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Assessment of *in situ* product recovery techniques to enhance 2-phenylethanol production by *Acinetobacter soli* ANG344B

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ABSTRACT

The 2-phenylethanol (2-PE) production process by the newly isolated *Acinetobacter soli* ANG344B is limited by product toxicity. To overcome this limitation and enhance 2-PE production process, various alternatives based in *in situ* product removal (ISPR) approaches were evaluated. The approaches selected for assessment were gas stripping using the air supplied to the bioreactor, liquid-liquid extraction and adsorption. Adsorption was found to be the most promising approach to increase 2-PE production. Amberlite XAD 4 was chosen from the different adsorbents tested since it has high affinity for 2-PE, being able to adsorb 205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$. In a batch cultivation process, in presence of 3 % (dry w/v) of Amberlite XAD 4, *A. soli* ANG344B was able to produce 6.99 ± 0.06 g/L of 2-PE with a volumetric productivity of 0.17 ± 0.00 g/L.h, which represents an improvement of 3.3-fold. To the best of our knowledge, this is the highest 2-PE production reported for a wild-type bacteria. These findings highlight the potential of *Acinetobacter soli* ANG344B as 2-PE producer, contributing to the development of natural 2-PE production process.

1. Introduction

2-Phenylethanol (2-PE) is an aroma compound characterized by its rose-like scent. This fragrance is widely used in cosmetic, perfume, home care, food and beverage industries. 2-PE can be extracted from plants, namely rose petals. However, the compound with botanical origin has high production costs due to the low recovery rates, weather dependence and trade restrictions and consequently, does not meet the high product demand. Further, this extraction process generates substantial amounts of organic waste. The majority of the commercialized aroma compound is obtained from chemical synthesis from benzene and styrene in harsh pH, temperature and pressure conditions. The chemical synthesis, besides having a negative impact in the environment, promotes the formation of undesirable byproducts that influence the final grade of the product. Biotechnological alternatives for 2-PE production have been developed to meet the disadvantages and limitations of the botanical extraction and chemical 2-PE production, required by the increasing concerns about the environment and demand for natural products. 2-PE produced by biotechnological approaches can be considered natural by US Food and Drug Administration and European legislation [1,2].

Acinetobacter soli ANG344B is a newly isolated bacterium with an exceptional ability to produce 2-phenylethanol (2-PE) from L-phenylalanine (L-Phe). This microorganism is able to produce 2-PE in concentrations of 2.35 g/L with a volumetric productivity up to 0.13 g/L.h, obtained in batch operational mode, a volumetric productivity that stands among the highest reported by a wild-type microorganism for 2-PE production [3,4]. This ability is typically reported in yeasts rather than bacteria, which have been reported to produce 2-PE in concentrations below 152 mg/L, while yeasts can produce the aroma in concentrations below 4 g/L, with volumetric productivities lower than 0.1 g/L.h [3]. Higher 2-PE production levels in bacteria are only reported in genetically modified microorganisms [5–8]. The short growth time and simple metabolism offered by bacteria, avoiding the production of ethanol, that presents a synergistic negative effect towards the microorganism in the presence of 2-PE, and can negatively influence the recovery process and might also impact the aroma quality, as well as the high 2-PE titers that can be achieved by *Acinetobacter soli* ANG344B, make this strain a valuable candidate to enhance the natural 2-PE production.

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The attempts to increase 2-PE microbial production through testing different cultivation conditions and different producing strains, have been impaired by the product inhibition towards the producing microorganism. Usually, 2-PE concentrations between 2 and 4 g/L are reported to inhibit cellular growth, depending on the specie [4,8,9]. In fact, 2-PE is reported as bacteriostatic agent due to its amphiphilic nature, interfering with the cell membrane and affecting cellular homeostasis [10].

To improve 2-PE production process, by reducing product inhibition, several *in situ* product removal (ISPR) techniques have been studied. These approaches consist of continuous removal of 2-PE from the cultivation broth, allowing the microorganism to continue the 2-PE production and cellular growth [11]. ISPR techniques can also minimize product loss by degradation or evaporation and facilitate the downstream processes [12,13]. The continuous removal of 2-PE can be done using several approaches such as gas stripping, liquid-liquid and solid-liquid extraction or membrane-based processes.

Gas stripping is a technique used for the recovery of volatile compounds from microbial cultivation broths. Those compounds are removed from the broth into a gas phase and recovered using a cooling device, for instance. This technique offers advantages as simplicity, continuous operation and the possibility of using gases supplied to or produced in the process. However, temperature, stirring speed, medium composition, gas flowrate and cooling temperature are factors that can affect the efficacy of the stripping process [11].

Liquid-liquid extraction is one of the most reported approaches attempted for improvement of 2-PE production process [1,2]. Solvents such as oleic acid, polypropylene glycol, ethyl acetate and ionic liquids have been tested to recover the 2-PE from the cultivation broth while it is being produced [1,2,13,14,15]. This can be achieved by mixing the solvents with the cultivation medium or using membrane contactors to avoid the direct contact of the solvents with the cells. The use of these solvents has been reported to successfully improve 2-PE production process in yeasts, improving the aroma production in 1.6–5-fold reaching 2-PE concentrations up to 16 g/L in *Saccharomyces cerevisiae* [13–16]. However, the use of most of these solvents present drawbacks as formation of stable emulsions, inhibitory effects towards the microorganisms due to the solvent interaction with the cellular membranes, interference with the organoleptic properties of the compound and, in the case of the ionic liquids, the worries about their toxicity.

Organophilic pervaporation has also been reported for process improvement, resulting in a 2-fold increase in 2-PE production to 2.20 g/L by *Kluyveromyces marxianus*, being cell separation and temperature, that influences the fluxes, limiting factors of this process [17].

Solid-liquid extraction approaches, specially using polymeric adsorbents have been also reported for enhancement of 2-PE production process. These adsorbents are reported to be used in direct contact with the cultivation broth or used in external columns connected to the bioreactor, through which the broth (with or without cells) can be recirculated allowing the contact with the resin. Non-polar polymeric adsorbents, as HZ818, D101 and FD0816, that interact with the aroma through the hydrophobic aromatic ring by π interactions are the most common adsorbents used [18–21]. Few polymers can interact by hydrogen bonding with the aroma, being Hytrel 8206 and Hytrel 63548 the reported polymers [9,22]. The amount of adsorbent in contact with the cultivation broth is usually between 2 % and 10 % (w/v), allowing an increase in 2-PE production by 1–3-fold up to 13.7 g/L [18,19,21]. Higher improvements have been reported when using the adsorbent coupled to the cultivation in an external column [9,21]. Even though this alternative covers some drawbacks of liquid-liquid extraction, as the toxicity or the organoleptic influence, the selectivity of the adsorbent, the product recovery from the polymer and the need for cell separation during production might be bottlenecks of the process.

In our previous work, the 2-PE production potential of *Acinetobacter soli* ANG344B was demonstrated, as well as the culture conditions that influence the aroma production. The toxicity of the product towards the producing microorganism was also described [3,4]. The present work focuses on the study of different alternatives for 2-PE recovery during the microbiological producing process by *Acinetobacter soli* ANG344B to improve the aroma production, avoiding its toxicity. Based on physical and chemical properties of the aroma compound, gas stripping, liquid-liquid extraction and adsorption approaches were evaluated for 2-PE extraction. For that, important features of the extractive processes were assessed: the evaporation of 2-PE under production conditions, the partition coefficients of the solvents towards 2-PE and the adsorption capacity and selectivity of the resins, with an extensive work regarding adsorption and recovery by desorption being detailed. The applicability of these alternatives to the 2-PE production by *A. soli* ANG344B was assessed, and the most suitable approach was applied in bioreactor production resulting in significant process improvement, achieving a 2-PE production not previously reported for a wild-type bacteria. The difficulties and problems encountered during the implementation of these approaches are also discussed, contributing to expand knowledge in the area. By improving the 2-PE production using this novel strain, we are contributing to a natural and sustainable aroma production, bringing process alternatives to the use of the chemically synthesized 2-PE.

