Solvent selection strategy for an ISPR (In Situ/In stream product recovery) process: The case of microbial production of p-coumaric acid coupled with a liquid-liquid extraction

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Solvent selection strategy for an ISPR (*In Situ*/In Stream Product Recovery) process: the case of microbial production of *p*-coumaric acid coupled with a liquid-liquid extraction

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Declaration of interest: none

Abstract

This work reports on a solvent selection for the liquid-liquid extraction of *p*-coumaric acid produced by an engineered strain of *Saccharomyces cerevisiae*. The solvent selection is a key point of liquid-liquid extraction processes and this work describes a simple strategy to choose a suitable solvent for an *in situ* or *in stream* product recovery (ISPR) process during bioconversion. ISPR processes allow to limit the inhibition caused by end-products accumulation in the fermentation medium. The strategy consists in scoring different solvents based on different criteria weighted according to their significance for the process. Extraction performance, solvent biocompatibility and compatibility with materials, were chosen as essential criteria and the first two were assessed experimentally using distribution coefficients and flow cytometry, respectively. Following this first step, three solvents were selected as candidates for the process of interest and ranked according to the process needs using secondary criteria, namely safety, sourcing and price. Finally, oleyl alcohol obtained the highest score and was therefore considered as the most suitable candidate for an ISPR process with the aim of continuously extracting *p*-coumaric acid from the fermentation medium. This work is a first step towards the implementation of integrated extractive bioconversion for the production of bio-based molecules such as *p*-hydroxycinnamic acids and derivatives.

Keywords

Solvent extraction, in situ/in stream product recovery, *p*-hydroxycinnamic acids, distribution coefficient, biocompatibility

1. Introduction

p-Hydroxycinnamic acids (*p*-HCAs) are widely used for their biological properties such as antioxidant [1]–[4], anti-UV [5]–[7], anti-inflammatory [8], [9] and antimicrobial activities [10]–[13]. As a result, they are considered as high value molecules and precursors of other molecules of interest for cosmetic [14], pharmaceutical and food industries. Moreover, recent developments in polymers present *p*-HCAs as building blocks of interest for the plastics industry [15]–[18].

p-HCAs are plant and fungi secondary metabolites. They can be produced by chemical synthesis such as Knoevenagel-Doebner condensation from *p*-hydroxybenzaldehydes [19] or extracted directly from plants using aqueous solution of ethanol, methanol and acetone as extractants [20]–[23]. Nevertheless, those techniques present many drawbacks such as the reagents cost, the availability of natural raw materials at low cost and large quantities, the low content of such molecules in the biomass, the use of hazardous solvents and the production of organic waste. On the other hand, synthetic biology and fermentation technologies showed promising results in producing *p*-HCAs using different simple carbon substrate such as glucose. Hence, there is an upsurge interest for the biotechnological of *p*-HCAs using microorganisms [24], [25].

The viability of biotechnological *p*-HCAs production depends not only on the fermentation step, but also on the development of a clean and efficient downstream process, on its technical feasibility and its economic viability. In this paper, we are interested in the production of *p*-coumaric acid (*p*-hydroxycinnamic acid, *p*-CA), the precursor of other *p*-HCAs (e.g., caffeic acid, ferulic acid, sinapic acid) and of various valuable secondary metabolites, using a modified strain of *Saccharomyces cerevisiae* (*S. cerevisiae*), ABG010 (details in section 2.4.1).

Microbial *p*-HCAs production still faces several challenges. First, *p*-CA and other *p*-HCAs are slightly soluble in water (0.78 $g.L^{-1}$ for ferulic acid and 0.98 $g.L^{-1}$ for caffeic acid, at 25 °C) [26]. As no water solubility value has yet been published for *p*-CA, we determined it experimentally and found 0.838 ±0.003 $g.L^{-1}$ at 30 °C. Their hydrophilic-lipophilic features can be deduced from the partition coefficient, usually measured between octanol and water ($K_{o/w}$) [27]. Databases or estimation software can be used to find or predict $K_{o/w}$ in order to get an idea about the solute nature. For *p*-CA, $Log(K_{o/w})$ is 1.59 when estimated using KOWWIN™ v 1.68, indicating its lipophilicity. As the fermentation medium is an aqueous-based solution, the hydrophobicity of *p*-CA is a production limiting factor. Secondly, as stated above, *p*-HCAs such as *p*-CA have anti-microbial properties that could harshly limit their production by microorganisms. Baranowski *et al.*, 1980, studied the inhibition of a *S. cerevisiae* strain by *p*-HCAs, and observed an increase of the growth lag phase with only 100 $mg.L^{-1}$ *p*-CA in the

culture medium, and a total inhibition at 1 $g.L^{-1}$ of *p*-CA in the culture medium [10]. Herald and Davidson, 1983, studied *p*-HCAs antibacterial activity as well but on different strains: *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) and *Bacillus cereus* (*B. cereus*). They found that *p*-CA caused more than 99.9% inhibition of *E. coli* at 1 $g.L^{-1}$ (at pH 5.0 and after 48 hours), *S. aureus* at 500 $mg.L^{-1}$ (at pH 5.0 and after 48 hours), and *B. cereus* at 500 $mg.L^{-1}$ (at pH 7.0 and after 9 hours) [11].

Continuous removal of a fermentation product using an *in situ* or *in stream* product recovery (ISPR, also called extractive bioconversion) process allows avoiding product retro-inhibition as well as antimicrobial effect of the product if applicable, as demonstrated for several molecules produced by fermentation [28] such as alcohols (ethanol [29], butanol [30], [31]) and organic acids [32] (3-hydroxypropionic acid [33], [34], lactic acid [35]). In the case of biotechnological production of *p*-HCAs, continuous product removal is expected to detoxify the fermentation medium, remove the solubility limitation and enhance the productivity. For hydrophobic molecules such as *p*-HCAs, produced by fermentation and thus in an aqueous medium, liquid-liquid extraction using a water-immiscible solvent seems to be a promising extraction technique [36].

The keystone of a liquid-liquid extraction process is the choice of solvent. It is necessary to establish beforehand the properties that the solvent must have for the process implementation. Some solvent selection strategies for extractive fermentations have been published, using theoretical or experimental data. First, Kollerup and Daugulis, 1985, and then Bruce and Daugulis, presented the first systematic and comprehensive screening strategy based on predicted solvent properties [37], [38]. They, among other things, developed a computer program allowing to screen a large quantity of solvent prior to experimental assessment. Dafoe and Daugulis, 2014, presented a review on extractant selection for direct removal of target molecules from the bioreactor over the last five years, and a part of this work reviews liquid-liquid extraction system using organic solvents and their selection [28]. In this paper, an original and simple score enabling solvent selection is proposed for an indirect removal of bioconversion product. The developed methodology is based on different criteria weighted according to their significance for the process with experimental demonstrations and assessments. This method is adaptable and applicable under process specifications and requires a pre-selection based on literature in order not to assess unreasonable solvents. For example, the first part of Grundtvig *et al.* selection proposition in 2018, regarding tabulated properties, could be used as so and would allow to narrow the list of pre-selected solvents [39].

Some of the criteria are essential for an extractive fermentation using liquid-liquid extraction. The first criterion is solvent biocompatibility towards the microorganism. Two toxicity

mechanisms must be taken into account when using solvent to extract a fermentation product: (i) the contact toxicity, related to the direct contact of the microorganism with the solvent, and (ii) the molecular toxicity caused by solvent saturation of the aqueous fermentation medium (i.e. solvent maximal water solubility) [40]. Several technologies, such as membrane contactors for a membrane-based solvent extraction or two-phase partitioning bioreactor with immobilized cells, allow limiting direct contact between the solvent and the fermentation medium. However, such an approach cannot prevent molecular toxicity. Therefore, the present work will focus only on the molecular toxicity of the tested solvents. This toxicity depends on several characteristics of the solvent, such as its nature, hydrosolubility, polarity and molecular weight. Laane *et al.*, 1985, [41], implied that Log($K_{o/w}$) is a meaningful parameter reflecting solvent polarity that can be used to predict biocompatibility. According to the authors, solvents with a Log($K_{o/w}$) > 4 are more likely to be biocompatible. A low hydrosolubility, a low polarity and a high molecular weight tend to favor a good biocompatibility but solvent chemical nature is also a crucial factor [42]. Moreover, as microorganisms have different solvent tolerances, it is therefore necessary to experimentally assess the solvent biocompatibility with the strain of interest.

The second, but equally important criterion, is the solvent capacity to extract selectively the molecule of interest. For the present application, a water-immiscible solvent is needed. Research on microporous membrane/solvent microextraction (MPMSME), a novel analytical method used for *p*-HCAs, gives a good indication of which water-immiscible solvents could be a good extractant for *p*-CA. Two research teams screened solvents and obtained the best results with long chain alcohols such as 1-heptanol, 1-octanol, and 1-decanol [43], [44]. Other authors found hexyl acetate as the best *p*-HCAs extractant [45]. A good *p*-HCAs extractant will be a polar solvent, protic or aprotic. It can be explained by the presence of the carboxyl group on *p*-HCAs, which confers polarity to these apolar molecules and thus a capacity to form hydrogen bonds. In order to experimentally measure the solvent's capacity to extract selectively the molecule of interest, the partition coefficient also called distribution coefficient (Log(K_D)) is a suitable parameter. As *p*-CA is a phenolic acid that possesses 2 pKa values, pH of the aqueous phase is an important factor for the extraction success. pKa of *p*-CA carboxylic acid function is 4.65 and that of the phenol is 9.92 [46], therefore at pH < 4.65 *p*-CA will be mostly in its undissociated form and less soluble in water, thus the extraction with an organic solvent would be more efficient. On the other hand, fermentation process allows a small window of working pH, depending on the microorganism, in consequence a trade-off should be made between fermentation and extraction processes and must be studied in detail. For example, *S. cerevisiae,* the strain used in this work, is an acidophilic microorganism, with optimal growth at pH 4.5-5 [47]. For these reasons, *p*-CA Log(K_D) will be measured between

solvents and water at four initial different pH (before the addition of *p*-CA): pH 1 in order to show the best case scenario for the extraction (99% of *p*-CA is in its undissociated form [48]), pH 3 to further assess the effect of more acidic pH than *p*-CA pKA, pH 7 as the reference and pH 11.55, conversely, to show the effect of a pH higher than *p*-CA pKa on the extraction (water at pH 11.55 with addition of 400 $mg.L^{-1}$ *p*-CA equate to a pH of 7.09, more information in section 2.2). Assessments at these pHs will show the beginning of the inflection point of the sigmoid function of extraction as function of pH due to deprotonation of *p*-CA carboxylic acid moiety.

