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Combinatorial pathway optimization in *Escherichia coli* by directed co-evolution of rate-limiting enzymes and modular pathway engineering†

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Abstract

Metabolic engineering of microorganisms for heterologous biosynthesis is a promising route to sustainable chemical production which attracts increasing research and industrial interest. However, the efficiency of microbial biosynthesis is often restricted by insufficient activity of pathway enzymes and unbalanced utilization of metabolic intermediates. This work presents a combinatorial strategy integrating modification of multiple rate-limiting enzymes and modular pathway engineering to simultaneously improve intra- and inter-pathway balance, which might be applicable for a range of products, using isoprene as an example product. For intra-module engineering within the methylerythritol-phosphate (MEP) pathway, directed co-evolution of DXS/DXR/IDI was performed adopting a lycopene-indicated high-throughput screening method developed herein, leading to 60% improvement of isoprene production. In addition, inter-module engineering between the upstream MEP pathway and the downstream isoprene-forming pathway was conducted via promoter manipulation, which further increased isoprene production by 2.94 fold compared to the recombinant strain with solely protein engineering and 4.7-fold compared to the control strain containing wild-type enzymes. These results demonstrated the potential of pathway optimization in isoprene overproduction as well as the effectiveness of combining metabolic regulation and protein engineering in improvement of microbial biosynthesis.

Keywords: isoprene, MEP pathway, directed co-evolution, modular pathway engineering

Introduction

Isoprenoids comprise the largest class of natural products which are valued as potential biofuels, biopharmaceuticals, flavors, nutraceuticals and agrichemicals. As one of the simplest members of isoprenoids, isoprene has wide applications in the synthesis of rubber, spices, medicines and pesticides, but its current manufacturing process is largely affected by the fluctuation in petroleum supply. Metabolic engineering offers a sustainable alternative route to manufacturing of natural products by manipulation of microorganisms, with advantages such as ease of pathway engineering and ability of large-scale fermentation. However, the efficiency of microbial biosynthesis is often restricted by insufficient activity of pathway enzymes (Jendresen et al. 2015; Kirby et al. 2008; Yan et al. 2005) and unbalanced utilization of metabolic intermediates (Ajikumar et al. 2010; Pitera et al. 2007; Xie et al. 2014a).

The isoprene synthetic pathway in engineered microorganisms typically consists of an upstream methylerythritol-phosphate (MEP) pathway or mevalonic acid (MVA) pathway commonly required for production of all isoprenoids (Maury et al. 2005; Rohmer et al. 1993) and a downstream heterologous isoprene-forming branch, catalyzed by isoprene synthase (ISPS) originated from plants (Miller et al. 2001; Sasaki et al. 2005; Sharkey et al. 2005). 1-deoxyxylulose-5-phosphate synthase (DXS) and 1-deoxyxylulose-5-phosphate reductoisomerase (DXR) are generally regarded as the main rate-limiting enzymes in the MEP pathway (Albrecht et al. 1999; Kim and Keasling 2001; Matthews and Wurtzel 2000), overexpression of which has been adopted to improve isoprene production in microbial systems such as *Escherichia coli* and *Bacillus subtilis* (Lindberg et al. 2010; Zhao et al. 2011). Our previous study revealed isopentenyldiphosphate (IPP)-dimethylallyldiphosphate (DMAPP) isomerase (IDI) as another key enzyme for isoprene biosynthesis ascribing to its role in adjusting the intracellular concentrations of IPP and DMAPP,

and found that ordered co-expression of genes *dxs, dxr* and *idi* significantly improved isoprene production in *E. coli* (Lv et al. 2013). The overexpression of these rate-limiting enzymes could effectively enhance the MEP pathway flux and thus the precursor supply for the target product. However, simple overexpression of multiple pathway enzymes has the risk of causing imbalance among enzymes within the pathway or between the upstream and downstream pathway modules, leading to growth inhibition or suboptimal production due to cytotoxicity of accumulated intermediates and generation of new bottleneck nodes in the metabolic pathway (Martin et al. 2003; SIVY et al. 2011). Therefore, further regulation of the metabolic balance among rate-limiting enzymes and pathway modules might provide hints for further improvement in the efficiency of biosynthesis.

The inherent insufficient enzyme activity is often a restricting factor leading to inefficient conversion of precursors and consequently limited production of the target compound. To achieve a well balanced and enhanced metabolic pathway, simultaneous modification of multiple enzymes is often required. Protein engineering, especially directed evolution, is widely used to alter the coding sequence and thus the properties of enzymes including catalytic activity and sometime also expression level, as proven by a number of studies (Ma et al. 2013; Wang et al. 2012). Independent of the enzyme structure and catalytic mechanism, directed evolution can be performed with specific screening methods that evaluate the performance of mutants in a sensitive and efficient manner to achieve the goal of "you get what you screen for" (Arnold 1998). However, for most enzymes in the metabolic pathway, direct high-throughput detection of their products is not possible. Besides, individual enzyme modification for multiple rate-limiting enzymes is labor-intensive and may generate new rate-limiting nodes that can be attributed to the unevenly modified activity. Directed co-evolution of multiple pathway enzymes coupled to synthesis of a colored downstream metabolite

as the indicator is therefore a potential solution.

