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In situ recovery of taxadiene using solid adsorption in cultivations with *Saccharomyces cerevisiae*

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ABSTRACT
In this study, an engineered strain of *Saccharomyces cerevisiae* was used to produce taxadiene, a precursor in the biosynthetic pathway of the anticancer drug paclitaxel. Taxadiene was recovered *in situ* with the polymeric adsorbent Diaion © HP-20. Here we tested two bioreactor configurations and adsorbent concentrations to maximize the production and recovery of taxadiene. An external recovery configuration (ERC) was performed with the integration of an expanded bed adsorption column, whereas the internal recovery configuration (IRC) consisted in dispersed beads inside the bioreactor vessel. Taxadiene titers recovered in IRC were higher to ERC by 3.4 and 3.5 fold by using 3% and 12% (w/v) adsorbent concentration respectively. On the other hand, cell growth kinetics were faster in ERC which represents an advantage in productivity (mg of taxadiene/L*h). High resin bead concentration (12% w/v) improved the partition of taxadiene onto the beads up to 98%. This result represents an advantage over previous studies using a 3% resin concentration where the partition of taxadiene on the beads was around 50%. This work highlights the potential of *in situ* product recovery to improve product partition, reduce processing steps and promote cell growth. Nevertheless, a careful design of bioreactor configuration and process conditions is critical.

KEYWORDS
Expanded bed adsorption; *In situ* product recovery; solid adsorbents; *Saccharomyces cerevisiae*; Taxadiene

Introduction

Paclitaxel (Taxol®) is a valuable chemotherapeutic agent that can be found naturally on the bark of different species of trees of the genus Taxus.[1] The current commercial routes for the production of this valuable and widely used anticancer drug are still not eco-friendly and suffer from low yields.[2] The development of a microbial production host could significantly reduce the production cost and increase the availability of this important compound. Such a host could also produce other medically important taxanes and diterpenoids that are not produced naturally or at levels insufficient to detect.[3,4] One of such precursors, taxadiene, has been synthesized in engineered strains of *E. coli*[5] and *S. cerevisiae*.[6–9] The expression of plant P450 enzymes is difficult to achieve in bacterial cells, which affects the biosynthesis of many molecules by recombinant bacteria.[10] On the other hand, yeast cells enable the functional expression of complex eukaryotic proteins like cytochrome p450s, therefore was considered as a suitable chassis for terpenoid production.[11]

Elevated concentrations of terpenoids like taxadiene cause inhibition of the enzymatic reactions and/or microbial growth, leading to low productivity.[12] Fortunately, the low water solubility and high volatility of terpenes can be an advantage for developing selective recovery methods.[13,14]

An approach to alleviate the accumulation of terpenes during cultivations is *in situ* product recovery (ISPR), i.e., where a solid adsorbent present in the culturing environment captures the target compound as it is produced, thus also enabling an initial separation step by compartmentalization of the biocatalyst and the product.[14] Appropriate selection of the methodology and process variables is important to maximize product recovery and avoid undesired removal of nutrients from the media.[14] In addition, ISPR can minimize product loss of highly volatile components such as terpenes. For example, it has been observed that the concentration of (+)-zizaene produced in a shake flask halves after 1 hr due to volatilization, with only traces detected after 4 hr.[15]

ISPR methodologies are classified on the basis of the separation method, the bioreactor configuration, and its operation mode.[16] Separation techniques used for ISPR include

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evaporation, extraction, permeation, immobilization, and precipitation.[16] The suitability of the technique will depend on the product properties such molecular weight, volatility, charge, hydrophobicity, and specific binding properties.[14,17] The bioreactor configurations are classified according to whether the removal of the product is within or outside the bioreactor, and if the contact between the biocatalyst and separation phase is direct or indirect.[16]

Thanks to their hydrophobic nature, terpenes can be recovered from aqueous media by liquid-liquid extraction with a hydrophobic organic solvent not miscible with the aqueous phase containing the cell biocatalyst and nutrients.[15] While dodecane has been used for ISPR of taxadiene,[7] liquid-liquid extraction requires vast quantities of solvent (20% of total volume) and the high boiling point of dodecane (216 °C) makes the recovery of taxadiene energy-intensive, highly contaminant and expensive.[18]

Solid phase extraction using hydrophobic polymers is an alternative to extraction with organic solvents.[19] The main advantages of solid-phase extraction are ease of separation of the solid adsorbent from the aqueous phase, the possibility to tailor the chemical structure of the polymer to enhance selectivity toward the target molecule, reusability, resistance to microbial degradation and no emulsion formation.[19] It is desirable that a polymeric adsorbent exhibits high adsorption capacity but also selectivity to avoid the removal of substrates and other metabolites.[20] Hydrophobic resins like Diaion HP-20 and HP-21 have proven to be effective and selective in the recovery of some hydrophobic natural products.[15] HP-21 was effective to selectively adsorb 98.5% of the virginiamycin contained in a *Streptomyces virginiae* culture broth, thus simplifying the recovery procedure during the fermentation.[21] Furthermore, HP-20 showed 98.4% recovery of (+)-zizaene on a reactor configuration where the adsorbent was dispersed inside the reactor vessel.[15] Sequestration of the natural product in the solid adsorbent can also increase productivity, e.g., the production of teicoplanin was increased by 4.2-fold by the addition of Diaion HP-20 beads in the fermenter at the start of the fermentation.[22]

