

Utilization of alkaline phosphatase PhoA in the bioproduction of geraniol by metabolically engineered *Escherichia coli*

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Geraniol is a valuable acyclic monoterpene alcohol and has many applications in the perfume industries, pharmacy and others. It has been hypothesized that phosphatases can convert geranyl diphosphate (GPP) into geraniol. However, whether and which phosphatases can transform GPP to geraniol has remained unanswered up till now. In this paper, the catalysis abilities of 4 different types of phosphatases were studied with GPP as substrate *in vitro*. They are bifunctional diacylglycerol diphosphate phosphatase (DPP1) and lipid phosphate phosphatase (LPP1) from *Saccharomyces cerevisiae*, ADP-ribose pyrophosphatase (NudF) and alkaline phosphatase (PhoA) from *Escherichia coli*. The results show that just PhoA from *E. coli* can convert GPP into geraniol. Moreover, in order to confirm the ability of PhoA *in vivo*, the heterologous mevalonate pathway and geranyl diphosphate synthase gene from *Abies grandis* were co-overexpressed in *E. coli* with PhoA gene and 5.3 ± 0.2 mg/l geraniol was produced from glucose in flask-culture. Finally, we also evaluated the fed-batch fermentation of this engineered *E. coli* and a maximum concentration of 99.3 mg/l geraniol was produced while the conversion efficiency of glucose to geraniol (gram to gram) was 0.51%. Our results offer a new option for geraniol biosynthesis and promote the industrial bio-production of geraniol.

Introduction

The monoterpene geraniol, which is emitted from flowers, has an important role in flavor and fragrance industries due to its pleasant rose-like odor.¹ Geraniol also exhibits huge potential in pharmacy and agrochemistry.^{2,3} Fractional distillation of plant essential oils is the major method for geraniol manufacture, but high cost and other limitations, such as weather dependence and plant diseases, limited the supplies of geraniol.⁴ Converting renewable resources into monoterpene products by engineered microorganisms was interesting technology and developed quickly recently year, which have the advantages of fast growth, no need for land during their growth and sustainable development.^{5,6}

Geraniol is likely to be synthesized from geranyl diphosphate (GPP), the universal precursor of all monoterpenes, which can be produced from both the methylerythritol 4-phosphate pathway and the mevalonate (MVA) pathway in plants.^{7,8} Although many microorganisms carry out the methylerythritol 4-phosphate pathway or MVA pathway to supply the intermediates dimethylallyl pyrophosphate and isopentenyl pyrophosphate, they are unable to produce the monoterpenes because of the absence of monoterpene synthase. In recent year, Iijima et al.⁹ first purified and characterized geraniol synthase (GES) from peltate glands of

Ocimum basilicum as a member of the terpene synthase family and proved that GES can catalyze geraniol formation from GPP by the addition of a hydroxyl group to a carbocation intermediate. Then, the generation of geraniol by GES has been demonstrated in engineered microbial and a recent study proved that geraniol could be generated at a level of 0.185 mg l^{-1} by simply over-expressing GES in *E. coli*.¹⁰ However, the structure of geraniol, whose carbon skeleton is identical to that of its precursor GPP, hypothetically allows for an alternative mechanism of simply breaking the phosphoester bond by a phosphatase to generate geraniol.⁹ Moreover, geraniol formation via endogenous dephosphorylation of GPP has been observed in engineered *E. coli* and yeast.^{11,12} But, whether and which phosphatases can transform GPP to geraniol has remained unanswered up till now. The catalysis ability of phosphatases toward geraniol formation was investigated in this study, and it will show a new option for geraniol biosynthesis.