Imbalance in expression level of enzymes between the upstream and downstream pathway modules is another key target for pathway optimization. To modulate the expression of enzymes in the metabolic pathway, regulation can be conducted at transcriptional, post-transcriptional, and post-translational levels (Seo et al. 2013), among which transcriptional control of gene expression is the most widely used because of its lower energy consumption. At the level of transcriptional regulation, one of the simplest and most direct methods is promoter manipulation. A recently proposed pathway optimization strategy is the multivariant-modular approach combining various promoters and gene copy-numbers, through which the diverse expression levels were balanced between the upstream and downstream pathways of taxadiene synthesis, achieving ~15,000-fold improvement in taxadiene production (Ajikumar et al. 2010). Since isoprene synthesis shares the same upstream pathway with isoprenoids including taxadiene, the effectiveness of modular pathway engineering in enhancing isoprene production is highly possible, but still needs to be experimentally verified.

In this work, the capacity of combinatorial pathway optimization integrating modification of multiple rate-limiting enzymes and modular pathway engineering in improving microbial biosynthesis was exemplified by isoprene overproduction in *E. coli*. First, the isoprene synthetic pathway was partitioned into two modules using DMAPP (the key intermediate in isoprenoids biosynthesis) as the connection node: the upstream module consisting of the native MEP pathway and the downstream module containing ISPS, and then intra-module and inter-module engineering was conducted using strategies of protein engineering and simplified modular pathway engineering, respectively. More specifically, directed co-evolution of the three rate-limiting enzymes in the upstream module was combined with promoter manipulation of the two pathway modules to

maximize precursor conversion and optimize the whole isoprene synthetic pathway. The approach proposed provides a possibility of solving intermediate imbalance at both intra- and inter-module levels. Considering the complex metabolic networking among the pathway enzymes as well as the pathways, such a comprehensive engineering strategy might help to achieve optimized product synthesis.

Materials and Methods

Materials

E. coli BL21 (DE3) (Novagen, USA) was used for gene cloning and expression. Plasmid pAC-LYC (harboring *CrtE*, *CrtB* and *CrtI*) was a kind gift from Francis X. Cunningham, Jr. (Cunningham et al. 1994). pET30a, pET32a, pTrc99a and pKD46 were purchased from Novagen (USA). Reagents for polymerase chain reaction (PCR), restriction enzymes, *Taq* DNA polymerase and T4DNA ligase used in cloning were purchased from Sangon Biotech (Shanghai, China). The plasmid isolation kit, genomic DNA extraction kit, PCR Cleanup kit and DNA gel extraction kit were purchased from Axygen Biotechnology Ltd. (Hangzhou, China). Primers were synthesized by Sangon Biotech (Shanghai, China).

Construction of mutant library

For construction of *dxs/dxr/idi* mutant library, *dxs/dxr/idi* was amplified with primers of PET-common-F(5'-GGAATTGTGAGCGGATAAC-3') and PET-common-R (5'-CAAAAAACCCC TCAAGACC-3'), using the previously constructed pET30a-*dxs/dxr/idi* as template (Lv et al. 2013). Error-prone PCR conditions were optimized by adjusting Mn^{2+} concentration to get an error frequency of 1-2 mutations/kb. The final PCR system contained 0.1 μg of template, 0.2 μM of eachprimer, 2.5 U of *Taq* polymerase,1×buffer, 0.2 mM dNTP, 2 mM Mg^{2+} and 0.3 mM Mn^{2+} in a total volume of 50 μL. PCR was performed with 32 cycles of 30 s at 95°C, 30 s at 55°C, and 3 min

at 72°C. The PCR products were purified and digested with *Xba*I and *Xho*I, and then cloned into the same sites of pET30a. The recombinant plasmids were transformed into *E. coli* BL21 harboring pAC-LYC and grown on LB medium plates (supplemented with 50 μg/ml kanamycin and 34 μg/ml chloromycetin) for high-throughput screening.

Construction of recombinant plasmids for inter-module engineering

In inter-module engineering, a total of 6 plasmids with varying promoter strength or antibiotic resistance, pET30a (P_{T7}, kanamycin resistance), pTrc99k (P_{Trc}, kanamycin resistance), pAra99k (P_{Ara}, kanamycin resistance), pET32a (P_{T7}, ampicillin resistance), pTrc99a (P_{Trc}, ampicillin resistance), and pAra99a (P_{Ara}, ampicillin resistance), were adopted to modulate the metabolic balance between the upstream and downstream pathways. The fragment containing P_{Ara} and araC was amplified from pKD46 using Ara-F (5'-ACGCGTCGACTTATGACAACTTGACGGCTACA-3') and Ara-R (5'-CATGCCATGGTTTTTTATAACCTCCTTAGAGCTCG-3') as primers. It was then digested with *Sal*I&*Nco*I and ligated with the pTrc99a-ΔTrc/lacI fragment (digested with *Xho*I&*Nco*I), amplified from pTrc99a using the primer pair pTrc99a-ΔTrc/lacI-F (5'-GCCGACATCATAACGGTT -CTGG-3') &pTrc99a-ΔTrc/lacI-R (5'-CCGCTCGAGCCTGAATTGACTCTCTTCCGGGC-3'), to generate pAra99a. Kana-ORF from pET30a was amplified with the primers Kana-F (CGCGGATCC ATGAGCCATATTCAACGGGA) and Kana-R (GGAAGTCGACTTAGAAAAACTCATCGAGCAT C). The PCR product was then digested with *BamHI*&*Sal*I and ligated with the pTrc99a-ΔAmp/pAra99a-ΔAmp fragment (digested with *Bgl*II&*Xho*I) amplified from pTrc99A/pAra99A using Δamp-F (5'-CCGCTCGAGCTGTCAGACCAAGTTTACTCATATATA-3') and Δamp-R (5'-GGAAGATCTACTCTTCCTTTTTCAATATTATTGAAG-3') as primers, to generate plasmids

of pTrc99k and pAra99k. Subsequently, the positive mutant *dxs/dxr/idi*-1-6 was subcloned into the above empty plasmids with kanamycin resistance, generating pET30a-*dxs/dxr/idi*-1-6, pTrc99k-*dxs/dxr/idi*-1-6 and pAra99k-*dxs/dxr/idi*-1-6 for regulation of the upstream MEP pathway. Meanwhile, the *ispS* gene from our previously constructed plasmid pET32a-*ispS* (Lv et al. 2013) was subcloned between the *Xba*I and *Xho*I sites of the other two plasmids with ampicillin resistance, resulting in pTrc99a-*ispS* and pAra99a-*ispS* for regulation of the downstream isoprene-forming branch.

