



HAL
open science

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

Thi-Lan-Phuong Nguyen, Catherine Béal, Sarrah Ghorbal, Claire Saulou-Bérion

► To cite this version:

Thi-Lan-Phuong Nguyen, Catherine Béal, Sarrah Ghorbal, Claire Saulou-Bérion. Environmental conditions during glycerol bioconversion affect 3-hydroxypropionic acid bioproduction by *Limosilactobacillus reuteri* DSM 17938. *Biotechnology Progress*, inPress, 39 (1), pp.e3299. 10.1002/btpr.3299 . hal-03774627

HAL Id: hal-03774627

<https://agroparistech.hal.science/hal-03774627>

Submitted on 11 Sep 2022

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.

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

3 Thi-Lan-Phuong Nguyen, Catherine Béal, Sarrah Ghorbal, Claire Saulou-Bérion

4 *Université Paris-Saclay, AgroParisTech, INRAE, UMR 0782 SayFood, F-91120, Palaiseau,*
5 *France*

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⁻¹ improved the
65 glycerol metabolism by *L. reuteri* DSM 17938 [14]. The addition of Na⁺ leads to a decrease
66 in the enzyme 1,3-propanediol oxidoreductase activity [15, 16]. However, as less NAD⁺
67 cofactor was synthesized in the presence of Na⁺, 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 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⁺, Dourdan,
117 France). Cells were inoculated at an initial concentration of 5.1×10^{-6} g of cell dry weight
118 (CDW) per liter ($\text{g}_{\text{CDW}} \cdot \text{L}^{-1}$), corresponding to 5.8×10^3 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 NH_4OH 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 (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 NH_4OH $1.48 \text{ mol} \cdot \text{L}^{-1}$. The volume variation in the bioreactor
144 was calculated on-line by considering both glycerol and NH_4OH 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 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 (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⁻¹) 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⁻¹. 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⁻¹) 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_G) 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 CO₂ 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_B) 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:

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

195 Where Y is the predicted value of the response variable; pH_c and q_{s_c} 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 ± 0.62 h),
209 the maximal specific growth rate (1.14 ± 0.04 h⁻¹), the final bacterial concentration (3.0 ± 0.3
210 g_{CDW}·L⁻¹) and the total duration of the growth phase (13.31 ± 0.36 h). The main bioproducts
211 were lactic acid (16.6 ± 1.1 g·L⁻¹), acetic acid (5.5 ± 0.6 g·L⁻¹) and ethanol (6.9 ± 0.9 g·L⁻¹),
212 thus confirming that bacteria used both EMP and PK pathways [6]. The carbon mass balance
213 during growth was equal to 109.2 ± 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_{gly}·g_{CDW}·h⁻¹ 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 ± 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 ± 0.1 g_{3-HP}·L⁻¹ and 8.6
225 ± 0.2 g_{1,3-PDO}·L⁻¹. The final 3-HP titer obtained here was lower than that reached in a previous
226 study (14.7 g_{3-HP}·L⁻¹) [6] which can be explained by the shorter duration of the bioconversion
227 (37.1 ± 3.8 h instead of 88.6 h). 3-HP was produced equimolarly to 1,3-PDO (molar ratio of
228 1.07 ± 0.01 mol_{3-HP}·mol_{1,3-PDO}⁻¹), which indicated that the redox balance between NAD⁺ 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 NH_4OH 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 NH_4OH that is volatile. A small amount of 3-HPA (0.11 ± 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 NH_4OH 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 NH_4OH 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 NH_4OH 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 g₃₋
332 HPA·L⁻¹ at a specific glycerol feeding rate of 190.5 mg_{gly}·g_{CDW}⁻¹·h⁻¹ 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_{gly}·g_{CDW}⁻¹·h⁻¹
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_{gly}·g_{CDW}⁻¹·h⁻¹.

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_{gly}·g_{CDW}⁻¹·h⁻¹ 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_{gly}·g_{CDW}⁻¹·h⁻¹, 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 NH_4OH 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.

