High-Level Production of Amorpha-4,11-Diene in a Two-Phase Partitioning Bioreactor of Metabolically Engineered *Escherichia coli*

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Abstract: Reconstructing synthetic metabolic pathways in microbes holds great promise for the production of pharmaceuticals in large-scale fermentations. By recreating biosynthetic pathways in bacteria, complex molecules traditionally harvested from scarce natural resources can be produced in microbial cultures. Here we report on a strain of *Escherichia coli* containing a heterologous, nine-gene biosynthetic pathway for the production of the terpene amorpha-4,11-diene, a precursor to the anti-malarial drug artemisinin. Previous reports have underestimated the productivity of this strain due to the volatility of amorphadiene. Here we show that amorphadiene evaporates from a fermentor with a half-life of about 50 min. Using a condenser, we take advantage of this volatility by trapping the amorphadiene in the off-gas. Amorphadiene was positively identified using nuclear magnetic resonance spectroscopy and determined to be 89% pure as collected. We captured amorphadiene as it was produced in situ by employing a two-phase partitioning bioreactor with a dodecane organic phase. Using a previously characterized caryophyllene standard to calibrate amorphadiene production and capture, the concentration of amorphadiene produced was determined to be 0.5 g/L of culture medium. A standard of amorphadiene collected from the off-gas showed that the caryophyllene standard overestimated amorphadiene production by approximately 30%.

Keywords: fermentation; amorphadiene; terpene; *Escherichia coli*; heterologous pathway

INTRODUCTION

The need for new, effective, and affordable anti-malarial drugs is on the rise as the malaria parasite has developed resistance to drugs in current use. Artemisinin, which is made by the plant *Artemisia annua*, is an effective anti-malarial drug. However, because extraction from the plant is costly, the developing nations most in need of anti-malarial therapy cannot afford artemisinin. Biosynthesis of plant pharmaceuticals in microbial fermentations can drastically reduce the cost of production while stabilizing the supply and enhancing the purity of the drug. Although the fermentation of bioactive compounds is commonplace, most processes rely on the fermentation of a microorganism that naturally produces the molecule of interest. To date, examples of the heterologous production of pharmaceuticals are largely restricted to protein therapeutics such as blood coagulation factors, insulin, and growth factors, which may be encoded by one or two genes (Schmidt, 2004). A more sophisticated strategy involving the expression of an optimized biosynthetic pathway for a small molecule drug (non-protein therapeutic) in a tractable host, such as *Escherichia coli*, could ensure a steady, consistent supply of new drugs at a greatly reduced cost.

However, the development of microbial strains for heterologous production of small molecule products from simple carbon sources has been hampered by the difficulty in expressing the large and complex pathways necessary for production of the non-native molecule. Despite the difficulties involved, progress has been made on the heterologous production of several classes of small molecule therapeutics such as polyketides, non-ribosomal peptides, and isoprenoids (Peiru et al., 2005; Pfeifer et al., 2001; Watts et al., 2005). These model systems prove that the production of non-native

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small molecules by large heterologous pathways in microorganisms is possible, although substantial engineering and optimization remains in order to transform the above examples into hosts for industrial production.

We recently reported the synthesis of the artemisinin precursor, amorpha-4,11-diene, by an engineered strain of *E. coli* containing a nine-gene heterologous pathway (Martin et al., 2003). We hypothesized that our reported production titers of amorphadiene, 24 mg/L, was an underestimate because amorphadiene was being lost from the cultures. Two-phase partitioning bioreactors (TPPB) have been used to separate hydrophobic products or reactants from growing cultures in the liquid phase (Daugulis and Janikowski, 2002). While many TPPBs have been employed to sequester toxic substrates for release to the aqueous phase in a controlled manner (Collins and Daugulis, 1999), TPPBs are also an attractive means for in situ product removal (ISPR) where the product is toxic or inhibits further production (Daugulis, 1997). For our purposes, the TPPB is useful for trapping an isoprenoid product that may be lost due to air-stripping from the fermentation. This is an issue that likely will affect the recovery of many microbially produced volatile isoprenoids and terpenes.

In this work, we show that amorphadiene can be lost from culture media and that an organic phase can trap amorphadiene and prevent some of the loss. We describe production of amorphadiene by an engineered strain of *E. coli* in a two-phase partitioning bioreactor that uses a hydrophobic *n*-dodecane overlay to trap amorphadiene. We also show that overall amorphadiene titers can be increased by supplementing the culture with increased carbon and complex nutrients. By increasing substrate and eliminating loss of product through volatilization, we improved the titers of amorphadiene 20-fold, generating approximately 0.5 g/L.

