1 *In situ* solid-liquid extraction enhances recovery of taxadiene

2 from engineered *S. cerevisiae* cell factories

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12 **Abstract**

13 A novel *in situ* solid phase adsorption strategy was investigated for enhanced recovery

14 of taxadiene, a precursor to the blockbuster anticancer drug, paclitaxel, from

15 engineered *Saccharomyces cerevisiae*. A synthetic adsorbent resin (HP-20) was

16 employed to capture taxadiene across a range of cultivation scales. Cultivations from 12

17 % (w/v) resin concentration resulted in bead fragmentation which were found to be

18 detrimental to cellular growth. After cultivation, the use of acetone for desorption

19 captured intracellular and secreted taxadiene, achieving an integration of the

20 bioprocess. Implementation of the proposed method at microscale (2 mL) and benchtop

21 bioreactor scale (250 mL) resulted in 1.9-fold and 1.4-fold increments in taxadiene titer,

22 respectively, compared to the extraction method using a dodecane overlay. Taxadiene

23 was found to be distributed between resin beads and biomass in a ratio of 50 %. Finally,

24 a maximum taxadiene titer of 76 ± 19 mg/L was achieved in the benchtop bioreactor

25 cultivations.

26 **Keywords:** Taxadiene, *In situ* solid phase adsorption, Bioprocess integration, HP-20

27 resin beads, *Saccharomyces cerevisiae*.

1. Introduction.

29 Downstream processing for the recovery and purification of valuable natural products

30 (NPs) typically accounts for 60-80% of the total operational costs at industrial scale [1].

31 Such processing steps are typically energy intensive and require vast quantities of highly

32 toxic solvents, resulting in major economic and environmental sustainability challenges.

33 NPs have extensive applications across the global food, cosmetics and pharmaceutical

34 industries [2,3]. As a result of such far-reaching potential benefits, NPs consumption has

35 tripled since the 1990s [4].

36 Over 60% of all known NPs are terpenoids, a large chemically diverse group of

37 compounds with important roles in both primary and secondary metabolism in their

38 natural hosts [5,6]. Some terpenoids in particular are drug candidates for the treatment

39 of human disease; examples include the sesquiterpenoid anti-malarial drug, artemisinin

40 and diterpenoid chemotherapy drug, paclitaxel [7,8]. Most terpenoids are hydrophobic

41 in nature and often become toxic to heterologous hosts at industrially feasible

42 concentrations [9]. To minimize the detrimental effects of terpenoids on the host, *in situ*

43 product recovery methods have become common practice at laboratory scale [10,11].

44 Through *in situ* extraction, terpenoids can be effectively removed from the cultivation

45   medium as they are produced, thereby minimizing the accumulation, product loss and

46   cell death [12].

47   1.1 *In situ* solid phase adsorption of natural products

48   *In situ* solid phase adsorption (SPA) is an alternative method, which could potentially

49   alleviate the aforementioned bottlenecks associated with LLE. During *in situ* SPA, an

50   inert adsorbent resin is added to the cultivation medium and the NP adsorbs to its

51   surface as it is secreted by the host [13]. This dramatically reduces product loss due to

52   air stripping or co-evaporation with the organic overlay [14].

53   Through *in situ* SPA, feedback inhibition and cytotoxic effects can be minimized

54   thereby enhancing productivity [15,16]. Adsorbent resins have been successfully

55   employed in this context to extract a wide range of NPs from a number of heterologous

56   hosts including *Escherichia coli*, *Aspergillus fumigatus* and *Streptomyces hygroscopicus*

57   via *in situ* or post-cultivation adsorption [15–18]. Different SPA processes have been

58   developed, one example is the extraction of chaetominine, an alkaloid compound with

59   anti-cancer activity, from *Aspergillus fumigatus*, where out of eight resins tested, XAD-

60   16 and HP-20 demonstrated the best extraction performance [17]. This superior

61   performance was attributed to greater adsorption capacity resulting from their larger

62   surface area. A number of polymeric resins have also been investigated recently for the

63   recovery of the sesquiterpene, (+)-zizaene, from engineered *E. coli* cultivations. Of those

64   tested, the highest (+)-zizaene titer was achieved using HP-20 in the *E. coli* cultivations,

65   where the resin structure was a key element for higher hydrophobic adsorption [19].

66   1.2 <u>Research scope: *In situ* solid phase adsorption for taxadiene biosynthesis and</u>

67   <u>recovery</u>

68 The biosynthesis and recovery of the highly effective chemotherapy drug, paclitaxel

69 (commercially known as Taxol ®), is a major ongoing research effort [20,21]. Despite

70 decades of study, the complex paclitaxel biosynthetic pathway is yet to be fully

71 elucidated. A robust precursor purification method therefore has the potential to

72 expedite cell factory development and hence progress towards a more sustainable

73 source of the drug [7,22]. Heterologous biosynthesis of the first committed precursor in

74 the paclitaxel pathway, taxadiene, has been successfully achieved in both *E. coli* [23,24]

75 and *S. cerevisiae* [25,26].

76   The use of a dodecane overlay has proven to be an effective method for

77 taxadiene recovery in the highest-yielding *E. coli* (Ajikumar et al., 2010; Biggs et al., 2016)

78 and *S. cerevisiae* at laboratory scale cultivations [21,25]. However, research into more

79 scalable recovery and purification methods for this critical precursor is scarce, despite

80 taxadiene itself being considered a valuable NP [22]. As taxadiene is a hydrophobic

81 molecule by nature, a non-polar resin is appropriate for its extraction. Of the wide range

82 of available non-polar resins, HP-20 beads (Diaion®) were deemed most suitable for

83 taxadiene recovery due to their very large surface area (850-1000 m^2/g) and high

84 adsorption capacity of NPs with similar chemical structure (chaetominine) [17].