2. Materials and methods

2.1. Gas stripping approach

The experimental setup consisted of two condensers-in-series immersed in a water bath at 0 °C for vapor condensation, connected to the gas outlet of the 2 L bioreactor (BioStat B-Plus, Sartorius, Germany), as represented in Fig. 1. The air stripping was carried out using the air supplied to the bioreactor cultivation medium, that was maintained constant at 1 vvm (volume of air per volume of reactor per minute) with variation of stirring speed from 300 to 800 rpm. The first experiment was performed using the bacterial cultivation media (Na2HPO4, 4 g/L; KH2PO4, 1 g/L; NaCl, 0.2 g/L; MgSO4.7H2O, 0.2 g/L; CaCl2, 0.05 g/L; yeast extract 5 g/L; glucose anhydrous, 10 g/L)(as previously optimized [4]) modified with 4 g/L of L-Phe and 1 g/L of 2-PE, without bacterial inoculation.

The second experiment was accomplished in the presence of bacterial cellular growth (5 % (v/v) of inoculum grown in Luria-Bertani (LB) media for 20 h, at 30°C, at 200 rpm in an orbital shaker) under the same conditions described above, with a pulse feeding of 160 mL of a 25 g/L L-Phe solution at 9 h of the experiment.

2.2. Liquid-liquid ISPR approach

2.2.1. Partition coefficient determination for liquid-liquid ISPR approach

2-PE partition coefficients in oleic acid and polypropylene glycol (PPG) 1200 were determined by mixing 50 % (v/v) of each solvent and solutions with different concentrations of 2-PE, ranging from 0 to 2 g/L, similar to the aroma concentrations achieved during a batch production process by *Acinetobacter soli* ANG344B (0, 0.3, 0.7, 1.0, 1.3, 1.7 and 2.0 $g_{2\text{-PE}}$/L). The vials (10 mL vials) were incubated in an orbital shaker

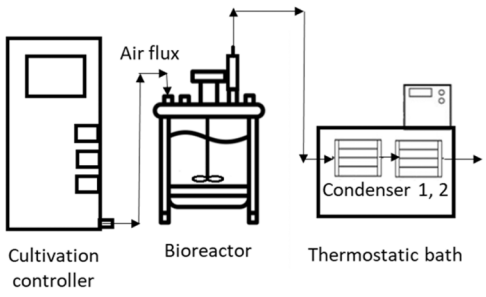

Fig. 1. Schematic representation of the setup for gas stripping ISPR approach.

at 30 °C (bacterial cultivation temperature), at 200 rpm, for 24 h, to achieve phase equilibrium. To assure phase separation, a centrifugation step was performed at 13 000 xg, 4 °C, for 15 min. The 2-PE concentration in each phase was analyzed by HPLC, as described below, Section 2.5.2. The 2-PE partition coefficients towards the solvents were calculated as the slope of 2-PE concentrations in solvent phase versus aqueous phase.

2.3. Polymeric adsorption approach

2.3.1. Polymeric resins screening

Three different resins, Amberlite XAD 4 (supplied by Thermo Scientific, USA), Macronet MN 102 (supplied by Purolite, USA) and Hytrel 8206 (supplied by DuPont Ibérica S.L, USA) were tested to be used as ISPR adsorbents on the 2-PE production process. Their main characteristics are summarized in Table 1.

Prior to use, resins were washed with ethanol 96 % (v/v) for 24 h under slight agitation (200 rpm in an orbital shaker), and afterwards washed with distilled water and vacuum filtered.

The dry weight of the resins was determined gravimetrically, after drying a 1 g of resin at 100 °C for 24 h.

To determine the best adsorbent ratio for 2-PE adsorption, different adsorbent dosages ranging from 1 % to 20 % (for Amberlite XAD 4, Macronet MN102) or 30 % (w/v) (for Hytrel 8206) in a dry basis were tested using a 2 g/L 2-PE model solution at pH 7 (the pH used for the 2-PE production process by *Acinetobacter soli* ANG344B). The different amounts of resin were incubated in 6 mL of the 2-PE solution at 30 °C (the cultivation temperature for the 2-PE production process by *Acinetobacter soli* ANG344B [4]), 200 rpm for 24 h to allow reaching the equilibrium. The 2-PE concentration before the adsorption and after the establishment of the equilibrium was measured by HPLC as described in Section 2.5.2. The adsorption capacity and the adsorption efficiency were calculated as follows:

Adsorption capacity:

$$Q = \frac{C_0 - C_{eq}}{m_0} \times V \quad (1)$$

Adsorption efficiency:

$$E(\%) = \frac{(C_0 - C_{eq})}{C_0} \times 100 \quad (2)$$

where Q is the adsorption capacity ($g_{adsorbate}/g_{dry\ resin}$), E is the adsorption efficiency (%), C_0 and C_{eq} are the concentrations of adsorbate in aqueous phase before the adsorption and after the equilibrium was reached, respectively (g/L), m_0 is the mass of dry resin (g) and V is the solution volume (L).

Using the best resin ratio for each adsorbent, the affinity for L-Phe and 2-PE was assessed by incubating the adsorbent with a model solution of 5 g/L of L-Phe and in a binary solution of 2 g/L of L-Phe and 2 g/L of 2-PE in the conditions described above.

Table 1
Summary of resins' characteristics, tested for 2-PE adsorption in this work.

Resins	Matrix	Functional group	Surface area (m^2/g)	Porosity
Amberlite XAD 4	Styrene-divinylbenzene	ND	≥ 750	≥ 0.50
Hytrel 8206	Copolymer polybutylene ester and polyether	ND	ND	ND
Macronet MN 102	Polystyrene-divinylbenzene	Tertiary amine	800	0.40

ND – not disclosed by supplier.

2.3.2. Desorption of 2-PE and L-Phe

The desorption of both 2-PE and L-Phe adsorbed in Amberlite XAD 4 from model solutions containing 2 g/L of L-Phe and 2-PE, was evaluated. Desorption was performed by incubating the resin containing 2-PE and L-Phe in ethanol 96 % (v/v) or in deionized water, overnight at room temperature and 200 rpm. The proportion of resin and desorbing solution was the same used in the adsorption experiments. The desorption process was repeated until the concentration of the compounds measured in the eluent phase was lower than 0.05 g/L. The desorption efficiency was determined as follows:

$$D(\%) = \frac{C_d \times V_d}{(C_0 - C_{eq}) \times V} \times 100 \quad (3)$$

where D is the desorption efficiency (%), C_d is the concentration of adsorbate in the eluent (g/L) and V_d is the volume of the eluent (L).

2.3.3. Dynamic adsorption and desorption

Dynamic fixed-bed column adsorption and desorption experiments were performed in a glass column with an inner diameter of 2 cm with 13.5 g of dry Amberlite XAD 4, with a fixed bed height of 19.5 cm, corresponding to a bed volume (BV) of 61.3 cm^3, calculated as follows:

$$Bed\ volume(cm^3) = bed\ height(cm) \times column\ crossectional\ area(cm^2) \quad (4)$$

The adsorption tests were conducted at 30 °C, maintained constant by a thermostatic water bath, and pH 7 to simulate the 2-PE production process by *Acinetobacter soli* ANG344B. A solution containing only 1 g/L of 2-PE or 1 g/L of 2-PE and 1 g/L of L-Phe was fed to the column from the bottom at 9.79 BV/h, equivalent to 10 mL/min, using a peristaltic pump, for 8 h, as represented in Fig. 2. Samples were collected periodically at the top of the column and analyzed by HPLC, as described below in the analysis section, to determine the breakthrough curves. The breakthrough curves were expressed in terms of normalized concentration of the compounds, the ratio of outlet to inlet concentration (C/C_0), as function of time.

