

RESEARCH ARTICLE

Definitive screening accelerates Taxol biosynthetic pathway optimization and scale up in *Saccharomyces cerevisiae* cell factories

Laura E. Walls^{1,2,3} | José L. Martínez³ | E. Antonio del Rio Chanona⁴ |
Leonardo Rios-Solis^{1,2} 

¹ Institute for Bioengineering, School of Engineering, University of Edinburgh, Edinburgh, UK

² Centre for Synthetic and Systems Biology (SynthSys), University of Edinburgh, Edinburgh, UK

³ Department of Biotechnology and Biomedicine, Section for Synthetic Biology, Technical University of Denmark, Kongens Lyngby, Denmark

⁴ Sargent Centre for Process Systems Engineering, Department of Chemical Engineering, Imperial College London, UK

Correspondence

Leonardo Rios-Solis, Institute for Bioengineering, School of Engineering, University of Edinburgh, Edinburgh, UK.
Email: Leo.Rios@ed.ac.uk

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Abstract

Background: Recent technological advancements in synthetic and systems biology have enabled the construction of microbial cell factories expressing diverse heterologous pathways in unprecedentedly short time scales. However, the translation of such laboratory scale breakthroughs to industrial bioprocesses remains a major bottleneck.

Methods and Major Results: In this study, an accelerated bioprocess development approach was employed to optimize the biosynthetic pathway of the blockbuster chemotherapy drug, Taxol. Statistical design of experiments approaches were coupled with an industrially relevant high-throughput microbioreactor system to optimize production of key Taxol intermediates, Taxadien-5 α -ol and Taxadien-5 α -yl-acetate, in engineered yeast cell factories. The optimal factor combination was determined via data driven statistical modelling and validated in 1 L bioreactors leading to a 2.1-fold improvement in taxane production compared to a typical defined media. Elucidation and mitigation of nutrient limitation enhanced product titers a further two-fold and titers of the critical Taxol precursors, Taxadien-5 α -ol and Taxadien-5 α -yl-acetate were improved to 34 and 11 mg L⁻¹, representing a three-fold improvement compared to the highest literature titers in *S. cerevisiae*. Comparable titers were obtained when the process was scaled up a further five-fold using 5 L bioreactors.

Conclusions: The results of this study highlight the benefits of a holistic design of experiments guided approach to expedite early stage bioprocess development.

KEYWORDS

bioprocess optimization, definitive screening design, high-throughput microbioreactor, *Saccharomyces cerevisiae*, taxol

Abbreviations: CPR, cytochrome P450 reductase; CYP725A4, taxadiene-5 α -hydroxylase; DO, dissolved oxygen; DoE, design of experiments; DSD, definitive screening design; GC-MS, gas chromatography-mass spectrometry; GGPP, geranylgeranyl diphosphate; HPLC, high performance liquid chromatography; OFAT, one-factor-at-a-time; SDG, synthetic defined media with galactose; T5 α Ac, taxadien-5 α -yl-acetate; T5 α ol, taxadien-5 α -ol; TASY, taxadiene synthase; TAT, taxadien-5- α -O-acetyltransferase; YNB, yeast nitrogen base; YPD/YPG, yeast peptone dextrose/yeast peptone galactose; YSM, yeast synthetic drop-out medium supplements without uracil

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1 | INTRODUCTION

The construction of microbial cell factories for the sustainable bio-conversion of renewable feedstocks into complex natural products represents an auspicious alternative approach to traditional organic synthesis routes. Recent advances in synthetic and systems biology have accelerated progress towards this through enabling the construction of strains expressing wide ranging heterologous pathways in unprecedentedly short timescales.^[1] However, the translation of such laboratory scale breakthroughs to industrial scale bioprocess remains a major challenge.^[2] Despite lacking infrastructure for online monitoring and control of parameters such as pH and dissolved oxygen (DO), which are deemed critical to product quality at industrial scale, simple batch microtiter plate or shake flask cultivations are ubiquitously employed in the early stages of bioprocess development. In addition, the largely discredited intuition led one-factor-at-a-time (OFAT) approach is typically employed to guide screening experiments, further limiting process insight during this crucial developmental stage.^[3-5]

Efficient, standardized methods for the detailed characterization of candidate strains under process relevant conditions are critical to alleviating key bottlenecks and accelerating bioprocess development.^[6-8] Quality by design is a strategic approach, which aims to maximize process insight and understanding from the outset to minimize the risks associated with bioprocess scale-up. Statistical design of experiments (DoE), a key tool in the implementation of quality by design, provide a structured approach to exploring relationships between factors of interest and the measured response.^[4] As factors are varied systematically and simultaneously, interaction effects, which are omnipresent in biological systems and neglected by OFAT approaches, can be estimated. The resulting data may be used to drive the derivation of statistical models to maximize process understanding and expedite the optimization process, whilst minimizing the experimental burden. Such DoE approaches can be readily coupled with recently developed advanced microbioreactor tools to facilitate high-throughput screening with dramatically increased process insight and closer mimicry of larger scale conditions compared to traditional microplates.^[9,10] The microscale BioLector platform, for example, enables the online monitoring of critical process parameters such as pH, DO, fluorescence and cell growth for 48 simultaneous fermentations. Such data is extremely beneficial for bioprocess characterization, rendering the BioLector an excellent instrument for the execution of DoE studies. The platform has been successfully employed to screen the effect of media and processing conditions on recombinant β -carotene production in *Yarrowia lipolytica*.^[11,12] Comparable trends were observed in the microscale platform and larger bench top bioreactor (0.5–1 L) cultivations, highlighting scalability.

The densely functionalized and intricate structure of the highly effective chemotherapy drug, Taxol, renders it an excellent model compound for metabolic engineering studies. As a result, its microbial synthesis has been a major research focus for decades.^[13-15] However, the pathway (Figure 1B) is yet to be fully elucidated and low and variable yields of early Taxol precursors have been achieved in microbial hosts

to date. The highly promiscuous and multi-specific enzyme, taxadiene-5 α -hydroxylase (CYP725A4), which is responsible for catalyzing the first oxidation step (Figure 1B), is a particular bottleneck. Both the activity and selectivity of the enzyme are highly sensitive to external conditions. Substantial differences in product spectra and overall titer have been observed in response to deviations in medium composition, pH, extraction solvent, and production host.^[16,17] The characterization of the superfluous side products generated during this initial oxidation step is desirable for future metabolic engineering efforts to improve enzyme selectivity. Nevertheless, current production titers are insufficient for purification, hindering such characterization. The development of an optimized and scalable bioprocess for the robust production of Taxol precursors and the corresponding side products is critical to accelerating pathway development.

Previous studies involving Taxol precursor production in *S. cerevisiae* have predominantly been performed in complex media.^[17,18] Chemically defined media are inherently more desirable as they allow precise metabolic analysis of the production host and greater reproducibility compared to complex media.^[19,20] Uncontrollable variables such as batch-batch variation in media can pose a major reproducibility challenge in complex media.^[21] It was therefore hypothesized that the development of an optimized, chemically defined cultivation medium could be beneficial for Taxol precursor overproduction.

In this work, a DoE guided accelerated bioprocess development approach (Figure 1A) was employed with the aim of alleviating a key bottleneck in the biosynthetic pathway of the complex diterpenoid chemotherapy drug, Taxol. Statistical DoE were coupled with a high-throughput microbioreactor screening platform to optimize production of taxadien-5 α -ol and the subsequent Taxol intermediate, taxadien-5 α -yl-acetate, in yeast cell factories. Critical process parameters including pH and DO were monitored online for 48 simultaneous cultivations to enhance process insight. Statistical modelling was employed to identify and optimize factors significantly affecting productivity and the optimal combination was validated at 1 L and 5 L bioreactor scales under industrially relevant, controlled conditions.