85   The metabolic pathway for heterologous production and subsequent adsorption

86 of taxadiene onto the HP-20 resin beads is summarized in Figure 1. Although HP-20 resin

87   beads have been employed for the adsorption and subsequent recovery of a variety of

88   NPs, their application for the extraction of microbially synthesised taxadiene is novel.
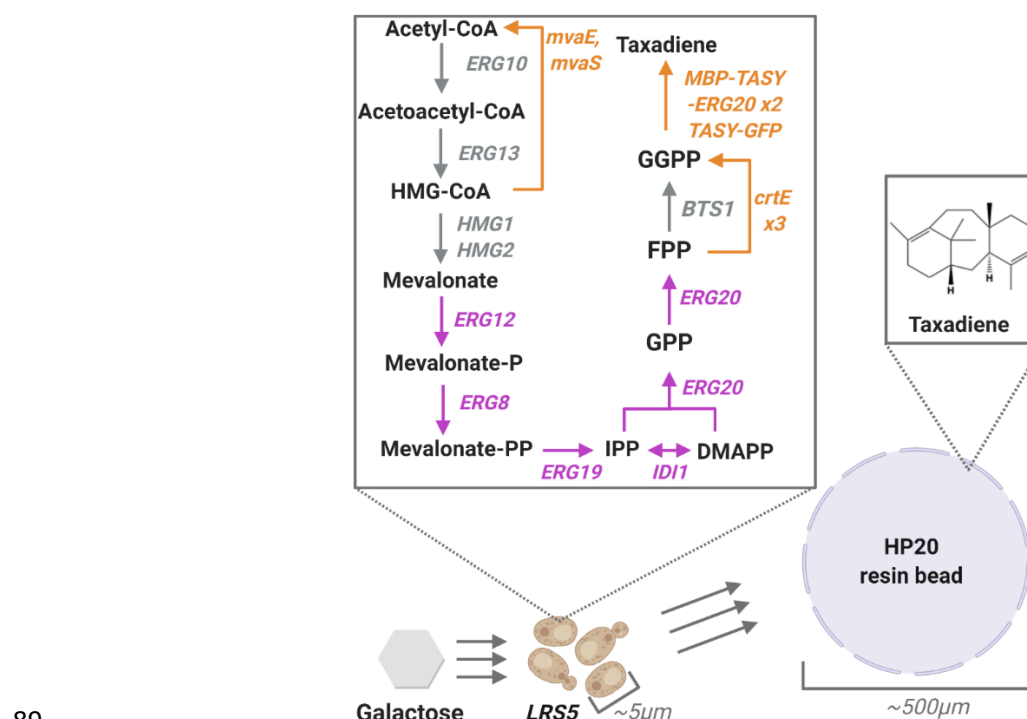

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90   Figure 1. Heterologous taxadiene biosynthetic pathway in *S. cerevisiae* engineered strain (*LRS5*) and

91   concurrent *in situ* adsorption of the secreted taxadiene to the surface of the HP-20 resin beads. Galactose

92   is metabolized via glycolysis yielding acetyl-CoA, which is then converted into the universal diterpenoid

93   precursor, geranylgeranyl diphosphate (GGPP), via the highlighted mevalonate pathway. Finally GGPP

94   undergoes cyclization by taxadiene synthase (TASY) yielding taxadiene. Native overexpressed genes are

95   highlighted in purple, whilst heterologous genes are shown in orange.

96   In this study, a HP-20 resin bead mediated SPA method for the recovery of

97   taxadiene produced by an engineered *S. cerevisiae* strain, *LRS5* [25], was developed and

98   optimized. The effect of key factors such as resin concentration and extraction solvent

99   on product recovery were investigated in detail across a range of bioreactor scales. The

100   traditional liquid-liquid extraction approach was used as a control to assess

101 performance. The system was tested across a range of cultivation scales from 2 mL high

102 throughput cultivations previously shown to be predictive of larger scale operations

103 [11], to controlled benchtop bioreactors (250 mL) to investigate scalability and

104 performance under more industrially relevant conditions. Finally, the bioprocess was

105 intensified by provoking cell lysis using cell-wall disruptive organic solvents in the final

106 extraction step.

2. Materials and Methods.

2.1 Yeast strains and media

109 The *S. cerevisiae* strain employed in this study was *LRS5 {mGTy116; ARS1014::GAL1p-*

110 *TASY-GFP; ARS1622b::GAL1p-MBP-TASY-ERG20; ARS1114a::TDH3p-MBP-TASY-ERG20}*

111 as described in detail in [25] was originally derived from laboratory strain CEN.PK2-1C

112 (EUROSCARF, Germany). All reagents, including extraction solvents were sourced from

113 Fisher Scientific UK at the highest available purity unless otherwise stated. The media

114 used for the yeast cultivations was yeast extract peptone (YP, yeast extract 1 % (w/v),

115 peptone 2 % (w/v)) supplemented with 2 % (w/v) galactose (YPG) or 2 % (w/v) glucose

116 (YPD) unless otherwise stated.

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

118 High-throughput microscale screening was performed using 24-well 10 ML deep well

119 plates (Axygen, USA) with a working volume of 2 mL. Inoculum cultures were prepared

120 by transferring a single colony of *LRS5* to 5 mL of YPD media and incubating at 30 °C and

121 250 rpm overnight. An aliquot of this culture was then diluted with YPG media to give a

122 2 mL culture with an initial OD_{600} = 1. Autoclaved HP-20 adsorbent resin beads were

123 added to each of the cultivations at concentrations between 3 to 12 % (w/v) as indicated

124 in the loading plan (see supplementary material). Prior to use, the beads were

125 autoclaved, rinsed with 100% ethanol and washed with sterile deionized water. The

126 resulting culture plate was incubated at 30 °C for 72 hours at 350 rpm and covered with

127 an adhesive gas permeable membrane (Thermo Fisher Scientific, UK). At the end of the

128 cultivation, the contents of each well was centrifuged. To remove water traces in wells

129 containing beads, the solid phase was recovered and dried under nitrogen gas at room

130 temperature before adding acetone at 1:1 ratio of the cultivation volume and incubating