Quantification of lycopene with HPLC

For analysis of lycopene production, BL21-*lyc/dxs/dxr/idi* and BL21-*lyc/dxs/dxr/idi*-1-6 were cultivated in 5 ml Luria-Bertani (LB) medium (supplemented with 50 μg/ml kanamycin and 34 μg/ml chloromycetin) at 37^{o}C with shaking (200 rpm). About 2% of the overnight-grown seed culture was inoculated into 20 ml fresh LB medium (supplemented with 50 μg/ml kanamycin and 34 μg/ml chloromycetin) and then cultured under the same condition for 24 h. Cells were harvested from 3 ml of culture by centrifugation at 4000 g, washed twice with ultrapure water, and then dried at 95 ^{o}C to constant weight for measuring dry cell weight (DCW). Another 3 ml of culture was used to extract the intracellular lycopene with acetone following the procedure described previously (Xie et al. 2014b). The analysis of lycopene was performed by HPLC (Agilent 20AT) equipped with a KromasilC18 column (4.6 mm × 250 mm) and the signals were detected by a UV/VIS detector at 475 nm. The mobile phase consisted of acetonitrile-methanol-isopropanol (50:30:20) with a flow rate of 1 mL/min at 40°C.

Quantification of isoprene with GC

For analysis of isoprene production, the strains containing recombinant vectors were grown in 50 mL LB medium (supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin) at 37°C with

shaking until OD_{600} reached 0.5. Five mL of culture were transferred to 17-mL sealed vials and supplemented with 0.1 mM IPTG before further incubation at 37°C with shaking (200 rpm) for 2.5 h. Isoprene qualification was performed using a modified procedure of A.LEHNING et al. (Lehning et al. 1999). The gas sample was collected from the headspace of sealed culture with a gas-tight syringe, and analyzed by a GC system equipped with an HP-FFAP column (30 m × 0.25 mm, 0.25 μm film thickness) and a flame ionization detector. The temperature of oven, injector and detector was held at 80°C, 180°C and 180°C, respectively.

Molecular dynamic (MD) simulation

The initial geometry of the wild-type DXR was obtained from the protein data bank (PDB ID: 1Q0Q) (Mac Sweeney et al. 2005). The mutant K37N/K217N was constructed using the Discovery Studio (DS, Accelrys Inc., San Diego, USA). Mg^{2+} was manually located into the active site according to the crystal structure of DXR with fosmidomycin and manganese (1ONP). Partial charges and force field parameters for the substrates were automatically generated by the antechamber program using the general AMBER force field (GAFF) (Wang et al. 2006; Wang et al. 2004). The non-polar hydrogen atoms were added to the enzyme by AMBER 11 simulation package using the ff10 force field. The protonation state of residues was set according to pH 7.0. Counterions of Na^{+} were added to neutralize the system and the whole system was immersed in a rectangle box of TIP3P water molecules, extended 10 Å from the dissolved atoms in all three dimensions (Jorgensen et al. 1983). Each MD simulation included minimization, heating, density equilibration at constant temperature, equilibration under constant pressure (NPT) and a final 6 ns productive run in the NPT ensemble as described in our previous work (Gu and Yu 2012). The temperature was maintained by coupling to the Langevin thermostat. The SHAKE algorithm was applied to constrain bond distances of the hydrogen atoms. The non-bonding cut-off distance was

set to 8 Å and the simulation snapshots were saved every 2 ps for analysis.

Results and discussion

Development of a lycopene-indicated high-throughput screening (HTS) method for *dxs/dxr/idi* co-evolution

Lycopene is a typical colored isoprenoid product. Since it shares the same upstream MEP pathway with isoprene, lycopene overproduction should also benefit from the enhanced expression level of *dxs*/*dxr*/*idi* as reported for isoprene synthesis (Kajiwara et al. 1997; Kim and Keasling 2001; Rad et al. 2012). To examine this assumption, a lycopene-producing *E. coli* strain was constructed by transformation of pAC-LYC harboring *CrtE*, *CrtB* and *CrtI* (Cunningham et al. 1994) into *E. coli* BL21, followed by overexpression of *dxs*/*dxr*/*idi* through introduction of pET30a-*dxs/dxr/idi* constructed in our previous study (Lv et al. 2013), resulting in BL21-*lyc/dxs/dxr/idi*. The lycopene production was then compared with that of BL21 (pAC-LYC) after 2-day cultivation on LB plate supplemented with kanamycin and chloromycetin. The distinct color difference of the strains before and after over-expression of *dxs*/*dxr*/*idi* (Fig. 1A) unambiguously pointed to a positive correlation between the MEP pathway flux and lycopene production, leading to the conclusion of consistent trends in correlation between MEP flux (*dxs*/*dxr*/*idi*) and isoprene/lycopene yield (Fig. 1B). Based on this consistency, a high-throughput screening method was developed for directed co-evolution of DXS/DXR/IDI so as to enhance isoprene production, using lycopene as a colorimetric reporter to screen the mutant library (Fig. 1B). Improved DXS/DXR/IDI variants from the mutant library could be easily screened out by the enhanced lycopene production, as illustrated by red coloration, which were then used as candidates to elevate isoprene production. The enhancement of lycopene production indicated the improved catalytic performance of the engineered enzymes, while the improved isoprene production adopting these engineered enzymes validated the feasibility of this

screening method.

