In-stream product recovery of p-coumaric acid heterologously produced: Implementation of a continuous liquid-liquid extraction assisted by hollow fiber membrane contactor

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Abstract

This work aims to intensify *trans*-*p*-coumaric acid (*p*-CA) heterologous production. *p*-CA exhibits antimicrobial properties, low hydrosolubility, and *retro*-inhibition activity, making its heterologous production limited due to its accumulation in the broth. To overcome these limitations, an in-stream product recovery process (ISPR) is proposed and consists in a liquid-liquid extraction assisted by a hollow fiber membrane contactor. pH, medium composition, and solvent impacts on extraction performances, were investigated prior to the implementation. The coupling of the fermentation and the membrane-assisted extraction was then studied. Although cells were impaired by shear stress with only 15% of viable cells at the end in the extractive fermentation, the final *p*-CA concentration was approximatively 89% of the control one**,** suggesting an intensification of *p*-CA heterologous production if one factors the proportion of viable cells.

Keywords : Trans-p-coumaric acid, Extractive fermentation, In-stream product recovery, Hollow fiber membrane contactor, Liquid-liquid extraction

1. Introduction

trans-*p*-Coumaric acid (*p*-CA, CAS 501–98-4) is a metabolite found in plants and fungi. It is a high added-value molecule, with multiple applications in food, cosmetic and health industries due to its numerous biological activities (i.e., antimicrobial, antioxidant, anti-inflammatory) [1]. Moreover, *p*-CA links the aromatic amino acid (AAA) pathway (the shikimate pathway) to the phenylpropanoid pathway [2], hence it can be the precursor of a wide range of natural molecules with high added-value, accessible through bioconversion (e.g., flavonoids, stilbenoids, coumarins) [3–5].

p-CA can be obtained by chemical synthesis, biomass recovery or engineered microbial production [6]. Although the first two routes are more mature technologies, they still have several drawbacks such as the consumption of substrate from the petrochemical industry, the use of toxic chemicals and the need of large quantities of biomass. Conversely, the production of natural molecules with high added-value by means of engineered microbial cell factories is gaining increasing interest [7–10]. Microorganisms such as *Saccharomyces cerevisiae* (*S. cerevisiae*) are AAA prototrophs. A single deamination of L-tyrosine (Tyr), or a deamination followed by a hydroxylation of L-phenylalanine (Phe), lead to *p*-CA production when genes encoding enzymes able to catalyze these reactions are expressed. Therefore, many studies explored the heterologous production of *p*-CA using microorganisms [4,11–16]. However, *p*- CA accumulation in the broth is limited by (i) its low hydrosolubility [17]; (ii) *retro*-inhibition of the enzymes catalyzing the deamination of L-phenylalanine and L-tyrosine to produce *trans*-cinnamic acid and *p*- CA, respectively, (i.e., L-tyrosine/L-phenylalanine ammonia lyase) by the products [18–20]; (iii) the antimicrobial activity of *p*-CA [1,21] (as microorganisms are the producers of *p*-CA, they could be impaired by *p*- CA accumulation in the broth) and (iv) *p*-CA decarboxylation by endogenous enzymes of *S. cerevisiae* [22]. To address the third limitation, several studies have used *Pseudomonas putida* as a more tolerant *p*- CA producer. However, this strategy only solves one impediment [23,24].

To address all issues at once, a solution consists in *p*-CA continuous extraction from the broth. Several reviews focused on *in situ* or in-stream (also called *ex situ*) product recovery (ISPR) implementation on whole cell bioprocess [25–27]. They highlighted the numerous benefits of ISPR processes such as increased cell growth and higher rates of product formation. *p*-CA hydrophobicity suggests that a liquid–liquid extraction (LLE) with an organic solvent is promising and the most practical ISPR process [25,28]. However, direct contact of the two phases (broth and solvent) allows a dispersion of the phases and hence, complicates the implementation of this process. Mixing, aeration, viscous solvents and surfactants in the fermentation broth promote rapid formation of stable emulsions and foam, which lead ultimately to difficult recovery of fermentation products [29–31]. Moreover, cells may be impaired by direct contact with the solvent at the interface [32]. Assisting LLE with a hollow fiber membrane contactor (HFMC) is one way to overcome these drawbacks (also called membrane-based solvent extraction or pertraction). By means of a hydrophobic membrane and the application of a constant low transmembrane pressure to the aqueous phase, the interface between the fermentation broth and the organic solvent is stabilized. Therefore, there is no direct contact between cells and organic solvent, no phase dispersion and hence, no emulsion formation [33]. Mass transfer occurs by diffusion at pores

mouth which allows a high interfacial area [34]. The use of HFMCs as an interface in a LLE process to reduce the toxicity of solvents and inhibition of fermentation products towards microorganisms has been studied by several authors [35–39]. The work of Jin and Yang in 1998 is a particularly successful and encouraging example, they presented an extractive fermentation process using an amine/oleyl alcohol extractant and a hollow-fiber membrane contactor to selectively remove propionic acid from the fermentation broth. Their process was stable and gave consistent long-term performance over the 1.5-month period studied and obtained a 5-fold increase in productivity [40]. The work in 2015 of Ge et al. is another interesting evidence of long-term stability of those processes. They improved the conversion complex yeast-fermentation beer from the corn kernel-to-ethanol industry into primarily *n*-caproic acid during 550 days using a membrane liquid-liquid extraction to prevent inhibition [41]. However, to the best of our knowledge, no reported study has dealt with the intensification of heterologous production of *p*-CA through coupling of the fermentation with the HFMC-assisted LLE process.

