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

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

Keywords

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

Abstract

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

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

Introduction

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

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

Focusing on non-genetically modified *L. reuteri* strains, several studies report the effect of environmental conditions on the performance of glycerol metabolism, namely the composition of the bioconversion medium, the bioconversion temperature and pH, the composition of the gaseous atmosphere, and the glycerol or specific glycerol feeding rates during the fed-batch process. However, whereas the conditions favoring the glycerol 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 $mg \cdot L^{-1}$ 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

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

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

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

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

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

Material and methods

Bacterial strain

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

Bacterial growth in batch mode

Growth of *L. reuteri* DSM 17938 was conducted in a 5-L bioreactor (Sartorius B^+, Dourdan, France). Cells were inoculated at an initial concentration of 5.1×10^{-6} g of cell dry weight (CDW) per liter ($g_{CDW} \cdot L^{-1}$), corresponding to 5.8×10^3 cells$\cdot mL^{-1}$. Growth conditions were defined from the results obtained by [6]. The growth medium was composed of MRS broth supplemented with 20 $g \cdot L^{-1}$ glucose (VWR, Fontenay-sous-Bois, France), 25 $g \cdot L^{-1}$ phytone peptone (Merck KGaA, Darmstadt, Germany), 3 $g \cdot L^{-1}$ 1,2-propanediol (1,2-PDO) (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 0.234 $g \cdot L^{-1}$ betaine (Sigma-Aldrich) plus 7.455 $g \cdot L^{-1}$ KCl (VWR), and 4 $g \cdot L^{-1}$ Tween 80 (VWR). The temperature was set at 37 °C, the agitation rate at 100 rpm and the pH was controlled at 6.0 by the addition of NH_4OH 14.8 $mol \cdot L^{-1}$. Neither gas nor air was transferred into the bioreactor in order to generate micro-aerobiosis conditions.

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

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

Glycerol bioconversion in fed-batch mode

Bioreactors of 500-mL total working volume (Global Process Concept, La Rochelle, France) were used with an initial working volume of 200 mL. The bacterial cells obtained from the growth step were diluted in osmosis water to reach a concentration of 21.6 ± 2.3 $g_{CDW} \cdot L^{-1}$ (2.5 ± 0.3 × 10^{10} cells·mL^{-1}) then introduced into the bioreactor. The bioconversion was started by feeding the resting cells with glycerol using a peristaltic pump (Watson Marlow 120U-DV, La Queue Lez Yvelines, France). The concentration of the glycerol solution was defined between 44 and 110 $g_{gly} \cdot L^{-1}$, depending on the targeted specific glycerol feeding rate, as defined by the experimental design. Temperature and agitation were controlled at 37 °C and 100 rpm, respectively. The partial pressure of dissolved oxygen (pO_2) was measured by an optical dissolved oxygen probe (Hamilton, Bonaduz, Switzerland). The pH was measured using a pH probe (Mettler Toledo, Viroflay, France) and controlled at a value defined by the experimental design by adding NH_4OH 1.48 mol·L^{-1}. The volume variation in the bioreactor was calculated on-line by considering both glycerol and NH_4OH addition. Bioconversion was interrupted when no more 3-HP was produced, which was evaluated by the cessation of base consumption and by the concomitant increase in pO_2 [6].

Analytical methods

Measurement of biomass concentration

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

$$CDW = 0.26 \times OD_{600} \qquad (R^2 = 0.99) \qquad \text{(eq. 1)}$$

The bacterial cell concentration (CFC, in cells·mL^{-1}) was also determined in triplicate by flow cytometry as described by [6]. The results were correlated according to the following equations:

$$CFC = 2.99.10^{8} \times OD_{600} \quad (R^2 = 0.99) \quad \text{(eq. 2)}$$

$$CDW = 8.82.10^{-10} \times CFC \quad (R^2 = 0.99) \quad \text{(eq. 3)}$$

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

Quantification of substrate and metabolite concentrations

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

Assessment of carbon mass balances

The carbon mass balance (in % mol·mol^{-1}) corresponds to the ratio of the sum of the molar quantities of carbon in metabolites and bacterial cells, to the molar quantity of carbon in substrate or precursor. During the growth step, the carbon mass balance (CMB_G) was calculated by considering that the central metabolism of *L. reuteri* used PK and EMP pathways, as described in [6]. The molar quantity of CO_2 produced was estimated as being equal to that of produced [ethanol + acetate], in agreement with the stoichiometry of PK pathway [10]. The molecular weight of *L. reuteri* DSM 17938 cells was previously

