Balancing glucose and oxygen uptake rates to enable high amorpha-4,11-diene production in *Escherichia coli* via the methylerythritol phosphate pathway

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Abstract
Amorpha-4,11-diene (AMD4,11) is a precursor to artemisinin, a potent antimalarial drug that is traditionally extracted from the shrubs of *Artemisia annua*. Despite significant prior efforts to produce artemisinin and its precursors through biotechnology, there remains a dire need for more efficient biosynthetic routes for its production. Here, we describe the optimization of key process conditions for an *Escherichia coli* strain producing AMD4,11 via the native methylerythritol phosphate (MEP) pathway. By studying the interplay between glucose uptake rates and oxygen demand, we were able to identify optimal conditions for increasing carbon flux through the MEP pathway by manipulating the availability of NADPH required for terpenoid production. Installation of an optimal $q_{O_2}/q_{glucose}$ led to a 6.7-fold increase in product titers and a 6.5-fold increase in carbon yield.

KEYWORDS
amorpha-4,11-diene, FBA, MEP pathway, NADPH, oxygen uptake, terpenoid

1 | INTRODUCTION

Malaria is a life-threatening disease caused by parasites within the *Plasmodium* group. In 2018, the World Health Organization (WHO) reported around 228 million cases of malaria, with 0.17% or 405,000 of these cases ultimately ending in death (WHO Report, 2019). Most of these deaths were attributed to infections by the parasite *P. falciparum*, which has unfortunately become resistant to most of the conventionally used drug therapies, such as mefloquine, chloroquine, and so forth (Bloland, 2001). In 2015, the WHO began recommending the use of artemisinin combination therapies for the treatment of malaria (Olumese, 2006). As the name suggests, artemisinin derivatives are the primary component of these therapies and are used in combination with other antimalarial drugs like lumefantrine and mefloquine to slow the progression of multidrug resistance.

Artemisinin is a sesquiterpene lactone peroxide which is primarily extracted from the *Artemisia annua* shrub. Despite its important use in antimalarial therapies, its availability and price fluctuate by as much as 10-fold from season to season owing to fickle agricultural dynamics related to supply and demand (Peplow, 2016). To stabilize the economics of artemisinin, alternate biotechnological routes have long been sought for its production. In 2013, Sanofi launched a semisynthetic artemisinin product after a more than decade-long and $43 M+ initiative to engineer the mevalonate (MVA) pathway in yeast to produce the precursor artemisinic acid...
followed by chemical conversion to artemisinin (University of California, Berkeley, 2004). However, at ~$400/kg, the price of semisynthetic artemisinin was still more than double that of its agricultural counterpart, and the process was terminated after just a year of production (Peplow, 2016). As such, there continues to be interest and renewed efforts in developing a more economical biotechnological route for artemisinin production.

In this study, we focus on the production of amorpha-4,11-diene (AMD4,11), the sesquiterpene precursor of artemisinin (Figure S1). AMD4,11 can be oxygenated into artemisinin alcohol, artemisinic aldehyde, and artemisinic acid via a cytochrome P450 enzyme, with the latter two reactions further enhanced by alcohol dehydrogenase and aldehyde dehydrogenase enzymes. While enzymes have been identified for the further biological conversion of artemisinic acid, the semisynthetic process for artemisinin relies on photochemical conversion of artemisinin into artemisinin (Kung et al., 2018).

AMD4,11 is produced from farnesyl pyrophosphate (FPP) using amorphanediene synthase (ADS). FPP can be produced from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the building blocks for terpenoids (Kirby & Keasling, 2009) which are generated in nature via two distinct biological pathways. The MVA pathway is native to yeast, and the methylenylthritol phosphate (MEP) pathway is native to bacteria, such as Escherichia coli. A stoichiometric analysis of these two pathways reveals that the MEP pathway is superior to the MVA pathway for two reasons. First, it has a higher maximum theoretical yield on carbon (1.255 mol glucose per mol of IPP for MEP vs. 1.5 mol glucose per mol of IPP for MVA), making it the most carbon-efficient pathway for terpenoid production (Yadav et al., 2012). Second, the MEP pathway has a zero-net balance of NADH in contrast to the MVA pathway which generates 4 NADH, resulting in a more robust, redox-balanced system (Appendix, Figure S2). It is important to note that the use of bacterial hosts also has clear advantages over eukaryotic platforms like yeast. Because of their faster growth rates, fermentation batch lengths for bacteria are significantly shorter than yeast (2–3 vs. 5–10 days), which enables both higher productivities and improved process economics. As such, there is significant interest in utilizing the MEP pathway with E. coli as the host for terpenoid production.