The maximum capacity of the column was calculated from the experimental breakthrough curve using the following equation [23]:

$$q_{total} = \frac{Q \times A}{1000 \times m_0} \quad (5)$$

Where q_{total} (g/g) is maximum capacity of the column, A is the area under the breakthrough curve, calculated by integrating the adsorbed 2-PE concentration (C_0 - C_t) as function of time, Q is the flow rate (mL/min) and m_0 is the mass (g) of dry Amberlite XAD 4.

The total mass of the compound adsorbed was calculated using the area under the breakthrough curve as follows [24]:

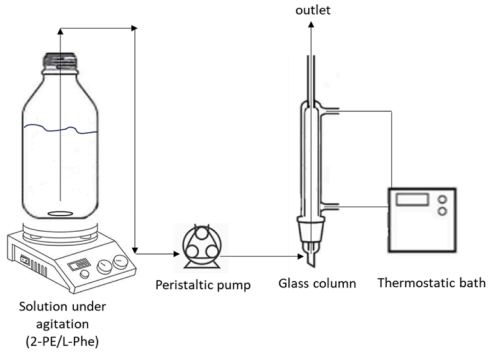

Fig. 2. Schematic representation of the dynamic fixed-bed column adsorption and desorption experiments setup.

$$m_{ads} = \frac{C_0 \times Q}{1000} \int_0^{t_{sat}} \left(1 - \frac{C}{C_0}\right) \quad (6)$$

Where the m_{ads} is the total mass of 2-PE adsorbed (g), C_0 is the inlet concentration of 2-PE, Q is the flow rate (mL/min), t_{sat} is the bed saturation time (min).

After adsorption, the compounds were desorbed in the same operating conditions used for adsorption, but at room temperature, with ethanol 96 %. Samples were taken periodically for 3 h and analyzed by HPLC, as described below in the analysis section.

2.4. Biotransformation experiments with extraction from cultivation broth

2.4.1. Microorganism and inoculum preparation

Acinetobacter soli ANG344B, isolated from the Catumbela River (Angola) as described in our previous work [3], is deposited in the Microbial Strain Collection of Latvia (MSCL) under accession number 1593. *Acinetobacter soli* ANG344B, preserved in cryovials containing glycerol (20 wt%), at –80 °C, was reactivated in Luria-Bertani (LB) agar medium plates (bacto-tryptone, 10 g/L; yeast extract, 5 g/L; sodium chloride, 10 g/L; agar, 18 g/L). The inoculum was grown in LB medium at 30 °C, 200 rpm, for 20 h in an orbital shaker, as described in previous work with this strain [3,4].

2.4.2. Shake flask cultivation

Different ISPR techniques were tested in cultivations performed in 250 mL baffled shake flasks with a working volume of 100 mL at 30 °C, pH 7 and stirred at 200 rpm, in an orbital shaker. Cultivation was performed in the standard cultivation media (previously described [3,4]): Na_2HPO_4, 4 g/L; KH_2PO_4, 1 g/L; NaCl, 0.2 g/L; $MgSO_4.7\ H_2O$, 0.2 g/L; $CaCl_2$, 0.05 g/L; yeast extract 5 g/L; glucose anhydrous, 10 g/L, L-Phe, 5 g/L. For cultivation using a liquid-liquid ISPR technique, 20 % (v/v) of oleic acid was added to the cultivation media after 3 h of cultivation. For cultivations using a ISPR polymeric adsorption approach, 20 % (v/v) of dry Hytrel 8206 and 3 % (v/v) of dry Amberlite XAD 4 were added at the beginning of cultivation. When the cultivation was performed in presence of Amberlite XAD 4, 7 g/L of L-Phe was used, taking in consideration the adsorption of that compound, to achieve an initial concentration of 5 g/L. Cultivations were performed for 24 or 48 hours, respectively for liquid-liquid or adsorption approaches. Samples were taken periodically for evaluation of cell growth, L-Phe and glucose consumption and 2-PE production.

2.4.3. Bioreactor cultivation

Bioreactor cultivations were performed in 2 L bioreactors (BioStat B-plus, Sartorius, Germany), using the standard cultivation media described in Section 2.4.2, modified with 17 g/L of L-Phe to achieve an initial concentration of 10 g/L of the precursor (considering the maximum adsorption of the compound) and 20 g/L of glucose. 3 % (w/v) of dry Amberlite XAD 4 was added to the bioreactor cultivation medium for ISPR by adsorption and then sterilized. The culture was cultivated at 30.0 ± 0.1 °C, with pH controlled at 7.00 ± 0.02 by automatic addition of 5 M NaOH or 5 M HCl. The aeration rate was maintained constant at 1 vvm (volume of air per volume of reactor per minute), by automatic variation of the stirring speed between 300 and 1000 rpm, with the minimum dissolved oxygen controlled at 30 %. To avoid evaporation losses, the bioreactor condenser was maintained at 3 °C. Foam formation was prevented by automatic addition of silicone antifoam liquid (AQ) (PanReac AppliChem). Samples were taken periodically during the cultivation, and centrifuged at 16000 xg, to measure cell growth, L-Phe and glucose consumption, and 2-PE production in aqueous phase. The aroma adsorbed to the polymer was quantified at the end of the experiment.

2.4.4. Product recovery from the resin

After the bioconversion, the resin was recovered from the cultivation broth, decanted and vacuum filtered. The resin was incubated with ethanol 96 % in an orbital shaker at 200 rpm, at room temperature overnight to desorb the 2-PE. The eluent (ethanol) was removed by vacuum filtration. Fresh ethanol was added to the resin for further 3 cycles until the 2-PE concentration in the resin was below 0.01 g/L. The ethanol containing 2-PE was evaporated by vacuum distillation using a rotatory evaporator (Büchi Rotavapor R-210, Switzerland), operating at a pressure of 31 mbar and a temperature of 30 °C, being the ethanol condensed at −11 °C, until the volume of the 2-PE phase was below 50 mL.

2.5. Analytical techniques

2.5.1. Cell separation and sample processing

Culture broth samples were taken periodically. The optical density was determined at 600 nm. Samples were centrifuged at 9056 xg, for 15 min at 4°C, for cell separation. Cell pellet was used for gravimetrical cell dry weight determination, after washing twice and lyophilization (ScanVac CoolSafe, LaboGene, Denmark). Cell-free supernatant was used for quantification of L-Phe, 2-PE, glucose.

2.5.2. L-Phe and 2-PE quantification

L-Phe and 2-PE were quantified by high-performance liquid chromatography (HPLC) (Alliance e2695, Waters, USA) equipped with a C18 3.9 ×150 mm column and a guard column (Nova-Pak®) maintained at 25 °C, coupled to a PDA (Photo Diode Array) detector, set at 216 nm. A gradient method comprising water/methanol was applied with a flow rate of 0.5 mL/min, as follows: 0–7 min 70/30; 7–10 min 50/50; 10–16 min 30/70; 16–25 min 70/30. L-Phe and 2-PE were used as standards in concentrations ranging from 0.01 to 0.2 g/L.

2.5.3. Glucose quantification

Glucose quantification was performed by HPLC (VWR Hitachi Chromaster, Japan), equipped with a Diode Array Detector 5430 and a Refractive Index Detector 5450. The column used was an Aminex HPX-87 H (300 × 7.8 mm) coupled to a pre-column Biorad 125–0129 (30 ×4.6 mm), operated at 30 °C. The mobile phase was 0.01 N H_2SO_4 and the flow rate 0.5 mL/min. D-Glucose was used as standard in concentrations between 0.03 and 1 g/L.

