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Organic phase screening for in-stream reactive extraction of bio-based 3-hydroxypropionic acid: biocompatibility and extraction performances

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Ana Karen Sanchez-Castañeda, Marwen Moussa, Luther Ngansop, Ioan-Cristian Trelea, Violaine Athès. Organic phase screening for in-stream reactive extraction of bio-based 3-hydroxypropionic acid: biocompatibility and extraction performances. *Journal of Chemical Technology and Biotechnology*, 2020, 7th European Bioremediation Conference (EBCVII), 95 (4), pp.1046-1056. 10.1002/jctb.6284 . hal-02500627

HAL Id: hal-02500627

<https://agroparistech.hal.science/hal-02500627>

Submitted on 6 Mar 2020

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1 **RESULTS:** Inert diluent addition to a trioctylamine (TOA)-decanol mixture decreased
2 its toxicity and viscosity but decreased extraction yield. The linear and ramified long-
3 chain alcohols tested showed that increasing the number of carbon atoms decreased
4 extraction performance as well as toxicity. Ramified alcohols showed the lowest
5 extraction performance. Didodecylmethylamine (DDMA) gave higher extraction yield
6 and lower solvent toxicity than TOA. Flow cytometry with dual staining for cell
7 membrane integrity and enzymatic activity proved to give concordant and
8 complementary information with cells bioconversion ability, being an adequate and
9 quick method for solvent toxicity assessment. The selected organic phase consisted of
10 20% DDMA, 47% dodecanol and 33% dodecane by volume, and can be used for in
11 stream extraction of 3-HP produced by *L. reuteri*.

12 **CONCLUSIONS:** The integrated selection criteria proposed in this study - extraction
13 yield, solvent viscosity and toxicity - provide key information for choosing an organic
14 phase with the best trade-off between extraction performance and biocompatibility.

15 **Keywords:** In stream reactive extraction, Bioconversion, Biocompatibility, Flow
16 cytometry, *Lactobacillus reuteri*, 3-hydroxypropionic acid (3-HP).

1 INTRODUCTION

2 Since fossil resource reserves are declining and because of their serious impact on the
3 environment, the need to move from the current fossil-based economy to a more
4 sustainable one using renewable resources has significantly increased. For the chemical
5 industry, this has generated an increasing interest in developing new processes for bio-
6 based chemical production. 3-hydroxypropionic acid (3-HP) was identified by the U.S.
7 Department of Energy (DoE) as one of the value-added chemicals that can be obtained
8 from biomass with the potential to be a key building-block.^{1,2} This molecule has two
9 functional groups that confer reactivity properties suitable for obtaining a wide range
10 of molecules of interest such as acrylic acid and bio-based polymers. Following this
11 report, research on 3-HP bio-production received a significant boost and has made
12 remarkable advances in the past few years.³

13 Among the few microorganisms that can naturally produce 3-HP, *Lactobacillus*
14 *reuteri*⁴ is able to perform the bioconversion of glycerol, yielding only 3-HP and 1,3-
15 propanediol (1,3-PDO).⁵ Although this pathway passes through 3-
16 hydroxypropionaldehyde (3-HPA) production as an intermediate, which is toxic for the
17 strain,⁶ its accumulation in the medium can be avoided by progressively supplying
18 glycerol.⁷ This pathway has the advantage that no other by-products are present, which
19 simplifies further purification of the desired product. However, one of the main
20 drawbacks of the process is inhibition by 3-HP accumulation,⁸ resulting in low
21 bioconversion performance with the best results so far being an overall process
22 productivity of 0.25 g/L·h and a final titre of 14 g/L,⁷ which is insufficient for
23 industrial-scale production.¹

1 *In-situ* or in stream product recovery (ISPR) is a promising strategy for the
 2 intensification of processes affected by end-product inhibition, such as glycerol
 3 bioconversion to 3-HP.^{9,10} Liquid-liquid extraction is frequently used in the
 4 downstream recovery of bioconversion products. Depending on the characteristics and
 5 needs of the bioproduction process, there are several configurations for putting both
 6 phases in contact (Figure 1).

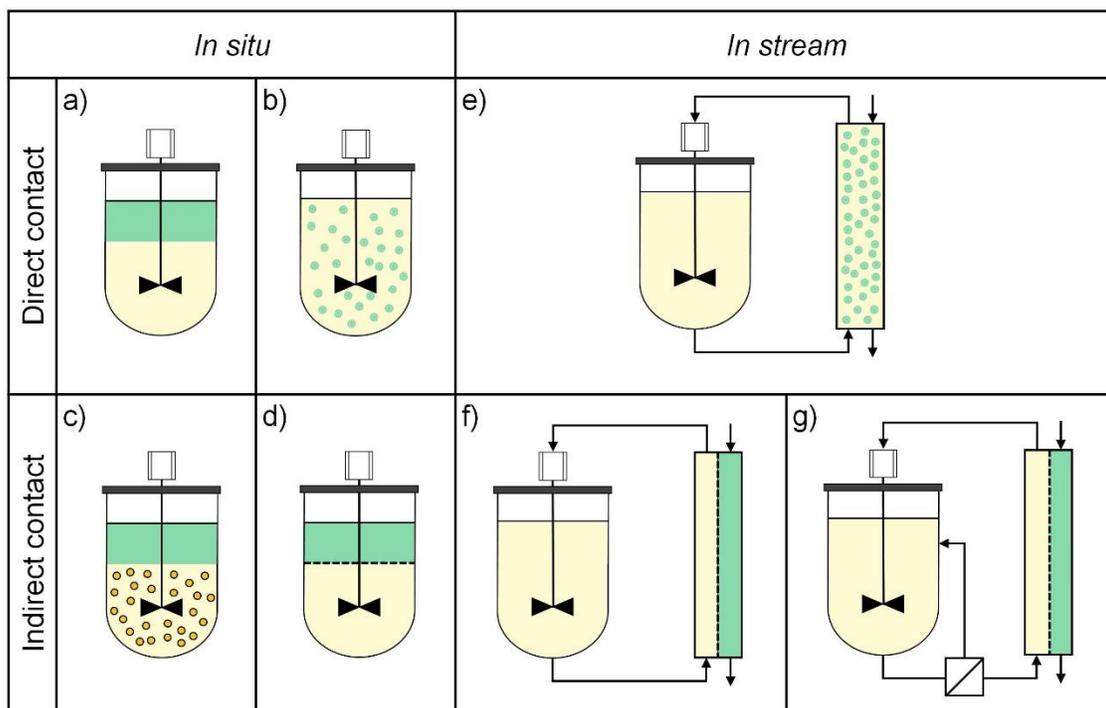


Figure 1. Different configurations for liquid-liquid extraction of compounds produced in bioreactors. *In situ* extraction occurs with a direct contact between the cells and the extraction phase: (a) two-phase partitioning bioreactors;^{33,50,51} (b) solvent-impregnated particles dispersed in bioconversion medium^{52,53} or with an indirect contact; (c) using immobilized cells in the bioconversion broth;^{47,54} and (d) using pertraction inside the bioreactor.^{31,55} In stream extraction with direct contact: (e) pumping bioconversion broth to a column packed with solvent-impregnated particles⁵⁶ and indirect contact; (f) pertraction outside the bioreactor;^{57,58} and (g) introducing a microfiltration unit to separate the biocatalyst from broth before contact.^{59,60}

