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In situ solid-liquid extraction enhances recovery of taxadiene from engineered *Saccharomyces cerevisiae* cell factories

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ABSTRACT

Microbial cell factories express diverse heterologous pathways for the production of a wide range of valuable natural products. However, the recovery and purification of such compounds is a major bottleneck in commercialization. In this study, a novel *in situ* solid phase adsorption strategy was investigated for enhanced recovery of taxadiene, a precursor to the blockbuster anticancer drug, paclitaxel, from engineered *Saccharomyces cerevisiae*. A synthetic adsorbent resin (HP-20) was employed to efficiently sequester taxadiene as it was secreted during growth and a carefully optimized desorption solvent was applied following cultivation to maximize recovery of both secreted and intracellular taxadiene, across a range of scales (2 – 250 mL). Resin concentration was found to have an impact on cellular growth, with the high concentration of 12 % (w/v) resulting in fragmentation of the resin beads, which was detrimental to growth. The optimal resin concentration and desorption solvent combination elucidated at microscale (2 mL) resulted in a two-fold improvement in taxadiene titer to 61 ± 8 mg/L, compared to the traditional liquid-liquid extraction approach (dodecane overlay). Taxadiene was found to be distributed evenly between resin beads and biomass. Performance of the optimal process was subsequently investigated through scale-up using controlled mini-bioreactors (250 mL). Here, a comparable taxadiene titer of 76 ± 19 mg/L was achieved despite a 125-fold scale-up in cultivation volume. This represented a 1.4-fold improvement in taxadiene recovery compared to previous mini-bioreactor scale cultivations using the dodecane overlay extraction approach.

1. Introduction

Downstream processing for the recovery and purification of valuable natural products (NPs) typically accounts for 60–80% of the total operational costs at industrial scale [1]. Such processing steps are typically energy intensive and require vast quantities of highly toxic solvents, resulting in major economic and environmental sustainability challenges. NPs have extensive applications across the global food, cosmetics and pharmaceutical industries [2,3]. As a result of such far-reaching potential benefits, NPs consumption has tripled since the 1990 s [4].

Over 60% of all known NPs are terpenoids, a large chemically diverse group of compounds with important roles in both primary and secondary metabolism in their natural hosts [5,6]. Some terpenoids in particular are drug candidates for the treatment of human disease; examples include the sesquiterpenoid anti-malarial drug, artemisinin and diterpenoid chemotherapy drug, paclitaxel [7,8]. Most terpenoids are hydrophobic in nature and often become toxic to heterologous hosts at industrially feasible concentrations [9]. To minimize the detrimental effects of terpenoids on the host, *in situ* product recovery methods have become common practice at laboratory scale [10,11]. Through *in situ* extraction, terpenoids can be effectively removed from the cultivation medium as they are produced, thereby minimizing the accumulation, product loss and cell death [12]. Liquid-liquid extraction (LLE) using a biocompatible solvent such as the long chain alkane, dodecane [10], to sequester secreted products from the cultivation medium, is one of the

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most common approaches employed in this context. The organic solvent layer is immiscible, facilitating straightforward separation from the culture at the end of the cultivation. However, vast quantities of solvent are required and high boiling point of dodecane (216 °C) renders purification energy intensive, hindering economic feasibility. Furthermore, as only secreted terpenoids can be recovered using this approach, intracellular terpenoids (up to 33% of total production [13]) are typically neglected.

1.1. *In situ* solid phase adsorption of natural products

In situ solid phase adsorption (SPA) is an alternative method, which could potentially alleviate the aforementioned bottlenecks associated with liquid–liquid extraction (LLE). During *in situ* SPA, an inert adsorbent resin is added to the cultivation medium and the NP adsorbs to its surface as it is secreted by the host [14]. This dramatically reduces product loss due to air stripping or co-evaporation with the organic overlay [15].

Through *in situ* SPA, feedback inhibition and cytotoxic effects can be minimized thereby enhancing productivity [16,17]. Adsorbent resins have been successfully employed in this context to extract a wide range of NPs from a number of heterologous hosts including *Escherichia coli*, *Aspergillus fumigatus* and *Streptomyces hygroscopicus* via *in situ* or post-cultivation adsorption [16-19]. Different *in situ* SPA processes have been developed, one example is the extraction of chaetominine, an alkaloid compound with anti-cancer activity, from *Aspergillus fumigatus*, where out of eight resins tested, XAD-16 and HP-20 demonstrated the best extraction performance [18]. This superior performance was attributed to greater adsorption capacity resulting from their larger surface area. A number of polymeric resins have also been investigated recently for the recovery of the sesquiterpene, (+)-zizaene, from engineered *E. coli* cultivations. Of those tested, the highest (+)-zizaene titer was achieved using HP-20 in the *E. coli* cultivations, where the resin structure was a key element for higher hydrophobic adsorption [20].

1.2. Research scope: *In situ* solid phase adsorption for taxadiene biosynthesis and recovery

The biosynthesis and recovery of the highly effective chemotherapy drug, paclitaxel (commercially known as Taxol®), is a major ongoing research effort [21,22]. Despite decades of study, the complex paclitaxel biosynthetic pathway is yet to be fully elucidated. A robust precursor purification method therefore has the potential to expedite cell factory development and hence progress towards a more sustainable source of the drug [7,23]. Heterologous biosynthesis of the first committed precursor in the paclitaxel pathway, taxadiene, has been successfully achieved in both *E. coli* [24,25] and *S. cerevisiae* [13,26].

The use of a dodecane overlay has proven to be an effective method for taxadiene recovery in the highest-yielding *E. coli* [27,28] and *S. cerevisiae* at laboratory scale cultivations [13,22]. However, research into more scalable recovery and purification methods for this critical precursor is scarce, despite taxadiene itself being considered a valuable NP [23]. As described in Section 1.1, *in situ* solid phase adsorption is an alternative method with the potential to improve recovery of natural products. To maximize the efficiency of SPA, an appropriate resin material must be selected according to the polarity of the product of interest. In the case of taxadiene, a non-polar resin is desirable as the molecule is hydrophobic by nature. Of the wide range of available non-polar resins, HP-20 beads (Diaion®) were deemed most suitable for taxadiene recovery due to their large surface area (590–850 m^2/g) and high adsorption capacity of NPs with similar chemical structure [18].

The metabolic pathway for heterologous production in *Saccharomyces cerevisiae* and subsequent adsorption of taxadiene onto the HP-20 resin beads is summarized in Fig. 1. Although HP-20 resin beads have been employed for the adsorption and subsequent recovery of a variety of NPs, their application for the extraction of microbially synthesised

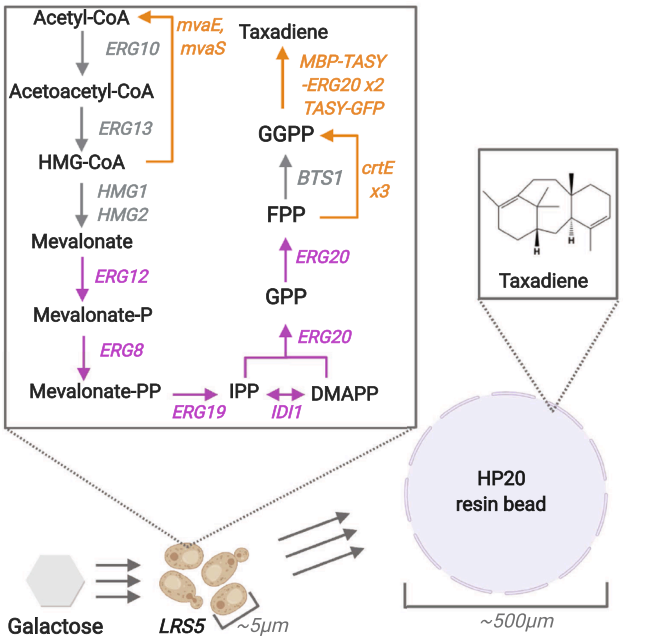

Fig. 1. Heterologous taxadiene biosynthetic pathway in *S. cerevisiae* engineered strain (*LRS5*) and concurrent *in situ* adsorption of the secreted taxadiene to the surface of the HP-20 resin beads. Galactose is metabolized via glycolysis yielding acetyl-CoA, which is then converted into the universal diterpenoid precursor, geranylgeranyl diphosphate (GGPP), via the highlighted mevalonate pathway. Finally GGPP undergoes cyclization by taxadiene synthase (TASY) yielding taxadiene. Native overexpressed genes are highlighted in purple, whilst heterologous genes are shown in orange.

taxadiene is novel.