Directed co-evolution of *dxs*/*dxr*/*idi*

Directed co-evolution of *dxs*/*dxr*/*idi* was conducted as shown in Fig. 2. As the first step, error-prone PCR was carried out using pET30a-*dxs/dxr/idi* (kanamycin resistance) as template for construction of DXS/DXR/IDI mutant library. The amplified products and pET30a were digested, ligated, and co-transformed into the lycopene-producing BL21 strain (containing pAC-LYC, chloromycetin resistance) and then spread on plate with kanamycin and chloromycetin for selection of positive colonies. After 2-day cultivation, the recombinant strains with darker color were selected, cultured in LB supplemented with kanamycin to achieve natural loss of pAC-LYC, followed by extraction of the pET30a-*dxs/dxr/idi* plasmids containing the positive variants, which were then re-transformed into an isoprene-producing BL21 strain (containing pET32a-*ispS*, ampicillin resistance). The recombinant strains were subsequently selected on LB plates with ampicillin and kanamycin, and the remaining unwanted transformants containing pAC-LYC (red color) could be readily excluded upon color screening. Finally, isoprene production was analyzed using GC to reconfirm the strains containing positively modified DXS/DXR/IDI proteins.

In the first round of mutagenesis, a library of about 8000 colonies was constructed. One positive variant (named *dxs/dxr/idi*-1-6) with improved lycopene production was selected, in which lycopene accumulation was increased by 80% (0.65 mg/L vs. 0.36 mg/L) compared with the control strain harboring pET30a-*dxs/dxr/idi*. After transformation of pET30a-*dxs/dxr/idi*-1-6 into BL21 (pET32a-*ispS*), isoprene production was increased by 60% (5.96 mg/L vs. 3.71 mg/L) (Fig. 3A). Besides, the cell growth of recombinant strains containing the *dxs/dxr/idi*-1-6 variant was slightly improved (Fig. 3B), indicating that intra-module engineering of the three limiting enzymes might to some extent eliminate the cytotoxicity of accumulated intermediate metabolites caused by

unbalanced pathway and result in the simultaneously improved yield of downstream products and biomass. In the second round of mutagenesis, pET30a-*dxs/dxr/idi*-1-6 was used as template for error-prone PCR. However, no positive mutant was found after screening of about 10,000 colonies, pointing to a low probability of positive mutation for large-size target (here the three genes together have a size of 3609 bp).

DNA sequencing of *dxs/dxr/idi*-1-6 revealed three point mutations in DXS (E289G, N455I, E598V), two point mutations in DXR (K37N, K217N), and no mutation in IDI. The locations of mutations in the enzyme as well as their potential effects on the catalysis were analyzed according to the crystal structure of the corresponding wild-type proteins and molecular dynamic (MD) simulation. In DXS, E289 locates in the substrate-binding pocket, while N455 and E598 are far away from the active site (Fig. S1A) (Xiang et al. 2007). It is possible that substitution of glutamate with a smaller glycine at site 289 might reduce the steric hindrance for substrate binding, and elimination of electrostatic effect among neighboring residues might change the local space structure leading to altered forces between the adjacent residues in binding pocket and substrate. However, we failed to construct the MD system due to the incomplete crystal structure of this enzyme (with about eighty amino acids missing).

In DXR, residue K37 is in the N-terminal NADPH binding domain, which plays an important role in conversion of the transition structure 2-C-methyl-D-erythrose-4-phosphate to 2-C-methyl-D-erythritol-4-phosphate, whereas residue K217 locates near the 206-216 region functioning as a flexible lid, which closes upon substrate binding to shield the active site from the solvent (Mac Sweeney et al. 2005) (Fig. S1B). To better illuminate the effect of K37N/K217N mutations on DXR structure as well as the interaction between the substrate and key residues in the active site, MD analysis was performed with wild-type and mutated DXR. Stronger hydrogen bond

interactions were found between the mutated residues (N37, N217), NADPH (cosubstrate) and water molecules in the DXR mutant than in the wild-type enzyme (Fig. S2, Table S1). Besides, the forces between the substrate 1-D-deoxyxylulose-5-phosphate (DXP) and some key amino acids (H209, S186, S222 and E231) involved in the substrate-binding pocket were also improved in the mutant K37N/K217N (Fig. S3, Table S2). These above results demonstrate that substitution of lysine with asparagine at sites 37/217 most likely directly strengthened their binding force with cosubstrate NADPH so as to indirectly affect the interactions between the substrate DXP and the DXP binding domain, and thus influence the activity of DXR.

The *in vitro* activity of individual enzyme variants was not measured considering that the *in vivo* conditions are dynamic and very different from the *in vitro* situation. Taking the complicated metabolic network into account, synthesis of target products (here lycopene and isoprene) is taken as the reporter for *in vivo* activities of the enzymes, using the strain harboring wild-type enzymes as the control, which is a generally accepted method for evaluating evolved enzymes in a metabolic pathway (Leonard et al. 2010; Wang et al. 2000). In addition, obtained by means of directed co-evolution of multiple enzymes, the improved production of isoprene and lycopene might be mainly attributed to the synergistic effect of DXS/DXR/IDI co-modification leading to more balanced intermediate utilization in the MEP pathway rather than individually improved enzyme activities.

