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Béal

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1	Culture conditions affect Lactobacillus reuteri DSM 17938 ability
2	to perform glycerol bioconversion into 3-hydroxypropionic acid
3	Thi-Lan-Phuong Nguyen, Claire Saulou-Bérion [*] , Jérôme Delettre, Catherine Béal
4	Université Paris-Saclay, AgroParisTech, INRAE, UMR 0782 SayFood, F-78850, Thiverval-
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6	Keywords:
7	Lactobacillus reuteri, Plackett and Burman experimental design, growth medium composition,
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9	yield
10	Abstract
11	The platform molecule 3-hydroxypropionic acid (3-HP) can be produced using Lactobacillus
12	reuteri through a two-step bioprocess that involves a growth phase followed by a bioconversion
13	phase. The bioproduction is performed by resting cells that convert glycerol into 3-HP and 1,3-
14	propanediol in fed-batch mode. This work aimed at studying the effect of the growth conditions
15	of L. reuteri DSM 17938 during the first step, on the glycerol bioconversion into 3-HP during
16	the second step. A Plackett and Burman design was carried out to test, in controlled bioreactors,
17	the effect of 11 growth conditions simultaneously, at fixed bioconversion conditions. The
18	supplementation of the growth medium with vitamin B12 and cysteine displayed a negative
19	effect on the 3-HP bioproduction. The addition of glucose, phytone peptone, Tween 80, 1,2-
20	propanediol and betaine in the growth medium, together with a low temperature and an optimal
21	pH of 6.0 during the growth phase increased the bioconversion duration from 56 h to 89 h at a
22	glycerol feeding rate of 0.5 $g \cdot h^{-1}$. A validating experiment displayed that the 3-HP titer, 3-HP

^{*} Corresponding author: Claire Saulou-Bérion. E-mail: claire.saulou@agroparistech.fr; phone number: +33 1 30 81 54 17; fax number: +33 1 30 81 55 97

production yield and 3-HP specific production rate were significantly improved by 25 %, 150

24 % and 61 %, respectively.

25 Introduction

Using microorganisms to produce valuable biomolecules from renewable resources has gained 26 27 great interest in the two last decades due to limitation of fossil fuel resources and overwhelming environmental issues. In such context, the development of a biotechnological process to 28 produce the platform chemical 3-hydroxypropionic acid (3-HP) is a key issue as this weak 29 organic acid displays many applications. Firstly, it can be used as preservative in the food or 30 feed industry (1, 2). But its major attraction comes from its bi-functionality, i.e. the presence 31 of a carboxyl group and a β -hydroxyl group, which makes 3-HP the precursor of a large variety 32 of chemicals for multipurpose applications. It thus serves as a versatile agent for chemical 33 reactions such as dehydration, oxidation, reduction, cyclization or polymerization that lead to 34 the formation of acrylic acid, malonic acid, 1,3-PDO, propiolactone, valuable homopolymers 35 (poly 3-HP) or copolymers (containing 3-HP), respectively (3). This explains why it was 36 ranked as one of the top value-added platform molecules to be produced from biomass by the 37 US Department of Energy (4). Some chemical synthesis routes have been proposed to obtain 38 3-HP from acrylic acid, 3-propiolactone, 3-hydroxypropionitrile, allyl alcohol, vinyl acetate 39 and 1,3-PDO (5). However, these processes display several drawbacks such as the use of 40 expensive raw materials, the high costs for operating processes and associated environmental 41 issues (3). 42

43 Nowadays, a better alternative to produce 3-HP is based on biotechnological processes, by 44 employing either natural microbial producers or genetically modified organisms (GMO) 45 working as cell factories, including GMOs that do not produce 3-HP naturally and 46 microorganisms producing 3-HP but genetically modified to improve their performances. 47 Detailed information about genetically modified microorganisms is available in recent reviews

(6, 7). L. reuteri is an attractive candidate due to its safety characteristics, its probiotic 48 properties and its anaerobic but aerotolerant attributes (8). In considering 3-HP bioproduction, 49 L. reuteri is naturally capable of converting glycerol into 3-HP and 1,3-PDO in equimolar 50 proportions, as it possesses all the enzymes of the metabolic pathway for 3-HP biosynthesis 51 encoded by the *pdu* operon (9). It has the ability to synthesize vitamin B12 that is an essential 52 co-factor for the first enzyme of the glycerol metabolism (10), but cannot use glycerol as a 53 54 carbon source (11), which allows glycerol to be completely valorized into 3-HP and 1,3-PDO through the bioconversion pathway. 55

The 3-HP bioproduction has to be achieved through a two-step process, which includes a 56 growth phase to produce a high cellular concentration and a second step to perform the glycerol 57 bioconversion into 3-HP by resting cells (12). Glycerol is firstly converted to 3-58 hydroxypropionaldehyde (3-HPA) by a vitamin-B12 dependent glycerol dehydratase. 3-HPA 59 is then either converted to 1,3-PDO or 3-HP through parallel reductive and oxidative branches 60 that maintain the redox balance between NADH and NAD⁺. 1,3-PDO is obtained from 3-HPA 61 using the enzyme 1,3-propanediol oxidoreductase (13), while 3-HP is the product of three 62 successive reactions using propionaldehyde dehydrogenase, phosphotransacylase and 63 64 propionate kinase (12, 14). The conditions to be employed during the bioconversion step have been identified with the aim of increasing the 3-HP concentration and productivity. The use of 65 fed-batch cultures with glycerol supply is required to prevent accumulation of the toxic 66 intermediate 3-HPA, since the rate of 3-HPA formation is higher than that of its subsequent 67 conversion (12). The maximum rate of glycerol supply to L. reuteri DSM 20016 has been 68 determined at 0.75 g \cdot h⁻¹ to avoid 3-HPA accumulation (16). The use of micro-aerobiosis was 69 recommended to improve the 3-HP bioproduction (17). Finally, the absence of glucose was 70 shown to reduce 1,3-PDO production due to a redox cofactor imbalance (12) and to prevent 71 72 the synthesis of undesirable fermentation products (i.e. lactic acid, ethanol, acetic acid, CO₂).

