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1 **Environmental conditions during glycerol bioconversion affect 3-hydroxypropionic acid**  
2 **bioproduction by *Limosilactobacillus reuteri* DSM 17938**

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6

7 **Keywords**

8 Lactic acid bacteria, central composite experimental design, platform molecule, production  
9 yield, specific production rate

10

11 **Abstract**

12 3-Hydroxypropionic acid (3-HP) is a platform molecule whose biological production was  
13 carried out by the bacterium *Limosilactobacillus reuteri* according to a two-step process:  
14 first, a growth phase in batch mode on glucose, then a glycerol bioconversion into 3-HP in  
15 fed-batch mode. With the objective of improving 3-HP bioproduction, this study aimed at  
16 defining the operating conditions during the bioconversion phase that increases the  
17 bioproduction performance. A central composite rotatable design allowed testing various pH  
18 levels and specific glycerol feeding rates. By establishing response surfaces, optimal  
19 conditions have been identified that were different depending on the considered output  
20 variable (final 3-HP quantity, 3-HP production yield and production rate). Of them, 3-HP  
21 final quantity and 3-HP production yield were maximized at pH 6.0 and at specific glycerol  
22 feeding rates of 60 and 55  $\text{mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ , respectively. The specific 3-HP production rate  
23 was the highest at the upper limit of the specific substrate feeding rate (80  $\text{mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ )  
24 but was not affected by the pH. An additional experiment was carried out at pH 6.0 and a  
25 specific glycerol feeding rate of 80  $\text{mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$  to validate the previous observations. In

26 conclusion, the results showed a significant improvement of 3-HP concentration by 13 %, of  
27 specific production rate by 34 % and of 3-HP volumetric productivity by 39 %, as compared  
28 to the initial values

29

## 30 **Introduction**

31 The platform molecule 3-hydroxypropionic acid (3-HP) is of great interest due to its various  
32 applications. It is considered as a building block of a great number of derivatives such as  
33 acrylic acid, 1,3-propanediol (1,3-PDO), 3-hydroxypropionaldehyde (3-HPA), malonic acid,  
34 as well as biodegradable polymers and polyesters [1, 2]. In the framework of growing the  
35 bio-economy, the bioproduction of 3-HP from renewable resources, especially glycerol [3],  
36 could help reduce the environmental impact of these chemicals and the use of oil reserves for  
37 their production.

38 *Limosilactobacillus reuteri* (*L. reuteri*), that was previously known as *Lactobacillus reuteri*  
39 [4], has the natural ability to convert glycerol into 3-HP via the propanediol-utilization Pdu  
40 pathway [5]. With this bacterial species, the growth phase on glucose must be separated from  
41 the 3-HP production step, in order to allow resting cells to convert glycerol into 3-HP instead  
42 of only 1,3-propanediol (1,3-PDO) [6]. Two main approaches are applied to improve this  
43 biotransformation. The first one targets the microorganism by itself, either by rebalancing the  
44 pathway using metabolic engineering, by improving enzyme performance or by increasing  
45 strain robustness as recently reviewed by [7]. The second strategy aims at improving the  
46 production process. This is done by implementing the bioconversion in fed-batch mode with  
47 a progressive supply of glycerol, to avoid the overproduction of the metabolic intermediate 3-  
48 hydroxypropionaldehyde (3-HPA) [5] and its inhibiting effect on bacterial cells [8].

49 Focusing on non-genetically modified *L. reuteri* strains, several studies report the effect of  
50 environmental conditions on the performance of glycerol metabolism, namely the  
51 composition of the bioconversion medium, the bioconversion temperature and pH, the  
52 composition of the gaseous atmosphere, and the glycerol or specific glycerol feeding rates  
53 during the fed-batch process. However, whereas the conditions favoring the glycerol  
54 bioconversion into 3-HPA have been studied extensively, only few works deal with those

55 improving 3-HP production from glycerol. By considering the medium composition, a non-  
56 growing medium is reported to be used for 3-HP bioproduction in order to prevent the  
57 synthesis of undesirable fermentation products (i.e. lactic acid, ethanol, acetic acid) and to  
58 balance the redox cofactors during bioconversion [5, 9]. Consequently, only glycerol shall be  
59 supplied as a carbon source during the fed-batch step [6, 10]. The glycerol concentration was  
60 shown to influence the bioproduction of 3-HPA by *L. reuteri* ATCC 53608 [11]. Vitamin  
61 B12 is mandatory for the glycerol bioconversion into 3-HP by *L. reuteri*, as a required  
62 cofactor of the B12-dependent glycerol dehydratase involved in the dehydration of glycerol  
63 into 3-HPA [12]. Although *L. reuteri* naturally synthesizes it [13], a supplementation of the  
64 bioconversion medium with an exogenous source of vitamin B12 at 0.1 mg·L<sup>-1</sup> improved the  
65 glycerol metabolism by *L. reuteri* DSM 17938 [14]. The addition of Na<sup>+</sup> leads to a decrease  
66 in the enzyme 1,3-propanediol oxidoreductase activity [15, 16]. However, as less NAD<sup>+</sup>  
67 cofactor was synthesized in the presence of Na<sup>+</sup>, it was less available for 3-HPA oxidation  
68 into 3-HP [5].