Previous work showed the potential of implementing an ISPR process for the heterologous production of *p*-CA, with respiratory productivities being enhanced by the continuous removal of *p*-CA in biphasic fermentations [22]. Thus, the objective of this work is to implement a continuous LLE process assisted by HFMC associated to fermentation for the heterologous production of *p*-CA using an engineered strain of *S. cerevisiae*. Particular attention should be paid to the medium composition that can impair the high recovery of the product of interest, and to the pH which impacts the fermentation and the LLE. The implementation of HFMC-assisted LLE to improve *p*-CA production was conducted in two steps. First, the impact of pH and medium composition was evaluated on the mass transfer of *p*-CA through the membrane with model solutions using three extractants: oleyl alcohol, *n*-butyl acetate and *n*-hexyl acetate. Then, the implementation of HFMC-assisted LLE to a controlled fermentation batch is presented with an evaluation of the strain viability and *p*-CA productivity compared to a control fermentation batch.

2. Material and Methods

2.1. Chemicals

cis-9-Octadecen-1-ol (oleyl alcohol, OA) (≥80%) was purchased from Merck KGaA, Germany. *n*-Butyl acetate (BA) (≥99%) and isopropanol (≥99%) were purchased from VWR, France. *n*-Hexyl acetate (HA) (≥99%) was purchased form Acros Organics, France. Standard of *trans*-*p*-coumaric acid (≥98%) was purchased from TCI, Belgium and *trans*-ferulic acid (≥98%) was purchased from Sigma-Aldrich, France. KH_2PO_4 was purchased from Fisher Scientific, Belgium and K_2HPO_4 from Alfa Aesar, Germany. CH_3COONa was purchased from Acros Organics, France and CH_3COOH was purchased from VWR, France.

2.2. ABG010: engineered *S. Cerevisiae* strain

An engineered *S. cerevisiae* (AGB010) was provided by Abolis, France for this work. This strain was engineered to produce *de novo p*-CA. Information regarding ABG010 relevant characteristics can be found in previous work [22].

2.3. Model aqueous phase and medium compositions

2.3.1. Model aqueous solutions

Binary solutions consisted in 400 mg/L of *p*-CA in pure water (purified with Elix® system from Merck Millipore, France). Binary solutions initial pH (pHi), if specified, were adjusted with KOH 2 M solution. "Medium solutions" consisted of 20 g/L of D-glucose (anhydrous, 99%, Alfa Aesar), 1 g/L of yeast extract (Fisher Scientific), 1 g/L of $(NH_4)_2SO_4$ (Acros Organics) and either 0.08 M of KH_2PO_4/K_2HPO_4 buffer at pH 6 or 0.08 M CH_3COONa/CH_3COOH buffer at pH 4.5, depending on the desired pH.
"Medium solutions + cells" consisted of end-batch broth. The initial medium is the optimized semi-defined medium described in section 2.3.2, and the solution consisted of 72 h fermented medium with ABG010 as described in part 2.3.2 without continuous extraction. Prior to extraction, *p*-CA concentration was adjusted to 400 mg/L following a quantification through HPLC and then pH was adjusted to 4.5 with KOH 2 M solution and a pH meter.

2.3.2. Real fermentation media

Yeast extract peptone dextrose medium (YEPD) consisted of 20 g/L of peptone (Fisher Scientific), 10 g/L of yeast extract and 20 g/L of D-glucose. This medium was sterilized by autoclaving at 121 °C for 20 min.
The optimized semi-defined medium consisted of 20 g/L of D-glucose (anhydrous, 99%, Alfa Aesar), 1 g/L of yeast extract, 3 g/L of KH_2PO_4, 1 g/L of $(NH_4)_2SO_4$.

2.4. Implementation of the HFMC-assisted LLE

Liquid-liquid extraction were performed using a membrane contactor pilot engineered by Seprosys (La Rochelle, France). It consisted in a hollow fiber membrane contactor: the 2.5x8 X50 Liqui-Cel™ module with hydrophobic polypropylene fibers (details are given in Table 1). Fig. 1 describes the two configurations operated in this work. Configuration I was used with aqueous model solutions and configuration II for fermentation batch coupled with LLE assisted by HFMC.

Table 1: Characteristics of 2.5x8 X50 Liqui-Cel™ membrane contactor (Liqui-Cel Module, Membrana, USA)

Characteristic	Data
Membrane/Plotting material	Polypropylene/Polyethylene
Number of fibers	~9800
Fiber length	146 mm
Porosity	40%
Fiber internal diameter	220 µm
Fiber external diameter	300 µm
Average pore diameter	0.04 µm