The last equally essential criterion is material compatibility. The solvent must be harmless to process equipment. Besides those three criteria, in order to rank solvents, other criteria such as safety, sourcing and price were chosen. These criteria are important but non-essential for the process implementation.

To the best of our knowledge, no work has been published on *p*-HCAs separation from an engineered strain of *S. cerevisiae* fermentation. This paper presents an original strategy to select solvents for an ISPR process to recover *p*-CA produced by an engineered microorganism, considering the aforementioned criterion. The results presented here are potentially relevant to work on other *p*-HCAs due to their similarity in structure and pKas, and this strategy is applicable with adjustments for other extractive bioconversions.

2. Materials & Methods

2.1. Solvent selection score

Beforehand, literature search was made in order to get indications of which solvents are good candidates for *p*-CA extraction so as to reduce the initial solvents number (around fifty articles-books were studied). Based on this literature (presented partially in section 1), 10 solvents were thus selected: 9 good potential candidates, namely 2-methyltetrahydrofuran (99% form Alfa Aesar), ethyl acetate (>99.7% from Sigma-Aldrich), hexyl acetate (99% from Sigma-Aldrich), *n*-butyl acetate (>99% from Alfa Aesar), 1-heptanol (98% from Acros organics), 1-octanol (99% from Acros Organics), 1-decanol (>98% from Alfa Aesar), 1-dodecanol (>99% from TCI) and oleyl alcohol (80-85% from Alfa Aesar). The last solvent selected as a negative control for the extraction capacity was *n*-hexane (>99% from Merck KGaA) as it is an apolar solvent.

The first step was an exclusion step. Essential criteria, namely extraction performance, biocompatibility and material compatibility were assessed in this step. For each essential criterion, the solvent is graded between 0 and 1.

For biocompatibility and material compatibility, solvents get 0 or 1: 0 if the solvent does not meet the expectation and 1 if it does. A grade equal to zero forces the solvent to be removed from the candidates list. In consequence, it will not be studied further. Biocompatibility was determined experimentally (*cf*. section 2.3) and for material compatibility, information was obtained from suppliers. For this work, the equipment consisted in a membrane contactor pilot plant manufactured by Seprosys (La Rochelle, France) with 3M™ Liqui-Cel™ EXF-2.5x8 membrane contactor. The pilot plant materials are stainless steel, polytetrafluoroethylene, polypropylene and polyethylene.

For the extraction performance, grades are between 0 and 1 as a result of experimental distribution coefficient determination (*cf.* section 2.2). Solvents are compared based on results obtained at pH_{exp} 3.66 and the score 1 is fixed with the best solvent on extraction performance. Then, the other solvents grades are expressed as ratio of their $Log(K_D)$ to the best one. As for the first two criteria, a grade equal to zero forces the solvent to be withdrawn from the list of candidates.

The next step consists in the weighting of extraction performance score and not essential criteria depending on their importance for the present application, particularly on process and production requirements. Table 1 presents this second step with the non-essential criteria and the defined weighting, with a maximum total score equal to 50.

Table 1: Weighting of extraction performance and non-essential criteria

Criteria	Implementation of extraction performance	Safety	Sourcing	Price
Weighting	20	15	10	5

For implementation of extraction performances, the results are experimental ones: solvents are compared on *p*-CA distribution results with yeast nitrogen base without amino acids (YNB). As for extraction performances, the best score, here 20, is fixed with the best distribution coefficient obtained. Then, for the other solvents, rates are expressed as percentage regarding the best one. For the non-essential criteria, scores are set based on scientific literature. For measuring solvent safety, Globally Harmonized System of classification and labelling of chemicals (GHS) was used [49]. More details on the method of calculation are presented in experimental supplementary information.

2.2. Extraction performance: *p*-CA distribution coefficient determination in water and in real fermentation medium

The distribution coefficient measurement method used in this work is based on shake-flask method [27]. *p*-CA was purchased from TCI (Tokyo Chemical Industry, purity: ≥ 98.0%).

For distribution coefficient determination, initial pH of water was first adjusted to 1.03, 3.01, 7.01 and 11.56 with solutions of HCl 1 M and KOH 1 M. Solvents were pre-saturated with water and water was also pre-saturated with the respective solvent at the initial pH (pH_i) and 30 °C. It is noteworthy to mention that, for ethyl acetate, butyl acetate and hexyl acetate, experimental results for initial pH of 11.56 could not be accurately obtained due to saponification reaction during the pre-saturation, therefore data are not presented. The pre-saturation step was performed in order to avoid volume bias and for this purpose initial pH was used. This means that initial pH do not correspond to those of water with *p*-CA solubilized inside. Instead, corresponding pH with 400 mg.L^{-1} of *p*-CA solubilized are : 1.01 (for pH_i 1.03), 2.98 (for pH_i 3.01), 3.66 (for pH_i 7.01) and 7.09 (for pH_i 11.56) and thereafter, those pH will be used as they are more representative of the process and referred to as pH_{exp}. The use of buffer was rejected as it could affect the distribution of *p*-CA by a salting-out effect.

All experiments, for each pH_{exp} and each solvent were made in triplicate. 2.00 ±0.03 mg of *p*-CA were added in a Pyrex falcon tube with 5 mL of saturated water at pH_{exp} and 30 °C (to reach an initial concentration of *p*-CA of 400 mg.L^{-1}). Then, 5 mL of solvent was added. The mixture was manually shaken for 2 minutes and then let to settle for a minimum of 3 hours at 30 °C and atmospheric pressure to reach equilibrium. For oleic alcohol experiments, a centrifugation was carried out at 4000 rpm and 30 °C for 10 min in order to perfectly separate both phases.

For the determination of distribution coefficient in real fermentation media (section 3.4), the method was the same but instead of water, sterilized fermentation media were used. YEPD was made of 20 g.L^{-1} yeast extract, 10 g.L^{-1} peptone, 20 g.L^{-1} D-glucose and yeast nitrogen base without amino acids (YNB), purchased from Sigma-Aldrich. For YEPD, pHi was 6.81 and pH_{exp} was 6.50. For YNB, pH_i was 5.40 and pH_{exp} 4.05.

The organic phase was discarded and the resulting *p*-CA concentration in the aqueous phase was measured (see section 2.5). Then, Log(K_D) was calculated as follows (equation 1):

$$\mathrm{Log}(K_D) = \mathrm{Log}\left(\frac{[pCA]_{aq}^{i} - [pCA]_{aq}^{eq}}{[pCA]_{aq}^{eq}}\right) \quad (1)$$

where $[pCA]_{aq}^{i}$ is the initial concentration of *p*-CA in the aqueous phase and $[pCA]_{aq}^{eq}$ is the *p*-CA concentration in the aqueous phase at equilibrium. Concentrations were measured according to analytical method presented in section 2.5.

2.3. Statistical analysis for distribution coefficients

An analysis of variance (ANOVA) was conducted for distribution coefficient results 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 ($p<0.05$).

2.4. Biocompatibility experiments with an engineered *S. cerevisiae* strain (ABG10)

2.4.1. ABG010: Engineered *S. cerevisiae* relevant characteristics

Saccharomyces cerevisiae S288C [50], uracil, tryptophan and leucine auxotrophic was used. This yeast model strain was engineered in order to produce *p*-CA with optimized performances. Towards this ultimate objective, *ARO10* (YDR380W) and *Thi3*(YDL080C) genes are deleted. *ARO4* (NP_009808) and *ARO7* (NP_015385) were amplified by PCR from the genomic DNA of *S. cerevisiae* and mutated to resist to feedback inhibition (FBR: Feed Back Resistance) [51]. *TAL, PAL, C4H* and *Cpr1* were optimized for yeast codon usage bias then synthesized by DC Biosciences, Dundee, UK. Characteristics of the final strain ABG010 are: *MATα, ura3-52, trp1∆63, leu2∆1, GAL2+, LEU2+, aro10Δ0, thi3Δ0, FAT3~MTR2::(ARO4^{fbr}-ARO7^{fbr}-RgTAL)::URA3, NCA3~ASF1::(AtPAL-AtC4H-CrCPR1)::TRP1*. ABG010 was engineered and provided by Abolis, France.

2.4.2. Biocompatibility assessment method

The studied strain, ABG010, producing *p*-CA was maintained at -80 °C in 30% glycerol-YEPD medium. ABG10 was inoculated in a 250 mL baffled flask containing 20 mL of YEPD medium (20 g.L^{-1} yeast extract, 10 g.L^{-1} peptone, 20 g.L^{-1} D-glucose), and cultivated overnight in a Thermo MaxQ 4000 shaker set at 30 °C and 220 rpm.

This culture was used to inoculate two new 250 mL baffled flasks to an initial $OD_{620\ nm}$ of 0.1: one containing 20 mL of YEPD (the control) and one with 20 mL of solvent saturated YEPD. The flasks were grown in the same conditions as described above. Four samples were taken for each flask over time.

For each sample, membrane cells integrity and esterasic activity were analysed by flow cytometry using a double cell staining with propidium iodide (PI) and carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). This method uses the particularity of PI to fix to DNA after penetrating damaged cell membranes. On the opposite, unaffected cells will not integrate PI and will not display any PI related fluorescence. On the other hand, CFDA-SE penetrates viable cells and becomes fluorescent when cleaved by esterase activity. Esterases are ubiquitous enzymes that are used here as a marker of cells viability. Yeasts suspension was diluted to approximately 10^6 cells.mL^{-1} in pH 4 McIlvaine buffer. One millilitre of the cell suspension was stained by adding 10 µL of 1 mg.mL^{-1} commercial PI solution (Sigma-Aldrich) and 10 µL CFDA-SE (Chemchrom V8, Biomérieux) diluted ten times in acetone. After incubation for 10 min at 30 °C, the stained suspension was analysed with a Sysmex CyFlow® Space flow cytometer (Partec, France) equipped with an argon 488 nm laser and four filters: a forward-angle light scatter (FSC), a side-angle light scatter (SSC), both combined with a diode collector, a 536 nm band-pass filter (526 to 546 nm) to collect the green fluorescence of carboxyfluorescein (FL1 channel) and a 670 nm band-pass filter to collect the red fluorescence of PI (FL2 channel) with photomultiplier tubes. Data acquisition, instrument control and data analysis were controlled and performed with FloMax® software (version 2.9, Partec, France).

