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In Situ Product Recovery of Bio-Based Industrial Platform Chemicals: A Guideline to Solvent Selection

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Abstract: In situ product recovery (ISPR), in the form of an extractive fermentation process, can increase productivity and product titers in the sustainable production of platform chemicals. To establish a guideline for the development of industrially relevant production processes for such bio-based compounds, a wide screening was performed, mapping the potential of an extensive range of solvents and solvent mixtures. Besides solvent biocompatibility with *Saccharomyces cerevisiae*, distribution coefficients of three organic acids (protocatechuic acid, adipic acid and *para*-aminobenzoic acid) and four fragrance compounds (2-phenylethanol, geraniol, *trans*-cinnamaldehyde and β-ionone) were determined. While for highly hydrophobic fragrance compounds, multiple pure solvents were identified that were able to extract more than 98%, reactive extraction mixtures were proven effective for more challenging compounds including organic acids and hydrophilic alcohols. For example, a reactive mixture consisting of 12.5% of the extractant CYTOP 503 in canola oil was found to be biocompatible and showed superior extraction efficiency for the challenging compounds as compared to any biocompatible single solvent. This mapping of biocompatible solvents and solvent mixtures for the extraction of various classes of industrial platform chemicals can be a tremendous step forward in the development of extractive fermentations.

Keywords: in situ product recovery (ISPR); biocompatibility; *Saccharomyces cerevisiae*; bio-based platform chemicals; reactive extraction

1. Introduction

The current pressure to shift towards a more sustainable bioeconomy has led to a search in both research and industry for efficient production strategies for bio-based 'drop-in' or novel compounds. To this end, fermentative production of industrial platform chemicals from second-generation biomass or waste streams could present a principal solution with substantial CO_2 abatement potential and a significantly reduced carbon footprint. Here, genetically engineered strains of e.g., *Escherichia coli* and *Saccharomyces cerevisiae* have been employed as well-studied and easily cultivatable microbial cell factories [1]. The advantages of using yeast, as compared to other microorganisms, include its robustness and tolerance to low pH, resulting in a reduced susceptibility to contamination. Efficient platform yeast strains have recently been developed aiming at maximal stress resistance and carbon utilization with second-generation feedstocks [2,3]. Further specific genetic engineering of these platform strains aims to deliver industrial superbugs capable of producing bio-based platform chemicals with great industrial relevance [4].

However, the production of many of these platform chemicals is currently hindered due to their inherent toxicity to the yeast strain, limiting the attained product titres and

productivities and thus compromising the overall process performance and industrial feasibility. A radical strategy to overcome this and enhance fermentative production involves in situ product recovery (ISPR). The latter technique allows for continuous withdrawal of toxic compounds and thus alleviates product inhibition [5]. During ABE fermentations, for example, ISPR improved substrate use and led to an increase in butanol yield and productivity of up to 67% and 357% respectively [6]. Besides this, an integrated process comprising both fermentation and product separation is anticipated to yield significant economic advantages by lowering product purification costs [7]. In the most prevalent ISPR configuration, extractive solvents are brought in direct contact with the microbial cells inside the bioreactor [8]. Therefore, an interplay of two principal parameters, i.e., biocompatibility with the producing organism and extractability for the target compound, determine the potential of an extractant for ISPR. Careful balancing of both parameters is then crucial to obtain an efficient extractive fermentation process and will be the core research topic of this paper.

Several authors have previously reported the use of solvents for in situ extraction of e.g., alcohols, organic acids, monoterpenes and ketones, to alleviate product inhibition and increase product titers or productivity [9–11]. And although back extraction of several target compounds such as lactic acid and propionic acid has been extensively studied [12,13], studies are generally limited to a small number of specific solvents and to the best of the authors' knowledge, a comprehensive overview covering different solvent classes for their extraction capacity for different industrially relevant target compounds, while also including solvent biocompatibility, is currently lacking. Therefore, this paper presents the results of an extensive screening of a wide range of solvents and solvent mixtures, covering the major solvent classes, for their potential as extractants for industrial platform chemicals, both in terms of extraction capacity and biocompatibility with an industrial platform *S. cerevisiae* strain. The target compounds in this paper cover the principal classes within industrially relevant molecules, including organic acids, aromatics, terpenes, aldehydes, and alcohols (Figure 1). Furthermore, the selected compounds have witnessed a recent surge of interest, though their bio-based production through fermentation is currently hindered by product inhibition.
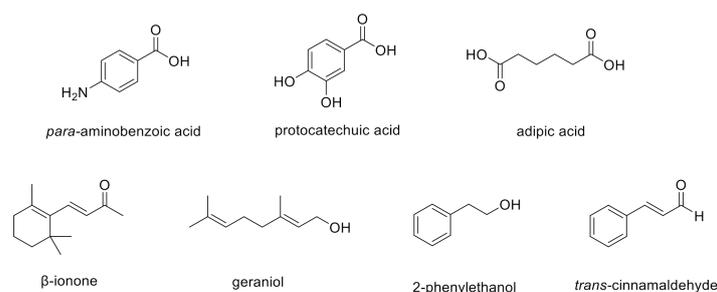

