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Significantly enhanced production of isoprene by ordered coexpression of genes *dxs*, *dxr*, and *idi* in *Escherichia coli*

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Abstract We constructed a biosynthetic pathway of isoprene production in *Escherichia coli* by introducing isoprene synthase (*ispS*) from *Populus alba*. 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*dxr*) and isopentenyl diphosphate (IPP) isomerase (*idi*) were overexpressed to enhance the isoprene production. The isoprene production was improved 0.65, 0.16, and 1.22 fold over the recombinant BL21 (pET-30a-*ispS*), respectively, and *idi* was found to be a key regulating point for isoprene production. In order to optimize the production of isoprene in *E. coli*, we attempted to construct polycistronic operons based on pET-30a with genes *dxs*, *dxr*, and *idi* in various orders. The highest isoprene production yield of 2.727 $mg\,g^{-1}h^{-1}$ (per dry weight) was achieved by *E. coli* transformed with pET-30a-*dxs*/*dxr*/*idi*. Interestingly, the gene order was found to be consistent with that of the metabolic pathway. This indicates that order of genes is a significant concern in metabolic engineering and a sequential expression pattern can be optimized according to the biosynthetic pathway for efficient product synthesis.

Keywords Isoprene · *Escherichia coli* · Biosynthesis · Ordered coexpression

Introduction

Isoprene is the monomeric building block of the most diverse and abundant groups of biological organic compounds referred to as isoprenoids. As a feedstock in the synthetic chemistry industry, it has significant potential and is widely used in the synthesis of rubber, spices, medicines, and pesticides. Currently, the industrial supply of isoprene is limited to petrochemical sources. Considering the growing energy crisis and environmental pressures, there is an urgent need to seek feasible substitute approaches.

The biosynthesis of isoprene was first described in plants in 1957 (Sanadze 1957). A series of plants were reported to emit 1–2 % of their fixed carbon as isoprene (Kesselmeier and Staudt 1999; Monson and Fall 1989). Nevertheless, as isoprene is gaseous above 34 °C, it is impractical to harvest this hydrocarbon from plants. With the development of biosynthesis, isoprene produced by microorganisms provides a feasible approach. Isoprene is poorly soluble in water, so it can be condensed from the gas phase in fully enclosed bioreactors, with no need for additional purification of the liquid product. Although no *ispS* (isoprene synthase) gene has been elucidated in any microorganism, over-expression of *ispS* gene from plants can effectively endow microorganisms with the property of isoprene production from their own metabolism. At present, *ispS* has been cloned and characterized from *Poplar* (*Populus alba*, *Populus tremuloides*) (Miller et al. 2001; Sasaki et al. 2005) and *kudzu vine* (*Pueraria montana*) (Sharkey et al. 2005).

There are two distinct pathways leading to the biosynthesis of key isoprenoid intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP): the mevalonic acid pathway, which operates in eukaryotes, archaea and the cytosol of higher plants; and the methylerythritol-4-phosphate (MEP) pathway, which is present in prokaryotic bacteria, green algae and the chloroplasts of higher plants (Kuzuyama 2002; Lichtenthaler 2000). As the common engineering strain, *Escherichia coli* uses the MEP pathway to produce isoprenoids. The MEP pathway, which initiates with the glycolytic intermediates pyruvate and glyceraldehyde-3-phosphate and ends with the DMAPP

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and IPP, consists of seven subsequent enzymatic steps. As numerous reports have shown, the main rate-limiting enzymes within the MEP pathway were found to be 1-deoxyxylulose-5-phosphate synthase, 1-deoxyxylulose-5-phosphate reductoisomerase, IPP–DMAPP isomerase (Albrecht et al. 1999; Kajiwara et al. 1997; Kim and Keasling 2001; Matthews and Wurtzel 2000). Success has been achieved in the enhancement of isoprenoid production through regulation methods such as eliminating the rate-limiting steps and engineering regulation networks. However, research on isoprene production by regulating the metabolic flux of the MEP pathway has been limited, and has focused only on the overexpression of *dxs* or *dxr* for isoprene production enhancement. Xue et al. reported that the yield of isoprene in *Bacillus subtilis* was increased by 40 % over the wild-type strain by overexpressing *dxs*, but no improvement was observed in the overexpression of the *dxr* (Xue and Ahring 2011). It was also found that heterologous expression of *dxs* and *dxr* from *B. subtilis* in the *E. coli* harboring *ispS* resulted in a 2.3-fold enhancement of isoprene production (Zhao et al. 2011). Until now, no report about the effect of *idi* on the production of isoprene has been released, nor has there been any coexpression of the genes *dxs*, *dxr*, and *idi* to study their synergetic effect.

Usually, two methods are applied to coexpress heterologous genes. One is using a vector with independent promoters or internal ribosome entry site (IRES). Promoter interference sometimes occurs with the use of heterologous promoters, i.e., transcription from one promoter suppresses transcription from another (Emerman and Temin 1984). Using the IRES method, more than two genes connected by IRES sequences can be efficiently expressed from a single promoter (Ghattas et al. 1991). Nevertheless, the target genes' number is limited to its restriction sites. The other one is using two compatible systems. ColE1-derived plasmid is the most attractive candidate due to its wide application and high copy number. However, many ColE1-compatible plasmids (such as p15A-derived plasmids) are of low copy numbers, which makes it difficult and time-consuming to construct vectors and also reduces the level of mRNA transcription (Johnston et al. 2000). Using two incompatible plasmids was reported as a new method for protein coexpression in *E. coli* and compensates for the deficiency of using two compatible plasmids (Yang et al. 2001). Therefore, a combination of using incompatible plasmids and the IRES method might provide a feasible strategy to coexpress several genes.

