



**HAL**  
open science

## Towards an extractive bioconversion of 3-hydroxypropionic acid: study of inhibition phenomena

Grégoire Burgé, Marwen Moussa, Claire Saulou-Berion, Florian Chemarin,  
Marianne Kniest, Florent Allais, Henry-Eric Spinnler, Violaine Athes-Dutour

### ► To cite this version:

Grégoire Burgé, Marwen Moussa, Claire Saulou-Berion, Florian Chemarin, Marianne Kniest, et al..  
Towards an extractive bioconversion of 3-hydroxypropionic acid: study of inhibition phenomena.  
Journal of Chemical Technology and Biotechnology, 2017, 92 (9), pp.2425-2432. 10.1002/jctb.5253 .  
hal-01566310

**HAL Id: hal-01566310**

<https://hal.science/hal-01566310v1>

Submitted on 20 Jul 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - ShareAlike 4.0 International License

# Towards an extractive bioconversion of 3-hydroxypropionic acid: study of inhibition phenomena

Gregoire Burgé,<sup>a,b</sup> Marwen Moussa,<sup>b</sup> Claire Saulou-Bérion,<sup>b</sup> Florian Chemarin,<sup>a,b</sup> Mariane Kniest,<sup>b</sup> Florent Allais,<sup>a,b</sup> Henry-Eric Spinnler<sup>b</sup> and Violaine Athès<sup>b\*</sup>

## Abstract

**BACKGROUND:** 3-hydroxypropionic acid (3-HP) microbial synthesis through glycerol bioconversion by *Lactobacillus reuteri* is at the moment characterized by too low performances to consider production at industrial scale. To avoid its toxic accumulation in the medium and to recover this molecule of interest, 3-HP *in situ* reactive extraction from bioconversion broth was investigated using a hollow fiber membrane contactor (HFMC) in order to intensify its production. The so-called integrated system was compared with the conventional bioconversion system. The impact of the extractive bioconversion on the overall production performance and on cell physiological state was studied.

**RESULTS:** Results underlined drastic inhibitory effects on the producing bacteria, especially under extractive bioconversion conditions despite the use of a HFMC supposed to avoid direct contact between organic phase and bacteria. Indeed, the extractant phase components (trioctylamine in *n*-decanol) were found to be toxic for the cells (due to solubility and by direct contact). These phenomena were increased by the presence of 3-hydroxypropionaldehyde (3-HPA) and 3-HP produced during glycerol bioconversion. These cumulative effects induced complete loss of the cell membrane integrity and esterase activity after 1.5 h of extractive bioconversion. When the bioconversion was conducted alone, the bacterial inhibition was lower, as around 50% of *L. reuteri* cells remained active with unaltered membrane after 3 h. Hypotheses concerning the mechanisms of action of the observed inhibitions were proposed and discussed.

**CONCLUSIONS:** This work highlighted the importance of performing such an integrative approach to address specific questions prior to the optimization of the whole process.

© 2017 Society of Chemical Industry

**Keywords:** 3-hydroxypropionic acid (3-HP); *Lactobacillus reuteri*; *in situ* product recovery (ISPR); hollow fiber membrane contactor (HFMC); inhibition; glycerol bioconversion

## INTRODUCTION

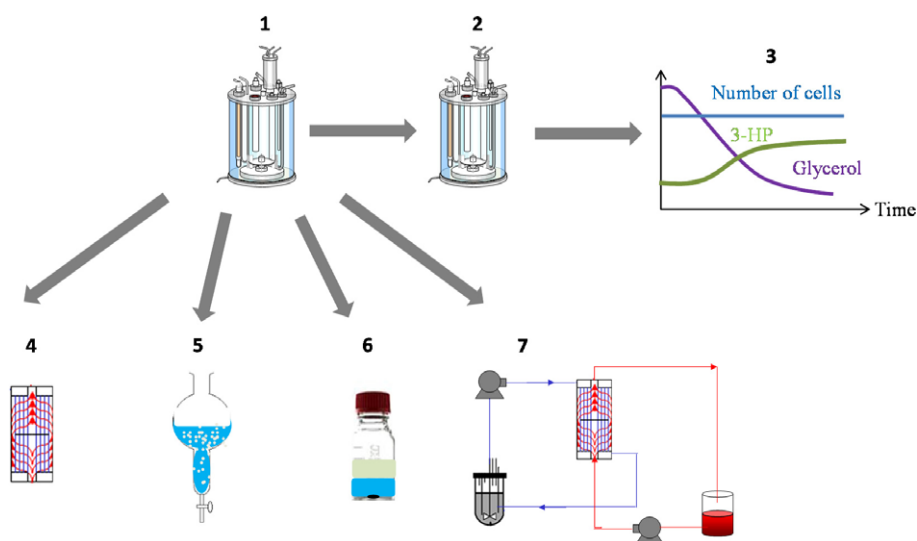
3-hydroxypropionic acid (3-HP) is a promising building block with many applications in the chemical industry and is currently exclusively synthesized at the industrial scale via chemical pathways.<sup>1,2</sup> Despite its promising and diverse applications, 3-HP remains so far of limited use due to the low yield and high production cost of these chemicals routes.<sup>3</sup> Nevertheless, several studies have demonstrated that a few microorganisms, including the Gram-positive *Lactobacillus reuteri*, are naturally able to produce 3-HP by glycerol bioconversion.<sup>4–8</sup> Several works on the biotechnological production of this organic acid from glucose or glycerol using recombinant strains of *Escherichia coli* and *Klebsiella pneumoniae* and more recently by modified yeast strains of *Saccharomyces cerevisiae* have also been investigated.<sup>9–13</sup> Even if the production performance of these genetically modified microorganisms (GMM) has been significantly improved (yield around 0.5 g g<sup>-1</sup> and final titer around 70 g L<sup>-1</sup>) compared with natural producers, 3-HP biotechnological production at industrial scale is still difficult to consider. Observed limitations can be in part attributed to the fact that bacteria are subjected to product inhibition.<sup>8–14</sup>

The implementation of an '*in situ* product recovery' (ISPR) approach, by which upstream bioconversion is combined with downstream extraction and 3-HP recovery, constitutes a potential strategy to: (i) reduce the cell inhibition associated with 3-HP accumulation; (ii) increase the performance of the producing cells; and (iii) recover selectively the molecule of interest. This strategy of extractive bioconversion has thereby been demonstrated to be advantageous for the production of lactic acid (i.e. 2-hydroxypropionic acid), an isomer of 3-HP leading to significantly improved productivity and yield.<sup>15–17</sup> Several techniques have been described for the *in situ* extraction of carboxylic acids. Among them, the reactive liquid–liquid extraction seems to