2.5.4. 2-PE extract analysis

The recovered extract desorbed from the resin XAD 4 was analyzed by Gas Chromatography (GC) with a Flame Ionization Detector (FID) (Agilent 6890) using a VF5-ms (30 m, 0.25 nmID, 0.25 mm film) column. Helium was used as carrier gas at 1 mL/min. Injector and detector temperatures were 250 °C. The temperature program applied started with 50 °C for 5 min and increased at 20 °C/min until 320 °C. For the identification of compounds present in the extract, samples were analyzed by GC-mass spectrometry (GC-MS). The sample was analyzed by GC-MS (Agilent 7890 GC) with an Agilent HP- MS UI fused silica capillary column (30 mx0.25 mm ID, 0.25 μm df – film thickness), in the same conditions as GC-FID. For GC-MS a Leco Pegasus® BT GC-TOFMS was used. The transfer line was set at 300 °C, source 250 °C. The MS was operated in electron ionization mode (70 eV) using a range of *m/z* 40–470. Data was processed using software ChromaTof v5.40.12.0 (LECO Corp., Saint Joseph, MI, USA). NIST MS Search Program Version 2.3 was used for spectra matching (NIST, 2015).

2.6. Statistical analysis

Experiments were performed at least in duplicates of independent samples. Data are expressed as the mean ± standard deviation.

3. Results and discussion

Acinetobacter soli ANG344B is a strain capable of producing 2-PE in

concentrations above 2 g/L, using L-Phe as precursor [3,4]. However, further 2-PE production is limited by the product toxicity [4]. Previous work developed with this strain found that a 2-PE concentration as low as 1 g/L has a negative influence on the cells, causing a decrease in bacterial growth of 50 % after 24 h of cultivation, when compared with the cultivation in absence of 2-PE [4]. This effect is stronger as the 2-PE concentration increases, being completely inhibitory for concentrations above 4 g/L [4]. So, in order to improve 2-PE production by this microorganism and to selectively recover the product of interest from the cultivation broth, several ISPR strategies were attempted.

3.1. Gas stripping and liquid-liquid extraction

During the cultivation process of 2-PE in bioreactor, the aeration supplied to the bioreactor together with the stirring can lead to the loss of the aroma in the off-gas [25]. In fact, during cultivation of *Acinetobacter soli* ANG344B for 2-PE production in bioreactor, a rose aroma was perceived in the surrounding of the system, which motivated a gas stripping approach as recovery technique. To evaluate the potential of using the air supplied to the bioreactor (1 vvm) to remove and recover the 2-PE as it is being produced, experiments were performed using condensers at 0 °C connected to the bioreactor's gas outlet. A 2 L bioreactor experiment, mimicking the standard conditions used for *A. soli* ANG344B cultivation [4], was performed in the presence of 1 g/L of 2-PE for 22 h but without bacterial cultivation, allowing for recovering only 1.2 % of the initial 2-PE present in the medium. Similar results were obtained during bacterial cultivation, indicating that evaporation driven by air flow and bioreactor stirring was not efficient for 2-PE removal.

Gas stripping is a typically effective for low molecular weight (lower than 1000 g/mol) and high volatile compounds, such as ethanol and flavors as limonene [11,12,26], but 2-PE, is as a semi-volatile compound, with low vapor pressure (8×10^{-5} atm) and high boiling point (225 °C) [25,27]. Despite 2-PE's high activity coefficient in aqueous media, the air stripping approach did not result in a sufficiently expedite aroma recovery, under the cultivation conditions used.

Moreover liquid-liquid extraction was also tested for 2-PE removal. Oleic acid and polypropylene glycol (PPG) 1200 were tested as extractive solvents for liquid-liquid extraction in the 2-PE production process by *A. soli* ANG344B. These solvents are commonly used in cosmetic applications and could potentially simplify 2-PE extraction [28,29].

Oleic acid shows a partition coefficient (P) of 6.18 ± 0.01, for a 2-PE concentration within 0–2 g/L, allowing for removing 74 % of the aroma compound to the oleic acid phase, considering the maximum 2-PE concentration tested. PPG 1200, with a P of 21.97 ± 1.82 (experimentally determined), extracted 96 % of the 2-PE present in the aqueous phase, but formed emulsions that complicated the phase separation.

Experiments with *A. soli* ANG344B using oleic acid showed a pH decrease, negatively impacting 2-PE production [4]. Attempts to correct the pH caused saponification, further hindering production. These results indicate a negative influence of oleic acid towards 2-PE production, although other studies showed distinct results depending on the microorganism used. For *Saccharomyces cerevisiae*, oleic acid sometimes enhanced 2-PE production, while in others, it interfered with cell viability and formed stable emulsions [13,16,30,31].

PPG (1200 and 1500) was also reported to be used as extractant solvent for ISPR of 2-PE production processes in other studies, being able to support high 2-PE productions, but emulsion formation remains a challenge [14,31]. Alternative examples like ethyl acetate, biodiesel, rapeseed oil and ionic liquids as extractants have been explored [15,30,32–36], along with the use of hollow fiber membrane contactors preventing direct contact between microorganisms and solvents to avoid emulsion formation and toxicity [37,38]. These alternative approaches aim to improve 2-PE production while minimizing negative effects on the production process.

3.2. Polymeric adsorption

3.2.1. Adsorbent screening

A solid-liquid extraction was also studied in order to develop an ISPR process for 2-PE production by *A. soli* ANG344B. The use of a polymeric adsorbent would avoid the inhibition problems caused by the solvents used in liquid-liquid recovery, also allowing a selective adsorption and recovery of the product. Thus, the adsorption capacity of three polymeric resins (selected by its characteristics detailed in Section 2.3.1) towards 2-PE was evaluated, namely Amberlite XAD 4, Macronet MN 102 and Hytrel 8206.

Amberlite XAD 4 and Macronet MN 102 have a matrix of styrene-divinylbenzene, being the last one functionalized with a tertiary amine and both have a high surface area (Table 1). Although Macronet MN 102 has an ionic matrix, it has a limited ion exchange capacity, being considered as an adsorbent resin. Due to the presence of the aromatic ring and the hydroxyl group, 2-PE can interact through π and/or hydrogen interactions with the matrix of the resin. This aroma compound can interact with the styrene-divinylbenzene matrix through π interactions and the functionalization of the resin with a tertiary amine can also improve the interactions with the hydrophilic group of the molecule. Hytrel 8206 is a block copolymer of polybutylene ester and polyethylene that offers the possibility of 2-PE interaction (through the hydroxyl group) with the polymer by hydrogen bonding due to the ester and ether linkages [9].

To assess which resin could have a better performance for the recovery of 2-PE from the cultivation broth, different dosages of each resin (from 1 % to 20 % for Amberlite XAD 4 and Macronet MN 102 and from 1 % to 30 % for Hytrel 8206) were evaluated, using pure solutions of 2-PE with 2 g/L, the concentration that is usually obtained in a batch production process. The three tested resins have the ability to adsorb 2-PE, being Amberlite XAD 4 and Macronet MN 102 the ones presenting a higher adsorption efficiency (Fig. 3). These resins are capable of adsorbing above 96 % of 2-PE with a resin ratio of 3 % (dry w/v), resulting in an adsorption of 65.2 ± 0.2 and 66.5 ± 0.3 $mg_{2\text{-}PE}/g_{dry\ resin}$, for Amberlite XAD4 and Macronet MN102, respectively. Above this ratio the adsorption efficiency was constant at 98 % for the 2-PE concentration tested. Regarding the resin Hytrel 8206, the adsorption efficiency is lower and seemed to start stabilizing at around 70 % for resin dosages above 25 % (dry w/v), resulting in an adsorption of 7.8 ± 0.6 $mg_{2\text{-}PE}/g_{dry\ resin}$, using a resin dosage of 20 % (dry w/v). Considering these results, 3 % (dry w/v) of Amberlite XAD 4 and Macronet MN 102 and 20 % (dry w/v) of Hytrel 8206 were the selected dosages for the next steps of this work.

