Fermentative production of enantiopure (S)-linalool using a metabolically engineered *Pantoea ananatis*

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**Abstract**

**Background:** Linalool, an acyclic monoterpene alcohol, is extensively used in the flavor and fragrance industries and exists as two enantiomers, (S)- and (R)-linalool, which have different odors and biological properties. Linalool extraction from natural plant tissues suffers from low product yield. Although linalool can also be chemically synthesized, its enantioselective production is difficult. Microbial production of terpenes has recently emerged as a novel, environmentally-friendly alternative. Stereoselective production can also be achieved using this approach via enzymatic reactions. We previously succeeded in producing enantiopure (S)-linalool using a metabolically engineered *Pantoea ananatis*, a member of the Enterobacteriaceae family of bacteria, via the heterologous mevalonate pathway with the highest linalool titer ever reported from engineered microbes.

**Results:** Here, we genetically modified a previously developed *P. ananatis* strain expressing the (S)-linalool synthase (AaLINS) from *Actinidia arguta* to further improve (S)-linalool production. AaLINS was mostly expressed as an insoluble form in *P. ananatis*; its soluble expression level was increased by N-terminal fusion of a halophilic β-lactamase from *Chromohalobacter* sp. 560 with hexahistidine. Furthermore, in combination with elevation of the precursor supply via the mevalonate pathway, the (S)-linalool titer was increased approximately 1.4-fold (4.7 ± 0.3 g/L) in comparison with the original strain (3.4 ± 0.2 g/L) in test-tube cultivation with an aqueous-organic biphasic fermentation system using isopropyl myristate as the organic solvent for in situ extraction of cytotoxic and semi-volatile (S)-linalool. The most productive strain, IP04S/pBLAAaLINS-ispA*, produced 10.9 g/L of (S)-linalool in "dual-phase" fed-batch fermentation, which was divided into a growth-phase and a subsequent production-phase. Thus far, this is the highest reported titer in the production of not only linalool but also all monoterpenes using microbes.

**Conclusions:** This study demonstrates the potential of our metabolically engineered *P. ananatis* strain as a platform for economically feasible (S)-linalool production and provides insights into the stereoselective production of terpenes with high efficiency. This system is an environmentally friendly and economically valuable (S)-linalool production alternative. Mass production of enantiopure (S)-linalool can also lead to accurate assessment of its biological properties by providing an enantiopure substrate for study.

**Keywords:** Linalool, Biphasic fermentation, Mevalonate pathway, Solubility tag, *Pantoea ananatis*

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**Background**

Terpenes are one of the most abundant classes of natural products with diverse structures and functions (over 50,000 known compounds) and have been widely used as pharmaceuticals, fragrances, and flavors. In plants,
terpenes are synthesized by terpene synthases (TPSs) from basic five-carbon precursor units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are supplied from either the methylerithritol phosphate pathway or mevalonate (MVA) pathway [1]. Microbial terpene production has recently emerged as an ecologically friendly alternative to extraction from natural vegetations (wood and leaf-derived essential oils), which tends to suffer from low product yield [2, 3]. Fermentation products from natural resources are also more economically valuable in the flavor market than their chemically synthesized counterparts because they can be labeled as "natural", aligning with emerging consumer preferences for natural substances [4, 5]. Additionally, microbial production allows for enantioselective production of terpenes, which is difficult via chemical synthesis, by exploiting the stereoselectivity (enantioselectivity) of enzymatic reactions such as enantioselective production of (R)-α-ionone [5] and (−)-α-bisabolol [6] in Escherichia coli. However, examples of fermentative terpene production reaching 10 g/L titer have been confined to four molecules, viridiflorol, amorpha-4,11-diene [7], β-farnesene [8] (sesquiterpenes), and isoprene (a hemiterpene) [9], according to our literature search [10].

Linalool, an acyclic monoterpene alcohol, has been widely used as a flavor additive and fragrance ingredient [11]. Linalool is synthesized by linalool synthases from geranyl pyrophosphate (GPP), which is generated by the condensation of IPP and DMAPP by GPP synthase in plants. Linalool exists as two enantiomers, (S)- and (R)-linalool, which are differentiated by the chiral properties of the hydroxylated third carbon; the different enantiomers show distinct odors and biological properties [11]. Since commercially available linalool is mainly racemate or (R)-linalool, enantiopure (S)-linalool is attractive to the flavor and fragrance industries. Enantiopure (S)-linalool production has already been reported in Saccharomyces cerevisiae [12] and Yarrowia lipolytica [13] expressing (S)-linalool synthase (AaLINS) from Actinida arguta [14]. We also successfully produced (S)-linalool in the cyanobacterium Synechocystis sp. PCC 6803 (Synechocystis) [15] by co-expressing AaLINS and the S80F mutant of farnesyl pyrophosphate synthase (IspA*; from E. coli, functioning as GPP synthase) [16]. However, production levels in these systems range from 240 µg/L to 11.6 mg/L, which cannot meet industrial needs.

Pantoea ananatis, a Gram-negative and yellow-pigmented bacterium, was identified in 1928 and has been mainly studied as a plant pathogen [17]. In the mid-1990s, a nonpathogenic P. ananatis strain AJ13355 was isolated by specialists from Ajinomoto Co., Inc. and has been demonstrated to be an excellent host for L-glutamate production because of its capability to grow at an acidic pH and resist the effects of high concentrations of L-glutamate [18]. The well-developed genetic tools [19, 20] and sequenced complete genome [18] has broadened the attractiveness of P. ananatis as a production host for various bio-based materials such as cysteine [21], dicarboxylic acids [22], and isoprene [23]. We constructed a metabolically engineered P. ananatis strain named SWITCH-PphoC, which contains heterologous genes of the MVA pathway (mvaE and mvaS from Enterococcus faecalis; MVA kinase gene from Methanocella paludicola [mvk]; phosphomevalonate kinase, diphosphomevalonate decarboxylase, and IPP isomerase genes from S. cerevisiae), to supply IPP/DMAPP for isoprene production [23]. This strain was designed to direct carbon flux to the MVA pathway only under external inorganic phosphate (Pi)-starved conditions by using the Pi-starvation-inducible phoC promoter for driving the expression of the mvaES operon, which encodes the enzymes that catalyze the conversion of acetyl-coenzyme A (CoA) to MVA. Furthermore, enantiopure production of both (S)- and (R)-linalool at a titer of greater than 1 g/L has been successful with a SWITCH-PphoC strain expressing IspA* and either AalINS or a (R)-linalool synthase from Streptomyces clavuligerus [24], whose genes were optimized based on the codon preference of Synechocystis, under an aqueous-organic biphasic fermentation system in which monoterpenes' cytotoxicity and product loss by its aircrapping were alleviated [25] using isopropyl myristate (IPM) as an organic solvent [26]. Additionally, the (S)-linalool titer was increased by deleting gcd (locus_tag PAJ_3473) encoding a glucose dehydrogenase in the SWITCH-PphoC strain (SWITCH-PphoC Δgcd) [26].