MATERIALS & METHODS

Strain and Media

The *E. coli* strain used in the study was W3110 (ATCC 27325) transformed with pMevT, pMBIS, and pADS (Martin et al., 2003), which we will refer to as the amorphadiene producing microbe (APM) since these three plasmids confer high-level amorphadiene production via the mevalonate pathway (Table I). Strains were cultivated at 37°C in Terrific Broth, TB (per liter: 12 g Tryptone, 24 g Yeast Extract, 14.9 g phosphate, and 1% glycerol), or Luria Broth, LB (per liter: 10 g Tryptone, 5 g Yeast Extract, 10 g NaCl) as indicated. The necessary antibiotics were added to all the media at the following concentrations: 100 mg/L ampicillin, 5 mg/L tetracycline, and 25 mg/L chloramphenicol. The transcription of the heterologous pathway was induced by adding 0.5 mM IPTG to the culture at in early log phase. *n*-dodecane was added to shake flask cultures as indicated at a 20% (v/v) ratio.

Bioreactor Studies

The 10-L Bioflo bioreactor (New Brunswick) was assembled as to manufacturer's instructions, including an ice water-cooled condenser. Six liters of TB with 1% glycerol (v/v) was inoculated with 50 mL of over-night culture of W3110 containing the three plasmids pMevT, pMBIS, pADS (Table I) grown in LB medium containing the same antibiotics at the same concentrations. Antifoam (Sigma-Aldrich, St. Louis, MO) was added at 10 µl/L. Oxygen was supplied as filtered air at 1 v/v/m and agitation was adjusted to maintain dissolved oxygen levels above 30%. The temperature of the culture in the fermentor was controlled at 37°C and pH was controlled at 7.0 using 10% NaOH. Cells were induced at an optical density of approximately 0.3, as measured at a wavelength of 600 nm (OD_{600}), with 0.5 mM IPTG. Dodecane was added aseptically to 20% (v/v) of the media volume. Two aliquots of carbon in the form of glycerol were added to a final concentration of 1% (v/v) as the culture entered stationary phase. One aliquot of TB (dry stock) was added in stationary phase as indicated (for 6 L: 72 g Tryptone, 144 g Yeast Extract, 1.39 g monobasic potassium phosphate, 7.5 g dibasic potassium phosphate). Fermentations were run approximately 60 h post-induction.

Collection of Amorphadiene in Two-Phase Partitioning Bioreactors, Abiotic TPPB, or Condensers

To collect amorphadiene in situ, a second organic layer of dodecane was added aseptically to the fermentations or shake flasks, to a final volume of 10% (v/v), 30 min after induction with IPTG. To sample the two phases, an aliquot (0.5 mL for shake flasks or 2–3 mL for the bioreactor) was collected and the emulsified dodecane/culture was separated in a microfuge tube by centrifugation at 10,000*g* for 2 min. The

Table I. Strains and plasmids used in this study.

Strain/plasmid	Genotype/description	Source/reference
W3110	F-, IN(rrnD-rrnE)1, lambda	ATCC
pADS	pTrc99A (Amann et al., 1988) containing synthetic *ADS* under control of P_{TRC}; Ap^r, pBR322 origin, $lacI^Q$; produces amorpha-4,11-diene from farnesyl pyrophosphate	Martin et al. (2003)
pMBIS	pBBR1MCS-3 (Kovach et al., 1995) containing *MK*, *PMK*, *MPD*, *idi*, and *ispA* under control of P_{LAC}; Tc^R; produces farnesyl pyrophosphate from mevalonate	Martin et al. (2003)
pMevT	pBad33 (Guzman et al., 1995) derivative containing the *atoB*, *HMGS*, and *tHMGR* genes under control of P_{LAC}; pACYC184 origin, Cm^r; produces mevalonate from acetyl coenzyme A	Martin et al. (2003)

organic sample was diluted 1 µl into 700 µl of ethyl acetate for analysis by gas chromatography-mass spectrometry (GC-MS) as described below. The aqueous layer from biphasic cultures was extracted 100 µl into 700 µl of ethyl acetate by vortexing for 20 min in 2-mL glass serum vials capped with Teflon tops; it has been demonstrated that >97% of the amorphadiene produced in TPPB is located in the organic phase and that amorphadiene is stable at 37°C in dodecane for at least 1 week (data not shown). Seven-hundred microliters of aqueous phase from single phase cultures or the abiotic TPPB was extracted with 700 µl of ethyl acetate by vortexing for 20 min as above and was analyzed by GC-MS.