7

1 Because of the hydrophilic nature of short-chain carboxylic acids, and especially the
2 hydroxy acids, traditional liquid-liquid extraction suffers from poor extraction yield.
3 Therefore, reactive extraction has been extensively studied for removal of organic acids
4 from aqueous media^{11,12} where the organic phase contains an extractant molecule able
5 to react with the acid. There are different types of extractants, but tertiary amines are
6 among the most effective thanks to their high extraction capacity, high selectivity and
7 low water solubility.¹³⁻¹⁶ Tertiary amines react with the non-dissociated form of the
8 acid and create an acid-base complex that is insoluble in the aqueous media. In order
9 to stabilise the complex in the organic phase and enhance the extraction yield, an active
10 diluent with a functional group able to interact with the acid-base complex is needed.
11 Long-chain alcohols are among the best active diluents due to their polarity and specific
12 H-bond donor character that favours their complex formation and solvation. This has
13 already been validated for 3-HP extraction¹⁷ and other organic acids.^{14,18,19} The
14 feasibility of 3-HP reactive extraction with different mixtures of extractants and active
15 diluents has been demonstrated and the extraction mechanism has been thoroughly
16 studied.^{17,20,21} Previous studies of 3-HP reactive extraction showed that an organic
17 phase made up of 20% (v/v) TOA and 80% decanol provided high extraction yield and
18 selectivity.^{9,20} However, its application for the ISPR of 3-HP during its production by
19 *L. reuteri* had a strong inhibitory effect on the cells, resulting in reduced 3-HP
20 production (56% of the total production compared to the process without ISPR), even
21 though the liquid-liquid extraction was assisted by a Hollow Fibre Membrane
22 Contactor (HFMC) that avoids the direct contact of the organic phase with the cells.⁸
23 The toxicity of solvents used for reactive extraction is often reported in the literature
24 concerning bacteria.²²⁻²⁴ It has been suggested that the addition of a biocompatible but

1 poor extractive solvent (inert solvent) to the active alcohol-type diluent can improve
2 the biocompatibility of the extracting phase while maintaining an adequate extraction
3 performance.^{24–28}

4 Solvents may affect cells at two different levels: by direct contact with the immiscible
5 part of the solvent (phase-level toxicity) and interaction with the water-soluble solvent
6 molecules (molecular-level toxicity).^{26,29,30} The toxicity of several organic solvents
7 commonly used for the reactive extraction of carboxylic acids has been assessed on
8 different strains of microorganisms,^{22,27,29,31,32} but the variable and often contradictory
9 results suggest that the selection of a biocompatible extraction phase strongly depends
10 on the microorganism strain used. Solvent selection according to the particular needs
11 of an ISPR strategy is therefore a key issue.

12 In comparison to other solvent screening studies, where the effect of different types of
13 organic solvents has been studied, either on the extraction yield^{17,33} or its toxicity
14 towards the producing microorganism only,^{27,29} this work describes an integrated
15 approach that considers the extraction performance (yield and viscosity) and toxicity
16 of the extraction phase towards microorganisms. The strategy consisted first in
17 evaluating the extraction yield at a given temperature and fixed initial 3-HP
18 concentration. The viscosity of the organic phases was then measured and taken into
19 account in the screening strategy because it affects mass transfer and, consequently,
20 extraction process performance. Since the mass transfer in a HFMC is mainly governed
21 by diffusion in membrane pores,³⁴ a low viscosity would lead to faster 3-HP
22 extraction.³⁵ This first approach provided a rapid screening of many solvents, the most
23 promising of which were retained for toxicity studies. Solvent toxicity was evaluated

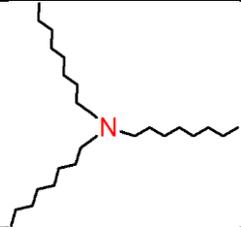
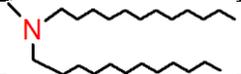
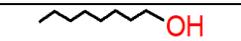
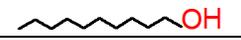
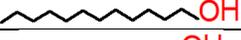
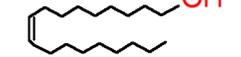
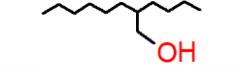
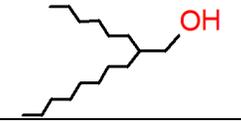
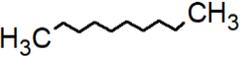
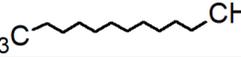
1 by molecular-level toxicity assessment, like for in stream extraction, since the use of a
2 HFMC avoids direct contact between the solvent and the microorganisms.^{36,37} Two
3 approaches were proposed: 1) flow cytometry was used with the dual staining of cells
4 as a novel and quick method to evaluate solvent toxicity, through enzymatic activity
5 and membrane integrity assessment; and 2) *L. reuteri*'s ability to convert glycerol into
6 3-HP was tested in an aqueous phase previously saturated with the soluble fraction of
7 the studied solvents. This strategy is proposed as a tool to find a compromise between
8 extraction performance and solvent toxicity in order to select an organic phase that
9 allows continuous recovery of 3-HP produced by bioconversion in an in stream
10 process.

11 **EXPERIMENTAL**

12 **Organic solvents for reactive extraction**

13 Commercial Trioctylamine (TOA, Sigma-Aldrich, Saint Louis, MO, USA) was
14 purified as described in a previous study¹⁷ and subsequently used as an *extractant* and
15 mixed with different long-chain alcohols as *active diluents*: decanol (Sigma-Aldrich,
16 Saint Louis, MO, USA) octanol, dodecanol, 2-butyl-1-octanol, 2-hexyl-1-decanol and
17 oleyl alcohol; and alkanes as *inert diluents*: decane and dodecane (TCI Europe,
18 Zwijndrecht, Belgium). Didodecylmethylamine (TCI Europe, Zwijndrecht, Belgium)
19 was also evaluated as an *extractant*, purified in the same way as TOA and diluted at
20 different concentrations with decanol in order to compare its extraction performance
21 with TOA. Each mixture was evaluated in terms of its 3-HP extraction performance
22 and its toxicity to the selected *Lactobacillus reuteri* strain. Table 1 shows the
23 physicochemical properties of the extractants and solvents used in this study.