69 The impact of the bioconversion temperature on glycerol metabolism has already been  
70 studied by some authors. The temperature of 37 °C was applied during bioconversions  
71 involving *L. reuteri* DSM 17938 [14] and *L. reuteri* DSM 20016 [5]. It was also identified as  
72 the optimal temperature, since it maximizes the glycerol dehydratase activity in *L. reuteri*  
73 CG001 [17].

74 The pH of the bioconversion medium also affects *L. reuteri* cells during bioconversion, as it  
75 acts directly on intracellular pH and enzymatic activities. The optimal pH of the enzymes  
76 involved in the Pdu pathway in *L. reuteri* ATCC 53608 was pH 7.0 in MRS and pH 5.0 in  
77 osmosis water [16]. The oxidation of 3-HPA to 3-HP thanks to the enzyme propionaldehyde  
78 dehydrogenase and the reduction of 3-HPA to 1,3-PDO through the propanediol  
79 oxidoreductase were maximized at pH 7.0 [18] and pH 6.2 [19], respectively. This was

80 confirmed by [5] who showed that increasing the pH from pH 5.0 to pH 7.0 enhanced the  
81 specific production rates of both 3-HP and 1,3-PDO by *L. reuteri* DSM 20016 and *L. reuteri*  
82 RPRB3007. Finally, a higher molar ratio of 3-HP to 1,3-PDO was achieved at pH 7.0 than at  
83 pH 5.0 ( $0.49 \text{ mol}\cdot\text{mol}^{-1}$  compared to  $0.43 \text{ mol}\cdot\text{mol}^{-1}$ ) [5].

84 The effect of gaseous atmosphere has already been studied on the production of 3-HP. Strict  
85 anaerobic conditions allowed increasing by 30 % the production of vitamin B12 by *L. reuteri*  
86 JCM1112 [20]. More recently, a study was dedicated to the definition of gaseous conditions  
87 for producing 3-HP and 1,3-PDO [21]. Micro-aerobiosis, carried out by gently mixing the  
88 bioconversion medium without any gas addition, was demonstrated to maximize 3-HP  
89 production by *L. reuteri* FXZ014 [21]. In fact, it was reported that a low dissolved oxygen  
90 level ensures the regeneration of  $\text{NAD}^+$  cofactor for glycerol bioconversion into 3-HP [22].

91 During the fed-batch process of 3-HP bioproduction by *L. reuteri*, the glycerol supply rate  
92 has to be accurately controlled. The aim is to avoid or minimize detrimental 3-HPA  
93 accumulation that is the consequence of the higher activity of glycerol dehydratase as  
94 compared to that of aldehyde dehydrogenase in the Pdu pathway [23]. Specific glycerol  
95 feeding rates of  $62.5$  and  $100 \text{ mg}_{\text{gly}}\cdot\text{g}_{\text{cell}}^{-1}\cdot\text{h}^{-1}$  were determined as maximum values to produce  
96 3-HP with *L. reuteri* DSM 20016, at pH 5.0 and 7.0 respectively, without undesirable  
97 accumulation of 3-HPA [23]. Increasing the glycerol feeding rate from  $0.6$  to  $1.6 \text{ g}_{\text{gly}}\cdot\text{h}^{-1}$   
98 enhanced the 3-HP titer and productivity by 218%, but with a low 3-HPA accumulation,  
99 during a multistep fed-batch performed with *L. reuteri* DSM 20016 at pH 7 [5]. A recent  
100 study performed with *L. reuteri* CICC 6118 reported a glycerol feeding rate of  $0.59 \text{ g}_{\text{gly}}\cdot\text{h}^{-1}$   
101 and a specific glycerol feeding rate of  $76 \text{ mg}_{\text{gly}}\cdot\text{g}_{\text{cell}}^{-1}\cdot\text{h}^{-1}$  to maximize the 3-HP titer [21].

102 From this literature review, the two environmental conditions pH and specific glycerol  
103 feeding rate appear to be highly important for 3-HP bioproduction, but they have never been  
104 considered together to figure out their possible interaction during bioconversion. This work

105 aims at studying the effect of these two factors on *L. reuteri* DSM 17938's ability to produce  
106 3-HP, in instrumented bioreactors by using a central composite rotatable design (CCRD).  
107 This experimental approach will allow defining optimized conditions of pH and specific  
108 glycerol feeding rate for the bioconversion step.

## 109 **Material and methods**

### 110 ***Bacterial strain***

111 The strain *Limosilactobacillus reuteri* DSM 17938 (BioGaia AB, Stockholm, Sweden) was  
112 stored at - 80 °C in cryotubes with glycerol 20 % (v/v). Bacterial cells were reactivated  
113 according to [6] by two successive cultures in MRS broth (Biokar Diagnostic, Beauvais,  
114 France) at 37 °C, 100 rpm for 16 h and then 8 h, respectively.