In this study, we chose (S)-linalool as the target product and aimed to further improve (S)-linalool production through several approaches: (1) by increasing carbon flux to IPP/DMAPP from acetyl-CoA through enhancement of the upper component of the MVA pathway; (2) by increasing intracellular AalINS activity with N-terminal fusion of a halophilic β-lactamase (BLA) from Chromohalobacter sp. 560 [27] joined to a hexahistidine (6×His); and (3) by adopting an external P(E)-dependent so-called "dual-phase" fed-batch fermentation [23], which separates the growth-phase from a subsequent production-phase to increase efficiency [28]. Guided by these approaches, the (S)-linalool titer finally reached 10.9 g/L with a 5.1% [w/w] yield from glucose in the biphasic fermentation system, which demonstrates the potential for industrial-scale enantioselective (S)-linalool production using P. ananatis.
Results
Search for putative rate-limiting reaction to increase (S)-linalool productivity in SWITCH-PphoC Δgcd/pAaLINS-ispA* strain
The previously constructed (S)-linalool-producing strain (SWITCH-PphoC Δgcd/pAaLINS-ispA*) was generated by integrating pACYC177-P\(_{tac}\)-AaLINS-ispA* (pAaL-INS-ispA*) [15], a co-expression plasmid for AaLINS and ispA*, into the strain SWITCH-PphoC Δgcd [26], which is equipped with genes comprising the whole MVA pathway (Fig. 1). This strain also has a metabolic switch to allow redirection of carbon flux to (S)-linalool via the MVA pathway by sensing Pi-starvation with the PhoB/PhoR two-component system [23]. As a result of test-tube cultivation under Pi-starved conditions using a biphasic fermentation system, SWITCH-PphoC Δgcd/pAaLINS-ispA* produced 3.4 ± 0.2 g/L of (S)-linalool from 60 ± 0.0 g/L of glucose in 48 h (Table 1), which was...
isoprene-producing expression-cassette led to higher productivity in an strain IP04 was also constructed from IP03 by integrating one mvk expression-cassette to increase carbon Δ gcd. The strain IP04 was also constructed from IP03 by integrating one mvk expression-cassette to increase carbon flux to IPP/DMAAPP. However, strains IP03 and IP04 harboring pAaLINS-ispA* (IP03/pAaLINS-ispA* and IP04/pAaLINS-ispA*) showed lower sugar consumption and (S)-linalool productivity than SWITCH-PphoC Δgcd/pAaLINS-ispA* in test-tube cultivation. IP03/pAaLINS-ispA* and IP04/pAaLINS-ispA* were unable to completely consume the initial glucose in 48 h (43 ± 0.6 and 36 ± 0.6 g/L), unlike SWITCH-PphoC Δgcd/pAaLINS-ispA*, and consequently produced only 0.8 ± 0.0 and 1.0 ± 0.0 g/L of (S)-linalool, respectively (Table 1). This result indicates that their lower sugar consumptions may result from intracellular accumulation of cytotoxic intermediates such as IPP and DMAAPP [7, 33]. This assumption was supported by the observation that SWITCH-PphoC Δgcd and IP04 harboring the empty vector (pACYC177), which are likely to accumulate IPP/DMAAPP intracellularly because of the lack of linalool synthase, were unable to completely consume all initial glucose in 48 h (43 ± 3.3 and 38 ± 1.7 g/L, Table 1). These results indicate that a rate-limiting step in (S)-linalool production exists among the reactions from IPP/DMAAPP to (S)-linalool.

Approaches to improve intracellular AaLINS activity
Since the lower sugar consumption in IP04/pAaL-INS-ispA* was thought to be attributed to isoprenoid precursor toxicity, which has been reported to be relieved by enhanced TPS activity [34], AaLINS activity was suspected of being a potential bottleneck in (S)-linalool production. Several TPSs have been identified as primary bottlenecks in terpene biosynthesis because of their poor in vivo properties [35, 36]. In the experiments described above, AaLINS and ispA*, which were codon-optimized for Synechocystis [15], were used even in P. ananatis; however, the codon usages of heterologous eukaryotic genes are commonly optimized for the prokaryotic host [6, 7, 31, 36] in order to improve their translation rate or efficiency. Therefore, the plasmid pAaLINS_pa-ispA*_pa expressing AaLINS_pa and ispA*_pa which were optimized to match the codon-preference of P. ananatis was constructed to increase AaLINS production or activity (Additional file 1: Figure S1 and S2). To confirm whether the expression level of AaLINS was increased by synonymous substitution of codons of AaLINS and ispA*, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted with samples of both SWITCH-PphoC Δgcd/pAaLINS-ispA* and SWITCH-PphoC Δgcd/pAaLINS-ispA*_pa. As a result, a putative band of AaLINS (molecular mass: 63 kDa) was observed in the crude homogenate containing soluble and insoluble proteins, whereas the band of AaLINS_pa was not visible even in the crude homogenate (Additional file 1: Figure S3). Contrary to our expectation, these data revealed that the total expression level of AaLINS_pa was lower than that of AaLINS, even after the codon-optimization for P. ananatis. Consistently, strain SWITCH-PphoC Δgcd/pAaLINS-ispA*_pa produced only 13 ± 1.2 mg/L of (S)-linalool in test-tube cultivation (Additional file 1: Table S1), indicating that