131 the resulting mixture at 30 °C and 350 rpm for four hours. Following incubation, the

132 mixture was centrifuged and the organic phase recovered for taxadiene analysis via GC-

133 MS. Biomass accumulation was monitored in offline samples as optical density at 600nm

134 using Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, UK). For

135 comparison, additional cultivations were performed using the widely used dodecane

136 overlay approach described in detail in section 2.7. Control cultivations with YPD only

137 and no extraction aid were also performed, taxadiene was extracted from the solid

138 phase (biomass only) after the cultivation time using acetone at 1:1 ratio of the

139 cultivation volume and incubating the resulting mixture at 30 °C and 350 rpm for four

140 hours. Microscope images were taken using an oil immersion light microscope that is

141 equipped with 100x Leica NPLAN objective lens (Leica Microsystems, Germany) using an

142 Andor-Zyla sCMOS camera (Oxford Instruments, UK). The SPA method using adsorbent

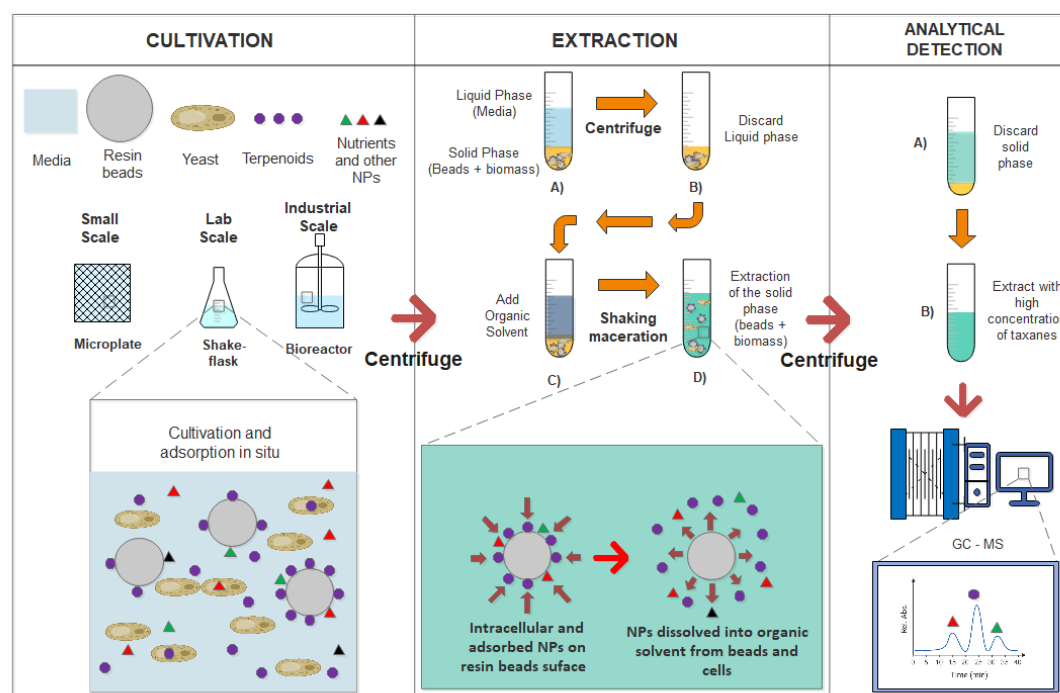143 HP-20 resin beads was summarized in Figure 2.

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Figure 2. HP-20 resin beads in situ SPA method. The methodology focuses on extracting taxadiene an early paclitaxel precursor produced by the engineered yeast (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.

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 *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 hours. At

8

157   the end of the cultivation, biomass accumulation and taxadiene extraction using

158   acetone was performed as described in section 2.2.

159   2.4 Taxadiene partition from solid and liquid phases.

160   To determine the taxadiene partition between the beads, biomass and liquid phase, an

161   additional experiment was conducted under the conditions detailed in Section 2.2. Once

162   the stationary phase of the growth had been reached at around 72 hours the beads were

163   removed from the cultivation using a nylon mesh cell strainer with a pore size of 70 μm

164   (Thermo Fisher Scientific, UK). The resulting solution was then centrifuged and the

165   supernatant transferred to a separate vessel. The biomass and beads were dried under

166   nitrogen gas before being suspended separately in same volume of acetone as they had

167   of cultivation volume and incubated at 30 $^{\circ}$C and 250 rpm for four hours. To recover

168   taxadiene from the supernatant, a dodecane overlay of 20 % of the cultivation volume

169   was added to the liquid phase at 30 $^{\circ}$C and 250 rpm for four hours. Following incubation,

170   the extracts were centrifuged and the dodecane organic phase was recovered for

171   taxadiene analysis via GC-MS.

172   2.5 Extraction solvent optimization

173   To investigate the effect of extraction solvent on recovery of taxadiene from the resin

174   beads, the following solvents were tested: ethanol, acetone, ethyl acetate and dodecane

175   were investigated. Following cultivation in small scale culture (5 ml) for 72 hours under

176   the conditions detailed in section 2.3, the solid phase was recovered by centrifugation

177   and re-suspended in a volume of the respective solvent equal to that of the original

178   culture. The resulting mixtures were incubated at 30 $^{\circ}$C and 250 rpm for four hours then

9

179   centrifuged to separate the organic and solid phases. The organic phases from each

180   organic solvent were subsequently extracted for GC-MS analysis.

181   2.6 Bioreactor cultivation

182   Larger scale cultivations were conducted in MiniBio 500 bioreactors (Applikon

183   Biotechnology, The Netherlands) with a working volume of 250 mL. Pre-inoculum

184   cultures were prepared by transferring from a single colony to 5 mL of YPD and

185   incubating at 30 °C and 250 rpm for eight hours. The resulting culture was subsequently

186   used to inoculate a secondary 10 mL culture to an OD_{600} = 1 and incubated overnight.