To collect amorphadiene by condensation, a cold trap cooled with dry ice and ethanol was attached in series to the first condenser of the bioreactor. Amorphadiene was condensed from the off-gas of a fermentation of the APM without the addition of *n*-dodecane. Initial bioreactor and media conditions were identical to those described above. Six liters of TB with 1% glycerol (v/v) was inoculated with 50 mL of overnight culture of the APM grown in LB. The cold trap was attached to the off-gas after the culture was induced with 0.5 mM IPTG at an OD_{600} of approximately 0.33. As the cell growth rate decreased at later times (approximately 10 h after induction) a pulse of 35 mL of 90% glycerol was added to the culture. Agitation rate and air flow was increased from 500 to 650 rpm and from 1 to 1.5 v/v/m, respectively, through the course of the fermentation to maintain dissolved oxygen above 30%.

Determination of Amorphadiene Loss

A 500-mL volume of TB in a 2-L baffled shake flask was inoculated with 5 mL of overnight growth of *E. coli* strain W3110 harboring pMEVT, pMBIS, and pADS, induced with 0.5 mM IPTG at an OD_{600} of approximately 0.25–0.3, and allowed to grow and produce amorphadiene for 24 h after induction. This culture was then centrifuged in 250 mL bottles at maximum speed for 1 h in a cold centrifuge and filter-sterilized into a container on ice. The long centrifugation time removed enough cells and particles that the supernatant could be easily filtered through a single 0.22 µm filter, which resulted in significant loss of amorphadiene, presumably due to processing of the medium, and adsorption to the nylon filter. The resulting cold, sterile medium was transferred via a siphon into a sterile 1 L Bioflo 110 fermentor, operating at the same conditions as typical growth experiments (temperature: 37°C, agitation: 500 rpm, air flow: 1 v/v/m). Samples of the medium were taken over time, extracted with ethyl acetate as described above, and the amorphadiene concentrations in the liquid medium were measured over time by GC-MS. The rate of volatilization was calculated as the rate of loss from the liquid medium.

Isolation of Amorphadiene

Amorphadiene was collected from the off-gas of fermentations. First, the water was condensed from the off-gas using a condenser cooled with 0°C water. The more volatile amorphadiene was condensed from the fermentor off-gas using a dry ice/ethanol cold trap connected after the water-cooled condenser. Amorphadiene was trapped as a frozen emulsion in the cold trap for the duration of the fermentation. After fermentation, the amorphadiene was removed from the trapped emulsion by extraction with ethyl acetate. The resulting emulsion was extracted three times with ethyl acetate. The combined organic layer was then dried over sodium sulfate and the ethyl acetate removed by rotary evaporation to give a clear oil, which was analyzed by GC-MS and NMR spectroscopy. Once the purity was estimated by GC-MS and NMR, the material was weighed and used to construct a true standard curve using dilutions of weighed material.

GC-MS Analysis

The amorphadiene product was analyzed in total ion scan mode using a D13-XL13 Universal Non-Polar column (J&W, 0.25 mm inner diameter × 0.25 µm film ×30 m length) on an HP G6890 gas chromatograph attached to an HP 6973 MSD. Samples of 1 µL were injected into the GC, which was run in splitless mode using helium as a carrier gas with a flow rate of 1 mL per minute. The injector temperature was 250°C and the oven program consisted of three steps: (1) 80–160°C, 10°C/min, (2) 160–175°C, 5°C/min, and (3) 175–300°C, 10°C/min. A solvent delay of 3 min was employed for samples containing dodecane.

Amorphadiene production by engineered *E. coli* was measured by GC-MS as previously described (Martin et al., 2003) by scanning only for two ions, the molecular ion (204 *m/z*) and the 189 *m/z* ion. Amorphadiene concentrations were converted to caryophyllene equivalents using a caryophyllene standard curve, and the relative abundance of ions 189 and 204 *m/z* to their total ions as described (Martin et al., 2003). The sesquiterpene caryophyllene was purchased from Sigma-Aldrich.