413 **Acknowledgments**

414 Mrs. Nguyen acknowledges the Vietnamese government for providing her scholarship.

415

416 **References**

- 417 1. Bozell, J.J. and G.R. Petersen, *Technology development for the production of*
418 *biobased products from biorefinery carbohydrates - the US Department of Energy's "Top*
419 *10" revisited*. Green Chemistry, 2010. **12**(4): p. 539-554.
- 420 2. Matsakas, L., E. Topakas, and P. Christakopoulos, *New trends in microbial*
421 *production of 3-hydroxypropionic acid*. Current Biochemical Engineering, 2014. **1**(2): p. 141-
422 154.
- 423 3. Ripoll, M. and L. Betancor, *Opportunities for the valorization of industrial glycerol*
424 *via biotransformations*. Current Opinion in Green and Sustainable Chemistry, 2021. **28**: p.
425 100430.
- 426 4. Zheng, J., S. Wittouck, E. Salvetti, C.M.A.P. Franz, H.M.B. Harris, P. Mattarelli,
427 P.W. O'Toole, B. Pot, P. Vandamme, J. Walter, K. Watanabe, S. Wuyts, G.E. Felis, M.G.
428 Gänzle, and S. Lebeer, *A taxonomic note on the genus Lactobacillus: Description of 23 novel*

- 429 *genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of*
430 *Lactobacillaceae and Leuconostocaceae.* International Journal of Systematic and
431 Evolutionary Microbiology 2020. **70**(4): p. 2782-2858.
- 432 5. Dishisha, T., L.P. Pereyra, S.-H. Pyo, R.A. Britton, and R. Hatti-Kaul, *Flux analysis*
433 *of the Lactobacillus reuteri propanediol-utilization pathway for production of 3-*
434 *hydroxypropionaldehyde, 3-hydroxypropionic acid and 1, 3-propanediol from glycerol.*
435 Microbial Cell Factories, 2014. **13**(1): p. 76-87.
- 436 6. Nguyen, T.L., C. Saulou-Bérion, J. Delettre, and C. Béal, *Culture conditions affect*
437 *Lactobacillus reuteri DSM 17938 ability to perform glycerol bioconversion into 3-*
438 *hydroxypropionic acid.* Journal of Bioscience and Bioengineering, 2021. **131**(5): p. 501-508.
- 439 7. Liang, B., G. Sun, X. Zhang, Q. Nie, Y. Zhao, and J. Yang, *Recent advances,*
440 *challenges and metabolic engineering strategies in the biosynthesis of 3-hydroxypropionic*
441 *acid.* Biotechnology and Bioengineering, 2022: p. 1-30.
- 442 8. Cleusix, V., C. Lacroix, S. Vollenweider, M. Duboux, and G. Le Blay, *Inhibitory*
443 *activity spectrum of reuterin produced by Lactobacillus reuteri against intestinal bacteria.*
444 BMC Microbiol, 2007. **7**: p. 101.
- 445 9. Chen, L. and R. Hatti-Kaul, *Exploring Lactobacillus reuteri DSM20016 as a*
446 *biocatalyst for transformation of longer chain 1, 2-diols: Limits with microcompartment.* Plos
447 One, 2017. **12**(9): p. e0185734.
- 448 10. Burgé, G., C. Saulou-Bérion, M. Moussa, B. Pollet, A. Flourat, F. Allais, V. Athès,
449 and H.-E. Spinnler, *Diversity of Lactobacillus reuteri strains in converting glycerol into 3-*
450 *hydroxypropionic acid.* Applied Biochemistry and Biotechnology, 2015. **177**(4): p. 923-939.
- 451 11. Doleyres, Y., P. Beck, S. Vollenweider, and C. Lacroix, *Production of 3-*
452 *hydroxypropionaldehyde using a two-step process with Lactobacillus reuteri.* Applied
453 Microbiology and Biotechnology, 2005. **68**(4): p. 467-474.