The influence of some conditions implemented during the growth step, prior to bioconversion, 73 has been studied, in the vast majority of cases with the aim of improving the growth 74 performances. By considering the nutritional requirements of L. reuteri, the influence of carbon 75 and nitrogen sources has been determined. Different carbon sources have been used to trigger 76 L. reuteri growth, such as glucose alone (18, 19), glucose plus fructose, saccharose (19), 77 galactose, lactose, melibiose, raffinose, saccharose (20) and industrial wheat or sugar beet 78 79 syrup by-products that contained glucose and fructose (21). From these studies, glucose was demonstrated as the better carbon source to maximize L. reuteri growth (21). The suggested 80 81 concentrations of glucose were comprised between 20 g·L⁻¹ (22) and 30 g·L⁻¹ (21). As L. *reuteri* displays a heterofermentative metabolism, glucose is catabolized through two pathways 82 (phosphoketolase (PK) and Embden-Meyerhof-Parnas (EMP) pathways) that operate 83 simultaneously and lead to the production of lactic acid, acetic acid, ethanol, CO₂ and energy 84 (19). 85

The effects of various nitrogen sources have been established on L. reuteri cell growth. Yeast 86 87 extracts and peptones are commonly used for growth of lactic acid bacteria due to their composition in amino acids and vitamins which meets cell needs. Yeast extract is the most 88 common source employed in the published studies concerning L. reuteri (21, 23). In addition, 89 phytone peptone, which is obtained from papaic digestion of soybean meal, displays an 90 interesting composition with 18 of the 20 natural amino acids (asparagine and glutamine are 91 absent) and is a cheaper nutrient source than yeast extract. Phytone peptone was demonstrated 92 to lead to the highest cell concentration by comparison with other nitrogen sources such as 93 94 peptones of animal origin, tryptone, proteose peptone, tryptic soy broth, yeast extract, and beef extract, in L. reuteri DSM 20016 (24) and other L. reuteri strains (PTA-4965, 23272, and 95 55730) (25). 96

97 The addition of the amino acid cysteine was also considered as this component is an essential
98 growth factor of some lactobacilli (26). For *L. reuteri* DSM 20016 and SD 2112 strains, it was

recommended at concentrations between 0.1 g·L⁻¹ (24) to 0.5 g·L⁻¹ (20). However, the addition 99 of 2 $g \cdot L^{-1}$ cysteine in the culture medium was mentioned to decrease the production of vitamin 100 B12 by L. reuteri JCM1112 by a factor 3 to 5 (10). This amino acid is also known as a potent 101 reducing agent, thus displaying antioxidative properties that help the cells to cope with the 102 oxidative stress encountered during the recovery and concentration steps, as demonstrated with 103 L. fermentum (27). 104

105 Tween 80 is a key growth factor in culture media for lactobacilli, as it brings unsaturated fatty acids that allow reducing intracellular energy consumption, thanks to the down-regulation of 106 107 de novo fatty acid synthesis in membrane phospholipids (28). It is for instance present at a concentration of 1 $g \cdot L^{-1}$ in MRS medium (12, 29) or added to reach 5 $g \cdot L^{-1}$ (21). 108

109 Besides, the influence of some other components in the culture medium of lactobacilli has been investigated. Supplementation with vitamin B12 ($0.1 \text{ mg} \cdot \text{L}^{-1}$), as a mandatory cofactor for the 110 first enzyme in the *pdu* pathway, although not influencing *L. reuteri* growth, positively impacts 111 the 3-HP bioproduction (21). The addition of 1,2-propanediol (1,2-PDO) has been studied as it 112 is involved in the activation of genes of the pdu pathway (16, 30). It was added at a 113 concentration of 5 g·L⁻¹ into L. reuteri DSM 20016 growth medium prior to glycerol 114 115 conversion (14). Betaine is a methylated derivative of the amino acid glycine that acts as an osmolyte to help lactic acid bacteria counteract the osmotic shocks they face during the 116 harvesting and concentration stages (31). By considering L. buchneri R1102, it was 117 demonstrated that the combined supplementation with 2 mmol \cdot L⁻¹ betaine and 0.1 mol \cdot L⁻¹ KCl 118 in the growth medium, together with a further addition of $0.6 \text{ mol} \cdot L^{-1}$ KCl at the end of growth, 119 led to intracellular accumulation of betaine and improved cell survival during freeze-drying.