2 | MATERIALS AND METHODS

2.1 | Yeast strains

The *Saccharomyces cerevisiae* strains used in this study were *LRS5* (MATa, leu2-3, 112::HIS3MX6-GAL1p-ERG19/GAL10p-ERG8;ura3-52::URA3-GAL1p-MvaSA110G/GAL10p-MvaE [codon optimized]; his3 Δ 1::hphMX4-GAL1p-ERG12/GAL10p-IDI1; trp1-289::TRP1_GAL1p-CrtE(X.den)/GAL10p-ERG20;YPRCdelta15::NatMX-GAL1p-CrtE(opt)/GAL10p-CrtE; ARS1014::GAL1p-TASY-GFP; ARS1622b::GAL1p-MBP-TASY-ERG20; ARS1114a::TDH3p-MBP-TASY-ERG20) and *LRS6* (*LRS5* ARS511b::GAL1p-CYP725A4-PGK1t/GAL3p-CPR-ENO2t; RKC3::GAL1p-TAT-CYC1t) as described previously,^[17,18] originating from CEN.PK2-1C (EUROSCARF collection). All reagents were obtained from Fisher Scientific UK at the highest available purity unless otherwise stated.

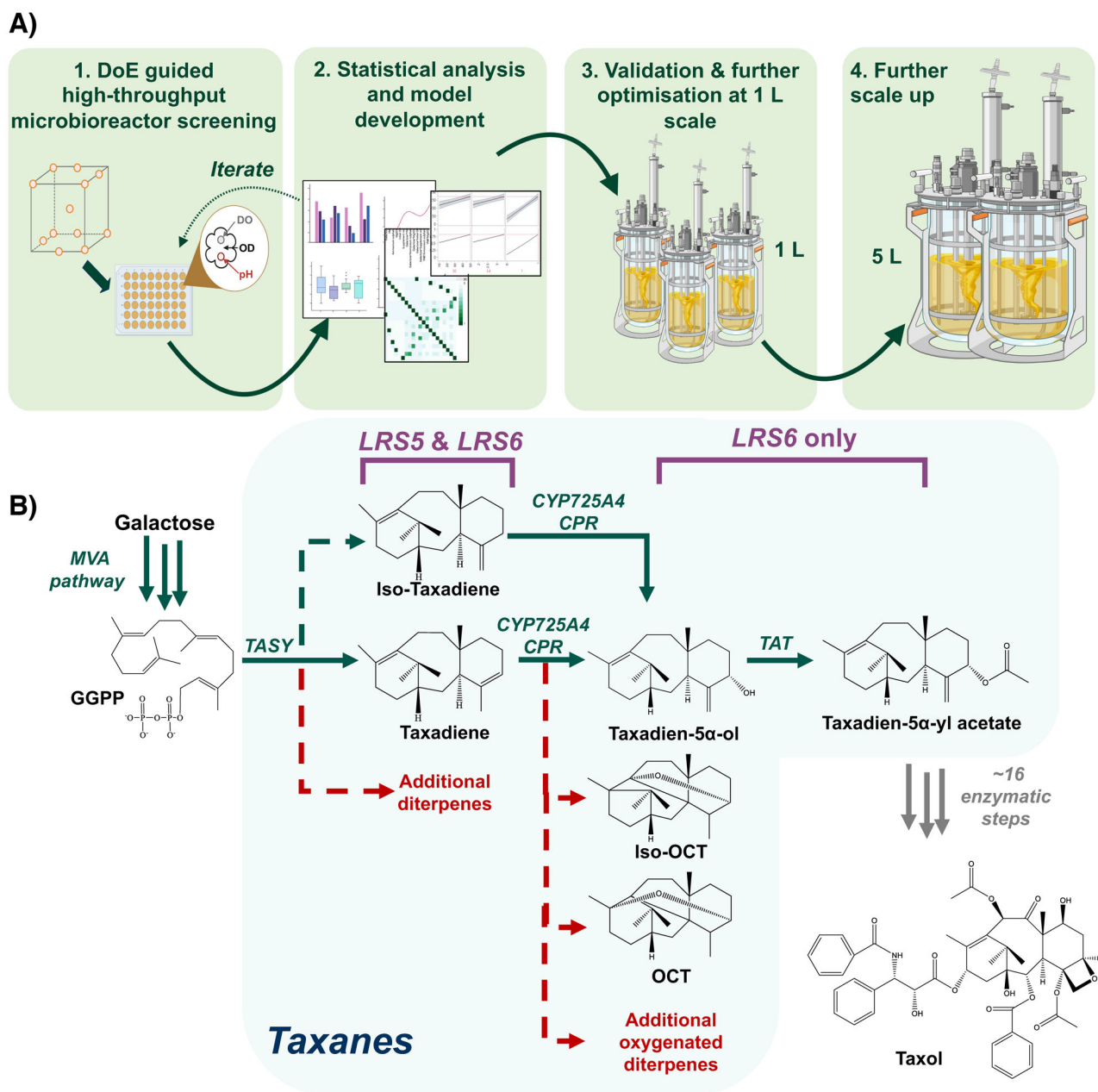


FIGURE 1 Summary of the accelerated bioprocess development approach and Taxol biosynthetic pathway. (A) The accelerated process optimization and scale up approach involved DoE guided screening of a range of process parameters using advanced high-throughput microbioreactors (1 mL). The optimal factor combination was then elucidated via statistical modelling and validated at 1 L and 5 L scale. (B) Heterologous Taxol pathway in *S. cerevisiae* cell factories. Galactose is converted into the universal diterpenoid precursor, GGPP, via the mevalonate pathway. Taxadiene synthase (*TASY*), expressed by both strains *LRS5* and *LRS6* catalyzes the cyclisation of GGPP to taxadiene, along with small amounts of iso-taxadiene and additional diterpene side products. *CYP725A4* and *TAT*, expressed by *LRS6* only, catalyze the subsequent hydroxylation and acetylation reactions, respectively. A further 16 enzymatic steps are believed to be required for the conversion of taxadiene-5 α -yl acetate to Taxol. Figure created with BioRender.com

2.2 | Experimental design and scoping

2.2.1 | Definitive screening design

Following the preliminary microscale cultivations (Section 2.3.1.), the CSM-URA was replaced with Yeast Synthetic Drop-out Medium Sup-

plements without uracil (YSM, Sigma-Aldrich, UK). Uracil was added at a concentration of 40 mg/g YSM. The effect of six factors: initial OD₆₀₀ along with the initial concentrations of galactose, Yeast Nitrogen Base without Amino Acids and Ammonium Sulphate (YNB), ammonium sulphate, additional MgSO₄ and YSM) on *LRS6* productivity was investigated. A three-level definitive screening design (DSD) was selected to

investigate the effect of the six factors on *LRS6* productivity as shown in Table S1.