\* Correspondence to: V Athès, UMR 782 Génie et Microbiologie des Procédés Alimentaires (GMPA), AgroParisTech, INRA, Université Paris-Saclay, F-78850, Thiverval-Grignon, France. E-mail: violaine.athes\_dutour@agroparistech.fr

a AgroParisTech, F-51110 Pomacle, France

b AgroParisTech, INRA, Université Paris-Saclay, Thiverval-Grignon, France



**Figure 1.** Overall diagram of the experimental methodology: 1. *L. reuteri* growth on MRS medium; 2. batch production of 3-HP from glycerol using *L. reuteri* resting cells; 3. influence of pH on *L. reuteri* bioconversion performance; 4. shear stress in the whole experimental device; 5. molecular level toxicity in separatory funnels; 6. phase level toxicity in static device (Schott bottles); 7. phase level toxicity in membrane contactor and first test of 3-HP extractive bioconversion.

be a promising approach. Recently, the feasibility of the 3-HP reactive extraction from real bioconversion media has been demonstrated.<sup>18,19</sup>

In reactive extraction, the aqueous phase containing the target acid is contacted with an organic phase containing an extractant diluted in an organic solvent. Nevertheless, the use of these organic molecules may lead to a series of physical, microbial and biochemical effects on the cell activity.<sup>20</sup> The toxicity level depends on the combination of involved micro-organisms and extractant mixture used. Moreover, strain dependent phenomena have also been underlined.<sup>21</sup> Two distinct types of toxicity linked to the use of organic extractant phases have been described in literature: toxicity due to the amount of organic molecules dissolved in aqueous phase (molecular toxicity) and that due to direct contact between cells and the immiscible organic phase (phase toxicity).<sup>22–24</sup> Several criteria have been used to characterize these toxic impacts on diverse micro-organisms, including the ability to grow or to produce a metabolite. The cell membrane has been pointed out as one of the major targets: disturbance of essential membrane functions, denaturation of membrane-bound enzymes and breakdown of transport mechanisms.<sup>25</sup> However, the distinctive mechanisms involved have not been thoroughly characterized.

Dispersive contact between aqueous and organic phases can be avoided by using a microporous membrane contactor while allowing interfacial liquid–liquid contact through membrane pores, which in turn may reduce or avoid the phase level toxicity.<sup>26–28</sup> This is made possible because of the larger size of bacterial cells compared with the membrane pores and thanks to the interface stability in the vicinity of the pores, between the two sides of the membrane, which avoids phase mixing. Membrane-assisted liquid–liquid reactive extraction has been reported for several organic acids such as 2-HP<sup>29,31</sup> and, more recently, 3-HP.<sup>19</sup> However, to the best of our knowledge, the integrated process including glycerol bioconversion to 3-HP and membrane-assisted liquid–liquid reactive extraction has not yet been reported. Furthermore, a major unanswered question about the possible feed-back mechanisms of the extraction process on *L. reuteri* cells remains.

The objective of the present work was to study an extractive bioconversion process for 3-HP production from glycerol by *L. reuteri* based on reactive liquid–liquid extraction assisted by a hollow fiber membrane contactor (HFMC) and to determine the impact of the so-called integrated process on the cells' physiological state. The process performance, in terms of productivity, production yield and final product titer, was compared with that reached with the conventional bioconversion process without extraction. Shear-induced physiological stress as well as molecular level and phase level toxicity of the extractant phase used were assessed.

## EXPERIMENTAL

Figure 1 shows a diagram explaining the experimental methodology.

### Microorganisms and growth conditions

*Lactobacillus reuteri* DSM 17938 was obtained from BioGaia AB (Stockholm, Sweden). Cells were cultivated anaerobically in batch mode at 37°C in MRS medium (Biokar diagnostics, Beauvais, France) supplemented with 20 g L<sup>-1</sup> of glucose (initial pH = 6.2) after inoculation at an initial cell concentration of about 10<sup>9</sup> CFU mL<sup>-1</sup> as detailed in a previous paper.<sup>8</sup> The resulting culture was used to inoculate the bioreactor.

### Production of the whole-cell biocatalyst for glycerol bioconversion

*Lactobacillus reuteri* cells were grown in batch mode in a 5 L bioreactor (Setric Génie Industriel, Toulouse, France) containing MRS medium supplemented with glucose (20 g L<sup>-1</sup>) inoculated at about 10<sup>5</sup> CFU mL<sup>-1</sup>. Monitoring and control of the operating parameters were done through a control unit (Setric Génie Industriel). Stirring speed was maintained at 100 rpm, temperature at 37°C and pH at 6 by addition of 10 N KOH (Sigma-Aldrich, Lyon, France). The cell growth was conducted for 16 h until the beginning of the stationary phase after which the broth was sterilely collected and centrifuged at 5000 × g and 4°C for 10 min. The supernatant was

discarded and the cell pellet was washed twice with 0.1 mol L<sup>-1</sup> potassium phosphate buffer (pH 6.5) to ensure no carry-over of metabolites, then re-suspended in distilled water to reach a final volume of 500 mL and subsequently used for the glycerol bioconversion step.

#### Batch production of 3-HP from glycerol using *L. reuteri* resting cells

A 3 L Biostat<sup>®</sup>-A bioreactor (Sartorius France, Dourdan, France) with a 2.5 L working volume was used. First, 500 mL of *L. reuteri* cell suspension obtained as described above was suspended in 2.0 L of sterile glycerol solution (to reach a final substrate concentration of 18 g L<sup>-1</sup> in distilled water) to a final cell concentration of about 10<sup>10</sup> CFU mL<sup>-1</sup>. Glycerol bioconversion was performed for 3 h at 37°C, 100 rpm and different pH levels (unregulated, 3, 4, 5 and 6) by addition of 10 N KOH and 5 N HCl. Samples were harvested every 30 min and immediately treated as described below.

#### Integrated system for extractive bioconversion of 3-HP

The experimental set-up consisted of two parts: a 3 L Biostat<sup>®</sup>-A bioreactor for free-cell glycerol bioconversion and a polypropylene hollow fiber membrane contactor (HFMC, Liqui-Cel<sup>®</sup> X50, Membrana, USA) for 3-HP reactive extraction. The bioconversion broth, containing the bacterial cells, was continuously fed to the lumen side of the contactor fibers at a flow rate of 10 mL s<sup>-1</sup> and circulated back to the bioreactor. The organic phase was composed of 20% v/v *n*-trioctylamine (TOA; 99.6%, Sigma-Aldrich, Lyon, France) in 80% v/v *n*-decanol (99%, Sigma-Aldrich). This phase crossed counter-currently through the shell side of the vertical module at a flow rate of 13 mL s<sup>-1</sup>. The pressure difference across the membrane was adjusted by two valves at the outlet of each phase, with the lumen side pressure being higher than that of the shell side to ensure interface stability. The pH regulation system through addition of base solution to the bioconversion medium was not activated since the pH was supposed to remain stable at the initial value of 6.0 thanks to the continuous extraction of 3-HP from the broth. Temperature was regulated at 37°C for both phases. Two independent extractive batch bioconversions were performed to test the process performance. Experiments were carried out with 2.5 L of bioconversion medium (cells in 18 g L<sup>-1</sup> glycerol) as aqueous phase and 500 mL of organic extractant phase. Samples were taken from both the bioreactor and the organic phase reservoir at regular time intervals of 15 min and analyzed as described below.