Formulating the environmental parameters (i.e. growth temperature, pH or the base used for 121 pH control) to improve cell growth and further bioconversion is also challenging. The 122 temperature for L. reuteri growth is generally established at 37 °C (18, 24, 32). However, the 123 optimum temperature for growth may differ from that maximizing a given metabolic activity. 124

For example, cultures of L. fermentum CRL 251 and Bifidobacterium longum CRL 849 125 implemented at 30 °C showed a slower growth but a stimulated alpha-galactosidase activity, 126 in comparison to that observed at 37 °C (33). In order to reduce inhibitory effects of acidic 127 conditions, the pH must be controlled during growth with an appropriate base solution. The 128 optimal pH for L. reuteri growth is generally between 6.0 and 6.8 (34). Recently, different pH 129 values between 3.7 and 6.7 were tested during batch cultures of L. reuteri DSM 12246 and the 130 final biomass concentration was enhanced at pH 5.5 (35). Finally, the use of two kinds of bases 131 have been reported for pH control during L. reuteri growth phase: NaOH (36, 37) and NH4OH 132 (12, 38). 133

134 However, they have never been compared for bacterial growth or 3-HP bioproduction.

From this information, it can be seen that most previous works studied the effects of culture 135 conditions on L. reuteri growth but not on 3-HP bioproduction. They show that many culture 136 conditions during the growth phase affect the growth performances of L. reuteri. However the 137 effects of these culture conditions were scarcely investigated on cell ability to subsequently 138 convert glycerol into 3-HP. In addition, the reported studies focus on a limited number of 139 factors, thus making it difficult to define the optimal conditions by taking all of them into 140 account. In such context, the present work aimed at quantifying the effects of relevant culture 141 conditions on L. reuteri capacity to produce 3-HP during the subsequent glycerol bioconversion 142 stage. As 11 factors were selected, a Plackett and Burman experimental design was retained as 143 it allowed identifying the key factors that act on 3-HP bioproduction and quantifying their main 144 effects, together with reducing the number of experiments. All experiments were performed in 145 bioreactors in order to evaluate the effects of the factors in well-controlled conditions. 146

147 Material and methods

148 Bacterial strain

The strain L. reuteri DSM 17938 was obtained from BioGaia AB, Stockholm, Sweden. It was 149 stored at -80 °C in cryotubes with glycerol 20 % as cryoprotectant. The inoculum was prepared 150 by cultivating the cells in MRS broth (Biokar Diagnostic, Beauvais, France) for 16 hours at 37 151 °C and 100 rpm and then in MRS broth supplemented with glucose 20 g·L⁻¹ (VWR BDH 152 Prolabo, Leuven, Belgium) for 8 hours in the same conditions. Inoculation of the culture 153 medium was performed at a concentration of 5.1.10⁻⁶ g of cell dry weight (CDW) per liter 154 $(g_{CDW}\cdot L^{-1})$, corresponding to 5.8.10⁴10³ cells·mL⁻¹. The specific growth rate was calculated by 155 plotting the natural logarithm of CDW against time. 156

157 Culture and bioconversion media

The reference culture medium used for bacterial growth was composed of MRS broth added 158 with 20 $g \cdot L^{-1}$ glucose. In accordance with the experimental design, it was supplemented or not 159 with the following components: glucose 50 g·L⁻¹ (instead of 20 g·L⁻¹), yeast extract 25 g·L⁻¹ 160 (Organotechnie S.A.S, La Courneuve, France), phytone peptone 25 g·L⁻¹ (Merck KGaA, 161 Darmstadt, Germany), Tween 80 4 g·L⁻¹ (VWR), vitamin B12 0.1 mg·L⁻¹ (Sigma-Aldrich, 162 Saint Louis, MO, USA), 1,2-PDO 3 g·L⁻¹ (Sigma-Aldrich), cysteine 1 g·L⁻¹ (Sigma-Aldrich), 163 betaine 0.234 g·L⁻¹ (Sigma-Aldrich) together with KCl 7.455 g·L⁻¹ (Prolabo, Fontenay-sous-164 Bois, France). All media were autoclaved at 110 °C for 20 minutes. Additional glucose was 165 autoclaved separately to prevent its degradation due to the Maillard reaction (18). 166

167 The bioconversion medium was composed of sterile osmosis water into which a glycerol

168 (Sigma-Aldrich) solution at 100 $g \cdot L^{-1}$ was added at a constant flow rate (29).

169 Two-stage process for 3-HP production

The two steps of 3-HP bioproduction were carried out in controlled bioreactors. Cell growth was conducted in batch mode in a 5-L Sartorius B bioreactor (Sartorius, Dourdan, France). The agitation rate was set at 100 rpm thanks to two Rushton propellers. The temperature and pH during growth were defined according to the experimental design (33 or 37 °C, pH 5.5 or 6.0).

174 The pH was controlled either with NH₄OH 14.8 mol·L⁻¹ (Sigma-Aldrich) or with NaOH 8.75

mol·L⁻¹ (VWR). The growth step was stopped when the cells reached stationary phase. Bacteria were harvested by centrifugation (Avanti[®] J-E centrifuge; Beckman Coulter, Fullerton, CA) at 6,200 g for 10 min at 4 °C. Cell pellets were then suspended in the same volume of sterile osmosis water to reach about 26.7 \pm 9.5 g_{CDW}·L⁻¹, corresponding to about 3.2.10¹⁰ \pm 1.3.10¹⁰ cells·mL⁻¹.

Bioconversion was achieved in a 2-L bioreactor (Sétric Génie Industriel, Toulouse, France) with an initial working volume of 0.8 L. It was performed in fed-batch mode, defined by a constant glycerol feeding rate of $0.5 \text{ g} \cdot \text{h}^{-1}$ to avoid 3-HPA accumulation. The pH was controlled at 6.0 by addition of NH4OH 1.48 mol·L⁻¹, the temperature was set at 37 °C and the agitation rate was maintained at 100 rpm with two Rushton propellers. Bioconversion was stopped when no more 3-HP was produced, which was evaluated by the stop of NH4OH consumption.