In this study, a HP-20 resin bead mediated SPA method for the recovery of taxadiene produced by an engineered *S. cerevisiae* strain, *LRS5* [13], was developed and optimized. The effect of key factors such as resin concentration and extraction solvent on product recovery were investigated in detail across a range of bioreactor scales. The traditional dodecane overlay approach was also used to compare the performance. The system was tested across a range of cultivation scales from 2 mL high throughput cultivations, previously shown to be predictive of larger scale operations [11], to controlled benchtop bioreactors (250 mL) to investigate scalability and performance under more industrially relevant conditions. Finally, the bioprocess was intensified by provoking cell lysis using cell-wall disruptive organic solvents in the final extraction step.

2. Materials and methods

2.1. Yeast strains and media

The *S. cerevisiae* strain employed in this study was *LRS5 {mGTy116; ARS1014::GAL1p-TASY-GFP; ARS1622b::GAL1p-MBP-TASY-ERG20; ARS1114a::TDH3p-MBP-TASY-ERG20}* as described in detail in [13] was originally derived from laboratory strain *CEN.PK2-1C* (EUROSCARF, Germany). All reagents, including extraction solvents were sourced from Fisher Scientific UK at the highest available purity unless otherwise stated. The media used for the yeast cultivations was yeast extract peptone (YP, yeast extract 1 % (w/v), peptone 2 % (w/v)) supplemented with 2 % (w/v) galactose (YPG) or 2 % (w/v) glucose (YPD) unless otherwise stated.

2.2. Microscale cultivation using the solid phase adsorption (SPA) method

High-throughput microscale screening was performed using 24-well

10 ML deep well plates (Axygen, USA) with a working volume of 2 mL. Inoculum cultures were prepared by transferring a single colony of *S. cerevisiae* strain *LRS5* to 5 mL of YPD media and incubating in an orbital shaker incubator (Innova 42, Eppendorf, UK) at 30 °C and 250 rpm overnight. An aliquot of this culture was then diluted with YPG media to give a 2 mL culture with an initial $OD_{600} = 1$. Diaion® HP-20 adsorbent resin beads with a diameter of 0.4–1 mm and pore size of 290 Å [16] (Merck, Germany) were added to each of the cultivations at concentrations between 3 and 12 % (w/v) as indicated in the loading plan (Table S1 of supplementary material). Prior to use, the resin beads were autoclaved, rinsed with 100% ethanol and washed with sterile deionized water. The resulting culture plate was incubated at 30 °C for 72 h at 350 rpm and covered with an adhesive gas permeable membrane (Thermo Fisher Scientific, UK). This cultivation time is based on previous cultivations with *LRS5* [22], as at this point the cells enter the stationary phase of growth and taxane production ceases. At the end of the cultivation, the contents of each well were centrifuged at 4500 rpm for 10 min. To remove water traces in wells containing beads, only the solid phase (cells + beads) was recovered and dried under nitrogen gas at room temperature before adding acetone (99.8 %, Thermo Fisher Scientific, UK) at 1:1 ratio of the cultivation volume and incubating the resulting mixture at 30 °C and 350 rpm for four hours. Following incubation, the mixture was centrifuged at 4500 rpm for 10 min and the organic phase recovered for taxadiene analysis via GC–MS. Biomass accumulation was monitored as optical density at 600 nm via offline sampling using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, UK). To ensure the presence of beads did not inflate optical density readings, controls containing culture media and resin beads in the absence of cells were ran in parallel. As the OD readings of these controls were not significantly different from the blank, correction was not deemed necessary in the culture readings. For comparison, additional cultivations were performed using the widely used dodecane overlay approach described in detail in section 2.7. Control YPG cultivations with no extraction aid were also performed, taxadiene was extracted from the solid phase (biomass only) at the end of the cultivation using acetone at a 1:1 ratio. The resulting mixture was incubated at 30 °C and 350 rpm for four hours. Microscope images were taken using an oil immersion brightfield microscope that is equipped with 100x Leica NPLAN objective lens (Leica Microsystems, Germany) using an Andor-Zyla sCMOS camera (Oxford Instruments, UK). The SPA method using adsorbent HP-20 resin beads is summarized in Fig. 2.

2.3. Small scale cultivations

Small scale cultivations were performed in 50 mL tubes and 250 mL shake flasks with a working volume of 10 % of their respective total volumes. Inoculum cultures were prepared by transferring a single colony of *S. cerevisiae* strain *LRS5* to 5 mL YPD media and incubating at 30 °C and 250 rpm overnight. An aliquot of this culture was then diluted with YPG media to give a 5 mL culture with an initial $OD_{600} = 1$. In the SPA cultivations, autoclaved HP-20 adsorbent resin beads were added at a concentration of 3, 6 or 12 % (w/v). All of the cultivations were incubated at 30 °C with a shaking speed of 250 rpm for 72 h. At the end of the cultivation, biomass accumulation and taxadiene extraction using acetone was performed as described in section 2.2.

2.4. Taxadiene partition from solid and liquid phases

To determine the taxadiene partition between the beads, biomass and liquid phase, an additional experiment was conducted under the conditions detailed in Section 2.2. Once the stationary phase of the growth had been reached at around 72 h, the beads were removed from