2.2.2 | Shake flask scoping experiment

The *LRS6* strain was cultivated in 250 mL Erlenmeyer flasks for 3 days. Inocula preparation was achieved by transferring single colonies to 5 mL of rich YPD medium (1% yeast extract; 2% peptone; and 2% glucose) and incubating at 30°C and 250 rpm overnight. The scoping trial involved the following three media compositions:

1. Least forcing: all factors set to their lowest level—Galactose 10 g L⁻¹, YNB 1.7 g L⁻¹, Nitrogen 1 g L⁻¹, MgSO₄ 0 g L⁻¹, YSM 2 g L⁻¹, Initial OD₆₀₀ = 0.1
2. Centre point: all factors set halfway between the low and high range—Galactose 30 g L⁻¹, YNB 2.55 g L⁻¹, Nitrogen 4.5 g L⁻¹, MgSO₄ 0.3 g L⁻¹, YSM 3 g L⁻¹, Initial OD₆₀₀ = 0.55
3. Most forcing: all factors set to their highest level—Galactose 50 g L⁻¹, YNB 3.4 g L⁻¹, Nitrogen 10 g L⁻¹, MgSO₄ 0.6 g L⁻¹, YSM 4 g L⁻¹, Initial OD₆₀₀ = 1

Each condition was tested in duplicate 250 mL flasks, each with a culture volume of 20 mL. Uracil was added at a concentration of 40 mg g⁻¹ YSM. A 5 mL dodecane overlay was also added giving a final working volume of 25 mL. Taxane production was analyzed via gas chromatography-mass spectrometry (GC-MS) at the end of the cultivation.

2.3 | Microbioreactor cultivation

Microscale cultivations were performed using a BioLector Pro (mp2-labs) microbioreactor-screening platform. Inocula were prepared as described for the shake flasks. The temperature was maintained at 30°C under agitation of 1000 rpm with a shaking diameter of 3 mm in 48-well FlowerPlates (mp2-labs). Temperature, biomass, DO, and pH were monitored online using the inbuilt optical sensors. Taxane production was analyzed via GC-MS at the end of the cultivation.

2.3.1 | Preliminary scoping trials

Preliminary cultivation experiments were performed in the BioLector FlowerPlates using a typical synthetic defined galactose (SDG) medium (Yeast Nitrogen Base without Amino Acids, 6.7 g L⁻¹; Complete Supplement Mixture minus Uracil (CSM-URA), 0.77 g L⁻¹; Uracil, 20 mg L⁻¹; galactose, 20 g L⁻¹). Control cultivations were performed with YPG (yeast extract, 10 g L⁻¹; peptone, 20 g L⁻¹; galactose 20 g L⁻¹). Aliquots of the preculture were diluted to give an 800 μL culture with an initial OD₆₀₀ = 1. A 200 μL dodecane overlay was also added to each well giving a total working volume of 1 mL.

2.3.2 | High-throughput screening

For the high-throughput screening experiments, aliquots of the preculture were diluted with each of the medium combinations indicated in Table S1 to give 800 μL cultures with the appropriate initial OD₆₀₀. A 200 μL dodecane overlay was also added to each well giving a total working volume of 1 mL. Each factor combination was tested in duplicate or triplicate in a 48-well FlowerPlate.

2.4 | Bioreactor cultivation

Larger scale cultivations were conducted in 1 L and 5 L BIOSTAT Q plus bioreactors (Sartorius-Stedim Biotech S.A.) with working volumes of 500 mL and 2.5 L, respectively. Pre-inoculum cultures were prepared by transferring from a single colony to 5 mL of YPD and incubating at 30°C and 250 rpm for 24 h. The resulting culture was subsequently used to inoculate a secondary 50 mL culture to an OD₆₀₀ = 1 and incubated overnight. An aliquot of the resulting culture was diluted with the optimized medium to give a culture with an initial OD₆₀₀ = 1.

Antifoam 204 (Sigma Aldrich, Denmark) was added to prevent excess foam production. A 20% dodecane overlay was also added. Temperature, DO, and pH were measured online. MFCS software (version 3.0, Sartorius-Stedim Biotech S.A.) was employed to control the cultivation, pH was maintained at six through the automatic addition of 2 M NaOH or 2 M H₂SO₄, and temperature was maintained at 30°C. A constant airflow of 1 vvm was maintained and stirrer speed was adjusted manually to maintain DO above 30%. Off-gas analysis was performed online via mass spectrometry (Prima Pro, Thermo Fisher Scientific). Samples were taken twice daily for taxane and metabolite quantification via GC-MS and high-performance liquid chromatography (HPLC).

2.5 | Taxane and metabolite identification and quantification

Taxane identification and quantification was achieved via GC-MS. The organic dodecane layer was separated from the culture medium through centrifugation and a 1 μL sample was injected into a TRACE 1300 Gas Chromatograph (Thermo Fisher Scientific) coupled to an ISQ LT single quadrupole mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was achieved using a Trace Gold TG-SQC gas chromatography column using a previously described method.^[22] To identify and quantify the production of compounds by *LRS5* and *LRS6*, pure standards of taxadiene, kindly supplied by Baran Lab (The Scripps Research Institute) and GGOH, obtained from Sigma Aldrich (UK), were used. Additional product concentrations were estimated relative to standard taxadiene concentrations. In the bioreactor cultivation, ethanol, acetate, and glycerol production were analyzed via ion-exchange HPLC. Following filtration using a 0.45 μm filter, 20 μL samples were injected into a Bio-Rad Aminex HPX-87H column for analysis. The eluent was 5 mM H₂SO₄, flowrate 0.6 mL min⁻¹,

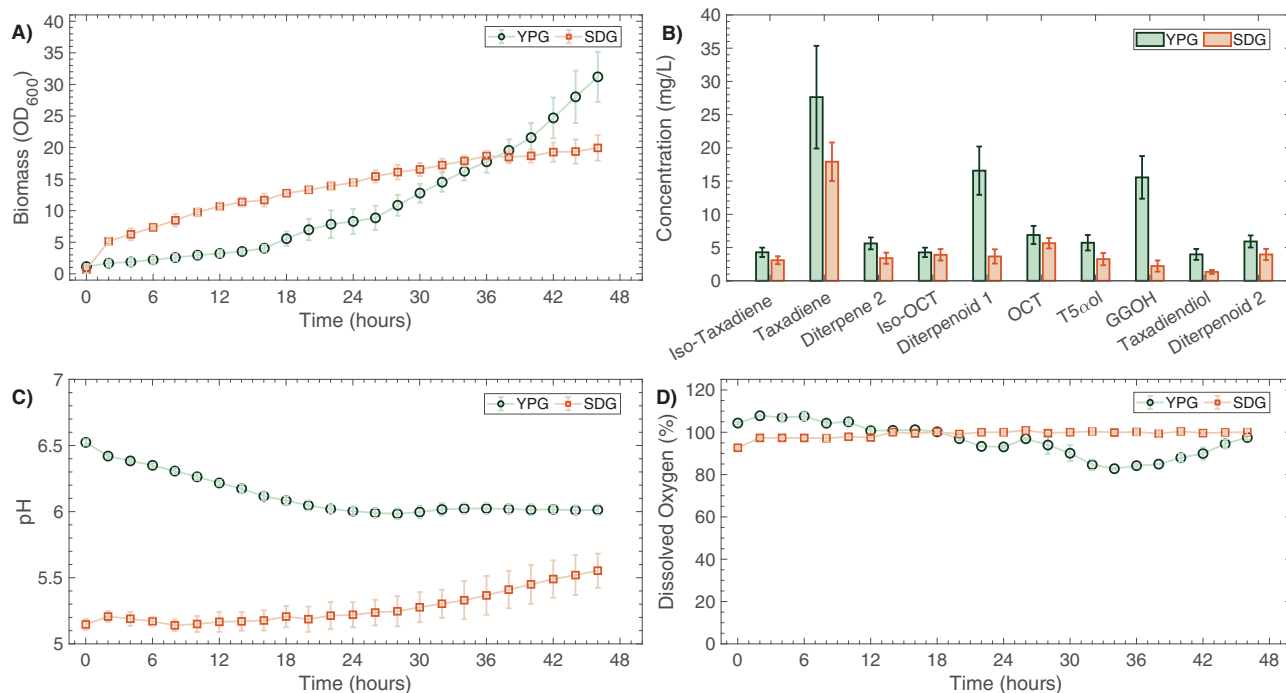


FIGURE 2 Preliminary microscale cultivation of *LRS6*. *LRS6* was cultivated in SDG (Yeast Nitrogen Base without Amino Acids, 6.7 g L⁻¹; Complete Supplement Mixture minus Uracil (CSM-URA), 0.77 g L⁻¹; Uracil, 20 mg L⁻¹; galactose, 20 g L⁻¹) and YPG (Yeast extract, 10 g L⁻¹; Peptone 20 g L⁻¹; Galactose, 20 g L⁻¹). (A) Biomass measured as optical density at 600 nm, (B) Taxane production, (C) pH, (D) DO. Measurements are mean \pm SD for triplicate cultivations

and the temperature was 60°C. A RID-detector was used for quantification.