186 Evaluation of cell concentration and analysis of cell physiological state by flow187 cytometry

The bacterial cell concentration was determined indirectly by measuring optical density at 600 nm (OD₆₀₀) (spectrophotometer UV-Vis EvolutionTM 201, Fisher Scientific SAS, Illkirch, France). Then, bacterial concentrations expressed in CDW were obtained from OD₆₀₀ values using the correlation: CDW = $0.2639 \times OD_{600}$ (R² = 0.99).

The physiological state of bacterial cells was assessed by flow cytometry after double 192 fluorescent staining with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) (39). 193 The cFDA allowed assessing cellular enzymatic activity after internalization and cleavage by 194 intracellular esterases, thus forming green fluorescent carboxyfluorescein (cF). Nucleic acid 195 dye PI was used to characterize cells with damaged membranes, in which it formed a 196 fluorescent DNA-complex. Samples were first diluted with McIlvaine buffer pH 7.3 (0.2 197 mol·L⁻¹ disodium dihydrogen phosphate (J. T. Baker, Deventer, NL) and 0.1 mol·L⁻¹ citric acid 198 (Fisher Chemical, Elancourt, France)) to reach a cell concentration of 10⁶ cells·mL⁻¹. One 199 milliliter of diluted sample was added simultaneously with 10 µL cFDA (Chemchrom V8, 200

Biomérieux, Marcy-l'Etoile, France) diluted at 100 mL·L⁻¹ in acetone (Fisher Scientific, 201 Leicestershire, UK) and 10 µL PI (1 g·L⁻¹ in distilled water, Sigma-Aldrich) before incubation 202 at 40 °C for 10 min. Flow cytometry analyses were performed with a BactiFlow cytometer 203 (Sysmex Partec, Roissy, France) equipped with the Flowmax software (Partec). Cell 204 fluorescence was measured at 530 nm for cF and 670 nm for PI after excitation by a 488-nm 205 emitting laser. Cells emitting in green only (530 nm) were considered as viable, while cells 206 emitting in red only (670 nm) were considered as dead. The data collected included cell 207 concentrations (in cells \cdot mL⁻¹) and percentages of viable and dead cells (29, 39). 208

209 Quantification of substrates and metabolites

Substrates used for growth (glucose) and bioconversion (glycerol) as well as growth products 210 (lactic acid, ethanol and acetic acid) and bioconversion metabolites (3-HP, 1.3-PDO and 3-211 HPA) were quantified by high-performance liquid chromatography (Waters Associates, 212 Molsheim, France). Growth samples were diluted twice with trichloroacetic acid (Sigma-213 Aldrich) at 60 g·L⁻¹, then centrifuged at 13,000 g for 10 min at 4 °C before filtration through a 214 0.22 µm pore-size filter (Sartorius Stedim Biotech, Gottingen, Germany). Bioconversion 215 samples were added with citric acid 5 $g \cdot L^{-1}$, centrifuged and filtered in the same conditions. 216 For each sample, a volume of 20 µL was injected (Waters 717 plus) in mobile phase (H₂SO₄, 217 Sigma-Aldrich) on a cation-exchange column Aminex HPX-87H (300 mm × 7.8 mm, Biorad, 218 Richmond, USA). Two sets of conditions including column temperature, H₂SO₄ concentration 219 and flow rate of the mobile phase, were applied to separate the components properly (Table 1). 220 Detection was done by a refractive index detector (Waters 2414) and an UV detector (Waters 221 222 2489). Quantification was achieved using the Empower software (Waters Associates) with the help of external standards. All analyses were duplicated and concentrations are given in $g \cdot L^{-1}$. 223

	Condition A	Condition B
Temperature (°C)	35	60
H_2SO_4 concentration in mobile phase (mmol·L ⁻¹)	5	0.5
Flow rate of mobile phase (mL·min ⁻¹)	0.6	0.4
Compounds detected in growth	Glucose (9.3), lactic	
samples (and corresponding	acid (13.2), acetic acid	
retention times, in min)	(15.6), ethanol (21.2)	
Compounds detected in	3-HPA (14.9), 1,3-	Lactic acid (18.7,
bioconversion samples (and	PDO (17.5), acetic acid	remaining from growth),
corresponding retention times,	(15.6), ethanol (21.2)	3-HP (19.7), glycerol
in min)	(the two latter remaining from growth)	(20.7)

Table 1. Conditions of HPLC analyses

225

224

226 Assessment of carbon mass balance

The carbon mass balance has been calculated during growth (CMB_G, in % mol·mol⁻¹) according to the central metabolism of *L. reuteri* via PK and EMP pathways and during bioconversion (CMB_B, in % mol·mol⁻¹), from the equations below:

230
$$CMB_G = (3.n_{Lactic acid} + 2.n_{Ethanol} + 2.n_{Acetic acid} + n_{CO2} + n_C in Biomass) / 6.n_{Glucose}$$
 (eq. 1)

231 $CMB_B = (n_{3-HP} + n_{1,3-PDO} + n_{3-HPA}) / n_{Glycerol}$ (eq. 2)

With n being the number of moles of consumed glucose (n_{Glucose}) and glycerol (n_{Glycerol}), produced lactic acid (n_{Lactic acid}), ethanol (n_{Ethanol}), acetic acid (n_{Acetic acid}), CO₂ (n_{CO₂} (hypothesized to be equal to the number of moles of produced [ethanol + acetate])), carbon in cells produced during growth (n_{C in Biomass}), 3-HP (n₃-HP), 1,3-PDO (n_{1,3}-PDO) and 3-HPA (n₃-HPA).