The objective of our study was to identify critical process parameters for optimizing AMD4,11 production via the MEP pathway in E. coli in 2 L bioreactors. We studied the impact of monophasic (aqueous) versus biphasic (10% oil) systems on mass transfer and cellular respiration. Through intracellular metabolite analysis and flux balance analysis (FBA), we were able to assess changes in cofactor (NADH/NADPH) and energy (adenosine triphosphate [ATP]) demands which influenced performance and flux through the pathway. Finally, by manipulating relative carbon and oxygen uptake rates, we were able to achieve sustained biomass specific productivities of >1.8 mg/DCW-h and a carbon yield of >5%, thus paving the way for the commercial use of the MEP pathway for terpenoid production.

2 | MATERIALS AND METHODS

2.1 | Strain E. coli AMD

E. coli AMD strain was provided by Manus Bio. This strain, derived from E. coli MG1655, was engineered for improved flux through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway toward the common terpenoid precursors IPP/DMAPP (Kumaran et al., 2019). The ADS gene derived from A. annua was integrated to enable the production of AMD4,11.

2.2 | Preculture cultivation

Preculture cultivation was started by inoculating 0.8 ml of frozen cell stock (30% glycerol) of E. coli strain AMD in 400 ml of batch media. Nonbaffled Erlenmeyer flask with total volume of 2000 ml was used for cultivation. One liter of batch media contained 15 g of glucose, 40 mg of FeSO4·7H2O, 1 ml of 1000× trace elements (10 mg MnCl2·4H2O, 3.2 mg of ZnSO4·7H2O, 1 mg of CuCl2·2H2O, 40 mg of CaCl2·2H2O, 0.50 mg of H3BO3, and 2 mg of Na2MoO4·2H2O), 300 mg of MgSO4, 4 mg of thiamine hydrochloride, 3.6 g of (NH4)2SO4, 12 g of KH2PO4, 4.58 g of K2HPO4, 5 g of yeast extract, and 1 g of citric acid. A total of 0.5 ml of antifoam (Struktol®J 647; Schill+Seilacher) was added to prevent foaming. The shake flask was incubated overnight at 37°C installing 110 RPM and was harvested during exponential growth with optical density (OD) of about 3–4 (detected with 600 nm) before inoculating the 2 L Dasgip bioreactor. Inoculum size was 5% of the bioreactor recipe volume.

2.3 | Cultivation in bioreactors

Cultivations in bioreactors were carried out in duplicate installing fed-batch mode. One liter of batch medium for 2 L Dasgip fermenter contained 12 g of glucose, 40 mg of FeSO4·7H2O, 1 ml of 1000× Trace Elements (10 mg MnCl2·4H2O, 3.2 mg of ZnSO4·7H2O, 1 mg of CuCl2·2H2O, 40 mg of CaCl2·2H2O, 0.50 mg of H3BO3, and 2 mg of Na2MoO4·2H2O), 300 mg of MgSO4, 4 mg of thiamine hydrochloride, 7.2 g of (NH4)2SO4, 12 g of KH2PO4, 4.58 g of K2HPO4, 10 g of yeast extract, and 1 g of citric acid. Ten percent (v/v based on initial volume) organic phase of safflower oil was added to the biphasic system. No such addition was made to the control condition yielding a monophasic system. Preliminary tests disclosed that the oil phase does not serve as carbon source for the E. coli cells (see Appendix). A total of 0.5 ml of antifoam agent (Struktol®J 647; Schill+Seilacher) was added in the batch media to avoid foaming during the process. When foaming occurred bolus of 0.2 ml of antifoam was added into the fermenter using a syringe at a regular interval. Temperature of 37°C was maintained throughout the process. Before inoculating, pH of the fermenter was adjusted to 7 using 6 N NaOH. pH was maintained at 7 until 42 h using 6 N NH4OH. It also acted as the source of nitrogen along with ammonium sulfate in the batch media. Base was switched to 6 N NaOH after addition of ~180
mmoles of NH₄OH to avoid accumulation of ammonia. Dissolved oxygen (pO₂) level was maintained at 40% of air saturation for first 24 h and then reduced to 15% linearly over the period of 6 h, which was unchanged for the rest of the process. pO₂ levels were regulated using agitation cascaded between 300 and 1200 RPM. Aeration was established at 12 L/h throughout the process.