In addition, optical density at 620 nm was measured for each sample and for the last sample, (at 24 h) *p*-CA production was measured (see section 2.5).

2.5. Analytical methods:

p-CA concentrations in aqueous phase were determined by high-performance liquid chromatography (HPLC) (Thermo scientific, Ultimate 3000) coupled with a diode array detector (DAD) using an accucore aQ C18 column (100x3 mm, Thermo scientific). The injection volume was 2.5 µL, the oven temperature was 48 °C and the flow rate was 0.8 mL.min^{-1}. The elution method was a 10 min gradient with acetonitrile (A) and formic acid 0.1% (B) as mobile phases: 0 min: 2% of A – 98% of B, 3 min: 10% of A – 90% of B, 8 min: 30% A – 70% of B and 9 min: 2% of A – 98% of B. The retention time of *p*-CA was 4.73 min. A *p*-CA calibration curve with 5 points was made using commercial *p*-coumaric acid (≥ 98% from TCI). Samples were diluted (1:1) in acetonitrile and filtered using regenerated cellulose 0.2 µm filters.

3. Results and discussion

Extraction performance and biocompatibility of ten solvents were experimentally assessed. Results are presented in Table 2 and discussed below.

Table 2: Solvent physico-chemical properties and distribution coefficients

Solvent	CAS number	Molecular weight ($g.mol^{-1}$)	Density ($g.cm^{-3}$) at 25 °C	Dynamic viscosity (mPa.s) at 25 °C	Solubility in water ($g.L^{-1}$) at 25 °C	Log ($K_{o/w}$)	Log (K_D) solvent-water of *p*-coumaric acid: mean ± SD*			
							pH_{exp} 1.01	pH_{exp} 2.98	pH_{exp} 3.66	pH_{exp} 7.09
2-MeTHF	96-47-9	86.13	0.849 [7]	0.475** [52]	140*** [53]	1.35	2.22 ± 0.08 a	1.83 ± 0.24 b	0.43 ± 0.14 c	-0.36 ± 0.04 d
Ethyl acetate	141-78-6	88.11	0.895 [54]	0.430 [54]	68.81 [55]	0.86	1.70 ± 0.01 a	1.64 ± 0.04 a,b	1.61 ± 0.02 b	-
Butyl acetate	123-86-4	116.16	0.875 [54]	0.662 [54]	8.330 [55]	1.85	1.65 ± 0.01 a	1.57 ± 0.03 b	1.43 ± 0.01c	-
Hexyl acetate	142-92-7	144.22	0.868 [56]	1.11 [56]	0.5105 [55]	2.83	1.69 ± 0,03 a	1.23 ± 0.06 b	1.23 ± 0.09 b	-
1-heptanol	111-70-6	116.21	0.819 [57]	6.00 [57]	1.797 [55]	2.31	2.07 ± 0.02 a	1.98 ± 0,11 a	1.76 ± 0,05 b	-0.17 ± 0.03 c
1-octanol	111-87-5	130.23	0.822 [57]	7.60 [57]	0.5353 [55]	2.81	1.84 ± 0,07 a	1.88 ± 0.10 a	1.56 ± 0.04 b	-0.32 ± 0.01 c
1-decanol	112-30-1	158.29	0.827 [57]	11.8 [57]	0.03704 [55]	3.79	1.63 ± 0.12 a	1.58 ± 0.17 a	1.47± 0.03 a	-0.69 ± 0.01 b
1-dodecanol	112-53-8	186.34	0.828 [58]	16.1 [59]	0.004286 [55]	4.77	1.16 ± 0.08 b	1.33 ± 0.04 a	1.18 ± 0.03 a	-0.76 ± 0.11 b
Oleyl alcohol	143-28-2	268.49	0.849 [60]	25.3** [61]	Insoluble	7.5	1.24 ± 0.04 b	1.34 ± 0.01 a	1.28 ± 0.01 b	-0.81 ± 0.01 c
Hexane	110-54-3	86.18	0.655 [62]	0.3 [62]	0.0123 [55]	3.29	-	-	-	-

*SD: standard deviation. Different letters (a, b) correspond to mean values statistically different within each row by analysis of variance (ANOVA) and Tukey HSD ($P< 0.05$).

**Calculated from kinematic viscosity ($m^2.s^{-1}$)

*** at 20 °C

3.1. Solvent extraction performances

Distribution coefficients of *p*-CA in five alcohols are presented in Figure 1 and in Table 2.

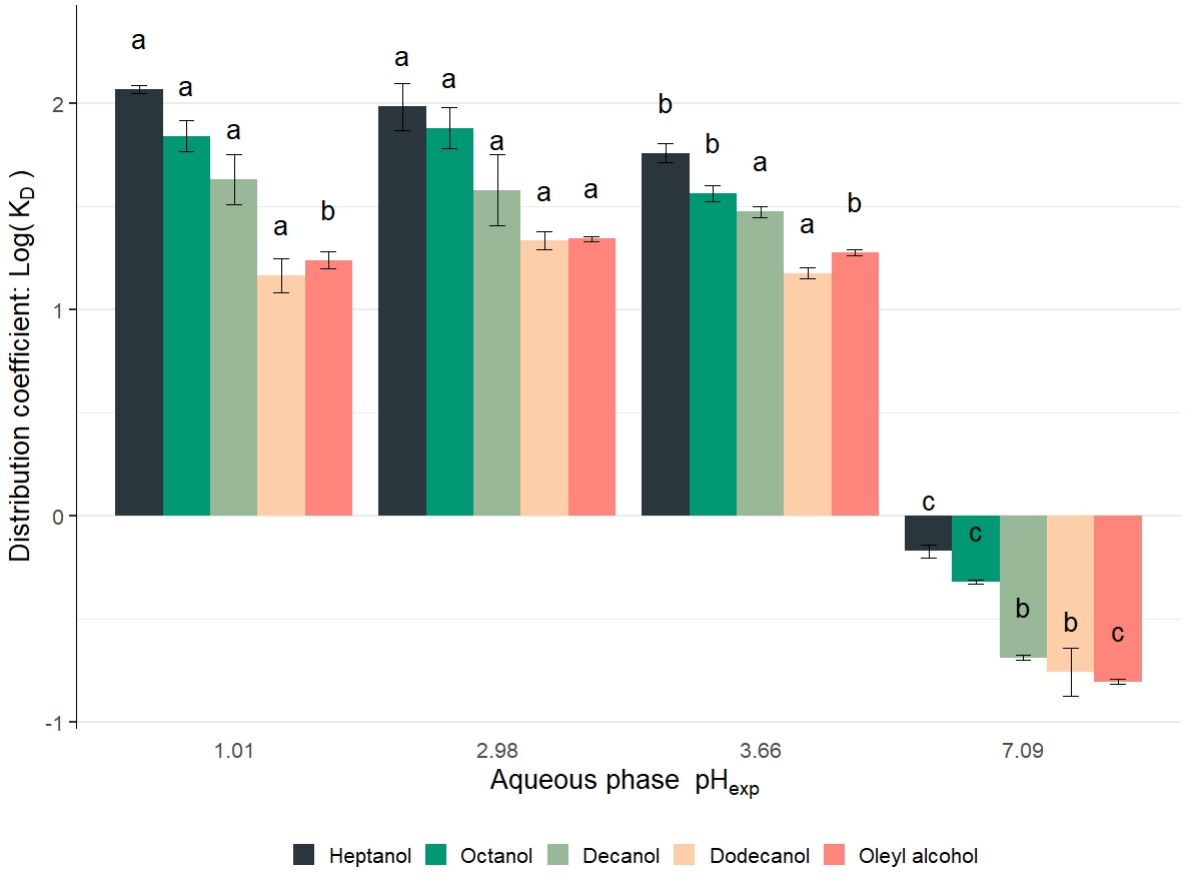

Figure 1: *p*-coumaric acid distribution coefficient between alcohols and water at 30 °C and different pH_{exp}. Different letters correspond to mean values statistically different within each pattern by analysis of variance (ANOVA) and Tukey HSD ($p < 0.05$).

Alcohols are protic solvents, *p*-CA experimental distribution coefficients ($Log(K_D)$) for pH_{exp} lower than *p*-CA pKa (i.e. 4.64) obtained were strictly above 1, which confirms that they are good candidates for the extraction. As expected, acidic pH_{exp} led to higher distribution coefficients due to protonation of *p*-CA carboxylic acid moiety. It must be emphasized that there were no statistically significant differences between pH_{exp} 1.01 and pH_{exp} 2.98 overall and that *p*-CA $Log(K_D)$ at pH_{exp} 3.66 while statistically lower, was still above 1. For pH_{exp} 7.09 *p*-CA $Log(K_D)$ were negatives, meaning that *p*-CA distribution is predominant in the aqueous phase, that can be explained by the large proportion of dissociated *p*-CA at this pH. Broadly, *p*-CA distribution coefficients decreased when pH increased due to the proportion of undissociated *p*-CA, but the trend is relatively weak at pH lower than *p*-CA pKa. It seems that alcohols with longer carbon chains have lower $Log(K_D)$ and the effect of pH on the extraction yield is smoothed (e.g. no significant differences between pH_{exp} 1.01, 2.98 and 3.66 $Log(K_D)$ for 1-decanol). The reduced capacity of solvents to extract *p*-CA with the increase of the carbon chain length may be explained by the difficulty to form the solvation shell around *p*-CA due to

steric hindrance. The smoothing of the pH effect could be a result of the solvent hydrosolubility diminution. This hypothesis is discussed further below in this section.

pH is a parameter influencing not only the extraction performance but also the fermentation process. Thus, it must be considered and discussed. At pH 1.01, *S. cerevisiae* will not grow and at pH 2.98 its growth will be affected as its optimal growth pH is 4.5-5 [47]. As shown in Figure 1, for pH_{exp} lower than *p*-CA pKa, pH does not seem limiting for the extraction performance with protic solvents; whereas for pH_{exp} higher than *p*-CA pKa, a liquid-liquid extraction may not be suitable as an ISPR. If a pH higher than *p*-CA pKa is desired for the fermentation process, a reactive liquid-liquid extraction using tertiary amine may be a good alternative [63], [64]. Here, as *S. cerevisiae* can tolerate pH lower than 4.64 (*p*-CA pKa), pH would be chosen as a compromise between extraction and fermentation needs, for example the process could be conducted at pH 4.5.