### 115 ***Bacterial growth in batch mode***

116 Growth of *L. reuteri* DSM 17938 was conducted in a 5-L bioreactor (Sartorius B<sup>+</sup>, Dourdan,  
117 France). Cells were inoculated at an initial concentration of  $5.1 \times 10^{-6}$  g of cell dry weight  
118 (CDW) per liter ( $\text{g}_{\text{CDW}} \cdot \text{L}^{-1}$ ), corresponding to  $5.8 \times 10^3$  cells  $\cdot \text{mL}^{-1}$ . Growth conditions were  
119 defined from the results obtained by [6]. The growth medium was composed of MRS broth  
120 supplemented with 20  $\text{g} \cdot \text{L}^{-1}$  glucose (VWR, Fontenay-sous-Bois, France), 25  $\text{g} \cdot \text{L}^{-1}$  phytone  
121 peptone (Merck KGaA, Darmstadt, Germany), 3  $\text{g} \cdot \text{L}^{-1}$  1,2-propanediol (1,2-PDO) (Sigma-  
122 Aldrich, Saint-Quentin-Fallavier, France), 0.234  $\text{g} \cdot \text{L}^{-1}$  betaine (Sigma-Aldrich) plus 7.455  
123  $\text{g} \cdot \text{L}^{-1}$  KCl (VWR), and 4  $\text{g} \cdot \text{L}^{-1}$  Tween 80 (VWR). The temperature was set at 37 °C, the  
124 agitation rate at 100 rpm and the pH was controlled at 6.0 by the addition of  $\text{NH}_4\text{OH}$  14.8  
125  $\text{mol} \cdot \text{L}^{-1}$ . Neither gas nor air was transferred into the bioreactor in order to generate micro-  
126 aerobiosis conditions.

127 The growth step was stopped when the base consumption ceased, i.e., when no more lactic  
128 acid was produced by bacteria. The broth was harvested aseptically, and the bacteria were

129 recovered by centrifugation (Avanti® J-26-XP centrifuge; Beckman Coulter, Fullerton, CA)  
130 at 6,200 g for 10 min at 4°C.

### 131 ***Glycerol bioconversion in fed-batch mode***

132 Bioreactors of 500-mL total working volume (Global Process Concept, La Rochelle, France)  
133 were used with an initial working volume of 200 mL. The bacterial cells obtained from the  
134 growth step were diluted in osmosis water to reach a concentration of  $21.6 \pm 2.3 \text{ g}_{\text{CDW}} \cdot \text{L}^{-1}$   
135 ( $2.5 \pm 0.3 \times 10^{10} \text{ cells} \cdot \text{mL}^{-1}$ ) then introduced into the bioreactor. The bioconversion was  
136 started by feeding the resting cells with glycerol using a peristaltic pump (Watson Marlow  
137 120U-DV, La Queue Lez Yvelines, France). The concentration of the glycerol solution was  
138 defined between 44 and 110  $\text{g}_{\text{gly}} \cdot \text{L}^{-1}$ , depending on the targeted specific glycerol feeding rate,  
139 as defined by the experimental design. Temperature and agitation were controlled at 37 °C  
140 and 100 rpm, respectively. The partial pressure of dissolved oxygen ( $\text{pO}_2$ ) was measured by  
141 an optical dissolved oxygen probe (Hamilton, Bonaduz, Switzerland). The pH was measured  
142 using a pH probe (Mettler Toledo, Viroflay, France) and controlled at a value defined by the  
143 experimental design by adding  $\text{NH}_4\text{OH}$   $1.48 \text{ mol} \cdot \text{L}^{-1}$ . The volume variation in the bioreactor  
144 was calculated on-line by considering both glycerol and  $\text{NH}_4\text{OH}$  addition. Bioconversion was  
145 interrupted when no more 3-HP was produced, which was evaluated by the cessation of base  
146 consumption and by the concomitant increase in  $\text{pO}_2$  [6].

### 147 ***Analytical methods***

#### 148 *Measurement of biomass concentration*

149 The optical density was assessed off-line by spectrophotometry measurements at 600 nm  
150 ( $\text{OD}_{600}$ , UV-Vis Evolution™ 201, Fisher Scientific SAS, Illkirch, France). They were  
151 performed in triplicate. Results were correlated to cell concentration, measured from cell dry  
152 weight (CDW) and expressed in  $\text{g}_{\text{CDW}} \cdot \text{L}^{-1}$ :

$$153 \text{CDW} = 0.26 \times \text{OD}_{600} \quad (\text{R}^2 = 0.99) \quad (\text{eq. 1})$$

154 The bacterial cell concentration (CFC, in cells·mL<sup>-1</sup>) was also determined in triplicate by  
155 flow cytometry as described by [6]. The results were correlated according to the following  
156 equations:

$$157 \text{ CFC} = 2.99 \cdot 10^8 \times \text{OD}_{600} \quad (\text{R}^2 = 0.99) \quad (\text{eq. 2})$$

$$158 \text{ CDW} = 8.82 \cdot 10^{-10} \times \text{CFC} \quad (\text{R}^2 = 0.99) \quad (\text{eq. 3})$$

159 Moreover, some experiments provided additional information as they were subjected to on-  
160 line medium turbidity measurements at 880 nm using a biomass probe EXcell 230 (CellD,  
161 Roquemaure France).