Depletion of glucose in the batch media was indicated by the spike in the pO₂ levels. With the exhaustion of glucose, fed-batch process was initiated using a feed solution of 500 g/L of glucose, 31.28 g/L of (NH)₂SO₄ and 18.75 ml/L of 25% NH₄OH started to control the rest of the process. Sodium acetate was removed from the bioreactor by centrifugation at 5430R (Eppendorf) at 4°C for 10 min was washed twice with 0.9% NaCl. A total of 0.9% NaCl was added again increasing the total volume to 1 ml which was vortexed to resuspend the pellet. OD₆₀₀ was measured using UV spectrophotometer at 600 nm. For accurate estimation samples were diluted to the optical density between 0.05 and 0.3 before measurement. For cell density measurement, 5 ml of well mixed sample was centrifuged at 1000g at 4°C (5430R; Eppendorf) for 10 min and the supernatant was discarded. The pellet was washed twice with 0.9% NaCl and dried at 105°C for 24 h in a convection oven (Heraeus). Pellet was weighed to estimate the cell-dry weight. Based on the OD measurement biomass to OD correlation was calculated to be 0.224.

### 2.6 Intracellular metabolite measurement

For intracellular measurements around 2 ml sample was withdrawn from the bioreactor using a syringe containing 6 ml of 60% methanol precooled at -30°C for quenching. Syringes were weighed before and after withdrawing the sample. The mixture was transferred to 15 ml falcon tubes and centrifuged at -11°C at 4000g (5430R; Eppendorf) for 10 min. Supernatant was discarded and the cell pellet was immediately flash froze in liquid nitrogen. It was then stored in -70°C freezer for further processing.

Based on the biomass concentration in the fermenter, the necessary extraction volume of 50% methanol was calculated to achieve biomass concentration of 50 g/L after extraction. Pellet was resuspended by repetition of 1 min vortexing and 1 min of cooling in the cryostat maintained at -30°C. After the resuspension 100% chloroform equivoluminal to 50% methanol was added. Then, samples were incubated at -20°C for 1 h. This was followed by further incubation at RT for 1 h. It was centrifuged 4000g for 10 min at 4°C and the topmost polar layer of aqueous methanol was pipetted out and stored at -70°C freezer for analysis.

ATP quantification was performed on the samples using 1200 Series Agilent HPLC equipped with diode array detector for detection of nucleotides. Method for qualification and quantification on HPLC is described in Löffler et al. (2016). For measurement for MEP pathway intermediates, samples were analyzed on Agilent 1290 Infinity II LC system with 6460 QQQ. Method for qualification and quantification on LC-MS system is described in Zhou et al. (2012).

### 2.7 AMD4,11 extraction and quantification

For extraction of AMD4,11, 400 µl of sample was transferred into glass tubes. A total of 1600 µl of ethyl acetate (99.9% purity) was added. The mixture was vortexed for 15 min and then centrifuged at 1000g for 10 min at RT. The topmost layer of ethyl acetate was pipetted out of the tube and transferred to the glass vials to be stored at -70°C for further analysis.

AMD4,11 quantification of samples was performed on Agilent 5840 GC equipped with flame ionization detector using Restek 5MS-Sil 0.25 mm ID, 0.1 µm df, and 30 m length column for separation. A total of 2 µl of sample was injected into the injection port maintained...
at 230°C. Oven temperature was maintained at 90°C for 4 min and then ramped up to 180°C at 5°C/min with the hold time of 3.5 min. It was further increased to 300°C at 50°C/min with the hold time of 5 min. Five-point calibration curve was developed as external reference standard.

2.8 Exhaust gas analysis

Dasgip bioreactors were attached with in line monitoring of exhaust gas composition using a GA4, four channel exhaust gas analyzers equipped with Blue Sens technology. The outlet stream through the fermenter was analyzed for concentration of oxygen, carbon dioxide, and nitrogen in terms of volume percentage.