Aprotic and polar solvents were tested as extractants, among them three esters (ethyl acetate, butyl acetate, hexyl acetate) and 2-methyltetrahydrofuran (2-MeTHF). Obtained *p*-CA distribution coefficients are presented in Figure 2 and in Table 2.

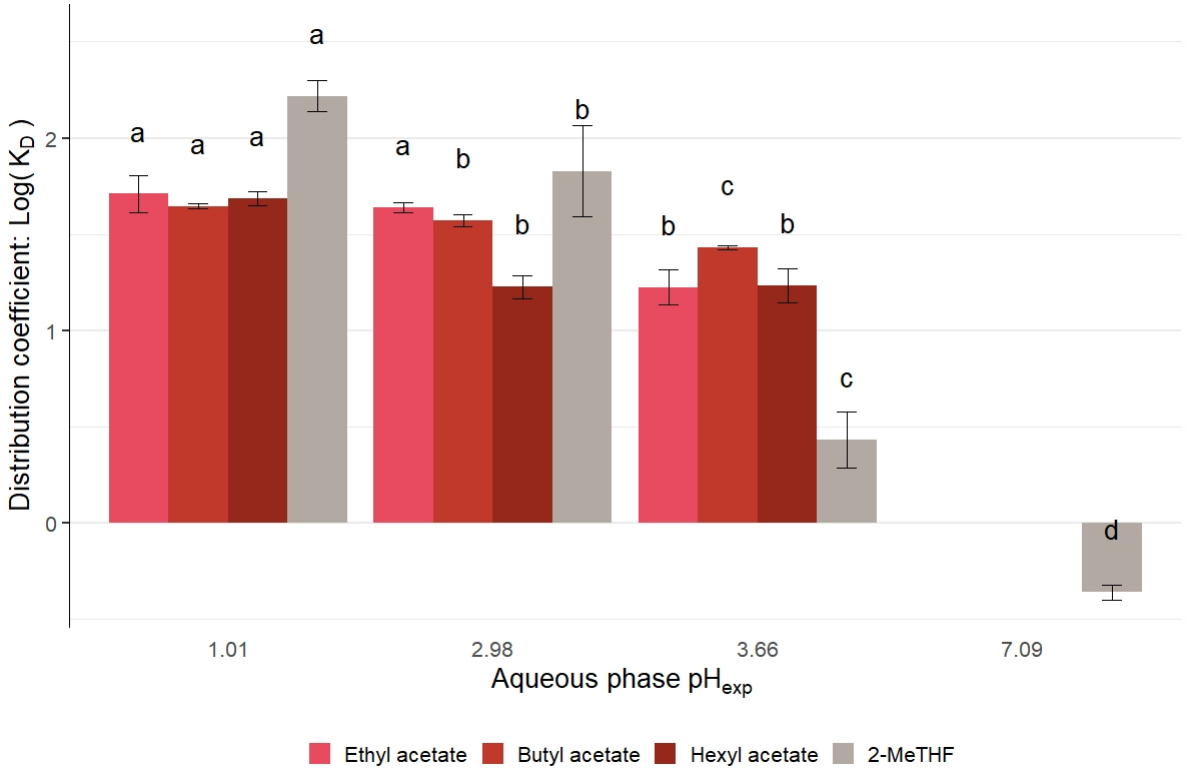

Figure 2: p-CA distribution coefficient between aprotic and polar solvents and water at 30 °C and different pH_{exp}. Different letters correspond to mean values statistically different within each pattern by analysis of variance (ANOVA) and Tukey HSD ($p < 0.05$).

As for the tested alcohols, *p*-CA Log(K_D) of the tested esters, for pH_{exp} more acidic than *p*-CA pKa, are all strictly above 1 and decrease broadly as the pH increases. Solvents such as esters, aprotic and polar, are good candidates for *p*-CA extraction and these results are consistent with the work of Saraji and Mousavi, 2010 [45] presented in section 1. In this case, the length of carbon chain is not associated with smoothing of pH effect since with ethyl acetate, the ester with the shortest carbon chain tested, there is no statistically significant differences between pH_{exp} 1.01 and pH_{exp} 2.98 Log(K_D). Results for esters at pH_{exp} 7.09 could not be obtained due to saponification reaction. This could limit their use, depending on the process.

The last aprotic and polar solvent tested was 2-MeTHF. Experimental results (see Figure 2 and Table 2) showed that this solvent is the best *p*-CA extractant at pH_{exp} 1.01 but on the contrary, the worst at pH_{exp} 3.66 compared to the other tested aprotic solvents. For this solvent, pH has an important effect on *p*-CA distribution, the hydrosolubility of 2-MeTHF may be the reason. The solubility of 2-MeTHF in water at 20 °C is 140 $g.L^{-1}$ (see Table 2), the highest hydrosolubility among the tested solvents. This could increase *p*-CA affinity with the aqueous phase and the differences between the different pH values could be due to different 2-MeTHF hydrosolubility with each tested pH. A high hydrosolubility suggests also a high solvent loss, which could impact the economic viability of the process. These results are consistent with those obtained for long carbon chain alcohols, for which the impact of pH might be limited by the low hydrosolubility of the solvent. Similarly to alcohols, for pH_{exp} higher than *p*-CA pKa, *p*-CA distribution is predominant in the aqueous phase due to the proportion of undissociated *p*-CA

To conclude on aprotic and polar solvent, as discussed for the protic ones, fermentation must be taken into consideration at this step. As observed for alcohols, in the case of esters, for pH_{exp} lower than *p*-CA pKa, pH does not seem to be a limiting factor for the extraction, but for more alkaline pH_{exp}, esters are not good candidates as saponification reaction can occur and this implies a solvent loss and a more problematic implementation of the process. For 2-MeTHF, although there is a high pH effect, at a pH_{exp} of 3.66: the Log(K_D) still higher than 0 and so its score will be higher than 0 (see Table 3). For a process for which acidic pH is not a limit, 2-MeTHF can be a good candidate.

As expected, the last tested solvent, *n*-hexane, was not suitable for *p*-CA extraction due to its apolarity. The determination of Log(K_D) was not feasible due to *p*-CA crystallisation at the water/hexane interface. This phenomenon may be explained by *p*-CA carboxylic acid moiety that imparts a localised polarity to this phenolic and apolar molecule. Indeed *p*-CA needs an organic solvent with a certain polarity to be extracted. *n*-Hexane and water have each an

affinity for a part of the molecule (hexane: aromatic ring, water: carboxylic acid moiety) and this could explain the appearance of this interfacial phenomenon. As a result, *p*-CA accumulates at the interface and when *p*-CA saturation is reached, the accumulation leads to crystallisation of the molecule. Even though this phenomenon is interesting, it raises the question of the implementation of an ISPR using hexane as the extractant phase for the recovery of *p*-CA.

The extraction performance scores are based on *p*-CA Log(K_D) at pH_{exp} 3.66, used as the reference pH and corresponding to a pHi of 7.01. The Table 3 presents the solvent score for this criterion, taking as a reference 1-heptanol, the solvent with the highest *p*-CA Log(K_D) at pH 3.66.

Table 3: Extraction performance score obtained at pH_{exp} 3.66

Solvent	Extraction performance score
1-heptanol	1
1-octanol	0.89
1-decanol	0.84
Butyl acetate	0.81
Oleyl alcohol	0.73
Hexyl acetate	0.70
Ethyl acetate	0.70
1-dodecanol	0.67
2-MeTHF	0.25
n-Hexane	0

Alcohols with a low molecular weight have the best scores, followed by esters and high molecular weight alcohols having similar score, then 2-MeTHF and *n*-hexane with a score of 0. Accordingly, *n*-hexane is the only solvent that will be excluded from the ranking for the first selection step due to its very poor extraction performance results.

The distribution of glucose, substrate of the fermentation process, was assessed with each solvent. It was not extracted by any of them (data not shown).

3.2. Solvent biocompatibility:

In order to determine whether solvents are biocompatible or not toward the strain of interest, the growth behavior of *S. cerevisiae* ABG10 in each solvent saturated cultivation medium was compared to the one obtained in the control medium (without solvent saturation). Strain growth with or without solvent saturation were compared according to four parameters measured after medium inoculation: (1) density of cells (measured by flow cytometry and correlated with optical density at 620 nm), (2) the produced *p*-CA concentration in the supernatant, (3) the percentage of PI labelled cells (membrane damaged cells), and (4) the percentage of CFDA-SE labelled cells (viable, enzymatically active cells). Figure 3 presents an example of growth parameters comparison between the oleyl alcohol saturated aqueous medium and the control medium. In this example, the strain growth as well as the cell viability are similar, which leads to the conclusion that oleyl alcohol is biocompatible towards *S. cerevisiae* ABG10.