Table 1 (S)-Linalool production in test-tube cultivation

| Host strain          | Plasmid          | (S)-linalool (g/L)* | Consumed glucose (g/L)* | Yield (%)b |
|----------------------|------------------|--------------------|-------------------------|-----------|
| SWITCH-PphoC Δgcd    | pACYC177         | NDc                | 43 ± 3.3                | –         |
| SWITCH-PphoC Δgcd    | pAaLINS-ispA*    | 3.4 ± 0.2          | 60 ± 0.0                | 5.6 ± 0.3 |
| SWITCH-PphoC Δgcd    | pBLAaLINS-ispA*  | 3.0 ± 0.0          | 60 ± 0.0                | 5.0 ± 0.0 |
| IP03                 | pAaLINS-ispA*    | 0.8 ± 0.0          | 43 ± 0.6                | 1.9 ± 0.0 |
| IP03                 | pBLAaLINS-ispA*  | 4.0 ± 0.2          | 60 ± 0.0                | 6.6 ± 0.4 |
| IP04                 | pACYC177         | ND                 | 38 ± 1.7                | –         |
| IP04                 | pAaLINS-ispA*    | 1.0 ± 0.0          | 36 ± 0.6                | 2.8 ± 0.1 |
| IP04                 | pBLAaLINS-ispA*  | 4.7 ± 0.3          | 60 ± 0.0                | 7.9 ± 0.2 |

All strains were cultivated for 48 h under biphasic fermentation using isopropyl myristate. Data are expressed as the mean ± SD of at least three biological replicates.

*Glucose and (S)-linalool concentrations are represented by dividing the total amounts by the volume of aqueous culture

b Yield was calculated as grams of product per grams of consumed glucose and is expressed as a percentage. Carbon sources contained in 2 g/L of Bacto yeast extract was not considered in this calculation

c ND, Not detected

greater than the reported linalool titers from yeasts [12, 13, 29, 30] and bacteria [15, 31]. As our previous study showed that additional integration of an mvaES operon expression-cassette led to higher productivity in an isoprene-producing P. ananatis strain [32], the strain preference of P. ananatis; however, the codon usages of heterologous eukaryotic genes are commonly optimized for the prokaryotic host [6, 7, 31, 36] in order to improve their translation rate or efficiency. Therefore, the plasmid pAaLINS_pa-ispA*_pa expressing AaLINS_pa and ispA*_pa which were optimized to match the codon-preference of P. ananatis was constructed to increase AaLINS production or activity (Additional file 1: Figure S1 and S2). To confirm whether the expression level of AaLINS was increased by synonymous substitution of codons of AaLINS and ispA*, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted with samples of both SWITCH-PphoC Δgcd/pAaLINS-ispA* and SWITCH-PphoC Δgcd/pAaLINS-ispA*_pa. As a result, a putative band of AaLINS (molecular mass: 63 kDa) was observed in the crude homogenate containing soluble and insoluble proteins, whereas the band of AaLINS_pa was not visible even in the crude homogenate (Additional file 1: Figure S3). Contrary to our expectation, these data revealed that the total expression level of AaLINS_pa was lower than that of AaLINS, even after the codon-optimization for P. ananatis. Consistently, strain SWITCH-PphoC Δgcd/pAaLINS-ispA*_pa produced only 13 ± 1.2 mg/L of (S)-linalool in test-tube cultivation (Additional file 1: Table S1), indicating that
codon-optimization of AaLINS and ispA* for Synechocystis unexpectedly led to higher AaLINS expression and (S)-linalool titers.

Meanwhile, SDS–PAGE revealed that AaLINS was mostly expressed as insoluble forms in *P. ananatis*; the band of AaLINS was mainly detected in the insoluble fraction (Additional file 1: Figure S3). Specific solubility-tag fusion to the N-terminus of the TPS improves its solubility in *E. coli*, as was found with fusion of the small ubiquitin-like modifier (SUMO) to the (+)-zizaene synthase from *Chrysopegon zizanioides* [37], and of the maltose binding protein (MBP) to the valencene synthase from *Callitropsis nootkatensis* [38]. Therefore, the N-terminal solubility-tag fusion approach was adopted for AaLINS to increase its soluble expression level. To identify an effective fusion partner protein for AaLINS, six solubility-tags joined to a 6×His (AFV1–99 protein from Acidianus filamentous virus 1, BLA, MBP, FKBP-type peptidyl-prolyl cis–trans isomerase, SUMO, and an *E. coli* elongation factor) were evaluated with the *P. ananatis* SC17(0) strain [19] using ready-to-use pSol vectors [39]. Each of the six constructed strains expressed AaLINS fused with one of six solubility-tags under control of a rhamnose-inducible promoter. Control strains capable of expressing untagged AaLINS or a 6×His-tagged AaLINS were also constructed (SC17(0)/pSol-AaLINS and SC17(0)/pSol-HisAaLINS). The results of SDS–PAGE reconfirmed that AaLINS was mostly expressed as insoluble forms in *P. ananatis*. Bands of 6×His-tagged AaLINS and untagged AaLINS were not visible in their soluble protein fractions, whereas they were observed in the crude homogenates (Fig. 2). A faint band appeared at approximately 60 kDa in the untagged control lane in the gel stained with anti-polyhistidine label, which was non-specific (Fig. 2). In contrast, a band of 6×His-BLA-fused AaLINS (molecular mass: 105 kDa) was observed in the soluble protein fraction by both Coomassie Brilliant Blue (CBB) staining and fluorescence staining of His-tagged proteins (Fig. 2b, d). The difference in the migration of 6×His-BLA-fused AaLINS between the crude homogenate and soluble fraction may have occurred due to overloading of AaLINS derived from the insoluble fraction. Fusion of other evaluated solubility-tags did not improve the solubility from baseline or showed a lower degree of improvement than the 6×His-BLA-fusion (Fig. 2). Aside from solubility, all seven AaLINS variants fused with each solubility-tag appeared to show higher total (insoluble and soluble forms) expression level than untagged AaLINS (Fig. 2a, b). These results indicate that the N-terminal 6×His-tag fusion itself influences the total AaLINS expression level and demonstrate that fusing 6×His-BLA to AaLINS is the most promising means of increasing its intracellular expression level, solubility, and activity.

