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ORIGINAL PAPER

Combining De Ley–Doudoroff and methylerythritol phosphate pathways for enhanced isoprene biosynthesis from D-galactose

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Abstract An engineered *Escherichia coli* strain was developed for enhanced isoprene production using D-galactose as substrate. Isoprene is a valuable compound that can be biosynthetically produced from pyruvate and glyceraldehyde-3-phosphate (G3P) through the methylerythritol phosphate pathway (MEP). The Leloir and De Ley–Doudoroff (DD) pathways are known existing routes in *E. coli* that can supply the MEP precursors from D-galactose. The DD pathway was selected as it is capable of supplying equimolar amounts of pyruvate and G3P simultaneously. To exclusively direct D-galactose toward the DD pathway, an *E. coli* Δ*galK* strain with blocked Leloir pathway was used as the host. To obtain a fully functional DD pathway, a dehydrogenase encoding gene (*gld*) was recruited from *Pseudomonas syringae* to catalyze D-galactose conversion to D-galactonate. Overexpressions of endogenous genes known as MEP bottlenecks, and a heterologous gene, were conducted to enhance and enable isoprene production, respectively. Growth test confirmed a functional DD pathway concomitant with equimolar generation of pyruvate and G3P, in contrast to the wild-type strain where G3P was limiting. Finally, the engineered strain with combined DD–MEP pathway exhibited the highest isoprene production. This suggests that the equimolar pyruvate and G3P pools resulted in a more efficient carbon flux toward isoprene production. This strategy provides a new platform for developing improved isoprenoid producing strains through the combined DD–MEP pathway.

Keywords Isoprene · *Escherichia coli* · D-Galactose · De Ley–Doudoroff pathway · Methylerythritol phosphate pathway

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Introduction

Isoprene (2-methyl-1,3-butadiene) is the smallest member of terpenoids, a group of naturally existing compounds. It serves as a common structural unit in various biological systems as well as a platform chemical for the production of rubber, thermoplastic elastomers and as a bridge molecule for other specialty chemicals [1]. Current commercial sources of isoprene include direct isolation from C_5 cracking fractions or through dehydrogenation of C_5 isoalkanes and isoalkenes [2]. These processes require large amounts of expensive raw materials but generate very low yields. Recently, microbial production of isoprene has become an alternative and feasible approach due to its ease in cultivation and convenient product recovery [3].

There are two known microbial pathways for the biosynthesis of isoprene and other isoprenoids, the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways [4]. The former exists in higher eukaryotes, while the latter (Fig. 1) was discovered to exist in some bacteria and plants [5]. The MEP pathway starts with the condensation

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of glyceraldehyde-3-phosphate (G3P) and pyruvate to 1-deoxy-D-xylulose-5-phosphate (DXP) [6]. Several downstream reactions convert DXP to dimethylallyl pyrophosphate (DMAPP), the common intermediate for the biosynthesis of several types of isoprenoids. Subsequently, conversion of DMAPP to isoprene is catalyzed by isoprene synthase encoded by the *ispS* gene which can be recruited from terrestrial plants [7].

The MEP pathway is present in *Escherichia coli* and has been used in the microbial production of several isoprenoids such as taxol, lycopene and isoprene [7–10]. These studies have focused on optimizing and balancing the expression of the genes involved in the MEP pathway to achieve higher titers of isoprenoids. However, the success of these techniques may be limited by the inherent genetic regulation mechanisms of this pathway in *E. coli* [11]. In another report, the proportion of G3P and pyruvate was considered to play a vital role in regulating the flux toward the formation of DMAPP [12]. The balanced distribution of these precursors was achieved by manipulating the central carbon metabolism involving several gene disruptions and overexpressions resulting in the formation of G3P from excess pyruvate. As a result, lycopene production dramatically increased using glucose as substrate [12]. Meanwhile, an earlier work demonstrated that simultaneous generation of G3P and pyruvate in Entner–Doudoroff (ED), a glycolytic pathway which served as an MEP feeding module, resulted in the highest isoprene titer from glucose [13]. This finding clearly indicates that MEP-dependent isoprenoid biosynthesis pathways can be optimized through G3P and pyruvate equalization.