Nuclear Magnetic Resonance (NMR) Characterization

All spectra were collected in $CDCl_3$ (Cambridge Isotope Laboratories, Andover, MA) at the University of California, Berkeley College of Chemistry NMR Facility. 1D ^1H spectra and all 2D spectra were collected using a Bruker AVQ-500 spectrometer while 1D ^{13}C spectra were collected using a Bruker AVB-400 spectrometer. All chemical shifts are reported using the standard δ notation in parts per million; positive chemical shifts are to higher frequency from the given reference. Assignments were made by COSY, HMQC, HMBC, and DEPT. When reported, multiplicity and coupling constants are derived from the 1D ^1H spectrum. ^1H NMR (500 MHz, $CDCl_3$, 25°C): $\delta = 5.08$ (1H, s, H_5), 4.88 (1 H, d, $J = 1.5$ Hz, H_{12a}), 4.66 (1H, s, H_{12b}), 2.57 (1H, s (broad), H_6), 2.01 (H_7), 1.99 (H_{2a}), 1.98 (H_{2b}), 1.92 (H_{3a}), 1.80 (H_{3b}), 1.78 (3H, s, H_{13}), 1.67 (H_{9a}), 1.62 (3H, d,

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$J = 0.5$ Hz, H_{15}), 1.54, H_{8a}), 1.42 (H_{10}), 1.33 (H_1), 1.27 (H_{8b}), 0.99 (H_{9b}), 0.90 (3H, d, $J = 6.5$ Hz, H_{14}). ^{13}C NMR (400 MHz, $CDCl_3$, 25°C): $\delta = 148.0$ (C_{11}), 134.6 (C_4), 120.9 (C_5), 109.8 (C_{12}), 47.6 (C_7), 41.8 (C_1), 37.6 (C_6), 35.4 (C_9), 27.9 (C_{10}), 26.5 (C_3), 26.1 (C_8), 25.8 (C_2), 23.6 (C_{15}), 22.6 (C_{13}), 19.8 (C_{14}).

RESULTS

Microbially Produced Amorphadiene Partitions to an Organic Phase

Previous work demonstrated amorphadiene production and loss from shake flask cultures of *E. coli* harboring pMevT, pMBIS, and pADS (Martin et al., 2003), the APM. We assumed loss due to volatilization; however, other mechanisms of loss have not been ruled out. To test the volatilization hypothesis, we attempted to trap amorphadiene in an organic phase in shake flask cultures of the APM. We chose the long-chain hydrocarbon dodecane due to its hydrophobicity as reflected in a high log*P* value [6.6 (Janikowski et al., 2002)] that would predict low toxicity to *E. coli* (Ramos et al., 2002). Furthermore, dodecane has low volatility that we determined to be negligible over the course of a 3-day cultivation (data not shown). We also determined empirically that dodecane does not affect the growth rate of *E. coli* in shake flasks (data not shown). Based on these characteristics, dodecane presented a reasonable choice of solvent for a TPPB, however an extensive survey of organic solvents would be useful in future studies to optimize this system.

The APM was grown in TB in shake flasks with and without dodecane, and amorphadiene was measured in the dodecane overlay and in the culture medium, respectively (Fig. 1). Indeed, 8.5-fold more amorphadiene was detected in APM cultures with a dodecane overlay (281.4 mg/L ± 51.4 SD) than from cultures without the overlay (33.3 mg/L ± 9.3 SD). These data confirm that, as expected, our previous estimate of 24 mg/L amorphadiene production by the APM was a significant underestimate due to the loss of amorphadiene from the culture.

Semi-Volatile Compound Amorphadiene Is Lost From Fermentations via Air-Stripping

Our results show that amorphadiene from cultures can be trapped in an extracting dodecane phase. However, it is possible that loss of amorphadiene from the culture occurred due to microbial degradation of the compound, rather than volatilization. We monitored amorphadiene levels during simulated bioreactor conditions with no cell present (sterile). Under standard bioreactor conditions, amorphadiene loss from the bioreactor demonstrates a first-order decay with a half-life of about 50 min (Fig. 2), thus confirming that amorphadiene loss occurs rapidly from clarified medium. Extraction from the off-gas condenser using ethyl acetate revealed high levels of amorphadiene. The majority (50–70%) of the amorphadiene initially added to the bioreactor

