *P. alba IspS* resulted in a 3.7-fold improvement over the MEP pathway engineering alone. The final titre was 260 mg/L isoprene, very similar to the first study. This result confirmed the importance of balancing precursor concentrations; however, it would not be feasible in an industrial setting due to the cost of galactose.

Other approaches for balancing GA3P and pyruvate that might increase isoprene production include overexpressing glucose-6-phosphate dehydrogenase (G6PDH, encoded by *zwf*) alone or in combination with the modifications described above, that is, limiting expression of *pgi* to redirect flux to PPP and EDP, and/or limiting expression of *gnd* to limit flux to the PPP and increase flux to the EDP [37].

3.3.3 Addressing Remaining MEP Flux Limitations

The fact that the MEP pathway delivers higher theoretical yields for isoprenoids from simple sugars than the MVA pathway [101, 133] has driven extensive research efforts towards improving MEP pathway flux. However, despite the significant engineering that has thus far been performed, the MEP pathway still delivers lower yields/titres than heterologously expressed MVA pathways (see below).

Flux is still clearly limited at the 4Fe–4S enzymes, and resolving the problems at these nodes will take further research. In addition, the MEP pathway relies heavily on reducing equivalents (required at both the 4Fe–4S nodes and at DXR), and it was anticipated early that increasing their availability would improve MEP pathway flux [4]. Ultimately, as flux bottlenecks are relieved, improving carbon channelling into MEP pathway precursors (in addition to keeping central carbon intermediates balanced, as described above) will then become limiting.

Avoiding loss of carbon through side pathway reactions may also help. In *E. coli*, DXP (the product of DXS) is used as a precursor for the B-group vitamins thiamine and pyroxidine. Deletion/downregulation of thiamine and pyridoxine pathways to minimise carbon loss/competition/feedback from those pathways might also improve MEP pathway flux for isoprene production [37]. However, given that these vitamins are essential, and that thiamine (as thiamine diphosphate, TPP) is a cofactor for DXS, care should be taken with this approach.

Finally, it has recently been shown that both IPP and DMAPP compete with TPP at the TPP binding site in a *Populus trichocarpa* DXS, thereby causing competitive inhibition of DXS [12]. Presumably, this is also the case for other DXS enzymes. Moreover, given that TPP is a cofactor for many different enzymes, and that IPP/DMAPP have very similar binding mechanisms to TPP, increased DMAPP/IPP pools might cause significant metabolic perturbation (this may indeed be the mechanism by which IPP/DMAPP exert toxicity). This feedback inhibition and the potential side-effects underline the importance of ensuring that prenyl phosphate pools do not accumulate in engineered strains. Further improvement of the IspS enzyme catalytic properties, in parallel with flux modification to ensure that isoprene production is balanced with essential pathway requirements downstream with DMAPP, may be required.

3.3.4 Engineering the MVA Pathway in *E. coli*

Although substantial advances have been achieved by engineering through the native MEP pathway, the best yields to date are significantly below calculated theoretical maxima for the pathway [101, 137]. It appears that MEP carbon flux is heavily regulated, and as yet we do not understand enough about this regulation to overcome it [135]. In order to circumvent this problem, either a partial MVA pathway (with mevalonate supplementation; [32]) or a complete MVA pathway [82] have been introduced into *E. coli* strains engineered for isoprenoid production. This approach was also successful for improving isoprene production [15, 31, 33,

57, 142, 157]. An MVA pathway was constructed by using *mvaE* (which encodes a dual function enzyme that has both acetyl-CoA acetyltransferase activity and HMG-CoA reductase activity; see Fig. 1) and *mvaS* (mevalonate synthase) from *Enterococcus faecalis* (referred to generically as the 'upper' MVA pathway) combined with mevalonate kinase (MK), phosphomevalonate kinase (PMK), diphosphomevalonate decarboxylase (MVD), and IDI from *Saccharomyces cerevisiae* (referred to generically as the 'lower' MVA pathway). In the presence of an engineered kudzu isoprene synthase, this engineered MVA pathway provided a 3-fold increase in isoprene production compared to the engineered MEP pathway, and was capable of producing 20 g/L in 40-h fermentation [33]. A next-generation strain that included a feedback-resistant version of MK from *Methanosarcina mazei* and an engineered *P. alba* isoprene synthase produced 35 g/L isoprene in fed-batch culture (see further comments regarding bioprocess considerations below; [15, 33]). The isoprene yield from glucose was 5.2 %. This strain provides a 73-fold improvement over IspS alone; compared to an engineered MEP pathway, the MVA pathway provides a 10–20-fold improvement in isoprene production [31]. Judicious selection of MVA pathway genes, including screening genes from different source organisms or improved catalytic activity and selection of feedback-resistant versions of hydroxymethylglutaryl-CoA reductase (HMGR) and MK (the enzymes catalysing the primary and secondary rate-limiting steps, respectively) can also increase MVA-produced isoprene [13, 15, 31, 142]. Examining MVA production from the 'upper MVA pathway' (steps up to MVA synthesis) and titrating copy number have demonstrated that the upper pathway flux is quite good when using *E. faecalis* enzymes, however, flux is limited by steps downstream of MVA when it is integrated onto the chromosome [31]. Varying the source of the upper pathway genes can have a significant effect on isoprene production, however, the effect is dependent on the catalytic properties of the isoprene synthase, as different IspS enzymes exhibit different levels of substrate inhibition [13].

3.3.5 Improving MVA Pathway Precursor Availability

Modifications to the central carbon metabolism in MVA pathway engineered strains can also improve isoprene production. The MVA pathway initiates at the central carbon metabolite acetyl-CoA, and the first two steps involve condensation of three acetyl-CoA molecules to produce HMG-CoA (see Fig. 1b). There are multiple competing pathways that use acetyl-CoA, and decreasing this competition where possible increases production of desirable biochemicals. Moreover, decreasing other indirect carbon sinks and increasing availability of reducing equivalents can also improve flux through desired pathways.

Overexpressing phosphogluconolactonase (*pgl*) to improve pentose phosphate pathway flux (and possibly also suppress posttranslational glucosylation of heterologously expressed proteins) results in a two- to threefold increase in specific productivity of isoprene over the parent strain, which contained a *P. alba IspS,* engineered MVA pathway, and an *M. masei* MK [16, 33, 142]. Titres of 80 g/L were

reported using a strain with a truncated, codon-optimised *P. alba* isoprene synthase engineered for improved catalytic properties in combination with an MVA pathway that included feedback-resistant enzymes, overexpression of *pgl*, and an optimised fed-batch process (see the section on bioprocess considerations below; [16]).

Further investigations into carbon and cofactor competition started with construction of a base strain with downregulated citrate synthase (*gltA*, a TCA-cycle enzyme that competes for acetyl-CoA; [17]). Combining this with knock-out of *pgl* and overexpressing phosphoketolase (*pkl*) to scavenge carbon from xylulose-5-phosphate into isoprenoid pathway precursors further increased isoprene titres [17]. This approach is thought to improve the balance or availability of pathway precursors, however, gene dose and the type of *pkl* are important. Combining overexpression of a plasmid-borne *Enterococcus gallinarum pkl* with other MVA pathway engineering steps and a truncated *P. alba IspS* provided titres of up to 123.6 g/L, overall volumetric productivities up to 2.21 g/L/h and 16.3–17.4 % yields of isoprene on glucose [17]. These are the highest isoprene titres thus far reported.