The concentration of adsorbent in the ISPR system is a key parameter to consider. For example, 126 and 134 mg/L of teicoplanin were recovered with 3 and 5% (w/v) HP-20 concentration respectively. However, in the same study, teicoplanin titer dropped to 95 mg/L if the concentration of HP-20 beads was increased to 10%.[22]

The addition of solid adsorbents directly into the bioreactor vessel of a stirred tank reactor (STR), also known as internal recovery configuration (IRC), is probably the most common ISPR technique in solid-phase extraction.[20] The turbulent fluid dynamics inside a STR can affect the morphology, metabolism, growth capacity, and viability of cells.[23,24] Although *S. cerevisiae* is usually considered a resistant microorganism, evidence suggests that it is susceptible to mechanical shear damage in fermenters.[23,25] The presence of solid adsorbents inside the STR contributes further to cell damage by the formation of sharp resin fragments produced from collisions.[18] Bead fragmentation inside the STR also results in changes on the adsorbent surface and its adsorption capacity.[26,27]

An option to avoid the problems associated with IRC is the external recovery configuration (ERC) where the adsorbent is placed in an external unit and the culture media with cells is recirculated during the cultivation process.[28] One suitable approach to use the ERC is to integrate the bioreactor with an expanded bed column. Expanded beds have been used for the *in situ* recovery of other biomolecules during fermentation processes, e.g., propionic acid,[29] butanol,[30] and succinic acid.[31]

In this study, integrated ISPR bioprocesses using engineered *S. cerevisiae* with external and internal solid-liquid extraction configurations (Figure 1) for the recovery of taxadiene were developed. The effect of different bioreactor configurations and adsorbent concentrations on cell growth, taxadiene recovery, and product partition was evaluated, and the most suitable bioreactor configuration for taxadiene recovery was determined. In particular, in the internal recovery configuration (IRC) solid resin HP-20 beads were dispersed inside the stirred bioreactor vessel (Figure 1a), whereas in the external recovery configuration (ERC) the beads were contained in a glass column separated from the bioreactor. In this configuration, the media and cells were recirculated from the bioreactor vessel through the column in a closed loop through the whole cultivation (Figure 1b). The column was operated as an expanded bed adsorption (EBA) unit to ease the recirculation of cells and to avoid clogging and channeling in the external column.

Materials and methods

Yeast strain and reagents

The *S. cerevisiae* strain used in this study was *LRS5* {*mGTy116; ARS1014::GAL1p-TASY-GFP; ARS1622b::GAL1p-MBP-TASY-ERG20; ARS1114a::TDH3p-MBP-TASY-ERG20*} which was previously described in Nowrouzi *et al.*[7] This strain was originally derived from the CEN.PK2-1C laboratory (EUROSCARF, Germany). The strain was maintained and stored in glycerol at −80 °C. Peptone from casein, glucose, and galactose were purchased from Sigma Aldrich UK. Yeast extract, Diaion HP-20, and all solvents employed for taxadiene desorption were obtained from Fisher Scientific UK. All of the chemicals were sourced at the highest available purity.

Pre-cultures and culture media

All pre-cultures were prepared in a media containing yeast extract (1% w/v) and peptone (2% w/v), supplemented with glucose (2% w/v). *S. cerevisiae* strain *LRS5* was obtained from a glycerol stock and cultivated overnight at 30 °C in a rotatory incubator at 250 rpm.

The culture media contained yeast extract (1% w/v), peptone (2% w/v) and was supplemented with galactose (2% w/v) that was required for the biosynthesis of taxadiene

