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Review Article

Metabolic Engineering of Microorganisms to Produce Isoprene

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ABSTRACT

Isoprene is an industrially important five carbon compound primarily used for production of high quality synthetic rubber. Two major pathways are involved in isoprene synthesis. The mevalonate pathway is present in eukaryotes, archaebacteria and cytosol of higher plants whereas the non-mevalonate pathway exists in many eubacteria and plastids in algae/plants. There have been continuous efforts to study and understand the phenomenon of biological production of isoprene for more than half a century. Although, the current feasibility and cost advantage of chemical processes leading to production of isoprene seems to be far from being dominated by a suitable biological substitute, the fear of extinction of non-renewable resources (raw material for chemical processes) in the near future prompts for a colossal expectation from the synthetic biology community. Technological advances in the field of metabolic engineering have made it possible to vigorously modify and swap genes among different organisms and push the limits for microorganisms to over-produce isoprene to an enormous extent. This review touches upon the limitations faced while improving isoprene titres and the meticulous strategies used to overcome them. It analyzes recent approaches that have resulted in significant improvement of biologically produced isoprene, summarizes the lessons learned from them, and compiles an exhaustive list of potential gene targets that could facilitate prospective research in this widespread arena.

Keywords: Isoprene; MEP/DXP pathway; MVA pathway; Metabolic engineering; Flux

INTRODUCTION

Isoprene, a naturally produced cell metabolite, is a volatile compound emitted from the leaves of many plant species. A brief history about the discovery of isoprene as a cell metabolite during the early second half of the twentieth century has been exquisitely described by Professor Guivi Sanadze, the person who published the first report of emission of isoprene from plants [1]. Speculations regarding the native role of isoprene dictates its function as a thermoprotectant and a potential plant defence mechanism against the invading parasites [2,3]. Isoprene is an important chemical used in the production of synthetic rubber, medicines and pesticides. The commercially valuable isoprenoid family of organic compounds are produced using isoprene as a monomeric building block. Chemical synthesis of such isoprenoids is hindered by factors which include depletion of fossil fuels and the complexity of the molecules. Harvesting of isoprene which is gaseous above 34°C from plants is not feasible and therefore isoprene is exclusively produced through chemical synthesis from petrochemicals [4,5]. With the recent advances in synthetic biology/metabolic engineering, isoprene production by microorganisms is a feasible and attractive alternative. Anticancer compound taxol and antimalarial drug artemisinin are both commercially produced by microorganisms with engineered/modified isoprenoid pathways [6,7]. Several studies and patents in recent times demonstrating genetic modifications enhancing production of isoprene from microorganisms have been published.

MECHANISM OF ISOPRENE PRODUCTION

Biosynthesis of isoprenoids is from the basic building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are synthesised from two naturally occurring pathways-methylerythritol 4-phosphate (MEP) pathway and mevalonate (MVA) pathway. The removal of pyrophosphate from DMAPP results in generation of isoprene and is catalysed by an enzyme, isoprene synthase. It is considered as a key enzyme involved in biosynthesis of isoprene and is usually found in the chloroplasts of various plant species [8-10]. Several variants

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of isoprene synthases have been identified and tested towards improving isoprene production [11-13]. Moreover, certain bacterial species such as *Bacillus subtilis* are also known to produce isoprene naturally despite lacking a homolog of isoprene synthase from plant sources [14-16].

METABOLIC ENGINEERING OF MEP PATHWAY

The MEP pathway is of prokaryotic origin and present in most bacterial species, including *Escherichia coli* and *B. subtilis*, as well as plastids in plants and blue green algae [17,18]. Intricate regulatory control of this pathway has given rise to a prodigious demand for its extensive in-depth exploration, particularly in heterologous hosts. Optimizations of the MEP pathway are worth pursuing since the calculated theoretical yields of isoprene from glucose are higher with MEP as compared to the MVA pathway. IPP and DMAPP synthesis via this pathway requires glyceraldehyde-3-phosphate and pyruvate as initial substrates. The common bacterial host in metabolic engineering viz. *E. coli*, uses the MEP pathway and it consists of seven enzymatic reactions (Figure 1). The genes identified as bottlenecks in various studies are dxs, dxr and idi [19-21]. Other studies have identified overexpression of IspD, IspF and IspE which resulted in increased production of isoprenoids [22-24]. The observed efflux of methylerythritol cyclodiphosphate (MEC) has also been noted as a rate limiting step in isoprenoid production. Overexpression of IspG, which is an iron-sulfur cluster protein in the MEP pathway, led to diminished MEC efflux thereby bypassing the bottleneck and enhancing the production of isoprenoids [25]. *Bacillus subtilis*