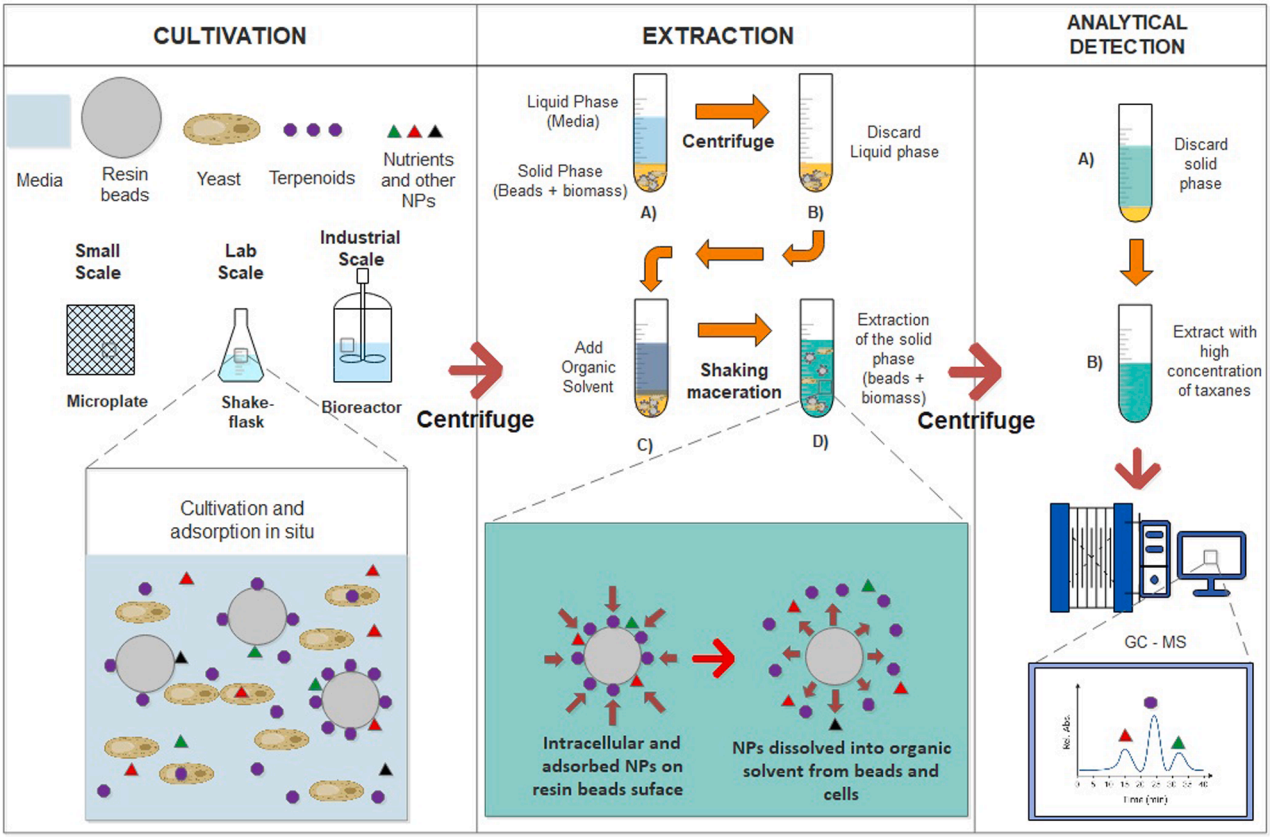

Fig. 2. HP-20 resin beads *in situ* SPA method. The methodology focuses on extracting taxadiene an early paclitaxel precursor produced by the engineered *Saccharomyces cerevisiae* strain, *LRS5*. Taxadiene is adsorbed onto the resin surface as it is secreted throughout the cultivation and extracted at the end using an appropriate organic solvent.

the cultivation using a nylon mesh cell strainer with a pore size of 70 μm (Thermo Fisher Scientific, UK). The cells were separated from the resulting solution via centrifugation at 4500 rpm for 10 min, the supernatant was transferred to a separate vessel for further analysis. The biomass and bead phases were both dried under nitrogen gas before being resuspended separately in a volume of acetone equal to the original cultivation volume. The resulting suspensions were incubated at 30 °C and 250 rpm for four hours. To recover taxadiene from the liquid phase, a dodecane overlay of 20 % of the cultivation volume was added to the recovered supernatant at 30 °C and 250 rpm for four hours. Following incubation, the extracts were centrifuged at 4500 rpm for 10 min and the dodecane organic phase was recovered for taxadiene analysis via GC–MS.

2.5. Extraction solvent optimization

To investigate the effect of extraction solvent on recovery of taxadiene from the resin beads, the following solvents were tested: ethanol, acetone, ethyl acetate and dodecane. Following cultivation at small scale (5 mL) for 72 h, under the conditions detailed in section 2.3, the solid phase was recovered by centrifugation at 4500 rpm for 10 min and resuspended in a volume of the respective solvent equal to that of the original culture. The resulting mixtures were incubated at 30 °C and 250 rpm for four hours then centrifuged at 4500 rpm for 10 min to separate the organic and solid phases. The organic phases from each organic solvent were subsequently extracted for GC–MS analysis.

2.6. Bioreactor cultivation

Larger scale cultivations were conducted in MiniBio 500 bioreactors (Applikon Biotechnology, The Netherlands) with a working volume of 250 mL. Pre-inoculum cultures were prepared by transferring from a single colony to 5 mL of YPD and incubating in an orbital shaker at 30 °C and 250 rpm for eight hours. The resulting culture was subsequently used to inoculate a secondary 10 mL YPD culture to an $OD_{600} = 1$ and incubated overnight under the same conditions as the pre-inoculum. An aliquot of this culture was then diluted with YPG media in the bioreactor to give a 250 mL culture with an initial $OD_{600} = 1$. To prevent excess foam production polypropylene glycol P2000 (Alfa Aesar, UK) was added to a concentration of 0.01 % (v/v) and an impeller with two Rushton turbines was placed at the medium-air interface (separation between turbines: 6.5 cm) at a stirring speed of 800 rpm. Autoclaved HP-20 adsorbent resin beads were added at a concentration of 3 % (w/v), the optimal bead concentration elucidated during small-scale experiments. A set point of 30 % saturation for DO was maintained through automated air sparging and the culture temperature was maintained at 30 °C. The pH was maintained above six through the automatic addition of 1 M NaOH. Biomass was measured offline twice daily using Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, UK). At this scale, biomass kinetics were monitored via offline sampling throughout the cultivation and the cultivation stopped moments before the stationary phase of growth was reached. Galactose concentration was monitored via offline sampling twice daily using the 3,5-Dinitrosalicylic acid (DNS) method [29].

2.7. Dodecane overlay method

Experiments using the widely used dodecane overlay method were performed to facilitate direct comparison of the traditional method with the proposed *in situ* SPA method. In this work, inocula, cultivations and extraction methods were as described previously by [13] for microscale cultivations. In the dodecane overlay experiments, dodecane (99 %, Acros Organics, UK) comprised 20 % (v/v) of the total cultivation volume and resin beads were not added. To determine the final taxadiene titre, the resulting cultures were centrifuged at 4500 rpm for 10 min to separate the aqueous and organic phases and an aliquot of the organic phase was subjected to GC–MS analysis.

2.8. Analytical methods

Taxadiene identification and quantification was achieved via GC–MS. A 1 μL sample of each organic solvent extract was injected into a TRACE™ 1300 Gas Chromatograph (Thermo Fisher Scientific, UK) coupled to an ISQ LT single quadrupole mass spectrometer (Thermo Fisher Scientific, UK). Chromatographic separation was achieved using a Trace Gold TG-SQC gas chromatography column (Thermo Fisher Scientific, UK) using a previously described method [26]. To identify and quantify production of compounds by *S. cerevisiae* strain *LRS5*, pure standards of taxadiene, kindly supplied by the Baran Lab (The Scripps Research Institute, California, USA) and GGOH, obtained from Sigma Aldrich (Gillingham, UK), were used. Characteristic gas chromatograms and taxadiene mass spectra can be found in supplementary information (Figure S5 of Supplementary material).

2.9. Statistical analysis

Statistical analyses were performed using MATLAB 2020a and Minitab 19 statistical software. One-way analysis of variance (ANOVA) was used to determine whether HP-20 resin beads concentration yielded a significant impact on biomass yield at microplate and shake flask scale. A Dunnett's multiple comparison test was used to compare each treatment to the control. One-way ANOVA was also used to determine whether the selected extraction solvent had an effect on taxadiene titers recovery. A Tukey's honest significant difference test was subsequently employed to compare each of the treatments. The null hypothesis considered that there was no significant difference between the treatments, hence if $p \leq 0.05$ the null hypothesis was rejected.