#### 162 *Quantification of substrate and metabolite concentrations*

163 The substrate of bacterial growth (glucose), the precursor of the bioconversion (glycerol), as  
164 well as the growth metabolites (lactic acid, ethanol and acetic acid) and the bioconversion  
165 products (3-HP, 1,3-PDO and 3-HPA) were quantified by high-performance liquid  
166 chromatography (HPLC, Waters Associates, Molsheim, France), as already described by [6].  
167 All analyses were duplicated, and the concentrations were given in g·L<sup>-1</sup>. The quantities of  
168 consumed glycerol, and produced 3-HP, 1,3-PDO and 3-HPA over time were calculated by  
169 multiplying the respective concentrations by the volume inside the bioreactor determined at  
170 each time.

#### 171 *Assessment of carbon mass balances*

172 The carbon mass balance (in % mol·mol<sup>-1</sup>) corresponds to the ratio of the sum of the molar  
173 quantities of carbon in metabolites and bacterial cells, to the molar quantity of carbon in  
174 substrate or precursor. During the growth step, the carbon mass balance (CMB<sub>G</sub>) was  
175 calculated by considering that the central metabolism of *L. reuteri* used PK and EMP  
176 pathways, as described in [6]. The molar quantity of CO<sub>2</sub> produced was estimated as being  
177 equal to that of produced [ethanol + acetate], in agreement with the stoichiometry of PK  
178 pathway [10]. The molecular weight of *L. reuteri* DSM 17938 cells was previously

179 determined to be equal to  $27.17 \text{ g}_{\text{cell}} \cdot \text{mol}_{\text{cell}}^{-1}$  [6]. During the bioconversion step, as no growth  
180 occurred, the carbon mass balance (CMB<sub>B</sub>) was established according to the Pdu pathway  
181 [6].

### 182 ***Central composite rotatable design***

183 A central composite rotatable design (CCRD) [24] was employed to search for optimized  
184 conditions for the 3-HP bioproduction from glycerol. The two studied factors were the pH  
185 and the specific glycerol feeding rate ( $q_s$ , in  $\text{mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ ), tested at five different levels  
186 (Table 1). The lower limit, the central point and the upper limit of each factor were defined  
187 from the literature and according to results of preliminary tests.

188 The two-factor CCRD allowed creating a matrix of 11 conditions, including a triplicate at the  
189 central point (Table 2).

190 The effects of the two factors were tested on three response variables that characterized the  
191 bioconversion performance: maximal 3-HP quantity ( $g_{3\text{-HP}}$ ), 3-HP production yield ( $g_{3\text{-HP}}$   
192  $\cdot \text{g}_{\text{CDW}}^{-1}$ ) and specific 3-HP production rate ( $g_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ ). They were expressed  
193 according to the general second order polynomial equation as follows:

$$194 \quad Y = a_0 + a_1 \times \text{pH}_c + a_2 \times q_{sc} + a_{12} \times \text{pH}_c \times q_{sc} + a_{11} \times \text{pH}_c^2 + a_{22} \times q_{sc}^2 \quad (\text{eq. 4})$$

195 Where  $Y$  is the predicted value of the response variable;  $\text{pH}_c$  and  $q_{sc}$  are the coded values of  
196 the factors pH and  $q_s$ , respectively;  $a_0$  is the constant coefficient corresponding to the central  
197 point (when all coded values of variables are set at 0);  $a_1$  and  $a_2$  are the linear coefficients;  $a_{11}$   
198 and  $a_{22}$  are the quadratic coefficients;  $a_{12}$  is the cross-product coefficient.

199 Analyses of variance (ANOVA) were performed to highlight the significant effects of the  
200 factors and their interaction at  $P < 0.05$ , with the Scheffe's procedure as post hoc test. The  
201 software Statgraphics 3.0 (Statistical Graphics Corp., Warrenton, USA) was used for  
202 designing the experiments and analyzing the data.

### 203 **Results and Discussion**

204 ***Bacterial growth and glycerol bioconversion under the conditions of the central point of***  
205 ***the experimental design***

206 Three repetitions of the central point were performed to check the reproducibility of the  
207 experiments conducted with *L. reuteri* DSM 17938. During the growth step, the bacterial  
208 growth was identified as reproducible by considering the lag phase duration ( $7.53 \pm 0.62$  h),  
209 the maximal specific growth rate ( $1.14 \pm 0.04$  h<sup>-1</sup>), the final bacterial concentration ( $3.0 \pm 0.3$   
210 g<sub>CDW</sub>·L<sup>-1</sup>) and the total duration of the growth phase ( $13.31 \pm 0.36$  h). The main bioproducts  
211 were lactic acid ( $16.6 \pm 1.1$  g·L<sup>-1</sup>), acetic acid ( $5.5 \pm 0.6$  g·L<sup>-1</sup>) and ethanol ( $6.9 \pm 0.9$  g·L<sup>-1</sup>),  
212 thus confirming that bacteria used both EMP and PK pathways [6]. The carbon mass balance  
213 during growth was equal to  $109.2 \pm 0.8$  % that is higher than 100 % due to the fact that a part  
214 of the carbon used for bacterial growth may come from other sources than glucose, i.e., yeast  
215 extract and phytone peptone from the culture medium [6].