#### Assessment of inhibitory effects due to 3-HP extraction on *L. reuteri* cells

##### *Shear stress in the whole experimental device*

The aqueous phase containing *L. reuteri* cells at about 10<sup>10</sup> CFU mL<sup>-1</sup> was fed through the fibers lumen in the membrane contactor at a flow rate of 10 mL s<sup>-1</sup> using a gear pump. The transmembrane pressure was equilibrated using distilled water circulating counter-currently through the shell-side of the module. Samples of 1 mL were collected each 15 min for 3 h and immediately prepared for flow cytometry analyses.

##### *Molecular level toxicity*

Stock solutions of various aqueous phases equilibrated with the two different tested organic phases (TOA 20% v/v or TOA 18% v/v + Aliquat 336<sup>®</sup> (Sigma-Aldrich, Lyon, France) 2% v/v in *n*-decanol or *n*-decanol 100% v/v) were first prepared. To this end, three

aqueous phases were used: pure distilled water and solutions of 3-HP (0.5 g L<sup>-1</sup>) and 3-HPA (1 g L<sup>-1</sup>) in distilled water. Then, the aqueous and organic phases were vigorously mixed in separatory funnels for 3 min. The mixture was stored at 37°C for 2 days to reach equilibrium. Contents of separatory funnels were then centrifuged at 5000 g for 10 min to separate the aqueous and organic phases. *Lactobacillus reuteri* cells, grown and washed as described above, were then suspended in the obtained aqueous phases to reach about 10<sup>10</sup> CFU mL<sup>-1</sup> and the suspensions were placed at 37°C. Samples of 1 mL were collected every 15 min for 3 h and bacteria physiological state was subsequently evaluated by flow cytometry.

##### *Phase level toxicity*

Equal volumes (80 mL) of *L. reuteri* cell suspension (at about 10<sup>10</sup> CFU mL<sup>-1</sup>) and organic phase (*n*-decanol 100% v/v or TOA 20% v/v in *n*-decanol) were put in contact in a 250 mL static device (i.e. Schott bottle) at 37°C. The aqueous phase was then gently stirred using a magnetic stirrer to renew the interface and generate contact between solvent and cells without causing dispersive mixture of the two phases. Samples of 1 mL of the aqueous phase were collected every 15 min for 3 h for flow cytometry analyses.

#### Evaluation of the cells physiological state by flow cytometry

The viability of *L. reuteri*, as reflected by the esterase activity of bacteria, itself assimilated to the whole enzymatic activity due to the widespread presence of esterase within cells, was assessed using carboxyfluorescein diacetate (cFDA) contained in Chemchrom V8 (Biomérieux, Marcy l'Etoile, France). The nucleic acid dye propidium iodide (PI, Sigma-Aldrich, Lyon, France), widely used to reveal the loss of cellular membrane integrity, made it possible to quantify the proportion of damaged and dead cells. In order to differentiate viable, dead and damaged cells, dual staining of each sample was performed and the concentration (in cells per mL) and percentages of each cell category were determined, as described previously.<sup>8</sup>

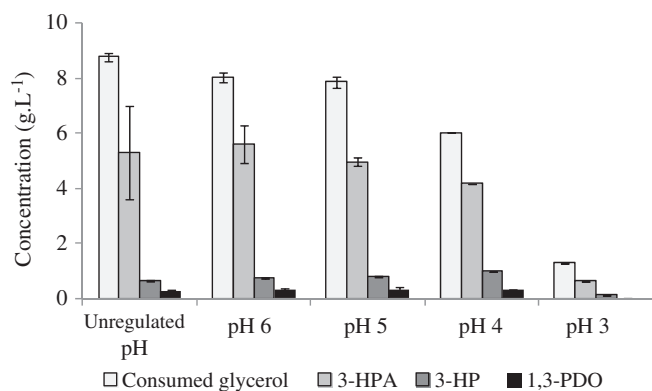
#### Quantitative analysis of substrates and metabolites

3-HP was purchased from TCI-Europe (Zwyndrecht, Belgium). Glycerol and 1,3-PDO were purchased from Sigma-Aldrich (Lyon, France). 3-HPA was chemically synthesized as described previously.<sup>32</sup> Residual glycerol and produced 3-HPA, 3-HP and 1,3-PDO were quantified by high performance liquid chromatography (HPLC) using a Biorad Aminex HPX-87H column (300 mm × 7.8 mm; Biorad, Richmond, USA) equipped with a cation H<sup>+</sup> Micro-Guard column (30 mm × 4.6 mm; Biorad) at a controlled temperature of 50°C, as described previously.<sup>8</sup> All quantifications were expressed as the means ± standard errors of two independent experiments.

Statistical significance of differences between data was assessed using analysis of variance at 0.05 level.

## RESULTS AND DISCUSSION

In order to develop an integrated process for glycerol bioconversion into 3-HP coupled to 3-HP reactive extraction, attention must be paid to the necessary compromise between the operating parameters of these two unit operations. Given the importance of pH for the mechanisms of organic acid reactive extraction by amines, the influence of this parameter on bioconversion performance was studied first. Before implementing the 3-HP extractive bioconversion, the biocompatibility of various organic extractant



**Figure 2.** Influence of pH on glycerol bioconversion performances after 2 h. Reported data correspond to the mean values of measurements from two independent experiments (n=2), except for pH 4 and 3 (n=1).

phases was assessed and the performance of the selected phase for extraction discussed. Finally, the global performance of the integrated process were characterized and the toxicity of the whole process on bacterial cells investigated.

### Influence of pH on *L. reuteri* bioconversion performance

Our previous studies have shown that initial pH of the aqueous phase when starting extraction in HFMC has a significant influence on the performance of 3-HP reactive extraction using TOA and Aliquat 336.<sup>19</sup> Briefly, it was shown that an increase in initial pH from 3 to 7 slowed down the extraction kinetics and decreased significantly the extraction yield. This is why the effects of pH on bioconversion performance and on *L. reuteri* physiological state were explored (Fig. 2), prior to implementation of the integrated extractive bioconversion process.