Results and Discussion

Phosphatases activity on geranyl diphosphate (GPP)

With the aim to study the catalysis ability of phosphatases to form geraniol from GPP, 4 different types of phosphatases genes

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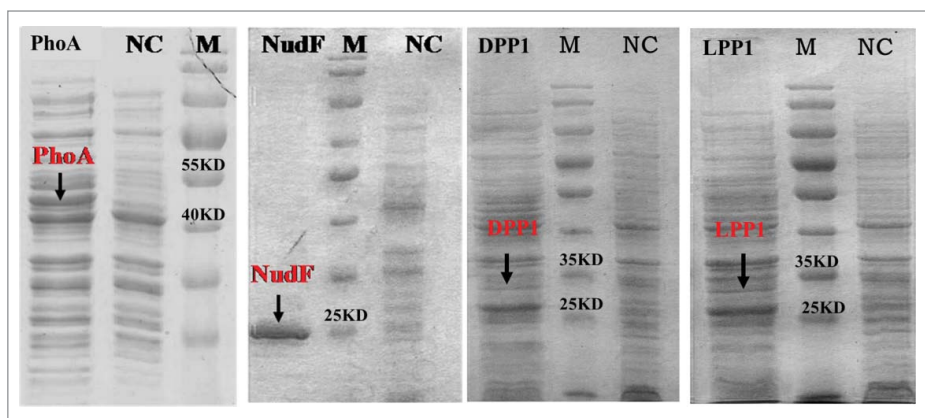


Figure 1. SDS-PAGE analysis of phosphatases protein expressed in *E. coli*. Lane M, unstained protein molecular weight marker; NC, crude protein extracts from *E. coli* BL21(DE3); PhoA, crude protein extracts from strain LWG2 4 h after induction; NudF, crude protein extracts from strain LWG5 4 h after induction; DPP1, crude protein extracts from strain LWG3 6 h after induction; LPP1, crude protein extracts from strain LWG2 6 h after induction.

were studied. These four phosphatases were bifunctional diacylglycerol diphosphate phosphatase (DPP1) from *Saccharomyces cerevisiae*, lipid phosphate phosphatase (LPP1) from *Saccharomyces cerevisiae*, ADP-ribose pyrophosphatase (NudF) from *E. coli* and alkaline phosphatase (PhoA) from *E. coli*. The crude protein samples taken from cells were analyzed by SDS-PAGE. The theoretical molecular mass of DPP1, LPP1, NudF and PhoA were known as 32 kDa, 30 kDa, 23 kDa and 52 kDa, which were determined from their amino acids.¹⁴ The results of SDS-PAGE show that all phosphatases were expressed and matched their theoretical molecular mass respectively (Fig. 1). Then, the crude proteins were used to detect the phosphatase activity toward GPP *in vitro*. The GC-MS result of reaction shows that only PhoA can act on geranyl diphosphate to form geraniol (Fig. 2), while geraniol was not detected in catalysis reactions by the crude proteins of DPP1, LPP1 and NudF. The control also didn't detect the generation of geraniol. Each reaction does 3 parallels.

This result shows the different catalysis ability of phosphatase. DPP1 and LPP1 were proved to produce geranylgeraniol by removing the phosphate of geranylgeranyl diphosphate.¹³ However, they cannot use GPP as substrate to form geraniol in this study, which is consistent with the result no significant increase in geraniol formation by overexpressed DPP1 *in vivo*, as proved by Oswald.¹¹ NudF can remove the pyrophosphate from IPP and DMAPP to form prenol and isoprenol by introducing NudF into the strain that employed the whole MVA pathway, but not useful in this reaction.¹⁴ Alkaline phosphatase (PhoA) is a nonspecific phosphomonoesterase which can remove the inorganic phosphate to form corresponding alcohols.¹⁵ The PhoA activity was studied and proved with other phosphorylated substrates, such as 4-nitrophenyl phosphate and p-nitrophenyl phosphate, but this paper first proves the catalysis ability of PhoA with GPP as substrate *in vitro*, and our finding opens the door to biosynthesis of geraniol with alkaline phosphatase.

Utilization of PhoA in the bioproduction of geraniol from glucose in *E. coli*

In order to biosynthesize geraniol from glucose by phosphatase, sufficient GPP should be obtained in *E. coli*. Considering the limited supply of IPP and DMAPP from the native methylerythritol 4-phosphate pathway, the heterologous MVA pathway and gene *GPPS2* from *Abies grandis* were co-overexpressed in *E. coli*, which have been confirmed can provide sufficient GPP in our pervious study (Fig. 3). To simplify the task of engineering a 7-gene pathway, they were separated into 2 operons and cloned into 2 compatible plasmids. The gene *phoA* was introduced into plasmid pYJM26, which made up of genes *GPPS2* and upper portion of MVA pathway with pACYCDuet-1 vector.