216 During the bioconversion step, glycerol was fed into the bioreactor at a constant specific rate  
217 of  $50$  mg<sub>gly</sub>·g<sub>CDW</sub>·h<sup>-1</sup> until the bioconversion stopped and the pH was controlled at pH 6.0 as  
218 defined by the CCRD matrix (Table 2). The kinetics of glycerol bioconversion into 3-HP and  
219 1,3-PDO are illustrated in Figure 1.

220 The results of the bioconversion step obtained from the three repetitions conducted under the  
221 central point conditions (experiments R9 to R11; Table 2) were considered as reproducible,  
222 taking into account the complexity of the experiments. The carbon mass balance of the three  
223 replicates was equal to  $99.7 \pm 1.6$  %, which was close to 100 %. After  $37.1 (\pm 3.8)$  h of  
224 bioconversion, 3-HP and 1,3-PDO reached final concentrations of  $10.8 \pm 0.1$  g<sub>3-HP</sub>·L<sup>-1</sup> and  $8.6$   
225  $\pm 0.2$  g<sub>1,3-PDO</sub>·L<sup>-1</sup>. The final 3-HP titer obtained here was lower than that reached in a previous  
226 study ( $14.7$  g<sub>3-HP</sub>·L<sup>-1</sup>) [6] which can be explained by the shorter duration of the bioconversion  
227 ( $37.1 \pm 3.8$  h instead of 88.6 h). 3-HP was produced equimolarly to 1,3-PDO (molar ratio of  
228  $1.07 \pm 0.01$  mol<sub>3-HP</sub>·mol<sub>1,3-PDO</sub><sup>-1</sup>), which indicated that the redox balance between NAD<sup>+</sup> and

229 NADH was well maintained during 3-HPA oxidation in 3-HP and reduction in 1,3-PDO.  
230 These results were in accordance with those obtained by [5]. The molar ratio between 3-HP  
231 and  $\text{NH}_4\text{OH}$  was equal to  $0.87 \pm 0.09 \text{ mol}_{3\text{-HP}} \cdot \text{mol}_{\text{NH}_4\text{OH}}^{-1}$  on average throughout the  
232 bioconversion, which indicated that base consumption can be used to monitor 3-HP  
233 production, as previously demonstrated [6]. The value lower than 1.0 could be explained by a  
234 slight evaporation of the base  $\text{NH}_4\text{OH}$  that is volatile. A small amount of 3-HPA ( $0.11 \pm 0.03$   
235  $\text{g} \cdot \text{L}^{-1}$ ) was accumulated when bioconversion stopped. This value was about 20 times lower  
236 than the minimum inhibition concentration reported for *L. reuteri* DSM 20016 ( $2.2 - 3.7 \text{ g}_{\text{HPA}} \cdot \text{L}^{-1}$  [8], thus indicating that 3-HPA did not inhibit bacterial cells in these conditions.

### 238 ***Combined effect of pH and specific glycerol feeding rate on the performance of 3-HP*** 239 ***bioproduction from glycerol***

240 By considering the 11 experiments of the CCRD, the bacterial concentration at the end of the  
241 growth step was equal to  $3.1 \pm 0.3 \text{ g}_{\text{CDW}} \cdot \text{L}^{-1}$  and the duration of the growth was  $13.7 \pm 0.7 \text{ h}$ .  
242 The results were not significantly different from one batch to another, which indicated that  
243 the 11 growth steps allowed obtaining bacterial cells in similar physiological state and  
244 quantity. This permitted to initiate further bioconversions with similar microbial cells to test  
245 the impact of the operating conditions on 3-HP production performances.

246 For each of the 11 bioconversions, the carbon mass balance has been verified over time. It  
247 was equal to  $97.2 \pm 6.3 \%$ , which was close to 100 % and considered as satisfactory. The  
248 molar ratio between 3-HP and 1,3-PDO was established over time for each set of conditions  
249 of the CCRD. The mean value for all experiments was equal to  $0.98 \pm 0.08 \text{ mol}_{3\text{-HP}} \cdot \text{mol}_{1,3\text{-PDO}}^{-1}$ ,  
250 which was close to the expected value of 1. The mean value of the bioconversion yield,  
251 obtained by relating the 3-HP concentration to the concentration of consumed glycerol, was  
252 calculated at  $0.47 \pm 0.03 \text{ mol}_{3\text{-HP}} \cdot \text{mol}_{\text{gly}}^{-1}$ , which was considered as reproducible. In addition,  
253 this result was consistent with the theoretical value of  $0.5 \text{ mol}_{3\text{-HP}} \cdot \text{mol}_{\text{gly}}^{-1}$  [10]. The molar

254 ratio between 3-HP and  $\text{NH}_4\text{OH}$  was lower than expected ( $0.89 \pm 0.19 \text{ mol}_{3\text{-HP}} \cdot \text{mol}_{\text{NH}_4\text{OH}}^{-1}$ ),  
255 which could be explained by the volatility of the  $\text{NH}_4\text{OH}$  solution used for pH control. At the  
256 end of the 11 fed-batches, the final volumes were comprised between 250 mL and 390 mL,  
257 depending on the glycerol feeding rate, the quantity of  $\text{NH}_4\text{OH}$  added to control the pH and  
258 the duration of bioconversion. As previously observed [6], the bioconversion ceased  
259 suddenly, irrespective of the glycerol feeding rate, pH or 3-HP and 3-HPA concentrations.