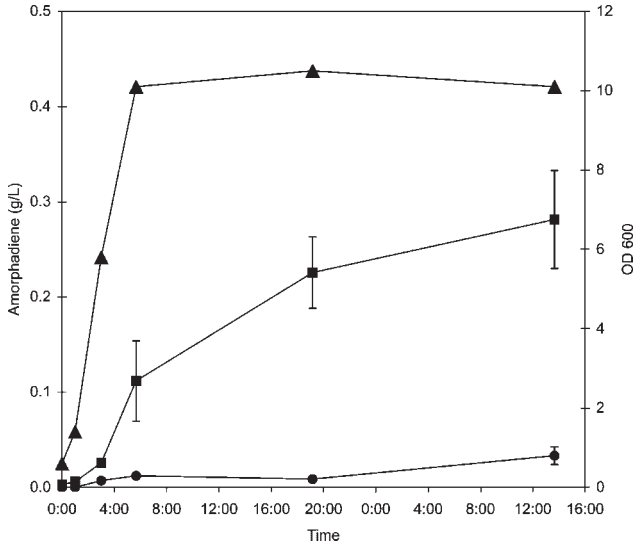

Figure 1. Production of amorphadiene in cultures with and without dodecane. *E. coli* W3110 cultures harboring pMevT, pMBIS, and pADS were grown in TB in shake flasks with (■) or without (●) dodecane, and amorphadiene production was monitored. Data are the average concentration of three cultures. Error bars represent standard deviation. Culture OD_{600} (▲) was measured at each time point.

was recoverable from the cold (0°C) condenser, which confirms that volatilization is the primary mechanism for loss of amorphadiene from this clarified medium and suggests that volatilization is a major mechanism of loss in liquid cultures.

Production of Amorphadiene in a Two-Phase Partitioning Bioreactor

Previous work had indicated that carbon supply limited the production of amorphadiene in shake flasks (Martin et al., 2003). To determine if additional carbon in larger scale

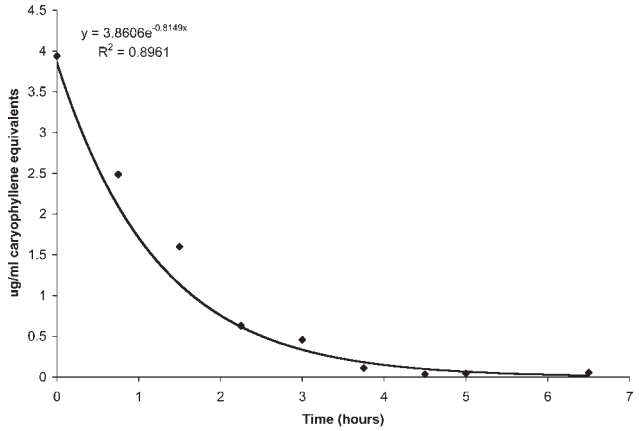

Figure 2. Amorphadiene loss from a bioreactor. Biologically produced amorphadiene in spent medium was filter sterilized and added to sterile bioreactor operating under standard parameters. Amorphadiene concentration in the liquid phase was monitored over time as described in Materials and Methods. The loss of amorphadiene with time successfully demonstrates a first order decay (solid curve). Experiments were performed in duplicate with an error of <5%.

cultures would increase amorphadiene titers, we supplemented a bioreactor cultivation of the APM with additional carbon and compared its production of amorphadiene to a bioreactor cultivation using standard TB medium. Additional carbon was supplied as pulses of glycerol (added to a final concentration of 1% v/v) once the culture reached a high density. Dodecane overlays were used in both cases to capture amorphadiene. Production with standard TB resulted in amorphadiene levels similar to those seen in shake flask experiments (Fig. 3). Additional pulses of glycerol (to 1% v/v) were added as respiration reached a plateau, which resulted in an increase in respiration (as measured by dissolved oxygen levels and CO_2 production, data not shown) for the first addition but not the second addition of glycerol. Additional yeast extract and tryptone, without glycerol was added (Fig. 3; as indicated in Materials and Methods), which also increased respiration. Addition of carbon and nutrients as the cells entered stationary phase increased the cell mass and nearly doubled overall the production of amorphadiene (0.48 g/L). Most of the increase in productivity occurred after the cells had ceased growing, indicating that carbon and nutrient availability was limiting production of amorphadiene at later time points. These data suggest that product volatilization and nutrient limitation both contribute to the diminished capacity reported for the heterologous mevalonate pathway expressed in this microbe. A trap set up on the out-gas in a dodecane fermentation demonstrated that less than 1% of the amorphadiene escaped capture in the organic layer (data not shown). Total production may still be underestimated since traps may not be 100% efficient in collecting amorphadiene. However, when two traps were placed in series, less than 1% of the total recoverable amorphadiene was found in the second trap in single- or two-phase fermentations, indicating that very little amorphadiene escapes the first trap. A detailed and systematic study of carbon and nutrient requirements as well as fermentation conditions and solvents will be the subject of future study.