3.3.6 Controlling Downstream Prenyl Phosphate Feedback

As discussed above (see the section, 'Addressing Remaining MEP Pathway Flux Constraints') MEP pathway-specific feedback issues are particularly problematic for DMAPP/IPP. However, as also discussed above, prenyl phosphates have been shown to drive feedback regulation of pathway flux in both the MVA and MEP pathways. Because of the high $K_{m(DMAPP)}$ of IspS relative to downstream prenyl transferases, in a strain with increased upstream flux to IPP and DMAPP there is likely to be a buildup of downstream prenyl phosphates. This can in turn feed back to control pathway flux. Approaches to minimise prenyl phosphate accumulation by coexpression of monoterpene and sesquiterpene synthases with IspS have been suggested to improve isoprene accumulation [37] and are integrated in the highest-producing MVA-based strains [17]. Appropriate balancing of IspS activity with upstream flux and downstream requirements for essential pathway products will be required for both MEP and MVA engineering going into the future. Titration of these complex factors requires high-throughput systems to analyse isoprenoid products [20].

3.4 Novel Isoprene Production Pathways

An alternative route for conversion of DMAPP to isoprene, presumably to circumvent the poor catalytic properties of isoprene synthase, has been proposed [27]. 3-Methyl-2-buten-l-ol has a similar carbon backbone to isoprene, and can be biotransformed into isoprene by *E. coli* engineered with a strawberry (*Fragaria* sp.) acyl-CoA transferase (SAAT) and a *Castellaniella defragrans* strain 65Phen linalool dehydratase-isomerase (LDI; [27]). The SAAT isomerises 3-methyl-2-buten-l-ol to the aldehyde 2-methyl-3-buten-l-ol (prenal), and the LDI dehydrates

2-methyl-3-buten-l-ol into isoprene. Although not directly demonstrated, it was proposed that DMAPP can be converted into 3-methyl-2-buten-l-ol through the action of a *Pinus sabiniana* methylbutenol synthase.

Both the MEP and MVA pathways operate optimally under aerobic conditions due to their energetic requirements. Moreover, their theoretical maxima are sub-optimal compared to other pathways. To address these issues, novel theoretical isoprene production pathways that operate effectively under anaerobic conditions have recently been proposed [40]. Four pathways that proceed via 2,3-di-hydroxyisovalerate (DHIV), an amino acid pathway intermediate which has an isoprene carbon skeleton and is produced from pyruvate via just two enzymatic steps, are described. Each pathway involves a series of reduction and dehydration reactions to produce isoprene and would require introduction of five to seven additional enzymes (many of which are theoretical activities). All four pathways are redox balanced (unlike the MVA pathway); one is ATP neutral and three of them are net ATP-positive (unlike the MEP pathway, which has a net cost of 3 ATP). Although the work is currently theoretical, it offers an exciting potential approach, especially given that ATP-producing pathways can be coupled to growth rate through increased pathway flux, thereby allowing the option of applying adaptive evolution for pathway improvement.

3.5 Engineering Isoprene Production in Other Organisms

Outside of *E. coli*, a handful of other microorganisms have been engineered for isoprene production, but the results have been somewhat disappointing in terms of titres/yields.

S. cerevisiae, which uses the MVA pathway, was engineered with truncated wild-type or codon-optimised *P. montana IspS* cDNA sequences [61]. Codon optimisation did improve isoprene production about twofold; however, although isoprene was detected in both strains, levels were very low, and the dominant products were hydroylated isoprene derivatives. Combined isoprene + derivatives in headspace measurements were estimated at ~500 μg/L culture (this excludes derivatives that were retained in the aqueous phase). A truncated *Mucuna bracteata IspS* was also tested in *S. cerevisiae*, but titres were only 16.1 μg/L under the conditions tested [57]. These results cannot be compared directly to the previous experiment, but they are not very encouraging. Overcoming both the low titres (relative to *E. coli*) and propensity for bioconversion will be necessary before yeast can become an attractive isoprene production organism.

Isoprene production by overexpression of an engineered kudzu IspS (truncated, codon-optimised for *E. coli*) in a variety of different constructs (different promoters and different copy numbers) was also tested in *Pantoea citra* [33]. The best production observed was about 10 μg/L. The truncated *M. bracteata IspS* yielded slightly better results in *Pantoea ananatis* (63 μg/L; [57]). The *M. bracteata IspS*

was also tested in *Corynebacterium glutamicum* (24.2 μg/L) and *Enterobacter aerogenes* (316 μg/L; [57]).

Upon expression of the kudzu construct in *B. subtilis*, isoprene titres were increased threefold over native production, from 400 μg/L to 1.2 mg/L in batch culture; in a fed-batch culture titres peaked at 30 mg/L. Similar titres were achieved using an engineered MVA pathway. Truncated kudzu and poplar IspS genes codon-optimised and expressed in *Yarrowia lipolytica* using a number of different approaches; titres ranged from 0.5–1.0 μg/L in the culture headspace [33]. The engineered kudzu IspS was also expressed in *Trichoderma reesei*; titres peaked at 0.5 μg/L. A codon-optimised version was also expressed in *Streptomyces alba*, which naturally produces low levels of isoprene; isoprene production was increased tenfold but levels were still extremely low (0.75 ppm in headspace samples; [33]).

Engineering photosynthetic organisms for isoprene production is an attractive proposition, as it provides the potential to circumvent feedstock production for bioprocesses. With this aim, a truncated (*sans* chloroplast targeting sequence), tagged *P. montana IspS* cDNA was introduced into a glucose-sensitive version of the photosynthetic bacterium *Synechocystis* sp. PC6803 [75]. Only very low levels of IspS protein were detected. Codon optimisation significantly enhanced protein production, and this latter strain produced ∼50 μg isoprene per gram dry cell weight per day. This was noted to be equivalent to ∼4 μg isoprene/L/h^{-1} [61], compared to production of 50 μg/L/h^{-1} in wild-type *B. subtilis* [64]. The same *IspS* construct was later used with a glucose-sensitive version of *Synechocystis* sp. PC6803 [21]. Isoprene production peaked at ∼100–130 μg/L culture [21, 22]. Introducing a heterologous MVA pathway improved production to slightly over 300 μg/L culture [22].

In an alternative approach to engineer photosynthetic isoprene production, the hybrid poplar IspS was tagged and codon-optimised for expression in the cyanobacterium *Thermococcus elongatus* [6]. This thermophilic organism has an optimal growth temperature of 55 °C, potentially rendering it a very attractive isoprene production organism inasmuch as (a) volatilisation is favoured, and (b) IspS enzymes have high temperature optima (see above). Mutations to improve thermostability were included in the gene design. However, isoprene production was low (25 μg/L per 30 min in the off-gas). As for yeast, and other production organisms, significant effort will be required to produce photosynthetic cyanobacterial strains that can compete with *E. coli* for isoprene production.

3.6 Bioprocess Considerations

A number of general and more specific considerations apply to microbial isoprene bioprocesses. First, as isoprene is a gas under typical bioprocess conditions, it volatilises into the headspace of the culture. This provides both advantages and disadvantages: purification is far more straightforward from the gas phase; however, it must be trapped from this phase, ideally in a continuous process.