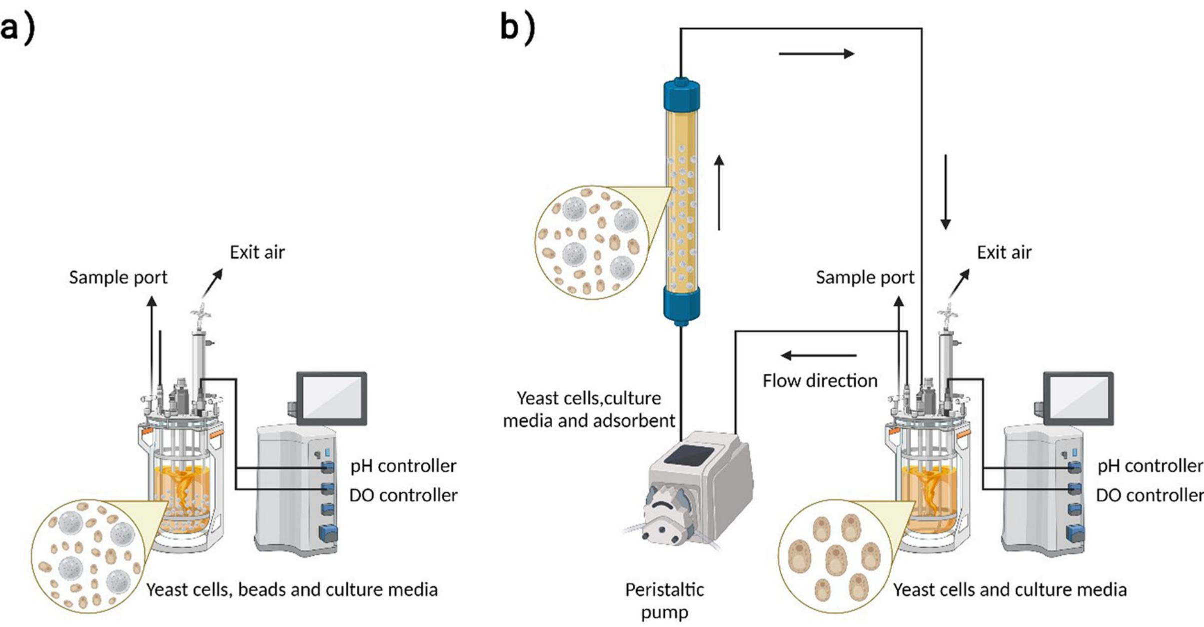

Figure 1. Bioreactor configurations for the production and *in situ* product recovery (ISPR) of taxadiene using adsorbent beads. (a) internal recovery configuration (IRC) and (b) external recovery configuration (ERC).

through the mevalonate pathway that relies on GAL promoters.[6]

Preparation of adsorbent resins

Diaion HP-20 was used as a solid adsorbent for ISPR of taxadiene (600 m^2/g surface area, 0.4–1 mm particle diameter, 29 nm pore size[18,32]). Prior to use, the beads were soaked in 99% ethanol (v/v) to remove impurities and enhance wettability. The solvent was removed by washing with deionized water for 5 times, every wash for 5 min to allow the beads to sediment to the bottom of the falcon tube and facilitate supernatant removal. Subsequently, 40 mL of culture media were mixed with the adsorbent before sterilization at 121 °C for 1h. Finally, the resin with media was added either to the reactor vessel (in the IRC) or the glass column (in the ERC). Diaion HP-20 beads were weighed before and after the preparation step in order to check that no more than 1% of the initial mass is loss in this procedure.

Bioreactor and column preparation

All the cultivations were conducted in a MiniBio500 bioreactor (Applikon Biotechnology, The Netherlands). In the IRC (Figure 1a), 19.2 g of pretreated sterile beads and 120 mL of culture media were introduced into the reactor vessel and left for 1 day to ensure no contamination of the system during handling. The inoculum was prepared by transferring a single colony to a tube containing 20 mL of pre-culture media and incubated overnight in a rotatory incubator (Eppendorf/Innova 42, UK) at 30 °C and 250 rpm. Finally, galactose, inoculum and a further 20 mL of culture media were added to reach a final working volume of 160 mL containing 2% w/v of galactose and 12% w/v adsorbent in culture media.

A similar procedure was employed for the ERC (Figure 1b) but with beads introduced inside a 50 mL glass superloop (15 mm inner diameter, 264 mm length, Cytiva, UK). A 200-µm mesh was glued to the inner end piece of the Superloop to avoid that beads would go out of the glass column. On this experimental set up, two extractant concentrations of 3% and 12% (w/v) were tested, corresponding to 4.8 g and 19.2 g of beads respectively. The cultivation broth was recirculated from the bioreactor vessel into the external column in a closed loop using a peristaltic pump (Cole Palmer Masterflex® L/S 77201-60, USA) at a superficial velocity of 237.7 (7 mL/min) and 101.9 cm/h (3 mL/min) for the 3% and 12% experiments, respectively. The volumetric flow rate passing through the 50 mL column, considering the volume occupied by the resin beads, correspond to residence times of 6.5 and 10.3 min for 3% ERC and 12% ERC, respectively. Such velocities were appropriate to expand the adsorbing bed to a degree of expansion of 2 and 1.5, respectively, where the degree of expansion is defined as the ratio between the expanded bed height (H) and the sedimented bed height (H_0). An expanded bed height should not cover above 80% of the total column length to avoid the accumulation of beads in the upper adapter that may eventually end up in clogging the system. Consequently, 12% ERC experiments was run using a degree of expansion of 1.5 due to length column limitations. The expansion of the adsorbent was carried out by slowly increasing the upward flow rate to obtain a stable bed rather than an uneven dispersion of beads that may result in back mixing and low adsorption efficiency.

Cultivation with no adsorbent was carried out as a control for comparison on cell growth and taxadiene recovery. In all cultivations, the pH of the culture media was kept above 6 by the automated addition of 1 M NaOH. The

automated gas sparging system maintained the DO level above 30%. Temperature was maintained at 30 °C and agitation was performed by stirring with a Rushton turbine at 800 rpm unless otherwise specified. The adaptive My-Control system (Applikon Biotechnology, The Netherlands) was employed to monitor and maintain all these parameters.