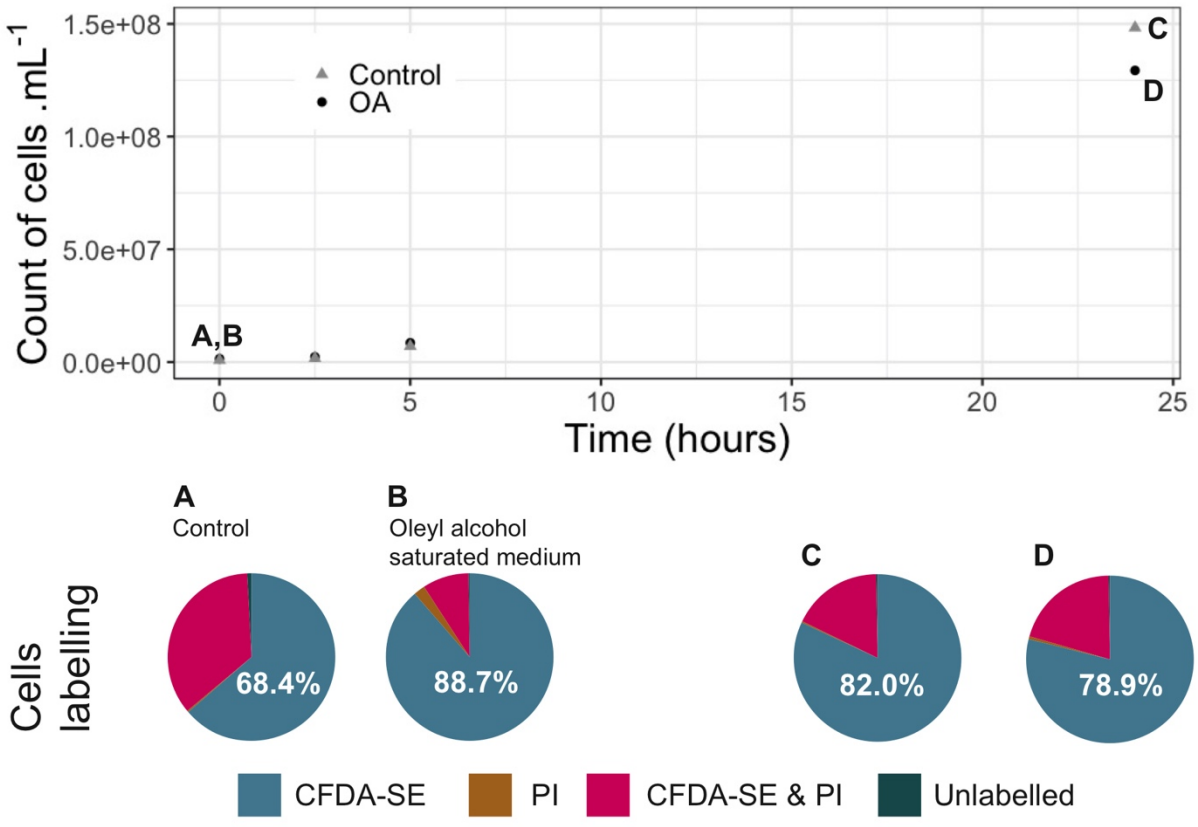

Figure 3: Oleyl alcohol (OA) biocompatibility assessment using cells labelling and flow cytometry. Numbers on the pie chart indicate the percentage of enzymatically active cells.

On the opposite, growth parameters presented on Figure 4 show that no growth occurs in 2-MeTHF saturated medium (cells density remains stable over 24h) compared to the control. There is a high proportion of PI-labelled cells in the presence of 2-MeTHF directly after inoculation, which reveals the high toxicity of this solvent to the strain in this cultivation configuration.

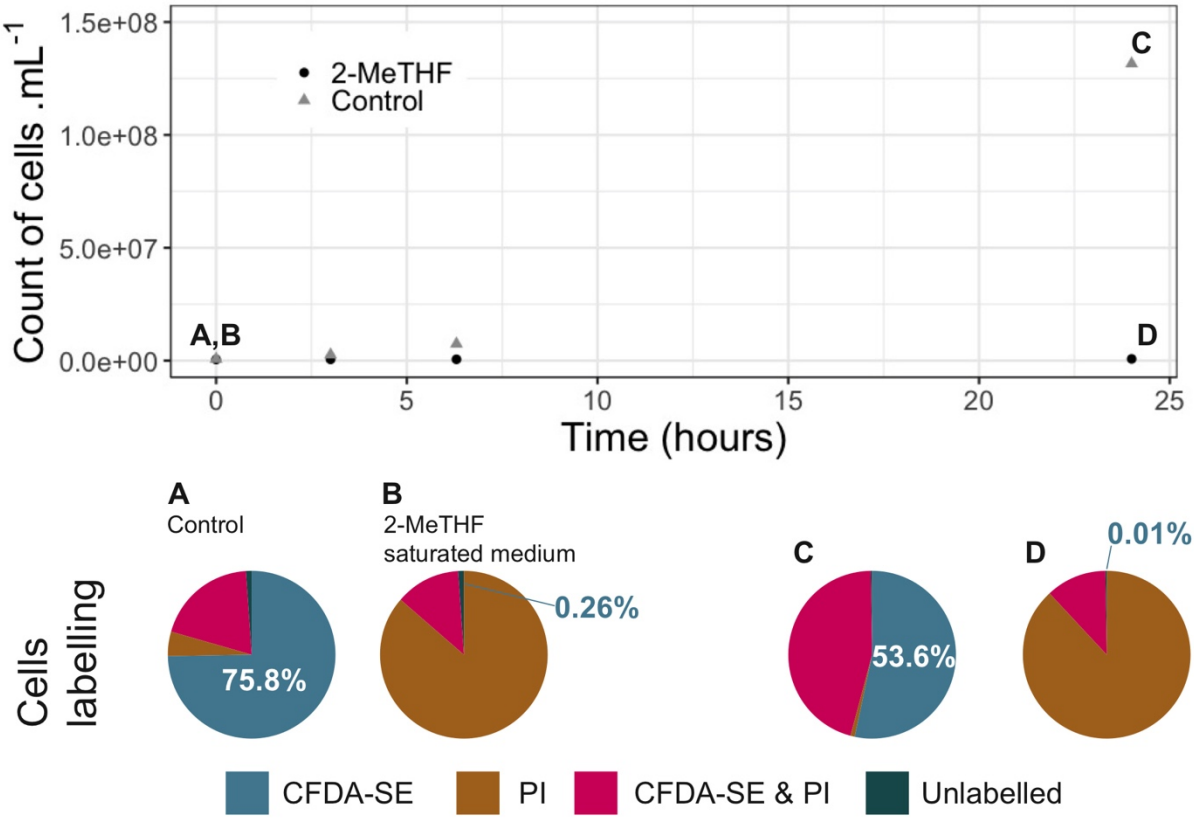

Figure 4: 2-methyltetrahydrofuran (2-MeTHF) biocompatibility assessment using cells labelling and flow cytometry. Numbers on the pie chart indicate the percentage of enzymatically active cells.

These kinetics have been performed for each solvent and, for the sake of clarity and concision, the 24 hours results of each solvent are presented and compared in four bar charts in Figure 5. In those charts, the differences towards the related control are reported. Each chart presents one of the four compared parameters: (a) *p*-CA production, (b) cell density, (c) percentage of viable cells, and (d) percentage of non-viable cells. Roughly, bars close to 0 indicate non-significant difference with the control and reflect the biocompatibility of the solvent.

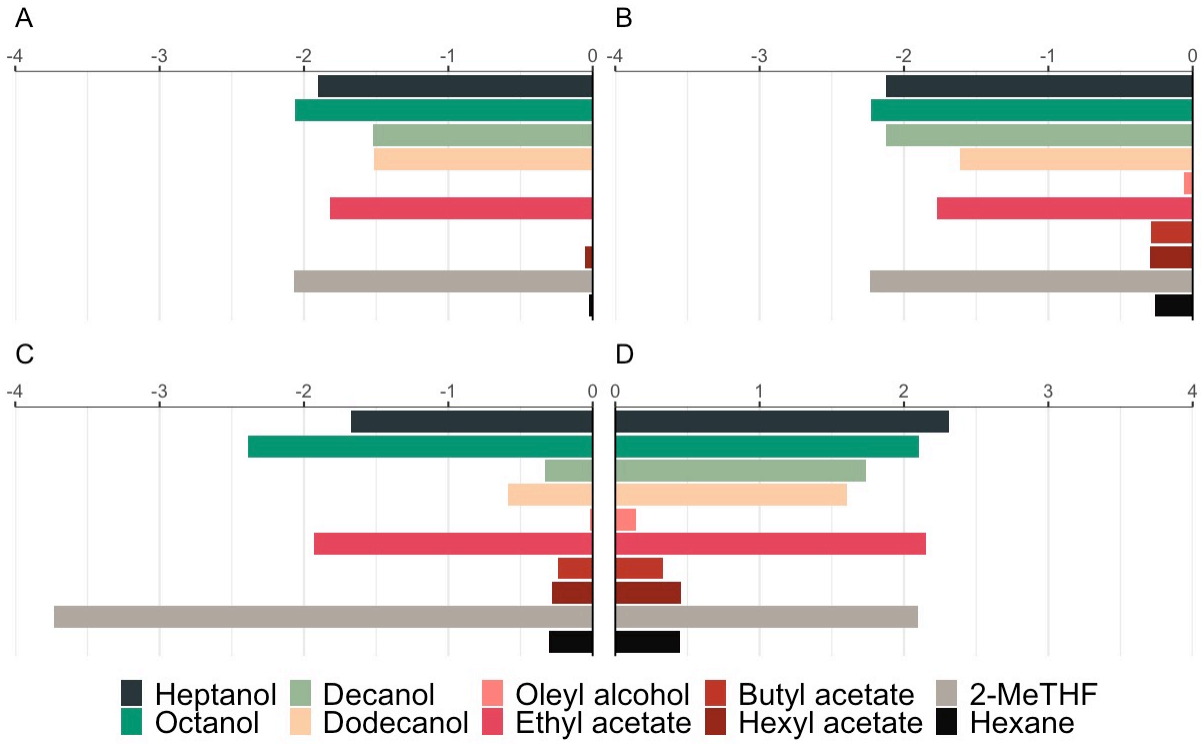

Figure 5: Results of solvents biocompatibility with the strain of interest.

A) Comparison of *p*-coumaric acid production after 24 h in solvent saturated YEPD with control: Log($[p\text{-CA}]_{experiment}$)-Log($[p\text{-CA}]_{control}$); B) Comparison of cell density after 24 h in solvent saturated YEPD with control: Log($count/mL_{experiment}$)-Log($count/mL_{control}$); C) Comparison of viable cells percentage after 24 h in solvent saturated YEPD with control: Log(%CFDA $labelled_{experiment}$)-Log(%CFDA $labelled_{control}$); D) Comparison of non-viable cells percentage after 24 h in solvent saturated YEPD with control: Log(%PI $labelled_{experiment}$)-Log(%PI $labelled_{control}$).

The results obtained are consistent with a toxicity towards the strain of interest. Indeed, the conditions leading to low biomass production were also the ones exhibiting lower *p*-CA production in comparison with the control fermentation, as well as higher PI labelled cells (non-viable). As for the CFDA labelling, toxicity is not as obvious as for PI labelling if only chart C from Figure 5 is considered. This can be explained by the nature of results format, labelling are presented as percentage of cells population and so CFDA-SE labelling has to be compared with cell density and PI labelling. The percentage of viable cells will attest of the biocompatibility of a solvent solely if the density of cells and the percentage of PI labelled cells are similar to the control.