Accumulation of isoprenoids is usually done in *E. coli* at decreased temperature (30 °C), as this minimises formation of inclusion bodies from overexpressed proteins. This may have a significant effect on IspS activity, depending on where the temperature optimum of the enzyme sits. As engineering to improve solubility and thermostability progresses (see above), titration of the bioprocess temperature—taking into account the cost-benefit analyses—may provide further improvements in yields.

Isoprene is flammable at high concentrations; consequently, it is necessary to engineer bioprocess conditions that maximise isoprene yields while keeping isoprene in the culture headspace at low enough levels to remain nonflammable and prevent serious hazards. A combination of computer modelling and experimental testing to determine the flammability limits of isoprene in the presence of various gases (among other considerations) was used to develop bioprocesses running outside the flammability envelope of isoprene [31].

Methods for recovering, purifying, and polymerising isoprene for industrial applications have also been developed [25, 31]. Isoprene can be recovered by adsorption to a solid phase, partition into a liquid phase, or direct compression/condensation. For off-gas methods, excess H_2O and CO_2 can be removed by passing the off-gas through sodium hydroxide pellets and isoprene condensed by cryotrapping in a continuous process. Both the nature and level of contaminants is important as polymerisation catalysts are sensitive to particular classes of compounds [142]. Isoprene produced via this biological route is purer than petrochemical-derived isoprene and therefore offers significant downstream processing advantages [31].

In bioprocesses it is often desirable to decouple the growth (biomass accumulation) phase from the production phase. When isoprene production genes (synthases and engineered isoprenoid pathway genes) are placed under inducible promoters, expression (and thus induction of isoprene production) can be titrated for optimal effect [31]. In this way, production of isoprene precursors (in this case, through the mevalonate pathway) and isoprene can be separated from cell growth. Different induction times can significantly affect titres.

Fed-batch processes produce significantly higher titres and yields than batch processes with the amount of isoprene being proportional to the amount of carbon source fed [33]. Optimising the feed times is important to maximise yields [31]. Isoprene yields are higher on rich media than on minimal media, with glucose/yeast extract fed-batch cultures providing the best results [31, 33].

For bulk biochemicals, the feedstock price is the primary bioprocess cost driver [101, 148]. This has driven research into alternative feedstocks, including sucrose [8, 9, 29, 104], which has a number of advantages over traditional glucose [7, 134, 137]; glycerol, a highly reduced industrial biofuel by-product [44]; and of course lignocellulosic feedstocks, which are challenging but have high potential [23, 42, 96]. A variety of different carbon sources, including glucose, lignocellulosic sources, glycerol, and the like, has also been tested for isoprene production [27, 31, 33, 38]. The advantages of glycerol are particularly applicable to isoprenoids, as they themselves are highly reduced. Use of glycerol provides improved yields of

isoprenoids [27, 66, 82, 149], most likely due to improved energy and redox balances [44]. Improved isoprene yields from glycerol were achieved by improving glycerol catabolism through introduction of either a glycerol kinase and a glycerol-3-phosphate dehydrogenase or a glycerol dehydrogenase and a dihydroxyacetone kinase [27].

For photosynthetic production of isoprene from cyanobacteria (and potentially, microalgae), a diffusion-based process allowing both uptake of carbon dioxide and emission of isoprene has been developed [21]. This involves use of a custom-designed two-phase (gas/aqueous) photobioreactor. The reactor is sealed, and the headspace is periodically flushed with carbon dioxide in parallel with recovery of the gas-phase products (isoprene). As discussed above, substantial engineering will be required to make such a process competitive with current *E. coli*-based processes; however, the benefit of photosynthetic conversion would be significant. The photobioreactor process can potentially be applied to other photosynthetically produced volatiles.

4 Engineering Isoprene Production in Plants

Although the exact mechanism remains a topic of research, it is now apparent that isoprene production provides protection to plants against a variety of abiotic stresses [80, 118, 136]. Naturally nonemitting model species (tobacco and *Arabidopsis*) emit isoprene and show resistance to oxidative and thermal stress when isoprene synthase is introduced [78, 110, 138]. This has opened the discussion on the possibility of exploiting the biological role of isoprene by engineering agricultural species (which do not typically produce isoprene) for improved stress resistance through introducing isoprene synthase [135].

However, although the complex emission patterns observed in naturally emitting plants can be reproduced in engineered plants [138, 140], the genetics and molecular regulation of isoprene synthase are relatively complex [139]. This may present unforeseen problems when trying to reproduce the protective effects observed in nature [135]. For example, protection of photosynthetic processes is observed in isoprene-emitting transgenic tobacco exposed to drought [103, 128], however, broad changes in isoprenoid, nonstructural carbohydrate, and phenylpropanoid metabolism [128], and reductions in plant productivity [103], may be observed. Moreover, although engineering tobacco plants with isoprene synthase can deter herbivores [71, 72], isoprene emission in engineered *Arabidopsis* can interfere with recruitment of herbivore parasites, thus upsetting complex tritrophic interactions [77]. This would obviously be undesirable in an agricultural setting.

Isoprene production appears to mediate in the cellular reactive oxygen balance/response system, particularly under stress conditions; but differences are also observed under nonstress conditions, suggesting that the response system is essentially 'primed' for a rapid effective response to oxidative stress [136, 138]. Moreover, loss of carbon in the form of isoprene represents a cost to the plant; this

may be only a few percent of recently fixed carbon, or may form up to 10–20 % under increased temperature [56, 86, 116]. When plants are suffering from abiotic stress, emissions are often increased/maintained even under photosynthetic limitation [28, 91, 130], thereby further increasing the relative proportion of lost carbon. Both of these elements could affect the observed metabolic and productivity perturbations [103, 128]. A final consideration is the possible effect of perturbed reactive oxygen response systems under pathogen attack, where carefully controlled and specific ROS responses are required for protection against different pathogens.

The opposite proposition—that is, engineering plants to decrease/remove isoprene emission—has also been considered [19]. Tree species, including many that are grown as forestry crops (e.g., pine, eucalyptus, and poplar; [65]), are contributors to the global atmospheric isoprene budget. Isoprene emission in plantation forests can affect local climate and air quality [59, 143]. Moreover, minimising carbon loss to isoprene might increase productivity in forestry species. When transgenic hybrid poplar plants that do not emit isoprene (emission suppressed) were compared to empty vector controls over two growing seasons, no differences in growth or biomass accumulation were observed under the growth conditions used. Modelling suggested a 2.2 % reduction in carbon loss in the absence of isoprene emission; this may contribute to improved biomass accumulation over the lifecycle of a tree. However, although the trees exhibited reduced susceptibility to the fungal pathogen *Pollaccia radiosa* (poplar shoot blight), they also became more attractive to herbivores. Moreover, given the demonstrated protective effects of isoprene under abiotic stress conditions (see above), this approach should be treated with caution, especially as it has been indicated that suppression of isoprene emission in these transgenic plants also has a negative effect on thermotolerance [18].

5 Summary, Conclusions, Outlook

The biology, atmospheric chemistry, and industrial applications of isoprene have proved rich grounds for discovery over a period of scientific investigation spanning over one and a half centuries from its discovery. As our society begins its shift from a petrochemical society to a biochemical society, biological engineering of isoprene production has emerged at the forefront of the new age of biotechnology. To date, engineering in the industrial workhorse *E. coli* has proved to be by far the most successful. Other organisms, including the lab/industry favourite, yeast, will require significant work (both in bioengineering and in bioprocess engineering) to approach the titres, rates, and yields observed in *E. coli*.