As depicted in Fig. 3, the lower dosage of resin (1 % (dry w/v)) resulted in the lowest adsorption efficiency for all the tested resins. These results indicate that 2-PE was not totally adsorbed into the resin, meaning that there was an excess of the aroma compound for the available amount of resin used. When the available aroma is not limiting it is possible to calculate the maximum resin adsorption capacity. The adsorption capacity of each resin was, therefore, estimated under resin-limiting conditions (no limitation of aroma available for adsorption). Amberlite XAD 4 could adsorb 155.0 ± 0.7 $mg_{2\text{-}PE}/g_{dry\ resin}$, Macronet MN 102 could adsorb 171.4 ± 5.9 $mg_{2\text{-}PE}/g_{dry\ resin}$ and Hytrel 8206 could adsorb 24.1 ± 0.4 $mg_{2\text{-}PE}/g_{dry\ resin}$. Hytrel 8206 presented the lowest adsorption capacity, and this might be caused by the large particle size of the resin (> 2 mm), that might offer a lower specific surface for adsorption and/or longer diffusion paths to adsorption sites. Further, the absence of regular pores and the type of uptake mechanism (hydrogen bonding) might be the additional cause for the low adsorption capacity. The results obtained are similar to the ones reported by Shu et al. for an analogous polymer, Hytrel G3548, which allows the same type of interactions with the aroma compound [22]. These authors reported an adsorption capacity of 20.4 $mg_{2\text{-}PE}/g$ with an efficiency of 60.8 %, when using 10 % (w/v) of the adsorbent in a solution of 3.4 g/L of 2-PE. Amberlite XAD 4 presented results comparable with other

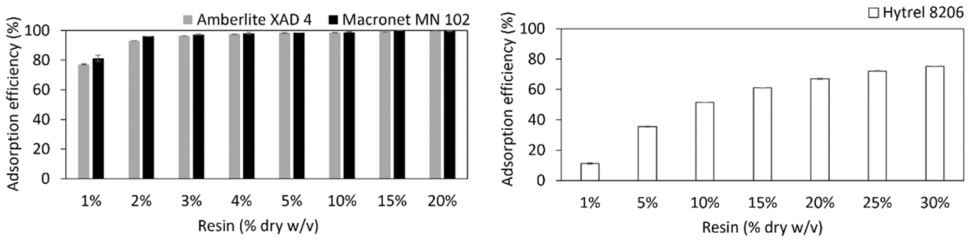

Fig. 3. Adsorption efficiency calculated from experimental results for 2-PE adsorption by Amberlite XAD 4, Macronet MN 102 and Hytrel 8206 at different resin ratios, from a solution of 2 g/L of 2-PE. The error bars represent the standard deviations from independent samples from duplicate experiments.

styrene-based resins, such as D101 and HZ818, that are described as having an adsorption capacity of around 140 $mg_{2\text{-PE}}/g_{resin}$ [19,39].

The adsorption efficiency of each resin (in the selected dosage for each resin) towards L-Phe was also evaluated using a pure solution of 5 g/L of L-Phe, the concentration of precursor that is usually used in the beginning of the cultivation process [4]. In these conditions 35.9 ± 0.1, 38.7 ± 0.7 and 3.2 ± 0.2 % of L-Phe were adsorbed by Amberlite XAD 4, Macronet MN 102 and Hytrel 8206, with an adsorption capacity of 60.6 ± 0.3, 64.2 ± 1.1 and 0.9 ± 0.0 $mg_{L\text{-Phe}}/g_{dry\ resin}$, respectively. These results show that all the resins tested had a lower affinity for L-Phe than for 2-PE, as expected and desirable.

Moreover, the co-adsorption of L-Phe and 2-PE was also evaluated using a binary solution of 2-PE and L-Phe at 2 g/L of each compound (Fig. 4). In these conditions, the adsorption efficiency for 2-PE was similar to the obtained for the single component solution for all the adsorbents tested, not being affected by the presence of L-Phe, having obtained similar adsorption yields: 66.4 ± 0.1, 61.4 ± 0.4 and 7.1 ± 0.0 $mg_{2\text{-PE}}/g_{dry\ resin}$, respectively for Amberlite XAD 4, Macronet MN 102 and Hytrel 8206. Regarding L-Phe, the adsorption efficiency was 29.8 ± 0.3 % for Amberlite XAD 4, 37.6 ± 0.2 % for Macronet MN 102 and 6.1 ± 0.1 % to Hytrel 8206, showing their higher selectivity of all the resins towards 2-PE (Fig. 4). In these conditions, using a binary solution of L-Phe and 2-PE, 20.0 ± 0.0, 25.4 ± 0.1 and 0.6 ± 0.0 $mg_{L\text{-Phe}}/g_{dry\ resin}$ were adsorbed respectively for Amberlite XAD 4, Macronet MN102 and Hytrel 8206. Macronet MN 102 has a hydrophilic functional group, that might contribute to the increase of L-Phe adsorption, without improving the aroma adsorption. All tested the adsorbents exhibited high specificity for 2-PE, while also demonstrating the ability to adsorb L-Phe. In fact, the majority of the polymeric adsorbent resins reported for 2-PE adsorption have also the ability to adsorb L-Phe in lower amounts.

Amberlite XAD 4 was reported by Lukito et al., to adsorb 96 % of 2-PE and 20 % of L-Phe with a resin dosage of 10 % (w/v) from solutions with 50 mM of 2-PE or L-Phe [35]. The results reported by these authors allowed us to determine an adsorption of 59 $mg_{2\text{-PE}}/g_{Amberlite\ XAD\ 4}$ and 17 $mg_{L\text{-Phe}}/g_{AmberliteXAD\ 4}$ that are similar to the obtained in our results. However, it is important to notice that the initial 2-PE and L-Phe concentrations and the resin dosage tested by these authors are different from those used in the present work, as well as the composition of the solution, which can influence product adsorption. Other non-polar resins, HZ818 and D101, were reported to have different capacity for L-Phe adsorption (14 and 80 $mg_{L\text{-Phe}}/g_{resin}$, respectively) despite of similar 2-PE adsorption capacity (140 $mg_{2\text{-PE}}/g_{resin}$), comparable to the obtained in the present work (60.6 ± 0.3 $mg_{L\text{-Phe}}/g_{dry\ resin}$ and 155.0 ± 0.7 $mg_{2\text{-PE}}/g_{dry\ resin}$) [18,19].

Considering an adsorbent functionalized with a tertiary amine, Macronet MN 100 (that is an adsorbent similar to the used in the present work), the maximum adsorption calculated towards 2-PE was 1.5 times higher than for L-Phe, reveling a low 2-PE selectivity [40].

Different from the results obtained in the present work, Gao and Daugulis reported that Hytrel 8206 has no affinity for L-Phe, a difference that can also be related with the environment in which the adsorbent contacts [9].

The adsorption efficiency and adsorption capacity of Amberlite XAD 4 and Macronet MN 102 were similar towards 2-PE. However, Amberlite XAD 4 has a lower adsorption efficiency towards L-Phe, so this adsorbent was selected to perform an ISPR cultivation of *A. soli* ANG344B in shake flasks to access the potential of the adsorbent as *in situ* extractant. Although Hytrel 8206 has a lower adsorption capacity for 2-PE than the other resins, the adsorption of L-Phe is also lower, a feature that can be advantageous for the aroma recovery, thus, this adsorbent was also evaluated on bacterial cultivation.