**Biotransformation assay with 6×His-BLA-fused AaLINS**

An in vitro biotransformation assay was conducted to confirm whether 6×His-BLA-fused AaLINS still retained the capability of converting GPP to (S)-linalool, and whether intracellular AaLINS activity could be increased by 6×His-BLA-fusion. The assay consisted of adding crude homogenate or soluble protein fraction of SC17(0)/pSol-AaLINS, SC17(0)/pSol-HisAaLINS, and SC17(0)/pSol-BLAAaLINS to the reaction mixture containing the substrate (GPP) and cofactor (Mg2+), respectively. After a 26-h reaction at 30 °C, 273±23, 241±3, and 550±45 µg/L of (S)-linalool was detected in the reaction buffer containing the soluble protein fraction of SC17(0)/pSol-AaLINS, SC17(0)/pSol-HisAaLINS, and SC17(0)/pSol-BLAAaLINS, respectively (Fig. 3). When the crude homogenates were used for the assay, 339±15, 514±26, and 669±80 µg/L of (S)-linalool was detected in the reaction buffer containing samples of SC17(0)/pSol-AaLINS, SC17(0)/pSol-HisAaLINS, and SC17(0)/pSol-BLAAaLINS, respectively (Fig. 3). This result shows that fusing only 6×His-tag to AaLINS increased its total activity despite nearly no solubility enhancement compared to untagged AaLINS (Fig. 2), which was supported by the result that the (S)-linalool titers of strain SWITCH-PphoC Δgcd expressing IspA*_pa and 6×His-tagged AaLINS (pa 306±49 mg/L) was approximately 30 times higher than that of SWITCH-PphoC Δgcd/pAaLINS_paispA*_pa (Additional file 1: Table S1). These data reveal that even with the 6×His-BLA-moiety attached, the (S)-linalool producing capability was maintained, and that N-terminal fusion of 6×His-BLA increased the total intracellular AaLINS activity.

**Collective impact of 6×His-BLA-fusion to AaLINS and enhancement of the MVA pathway on (S)-linalool production**

To evaluate the impact of 6×His-BLA-fusion to AaLINS on (S)-linalool production, strains SWITCH-PphoC Δgcd, IP03, and IP04 harboring pBLAAaLINS-ispA*, a plasmid for over-expressing IspA* and 6×His-BLA-fused AaLINS, were cultured in test tubes. IP03/pBLAAaLINS-ispA* and IP04/pBLAAaLINS-ispA* produced 4.0±0.2 g/L of (S)-linalool with a 6.6±0.2% yield, respectively (Table 1). Both IP03/pBLAAaLINS-ispA* and IP04/pBLAAaLINS-ispA* completely consumed the initial glucose, unlike IP03/pAaLINS-ispA* and IP04/pAaLINS-ispA*. These data indicate that the bottleneck of (S)-linalool production in IP04/pAaLINS-ispA*
untagged AaLINS, was intracellular AaLINS activity. In contrast, SWITCH-PphoC Δgcd/pBLAAaLINS-ispA* produced only 3.0 ± 0.0 g/L of (S)-linalool, which was almost the same as that produced by SWITCH-PphoC Δgcd/pAaLINS-ispA* (3.4 ± 0.2 g/L) (Table 1). Therefore, the putative bottleneck for (S)-linalool production in SWITCH-PphoC Δgcd/pBLAAaLINS-ispA* was the supply of IPP/DMAPP or GPP, and this bottleneck was eliminated by the additional integration of the mvaES operon and mvk expression cassette in a step-by-step manner. As a result of the collective effects of both the higher intracellular AaLINS activity imparted by fusion to 6×His-BLA and higher GPP supply by enhancement of the MVA pathway, the (S)-linalool titer of IP04/pBLAAaLINS-ispA* was approximately 1.4-fold greater than that of SWITCH-PphoC Δgcd/pAaLINS-ispA*.

In addition, to confirm whether the linalool synthesized from GPP by 6×His-BLA-fused AaLINS was still exclusively (S)-enantiomer, the culture sample of SWITCH-PphoC Δgcd/pBLAAaLINS-ispA* was analyzed by gas
chromatography mass spectrometry (GC–MS) with a chiral column. Only one enantiomer (retention time: 6.5 min) was detected in the sample (Fig. 4a); its peak corresponded to the (S)-enantiomer peak of racemic linalool. Furthermore, the fragment ion mass spectrum of this product matched the spectral data of the linalool
standard (Fig. 4b, c). These data reveal that $6 \times$ His-BLA-fusion did not affect the enantioselectivity of AaLINS.

**Fed-batch fermentation with IP04/pBLAAaLINS-ispA* strain**

To investigate the (S)-linalool-producing ability of IP04S/pBLAAaLINS-ispA*, a fed-batch fermentation, which is relevant to industrial processes, was conducted in a 1-L scale fermenter; SWITCH-PphoC Δgcd/pAaLINS-ispA* was also cultured as a control strain. One prominent strategy for high terpene production is to divide fermentation into a growth-phase and a subsequent production-phase (dual-phase fermentation), as this method can bypass the allocation of substrate between cell-growth and the target product and alleviate the effects of the accumulation of cytotoxic intermediates from the MVA pathway during the growth phase [28].

An external P$_i$-dependent dual-phase fed-batch fermentation process was previously established for isoprene production using the SWITCH-PphoC strain [23]. The P$_i$-starvation-inducible metabolic switch enables cells to grow efficiently under the P$_i$-saturated phase and efficiently produce (S)-linalool under the subsequent P$_i$-starved phase. Thus, a P$_i$-starved fed-batch fermentation (1.8 g/L of KH$_2$PO$_4$) was conducted using the biphasic fermentation system. The culture temperature was optimized from the previous study [23] in which the culture temperature was set at 33 °C. The results are summarized in Fig. 5. As the optical density at 600 nm (OD$_{600}$) could not represent the biomass concentration accurately owing to oil-in-water emulsion formation in biphasic fermentation [40], the profile of the CO$_2$ concentration in the exhausted gas (ExCO$_2$) was exploited as an index of total cell activity in the fermenter. The patterns of ExCO$_2$ showed almost no difference between the two strains for