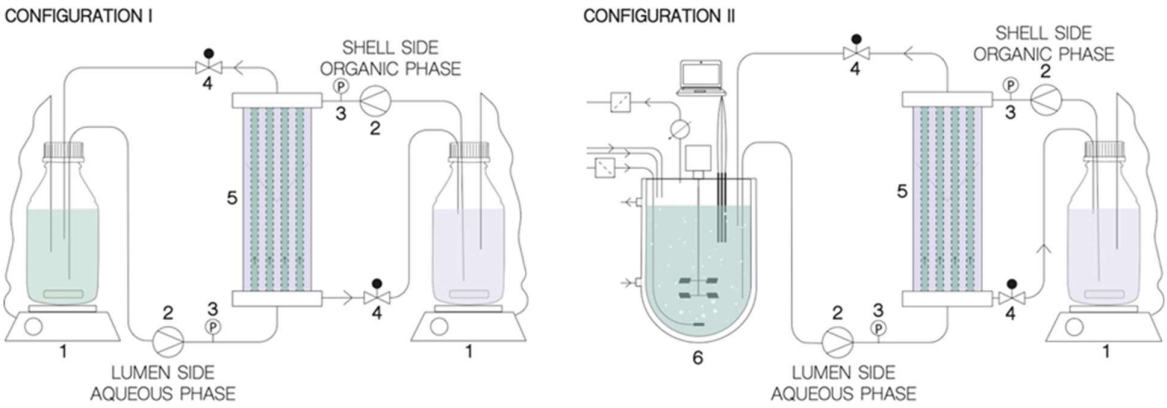

Fig. 1. Schematic experimental setup diagrams. Configuration I: Experiments with model solutions; Configuration II: Final coupling experiment with real fermentation; 1: stirring and heating plate; 2: pump; 3: manometer; 4: pressure valve; 5: HFMC; 6: controlled bioreactor.

2.4.1. Membrane-assisted LLE with aqueous model solutions

Liquid-liquid extractions were operated in crossflow countercurrent mode (Fig. 1). Aqueous phases were pumped through the fiber lumen side while organic phases were pumped through the module shell side. The aqueous phase was pumped first in the system and then the organic phase. The aqueous phase pressure (P_{lumen}) was maintained higher than the organic phase pressure (P_{shell}) to prevent the organic phase (the wetting phase) from permeating. A constant pressure difference (ΔP) between the two phases was kept at 0.5 bar and the corresponding pressure valve was used to this end. This value was fixed through preliminary experiments to determine breakthrough pressure. Flow rates of the two phases were set at around 10 mL/s.

Each phase was maintained at 30 °C and homogenized continuously by using magnetic stirrer (500 rpm) and heating plate. The starting volume for each phase was 1 L. Samples of 1 mL were periodically collected from both phases. In this configuration, only aqueous phases were analyzed for *p*-CA concentration determination. The pH of aqueous phases was measured prior to the extraction and at equilibrium. Experiments were made at least in duplicate.

2.4.2. Coupling of fermentation with HFMC-assisted LLE

In the configuration II, the membrane-assisted LLE was coupled with batch mode fermentation (Fig. 1). Differences with configuration I was primarily on aqueous phase, thus, focus will be on fermentation methodology here. In order to compare the performance of the ISPR configuration with a conventional batch fermentation, a control fermentation (batch mode) was carried out under the same conditions.

For each experiment, the strain was pre-cultured in 50 mL of YEPD in a baffled Erlenmeyer overnight at 30 °C and 180 rpm from an inoculum kept at -80 °C. Bioreactors were inoculated with pre-culture to reach an initial optical density at 620 nm ($OD_{620\ nm}$) of 0.2.

Fermentations were conducted in 1.5 L bioreactors with a PRO-LAB™ controller unit, C-BIO2™ operator and control software from Global Process Concept (GPC, La Rochelle, France). Bioreactors

were equipped with pH and dissolved oxygen (DO_2) probes from Hamilton Company, France. Temperature was regulated at 30 °C. Air was delivered through a nut-sparger in bioreactors and the airflow was maintained at 0.5 L/min. The DO_2 was set up at 30% saturation level and was controlled by stirring at a rate between 350 and 900 rpm. pH was maintained at 6.0 using KOH 1 M or H_2SO_4 0.5 M solutions. The initial volume of medium was 1 L for controls and experiments. Oleyl alcohol (OA) was used as extractant, with an initial volume of 1 L.

In this configuration, 2 mL were collected at least every 2 h in each phase, for 72 h. *p*-CA content was analyzed as described previously [22]. Yeast growth in the fermentation broth was measured by $OD_{620\ nm}$ using a spectrophotometer Cary 60 UV–Vis from Agilent, France. Membrane cells integrity and esterasic activity were analyzed twice a day by flow cytometry.

2.5. Determination of ISPR monitoring parameters

2.5.1. Extraction yield (Yexp)

The extraction yield illustrates the proportion of *p*-CA recovery in the organic phase at equilibrium in percentage and was calculated as follows (equation (1)):

$$Y_{exp} = \frac{[pCA]_{eq}^{org} \times V_{eq}^{org}}{[pCA]_{eq}^{org} \times V_{eq}^{org} + [pCA]_{eq}^{aq} \times V_{eq}^{aq}} \times 100 \quad (1)$$

where $[pCA]_{eq}^{x}$ and V_{eq}^{x} are respectively *p*-CA concentration and volume of the phase x: "org" for organic phase and "aq" for aqueous phase and at equilibrium, determined experimentally.

$[pCA]_{t}^{aq}$ represent the concentration of the acid dissociated and undissociated in the aqueous phase.