Another biomass-derived sugar that has recently gained popularity is D-galactose, which can be acquired from red macroalgae [14, 15]. D-Galactose can be metabolized by microorganisms through two known pathways, the Leloir and the De Ley–Doudoroff pathways (DD) (Fig. 1) [16, 17]. The DD pathway is incomplete in *E. coli* since it does not possess galactose dehydrogenase activity. However, the subsequent reactions of the DD pathway are present since *E. coli* can metabolize D-galactonate [18]. Coincidentally, the DD pathway also generates G3P and pyruvate simultaneously (Fig. 1), similar to the ED pathway [13]. Thus, diversion of D-galactose toward the DD pathway may lead to a higher isoprene titer than that toward the Leloir pathway.

In this study, the effect of combining the DD pathway with the MEP pathway for increased isoprene production was investigated. To accomplish this, an *E. coli* BW25113 mutant strain with a non-functional Leloir pathway (Δ*galK*) was engineered to convert D-galactose to D-galactonate by recruiting galactose dehydrogenase (GalDH) from *Pseudomonas syringae*. Intracellular concentrations of the precursors were also monitored for both wild-type and engineered strains. Furthermore, heterologous isoprene

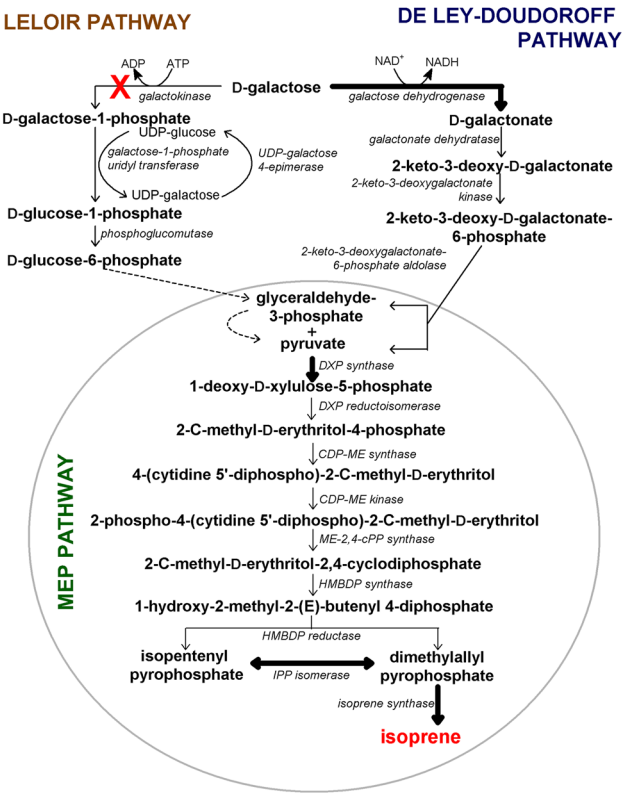

Fig. 1 Galactose metabolic pathways and the methylerythritol phosphate (MEP) pathway. *Dashed arrows* represent multiple reactions; *bold arrows* represent gene overexpression; *X* represents gene deletion

synthase (*ispS*) from *Populus alba* was overexpressed to enable the host strain to produce isoprene, in addition to overexpressions of the endogenous 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) and isopentenyl diphosphate isomerase (*idi*), which were previously identified as MEP bottlenecks [3].

Materials and methods

Strains and plasmids construction

The strains and plasmids used in this study are listed in Table 1. *E. coli* BW25113 and *E. coli* BW25113*ΔgalK*::*kan* were obtained from the National BioResource Project (NIG, Japan) [19]. The expression vector pACYC-Duet1 was purchased from Novagen (South Korea).

Standard procedures were followed in all DNA manipulations [20]. To place the cloned genes under the control of the T7 promoter in *E. coli* hosts, the 69734 λDE3 Lysogenization Kit (Novagen, EMD Millipore, USA) was used to integrate the λDE3 prophage into the host chromosome. Lysogens were verified using the T7 tester phage according to Novagen user protocol TB031 [21]. The

Table 1 Plasmids and strains used in this work

Plasmid/strain	Function/characteristic	Source/reference
pET28a-*gld*	D-galactose dehydrogenase expression vector	22
pET28a-*galK*	Galactokinase expression vector	This work
pACYC-*Duet1*	Expression vector, T7 promoter	Novagen
pACYC-*dxs-idi-ispS*	DXP synthase, IPP isomerase, and isoprene synthase expression vector	This work
BW25113	*E. coli* BW25113F$^-$, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	19
BW25113 *ΔgalK*	*E. coli* BW25113 *ΔgalK*	19
EWG2	*E. coli* BW25113 *ΔgalK* (DE3)/pET28a-*gld*	22
EBWI3	*E. coli* BW25113 (DE3)/pET28a; pACYC-*dxs-idi-ispS*	This work
EBWI3.1	*E. coli* BW25113 Δ*galK* (DE3)/pET28a-*galK;* pACYC-*dxs-idi-ispS*	This work
EBWI4	*E. coli* BW25113 Δ*galK* (DE3)/pET28a-*gld;* pACYC-*dxs-idi-ispS*	This work