![Fig. 5](image-url)
up to 13 h of cultivation (Fig. 5a), which indicates that both strains grew with the same efficiency regardless of their genotypic differences. This result demonstrates that our $P_f$-dependent dual-phase process contributed to overcoming both the competition for acetyl-CoA between cell-growth and (S)-linalool and intracellular accumulation of cytotoxic compounds in the growth phase. This feature of dual-phase fermentation also enables estimation of the approximate cell density when transiting to (S)-linalool production phase ($P_f$-starved phase), despite the difficulty of monitoring the actual cell density with OD$_{600}$ values in biphasic fermentation. The growth profile during the $P_f$-starved phase should be nearly the same regardless of the existence of IPM and genotypic differences. When the strain SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA* was cultured (1.8 g/L of KH$_2$PO$_4$) without IPM, its OD$_{600}$ value at 13 h was 33. The ExCO$_2$ of both strains declined at 13 h of cultivation (Fig. 5a), demonstrating that external $P_f$-starvation started at this time. After entering the $P_f$-starved phase, the ExCO$_2$ profile of IP04/pBLAAaLINS-ispA* was lower than that of SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA* (Fig. 5a), indicating that IP04/pBLAAaLINS-ispA* redirected higher carbon flux from acetyl-CoA to the MVA pathway from the tricarboxylic acid (TCA) cycle, which generates CO$_2$ and NADH for cell respiration, as compared to SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA*. SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA* accumulated higher levels of (S)-linalool than IP04/pBLAAaLINS-ispA* until approximately 60 h of cultivation (Fig. 5b). However, at 72 h of cultivation, despite less sugar consumption (63.5 g, Fig. 5c), IP04/pBLAAaLINS-ispA* produced larger amounts of (S)-linalool (9.3 g/L) than SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA*, which produced 7.7 g/L of (S)-linalool from 81.4 g of glucose (3.7% yield). A similar fermentation profile was observed in the other fed-batch fermentation (Additional file 1: Figure S4), though the culture temperature and initial KH$_2$PO$_4$ concentration (1.6 g/L) were different. Since the (S)-linalool titer of IP04/pBLAAaLINS-ispA* was still linearly increasing at 72 h of cultivation unlike SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA* (Fig. 5b), the culture time of IP04/pBLAAaLINS-ispA* was elongated to 81 h. As a result, IP04/pBLAAaLINS-ispA* produced a total of 10.9 g/L (final concentration) of (S)-linalool from 72.4 g of glucose (5.1% yield), whereas it accumulated 7.2 g/L of MVA in the aqueous culture medium as a main by-product.

**Discussion**

Although advancements in the field of metabolic engineering have improved the production level of terpenes, linalool production has been confined to the mg/L-scale [12, 13, 15, 29–31]. However, our metabolically engineered *P. ananatis* strain IP04/pBLAAaLINS-ispA* produced enantiopure (S)-linalool with a 10.9 g/L titer under $P_f$-dependent dual-phase fed-batch fermentation. This is the highest reported titer for microbial production of not only (S)-linalool but also all monoterpenes [10].

In this study, the use of *AaLINS* (codon-optimized for *Synechocystis*) unexpectedly led to higher AaLINS expression compared to the use of *AaLINS_pa* (codon-optimized for *P. ananatis*). When we previously evaluated 8 homologs of AaLINS, the codons of which were optimized for *P. ananatis* based on the OptimumGene algorithm [41], in SWITCH-PphoC $\Delta gcd$, the linalool titers were less than 120 mg/L, although SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA* produced 1.6 g/L of (S)-linalool [26]. Further studies of the codon-optimization of linalool syntheses are required. We also found that AaLINS was mainly expressed as insoluble form in *P. ananatis*. To improve AaLINS solubility, a solubility-tag fusion approach was applied to AaLINS. This approach is one of the commonly used methods for increasing the solubility of “difficult-to-express” heterologous proteins in bacterial cells. As a result of screening commonly used solubility-tags, 6×His-BLA was identified as a suitable one for AaLINS to increase its soluble production level; however, AaLINS aggregation could not be completely avoided by the N-terminal 6×His-BLA-fusion as observed in SDS–PAGE analysis, which demonstrates the positive effect of 6×His-BLA-fusion on solubility improvement is limited. Additionally, the mechanism of action underlying differences in solubility-tag efficacy has not been investigated. Furthermore, it has been revealed that 6×His-fusion may increase the expression level and activity, as seen in a previous study of a hyaluronidase production in a methylobacterial yeast [42].

Unlike SWITCH-PphoC $\Delta gcd/pAaLINS$-ispA*, IP04/pAaLINS-ispA* was unable to completely consume the initial glucose in 48 h as well as IP04/pACYC177 in test-tube cultivation. IP04/pAaLINS-ispA* likely stopped consuming glucose after the start of $P_f$ starvation because of over-accumulation of cytotoxic IPP/DMAPP, which led to a lower (S)-linalool titer. Compared to SWITCH-PphoC $\Delta gcd$, IP04 is more likely to accumulate these cytotoxic intermediates intracellularly because of enhanced carbon flux to the MVA pathway when AaLINS activity was not high enough to avoid their excess accumulation. This issue was attenuated by increased intracellular AaLINS activity via 6×His-BLA-fusion. IP04/pBLAAaLINS-ispA* completely consumed the initial glucose and showed higher productivity compared to SWITCH-PphoC $\Delta gcd/pBLAAaLINS$-ispA*.

Our $P_f$-exploiting dual-phase fed-batch fermentation process has advantages for industrial-scale production,
including simple operation to transit cells to the production phase, no requirement for exogenous inducers, and restricted respiratory activity, which is required for aerobic fermentation at industrial scale in order to fulfill technical constraints such as oxygen and heat transfer. Furthermore, we can aim to achieve the theoretical (S)-linalool yield from glucose in the production (P_i-starved) phase. This is because ATP production and consumption can be stoichiometrically balanced from glucose to (S)-linalool via standard Embden-Meyerhof-Parnas (EMP) glycolysis and the MVA pathway [9, 43]. Four moles of NADPH, which are required to yield one mole of (S)-linalool from 6 acetyl-CoA via the MVA pathway, can be supplied via the EMP glycolysis by providing 12 NADH to yield 6 acetyl-CoA from 3 glucose as long as NAD(P) transhydrogenase (locus tag PAJ_1324 and 1325) is functional [9, 43]. To realize this, we aim to improve our P_i-dependent metabolic switch to allow for dynamic metabolic control by developing conditional metabolic on/off systems [44] to shut off carbon flux to competing pathways such as TCA cycle and oxidative pentose-phosphate pathway, which is estimated to be upregulated by P_i-starvation [45].