2.6 | Statistical analysis

DoE and statistical modelling were performed using JMP Pro 15 statistical software. Forward stepwise regression with a p -value to enter of 0.1 was performed using JMP to fit the regression model for the DSD. A full quadratic analysis was performed, thereby considering all main effects and any second order interactions. The resulting model contained those factors which produced a significant effect on the response. The prediction profiler was then used to optimize the settings of these factors. Pearson's correlation coefficient was used to assess linear relationships between variables.

3 | RESULTS AND DISCUSSION

3.1 | Preliminary microscale characterization

Previous studies at micro (1 mL) and bioreactor (0.5–1 L) scale involving the cultivation of *S. cerevisiae* strain, *LRS6*, which has been engineered for heterologous expression of the first three genes of the Taxol biosynthetic pathway (Figure 1B), were performed exclusively in complex media.^[17] In order to characterize performance in defined media

preliminary microscale growth experiments were performed using a typical synthetic defined media (SDG, Section 2.3.1). Galactose was selected as the carbon source to maximize expression of the heterologous pathway, which was under the control of galactose inducible promoters (Figure 1B) Control experiments using a typical complex media (YPG, Section 2.3.1.) were included for comparison. The results of this investigation are summarized in Figure 2.

The initial pH of the SDG media was significantly lower than that of YPG at around 5.2 compared to 6.5. Previous studies revealed that the CYP725A4 is sensitive to deviations in external pH,^[17] indicating pH adjustment may be beneficial in future runs. CYP725A4 has an optimal pH of 7.2^[23] and little activity was observed in cultivations where the pH dropped below 5.^[17] However, as *LRS6* growth has been found to be dramatically reduced at neutral pH,^[17] a pH of 6 was deemed adequate for this process to maximize productivity. After 48 h of cultivation the OD₆₀₀ values were 31.2 \pm 4.0 and 19.9 \pm 2.0 for the YPG and SDG cultures respectively. Although SDG was supplemented with a source of amino acids and vitamins, the complex YPD media is a much richer source. The reduced nutrient availability likely contributed the reduced biomass accumulation observed. This is consistent with previous studies which demonstrated that the growth rate of *S. cerevisiae* is reduced in standard defined media compared to complex YPD.^[24] Total taxane yields were 81 \pm 17 and 46 \pm 8 mg L⁻¹ for the YPG and SDG cultures, respectively. The lower taxane titer observed in the SDG cultures was expected as a strong positive correlation has been elucidated between biomass and taxane accumulation.^[17,18]

3.2 | Definitive screening design development and validation

In order to improve taxane productivity, a range of defined media compositions were screened. A DSD was selected for this step, due to the improved optimization efficiency of the design compared to traditional screening designs. DSDs are a class of three level designs which combine the efficiency of a screening design with the capacity to characterize non-linearity and second-order interactions.^[4,25] In just $2N + 1$ runs for N factors, main effects can be reliably estimated without confounding with second order effects. In addition, designs with 6–12 factors are capable of efficient estimation of all possible full quadratic models involving ≥ 3 active factors.^[25] In such cases it is possible to progress to optimization without further experimentation. Should the number of active effects exceed three the full quadratic model cannot be reliably fitted, however, this can be mitigated through augmenting the design. Using a synthetic complete media as a basis, a range of media compositions were screened to determine the optimal for taxane accumulation. Preliminary studies revealed a significantly reduced growth rate in synthetic compared to complex media (Figure 2A). As the reduced amino acid and nutrient availability likely contributed, an alternative supplement mixture (YSM, Sigma-Aldrich, UK) with a richer supply of amino acids was used and its concentration along with that of yeast nitrogen base were included as factors in the DSD. As the critical taxadiene synthase enzyme relies on the co-factor Mg^{2+} for activation, it was hypothesized that supplementing the media with an additional magnesium supply may improve activity. The highest reported oxygenated taxane yields reported in *E. coli* were achieved using an optimized media supplemented with around $0.6 \text{ g L}^{-1} \text{ MgSO}_4$.^[26] This concentration was therefore selected for this optimization study. As taxane accumulation has been found to be strongly correlated to *LRS6* growth,^[17] it was hypothesized that the initial OD_{600} of the cultivation may influence taxane accumulation and initial OD_{600} was therefore included as a factor. The carbon to nitrogen (C/N) ratio of the growth media can have a major influence on cellular metabolism. It is widely accepted that increasing the C/N ratio has a positive effect on lipid accumulation by oleaginous yeast;^[27] however, the effect on isoprenoid production is more ambiguous.^[28] Like lipids, isoprenoids rely on acetyl-CoA as a universal precursor. In addition, as hydrophobic isoprenoids such as the triterpene, squalene^[29] and carotene, β -carotene^[30] have been found to accumulate in cytoplasmic lipid particles, increasing the C/N ratio may be beneficial. In a study by Olson et al. 2016, β -carotene production was found to increase up to four-fold when the C/N ratio was increased 5.7-fold in an engineered *S. cerevisiae* strain. Similarly, increasing lipid accumulation has been found to improve lycopene accumulation in *S. cerevisiae*.^[31] The concentrations of the carbon (galactose) and nitrogen (ammonium sulphate) were therefore deemed important factors in this study. A six factor, three-level DSD was created as summarized in Table S1 (Section 2.2.1). In order to validate the design a scoping experiment was performed in shake flasks. This involved three conditions in which, the levels of each factor were set to their low (least forcing), medium (center point) and high (most forcing) levels, respectively, as described in section 2.2.2.

The results of the scoping trial are summarized in Figure S1 and Figure 3B.

The least and most forcing conditions gave rise to the lowest and highest taxane titers, respectively. The center point titer was between that of the least and most forcing condition (Figure S1). In addition, there was little variation between the duplicate shake flasks, the experimental factors were therefore deemed appropriate. The use of defined media resulted in substantial changes in product spectra in comparison to complex media (Figure 3B). The titer of diterpenoid 1, an isomer of the first oxygenated Taxol intermediate, taxadien-5 α -ol (T5 α ol), which was the major oxygenated product of the strain in complex media investigations,^[17] was negligible. The main oxygenated products were OCT and Iso-OCT. Relative production of the di-oxygenated taxadiendiol compound was enhanced, and additional products were observed at 8.67 and 9.70 min as shown in Figure 3B and Figure S2. Although the mass spectra corresponding to these peaks was similar to the other oxygenated compounds it is possible that the compounds at 8.67 and 9.7 min could be endogenous products. In order to determine whether the compounds were products of the heterologous CYP725A4 or TAT enzymes rather than endogenous gene products a further experiment was performed. The *LRS5* strain which expresses TASY alone was also cultivated in the most forcing media as a control. The resulting gas chromatogram is shown in Figure 3A. The product spectra for *LRS5* grown in the most forcing synthetic media was very similar to that observed in complex media.^[17,18] Taxadiene was the main product of the strain and the additional products observed in the *LRS6* cultures were not observed. This indicates that the additional peaks likely resulted from CYP725A4 or TAT enzymatic activity or products.