Figure 1. Target industrial platform chemicals for ISPR in the present study.

para-Aminobenzoic acid (pABA) is a versatile aromatic compound with applications as a pharmaceutical precursor and crosslinking agent for resins and dyes [4]. Protocatechuic acid (PCA) shows interesting functionality and is promising as a platform molecule for bioplastics production [14], while adipic acid (AA) represents a one billion dollar market owing to its use for the synthesis of nylon 6,6. Currently, significant research efforts are directed towards the development of a feasible biological production pathway in *S. cerevisiae* for these organic acids, and ISPR has been suggested as an attractive technique to increase productivity and titer [11,15]. In the flavor and fragrance industry, isoprenoids present a major class of components and their biological production has become increasingly important [16–18]. However, being lipophilic compounds, they typically show strong toxicity towards microorganisms due to their interference with microbial membranes [19]. Geraniol (GE) and β-ionone (ION) present principal fragrance compounds in this class, while *trans*-cinnamaldehyde (CA) is applied as a bioactive flavoring agent, owing to its distinctive

taste and odor along with its antimicrobial and anti-inflammatory properties [20]. With a global market demand exceeding 10,000 tons, 2-phenylethanol (2-PE) is an important industrial aroma compound and many research efforts have been made to enhance its microbial production using ISPR strategies [21]. In this respect, in situ extraction techniques, particularly using ionic liquids (ILs) and polypropylene glycol (PPG) polymers, seem to be very promising [22,23]. Both solvent classes were closely examined in this paper and their potential and industrial feasibility was compared to alternative solvent candidates as well as solvent mixtures.

2. Materials and Methods

2.1. Chemicals

para-Aminobenzoic acid (pABA, 99%), *trans*-cinnamaldehyde (CA, 99%), β-ionone (ION, 96%), geraniol (GE, 99%) and 2-phenylethanol (2-PE, >98%) were purchased from VWR (Radnor, PA, USA). Protocatechuic acid (PCA, >97%) and adipic acid (AA, >99%) were sourced from Sigma-Aldrich (Saint Louis, MO, USA). The ionic liquids 1-n-butyl-3-methylimidazolium hexafluorophosphate (BMIM[PF_6], 99%), 1-n-butyl-3-methylimidazolium-bis(trifluoromethylsulfonyl)imide (BMIM[Tf_2N], 99%), 1-methyl-1-propylpiperidinium bis(trifluoromethylsulfonyl)imide (MPPyr[Tf_2N], 99%), methyltri-octylammonium bis(trifluoromethylsulfonyl)imide (MOA[Tf_2N], 99%), trihexyltetrade-cylphosphonium chloride (CYPHOS IL-101, >95%) and trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate (CYPHOS IL-104, >90%) were purchased from Io-LiTec (Heilbronn, Germany). CYTOP 503 was kindly provided by Solvay (Brussels, Belgium). FAMEs were obtained from Mosselman (Ghlin, Belgium). Canola oil and sunflower oil were purchased from Vandemoortele (Ghent, Belgium). All other chemicals used in this study were at least analytical grade and were obtained from Sigma-Aldrich, VWR, Thermo Fisher Scientific (Waltham, MA, USA) or TCI Europe (Zwijndrecht, Belgium).

2.2. Microorganisms and Cultivation

A genetically modified platform yeast strain, *Saccharomyces cerevisiae* MDS130, in which xylose utilization and enhanced inhibitor tolerance was introduced, was provided by the KUL-VIB research institute (Leuven, Belgium). Yeasts were cultured in a complex medium (YPD) consisting of 10 g L^{-1} yeast extract (Kerry Ingredients & Flavours Ltd, Tillburg, The Netherlands), 20 g L^{-1} bacto peptone (Thermo Fisher Scientific) and 20 g L^{-1} glucose (Brenntag, Essen, Germany), where pH was adjusted to 4.25 ± 0.05. For biocompatibility trials, a single seed step in 500 mL baffled shake flasks filled with 20% complex medium was applied. Inoculation was done using 1 mL from a cryovial stored at −80 °C containing 30% glycerol stock solution, after which it was incubated (Innova S44i, Eppendorf incubator, Hamburg, Germany) at 200 rpm (orbit 51 mm) and 30 °C to reach an OD_{600} of 30.