3. Results and discussion

3.1. Effect of bead concentration on LRS5 growth and taxadiene accumulation kinetics

In situ solid phase adsorption (SPA) using HP-20 resin beads has proven to be an effective method for improving NP recovery in microbial cultivations [17,30]. However, high bead concentrations have been found to inhibit microbial growth [31]. As the rates of biomass and taxane accumulation have been shown to be highly correlated in the engineered *S. cerevisiae* strain *LRS5* used in this study [13], optimization was necessary to maximize both growth and product recovery. The *S. cerevisiae* strain *LRS5* was cultivated in the absence and presence of a range of bead concentrations in order to determine the effect on growth kinetics at microscale. A further study was conducted using shake flasks to investigate the effect of concentrations at an increased scale. Control cultivations of *S. cerevisiae* strain *LRS5* in the absence of the resin were included in the study, both with or without (no extraction aid) a dodecane overlay. The results of these studies are summarized in Fig. 3.

At microscale, the addition of 3 % (w/v) HP-20 resin beads was found to enhance the growth rate of *S. cerevisiae* strain *LRS5,* with the stationary phase of growth reached earlier than for the control cultivation with no extraction aid (Fig. 3A). An OD_{600} of 21 ± 3 was observed after 55 h, significantly greater than the control cultivation with no extraction aid ($p = 0.0005$, Fig. 3A). However, the addition of 3 % (w/v) beads had no significant effect on the final OD_{600} ($p = 0.747$, Fig. 3A) with highly similar OD_{600} values of 23 ± 3 and 22 ± 8 obtained for the 3 % and no extraction aid cultivations. This behavior was similar in the cultivations using 6 % (w/v) resin beads where no significant statistical effect at 72 h was observed with an OD_{600} value of 20 ± 1 ($p = 0.280$, Fig. 3A). The final biomass yield of the cultures treated with a dodecane overlay was also not statistically different from the control with no extraction aid ($p = 0.654$, Fig. 3A) at 20 ± 1. At the higher concentration of 12 % (w/v), however, the addition of beads was highly detrimental to

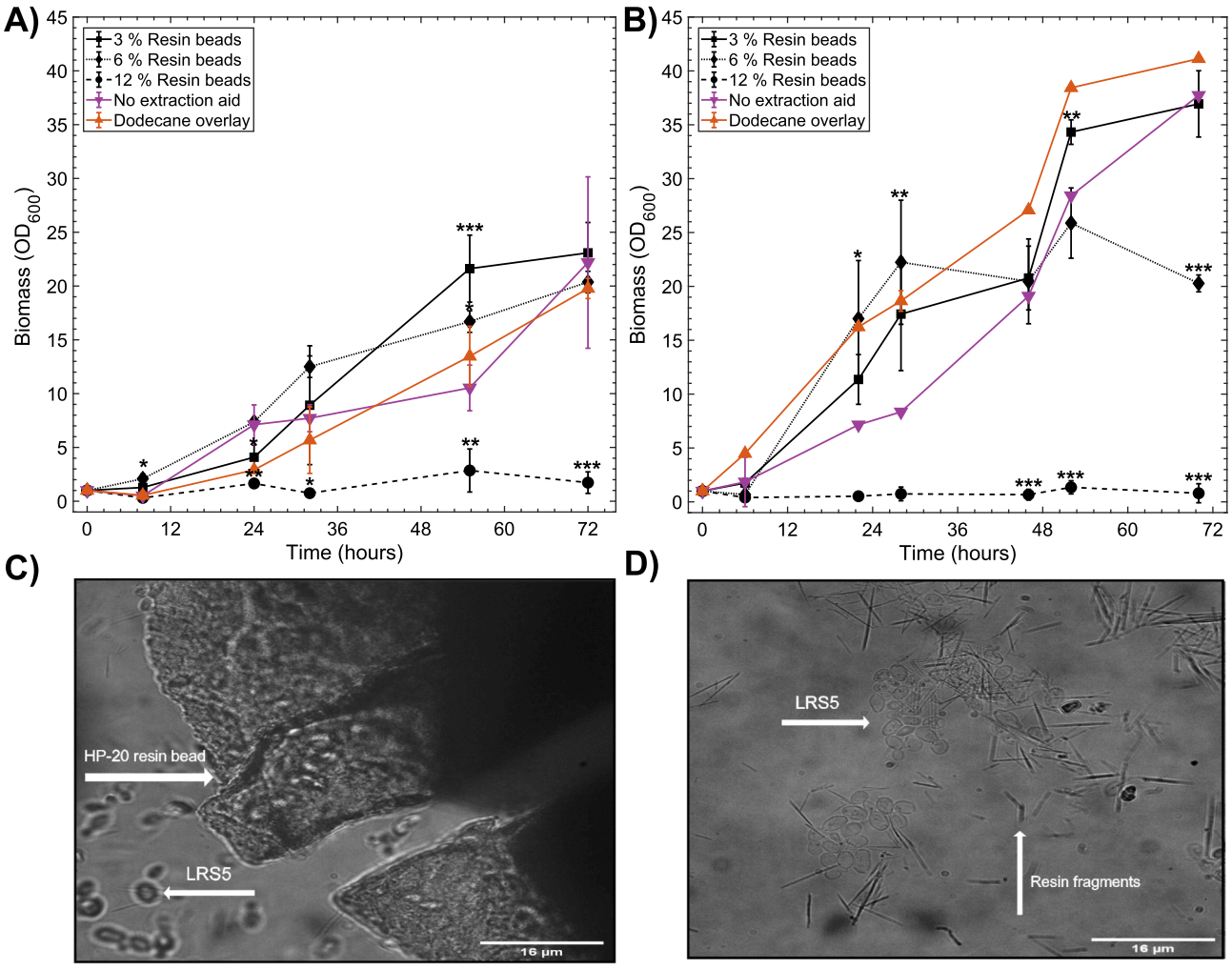

Fig. 3. Growth kinetics of *S. cerevisiae* strain *LRS5* in presence of different concentrations of HP-20 resin beads (3A & 3B), microscope images of *S. cerevisiae* strain *LRS5* cultivated at 3 % (w/v) resin beads at small scale (3C & 3D). 3A) Experiments were carried out using microscale cultivations (2 mL cultivation) and 3B) small scale cultivations (25 mL cultivation). All cultivations were performed with YP media supplemented with 2 % galactose at 30 °C and 250 rpm. Values represent mean ± standard deviation ($n = 3$) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Dunnett's multiple comparison test with no extraction aid (0 % resin bead) as control). 3C) Interaction between HP-20 resin bead and *S. cerevisiae* strain *LRS5* cells. 3D) Sharp resin fragments observed in the culture medium after 72 h.

S. cerevisiae strain *LRS5* growth ($p = 0.0003$, Fig. 3A) with a final OD_{600} of just 3 ± 1 achieved at the end of the 72 h cultivations. This indicated that at low concentrations, the beads were not detrimental to *S. cerevisiae* growth. As for the microscale cultivations, the final OD_{600} of the 3 % (w/v) bead and control cultures, which had no extraction aid were comparable ($p = 0.883$, Fig. 3B). At the higher bead concentration of 6 % (w/v) a 46 % reduction in biomass accumulation was observed compared to the control ($p < 0.0005$, Fig. 3B). Increasing the concentration further to 12 % (w/v) inhibited growth almost entirely with a final $OD_{600} = 1 \pm 0.5$ ($p < 0.0005$, Fig. 3B). The optimal bead concentration was therefore deemed to be 3 % (w/v) with maximum OD_{600} values of 21 ± 3 and 37 ± 3 at micro and shake flask scale, respectively. These results were in agreement with previous studies using *E. coli*, *Streptomyces hygroscopicus* and *Streptomyces virginiae* [17,20,33], which found high bead concentrations were detrimental to microbial growth. *E. coli* was able to tolerate higher bead concentrations with an optimal of 5 % (w/v) [20], whilst the optima for *Streptomyces hygroscopicus* and *Streptomyces virginiae* were similar to that found in this study (Fig. 3A and Fig. 3B) at between 2 % and 5 % (w/v) respectively [17,33].