Table 1. Physicochemical properties of tested extractants and solvents.⁴⁹

Compound	Purity %	CAS number	Number of carbons	Log P _{o/w}	Solubility in water (mg/L) at 25°C	Molecular weight (g/mol)	Density (g/mL)	Chemical structure
Extractants								
Trioctylamine	99.7	1116-76-3	24	11.22	0.05	353.7	0.809	
N,N-Didodecylmethylamine	99.2	2915-90-4	25	8.76	-	367.7	0.820	
Active diluents: alcohols								
Octanol	99.5	111-87-5	8	2.68	540	130.2	0.826	
Decanol	98.9	112-30-1	10	4.57	37	158.3	0.829	
Dodecanol	99.6	112-53-8	12	5.13	4	186.3	0.831	
Oleyl alcohol	75.3	143-28-2	18	7.40	0.07	268.5	0.848	
2-Butyl-1-Octanol	99	3913-02-8	12	4.80	16.18	186.3	0.833	
2-Hexyl-1-Decanol	99	2425-77-6	16	7.10	0.1727	242.4	0.836	
Inert diluents: alkanes								
Decane	99	124-18-5	10	5.01	0.052	142.3	0.730	
Dodecane	99	112-40-3	12	6.10	0.0037	170.3	0.748	

1 **Extraction performance evaluation**

2 *Extraction yield*

3 3-HP solutions at 5 g/L (0.055 mol/L) were prepared from a commercial solution at
4 28.9% w/w (TCI Europe, Zwijndrecht, Belgium). The organic phases were prepared
5 by mixing the purified TOA with the different alcohols and alkanes, then washed with
6 an equal volume of deionised water and separated by centrifugation at 15000×g and
7 25°C for 20 min. Ten mL of the aqueous 3-HP solution and organic phase were put in
8 contact, vigorously shaken and then left to achieve equilibrium at 25°C for 48 h. They
9 were subsequently separated by centrifugation as described above. The volume ratio
10 between the aqueous and organic phases for yield determination was 1:1 throughout
11 this study.

12 In addition, an aqueous phase composed of 5 g/L 3-HP, 5 g/L 1,3-PDO and
13 *Lactobacillus reuteri* cells was prepared. This composition mimics the real medium in
14 glycerol bioconversion into 3-HP and 1,3-PDO by *Lactobacillus reuteri*.⁸ This aqueous
15 phase was put in contact finally with the selected organic phase, for 3-HP extraction
16 yield determination.

17 *HPLC analysis*

18 The aqueous phase was recovered and analysed by High Performance Liquid
19 Chromatography (HPLC). Citric acid (10 g/L in deionised water) was used as an
20 internal standard and added at 50% (v/v) to every sample just before analysis.
21 Separation was performed using a Biorad Aminex HPX-87H column (300 mm×7.8
22 mm; Biorad, Richmond, VA, USA) equipped with a cation H⁺ Micro-Guard column

1 (30 mm×4.6 mm; Biorad), as previously described.²⁰ Extraction yield was calculated
2 with Equation 1 (volume ratio 1:1):

$$Y\% = \frac{[AH]_{HPLC}^{ini} - [AH]_{HPLC}^{eq}}{[AH]_{HPLC}^{ini}} \times 100\% \quad (1)$$

3 where $[AH]_{HPLC}^{ini}$ is the total initial acid concentration in the aqueous phase and
4 $[AH]_{HPLC}^{eq}$ the acid concentration at equilibrium, as measured by HPLC. This value is
5 related to the distribution coefficient K_D of the acid according to Equations 2 and 3.

$$K_D = \frac{[AH]_{org}^{eq}}{[AH]_{aq}^{eq}} = \frac{[AH]_{HPLC}^{ini} - [AH]_{HPLC}^{eq}}{[AH]_{HPLC}^{eq}} \quad (2)$$

$$Y\% = \frac{K_D \times \frac{V_{org}}{V_{aq}}}{1 + K_D \times \frac{V_{org}}{V_{aq}}} \times 100\% \quad (3)$$

6 where the volume ratio of the organic and the aqueous phase $\frac{V_{org}}{V_{aq}} = 1$.

7 *Viscosity of the organic mixtures*

8 The viscosity of each organic phase mixture was measured with a cone and plate
9 rheometer (Rheostress 600, Thermo Scientific), using a linear increase of shear rate
10 from 0 to 100 s⁻¹ at 25°C. Measurements were made in triplicate.

11 **Solvent toxicity evaluation**

12 *Microorganism*

13 *Lactobacillus reuteri* DSM 17938 was obtained from BioGaia AB (Stockholm,
14 Sweden). Cells were grown in batch mode at 37°C in MRS broth, as previously
15 described,⁸ for 16 h until the beginning of the stationary phase, from an initial optical

1 density (OD) of 1.67×10^{-5} (around 4×10^3 cells/mL) to a final OD of 6 (around 1.5×10^9
2 cells/mL).

3 *Cell preparation for toxicity tests*

4 The culture medium was centrifuged at $5000 \times g$ and 4°C for 10 min. The supernatant
5 was then discarded and the cell pellet was washed once with sterilised reverse osmosis
6 (RO) water and re-suspended to a final OD of 70 (1.8×10^{10} cells/mL) in RO water.

7 *Evaluation of solvent molecular toxicity by flow cytometry*

8 Equal volumes of sterilised RO water and of each organic phase were mixed and left
9 to achieve equilibrium at 25°C for 48 h. The phases were then separated by
10 centrifugation at $15000 \times g$ for 20 min at 25°C and the aqueous phase saturated with the
11 soluble fraction of the solvents was recovered. Around 1.2 mL of the washed cell
12 suspension was diluted in 20 mL of the aqueous phase for an initial concentration of
13 1.2×10^9 cells/mL (OD of 4) and placed in optimal living conditions at 37°C and
14 agitated at 100 rpm. Samples were taken at time 0, after 30 min, and every hour for 5
15 hours, to evaluate the physiological state of the cells by flow cytometry. A control
16 experiment was made in parallel for each solvent tested, placing the cells at 37°C and
17 100 rpm in pure RO water (without previous contact with the solvent). Experiments
18 were made in duplicate.

19 *Flow cytometry*

20 Cell enzymatic activity was evaluated through esterase activity assessment using the
21 Carboxyfluorescein diacetate (cFDA) contained in the commercial solution
22 Chemchrom V8 (Biomérieux, Marcy l'Étoile, France) diluted in acetone at 10%. The

1 loss of membrane integrity was also evaluated using the dye propidium iodide (PI)
 2 (Sigma-Aldrich, Lyon, France). This dual staining and subsequent analysis in a flow
 3 cytometer using FloMax© 2.52 software (Partec, 2007), as previously described,⁴
 4 made it possible to distinguish between the viable (Q4, stained with cFDA), altered
 5 (Q2, stained with cFDA and PI) and dead cells (Q1, stained with PI) in the samples
 6 (Figure 2).

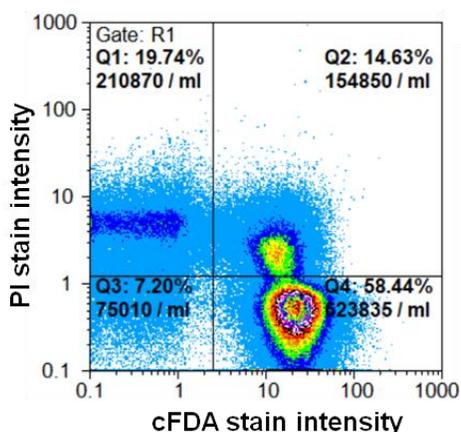


Figure 2. Cytogram of *L. reuteri* using a dual stain with propidium iodide (PI) and carboxy fluorescein diacetate (cFDA). Q1: Dead cells (stained with PI); Q2: altered cells (PI and cFDA); Q3: unstained particles; and Q4: viable cells (cFDA).