260 Multivariable second order linear regressions were carried out to represent the final 3-HP  
261 quantity, the 3-HP production yield and the specific 3-HP production rate as a function of pH  
262 and specific glycerol feeding rate. The coefficients of the polynomials are summed up in  
263 Table 3. The multiple correlation coefficients  $R^2$  were comprised between 0.85 and 0.94. By  
264 considering the complexity of the experiments, which included two successive steps of  
265 growth and bioconversion with a concentration step between them, these results were  
266 considered as satisfactory. The coefficients displayed in Table 3 indicated that 3-HP final  
267 quantity and production yield were affected by pH that displayed a quadratic effect and by  
268 specific glycerol feeding rate that showed linear and quadratic effects, with no interaction  
269 between these two factors. Meanwhile, the specific 3-HP production rate was only affected  
270 by a linear effect of the specific glycerol feeding rate but not by the pH, nor by an interaction.

271 This is the first demonstration of the absence of interaction between pH and specific glycerol  
272 feeding rate on the 3-HP bioproduction performance by a strain of *L. reuteri*, on the ranges  
273 tested for these two environmental conditions (i.e., pH from 4.8 to 7.2 and specific substrate  
274 feeding rate between 20 and  $80 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ ).

275 *Effect of pH and specific glycerol feeding rate on 3-HP final quantity and 3-HP production*  
276 *yield*

277 The three-dimensional representations of the effects of the specific glycerol feeding rate and  
278 pH on 3-HP final quantity and production yield are displayed on Figure 2 and Figure 3,

279 respectively. The 3-HP final quantity was calculated from the 3-HP final concentrations that  
280 ranged between 6.48 and 10.88  $\text{g}_{3\text{-HP}}\cdot\text{L}^{-1}$  and from the volumes of the corresponding  
281 experiments, thus ranging between 1.65 and 4.15  $\text{g}_{3\text{-HP}}$ . The production yields were calculated  
282 by relating the final 3-HP quantities to the corresponding final biomass quantities. They  
283 ranged between 0.39 and 1.15  $\text{g}_{3\text{-HP}}\cdot\text{g}_{\text{CDW}}^{-1}$ . These values were consistent with those obtained  
284 by [6] with the same strain at pH 6.0 and by [5] with the strain DSM 20016 at pH 7.0 and 5.0.  
285 The optimum pH for these two variables was equal to pH 6.0, whatever the specific glycerol  
286 feeding rate value. This value is close to pH 6.2, which is the optimal pH of the enzyme  
287 propanediol oxidoreductase involved in the reduction of 3-HPA into 1,3-PDO [19] but below  
288 the optimal pH (pH 7.0) of the enzyme propionaldehyde dehydrogenase [18]. In addition, this  
289 pH is unfavorable for 3-HPA accumulation [25] thus preventing any detrimental effects by  
290 this compound. The final quantity and production yield of 3-HP were close to zero at pH 4.8  
291 and pH 7.2. This can be explained by the fact that pH 4.8 is far from the optimum pH of the  
292 enzymes involved in the Pdu pathway [18, 19, 25]. In addition, at pH 7.2, the activity of  
293 glycerol dehydratase that catalyzes the dehydration of glycerol into 3-HPA is high [25], thus  
294 leading to 3-HPA accumulation, that reached 0.4  $\text{g}_{3\text{-HPA}}\cdot\text{L}^{-1}$  in this study.

295 The specific glycerol feeding rate displayed both linear and quadratic effects on the 3-HP  
296 final quantity (Figure 2) and 3-HP production yield (Figure 3). These variables reached their  
297 maximum values for specific glycerol feeding rates respectively equal to 60 and 55  
298  $\text{mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$ . These results are in accordance with those obtained by [5] with *L. reuteri*  
299 DSM 20016 (62.5  $\text{mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$ ) and by [6] with *L. reuteri* DSM 17938 (51  $\text{mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}$   
300  $\cdot\text{h}^{-1}$ ). At low specific glycerol feeding rates (i.e., 20  $\text{mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$ ), the two response  
301 variables remained very low, indicating a glycerol limitation.

302 The maximal values of 3-HP final quantity and 3-HP production yield by *L. reuteri* DSM  
303 17938 were estimated from the coefficients of the polynomial equations given in Table 3.

304 The maximal 3-HP final quantity was calculated at  $4.1 \pm 0.2 \text{ g}_{3\text{-HP}}$  at pH 6.0 and 55  
305  $\text{mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$  and the maximal 3-HP production yield was estimated at  $1.11 \pm 0.07 \text{ g}_{3\text{-HP}}$   
306  $\text{HP} \cdot \text{g}_{\text{CDW}}^{-1}$  at pH 6.0 and  $60 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ . These estimations were close to the experimental  
307 values obtained for the three repetitions of the central point (i.e.,  $4.0 \pm 0.2 \text{ g}_{3\text{-HP}}$  and  $1.10 \pm$   
308  $0.07 \text{ g}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1}$  respectively) conducted at pH 6.0 and  $50 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ .

309 *Effect of specific glycerol feeding rate on specific 3-HP production rate of L. reuteri DSM*  
310 *17938*

311 The specific 3-HP production rate represents the intrinsic capacity of the bacterial cells to  
312 produce 3-HP. It was determined by dividing the 3-HP production rates by the biomass  
313 quantity. The values ranged between 11.25 and  $41.50 \text{ mg}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ . This result is in  
314 agreement with those obtained with *L. reuteri* DSM 20016 by [5]. Figure 4 shows the  
315 response surface corresponding to the polynomial equation characterizing the effects of pH  
316 and specific glycerol feeding rate on specific 3-HP production rate. As shown in Table 3 and  
317 Figure 4, no effect of pH was observed.

318 Whatever the pH, the specific 3-HP production rate increased with the specific glycerol  
319 feeding rate, the highest value of  $41.50 \text{ mg}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$  being obtained at  $80 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1}$   
320  $\cdot \text{h}^{-1}$ . This was a great improvement compared to the value of  $28.4 \text{ mg}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$   
321 previously reported by [23] at  $62.5 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ . In addition, we observed that a low  
322 specific substrate-feeding rate is limiting for 3-HP bioproduction, as previously hypothesized  
323 [6]. It is also consistent with the results reported by [5] with *L. reuteri* DSM 20016.

324 However, a slowdown of the increase of the specific production rate was observed between  
325  $70$  and  $80 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$  (Figure 4), which could be explained by the accumulation of the  
326 inhibitory intermediate 3-HPA. Indeed, 3-HPA accumulation was not significant in all  
327 experiments of the CCRD ( $0.05$  to  $0.62 \text{ g}_{3\text{-HPA}} \cdot \text{L}^{-1}$ ). This range was lower or similar to the  
328 minimum 3-HPA inhibitory concentration ( $0.60 \text{ g} \cdot \text{L}^{-1}$ ) that was reported as deleterious for the

329 activity of the enzyme propionaldehyde dehydrogenase responsible for the transformation of  
330 3-HPA into 3-hydroxypropionyl-CoA [8]. Nevertheless, 3-HPA accumulation remained  
331 lower than that observed by [5], who detected final concentrations between 1.9 to 2.1 g<sub>3-</sub>  
332 HPA·L<sup>-1</sup> at a specific glycerol feeding rate of 190.5 mg<sub>gly</sub>·g<sub>CDW</sub><sup>-1</sup>·h<sup>-1</sup> and at pH 5.0 and pH 7.0,  
333 respectively.

334 The optimal conditions of pH and specific glycerol feeding rate that maximized the specific  
335 3-HP production rate of *L. reuteri* DSM 17938 were identified from the equation given in  
336 Table 3. As no optimal pH could be identified from the polynomial equation, the  
337 bioconversion may be carried out at any pH comprised between pH 4.8 and pH 7.2. This  
338 result is interesting as it indicates that the specific rate will be maintained, even at low pH  
339 values. The real optimal specific glycerol feeding rate could not be calculated, but for another  
340 reason. From the polynomial equation, its value may be higher than 80 mg<sub>gly</sub>·g<sub>CDW</sub><sup>-1</sup>·h<sup>-1</sup>  
341 which is out of the tested range. However, too high values of specific glycerol feeding rate  
342 may lead to 3-HPA accumulation, thus leading to an inhibition of 3-HP bioproduction [8]. It  
343 can therefore be hypothesized that the optimum specific glycerol feeding rate should be close  
344 to 80 mg<sub>gly</sub>·g<sub>CDW</sub><sup>-1</sup>·h<sup>-1</sup>.

345 To summarize the results obtained from the experimental design about the effects of pH and  
346 specific glycerol feeding rate on 3-HP bioproduction performance of *L. reuteri* DSM 17938,  
347 two sets of conditions have been highlighted. By considering 3-HP final quantity and  
348 production yield, specific glycerol feeding rates of 60 and 55 mg<sub>gly</sub>·g<sub>CDW</sub><sup>-1</sup>·h<sup>-1</sup> respectively,  
349 and pH 6.0 were found to be optimal conditions. To maximize the specific 3-HP production  
350 rate, the specific substrate feeding rate has to be higher than 80 mg<sub>gly</sub>·g<sub>CDW</sub><sup>-1</sup>·h<sup>-1</sup>, irrespective  
351 of the pH in the range of pH 4.8 to pH 7.2.

352 ***Glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 in the optimal conditions***  
353 ***defined from the experimental design***

354 In order to validate optimal conditions obtained from the models and with a view of  
355 maximizing 3-HP bioproduction, a validation experiment was performed at  $80 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$   
356  $^1 \cdot \text{h}^{-1}$  and pH 6.0 and compared to the results obtained in the central point condition that was  
357 taken as a reference. The growth phase was conducted under the same conditions as in the  
358 previous experiments, thus allowing to reach a similar final bacterial concentration as in  
359 previous experiments. During the bioconversion phase, the pH was controlled at pH 6.0, as it  
360 was the optimal pH found for maximizing the 3-HP final quantity and the 3-HP production  
361 yield without affecting the specific 3-HP production rate. As the experimental design cannot  
362 be extrapolated, a specific glycerol feeding rate of  $80 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$  was selected to  
363 maximize the specific 3-HP production rate, while limiting 3-HPA accumulation.