The yield (5.6%) in fed-batch fermentation was lower than that in the test-tube cultivation (7.9 ± 0.2%). However, the yield could be increased by elongating cultivation time (production-phase), as the cumulative yield continued to increase along with the culture time at the termination of production (Fig. 5d). Our next target is to further increase the (S)-linalool yield and titer; however, the upper limit of the titer is closely related to the properties and amount of in situ extraction organic solvent (IPM). Higher production of (S)-linalool by the P. ananatis strain leads to a higher (S)-linalool titer in the aqueous medium, according to the (S)-linalool distribution coefficient for IPM. Once more than 1 g/L of cytotoxic (S)-linalool accumulated in the aqueous phase, the growth and metabolic activity of P. ananatis were significantly reduced (Additional file 1: Figure S5), as reported for other Gram-negative bacteria [11, 46], and (S)-linalool production would be hampered. IPM has been used as an organic solvent in our studies [15, 26] because of its high biocompatibility with microbes and high distribution coefficient of monoterpenes [25]. However, IPM is not an economically viable solvent; thus, not only increasing the titer but also enhancing the resistance to monoterpene toxicity of P. ananatis [46, 47] to decrease the amount of IPM are necessary to reduce the production cost for industrial production.

IP04/pBLLaaLINS-ispA® accumulated 7.2 g/L of MVA into the medium at the end of fed-batch fermentation, which suggests that a pathway downstream of (S)-linalool biosynthesis, particularly AaLINS activity, may still be a potent bottleneck in this strain. Therefore, additional means may be required to increase intracellular (S)-linalool synthase activity. Lowering the culture temperature or co-expression of chaperones, which is known to improve heterologous protein solubility [48], should be considered; using protein engineering to screen mutant variants of AaLINS with improved performance is also an option [35, 36]. Another prominent option is using other linalool synthases that are known to produce only the (S)-enantiomer such as those from Cinnamomum osmophloeum [49] or Malus domestica [50] if their kinetic parameters and solubilities in bacteria are superior to those of AaLINS. Additionally, our platform strain and fermentation process can theoretically synthesize a multitude of different monoterpenes with almost the same productivity as (S)-linalool by changing only AaLINS to other mono-TPS, although it would depend on the solubility/stability and kinetic parameters of mono-TPS.

Conclusions
We achieved a 10.9 g/L titer of (S)-linalool on the basis of SWITCH-PphoC Δgcd/pAaLINS-ispA® via three main approaches: (1) improving intracellular activity of AalINS, (2) increasing the precursor (GPP) supply, and (3) applying dual-phase fed-batch fermentation. Our results demonstrate that fermentative enantiopure “natural” (S)-linalool production with a metabolically engineered P. ananatis strain is a promising system that is environmentally-friendly and can be readily industrialized, although additional studies are needed to improve the economic viability of this process. Mass production of enantiopure (S)-linalool may contribute to accurate assessment of its biological properties, as most studies have been performed with (R)-linalool or linalool racemate [11].

Methods
Bacterial strains, plasmids, and growth conditions
The primary bacterial strains and plasmids used in this study are listed in Table 2. Other strains and plasmids used as materials for strain construction are listed in Additional file 1: Table S2. The primers used in this study are listed in Additional file 1: Table S3. E. coli strain JM109 (Takara Bio, Otsu, Japan) was primarily used for plasmid cloning and propagation. The DNA fragment was cloned into a linearized vector with an In-Fusion® HD cloning kit (Takara Bio). The plasmid was transformed into P. ananatis as previously reported [19]. Antibiotics were used to maintain plasmids or screen antibiotic-resistant transformants with the following concentrations: chloramphenicol (Cm: 60 mg/L), kanamycin (Km: 50 mg/L), and tetracycline (Tet: 10 mg/L).
Table 2  Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Antibiotic resistance | Source or reference |
|------------------|-------------|-----------------------|---------------------|
| Pantoea ananatis | SC17(0) λ Red resistant strain | – | [19] |
| SWITCH-PphoC Δgcd | SC17(0) ΔampC::P_tac XDyl ΔampH.P_phec-mvaES ΔcrtEXYB-criZ P_tac-mvk Δgcd | – | [26] |
| IP03 | SWITCH-I-PphoC Δgcd ΔL-ldh::P_phec-mvaES | – | This study |
| IP04 | IP03 ΔadhE.P_tac-φ10-mvk | – | This study |
| Plasmid | pSol-AaLINS | pSol plasmid for expression of AaLINS under the control of a rhamnose-inducible promoter | Km | This study |
| | pSol-HisAaLINS | pSol plasmid for expression of hexahistidine-tagged AaLINS | Km | This study |
| | pSol-BSLAaLINS | pSol plasmid for expression of AaLINS fused with β-lactamase from Chromohalobacter sp. 560 joined to hexahistidine | Km | This study |
| | pACYC177-P_tac-AaLINS-ispA* (pAaLINS-ispA*) | pACYC177 derivative for expression of AaLINS and ispA* under the control of tac promoter | Km | [15] |
| | pBLSAaLINS-ispA* | pACYC177 derivative for expression of the gene of AaLINS fused with β-lactamase from Chromohalobacter sp. 560 joined to hexahistidine and ispA* under the control of tac promoter | Km | This study |

*Km* kanamycin

**Construction of plasmids for expressing solubility-tag fused AaLINS**

An Expresso Solubility and Expression Screening System (Lucigen Corp., Middleton, WI, USA) was used to fuse each solubility-tag to AaLINS in *E. coli* strain E. coli® 10G, according to the manufacturer’s protocol [39]. A DNA fragment of AaLINS was PCR-amplified from pAaLINS-ispA* using primers Lin-fw/Lin-rv and cloned into the linear pSol vectors [39]; the obtained plasmids are listed in Table 2 and Additional file 1: Table S2. A DNA fragment of AaLINS was PCR-amplified from pAaLINS-ispA* using primers P19/Lin-rv and then ligated to a vector fragment, which was PCR-amplified from pSol-BSLAaLINS using primers pSOL-fw/pSOL-rv, to yield pSol-AaLINS. A DNA fragment of the gene of 6×His-BLA fused AaLINS was PCR-amplified from pSol-BSLAaLINS using primers His-fw/LIS-rv and then ligated to a vector fragment, which was PCR-amplified from pAaLINS-ispA* using primers P-fw/P-rv to yield pBSLAAaLINS-ispA*. The sequence data for the genes of AaLINS and IspA*, which were optimized based on the codon-preference of *Synechocystis*, are available in GenBank (GenBank accession numbers: LX078595.1 and LX078599.1).