The resulting plasmid was named as pLWG9. The other plasmid named pYJM14 contains bottom portion of MVA pathway. The plasmids pLWG9 and pYJM14 were transformed into *E. coli* BL21 (DE3) strain simultaneously, resulting in strain LWG9. Growing in 100 ml shake-flask, about 5.3 ± 0.2 mg/l geraniol was produced from glucose when LWG9 was grown in 100 ml shake flask for 48h at pH7, 180 rpm and 30°C. As control, strain LWG10 was constructed by transforming plasmids pYJM26 and pYJM14 into *E. coli* BL21 (DE3). Geraniol production was not detected during 48h of culture by strain LWG10, though geraniol formation via endogenous dephosphorylation of GPP has been observed in *E. coli* with supplementation of mevalonate, which bearing both *GPPS* mutated form *E. coli* and bottom portion of MVA pathway.¹² The failure of geraniol production by strain LWG10 might be ascribed to that the supply of GPP by whole MVA pathway from glucose was lower than by the bottom portion of MVA pathway from mevalonate and the low native PhoA catalytic activity limited the conversion of GPP to geraniol in *E. coli*.

Fed-batch fermentation was carried out using the engineered *E. coli* strain LWG9. As is shown in Figure 4, geraniol production increased rapidly from 0 h to 10 h after induction and reached 98.7 mg/l at 14 h of culture with an average productivity of $16.7 \text{ mg g cdw}^{-1} \text{ h}^{-1}$. Then, geraniol production keeps relatively stable till 32h of culture. Considering that geraniol could be dehydrogenated and transformed into other geranoids in *E. coli* by YjgB, geranoids were analyzed by GC-MS.¹² Geranial, the oxidative product of geraniol, was detected after 6h of culture, while nerol, the *cis*-isomer of geraniol, was detected after 24h of culture in this study. Geraniol, geranial and nerol formations were sequentially observed during culture, which is consistent with the geraniol formation mechanism that geranial and nerol are formed via geraniol as proposed by Iijima et al.¹⁶ and Zhou et al.^{12,16} Moreover, considering the mechanism of phosphatase, it is hard to form nerol and geranial directly from GPP,