Figure 2 displays the influence of different pH levels (unregulated, 6, 5, 4 and 3) on glycerol consumption and metabolites production by *L. reuteri* DSM 17938. For all the conditions tested, molar balances were checked and included in a range between 80 and 105%. At pH 3, glycerol consumption was low (1.3 g L<sup>-1</sup>) and few 3-HPA (0.6 g L<sup>-1</sup>) and 3-HP (0.1 g L<sup>-1</sup>) were produced while no 1,3-PDO was detected in the medium. By maintaining the pH of the bioconversion medium at higher values (between 4 and 6), results showed an increase in glycerol consumption (6.0, 7.9 and 8.0 g L<sup>-1</sup> at pH 4, 5 and 6, respectively) and 3-HPA production (4.2, 5.0 and 5.6 g L<sup>-1</sup> at pH 4, 5 and 6, respectively). Concerning 3-HP and 1,3-PDO, pH between 4 and 6 induced little effect on the production performance (between 0.7 and 0.9 g L<sup>-1</sup> of 3-HP and 0.25 to 0.35 g L<sup>-1</sup> of 1,3-PDO produced depending on the conditions; Fig. 2). At unregulated pH (i.e. pH decreased from 6.5 to 4.5 after a 2 h bioconversion), glycerol consumption (8.8 g L<sup>-1</sup>) and 3-HPA production (5.3 g L<sup>-1</sup>) were significant. Concerning 3-HP and 1,3-PDO, few differences were observed in comparison with experiments at regulated pH (Fig. 2). The molar balance analysis evidenced a higher 3-HP production than that of 1,3-PDO for all the tested pH (molar ratios 3-HP/1,3-PDO > 1), which aligns with previous results.<sup>8</sup>

Although the impact of pH of bioconversion medium on 3-HPA production capabilities by *L. reuteri* has been underlined<sup>33,34</sup> showing pH 6 as optimal, its influence on 3-HP production has rarely been studied. Our results on the DSM 17938 strain are consistent with those of Dishisha et al.<sup>4</sup> showing better 3-HP accumulation at pH 7 than at pH 5 with *L. reuteri* DSM 20016. This is also in line with the optimal pH values reported in the literature for several

**Table 1.** Molecular level toxicity of various extractant phases with or without additional metabolites on *L. reuteri*. Reported data correspond to the mean values of measurements from two independent experiments of the percentage viability compared with the control (cells in distilled water) after 3 h of contact (n=2)

Conditions	Viability (%)
<i>n</i> -decanol (100%)	84
<i>n</i> -decanol (80%) + TOA (20%)	93
<i>n</i> -decanol (80%) + TOA (20%) + 3-HP (0.5 g L <sup>-1</sup> )	79
<i>n</i> -decanol (80%) + TOA (20%) + 3-HPA (1 g L <sup>-1</sup> )	59
<i>n</i> -decanol (80%) + TOA (18%) + Aliquat 336 (2%)	39

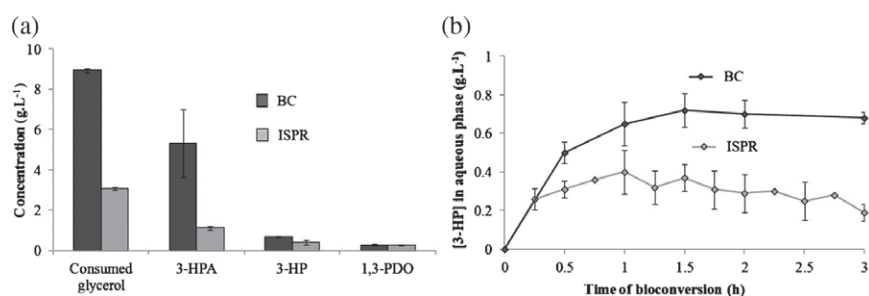
enzymes of the bioconversion pathway (i.e. 7.2 for the glycerol dehydratase GDH<sup>35</sup> and 7.0 for the propionaldehyde dehydrogenase PduP<sup>36</sup>).

For the set-up of extractive bioconversion, pH 5 and 6 seem to be most appropriate because of better glycerol consumption compared with lower pH levels. Unregulated pH could also be adopted, because this operating parameter would be regulated due to 3-HP extraction from the broth.

### Biocompatibility assessment of various extractant phases

It is known that mixed extractants in active diluents show a positive synergistic effect on the reactive extraction performance of organic acids.<sup>37,38</sup> This effect was recently demonstrated in the case of 3-HP using TOA and Aliquat 336 as extractants in *n*-decanol.<sup>18</sup> However, regarding the ultimate objective of integrated extractive bioconversion, the biocompatibility of this extractant mixture should be checked. Experiments were therefore performed with *L. reuteri* cells to quantify the possible inhibition caused by the soluble fraction of several extractant phases. Results of the evaluation of this molecular level toxicity are given in Table 1.

It appears that *n*-decanol alone or in mixture with TOA (20% v/v) had limited impact on cell viability (84% and 93% of viability, respectively). Conversely, the addition of only a minimal amount of Aliquat 336 (2% v/v) to the mixture led to a greater impact on *L. reuteri*, with only 39% of residual viability. Toxic effects of Aliquat 336 have previously been reported on *L. delbrueckii* (no glucose consumption detected when 0.1% of Aliquat 336 was added in the medium).<sup>39</sup> Several hypotheses have been proposed to explain this phenomenon such as interaction of organic molecules with cell membrane lipids leading to disturbance of essential membrane functions, inactivation or denaturation of membrane-bound enzymes, breakdown of transport mechanisms.<sup>40,41</sup> Flow cytometry results obtained through the present work corroborate these suppositions, as the membrane integrity of about 60% of bacterial cells was disrupted in the presence of organic extractant phase including Aliquat 336 (Table 1). Given the loss of esterase activity observed, it can be supposed that the global enzymatic activity of bacterial cells was also affected and therefore membrane-bound enzymes could be damaged. Different solvents and extractants have previously been assessed (*n*-octanol, MIBK and TOA on *L. rhamnosus*;<sup>24</sup> TOA and Aliquat 336 on *L. delbrueckii*;<sup>42</sup> TOA, Aliquat 336, Alamine 336 and *n*-octanol on *L. casei*<sup>39</sup>), but data available in the literature are variable and can be contradictory. Toxicity seems to be closely related to the type of micro-organism used and could even be strain dependent.<sup>21</sup> Given the observed strong toxicity of Aliquat 336 on *L. reuteri* strain DSM 17938, it



**Figure 3.** Results of the glycerol bioconversion by *L. reuteri* ( $10^{10}$  CFU mL<sup>-1</sup>) at unregulated pH and 37°C performed alone (BC) or coupled with 3-HP reactive extraction assisted by HFMC using TOA 20% v/v in *n*-decanol (ISPR). (a) Glycerol consumption and metabolites production after 2 h of bioconversion. (b) Evolution of 3-HP concentration in the bioconversion medium. Reported data correspond to the mean values of measurements from two independent experiments ( $n=2$ ).