As reviewed above, many different engineering steps and approaches have been applied to isoprene production in *E. coli*. A wide variety of other gene targets and engineering approaches that have been shown to influence production of related isoprenoid compounds might also increase flux towards isoprene. These include targets that increase flux to pathway precursors, balance cofactor/precursor

concentrations, regulate overall isoprenoid accumulation, balance expression of pathway enzymes, and avoid accumulation of toxic intermediates [1, 2, 5, 17, 26, 36, 62, 67, 81, 124, 141, 153, 155]. As of 2010, the tires produced using isoprene from the Genencor process cost $150,000 each [54], obviously an unattractive proposition for the average motorist. No current estimates are available, but significant advances in bioengineering and bioprocess conditions have been made since then, and current yields are much higher.

With respect to engineering isoprene production in plants, the cost and benefit of the pleiotropic effects discussed above would need to be carefully evaluated in agricultural/silvicultural settings. It was recently suggested that the patchy taxonomic distribution of isoprene emission might be due to isoprene emission only being advantageous under a narrow range of environmental and phenotypical conditions [87]; if this is the case, a better understanding of those conditions is required. Changing climate conditions in the future, which are now accepted as a scientific reality regardless of the political debate on the cause, may have a significant effect on this cost-benefit analysis. All of these considerations should be taken into account, and potential mitigating strategies should be investigated, when designing potential agronomic engineering strategies involving isoprene emission [135].

References

1. Ajikumar PK, Tyo K, Carlsen S, Mucha O, Phon TH, Stephanopoulos G (2008) Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. Mol Pharm 5(2):167–190. doi:10.1021/mp700151b
2. Ajikumar PK, Xiao W-H, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. Science 330(6000):70–74. doi:10.1126/science.1191652
3. Albrecht M, Misawa N, Sandmann G (1999) Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. Biotechnol Lett 21(9):791–795
4. Alper H, Jin YS, Moxley JF, Stephanopoulos G (2005) Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. Metab Eng 7 (3):155–164. doi:10.1016/j.ymben.2004.12.003
5. Alper H, Miyaoku K, Stephanopoulos G (2005) Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets. Nat Biotechnol 23(5):612–616
6. Anderson S (2013) Methods for the direct conversion of carbon dioxide into a hydrocarbon using a metabolically engineering photosynthetic organism. USA Patent US8609385, 17 Dec 2013
7. Archer C, Kim J, Jeong H, Park J, Vickers C, Lee S, Nielsen L (2011) The genome sequence of *E. coli* W ATCC 9637: comparative genome analysis and an improved genome-scale model of *E. coli*. BMC Genomics 12(9):9. doi:10.1186/1471-2164-1112-1189
8. Arifin Y, Archer C, Lim S, Quek L-E, Sugiarto H, Marcellin E, Vickers C, Krömer J, Nielsen L (2014) *Escherichia coli* W shows fast, highly oxidative sucrose metabolism and low acetate formation. Appl Microbiol Biotechnol 98(21):9033–9044. doi:10.1007/s00253-014-5956-4

9. Arifin Y, Sabri S, Sugiarto H, Krömer JO, Vickers CE, Nielsen LK (2011) Deletion of *cscR* in *Escherichia coli* W improves growth and poly-3-hydroxyburyrate (PHB) production from sucrose in fed batch culture. J Biotechnol 156:275. doi:10.1016/j.jbiotec.2011.07.003
10. Artsatbanov VY, Vostroknutova GN, Shleeva MO, Goncharenko AV, Zinin AI, Ostrovsky DN, Kapreliants AS (2012) Influence of oxidative and nitrosative stress on accumulation of diphosphate intermediates of the non-mevalonate pathway of isoprenoid biosynthesis in corynebacteria and mycobacteria. Biochemistry 77(4):362–371. doi:10.1134/S0006297912040074
11. Banerjee A, Sharkey TD (2014) Methylerythritol 4-phosphate (MEP) pathway metabolic regulation. Nat Prod Rep. doi:10.1039/C3NP70124G
12. Banerjee A, Wu Y, Banerjee R, Li Y, Yan H, Sharkey TD (2013) Feedback inhibition of deoxy-D-xylulose 5-phosphate synthase regulates the methyl erythritol 4-phosphate pathway. J Biol Chem 288(23):16926–16936. doi:10.1074/jbc.M113.464636
13. Beatty MK, Hayes K, Hou Z, Meyer DJ, Nannapaneni K, Rife CL, Wells DH, Zastrow-Hayes GM (2013) Legume isoprene synthase for production of isoprene. USA Patent US 20130330709, 12 Dec 2013
14. Beck ZQ, Bott RR, Rife CL, Wells D, Miller JV (2013) Three-dimensional structure of isoprene synthase and its use thereof for generating variants. USA Patent US8518686, 27 Aug 2013
15. Beck ZQ, Calabria AR, Miller MC, Vaviline DV, Nielsen AT (2013) Increased isoprene production using the archaeal lower mevalonate pathway. USA Patent US8361762, 29 Jan 2013
16. Beck ZQ, Cervin MA, Nielsen AT, Peres CM (2013) Compositions and methods of PGL for the increased production of isoprene. USA Patent US8455236B2, 4 Jun 2013
17. Beck ZQ, Eliot AC, Peres CM, Vaviline DV (2013) Utilization of phosphoketolase in the production of mevalonate, isoprenoid precursors, and isoprene. Application US20130089906, 11 April 2013
18. Behnke K, Ehlting B, Teuber M, Bauerfeind M, Louis S, Hansch R, Polle A, Bohlmann J, Schnitzler JP (2007) Transgenic, non-isoprene emitting poplars don't like it hot. Plant J 51:485–499
19. Behnke K, Grote R, Brüggemann N, Zimmer I, Zhou G, Elobeid M, Janz D, Polle A, Schnitzler J-P (2012) Isoprene emission-free poplars—a chance to reduce the impact from poplar plantations on the atmosphere. New Phytol 194(1):70–82. doi:10.1111/j.1469-8137.2011.03979.x
20. Behrendorff JBYH, Vickers CE, Chrysanthopoulos P, Nielsen LK (2013) 2,2-Diphenyl-1-picrylhydrazyl as a screening tool for recombinant monoterpene biosynthesis. Microb Cell Fact 12:76. doi:10.1186/1475-2859-12-76
21. Bentley FK, Melis A (2012) Diffusion-based process for carbon dioxide uptake and isoprene emission in gaseous/aqueous two-phase photobioreactors by photosynthetic microorganisms. Biotechnol Bioeng 109(1):100–109. doi:10.1002/bit.23298
22. Bentley FK, Zurbriggen A, Melis A (2014) Heterologous expression of the mevalonic acid pathway in cyanobacteria enhances endogenous carbon partitioning to isoprene. Mol Plant 7(1):71–86. doi:10.1093/mp/sst134
23. Blanch HW, Simmons BA, Klein-Marcuschamer D (2011) Biomass deconstruction to sugars. Biotechnol J 6(9):1086–1102. doi:10.1002/biot.201000180
24. Boghigian BA, Salas D, Ajikumar PK, Stephanopoulos G, Pfeifer B (2012) Analysis of heterologous taxadiene production in K- and B-derived *Escherichia coli*. Appl Microbiol Biotechnol 93(4):1651–1661. doi:10.1007/s00253-011-3528-4
25. Bott RR, Cervin MA, Kellis JT, Jr, McAuliffe JC, Miasnikov A, Peres CM, Rife CL, Wells DH, Weyler W, Whited GM (2012) Isoprene synthase variants for improved microbial production of isoprene. USA Patent US8173410, 8 May 2012
26. Bramucci MG, Brzostowicz PC, Cheng Q, Kostichka KN, Rouviere PE, Nagarajan V, Tao L, Thomas SM (2006) Genes involved in isoprenoid compound production. USA Patent US7034140, 25 April 2006