Table 2 Primers used in this study

Name	Sequence (5–3′)	Function
IspS-F	CGATCCATGGATGAGATGTAGCGTGT	For PCR amplification of *ispS*
IspS-R	CGTAGATCT TTAGCGAACAAACGGC	
Dxs-F	CGTCGGATCCATGAGTTTTGATATTGCCA	For PCR amplification of *dxs*
Dxs-R	CGGGAATTCTTATGCCAGCCAGGCCTTG	
Idi-F	CGTAGATCT*AAGGAGATA*TAATGCAAACGGAACA	For PCR amplification of *idi*
Idi-R	CGGCTCGAGTTATTTAAGCTGGGT	
GalK-F	CTCCATATGGTGTAAGAAATGAGTCTGAA	For PCR amplification
GalK-R	CTCGGATCCTCAGCACTGTCCTGCTCCTT	of *galK*

Straight underline denotes restriction site, italics denotes ribosome binding site

plasmid pET28a-*gld* and strain EWG2 were constructed as reported elsewhere [22]. Construction of the pACYC-*dxs-idi-ispS* plasmid was done as follows: from the *E.* coli genomic DNA, the *dxs* and *idi* genes were amplified using appropriate primers listed in Table 2. The codon-optimized *ispS* gene of *Populus alba* (GeneBank: AB198180.1) was synthesized by Bioneer (South Korea). The amplified sequences were inserted into the pACYC-Duet1 vector at the *Bam*HI and *Eco*RI restriction sites for *dxs*, *Bgl*II and *Xho*I for *idi*, and *Nde*I and *Bgl*II for *ispS*.

The kanamycin resistance gene in the *galK*-deletion mutant was removed using pCP20 [23]. EBWI3 was constructed by transforming pET28a and pACYC-*dxs-idi-ispS* into *E. coli* BW25113, while EBWI4 was constructed by transforming pET28a-*gld* and pACYC-*dxs-idi-ispS* into *E. coli* BW25113 *ΔgalK*.

Phenotype analysis

Overnight cultures with optical densities of 1 absorbance unit (AU) at 600 nm were harvested and washed twice with sterile deionized water. The cell pellets were re-suspended in 1-mL sterile deionized water and streaked onto M9 minimal salts agar medium containing 4 g L^{-1} D-galactose as the sole carbon source. The plates were incubated at 37 °C until colonies were visible. For growth analysis, D-galactose consumption and D-galactonate accumulation in the wild-type and EWG2 cultures were monitored in 300-mL shake flasks containing 100-mL M9 minimal salts and 10 g L^{-1} D-galactose.

Quantification of pyruvate and G3P

The wild-type and EWG2 strains were inoculated in M9 minimal medium with D-galactose (10 g L^{-1}) as sole carbon source. After 6 h cultivation, 0.5 mM IPTG (isopropyl ß-D-1-thiogalactopyranoside) was added and the cultures were further incubated for another 24 h to induce gene expressions. Cell-free lysates were prepared via collection and centrifugation of the broth at 13,000 rpm for 10 min at 4 °C. Cell pellets were re-suspended in 350 µL STET buffer (10 mM Tris–HCl, pH 8.0, with 0.1 M NaCl, 1 mM EDTA, and 5 % [w/v] TRITON® X-100) and then a freshly prepared lysozyme solution (10 mg mL^{-1} in 10 mM Tris–HCl, pH 8.0) was added. Samples were mixed by vortexing for 3 s. The lysis mixtures were incubated at 37 °C for 30 min. After incubation, the mixtures were placed in a boiling water bath for exactly 40 s and then centrifuged at 13,000 rpm for 10 min at 4 °C. Cell debris was removed using a sterile toothpick [20].