237 Experimental design and statistical analyses

A Plackett and Burman matrix (40) allowed creating an experimental design to test the effects of 11 medium components and culture conditions on growth and subsequent 3-HP bioproduction by *L. reuteri* DSM 17938. The general form of the model is given as follows: Growth conditions impact 3-HP production by L. reuteri

- 241 $Y_i = k_i + \Sigma a_i \cdot Y_{(+)i}$ (eq. 3)
- Where Y_i is the response variable; k_i , the constant value of Y_i when all 11 variables are at their Minus levels; a_i , the linear coefficient of each variable to express the effect of the Plus level; and $Y_{(+)i}$, the value of the Plus level of each variable i.
- 245 The multiple regression analysis was performed using the XLSTAT software (Paris, France).

The 11 experimental factors tested in this study are listed in Table 2 that specifies the 246 concentrations or values characterizing the Minus and Plus levels in the experimental design. 247 The conditions used in each experiment are listed in Table S1. They were randomized and the 248 reference condition was performed in triplicate. The final cell concentration $(g_{CDW} \cdot L^{-1})$ was 249 selected as the response variable to describe the effect of the factors on the growth step. The 250 percentage of viable cells at the beginning of the bioconversion stage was included in the 251 analysis. Three response variables were chosen to characterize the effect of the experimental 252 factors on the bioconversion performance: total quantity of 3-HP produced at the end of the 253 fed-batch bioconversion (g3-HP), 3-HP production yield (g3-HP·gCDW⁻¹) and duration of the 254 bioconversion (h). 255

256 Table 2. Factors included in the Plackett-Burman experimental design to study the

257 effects of culture conditions of <i>L. reuteri</i> DSM 17938 on its bioconversion per	formances
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Medium components	Minus level	Plus level
Additional glucose (g·L ⁻¹)	20	50
Additional yeast extract (g·L ⁻¹)	0	25
Phytone peptone (g·L ⁻¹)	0	25
Tween 80 (g·L ⁻¹)	1	5
Vitamin B12 (mg·L ⁻¹)	0	0.1
1,2-propanediol (3 g·L ⁻¹)	0	3
Cysteine (g·L ⁻¹)	0	1
Betaine $(g \cdot L^{-1})$ and	0	0.234
$\operatorname{KCl}(g \cdot L^{-1})$	0	0.745
Culture conditions		
Temperature (°C)	33	37
pH	5.5	6.0
Base type (and concentration, in $mol \cdot L^{-1}$)	NH4OH (14.8)	NaOH (8.75)

²⁵⁸

259 **Results and Discussion**

260 Kinetics of cell growth and glycerol bioconversion in the reference condition of the

261 experimental design

262 In order to assess the kinetics of L. reuteri DSM 17938 in the reference condition, three

replicates were performed. The experimental conditions are summarized with the code T1 in

the Table S1. Figure 1 shows the cell growth kinetics during the first phase, whereas Figure 2

265 displays the bioconversion kinetics of during the second step.



Figure 1. Kinetics of *L. reuteri* DSM 17938 growth and acidification in the reference
 condition
 Blue circle: cell dry weight (CDW) expressed in g_{CDW}·L⁻¹; red square: consumed base; error

bars correspond to standard errors from 3 replicates.

From Figure 1, two successive exponential growth phases were observed, with different 272 specific growth rates that were calculated at 0.99 (\pm 0.002) h⁻¹ from 6 h to 9.5 h, and then 0.13 273 (± 0.01) h⁻¹, between 10.5 h and 14 h, indicating that a substrate limitation occurred. This 274 limitation cannot be due to a lack of carbon source as the glucose concentration was equal to 275 8.5 (\pm 1.7) g·L⁻¹ at 10 h. It may be caused by a lack of another nutrient source that was not 276 searched in this study or by an inhibition of bacterial growth by the accumulation of lactic acid 277 (41) and ethanol (42) that reached 4.6 (\pm 0.9) and 2.0 (\pm 0.4) g·L⁻¹, respectively after 10 h. 278 Meanwhile, no limitation was observed on organic acid production since the base was 279 consumed regularly during the logarithm phase. However, when no more glucose was available 280 281 in the medium, the base consumption stopped suddenly, together with growth, indicating that no more organic acid was synthesized. These results were confirmed by HPLC analysis. The 282 base profile was then used to precisely detect the growth stop. Quantitative results obtained 283 within the three reference experiments are summarized with the codes T1a, T1b and T1c in 284 Table S2. The carbon mass balance during growth was verified at 104.33 (\pm 3.82) % for all the 285

experiments performed. This value was slightly higher than 100%, which could be explained 286 by the fact that some carbon used for the growth may come from other sources than glucose 287 (for instance yeast extract) that could not be quantified. A final CDW value of 3.3 (\pm 0.3) 288 $g_{CDW} \cdot L^{-1}$ was achieved, which was similar to previous results obtained with the same *L. reuteri* 289 strain at pH 5.5 (3.1 g_{CDW}·L⁻¹) (12). From HPLC analyses, the presence of lactic acid (16.4 (\pm 290 1.2) g·L⁻¹), acetic acid (3.7 (± 0.4) g·L⁻¹) and ethanol (7.6 (± 0.8) g·L⁻¹) was detected. The 291 molar ratio of the sum of (ethanol + acetic acid) to lactic acid was close to 1 (0.97 (\pm 0.05)) 292 mol·mol⁻¹). This value indicated that the PK pathway was prevalent in this heterofermentative 293 L. reuteri strain, as previously observed by Burgé et al. (18). This value indicated that the PK 294 pathway was prevalent in this heterofermentative L. reuteri strain, as previously observed by 295 Burgé et al. (2015) (18). This result, obtained at controlled pH, is consistent with previous 296 studies on L. reuteri ATCC 55730 (19) and L. reuteri DSM 17938 grown in free-pH conditions 297 (18). Regarding the efficiency of ATP synthesis, the domination of PK pathway is unfavorable 298 compared to EMP pathway (production of 1 instead of 2 ATP). Nevertheless, by producing 299 300 less organic acid, the PK pathway reduced the inhibition of the bacteria by lactic acid, thus improving their survival in an acidic environment (18). 301