2.5.2. Prediction of the extraction yield

A predicted extraction yield (Ypr) can be calculated with the following equation (2) based on pH and so on the proportion of undissociated *p*-CA (details are given in supplementary data):

$$Y_{pr} = \frac{1}{\frac{1}{P} + K_a \times \frac{10^{pH}}{P} + 1} \quad (2)$$

where Ka is the acid dissociation constant, and P is the partition coefficient defined by:

$$P = \frac{[pCA]_{eq}^{org} \times V_{eq}^{org}}{[pCA]_{eq}^{aq-AH} \times V_{eq}^{aq}} \quad (3)$$

In equation (3), the denominator is the amount of undissociated *p*-CA at the equilibrium, and values were obtained from previous work and available in supplementary data [17].

2.5.3. Time needed to extract 63% of extractable p-CA

The extraction characteristic time, τ, was used. Here, it is referred as the time needed to extract 63% of the total *p*-CA extractable fraction [42]. As extraction curve shape correspond to first order linear time-invariant system described by the equation (4):

$$[pCA]^{org}(t) = [pCA]_{eq}^{org} \times (1 - e^{-\frac{t}{\tau}}) \quad (4)$$

where τ corresponds to the time constant and can be graphically determined.
Experiments with binary solutions at pHi of 6.0 did not show same trend curves and thus, τ could not be determined for those experiments.

2.5.4. Determination of total concentrations of solute in extractive fermentations

Total final concentrations of *p*-CA or 4-vinylphenol (4-vp) in extractive fermentations were calculated as follows (equation (5)):

$$Total\ [i]_{final} = \frac{[i]_{72\,h}^{OA} \times V_{72\,h}^{OA}}{V_{72\,h}^{brot}} + [i]_{72\,h}^{broth} \quad (5)$$

where $[i]_{72\,h}^{x}$ is the solute of interest concentration at 72 h in the phase x and $V_{72\,h}^{x}$ is the volume of the phase x ("OA" for oleyl alcohol) at 72 h.

2.5.5. Assessment of the cell viability and esterasic activity using flow cytometry

Membrane cell integrity and esterasic activity were analyzed twice a day by flow cytometry using a double cell staining with propidium iodide (PI) and carboxyfluorescein diacetate (CFDA). This method uses the particularity of PI to fix to DNA after penetrating damaged cell membranes. On the opposite, unaffected cells did not integrate PI, displaying no PI related fluorescence. On the other hand, CFDA penetrates viable cells and becomes fluorescent when cleaved by esterase. Esterases are ubiquitous enzymes that are used here as a marker of cells viability. Detailed methodology is given in previous work [17].

2.6. Statistical analysis

Through each experiment, the data set size was n = 2 and so, mid-range (MR) was used to illustrate statistical dispersion. An analysis of variance (ANOVA) was conducted for Y_{exp} and τ values for each solvent. The achievement of the one-way ANOVA requirements, the normal distribution of the residuals and the homogeneity of variance, were tested by the Shapiro–Wilk's and the Bartlett's tests, respectively. In the cases where statistical significance differences were identified, the dependent variables were compared using Tukey HSD (honestly significant difference) test ($p < 0.05$).

3. Results & discussion

The first part of this study dealt with the effects of pH, medium composition and solvent on LLE assisted by HFMC with model solutions, before the study of the coupling between the fermentation and the process of HFMC-assisted LLE.

3.1. Effects of pH and medium composition on p-CA recovery using different solvents in HFMC

Table 2 presents the extraction yield of *p*-CA (Y_{exp}) and the time necessary to extract 63% of extractable *p*-CA (τ) according to the medium composition, the pH and the solvent used.
The extraction kinetics for the different experiments are presented in Fig. 2.

Table 2: Characteristics and parameters of experiments with model solutions

Entry	Aqueous phase	Solvent [1]	Y_{exp} ± MR (%) [2]	τ ± MR (min) [2,3]
1	**Binary solution pHi 3.7**	**OA**	**90.9±1.3^{a}**	**8.64±0.38^{b}**
2	Medium pH 4.5	OA	90.2±0.2^{a,b}	17.23±0.27^{b}
3	Medium + cells pH 4.5	OA	85.0±2.0^{b}	19.22±1.72^{b}
4	Medium pH 6.0	OA	43.8±0.0^{c}	63.92±6.02^{a}
5	Binary solution pH_i 6.0	OA	5.5±1.6^{d}	N/A
6	**Binary solution pHi 3.7**	**BA**	**96.7±1.1^{a}**	**0.88±0.03^{c}**
7	Medium pH 4.5	BA	94.3±0.7^{b}	1.20±0.02^{b}
8	Medium + cells pH 4.5	BA	92.5±0.4^{b}	1.18±0.02^{b}
9	Medium pH 6.0	BA	61.1±0.2^{c}	2.39±0.02^{a}
10	Binary solution pH_i 6.0	BA	7.3±0.2^{d}	N/A
11	**Binary solution pHi 3.7**	**HA**	**95.5±1.9^{a}**	**0.93±0.09^{c}**
12	Medium pH 4.5	HA	89.8±0.1^{b}	1.57±0.07^{b}
13	Medium + cells pH 4.5	HA	82.4±1.0^{c}	1.63±0.03^{b}
14	Medium pH 6.0	HA	45.0±0.6^{d}	4.24±0.15^{a}
15	Binary solution pH_i 6.0	HA	10.9±0.8^{e}	N/A

[1] OA: oleyl alcohol, BA: butyl acetate, HA: hexyl acetate; [2] Different letters (a, b, c, d) correspond to mean values statistically different within each solvent and parameter assessed by analysis of variance (ANOVA) and Tukey HSD ($p < 0.05$); [3]N/A stands for "Not available".