2.9 Flux balance analysis

We used Cobra Toolbox to perform FBA to optimize ATP4sr reaction, primary ATP synthesis reaction, which was considered as the objective function. iJR904 was used as the base E. coli model and further modifications were implemented according to strain construct (Reed et al., 2003). For synthesis of AMD4,11 from farnesyl diphosphate, AMD was added to the model along with export sink Ex_AMD. Reactions associated with quinol oxidase cytochrome bd (CYTBD) and pyruvate formate lyase (PFL) respectively were deleted for aerobic condition. Reactions representing quinol oxidase cytochrome bo3 (CYTBO3) and pyruvate dehydrogenase (PDH) were deleted for micro-aerobic condition (Partridge et al., 2007). Isocitrate lyase was deactivated, as glyoxylate shunt cycle is repressed by growth on glucose (Cortay et al., 1989). Reactions associated with idonate oxidation IDOND and IDON2, involving NADH and NADPH respectively, acted as a sink for redox molecules. They were identified as futile reactions and hence were deleted. Our model consisted of 1074 reactions and 762 metabolites. P/O ratio of 2 was assumed for phosphorylation. Cellular transport of glucose ($q_{\text{glucose}}$), oxygen ($q_{\text{O2}}$), AMD4,11 ($q_{\text{AMD4,11}}$), and specific growth rate ($\mu$) were limited by the constraints quantified from experimental observations. Experimental observations were parsed out from two different time point 16.75 and 26 h during the fermentation from both biphasic and monophasic system. Based on the constraints, objective function was optimized to predict fluxes using LP solver.

3 RESULTS

3.1 Impact of overlay addition on fermentation performance

The use of organic overlays during sesquiterpene production is a common practice, as it both facilitates the capture and subsequent purification of volatile products and sequesters product from cells to mitigate potential inhibition or toxicity. However, the addition of oil affects the surface tension of the system, thus modifying air bubble breakage and mass transfer coefficients (Dumont & Delmas, 2003). Because this change directly impacts cellular respiration, we sought to first characterize the effect of oil addition on the fermentation process.

We set up two duplicate conditions in 2L DASGIP bioreactors—a monophasic system with no organic overlay and a biphasic system with the addition of 10% (v/v) safflower oil to facilitate product capture. These bioreactors were ran as fed-batch fermentations with a glucose and phosphate feed which was initiated after the initial depletion of glucose in the batch media at 11 h. A glucose-restricted feeding
strategy was implemented to avoid glucose accumulation and byproduct formation (Han et al., 1992), and all fermenters were subjected to the same feed rates.

As seen in Figure 1, we observed differences in both biomass and AMD4,11 accumulation between the two conditions, with the monophasic system outperforming the biphasic system with respect to both overall titer and cell specific productivities. Peak biomass concentrations were 40% higher in the monophasic system compared to the biphasic system (26 vs. 18 g DCW/L), and AMD4,11 titers were almost sixfold higher in the monophasic system at 71 h (3.5 vs. 0.6 g/L; Figure 1a,b).

Although we observed large differences in overall performance, our time course analysis reveals that the deviations between the two systems began with the onset of the fed-batch phase. Despite having established the same feed rates for both systems, biomass specific uptake rates of glucose were higher in the biphasic approach than in the monophasic system (0.29 vs. 0.23 g/DCW·h at 17 h; Figure 2a). Specific oxygen uptake rates were also 20–25% higher for the biphasic system throughout the fed-batch portion of the run (Figure 2b). Interestingly, these higher glucose and oxygen uptake rates correlated with both lower biomass formation and a sharp decline in the specific productivity of AMD4,11. For the oil-free system, AMD4,11 productivities remained at around 4 mg/DCW·h in growing cells and then continued at a low level of 1–2 g/DCW·h until the end of the fermentation. In contrast, we observed a large drop from 5.5 mg/DCW·h after only 19 h into the run for the biphasic condition (Figure 2c).

### 3.2 Intracellular ATP and metabolite measurements

To gain insight into the dynamics of the system, we measured the intracellular levels of three key metabolites—ATP, 1-deoxy-d-xylulose (DOX), and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (MEcPP), the latter two of which serve as important indicators of MEP pathway flux and functionality. Although we attempted to collect data from four time points throughout the run, the final time point (71 h) was excluded for the biphasic system due to severe foaming which impaired our ability to obtain representative samples.