Inter-module pathway engineering

Following intra-module engineering which widens the bottleneck of the MEP pathway by simultaneous modification of the three rate-limiting enzymes, inter-module engineering was conducted employing various promoters to modulate diverse expression levels of upstream and downstream pathway modules to achieve an optimally balanced synthetic pathway, and thus the

maximized isoprene production, as depicted in Fig. 4A. More specifically, three promoters with strength gradient, P_{T7}, P_{Trc}, P_{Ara}, were adopted in each module, the combination of which resulted in 9 strains. For the upstream module, the three genes deemed to be rate-limiting in the MEP pathway, here in their modified form, namely *dxs/dxr/idi*-1-6 were overexpressed in the form of polycistronic operon and controlled under P_{T7}, P_{Trc}, P_{Ara} using pET30a, pTrc99k, pAra99k (with the same copy number and distinct gene expression levels; all with kanamycin resistance) as vectors, respectively. For the downstream isoprene-forming module, pET32a, pTrc99a, pAra99a with ampicillin resistance were adopted for promoter replacement of P_{T7}, P_{Trc}, P_{Ara}, respectively.

The dependence of isoprene accumulation on the upstream pathway flux was determined under constant flux of the downstream pathway, and the data are shown in Fig. 4B, together with the result of dependence on the downstream pathway flux at constant upstream pathway strength. With constant downstream pathway expression, as the promoter of upstream pathway expression changed from P_{Ara} to P_{Trc} with increasing strength, isoprene production increased correspondingly due to the enhanced precursor supply to the whole pathway. After further increase of upstream pathway expression by using the even stronger promoter P_{T7}, isoprene production showed an obvious decline, suggesting that excessive upstream metabolic flux exerted negative effect on the accumulation of target product subjected to the limited capacity of the downstream pathway or potential cytotoxicity of accumulated intermediate metabolites. When the upstream pathway expression was kept constant and controlled under weak promoters such as P_{Ara} and P_{Trc}, isoprene production declined gradually with the increasing promoter strength of the downstream pathway. The limited isoprene production might be ascribed to deficient precursor supply and competitive consumption of energy essential for cell growth and metabolism by excessive expression of the downstream genes. When the upstream pathway flux was controlled under the strong T7 promoter, higher isoprene production was obtained

in the strain with P_{trc}-controlled downstream pathway as compared to that controlled by P_{Ara}, suggesting that stronger downstream flux could take full advantage of the rich precursor supply leading to enhanced accumulation of the target product.

In addition to promoter variation, the effect of inducer concentrations on isoprene production was also investigated for further balancing between the upstream/downstream pathways. Four inducer concentrations, 0.02 mM, 0.05 mM, 0.1 mM and 0.2 mM of isopropyl-β-d-thiogalactoside (IPTG) for P_{T7}, P_{Trc} and/or 0.2 mM, 0.5 mM, 2 mM and 5 mM of arabinose for P_{Ara}, were applied for each strain, generating a total of 84 samples (Fig. 5). As shown in Fig. 5A, when the upstream pathway was controlled under the strong T7 promoter, isoprene production in most strains was less than 8 mg/L except P_{T7}-P_{Trc}0.02 (BL21-pET30a-*dxs/dxr/idi*-1-6-pTrc99a-*ispS* with addition of 0.02 mM IPTG, producing 9.6 mg/L of isoprene) and P_{T7}-P_{Trc}0.05 (BL21-pET30a-*dxs/dxr/idi*-1-6-pTrc99a-*ispS* with addition of 0.05 mM IPTG, producing 8.8 mg/L of isoprene). After adjustment of the upstream pathway to a weaker level by switching to Trc promoter (Fig. 5B), isoprene production gradually increased with the decline in promoter strength of the downstream pathway, in which the highest production of 17.5 mg/L was achieved in P_{Trc}-P_{Ara}0.02-5 (BL21-pTrc99k-*dxs/dxr/idi*-1-6-pAra99a-*ispS* with addition of 0.02 mM IPTG and 5mM arabinose). With decreased expression of the upstream pathway under the control of Ara promoter (Fig. 5C), the highest isoprene production of 12.4 mg/L was achieved in P_{Ara}-P_{Ara}0.5 (BL21-pAra99k-*dxs/dxr/idi*-1-6-pAra99a-*ispS* with addition of 0.5 mM arabinose). In general, under the control of the same promoter, higher isoprene production (Fig. 5A-C) and cell biomass (Fig. S4) were obtained in the strains with lower concentrations of IPTG, indicating higher concentrations of IPTG might impair cell growth and activity so as to decrease isoprene production. The slightly higher cell biomass in P_{Trc}-P_{Ara}, compared with P_{Trc}-P_{T7}/P_{Trc}-P_{Trc}, implicating the

introduction of arabinose benefited cell growth probably as an additional carbon source, which was also observed by other researchers (Kim et al. 2009). Finally, the highest yield of isoprene (17.5 mg/L) was achieved in the combinatorially engineered strain BL21-pTrc99k-*dxs/dxr/idi*-1-6-pAra99a-*ispS* induced with 0.02 mM IPTG and 5 mM arabinose, which was increased by 4.72 fold as compared to that of the control strain BL21-pET30a-*dxs/dxr/idi*-pET32a-*ispS* (3.71 mg/L), and 2.94 fold as compared to that of BL21-pET30a-*dxs/dxr/idi*-1-6-pET32a-*ispS* with solely protein engineering (5.96 mg/L). These results demonstrated the efficiency of combining protein engineering and modular pathway engineering in pathway optimization towards enhanced yield of the target product.