3.3 | Efficient defined medium optimization using definitive screening DoE

Following validation, the DSD outlined in Table S1 was implemented at microscale using the BioLector microbioreactor screening platform. The key response variable, total taxane accumulation was evaluated at the end of the cultivation as summarized in Figure 3D.

Of the 17 conditions tested, factor combinations six, nine, and 11 gave rise to the highest total taxane titers of 155 ± 3 , 194 ± 46 and $145 \pm 12 \text{ mg L}^{-1}$, respectively. The product profile obtained for these factor combinations were highly similar to those observed in the most forcing shake flask cultivations (Figure 3B). The effect of each of the six factors on taxane production was evaluated via forward stepwise regression using JMP with a p-value to enter of 0.1. A full quadratic model was derived, thereby considering all main effects and any second order interactions. The resulting statistical model revealed initial OD_{600} ($p = 6 \times 10^{-6}$) along with initial YNB ($p = 0.021$) and galactose ($p = 0.011$) concentrations were significant main effects. As just three factors were active a full quadratic model could be derived with high statistical efficiency,^[25] facilitating optimization without augmentation of the design. According to the statistical model the optimal galactose, YNB and initial OD_{600} settings were 50 g L^{-1} , 3.4 g L^{-1} and 1,

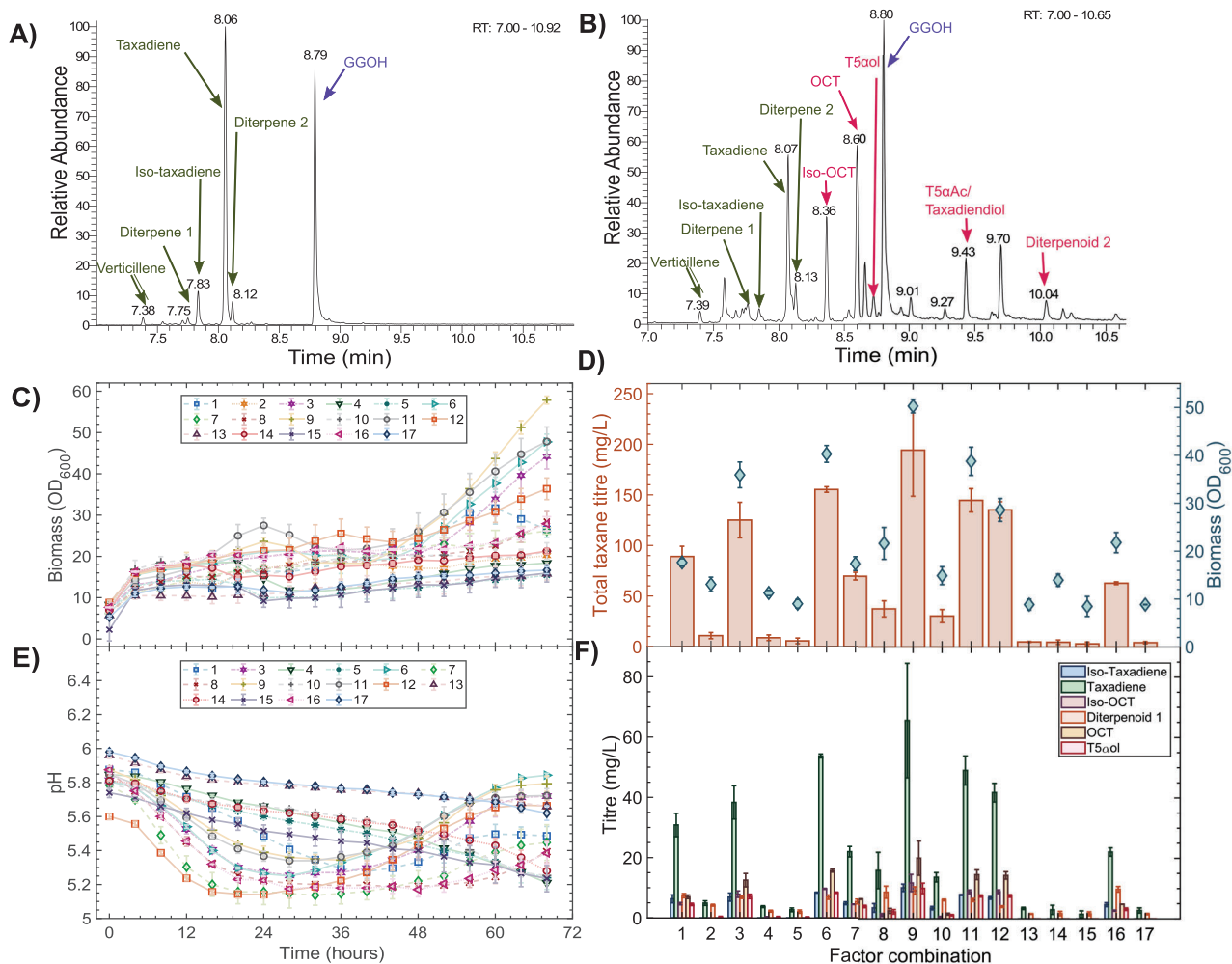


FIGURE 3 Scoping and definitive screening design results. Gas chromatographs resulting from scoping cultivation under most forcing condition in shake flasks. (A) Gas chromatograph resulting from control cultivation of *LRS5* (expressing *TASY* only) in the most forcing medium, (B) Gas chromatograph resulting from cultivation of *LRS6* (expressing *TASY*, *CYP725A4* + *CPR*, and *TAT*) in the most forcing medium. Results of the definitive screening design conducted using the BioLector FlowerPlate. (C) Online biomass data, (D) Final total taxane titer and optical density values, (E) Online pH data, (F) Final titer of each of the key pathway intermediates. Data is mean \pm SD for duplicate or triplicate cultivations under each of the 17 factor combinations of the DSD as described in Table S1 (Section 2.2.1.) at microscale

respectively (Figure S3). As the concentrations of ammonium sulphate ($p = 0.285$), YSM ($p = 0.990$) and $MgSO_4$ ($p = 0.690$) did not significantly affect productivity, the lowest values of 1, 2, and 0 g L⁻¹ were used in subsequent cultivations to conserve resources.

The final titer of the key products, iso-taxadiene, taxadiene, iso-OCT, diterpenoid 1, OCT and T5 α ol were plotted for each of the 17 factor combinations as shown in Figure 3F. The observed product spectra in the high yielding defined media (factor combinations 6, 9, 11, and 12; Figure 3F) were highly different to those observed in the YPG control (Figure 2) and previous cultivations with complex media in BioLector FlowerPlates.^[17] *CYP725A4* activity and selectivity has been previously found to be highly sensitive to media composition. Edgar et al. 2016, reported a relative increase in iso-OCT production in minimal media compared to rich media when the pathway was expressed in *S. cerevisiae* and *Yarrowia lipolytica*. Differences in media composition may have therefore contributed to the deviations in prod-

uct spectra observed in this study. As in the shake flask cultivations (Figure 3B), the major oxygenated products were OCT and Iso-OCT along with smaller quantities of diterpenoid 1 and T5 α ol. The novel diterpenoids with retention times of 8.67 and 9.70 min were also detected along with two additional peaks at 10.23 and 10.57 min (Figure S4). The diagnostic T5 α Ac peaks at m/z 287 ($P^+ - CH_3CO$), 270 ($P^+ - CH_3COOH$) and 255 ($P^+ - CH_3COOHCH_3$)^[14] were observed in the mass spectra of the peak at 9.43 (Figure S5). However, a large peak at 245 was also detected along with peaks at m/z 304 and 286 ($P^+ - H_2O$), which are characteristic of taxadiendiol. Therefore, although T5 α Ac was likely produced in the BioLector cultivations, accurate quantification was not possible due to co-elution with the di-oxygenated compound.^[17]