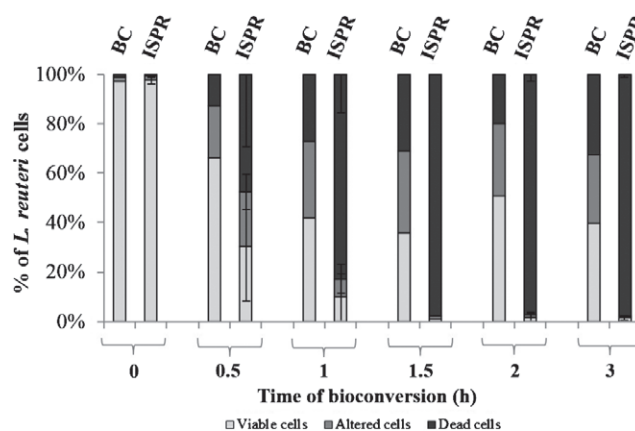
would be interesting to test the resistance capabilities of other *L. reuteri* strains to this quaternary ammonium. Accordingly, Aliquat 336 was removed from the extractant phase composition for the next parts of the present work, despite its known synergistic effect in combination with TOA on extraction performance.<sup>18,19</sup>

### Study of the integrated extractive bioconversion for the *in situ* 3-HP recovery

The glycerol consumption and metabolites production after 2 h of bioconversion as well as the evolution of 3-HP concentration in the bioconversion medium with or without *in situ* reactive extraction of the produced 3-HP are shown in Fig. 3.

The glycerol consumption during extractive bioconversion ( $3.0$  g L<sup>-1</sup>, Fig. 3(a)) was lower than during the standard bioconversion ( $8.8$  g L<sup>-1</sup>, Fig. 3(a)). Similarly, the production of 3-HPA, 1,3-PDO and 3-HP were lower. However, the 3-HPA production yield ( $0.5 \pm 0.1$  mol mol<sup>-1</sup>) was slightly lower than that reached during single bioconversion ( $0.7 \pm 0.2$  mol mol<sup>-1</sup>), while the 3-HP and 1,3-PDO yields increased ( $0.15 \pm 0.02$  vs.  $0.07 \pm 0.01$  mol mol<sup>-1</sup> and  $0.10 \pm 0.01$  vs.  $0.04 \pm 0.01$  mol mol<sup>-1</sup>, respectively). In addition, focusing on the 3-HP concentration in the aqueous phase (Fig. 3(b)), a slight decrease was observed after 1 h of extractive bioconversion ( $0.4$  g L<sup>-1</sup> after 1 h and  $0.2$  g L<sup>-1</sup> after 3 h), showing low 3-HP extraction. The low extraction performance observed in the case of the integrated process can be explained by several environmental factors known to affect liquid–liquid reactive extraction, such as the high pH of the bioconversion medium regarding the optimal value for TOA as extractant, or the presence of cell originating macromolecules such as proteins or phospholipids that could interfere with the reactive extraction of 3-HP and lower the distribution coefficient ( $K_D$ ).<sup>18</sup> In our previous work<sup>19</sup> we observed that the extraction kinetics of 3-HP from real bioconversion media using a membrane contactor was comparable with model media (3-HP in water). This result suggests that mass transfer could not be limiting in the case of bioconversion medium, and only the equilibrium  $K_D$  is affected.

The physiological state of *L. reuteri* cells was evaluated in the case of the integrated extractive bioconversion process (ISPR) and compared with the standard bioconversion (BC; Fig. 4). For ISPR configuration, a significant decrease in the percentage of viable cells was observed after 30 min and all cells lost their membrane integrity after 1.5 h. When the bioconversion was conducted alone, the bacterial inhibition was lower as around 50% of bacterial cells remained viable from 1 h until the end of the experiment (Fig. 4). The higher cell alteration observed during extractive bioconversion was certainly mostly responsible for the low 3-HP production

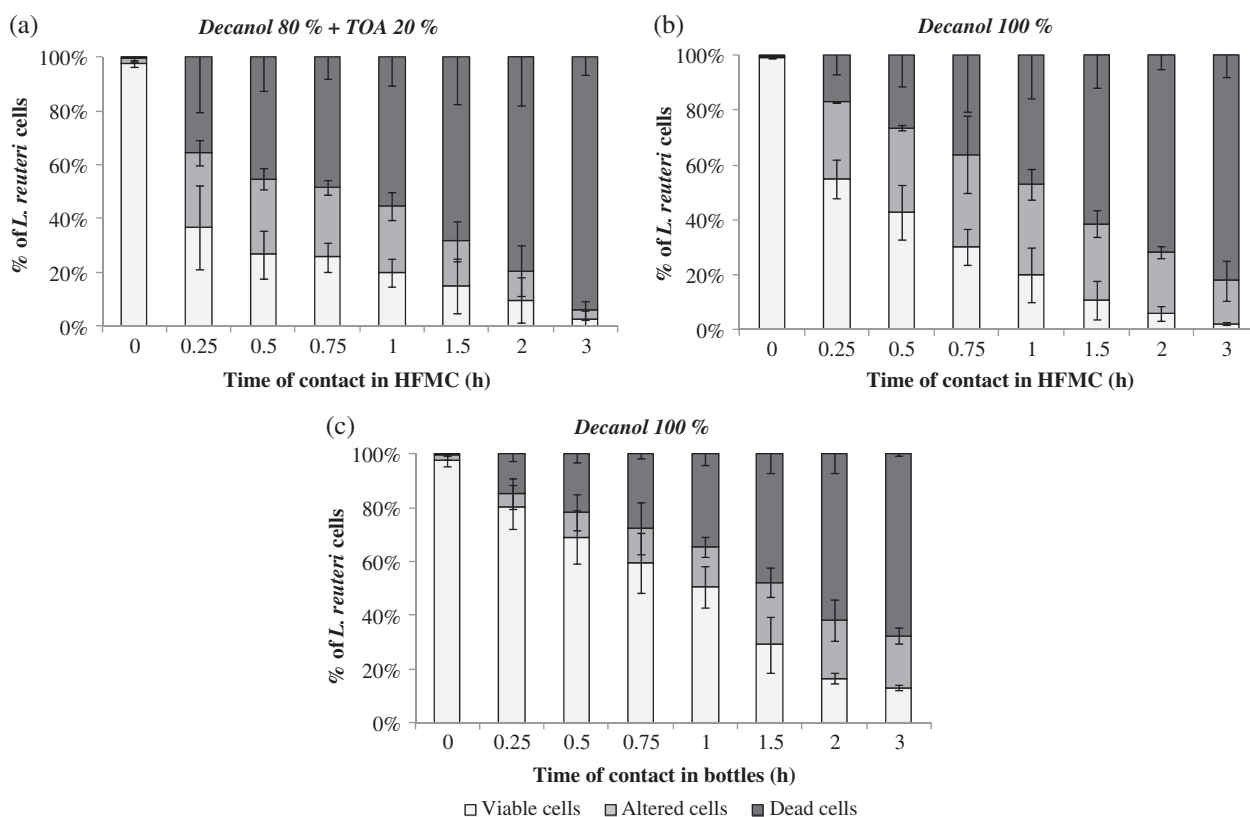


**Figure 4.** Evaluation of the physiological state of *L. reuteri* ( $10^{10}$  CFU mL<sup>-1</sup>) during bioconversion at unregulated pH and 37°C performed alone (BC) or coupled with *in situ* 3-HP reactive extraction assisted by HFMC using TOA 20% v/v in *n*-decanol (ISPR). Reported data for ISPR correspond to the mean values of measurements from two independent experiments ( $n=2$ ), the control was not repeated.

performances. As the global cell enzymatic activity was probably disturbed, enzymes of the glycerol bioconversion pathway may also be affected.