Kinetics of glycerol bioconversion into 3-HP and 1,3-PDO are illustrated in Figure 2. The 302 carbon mass balance during bioconversion was verified at 100.12 (\pm 4.39) %. This step was 303 performed using resting cells of L. reuteri, to allow the NAD⁺ regeneration through 3-HP 304 production in place of lactate production. The glycerol was fed into the bioreactor at a constant 305 feeding rate 0.5 g_{glycerol}·h⁻¹ until the bioconversion stopped. The slope of glycerol consumption 306 307 was constant (Figure 2), which indicated that all substrate was consumed as and when it was supplied. The glycerol consumption ceased at the same time as the base consumption, which 308 was confirmed from HPLC analyses. This indicated that the base profile was a relevant 309 310 indicator of the cessation of the bioconversion.



Figure 2. Kinetics of glycerol bioconversion into 3-HP and 1,3-PDO by *L. reuteri* DSM
 17938 in the reference condition
 Yellow square with cross: consumed glycerol; green square: 3-HP; blue triangle: 1,3-PDO;
 red diamond: 3-HPA; white circle: consumed NH4OH; grey circle: dissolved O₂; error bars

correspond to standard errors from 3 replicates.

316 317

After 55.8 (\pm 2.2) h of bioconversion, two final products 3-HP and 1,3-PDO were obtained at 318 final concentrations of 11.8 (\pm 0.5) g·L⁻¹ and 9.5 (\pm 0.5) g·L⁻¹, respectively. In comparison to 319 the previous study of Dishisha et al. (2015) (16) using L. reuteri DSM 20016, the duration of 320 bioconversion was similar (56 h compared to 58 h), but the final 3-HP titer was 16 % lower 321 which was explained by the lower substrate feeding rate used in our study (0.5 $g_{glycerol}$ ·h⁻¹ 322 compared to 0.75 g_{glycerol}·h⁻¹). The maximal 3-HPA content detected into the bioconversion 323 broth was equal to 0.4 ± 0.1 g·L⁻¹. This value was about 10 times lower than the minimum 324 inhibition concentration reported for L. reuteri DSM 20016 (2.2 - 3.7 g·L⁻¹) (43), thus 325 protecting it from detrimental effects (44). 3-HP was produced equimolarly to 1,3-PDO, as the 326 molar ratio was equal to $1.04 \pm 0.06 \text{ mol}^{-1}$, which indicated that the redox balance between 327 NAD⁺ and NADH was well maintained during the glycerol bioconversion. These results were 328 close to those obtained by (12) who obtained a molar ratio of 1 mol·mol⁻¹. The molar ratio 329 between 3-HP and NH₄OH was equal to $1.08 \pm 0.01 \text{ mol}^{-1}$, which is a little bit higher than 330

1. As no other organic acid was detected from HPLC analyses, this small divergence can be explained by a slight volatilization of the ammonia solution during the bioconversion. The total amount of 3-HP and 1,3-PDO produced at the end of the fed-batches were respectively equal to 13.4 ± 0.9 g and 10.8 ± 0.7 g. These values were lower than those obtained by (16) due to the lower supply rate of glycerol as aforementioned. The 3-HP production yield was equal to 0.78 ± 0.11 g_{3-HP}.gcDw⁻¹, which was also lower than the higher value reported by (16).

Micro-aerobic conditions were applied in this study to facilitate glycerol metabolism toward 3-337 HP production (17). The partial pressure of dissolved oxygen (pO₂) in the medium remained 338 at a value lower than 0.5 % until 51 h (Figure 2). At that time, the base consumption started to 339 decrease, 3-HP and 1,3-PDO production ceased and pO₂ increased. This link between the pO₂ 340 increase and the cessation of bioconversion is consistent with previous works (17, 45). This 341 pO₂ increase may be due to a reduction of the activity of the enzyme NAD(P)H oxidase that 342 uses molecular oxygen as a substrate (45). We may hypothesize that the stopping of the 343 bioconversion corresponded to a global dysfunction of the whole enzyme pool of bacterial 344 cells. In that case, the catalytic work of the NAD(P)H oxidase stopped concomitantly with that 345 of the enzymes of the *pdu* operon that drives the 3-HP bioproduction. The decrease in oxygen 346 consumption is thus observed simultaneously to the reduction in 3-HP bioproduction. 347 Moreover, our results indicate that the pO₂ measurement may constitute an early on-line 348 indicator of the cessation of bioconversion, which is an original approach for controlling the 349 fed-batch bioconversion of glycerol into 3-HP. 350

Effect of culture conditions on *Lactobacillus reuteri* DSM 17938 growth performances According to the Plackett and Burman experimental design (Table S1), various growth medium recipes and culture conditions were designed to screen, among 11 factors, the best combination to improve bacterial growth. The final biomass concentration ($g_{CDW}\cdot L^{-1}$) was retained as the variable to characterize the bacterial growth performance. Indeed, as the growth occurred during night without being monitored by a biomass probe, the effect of the conditions on the