G3P concentration was measured at room temperature in a 1-mL reaction mixture containing 0.5 mM Tris, pH 7.6, 280 mM triethanolamine, 0.132 mM NADH, 100 µL crude cell extract and deionized water. The mixture was incubated at room temperature for 30 min to allow endogenous NADH oxidation. The reaction was initiated upon addition of 0.02–0.04 U triosephosphate isomerase and 4 U α-glycerophosphate dehydrogenase. The absorbance at 340 nm was monitored for 5–10 min [24]. On the other hand, pyruvate concentration was measured at room temperature in a 1-mL reaction mixture comprised of 0.25 M Trizma Base solution (Sigma-Aldrich, USA), 0.106 mM NADH, 100 µL crude cell extract and deionized water. The mixture was initially incubated for 30 min at room temperature to allow endogenous NADH oxidation. Reaction was started upon addition of 16.7 U lactic dehydrogenase. Similarly, absorbance at 340 nm was monitored for 5–10 min [25]. The metabolite contents were calculated on the basis of the molar extinction coefficient of NADH [26].

Isoprene production cultures

Cultivations were performed in 500-mL serum bottles sealed with butyl stoppers each containing 100-mL M9 minimal salts with 10 g L^{-1} D-galactose (Sigma-Aldrich, USA), 5 g L^{-1} yeast extract (Becton–Dickinson, USA), 50 mg L^{-1} kanamycin, 35 mg L^{-1} chloramphenicol, and 1 mM thiamine pyrophosphate (TPP). The medium was inoculated with 2 mL of overnight culture and incubated at 37 °C with 200 rpm agitation. After 6 h of cultivation, the cultures were induced with 0.5 mM IPTG and incubated further for 48 h. Samples were taken at regular intervals to measure cell density and isoprene production. The liquid-to-headspace ratio (1:5) of the cultures ensured the presence of sufficient oxygen to maintain the aerobic conditions throughout the cultivation period. The dissolved oxygen measured at the end of each run was within a range of 5.3–81.0 % saturation.

Cellular growth and metabolite analysis

Cell growth was measured in terms of OD_{600} values based on a standard curve correlated with measured dry cell weights as described previously [22]. Intracellular G3P and pyruvate were analyzed using the aforementioned methods. Extracellular metabolites such as D-galactose and D-galactonate were analyzed by high-pressure liquid chromatography (HPLC) equipped with Bio-Rad Aminex HPX-87H Column (300 × 7.8 mm). The mobile phase used was 5 mM H_2SO_4 and was pumped at a flow rate of 0.4 mL min^{-1}. Column temperature was maintained at 55 °C and the peaks were detected using a Waters 214 refractive index detector. D-Galactonate concentrations were also measured using the modified hydroxamate method described previously [22, 27].

Isoprene concentrations were determined by gas chromatography (GC). Gas samples (1 mL) collected at the headspace of the serum bottles were injected into GC HP6890 equipped with a flame ionization detector (FID) and a HP-FFAP column (50 m × 20 µm × 34 µm). The inlet and column temperatures were maintained at 50 °C, while the detector was set at 250 °C. High-purity N_2 gas was used as a carrier with a linear velocity of 1 mL min^{-1}. The product was identified and quantified against isoprene standard (Sigma-Aldrich, USA).

Results

D-Galactose catabolism through the De Ley–Doudoroff pathway

To exclusively direct D-galactose toward the DD pathway, a *galK*-deletion strain was used as described in an earlier work [22]. This host was unable to grow on D-galactose, indicating that the Leloir pathway was successfully inactivated by the said deletion (Fig. 2a). To complete the DD pathway in *E. coli*, the D-galactose dehydrogenase gene (*gld*) from *P. syringae* was recruited to catalyze the conversion of D-galactose to D-galactonate. This enzyme has been thoroughly characterized in a previous work [22]. Introduction of *gld* into the Δ*galK* strain (denoted as EWG2) partially restored its ability to grow on D-galactose (Fig. 2a,b), wherein it accumulated up to 0.57 ± 0.03 g L^{-1} of D-galactonate after 12 h of cultivation. Furthermore, the accumulated D-galactonate was slowly consumed for the rest of the cultivation period giving a final concentration of 0.49 ± 0.01 g L^{-1} at 48 h (Fig. 2b). These results demonstrated that the EWG2 strain was capable of metabolizing D-galactose for cell growth through the completed DD pathway.

G3P and pyruvate pools in DD pathway of strains

The intracellular concentrations of pyruvate and G3P from the wild type and EWG2 were analyzed. Both strains were cultivated in M9 minimal salts medium with D-galactose as sole carbon source. Results revealed that the wild-type strain had a higher pyruvate pool than G3P, with G3P/pyruvate ratio of 0.42 (Fig. 2c). On the other hand, EWG2 showed a more balanced distribution of G3P and pyruvate with a ratio of 0.90 (Fig. 2c). Interestingly, the total G3P and pyruvate concentration for EWG2 is lower by 29 % compared to the wild type.