179 determined to be equal to 27.17 $g_{cell}.mol_{cell}^{-1}$ [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 $mg_{gly}.g_{CDW}^{-1}.h^{-1}$), 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 ($g_{3\text{-}HP}\cdot g_{CDW}^{-1}$)
192 and specific 3-HP production rate ($g_{3\text{-}HP}\cdot g_{CDW}^{-1}\cdot h^{-1}$). They were expressed
193 according to the general second order polynomial equation as follows:

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

195 Where *Y* is the predicted value of the response variable; 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**

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

Three repetitions of the central point were performed to check the reproducibility of the experiments conducted with *L. reuteri* DSM 17938. During the growth step, the bacterial growth was identified as reproducible by considering the lag phase duration (7.53 ± 0.62 h), the maximal specific growth rate (1.14 ± 0.04 h^{-1}), the final bacterial concentration (3.0 ± 0.3 $g_{CDW}·L^{-1}$) and the total duration of the growth phase (13.31 ± 0.36 h). The main bioproducts 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 $g·L^{-1}$), thus confirming that bacteria used both EMP and PK pathways [6]. The carbon mass balance during growth was equal to 109.2 ± 0.8 % that is higher than 100 % due to the fact that a part of the carbon used for bacterial growth may come from other sources than glucose, i.e., yeast extract and phytone peptone from the culture medium [6].

During the bioconversion step, glycerol was fed into the bioreactor at a constant specific rate of 50 $mg_{gly}·g_{CDW}·h^{-1}$ until the bioconversion stopped and the pH was controlled at pH 6.0 as defined by the CCRD matrix (Table 2). The kinetics of glycerol bioconversion into 3-HP and 1,3-PDO are illustrated in Figure 1.

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

NADH was well maintained during 3-HPA oxidation in 3-HP and reduction in 1,3-PDO. These results were in accordance with those obtained by [5]. The molar ratio between 3-HP and NH_4OH was equal to 0.87 ± 0.09 $mol_{3\text{-}HP} \cdot mol_{NH4OH}^{-1}$ on average throughout the bioconversion, which indicated that base consumption can be used to monitor 3-HP production, as previously demonstrated [6]. The value lower than 1.0 could be explained by a slight evaporation of the base NH_4OH that is volatile. A small amount of 3-HPA (0.11 ± 0.03 $g \cdot L^{-1}$) was accumulated when bioconversion stopped. This value was about 20 times lower than the minimum inhibition concentration reported for *L. reuteri* DSM 20016 (2.2 – 3.7 $g_{3\text{-}HPA} \cdot L^{-1}$ [8], thus indicating that 3-HPA did not inhibit bacterial cells in these conditions.

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

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

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

ratio between 3-HP and NH_4OH was lower than expected (0.89 ± 0.19 $mol_{3\text{-}HP} \cdot mol_{NH4OH}^{-1}$), which could be explained by the volatility of the NH_4OH solution used for pH control. At the end of the 11 fed-batches, the final volumes were comprised between 250 mL and 390 mL, depending on the glycerol feeding rate, the quantity of NH_4OH added to control the pH and the duration of bioconversion. As previously observed [6], the bioconversion ceased suddenly, irrespective of the glycerol feeding rate, pH or 3-HP and 3-HPA concentrations.