7 In order to compare the impact of the different solvents on the physiological state of
 8 the cells, a parameter referred to as Excess Mortality Rate (EMR) compared to control
 9 conditions was calculated (h^{-1}). To do this, the concentration of viable cells (stained
 10 with cFDA) in the solvent-saturated aqueous phase (X) was divided by the
 11 concentration of viable cells in the control (X^*). These values were plotted vs. time,
 12 and Equation 4 was adjusted to the data:

$$\frac{X}{X^*} = \frac{X_0}{X_0^*} e^{-EMR \cdot t} \quad (4)$$

1 where $\frac{X_0}{X^*_0}$ is the fraction of viable cells at time 0.

2 *Assessment of 3-HP production ability*

3 Bioconversions of 5 g/L of glycerol solubilised in 100 mL of RO water previously
4 saturated with the organic phases were performed in Schott bottles at 37°C and 100
5 rpm. Aqueous solutions were prepared as described in the molecular-level toxicity
6 evaluation. For each set of experiments, a control was performed simultaneously with
7 a bioconversion with glycerol at 5 g/L in RO water. Samples were taken at the initial
8 time, after 30 min, and every hour for 5 hours. Experiments were made in triplicate,
9 samples were centrifuged and filtered to eliminate cells and then analysed by HPLC to
10 measure glycerol, 3-HP, 1,3-PDO and 3-HPA concentration, as described above.
11 Glycerol and 1,3-PDO were purchased from Sigma-Aldrich (Lyon, France), 3-HP from
12 TCI Europe (Zwijndrecht, Belgium), and 3-HPA was synthesized as described by
13 Burgé *et al.*³⁸

14 *Bioconversion parameter determination*

15 Glycerol consumption and product formation data was fitted to an exponential function
16 according to Equation 5:

$$P = a(1 - e^{-kt}) \quad (5)$$

17 This equation describes an increasingly asymptotic behavior that approaches an upper
18 limit. It appears appropriate to describe a production affected by inhibition, as is the
19 case of glycerol bioconversion to 3-HP. The parameter a represents the maximum
20 produced concentration (mol/L), and k (h^{-1}) is related to the maximum production rate:

1 the first derivative of Equation 3 gives the production/consumption rate r , which is
2 maximal at $t=0$ (Equation 6):

$$r_0 = P'(0) = ak \quad (6)$$

3 In order to compare the different sets of experiments, the parameters a , k and r_0 for
4 each tested organic phase were divided by their respective values obtained for the
5 control (a^* , k^* and r_0^*), corresponding to bioconversion without contact with the
6 solvents.

7 *Statistical analysis*

8 Statistical significance of differences between data was assessed using analysis of
9 variance (ANOVA) tests at a 0.05 level.

10 **RESULTS AND DISCUSSION**

11 **Effect of inert diluent addition on extraction performances and toxicity towards**

12 ***L. reuteri* cells**

13 The first step in solvent screening was to determine the effect of concentration and the
14 type of inert diluent addition on 3-HP extraction performance and toxicity. The organic
15 phase was composed of 20% (v/v) of TOA as the extractant, diluted in 80% decanol as
16 the active diluent or in a mixture containing 20%, 40% or 60% of decanol, where the
17 remaining part was either decane or dodecane as the inert diluent.

18 *Extraction performance*

19 It appears from Figure 3a that increasing decane and dodecane concentration
20 dramatically decreases the extraction yield. There is no significant difference ($p>0.05$)
21 in extraction yield using either of the two inert diluents at the same concentration.

1 Figure 3b shows that decane or dodecane addition reduces the viscosity of the mixture,
 2 with relatively minor differences between decane and dodecane. It should be recalled
 3 that low viscosities are desirable for accelerating mass transfer.³⁵ A compromise
 4 between low viscosities and high extraction yield is quite difficult to find when looking
 5 at Figure 3b. Nevertheless, following the ideal tendency of the solvent properties (high
 6 extraction yield and low viscosity) shown on Figure 3b, the use of either decane or
 7 dodecane at 20 and 40% seems to be the most promising composition. Indeed, the
 8 extraction yield of the mixture with 60% of inert diluent is very low, and the mixture
 9 of 20% TOA and 80% decanol has proven to be toxic for *L. reuteri*.⁸ For this reason,
 10 toxicity to the producing strain was further studied.

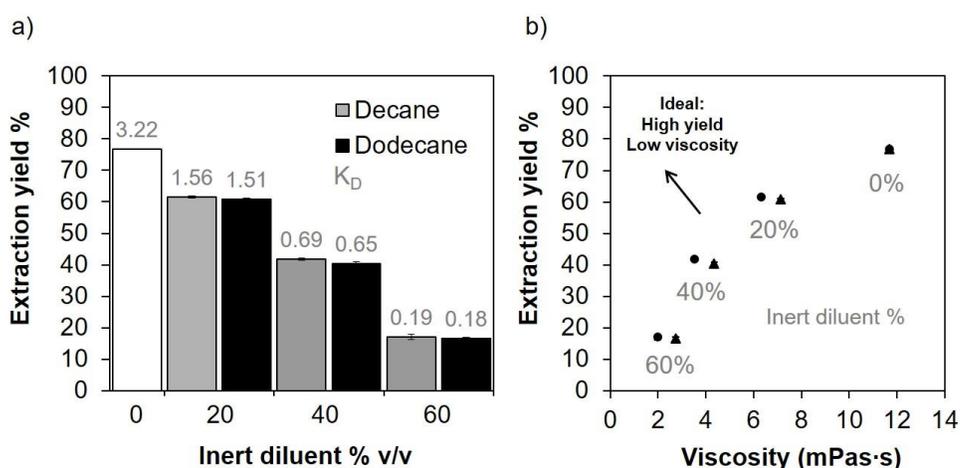


Figure 3. Effect of inert diluent addition to a TOA-decanol mixture on 3-HP extraction yield and organic phase viscosity. (a) Extraction yield (volume ratio 1:1) with corresponding distribution coefficient K_D , and (b) relationship between extraction yield and viscosity for decane (●) and dodecane (▲) at different concentrations (% v/v). Initial [3-HP] = 5 g/L. Error bars are masked by symbols.

11 Solvent toxicity

12 For this study, dodecane was selected as the inert diluent regarding its Log P value
 13 (6.10), which is higher than that of decane (Log P = 5.01, Table 1). Indeed, the results
 14 in the literature on solvent toxicity towards microorganisms often link the toxicity level

1 to the Log P of the solvent,³⁹ and dodecane is therefore expected to be less toxic. In
 2 order to choose the adequate dodecane concentration, the molecular toxicity of the
 3 mixtures was evaluated by flow cytometry. Figure 4 shows the physiological state of
 4 *L. reuteri* in contact with TOA-decanol mixtures containing 0, 20 and 40% of
 5 dodecane. Figure 4a shows how the number of *L. reuteri* cells with different
 6 physiological states after molecular contact with the mixture of 20% (v/v) TOA, 40%
 7 decanol and 40% dodecane varies over time compared to the control. It appears that
 8 the number of viable cells X^* essentially remains constant in the control, whereas the
 9 number of viable cells X decreases when in contact with the solvent molecules. Figure
 10 4b shows the X/X^* ratio vs. time for three dodecane concentrations between 0 and 40%
 11 in mixture with TOA and decanol, and compares them using the EMR value. As
 12 expected, the addition of dodecane to the TOA-decanol mixture makes it possible to
 13 significantly decrease its toxicity towards *L. reuteri*. However, even with 40%