Samples were regularly withdrawn during all cultivation runs to measure the kinetics of cell growth and galactose concentration. Galactose was quantified using the 3,5-dinitrosalicylic acid (DNS) method for reducing sugar. Briefly, 40 µL of the sample were mixed with 500 µL and 460 µL of DNS reagent and water respectively. Subsequently, the resulting mixture was heated at 100 °C for 5 min. Finally, 167 µL of Rochelle salt was added to the mixture and analyzed at 575 nm in a spectrophotometer (NanoDrop™ 2000c ThermoFisher, UK). The galactose calibration curve can be found in the SI (Figure S1). Cell growth was measured in terms of optical density (OD) at 600 nm, and the correlation between dry biomass concentration (g/L) and OD is found in the SI (Figure S2).

Extraction and recovery of taxadiene from the biomass, adsorbent beads and cell free media fractions

At the end of the cultivation runs the adsorbent and culture media were separated to extract and quantify taxadiene from the different fractions (Figure 2). In the IRC the beads were recovered from the culture media using a 250 µm sieve mesh. In the ERC all the culture media was drained out of the column and collected into the bioreactor vessel. 150 mL of deionized water was used to recover the beads in the column and a 250 µm sieve mesh was used to recover the solids.

Culture media was also centrifuged at 4000 rpm for 10 min. The supernatant was decanted and filtrated to obtain the cell-free media. All the biomass recovered, namely from cell media and beads, was gently dried with nitrogen gas and weighted to quantify the total mass of cells in the reactor. Taxadiene was recovered from the different fractions by mixing and shaking the beads, cells, and cell-free media with an extraction solvent for 12 hr in a rotary incubator at 30 °C and 250 rpm (Eppendorf/Innova 42, UK). Acetone was used to desorb and extract taxadiene from beads and cells using a volume ratio of 1:1 as previously described.[18] Taxadiene extraction from cell-free media was achieved using dodecane as it is not miscible in water and has been successfully used for taxadiene extraction.[7] Extraction with dodecane was performed with a volume ratio of 1:0.2. Volume ratio is defined as the ratio between the total cultivation volume and the extraction solvent.

Taxadiene identification and quantification

Aliquots of 100 µL from extraction samples were analyzed via GC-MS using a TRACE 1300 Gas Chromatography system, equipped with a TG-SQC column (15 m × 0.25 mm × 0.25 µm) and coupled to an ISQ LT single quadrupole mass spectrometer (ThermoFisher Scientific, UK). Chromatographic separation and mass spectra were obtained using the procedure previously described by Nowrouzi *et al.*[7] Briefly, the gas chromatography program started at 120 °C, and the temperature was increased at a rate of 20 °C/min with 3 min hold time as 250 °C were reached. The mass spectra was operated in the range of 50–650 m/z using an EI ionization mode and scanning time of 0.204 s. Pure standards of taxadiene, kindly supplied by Baran Lab (The Scripps Research Institute, California, USA) were used

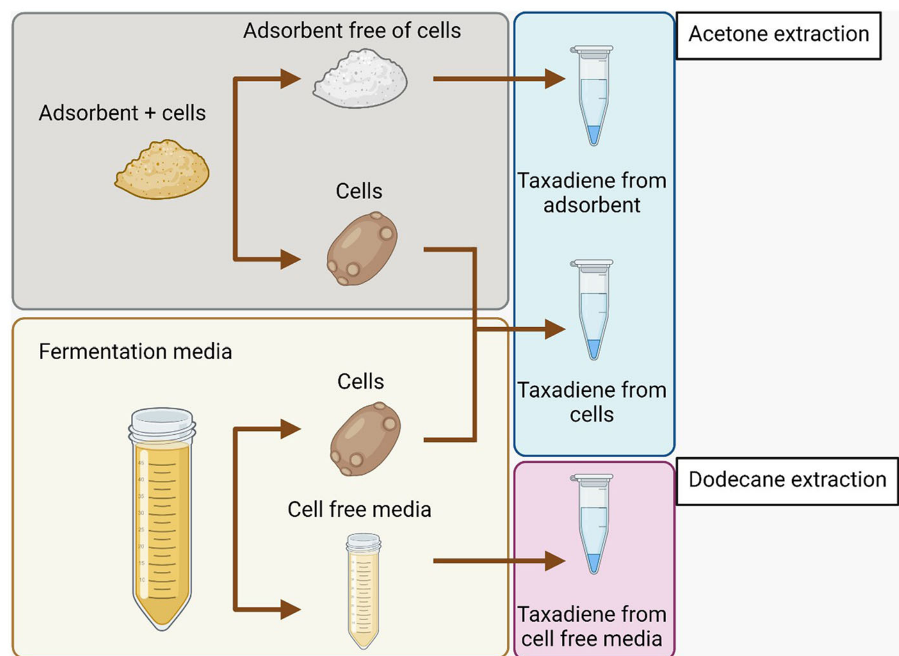

Figure 2. Schematic diagram illustrating the experimental procedure to extract and quantify taxadiene from the three different fractions: adsorbent beads, cells, and cell-free culture media.

as a reference to identify and quantify the taxadiene produced by our *S. cerevisiae LRS5*. Xcalibur™ software was used to compare the ions and their relative abundance with a library to verify that the sample corresponded to taxadiene. Mass spectra for taxadiene identification can be found in the SI (Figure S3).