The highest extraction yields and the fastest extraction kinetics were obtained with the binary solution without adjustment of initial pH for the three solvents studied (Fig. 2 and Table 2).

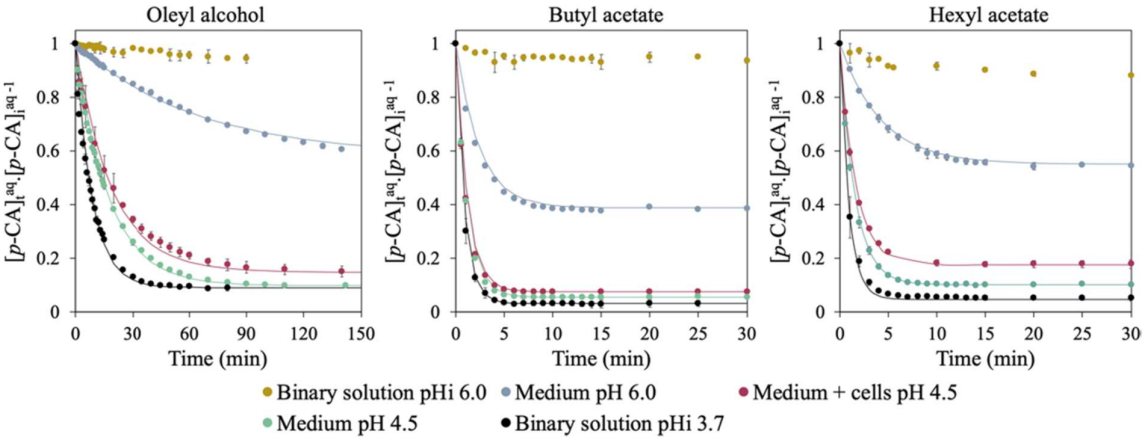

Fig. 2. Dimensionless *p*-CA concentrations in the aqueous phase in function of time through HFMC-assisted LLE with three solvents. Lines correspond to applications of the function in equation (4) for aqueous content of *p*-CA with respective τ.

The increase in pH and the changes in medium composition seem to have a negative impact on the yield and/or the extraction kinetics for each solvent. These observations will be detailed and discussed afterwards as well as differences between the three solvents and their extraction performances.

3.1.1. Impact of pH on p-CA HFMC-assisted LLE

According to Table 2, significantly greater extraction yields (Yexp) were obtained with medium at pH 4.5 compared to medium at pH 6.0 (90.2% vs. 43.8% for OA, 94.3% vs. 61.1% for BA, and 89.8% vs. 45.0% for HA, Table 2, entries 2, 4, 7, 9, 12, and 14). As previously described (Fig. 2), pH more acidic than *p*-CA acid moiety pKa (4.65) favors extraction of *p*-CA in organic phases [17]. At pH < 4.65, *p*-CA is mainly in its undissociated form, which favors its extraction. In addition, *p*-CA at pH 4.5 than in experiment with medium at pH 6.0, as illustrated by τ (Table 2, entries 2, 4, 7, 9, 12 and 14). It is noteworthy to mention that this behavior seems particularly amplified with OA (17.23 min with medium at pH 4.5 vs 63.92 min with medium at pH 6.0, Table 2 entries 2 and 4). As at pH 6.0 the continuous undissociated *p*-CA fraction is lower than at pH 4.5, the driving force throughout the extraction is lower and explains the slower kinetics observed.

Significantly greater Y_{exp} was always obtained in LLE with medium at pH 6.0 as aqueous phase compared to binary solutions with an initial pHi of 6.0 (43.8% vs. 5.5% for OA, 61.1% vs. 7.3% for BA, 45.0% vs. 10.9% for HA, Table 2, entries 4, 5, 9, 10, 14 and 15). Medium solutions without cells were buffered, thus the pH of 6.0 was constant throughout the extraction, and so was the proportion of undissociated *p*-CA unlike in binary solutions where the pH increased during the course of extraction. Thus, the difference in behavior are believed to be pH-related. From this assumption and the hypothesis that only undissociated *p*-CA is extracted by the organic phase, a predicted yield (Y_{pr}) can be calculated with equation (2). Table 3 compares the predicted Y_{pr} and the experimental Y_{exp}.