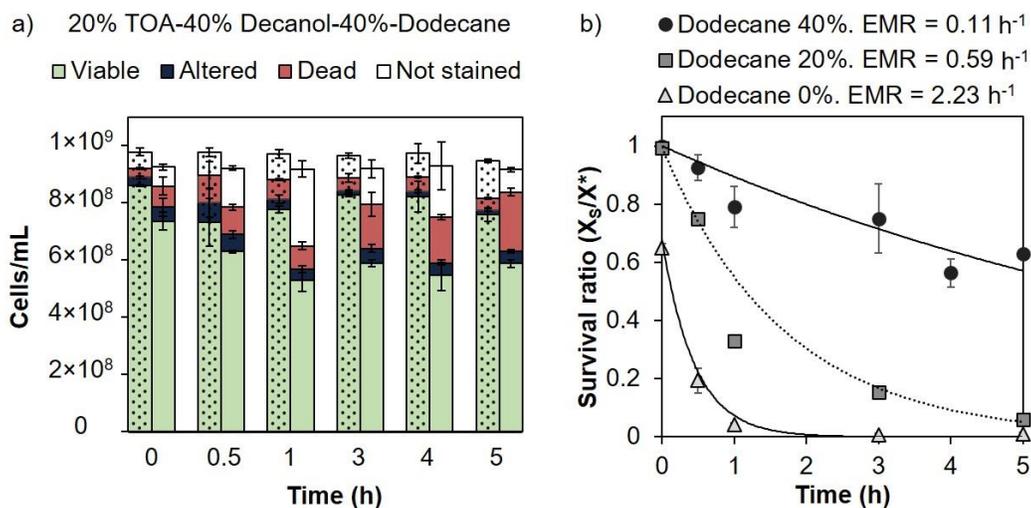


Figure 4. Evolution of the physiological state of *L. reuteri* in contact with three organic phases. (a) Number of stained cells analysed by flow cytometry: Control with *L. reuteri* suspended in RO water (dotted bars, X^*) and molecular-level toxicity of a 20% TOA-40% decanol-40% dodecane mixture (plain bars, X_S). (b) Effect of dodecane addition to the mixture of TOA and decanol on cell viability.

1 dodecane, a significant viability loss after 5 h was observed, even though the EMR
 2 value is lower compared to the other conditions. Decanol seems to be responsible for
 3 this loss in viability since the TOA concentration is the same in all of these experiments.
 4 Based on these results, the mixture of 20% TOA, 40% decanol and 40% dodecane was
 5 chosen as the reference composition for the next step in order to compare different
 6 active diluents to replace the decanol.

7 **Evaluation of the replacement of decanol with alternative long-chain active**
 8 **diluents**

9 In a previous study it was determined that long-chain alcohols were the best active
 10 diluents for 3-HP reactive extraction with TOA thanks to their H-bond donor
 11 characteristic that provides good stabilisation of the acid-amine complex in the organic
 12 phase.¹⁷ Thus, alcohols of different carbon chain lengths were evaluated while
 13 maintaining the same TOA/alcohol molar proportion as in the reference composition
 14 to make a stoichiometric comparison of extraction yields, the rest being completed with
 15 dodecane (Table 2). Oleyl alcohol was also evaluated at 80% with TOA without the
 16 addition of dodecane.

Table 2. Organic phase composition for active diluent evaluation.

#	Active diluent				TOA		Dodecane	
	Name	Carbon number	% (v/v)	mol/L	% (v/v)	mol/L	% (v/v)	mol/L
1	Octanol	8	33	2.10	20	0.46	47	2.06
2	Decanol	10	40	2.10	20	0.46	40	1.75
3	Dodecanol	12	47	2.10	20	0.46	33	1.45
4	2-Butyl-1-octanol	12	47	2.10	20	0.46	33	1.45
5	2-Hexyl-1-decanol	16	61	2.10	20	0.46	19	0.85
6	Decanol	10	80	4.20	20	0.46	-	-
7	Oleyl alcohol	18	80	2.51	20	0.46	-	-

1 Table 3 shows the molecular toxicity of each mixture sorted according to its EMR value
 2 and its survival ratio after 5 h of contact with aqueous solutions equilibrated with the
 3 selected solvent mixtures. Similar to the classification proposed by Marták *et al.*,⁴⁰ we
 4 can consider a biocompatible solvent with a survival ratio higher than 0.75, a toxic
 5 solvent with a value lower than 0.25 and a medium toxicity in between.

Table 3. Molecular level toxicity of the selected solvent mixture with 20% TOA and dodecane.

#	Active diluent	% (v/v)	[mol/L]	Excess Mortality Rate (EMR) h ⁻¹	Survival ratio X/X* at 5 h	Toxicity classification ⁴⁰
5	2-Hexyl-1 decanol	61	2.1	0.004 ± 0.002	0.833 ± 0.027	Low
7	Oleyl alcohol	80	2.5	0.039 ± 0.011	0.867 ± 0.126	Low
2	Decanol	40	2.1	0.112 ± 0.004	0.566 ± 0.011	Medium
3	Dodecanol	47	2.1	0.146 ± 0.019	0.501 ± 0.007	Medium
6	Decanol	80	4.2	2.231 ± 0.205	0.013 ± 0.006	High
1	Octanol	33	2.1	2.319 ± 0.242	0.002 ± 0.001	High

6 The selected markers for flow cytometry provide valuable information about the
 7 physiological state of the cells based on esterase activity and membrane integrity. This
 8 information is relevant in terms of the ultimate goal of identifying biocompatible
 9 organic phases that do not hinder the bioconversion step during the integrated process.
 10 In addition, it is necessary to determine if there is a correlation with their bioconversion
 11 capacity. Therefore, solvent toxicity was also evaluated by comparing the
 12 bioconversion of 5 g/L of glycerol in contact with the soluble fraction of the organic
 13 phases. To do this, three bioconversion parameters were calculated for glycerol

1 consumption: 3-HP, 1-3-PDO production and their metabolic intermediate (3-HPA)
2 accumulation. All parameters were expressed as ratios relative to a control without
3 contact with the solvents (Figure 5).

4 These parameters provide an interesting description of metabolite production and
5 glycerol consumption in the presence of the different solvent molecules. Results for the
6 ratio a/a^* show that the mixture with octanol is the most toxic of all the organic phases.
7 This can be explained by its relatively high solubility value (540 mg/L; Table 1). The
8 ratio k/k^* shows an interesting behaviour for mixtures with decanol. It appears that
9 the 3-HP production rate is higher than the control at the beginning of the
10 bioconversion, as confirmed by the ratio of initial production rates (r_0/r_0^*), but it is
11 inhibited by the solvent molecules later on. This phenomenon is not observed with the
12 octanol mixture, however, probably because of its very high toxicity that does not allow
13 much metabolic activity. Bioconversion parameters indicate that the mixtures with 2-
14 hexyl-1-decanol, oleyl alcohol and dodecanol are not toxic since they have
15 bioconversion indicators similar to the control.