2.3. Biocompatibility Testing

Biocompatibility experiments were conducted in 24-square deepwell plates obtained from Enzyscreen (Heemstede, The Netherlands). For inoculation of the deepwell plates, 80 μL inoculum from the seed culture was transferred to a well containing 1.6 mL of the same complex medium. After 3 h of incubation, 0.4 mL (20 *v*/*v*%) of solvent or solvent mixture was added to each well. After 8 h of cultivation, the wells were sampled and centrifuged (Eppendorf 5427 R, 14,000 rpm, 3 min) prior to analysis. Biocompatibility was evaluated by determining the glucose concentration in the aqueous phase by HPLC and by measuring the optical density of the resuspended cells (physiological water, 9 g L^{-1} NaCl) at 600 nm (OD_{600}) using an Agilent Cary 60 spectrophotometer (Santa Clara, CA, USA). Results were compared to blank cultures containing no solvent. Each deepwell plate included four positive controls (i.e., without solvent addition) as well as two negative controls (i.e., without inoculation) to assess for potential contamination of the deepwell. To effectively compare the results across multiple deepwell trials, glucose consumption (0–100%) and relative OD_{600} (0–1) are expressed relative to their positive control. During

the initial screening, biocompatibility was determined in single experiments, while for solvent mixtures, experiments were carried out in duplicate and results are presented as mean values.

2.4. Distribution Coefficients

The distribution coefficient K_d is defined as the ratio of the concentration of a target compound in the organic phase to the aqueous phase according to Equation (1). For this, 4 mL of YPD medium, pH-adjusted to 4.25 ± 0.05 using 2M H_2SO_4 and spiked with a known amount of each specific target compound (2 g L^{-1} for AA and 2-PE, 1 g L^{-1} for pABA and PCA, 0.5 g L^{-1} for CA and 50 mg L^{-1} for ION and GE), and 1 mL (20 $v/v\%$) of solvent or solvent mixture, were added to a 5 mL Eppendorf tube. After vigorous shaking for 30 min at 30 °C in an Eppendorf ThermoMixer, samples were centrifuged at 14,000 rpm for 3 min. The aqueous phase was sampled and analysed by HPLC as described below.

$$K_d = \frac{c_{org}}{c_{aq}} \tag{1}$$

where K_d is the distribution coefficient, c_{org} is the concentration of the target compound in the organic phase and c_{aq} is the concentration of the target compound in the aqueous phase. c_{org} was calculated based on the concentration of the target compound in the aqueous phase before and after extraction, taking into account the applied volumetric solvent ratio. During the initial screening, distribution coefficients were determined in single experiments, while for solvent mixtures, experiments were carried out in duplicate and results are presented as mean values.

2.5. Analytics

Glucose concentrations in the aqueous phases obtained from biocompatibility trials were determined by HPAEC-PAD using a Dionex ICS-6000 system (Thermo Fisher Scientific, Sunnyvale, CA, USA), equipped with a Dionex Electrochemical Detector (ED). Samples (2 μL) were injected using a Dionex AS-AP Autosampler into a CarboPac PA20 Analytical column (3 × 150 mm) with a PA20G guard column (3 × 30 mm) at 30 °C. The mobile phase was a gradient of 250 mM NaOH solution in milli-Q water (0.5 mL min^{-1}). Results were processed using Chromeleon 7 software. Glucose concentration was linear between 1.25 and 25 mg L^{-1}. Prior to analysis, samples were heated to 99 °C for 10 min, cooled and centrifuged (Eppendorf 5427 R) at 14,000 rpm for 3 min to remove proteins and other debris. The supernatant was filtered using a 0.2 μm PES filter and diluted to be within the linear range.

Concentrations of pABA, PCA, ION, GE, 2-PE and CA in the aqueous phases from extractability trials were determined using an Agilent 1260 Infinity HPLC system equipped with a C18 column (Zorbax eclipse plus, 4.6 × 100 mm, 3.5 μm), with a mobile phase at 1.0 mL min^{-1} consisting of a gradient of acetonitrile and 0.05% acetic acid in milli-Q water, a column temperature of 40 °C and a DAD detector at 210 nm. AA was analysed using an Agilent 1260 Infinity HPLC with a Metacarb 67H column (300 × 6.5 mm, connected to a varia 5244GC precolumn), a mobile phase of 2.5 mM H_2SO_4 at 0.8 mL min^{-1} and a column temperature of 40 °C. AA was detected by a refractive index detector (RID). HPLC results were processed using Chemstation software (version C.01.05).

3. Results

3.1. Solvent Screening

For 63 solvents, biocompatibility with a platform yeast strain of *Saccharomyces cerevisiae* and the extraction potential for seven industrial platform chemicals were evaluated. Given the importance of pH when assessing the extractability of organic acids such as PCA, AA and pABA, the pH was adjusted to a value of 4.25 (±0.05) for each of the spiked media. This allows for a higher extraction efficiency using nonpolar solvents, since it is below the pKa value for each evaluated acid. Yet it is common practice to perform yeast fermentations

at these lower pH values and further reduces the risk of contamination, emphasizing the potential of *S. cerevisiae* as microbial cell factories.