364 During the bioconversion step, the carbon mass balance was calculated at  $104.0 \pm 7.0 \%$ . The  
365 molar ratio between 3-HP and 1,3-PDO was equal to  $1.01 \pm 0.001 \text{ mol}_{3\text{-HP}} \cdot \text{mol}_{1,3\text{-PDO}}^{-1}$  which  
366 confirmed that the redox balance was well maintained inside bacterial cells.

367 The kinetics of glycerol consumption, 3-HP production and  $\text{NH}_4\text{OH}$  consumption are  
368 displayed in Figure 5, together with the three replicates of the central point.

369 The bioconversion lasted 24.8 h at  $80 \text{ mg}_{\text{gly}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ , which was shorter than in the central  
370 point conditions ( $37.0 \pm 4.0 \text{ h}$ ). Even if 3-HPA accumulated from 21 h of bioconversion to  
371 reach a final concentration of  $0.31 \text{ g}_{3\text{-HPA}} \cdot \text{L}^{-1}$ , this value remained lower than the inhibitory  
372 concentration [18]. The 3-HP concentration was higher in the validation experiment ( $12.2 \text{ g}_{3\text{-HP}} \cdot \text{L}^{-1}$ )  
373 than in the reference one ( $10.8 \text{ g}_{3\text{-HP}} \cdot \text{L}^{-1}$ ). Additionally, the 3-HP final quantity  
374 reached  $4.9 \text{ g}_{3\text{-HP}}$  in the validation conditions, compared to  $4.0 \text{ g}_{3\text{-HP}}$  in the central point  
375 conditions. The specific 3-HP production rate reached  $40.0 \text{ mg}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$  which was  
376 remarkably higher than in the case of the central point ( $29.8 \pm 1.1 \text{ mg}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ ).  
377 Moreover, it was close to the predicted value given by the model ( $39.4 \pm 4.0 \text{ mg}_{3\text{-HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ )  
378  $^1$ ). Finally, the 3-HP volumetric productivity increased from  $0.36 \pm 0.01 \text{ g}_{3\text{-HP}} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$  in the

379 central point conditions to  $0.50 \text{ g}_{3\text{-HP}}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$  in the validation experiment. This increase by 39  
380 % was noticeable and confirmed that the validation conditions improved the performance of  
381 3-HP bioproduction. In addition, the specific 3-HP production rate and the 3-HP volumetric  
382 productivity reached in this validation experiment were respectively enhanced by 41 % and  
383 208 % when compared with those reported by [23].

384 Finally, the validation experiment derived from the results of the experimental design  
385 allowed obtaining a higher 3-HP final concentration and quantity as well as greater specific  
386 3-HP production rate and volumetric productivity, together with a shorter duration. It  
387 confirmed that this experimental approach was relevant to improve the performance of this  
388 bioprocess.

### 389 **Conclusion**

390 The performance of the glycerol bioconversion into 3-HP by *L. reuteri* DSM 17938 has been  
391 improved at the bioreactor-scale by implementing an experimental design to quantify the  
392 effects of two environmental factors, the bioconversion pH (between pH 4.8 and pH 7.2), and  
393 the specific glycerol feeding rate (between 20 and  $80 \text{ mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$ ). Statistical analyses  
394 allowed identifying optimal values for these factors by considering three performance  
395 indicators. The pH 6.0 was identified as the optimum value by considering 3-HP final  
396 quantity and 3-HP production yield. These two variables were maximized at this pH and at  
397 specific feeding rates of 60 and  $55 \text{ mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$ , respectively. The specific 3-HP  
398 production rate was the highest at the upper limit of the specific substrate feeding rate ( $80$   
399  $\text{mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$ ) without significant accumulation of deleterious 3-HPA. Interestingly, the  
400 pH could be chosen equally in the range between pH 4.8 and pH 7.2 without affecting the  
401 specific 3-HP production rate, which is valuable in the perspective of recovering this building  
402 block for further applications. This higher specific feeding rate of  $80 \text{ mg}_{\text{gly}}\cdot\text{g}_{\text{CDW}}^{-1}\cdot\text{h}^{-1}$  has  
403 been implemented in an additional experiment conducted at pH 6.0, to confirm the

404 improvement of the bioprocess. The significant improvement of the 3-HP bioproduction  
405 performance together with the small 3-HPA accumulation suggested that these conditions  
406 might be close to their optimum by considering *L. reuteri* DSM 17938. However, as the  
407 bioconversion suddenly ceased whereas the glycerol was still supplied, further experiments  
408 have to be performed to better understand the physiological mechanisms that may explain  
409 this behavior. The implementation of the production process at the bioreactor-scale paves the  
410 way towards a scale-up strategy. Moreover, the approach of optimizing the environmental  
411 conditions to improve production performance can be used with genetically modified  
412 microbial hosts to efficiently produce bio-based 3-HP.

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415

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