27. Bresdow S, Doneske S, Li M, Zhou H, Monticello DJ, Campbell P (2014) Microorganisms and processes for the conversion of glycerol to isoprene. Application WO2014015210 A2, 23 Jan 2014
28. Brilli F, Barta C, Fortunati A, Centritto M, Loreto F (2006) The contribution of different carbon sources to isoprene synthesis during and after drought in *Populus alba* leaves. Geophys Res Abs 8:EGU06-A-04113
29. Bruschi M, Boyes S, Sugiarto H, Nielsen LK, Vickers CE (2011) A transferrable sucrose utilization approach for non-sucrose-utilizing *Escherichia coli* strains. Biotech Adv 30 (5):1001–1010. doi:10.1016/j.biotechadv.2011.08.019
30. Burke C, Croteau R (2002) Geranyl diphosphate synthase from Abies grandis: cDNA isolation, functional expression, and characterization. Arch Biochem Biophys 405(1):130–136. doi:10.1016/S0003-9861(02)00335-1
31. Calabria AR, Cervin MA, Chotani GK, La Duca R, McAuliffe JC, Miller MC, Sabo TA, Sanford KJ, Webster EL, Whited GM (2013) Compositions and methods for producing isoprene free of C5 hydrocarbons under decoupling conditions and/or safe operating ranges. USA Patent US8420360, 16 April 2013
32. Campos N, Rodríguez-Concepción M, Sauret-Gueto S, Gallego F, Lois LM, Boronat A (2001) *Escherichia coli* engineered to synthesize isopentenyl diphosphate and dimethyl allyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis. Biochem J 353:59–67
33. Cervin MA, Chotani GK, Feher FJ, La Duca R, McAuliffe JC, Miasnikov A, Peres CM, Puhala AS, Sanford KJ, Valle F, Whited GM (2012) Compositions and methods for producing isoprene. USA Patent US8420360 B2, 16 Oct 2012
34. Chae HS, Kim KH, Kim SC, Lee PC (2010) Strain-dependent carotenoid productions in metabolically engineered *Escherichia coli*. Appl Biochem Biotechnol 162(8):2333–2344. doi:10.1007/s12010-010-9006-0
35. Chameides WL, Lindsay RW, Richardson J, Kiang CS (1998) The role of biogenic hydrocarbons in urban photochemical smog: atlanta as a case study. Science 241:1473–1475
36. Chandran SS, Kealey JT, Reeves CD (2011) Microbial production of isoprenoids. Process Biochem 46(9):1703–1710. doi:10.1016/j.procbio.2011.05.012
37. Chotani GK, McAuliffe JC, Miller MC, Muir RE, Vaviline DV, Weyler W (2013) Isoprene production using the DXP and MVA pathway. US8507235 B2, 13 Aug 2013
38. Chotani GK, Nielsen A, Sanford KJ (2013) Reduction of carbon dioxide emission during isoprene production by fermentation. WO/2010/031068
39. Claeys M, Graham B, Vas G, Wang W, Vermeylen R, Pashynska V, Cafmeyer J, Guyon P, Andreae MO, Artaxo P, Maenhaut W (2004) Formation of secondary organic aerosols through photooxidation of isoprene. Science 303(5661):1173–1176
40. Coelho PS, Farrow MF, Smith MA (2014) *De novo* metabolic pathways for isoprene biosynthesis. World intellectual property organisation patent application WO2014066892 A1, 1 May 2014
41. Dellas N, Thomas ST, Manning G, Noel JP, Weigel D (2013) Discovery of a metabolic alternative to the classical mevalonate pathway. eLife 2:e00672. doi:10.7554/eLife.00672
42. Dionisi D, Anderson JA, Aulenta F, McCue A, Paton G (2014) The potential of microbial processes for lignocellulosic biomass conversion to ethanol: a review. J Chem Technol Biotechnol. doi:10.1002/jctb.4544
43. Djaman O, Outten FW, Imlay JA (2004) Repair of oxidized iron-sulphur clusters in *Escherichia coli*. J Biol Chem 279(43):44590–44599. doi:10.1074/jbc.M406487200
44. Durnin G, Clomburg J, Yeates Z, Alvarez PJJ, Zygourakis K, Campbell P, Gonzalez R (2009) Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol Bioeng 103(1):148–161. doi:10.1002/bit.22246
45. Eisenreich W, Bacher A, Arigoni D, Rohdich F (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell Mol Life Sci V61(12):1401–1426
46. Fall R, Copley SD (2000) Bacterial sources and sinks of isoprene, a reactive atmospheric hydrocarbon. Environ Microbiol 2(2):123–130

47. Fall R, Wildermuth MC (1998) Isoprene synthase: from biochemical mechanism to emission algorithm. J Geophys Res 103(D19):25599–25610
48. Farmer WR, Liao JC (2001) Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. Biotechnol Progr 17(1):57–61
49. Fortunati A, Barta C, Brilli F, Centritto M, Zimmer I, Schnitzler JP, Loreto F (2008) Isoprene emission is not temperature-dependent during and after severe drought-stress: a physiological and biochemical analysis. Plant J 55(4):687–697
50. Goldstein JL, Brown MS (1990) Regulation of the mevalonate pathway. Nature 343 (6257):425–430
51. Gräwert T, Kaiser J, Zepeck F, Laupitz R, Hecht S, Amslinger S, Schramek N, Schleicher E, Weber S, Haslbeck M, Buchner J, Rieder C, Arigoni D, Bacher A, Eisenreich W, Rohdich F (2004) IspH protein of *Escherichia coli*: studies on iron–sulfur cluster implementation and catalysis. J Am Chem Soc 126(40):12847–12855. doi:10.1021/ja0471727
52. Gray DW, Breneman SR, Topper LA, Sharkey TD (2011) Biochemical characterization and homology modelling of methylbutenol synthase and implications for understanding hemiterpene synthase evolution in plants. J Biol Chem 286(23):20582–20590. doi:10.1074/jbc.M111.237438
53. Guenther A, Karl T, Harley P, Wiedinmyer C, Palmer PI, Geron C (2006) Estimates of global terrestrial isoprene emissions using MEGAN (model of emissions of gases and aerosols from nature). Atmos Chem Phys 6:3181–3210
54. Hansegard J (2010) Sugar plus science equals $150,000 tires. Wall Street J, 8 Dec 2010, http://online.wsj.com/news/articles/SB10001424052748703493504576007112633560914
55. Harker M, Bramley PM (1999) Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. FEBS Letters 448(1):115–119. doi:10.1016/s0014-5793(99)00360-9
56. Harley P, Guenther A, Zimmerman P (1996) Effects of light, temperature and canopy position on net photosynthesis and isoprene emission from sweetgum (*Liquidambar styraciflua*) leaves. Tree Physiol 16(1–2):25–32
57. Hayashi Y, Harada M, Takaoka S, Fukushima Y, Yokoyama K, Nishio Y, Tajima Y, Mihara Y, Nakata K (2014) Isoprene synthase and gene encoding the same, and method for producing isoprene monomer. USA Patent Application Number US2014/0113344
58. Hemmerlin A (2013) Post-translational events and modifications regulating plant enzymes involved in isoprenoid precursor biosynthesis. Plant Sci 203–204:41–54
59. Hewitt CN, MacKenzie AR, Di Carlo P, Di Marco CF, Dorsey JR, Evans M, Fowler D, Gallagher MW, Hopkins JR, Jones CE, Langford B, Lee JD, Lewis AC, Lim SF, McQuaid J, Misztal P, Moller SJ, Monks PS, Nemitz E, Oram DE, Owen SM, Phillips GJ, Pugh TAM, Pyle JA, Reeves CE, Ryder J, Siong J, Skiba U, Stewart DJ (2009) Nitrogen management is essential to prevent tropical oil palm plantations from causing ground-level ozone pollution. Proc Natl Acad Sci USA 106(44):18447–18451. doi:10.1073/pnas.0907541106
60. Hewitt CN, Street RA, Scholefield PA (2004) Biogenic VOC Emission Database. Lancaster University. http://www.es.lancs.ac.uk/cnhgroup/download.html. Accessed on 6 Oct 2014
61. Hong S-Y, Zurbriggen AS, Melis A (2012) Isoprene hydrocarbons production upon heterologous transformation of *Saccharomyces cerevisiae*. J Appl Microbiol 113(1):52–65. doi:10.1111/j.1365-2672.2012.05319.x
62. Jiang M, Stephanopoulos G, Pfeifer BA (2012) Toward biosynthetic design and implementation of *Escherichia coli*-derived paclitaxel and other heterologous polyisoprene compounds. Appl Environ Microbiol 78(8):2497–2504. doi:10.1128/aem.07391-11
63. Jones CA, Rasmussen RA (1975) Production of isoprene by leaf tissue. Plant Physiol 55 (6):982–987
64. Julsing M, Rijpkema M, Woerdenbag H, Quax W, Kayser O (2007) Functional analysis of genes involved in the biosynthesis of isoprene in *Bacillus subtilis*. Appl Microbiol Biotechnol 75:1377–1384
65. Kesselmeier J, Staudt M (1999) Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J Atmos Chem V33(1):23–88