In order to explain the high toxicity observed on *L. reuteri* cells during the extractive bioconversion assisted by HFMC and to determine more precisely the underlying causes, supplementary experiments were performed. First, the possible shear stress was studied and its impact on bacterial cells was assessed by flow cytometry. It appeared that even after 3 h of circulation in the aqueous circuit (i.e. the HFMC module, pipes and pumping system), neither loss of membrane integrity nor of esterase activity were detected ( $P=0.75$ ).

Then, the effect of the soluble fraction of the organic extractant phase (molecular level toxicity) was investigated together with the cumulative impacts of 3-HP or 3-HPA. The concentration levels of 3-HP and 3-HPA were chosen in the range reached after the extractive bioconversion ( $0.5$  and  $1$  g L<sup>-1</sup>, respectively). As depicted in Table 1, the presence of 3-HP or 3-HPA induced an additional decrease in cell viability (79% and 59% of viability with  $0.5$  g L<sup>-1</sup> of 3-HP or  $1$  g L<sup>-1</sup> of 3-HPA, respectively) compared with the molecular level toxicity alone. However, when only 3-HP or 3-HPA were added to bacterial cell suspensions at this range of concentrations, no significant impact was observed on *L. reuteri* viability (data not shown). Accordingly, these two kinds of stress conditions, namely the soluble fraction of the extractant phase and the presence of



**Figure 5.** Phase level toxicity on *L. reuteri*. Direct contact of the aqueous phase containing *L. reuteri* ( $10^{10}$  CFU.mL<sup>-1</sup>) with (a) *n*-decanol with TOA (20% v/v) in HFMC, (b) *n*-decanol alone in HFMC, and (c) *n*-decanol alone in static device. Reported data correspond to the mean values of measurements from two independent experiments ( $n=2$ ).

metabolites, were not significant individually but became toxic for bacterial cells in combination, probably due to a synergistic effect.

To complete the study on solvent toxicity encountered during ISPR, two different types of contact were performed, i.e. directly in the membrane contactor and in a static device, using two organic phases: *n*-decanol alone or *n*-decanol with TOA (20% v/v). Results are displayed in Fig. 5.

The use of *n*-decanol with TOA (20% v/v) as the organic extractant phase in the shell side of the HFMC induced a drastic loss of cell viability (27% of viable cells after 0.5 h, 20% after 1 h, 10% after 2 h and only 3% after 3 h; Fig. 5(a)). This organic phase was the one used for the 3-HP extractive bioconversion experiments and the observed kinetics as well as the final impact on cell viability (Fig. 4) were similar after 3 h ( $P=0.52$ ). To better understand which molecule of the extractant phase is responsible for the observed inhibition, the same experiment was made using *n*-decanol alone (Fig. 5(b)). Once again, kinetics and final toxic impact were close to those obtained in the presence of 20% v/v TOA although slightly lower (43% of viable cells after 0.5 h, 20% after 1 h, 6% after 2 h and only 2% after 3 h). In fact, the presence of TOA in the organic phase induced an additional impact on bacterial cells, leading to a more important cell death from 0.25 h of experiment compared with *n*-decanol alone.

In order to assess whether the observed inhibition would be related to direct contact of bacterial cells with the organic phase, close to the membrane pores, the phase level toxicity of *n*-decanol alone was then investigated in a static device (Fig. 5(c)). As presented in the experimental section, the non-dispersive contact in a static device (Schott bottles) between the aqueous

cell suspension and the organic phase was designed to mimic the interface at the membrane pores level and hence possible direct contact between cell suspension and organic phase.

A strong decrease in cell viability due to this direct contact was observed (Fig. 5(c)). Nevertheless, it should be mentioned that the impact evidenced in this set of experiments is not only due to the phase level toxicity but also to the molecular level toxicity, as it is not possible to differentiate both effects. Comparing the impacts due to both toxicity levels of the organic phase (Fig. 5 and Table 1), the toxicity due to direct contact was more deleterious than that due to organic molecules dissolved in the aqueous phase. Similar results have previously been reported concerning *n*-decanol phase level toxicity, as a great impact was observed on biomass and lactic acid production by *L. casei* cells (only 11% of bacterial growth and 32% of lactic acid production after 24 h compared with the control sample).<sup>25</sup> Under direct contact conditions, the deleterious interactions mentioned above<sup>40,41</sup> between the organic molecules and the cells would be enhanced.

The higher impact on *L. reuteri* physiological state observed during the HFMC experiment (Fig. 5(b)) compared with that in the static device (Fig. 5(c)) was probably due to the different specific exchange areas (730 vs. 36 m<sup>2</sup> m<sup>-3</sup> of contact area per volume unit of the aqueous phase, respectively, in the HFMC and static device), as well as differences in the aqueous/organic interface renewal between both devices. The cumulative effects of the organic phase at the molecular and phase levels and the presence of 3-HPA and 3-HP during glycerol bioconversion may explain the low bacterial cell viability noticed during extractive bioconversion

assisted by HFMC and therefore the low performances of the integrated process.

As one would expect, the use of a membrane contactor normally allows minimizing the direct contact between cells in the aqueous phase and the organic phase, compared with classical dispersive liquid–liquid extraction in separating funnel. But given the results observed here, direct contact of *L. reuteri* bacteria with the organic phase close to the membrane pores is suspected. The average pore size of the polypropylene membrane used in our study is theoretically around 0.04  $\mu\text{m}$  (X50 extra-flow product data sheet, Membrana), which ensures bacteria exclusion from the pores. Preliminary experiments to get membrane pore size distribution (mercury intrusion performed for the X50 membrane of the Liqui-Cel® module) allowed us to suspect the existence of some larger pores. These latter may affect the stability of the interface and therefore provoke the passage of organic phase over the membrane into the aqueous phase. In addition, the organic phase/water interfacial tension has been measured experimentally at 8.5  $\text{mN m}^{-1}$  for *n*-decanol alone and 9.3  $\text{mN m}^{-1}$  for TOA 20% v/v in *n*-decanol. These quite low values could also promote the presence of solvent drops at the vicinity of larger pores mouths, because of a low critical pressure, due both to the maximum pore radius and low interfacial tension of the system. This is consistent according to the Young–Laplace equation: the interface is more difficult to stabilize for larger pore size and lower interfacial tension between aqueous and organic phases.<sup>43</sup> In order to perform ISPR with membrane contactors in better conditions, the critical pressure of the system needs to be enhanced to avoid direct contact between cells and solvent, either by changing the HFMC membrane material (nature, pore size and distribution) or choosing a solvent allowing higher interfacial tension.