Conclusion

In this work, protein engineering and metabolic regulation were combined for enhancement of isoprene production in *E. coli*. For intra-module engineering within the MEP pathway, a novel HTS strategy was developed for directed co-evolution of multiple rate-limiting enzymes (DXS, DXR, and IDI) based on analysis of the red-colored metabolite lycopene. Simultaneous directed evolution of multiple pathway enzymes was proven to be an efficient method for improving isoprene production in *S. cerevisiae*, and this method might be generalized to optimize the carbon flux of various metabolic pathways by changing the indicator metabolite. The advantages of this method can be summarized as following: 1) the plate screening method with lycopene as the indicator is simple, direct and convenient; 2) as a successful example, it provides reference for directed evolution of multiple enzymes in biosynthesis pathways, avoiding onerous task required for individual modification of three enzymes and extra expression balancing among these enzymes; 3) this method has wide applicability not only in biosynthesis engineering of isoprenoids but also in other secondary metabolites, especially those with cytotoxicity after accumulated to a certain

concentration. In addition to protein engineering, the isoprene biosynthesis pathway was further optimized via modular engineering. By tuning the metabolic flux between the upstream and downstream pathway modules upon promoter replacement and inducer adjustment, in addition to co-engineering of DXS/DXR/IDI, the final production of isoprene was improved by 4.7-fold, demonstrating the potential of combinatorial pathway optimization in enhancing the efficiency of biosynthesis. This strategy might also be applicable for a wide range of natural products, in particular, the various isoprenoids sharing the same MEP pathway with isoprene.

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Figure captions

Fig. 1 High-throughput screening (HTS) method for directed co-evolution of DXS/DXR/IDI. A, Pre-experiment to evaluate the correlation between lycopene production and *dxs/dxr/idi* overexpression: color difference of lycopene producing strains (BL21 harboring pAC-LYC) before and after over-expression of *dxs/dxr/idi*; B, Schematic of lycopene-indicated HTS method for co-directed evolution of *dxs/dxr/idi*. The enclosed square indicates the principle of the HTS method: the consistent trends in correlation between MEP flux (*dxs/dxr/idi*) and isoprene/lycopene yield. The biosynthetic pathways of isoprene and lycopene are presented with the overexpressed or introduced genes highlighted in different colors.

Fig. 2 The flow chart of directed co-evolution of *dxs*/*dxr*/*idi*. Error-prone PCR was performed for construction of mutant library, and lycopene was used as the colormetric reporter for high-throughput screening, finally, the positive mutant was confirmed by GC analysis of isoprene production.

Fig. 3 Effects of modified *dxs/dxr/idi* on isoprene/lycopene production and cell growth. A, Isoprene/lycopene production of the isoprene or lycopene producing strains, transformed with pET30a-*dxs/dxr/idi* and pET30a-*dxs/dxr/idi*-1-6, respectively. B, Biomass of the isoprene or lycopene producing strains transformed with pET30a-*dxs/dxr/idi* and pET30a-*dxs/dxr/idi-1-6*, respectively. Lycopene producing strains (BL21-*lyc/dxs/dxr/idi* and BL21-*lyc/dxs/dxr/idi*-1-6) were cultivated in 20 ml Luria-Bertani (LB) medium (supplemented with 50 μg/ml kanamycin and 34 μg/ml chloromycetin) at 37^{o}C for 24 h. Isoprene producing strains (BL21-pET32a-*dxs/dxr/idi*-pET32a-*ispS* and BL21-pET32a-*dxs/dxr/idi*-1-6-pET32a-*ispS*) were grown in 50 mL LB medium (supplemented with 100 μg/mL ampicillin and 50 μg/mL kanamycin) at 37°C with shaking until OD_{600} reached 0.5. Five mL of culture were transferred to 17-mL sealed

vials, supplemented with 0.1 mM IPTG before further incubation at 37°C with shaking (200 rpm) for 2.5 h and analyzed with GC.

Fig. 4 Inter-module engineering of the isoprene synthetic pathway. A, Schematic of modular regulation approach for optimization of the isoprene biosynthesis pathway. This pathway was partitioned into two modules using DMAPP as the connection node: the upstream module consisting of the native MEP pathway and the downstream module containing ISPS. To increase the flux of the upstream MEP pathway, the reported bottlenecks (*dxs*, *dxr*, *idi*) were overexpressed with the polycistron method, in which the three genes were under control of the same promoter but each gene can be expressed separately using their own ribosome binding site (RBS). Three promoters with strength gradient, P_{T7}, $P_{\underline{Trc}}$, P_{Ara}, were adopted in each module to modulate diverse expression levels of the upstream and downstream pathways. The concentration of IPTG and arabinose was 0.05 mM and 0.5 mM, respectively. B. Response of isoprene accumulation to changes in promoter strength of the upstream and downstream pathway modules. Blue columns indicate strains with constant P_{Ara}-downstream pathway; red columns indicate strains with constant P_{Trc}-downstream pathway; black columns indicate strains with constant P_{T7}-downstream pathway.

Fig. 5 Optimization of isoprene production through regulating expression of the upstream and downstream pathway modules with different promoters (P_{Ara}, P_{Trc}, P_{T7}) and inducer concentrations (mM). A, Isoprene production of strains with the constant P_{T7}-MEP pathway. B, Isoprene production of strains with the constant P_{Trc}-MEP pathway. C, Isoprene production of strains with the constant P_{Ara}-MEP pathway. X-coordinate represents the strains with different promoters and inducer concentrations: P1-P2, BL21-P1-*dxs/dxr/idi*-1-6-P2-*ispS*; I1-I2, inducer concentration of upstream pathway (mM)-inducer concentration of downstream pathway (mM). Y-coordinate stands for isoprene production.