The concentration of taxadiene in the sample (mg/L) was determined using a calibration curve obtained by measuring the peak area of a reference at a known concentration. The concentration of taxadiene was used to calculate the mass of taxadiene in the total cultivation volume (150 mL), and the mass of taxadiene was divided by the mass of cells or adsorber to calculate the ratio of the mass of taxadiene per mass of cells or adsorber (mg/g).

3. Results and discussion

Operation of expanded bed adsorption column

The adsorption of natural products with HP-20 beads has been tested at different concentrations and bioreactor setups such as expanded bed adsorption,[15] dispersed resin,[18,22] and an internal column consisting in a mesh containing the beads inside the bioreactor vessel to prevent direct contact between the biocatalyst (cells) and the adsorbent.[33]

According to Santoyo-Garcia *et al.*,[18] *S. cerevisiae LRS5* is susceptible to beads concentrations higher than 6%, in microscale (2 mL) and small scale (25 mL) cultivations with inhibited cell growth at 12% beads. This inhibition was attributed to the adsorption of fatty acids and non-polar amino acids from the culture media onto the HP-20 beads, resulting in lower availability of essential nutrients for cell growth. In addition, microscope images revealed beads fragments in the cell cultures, likely contributing to cell mortality due to mechanical cell lysis. To further investigate the effect of bead concentration on cell growth, cultivations were carried out on the IRC and ERC bioreactor configurations to provide a different environment to the cells and interactions with the adsorbent (Figure 3a). Table 1 summarizes the parameters employed in the IRC and ERC experiments.

The external recovery unit was operated as an expanded bed to guarantee the recirculation of culture media and cells throughout the cultivation process while avoiding column clogging due to cell aggregates in the small voids of a packed bed column. In fact, the average channel size in a packed bed is approximately 0.3 times the diameter of the spherical packing material. Based on this, and considering that Diaion HP-20 beads have a particle size of 250–850 µm, the smallest channels in the settled bed would be in the order of 75 µm. Given that *S. cerevisiae* cells have a diameter ranging from 5 to 10 µm, a conglomerate of 10 or more cells may reach a size large enough to clog a packed bed column. Therefore, the bed was operated with a degree of expansion of 2 and 1.5 in the 3% and 12% experiments, respectively, in order to increase the voidage into the column and hence the channel size between particles.

The expanded bed format also enables a laminar flow regime in the external column, as opposed to the turbulent conditions in the bioreactor vessel (see Reynolds number in the different bioreactor systems in Table 1). Laminar flow inside the column avoids damage to the adsorber beads caused by the impeller or collision between beads inside the bioreactor vessel. For example, Oslan and collaborators observed cracks on the surface of Amberlite IR86 beads that were used in cultivation with dispersed resin.[27] On the other hand, only light scratches were detected on the surface of beads that were packed in an internal adsorption column.

The minimum fluidization velocity (*Umf*) was estimated combining the Ergun equation with Wen and Yu relation[34] to establish a flow rate range in which to operate the column (the equation can be found in the SI). The

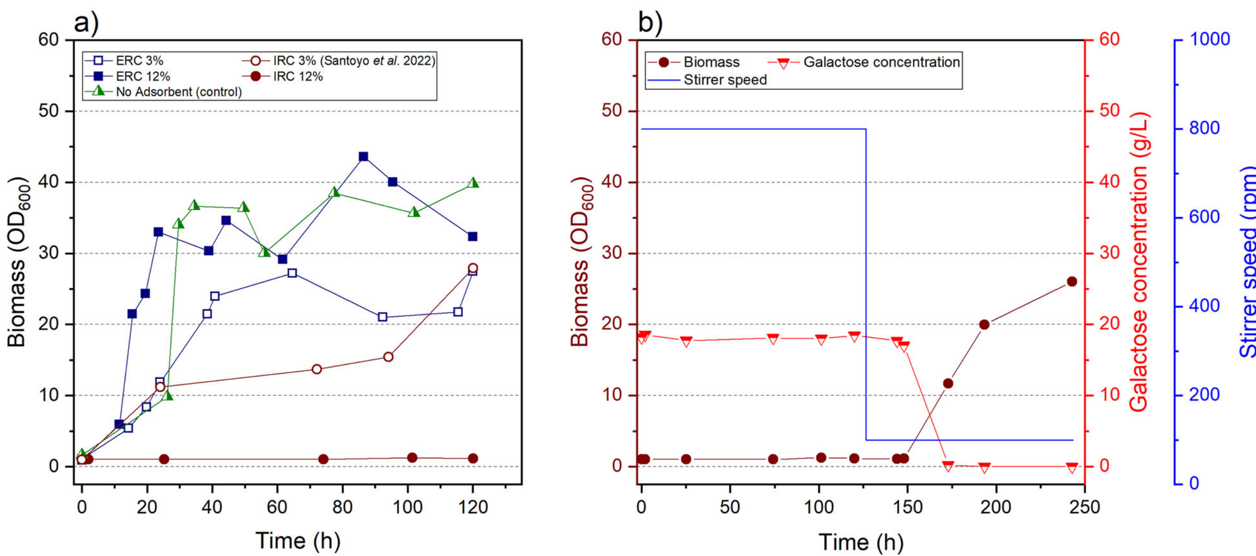

Figure 3. (a) Comparison of cell growth in bioreactor scale cultivations (160 mL) using different adsorbent concentrations and recovery configurations. (b) Effect of agitation speed on cell growth in cultivation with 12% adsorbent using the IRC. All cultivations were performed with YP media supplemented with 2% galactose. Temperature was controlled at 30 °C, pH was controlled with the addition of NaOH 1 M to keep the value at 6 and the stirring speed was 800 rpm.