Although the initial pH of the cultivation medium was adjusted to pH 6, acidification of the cultivation medium was observed (Figure 3E). The rate of acidification was greater in the higher yielding

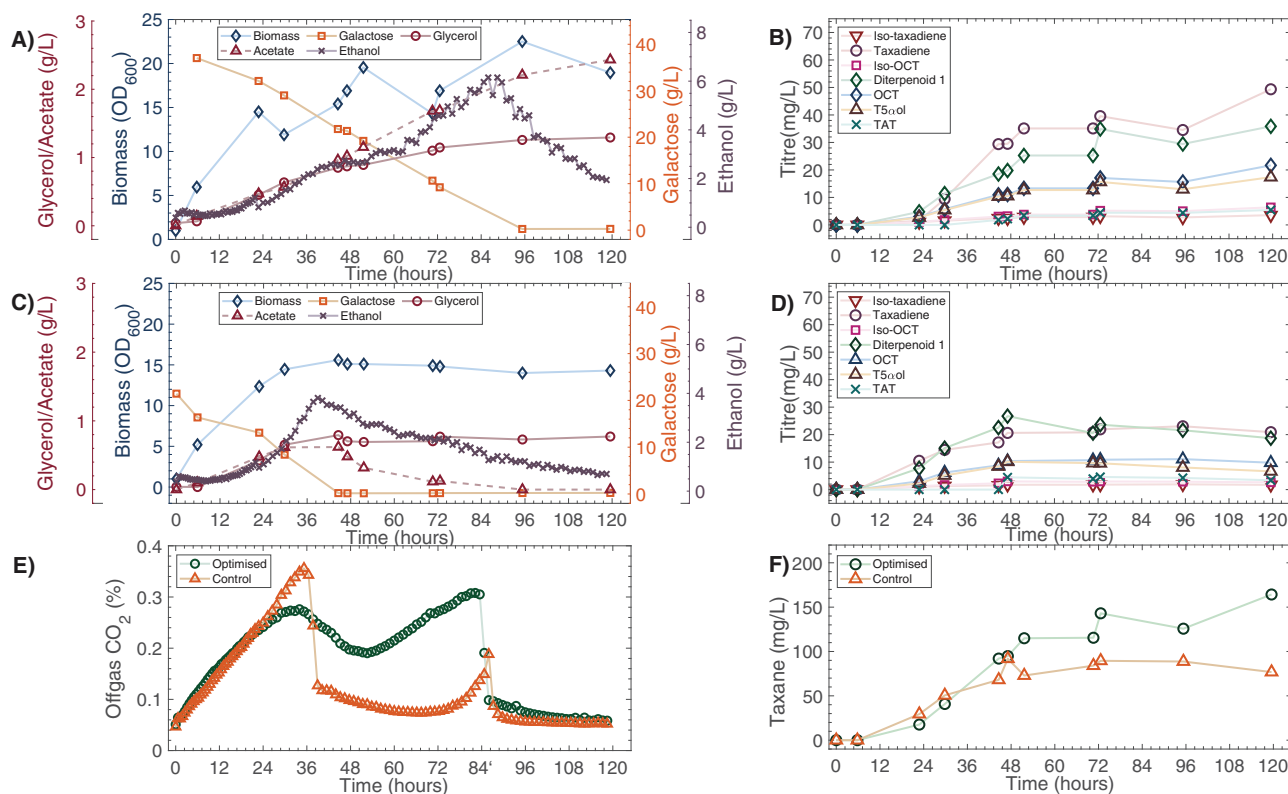


FIGURE 4 Defined media validation in 1 L bioreactors. Biomass, galactose and secondary metabolite concentrations were monitored for *LRS6* cultivations using (A) optimized defined (YNB 3.4 g L⁻¹, ammonium sulphate 1 g L⁻¹, YSM 2 g L⁻¹, galactose 50 g L⁻¹) and (C) standard defined media (YNB 1.7 g L⁻¹, ammonium sulphate 5 g L⁻¹, YSM 2 g L⁻¹, galactose 20 g L⁻¹). Total taxane production was also monitored for the two cultivations (F). Accumulation of the key Taxol precursors and side products was also plotted for the optimized (B) and control (D) cultivations. Offgas CO₂ data is also shown for both the control and optimized cultivations (E)

treatments (6,9,11,12), with a rapid decrease in pH to between 5.1 and 5.3 observed in the first 30 h of cultivation. This was considerably lower than the minimum pH of 5.98 ± 0.04 detected in the standard YPG cultivation (Figure 2). Metabolism of galactose by *LRS6* is respiratory-fermentative, during fermentative growth the yeast acidifies the cultivation medium through a combination of proton pumping and organic acid secretion.^[32] As the initial concentrations of galactose and biomass were at the high level of 50 g L⁻¹ and OD₆₀₀ = 1 for factor combinations 6, 9, 11, and 12, the rate of fermentation was likely greater in these cultivations resulting in an increased rate of acidification. The rate of acidification was much lower during the lower yielding treatments (4, 5, 13, 14, 15, 17), likely due to the reduced growth and fermentation rates, with the minimum pH observed at the end of the 72 h cultivation. Interestingly, diterpenoid 1 was the sole oxygenated product detected in these cultivations (4, 5, 13, 14, 15, 17; Figure 3F and Figure S6A). In addition, the buffering capacity of yeast extract peptone medium has been found to be greater than that of defined medium supplemented with amino acids^[33], which may have contributed to the increased acidification. The *CYP725A4* enzyme has been previously found to be highly sensitive to deviations in pH. A dramatic reduction in enzyme activity was observed when the pH of the cultivation medium dropped to 4.9 during an uncontrolled bioreactor cultivation of the strain.^[17] The fluctuations in the external pH of

the cultivation medium may have therefore contributed to the deviations in enzyme selectivity observed in defined medium at microscale. Between 30 and 72 h a gradual increase in pH toward the initial pH of 6 was observed for treatments 6, 9, 11, and 12. This was likely due to the assimilation of less favorable carbon and nitrogen sources in the later stages of the cultivation.

3.4 | Scale up of optimized defined media in 1 L bioreactors

The optimal conditions elucidated at microscale (Figure S3) were subsequently scaled up using 1 L bioreactors. A control was also ran with a standard defined media similar to the SDG media (Figure 2), except the CSM supplement was replaced with the more nutrient rich YSM. The results of this investigation are summarized in Figure 4.