In this work, an isoprene synthase from *P. alba* was introduced to construct a pathway of isoprene production in *E. coli*. To achieve the coexpression of multiple genes, an expression system was established by combining two incompatible plasmids (pET-32a and pET-30a) and the IRES method together. Based on this expression system, the genes *dxs*, *dxr*, and *idi* were overexpressed and coexpressed for enhancing the production of isoprene. In addition, we attempted to optimize the isoprene production in transformed *E. coli* by constructing polycistronic operons with rearranged gene orders to investigate whether the gene order affects the final product yield.

Materials and methods

Bacterial strains, plasmids, and culture conditions

E. coli BL21 (DE3) (Novagen, USA) was used for gene cloning and expression. *E. coli* K12 AB200068 (CCTCC) was used for amplification of *dxs*, *dxr*, and *idi* genes. Plasmids pET-32a and pET-30a (Novagen, USA) were used as vectors for *ispS*, *dxs*, *dxr*, and *idi* expression. All *E. coli* strains were cultivated in Luria–Bertani (LB) medium at 37 °C with shaking (200 rpm), supplemented with antibiotics (100 μg/ml of ampicillin or 50 μg/ml of kanamycin) as needed.

Construction of recombinant plasmids and transformation

The nucleotide sequence of the *P. alba* isoprene synthase (GenBank accession No. AB198180) without its predicted chloroplast transit peptide was optimized to the preferred codon usage of *E. coli* and synthesized by Sangon Biotech (Shanghai. China). The gene was amplified using *ispS*-*Bam*HI (F) and *ispS*-*Hind*III(R) as primers and cloned into pET-32a between restriction sites of *Bam*HI and *Hind*III. The resulting plasmid was designated as pET-32a-*ispS* (1, Fig. 1). The genes *dxs*, *dxr*, and *idi* were amplified by PCR using *E. coli* K12 genomic DNA as a template. The *dxs* gene was amplified using primers *dxs*-*Nco*I (F) and *dxs*-*Bam*HI (R) and then ligated into the pET-30a digested with *NcoI* and *BamH*I to create pET-30a-*dxs* (2, Fig. 1). The *dxr* gene was amplified using primers *dxr*-*Bam*HI (F) and *dxr*-*Hind*III (R) and inserted into pET-30a between the restriction sites of *Bam*HI and *Hind*III to create pET-30a-*dxr* (3, Fig. 1). The *idi* gene was amplified using primers *idi*-*Hind*III (F) and *idi*-*Xho*I (R) and inserted into pET-30a between the restriction sites of *Hind*III and *Xho*I to create pET-30a-*idi* (4, Fig. 1).

In the construction of these polycistronic operons (5–10, Fig. 1), the RBS and its surrounding sequence originated from pET-30a (AATAATTTTGTTTAACTTTAAGA AGGAGATATACAT) (Liu and Yu 2012) were introduced upstream of the second and third gene for coexpressing all the genes as separate proteins. These genes *dxs*, *dxr*, and *idi* were inserted into pET-30a among the restriction sites of *Kpn*I, *Bam*HI, *Sac*I, and *Xho*I randomly, generating the operons: pET-30a-*dxs*/*dxr*/*idi*, pET-30a-*dxs*/*idi*/*dxr*, pET-30a-*dxr*/*dxs*/*idi*, pET-30a-*dxr*/*idi*/*dxs*, pET-30a-*idi*/*dxs*/*dxr* and pET-30a-*idi*/*dxr*/*dxs* (5–10, Fig. 1). For the genes cloning, *dxs* was amplified by PCR from *E. coli* K12 genomic