Recovery of Microbially Produced Amorphadiene

We next sought to recover pure amorphadiene produced during a fermentation of the APM. To determine if pure amorpha-4,11-diene could be obtained from a simple condensation of the off-gas, we performed a single-phase fermentation with a condenser and a cold trap. GC-MS analysis indicated that the major product, amorphadiene, was found in 89% abundance with other sesquiterpene side-products. This profile is consistent with the literature value of 91% as reported for in vitro reactions using purified amorphadiene synthase and FPP (Picaud et al., 2005). The identity of the major product was established conclusively using 1D- and 2D-NMR spectroscopy (Fig. 4). The 1D ^{13}C spectrum was identical to that reported in the literature (Ngo et al., 1999) within 0.1 ppm and confirms the identity of the major sesquiterpene product as amorpha-4,11-diene. Proton and carbon assignments were made using COSY, HMQC, HMBC, and DEPT experiments. The assignments were consistent with previous reports, except for the assignments of C_2, C_8, and H_2 and H_8. The present assignments of C_2 and C_8 are supported both by the HMQC and HMBC experiments. In addition, H_2 and H_8 assignments were changed based on the C_2 and C_8 assignments, as well as the HMQC spectrum.

DISCUSSION

We previously speculated that the observable loss of amorphadiene from shake flask cultures was due to the volatility of the terpene product. Here our data from multiple

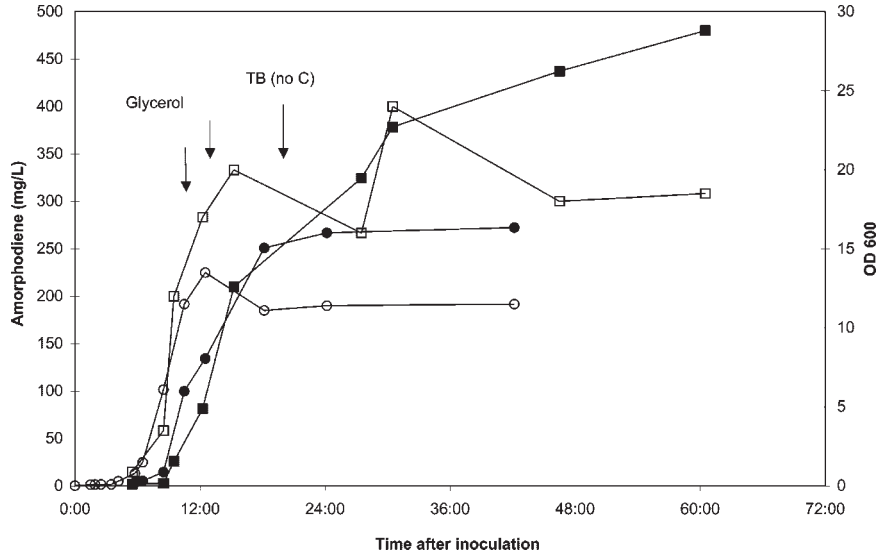

Figure 3. Production of amorphadiene in a partitioning (two-phase) bioreactor. *E. coli* W3110 harboring pMevT, pMBIS, and pADS grown in two 10-L bioreactors with 10% (v/v) dodecane. The engineered strains were grown on TB or TB with additional carbon in the form of glycerol or TB (arrows). Cultures with standard TB: OD_{600} (○), amorphadiene concentration (●). Cultures with additional carbon: OD_{600} (□), amorphadiene concentration (■).