The biocompatibility of all pure solvents and their respective distribution coefficients for each target compound are shown in Table 1.

Table 1. Biocompatibility and distribution coefficients of single solvents for *para*-aminobenzoic acid (pABA), protocatechuic acid (PCA), adipic acid (AA), *trans*-cinnamaldehyde (CA) and 2-phenylethanol (2-PE).

Solvent	Biocompatibility		Distribution Coefficient (K_d)				
	Relative OD_{600}	Glucose Consumed (%)	pABA	PCA	AA	CA	2-PE
ALKANES							
Octane	0.06	0.0	0.34	0.29	0.00	13.5	0.63
Nonane	0.13	0.0	0.00	0.32	0.00	14.7	0.69
Dodecane	>0.99	>98	0.04	0.26	0.00	13.4	0.65
Hexadecane	0.84	>98	0.05	0.10	0.00	12.5	0.42
ALCOHOLS							
Butanol	0.08	0.0	4.24	1.82	1.40	50.9	15.2
Octanol	0.10	0.0	2.32	1.65	0.45	49.9	19.4
Decanol	0.07	0.0	1.39	1.09	0.29	62.9	11.8
Undecanol	0.26	0.0	1.50	0.58	0.24	57.5	12.5
Dodecanol	0.29	0.0	1.46	0.66	0.19	54.7	9.04
Oleyl alcohol	0.96	>98	1.58	4.19	0.12	53.5	6.75
Isoamyl alcohol	0.00	0.0	3.75	22.7	1.18	76.7	18.7
2-Ethyl-1-hexanol	0.16	0.0	2.14	0.94	0.50	76.2	16.8
2-Butyl-1-octanol	>0.99	>98	1.15	0.59	0.16	21.9	8.15
2-Hexyl-1-decanol	n.a. †	>98	1.11	0.00	0.04	22.6	5.36
ETHERS							
tert-Butyl methyl ether	0.04	0.0	0.30	0.54	0.44	67.4	7.62
Diisopentyl ether	n.a. †	0.0	0.00	0.00	0.00	31.1	2.02
Dihexyl ether	n.a. †	0.0	0.00	0.29	0.00	32.2	1.95
Didecyl ether (decyl ether)	n.a. †	>98	0.00	0.54	0.00	23.3	1.22
ESTERS							
Ethyl caprylate	0.06	10.5	1.06	0.00	0.00	81.6	1.76
Ethyl decanoate	0.70	>98	0.71	0.14	0.00	108	1.26
Ethyl laurate	0.89	>98	0.69	0.29	0.01	115	1.02
Isopropyl myristate	>0.99	>98	0.58	0.33	0.00	71.8	0.83
Ethyl oleate	>0.99	>98	0.53	0.00	0.00	76.9	0.75
Dibutyl maleate	n.a. †	>98	2.17	0.20	0.01	231	2.14
Diisobutyl adipate	n.a. †	>98	2.52	0.26	0.00	181	2.29
Bis-2-ethylhexyl adipate	0.95	>98	0.85	0.05	0.00	118	1.00
Tributyrin	0.87	>98	2.73	0.50	0.04	230	2.82
Tributyl citrate	0.80	94.7	1.83	0.34	0.03	173	2.98
Methyl phenyl acetate	n.a. †	0.0	2.39	0.57	0.00	252	1.43
Benzyl benzoate	n.a. †	>98	0.00	0.29	0.00	224	1.62
Bis-2-ethylhexyl phthalate	>0.99	>98	0.64	0.00	0.00	111	5.13
Diisononyl phthalate	>0.99	>98	0.00	0.42	0.00	236	3.96
KETONES							
Methyl isobutyl ketone	n.a. †	0.0	4.20	1.54	0.34	143	16.8
4-decanone	0.00	0.0	0.82	0.87	0.00	139	7.63
VEGETABLE OILS							
Canola oil	0.76	>98	0.00	0.23	0.00	58.3	2.10
Sunflower oil	0.81	>98	0.00	0.00	0.01	49.4	2.00
FATTY ACID METHYL ESTERS							
Castor oil FAME	>0.99	>98	5.64	2.95	0.15	53.8	2.21
Linseed oil FAME	>0.99	>98	3.67	1.15	0.00	40.2	0.83
Soybean oil FAME	0.65	67.4	1.35	1.14	0.00	40.2	0.67
Sunflower oil FAME	>0.99	>98	1.60	1.08	0.00	37.8	0.56
Methyl oleate	>0.99	91.9	1.96	1.21	0.00	34.4	0.64
C16-C18 mixture of FAME	>0.99	91.9	1.35	1.16	0.00	35.6	0.57