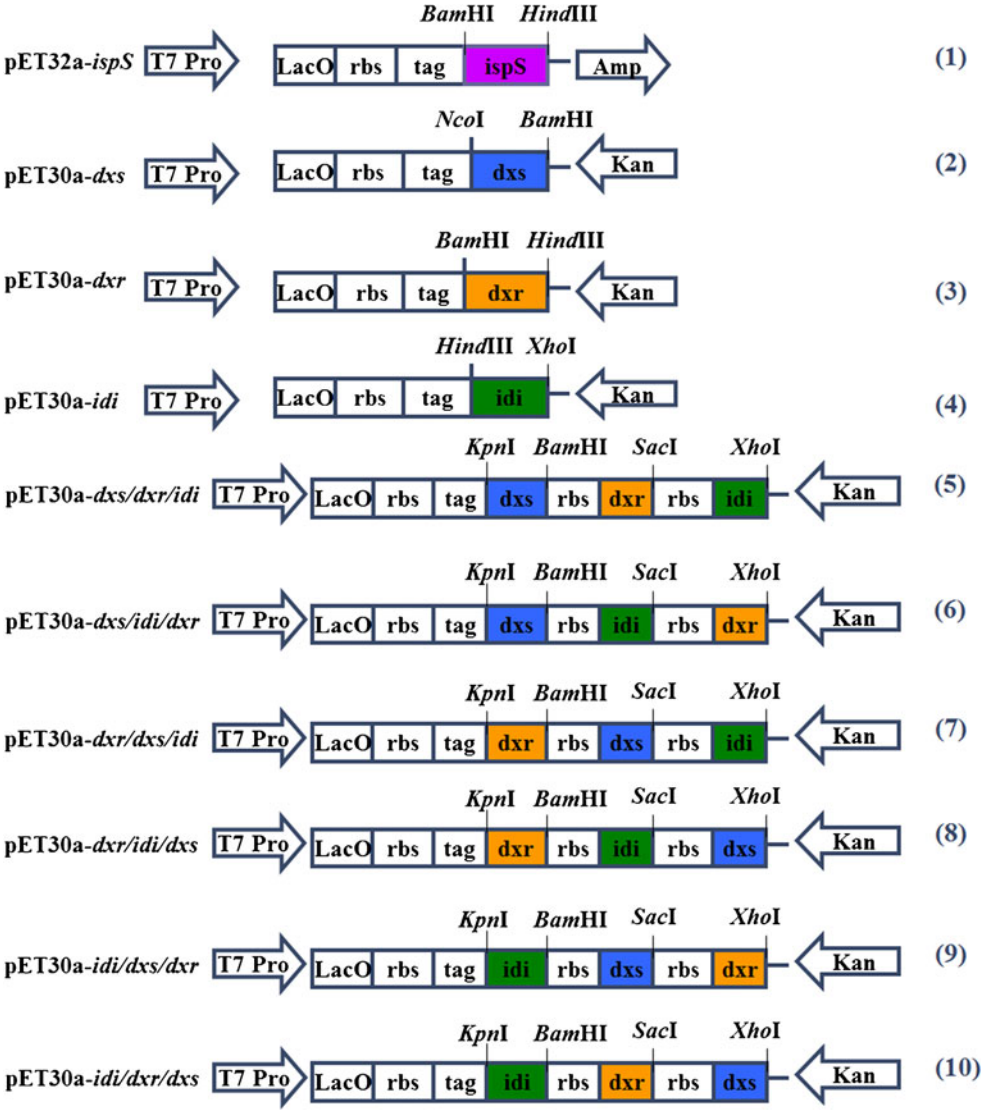

Fig. 1 Construction of recombinant vectors. (*1*)–(*4*): monocistronic operons, (*5*)–(*10*): polycistronic operons

DNA using primers *dxs-Kpn*I (F) and *dxs-Bam*HI (R) (for pET-30a-*dxs*/*dxr*/*idi*, pET-30a-*dxs*/*idi*/*dxr*), rbs-*dxs-Bam*HI (F) and rbs-*dxs-Sac*I (R) (for pET-30a-*dxr*/*dxs*/*idi*, pET-30a-*idi*/*dxs*/*dxr*), rbs-*dxs-Sac*I (F) and rbs-*dxs-Xho*I (R) (for pET-30a-*dxr*/*idi*/*dxs*, pET-30a-*idi*/*dxr*/*dxs*); *dxr* was amplified by PCR from *E. coli* K12 genomic DNA using primers *dxr-Kpn*I (F) and *dxr-BamH*I (R) (for pET-30a-*dxr*/*dxs*/*idi*, pET-30a-*dxr*/*idi*/*dxs*), rbs-*dxr-Bam*HI (F) and *rbs-dxr-Sac*I (R) (for pET-30a-*dxs*/d*xr*/*idi,* pET-30a-*idi*/*dxr*/*dx s*), rbs-*dxr-Sac*I (F) and rbs-*dxr-Xho*I (R) (for pET-30a-*dxs*/*idi*/*dxr*, pET-30a-*idi*/*dxs*/*dxr*); *idi-Kpn*I (F) and *idi-BamH*I (R) (for pET-30a-*idi*/*dxs*/*dxr*, pET-30a-*idi*/*dxr*/*dxs*), *idi* was amplified by PCR from *E. coli* K12 genomic DNA using primers rbs-*idi-Bam*HI (F) and rbs-*idi-Sac*I (R) (for pET-30a-*dxs*/*idi*/*dxr*, pET-30a-*dxr*/*idi*/*dxs*), rbs-*idi-Sac*I (R) and *idi-Xho*I (R) (for pET-30a-*dxs*/*dxr*/*idi*, pET-30a-*dxr*/*dxs*/*idi*), respectively. All the primers were shown in Table 1.

These recombinant plasmids with kanamycin resistance were separately transformed into *E. coli* BL21 (pET-32a-*ispS*) competent cells using a heat pulse at 42 °C and then spread on LB agar plate containing kanamycin (selection for pET-30a) and ampicillin (selection for pET-32a).

Method to confirm incompatible plasmids' coexistence and coexpression under antibiotic pressure in *E. coli*

The method for confirming the coexistence of incompatible plasmids is based on the method reported by Yang et al. (2001). Colonies (harboring incompatible plasmids) were picked from plates containing fresh cotransformed cells and cultured in liquid LB medium with selective pressure (ampicillin and kanamycin). Samples were taken from liquid cultures every 4 h and diluted appropriately. Each sample was spread both on selective LB agar plate and non-