187   An aliquot of the resulting culture was diluted with 1 % (v/v) medium to give a 200 mL

188   culture with an initial OD_{600} = 1. To prevent excess foam production polypropylene glycol

189   P2000 (Alfa Aesar, UK) was added to a concentration of 0.01 % (v/v) and a Rushton

190   turbine was placed at the medium-air interface. Autoclaved HP-20 adsorbent resin

191   beads were added at a concentration of 3 % (w/v). A set point of 30 % saturation for DO

192   was applied and the culture temperature was maintained at 30 °C. The pH was

193   maintained above six through the automatic addition of 1M NaOH. Biomass was

194   measured offline twice daily using Nanodrop 2000c spectrophotometer (Thermo Fisher

195   Scientific, UK). Galactose concentration was monitored via offline sampling twice daily

196   using the 3,5-Dinitrosalicylic acid (DNS) method [28].

197   2.7 Dodecane overlay method.

198   Dodecane overlay method was made as point of comparison for our *in situ* SPA method,

199   as it is a methodology widely used to recover taxanes. In this work, the culture, seed

200   culture and extraction were prepared as described before [25] for microscale

201 cultivations. After the cultivation time, an aliquot of dodecane was recovered for

202 taxadiene analysis via GC-MS.

203   2.8 <u>Analytical methods</u>

204 Taxadiene identification and quantification was achieved via GC-MS. A 1 μL sample of

205 each taxadiene containing organic solvent extract was injected into a TRACE™ 1300 Gas

206 Chromatograph (Thermo Fisher Scientific, UK) coupled to an ISQ LT single quadrupole

207 mass spectrometer (Thermo Fisher Scientific, UK). Chromatographic separation was

208 achieved using a Trace Gold TG-SQC gas chromatography column (Thermo Fisher

209 Scientific, UK) using a previously described method [26]. To identify and quantify

210 production of compounds by *LRS5*, pure standards of taxadiene, kindly supplied by the

211 Baran Lab (The Scripps Research Institute, California, USA) and GGOH, obtained from

212 Sigma Aldrich (Gillingham, UK), were used. Characteristic gas chromatograms and

213 taxadiene mass spectra can be found in supplementary information (see supplementary

214 material).

215   2.9 <u>Statistical analysis</u>

216 Statistical analyses were performed using MATLAB 2020a and Minitab 19 statistical

217 software. One-way analysis of variance (ANOVA) was used to determine whether HP-20

218 resin beads concentration yielded a significant impact on biomass yield at microplate

219 and shake flask scale. A Dunnett's multiple comparison test was used to compare each

220 treatment to the control. One-way ANOVA was also used to determine whether the

221 selected extraction solvent had an effect on taxadiene titers recovery. A Tukey's honest

222 significant difference test was subsequently employed to compare each of the

11

223   treatments. The null hypothesis considered that there was no significant difference

224   between the treatments, hence if $p \leq 0.05$ the null hypothesis was rejected.

3. Results and discussion.

3.1 Effect of resin bead concentration on *LRS5* cell growth

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 [16,29]. However, high bead concentrations have been found to inhibit microbial growth [30]. 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 [25], optimization was necessary to maximize both growth and product recovery. The *LRS5* strain was cultivated in the 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 *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 Figure 3.
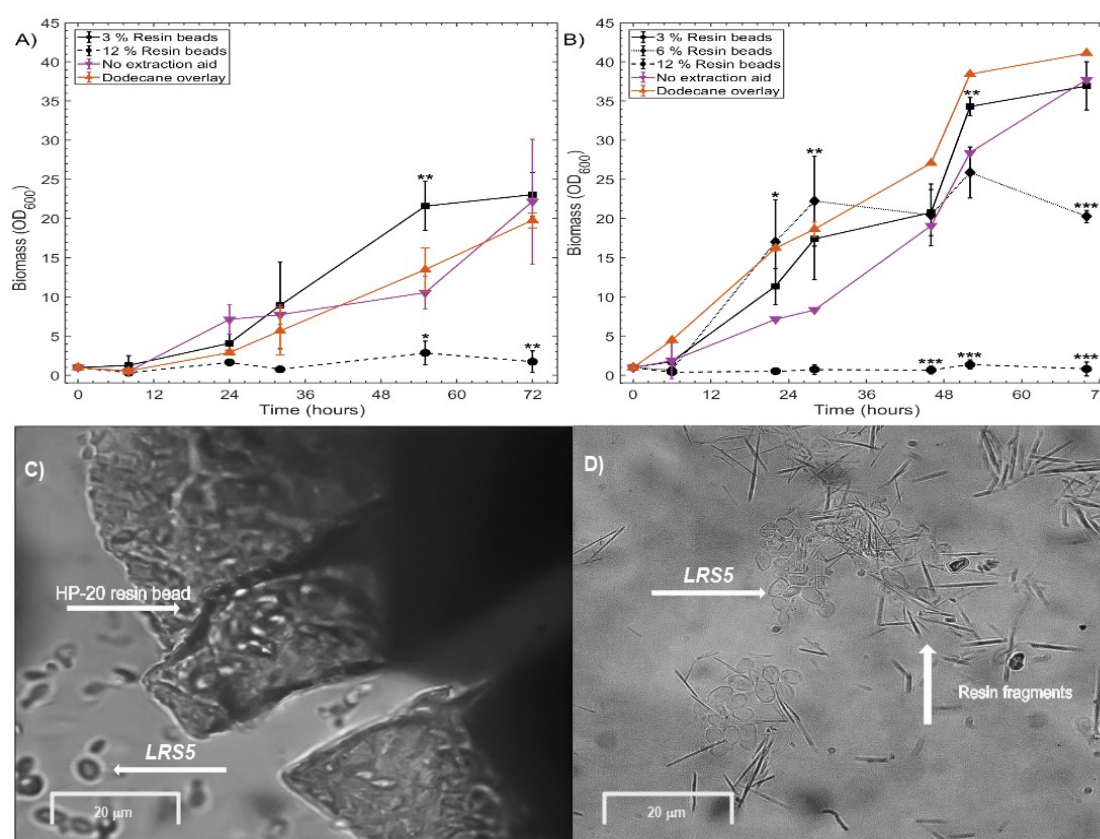
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Figure 3. Growth kinetics of yeast strain *LRS5* in presence of different concentrations of HP-20 resin beads (TOP), microscope images of *LRS5* cultivated at 6% (w/v) resin beads at small scale (BOTTOM). 3A) Experiments were carried out using microscale 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 *LRS5* cells. 3D) Sharp resin fragments observed in the culture medium after 72 hours.