Table 1. *Cont.*

Solvent	Biocompatibility		Distribution Coefficient (K_d)				
	Relative OD_{600}	Glucose Consumed (%)	pABA	PCA	AA	CA	2-PE
POLYMERS							
PPG1000	>0.99	>98	5.72	10.18	1.73	46.8	25.5
PPG2000	0.89	>98	11.1	8.23	0.40	102	14.1
PPG4000	0.77	>98	9.51	4.88	0.58	102	12.7
IONIC LIQUIDS							
BMIM[TF2N]	n.a. †	>98	0.94	0.16	0.06	166	10.2
BMIM[PF6]	n.a. †	>98	4.39	0.00	0.05	93.3	0.27
MPPyr[Tf2N]	n.a. †	>98	0.61	0.11	0.03	140	7.02
MOA[Tf2N]	n.a. †	>98	0.00	0.12	0.00	102	4.22
CYPHOS IL-101	n.a. †	0.0	22.7	47.1	14.7	165	108
CYPHOS IL-104	n.a. †	5.4	3.70	54.2	2.25	119	43.8
Aliquat 336	n.a. †	0.0	12.7	52.9	9.75	140	53.3
AMINES							
Tributylamine	0.00	0.0	0.00	0.00	0.01	21.2	2.63
Trioctylamine	n.a. †	5.4	0.00	1.06	0.61	27.6	1.38
Tridodecylamine	0.06	26.3	0.16	1.35	0.40	27.1	27.2
PHOSPHOROUS COMPOUNDS							
CYTOP 503	n.a. †	55.9	15.4	47.2	13.2	144	63.6
Tributyl phosphate	n.a. †	26.0	33.7	40.3	4.86	221	42.4

† not applicable. Residual solvent interference with OD_{600} measurement or impossible to isolate cell pellet. Note: Tween 20, Tween 80, PEG400, PPG425, tetrahydrofuran and acetophenone were also evaluated during the biocompatibility and extractability experiments, though no effective phase separation was obtained and therefore no measurements of OD, glucose or extraction were possible.

No growth inhibition was observed for dodecane and hexadecane, whereas smaller alkanes (<C9) showed almost complete growth inhibition upon addition. Similarly, the larger and branched alcohols exhibited improved biocompatibility as compared to the smaller alcohols, e.g., isoamyl alcohol, which in turn showed higher extraction capacity. While dodecane was found to be biocompatible, dodecanol, the alcoholic counterpart of dodecane, was not. However, a higher distribution coefficient was obtained for the alcohol. For all ethers evaluated, only the largest, decyl ether, was found to be biocompatible. Out of the 13 esters evaluated in this study, 11 showed little to no growth inhibition, making esters a suitable class to use as solvent or diluent for ISPR processes. Remarkably, none of the ketones were found biocompatible. Both vegetable oils, canola oil and sunflower oil, showed good biocompatibility, as well as fatty acid methyl esters (FAME). In the latter class, only soybean oil-derived FAME showed a slight growth-inhibiting effect. The group of the amine and phosphorous compounds, commonly used extractants, were typically non-biocompatible. Yet, for CYTOP 503, a phosphine-oxide based extractant, a biocompatibility of 56% was found. Of the seven ionic liquids (IL) selected in this study, four showed to be biocompatible with the yeast strain. More specifically, BMIM[TF2N], BMIM[PF6], MPPyr[Tf2N] and MOA[Tf2N] were found to be biocompatible while CYPHOS IL-101, CYPHOS IL-104 and Aliquat 336 were not.

The hydrophobic compounds CA, ION and GE were well extracted by almost every solvent evaluated, whereas the extraction of 2-PE and particularly the organic acids was found to be much less efficient. For ION and GE, extraction efficiencies above 98% were determined for all solvents, with the exception of large alkanes (>C12) for GE. Here, extraction efficiency was still above 95%. At the same time, good extractability of 2-PE was observed with several biocompatible solvents such as long-chain alcohols oleyl alcohol, 2-butyl-1-octanol, and 2-hexyl-1-decanol, but also polymers including polypropylene glycol (PPG). High extraction efficiencies were obtained for the ionic liquids CYPHOS IL-101, CYPHOS IL-104 and Aliquat 336, and for phosphorous-based extractants tributyl phosphate (TBP) and CYTOP 503, while amine-based extractants showed limited extraction

capacity. For Tween 20, Tween 80, PEG400, PPG425, tetrahydrofuran, and acetophenone, no phase separation was obtained, limiting the applicability of the latter solvents.