Table 1. Comparison of process variables between different bioreactor configurations.

Bioreactor configuration	Volumetric flow rate (mL/min)	Stirring speed (rpm)	Linear speed (cm/h)[a]	Reynolds number	Sedimented bed height (H_0, mm)	Expanded bed height "H" (mm)	Degree of expansion
IRC 3%[a,b]	–	800	4.22E + 05	13042.4	–	–	–
IRC 12%	–	800	4.22E + 05	13042.4	–	–	–
IRC 12%	–	100	5.28E + 04	1630.3	–	–	–
ERC 3%	7	800	2.38E + 02	12.4	35.2	71.4	2.0
ERC 12%	3	800	1.02E + 02	5.3	143.1	216.7	1.5

[a]Linear speed in IRC experiments is calculated from stirring speed, where in ERC experiments from the volumetric flow rate through the external column.
[b]Data from Santoyo-Garcia *et al.*[18]

experimental and calculated values for *Umf* were within one order of magnitude (1.6 and 0.2 mL/min respectively) as it was deemed appropriate for back-of-the-envelope estimations.

The degree of expansion in the 12% ERC experiment was lower than the 3% ERC because the column available was not long enough to accommodate a degree of expansion of 2.

Cell growth kinetics

S. cerevisiae LRS5 was cultivated in two different bioreactor configurations and two beads concentrations in order to determine their effect on cell growth kinetics. A control experiment with no adsorbent beads in the bioreactor system was conducted for comparison. The results of this study is summarized in Figure 3.

Figure 3a,b show that cells did not grow as well as no galactose was consumed using 12% beads in the IRC configuration. This result is in agreement with the hypothesis that relatively high concentrations of HP-20 beads are detrimental to cell growth. To investigate if shear stress contributes to growth inhibition, the stirring speed was reduced to 100 rpm after the bioreactor was run for 125 hr at 800 rpm, changing the flow regime from turbulent to laminar (see Reynolds numbers in Table 1). Cell growth started after 46 hr following the reduction in stirring speed, indicating that shear stress was the contributing factor for cell growth inhibition at relatively high concentrations of beads, while no growth inhibition was observed at lower resin concentrations.

Agitation speed is important to ensure the homogeneous dispersion of resins inside the bioreactor vessel and avoid sedimentation.[26] However, agitation speed may also induce shear stress to the cells, in addition to affect the physical integrity of the resin.[22,33,35] Different authors have reported negative effects on cell growth[35–37] and viability[27,38] when an adsorbent resin is added into the bioreactor. The most accepted hypothesis is that resin induces shear stress to the microorganisms due to collisions with cells from stirring agitation. This has been supported by experiments evaluating the effect of stirring speed. Othman *et al.* observed a reduction in the cell viability of *Pediococcus acidilactici* after increasing agitation speed from 300 to 400 rpm in a bioreactor with 1% (w/v) dispersed Amberlite IRA 67 resin.[26] The effect of agitation speed was also studied in a dispersed resin system with Amberlite IRC 86 to recover ammonium and increase the cell viability of *Pasteurella multocida*. In the dispersed resin system, cell viability decreased after increasing the agitation speed from 300 to 400 rpm. On the other hand, a system that contained the resin in an internal column, viability increased in proportion to increasing agitation speed until 500 rpm.[27]

Bead concentration showed an important effect on cell growth kinetics. 12% concentration displayed the fastest kinetics on both reactor configurations. ERC experiments showed higher cell growth extent in comparison to IRC, regardless of adsorbent concentration.

Growth rate was calculated in all experiments using the logistic model and the values are shown in Table 2. The growth rate was higher in the experiments with the external adsorption column in comparison to the bioreactor with dispersed resin. Cell growth kinetics in the 12% ERC were 1.9, 5, 5, and 1.5 fold higher in comparison to 3% ERC, 12% IRC, 3% IRC, and control, respectively. An increment in cell growth rate has been observed in operations using EBA systems for in situ product recovery. Wang *et al.* used ZGA330 resin to recover propionic acid from culture media and observed faster cell growth kinetics in a fluidized bed systems in comparison to a bioreactor with internal column, dispersed resin, and control experiment with no resin.[36] Tan *et al.* integrated a stirred tank bioreactor with an expanded bed adsorption column (STR/EBA) to remove acetate from culture media and improve the production of α-PrIFN-2b. They indicated that the growth rate cycle was faster in the STR/EBA configuration in comparison to dispersed resin and control.[35]

Taxadiene titers

Product partitioning was investigated by the extraction of taxadiene from the different fractions, i.e., adsorbent beads, cells, and cell-free media at the end of the cultivation. The results of product partition in all tested bioreactor configurations and beads concentrations is summarized in Figure 4.