Escherichia coli

Efforts to improve isoprene and isoprenoid production in *E. coli* have focused on the overexpression of (a) endogenous MEP pathway genes as well as (b) heterologous genes [26,27]. In case of the native MEP genes, the pathway constructs were cloned as one super operon into a suitable expression plasmid where transcription was driven from a strong promoter and a translation initiation region placed in front of each gene. In this study, isoprene production titre with modifications of the MEP pathway improved up to 3.04 mg/L [26]. Overexpression of dxs/dxr/idi in the specific order consistent with that of the metabolic pathway resulted in a production yield of 2.7 mg/g/h [19]. In case of heterologous expression, overexpression of dxs and dxr genes from *B. subtilis* in *E. coli* resulted in an enhancement of isoprene production giving a yield of 314 mg/L [27]. Besides, Type II idi when expressed in *E. coli* was found to enhance the production of lycopene in comparison with the Type I idi [28]. Yang et al. reported an approach with hybrid MVA pathway utilizing upper pathway genes from *Enterococcus faecalis* possessing mvaS A110G mutant and achieved 6.3 g/L isoprene titre [29]. Combinatorial approaches towards combining MVA and MEP pathways have also yielded promising results producing 24 g/L isoprene [30].

Bacillus subtilis

This organism's fast growth rate and GRAS (generally recognised as safe) status makes it a promising microbial host for the production of isoprenoids. Overexpression of DXS resulted in production of 3.73 ng/ml/OD600 while overexpression of both DXS and DXR gave nearly identical yields but resulted in the loss of diauxic growth of the strain [31]. Amorphadiene, the precursor of the anti-malarial