At bioreactor scale the total taxane titer was 164 mg L⁻¹, this represented a 2.1-fold improvement in titer compared to the control (Figure 4F) and was just 13% lower than that predicted by the statistical model (Figure S3) derived from the microscale cultivation data. However, the maximum OD₆₀₀ value was just 22.5, significantly lower than those obtained in the higher yielding BioLector cultivations (Figure 3). Biomass accumulation (Figure 4A and C) and CO₂

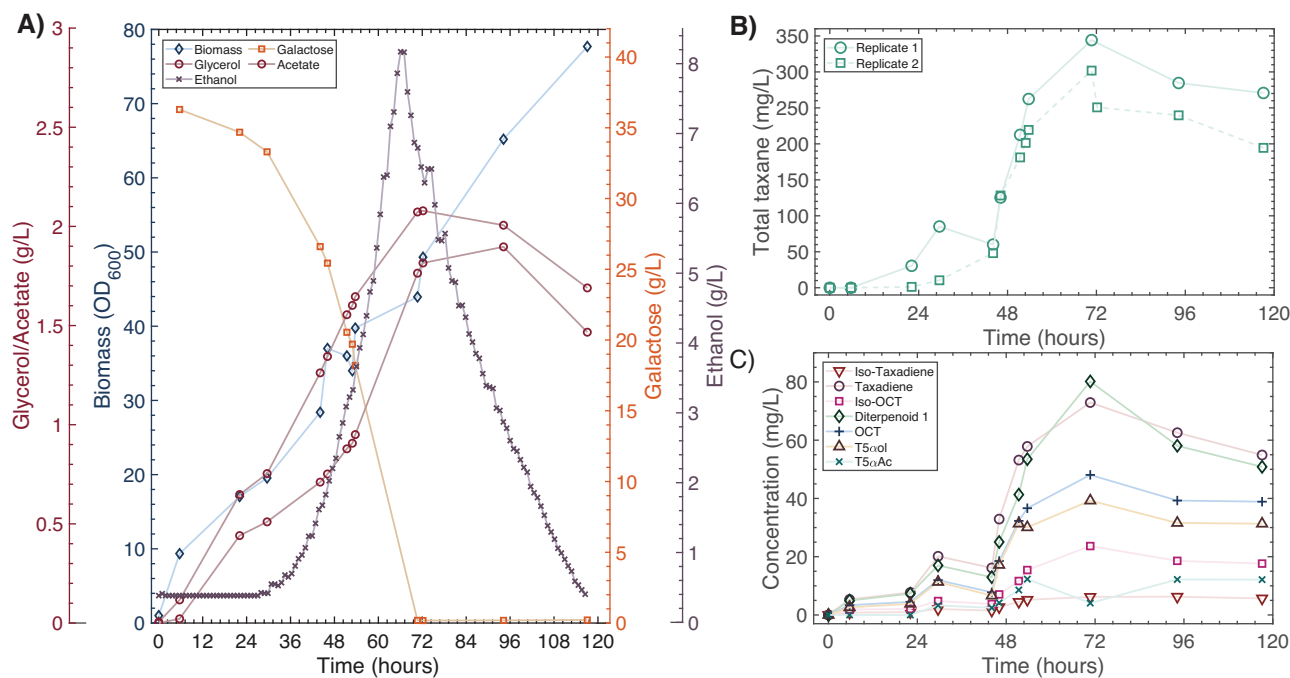


FIGURE 5 Optimized defined media with increased ammonium sulphate. *LRS6* was cultivated in the optimal defined media with a higher initial ammonium sulphate concentration (YNB 3.4 g L⁻¹, ammonium sulphate 10 g L⁻¹, YSM 2 g L⁻¹, galactose 50 g L⁻¹). (A) Biomass, galactose, and secondary metabolite concentration. (B) Total taxane accumulation for two cultivations, the lag phase has been normalized for replicate two to allow comparison. (C) Accumulation of key products in the Taxol pathway

production (Figure 4E) were similar for the control and optimized cultivations during the first 24 h. However, after 24 h a drop in CO₂ production was observed in the optimized cultivation (Figure 4E) despite excess galactose availability (Figure 4A), this was indicative of nutrient limitation. As ammonium sulphate concentration did not have a significant effect on taxane accumulation at microscale ($p = 0.285$), the concentration was set to the low level of 1 g L⁻¹ at this scale. As all other nutrients were in excess it was hypothesized that nitrogen limitation may have been responsible for the reduced growth rate observed. A second fermentation was therefore performed with an increased initial ammonium sulphate as summarized in Figure 5.

Through increasing the initial ammonium sulphate concentration, the nutrient limitation was effectively eliminated. Biomass accumulation was improved 3.5-fold with a maximum OD₆₀₀ of 78 at the end of the 120 h cultivation (Figure 5A). Total taxane production was also improved 2.1-fold, with a maximum titer of 344 mg L⁻¹ obtained at 70.7 h (Figure 5B). At 5.7 h the galactose concentration was considerably lower than the initial concentration of 50 g L⁻¹ at 36.3 g L⁻¹. Although some of this difference could be attributed to uptake by the cells for growth and fermentation, the OD₆₀₀ increased only slightly to around 9.4 and the ethanol concentration was just 0.3 g L⁻¹. It is therefore likely that the initial concentration of galactose was slightly lower than expected due to attrition during transfer to the reactor. To prevent loss of sugar due to the Maillard reaction during sterilization, the galactose was autoclaved separately and added just before inoculation via sterile tubing. It is therefore likely that some of the galactose was retained in the tubing resulting in a lower initial galactose concentration. At 70.7 h the galactose had been depleted and the yeast metabolized the pro-

duced ethanol in the subsequent 46 h of cultivation (Figure 5A). As the heterologous pathway was expressed under the control of galactose inducible promoters, no additional taxanes were produced during growth on ethanol and the total titer declined to around 271 mg L⁻¹ at the end of the cultivation. As this drop in titer was proportional for each of the key products (Figure 5C), it may have been due to loss via air stripping. Although the addition of a dodecane overlay has been found to reduce dramatically reduce air-stripping of volatile taxane compounds, with the relatively high air flow rate (1 vvm) and long cultivation time in the bioreactor, its application may not have been sufficient to eliminate the phenomenon entirely.

Interestingly, the nature of the taxane products generated in the 1 L bioreactors (Figure 5) was highly similar to those obtained for *LRS6* grown in complex media in 1 L BIOSTAT reactors.^[17] The major product of CYP725A4 was diterpenoid 1 under each of the three conditions tested in the bioreactor (Figure 4B and D, Figure 5C). Under the optimized condition with increased ammonium sulphate a maximum diterpenoid 1 titer of 73 mg L⁻¹ was achieved (Figure 5C). The confirmed Taxol intermediates taxadiene, iso-taxadiene, T5 α ol and T5 α Ac were also detected with maximum titers of 63, 6, 35, and 12 mg L⁻¹, respectively. This represented 1.5 and 3.2-fold improvements in titers of the critical Taxol precursors, T5 α ol and T5 α Ac, compared to the highest reported literature titers in *S. cerevisiae*.^[17] OCT and iso-OCT were also produced, however, the additional compounds observed in the BioLector and shake flask cultivations were not detected. A further diterpenoid compound, diterpenoid 4 (Figure S7), which was previously produced by the strain in bioreactor cultivations with complex media^[17] was also detected. The differences in product spectra observed in the