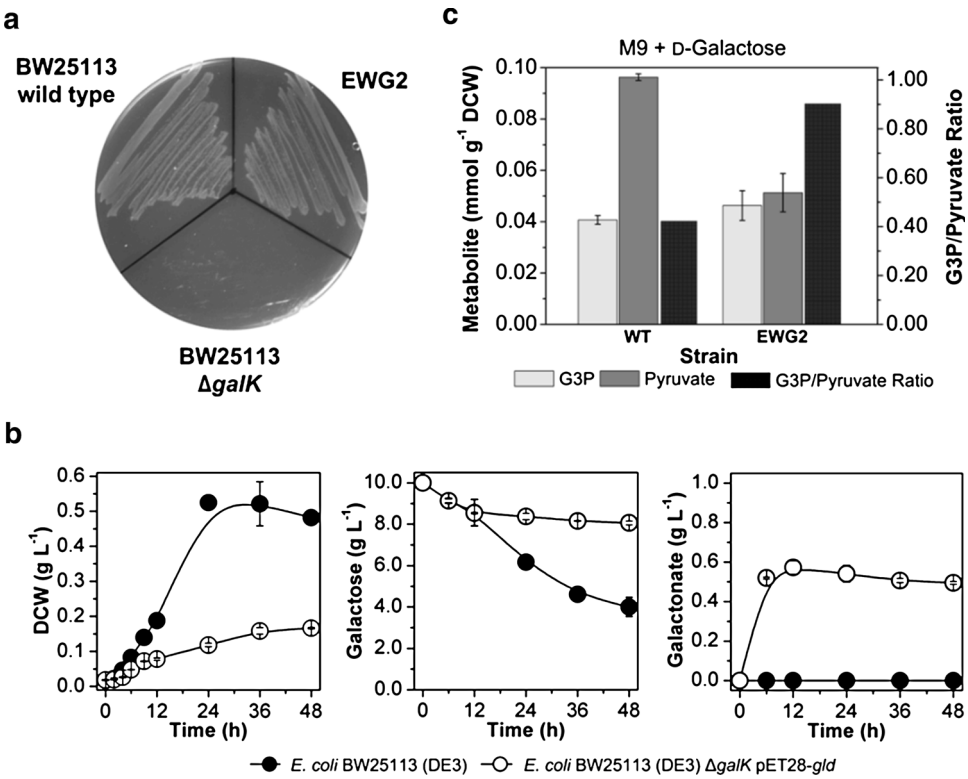

Fig. 2 **a** Growth on solid medium with D-galactose as sole carbon source, **b** biomass accumulation, D-galactose consumption, and D-galactonate accumulation. **c** Intracellular G3P and pyruvate concentrations after 24 h of cultivation of *E. coli* BW25113 and EWG2

Isoprene production via DD pathway

To test the performance of the completed DD pathway for isoprene production, a functional MEP-dependent isoprene synthesis pathway in *E. coli* was constructed. Based on previous studies, overexpressing the native 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) and isopentenyl diphosphate isomerase (*idi*) genes, and the isoprene synthase gene from *P. alba* (*ispS*) were necessary to achieve detectable isoprene concentrations [3, 7]. The plasmid pACYC-*dxs-idi-ispS* was constructed and transformed into the wild-type strain carrying pET28a empty vector (as the control) and into EWG2. The resulting strains were denoted as EBWI3 and EBWI4, respectively. The expressions of the cloned genes were tested through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Supplementary Fig. S1). Subsequently, the isoprene production of EBWI3 and EWBI4 was tested in M9 minimal salt medium with D-galactose as sole carbon source. EBWI4 produced a slightly higher isoprene titer despite its sixfold lower growth than EBWI3 (Table 3). Thus considering the biomass accumulation of both strains, EBWI4 had superior specific isoprene yield. In addition, the isoprene produced by EBWI4 accounted for up to 1.2 % (mol/mol) of the theoretical yield from the consumed D-galactose compared to the 0.5 % (mol/mol) by EBWI3.