	Cell concentration at the end of growth (g _{CDW} ·L ⁻¹)	Total quantity of 3- HP produced (g)	Bioconversion duration (h)	3-HP production yield (g _{3-HP} ·g _{CDW} ⁻¹)
Constant	3.90	14.12	64.1	0.75
Additional glucose	0.58**	ns	4.0**	-0.23**
Additional yeast extract	ns	ns	ns	ns
Phytone peptone	ns	0.94*	5.7**	0.11*
Tween 80	ns	0.84*	2.1*	0.10*
Vitamin B12	ns	-2.16**	-10.6***	-0.15**
1,2-propanediol	ns	1.03*	4.5**	0.093*
Cysteine	ns	ns	ns	-0.10*
Betaine and KCl	ns	ns	4.6**	0.10*
Temperature	0.53**	ns	2.1*	-0.15**
pH	ns	ns	ns	0.096*
Base	ns	ns	ns	ns
R ²	0.873	0.893	0.978	0.919

 Table 3. Effect of growth conditions on cell concentration and on glycerol bioconversion performances of L. reuteri DSM 17938

Confidence level: * 90 %; ** 95 %; *** 99 %; ns: not significant; R² adjusted for degree of freedom.

specific growth rate could not be established. The results obtained from the 14 experiments are 357 summarized in Table S2 and were used for statistical analyses. From Table 3, only two factors 358 displayed a significant effect on L. reuteri DSM 17938 final concentration: the addition of 359 glucose and the higher temperature, which positively influenced the final biomass 360 concentration, by 15 % and 20 %, respectively. This result was confirmed by considering the 361 number of viable cells at the end of the culture, enumerated by flow cytometry. It was improved 362 by 34 % in the presence of 50 $g \cdot L^{-1}$ glucose, which matches with the aforementioned result. 363 This positive effect of glucose on *L. reuteri* growth is consistent with early studies (18, 19, 21). 364 Conversely, the addition of yeast extract or phytone peptone to the MRS medium as nitrogen 365 sources did not improved neither the biomass concentration nor the cell viability. This result is 366 contrary to those obtained in previous studies (21, 24) and suggested that the growth limitation 367 previously observed in the growth phase cannot be justified by a lack of these nutrients. The 368 existence of interactions between factors, which cannot be considered by the type of 369 experimental design used in our study, can partly explain this observation. 370

Tween 80 and cysteine are both growth factors for lactobacilli. However, the current supplementation of these compounds at 5 $g \cdot L^{-1}$ and 1 $g \cdot L^{-1}$ respectively, seemed not enough to increase the cell concentration. The addition of vitamin B12, 1,2-PDO and betaine together with KCl did not affect the growth, which was expected as they were added to act during the bioconversion stage.

The temperature positively affected the biomass concentration that was higher at 37 °C than at 377 33 °C, which was confirmed by flow cytometry measurements ($5.6.10^9$ cell·mL⁻¹ instead of 378 $4.5.10^9$ cell·mL⁻¹). This result is consistent with the conditions found in the digestive tract, the 379 ecological niche of *L. reuteri* (46).

Finally, the pH value (pH 5.5 or pH 6.0) and the base used for pH control (NH4OH or NaOH)
did not significantly modify the final cell concentration of *L. reuteri* DSM 17938. Therefore,
they can be used equally to maintain the pH during the growth phase.

Effect of culture conditions on *L. reuteri* DSM 17938 ability to convert glycerol into 3-

384 **HP**

The Plackett and Burman experimental design allowed identifying the growth medium 385 composition and culture conditions that affected the ability of L. reuteri DSM 17938 to further 386 perform glycerol bioconversion into 3-HP. During all experiments, no residual glycerol was 387 detected in the bioconversion broth. Table 3 summarizes the results obtained from the 14 388 different experiments (including three replicates of the reference condition), to explain the 389 effects of the 11 factors on three variables that characterized the bioconversion step: quantity 390 of 3-HP produced, bioconversion duration and 3-HP production yield. From Table 3, nine 391 factors displayed a statistically significant effect on these variables. Only two factors did not 392 show any significant effect on the characteristic variables of the 3-HP production step: the 393 addition of yeast extract and the type of base used for pH control. The three variables were 394 positively influenced by the addition of phytone peptone, Tween 80 and 1,2-PDO but 395 negatively by a vitamin B12 supplementation. A high glucose concentration and a high 396 temperature positively affected the bioconversion duration but negatively the 3-HP production 397 yield. The addition of betaine enhanced the bioconversion duration and the 3-HP production 398 yield. Finally, the addition of cysteine and a low pH value negatively influenced the 3-HP 399 production yield. In addition to these three variables, the quantity of 1,3-PDO produced and 400 the molar ratio between 3-HP and 1,3-PDO were also determined and will be analyzed when 401 required. 402

As explained above, the growth medium supplementation with a higher glucose concentration led to a higher quantity of harvested biomass and a higher percentage of viable cells at the beginning of bioconversion. However, in the experimental conditions chosen during the bioconversion step, no increase of 3-HP production was observed, which is explained by the moderate glycerol feeding rate (0.5 g_{glycerol}·h⁻¹) used in this study. In other words, the quantity of glycerol provided was insufficient to feed the higher cell quantity obtained in this condition.