Table 1 Primers used in the work

Primers[a]	Sequences of oligonucleotides(5′–3′)[b]
*ispS-Bam*HI (F)	CGCGGATCCGATGTGTTCTGTTAGCACT
*ispS-Hind*III (R)	CCCAAGCTTTTAACGTTCAAACGGCAGG
*dxs-Nco*I (F)	CATGCCATGGCTATGAGTTTTGATATTGCCAAAT
*dxs-Bam*HI (R)	CGCGGATCCTTATGCCAGCCAGGCCTTGATT
*dxr-Bam*HI (F)	CGCGGATCCATGAAGCAACTCACCATTCTG
*dxr-Hind*III (R)	CCCAAGCTTTCAGCTTGCGAGACGCATCAC
*idi-Hind*III (F)	CCCAAGCTTGCATGCAAACGGAACACGTCATT
*idi-Xho*I (R)	CGGCTCGAGTTATTTAAGCTGGGTAAATGCA
*dxs-Kpn*I (F)	CGGGGTACCATGAGTTTTGATATTGCCAAAT
rbs-*dxr-Bam*HI (F)	CGCGGATCCAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACATATGAAGCAACTCACCATTCTG
*rbs-dxr-Sac*I (R)	CGAGCTCTCAGCTTGCGAGACGCATCAC
rbs-*idi-Sac*I (F)	CGAGCTCAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCAAACGGAACACGTCATT
rbs-*idi-Bam*HI (F)	CGCGGATCCAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCAAACGGAACACGTCATT
rbs-*idi-Sac*I (R)	CGAGCTCAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCAAACGGAACACGTCATT
rbs-*dxr-Sac*I (F)	CGCGGATCCAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACATATGAAGCAACTCACCATTCTG
rbs-*dxr-Xho*I (R)	CGGCTCGAGTCAGCTTGCGAGACGCATCAC
*dxr-Kpn*I (F)	CGGGGTACCATGAAGCAACTCACCATTCTG
*dxr-BamH*I (R)	CGCGGATCCTCAGCTTGCGAGACGCATCAC
rbs-*dxs-Bam*HI (F)	CGCGGATCCAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACATATGAGTTTTGATATTGCCAAAT
rbs-*dxs-Sac*I (R)	CGAGCTCTTATGCCAGCCAGGCCTTGATT
rbs-*dxs-Sac*I (F)	CGAGCTCAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACATATGAGTTTTGATATTGCCAAAT
rbs-*dxs-Xho*I (R)	CGGCTCGAGTTATGCCAGCCAGGCCTTGATT
*idi-Kpn*I (F)	CGGGGTACCATGCAAACGGAACACGTCATT
*idi-BamH*I (R)	CGCGGATCCTTATTTAAGCTGGGTAAATGCA

[a] *F* forward primer; *R* reverse primer

[b] Restriction sites were underlined

selective control plate. The ratio of the number of colonies on the selective agar plate to that on the non-selective agar plate represents the proportion of *E. coli* cells carrying both plasmids to total *E. coli* cells when the samples were taken. Co-transformants were also inoculated into liquid LB medium without antibiotics as control. Plating was done on the two kinds of plates in the same way as described above, and the colonies were also counted. Coexpression of recombinant plasmids was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Expression and purification of recombinant proteins

E. coli BL21 (DE3) strains containing recombinant vectors were grown in 50 ml LB medium to an OD_{600} of 0.5 and induced by 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h. After cultivation, cells were harvested by centrifugation (4,000 rpm for 10 min), washed with PBS buffer and resuspended in 50 mM Tris–HCl (pH 8.0). Cells were mechanically disrupted by ultrasonication. The supernatants were then analyzed by 15 % SDS-PAGE. Gel band intensity was determined using a Gel-Pro Analyzer 6.0 (Media Cybernetics, Bethesda, MD). For isoprene synthase purification, the supernatants were pretreated with filters, purified with ÄKTA Purifier FPLC purification system equipped with HisTrap™ HP column (GE Healthcare, Sweden) and desalted with gel column.

Isoprene synthase assay

Isoprene synthase activity was assayed using a modified procedure of Lehning et al. (1999): by mixing 0.1 ml of 1 M $MgCl_2$, 2.2 ml of ISB, 0.5 ml of 0.1 M DMAPP (in ISB) and 2.2 ml of purified isoprene synthase. ISB consists of 50 mM Tris–HCl, 12 mM $MgCl_2$, 5 % glycerol and 2 mM dithiothreitol. The mixture was incubated in 17 ml gas-tight vial at 37 °C for 2 h. Gas from the headspace of sealed cultures was sampled with gas-tight syringe and analyzed by gas chromatography (GC) equipped with a flame ionization detector and HP-FFAP column.

Quantification of isoprene production

The strains containing recombinant vectors were grown in 50 ml LB culture (100 μg/ml ampicillin and 50 μg/ml kanamycin) at 37 °C with shaking until OD_{600} reached 0.5. 2 ml of culture were transferred to sealed vials and added with 0.1 mM IPTG at 37 °C with shaking (200 rpm) for 2 h. Gas sample from the headspace of the sealed cultures was analyzed with GC.

Growth curve experiments of recombinant strains

The recombinant strains were grown in 5 ml LB medium (100 μg/ml of ampicillin or 50 μg/ml of kanamycin) overnight at 37 °C with shaking and then diluted into 50 ml LB medium (100 μg/ml of ampicillin or 50 μg/ml of kanamycin) to an OD of 0.015. The OD was tested at 600 nm every 30 min. Each sample was measured in triplicate. And when OD_{600} of the culture reached approximately 0.3, IPTG was added to a final concentration of 0.1 mM.