Among the ten assessed, four solvents were found biocompatible with the strain of interest: *n*-hexane, oleyl alcohol, butyl acetate and hexyl acetate. Contrary to the results obtained in this

work, Minier and Goma, 1982 [65], found dodecanol as a biocompatible solvent in an extractive fermentation with *S. cerevisiae* UG5, however *S. cerevisiae* ABG10 seems highly sensitive to this solvent. Likewise, in this work, *n*-Hexane was found biocompatible whereas Kollerup and Daugulis, 1985 [37], found it toxic toward *S. cerevisiae* NCYC716. In contrast, oleyl alcohol is a well-known and widely used biocompatible solvent for different strains [66]–[68], which is in accordance with this study. These results highlight that solvent biocompatibility is strain dependent and must be assessed experimentally.

As stated in section 1, it can be observed that there is broadly a trend towards biocompatibility increasing with solvent Log($K_{o/w}$) (see in Table 2) but Log($K_{o/w}$) has limits as a biocompatibility indicator. 1-Decanol and 1-dodecanol have a Log($K_{o/w}$) close to 4 but in Figure 5 it can be seen that they have a significant negative impact on both the strain development and production. The solvent chemical nature seems to have an important role in the solvent toxicity. Butyl acetate and hexyl acetate esters Log($K_{o/w}$) are 1.85, 2.83, respectively, lower than those of 1-decanol and 1-dodecanol, though the strain production is not impacted when growing in a saturated media with these solvents. Furthermore, the effect on the growth and viability of the cells seems negligible.

Organic solvents tend to increase microorganism membrane fluidity. The amount of solvent solubilised in the fermentation medium is a key factor, and as demonstrated by Osborne *et al.*, 1990 [69], it may be the solvent volume in the membrane that is critical, and when it is reached, it leads to loss of bioactivity. Solvents with high hydrosolubility reach more easily the critical threshold leading to complete loss of activity. Furthermore, it is dependent on solvent chemical nature, and its distribution between the membrane and the fermentation medium. This can explain the differences observed between the different class of solvent and between strains.

Therefore, at this step, only four solvents get a score of 1 for biocompatibility criterion, namely hexane, oleyl alcohol, butyl acetate and hexyl acetate.

3.3. Outcome for the exclusion step for the solvent selection

Table 4 presents solvents ranking for the first step of the selection strategy.

Table 4: Exclusion step scores

	Extraction performance	Biocompatibility	Material compatibility	Total
Butyl acetate	**0.81**	**1**	**1**	**2.81**
Oleyl alcohol	**0.73**	**1**	**1**	**2.73**
Hexyl acetate	**0.70**	**1**	**1**	**2.70**
n-Hexane	0	1	1	2
1-heptanol	1	0	1	2
1-octanol	0.89	0	1	1.89
1-decanol	0.84	0	1	1.84
Ethyl acetate	0.70	0	1	1.70
1-dodecanol	0.67	0	1	1.67
2-MeTHF	0.25	0	0	0.25

After this step of exclusion, only three solvents among the ten initially studied are still in the candidates list (see in Table 4 the ones with a total score strictly higher than 2, in bold text).

3.4. Assessment of the extraction performances in fermentation media

p-CA distribution coefficient was measured between the three previously selected solvents and two fermentation media in order to go further in the assessment and process implementation. One complex medium, YEPD and one defined synthetic medium, YNB were assessed. Figure 6 presents the *p*-CA distribution coefficients obtained.

These results are used in the second part of the strategy to rank the selected solvents in the next section.

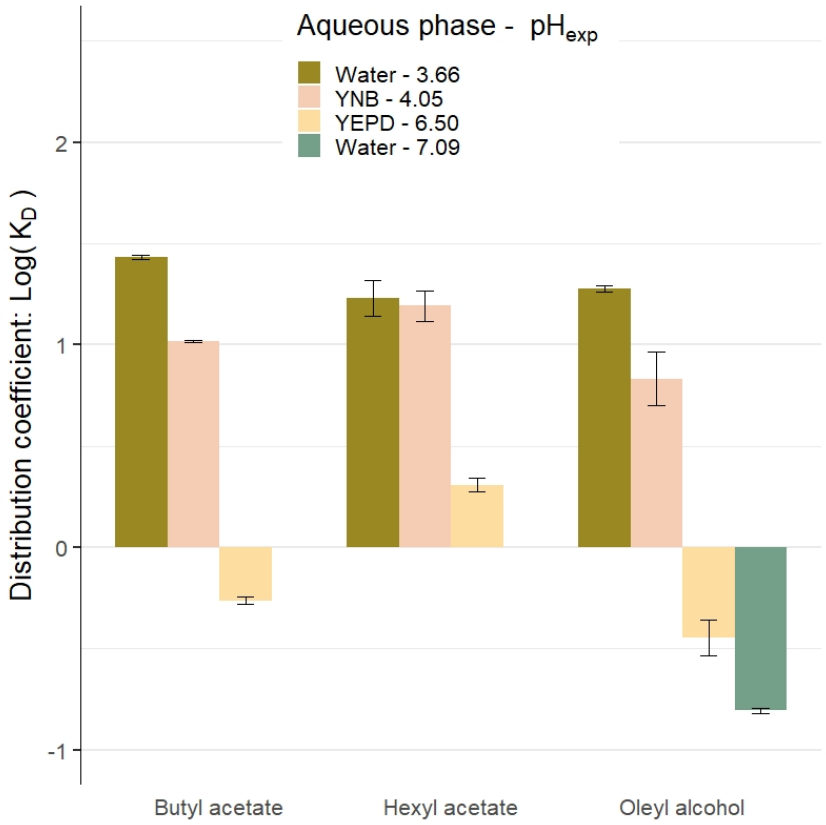

Figure 6: *p*-CA distribution coefficients at 30 °C with two different fermentation media (YEPD: complex medium, pH_{exp} = 6.50; YNB: defined synthetic medium, pH_{exp} = 4.05). Log(K_D) values for pH_{exp} 3.66 and pH_{exp} 7.09 aqueous phase are shown for comparison purposes.

The *p*-CA Log(K_D) obtained are consistent with the observation made in section 3.1 regarding the pH: *p*-CA distribution is higher at acidic pH and especially at pH lower than *p*-CA pKa (4.64) due to the proportion of undissociated acid. YEPD is a strongly buffered medium as pH_i (6.81) and pH_{exp} (6.50) illustrate, whereas YNB has a lower buffering capacity. This characteristic of YNB can explain partially the good capacity of extraction of solvents with this medium, even if pH_i were not the same at first. The assessment of *p*-CA distribution between oleyl alcohol and YEPD was very arduous due to a strong emulsion and showed the limits of a complex medium for the process implementation.

As assumed by Zhang et Greasham, 1999 [70], these data indicate that the less complex the fermentation medium is, the easier the product recovery, this would be the case of *p*-CA recovery. In biotechnological production there is always a compromise to make between the fermentation process and the downstream process to achieve the highest production and working on the definition of an optimized and better-defined medium is one of the potential strategies. Medium complexity serves the interests of the fermentation, by providing biosynthetic precursors to the strain while allowing energy savings. Those media are

inexpensive ones, and this explains their large industrial use. Nevertheless, with the increase of research in fermentation biotechnology, there is a growing interest in defined medium, allowing replication and simpler product recovery.

3.5. Final scores

The final scoring step for the solvent selection is presented in Table 5.

Table 5: Final solvent classification

	Implementation of extraction performances (/20)	Safety (/15)	Sourcing (/10)	Price (/5)	Total (/50)
Oleyl alcohol	14	15	10	5	**44**
Butyl acetate	17	10	8	5	**40**
Hexyl acetate	20	12	4	3	**39**

For the implementation of extraction performance criteria, a score of 20 was given to hexyl acetate, having the highest distribution coefficient for *p*-CA extraction when using YNB medium (Figure 6). The score of the two other solvents were obtained as a function of the hexyl acetate score and their corresponding distribution coefficient (*cf.* section 2.1). Safety and sourcing scores were given using the following information: oleyl alcohol is considered as the safest of the three solvent candidates [49] and is produced from animal and vegetable fats and oils. Butyl acetate can be bio-sourced from *n*-butanol and acetic acid, two chemicals that can be produced by fermentation. Hexyl acetate is produced from acetic acid and 1-hexanol. Nevertheless, 1-hexanol is synthesized generally from ethylene, which is mostly produced by steam cracking of hydrocarbons, so petro-sourced. Price score was given following suppliers information. According to the total score (Table 5), the most suitable solvent for an ISPR process to continuously extract *p*-CA from a fermentation medium having the YNB characteristics, is the oleyl alcohol followed by butyl acetate and then hexyl acetate.

4. Conclusion

The reported strategy allows a quick, simple and adaptable selection of solvents for an ISPR coupled with a fermentation process limited by end-product accumulation. Methodologies to

assess the solvent capacity of recovery and its compatibility with microorganisms are described and can easily be implemented for a similar process. If the molecule chemical nature is different than that of *p*-CA, adjustment must be made and the pre-selected list of solvent should be adapted. As for the strain, the implementation of biocompatible assessment has to be adjusted to strain properties (i.e. temperature, medium, pH). Towards the ISPR of *p*-CA produced by an engineered strain of *S. cerevisiae* using liquid-liquid extraction, three solvents can be used when working with an acidic aqueous phase, by preference order: oleyl alcohol, butyl acetate and hexyl acetate. In order to achieve the set-up of an effective downstream process, it has been highlighted that a trade-off between liquid-liquid extraction and fermentation regarding the pH must be made and definition and optimization of fermentation medium can be necessary.

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References

[1] E. Graf, « Antioxidant potential of ferulic acid », *Free Radic. Biol. Med.*, vol. 13, n° 4, p. 435-448, oct. 1992.

[2] H. Kikuzaki, M. Hisamoto, K. Hirose, K. Akiyama, et H. Taniguchi, « Antioxidant Properties of Ferulic Acid and Its Related Compounds », *J. Agric. Food Chem.*, vol. 50, n° 7, p. 2161-2168, mars 2002, doi: 10.1021/jf011348w.

[3] I. Kiliç et Y. Yeşiloğlu, « Spectroscopic studies on the antioxidant activity of p-coumaric acid », *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.*, vol. 115, p. 719-724, nov. 2013, doi: 10.1016/j.saa.2013.06.110.