Table 3: Predicted and experimental extraction yields

Organic phase – aqueous phase	Y_{pr} ± SD (%)	Y_{exp} ± MR (%)	Welch's t-test (p-value)
OA – medium buffered at pH 4.5	94.1 ± 0.2	90.2±0.2	<0.01*
OA – medium buffered at pH 6	53.7 ± 0.9	43.8±0.0	<0.01*
BA – medium buffered at pH 4.5	96.0 ± 0.1	94.3±0.7	0.18
BA – medium buffered at pH 6	63.5 ± 0.6	61.1±0.2	0.02*
HA – medium buffered at pH 4.5	93.3 ± 1.5	89.8±0.1	0.06
HA – medium buffered at pH 6	50.7 ± 5.7	45.0±0.6	0.23

* corresponds to yield mean values statistically different within each raw (Welch's t-test, p-value < 0.05)

For experiments with HA and BA as solvents, Y_{pr} seems consistent with Y_{exp} (p-value > 0.01, Table 3). For these conditions, one can assume that improved Yexp with medium solutions at pH 6.0 compared to binary solutions with a pHi of 6.0 is solely due to pH. However, for OA experiments, Y_{pr} are found

significantly greater than respective Y_{exp}. It will be discussed in section 3.1.3, as lower Y_{exp} could be due to slower extraction kinetics, suggesting that experimental equilibria are not reached.

pH seems to be the major factor impacting extraction performances for the three solvents as it defines the proportion of extractable *p*-CA (undissociated *p*-CA). The most acidic pH allowed the greatest yield, while constant pH (by buffering), allowing a constant proportion of undissociated *p*-CA, increased significantly the final Y_{exp} at pH 6.0.

3.1.2. Effects of cells on *p*-CA extraction in HFMC

As a membrane contactor allows the direct use of the fermentation broth through a LLE without cell separation beforehand, the study of the impact of cells on *p*-CA extraction is essential. According to the data in Table 2, the addition of cells to the medium at pH 4.5 seems to decrease the extraction yield. Indeed, for HA the Y_{exp} obtained with medium at pH 4.5 was significantly greater than the Y_{exp} obtained with cells at the same pH (89.8 ± 0.1% vs. 82.4 ± 1.0%, Table 2, entries 12 and 13). For OA and BA, such an observation was not statistically validated (90.2 ± 0.2% vs. 85.0 ± 2.0% for OA; 94.3 ± 0.7% vs. 92.5 ± 0.4% for BA; Table 2, entries 2, 3, 7 and 8). A decrease in yield by addition of cells could be explained by the fact that the model solutions with cells were not as buffered as media without cells, and therefore, the lower Y_{exp} observed could be due to an increase in pH. As an example, for the LLE with HA, the pH increased through the experiment and reached 5.1 at the end of extraction. A pH of 5.1 gives a Y_{pr} of 86.1% (calculated from equation (2)) and so, closer to the obtained Y_{exp} of 82.4% than Y_{pr} for a medium buffered at pH 4.5: 93.3% (Table 2). The adsorption of *p*-CA on cell membrane could also explain the reduced yields observed. Indeed, the interaction of *p*-CA and other hydroxycinnamic acids with microorganism membrane is believed to be one of the antimicrobial mechanisms of these molecules [21]. It is noteworthy to mention that yields were slightly affected by cell presence, as yields obtained with cells amounted to at least 90% of yields without cells.

τ were equivalents for experiments with medium at pH 4.5 and experiments with medium at pHi 4.5 and cells for each solvent (Table 3, entries 2, 3, 7, 8, 12 and 13). Thus, cells do not seem to impact *p*-CA extraction kinetics in membrane contactors in those experiences. Cells could affect the diffusion of the solute through adsorption to the interface, their production of biosurfactants and the increase of the apparent viscosity of the broth [43,44], however, the cell density and yeast strain used in our work seemed not limiting. The nature of the membrane material and the pilot specificities may also explain those results.

3.1.3. Comparison of extraction capacities of solvents

Fig. 3 presents the kinetics of *p*-CA extraction with the three solvents tested with binary solution without adjustment of the pHi in the aqueous phase.

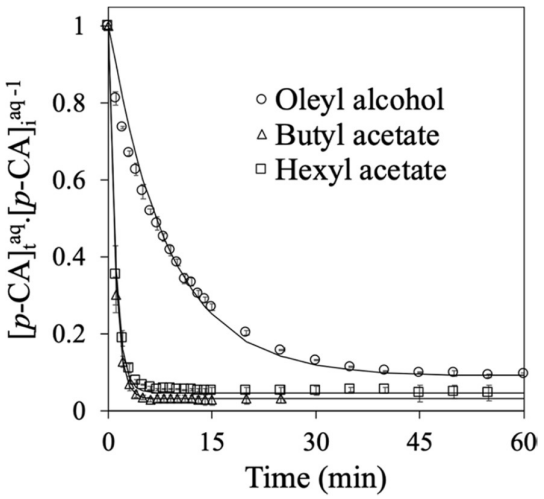

Fig. 3. Kinetics of *p*-CA extraction from binary solution pHi 3.7 with the studied solvents. Lines correspond to equation (4) for aqueous content of *p*-CA with respective τ (Table 3).

The kinetics of *p*-CA extraction obtained with OA was slower than those obtained with BA and HA (Fig. 3). The corresponding τ were 8.64 ± 0.38 min, 0.88 ± 0.03 min, 0.93 ± 0.09 min for OA, BA and HA, respectively (Table 3, entries 1, 6 and 11). As the diffusion rate is inversely proportional to the viscosity of the solvent, the difference in viscosity between the 3 solvents can explain this difference [45]. Indeed, OA viscosity is 28.32 mPa.s at 25 °C [46] while those of BA and HA are 0.677 mPa.s and 1.036 mPa.s at 25 ◦C, respectively [47]. The slower kinetics of OA, especially with a medium at pH 6.0, may explain the significant differences obtained between the experimental extraction and predicted yields in part 3.1.1 as experimental equilibria may not be accurately reached during the experiments.