At microscale, the addition of 3 % (w/v) HP-20 resin beads was found to enhance the growth rate of *LRS5,* with the stationary phase of growth reached earlier than for the control cultivation with no extraction aid (Figure 3A). An OD_{600} of 21 ± 3 was observed after 55 hours, significantly greater than the control cultivation with no

13

251   extraction aid (p = 0.002). However, the addition of 3 % (w/v) beads had no significant

252   effect on the final OD_{600} (p = 0.964) with highly similar OD_{600} values of 23 ± 3 and 22 ± 8

253   obtained for the 3 % and no extraction aid cultivations. The final biomass yield of the

254   cultures treated with a dodecane overlay was also not statistically different from the

255   control with no extraction aid (p = 0.654) at 20 ± 1. At the higher concentration of 12 %

256   (w/v) the addition of beads was highly detrimental to *LRS5* growth and final biomass

257   yield (p = 0.004) with an OD_{600} of just 3 ± 1 achieved at the end of the 72 hour

258   cultivations. This indicated that at low concentrations, the beads were not detrimental

259   to *LRS5* growth. To investigate this effect further and determine the optimal

260   concentration of beads a second experiment involving larger shake flask cultivations was

261   performed, here an additional bead concentration of 6 % (w/v) was tested.

262   As for the microscale cultivations, the final OD_{600} of the 3 % (w/v) bead (p = 0.883)

263   cultivations were very similar to the control cultivation with no extraction aid (Figure

264   3B). At the higher bead concentration of 6 % (w/v) a 46 % reduction in biomass

265   accumulation was observed compared to the control (Figure 3B, p < 0.0005). Increasing

266   the concentration further to 12 % (w/v) inhibited growth almost entirely with a final

267   OD_{600} = 1 ± 0.5 (p < 0.0005). The optimal bead concentration was therefore deemed to

268   be 3 % (w/v) with maximum OD_{600} values of 21 ± 3 and 37 ± 3 at micro and shake flask

269   scale, respectively.

270   These results were in agreement with previous studies using *E. coli*,

271   *Streptomyces hygroscopicus* and *Streptomyces virginiae* [16,19,31], which found high

272   bead concentrations were detrimental to microbial growth. *E. coli* was able to tolerate

273   higher bead concentrations with an optimal of 5 % (w/v) [19], whilst the optima for

14

274   *Streptomyces hygroscopicus* and *Streptomyces virginiae* were similar to that found in

275   this study (Figure 3) at between 2 % and 5 % (w/v) respectively [16,31].

276   Adsorption to the resin surface is not exclusive to the desired taxadiene

277   compound [32], additional hydrophobic compounds present within the culture medium

278   therefore likely adsorbed to its surface. Such compounds include fatty acids and non-

279   polar amino acids, which are essential for yeast growth [33]. A previous study revealed

280   that 55 % of the casein present within the culture medium adsorbed to the HP-20 beads

281   when a concentration of 20 % (v/v) beads was used compared to just 17 % at a 5 % (v/v)

282   bead concentration [34]. This resulted in a reduced availability of amino acids for cell

283   growth, the adsorption of essential nutrients may have therefore contributed to the

284   poor growth observed in the 12 % (w/v) cultivations of this study. This highlights the

285   importance of carefully optimizing the concentration of resin to ensure sufficient

286   capacity to maximize product recovery whilst minimizing nutrient loss and hence growth

287   inhibition [16]. In addition, visualization of the cultures using oil immersion light

288   microscopy (Figure 3C and 3D) revealed physical degradation of the beads, generating

289   sharp fragments as shown in Figure 3D, which may have caused some mechanical lysis

290   of the cells.

291   Such degradation was likely enhanced by culture agitation as it has been noted

292   in previous works using similar type of resins [35]. Furthermore, as the resin beads are

293   around 100-fold larger than the yeast cells, their presence within the culture likely

294   contributed to shear stress, which may have hindered cell viability at the higher bead

295   concentrations (>6 % (w/v)). To our knowledge, this is the first report of the contribution

15

296   of HP-20 bead degradation and subsequent sharp fragment generation to reduced cell

297   growth at higher bead concentrations.

298   3.1 <u>Solid – Liquid Extraction optimization using different organic solvents</u>

299   The partition of taxadiene between the solid (beads + biomass) and aqueous (culture

300   medium) phases was subsequently investigated in small scale (5 mL) cultures of *LRS5*.

301   Following cultivation, taxadiene was extracted from each phase using dodecane and

302   quantified via GC-MS. Analysis revealed 97 % of the detected taxadiene was present in

303   the solid phase (see supplementary material). As a result, subsequent experiments

304   focused on extraction and purification of taxadiene from the solid phase exclusively. This

305   ensured recovery of the vast majority of the product of interest, whilst minimizing

306   solvent requirements.