Table 3 Biomass, product titers, and specific yield of isoprene producing strains on M9 defined media and D-galactose

Strain	Isoprene, mg L^{-1}	Specific yield, mg isoprene g^{-1} DCW	% Yield, mol isoprene mol^{-1} D-galactose consumed
EBWI3	6.16 ± 0.09	17.42 ± 2.26	0.56 ± 0.08
EBWI4	7.14 ± 0.71	124.29 ± 10.16	1.21 ± 0.12

In addition to the pathway differences between EBWI3 and EBWI4, the *gld* in EBWI4 was plasmid-borne with a high copy number and under the T7 promoter, whereas induction of *galK* in EBWI3 relied on its single chromosomal copy under its native regulation in the host. To provide a more standardized comparison between DD and Leloir as pathways that supply the MEP precursors, another strain was constructed such that it carries pET28a-*galK*, with the chromosomal *galK* intentionally deleted (EBWI3.1). The GalK activity of EBWI3.1 was 51 % lower than the wild type, while its galactose consumption was also lower than EBWI3 and EBWI4 (Supplementary Table 1 and Supplementary Fig. 2). Furthermore, isoprene production of the GalK overexpressing strain was greatly reduced compared to EBWI3 (Supplementary Fig. 3). Since both strains EBWI3.1 and EBWI4 are pET28a-based, factors such as promoter and copy number are ruled out and any observed difference can be solely attributed to the corresponding pathway differences.

Improved isoprene production from DD pathway strain

The considerably poor growth of the EBWI4 strain was a major setback in the target product synthesis from D-galactose through the DD pathway. An additional non-sugar carbon source was needed to support the growth of the strain as well as to supply the necessary co-factor (i.e., NAD^+) in the first enzymatic reaction of the DD pathway. A suitable non-sugar source must have minimal contribution to the formation of pyruvate and G3P, and can be used directly to build cellular components by the host, hence yeast extract (YE) was chosen for such purpose.

To test the level of isoprene production on YE, EBWI3 and EBWI4 were cultivated in M9 minimal salt medium

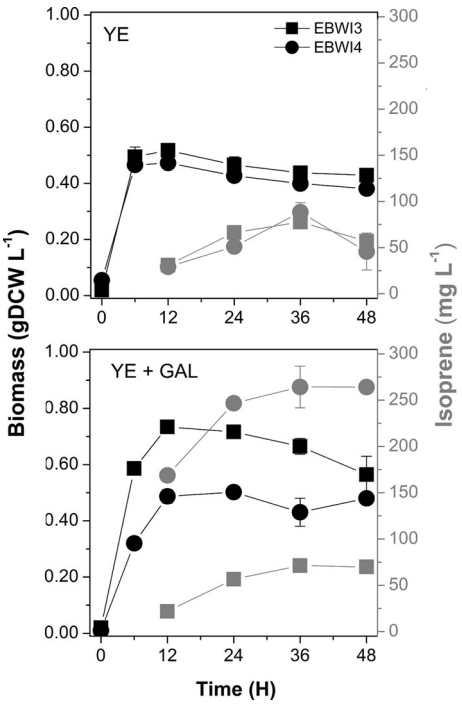

Fig. 3 Biomass and isoprene accumulation of EBWI3 and EBWI4 on minimal M9 media with yeast extract only (*Y.E. top frame*) or D-galactose and yeast extract (*GAL + Y.E. bottom frame*)

and 5 g L^{-1} YE. After 48 h of cultivation, the growth profile of EBWI4 has significantly improved and was similar to that of EBWI3 (Fig. 3). The strains were capable of producing isoprene up to 56.86 ± 8.80 mg L^{-1} for EBWI3 and 45.62 ± 19.84 mg L^{-1} for EBWI4. Thereafter, isoprene production on 10 g L^{-1} D-galactose and 5 g L^{-1} YE was tested under the same culture conditions. EBWI3 produced a slightly higher isoprene titer of 69.82 ± 6.07 mg L^{-1} and has an increased biomass accumulation compared to cultures grown on YE only (Fig. 3). The presence of D-galactose mainly contributed to the increased growth of the strain but had less effect on isoprene production. On the other hand, isoprene production of EBWI4 was significantly higher at 264.21 ± 4.60 mg L^{-1} while the growth was similar to cultures grown on YE only (Fig. 3). Galactose consumption by EBWI4 was lower than that of EBWI3; 2.67 ± 0.01 vs. 3.68 ± 0.03 g L^{-1}. A conservative estimate of the carbon balance in both strains indicated that 95.6 and 24.8 % (mol/mol) of the consumed D-galactose was utilized for growth and other cellular functions by EBWI3 and EBWI4, respectively. In addition, EBWI4 secreted 54.4 % (mol/mol) of the consumed D-galactose as D-galactonate (Fig. 4a).