[4] S. Mathew, T. E. Abraham, et Z. A. Zakaria, « Reactivity of phenolic compounds towards free radicals under in vitro conditions », *J. Food Sci. Technol.*, vol. 52, n° 9, p. 5790-5798, sept. 2015, doi: 10.1007/s13197-014-1704-0.

[5] A. Saija *et al.*, « In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents », *Int. J. Pharm.*, vol. 199, n° 1, p. 39-47, avr. 2000.

[6] M. Larrosa, M. Lodovici, L. Morbidelli, et P. Dolara, « Hydrocaffeic and p-coumaric acids, natural phenolic compounds, inhibit UV-B damage in WKD human conjunctival cells in vitro and rabbit eye in vivo », *Free Radic. Res.*, vol. 42, n° 10, p. 903-910, janv. 2008, doi: 10.1080/10715760802510077.

[7] J. C. Dean, R. Kusaka, P. S. Walsh, F. Allais, et T. S. Zwier, « Plant Sunscreens in the UV-B: Ultraviolet Spectroscopy of Jet-Cooled Sinapoyl Malate, Sinapic Acid, and

Sinapate Ester Derivatives », *J. Am. Chem. Soc.*, vol. 136, n° 42, p. 14780-14795, oct. 2014, doi: 10.1021/ja5059026.

[8] P.-C. Chao, C.-C. Hsu, et M.-C. Yin, « Anti-inflammatory and anti-coagulatory activities of caffeic acid and ellagic acid in cardiac tissue of diabetic mice », *Nutr. Metab.*, vol. 6, p. 33, août 2009, doi: 10.1186/1743-7075-6-33.

[9] S. J. Pragasam, V. Venkatesan, et M. Rasool, « Immunomodulatory and Anti-inflammatory Effect of p-Coumaric Acid, a Common Dietary Polyphenol on Experimental Inflammation in Rats », *Inflammation*, vol. 36, n° 1, p. 169-176, févr. 2013, doi: 10.1007/s10753-012-9532-8.

[10] J. D. Baranowski, P. M. Davidson, C. W. Nagel, et A. L. Branen, « INHIBITION OF Saccharomyces cerevisiae BY NATURALLY OCCURRING HYDROXYCINNAMATES », *J. Food Sci.*, vol. 45, n° 3, p. 592-594, 1980, doi: 10.1111/j.1365-2621.1980.tb04107.x.

[11] P. J. Herald et P. M. Davidson, « Antibacterial Activity of Selected Hydroxycinnamic Acids », *J. Food Sci.*, vol. 48, n° 4, p. 1378-1379, juill. 1983, doi: 10.1111/j.1365-2621.1983.tb09243.x.

[12] M. S. Barber, V. S. McConnell, et B. S. DeCaux, « Antimicrobial intermediates of the general phenylpropanoid and lignin specific pathways », *Phytochemistry*, vol. 54, n° 1, p. 53-56, mai 2000, doi: 10.1016/S0031-9422(00)00038-8.

[13] Z. Lou, H. Wang, S. Rao, J. Sun, C. Ma, et J. Li, « p-Coumaric acid kills bacteria through dual damage mechanisms », *Food Control*, vol. 25, n° 2, p. 550-554, juin 2012, doi: 10.1016/j.foodcont.2011.11.022.

[14] O. Taofiq, A. González-Paramás, M. Barreiro, et I. Ferreira, « Hydroxycinnamic Acids and Their Derivatives: Cosmeceutical Significance, Challenges and Future Perspectives, a Review », *Molecules*, vol. 22, n° 2, p. 281, févr. 2017, doi: 10.3390/molecules22020281.

[15] M. Janvier *et al.*, « Syringaresinol: A Renewable and Safer Alternative to Bisphenol A for Epoxy-Amine Resins », *ChemSusChem*, vol. 10, n° 4, p. 738-746, 2017, doi: 10.1002/cssc.201601595.

[16] F. Pion, A. F. Reano, P.-H. Ducrot, et F. Allais, « Chemo-enzymatic preparation of new bio-based bis- and trisphenols: new versatile building blocks for polymer chemistry », *RSC Adv.*, vol. 3, n° 23, p. 8988-8997, mai 2013, doi: 10.1039/C3RA41247D.

[17] A. F. Reano, S. Domenek, M. Pernes, J. Beaugrand, et F. Allais, « Ferulic Acid-Based Bis/Trisphenols as Renewable Antioxidants for Polypropylene and Poly(butylene succinate) », *ACS Sustain. Chem. Eng.*, vol. 4, n° 12, p. 6562-6571, déc. 2016, doi: 10.1021/acssuschemeng.6b01429.

[18] A. C. Fonseca, M. S. Lima, A. F. Sousa, A. J. Silvestre, J. F. J. Coelho, et A. C. Serra, « Cinnamic acid derivatives as promising building blocks for advanced polymers:

synthesis, properties and applications », *Polym. Chem.*, vol. 10, n° 14, p. 1696-1723, 2019, doi: 10.1039/C9PY00121B.

[19] C. Peyrot, A. A. M. Peru, L. M. M. Mouterde, et F. Allais, « Proline-Mediated Knoevenagel–Doebner Condensation in Ethanol: A Sustainable Access to p-Hydroxycinnamic Acids », *ACS Sustain. Chem. Eng.*, vol. 7, n° 10, p. 9422-9427, mai 2019, doi: 10.1021/acssuschemeng.9b00624.

[20] Y. S. Velioglu, G. Mazza, L. Gao, et B. D. Oomah, « Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products », *J. Agric. Food Chem.*, vol. 46, n° 10, p. 4113-4117, oct. 1998, doi: 10.1021/jf9801973.

[21] A. Tilay, M. Bule, J. Kishenkumar, et U. Annapure, « Preparation of Ferulic Acid from Agricultural Wastes: Its Improved Extraction and Purification », *J. Agric. Food Chem.*, vol. 56, n° 17, p. 7644-7648, sept. 2008, doi: 10.1021/jf801536t.

[22] C. M. Galanakis, V. Goulas, S. Tsakona, G. A. Manganaris, et V. Gekas, « A Knowledge Base for The Recovery of Natural Phenols with Different Solvents », *Int. J. Food Prop.*, vol. 16, n° 2, p. 382-396, janv. 2013, doi: 10.1080/10942912.2010.522750.

[23] Q. D. Do *et al.*, « Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica », *J. Food Drug Anal.*, vol. 22, n° 3, p. 296-302, sept. 2014, doi: 10.1016/j.jfda.2013.11.001.

[24] A. Vargas-Tah et G. Gosset, « Production of Cinnamic and p-Hydroxycinnamic Acids in Engineered Microbes », *Front. Bioeng. Biotechnol.*, vol. 3, 2015, doi: 10.3389/fbioe.2015.00116.

[25] Q. Liu *et al.*, « Rewiring carbon metabolism in yeast for high level production of aromatic chemicals », *Nat. Commun.*, vol. 10, n° 1, p. 4976, déc. 2019, doi: 10.1038/s41467-019-12961-5.

[26] F. L. Mota, A. J. Queimada, S. P. Pinho, et E. A. Macedo, « Aqueous Solubility of Some Natural Phenolic Compounds », *Ind. Eng. Chem. Res.*, vol. 47, n° 15, p. 5182-5189, août 2008, doi: 10.1021/ie071452o.

[27] J. Sangster, « Octanol-Water Partition Coefficients of Simple Organic Compounds », *J. Phys. Chem. Ref. Data*, vol. 18, n° 3, p. 1111-1229, juill. 1989, doi: 10.1063/1.555833.

[28] J. T. Dafoe et A. J. Daugulis, « In situ product removal in fermentation systems: improved process performance and rational extractant selection », *Biotechnol. Lett.*, vol. 36, n° 3, p. 443-460, mars 2014, doi: 10.1007/s10529-013-1380-6.

[29] C. Weilnhammer et E. Blass, « Continuous fermentation with product recovery by in-situ extraction », *Chem. Eng. Technol.*, vol. 17, n° 6, p. 365-373, 1994, doi: 10.1002/ceat.270170602.

[30] S. R. Roffler, H. W. Blanch, et C. R. Wilke, « In-situ recovery of butanol during fermentation », *Bioprocess Eng.*, p. 12, 1987.

[31] M. A. Khedkar, P. R. Nimbalkar, S. G. Gaikwad, P. V. Chavan, et S. B. Bankar, « Solvent extraction of butanol from synthetic solution and fermentation broth: Batch and continuous studies », *Sep. Purif. Technol.*, vol. 249, p. 117058, oct. 2020, doi: 10.1016/j.seppur.2020.117058.

[32] A. Gössi *et al.*, « In-situ recovery of carboxylic acids from fermentation broths through membrane supported reactive extraction using membrane modules with improved stability », *Sep. Purif. Technol.*, vol. 241, p. 116694, juin 2020, doi: 10.1016/j.seppur.2020.116694.

[33] F. Chemarin, V. Athès, M. Bedu, T. Loty, F. Allais, et I. C. Trelea, « Towards an *in situ* product recovery of bio-based 3-hydroxypropionic acid: influence of bioconversion broth components on membrane-assisted reactive extraction: Towards an *in situ* product recovery of bio-based 3-hydroxypropionic acid », *J. Chem. Technol. Biotechnol.*, vol. 94, n° 3, p. 964-972, mars 2019, doi: 10.1002/jctb.5845.

[34] F. Chemarin *et al.*, « New insights in reactive extraction mechanisms of organic acids: An experimental approach for 3-hydroxypropionic acid extraction with tri-n-octylamine », *Sep. Purif. Technol.*, vol. 179, p. 523-532, mai 2017, doi: 10.1016/j.seppur.2017.02.018.

[35] V. M. Yabannavar et D. I. C. Wang, « Extractive fermentation for lactic acid production », *Biotechnol. Bioeng.*, vol. 37, n° 11, p. 1095-1100, 1991, doi: 10.1002/bit.260371115.

[36] A. Freeman, J. M. Woodley, et M. D. Lilly, « In Situ Product Removal as a Tool for Bioprocessing », *Bio/Technology*, vol. 11, n° 9, p. 1007-1012, sept. 1993, doi: 10.1038/nbt0993-1007.