3.2. Solvent Mixtures

Effective reactive extraction with concurrent good biocompatibility can be achieved by combining a reactive extractant with a highly biocompatible diluent. Considering the data from the screening of pure solvents, mixtures of the most promising biocompatible solvents and efficient extractants were prepared and examined at extractant concentrations ranging between 2.5% and 25%. Here, the extractants CYPHOS IL-101, CYPHOS IL-104, trioctylamine (TOA), TBP, CYTOP 503, and Aliquat 336 were evaluated in combination with five diluents, i.e., canola oil, oleyl alcohol, PPG1000, dodecane, and sunflower oil FAME. Additionally, three alternative solvent mixtures were considered aiming to optimize both extraction and biocompatibility, more specifically isoamyl alcohol with oleyl alcohol, octanol with oleyl alcohol and methyl isobutyl ketone (MIBK) with tributyrin. The results are summarized in Table 2. Due to solvent interference with OD_{600} measurement, only glucose consumption was considered for biocompatibility. Promising biocompatible mixtures were evaluated at higher extractant concentrations aiming to improve the extraction efficiency, while non-biocompatible mixtures were evaluated at a lower concentration to increase biocompatibility.

Table 2. Biocompatibility of the composed solvent mixtures.

Reactive extraction Mixtures							
Extractant	Diluent	Extractant Ratio (% *v/v*)	Glucose Consumed (%)	Extractant	Diluent	Extractant Ratio (% *v/v*)	Glucose Consumed (%)
CYPHOS IL-101	Canola oil	2.5	7.74	Tributyl phosphate	Canola oil	15	>98
		5	9.31			20	50.4
		12.5	11.5				
	Oleyl alcohol	25	>98		Oleyl alcohol	25	>98
	PPG1000	5	2.03		PPG1000	12.5	>98
		12.5	2.33		Dodecane	5	>98
	Dodecane	5	0.00			12.5	11.6
	Sunflower oil FAME	5	0.00		Sunflower oil FAME	12.5	>98
CYPHOS IL-104	Canola oil	2.5	9.25	CYTOP 503	Canola oil	5	>98
		5	18.9			12.5	97.1
		12.5	18.0			25	57.2
	Oleyl alcohol	25	>98			50	24.1
	PPG1000	2.5	70.4		Oleyl alcohol	5	>98
		5	53.6			12.5	91.3
		12.5	20.4		PPG1000	5	>98
	Dodecane	5	15.9			12.5	87.7
		12.5	0.00		Dodecane	5	>98
	Sunflower oil FAME	5	0.00			12.5	90.4
					Sunflower oil FAME	12.5	>98

Table 2. *Cont.*

				Reactive extraction Mixtures			
Extractant	**Diluent**	**Extractant Ratio (% *v/v*)**	**Glucose Consumed (%)**	**Extractant**	**Diluent**	**Extractant Ratio (% *v/v*)**	**Glucose Consumed (%)**
Trioctyl-amine	Canola oil	2.5	73.4	Aliquat 336	Canola oil	1	12.5
		5	6.73			2.5	0.00
		12.5	0.00		Oleyl alcohol	2.5	>98
	Oleyl alcohol	12.5	>98			5	0.00
	PPG1000	5	7.33		PPG1000	5	10.1
		12.5	10.7			12.5	0.00
	Dodecane	5	91.5		Dodecane	5	0.00
		12.5	0.00		Sunflower oil FAME	5	0.00
	Sunflower oil FAME	5	>98				
		12.5	85.6				

		Alternative Solvent Mixtures	
Solvent 1	**Solvent 2**	**Solvent Ratio (% *v/v*) (Solvent 1 : Solvent 2)**	**Glucose Consumed (%)**
Isoamyl alcohol	Oleyl alcohol	20 : 80	6.59
		40 : 60	1.90
Octanol	Oleyl alcohol	20 : 80	37.8
		40 : 60	4.76
MIBK	Tributyrin	20 : 80	46.5
		40 : 60	4.22

Note: reactive extraction mixtures are composed of a reactive extractant (conc. 1 to 50% *v/v*) and a highly biocompatible diluent.

The most promising biocompatible solvent mixtures were evaluated for their extraction efficiency of pABA, PCA, AA and 2-PE (Table 3). The biocompatible mixtures with PPG1000 as a diluent showed the highest distribution coefficients, however, difficulties in phase separation were observed. Alternatively, for all three organic acids, good extractability ($K_d > 1.5$) was obtained with biocompatible mixtures of 12.5% CYTOP 503 in canola oil or sunflower oil FAME, and 25% CYPHOS IL-101 or CYPHOS IL-104 in oleyl alcohol. The biocompatible mixture of 25% TBP in oleyl alcohol showed the highest extraction capacity for 2-PE but was found less effective for the organic acids.

Table 3. Distribution coefficients of the most promising biocompatible solvent mixtures for reactive extraction of *para*-aminobenzoic acid (pABA), protocatechuic acid (PCA), adipic acid (AA), and 2-phenylethanol (2-PE).