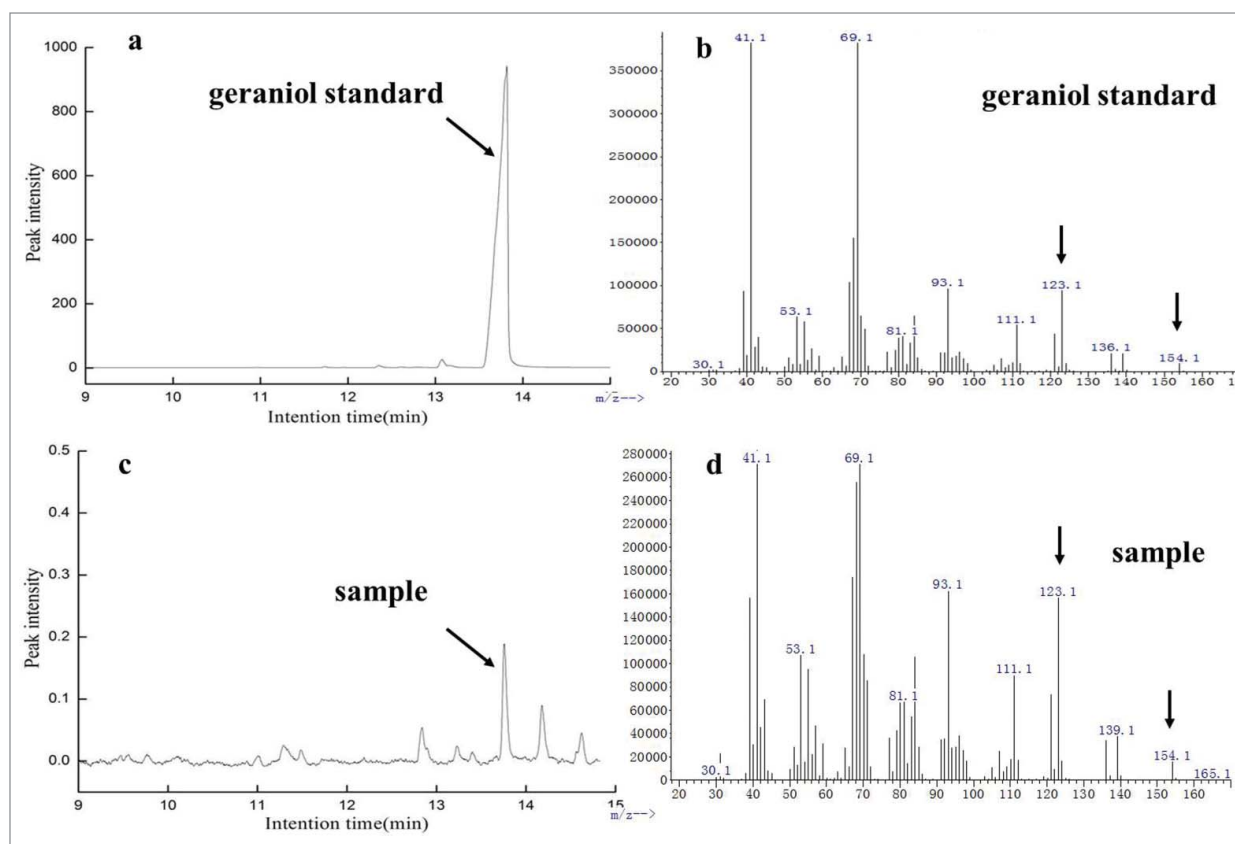


Figure 2. GC-MS analysis of the product of reaction catalyzed by phosphatases with geranyl diphosphate as substrate. By comparing with the authoritative geraniol (**a, b**), the capacity of geraniol formation was verified in the reaction catalysis by alkaline phosphatase PhoA from *E. coli* (**c, d**). (**a, c**) total ion chromatogram (TIC); (**b, d**) mass spectrum. Geraniol was not detected either in control or reactions catalyzed by bifunctional diacylglycerol diphosphate phosphatase (DPP1), lipid phosphate phosphatase (LPP1) and ADP-ribose pyrophosphatase (NudF) respectively. The experiment was performed in triplicate.

which supported above geraniol formation mechanism from another aspect. At 28 h of culture, the total geraniol production was increased to 151.9 mg/l while the geraniol production reached 99.3 mg/l and the geraniol dehydrogenation rate in strain LWG9 was $1.88 \text{ mg l}^{-1} \text{ h}^{-1}$. The conversion efficiency of glucose to geraniol (gram to gram) just reached 0.51%.

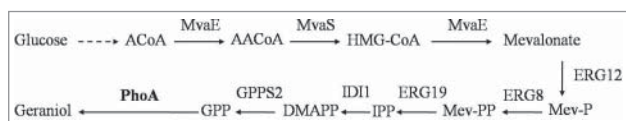


Figure 3. Production of geraniol from glucose with PhoA via the MVA pathways used in this study. The enzymes involved in this pathway: MvaE, *Enterococcus faecalis* acetyl-CoA acetyltransferase /HMG- CoA reductase; MvaS, *Enterococcus faecalis* HMG-CoA synthase; ERG12, *Saccharomyces cerevisiae* mevalonate kinase; ERG8, *Saccharomyces cerevisiae* phosphomevalonate kinase; ERG19, *Saccharomyces cerevisiae* mevalonate pyrophosphate decarboxylase; IDI1, *Saccharomyces cerevisiae* IPP isomerase; GPPS2, *Abies grandis* geranyl diphosphate synthase; PhoA, *Escherichia coli* alkaline phosphatase. Pathway intermediates: A-CoA, acetyl-CoA; AA-CoA, acetoacetyl-CoA; HMG-CoA, hydroxymethylglutaryl-CoA; Mev-P, mevalonate 5-phosphate; Mev-PP, mevalonate pyrophosphate. IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl diphosphate.