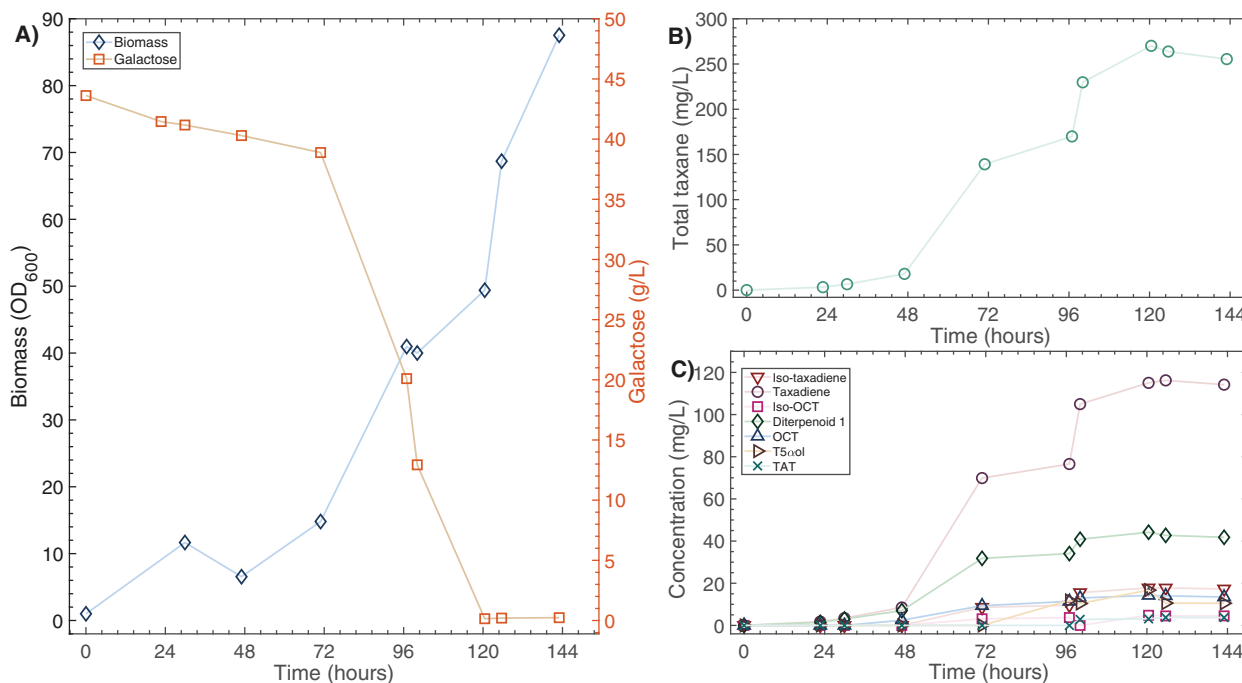


FIGURE 6 Optimized defined media with increased ammonium sulphate at 5 L scale. *LRS6* was cultivated in the optimal defined media with a higher initial ammonium sulphate concentration (YNB 3.4 g L⁻¹, ammonium sulphate 10 g L⁻¹, YSM 2 g L⁻¹, galactose 50 g L⁻¹). (A) Biomass and galactose concentration, (B) Total taxane concentration, (C) Accumulation of key products in the Taxol pathway

smaller scale cultivations were therefore unlikely to be solely the result of the differences in medium composition. The acidification of the cultivation medium in the absence of pH control at smaller scale likely contributed to the deviations in selectivity.

In order to validate the results of the experiment summarized in Figure 5, an additional bioreactor cultivation was performed under the same conditions as summarized in Figure S8. In the replicate cultivation, a shorter lag phase of growth was observed and the galactose was depleted after just 53.7 h (Figure S8) compared to around 70.7 h in the first replicate (Figure 5A). Despite this, the exponential growth phases were found to be highly comparable for the two cultivations. To facilitate comparison of the total taxane kinetics, the lag phase of the second cultivation was normalized to that of the first as shown in Figure 5B. Total taxane accumulation was strongly correlated in the two replicates (Pearson's $R = 0.9759$) with maximum titers of 344 and 302 mg L⁻¹, respectively, achieved upon depletion of galactose. This further demonstrates that the optimized medium with increased ammonium sulphate effectively alleviated nutrient limitation and successfully improved taxane titers to around 300 mg L⁻¹ in *S. cerevisiae* strain *LRS6* at 1 L scale.

3.5 | Further scale up of the optimized defined media cultivation in 5 L bioreactor

To further investigate the scalability of the optimized 1 L process, a further five-fold scale up was performed using a 5 L bioreactor. The results of this experiment are summarized in Figure 6.

At 5 L scale the lag phase of growth was increased to around 72 h (Figure 6A). Despite this, the final OD₆₀₀ was 87.5, highly comparable to the final OD₆₀₀ values of 77.7 and 84 obtained in the 1 L bioreactor cultivations (Figure 5A and Figure S8). As described in Section 3.4, it was hypothesized that the initial concentration of galactose may be lower than the intended 50 g L⁻¹ due to some of the galactose being retained within the tubing. In order to investigate this, the initial galactose concentration was measured prior to inoculation of the 5 L bioreactor as shown in Figure 6A. The resulting concentration was 44 g L⁻¹, corresponding to a loss of around 10%. A maximum total taxane titer of 270 mg L⁻¹ was observed at 126 h (Figure 6B), just 10% lower than the maximum titer of 302 mg L⁻¹ obtained at 1 L scale (Figure 5B). A slight decrease in titer to 260 mg L⁻¹ was observed in the final 17 h of the cultivation. Interestingly, although total product titers were comparable at 1 and 5 L scale, considerable differences in product spectra were observed despite analogous operating conditions. In the 1 L cultivation (Figure 5), the oxygenated and acetylated products of the CYP725A4/TAT enzymes accounted for 65.4% of the total products compared to just 36.4% at 5 L scale (Figure 6). The activity of the critical CYP725A4 enzyme has been found to be sensitive to deviations in pH in both defined (Section 3.4) and complex media [17]. However, here it can be seen that CYP725A4 was also sensitive to differences in processing conditions between the two scales, despite careful control of pH and other key process parameters. This, further highlights that this enzyme is a key bottleneck in Taxol biosynthetic pathway development. Despite this, the investigated process was demonstrated to be robust with comparable total taxane titers observed despite a 2500-fold scale up in cultivation volume from micro- to 5 L bioreactor scale.

To our knowledge this represents the greatest scale-up in production of oxygenated and acetylated taxanes by yeast microbial cell factories.

4 | CONCLUDING REMARKS

In this study, a DoE guided approach was applied to develop an optimized bioprocess to produce Taxol precursors using an engineered *S. cerevisiae* strain. A DSD elucidated key factors affecting taxane accumulation in the strain at microscale, facilitating statistical model derivation. The optimal factor combination predicted by the statistical model was validated in highly instrumented 1 L bioreactors with comparable total taxane titers of 164 mg L⁻¹ and 189 mg L⁻¹ achieved. This titer was 2.1-fold higher than that in the parallel control cultivation in standard defined media. However, the final OD₆₀₀ was just 19.0, 2.6-fold lower than the highest yielding microscale cultivation and closer inspection of the offgas data revealed nitrogen limitation. Mitigation of this nutrient limitation led to maximum total and oxygenated taxane titers of 344 and 197 mg L⁻¹, respectively. This represented 2.1 and 2.5-fold improvements compared to the highest reported titers for yeast, achieved in rich media. A further five-fold scale up of this optimized process was performed using 5 L bioreactors. At this scale a final OD₆₀₀ of 87.5 and maximum total taxane titer of 270 mg L⁻¹ was achieved, comparable to the 77.7 and 344 mg L⁻¹ obtained at 1 L scale. The ability of strategic quality by design approaches to dramatically improve the efficiency of the early phases of bioprocess development was demonstrated in this study. Production of critical oxygenated and acetylated Taxol intermediates was successfully scaled up 2500-fold in yeast cell factories. Optimization of the cultivation medium resulted in substantial improvements in taxane titers in this study, however, a large proportion of the galactose carbon source was fermented to the undesirable side product ethanol, due to the overflow metabolism of *S. cerevisiae*. Future work should therefore focus on minimizing the accumulation of ethanol, for example through transitioning the mode of operation from batch to fed-batch. Through feeding the substrate at the rate of uptake, accumulation can be minimized and the overflow metabolism circumvented. In addition, as the expression of the heterologous pathway was under the galactose inducible promoters, further improvements in titer could potentially be achieved through knockout of the GAL80 gene, which would facilitate constitutive expression and ensure taxane biosynthesis by LRS6 irrespective of the carbon source. Furthermore, the optimization methods presented in this study should be incorporated during microbial cell factory construction and optimization to ensure robust growth under industrially relevant conditions and further reduce the risks associated with bioprocess scale up.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

ORCID

Leonardo Rios-Solis  <https://orcid.org/0000-0002-4387-984X>

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