Y_{exp} on experiment with binary solutions (Table 3, entries 1, 6 and 11) are consistent with previous work as BA gave the best performance of extraction [17]. However, OA is the safest solvent out of the three, and thus, the easiest to implement in the process [17]. For this reason, the fermentation coupled to HFMC-assisted LLE (extractive fermentation) described below was conducted with OA as extractant.

3.2. Extractive fermentation experiments

In a previous work, extractive fermentations were conducted through biphasic fermentations, a dispersive LLE using oleyl alcohol [22], where pH 6.0 gave the best productivities with ABG010 strain. Thus, the fermentations coupled to LLE in HFMC (extractive fermentations) presented here were continuously regulated at this pH. Moreover, since ABG010 productions do not reach hydrosolubility limit nor toxicity limit, the high continuous distribution of *p*-CA in the organic phase due to continuous pH 6.0 is not critical for the process.

Fig. 4A presents the growth of cells and the production of *p*-CA during the extractive and control fermentation and Fig. 4B presents the distribution of *p*-CA in the extractive fermentation between the two phases.

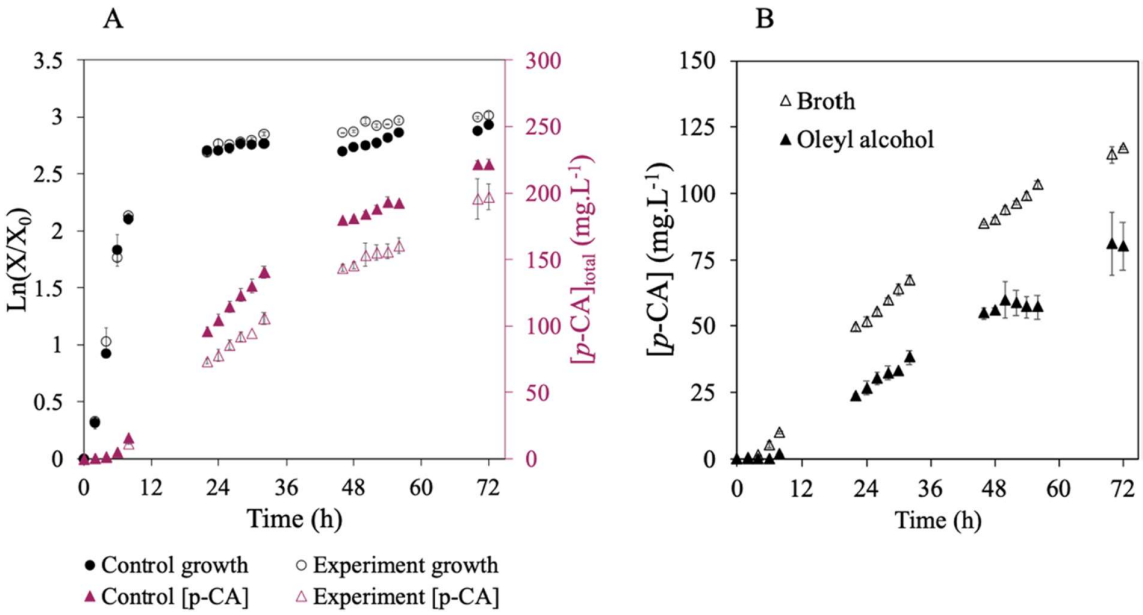

Fig. 4. A: Growths and *p*-CA total concentrations in control and extractive fermentations/experiment ([*p*-CA]aq + [*p*-CA]org). B: Distribution of *p*-CA in the extractive fermentation.

Table 4: Parameters of extractive and control fermentation

Experiment	Fermentative μ_{max} ± MR (h^{-1})	Global *p*-CA productivity ($r_{p\text{-}CA}$) ± MR ($mg.L.h^{-1}$)	Final Y_{exp} of extraction ± MR (%)[1]	Total $[p\text{-}CA]_{final}$ ± MR ($mg.L^{-1}$)	Total $[4\text{-}vp]_{final}$ ± MR ($mg.L^{-1}$)
Extractive fermentation	0.31±0.01	2.7±0.1	40.5±2.6	197.1±9.7	3.0±0.5
Control	0.30±0.01	3.1±0.1	N/A	221.7±3.9	17.5±0.5

[1] N/A stands for "Not available"; [2]Calculation details given in equation (4)

Key parameters calculated from the data are given in Table 4.

According to Fig. 4A and fermentative specific growth (μmax) in Table 4, cell growths appeared equivalent for the extractive fermentation and the control one (μmax = 0.31 ± 0.01 h^{-1} for the extractive fermentation and 0.30 ± 0.01 h^{-1} for the control fermentation).

The cells physiological state was assessed by flow cytometry and results are presented in Fig. 5. Significant differences in cell viability were noticed between the control and the extractive fermentation. Indeed, the extractive fermentation significantly and rapidly impaired cells. After 8 h, 12% of cells in the extractive fermentation were labelled by PI only (cells with damaged membrane), compared to 3% in control fermentation (Fig. 5). The fraction of cells labelled by PI increased over time to reach a maximum value of about 58 to 65% while the maximum of PI-labelled cells in the control was 18%.