Bacterial cultivations were performed with the selected dosages of each resin (3 % w_{dry}/v for Amberlite XAD 4 and 20 % w_{dry}/v for Hytrel 8206) and compared to the production without the use of *in situ* extractant. Standard conditions (without *in situ* product adsorption) allowed the production of 2.00 g/L of 2-PE with 2.9 g_{CDW}/L, consuming 6.80 g/L of glucose and at the end of the cultivation 0.7 g/L of L-Phe was still present in the cultivation broth. In the experiments using the adsorbents, 2-PE overall production increased to 2.80 and 3.86 g/L for Hytrel 8206 and Amberlite XAD 4, respectively, with all the L-Phe being consumed, representing an increase in aroma production of 40 and 93 %, respectively. In these conditions, it was possible to achieve a 2-PE production yield from L-Phe of 0.51 and 0.64 $g_{2\text{-PE}}/g_{L\text{-Phe}}$, respectively for Hytrel 8206 and Amberlite XAD 4. Furthermore, in the presence of the resins, 2-PE concentration in aqueous phase decreased to 0.71 and 0.17 g/L, for Hytrel 8206 and Amberlite XAD 4, respectively. The lower 2-PE concentration in the cultivation media led to an increase in cellular growth when using the adsorbent resins (4.8 and 7.4 g_{CDW}/L, respectively for Hytrel 8206 and Amberlite XAD 4) and total glucose consumption in both assays (resins seem to have no affinity for glucose). Similar behavior was reported for *Kluyveromyces marxianus* and *Saccharomyces cerevisiae,* that in the presence of the adsorbents (Hytrel 8206 and D101, respectively), were able to increase cell density, L-Phe consumption and consequently 2-PE production [9,19].

Therefore, based on these results Amberlite XAD 4 was selected for further investigation.

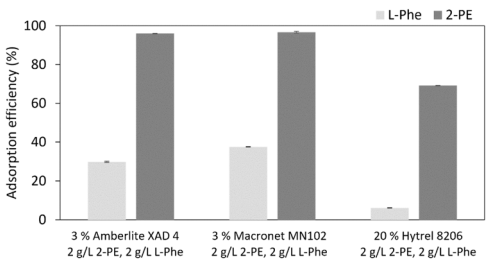

Fig. 4. Adsorption efficiency calculated from experimental results for 2-PE and L-Phe adsorption by Amberlite XAD 4, Macronet MN 102 and Hytrel 8206, from a solution of 2 g/L of 2-PE and 2 g/L of L-Phe. The error bars represent the standard deviations from independent samples from duplicate experiments.

3.2.2. Dynamic adsorption

In order to determine the adsorption capacity, desorption and operating behavior that allow a process scale up, a dynamic adsorption

experiment was performed using Amberlite XAD 4, a single component solution with 1 g/L of 2-PE and a binary solution containing L-Phe and 2-PE with 1 g/L of each component.

The breakthrough curve of 2-PE is represented in Fig. 5a. It was obtained in a fixed bed column of Amberlite XAD 4 and expressed in terms of normalized concentration of 2-PE (ratio of outlet to inlet 2-PE concentration, C/C_0) as function of time.

In the conditions tested, the resin saturation ($C/C_0 = 1$) was achieved at 340 min, that corresponds to 55 BV (bed volume, 1 BV = 61.3 mL). At that time 2.7 ± 0.2 g of 2-PE have been adsorbed, corresponding to a maximum adsorption capacity of 205.8 ± 8.1 $mg_{2\text{-PE}}/g_{dry\ resin}$, higher than the estimated in batch experiments described above (155.0 $mg_{2\text{-PE}}/g_{dry\ reisin}$).

Considering the breakthrough curves for 2-PE and L-Phe in a binary solution, illustrated in Fig. 5b., the adsorption of 2-PE does not seem to be affected by the presence of L-Phe, since the 2-PE breakthrough curve is similar to the one obtained using the single component solution as clearly shown in Fig. 5c by the overlay of both breakthrough curves. L-Phe was detected in the outlet of the column from the beginning of the experiment, reaching the saturation of the resin for this compound after 60 min, corresponding to 10.4 BV. In these conditions, Amberlite XAD 4 adsorbed 21.8 ± 0.0 $mg_{L\text{-Phe}}/g_{dry\ resin}$, that was lower than the estimated in the batch experiments which was limited in the adsorbent available using a single component solution (5 g/L of L-Phe), 60.6 ± 0.3 $mg_{L\text{-Phe}}/g_{dry\ resin}$. The adsorption capacity for 2-PE calculated using a binary solution was 219.2 ± 0.3 $mg_{2\text{-PE}}/g_{dry\ resin}$, which is similar to that obtained using a single component solution in a dynamic adsorption experiment (205.8 ± 8.1 $mg_{2\text{-PE}}/g_{dry\ resin}$). These results suggest that the adsorption of 2-PE remains unaffected by the presence of L-Phe, while the reverse does not hold true.

Based on these results, Amberlite XAD 4 seems to be a good alternative to enhance 2-PE production process, since it has a high adsorption capacity for the product, higher than the reported for other non-polar macroporous resins or copolymers of polybutylene ester and polyether. In fact, HZ818 was reported to adsorb 140 $mg_{2\text{-PE}}/g_{dry\ resin}$, D101 could adsorb 136.2 $mg_{2\text{-PE}}/g_{dry\ resin}$, FD0816 allowed an adsorption of 190.9 $mg_{2\text{-PE}}/g_{dry\ resin}$, Hytrel 8206 resulted in calculated maximum adsorption capacity of 133 $mg_{2\text{-PE}}/g_{dry\ resin}$ and Hytrel G3548 ensured an adsorption of 20.4 $mg_{2\text{-PE}}/g_{dry\ resin}$ [9,18,19,21,22]

For the development of a continuous ISPR production process, the determination of the breakthrough point is crucial. The breakthrough point corresponds to the time at which 5 % of the initial product concentration is reached in the effluent, coming out from the column. At that moment, the column should be replaced to avoid product loss, that passes through the resin and is not completely adsorbed. Considering this, the maximum adsorbent capacity cannot be reached [24,41,42]. Regarding results depicted in Fig. 5a, the breakthrough point is achieved at 190 min, corresponding to 31.0 BV. At that time, 2.1 ± 0.0 g of 2-PE had been adsorbed by Amberlite XAD 4, with an adsorption capacity of 160.2 ± 3.9 $mg_{2\text{-PE}}/g_{dry\ resin}$, which corresponds to 78 % of the maximum capacity of the adsorbent. It is worth note that, considering the adsorption of L-Phe and 2-PE in a binary solution, at the time the breakthrough point for 2-PE was reached (190 min), a low L-Phe adsorption is achieved (21.8 ± 0.0 $mg_{L\text{-Phe}}/g_{dry\ resin}$). However, the breakthrough curves are dependent on the flow rate, the bed length, the initial product concentration and the amount of adsorbent used. Thus, to develop a continuous process, these parameters need to be optimized, although these results offer a good basis for further development [23, 41].

3.2.3. *Product recovery from the resin*

Ethanol 96 % and deionized water were evaluated as eluents for product recovery from Amberlite XAD 4, in batch experiments. For that, Amberlite XAD 4 contacted with a binary solution with 2 g/L of 2-PE and L-Phe or with a single component solution containing 2 g/L of 2-PE for product adsorption and was further eluted with ethanol 96 % or water.

The elution with ethanol 96 % could desorb 100.0 ± 0.1 % of the adsorbed L-Phe and 88.8 ± 0.4 % of the adsorbed 2-PE in only one step of desorption (Fig. 6). The elution with water could desorb 62.5 ± 0.1 % of the adsorbed L-Phe and 2.6 ± 0.0 % of the adsorbed 2-PE (Fig. 6). Similar results were reached for the desorption of 2-PE, adsorbed from a single component solution, that reached a desorption efficiency of 89.6 ± 0.2 % using ethanol 96 % and 2.3 ± 0.0 % using water as eluent. These results show that the presence of L-Phe adsorbed in the resin does not affect the desorption of the aroma. However, using ethanol as eluent, the adsorbed L-Phe will be part of the extract recovered from the resin, since it is completely recovered. Even so, Amberlite XAD 4 has a lower adsorption efficiency for L-Phe and the extract will be mostly composed of 2-PE.