Adsorption to the resin surface is not exclusive to the desired taxadiene compound [34], additional hydrophobic compounds present within the culture medium therefore likely adsorbed to its surface. Such compounds include fatty acids and non-polar amino acids, which are essential for yeast growth [35]. A previous study revealed that 55 % of the casein present within the culture medium adsorbed to the HP-20 beads when a concentration of 20 % (v/v) beads was used compared to just 17 % at a 5 % (v/v) bead concentration [36]. This resulted in a reduced availability of amino acids for cell growth, the adsorption of essential nutrients may have therefore contributed to the poor growth observed in the 12 % (w/v) cultivations of this study. This highlights the importance of carefully optimizing the concentration of resin to ensure sufficient capacity to maximize product recovery whilst minimizing nutrient loss and hence growth inhibition [17]. In addition, visualization of the cultures using oil immersion light microscopy (Fig. 3C and 3D) revealed physical degradation of the beads, generating sharp fragments as shown in Fig. 3D, which may have caused some mechanical lysis of the cells.

Such degradation was likely enhanced by culture agitation as it has been noted in previous works using similar type of resins [37]. Furthermore, as the resin beads were around 100-fold larger than the yeast cells, their presence within the culture likely contributed to shear stress, which may have hindered cell viability at the higher bead concentrations (>6 % (w/v)). To our knowledge, this is the first report of the contribution of HP-20 bead degradation and subsequent sharp fragment generation to reduced cell growth at higher bead concentrations.

To further investigate the effect of bead concentration on cellular

fitness, additional microplate cultivations were conducted under the same conditions as described in section 2.2, except the parental strain, *CEN.PK2-1C*, was used in place of strain *LRS5*. As this strain has not been engineered for taxadiene biosynthesis or mevalonate pathway overexpression, the effect of heterologous gene expression could be evaluated. When *CEN.PK2-1C* was cultivated in the presence of 3 % (w/v) resin beads, a maximum OD_{600} value of 40 ± 3 was reached after 72 h (Figure S1, Supplementary material), Considerably higher than the 23 ± 3 observed for strain *LRS5* under the same conditions. Interestingly, even at the highest bead concentration of 12% (w/v), no significant effect on growth was observed in the wild-type strain, with a maximum OD_{600} value of 38 ± 4.0. These results highlight the detrimental effect of gene editing on *S. cerevisiae* fitness, as the tolerance to mechanical stress caused by the resin beads was reduced considerably in *LRS5*.

To investigate this effect further and determine the optimal concentration of beads, a second experiment involving larger shake flask cultivations was performed, here the bead concentration of 3 and 6 % (w/v) were tested. As the increased volumes at this scale facilitated more frequent manual sampling, an investigation into taxadiene accumulation kinetics was also performed as summarized in Fig. 4.

In these experiments, final OD_{600} values were 30 ± 1 and 20 ± 0.5 for the 3 % (w/v) and 6 % (w/v) bead cultivations, respectively, indicating that the lower bead concentration of 3 % (w/v) was more suitable at this scale. In addition, a strong correlation between biomass and taxadiene accumulation was observed in 3 % (w/v) (Pearson's r = 0.962) and 6 % (w/v) (Pearson's r = 0.943). This was in agreement with previous studies [13,22], which also observed a strong correlation between biomass accumulation and taxane production. It was therefore deemed important to optimize the HP-20 resin beads concentration such to minimize any detrimental effects on cell growth and productivity. It was also seen that there was no statistical difference in taxadiene titer using 3% and 6% (w/v) of resin beads.

Although the exact adsorption capacity of the HP-20 beads for taxadiene is unknown, previous studies have investigated their adsorption capacity for the final paclitaxel compound [38]. The maximum adsorption capacity was found to be 45 – 57 mg/g under controlled conditions. Assuming the capacity for taxadiene were in the same range to that of taxadiene, the resin bead concentration could theoretically be reduced to as low as 0.1 % (w/v) to recover the taxadiene produced by *S. cerevisiae* strain *LRS5*. To investigate this further, additional microscale cultivations were performed using lower bead concentrations of 0.1 and 1 % (w/v) (Figure S4, Supplementary information). There was no significant difference in the final OD_{600} values observed for the 0.1, 1 and 3 % (w/v) bead cultivations at 26 ± 2, 32 ± 4 and 28 ± 0.3, respectively (Figure S4A, Supplementary information). Although bead concentration had little effect on growth of *S. cerevisiae* strain *LRS5* between 0.1 and 3 % (w/v), taxadiene recovery was enhanced by 17 and 76 % at 1 and 3 % (w/v) beads, respectively, compared to 0.1 % (w/v) (Figure S4B, Supplementary information). This was likely due to the aforementioned lack of specificity of the resin, which may have resulted in the adsorption of additional non-polar compounds present in the culture medium, reducing the capacity available for taxadiene adsorption. A resin bead concentration of 3 % (w/v) was therefore deemed optimal for taxadiene recovery and applied in subsequent experiments.

3.2. Solid – Liquid extraction optimization using different organic solvents

The partition of taxadiene between the solid (beads + biomass) and aqueous (culture medium) phases was subsequently investigated in small scale (5 mL) cultures of *S. cerevisiae* strain *LRS5*. Following cultivation, taxadiene was extracted from each phase using dodecane and quantified via GC–MS. Analysis revealed 97 % of the detected taxadiene was present in the solid phase (Figure S2 of supplementary material). As a result, subsequent experiments focused on extraction and purification of taxadiene from the solid phase exclusively. This ensured recovery of the vast majority of the product of interest, whilst minimizing solvent requirements.

A key benefit of the application of an adsorbent resin is the elimination of reliance on biocompatible extraction solvents, which with high boiling points render the purification stage both energy intensive and costly (*i.e.* dodecane). A low cost extraction solvent with a low boiling point is preferable to maximize the environmental and economic