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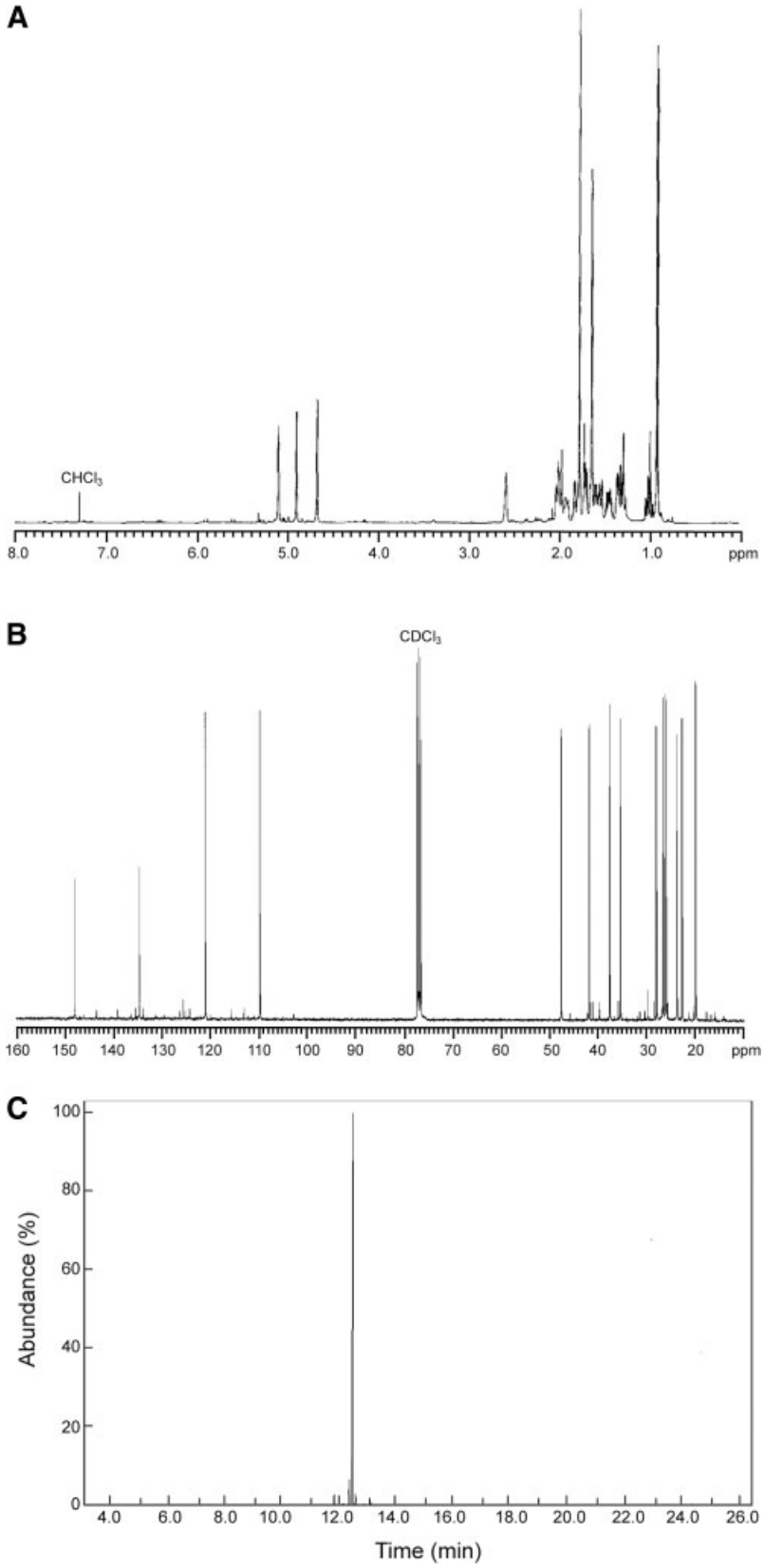

Figure 4. Preparative recovery of amorphadiene. 1D NMR spectra of amorphadiene. **A**: ^{1}H NMR spectrum collected at 500 MHz in $CDCl_3$. **B**: ^{13}C NMR spectrum collected at 400 MHz in $CDCl_3$. **C**: Gas chromatogram of product extracted from an APM bioreactor culture. Amorphadiene was present in 89% abundance.

bioreactor studies as well as from shake flask cultures clearly demonstrate that volatilization of amorphadiene from the culture is significant, and that a significant increase in recovered product occurs when amorphadiene production is carried out in the presence of a dodecane phase. While these data do not rule out the possibility of loss due to other mechanisms, our findings strongly suggest that evaporative loss is a major cause of low amorphadiene titers in single phase fermentations.