Fig. 1

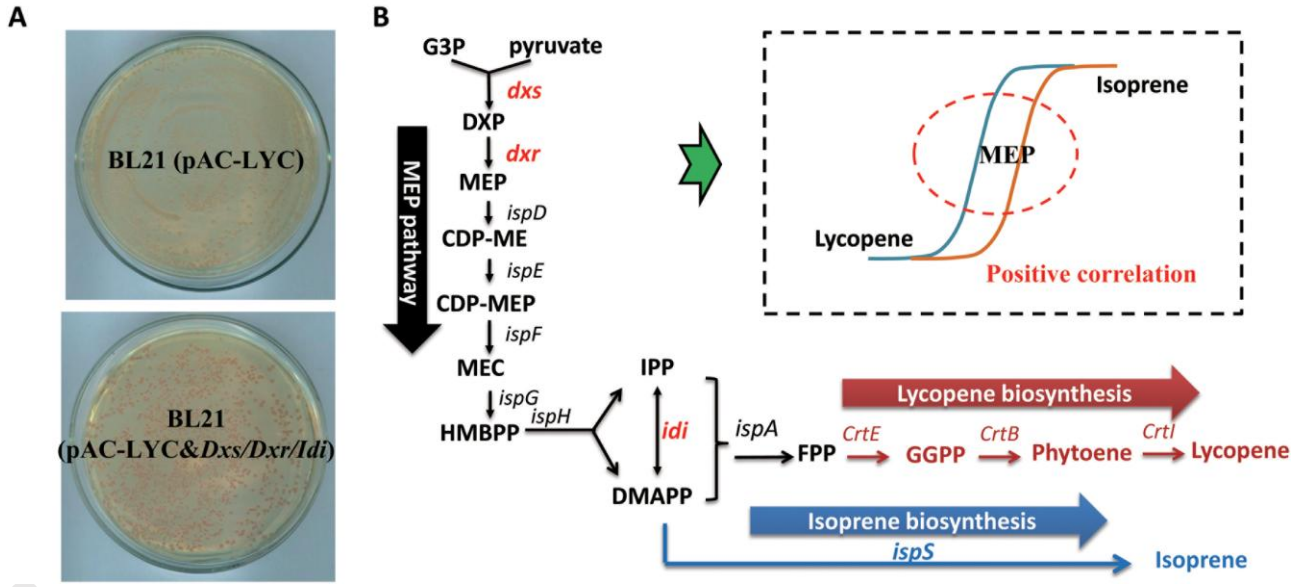


Fig. 2

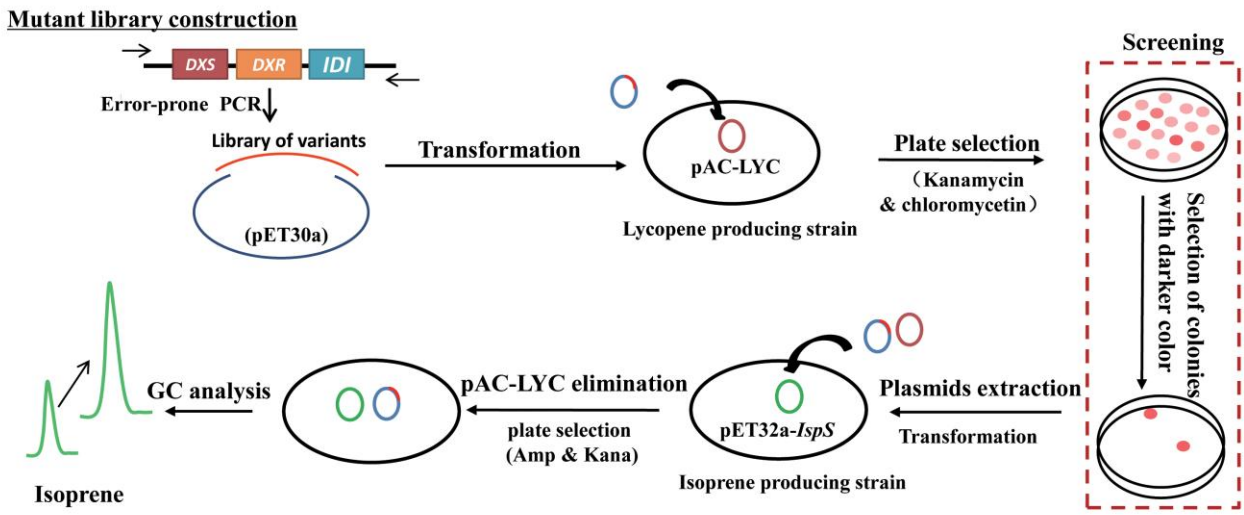


Fig. 3