66. Kim YS, Lee JH, Kim NH, Yeom SJ, Kim SW, Oh DK (2011) Increase of lycopene production by supplementing auxiliary carbon sources in metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 90(2):489–497. doi:10.1007/s00253-011-3091-z
67. Klein-Marcuschamer D, Ajikumar PK, Stephanopoulos G (2007) Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. Trends Biotechnol 25 (9):417–424
68. Köksal M, Zimmer I, Schnitzler JP, Christianson DW (2010) Structure of isoprene synthase illuminates the chemical mechanism of teragram atmospheric carbon emission. J Mol Biol 402(2):363–373
69. Kuzma J, Fall R (1993) Leaf isoprene emission rate is dependent on leaf development and the level of isoprene synthase. Plant Physiol 101(2):435–440
70. Kuzma J, Nemecek M, Pollock WH, Fall R (1995) Bacteria produce the volatile hydrocarbon isoprene. Curr Microbiol V30(2):97–103
71. Laothawornkitkul J, Paul ND, Vickers CE, Possell M, Mullineaux PM, Hewitt CN, Taylor JE (2008) The role of isoprene in insect herbivory. Plant Signal Behav 3(12):1141–1142
72. Laothawornkitkul J, Paul ND, Vickers CE, Possell M, Taylor JE, Mullineaux PM, Hewitt CN (2008) Isoprene emissions influence herbivore feeding decisions. Plant Cell Environ 31 (10):1410–1415. doi:10.1111/j.1365-3040.2008.01849.x
73. Lehning A, Zimmer I, Steinbrecher R, Brüggemann N, Schnitzler JP (1999) Isoprene synthase activity and its relation to isoprene emission in *Quercus robur* L. leaves. Plant Cell Environ 22(5):495–504. doi:10.1046/j.1365-3040.1999.00425.x
74. Leonard E, Ajikumar PK, Thayer K, Xiao W-H, Mo JD, Tidor B, Stephanopoulos G, Prather KLJ (2010) Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. Proc Nat Acad Sci 107(31):13654–13659. doi:10.1073/pnas.1006138107
75. Lindberg P, Park S, Melis A (2010) Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. Metab Eng 12 (1):70–79. doi:10.1016/j.ymben.2009.10.001
76. Liu H, Sun Y, Ramos KRM, Nisola GM, Valdehuesa KNG, Lee WK, Park SJ, Chung W-J (2013) Combination of entner-doudoroff pathway with MEP increases isoprene production in engineered *Escherichia coli*. PLoS ONE 8(12):e83290. doi:10.1371/journal.pone.0083290
77. Loivamäki M, Mumm R, Dicke M, Schnitzler JP (2008) Isoprene interferes with the attraction of bodyguards by herbaceous plants. Proc Natl Acad Sci USA 104 (45):17430–17435
78. Loivamäki M, Gilmer F, Fischbach RJ, Sorgel C, Bachl A, Walter A, Schnitzler JP (2007) Arabidopsis, a model to study biological functions of isoprene emission? Plant Physiol 144:1066–1078
79. Lombard J, Moreira D (2011) Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. Mol Biol Evol 28(1):87–99. doi:10.1093/molbev/msq177
80. Loreto F, Mannozzi M, Maris C, Nascetti P, Ferranti F, Pasqualini S (2001) Ozone quenching properties of isoprene and its antioxidant role in leaves. Plant Physiol 126 (3):993–1000
81. Marienhagen J, Bott M (2013) Metabolic engineering of microorganisms for the synthesis of plant natural products. J Biotechnol 163(2):166–178
82. Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21 (7):796–802. doi:10.1038/nbt833
83. McCoy M (2008) Biobased isoprene: goodyear and genencor seek a fermentation route to important synthetic rubber raw material. Chem Eng News 86:15
84. Meskhidze N, Nenes A (2006) Phytoplankton and cloudiness in the southern ocean. Science 314(5804):1419–1423. doi:10.1126/science.1131779