## CONCLUSION

Extractive fermentation has been shown in the literature to be advantageous for organic acid production (e.g. butyric, propionic, lactic acids) leading to significant improvements in productivity, product yield and final titer. However, in the case of 3-HP production performed by *L. reuteri* DSM 17938 from glycerol, the integration of *in situ* reactive extraction led so far to lower performance over the conventional single bioconversion mostly because of the toxicity of TOA and *n*-decanol against producing cells. Similar results have already been obtained in the case of lactic acid extractive fermentation with tri-*n*-decylamine, which did not provide high production relative to fermentation without extraction.<sup>44</sup> In our work, direct contact between the cells in aqueous medium and the organic extractant phase near the pores of the membrane is suspected. It was shown that this contact is more deleterious than the toxicity of the small amount of solvent in solution in the aqueous phase. Better control of the interfacial stability at the membrane contactor level would help in reducing these effects, either by changing the membrane material or by lowering the interfacial tension of the system. Over the two components of the extractant phase (*n*-decanol and TOA), *n*-decanol seemed to be the most toxic. Methods to reduce solvent toxicity should be investigated. This may include the use of immobilized cells in bioreactors, which has been demonstrated to be promising,<sup>45,46</sup> the use of less toxic solvents such as oleyl alcohol<sup>25</sup> or sunflower oil,<sup>47,48</sup> or the purification of solvent and amines before use.<sup>49</sup> However, a compromise between the efficiency of these solvents for reactive extraction and their biocompatibility should be found.

This work thoroughly investigated the toxicity of the organic extractant phase on bacterial cells. Flow cytometry results thus corroborated the hypotheses previously suggested in the literature concerning the mechanisms of action of these molecules. This technique indeed enabled identification of the cell membrane as one of the main targets of the observed inhibition. Moreover, it seems that there is also an effect of the organic extractant phase on the global cell enzymatic activity. The implementation of transcriptomic or proteomic analyses could improve understanding of the mechanisms at the root of these toxicity phenomena, to clarify the biological impact of the toxicity and finally identify the reaction by the expression of genes involved in stress resistance.<sup>41,50,51</sup>

## ACKNOWLEDGEMENTS

The authors are grateful to the Region Champagne-Ardenne, the Conseil Départemental de la Marne and Reims Métropole for their financial support.

## REFERENCES

- 1 Werp T and Petersen G, Top value added chemicals from biomass. Volume 1 – Results of Screening for Potential Candidates from Sugars and Synthesis Gas. USA DOE 76 2004).
- 2 Smithers-Rapra, *Ten-Year Forecast of Disruptive Technologies in Industrial Biotechnology to 2021*, 1–124 (2011).
- 3 Della Pina C, Falletta E and Rossi M, A green approach to chemical building blocks. The case of 3-hydroxypropanoic acid. *Green Chem* **13**:1624–1632 (2011).
- 4 Dishisha T, Pereyra LP, Pyo SH, Britton RA and Hatti-Kaul RH, Flux analysis of the *Lactobacillus reuteri* propanediol-utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol. *Microbiol Cell Fact* **13**:76–85 (2014).
- 5 Dishisha T, Pyo SH and Hatti-Kaul RH, Bio-based 3-hydroxypropionic acid production from biodiesel glycerol via integrated microbial and chemical catalysis. *Microbiol Cell Fact* **14**:200 (2015).
- 6 Ramakrishnan Gopi G, Ganesh N, Pandiaraj S, Sowmiya B, Brajesh RG and Ramalingam S, A study on enhanced expression of 3-hydroxypropionic acid pathway genes and impact on its production in *Lactobacillus reuteri*. *Food Technol Biotechnol* **53**:331–336 (2015a).
- 7 Ramakrishnan Gopi G, Ganesh N, Pandiaraj S, Sowmiya B, Brajesh RG and Ramalingam S, Bio-transformation of glycerol to 3-hydroxypropionic acid using resting cells of *Lactobacillus reuteri*. *Curr Microbiol* **71**:517–523 (2015b).
- 8 Burgé G, Saulou-Bérion C, Moussa M, Pollet B, Flourat A, Allais F *et al.*, Diversity of *Lactobacillus reuteri* strains in converting glycerol into 3-hydroxypropionic acid. *Appl Biochem Biotechnol* **177**:923–939 (2015a).
- 9 Jiang X, Meng X and Xian M, Biosynthetic pathways for 3-hydroxypropionic acid production. *Appl Microbiol Biotechnol* **82**:995–1003 (2009).
- 10 Kumar V, Ashok S and Park S, Recent advances in biological production of 3-hydroxypropionic acid. *Biotechnol Adv* **31**:945–961 (2013).
- 11 Valdehuesa KNG, Liu H, Nisola GM, Chung WJ, Lee SH and Park SJ, Recent advances in the metabolic engineering of microorganisms for the production of 3-hydroxypropionic acid as C3 platform chemical. *Appl Microbiol Biotechnol* **97**:3309–3321 (2013).
- 12 Borodina I, Kildegaard KR, Jensen NB, Blicher TH, Maury J, Sherstky S *et al.*, Establishing a synthetic pathway for high-level production of 3-hydroxypropionic acid in *Saccharomyces cerevisiae* via  $\beta$ -alanine. *Metab Eng* **27**:57–64 (2015).
- 13 Kildegaard KR, Wang Z, Chen Y, Nielsen J and Borodina I, Production of 3-hydroxypropionic acid from glucose and xylose by metabolically engineered *Saccharomyces cerevisiae*. *Metab Eng Com* **2**:132–136 (2015).
- 14 van Maris AJA, Konings WN, van Dijken JP and Pronk JT, Microbial export of lactic acid and 3-hydroxypropanoic acid: implications for industrial fermentation processes. *Metab Eng* **6**:245–255 (2004).