[37] F. Kollerup et A. J. Daugulis, « Screening and identification of extractive fermentation solvents using a database », *Can. J. Chem. Eng.*, vol. 63, n° 6, p. 919-927, 1985, doi: 10.1002/cjce.5450630608.

[38] L. J. Bruce et A. J. Daugulis, « Solvent selection strategies for extractive biocatalysis », *Biotechnol. Prog.*, vol. 7, n° 2, p. 116-124, mars 1991, doi: 10.1021/bp00008a006.

[39] I. P. Rosinha Grundtvig *et al.*, « Screening of organic solvents for bioprocesses using aqueous-organic two-phase systems », *Biotechnol. Adv.*, vol. 36, n° 7, p. 1801-1814, nov. 2018, doi: 10.1016/j.biotechadv.2018.05.007.

[40] R. Bar et J. L. Gainer, « Acid Fermentation in Water-Organic Solvent Two-Liquid Phase Systems », *Biotechnol. Prog.*, vol. 3, n° 2, p. 109-114, 1987, doi: 10.1002/btpr.5420030208.

[41] C. Laane, S. Boeren, et K. Vos, « On optimizing organic solvents in multi-liquid-phase biocatalysis », *Trends Biotechnol.*, vol. 3, n° 10, p. 251-252, oct. 1985, doi: 10.1016/0167-7799(85)90023-X.

[42] J. Frenz, C. Largeau, E. Casadevall, F. Kollerup, et A. J. Daugulis, « Hydrocarbon recovery and biocompatibility of solvents for extraction from cultures of Botryococcus

braunii », *Biotechnol. Bioeng.*, vol. 34, n° 6, p. 755-762, 1989, doi: 10.1002/bit.260340605.

[43] R. R. Xing, X. Chen, S. Hu, et X. H. Bai, « Novel Microporous Membrane/Solvent Microextraction for Preconcentration of Cinnamic Acid Derivatives in Rhizoma Typhonii », *Chromatographia*, vol. 77, n° 7, p. 553-559, avr. 2014, doi: 10.1007/s10337-014-2635-7.

[44] S. Li, S. Hu, X. Chen, X. Bai, et S. Zhang, « Two-phase/Three-phase Hollow Fibre Liquid-Phase Simultaneous Microextraction Combined with HPLC for Analysis of Phenolic Acids and Flavonoids in Traditional Chinese Medicine », *Chromatographia*, vol. 78, n° 17, p. 1159-1167, sept. 2015, doi: 10.1007/s10337-015-2930-y.

[45] M. Saraji et F. Mousavi, « Use of hollow fibre-based liquid–liquid–liquid microextraction and high-performance liquid chromatography–diode array detection for the determination of phenolic acids in fruit juices », *Food Chem.*, vol. 123, n° 4, p. 1310-1317, déc. 2010, doi: 10.1016/j.foodchem.2010.06.012.

[46] A. Benvidi *et al.*, « Experimental and computational study of the p K_a of coumaric acid derivatives », *J. Chin. Chem. Soc.*, vol. 66, n° 6, p. 589-593, juin 2019, doi: 10.1002/jccs.201800265.

[47] G. Reed et T. W. Nagodawithana, *Yeast Technology*. Dordrecht: Springer Netherlands, 1990.

[48] N. R. Ronco, L. G. Gagliardi, et C. B. Castells, « Chapter 3 - Aqueous-Organic Biphasic Systems: Extraction of Organic Compounds », in *Liquid-Phase Extraction*, C. F. Poole, Éd. Elsevier, 2020, p. 91-119.

[49] United Nations, Economic Commission for Europe, *Globally Harmonized System of Classification and Labelling of Chemicals (GHS, Rev 8)*. 2019.

[50] R. K. Mortimer et J. R. Johnston, « Genealogy of Principal Strains of the Yeast Genetic Stock Center », *Genetics*, vol. 113, n° 1, p. 35-43, mai 1986.

[51] N. D. Gold, C. M. Gowen, F.-X. Lussier, S. C. Cautha, R. Mahadevan, et V. J. J. Martin, « Metabolic engineering of a tyrosine-overproducing yeast platform using targeted metabolomics », *Microb. Cell Factories*, vol. 14, n° 1, déc. 2015, doi: 10.1186/s12934-015-0252-2.

[52] L. De Lorenzi, M. Fermeglia, et G. Torriano, « Density and Viscosity of 1-Methoxy-2-Propanol, 2-Methyltetrahydrofuran, α,α,α-Trifluorotoluene, and Their Binary Mixtures with 1,1,1-Trichloroethane at Different Temperatures », *J. Chem. Eng. Data*, vol. 41, n° 5, p. 1121-1125, janv. 1996, doi: 10.1021/je9601220.

[53] D. F. Aycock, « Solvent Applications of 2-Methyltetrahydrofuran in Organometallic and Biphasic Reactions », *Org. Process Res. Dev.*, vol. 11, n° 1, p. 156-159, janv. 2007, doi: 10.1021/op060155c.

[54] M. I. Aralaguppi, C. V. Jadar, et T. M. Aminabhavi, « Density, Viscosity, Refractive Index, and Speed of Sound in Binary Mixtures of 2-Chloroethanol with Methyl Acetate, Ethyl Acetate, *n* -Propyl Acetate, and *n* -Butyl Acetate », *J. Chem. Eng. Data*, vol. 44, n° 3, p. 441-445, mai 1999, doi: 10.1021/je980218p.

[55] S. H. Yalkowsky, Y. He, P. Jain, Y. He, et P. Jain, *Handbook of Aqueous Solubility Data*. CRC Press, 2016.

[56] M. M. El-Banna, « Densities and Viscosities for Mixtures of Pentyl Acetate and Hexyl Acetate with Normal Alkanols at 298.15 K », *J. Chem. Eng. Data*, vol. 42, n° 1, p. 31-34, janv. 1997, doi: 10.1021/je960193z.

[57] A. S. Al-Jimaz, J. A. Al-Kandary, et A.-H. M. Abdul-Latif, « Densities and viscosities for binary mixtures of phenetole with 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, and 1-decanol at different temperatures », *Fluid Phase Equilibria*, vol. 218, n° 2, p. 247-260, avr. 2004, doi: 10.1016/j.fluid.2003.12.007.

[58] N. V. Sastry et M. K. Valand, « Volumetric Behaviour of Acrylic Esters (Methyl-, Ethyl-, and Butyl Acrylate) + 1-Alcohol (Heptanol, Octanol, Decanol and Dodecanol) at 298.15 K and 308.15 K », *Phys. Chem. Liq.*, vol. 38, n° 1, p. 61-72, janv. 2000, doi: 10.1080/00319100008045297.

[59] N. V. Sastry et M. K. Valand, « Viscosities and Densities for Heptane + 1-Pentanol, +1-Hexanol, +1-Heptanol, +1-Octanol, +1-Decanol, and +1-Dodecanol at 298.15 K and 308.15 K », *J. Chem. Eng. Data*, vol. 41, n° 6, p. 1426-1428, janv. 1996, doi: 10.1021/je9601725.

[60] T. P. Thomas, D. M. Birney, et D. L. Auld, « Viscosity reduction of castor oil esters by the addition of diesel, safflower oil esters and additives », *Ind. Crops Prod.*, vol. 36, n° 1, p. 267-270, mars 2012, doi: 10.1016/j.indcrop.2011.06.021.

[61] G. Knothe et K. R. Steidley, « Kinematic viscosity of biodiesel components (fatty acid alkyl esters) and related compounds at low temperatures », *Fuel*, vol. 86, n° 16, p. 2560-2567, nov. 2007, doi: 10.1016/j.fuel.2007.02.006.

[62] T. M. Aminabhavi, V. B. Patil, M. I. Aralaguppi, et H. T. S. Phayde, « Density, Viscosity, and Refractive Index of the Binary Mixtures of Cyclohexane with Hexane, Heptane, Octane, Nonane, and Decane at (298.15, 303.15, and 308.15) K », *J. Chem. Eng. Data*, vol. 41, n° 3, p. 521-525, janv. 1996, doi: 10.1021/je950279c.

[63] A. Krzyżaniak, M. Leeman, F. Vossebeld, T. J. Visser, B. Schuur, et A. B. de Haan, « Novel extractants for the recovery of fermentation derived lactic acid », *Sep. Purif. Technol.*, vol. 111, p. 82-89, juin 2013, doi: 10.1016/j.seppur.2013.03.031.

[64] F. Chemarin, M. Moussa, F. Allais, I. C. Trelea, et V. Athès, « Recovery of 3-hydroxypropionic acid from organic phases after reactive extraction with amines in an

alcohol-type solvent », *Sep. Purif. Technol.*, vol. 219, p. 260-267, juill. 2019, doi: 10.1016/j.seppur.2019.02.026.

[65] M. Minier et G. Goma, « Ethanol production by extractive fermentation », *Biotechnol. Bioeng.*, vol. 24, n° 7, p. 1565-1579, 1982, doi: 10.1002/bit.260240710.

[66] P. J. Evans et H. Y. Wang, « Effects of extractive fermentation on butyric acid production byClostridium acetobutylicum », *Appl. Microbiol. Biotechnol.*, vol. 32, n° 4, p. 393-397, janv. 1990, doi: 10.1007/BF00903771.

[67] R. D. Offeman, S. K. Stephenson, D. Franqui, J. L. Cline, G. H. Robertson, et W. J. Orts, « Extraction of ethanol with higher alcohol solvents and their toxicity to yeast », *Sep. Purif. Technol.*, vol. 63, n° 2, p. 444-451, oct. 2008, doi: 10.1016/j.seppur.2008.06.005.

[68] A. J. Daugulis, D. B. Axford, B. Ciszek, et J. J. Malinowski, « Continuous fermentation of high-strength glucose feeds to ethanol », *Biotechnol. Lett.*, vol. 16, n° 6, p. 637-642, juin 1994, doi: 10.1007/BF00128614.

[69] S. J. Osborne, J. Leaver, M. K. Turner, et P. Dunnill, « Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane », *Enzyme Microb. Technol.*, vol. 12, n° 4, p. 281-291, avr. 1990, doi: 10.1016/0141-0229(90)90100-5.

[70] J. Zhang et R. Greasham, « Chemically defined media for commercial fermentations », *A@ppl. Microbiol. Biotechnol.*, vol. 51, n° 4, p. 407-421, avr. 1999, doi: 10.1007/s002530051411.