307   A key benefit of the application of an adsorbent resin is the elimination of

308   reliance on biocompatible extraction solvents, which with high boiling points render the

309   purification stage both energy intensive and costly (*i.e*. dodecane). A low cost extraction

310   solvent with a low boiling point is preferable to maximize the environmental and

311   economic sustainability of the process. Previous studies have shown solvents with

312   intermediate polarity such as acetone can improve terpenoid recovery in *in situ* SPA

313   systems compared to dodecane [36,37]. Aguilar et al. (2019), recently employed HP-20

314   resin beads for the *in situ* extraction of the sesquiterpene zizaene from engineered *E.*

315   *coli* cultivation. In that study, of the seven extraction solvents tested in the study, ethyl

316   acetate, decane and isooctane displayed superior zizaene recovery to dodecane. Ethyl

317   acetate has also been successfully employed for the recovery of limonene [38] and

318   Fusicocca-2,10(14)-Diene [39] from resins resulting from *E. coli* and *S. cerevisiae*

16

319   cultivations, respectively. Ethyl acetate was therefore selected as a potential solvent in

320   this study, as in addition to its observed affinity for terpene compounds, it is a volatile

321   and relatively low-cost solvent with low toxicity. Acetone and ethanol with similar and

322   higher polarities compared to ethyl acetate, respectively, are commonly used organic

323   solvents for the extraction of carotenoids from yeast cells [40]. The efficacy of these

324   solvents for eluting the shorter chain diterpene, taxadiene, from the HP-20 resin was

325   investigated in this study, as summarized in Figure 4.
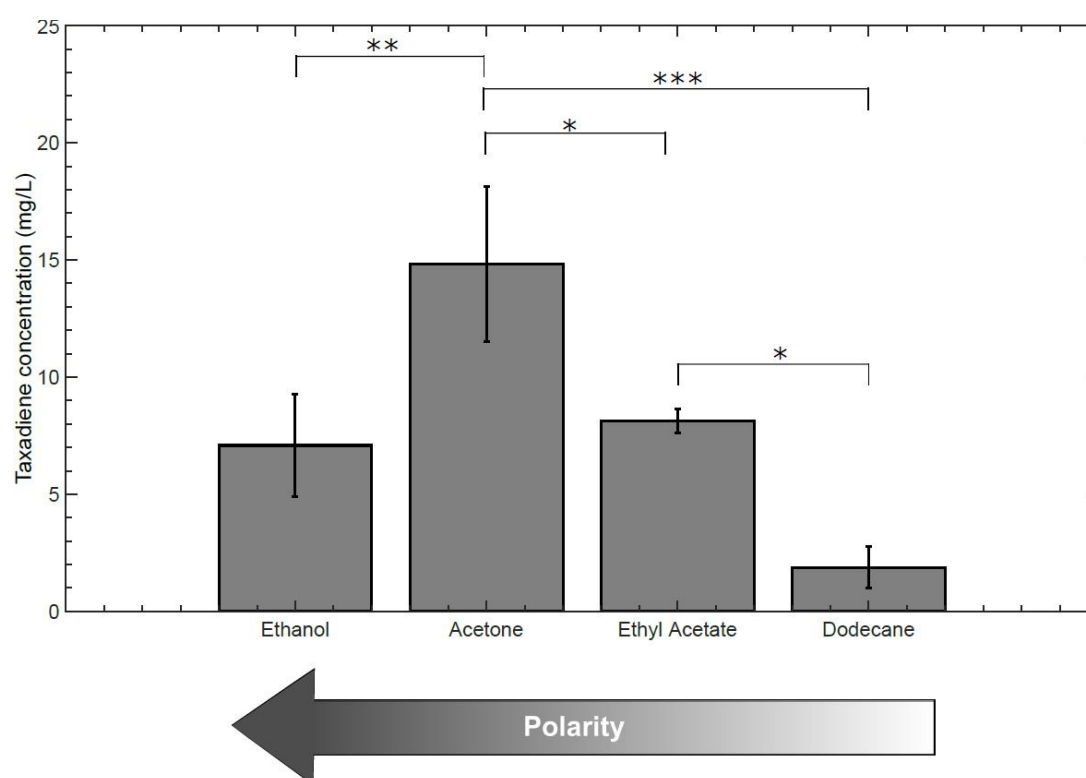

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327   Figure 4. Effect of different organic solvents on taxadiene recovery in Solid phase adsorption (SPA)

328   cultivation method. All the solvents were used in the extraction stage, following resin bead cultivation at

329   small scale (5 ml). Values represent mean ± standard deviation (n = 3) (* $p < 0.05$, ** $p < 0.01$, $p < 0.001$;

330   Tukey's honestly significant difference multiple comparison test).

17

331   Of the tested solvents acetone ($p = 0.007$) and ethyl acetate ($p = 0.023$) enhanced

332 taxadiene recovery significantly with respect to dodecane as shown in Figure 4. Unlike

333 dodecane, the three other solvents tested in this investigation are not biocompatible

334 and are known to affect yeast-cell viability and metabolic activity retention at

335 concentrations from 6 % (v/v) [41]. Organic solvents including ethanol, acetone and

336 ethyl acetate, penetrate the lipid portion of the cell membrane, which causes

337 permeabilization and can ultimately lead to cell lysis [42–44]. Biocompatible solvents

338 such as dodecane are commonly used for *in situ* liquid-liquid extraction to minimize this

339 effect during cultivation and maximize cell viability. However, permeabilization is

340 desirable for maximizing product recovery as it ensures access to intracellular taxadiene

341 [42,45]. The higher titers observed with the alternative solvents in this study (Figure 4)

342 was likely attributed to increased membrane permeability and intracellular taxadiene

343 recovery. The greatest taxadiene titer of 15 ± 2.0 mg/L was achieved using acetone (5

344 ml cultivations), four-fold greater than that using the highly non-polar and

345 biocompatible dodecane solvent. This solvent was therefore selected for subsequent

346 studies.

347   3.2 <u>Synergic effect of HP-20 resin beads and selected solvent to improve</u>

348   <u>taxadiene extraction</u>

349 The results of the desorption step indicated that the use of ethanol, acetone or ethyl

350 acetate as the extraction solvent, resulted in higher taxadiene titres than dodecane.