85. Miller B, Oschinski C, Zimmer W (2001) First isolation of an isoprene synthase gene from poplar and successful expression of the gene in *Escherichia coli*. Planta 213(3):483–487. doi:10.1007/s004250100557
86. Monson RK, Fall R (1989) Isoprene emission from aspen leaves: influence of environment and relation to photosynthesis and photorespiration. Plant Physiol 90(1):267–274
87. Monson RK, Jones RT, Rosenstiel TN, Schnitzler JP (2013) Why only some plants emit isoprene. Plant Cell Environ 36(3):503–516
88. Muir RE, Weyler W (2014) Compositions and methods for improved isoprene production using two types of ispg enzymes. USA Patent Application US20140147902 A1
89. Okada K, Hase T (2005) Cyanobacterial non-mevalonate pathway. (*E*)-4-Hyddroxy-3-methylbut-2-enyl diphosphate synthase interacts with ferredoxin in *Thermosynechococcus elongatus* BP-1. J Biol Chem 280(21):20672–20679. doi:10.1074/jbc.M500865200
90. Okamura E, Tomita T, Sawa R, Nishiyama M, Kuzuyama T (2010) Unprecedented acetoacetyl-coenzyme a synthesizing enzyme of the thiolase superfamily involved in the mevalonate pathway. Proc Natl Acad Sci USA 107(25):11265–11270. doi:10.1073/pnas.1000532107
91. Pegoraro E, Rey A, Bobich EG, Barron-Gafford G, Grieve KA, Malhi Y, Murthy R (2004) Effect of elevated CO_2 concentration and vapour pressure deficit on isoprene emission from leaves of *Populus deltoides* during drought. Funct Plant Biol 31(12):1137–1147
92. Poisson N, Kanakidou M, Crutzen PJ (2000) Impact of non-methane hydrocarbons on tropospheric chemistry and the oxidizing power of the global troposphere: 3-dimensional modelling results. J Atmos Chem 36(2):157–230
93. Ramos K, Valdehuesa K, Liu H, Nisola G, Lee W-K, Chung W-J (2014) Combining De Ley–Doudoroff and methylerythritol phosphate pathways for enhanced isoprene biosynthesis from d-galactose. Bioproc Biosys Eng, pp 1–9. doi:10.1007/s00449-014-1228-z
94. Rasmussen R (1972) Survey of plant species that release isoprene to atmosphere. Abs Pap Am Chem Soc, 13
95. Rasmussen RA (1970) Isoprene: identified as a forest-type emission to the atmosphere. Envir Sci Tech 4(8):667–671. doi:10.1021/es60043a008
96. Reardon K (2014) Lignocellulosic biorefineries: concepts and possibilities. In: McCann MC, Buckeridge MS, Carpita NC (eds) Plants and bioenergy, Vol 4. Adv Plant Biol. Springer, New York, pp 255–265. doi:10.1007/978-1-4614-9329-7_15
97. Rife CL, Wells DH (2014) Isoprene synthase variants with improved solubility for production of isoprene. USA Patent US8735134, 27 May 2014
98. Rodríguez-Concepción M, Boronat A (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol 130(3):1079–1089
99. Rodríguez-Villalón A, Perez-Gil J, Rodríguez-Concepción M (2008) Carotenoid accumulation in bacteria with enhanced supply of isoprenoid precursors by upregulation of exogenous or endogenous pathways. J Biotechnol 135(1):78–84. doi:10.1016/j.jbiotec.2008.02.023
100. Rohmer M (2003) Mevalonate-independent methylerythritol phosphate pathway for isoprenoid biosynthesis elucidation and distribution. Pure Appl Chem 75(2–3):375–387
101. Rude MA, Schirmer A (2009) New microbial fuels: a biotech perspective. Curr Opin Microbiol 12(3):274–281. doi:10.1016/j.mib.2009.04.004
102. Ruzicka L (1953) The isoprene rule and the biogenesis of terpenic compounds. Experientia 9 (10):357–367
103. Ryan AC, Hewitt CN, Possell M, Vickers CE, Purnell A, Mullineaux PM, Davies WJ, Dodd IC (2014) Isoprene emission protects photosynthesis but reduces plant productivity during drought in transgenic tobacco (*Nicotiana tabacum*) plants. New Phytol 201(1):205–216. doi:10.1111/nph.12477
104. Sabri S, Nielsen LK, Vickers CE (2013) Molecular control of sucrose utilization in *Escherichia coli W*, an efficient sucrose-utilizing strain. Appl Environ Microbiol 79 (2):478–487. doi:10.1128/aem.02544-12

105. Saltman W (ed) (1985) Isoprene Kirk-othmer concise encyclopaedia of chemical technology. Wiley, New York
106. Sanadze GA (1957) The nature of gaseous substances emitted by leaves of *Robina pseudoacacia*. Soobshch Akad Nauk Gruz SSR 19:83–86
107. Sanadze GA (1969) Light-dependent excretion of molecular isoprene by leaves. Prog Photosynth Res II:701–706
108. Sasaki K, Ohara K, Yazaki K (2005) Gene expression and characterization of isoprene synthase from *Populus alba*. FEBS Lett 579(11):2514–2518. doi:10.1016/j.febslet.2005.03.066
109. Sasaki K, Ohara K, Yazaki K (2005) Gene expression and characterization of isoprene synthase from *Populus alba*. FEBS Lett 579(11):2514–2518
110. Sasaki K, Saito T, Lamsa M, Oksman-Caldentey KM, Suzuki M, Ohyama K, Muranaka T, Ohara K, Yazaki K (2007) Plants utilize isoprene emission as a thermotolerance mechanism. Plant Cell Physiol 48(9):1254–1262
111. Schnitzler JP, Lehning A, Steinbrecher R (1997) Seasonal pattern of isoprene synthase activity in *Quercus robur* leaves and its significance for modeling isoprene emission rates. Bot Acta 110(3):240–243
112. Seemann M, Bui BT, Wolff M, Tritsch D, Campos N, Boronat A, Marquet A, Rohmer M (2002) Isoprenoid biosynthesis through the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (GcpE) is a [4Fe-4S] protein. Angewandte Chemie 41(22):4337–4339. doi:10.1002/1521-3773(20021115)41:22<4337:AID-ANIE4337>3.0.CO;2-K
113. Shallcross DE, Monks PS (2000) New Directions: a role for isoprene in biosphere-climate-chemistry feedbacks. Atmos Environ 34(10):1659–1660
114. Sharkey TD (1996) Isoprene synthesis by plants and animals. Endeavour 20(2):74–78
115. Sharkey TD, Gray DW, Pell HK, Breneman SR, Topper L (2013) Isoprene synthase genes form a monophyletic clade of acyclic terpene synthases in the Tps-b terpene synthase family. Evolution 67(4):1026–1040. doi:10.1111/evo.12013
116. Sharkey TD, Loreto F (1993) Water stress, temperature, and light effects on the capacity for isoprene emission and photosynthesis of kudzu leaves. Oecologia 95(3):328–333
117. Sharkey TD, Monson RK (2014) The future of isoprene emission from leaves, canopies and landscapes. Plant Cell Environ 37(8):1727–1740. doi:10.1111/pce.12289
118. Sharkey TD, Singsaas EL (1995) Why plants emit isoprene. Nature 374:769
119. Sharkey TD, Yeh S, Wiberley AE, Falbel TG, Gong D, Fernandez DE (2005) Evolution of the isoprene biosynthetic pathway in kudzu. Plant Physiol 137:700–712
120. Silver GM, Fall R (1991) Enzymatic synthesis of isoprene from dimethylallyl diphosphate in aspen leaf extracts. Plant Physiol 97(4):1588–1591
121. Silver GM, Fall R (1995) Characterization of aspen isoprene synthase, an enzyme responsible for leaf isoprene emission to the atmosphere. J Biol Chem 270(22):13010–13016
122. Sivy TL, Fall R, Rosenstiel TN (2011) Evidence of isoprenoid precursor toxicity in *Bacillus subtilis*. Biosci Biotechnol Biochem 75(12):2376–2383
123. Sivy TL, Shirk MC, Fall R (2002) Isoprene synthase activity parallels fluctuations of isoprene release during growth of *Bacillus subtilis*. Biochem Biophys Res Commun 294(1):71–75
124. Stephanopoulos G, Alper HS, Jin YS (2006) Gene targets for enhanced carotenoid production. USA Patent US7741070, 22 June 2010
125. Stephanopoulos G (2007) Challenges in engineering microbes for biofuels production. Science 315:801–804
126. Taalman RDFM (1996) Isoprene: background and issues. Toxicology 113(1–3):242–246
127. Takahashi S, Yeo Y, Greenhagen B, McMullin T, Song L, Maurina-Brunker J, Rosson R, Noel JP, Chappell J (2007) Metabolic engineering of sesquiterpene metabolism in yeast. Biotechnol Bioeng 97(1):170–181
128. Tattini M, Velikova V, Vickers C, Brunetti C, Di Ferdinando M, Trivellini A, Fineschi S, Agati G, Ferrini F, Loreto F (2014) Isoprene production in transgenic tobacco alters