Despite a high boiling point of approximately 270°C, the amorphadiene readily volatilizes from the aqueous medium. Based on calculations using Advanced Chemistry Development (ACD/Labs) Software Solaris V4.67, amorpha-4,11-diene and sesquiterpene isomers are only sparingly soluble in water. As expected in such an immiscible liquid mixture, the vapor phase is enriched for the dilute component of the system, explaining the short half-life of amorphadiene in the culture.

To overcome the rapid loss of amorphadiene from the culture, an organic layer of dodecane was added to the medium. This alkane is also only sparingly soluble in water. Any amorphadiene produced from the cells quickly partitioned to dodecane, as expected from a large calculated water:octanol partition coefficient (Log *P*-value) of 6.6, which causes amorphadiene to become trapped in the hydrophobic layer. The multi-component system continues to favor the volatilization of the organics. However, the second liquid phase, in which the amorphadiene is solubilized, is of significant quantity to prevent a rapid loss to the air. The two-phase fermentation therefore retains the bulk of the produced amorphadiene, which is concentrated in the dodecane.

Use of a second phase of dodecane to trap amorphadiene allows a more accurate measurement of product formation from our engineered strains. By decreasing product loss to the headspace we can achieve approximately 8.5-fold higher titers of sesquiterpenes. Although we have demonstrated this method for microbially produced amorphadiene, the partitioning bioreactor provides a more accurate quantification of many different terpenes produced from the fermentation of a variety of recombinant microbes and yeast. Experiments are underway to move to a defined medium and to identify the exact nutrient requirements of the APM as well as to investigate a solvent layer most appropriate to downstream purification and processing of amorphadiene.

Although amorphadiene volatility from aqueous culture presents an additional complication in accurate product measurement, we have used it to our advantage to produce and purify a sesquiterpene in essentially one unit step. Such purification is further eased in a bioreactor where the half-life of amorphadiene is only 50 min, in comparison to a shake flask where it has a half-life of 3 h (data not shown). The rapid loss is due in part to the fact that the high aeration rate in the bioreactor equates to a high rate of air stripping.

Both present and previous work (Martin et al., 2003) reports the production titers of amorphadiene in units of caryophyllene equivalents because of the lack of a commercial source of amorpha-4,11-diene standard. Pure amorpha-4,11-diene recovered from the microbial cultivation was subsequently used to construct a true standard curve. In doing so, we found that the substitution of caryophyllene as a calibrant for amorphadiene was sufficiently accurate, as we were overestimating amorphadiene concentration by only 30%. With the use of this new standard, we can now determine amorphadiene concentrations more accurately and thereby more accurately calculate the metabolic fluxes within the APM. This information will then guide further engineering and optimization of the microbe.

CONCLUSIONS

From our partitioning bioreactor cultivations, we have demonstrated that two-phase culture can successfully trap volatile terpenes, like the sesquiterpene amorpha-4,11-diene, thereby allowing a more accurate quantification of product formation. By implementing this method and increasing the carbon substrate levels, we have shown that expression of a heterologous yeast mevalonate pathway and codon-optimized amorphadiene synthase in *E. coli* yields approximately 0.4 g/L of amorphadiene product in 30 h from standard media. To the best of our knowledge, this remains the highest reported heterologous production of a sesquiterpene. We plan to continue optimizing bioreactor media and conditions to use a minimal medium with balanced nutrients and optimal carbon delivery. A future, detailed study of medium development should further increase product concentrations.

Using the volatility of our terpene product, we have created a pure standard of amorpha-4,11-diene, which is useful for more accurately measuring production from our engineered strains. While the loss of amorphadiene from aqueous cultures may confound measurements of production, recovering the amorphadiene material from the condensate may be a simple method to obtain purified material from microbial productions. Of equal or greater importance, the pure amorphadiene will also prove useful as an enzyme assay substrate for elucidating the remaining steps in the pathway to the anti-malarial compound, artemisinin. Ample pure hydrocarbon terpene substrate is often necessary to prove the function of hydroxylating enzymes expressed heterologously in both *in vitro* and *in vivo* experiments (Carter et al., 2003).

With this method in place, we can continue our efforts of engineering multigene enzymatic pathways in both microbes and yeast for the production of heterologous terpenoid products.

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