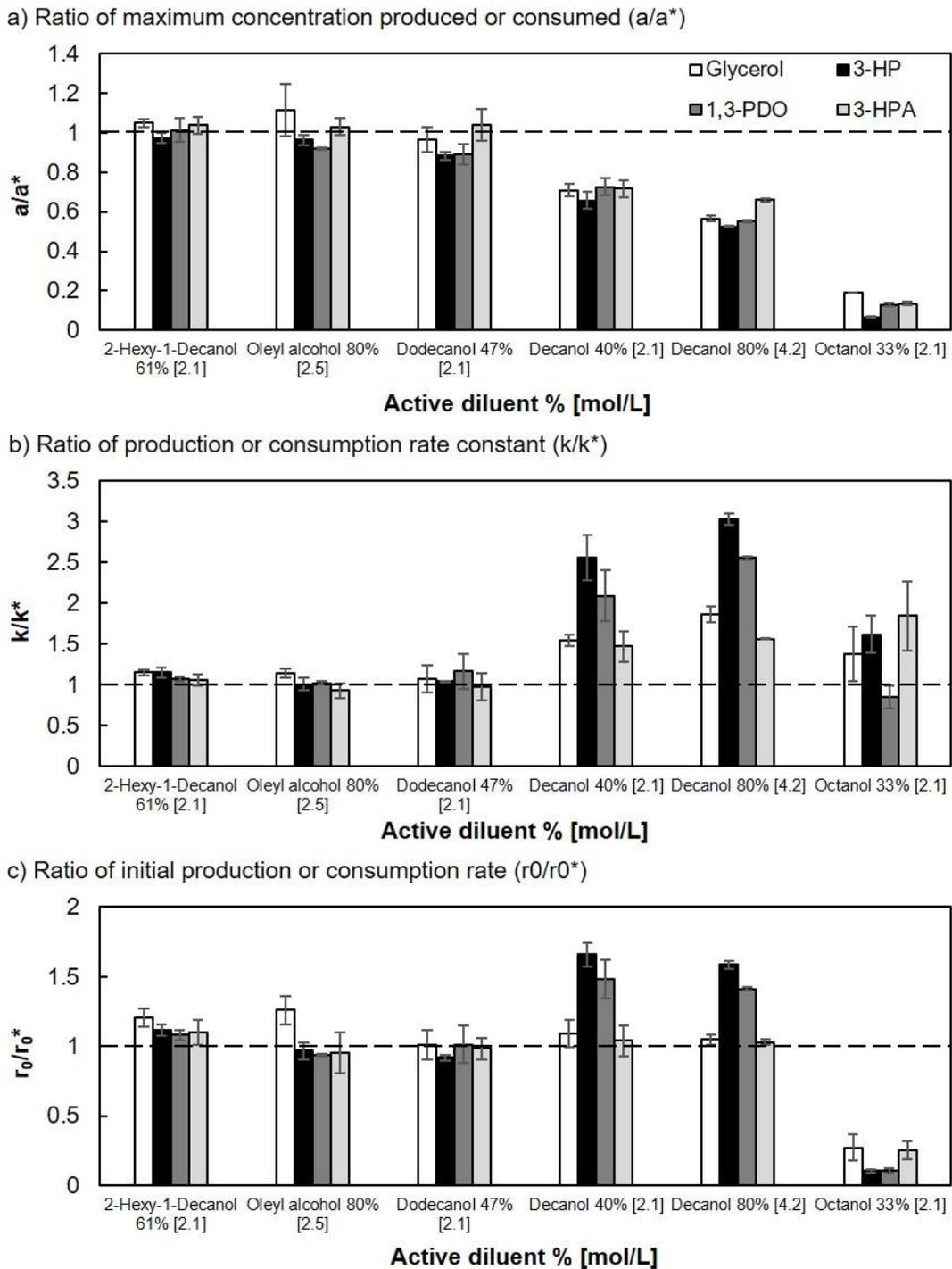


Figure 5. Bioconversion parameters of *L. reuteri* in contact with the soluble fraction of the different solvents, compared to a control (bioconversion in pure water).

1 Figure 6 shows the time evolution of the ratio of 3-HP production in contact with the
 2 soluble fraction of the solvent ($[3 - HP]_S$) compared to the control ($[3 - HP]^*$),
 3 represented by the ratio $[3 - HP]_S/[3 - HP]^*$. Once again, the difference between
 4 octanol and the other alcohols is clear. In the case of the two mixtures with decanol,
 5 there is a higher initial production of 3-HP than in the control, which is consistent with
 6 the bioconversion indicators represented in Figure 6. However, this increase in
 7 production rate is followed by an inhibition effect during the first 2-3 hours before the
 8 production rate stabilises at 0.70 for 40% decanol and 0.55 for 80% decanol. The
 9 mixtures with oleyl alcohol and 2-hexyl-1-decanol seem to be quite biocompatible,
 10 while the mixture with dodecanol shows a slight reduction of 3-HP production after 4
 11 h, compared to the control. However, a similar trend between the impact of solvents on
 12 cell viability and their bioconversion ability was observed overall.

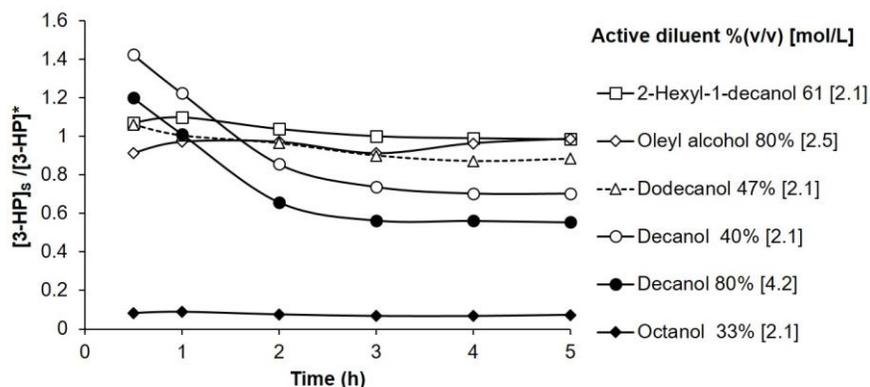


Figure 6. Evolution of 3-HP concentration ratio between bioconversion in contact with the solvent's soluble fraction $[3 - HP]_S$ and control $[3 - HP]^*$.

13 Figure 7 shows the extraction yield and viscosity of the organic phases presented in
 14 Table 3 and includes information about their toxicity towards *L. reuteri*. For the same
 15 alcohol molar concentration, extraction yield decreases with the number of carbons in

1 the alcohol chain, whereas viscosity increases. The Log P value also increases with the
 2 carbon number (Table 1), which may be related to a better biocompatibility, as
 3 observed in Table 3. This is consistent with what has been widely described in the
 4 literature, i.e., that attempts to improve the biocompatibility of the extraction phase
 5 usually decrease its extraction performance.^{11,18,41} Thus, the evaluation of the solvent
 6 toxicity on the studied microorganism is mandatory in order to find a good
 7 compromise. Extraction performance of 2-butyl-1-octanol was tested to determine the
 8 difference between linear and ramified alcohol of the same carbon length (dodecanol)