Given the moderate cost of ethanol and its widespread acceptance in industrial applications, particularly in the food and cosmetics sectors, it can be deemed a suitable elution solvent for the recovery of 2-PE from the resin. Therefore, this solvent was selected as eluent in the next steps

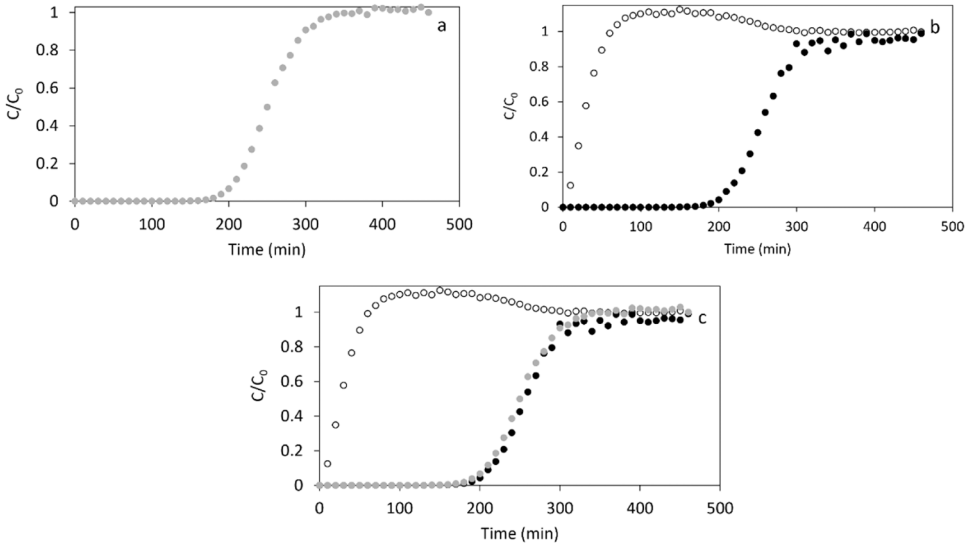

Fig. 5. Breakthrough curve of 2-PE (●) in a single component solution (a), 2-PE (●) and L-Phe (○) in a binary solution (b) on Amberlite XAD 4 at 30 °C and the overlay of the curves (c).

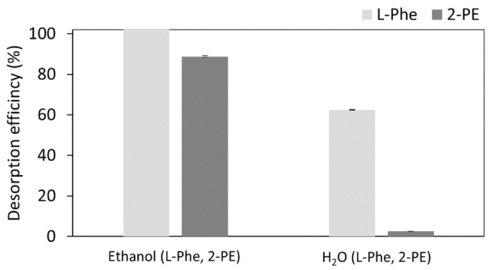

Fig. 6. Desorption efficiency of L-Phe and 2-PE from Amberlite XAD 4 in one desorption cycle using water and ethanol. A previous adsorption step was performed using a binary solution of L-Phe and 2-PE with 2 g/L of each component. The error bars represent the standard deviations from independent samples from duplicate experiments.

of this work.

After the dynamic adsorption experiments (after reaching the saturation point of the resin towards 2-PE), a desorption procedure was performed, aiming to determine the volume of eluent needed for total recovery of 2-PE from the resin. The desorption was performed with ethanol 96 %, at the same flow rate as the adsorption but at room temperature, the results are represented in Fig. 7. A total of 99 % of the adsorbed 2-PE could be recovered from Amberlite XAD 4 after 5 BV (1 BV = 61.3 mL) (Fig. 7a) and this behavior did not change when L-Phe was also adsorbed in the resin (Fig. 7b), as anticipated from the batch desorption experiments. Regarding L-Phe, 35 % of the adsorbed compound was eluted from the resin, during the tested period. The high recovery percentage of 2-PE from the resin further confirm the suitability of this resin to adsorb the 2-PE, since the aroma is not lost in this recovery step.

3.3. 2-PE bioproduction with ISPR using Amberlite XAD 4

Considering the behavior of the selected resin towards 2-PE and L-Phe, an *A. soli* ANG344B batch cultivation in 2 L bioreactor experiment was performed using 3 % (w_{dry}/v) of Amberlite XAD 4 (Fig. 8). The cultivation was performed using 12 g/L of soluble L-Phe, taking into account the adsorption of that compound, considering the adsorption capacity of the resin towards 2-PE (205.8 ± 8.1 $mg_{2\text{-PE}}/g_{dry\ resin}$ determined in dynamic adsorption experiments) and the expected production yield (0.49 ± 0.07 $g_{2\text{-PE}}/g_{L\text{-Phe}}$ [4]), aiming to increase the aroma production.

In these conditions, at the end of the experiment, 14.33 ± 0.11 g of 2-PE (considering the 2-PE in solution and the amount recovered from the resin) was achieved, corresponding to 6.99 ± 0.06 g/L of 2-PE, taking in consideration the total volume of the experiment, with a volumetric productivity of 0.17 ± 0.00 g/L.h. From the total amount of 2-PE produced, 84 % was adsorbed into the resin and the concentration in the aqueous phase was kept around 1 g/L through all the cultivation period. The use of Amberlite XAD 4 as *in situ* product adsorbent avoided the accumulation of 2-PE in toxic concentrations, allowing to reach 8.2 ± 0.0 g/L of biomass (dry weight), that is the double of the obtained in

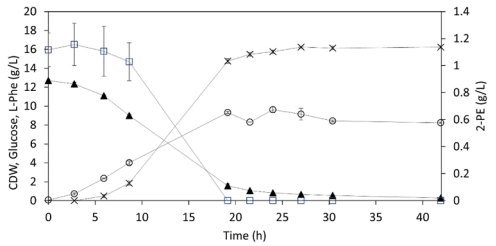

Fig. 8. Cultivation profile of *Acinetobacter soli* ANG344B in 2 L bioreactor using 3 % Amberlite XAD 4 from the beginning of the cultivation, starting with 12 g/L of L-Phe. CDW (○), L-Phe (▲), 2-PE in the broth (x) and glucose (□).

standard conditions (without ISPR). In the presence of the resin, L-Phe was totally consumed, which has not happened in standard conditions due to the loss of cell viability by contact with toxic aroma concentrations [4]. The comparison between standard conditions and the use of the selected ISPR technique are presented in Table 2. With this ISPR approach, the production yield from L-Phe was 0.58 ± 0.01 $g_{2\text{-PE}}/g_{L\text{-Phe}}$, similar to the obtained in standard conditions, as expected, and the volumetric productivity, increased from to 0.12 $g_{2\text{-PE}}$/L.h to 0.17 ± 0.00 g/L.h. However, the volumetric productivity obtained in standard conditions was calculated at 15 h of assay, by the time that the aroma produced started to stabilize. With ISPR approach, the volumetric productivity was calculated at the end of the experiment, since the resin was not removed during sample withdrawal. In fact, the use of this ISPR technique resulted in a faster L-Phe consumption. L-Phe consumption rate using this ISPR approach was 0.54 ± 0.01 g/L.h until 22 h, by the time that the production process started to slow down, suggesting a much higher volumetric productivity of 2-PE for the same period, as expected since *Acinetobacter soli* ANG344B is reported to achieve a 2-PE volumetric productivity among the highest for a wild-type 2-PE producer [3]. In the standard conditions (without ISPR techniques), the L-Phe was consumed at 0.26 ± 0.05 g/L.h during the first 16 h, half than the rate achieved using the resin. These results indicates that the use of Amberlite XAD 4 might have a higher impact in the process than the suggested by the calculated volumetric productivity for the 42 h of the

Table 2
Kinetic parameters calculated for 2-PE production using an ISPR approach with 3 % of Amberlite XAD 4 at 42 h and in standard conditions at 16 h. $2\text{-PE}_{overall}$ is calculated having in consideration the total mass of the aroma (2-PE present in solution and recovered from the resin) and the total volume of media used in the experiment.