Although the ability of PhoA to form geraniol was proved in this study, the geraniol production and productivity need to be improved in the future. The main reason for the poor status of strain LWG9 may lie in the low activity of wild type of PhoA. Meanwhile, overexpression of many heterologous genes may be another reason. To resolve the above-mentioned problems, many possible improvements can be used to enhance geraniol production. One approach is to mutate and screen the high activity PhoA for geraniol production. The previous studies about PhoA including the structural and mutant studies will provide a large amount of data for PhoA optimizing. Another approach is engineering of the host including: employing a chromosome integration technique to decrease the cell growth burden on the host that results from overexpression of heterologous genes.

Conclusions

In conclusion, the catalysis ability of phosphatases with GPP as substrate to produce geraniol was first studies. Four different types of phosphatases, DPP1, LPP1, NudF and PhoA were screened and only PhoA from *E. coli* can form geraniol from GPP *in vitro*. Moreover, a new pathway with PhoA for geraniol

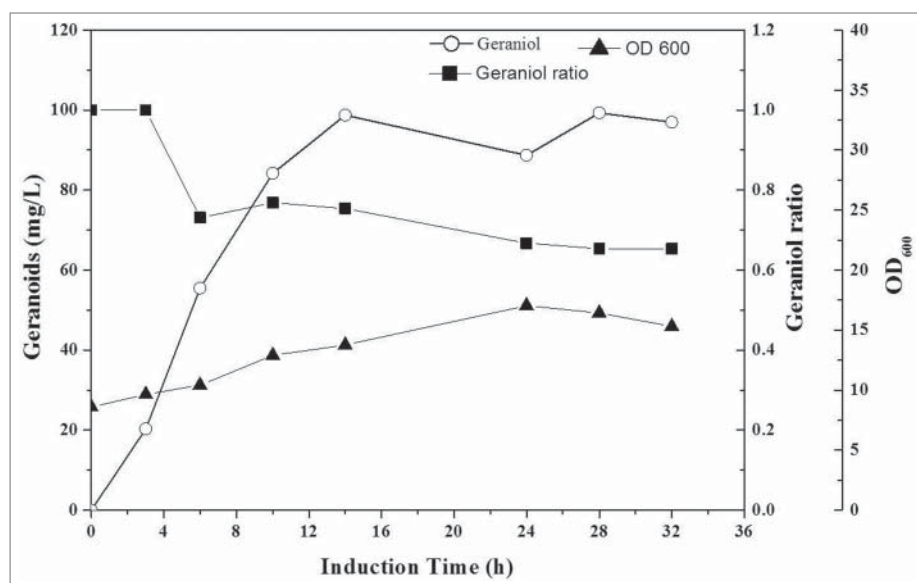


Figure 4. The fermentation course of geraniol (□) production by LWG9. Induction was carried out when OD₆₀₀ (▲) reached 20 using 1mM IPTG. Nerol and geraniol were observed during fermentation with geraniol biosynthesis and (■) representing the ratio of geraniol to these geraniols.

bioproduction from glucose was established in engineered *E. coli* and 5.3 mg/l geraniol was biosynthesized under shake-flask culture. Finally, we also evaluated the fed-batch fermentation of geraniol and a maximum concentration of 99.3 mg/l was reached. This study provided a new sustainable production strategy for geraniol in *E. coli*.

Materials and Methods

Medium and culture conditions

The Luria Broth (LB) medium (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract) was used for gene cloning and

shake-flask fermentation. For geraniol production, recombinant strains were cultured in shake-flask or fed-batch fermentation with the medium (pH 7) containing glucose 20 g/l, K₂HPO₄ 9.8 g/l, beef extract 5 g/l, ferric ammonium citrate 0.3 g/l, citric acid monohydrate 2.1 g/l, MgSO₄ 0.06 g/l and 1 ml trace element solution which includes (NH₄)₆ Mo₇O₂₄·4H₂O 0.37 g/l, ZnSO₄·7 H₂O 0.29 g/l, H₃BO₄ 2.47 g/l, CuSO₄·5H₂O 0.25 g/l, and MnCl₂·4H₂O 1.58 g/l. Appropriate antibiotics were added to the culture medium according to selectable marker gene of each plasmid listed in Table 1 at the following concentration: ampicillin (Amp, 100 mg/ml), kanamycin (Kan, 50 mg/ml), and chloramphenicol (Cm, 34 mg/ml).