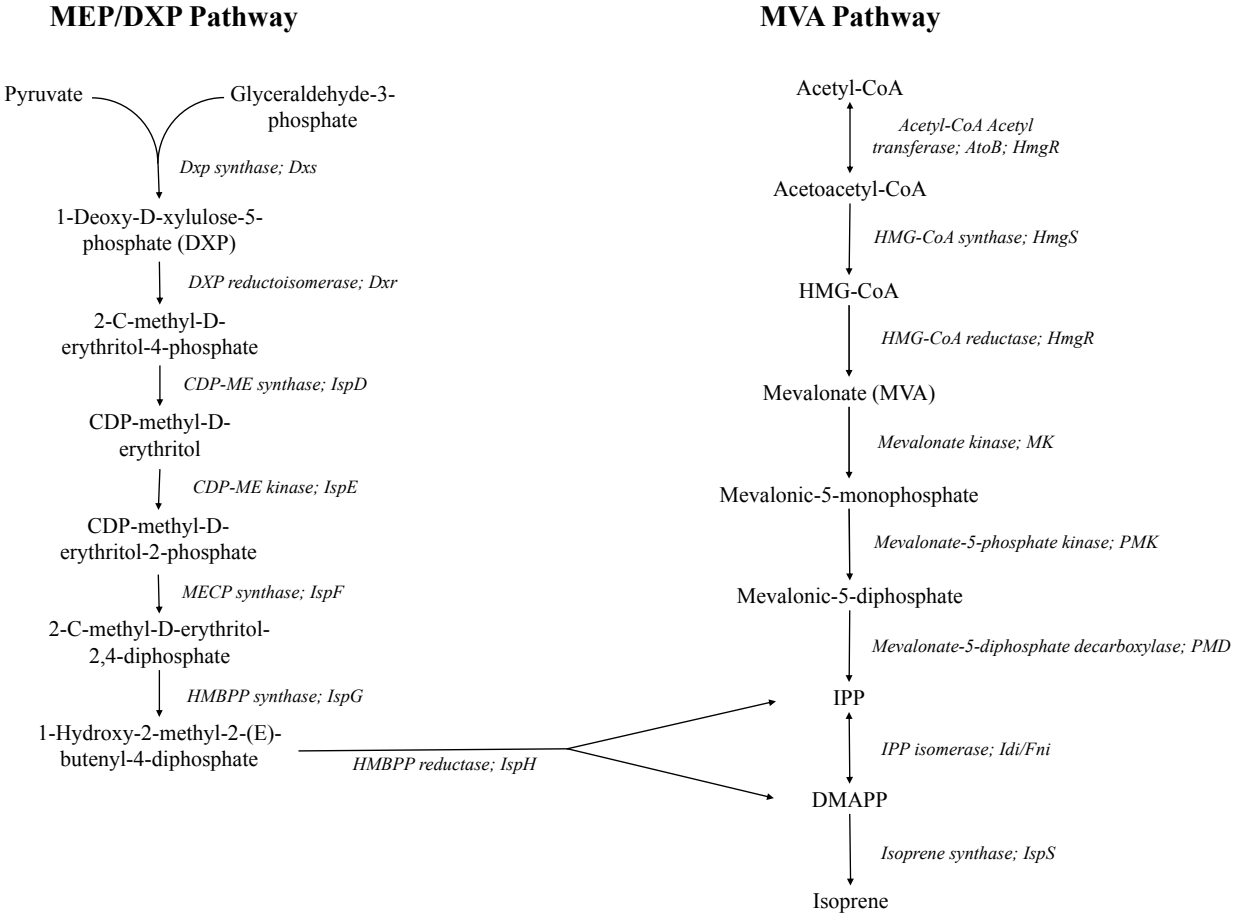

Figure 1: Pathways.

drug Artemisinin, was produced at ~20 mg/L with overexpression of DXS, IDI and ADS (Amorpha-4,11-diene Synthase) coupled with protein translation engineering and systematic media optimization [32].

Saccharomyces cerevisiae

Replacing the endogenous MVA pathway with a synthetic bacterial MEP pathway while using an integration based approach was unsuccessful and growth could not be restored. However, bacterial MEP genes on an expression plasmid were able to sustain *S. cerevisiae* growth in the presence of very well-known mevalonate pathway inhibitor lovastatin [33]. Amorphadiene, the precursor of the anti-malarial drug Artemisinin has been successfully produced in *S. cerevisiae* with manipulations of the MVA pathway with yields of 40 g/L [34]. Directed evolution of isoprene synthase coupled with perturbation of gal regulon in *S. cerevisiae* resulted in 3.7 g/L of isoprene [35].

***Synechocystis* sp. PCC 6803**

Recently, cyanobacteria have been explored as "green" environment friendly alternate hosts for production of isoprene [36]. Heterologous overexpression of fni from *S. pneumoniae* along with the overexpression of ispS gene as a fusion construct with the highly expressed cyanobacterial cpcB gene encoding the β-subunit of phycocyanin resulted in more than 60 fold increase in isoprene production in the type strain Synechocystis sp. PCC 6803 [37]. A benchmark level of 12.3 mg isoprene per gram dry cell weight was achieved using the autotrophic photosynthetic route. In anticipation of freshwater becoming a limiting factor for autotrophic mass production of biofuels in future, research on cultivation of these freshwater cyanobacteria at different concentrations of NaCl has also been instigated [38-40].

The metabolic regulatory constraints of the MEP pathway restrict it to a confined boundary despite the multidimensional efforts in diverse arenas; some of which are listed above. There exists an immediate requirement for better understanding of cellular functioning and concomitant development of advanced molecular engineering tools that could precisely predict and bypass the possible bottlenecks to fast-track the scientific progress made so far in the field.

METABOLIC ENGINEERING OF MVA PATHWAY

The predominant metabolic pathway engineering attempts for the microbial production of isoprenoid family of compounds have focused on the MVA pathway due to the obvious advantage of not being subject to as tight regulation as for the MEP pathway. It is further classified as upper pathway that leads to synthesis of mevalonate followed by the lower pathway that consumes mevalonate to synthesize IPP/DMAPP. In one attempt, the upper pathway was cloned from *E. faecalis* and the lower pathway from *S. pneumoniae* along with the addition of an extra thiolase (atoB) which resulted in the increased yield of isoprene [26,41].