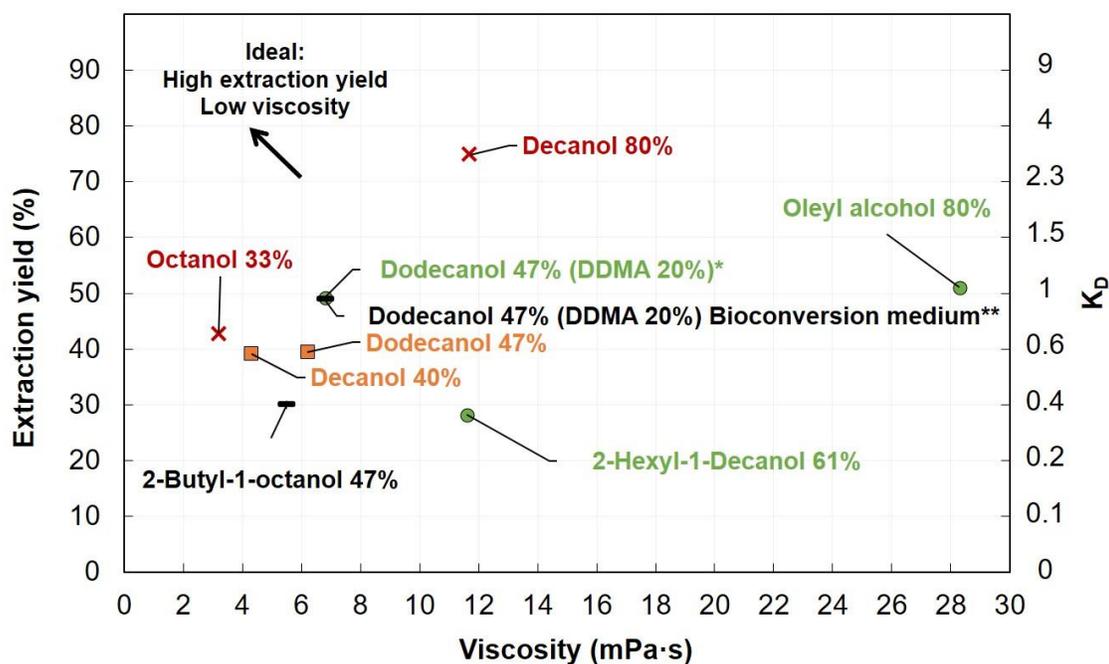


Figure 7. Extraction performance: extraction yields (volume ratio 1:1) and corresponding partition coefficients K_D vs. viscosity. Solvent toxicity classification of mixtures containing 20% v/v TOA (unless otherwise specified) with different active diluents and dodecane concentrations. Initial [3-HP] = 5 g/L. Classification was according to survival ratio reported in Table 3. Green (●): low toxicity; orange (■): medium toxicity; red (x): high toxicity; black (—): toxicity was not determined. *Didodecylmethylamine (DDMA) as the extractant. **3-HP in bioconversion-like medium.

1 on 3-HP extraction. The extraction yield is lower with the ramified alcohol and the
2 viscosity is similar. If the Log P value of both alcohols (dodecanol Log P = 5.13; 2-
3 butyl-1-octanol Log P = 4.8, Table 1) is considered, the toxicity of the ramified alcohol
4 can be similar to that of the linear alcohol or even higher. Therefore, 2-butyl-1-octanol
5 was not considered for solvent toxicity tests.

6 Flow cytometry results gave consistent information with 3-HP bioconversion ability.
7 However, solvents with medium toxicity as the mixture with dodecanol, showed a
8 stronger effect on cells viability and integrity (Table 3) than on 3-HP bioconversion
9 ability, since it was very similar to the control (Figure 6). The same behaviour was
10 observed with 20% (v/v) TOA in decanol, implying that even if the physiological state
11 of cells is affected by the solvents, they are still capable of producing 3-HP.
12 Bioconversion in contact with this organic phase in the ISPR system, gave lower 3-HP
13 production than bioconversion without extraction.⁸ This concordant and
14 complementary information given by flow cytometry suggests that it is a relevant and
15 quick method to monitor bioconversion with *L. reuteri*.

16 Taking into account the compromise between the extraction yield, viscosity and solvent
17 toxicity related to cell viability and 3-HP production ability, the mixture of 20% (v/v)
18 TOA, 47% dodecanol and 33% dodecane seems to be the best choice for the in stream
19 3-HP recovery from bioconversion by *L. reuteri*. However, this mixture shows
20 relatively low extraction performance (extraction yield = $39.5 \pm 0.5\%$). A possible
21 strategy to solve this issue is the use of an alternative extractant while maintaining the
22 biocompatibility of the organic phase. This strategy was investigated using
23 didodecylmethylamine (DDMA) to replace TOA.

1 **Impact of the amine extractant: comparison between DDMA and TOA**

2 DDMA consists of an amine group linked to two chains of 12 carbons each and one
3 methyl group (Table 1). It was chosen because the N atom in this configuration is
4 expected to exhibit better reactivity than in the case of TOA where the amine group is
5 more affected by steric hindrance of the three chains of eight carbons. In addition,
6 having a similar number of carbon atoms as TOA and a Log P value of 8.76 is expected
7 to result in low water solubility and low molecular level toxicity.

8 Figure 8 shows the extraction yield of DDMA diluted in decanol compared with TOA
9 at the same molar concentrations determined by Chemarin *et al.*¹⁷ A similar bell-shaped
10 behaviour is observed for both extractants, with higher extraction yield for DDMA than
11 for TOA. Others studies that tested different amines as extractants have shown a
12 decrease in the extraction yield caused by steric hindrance of the carbon chains linked
13 to the N atom. For example, the results of Kyuchoukov and Yankov⁴² show that when
14 different amines are used for lactic acid extraction, yields decrease when the steric
15 hindrance of amine increases. The presence of longer alkyl chains next to the N atom
16 hinders the access of the H atom from the acid to form the ion pair in the acid-base
17 complex. Matsumoto *et al.*⁴³ also observed a decrease in extraction yields of TOA
18 compared with two other tertiary amines with the same carbon number but in ramified
19 configurations. They mentioned that the stability of the complex becomes impaired due
20 to steric hindrance.

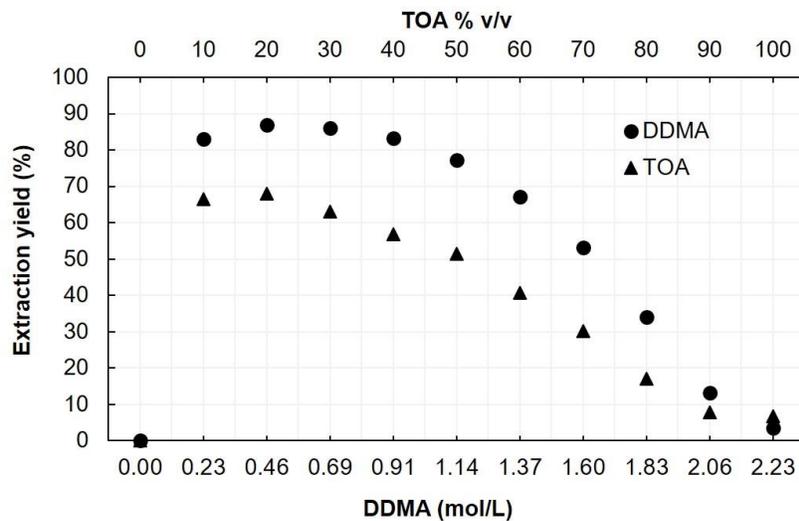


Figure 8. Extraction yields (volume ratio 1:1) of DDMA diluted in decanol at different concentrations compared with TOA with a 1 g/L 3-HP solution. (●) DDMA, (▲) TOA. Error bars masked by symbols.