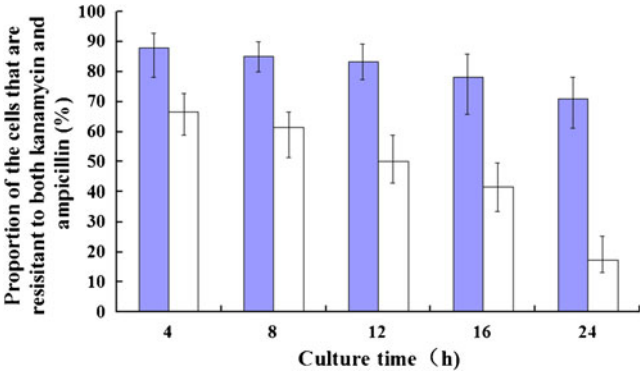

Fig. 3 Effect of antibiotic pressure on coexistence of pET-32a-*ispS* and pET-30a-*dxs* in *E. coli*. The columns show the proportions of cells that are resistant to both kanamycin and ampicillin in total *E. coli* cells after culturing the cotransformants for certain time: the *blue* represents liquid culture medium containing 50 μg/ml kanamycin and 100 μg/ml ampicillin; the *blank* represents liquid culture medium without antibiotics. *Error bars* represent standard deviations from the means

Results

Construction of the isoprene metabolic pathway in *E. coli*

IspS from *P. alba* was cloned into pET-32a and the expression was analyzed by SDS-PAGE. The band corresponding to the size of *ispS* (81KDa), which harboring the N-terminal Trxis•Tag™/His•Tag®/S•Tag™/enterokinase configuration of pET-32a, was visible in SDS-PAGE gel (Fig. 2a, lane 2). The enzyme assay was performed as described. After a 2-h incubation at 37 °C, an isoprene-specific peak was detected with the same retention time (4.1 min) as the authentic standard. To identify whether the isoprene metabolic pathway is constructed successfully in *E. coli* (pET-32a-*ispS*), gas samples from the headspace of sealed vial cultures were analyzed by gas chromatography. It was found that *E. coli* BL21 (pET-32a-*ispS*) can accumulate 1.0 mg/g isoprene in 2 h, while the control strain produced little isoprene about 0.05 mg/g (Fig. 2b). Then, the enhancement of isoprene production should be attributed to the codon optimized isoprene synthase introduced.

Coexistence of pET-32a and pET-30a

Due to having the same pBR322 origin, pET-32a and pET-30a cannot coexist without selected pressure. Therefore, we maintained them in the same cell by taking advantage of different selective antibiotic resistance. As shown in Fig. 3, after continuous 24 h incubation, the two types of vectors in

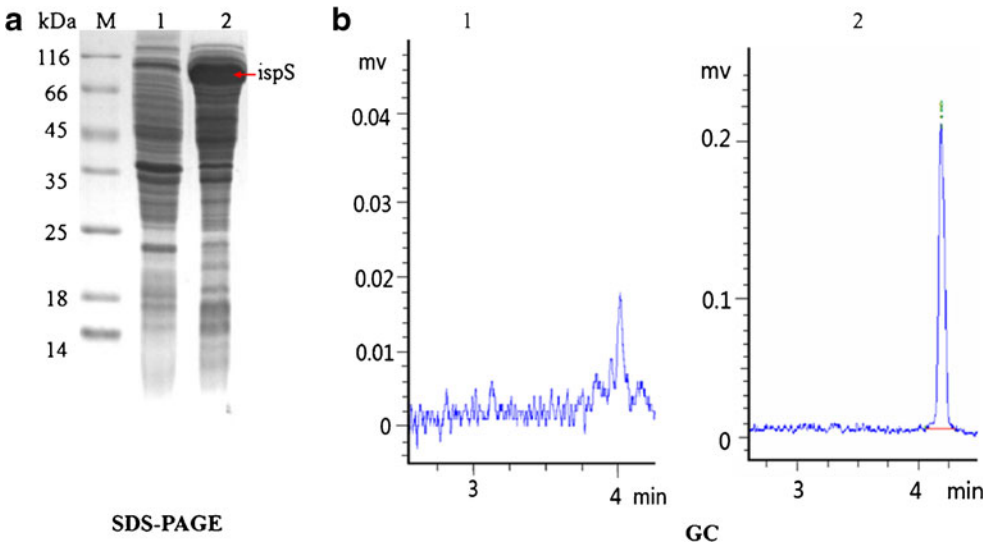

Fig. 2 SDS-PAGE analysis of *ispS* and isoprene production assays. **a** *M* Protein molecular weight marker; *Lane 1*: *E. coli* BL21; *Lane 2*: *E. coli* BL21 (PET-32a-*ispS*). **b** *1* GC profile of isoprene production in BL21; *2* GC profile of isoprene production in BL21 (pET-32a-*ispS*)

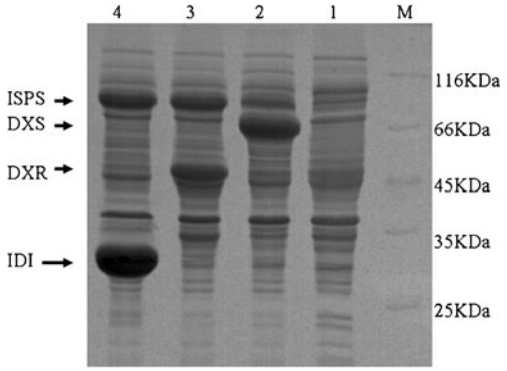

Fig. 4 SDS-PAGE analysis of crude protein fractions coexpressed in recombinant *E. coli* BL21. *M* Protein molecular weight marker; *Lane 1* *E. coli* BL21; *Lane 2* BL21 (pET-32a-*ispS*&pET-30a-*dxs*). *Lane 3* BL21 (pET-32a-*ispS*&pET-30a-*dxr*); *Lane 4* BL21 (pET-32a-*ispS*&pET-30a-*idi*)