- 454 12. Toraya, T., *Enzymatic radical catalysis: coenzyme B12-dependent diol dehydratase*.
455 The Chemical Record, 2002. **2**(5): p. 352-366.
- 456 13. Matsakas, L., K. Hrůzová, U. Rova, and P. Christakopoulos, *Biological production of*
457 *3-hydroxypropionic acid: an update on the current status*. Fermentation, 2018. **4**(1): p. 13-34.
- 458 14. Couvreur, J., A. Teixeira, F. Allais, H.-E. Spinnler, C. Saulou-Bérion, and T.
459 Clément, *Wheat and sugar beet coproducts for the bioproduction of 3-hydroxypropionic acid*
460 *by Lactobacillus reuteri DSM17938*. Fermentation, 2017. **3**(3): p. 32-44.
- 461 15. Malaoui, H. and R. Marczak, *Purification and characterization of the 1-3-*
462 *propanediol dehydrogenase of Clostridium butyricum E5*. Enzyme and Microbial
463 Technology, 2000. **27**(6): p. 399-405.
- 464 16. Lüthi-Peng, Q., S. Schärer, and Z. Puhan, *Production and stability of 3-*
465 *hydroxypropionaldehyde in Lactobacillus reuteri*. Applied Microbiology and Biotechnology
466 2002. **60**(1-2): p. 73-80.
- 467 17. Ma, H.-Y., G. Chen, and J. Zhao, *Characteristics and in situ reactivation of glycerol*
468 *dehydratase in Lactobacillus reuteri*. Chinese Journal of Applied and Environmental
469 Biology, 2013. **19**: p. 30-36.
- 470 18. Sabet-Azad, R., J.A. Linares-Pastén, L. Torkelson, R.R. Sardari, and R. Hatti-Kaul,
471 *Coenzyme A-acylating propionaldehyde dehydrogenase (PduP) from Lactobacillus reuteri:*
472 *Kinetic characterization and molecular modeling*. Enzyme and Microbial Technology, 2013.
473 **53**(4): p. 235-242.
- 474 19. Talarico, T.L., L.T. Axelsson, J. Novotny, M. Fiuzat, and W.J. Dobrogosz, *Utilization*
475 *of glycerol as a hydrogen acceptor by Lactobacillus reuteri: Purification of 1,3-propanediol:*
476 *NAD⁺ oxidoreductase*. Applied and Environmental Microbiology, 1990. **56**(4): p. 943-948.

- 477 20. Santos, F., B. Teusink, D. Molenaar, M. van Heck, M. Wels, S. Sieuwerts, W.M. de
478 Vos, and J. Hugenholtz, *Effect of amino acid availability on vitamin B12 production in*
479 *Lactobacillus reuteri*. Applied and Environmental Microbiology, 2009. **75**(12): p. 3930-3936.
- 480 21. Zabed, H.M., Y. Zhang, Q. Guo, J. Yun, M. Yang, G. Zhang, and X. Qi, *Co-*
481 *biosynthesis of 3-hydroxypropionic acid and 1,3-propanediol by a newly isolated*
482 *Lactobacillus reuteri* strain during whole cell biotransformation of glycerol. Journal of
483 Cleaner Production, 2019. **226**: p. 432-442.
- 484 22. Ashok, S., M. Sankaranarayanan, Y. Ko, K.E. Jae, S.K. Ainala, V. Kumar, and S.
485 Park, *Production of 3-hydroxypropionic acid from glycerol by recombinant Klebsiella*
486 *pneumoniae ΔdhaTΔyqhD which can produce vitamin B12 naturally*. Biotechnology and
487 Bioengineering, 2013. **110**(2): p. 511-524.
- 488 23. Dishisha, T., S.-H. Pyo, and R. Hatti-Kaul, *Bio-based 3-hydroxypropionic-and acrylic*
489 *acid production from biodiesel glycerol via integrated microbial and chemical catalysis*.
490 Microbial Cell Factories, 2015. **14**(1): p. 200-211.
- 491 24. Box, G.E.P. and K.B. Wilson, *On the experimental attainment of optimum conditions*.
492 1951. **13**(1): p. 1-38.
- 493 25. Talarico, T.L. and W.J. Dobrogosz, *Purification and characterization of glycerol*
494 *dehydratase from Lactobacillus reuteri*. Applied Environmental Microbiology, 1990. **56**(4):
495 p. 1195-1197.
- 496