isoprenoid, non-structural carbohydrate and phenylpropanoid metabolism, and protects photosynthesis from drought stress. Plant Cell Environ 37(8):1950–1964. doi:10.1111/pce.12350
129. Thompson AM (1992) The oxidizing capacity of the earth's atmosphere—probable past and future changes. Science 256(5060):1157–1165
130. Tingey DT, Evans R, Gumpertz M (1981) Effects of environmental conditions on isoprene emission from live oak. Planta 152(6):565–570
131. Tokumoto U, Takahashi Y (2001) Genetic analysis of the *isc* operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. J Biochem 130(1):63–71
132. Trainer M, Williams EJ, Parrish DD, Buhr MP, Allwine EJ, Westberg HH, Fehsenfeld FC, Liu SC (1987) Models and observations of the impact of natural hydrocarbons on rural ozone. Nature 329(6141):705–707
133. Vickers CE, Behrendorff JBYH, Bongers M, Brennan TCR, Bruschi M, Nielsen LK (2014) Production of industrially-relevant isoprenoid compounds in engineered microbes. In: Kamm B (ed) Microorganisms in biorefineries. Springer, Heidelberg
134. Vickers CE, Blank LM, Kromer JO (2010) Chassis cells for industrial biochemical production. Nat Chem Biol 6(12):875–877. doi:10.1038/Nchembio.484
135. Vickers CE, Bongers M, Liu Q, Delatte T, Bouwmeester H (2014) Metabolic engineering of volatile isoprenoids in plants and microbes. Plant Cell Environ Online Early. doi:10.1111/pce.12316
136. Vickers CE, Gershenzon J, Lerdau MT, Loreto F (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nat Chem Biol 5(5):283–291. doi:10.1038/Nchembio.158
137. Vickers CE, Klein-Marcuschamer D, Krömer JO (2012) Examining the feasibility of bulk commodity production in *Escherichia coli*. Biotechnol Lett 34(4):585
138. Vickers CE, Possell M, Cojocariu CI, Velikova VB, Laothawornkitkul J, Ryan A, Mullineaux PM, Hewitt CN (2009) Isoprene synthesis protects transgenic tobacco plants from oxidative stress. Plant Cell Environ 32(5):520–531. doi:10.1111/j.1365-3040.2009.01946.x
139. Vickers CE, Possell M, Hewitt CN, Mullineaux PM (2010) Genetic structure and regulation of isoprene synthase in poplar (*Populus* spp.). Plant Mole Biol 73(4–5):547–558
140. Vickers CE, Possell M, Laothawornkitkul J, Ryan AC, Hewitt CN, Mullineaux PM (2011) Isoprene synthesis in plants: lessons from a transgenic tobacco model. Plant Cell Environ 34(6):1043–1053. doi:10.1111/j.1365-3040.2011.02303.x
141. Wang HH, Isaacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, Church GM (2009) Programming cells by multiplex genome engineering and accelerated evolution. Nature 460(7257):894–898. doi:10.1038/nature08187
142. Whited GM, Feher FJ, Benko DA, Cervin MA, Chotani GK, McAuliffe JC, LaDuca RJ, Ben-Shoshan EA, Sanford KJ (2010) Development of a gas-phase bioprocess for isoprene-monomer production using metabolic pathway engineering. Ind Biotechnol 6(3):152–163. doi:10.1089/ind.2010.6.152
143. Wiedinmyer C, Tie X, Guenther A (2006) Future changes in biogenic isoprene emissions: how might they affect regional and global atmospheric chemistry? Earth Interact 10:1–9
144. Wildermuth MC, Fall R (1996) Light-dependent isoprene emission (characterization of a thylakoid-bound isoprene synthase in *Salix discolor* chloroplasts). Plant Physiol 112(1):171–182
145. Wildermuth MC, Fall R (1998) Biochemical characterization of stromal and thylakoid-bound isoforms of isoprene synthase in willow leaves. Plant Physiol 116(3):1111–1123
146. Williams CG (1859) On isoprene and caoutchine. Proc R Soc London 10:516–519. doi:10.1098/rspl.1859.0101
147. Williams DC, McGarvey DJ, Katahira EJ, Croteau R (1998) Truncation of limonene synthase preprotein provides a fully active 'Pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochemistry 37(35):12213–12220

148. Willke T, Vorlop K (2008) Biotransformation of glycerol into 1,3-propanediol. Eur J Lipid Sci Tech 110(9):831–840. doi:10.1002/ejlt.200800057
149. Willrodt C, David C, Cornelissen S, B BH, Julsing MK, Schmid A (2014) Engineering the productivity of recombinant *Escherichia coli* for limonene formation from glycerol in minimal media. Biotechnol J. doi:10.1002/biot.201400023
150. Withers ST, Gottlieb SS, Lieu B, Newman JD, Keasling JD (2007) Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. Appl Environ Microbiol 73(19):6277–6283
151. Wolfertz M, Sharkey TD, Boland W, Kühnemann F (2004) Rapid regulation of the methylerythritol 4-phosphate pathway during isoprene synthesis. Plant Physiol 135(4):1939–1945
152. Wolff M, Seemann M, Tse Sum Bui B, Frapart Y, Tritsch D, Garcia Estrabot A, Rodriguez-Concepcion M, Boronat A, Marquet A, Rohmer M (2003) Isoprenoid biosynthesis via the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB/IspH) from *Escherichia coli* is a (4Fe-4S) protein. FEBS Lett 541 (1–3):115–120
153. Zhao J, Li Q, Sun T, Zhu X, Xu H, Tang J, Zhang X, Ma Y (2013a) Engineering central metabolic modules of *Escherichia coli* for improving β-carotene production. Metab Eng 17 (0):42–50. doi:http://dx.doi.org/10.1016/j.ymben.2013.02.002
154. Zhao L, W-c Chang, Xiao Y, H-w Liu, Liu P (2013) Methylerythritol phosphate pathway of isoprenoid biosynthesis. Ann Rev Biochem 82(1):497–530. doi:10.1146/annurev-biochem-052010-100934
155. Zhao Y, Yang J, Qin B, Li Y, Sun Y, Su S, Xian M (2011) Biosynthesis of isoprene in *Escherichia coli* via methylerythritol phosphate (MEP) pathway. Appl Microbiol Biotechnol 90(6):1915–1922. doi:10.1007/s00253-011-3199-1
156. Zhou K, Zou RY, Stephanopoulos G, Too HP (2012) Metabolite profiling identified methylerythritol cyclodiphosphate efflux as a limiting step in microbial isoprenoid production. PLOS ONE 7(11):e47513. doi:10.1371/journal.pone.0047513
157. Zurbriggen A, Kirst H, Melis A (2012) Isoprene production via the mevalonic acid pathway in *Escherichia coli* (Bacteria). BioEnergy Res 5:814–828. doi:10.1007/s12155-012-9192-4