As seen in Figure 3a, we again observed significant deviations between the two conditions tested, with biomass specific ATP levels between 2- and 12-fold higher for the biphasic system compared to the monophasic system throughout the run (Figure 3a). The dynamics of the MEP pathway also varied based on both DOX and MEcPP measurements. DOX is the dephosphorylated product of 1-deoxy-d-xylulose 5-phosphate (DOXP), the first intermediate of

![Figure 2](https://example.com/figure2.png) **Figure 2** Biomass specific dynamics for series 1. Biomass specific uptake rate of (a) glucose ($q_{glucose}$) and (b) oxygen ($q_{O2}$). (c) Specific productivity of amorpha-4,11-diene ($q_{AMD}$) and (d) specific growth rate ($\mu$) for biphasic (10% oil) and monophasic (control) in series 1. Units: $q_{glucose}$, g/DCW·h; $q_{O2}$, mmol/DCW·h; $q_{AMD}$, mg/DCW·h; $\mu$, 1/h [Color figure can be viewed at wileyonlinelibrary.com]
the MEP pathway, and its level gives clues into both the amount of carbon flowing into the MEP pathway and the ability of subsequent enzymes in the pathway to pull this carbon downstream (Figure 4).

MEcPP is a product of IspF and is a double phosphorylated intermediate. Its accumulation also serves as a proxy for further bottlenecks downstream in the pathway. In Figure 3c, we see that DOX is at least 10-fold higher for the biphasic (10% oil) system compared to the monophasic system at both 25 and 50 h. In contrast, MEcPP deviates only after 26 h, with the monophasic system showing a sharp increase to 151.5 µM/DCW at 50 h, and the biphasic system showing a steady level of ~40 µM/DCW throughout (Figure 3b). We note that the large error bars seen at 71 h is reflective of foaming that was observed at this stage of the fermentation.

3.3 | Stoichiometric modeling to estimate carbon distribution and cofactor demands

To estimate the distribution of carbon flux in our two systems, FBA was performed for two time points (16.75 and 24 h) using...
FIGURE 4  MEP pathway. GA-3P reacts with pyruvate to form DOXP. If accumulating, DOXP gets converted into DOX. Metabolites: MEP, CD-ME, CD-MEP, MECPP, HMBPP, IPP, and DMAPP. Enzymes: dxs, dxr, ispD, ispE, ispF, ispG, and idi. CD-ME, 4-diphosphocytidyl-2-C-methylerythritol; CD-MEP, 4-diphosphocytidyl-2-C-methyl-o-erythritol 2-phosphate; DMAPP, dimethylallyl pyrophosphate; DOX, 1-deoxy-o-xylulose; DOX, 1-deoxy-o-xylulose 5-phosphatase; dxr, DXP reductoisomerase; dxs, DOXP synthase; GA-3P, glyceraldehyde 3-phosphate; HMBPP, (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; idi, isopentenyl pyrophosphate isomerase; IPP, isopentenyl pyrophosphate; ispD, 2-C-methyl-o-erythritol-4-phosphate cytidylyltransferase; ispE, 4-diphosphocytidyl-2-C-methyl-o-erythritol kinase; ispF, 2-C-methyl-o-erythritol 2,4-cyclodiphosphate synthase; ispG, HMB-PP synthase; ispH, HMB-PP reductase; MECPP, 2-C-methyl-o-erythritol 2,4-cyclodiphosphate; MEP, 2-C-methylerythritol 4-phosphate [Color figure can be viewed at wileyonlinelibrary.com]

experimentally-generated data ($q_{\text{O}_2}$, $q_{\text{glucose}}$, $q_{\text{AMD}}$) as model inputs/ constraints (Table 1). Since the MEP pathway consumes 1 ATP but does not provide opportunities for regeneration, we chose to maximize the ATP synthesis reaction ATPS4r as our objective function, resulting in

monophasic and biphasic systems. Interestingly, the net NADPH formation of growing cells was 17.6% lower with oil than without oil, a trend that was further amplified at 24 h (Figure 3d). Inversely, fluxes via NAD transhydrogenase (Figure 3e) were highest in the biphasic system at both time points (3.08 mmol/DCW-h at 16 h and 2.5 mmol/DCW-h at 24 h). Fluxes for the ATP synthesis reaction, ATPS4r (Figure 3f) were highest during the growth phase (16 h) in the biphasic system (33.47 mmol/DCW-h) but were reduced by almost half for the other three data points.