Bioreactor configuration and adsorbent concentration had an important effect on the amount of taxadiene recovered. In general, IRC showed higher taxadiene titers compared to ERC, with total taxadiene titers in IRC 3.4 and 3.5 fold higher than ERC when using 3% and 12% beads, respectively. Similar results were obtained in the production of (+)-zizaene[15] and prodigiosin-like red pigment[33] where titer obtained with an IRC system were higher than ERC due to the more intimate contact of the solid adsorbent with the culture broth. In particular, in IRC the adsorbent was constantly in contact with culture media, while residence

Table 2. Comparison of growth rate and cell dry weight on different bioreactor configurations.

Bioreactor configuration	Growth rate (h^{-1})	OD_{600}	Cell dry weight (g/L)
IRC 3%[a]	0.03	27.9	4.57
IRC 12%	0.03	26.0	4.27
ERC 3%	0.08	27.5	4.51
ERC 12%	0.15	32.3	5.30
No adsorbent (control)	0.10	39.8	6.51

[a]Data from Santoyo-Garcia *et al.*[18]

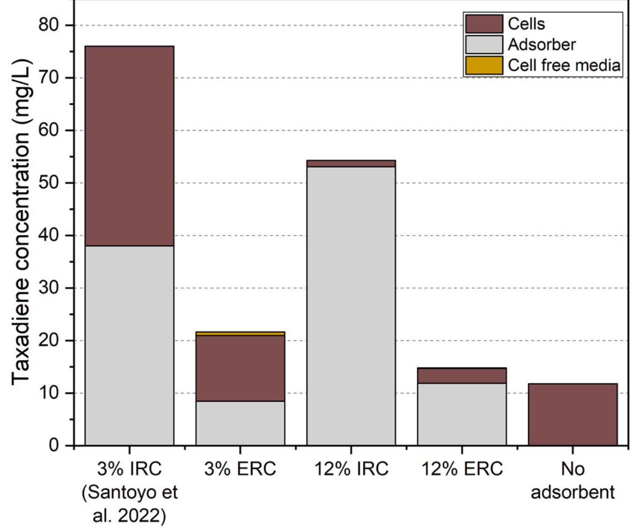

Figure 4. Total taxadiene titer (mg/L) and partition across the different fractions on the two different bioreactor configurations and adsorbent concentrations investigated.

time in ERC were 6.5 and 10.35 min in 3% ERC and 12% ERC respectively, with possible, taxadiene loss due to volatilization in the ERC. This hypothesis is supported by the partition of taxadiene in the control experiment, where the cultivation was carried out without adsorbent. In this experiment there was no taxadiene in the cell-free media, indicating product loss due to volatilization. Aguilar *et al.*, also observed the volatilization of (+)-zizaene if no solid adsorbent was used during fermentation. In this particular experiment, an initial (+)-zizaene concentration of 2.6 mg/L in the cell-free media decreased to 0.5 mg/L in 1-hr and only traces were detected after 4 hr.[15] Taxadiene loss by volatilization has also been observed by Santoyo-Garcia *et al.*, who mentioned the accumulation of hydrophobic metabolites in the gas-liquid interphase further facilitating the volatilization process.[18]

Resin concentration also had an influence on taxadiene titers, with 3% beads having 1.5 and 1.4 fold higher total taxadiene titer at the end of the cultivation, than 12% beads in ERC and IRC, respectively (Figure 4). It has been reported that a larger concentration of resin may interfere in the metabolism of the yeast. For example, teicoplanin production using 3% and 5% (w/v) resin concentration (Diaion HP-20) was 126 and 134 mg/L respectively, but, production decreased to 95 mg/L with 10% resin.[22] Wang *et al.* used ZGA330 resin on different bioreactor configuration to recover propionic acid produced by *Propionibacterium freudenreichii*.[36] They observed that the interaction of cells with the resin and bioreactor configuration influenced the metabolism flows with more substrate converted into biomass instead of the target metabolite. Also, their control showed the highest dry cell biomass but lowest propionic acid concentration compared to experiments with ISPR.

Both experiments with 12% of adsorbent showed an almost complete partition of taxadiene on the beads (Figure 4). On the other hand, partition in the 3% ERC experiment was 39% from the adsorbent and 58% from cells. A similar distribution to IRC cultivations with 3% beads where the distribution of taxadiene was 51% in the cells and 49% on the adsorbent.[18] These results suggest that increasing adsorbent concentration enhanced the partition on the beads by around twofold on both bioreactor configurations. Therefore, more adsorbent in the system increases the availability of adsorption sites, directly influencing the partition of taxadiene between cells, cell-free media and beads.

The increment in beads partition is economically advantageous for an industrial-scale process because most of the taxadiene produced is recovered by the adsorbent thus easing the subsequent desorption process.

Taxadiene productivity

The adsorption loading of Diaion HP-20 for taxadiene was calculated as the quotient of the mass of taxadiene extracted from the beads per mass of the beads used in the experiment (q). The q values obtained in all of the experiments is summarized in Figure 5a. Kinetic experiments have revealed that taxadiene production in *S. cerevisiae LRS5* increases during the exponential growth phase, with concentration remaining steady during the stationary phase of growth.[18] Experiments in the ERC configuration showed faster growth kinetics than IRC. Defining productivity as the quotient between total taxadiene titer and time needed to reach stationary phase, then productivity for the different experiments were in a similar range from 0.21 to 0.51 mg/L*h. The results of productivity on the different experiments is shown in Figure 5b.