Multivariable second order linear regressions were carried out to represent the final 3-HP quantity, the 3-HP production yield and the specific 3-HP production rate as a function of pH and specific glycerol feeding rate. The coefficients of the polynomials are summed up in Table 3. The multiple correlation coefficients R^2 were comprised between 0.85 and 0.94. By considering the complexity of the experiments, which included two successive steps of growth and bioconversion with a concentration step between them, these results were considered as satisfactory. The coefficients displayed in Table 3 indicated that 3-HP final quantity and production yield were affected by pH that displayed a quadratic effect and by specific glycerol feeding rate that showed linear and quadratic effects, with no interaction between these two factors. Meanwhile, the specific 3-HP production rate was only affected by a linear effect of the specific glycerol feeding rate but not by the pH, nor by an interaction. This is the first demonstration of the absence of interaction between pH and specific glycerol feeding rate on the 3-HP bioproduction performance by a strain of *L. reuteri*, on the ranges tested for these two environmental conditions (i.e., pH from 4.8 to 7.2 and specific substrate feeding rate between 20 and 80 $mg_{gly} \cdot g_{CDW}^{-1} \cdot h^{-1}$).

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

The three-dimensional representations of the effects of the specific glycerol feeding rate and 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 $g_{3\text{-HP}} \cdot L^{-1}$ and from the volumes of the corresponding
281 experiments, thus ranging between 1.65 and 4.15 $g_{3\text{-HP}}$. 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 $g_{3\text{-HP}} \cdot g_{CDW}^{-1}$. 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 $g_{3\text{-HPA}} \cdot L^{-1}$ 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 $mg_{gly} \cdot g_{CDW}^{-1} \cdot h^{-1}$. These results are in accordance with those obtained by [5] with *L. reuteri*
299 DSM 20016 (62.5 $mg_{gly} \cdot g_{CDW}^{-1} \cdot h^{-1}$) and by [6] with *L. reuteri* DSM 17938 (51 $mg_{gly} \cdot g_{CDW}^{-1} \cdot h^{-1}$). At low specific glycerol feeding rates (i.e., 20 $mg_{gly} \cdot g_{CDW}^{-1} \cdot h^{-1}$), 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.

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

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

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

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

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

activity of the enzyme propionaldehyde dehydrogenase responsible for the transformation of 3-HPA into 3-hydroxypropionyl-CoA [8]. Nevertheless, 3-HPA accumulation remained lower than that observed by [5], who detected final concentrations between 1.9 to 2.1 $g_{3\text{-}HPA}\cdot L^{-1}$ at a specific glycerol feeding rate of 190.5 $mg_{gly}\cdot g_{CDW}^{-1}\cdot h^{-1}$ and at pH 5.0 and pH 7.0, respectively.

The optimal conditions of pH and specific glycerol feeding rate that maximized the specific 3-HP production rate of *L. reuteri* DSM 17938 were identified from the equation given in Table 3. As no optimal pH could be identified from the polynomial equation, the bioconversion may be carried out at any pH comprised between pH 4.8 and pH 7.2. This result is interesting as it indicates that the specific rate will be maintained, even at low pH values. The real optimal specific glycerol feeding rate could not be calculated, but for another reason. From the polynomial equation, its value may be higher than 80 $mg_{gly}\cdot g_{CDW}^{-1}\cdot h^{-1}$ which is out of the tested range. However, too high values of specific glycerol feeding rate may lead to 3-HPA accumulation, thus leading to an inhibition of 3-HP bioproduction [8]. It can therefore be hypothesized that the optimum specific glycerol feeding rate should be close to 80 $mg_{gly}\cdot g_{CDW}^{-1}\cdot h^{-1}$.

To summarize the results obtained from the experimental design about the effects of pH and specific glycerol feeding rate on 3-HP bioproduction performance of *L. reuteri* DSM 17938, two sets of conditions have been highlighted. By considering 3-HP final quantity and production yield, specific glycerol feeding rates of 60 and 55 $mg_{gly}\cdot g_{CDW}^{-1}\cdot h^{-1}$ respectively, and pH 6.0 were found to be optimal conditions. To maximize the specific 3-HP production rate, the specific substrate feeding rate has to be higher than 80 $mg_{gly}\cdot g_{CDW}^{-1}\cdot h^{-1}$, irrespective of the pH in the range of pH 4.8 to pH 7.2.

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

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

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

The kinetics of glycerol consumption, 3-HP production and NH_4OH consumption are displayed in Figure 5, together with the three replicates of the central point.

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

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

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

Conclusion

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

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

Acknowledgments

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

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