3.5 Optimizing glucose and oxygen uptake rates to improve performance of biphasic system

Based on our previous FBA analysis, we postulated that a high $q_{\text{O}_2}$/$q_{\text{glucose}}$ ratio may lead to limited availability of both pyruvate and NADPH, two critical substrates for high MEP pathway functionality. We therefore anticipated that balancing the biomass specific glucose and oxygen uptake rates may improve cell productivities in biphasic systems. To test this, we performed another series of monophasic and biphasic fed batch fermentations. Although the monophasic fermentations were conducted as before, glucose uptake rates were lowered for the biphasic system by feeding glucose at a 10% reduced rate. The aeration regime was left unchanged.

As before, glucose in the batch media was depleted after about 11 h for both systems, after which glucose and phosphate feeding

| Condition | System  | EFT (h) | $q_{\text{O}_2}$ (mmol/DCW-h) | $q_{\text{glucose}}$ (mmol/DCW-h) | $q_{\text{AMD}}$ (mmol/DCW-h) | $\mu$ (1/h) |
|-----------|---------|--------|-------------------------------|---------------------------------|-------------------------------|-------------|
| B-16.75   | Biphasic| 16.75  | 5.38                          | 1.61                            | 0.014                         | 0.074       |
| M-16.75   | Monophasic| 16.75 | 3.42                          | 1.31                            | 0.02                          | 0.09        |
| B-24      | Biphasic| 24     | 3.52                          | 1.25                            | 0.011                         | 0.016       |
| M-24      | Monophasic| 24    | 2.09                          | 0.87                            | 0.019                         | 0.039       |

Note: Values are obtained from the experimental observation for both monophasic and biphasic systems.
was initiated. This time, we observed similar performance between both conditions, with the key parameters, including biomass production, glucose uptake rates, and oxygen uptake rates tracking closely throughout the run (Figure 6). The biphasic fermentations reached 3.5 g/L AMD in 64 h with a sustained cell specific productivity of ~4 mg/DCW·h throughout most of the run, rivaling the performance of the monophasic system (Figure 6c). Similar trends were also seen for intracellular ATP, DOX, and MEcPP measurements as depicted in Figure 7, with DOX and MEcPP levels rising steadily over the course of the fermentation.

In light of above hypothesis, we conducted series 3 and 4 to further consolidate our findings. Series 3 was conducted with mono- and biphasic (10% oil) compositions in duplicates. Both settings were investigated to mirror specific glucose uptake profiles of the biphasic system of series 1 (~0.24 g/DCW/h). As a result, both settings operated at $q_{O2}$ around 5 mmol/DCW·h for most part of the cultivation (Figure S5). As expected, amorphadiene titer was impacted negatively with the monophasic system achieving 1.09 ± 0.02 g/L and biphasic system producing only 0.74 ± 0.06 g/L (Figure S4).

For complementation, series 4 was conducted checking the biphasic approach by installing 2.5% and 10% oil in duplicates. We aimed at mimicking specific glucose uptake rates with those of series 2. Highest $q_{O2}$ for all the three systems was around 2 mmol/DCW·h after 20 h. At the end, that is, after 68.5 h, the monophasic approach reached 3.37 ± 0.16 g/L, 10% oil was 3.82 ± 0.35 g/L and 2.5% oil system was 3.38 ± 0.34 g/L. Figure 8 gives an outline of all the four studies illustrating a relationship between $q_{glucose}$, $q_{O2}$ attained after 20 h of elapsed fermentation time and final AMD4,11 titer of the cultivation. Two clusters were formed indicating the correlation between $q_{glucose}$ and $q_{O2}$. $q_{O2}$ of less than ~2.5 mmol/DCW·h was identified as a region with balanced NADPH supply, thus improving its availability for MEP pathway (indicated by checkered area). On the contrary, $q_{O2}$ above 3 mmol/DCW·h drained NADPH into NADH lowering its accessibility for AMD4,11 production. Lowering the specific glucose uptake rate resulted in lower $q_{O2}$ and improvement in AMD4,11 titer.