**Construction of φ80-integrative conditional replication, integration, and modular (CRIM) plasmid, pAH162-P_tac-φ10-mvk**

The DNA fragment containing mvk from *M. paludicola* was PCR-amplified from pAH162-P_tac-mvk [23] using primers P1/P2, and then cloned into a linearized vector that was PCR-amplified with primers P3/P4 from pIspSM [23] to yield pSTV28-P_tac-φ10-mvk. The DNA fragment containing the P_tac-φ10-mvk region was PCR-amplified from pSTV28-P_tac-φ10-mvk using primers P5/P6, and then ligated to the *PstI/HindIII*-digested pAH162-λattL-Tc⁸-λattR [51] and transformed into *E. coli* strain PIR2 (Thermo Fisher Scientific, Waltham, MA, USA).

**Construction of strains IP03 and IP04 using the Dual-In/Out strategy**

Strains IP03 and IP04 were constructed from SWITCH-PphoC Δgcd [26] by the Dual-In/Out strategy [20, 51]. The φ80attl·Km⁸-φ80att DNA fragment flanked with 50-bp homologous to the *L-ldh* (locus tag PAJ_p0276) and the *adhE* (locus tag PAJ_1411) site was PCR-amplified from pMWatphi [51] using primers Ldh-F/Ldh-R and adhE-F/adhE-R, respectively. To create a φ80att site in SC17(0), the PCR product was transformed by λRed-dependent homologous recombination [19]. After the removal of helper plasmid and Km⁸-marker gene [20], SC17(0) ΔL-ldh::φ80attB and SC17(0) ΔadhE::φ80attB were generated, respectively. To obtain strains SC17(0) ΔL-ldh::pAH162-P_phec-mvaES and SC17(0) ΔadhE::pAH162-P_tac-φ10-mvk, the CRIM plasmid pAH162-P_phec-mvaES was integrated into SC17(0) ΔL-ldh::φ80attB, and pAH162-P_tac-φ10-mvk was integrated into SC17(0) ΔadhE::φ80attB by φ80-dependent recombination with pAH123-cat [20]. The genomic DNA of SC17(0) ΔL-ldh::pAH162-P_phec-mvaES was purified, fragmented, and then electroporated into SWITCH-PphoC Δgcd to transfer the chromosomal modification
**Preparation of the bacterial lysate**

The SC17(0) strains harboring each pSol plasmid for expressing each AaLINS variant or AaLINS were grown on Luria–Bertani (LB)-agar for 16 h at 34 °C. A single colony was inoculated into 3 mL of LB liquid medium containing 0.4 g/L glucose and 4 g/L of rhamnose. After cultivation at 30 °C for 21 h on a reciprocal shaker at 120 rpm (OD600 was approximately 6), the harvested cells were washed twice with extraction buffer (50 mM MOPS [pH 7.0], 10 mM MgSO4·5H2O, 10% [v/v] glycerol, and 1 mM dithiothreitol), and re-suspended in the same buffer. The cells were disrupted by sonication at 4 °C to obtain the crude homogenate including soluble and insoluble proteins. The crude homogenate was centrifuged (21,600 × g, 10 min, 4 °C) to obtain the supernatant or soluble protein fraction.

**SDS–PAGE analysis**

Protein concentration was quantified with a Pierce BCA protein assay kit (Thermo Fisher Scientific). The soluble protein fraction containing 10 µg of protein was subjected to SDS–PAGE, and the crude homogenate was applied with the same volume (µL) of the corresponding soluble protein fraction. The samples were reduced at 70 °C for 10 min with NuPAGE Sample Reducing Agent (Thermo Fisher Scientific). Proteins were separated on a NuPAGE 4–12% Bis–Tris protein gel (Thermo Fisher Scientific) at 200 V for 90 min, and then stained with InVision His-Tag In-Gel Stain (Thermo Fisher Scientific) to visualize the His-tagged fusion proteins with an anti-polyhistidine label (nickel-nitrilotriacetic acid), according to the manufacturer’s protocol. Fluorescence images were obtained at an excitation wavelength of 520 nm. The gel was re-stained with CBB. An XL-Ladder Broad (intégrale Co., Ltd, Tokyo, Japan) was used as the molecular weight marker.

**Single-vial biotransformation assay**

The protein concentration of the soluble protein fraction was diluted to 300 mg/L in a final volume of 1 mL extraction buffer, which was previously supplemented with 15 µM (final concentration) GPP lithium salt (Sigma-Aldrich, St. Louis, MO, USA), in a 22-mL Crimp Top vial (PerkinElmer, Waltham, MA, USA). For the assay with the crude homogenate, the same volume (µL) of the corresponding soluble protein fraction was applied in 1 mL extraction buffer with GPP. The vials were tightly capped with a 20 mm Crimp Top Aluminum Silver Cap with PTFE/Butyl Septa (PerkinElmer) and incubated at 30 °C for 26 h on a reciprocal shaker (120 rpm). Two hundred microliters of IPM were injected into the vial, which was thereafter vigorously shaken to extract (S)-linalool from the reaction buffer. The IPM layer was diluted by five-fold with ethanol, which was used to quantify (S)-linalool using a gas chromatography flame-ionization detector (GC–FID) as described below. Triplicate reactions were performed with bacterial lysates from three independent transformant colonies.

**(S)-linalool production in test tubes**

Cultivation was conducted essentially as previously reported [26]. The concentrations of glucose and KH2PO4 in the medium were set at 60 and 0.5 g/L, respectively. The test tubes were shaken at 30 °C for 48 h in a reciprocal shaker (120 rpm). At least triplicate cultivations with independent transformant colonies were evaluated.