Extractant	**Diluent**	**Extractant Ratio (% *v/v*)**	**Distribution Coefficient (K_d)**			
			pABA	**PCA**	**AA**	**2-PE**
CYPHOS IL-101	Oleyl alcohol	5	1.90	2.52	0.50	11.6
		12.5	2.42	8.03	1.03	12.7
		25	4.83	19.1	1.53	15.6
CYPHOS IL-104	Oleyl alcohol	5	1.63	1.26	0.45	10.9
		12.5	1.95	3.39	0.66	11.4
		25	3.05	14.1	1.54	13.0
	PPG1000	2.5	8.51	13.9	1.74	20.3
Aliquat 336	Oleyl alcohol	2.5	1.57	1.02	0.37	10.8

Table 3. *Cont.*

Extractant	Diluent	Extractant Ratio (% *v/v*)	Distribution Coefficient (K_d)			
			pABA	PCA	AA	2-PE
Tributyl phosphate	Canola oil	15	1.43	1.04	0.00	8.24
	Oleyl alcohol	12.5	1.74	0.92	0.24	13.1
		25	3.29	2.60	0.47	16.2
	PPG1000	12.5	8.06	14.1	1.54	23.8
	Sunflower oil FAME	5	0.91	0.18	0.04	1.88
		12.5	2.27	0.82	0.17	1.87
CYTOP 503	Canola oil	12.5	5.99	17.7	2.05	11.7
	Oleyl alcohol	12.5	2.34	1.43	0.36	12.1
	PPG1000	12.5	9.53	19.3	2.60	23.9
	Sunflower oil FAME	5	2.65	2.03	0.65	2.29
		12.5	7.90	14.0	2.25	3.43

Note: reactive extraction mixtures are composed of a reactive extractant (conc. 5 to 25% *v/v*) and a highly biocompatible diluent.

4. Discussion

Overcoming product inhibition in fermentation through in situ solvent extraction has gained increasing attention over the past decade owing to its potential to alleviate product inhibition and its readily scalable process design [10]. When selecting an appropriate solvent, biocompatibility, next to extraction efficiency, is one of the decisive factors when developing an ISPR process [24]. Currently, a comprehensive overview of the applicability, both in terms of biocompatibility and extraction capacity, of different solvent classes for a variety of target compounds is not at hand. Yet such an overview is crucial to push industrialization of a large number of novel or drop-in bio-based compounds whose efficient production is currently hampered by their inherent toxicity. Therefore, this study aims to close this gap by mapping the potential of the principal solvent classes for several relevant classes of industrial platform chemicals. To allow screening of an extensive array of solvents, deepwell plates were used. Owing to the higher data output achieved through deepwell plates, this type of microtiter plate (MTP) has enabled faster development times for bioprocesses [25]. After this initial screening, promising solvent mixtures were evaluated, combining highly biocompatible diluents with the most potent extractants.

The potential interaction of a solvent with the cell membrane and its ability to enter the cell mainly determines its biocompatibility. In that respect, molecular size has a considerable impact as large, non-polar molecules, e.g., long-chain alkanes, are rather inert to the cell membrane, while smaller molecules, e.g., <C12 alkanes, can more easily migrate into the membrane, affecting its integrity and causing growth inhibition [26]. Indeed, owing to their biocompatibility, larger alkanes such as dodecane (C12) have been applied as biocompatible diluents for the extraction of organic acids and monoterpenes [15,27,28]. Besides this, while the addition of a functional group such as a hydroxyl group increases solvent-membrane interaction and thus limits biocompatibility, the proton-donating effect of alcohols assists in solvating organic acids, resulting in an improved extraction of these compounds as compared to their extraction using alkanes [29,30]. This was validated by comparing dodecanol with dodecane, where the addition of a hydroxyl group was found to decrease biocompatibility but increase extraction efficiency for pABA by a factor of 37. In view of this, oleyl alcohol (C18) presents an interesting solvent exhibiting high biocompatibility and improved extraction capacity as compared to hexadecane, a slightly smaller (C16) alkane (Table 1). Indeed, oleyl alcohol has, for example, been applied as an effective solvent for in situ removal of fermentation inhibitors such as acetic acid or furfural to increase ethanol productivity [31]. Even though ketones such as methyl isobutyl ketone (MIBK) are commonly used diluents for the reactive extraction of organic acids [32,33],

all ketones evaluated in this study showed poor biocompatibility with *S. cerevisiae*. This demonstrates the potential for improvement of these processes using alternative diluents, e.g., esters, which show good biocompatibility. As an example, isopropyl myristate has been used as a diluent with trioctylamine (TOA) for in situ reactive extraction of itaconic acid [34]. Polypropylene glycol (PPG) polymers such as PPG4000 have been described as biocompatible, which was confirmed in this study, resulting from a reduced interaction of such high molecular weight compounds with microbial cells [35]. Owing to their relatively hydrophilic properties, PPG1000 up to PPG4000 showed the best extraction efficiencies of all biocompatible solvents evaluated in this study. However, their inherent high viscosity, and as a result, difficult phase separation, limits their practical and industrial feasibility.