4 DISCUSSION

4.1 Use of an organic overlay leads to glucose overfeeding

Because of the hydrophobicity of sesquiterpene molecules, an organic oil overlay is commonly added to the fermentation broth to capture the products in situ from the aqueous phase. To maintain the same overall reactor volume, we reduced the aqueous volume of the biphasic system of series 1 (~0.24 g/DCW/h). As a result, both settings operated at $q_{O2}$ around 5 mmol/DCW·h for most part of the cultivation (Figure S5). As expected, amorphadiene titer was impacted negatively with the monophasic system achieving 1.09 ± 0.02 g/L and biphasic system producing only 0.74 ± 0.06 g/L (Figure S4).
nutrient consumption were more impacted by the utilization of yeast extract, as cells prefer to import amino acids rather than synthesizing it (Lyubetskaya et al., 2006). However, we observed a shift upon the start of glucose feeding, with the biphasic fermentations displaying 20% higher specific glucose uptake rates than the oil-free reactors despite maintaining the same volumetric glucose feed rates for both systems.

4.2 | Glucose overfeeding increases oxygen demands

The rise in specific glucose uptake rates within the biphasic system coincided with increased specific oxygen demands, reduced growth rates, and declining AMD4,11 productivities (Figure 2). Interestingly, the rise in oxygen uptake rates did not just increase proportionally with the glucose uptake rates but resulted in a higher $q_{O2}/q_{\text{Glucose}}$ ratio for the biphasic system. As predicted by our FBA model, this increase leads to a fundamental shift in the distribution of carbon, resulting in lower flux at the pyruvate node which is critical for high MEP pathway functionality (Figure 5). During aerobic respiration, more carbon enters the TCA cycle, pulling from the essential substrate pyruvate and leading to the generation of CO$_2$ which decreases the carbon yield for both biomass and AMD4,11 production. When the biphasic system shifts into a microaerobic state at 24 h (SSB-2), the flux into the TCA cycle is lowered by 96%. Pyruvate is partially diverted toward acetate production, resulting in a fivefold increase in acetate concentration and a 12-fold higher accumulation for the biphasic system compared to the oil-free condition (Figure 1c). These findings reflect the well-known property of E. coli to produce acetate once cells surpass threshold specific rates of glucose consumption (Eiteman & Altman, 2006). The presence of acetate coupled with oxygen limitation adversely affects the specific growth rate of E. coli (Luli & Strohl, 1990).

4.3 | Increased oxygen uptake also lowers NADPH availability

Under aerobic conditions, oxygen is the preferred electron acceptor, linking electron uptake from NADH with the stoichiometric formation of NAD$^+$, ATP, and H$_2$O. Because of the high reactivity of oxygen, its utilization should be safely controlled to prevent the formation of nonwanted radicals and toxic compounds (Imlay, 2013). Accordingly, increased oxygen uptake rates require a proper supply of NADH which is predominately produced in the TCA cycle. In cases of high NADH demand and insufficient supply, cells are also known to use their native transhydrogenase activity to convert NADPH to NADH (Spaans et al., 2015). In Figure 3d, we show that the net NADPH flux availability was 18% lower for the biphasic system.
compared to the oil-free approach. As such, AMD4,11 production accounted for a higher proportion of the NADPH flux in the biphasic system (32% vs. 19%). This suggests that increased glucose feeding and uptake rates in the biphasic system also had the effect of decreasing the availability of NADPH required for AMD4,11 production.

This hypothesis is further supported by the pattern of accumulation observed for key MEP pathway intermediates. As seen in Figure 3, the biphasic fermentations showed significant accumulation of DOX, the dephosphorylated derivative of DOXP. The subsequent conversion of DOXP to MEP via the Dxr enzyme in the MEP pathway requires NADPH as a cofactor; thus, the 10-fold higher accumulation of DOX in our biphasic system suggests limited NADPH availability in this system. As seen in both Figures 2c and 5, this shortage ultimately results in much lower AMD4,11 titers and productivities.

4.4 | Reduction in NADPH availability coincides with increased ATP supply

As seen in Figure 3e, we predict that the reduction in NADPH availability is caused by elevated NADH transhydrogenase activity, which consumes between 69% and 82% of NADPH in the biphasic system compared to 51–62% in the oil-free approach. The resulting NADH enters respiration, which leads to both higher ATP formation rates (Figure 3f) and elevated levels of ATP (Figure 3a) in the
biphasic system. The decrease in ATP concentrations toward 50 h mirrors the decrease in $q_{O_2}$, resulting in inefficient ATP synthesis due to the microaerobic condition (Chapman et al., 1971).