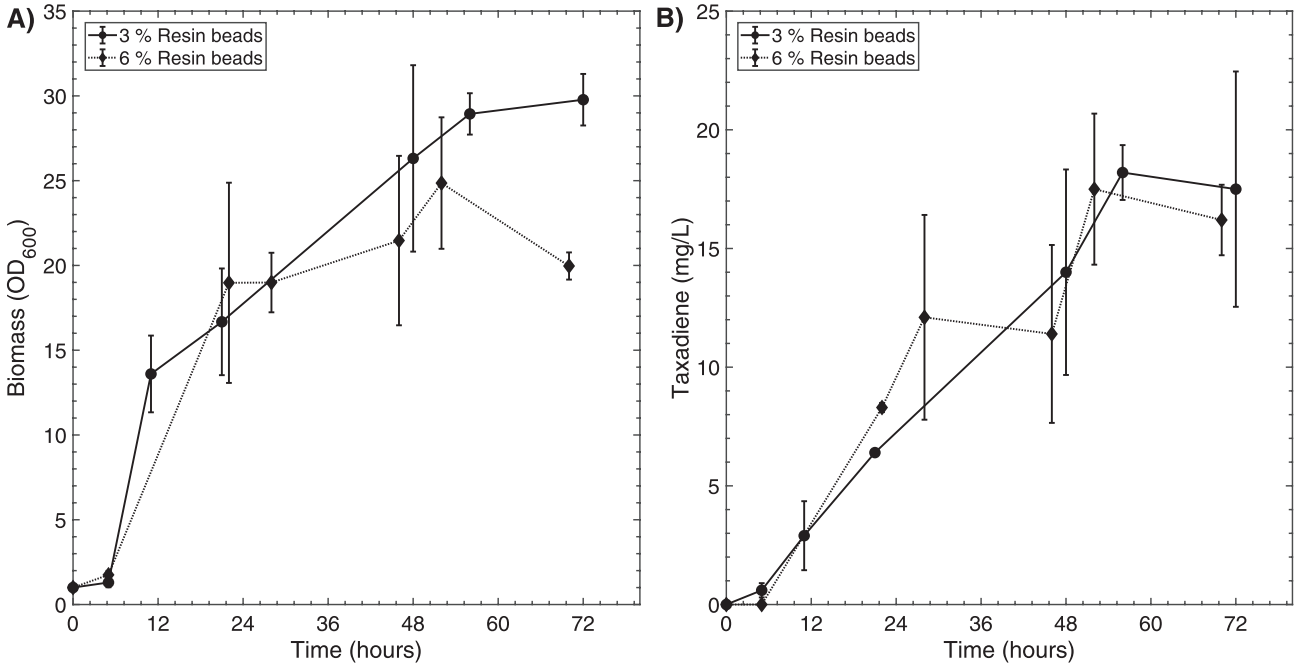

Fig. 4. Taxadiene concentration and yeast strain *LRS5* kinetics using *in situ* solid phase adsorption method, both cultivations were made in shake flask (25 mL) at 30 °C. A) Showcase the growth kinetics of the yeast at 3 and 6 % (w/v) beads concentration and B) Display the taxadiene concentration kinetics of the same cultivations. Solid phase (cells + beads) was treated with acetone in the extraction every specified hour (see section 2.3 for more details). Values represent mean ± standard deviation (n = 2).

sustainability of the process. Previous studies have shown solvents with intermediate polarity such as acetone can improve terpenoid recovery in *in situ* SPA systems compared to dodecane [39,40]. Aguilar et al. (2019), recently employed HP-20 resin beads for the *in situ* extraction of the sesquiterpene zizaene from engineered *E. coli* cultivation. In that study, of the seven extraction solvents tested in the study, ethyl acetate, decane and isooctane displayed superior zizaene recovery to dodecane. Ethyl acetate has also been successfully employed for the recovery of limonene [41] and Fusicocca-2,10(14)-Diene [42] from resins resulting from *E. coli* and *S. cerevisiae* cultivations, respectively. Ethyl acetate was therefore selected as a potential solvent in this study, as in addition to its observed affinity for terpene compounds, it is a volatile and relatively low-cost solvent with low toxicity. Acetone and ethanol with similar and higher polarities compared to ethyl acetate, respectively, are commonly used organic solvents for the extraction of carotenoids from yeast cells [43]. The efficacy of these solvents for eluting the shorter chain diterpene, taxadiene, from the HP-20 resin was investigated in this study, as summarized in Fig. 5.

Of the tested solvents acetone ($p = 0.007$) and ethyl acetate ($p = 0.023$) enhanced taxadiene recovery significantly with respect to dodecane as shown in Fig. 5. Unlike dodecane, the three other solvents tested in this investigation are not biocompatible and are known to affect yeast-cell viability and metabolic activity at concentrations ≥ 6 % (v/v) [44]. Organic solvents including ethanol, acetone and ethyl acetate, disrupt the phospholipid bilayer of the cell membrane, which increases permeability and can ultimately lead to cell lysis [45-47]. Biocompatible solvents such as dodecane are commonly used for *in situ* liquid-liquid extraction to minimize this effect during cultivation and maximize cell viability. However, permeabilization is desirable for maximizing product recovery as it ensures access to intracellular taxadiene [45,48]. Currently, limited data is available on the nature of the intracellular accumulation of taxadiene in *S. cerevisiae,* as previous localization studies focused on the taxadiene synthase (TASY) enzyme rather than the taxadiene product [26]. However, recent studies indicate that cells have limited capacity to store high concentrations of terpenoids [49,50] and any terpenoids that are not secreted likely accumulate in the cytoplasm.

The higher titers observed with the alternative solvents in this study (Fig. 5) was likely attributed to increased membrane permeability and intracellular taxadiene recovery. The greatest taxadiene titer of 15 ± 2.0 mg/L was achieved using acetone (5 mL cultivations), four-fold greater than that using the highly non-polar and biocompatible dodecane solvent. This solvent was therefore selected for subsequent studies.

3.3. Synergic effect of HP-20 resin beads and selected solvent to improve taxadiene extraction

The results of the desorption step indicated that the use of ethanol, acetone or ethyl acetate as the extraction solvent, resulted in higher taxadiene titres than dodecane. Such solvents are known to increase cell membrane permeability [46,47] and previous studies have shown that some taxadiene is retained intracellularly [13]. It was therefore hypothosised that improved recovery achived using ethanol, acetone or ethyl acetate may be partially due to increased cell memebrane permeability. In order to investigate this further, an additional experiment was performed using the highest yielding solvent, acetone (Fig. 5). *S. cerevisiae* strain *LRS5* was cultivated in the absence of resin beads or a dodecane overlay (no extraction aid) and acetone was applied at the end of the cultivation to extract taxadiene from the solid phase (biomass). This was compared to the optimal *in situ* solid phase (beads + biomass) cultivation with 3 % (w/v) HP-20 beads and the traditional dodecane overlay method as shown in Fig. 6.

Biomass accumulation was similar for the SPA, no extraction aid and dodecane overlay cultivations with final OD_{600} values of 23 ± 1, 22 ± 3 and 20 ± 1, respectively. When acetone was applied to extract taxadiene from the biomass resulting from the control treatment, in which *S. cerevisiae* strain *LRS5* was grown in the absence of an *in situ* extraction aid, the final taxadiene titer was 26 ± 6 mg/L at microscale as shown in Fig. 6. Interestingly, this was comparable to the 31 ± 3 mg/L obtained using the traditional dodecane liquid-liquid extraction method. This suggested that, in the absence of an *in situ* extraction aid, a significant proportion of the taxadiene was intracellular or adsorbed onto cell