Reactive extractants, such as amines and phosphorous compounds, are highly effective extracting agents as they interact with target compounds and form a complex that is more readily extracted to the organic phase. However, they typically also show high toxicity and are therefore commonly used with a biocompatible diluent, e.g., vegetable oils, which act as a protective layer [36–38]. However, the viscosity of oils and their tendency to form emulsions with typical fermentation media may hinder their applicability [39]. Owing to their low price, good availability, low viscosity and biocompatibility, FAME or biodiesels show high potential as renewable diluents and have been used as more economical alternatives to oleyl alcohol for ISPR of butanol or as a diluent in phenol extraction [40–43]. In this study, high extraction capacity was found for castor oil FAME and results from the predominance of ricinoleic acid methyl ester, a C18 FAME containing a hydroxyl group [39]. In the past two decades, substantial research efforts have been put in studying the biocompatibility of ionic liquids (ILs). These alternative solvents have been emerging as promising green solvents with good biocompatibility and extraction potential for organic compounds [23,44]. Additionally, ILs can be extensively fine-tuned and optimized by adjusting the cationic and/or anionic parts. Though as the mechanisms behind ILs and their properties are still not fully understood, this complexity renders ILs highly unpredictable, as observed from the variability in biocompatibility and extraction for the ILs in this study. Additionally, their high price and viscosity are important drawbacks.

The fragrance compounds *trans*-cinnamaldehyde (CA), geraniol (GE), and β-ionone (ION) are poorly soluble in water though show very strong toxicity towards micro-organisms and thus extreme product inhibition in fermentations. The fact that they were well extracted by the majority of the solvents makes them ideal compounds for ISPR. A comprehensive list of biocompatible solvents is presented in Table 1 and can serve as a practical guideline for designing an ISPR process. Specific solvent selection can then be based on economic and environmental considerations, besides (back-)extraction efficiency. On the contrary, to extract hydrophilic compounds such as organic acids and alcohols, the selection of a suitable solvent has proven more challenging given their limited extractability with common organic solvents. To effectively extract these compounds, advanced strategies such as solvent mixtures and reactive extraction techniques are required. From the results of the initial screening of pure solvents, mixtures were composed and evaluated for their biocompatibility and extraction of *para*-aminobenzoic acid (pABA), protocatechuic acid (PCA), adipic acid (AA), and 2-phenylethanol (2-PE).

Biocompatibility was found to decrease with an increasing extractant concentration. Yet, interestingly, biocompatibility was also strongly impacted by the diluent used. Oleyl alcohol emerged as the most promising diluent to relieve the toxicity of extractants. With the exception of Aliquat 336, using oleyl alcohol as a diluent resulted in biocompatible solvent mixtures for extractant concentrations up to 12.5%. While Aliquat 336 and TOA have commonly been regarded as efficient extractants and their application in ISPR has been widely studied, this study shows that these extractants show poor biocompatibility. As a result, their use in situ is restricted to low concentrations and, in turn, the extraction efficiency of such mixtures is limited. With the use of PPG1000 as a diluent, superior extraction efficiencies were consistently obtained. However, difficulties in phase separation render the use of PPG1000 impractical with respect to technical feasibility and scaling as

compared to other diluents. Due to the higher price and lower availability of castor oil FAME, sunflower oil FAME was chosen as a broadly applicable diluent, despite its lower extraction efficiency for all compounds in this study. Sunflower oil FAME with 12.5% of the phosphine oxide-based CYTOP 503 has proven to be the most effective biocompatible solvent mixture for the extraction of pABA and AA, whereas the solvent mixture of 25% TBP in oleyl alcohol was found to have the highest extraction efficiency for 2-PE. With a distribution coefficient of 19.1, the biocompatible mixture consisting of 25% CYPHOS IL-101 in oleyl alcohol showed the highest extraction efficiency for PCA.

Although several pure solvents were identified as potent ISPR candidates for hydrophobic fragrance compounds, high-performing reactive solvent mixtures were required for the extraction of more challenging compounds. The composed mixtures showed higher distribution coefficients than any pure biocompatible solvent, thus demonstrating the potential of reactive extraction for ISPR. By providing a comprehensive list of biocompatible solvents and solvent mixtures, completed with their respective extraction efficiencies for different industrially interesting compounds, this research has laid the foundation for the development of efficient production processes for a wide range of bio-based products where product inhibition is currently limiting industrial feasibility and commercialization.

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