The efficiency of MVA pathway was improved in order to increase MVA production; the source of the "upper pathway" which contains HMG-CoA synthase, acetyl-CoA acetyltransferase and HMG-CoA reductase to convert acetyl-CoA into MVA was changed from *S. cerevisiae* to *E. faecalis* [29]. Replacing the *S. cerevisiae* MVA upper pathway genes with those from *Staphylococcus aureus* resulted in doubling of production titres of Amorpha-4,11-diene [42]. Comparison of upper and lower MVA pathway genes from *S. pneumoniae*, *E. faecalis*, *S. aureus*, *Streptococcus pyogenes* and *S. cerevisiae* was carried out and the highest production of β-carotene was seen where the upper pathway was from *E. faecalis* and lower pathway from *S. pneumoniae* [43]. In the case of isoprene production, yields of up to 60 g/L were achieved with upper and lower pathway from *S. cerevisiae* with an additional copy of the mvk gene from *Methanosarcina mazei* [5]. Control systems for heterologous metabolic pathways predominantly rely on swapping of promoters [6,44]. The pmk and mk genes, previously identified as the bottlenecks were placed under a much stronger promoter as compared to the other genes [45]. To further enhance the production of isoprene, mvaS gene was modified replacing an alanine 110 with glycine. With these modifications, isoprene was produced up to 6.3 g/L after 40 h of fed-batch cultivation [29].

STRATEGIES BASED ON PLASMIDS AND CHROMOSOMAL INTEGRATIONS

The metabolic burden from DNA, RNA and protein synthesis of the cell is increased if it has to maintain multiple plasmids [46]. It is further increased due to the total number of antibiotic resistance proteins that the cell has to produce [47]. This often leads to low yields of the desired metabolite, therefore endeavours to have all the genes on a single plasmid have proved more efficient [45]. Successful isoprene production was seen with some genes of the pathway integrated into the chromosome while the remaining pathway plus additional accessory genes were expressed on two different plasmids [5].

Plasmid based expression systems have several drawbacks which include segregational instability or allele segregation and possible structural instability which may reduce the amount of production of compound of interest [48]. Additionally, antibiotics required for selecting and maintaining plasmids in the host during fermentation result in increased costs. A more stable and reliable approach is integration of heterologous genes or multiple copies of the host genes using suitable integration vector into the bacterial attachment (attB) site of *E. coli* using helper plasmids which express the phage integrase, by direct transformation [5,22,49]. Another strategy available for chromosomal integration is the Lambda-Red recombinase system in combination with the Flp/FRT site-specific recombination system for marker excision [50,51].

To achieve the high copy numbers for the production of metabolites, the desired pathway genes are first integrated into the genome and then can be evolved to the desired gene copy numbers by the process of chemical induction resulting in chemically induced chromosomal evolution (CIChE) [50]. To further remove the drawback of the presence of the antibiotic selection marker, existing variants of the CIChE technique could be readily employed [49].

ENHANCING FLUX TOWARDS ISOPRENE

Distinct studies have been attempted to increase the flux of substrates towards the relevant pathway and to prevent the efflux of intermediates from them. Some of the examples include attempts to increase the amount of acetyl CoA substrate for the MVA pathway. atoB overexpression has also been shown to be effective to an extent [26]. Overexpression of aceto-acetyl transferase (pho) from *R. eutropha* was found to be effective in increasing acetyl CoA substrate flux [43]. In certain host strains such as *E. coli* BL21, which has low phosphogluconolactonase (PGL) activity resulting in low

carbon flux through the pentose phosphate pathway, constitutive overexpression of PGL along with the rest of the MVA and ispS gave titres of 60 g/L [5].

Overexpression of certain MEP pathway enzymes resulted in the efflux of MEC indicating the existence of a novel competing pathway branch in DXP metabolism. To overcome this, overexpression of ispG was found to effectively reduce the efflux of MEC outside the cells [25]. Proteins encoded by ispG and ispH are metalloproteins with Fe-S clusters. Overexpression of fpr (flavodoxin reductase) and fldA (flavodoxin I), iron-sulfur cluster-interacting redox polypeptide along with ispG and ispH increased isoprene productivity to 600 µg/L/h [52-55]. A list of potential overexpression targets compiled from several references is summarized in Table 1 [52-66].

Systematic and combinatorial analysis to ascertain potential gene knockout targets for improving lycopene production in *E. coli* led to the identification of three genes viz. glutamate dehydrogenase (gdh), pyruvate dehydrogenase (aceE) and formate dehydrogenase (fdh) [61]. A combinatorial knockout of all the three genes resulted in 40% improved yield [67,68]. A list of potential knockout targets compiled from several references is summarized in Table 2 [69-78].