- 15 Ataei SA and Vasheghani-Farahani E, *In situ* separation of lactic acid from fermentation broth using ion exchange resins. *J Ind Microbiol Biotechnol* **35**:1229–1233 (2008).
- 16 Boontawan P, Kanchanathawee S and Boontawan A, Extractive fermentation of L-(+)-lactic acid by *Pediococcus pentosaceus* using electrodeionization (EDI) technique. *Biochem Eng J* **54**:192–199 (2011).
- 17 Xu K and Xu P, Efficient calcium lactate production by fermentation coupled with crystallization-based *in situ* product removal. *Biore-source Technol* **163**:33–39 (2014).
- 18 Moussa M, Burgé G, Chemarin F, Bounader R, Saulou-Bérion C, Allais F et al., Reactive extraction of 3-hydroxypropionic acid from model aqueous solutions and real bioconversion media. Comparison with its isomer 2-hydroxypropionic (lactic) acid. *J Chem Technol Biotechnol* **91**:2276–2285 (2015).
- 19 Burgé G, Moussa M, Chemarin F, Saulou-Bérion C, Allais F, Spinnler E et al., Reactive extraction of bio-based 3-hydroxypropionic acid assisted by hollow fiber membrane contactor using TOA and Aliquat 336 in *n*-decane. *J Chem Technol Biotechnol* **91**:2705–2712 (2016).
- 20 Yabannavar VM and Wang DIC, Strategies for reducing solvent toxicity in extractive fermentations. *Biotechnol Bioeng* **37**:716–722 (1991).
- 21 Matsumoto M, Mochiduki K and Kondo K, Toxicity of ionic liquids and organic solvents to lactic acid-producing bacteria. *J Biosci Bioeng* **98**:344–347 (2004).
- 22 Bar R and Gainer JL, Acid fermentation in water-organic solvent two-liquid-phase systems. *Biotechnol Prog* **3**:109–114 (1987).
- 23 Bar R, Effect of interphase mixing on a water-organic solvent two-liquid-phase microbial system: ethanol fermentation. *J Chem Technol Biotechnol* **43**:49–62 (1988).
- 24 Choudhury B, Basha A and Swaminathan T, Study of lactic acid extraction with higher molecular weight aliphatic amines. *J Chem Technol Biotechnol* **72**:111–116 (1998).
- 25 Marinova NA and Yankov DS, Toxicity of some solvents and extractants towards *Lactobacillus casei* cells. *Bulgarian Chem Com* **41**:368–373 (2009).
- 26 Fernandes P, Prazeres DMF and Cabral JMS, Membrane-assisted extractive bioconversions. *Adv Biochem Eng Biotechnol* **80**:115–148 (2003).
- 27 Sirkar KK, Membranes, phase interfaces, and separations: novel techniques and membranes-an overview. *Ind Eng Chem Res* **47**:5250–5266 (2008).
- 28 Bringas E, San Román MF, Urtiaga AM and Ortiz I, Integrated use of liquid membranes and membrane contactors: enhancing the efficiency of L-L reactive separations. *Chem Eng Process* **67**:120–129 (2013).
- 29 Coelho IM, Silvestre P, Viegas RMC, Crespo J and Carrondo MJT, Membrane-based solvent extraction and stripping of lactate in hollow fiber contactors. *J Membrane Sci* **134**:19–32 (1997).
- 30 Huang H, Yang ST and Ramey DE, A hollow fiber membrane extraction process for recovery and separation of lactic acid from aqueous solution. *Appl Biochem Biotechnol* **113–116**:671–688 (2004).
- 31 Harington T and Hossain MM, Extraction of lactic acid into sunflower oil and its recovery into an aqueous solution. *Desalination* **218**:287–296 (2008).
- 32 Burgé G, Flourat A, Pollet B, Spinnler HE and Allais F, 3-Hydroxypropionaldehyde (3-HPA) quantification by HPLC using synthetic acrolein-free 3-hydroxypropionaldehyde system as analytical standard. *RSC Adv* **5**:92619–92627 (2015b).
- 33 Lüthi-Peng Q, Schärer S and Puhani Z, Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*. *Appl Microbiol Biotechnol* **60**:73–80 (2002).
- 34 Bauer R, du Toit M and Kossmann J, Influence of environmental parameters on production of the acrolein precursor 3-hydroxypropionaldehyde by *Lactobacillus reuteri* DSMZ 20016 and its accumulation by wine lactobacilli. *Int J Food Microbiol* **137**:28–31 (2010).
- 35 Talarico TL and Dobrogosz WJ, Purification and characterization of glycerol dehydratase from *Lactobacillus reuteri*. *Appl Environ Microbiol* **56**:1195–1197 (1990).
- 36 Sabet-Azad R, Linares-Pasten JA, Torkelson L, Sardari RRR and Hatti-Kaul RH, Co-enzyme A-acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: kinetic characterization and molecular modeling. *Enzyme Microbiol Technol* **53**:235–242 (2013).
- 37 Kyuchoukov G, Marinova M, Molinier J, Albet J and Malmay G, Extraction of lactic acid by means of a mixed extractant. *Ind Eng Chem Res* **40**:5623–5639 (2001).
- 38 Matsumoto M, Takahashi T and Fukushima K, Synergistic extraction of lactic acid with alkylamine and tri-*n*-butylphosphate: effects of amines, diluents and temperature. *Sep Purif Technol* **33**:89–93 (2003).
- 39 Seevaratnam S, Holst O, Hjørlefsdóttir S and Mattiasson B, Extractive bioconversion for lactic acid production using solid sorbent and organic solvent. *Bioprocess Eng* **6**:35–41 (1991).
- 40 Leon R, Fernandes P, Pinheiro HM and Cabral JMS, Whole-cell biocatalysis in organic media. *Enzyme Microbiol Technol* **23**:483–500 (1998).
- 41 Sardesai Y and Bhosle S, Tolerance of bacteria to organic solvents. *Res Microbiol* **153**:263–268 (2002).
- 42 Demirci A, Pometto III AL and Harkins KR, Rapid screening of solvents and carrier compounds for lactic acid recovery by emulsion liquid extraction and toxicity on *Lactobacillus casei* (ATCC 11443). *Bioseparation* **7**:297–308 (1999).
- 43 Dupuy A, Athès V, Schenk J, Jenelten U and Souchon I, Experimental and theoretical considerations on breakthrough pressure in membrane-based solvent extraction: focus on citrus essential oil/hydro-alcoholic solvent systems with low interfacial tension. *J Membr Sci* **378**:203–213 (2011).
- 44 Gao MT, Shimamura T, Ishida N, Nagamori E, Takahashi H, Umemoto S et al., Extractive lactic acid fermentation with tri-*n*-decylamine as the extractant. *Enzyme Microbiol Technol* **44**:350–354 (2009).
- 45 Trauth E, Lemaître JP, Rojas C, Diviès C and Cachon R, Resistance of immobilized lactic acid bacteria to the inhibitory effect of quaternary ammonium sanitizers. *Lebensm-Wiss u-Technol* **34**:239–243 (2001).
- 46 Tik N, Bayraktar E and Mehmetoglu U, *In situ* reactive extraction of lactic acid from fermentation media. *J Chem Technol Biotechnol* **76**:764–768 (2001).
- 47 Keshav A, Wasewar KL and Chand S, Reactive extraction of propionic acid using tri-*n*-octylamine, tri-*n*-butyl phosphate and Aliquat 336 in sunflower oil as diluent. *J Chem Technol Biotechnol* **84**:484–489 (2009).
- 48 Wasewar KL, Shendea D and Keshav A, Reactive extraction of itaconic acid using tri-*n*-butyl phosphate and Aliquat 336 in sunflower oil as a non-toxic diluent. *J Chem Technol Biotechnol* **86**:319–323 (2011).
- 49 Chemarin F, Moussa M, Chadni M, Pollet B, Lieben P, Allais F 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*. DOI: 10.1016/j.seppur.2017.02.018
- 50 Torres S, Pandey A and Castro GR, Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. *Biotechnol Adv* **29**:442–452 (2011).
- 51 Segura A, Molina L, Fillet S, Krell T, Bernal P, Munoz-Rojas J and Ramos JL, Solvent tolerance in Gram-negative bacteria. *Curr Opin Biotechnol* **23**:415–421 (2012).