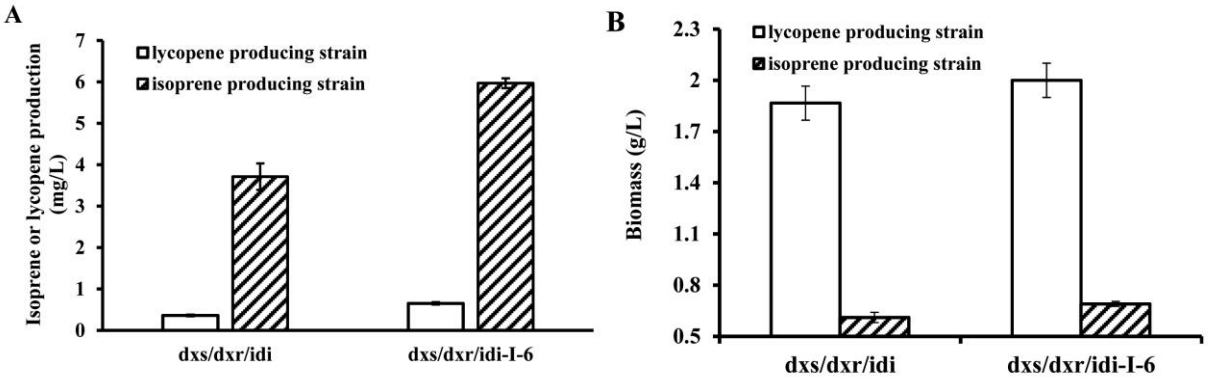
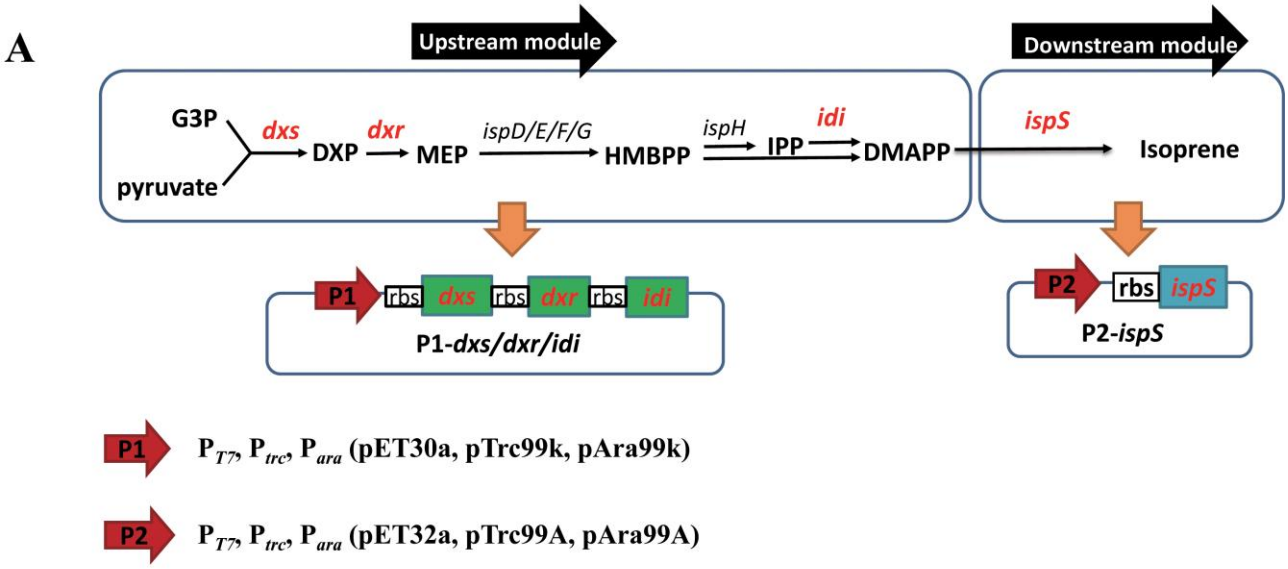


Fig. 4



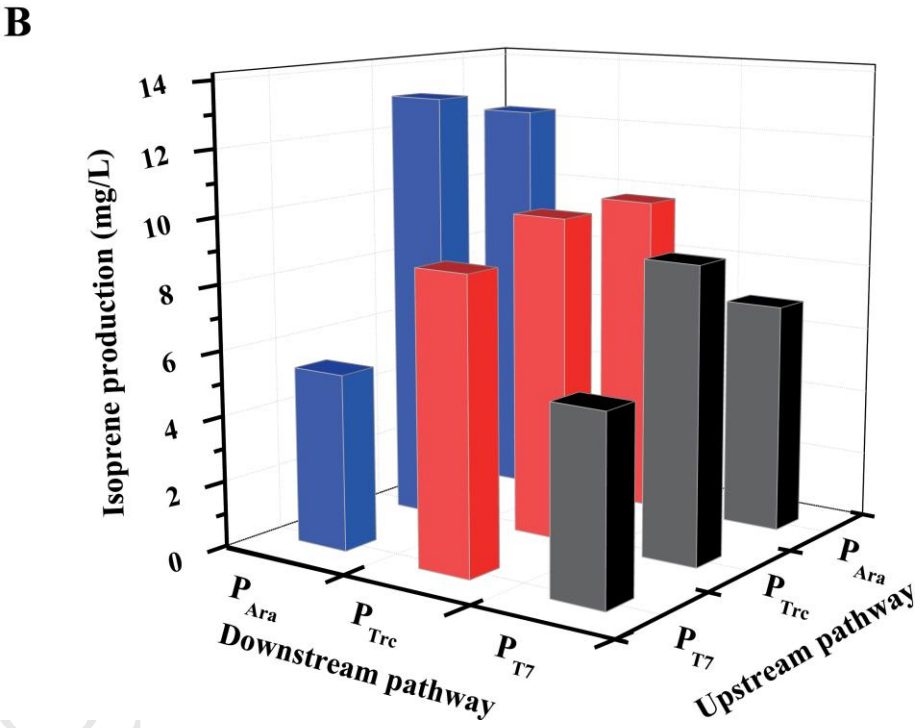


Fig. 5