1 Therefore, the replacement of TOA with DDMA was evaluated in the selected
 2 composition of the organic phase with 20% TOA, 47% dodecanol and 33% dodecane.
 3 The extraction yield, viscosity and solvent toxicity of the mixture are given in Figure
 4 5. As expected, a higher extraction yield is obtained with DDMA at the same diluent
 5 concentrations, with a slightly higher viscosity. Toxicity evaluated by flow cytometry
 6 was very low ($EMR = 0.004 \pm 0.001$, survival ratio at 5 h = 0.905 ± 0.041) and the
 7 bioconversion parameters were very similar to the control since this solvent mixture
 8 was classified as biocompatible.

9 Although it was possible to find an organic phase composition with a higher extraction
 10 yield and low toxicity, it was observed that dispersive extraction using DDMA formed
 11 a very stable emulsion (even after centrifugation at $15000 \times g$ for 15 min) where the
 12 stability of the emulsion increases with the amine concentration. This behaviour is
 13 probably caused by the strong surfactant properties of the acid-base complex formed

1 by DDMA. This issue can be solved using a non-dispersive in stream extraction system
2 like HFMC with careful interface stabilisation, which avoids the formation of
3 emulsion.

4 **Extraction performance of the selected organic phase with a bioconversion-like** 5 **medium**

6 Real bioconversion broth composition has an important effect on 3-HP reactive
7 extraction.²¹ However, one of the advantages of a fed-batch glycerol bioconversion into
8 3-HP by resting cells of *L. reuteri*, is the simplicity of the medium: a glycerol solution
9 is fed to a cell suspension in RO water only. The use of physiological water is avoided
10 because the presence of ions from dissolved salts decreases the distribution coefficient
11 of the acid.^{21,44} 3-HP and 1,3-PDO are the only products, at equimolar proportions.⁸
12 The feeding rate is adjusted to maintain a low glycerol concentration and avoid 3-
13 hydroxypropionaldehyde (3-HPA) accumulation, which is highly toxic for the strain.^{6,7}
14 It was previously observed that glycerol and 3-HPA did not have a significant effect
15 on 3-HP reactive extraction yield.^{9,20} In addition, as continuous removal of the acid is
16 expected, no base solution for pH control will be used. Therefore, the only components
17 considered to have a significant effect on extraction performance were 1,3- PDO and
18 *L. reuteri* cells.

19 In order to mimic the bioconversion medium, *L. reuteri* cells and 5 g/L of 1,3-PDO
20 were added to a 5 g/L 3-HP solution. This aqueous phase was put in contact with the
21 selected organic phase consisting of 20% DDMA, 47% dodecanol and 33% dodecane.
22 As expected, the obtained extraction yield (48.8 ± 0.1 %) was similar to the value with
23 the model solution at 5 g/L (49.3 ± 0.2 %, Figure 7). In addition, 1,3-PDO was extracted

1 only at a yield of 1.8 ± 0.5 %, proving that this organic phase has a high selectivity for
2 3-HP, even with the addition of dodecane to the organic mixture.

3 The presence of cells did not have an important effect on extraction yield, even though
4 there was an increase in the initial pH from 2.89 to 3.16, compared to the model 3-HP
5 solution. This is expected at values below the pKa of the acid (4.51).²¹ However, it has
6 been reported that cells decrease the mass transfer coefficient, acting as a physical
7 barrier at the liquid-liquid interface.⁴⁵ In this respect, low viscosity is an advantageous
8 property than can alleviate this effect for the global mass transfer coefficient.

9 Chen and Lee³¹ used a biocompatible organic phase composed of 20% Alamine 336,
10 40% oleyl alcohol and 40% kerosene by volume, for extractive fermentation of lactic
11 acid. They obtained a partition coefficient of 0.30, lower than the one obtained in this
12 study with the selected organic phase ($K_D = 0.95 \pm 0.004$), but it allowed continuous
13 acid extraction. Other successful studies of extractive fermentations assisted by HFMC
14 used biocompatible organic phases composed of a tertiary amine at concentrations from
15 4 to 10% (v/v), diluted in oleyl alcohol,⁴⁶⁻⁴⁸ similar to the composition of 20% (v/v)
16 TOA diluted in oleyl alcohol tested in this study ($K_D = 1.04 \pm 0.007$). Comparisons
17 with different systems in the literature is delicate because distribution coefficient
18 depends on initial acid concentration, pH, the nature of the acid and microbial medium
19 composition.^{9,21} However, assuming that distribution coefficient could be similar to the
20 one obtained in this study, the selected organic phase had a significantly lower viscosity
21 (6.4 vs. 28.3 mPa·s). As the partition coefficients are similar, a higher mass transfer
22 coefficient can be expected in extractive bioconversion.

23

1 **CONCLUSION**

2 Different solvent mixtures (extractants, active diluents and inert diluents) were
3 evaluated in terms of their extraction performance and toxicity in order to select an
4 adequate composition of the organic phase for in stream extraction of 3-HP produced
5 by *L. reuteri*. Inert diluent addition to a 20% TOA-decanol mixture decreased its
6 toxicity and viscosity. However, it also decreased extraction yield, so it was necessary
7 to determine a compromise between the extraction performance and the overall solvent
8 toxicity towards cells. The combination of the extraction yield, viscosity and solvent
9 toxicity criteria provided valuable information for the evaluation of different long-
10 chain alcohols as active diluents. For linear alcohols, extraction performance decreased
11 with the number of carbons (extraction yield decreased and viscosity increased). As for
12 solvent toxicity, the opposite behaviour was observed. Ramified alcohols resulted in
13 the lowest extraction yields, which can be explained by the steric hindrance of the OH
14 group.

15 Two different methods for the evaluation of solvent toxicity were tested: assessment of
16 the physiological state of the cells by flow cytometry and evaluation of glycerol
17 bioconversion ability. Both methods provided consistent and complementary
18 information about solvent toxicity and were found to be relevant and quick.

19 Among the most biocompatible solvent mixtures, the 20% TOA-47% dodecanol- 33%
20 dodecane solution gave the best trade-off between extraction yield and viscosity.
21 Replacing TOA with DDMA, a tertiary amine with a higher reactivity, made it possible
22 to improve the extraction yield while maintaining the benefit of low solvent toxicity. It
23 was verified that the extraction performance of the selected organic phase is not

1 expected to be highly affected by the components of the real bioconversion broth.
2 These results were observed in bioconversion-like conditions that mimic the broth
3 composition. They open promising prospects towards a coming study on the integrated
4 extractive bioconversion process for the fed-batch production of 3-HP with *L. reuteri*
5 from glycerol.

6 This study brings new insights into the implementation of a robust and intensified
7 extractive bioconversion process for the production of biobased 3-HP. The proposed
8 selection strategy is applicable for similar integrated bioprocesses.

9 **ACKNOWLEDGEMENT**

10 The authors would like to thank the National Council of Science and Technology
11 (CONACyT) of Mexico for Ana Karen Sánchez Castañeda's PhD scholarship.

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