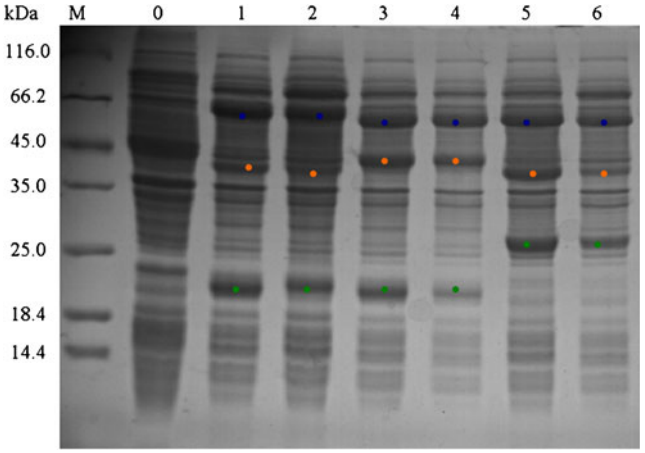

Fig. 6 SDS-PAGE analysis of crude protein fractions coexpressed in recombinant *E. coli* BL21. *M* Protein molecular weight marker; *0 E. coli* BL21; *1* pET-30a-*dxs*/*dxr*/*idi*&pET-32a-*ispS*; *2* pET-30a-*dxs*/*idi*/*dxr*&pET-32a-*ispS*; *3* pET-30a-*dxr*/*dxs*/*idi* &pET-32a-*ispS*; *4* pET-30a-dxr/idi/dxr&pET-32a-*ispS*; *5* pET-30a-*idi*/*dxs*/*dxr* &pET-32a-*ispS*; *6* pET-30a-*idi*/*dxr*/*dxs* &pET-32a-*ispS*. *filled blue circle* dxs, *filled orange circle* dxr, *filled green circle* idi

70 % of strains cultured with two antibiotics were found to coexist stably, while 80 % of the strains cultured in liquid LB medium without antibiotics lost the vectors. The gene expressions were confirmed by SDS-PAGE analysis. Figure 4 showed that the genes *ispS*, *dxs*, *dxr*, and *idi* were all well expressed in recombinant strains. All the above results demonstrated the availability of incompatible plasmids system for heterologous genes' coexpression.

Overexpression of *E. coli dxs*, *dxr*, and *idi*

To identify the effect of *dxs*, *dxr*, and *idi* on isoprene production, vectors pET-30a-*dxs*, pET-30a-*dxr* and pET-30a-*idi* were constructed and introduced into the strain BL21 (pET-32a-*ispS*) respectively. The expression bands of *dxs*, *dxr*, and *idi* were visible on SDS-PAGE at the predicted sizes of 72, 47, and 24 kDa. The sizes were all 5 kDa larger than that of the natural proteins due to the configuration of N-terminal His•Tag®/thrombin/S•Tag™/enterokinase in PET-30a. As shown in Fig. 4, the relative expression of the genes in these recombinant vectors were present as follows: *dxs* (32.7 %), *ispS* (8.6 %) in pET-32a-*isps*&pET-30a-*dxs*; *dxr* (20.5 %), *ispS* (17.2) in pET-32a-*isps*&pET-30a-*dxr*; *idi* (55.2 %) and *ispS* (11.3 %) in pET-32a-i*sps*&pET-30a-*idi*. The production of isoprene was analyzed with GC. Overexpression of *dxs*, *dxr*, and *idi* can produce 0.77, 0.54, 1.04 $mgg^{-1}h^{-1}$ isoprene respectively (Fig. 5), increasing isoprene production 0.65, 0.16, 1.22 fold over the BL21 (pET-32a-*ispS*). The order of these genes effect on the isoprene production was found to follow the corresponding trend: *dxr*<*dxs*<*idi*.

Ordered coexpression of *dxs*, *dxr*, and *idi* using polycistronic operons

In this study, polycistronic operons consisting of three isoprene biosynthetic genes (*dxs*, *dxr*, and *idi*), in which the genes can be assembled as one transcriptional unit, were constructed based on pET-30a. As shown in the Fig. 6, the genes *dxs*, *dxr*, and *idi* were coexpressed efficiently as

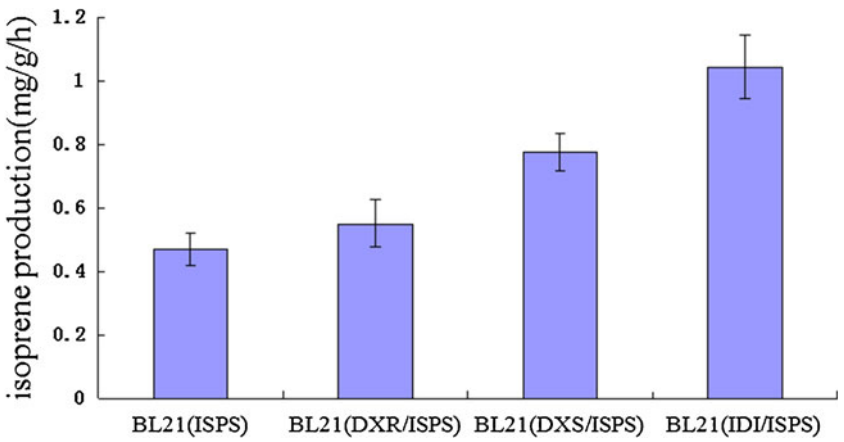

Fig. 5 Isoprene production by various engineered *E. coli* strains. When the OD reached 0.5, 5 ml culture was transferred to 17 ml headspace vials for 16 h. Gas sample from the headspace of the sealed cultures was analyzed with GC. Each independent measurement was repeated in triplicate