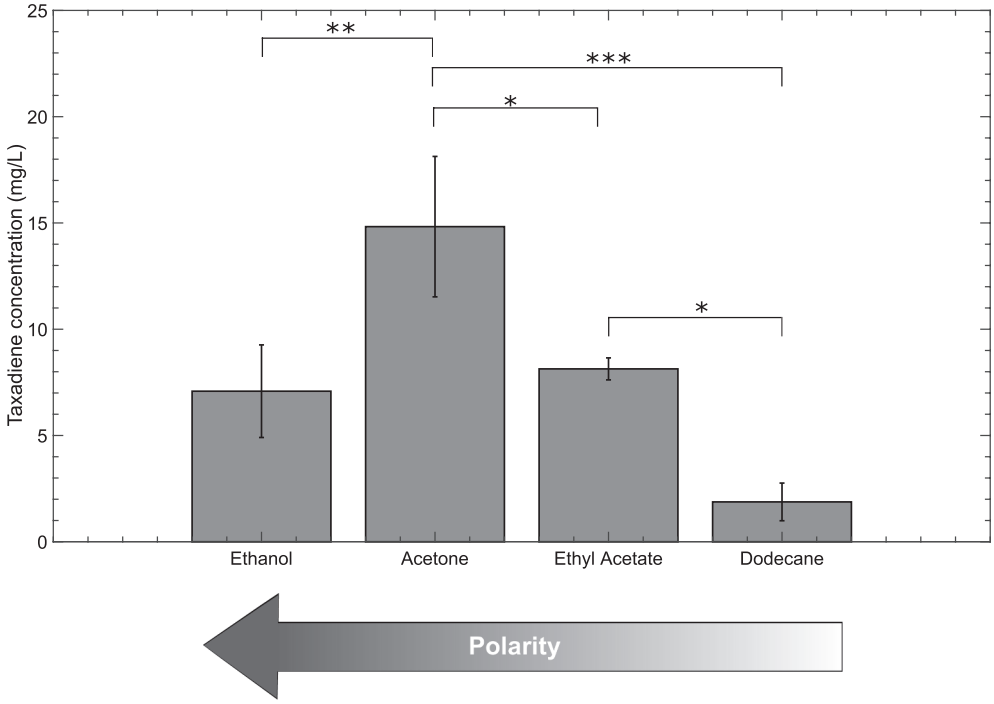

Fig. 5. Effect of different organic solvents on taxadiene recovery in Solid phase adsorption (SPA) cultivation method. All the solvents were used in the extraction stage, following resin bead cultivation at small scale (5 mL). Values represent mean ± standard deviation ($n = 3$) (* $p < 0.05$, ** $p < 0.01$, $p < 0.001$; Tukey's honestly significant difference multiple comparison test).

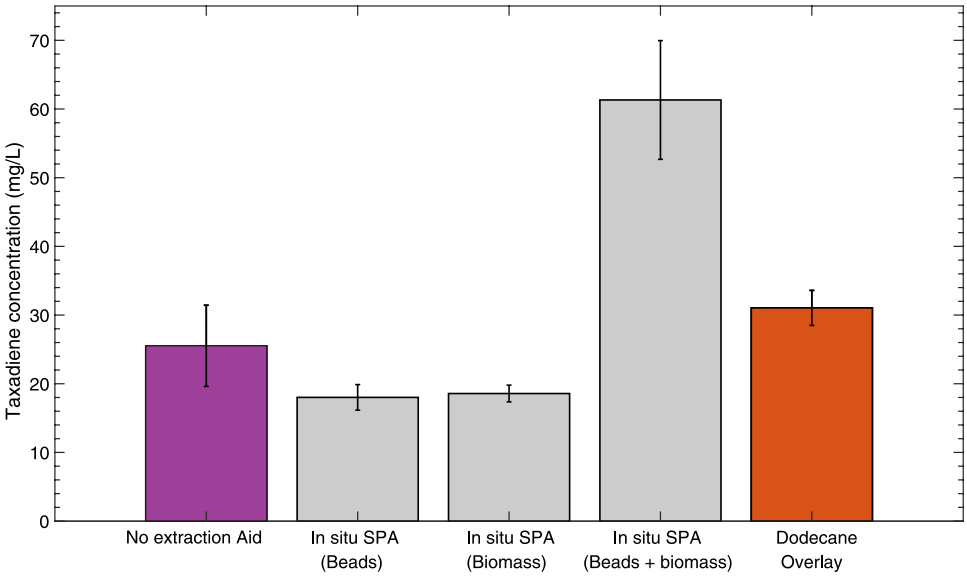

Fig. 6. Taxadiene recovery titers (bars) from microplate cultivations (2 mL cultivation volume) after 72 h of cultivation at 30 °C and 350 rpm. Purple bar represents the cultivation made with no extraction aid where the extraction was made to the biomass using acetone. Colored grey bars represent *in situ* Solid phase adsorption (SPA) cultivation using 3 % (w/v) bead concentration with further extractions made with acetone whilst orange bar represents extraction made with dodecane using the dodecane overlay method. *S. cerevisiae* strain *LRS5* reached similar OD at 600 nm in all treatments after 72 h. Beads + Biomass value could be higher due to less manipulation in the extraction steps. Values represent mean ± standard deviation ($n = 2$).

surface rather than secreted, likely due to the hydrophobic nature of the diterpene. In the *in situ* SPA cultivations, taxadiene was distributed equally between the resin beads and biomass with titers of 18 ± 2 mg/L and 19 ± 1 mg/L observed, respectively. This indicated that the cells retained up to near 50 % of the taxadiene produced during the cultivation. However, it was found that during the separation and desorption steps, around 30% of the bead volume was lost compared to parallel cultivations with the same bead concentration, in which the beads and biomass were not treated separately (Figure S3 of supplementary material). This taxadiene partition between biomass and resin beads provides an important piece of information, as it showcases where the taxadiene that is being detected in the system is being captured. To our knowledge, this information has not been commonly reported as the extraction is generally made to the biomass and resin in conjunction [17,30,51] or to the single beads without biomass [18,52]. These results could have an important impact into determining the capacity of the resin beads to relieve the cells from intracellular toxicity of selected NP among other phenomena.

Simultaneous extraction of taxadiene from the resin beads and biomass, without separation, resulted in a taxadiene titer of 61 ± 8 mg/L, at this scale. This suggests that around 39 % of the recoverable taxadiene was lost during the separation step, likely due to the loss of beads during the biomass and beads separation, indicating that the actual fraction of taxadiene retained by the cells was 51 %, an almost evenly partition ratio. This is in agreement with a previous study using *S. cerevisiae* strain *LRS5*, which demonstrated that up to one third of the total taxadiene was retained intracellularly using dodecane [13]. Using the optimal bead concentration (3 % (w/v)) and extraction solvent (acetone) resulted in a 1.8-fold improvement in taxadiene recovery compared to the widely used dodecane overlay approach (Fig. 6). Therefore, to maximize intracellular and secreted taxadiene titer, an extraction solvent with a high affinity for taxadiene and a high permeabilization ability should be selected following the cultivation. Differences in results between Fig. 5 and Fig. 6 were likely the result of an increased shaking speed of 350 rpm (Fig. 6) compared to the 250 rpm applied for the 5 mL cultures (Fig. 5). In addition, the microtiter plates were sealed with a gas permeable membrane whereas the tubes used for the 5 mL cultures were airtight. Oxygen mass transfer was therefore likely enhanced significantly at microscale, promoting aerobic growth and thus improving taxadiene titers. A similar effect was observed in a previous study where taxadiene titers were improved two-fold in baffled microtiter plate compared to shake flask cultivations of the strain [22].

A significant quantity of taxadiene was recovered from the cells cultured without an extraction aid, when acetone was used as the desorption solvent (Fig. 5). This indicated that, in addition to promoting elution of secreted taxadiene from the HP-20 resin, acetone also enhanced the recovery of intracellular taxadiene, likely due to its effect on cellular permeability. The proposed SPA method is therefore an integrated bioprocess, eliminating the requirement for an additional unit operation for cellular lysis [1,53] or further metabolic engineering to introduce efflux pumps (e.g. outer membrane protein TolC) [54] to enhance the product secretion.

3.4. Scale up bioprocess of the in situ SPA method

In order to validate the proposed *in situ* SPA method under industrially relevant conditions, the process was scaled up using MiniBio 500 bioreactors (Applikon Biotechnology, The Netherlands). Here industrially relevant process parameters including pH, temperature and dissolved oxygen concentration were monitored and controlled online in real-time, allowing industrial scale conditions to be more effectively mimicked [55]. The small- (Fig. 5) and micro-scale cultivations (Fig. 6) revealed that a resin beads concentration of 3 % (w/v) was optimal for biomass and taxane accumulation, this condition was therefore selected for investigation at larger scale. A stirring speed of 800 rpm was used to guarantee homogeneous distribution of the resin beads. The results of this experiment are summarized in Fig. 7.