351 Such solvents are known to increase cell membrane permeability [43,44] and previous

352 studies have shown that some of the taxadiene is retained intracellularly [25]. It was

353 therefore hypothosised that improved recovery achived using ethanol, acetone or ethyl

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354 acetate may be partially due to increased cell memebrane permeability. In order to

355 investigate this further, an additional experiment was performed using the highest

356 yielding solvent, acetone (Figure 4). *LRS5* was cultivated in the absence of resin beads

357 or a dodecane overlay (no extraction aid) and acetone was applied at the end of the

358 cultivation to extract taxadiene from the solid phase (biomass). This was compared to

359 the optimal *in situ* solid phase (beads + biomass) cultivation with 3 % (w/v) HP-20 beads

360 and the traditional dodecane overlay method as shown in Figure 5.

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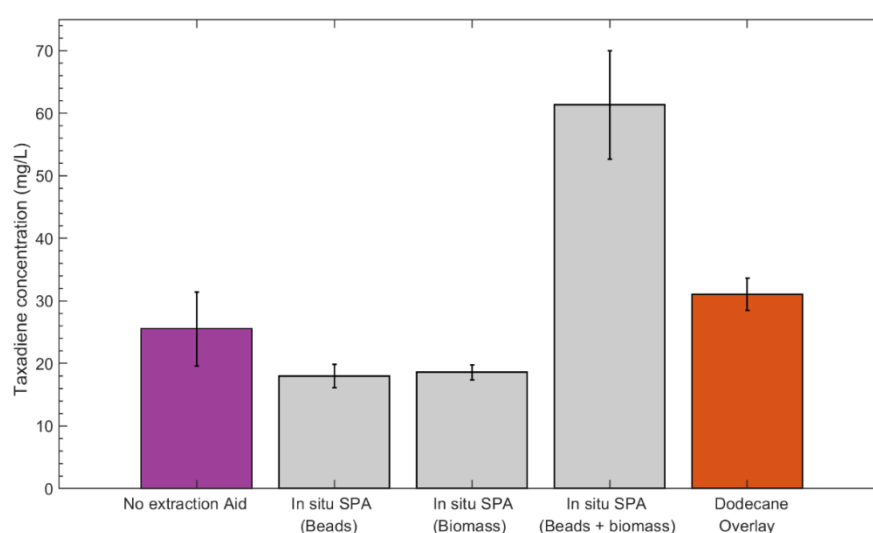
362 Figure 5. Taxadiene recovery titers (bars) from microplate cultivations (2 mL cultivation volume) after 72

363 hours of cultivation at 30 °C and 350 rpm. Purple bar represents the cultivation made with no extraction

364 aid where the extraction was made to the biomass using acetone. Colored grey bars represent *in situ* Solid

365 phase adsorption (SPA) cultivation using 3 % (w/v) bead concentration with further extractions made with

366 acetone whilst orange bar represents extraction made with dodecane using the dodecane overlay

367 method. *LRS5* reached similar OD at 600 nm in all treatments after 72 hours. Beads + Biomass value could

368 be higher due to less manipulation in the extraction steps. Values represent mean ± standard deviation

369 (*n* = 2).

370   Biomass accumulation was similar for the SPA, no extraction aid and dodecane

371   overlay cultivations with final OD_{600} values of 23 ± 1, 22 ± 3 and 20 ± 1, respectively.

372   When acetone was applied to extract taxadiene from the biomass resulting from the

373   control treatment, in which *LRS5* was grown in the absence of an *in situ* extraction aid,

374   the final taxadiene titer was 26 ± 6 mg/L at microscale as shown in Figure 5. Interestingly,

375   this was comparable to the 31 ± 3 mg/L obtained using the traditional dodecane liquid-

376   liquid extraction method. This suggested that, in the absence of an *in situ* extraction aid,

377   a significant proportion of the taxadiene was intracellular or adsorbed onto cell surface

378   rather than secreted, likely due to the hydrophobic nature of the diterpene. In the *in*

379   *situ* SPA cultivations, taxadiene was distributed almost equally between the resin beads

380   and biomass with titers of 18 ± 2 mg/L and 19 ± 1 mg/L observed, respectively. This

381   indicated that the cells retained up to 50 % of the taxadiene produced during the

382   cultivation. However, it was found that during the separation and desorption steps,

383   around 30% of the bead volume was lost compared to parallel cultivations with the same

384   bead concentration, in which the beads and biomass were not treated separately (see

385   supplementary material). The 50 % taxadiene repartition between biomass and resin

386   beads provides an important piece of information, as it showcases where the taxadiene

387   that is being detected in the system is being captured. To our knowledge, this

388   information has not been commonly reported as the extraction is generally made to the

389   biomass and resin in conjunction [16,29,46] or to the single beads without biomass

390   [17,47]. These results could have an important impact into determining the capacity of

391   the resin beads to relieve the cells from intracellular toxicity of selected NP among other

392   phenomena.

393   Simultaneous extraction of taxadiene from the resin beads and biomass, without

394   separation, resulted in a taxadiene titer of 61 ± 8 mg/L, at this scale. This suggests that

395   around 39 % of the recoverable taxadiene was lost during the separation step, likely due

396   to the loss of beads during the biomass and beads separation, indicating that the actual

397   fraction of taxadiene retained by the cells was likely around 50 %. This is in agreement

398   with a previous study using *LRS5*, which demonstrated that up to one third of the total

399   taxadiene was retained intracellularly using dodecane [25]. Using the optimal bead

400   concentration (3 % (w/v)) and extraction solvent (acetone) resulted in a 1.8-fold

401   improvement in taxadiene recovery compared to the widely used dodecane overlay

402   approach (Figure 5). Therefore, to maximize intracellular and secreted taxadiene titer,

403   an extraction solvent with a high affinity for taxadiene and a high permeabilization

404   ability should be selected following the cultivation. Differences in results between Figure

405   4 and Figure 5 were likely the result of an increased shaking speed of 350 rpm (Figure 5)

406   compared to the 250 rpm applied for the 5 mL cultures (Figure 4). In addition, the

407   microtiter plates were sealed with a gas permeable membrane whereas the tubes used

408   for the 5 mL cultures were airtight. Oxygen mass transfer was therefore likely enhanced

409   significantly at microscale, promoting aerobic growth and thus improving taxadiene

410   titers. A similar effect was observed in a previous study where taxadiene titers were

411   improved two-fold in baffled microtiter plate compared to shake flask cultivations of the

412   strain [21].