Table 2 Relative expression of *dxs*, *dxr*, and *idi* in recombinant strains with polycistronic operons

Relative expression	pET-30a-*dxs*/*dxr*/*idi*	pET-30a-*dxs*/*idi*/*dxr*	pET-30a-*dxr*/*dxs*/*idi*	pET-30a-*dxr*/*idi*/*dxs*	pET-30a-*idi*/*dxs*/*dxr*	pET-30a-*idi*/*dxr*/*dxs*
dxs (%)	17.2	14.7	14.3	14.4	11.5	12.2
dxr (%)	7.1	4.4	12.3	14.3	9.8	6.2
idi (%)	7.0	5.8	6.3	5.5	8.1	7.1

separate proteins. Due to the configuration of N-terminal His•Tag®/thrombin/S•Tag™/enterokinase upstream of the MCS in PET-30a, the top gene of each polycistronic operon is 5 kDa larger than its natural size. For example, the size of *dxs* is 72 kDa in pET-30a-*dxs*/*dxr*/*idi* and pET-30a-*dxs*/*dxr*/*idi*, 5 kDa larger than that in the other operons. The natural sizes of *dxs*, *dxr*, and *idi* were 67, 42, and 18 kDa, respectively. The relative expressions of the genes in these recombinant operons were present in Table 2. For all transformants no matter of the genes order, the *dxs* relative expression is higher than that of *dxr* and *idi*. Therefore, the gene expression is related to the protein structure to some extent. The expression was high when the gene was ordered at the top rather than at the downstream. For example, the *dxs* relative expression in pET-30a-*dxs*/*dxr*/*idi* and pET-30a-*dxs*/*dxr*/*idi*, in which *dxs* was ordered as the top gene, was more than that of the other four operons; the *dxr* relative expression in pET-30a-*dxr*/*dxs*/*idi* and pET-30a-*dxr*/*idi*/*dxs*, in which *dxr* was ordered as the top gene, was more than that of the other 4 operons and so did *idi*. For isoprene production, it was found that tandem coexpression of these limiting genes can stimulate the isoprene production up to 2.727, 2.463, 2.461, 1.647, 1.046, and 1.142 $mgg^{-1}h^{-1}$ in recombinant vectors ordered 1–6, respectively (Fig. 7), increasing isoprene production 4.8, 4.23, 4.23, 2.5, 1.2, 1.4 fold over the BL21 (pET-32a-*ispS*). Compared with single gene overexpression, the synergy of multiple limiting enzymes contributed more to the final product yield. Interestingly, the gene order of transform (pET-30a-*dxs*/*dxr*/*idi*) with the highest isoprene production corresponded to that of the metabolic pathway.

The effect of protein expression on cell growth

To test the effect caused by expression of *isps*, *dxs, dxr*, *idi*, and *isps*/*dxs*/*dxr*/*idi*, a growth curve experiment was performed. As shown in Fig. 8, the growth of strains with protein overexpression was significantly slower than the control, especially the BL21 (*isps*/*dxs*/*dxr*/*idi*). This is surmised to be caused by great amount of energy and resource consumption for protein synthesis.

Discussion

Applications in fuel and product biosynthesis require efficient gene expression and robust performance. Therefore, the method for the effective coexpression of multiple genes is particularly important. In this work, we established an expression system by combining incompatible plasmids system with IRES method, and achieved the efficient coexpression of the genes (*ispS*, *dxs*, *dxr*, *idi*). In IRES method, the genes connected by IRES sequences can be efficiently expressed from a single promoter (Ghattas et al. 1991). Nevertheless, the target genes' number is generally limited to restriction sites. The combination of incompatible plasmid system (Yang et al. 2001) not only increased target genes' number but also guaranteed the high copy number of both plasmids. This strategy can generally also be applied for the coexpression of multiple genes (more than three) in other product biosynthesis.

Based on this expression system, the genes *dxs*, *dxr*, and *idi* were overexpressed to eliminate the repression of these

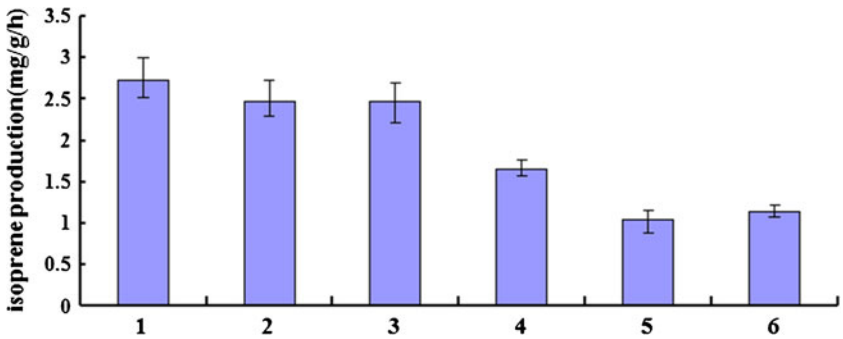

Fig. 7 Isoprene production in *E. coli* (pET-32a-*ispS*) transformants with pET-30a series plasmids, *1* pET-30a-*dxs*/*dxr*/*idi*; *2* pET-30a-*dxs*/*idi*/*dxr*; *3* pET-30a-*dxr*/*dxs*/*idi*; *4* pET-30a-*dxr*/*idi*/*dxr*; *5* pET-30a-*idi*/*dxs*/*dxr*; *6* pET-30a-*idi*/*dxr*/*dxs*