Strains and plasmids

All strains and plasmids used in this study are listed in Table 1. Four different phosphatase genes, alkaline phosphatase (PhoA) gene from *E. coli*, ADP-ribose pyrophosphatase (NudF) gene, bifunctional diacylglycerol diphosphate phosphatase (DPP1) gene and lipid phosphate phosphatase (LPP1) gene were cloned into pCOLA-Duet-1 creating pYY11, pYY12, pYY13 and pYY16 respectively in our previous study,¹⁴ and LWG2, LWG3, LWG4, LWG5 were formed by transferred these plasmid into *E. coli* BL21 (DE3) separately.

For geraniol production from glucose, plasmids of the whole pathway for geraniol synthesis were constructed. Plasmid pYJM26 and pYJM14, which harboring the heterologous mevalonate pathway and geranyl diphosphate synthase gene from *Abies grandis*, were constructed in our lab's early study.¹⁷ The *phoA*

Table 1. Strains and plasmids used in this study

Name	Relevant characteristics	References
Strains		
BL21(DE3)	F ⁻ ompT hsdS _B (r _B ⁻ m _B ⁻) gal dcm rne131 λ(DE3)	Invitrogen
LWG2	<i>E. coli</i> BL21(DE3)/ pYY11	This study
LWG3	<i>E. coli</i> BL21(DE3)/ pYY12	This study
LWG4	<i>E. coli</i> BL21(DE3)/ pYY13	This study
LWG5	<i>E. coli</i> BL21(DE3)/ pYY16	This study
LWG9	<i>E. coli</i> BL21(DE3)/ pLWG9, pYJM14	This study
LWG10	<i>E. coli</i> BL21(DE3)/ p YJM26, pYJM14	This study
Plasmids		
pACYCDuet-1	P15A (pACYC184), Cm ^r	Novagen
pLWG9	pACYCDuet-1 carrying <i>mvaE</i> and <i>mvaS</i> from <i>Enterococcus faecalis</i> , <i>GPPS2</i> from <i>Abiesgrandis</i> , ' <i>phoA</i> from <i>Escherichia coli</i> , Cm ^r	This study
pYY11	pCOLADuet-1 carrying ' <i>phoA</i> from <i>Escherichia coli</i> , Kan ^r	14
pYY12	pCOLADuet-1 carrying ' <i>DPP1</i> from <i>Saccharomyces cerevisiae</i> , Kan ^r	14
pYY13	pCOLADuet-1 carrying ' <i>LPP1</i> from <i>Saccharomyces cerevisiae</i> , Kan ^r	14
pYY16	pCOLADuet-1 carrying <i>nudF</i> from <i>Escherichia coli</i> , Kan ^r	14
pYJM26	pACYCDuet-1 carrying <i>mvaE</i> and <i>mvaS</i> from <i>Enterococcus faecalis</i> , <i>GPPS2</i> from <i>Abiesgrandis</i> , Cm ^r	17
pYJM14	pTrcHis2B carrying <i>ERG12</i> , <i>ERG8</i> , <i>ERG19</i> and <i>IDI1</i> from <i>Saccharomyces cerevisiae</i> , Amp ^r	17

gene was PCR-amplified from plasmid DNA of pYY11 with the primer sets *phoA-rbs-F* (GGAAGATCTAGGAGGTAAAAA-TATGCGGACACCAGAAATGCCTGTTC) and *phoA-R* (CACCTCGAGTTATTTTCAGCCCCAGAGCGGC). The PCR product was digested with BglII and XhoI respectively and then ligated into the corresponding sites of pYJM26 cut with the same restriction enzymes, creating pLWG9. The pLWG9 and pYJM14 were transformed into the BL21 (DE3) competent cell to form LWG9.