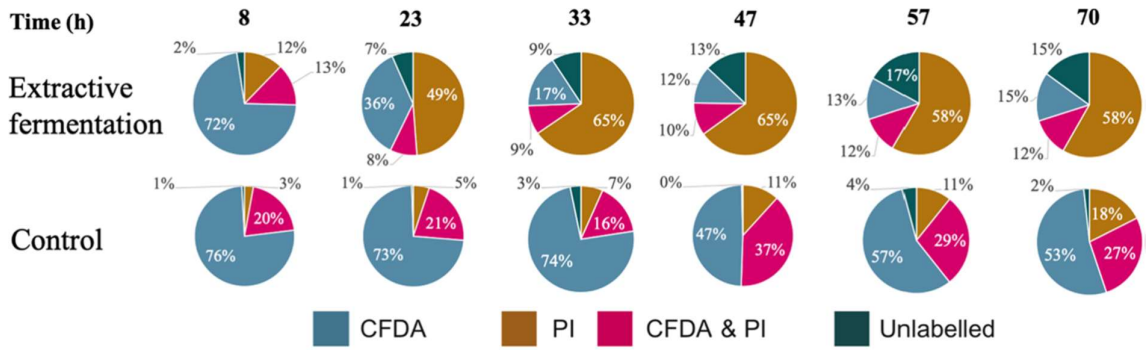

Fig. 5. Flow cytometry and cell labelling results on assessment of their viability in function of time for extractive fermentation and control fermentations. CFDA-labelled cells: good esterasic activity, viable cells; PI-labelled cells: porous membrane, unviable. Percentage are means of n = 2 independent samples.

The observations on CFDA-labelled cells (enzymatically active, viable, cells) are consistent with PI-labelling, as the opposite phenomenon is observed. Indeed, there was about 50% of viable cells at the end in the control fermentation while only 15% of cells were viable at the end of the extractive fermentation. Moreover, numerous cell debris were observed during flow cytometry analysis in the extractive fermentation compared to the control fermentation (data not shown). These observations are believed to be caused by shear stress as the cells pass through a gear pump. Burgé et al. in 2017 investigated a possible shear stress caused using the same system, however they did not find neither loss of membrane integrity nor of esterase activity with their strain (*Lactobacillus reuteri*) after 3 h [35]. Therefore, the impact of shear stress may be strain-specific. The use of a pump with less shear stress could decrease the observed mechanical damage on cells. Moreover, cell immobilization was shown as a safer approach for cells and could be another solution [29,34].

p-CA productivity (r*p*-CA) was lower in the extractive fermentation than in the control fermentation (2.7 ± 0.1 mg/(L.h) vs. 3.1 ± 0.1 mg/(L.h), Table 4 and Fig. 4). At the end, with a significantly lower proportion of viable cells through the process, the final total *p*-CA concentration obtained in the extractive fermentation was approximatively 89% of the control one (197.1 ± 9.7 mg/L of *p*-CA for the extractive fermentation and 221.7 ± 3.9 mg/L of *p*-CA for the control fermentation). It is worth mentioning that, with at least 50% of damaged cells since 23 h in the extractive fermentation, one could have expected a much lower final concentration *p*-CA. These quite unexpected results thus suggest an intensification of *p*-CA production. As the assessment of cells viability was not an online measurement, a specific productivity per viable cells could not be estimated.

The previous Yexp obtained during LLE assisted by HFMC with model solutions (Table 4, entry 4: 43.8 ± 0.0%) is consistent with the final yield obtained here, 40.5 ± 2.6%. Furthermore, this yield is greater than the one previously obtained in biphasic fermentations with OSD medium: 38.5 ± 1.9% [48] thanks to the HFMC. The enhanced mass transfer by the use of HFMC may explain the very low final 4-vinylphenol (4-vp) production at the end of the extractive fermentation (3.0 ± 0.5 mg/L of 4-vp in the extractive fermentation, vs. 17.5 ± 0.5 mg/L in the control fermentation, Table 4). Indeed, 4-vp is the product of *p*-CA decarboxylation through endogenous decarboxylase, and the distribution of *p*-CA in

the organic phase seems to prevent its decarboxylation (Fig. 4B). No fouling seemed to have impacted LLE capacity during the 72 h of fermentation and did not limit *p*-CA diffusion (distribution of *p*- CA presented in Fig. 4B). pH 6.0 seems interesting as it will allow<60% of total *p*-CA produced in the broth. ISPR processes aim to keep the concentration of the inhibiting solute below the critical value, thus it will depend on the strain productivity [49]. Concentrating *p*-CA in OA could be conducted at the end by a shift to acidic pH in order to ease purification step.

4. Conclusion

The implementation of *p*-CA heterologous production with an engineered *S. cerevisiae* with an integrated liquid–liquid extraction (LLE) assisted by a hollow fiber membrane contactor (HFMC) was achieved for the first time. This work showed the importance of pH through the process in LLE for the recovery of ionisable hydrophobic compound. The use of HFMC allowed a higher yield of *p*-CA extraction compared to previous biphasic fermentations and the lowest decarboxylation of *p*-CA. Although flow cytometry analysis revealed major impairment of cells during the extractive fermentation using a HFMC, the final production did not suffer from it and reached nearly 90% of the control one. Thus, if one can ensure cell viability throughout the process (e.g., using a peristaltic pump or cell immobilization), it would lead to an intensification of *p*-CA production.

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