**Fed-batch fermentation in 1-L fermenter**

The cells were incubated at 34 °C for 16 h on an LB-agar plate including Km (dish diameter: 88 mm). Whole cells on a plate were harvested with a 10-µL inoculation loop and then transferred into 270 mL of medium (44 g/L glucose, 1.1 g/L MgSO4·7H2O, 2.2 g/L Bacto yeast extract [BD Biosciences, Franklin Lakes, NJ, USA], 1.8 g/L KH2PO4, 1.1 g/L (NH4)2SO4, 1.1 g/L trisodium citrate, 11 mg/L MnSO4·5H2O, 11 mg/L FeSO4·7H2O, 0.11 mL/L antifoam reagent GD-113 K [NOF Corporation, Tokyo, Japan] and Km) overlaid with 30 mL of IPM in a 1-L fermenter. The glucose solution (700 g/L) containing 0.7 mL/L of GD-113 K was continuously fed from 9 h to the end of cultivation to maintain the glucose concentration at more than 5 g/L. The feeding rate was at approximately 1.5 mL/h for IP04/pBLAAaLINS-ispA* and at approximately 2.0 mL/h for SWITCH-PphoC Δgcd/pAaLINS-ispA*. Fermentation was aerobically conducted with 300 mL/min aeration; the culture temperature was set at 34 °C for 15 h, and then shifted to 30 °C and kept at 30 °C until the end of cultivation; the culture pH was maintained at 6.8 with ammonia gas. The oxygen and CO2 concentrations in the exhausted-gas were measured every hour with an exhaust oxygen CO2 meter Model EX-1562–1 (Able & Biott Co., Tokyo, Japan).

**Analysis of metabolites**

The OD600 was measured using a spectrometer (U-2900; Hitachi, Tokyo, Japan). The (S)-linalool concentration
was quantified as follows. After vigorously vortexing the culture samples (mixture of cells, medium, and IPM), 100 µL of the aliquot was added to 900 µL of ethanol. These diluted samples were centrifuged (21,600 × g, 5 min, 4 °C). The supernatants were used for (S)-linalool quantification with a GC-2025AF (Shimadzu, Kyoto, Japan) equipped with DB-5 capillary column (diameter, 0.25 mm; length, 30 m; thickness, 0.25 µm) (Agilent Technologies, Santa Clara, CA, USA) and FID as previously reported [15, 26]. The concentration of (S)-linalool produced was quantified using a standard curve. (S)-linalool concentration is represented, being assumed to completely exist in aqueous culture. After fractionizing the biphasic fermentation broth into a cell pellet, aqueous supernatant, and IPM fraction by centrifugation (21,600 × g, 10 min, 4 °C), glucose and MVA concentrations in the aqueous supernatant were quantified as previously reported [23]. The IPM fraction was used to identify the product, and the type of enantiomer was determined by GC–MS (Agilent 7890A GC and 5975C MSD, Agilent Technologies) equipped with a chiral GC capillary column Rt-bDEx3 (RESTEK Corporation, Bellefonte, PA, USA) (diameter, 0.25 mm; length, 30 m; thickness, 0.25 µm) using helium as the carrier gas. The injection temperature was maintained at 230 °C. The GC oven temperature gradient was as follows: 115 °C hold for 10 min, increased to 225 °C (10 °C/min), and held for 9 min. (R)- and (S)-linalool racemic reagent (Fujifilm Wako Pure Chemical, Osaka, Japan, catalog number: 126–00,993) and (R)-linalool reagent (Sigma-Aldrich, catalog number: 62139-25ML) were used to confirm the retention times of the (R)- and (S)-enantiomers.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01543-0.

Additional file 1: Figure S1. Nucleotide sequences of AaLINS_pa and ispA*_pa. Figure S2. Construction of plasmids for over-expression of both AaLINS_pa and IspA*_pa. Figure S3. SDS–PAGE gel illustrating total, soluble, and insoluble expression of AaLINS and AaLINS_pa. Figure S4. Typical time-course profiles of fed-batch fermentation (1.6 g/L of KH2PO4) of SWITCH-PpHCoL &amp; pAaLINS-ispA* and pIP4/pBLAAaLINS-ispA*. Figure S5. Growth inhibition of P. ananatis SC17 strain by exogenous linalool. Table S1. (S)-Linalool production in test-tube cultivation. Table S2. Bacterial strains and plasmids used for strain construction. Table S3. Primers used in this study.

Abbreviations

AaLINS, (S)-linalool synthase from Actinidia arguta; AFV: AFV-1-99 protein from Acidaminococcus filamentous virus 1; BLA: Halophilic β-lactamase from Chromohalobacter sp. 560; CB8: Coomassie Brilliant Blue; CoA: Coenzyme A; CRIM: Conditional replication, integration, and modular; DMAPP: Dimethylallyl pyrophosphate; EMP: Embden–Meyerhof–Parnas; EnCo2: Carbon dioxide in exhausted gas; FID: Flame-ionization detector; 6× His: Hexahistidine; GC: Gas chromatography; GPP: Geranyl pyrophosphate; Int: Integrase; IPA: Isopropyl myristate; IPP: Isopentenyl pyrophosphate; IspA*: S80F mutant of farnesyl pyrophosphate synthase from E. coli; Km: Kanamycin; LB: Luria–Bertani; MBP: Maltose binding protein; MOPS: 3-(N-morpholino)propanesulfonic acid; MS: Mass spectrometry; MVA: Mevalonate; OD: Optical density; PCR: Polymerase chain reaction; P: Inorganic phosphate; SDS–PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SlyD: FKBP-type peptidyl-prolyl cis–trans isomerase; SUMO: Small ubiquitin-like modifier; TCA: Tricarboxylic acid; Tet: Tetracycline; TPS: Terpene synthase; Tsf: E. coli Elongation factor; Xis: Excisionase.

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Authors’ contributions

NN drafted the manuscript and performed solubility tag screening, SDS–PAGE analysis, biotransformation assay, and fed-batch fermentation. YT and YN designed strains. YY constructed P. ananatis strains. YH designed plasmids for expressing (S)-linalool synthase and established the condition of test-tube cultivation. AM and MM constructed plasmids and conducted test-tube cultivation. YT, YN, and YU supervised the work and reviewed the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional file.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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