	3 % Amberlite XAD 4	Standard conditions [4]
CDW (g/L)	8.2 ± 0.1	4.5 ± 1.0
$L\text{-Phe}_{cons}$ (g/L)	12.12 ± 0.09	4.00 ± 0.74
$2\text{-PE}_{overall}$ (g/L)	6.99 ± 0.06	1.92 ± 0.13
$2\text{-PE}_{in\ solution}$ (g/L)	1.14 ± 0.01	1.92 ± 0.13
$2\text{-PE}_{desorbed}$ (g)	11.98 ± 0.10	-
$Y_{(P/S)}$ (g/g)	0.58 ± 0.00	0.49 ± 0.07
R_p (g/L.h)	0.17 ± 0.00	0.12 ± 0.01

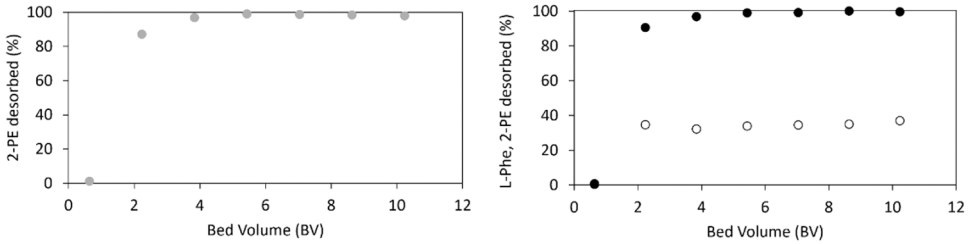

Fig. 7. Desorption of 2-PE (●) (a) and L-Phe (○) and 2-PE (●) (b) from Amberlite XAD 4 represented as percentage of desorbed compound as function of bed volume (1 BV = 61.3 mL) of ethanol 96 % at room temperature.

cultivation. Using this approach, it was possible to increase by 3.3-fold the aroma production when compared to the standard conditions.

The use of ISPR techniques was also reported for 2-PE production by different microorganisms, with 2-PE production improvements. *Kluyveromyces marxianus* cultivation with 100 g of Hytrel 8206, could improve aroma production from 1.45 g/L to 3.82 g/L of 2-PE with a volumetric productivity of 0.10 g/L.h by fed-batch cultivation in a 3 L bioreactor, and this production was further enhanced by increasing the amount of resin in contact with the cultivation broth [9]. *Saccharomyces cerevisiae* R-UV3 could produce 13.7 g/L of 2-PE using 300 g of FD0816 in contact with cultivation broth through filter-cloth bags in a 5 L bioreactor with 3 L working volume, resulting in an increase of 213 % of aroma production [20]. *Saccharomyces cerevisiae* P-3 cultivated with 7 % (w_{dry}/v) of the non-polar macroporous resin HZ818, could increase the aroma production in 66.2 % to 6.6 g/L, in a 1 L flask with 100 mL working volume [39]. The bioconversion in the presence of 2 g of resin D101 allowed the increase in 2-PE production from 4.65 to 6.17 g/L by *Saccharomyces cerevisiae* BD cultivated in a 250 mL flask with 30 mL of working volume [19]. As reported for other microorganisms, the use of a polymeric adsorbent as an ISPR technique resulted in significant improvements in 2-PE production by *A. soli* ANG344B, that achieved a 2-PE production comparable to the titers obtained by yeasts. Hence, the intrinsic advantages offered by bacteria, in terms of shorter growth time and simple metabolism, make *Acinetobacter soli* ANG344B a strong candidate to advance the 2-PE natural production. To the best of our knowledge, this is the highest 2-PE production reported for a wild-type bacteria.

The 2-PE adsorbed into Amberlite XAD 4 during the production process was 203.3 $mg_{2\text{-}PE}/g_{dry\ resin}$, that is very close to the maximum adsorption capacity of the resin (205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$), proving that the production environment does not affect the adsorption. These results, besides representing a great improvement in 2-PE production process by *A. soli* ANG344B, suggest that the process productivity and the amount of 2-PE produced might be further improved by increasing both the L-Phe fed to the cultivation broth and the amount of adsorbent in contact with the broth, paving the way for additional improvement in aroma production. This procedure might be implemented through the use of the resin Amberlite XAD 4 packed in external columns connected to the bioreactor (external loop), through which the cultivation broth, free of cells, is circulated, avoiding blocking of the column. Under these conditions, nutrients and L-Phe must be fed to the bioreactor to assure cell maintenance.

Based on the results described, 2-PE produced during the bioconversion process was recovered from the adsorbent using ethanol. Due to the difference in the boiling points of these two components, ethanol could be evaporated to obtain a highly concentrated 2-PE extract. In the conditions tested it was possible to obtain a 44 mL extract with a 2-PE concentration of 217.56 ± 0.31 g/L and 6.16 ± 1.42 g/L of L-Phe. To avoid the presence of L-Phe in the extract, the addition of a washing step with water before elution with ethanol might be evaluated, since the 2-PE is poorly recovered with water. From the total 2-PE desorbed, only a small percentage of 2.3 % was lost for the evaporated phase. The evaporated ethanol was recovered and can be further used in desorption steps of 2-PE production processes.

GC analysis of this concentrated extract revealed a chromatographic purity of 98 % of the 2-PE in a matrix of ethanol. The chromatographic purity was calculated considering the area of all the peaks in the chromatogram and the area of the 2-PE peak.

4. Conclusion

The use of ISPR techniques aims to improve 2-PE production process by avoiding the accumulation of the aroma in the culture media, which inhibits the producing microorganism. This work studied different approaches for 2-PE recovery from the cultivation broth, based on physical and chemical properties of the product, in cultivation of *Acinetobacter soli* ANG344B. In view of this, gas stripping, liquid-liquid extraction and adsorption approaches were studied, and their implementation assessed in *Acinetobacter soli* ANG344B cultivation, to verify the applicability of each process for enhancement of 2-PE production and its recovery. The gas stripping approach did not improve the process due to the slow volatilization of the product. Liquid-Liquid extraction-based approaches, using oleic acid and PPG 1200 showed that these solvents are not suitable for 2-PE production process by *A. soli* ANG334 due to the negative influence on bacterial growth and the formation of emulsions, respectively. On the other hand, the use of macroporous adsorbents is a promising alternative for improving 2-PE production processes. Amberlite XAD 4 was the adsorbent selected, having high affinity for 2-PE, with an adsorption capacity of 205.8 ± 8.1 $mg_{2\text{-}PE}/g_{dry\ resin}$. The use of 3 % (dry w/v) Amberlite XAD 4 in a batch bioconversion greatly improved the production process, being possible to achieve 6.99 ± 0.06 g/L of 2-PE with a volumetric productivity of 0.17 ± 0.00 g/L.h, compared to the 2.14 ± 0.18 g/L of 2-PE obtained in a conventional process without 2-PE removal, a remarkable result for a wild-type bacteria. During the batch bioconversion process, the maximum adsorption capacity of the resin was achieved with all the L-Phe consumed, suggesting that the process can be further improved by increasing the amount of adsorbent in contact with the cultivation broth and the amount of L-Phe fed to the bioreactor. This study constitutes a useful contribution to the field by assessing three different *in situ* extractive approaches - air stripping, liquid-liquid extraction and adsorption -, discussing the difficulties encountered in their application to the *Acinetobacter soli* ANG344B cultivation. This work also highlights the potential of *Acinetobacter soli* ANG344B as a viable 2-PE producer, paving the way for the development of a robust 2-PE bioproduction process.

CRediT authorship contribution statement

Ana Bernardino: Writing – original draft, Methodology, Investigation, Conceptualization. **Cristiana Torres**: Writing – review & editing, Supervision, Conceptualization. **João Crespo**: Writing – review & editing, Supervision. **Maria Reis**: Writing – review & editing, Supervision.

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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.

Data availability

Data will be made available on request.

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