Moreover, computational analysis performed using genome scale modelling (data not shown) suggested certain DNA-binding transcriptional regulators as targets that could largely improve the isoprene titres [69,70]. These targets include global regulators like cra (Catabolite Repressor Activator), fis (Factor for Inversion Stimulation), arcA (Regulator for respiratory and fermentative metabolism under microaerobic/anaerobic conditions) and iclR (Isocitrate Lyase Regulator); the expression of which could be delicately modulated in combination with the above mentioned overexpression and knockout targets accordingly [71-73].

RATIONAL PERTURBATIONS OF METABOLIC PATHWAYS FOR YIELD ENHANCEMENT

Increasing copy numbers of heterologous or homologous genes for the desired product will result in increased titres only up to a certain point. Beyond this, to improve the yields, other strategies have to be employed. The efficiency of expression will also be affected as the cell has to maintain many copies within the cell. By chromosomally integrating the desired genes, the problem of vector load and maintenance are possibly bypassed. However, this does not rule out the cells own expression apparatus or precursor limitation. Various strategies proposed to optimize yields include:

Precursor balancing: Precursor balancing is an indispensable tool towards achieving increased yield of desired metabolites. A few examples and strategies applied in the past for precursor balancing are described. Isoprenoid production via MEP pathway requires equimolar quantities of G3P and pyruvate [74-76]. The imbalanced supply of G3P and pyruvate precursors persists to be the main bottleneck of the MEP pathway. One possible way to manipulate the ratio between G3P and pyruvate, is to alter the flux of the phosphoenolpyruvate (PEP) to pyruvate interconversion, which is controlled by the enzymes pyruvate kinase (Pyk) and PEP synthase (Pps). Pps converts pyruvate to PEP and thus overexpression of pps resulted in a five-fold increase in lycopene yield over the wild type strain (25 mg/g dried cell weight). Moreover, the deletion of pyk also increased lycopene production with similar enhancements as observed with overexpression of other gluconeogenic and glycolytic enzymes [77-97]. In a separate study, the deletion of competing phosphotransferase system which otherwise consumes PEP also resulted in enhanced lycopene production [82]. It has also been shown that mere deletion of gap A gene which prevents conversion of G3P to glycerate 1,3-bisphosphate makes more G3P available for funnelling into the MEP pathway [77-80]. On the other side, overexpression of gapB (NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase) and fbp (fructose-1,6-bisphosphatase) resulted in increased yield of riboflavin [81,82]. It is imperative to remember that the relative regulation of metabolic flux through the glycolytic and the gluconeogenic pathways play an important role in central carbon metabolism.

There are four main glycolytic pathways that serve as feeding modules which generate pyruvate and G3P from sugar substrates:

Sr. no.	Gene name	Process / Molecule targeted	Reference
1	*galP*; *glk*	Metabolism	[52]
2	*gld*	Isoprene	[53]
3	*ompF*, *ompE*, *ndk*, *cmk*, *fbaA*, *fbaB*, *ompC*, *adk*, *pfkA*, *pfkB*, *pgi*, *pitA*, *tpiA*, *ompN*	Lycopene	[54]
4	*gapB*, *fbp*, *pckA*	Riboflavin	[55]
5	*ppc*, *pck*	Succinate	[56]
6	PEPCK	Succinate	[57]
7	*yhfR*, *nudF*	IPP, DMAPP, Isopentenol	[58]
8	*pck*, *pps*, *rpoS*, *appY*, *yjiD*, *ycgW*, *wrbA*, *atpE*	Lycopene	[49]
9	*appY*, *crl*, *rpoS*	Lycopene	[59]
10	*yggV* + *lpxH* + *hisL* + *ppa* + *cdh*	Isoprenol/prenol	[60]
11	*nuo*, *cyoABCD*, *cyAB*, *sucAB*, *talB*, *tktA*, *gltA*, *sdhABCD*	β-Carotene	[61]
12	*yajO*, *rib*	Terpene	[62]
13	*zwf*, *gnd*	Riboflavin	[63]
14	*pps*	Lycopene	[64]
15	*glf*, *glk*	Shikimic acid	[65]
16	*erpA*, *fldA*, *fpr*, *iscA*	Fe-S cluster	[66]
17	TpiA; OmpN	Lycopene	[54]

Table 1: List of potential targets to be overexpressed.