Changes in the intracellular concentration of G3P and pyruvate were monitored during the cultivation period. It was observed that the intracellular pools of G3P and pyruvate were maintained in an almost 1:1 ratio in EBWI4 despite the presence of yeast extract whereas in EBWI3, the pyruvate pool was consistently higher than G3P (Fig. 4b). Moreover, the total precursor concentration (G3P + pyruvate) in EBWI4 is 52.23 % lower than EBWI3, and the individual precursor concentrations in EBWI4 are consistently lower than EBWI3.

Discussion

It is well known that the MVA and MEP pathways both culminate in the synthesis of IPP and DMAPP, the

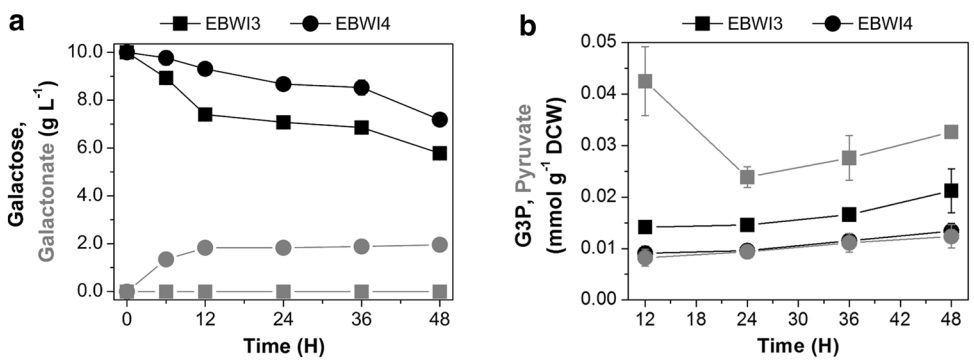

Fig. 4 **a** D-galactose and D-galactonate accumulation, and **b** intracellular G3P and pyruvate concentrations of EBWI3 and EBWI4

universal precursors of isoprenoid compounds. However, they differ in the required starting molecules: acetyl-CoA for MVA, and pyruvate + G3P for MEP. This difference also meant variations in carbon recovery and redox balance from one pathway to the other. In this case, MEP appears to be more advantageous since it is redox balanced and has a higher carbon recovery compared to MVA [7, 28]. Thus, researchers have focused on the MEP pathway for the production of various types of isoprenoids and adapted various techniques such as gene overexpression(s), use of different promoters, and acquisition of gene homologues from different microbial sources [8, 29]. Although substantial enhancement in isoprenoid production has been attained in studies involving the MEP pathway, the titers achieved are not sufficient enough to be considered for commercial production [3, 7–11, 30]. The MEP route is not only limited by the reactions involved within the pathway as it is also affected by the availability of the starting materials which contributes to the regulation of this route [31]. Another viable approach is to engineer the host microorganism to provide a balanced distribution of pathway intermediates. This technique can be executed in two ways: (1) modulating the central sugar metabolism (Embden–Meyerhof–Parnas pathway or EMP) to equalize pyruvate and G3P, or (2) directing the sugar metabolism toward an alternative pathway (e.g., Entner–Doudoroff/ED or DD pathway), which can simultaneously generate equimolar concentrations of the precursors. The former was used in an earlier study by Farmer and Liao which resulted in an increased lycopene production [12]. The latter has been implemented in an earlier work [13], in which diversion of carbon flux through the ED pathway, which directly generates equimolar amounts of G3P and pyruvate, resulted in the highest isoprene titer from glucose. Meanwhile, the current study has shown that the DD pathway, which produces equimolar pyruvate and G3P, greatly enhanced MEP-dependent isoprene production from galactose.

The naturally predominant pathway for galactose metabolism is the Leloir pathway, with glucose-6-phosphate as the final intermediate compound that enters the EMP [32]. The DD pathway is analogous to ED; both pathways have an aldolase reaction which results in the cleavage of a phosphorylated aldonic acid, which produces equimolar G3P and pyruvate. Energetically, EMP is superior to ED since it produces twice as much ATP. However, the ubiquity of ED suggests that microorganisms will utilize either pathway in response to adaptation to environmental or physiological conditions. In addition, it has been postulated that the chief function of the ED, which is on par with the DD pathway, is the catabolism of sugar acids that cannot be otherwise broken down through glycolysis [33, 34].