Final taxadiene titers were comparable at micro and bioreactor scale at 61 ± 8 mg/L and 76 ± 19, respectively (Fig. 7B), demonstrating that the proposed process was robust despite a 125-fold increase in cultivation volume. This result is promising as shear stress was likely exacerbated at this scale due to the relatively high agitation rate of 800 rpm and use of air sparging for dissolved oxygen control. The resulting titer was 1.4-fold higher than the 53 mg/L achieved for *S. cerevisiae* strain *LRS5* using the dodecane overlay method under similar cultivation conditions [13]. LLE using dodecane overlay was also employed for extraction of taxadiene from the highest yielding heterologous *E. coli* expression system [27], suggesting that the impressive titer of 1000 mg/L could possibly be improved further through the use of *in situ* SPA. The maximum taxadiene titers achieved in *S. cerevisiae* strain *LRS5* [22] were around sevenfold lower than those in *E. coli* [27]. Despite this, the eukaryotic host is desirable as unlike *E. coli*, it possesses the necessary biosynthetic and redox capabilities for the expression of the subsequent cytochrome P450 genes, which constitute around 50 % of the 19 steps of the paclitaxel biosynthetic pathway [56,57]. Lastly, a similar method for paclitaxel recovery (using XAD resin) showed 40–70 % enhanced

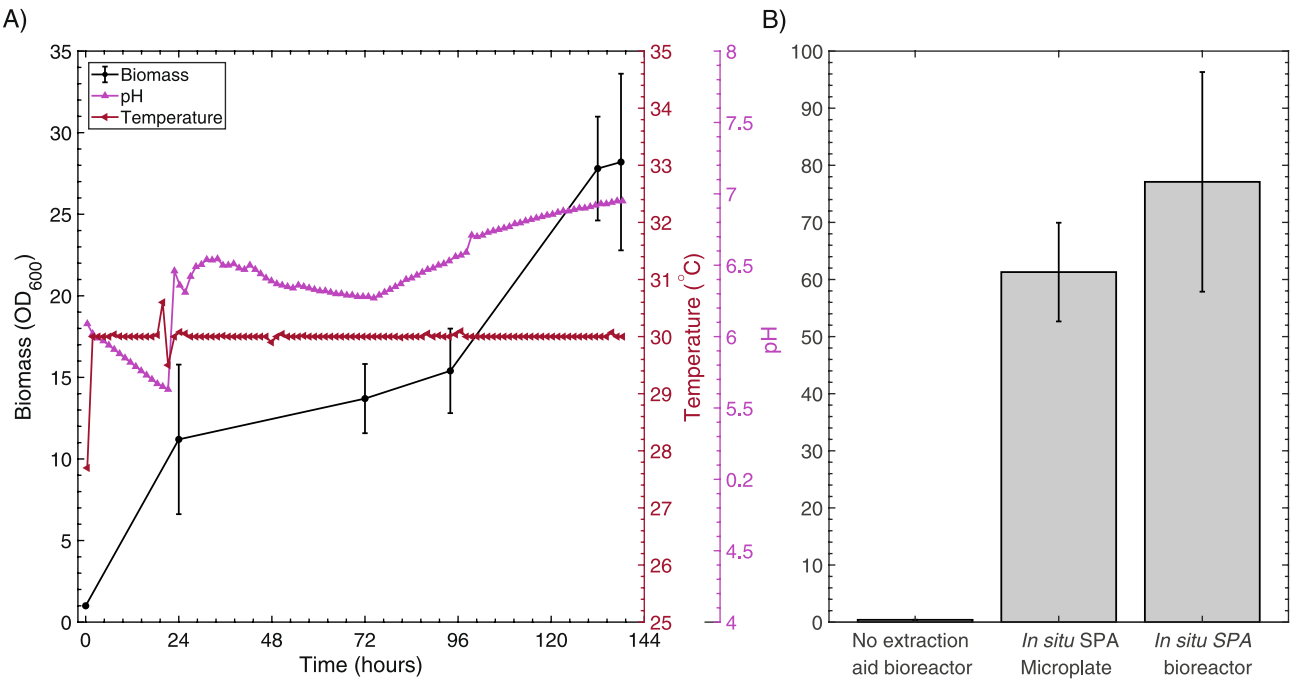

Fig. 7. Bioreactor cultivation kinetic parameters of *S. cerevisiae* strain *LRS5* using the *in situ* Solid phase adsorption (SPA) cultivation method. Fig. 7A) shows the cultivation parameters using the *in situ* SPA method at 250 mL cultivation volume. The controlled parameters were; pH and temperature. Fig. 7B) shows the taxadiene titers for microplate cultivation and bioreactor using the *in situ* SPA method using 3 % (w/v) and acetone in the extraction step as well as the cultivation with no extraction aid. Values in Fig. 7B represent mean ± standard deviation ($n = 2$).

paclitaxel biosynthesis in *Taxus cuspidate* cell cultures [58], which reinforce that the use of adsorbent resin beads could augment the recovery yields of different targeted taxanes.

One possible reason for the enhanced recovery using the *in situ* SPA method compared to the dodecane overlay method is the positive effect of acetone for solid phase (biomass + beads) extraction, allowing recovery of both the intracellular and secreted taxadiene. Moreover, the loss of taxadiene due to air stripping or co-evaporation with dodecane was likely reduced through the use of HP-20 resin beads. In the control cultivations with no resin beads or dodecane overlay, taxadiene titers were negligible (0.44 ± 0.01 mg/L , Fig. 7B) due to air stripping, highlighting the importance of *in situ* product recovery at bioreactor-scale.

This synergic combination of sequestering secreted and inner taxadiene through coupling non-polar resin beads with a disruptive desorption solvent is a key benefit of the proposed bioprocess. Through further optimization of key process parameters such as organic solvents, resin material, cultivation temperature and pressure [59], there is great potential to further improve recovery of taxadiene and other paclitaxel precursors.

These results demonstrate the potential of the *in situ* SPA cultivation method for effective recovery of taxadiene under industrially relevant conditions with considerable taxadiene yield. Nevertheless, further techno-economic assessment is needed to evaluate the cost effectiveness of the method at industrial scale.

4. Conclusions

The application of the *in situ* SPA method using HP-20 resin beads at micro and bioreactor scale improved taxadiene recovery 1.4 and 1.9-fold, respectively, compared to the traditional liquid-liquid extraction approach using dodecane overlay. Higher resin bead concentrations resulted in bead fragmentation (mechanical cell disruption) which were found to be detrimental to cellular growth in *S. cerevisiae* strain *LRS5*. As the biomass growth is heavily correlated with taxadiene production, it was found that by using 3 % (w/v) beads concentration, the biomass growth was not affected and the taxadiene time-profile recovery was the higher obtained at this resin bead concentration. Acetone ensured the sequestration of both the intracellular and secreted taxadiene at the extraction step, achieving process integration and taxadiene yield increment. In addition, it was found that by using the *in situ* SPA method with 3 % (w/v) resin beads, of the total taxadiene synthesized, 49 % was adsorbed by the resin beads with the remainder retained intracellularly.

CRediT authorship contribution statement

Jorge H. Santoyo-Garcia: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – original draft, Data curation, Writing – review & editing. **Laura E. Walls:** Formal analysis, Writing – review & editing, Data curation, Visualization. **Behnaz Nowrouzi:** Writing – review & editing, Visualization. **Giuseppe R. Galindo-Rodriguez:** Investigation. **Marisol Ochoa-Villareal:** Visualization. **Gary J. Loake:** Supervision, Visualization. **Simone Dimartino:** Supervision, Visualization. **Leonardo Rios-Solis:** Resources, Writing – review & editing, Supervision, Visualization, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2022.120880.

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