Sr. no.	Gene Name	Process / Molecule targeted	Reference
1	*iclr*; *arcA*	Metabolism	[69]
2	*cra*	Sugars	[70]
3	*atpFH*; *adhE*; *sucA*; *poxB*; *ldhA*; *frdBC*; *pflB*; *ackA*	Pyruvate	[71]
4	*ldhA*; *pflB*	Metabolism	[72]
5	*pts1*	Metabolism	[52]
6	*pts*; *pgi*; *zwf*; *gnd*; *pyk*; *ppc*; *pckA*; *lpdA*, *pfl*	Metabolism	[73]
7	*cyaA*; *pts1*; *crr*; *pfkA*; *pgi*; *ptsG*; *ihfA*; *ihfB*; *fis*; *pstH*; *atpCDEF*; *sucA*; *sucB*; *lpdA*; *sdhCDAB*	Metabolism	[74]
8	*maeB*; *frdA*; *pta*; *poxB*; *ldhA*; *zwf*; *ndh*; *mdh*; *sfcA*	Ethanol	[75]
9	*galK*	Isoprene	[53]
10	*tdh*; *tdC*; *sst*; *rhtA23*	Threonine	[76]
11	*deoB*; *yhfW*; *yahI*; *pta*; *eutD*; *arcC*; *yqeA*; *gdhA*; *ppc*; *pta*; *serA*; *thrC*	Lycopene	[54]
12	*sr1*; *gapB*; *pckA*; *gapA*; *ccpN*	Metabolism	[77]
13	*ldhA*; *pflB*; *ptsG*; *pepCK*	Succinate	[57]
14	*ppsA*; *poxB*; *aceBA*	Metabolism	[78]
15	*iclR*; *gdhA*; *aceE*	Lycopene	[49]
16	*cra*; *edd*; *iclR*	Metabolism	[79]
17	*hnr*; *yliE*	Lycopene	[80]
18	*arcA*	Metabolism	[81]
19	*nudF*	Isoprenol/Prenol	[60]
20	*ptsHIcrr* operon	Isoprenoids	[82]
21	*pgi*; *gnd*	Isoprene	[83]
22	*gdhA*; *gpmA*; *gpmB*; *aceE*; *fdhF*; *talB*; *fdhF*	Lycopene	[67]
23	*eno*	Lycopene	[67]
24	*gapA*; *mgsA*; *gapB*; *pgk*; *zwf*; *edd*; *eda*	Metabolism	[84]
25	*pgm*	Metabolism	[85]
26	*gapA*	Coenzyme Q10	[86]
27	*ptsG*	Metabolism	[87]
28	*thrA*	Metabolism	[88]
29	*glnA*	Bio-fuels	[89]
30	*serA*	Succinate	[90]
31	*fabD*	Metabolism	[91]
32	*rpiA*	Metabolism	[92]
33	*talA*; *talB*	Metabolism	[93]
34	*tktB*	Metabolism	[94]
35	*rpe*	Metabolism	[95]
36	*gntK*	Metabolism	[96]

Table 2: List of potential targets to be knocked out.

- EMP (Embden–Meyerhof–Parnas)
- ED (Entner-Doudoroff)
- PP (Pentose phosphate)
- Dhams

These pathways were investigated as feeding modules for increasing isoprene production. Highest isoprene production was seen with overexpression of the EDP in which pyruvate and G3P were generated simultaneously in contrast to EMP. In terms of precursor generation and energy/reducing-equivalent supply, overexpression of both EDP and PPP was found to be the ideal feeding module for MEP. Blocking EDP by knocking out pgi almost completely channelled the glucose through PPP and resulted in a significantly increased isopentanol production [83].

Reducing carbon loss: It is well established that bacterial growth using sugar substrates lead to production of secondary metabolites such as acetic acid, lactic acid, formic acid and ethanol; hence it is desirable to redirect this wasteful carbon towards the MEP pathway for higher production of isoprenoids. The genes which control the production of these secondary metabolites include ackA, pta, ldh, pflB, poxB and adhE. Reducing expression or knockout of these genes can result in making more carbon available for enhanced production of the desired metabolite. One such example was demonstrated for the production of pyruvate from glucose. Multiple gene deletions (ackA, pflB, ldh, adhE) resulted in an increase in pyruvate while acetate production was reduced by 85% [84]. For the production of ethanol from glycerol, knockout of the ldh gene resulted in up to 90% of its theoretical yield while lactate was reduced [85-88].