Enzyme extraction and assay

The four strains LWG2, LWG3, LWG4 and LWG5 were cultured in LB broth respectively. When the OD₆₀₀ of the bacterial culture reached 0.6–0.8, the cells were induced by IPTG at a final concentration of 0.1 mM and further incubated at 30°C for 4 h–6 h. Cells were harvested by centrifugation at 6000 g for 5 min, washed with distilled water, and then resuspended in reaction buffer of each enzyme. All the extraction procedures were carried out at 4°C. The cells were sonicated on ice for 10 min (3 s pulse on, 3 s pulse off, 40 W, Sonics VCX130, China). The corresponding supernatants were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and prepared for activity assays.

The phosphatase activity toward geranyl diphosphate (GPP) was assayed as the formation of geraniol. The activity of alkaline phosphatase (*PhoA*) was measured as described by Kojima et al.¹⁸ with the following modification: the incubation mixture contained 1 M Tris-Cl, pH 8.0, 10 mM MgSO₄, 50 μM ZnSO₄, 5 μM GPP and the enzyme extract (about 0.5 mg protein), incubated for 30 min at 37°C. The activity of bifunctional diacylglycerol diphosphate phosphatase (DPP1) and lipid phosphate phosphatase (LPP1) were assayed using a modification of existing methods.^{13,19} The assay mixture (100 μl) was comprised of citrate buffer (120 mM, pH 4.3), 5 mM MgCl₂, the enzyme extract (about 0.5 mg protein), and 5 μM GPP and was incubated for 30 min at 37°C. The activity of ADP-ribose pyrophosphatase (*NudF*) was measured as described by Dunn et al.²⁰ with the following modification: the incubation mixture contained 50 mM Tris-Cl (pH 8.0), 2 mM MgCl₂, 5 μM GPP and the enzyme extract (about 0.5 mg protein) and was incubated for 15 min at 37°C. The products of the reactions were analyzed by GC-MS. Every reaction has 3 parallels and cell extract with empty pCOLADuet-1 was used as control.

Shake-flask cultures

A single colony of LWG9 was grown up in LB broth overnight at 37°C. The culture was used to inoculate the same medium (1:100 dilution) and grown at 37°C until the culture reached an OD₆₀₀ of 0.6–0.8. IPTG was added to a final concentration of 0.1 mM, and the culture was further incubated at 30°C for 48 h at 180 rpm. The samples were added with the

same volume of ethyl acetate, vortexed briefly, and centrifuged to separate the phases and the organic phase was analyzed by GC-MS. The strain LWG10 was used as control. The experiment was performed in triplicate.

Fed-batch fermentation

The strain LWG9 grew up overnight at 37°C in 100 ml of fermentation medium. The overnight culture was used to inoculate a 5L fermentor (BIOSTAT Bplus MO 5 L, Sartorius, Germany) containing 2L fermentation medium. The temperature was maintained at 30°C, the pH was maintained at 7.0 via automated addition of ammonia. The stirring speed was first set at 400 rpm and then linked to the dissolved oxygen (DO) concentration to maintain a 20% saturation of DO. The expression of plasmid-born exogenous genes for geraniol production was initiated at an OD₆₀₀ about 20 by adding IPTG at a final concentration of 1 mM. During the course of fermentation, the residual glucose was measured using a glucose analyzer (SBA-40D, China) and was maintained below 1 g/l by adding 70% glucose solution during the course of fermentation. To harvest geraniol from the culture broth during fermentation, 2-phase culture was carried out by adding 200 ml isopropyl myristate at 4 h after induction. The samples were collected on time and the organic phase was separated by centrifugation at 13000 rpm for 10 min, then added with 10 volume of ethyl acetate and analyzed by GC-MS.

Geraniol characterization by GC-MS

Putative geraniol products were identified by GC-MS. A HP-INNOWAX capillary column (30 m×0.25 mm; 0.25 μm film thickness; Agilent Technologies) was used. The separation conditions were an initial column temperature of 50°C for 1 min, an increase of 10°C/min to 250°C, where it was held for 5 min. Peak identification was based on a relative retention time and total ion mass spectral comparison with an external standard (Sigma-Aldrich, USA). The peak areas were converted into geraniol or nerol concentrations in comparison with standard curves plotted with set of known concentrations of standards.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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