4.5 | Summary of key process parameters influencing AMD4,11 production

Through these fermentations, we have established that increase in $q_{O_2}/q_{glucose}$ negatively impacts MEP pathway flux and hence, AMD4,11 productivities. Taking this data and observations in aggregate, we propose a series of biochemical mechanisms that explains this phenomenon as shown in Figure 9.

We observed that the presence of an organic overlay reduced the aqueous volume in the bioreactor, thus leading to a slight increase in glucose concentrations available to the cells during the fed-batch phase despite the installation of equivalent feed rates. This led to an increase in glucose uptake rates and a corresponding rise in oxygen uptake rates, which positively impacted flux through PDH into the TCA cycle. This shift in carbon flux had the effect of reducing the amount of pyruvate for the MEP pathway. Simultaneously, higher oxygen uptake rates led to a higher utilization of NADH which was partially provided by the transhydrogenase-mediated conversion of NADPH to NADH. Lower overall availability of NADPH also had the effect of reducing the flux through the MEP pathway and into AMD4,11.

The observed phenomenon is specific for the MEP pathway, as the MVA pathway differs in its use of substrates and cofactors and results in the generation of excess NADH (Yadav et al., 2012). Interestingly, other groups have observed synergies between the MEP and MVA pathways and have shown the ability to increase flux through the MEP pathway in NADPH-limiting conditions when both are expressed together in E. coli (Yang et al., 2016). This finding is in agreement with our proposed mechanism in Figure 9.

4.6 | Maximizing yield and productivity by balancing glucose and uptake rates

In a subsequent set of fermentations, we found that lowering the glucose feed rates in our biphasic system by 10% (proportional to the increase seen in oil addition) was successful in completely recovering AMD4,11 production to levels that matched or even exceeded the monophasic condition (Figure 10b). All other measured and

FIGURE 9 Schematic illustration of dependence of flux dynamics on specific uptake rate of glucose ($q_{glucose}$). Green arrows indicate positive effect and red indicate negative effect. Hexagonal boxes represent concentrations while rectangles represent rates [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 10 Cell density, production, and acetic acid for series 2. (a) Biomass concentration (g/L), (b) AMD4,11 titer (g/L), and (c) acetic acid concentration (g/L) for biphasic (10% oil) and monophasic (control) in series 2. AMD4,11, amorpha-4,11-diene [Color figure can be viewed at wileyonlinelibrary.com]
calculated parameters also followed similar trends between both monophasic and biphasic conditions (Figures 10 and 6). Importantly, we observed a six-fold increase in AMD4,11 titers, an almost seven-fold increase in conversion yield, and a 67% drop in acetate accumulation in our biphasic fermentations from the first to second run, showing that we were able to relieve the constraints on both pyruvate and NADPH availability to enable higher fluxes through the MEP pathway to AMD4,11 (Figure 10c). Since we still see a rise in uvaT and NADPH availability to enable higher fluxes through the accumulation in our biphasic fermentations from the first to second run, this was attributed to a short, unintended limitation in oxygen which occurred at 48 h).

Our results compare favorably to other examples of AMD4,11 production in the literature. Tsuruta et al. (2009) used the nonnative MVA pathway in E. coli and achieved 1.94 mg/DCW·h of AMD4,11 in a process lasting 160 h. Our approach reached 2.44 mg/DCW·h of AMD4,11 in just 60 h. Westfall et al. (2012) were able to attain production of 5.5 g/L of AMD4,11 in 95 h with a 3.23 C-mol% yield on glucose in Saccharomyces cerevisiae. If we extrapolate the volumetric productivity of our approach out to 95 h, we estimate reaching titers of about 5.2 g/L AMD4,11. By installing the proper qO2/qglucose conditions in our fermentations, our E. coli AMD4,11 producer achieved a 5.08% C-mol yield which outperforms the results using the MVA pathway.

5 | CONCLUSION

By performing an in-depth multilayered characterization of our work that merged standard fermentation monitoring with metabolomics and stoichiometric modeling (FBA), we were able to unravel the complex metabolic interplay of glucose feeding, oxygen uptake, carbon redistribution, and cofactor availability on MEP pathway functionality and sesquiterpene production. Although the improvements enabled by process parameter tuning were demonstrated for AMD4,11 production, these findings are likely to apply generally to the production of other terpenoid products because they address fundamental problems related to the regulation and flux through the MEP pathway. We anticipate that further tuning of these key parameters will allow us to tap the full potential of the most carbon-efficient pathway for terpenoid production.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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