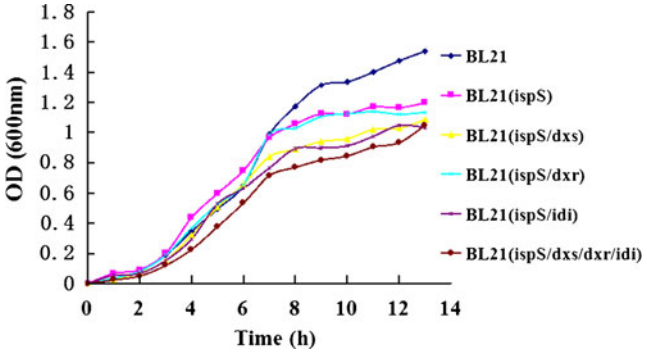

Fig. 8 Growth curve of the wild-type strain BL21 and recombinant strains in LB medium at 37 °C. The *curves* indicate the average OD600 of each culture over time. Each sample was measured in triplicate

limiting genes on IPP and DMAPP supply, which is a limiting factor on isoprenoids production. Overexpression of *dxs*, the first bottleneck in MEP pathway (Lois et al. 2000; Miller et al. 2000), resulted in significant enhancement of isoprene production, which indicated that adequate precursor was the base of high metabolic flux. In the previous study, the effect of *idi* on isoprene production was generally neglected (Xue and Ahring 2011; Zhao et al. 2011). Interestingly, our results showed that *idi* exhibited more significant effect on isoprene production than *dxs* and *dxr*. In the MEP pathway, *idi* appeared function as a salvage enzyme, adjusting the intracellular concentrations of IPP and DMAPP (Kajiwara et al. 1997). It has been extensively reported that IPP and DMAPP were produced in a 5:1 ratio by the catalysis of *IspH* (MEP pathway branch enzyme) in *E. coli* (Gräwert et al. 2004; Rohdich et al. 2002; Xiao et al. 2008). It has been proposed that the *idi* favors the production of DMAPP, adjusting this ratio to physiologically balanced level (Ajikumar et al. 2010). Since DMAPP is the immediate precursor of isoprene, overexpression of *idi* might increase the DMAPP concentration so as to significantly enhance the isoprene production. It is suggested that *idi* could be regarded as a key regulating point for isoprene production.

To further enhance isoprene production, genes *dxs*, *dxr*, and *idi* were coexpressed. The results showed that the isoprene production was further improved. It was revealed that the synergy of several rate-limiting enzymes made more contribution to the overall yield of final product than overexpression of any single gene.

When the limiting genes were overexpressed, the balance of the MEP pathway was important for high isoprene yield. A series of polycistronic operons were constructed via rearranging the gene (*dxs*, *dxr*, and *idi*) order, which resulted in different isoprene production. Moreover, among the six combinatorial orders of the three genes, the gene order with the highest isoprene production yield was consistent with that of the metabolic pathway. This phenomenon also appeared in the research on *crtE*, *crtB*, *crtI*, *crtY*, *crtZ* order assembly for carotenoid biosynthesis reported by Nishizaki et al. (2007). Because the gene transcription and translation are generally coupled in prokaryotic expression system, the gene ranked in front will be translated primarily, followed by the translation of the subsequent genes along with the mRNA transcription for polycistronic operons in *E. coli*. When the order of the limiting genes in polycistronic operons corresponds to that of isoprene metabolic pathway (*dxs–dxr–idi*), the upstream bottleneck could be eliminated, followed by the downstream flux enhancement. Thus, the quantity of final product could be improved. It fits the natural fine regulation mechanism of microorganisms that the closer the enzyme to the beginning of the pathway, the shorter the response time of the activation of its promoter (Zaslaver et al. 2004). Additionally, as shown in Fig. 7 and Table 2, isoprene production was closely related to relative *dxs* expression. This suggested that *dxs* became a key regulating point when downstream bottlenecks (*dxr* and *idi*) were eliminated. In conclusion, the gene order in this biosynthetic pathway is a very important determinant for final product yield. In addition, a sequential expression pattern according to the biosynthetic pathway could be optimized for more efficient synthesis of the product in metabolic pathway.

Although isoprene production was improved significantly in this study, many efforts are also needed to achieve the industrial production with higher yield and lower cost. On one hand, the productivity can be further improved by metabolic flux regulation (such as blocking the competing pathway through gene knockout method) and improving isoprene enzyme activity. On the other hand, to decrease the process cost, the fermentation conditions can be optimized. For example, LB medium is not suitable for industrial processes due to its price, and should be replaced by a more industrial medium such as marine mineral culture (M9). Moreover, although incompatible plasmids system can be used for the coexpression of several genes, different antibiotics led to the growth inhibition for industrial long-term productivity. To overcome this problem, the recombinant genes may be integrated into the chromosome replicon of a host for industrial fermentation which will be conducted in our future work.

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