The first step of the DD pathway is the oxidation of D-galactose to D-galactonate, followed by the D-galactonate catabolic pathway which is present in a number of non-pathogenic mycobacteria, archaea, and *E. coli* [17, 35]. Considering that only the initial oxidation step is missing in *E. coli*, introduction of an exogenous galactose dehydrogenase is required to complete the DD pathway. It has been previously demonstrated that *E. coli* BW25113 overexpressing *gld* from *P. syringae* was able to produce D-galactonate [22]. The subsequent conversion of D-galactonate to G3P and pyruvate was expected to be catalyzed by the inherent D-galactonate catabolic pathway in *E. coli*. Completion of the DD pathway in the *galK*-deletion mutant host only partially restored its growth on D-galactose. This relatively poorer growth may have been caused by some unknown cellular regulatory network related to the native D-galactonate metabolism in *E. coli*. It should be noted that when EWG2 was grown solely on D-galactose, 24 % (mol/mol) of the consumed substrate was secreted as D-galactonate. The same behavior was also observed when EWG2 was grown on two carbon sources, D-glucose and D-galactose [22].

The metabolism of D-galactose through the Leloir pathway in EBWI3 produced considerable amount of isoprene titers. Measurement of intracellular precursor concentrations showed that pyruvate was consistently higher than G3P which suggested that G3P is the limiting factor. These results are consistent with the report by Farmer and Liao [12] showing that changes in the central metabolic pathway which diverts the flux from pyruvate back to G3P led to increased lycopene production, while the reverse resulted to decreased production. On the other hand, the premise that directing the sugar metabolism through the DD pathway would generate equimolar supplies of pyruvate and G3P was validated by the current results. This hypothesis concurs with previous reports wherein enteric and soil microorganisms that metabolize D-galactose or D-galactonate through the DD pathway displayed intracellular pyruvate and G3P concentration ratios near to one [36–38]. Upon combination of the functional MEP pathway with the complete DD pathway in EBWI4, isoprene production was considerably improved. Apparently, the increase in titer is the result of a more balanced distribution of precursors rather than increased intracellular pools since the combined precursor concentration for EBWI4 is lower than EBWI3. These precursors are also key metabolic intermediates in the cell, often used for growth and other cell functions. Since these precursors were diverted toward producing more isoprene, a concomitant decrease in cell growth was expected and observed in EBWI4. These results are also in agreement with an earlier work which demonstrated the effect of the ED pathway (analogous to DD pathway) in supplying equimolar G3P and pyruvate

into the MEP pathway from glucose [13]. The highest isoprene titer (219.42 ± 6.24 mg L^{-1}) was achieved when the ED pathway was employed as the feeding module for the MEP route. This further proves that a balanced supply of these precursors is necessary to increase carbon flux toward the MEP pathway.

The Leloir pathway operates differently from the DD pathway and their corresponding gene regulatory systems also vary. The Leloir pathway consists of enzymes coded by the *gal* operon where its regulation has been extensively studied and is believed to be one of the most intricate control systems in *E. coli* [39]. Optimization of the Leloir pathway was attempted by overexpression of *galK* in EBWI3.1. But instead of achieving increased enzyme activity, improved substrate consumption and higher isoprene production, the effect was opposite. This indicates that *galK* overexpression in the Leloir pathway was found ineffective in improving the strain performance or can be even detrimental to the cell. Moreover, augmentation of the Leloir pathway still necessitates modulation of the lower glycolysis to provide balanced distribution of G3P and pyruvate.

The control of expression of the DD pathway genes in *E. coli* has not been fully explored, but it has been proposed that these genes are induced by the presence of D-galactonate [18, 37]. Certainly, maximization of the D-galactose metabolism through the DD pathway is still limited due to the lack of knowledge on the inherent regulatory system of the D-galactonate metabolism in *E. coli*. In addition, G3P and pyruvate participate in a multitude of pathway and thus subject to various layers of regulation and competing pathways [31]. Elucidation of these inherent regulatory mechanisms is beyond the scope of this study and thus will be dealt with in future works.

Conclusion

This work has shown the effectiveness of manipulating the supply of G3P and pyruvate for the MEP-dependent isoprene synthesis by changing the main substrate metabolism from EMP to DD pathway. Equimolar amounts of these precursors are supplied from D-galactose through the DD pathway which resulted in an increased isoprene production. This provides a new platform for developing an improved isoprenoid producing strain with combined DD–MEP pathway. Further studies are still required to improve the D-galactose metabolism through the DD pathway and minimize accumulation of intermediates such as D-galactonate. In addition, this strategy can be applied not only to isoprene but also to the production of other isoprenoids.

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