413   A significant quantity of taxadiene was recovered from the cells resulting from

414   the cultivation in the absence of an extraction aid using acetone (Figure 5). This indicated

415   that, in addition to promoting elution of secreted taxadiene from the HP-20 resin,

416   acetone also enhanced the recovery of intracellular taxadiene, likely due to its effect on

417   cellular permeability. The proposed SPA method is therefore an integrated bioprocess,

418   eliminating the requirement for an additional unit operation for cellular lysis [1,48] or

419   further metabolic engineering to introduce efflux pumps (e.g. outer membrane protein

420   TolC) [49] to enhance the product secretion.

421   3.3 Scale up bioprocess of the *in situ* SPA method

422   In order to validate the proposed *in situ* SPA method under industrially relevant

423   conditions, the process was scaled up using MiniBio 500 bioreactors (Applikon

424   Biotechnology, The Netherlands). Here industrially relevant process parameters

425   including pH, temperature and dissolved oxygen concentration were monitored and

426   controlled online in real-time, allowing industrial scale conditions to be more effectively

427   mimicked [50]. The results of this experiment are summarized in Figure 6.
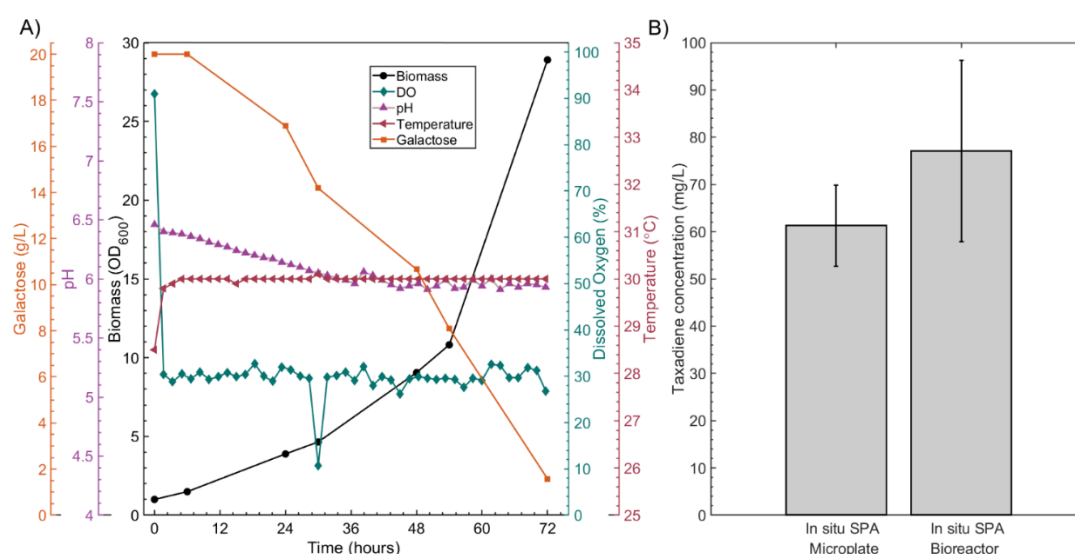
428

429   Figure 6. Bioreactor cultivation kinetic parameters of *LRS5* using the *in situ* Solid phase adsorption (SPA)

430   cultivation method. Figure 6A) shows the cultivation parameters using the *in situ* SPA method in YPD

431   media for 250 ml cultivation volume. The controlled parameters were; pH, temperature and dissolved

432   oxygen. Figure 6B) shows the taxadiene titers for microplate cultivation and bioreactor using the *in situ*

433   SPA method using 3 % (w/v) and acetone in the extraction step. All cultivations conditions were the same

434   in these experiments, with the difference that in the bioreactor cultivation pH, temperature and dissolved

435   oxygen parameters were controlled by the equipment. Values in figure 6B represent mean ± standard

436   deviation (*n* = 2).

437   Taxadiene titers were enhanced at bioreactor scale compared to previous

438   microscale (Figure 6) cultivations using the *in situ* SPA method. Here, the oscillations in

439   key process parameters such as pH and dissolved oxygen could be minimized through

440   automated feedback control facilitating improved taxadiene production. A taxadiene

441   titer of 76 ± 19 mg/L was achieved, compared to 61 mg/L at microscale (Figure 6). This

442   also represented a 1.4-fold improvement in titer compared to liquid-liquid extraction

443   using a dodecane overlay under similar cultivation conditions [25]. A taxadiene titer of

444   53 mg/L was observed for the *LRS5* strain cultivated in the bioreactor with a dodecane

445   overlay at 30 °C [25]. One possible reason for the enhanced recovery is the positive effect

446   of acetone for solid phase (biomass + beads) extraction, allowing recovery of both the

447   intracellular and secreted taxadiene. Moreover, the loss of taxadiene due to air stripping

448   or co-evaporation with dodecane was avoided through the use of HP-20 resin beads.

449   These results demonstrate the potential of the *in situ* SPA cultivation method for

450   effective recovery of taxadiene under industrially relevant conditions with improved

451   sustainability.

4. Conclusions.

453   The application of this novel method at micro and bioreactor scale improved taxadiene

454   recovery 1.4 and 1.9-fold, respectively, compared to the traditional liquid-liquid

455   extraction approach. Higher resin bead concentrations (from 12 % w/v) resulted in bead

456   fragmentation (mechanical cell disruption) which were found to be detrimental to

457   cellular growth. Acetone ensured the sequestration of both the intracellular and

458   secreted taxadiene at the extraction step achieving process integration. In addition, it

459   was found that from the total taxadiene that is being synthesized, 50 % is adsorbed by

460   the resin beads and the rest is retained in the biomass.

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