Increasing energy equivalents: Three reducing equivalents (NADH/NADPH) and three moles of ATP are required for biosynthesis of isoprene via the MEP pathway. A few of the recent studies have shown that moving the flux towards PPP results in an increase in the NADPH pool which facilitates isoprenoid production. Overexpression of genes controlling ATP synthesis and NADPH production led to increase in β-carotenoid production by 21% and 17% respectively [89-92]. Overexpression of zwf and gnd which enhance NADPH production led to an increase in riboflavin by 18% and 22%, respectively [93-96]. Some of the strategies applied for other metabolites such as arginine could be adapted for isoprene production in *E. coli*, for example, increasing the reducing equivalents pool (NADPH) within the cell was found to improve ornithine production [97]. In a study, the use of *B. subtilis* rocG which encodes NAD-dependent glutamate dehydrogenase allowed conversion of α-ketoglutarate to glutamate in an NADPH-independent manner and making more NADPH for ornithine biosynthesis [98]. Increasing the NADPH level was also achieved by inactivating two putative gluconate kinases (gntK) in *C. glutamicum* [99]. Overexpression of the ATP-dependent NAD kinase encoded by ppnK also led to enhance ornithine production in case of the same organism mentioned above [100].

Optimizing growth rate: Isoprene production is growth associated. Manipulation of global gene regulators affects cellular growth. For example, deletion of iclR and arcA genes resulted in 47% increase in biomass (in glucose abundant conditions). Modulation of the NADH: Ubiquinone oxidor-eductase (nuo), cytochrome bd-I oxidase (cyd), cytochrome b oxidase (cyo) and ATP synthase (atp) gene operons resulted in 20%, 16%, 5% and 21% increase in β carotene production respectively. Modulation of the nuo operon resulted in 29-40% decrease in cell mass [61].

GROWTH CONDITIONS AND MEDIA STANDARDIZATION

An effective method to increase cell density and production of metabolites is optimization of growth medium. The organic carbon supplement has an impact on the isoprene yield. Maximum production of isoprene was seen with glycerol as compared to other sources such as fructose and xylose [26]. Also, glycerol resulted in maximum production of β-carotene and lycopene in discrete studies and was shown to be superior to other carbon sources such as glucose, galactose, xylose and maltose [43,82]. Pyruvate and dipotassium phosphate as supplements were found to be beneficial for isoprenoid production possibly indicating enhanced pathway flux [32]. However, monopotassium phosphate was found to be dominant factor for the production of lycopene [82].

The source of nitrogen in the growth medium also plays an important role is improving biosynthesis of the product. Beef extract from a particular source was found to significantly increase α-pinene production as compared to other nitrogen sources [44]. Carbon and nitrogen restrictions during the process of fermentation resulted in significant enhancement of amorpha-4,11-diene production [42,101-104].

CONCLUSION

If we look back and attempt to estimate the progress achieved in terms of basic research on the subject, despite more than sixty years of intense research, the exact reason behind how evolutionary selection pressure has rendered some plants capable of synthesizing isoprene and others not still remains to be unveiled. This understanding could help us to predict how the future environmental changes would affect the capacity of plants to produce isoprene. The MEP pathway in particular seems to be yet in its nascent stage and demands a lot more effort to understand the overall fine-tuned regulation of the entire pathway and its significance. In terms of production titres, metabolic engineering of MEP and MVA pathways have significantly aided an upsurge in the production of isoprene from microbial sources and still has a lot more potential for further improvement with an optimal control guided in tactful manner. It is a continuous learning and development process. The design-build-test-debug approach for driving isoprenoid production in *E. coli* described recently by Wang et al. exemplifies one such continuous process towards achieving maximally improved production titres rapidly approaching the theoretical limits. An interesting synergistic approach combining the MVA and MEP pathways was recently pursued to utilize the merits of both the pathways in a well-balanced manner towards producing isoprene. Looking at the current societal consumption of non-renewable resources, there exists a huge demand for isoprene based alternate biofuel candidates. Scientists are developing novel strategies to explore current know-how about the biological functioning of the microorganisms and expand the limits further.

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