Productivity could also be used to select the most suitable conditions for taxadiene production because the cultivation followed by immediate recovery of taxadiene could be stopped right after reaching the cell growth stationary phase. In industry practice, metabolites produced in an IRC configuration would be recovered only from the adsorbent fraction as processing from the culture media would be unpractical and not economical. Therefore, the 12% ERC was the most productive system as it displayed faster cell growth kinetics and high partition on the beads. A 12% ERC system can be improved using bigger columns that allow higher flow rate and bed expansion, parameters that are important to increase taxadiene titers and productivity.

The integration of an external adsorption column for in situ product recovery has been reported to improve productivity. For example, Wang *et al.* used a fluidized bed column to remove propionic acid during fermentation, resulting in a 1.4 and 1.2-fold higher productivity compared to a

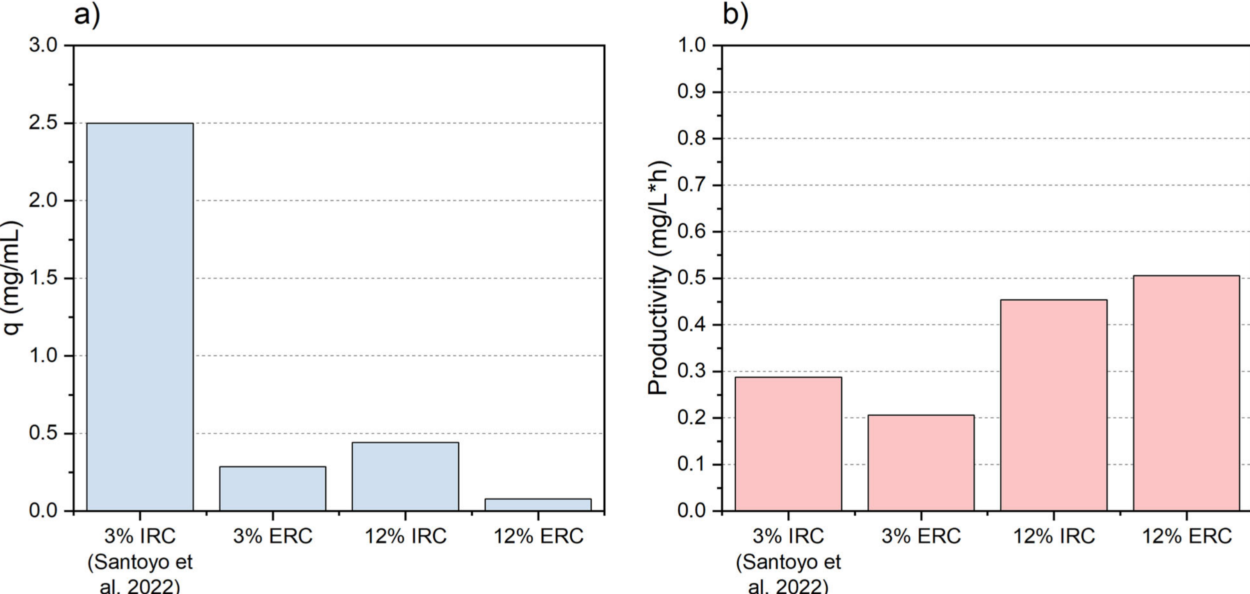

Figure 5. Comparison of different bioreactor configurations and adsorbent concentrations. (a) Taxadiene loading on the adsorbent; (b) Productivity.

bioreactor with an internal column and a bioreactor with dispersed resin, respectively.[36] Tan *et al.* compared the performance of an expanded bed adsorption column with a dispersed resin system for the in situ removal of acetate to improve α-interferon-2b production and observed higher productivity in the expanded bed adsorption column compared to a system with dispersed resin in the bioreactor.[35]

Conclusions

Production and recovery of taxadiene was carried out using two bioreactor configurations. Cell growth kinetics were significantly faster using the external recovery configuration, which could be seen as an advantage to obtaine higher productivities (mg of taxadiene/L*h) as taxadiene production stops after reaching the stationary phase. On the other hand, taxadiene titers recovered with the internal recovery configuration were around 3.5-fold higher in comparison to the external configuration regardless of the adsorbent concentration used. Here we also studied the effect of adsorbent concentration and observed that increasing adsorbent concentration to 12% (w/v) improved the partition of taxadiene into the beads by twofold. This represents an advantage for an industrial scale process as this facilitates the operation by extracting the products after the cultivation only from the solid adsorbents and reduce costs. It is expected that taxadiene production in the ERC systems could be improved by using columns with higher volume that could allow to increase residence time. In addition, cell growth of *S. cerevisiae LRS5* showed to be susceptible to shear stress induced by a combination of high concentration of resin beads (12%) and stirring speed (800 rpm). Results here presented demonstrate the importance of bioreactor configuration and process conditions like adsorbent concentration and agitation speed in the production and recovery of metabolites in bioprocesses.

Disclosure statement

No potential conflict of interest was reported by the authors.

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