As a consequence, the specific feeding rate of glycerol was lower in the condition of the higher 409 level of glucose addition $(18.1 \pm 3.5 \text{ mg}_{\text{glycerol}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1})$ as compared to that observed with 410 the lower level of glucose addition $(32.9 \pm 8.6 \text{ mgglycerol} \cdot \text{gcDw}^{-1} \cdot \text{h}^{-1})$. This observation was 411 supported by the decrease of the 3-HP production yield (- 30.7 %). The longer duration of 412 bioconversion (+ 6.2 %) noticed when the cells were previously cultivated in the presence of a 413 higher glucose concentration might be explained by a better enzymatic activity of the total 414 bacterial population at the beginning of bioconversion stage, as observed by cF fluorescence 415 measurements. This better activity allowed the cells to convert glycerol for a longer time. 416

Results showed that phytone peptone slightly promoted the 3-HP bioproduction whereas yeast 417 extract did not (Table 3). As these two compounds act as amino acid sources, the positive effect 418 of phytone peptone can be related to a specific amino acid composition that differs from that 419 of yeast extract. From the information available from the suppliers, phytone peptone contained 420 more arginine, histidine, tyrosine (+ 200 %) and glycine (+ 90 %) than yeast extract. 421 Particularly, the amino acids arginine and glycine have been shown to be used for energy 422 production and were linked to the survival of Lactococcus lactis under starvation (47) and 423 Lactobacillus sakei under acidic conditions (48). 424

A positive effect of Tween 80 supplementation was observed on cell ability to produce 3-HP (Table 3) that is consistent with (21). This component is known to support cell division of lactobacilli by modifying the cell membrane fatty acids composition (49). In addition, it induces a down-regulation of the *de novo* fatty acid synthesis that helps the cells to save intracellular energy (28).

Addition of 1,2-PDO significantly improved the three variables characterizing the bioconversion step (Table 3), in agreement with (16). Here we consider that due to the structural similarity between glycerol and 1,2-PDO, the addition of the latter in the growth medium might help the cells to prepare their enzymatic machinery.

A negative effect of the addition of 0.1 mg \cdot L⁻¹ vitamin B12 in the growth medium was observed 434 on the quantity of 3-HP produced, the bioconversion duration and the 3-HP production yield 435 (Table 3). This result was an unintended outcome because this compound is a required cofactor 436 of the first enzyme of the *pdu* metabolic pathway (12). It was reported that a supplementation 437 of vitamin B12 at 0.1 mg \cdot L⁻¹ in the bioconversion medium showed a positive impact on 3-HP 438 production by L. reuteri DSM 17938 (21). Thus, it could be hypothesized that the early addition 439 440 of exogenous vitamin B12 in the growth medium may reduce the ability of the cells to produced endogenous vitamin B12 during the further step of bioconversion. 441

The addition of betaine together with KCl led to a longer bioconversion, together with a higher production yield (Table 3) that can be explained by the protective effect of betaine to osmotic stress (50). This result can be linked to that of (31) who related a higher intracellular betaine content to a more rigid membrane that led to the reduction of water exchange between the intracellular and extracellular compartments in *L. buchneri* R1102 (31).

The addition of cysteine was related to a reduction of the 3-HP production yield (Table 3). This observation can be explained by its negative effect on vitamin B12 biosynthesis by *L. reuteri*, as previously demonstrated (10). As another hypothesis, cysteine may counteract the oxygen utilization by the cells, as suggested by (27).

Regarding the influence of the growth temperature on the further bioconversion step, a higher 3-HP production yield but shorter bioconversion duration was observed when the growth was conducted at 33 °C instead of 37 °C (Table 3). As the glycerol feeding rate was fixed at a moderate value of 0.5 $g_{glycerol}$ ·h⁻¹ during bioconversion, this higher 3-HP production yield was the consequence of the lower cell concentration achieved at the end of the growth phase.

The pH value during growth had a very little effect on the bioconversion step. Only the 3-HP production yield decreased slightly when the pH was reduced from 6.0 to 5.5 (Table 3). This difference could be ascribed to a small, even not significant, increase in the final biomass concentration when the growth was conducted at pH 5.5. Finally, it was demonstrated that the 460 type of base used had no effect, thus allowing us to use them indiscriminately. However, as the 461 base NH₄OH prevents the formation of salt in the medium, this base might be preferred to 462 facilitate further downstream processes.

463 Selected growth conditions that improve *L. reuteri* DSM 17938 ability to convert 464 glycerol into 3-HP

In order to enhance the capacity of L. reuteri DSM 17938 to perform 3-HP bioproduction at a 465 glycerol feeding rate of 0.5 g_{glycerol}·h⁻¹, the growth conditions leading to the best 3-HP titer, 466 bioconversion duration and 3-HP production yield have been identified. They consist in MRS 467 medium added with glucose (20 g·L⁻¹), phytone peptone (25 g·L⁻¹), Tween 80 (4 g·L⁻¹), 1,2-468 PDO (3 g·L⁻¹), betaine (0.234 g·L⁻¹) plus KCl (0.745 g·L⁻¹), temperature (33 °C) and pH 6.0. 469 No additional yeast extract, no supplementation with vitamin B12 nor cysteine were required, 470 and the base to control the pH was likely unimportant. This set of conditions has been assayed 471 to demonstrate its positive effects on the bioconversion performances. In comparison to the 472 reference condition, the 3-HP titer was increased from 11.8 ± 0.5 g·L⁻¹ to 14.7 g·L⁻¹, the 473 bioconversion duration from 55.8 \pm 2.2 h to 88.6 h, and the 3-HP production yield from 0.8 \pm 474 0.1 g_{3-HP}·g_{CDw}⁻¹ to 2.0 g_{3-HP}·g_{CDw}⁻¹. In addition, the specific 3-HP production rate was 475 improved from $14.0 \pm 2.5 \text{ mg}_{3-\text{HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$ to $22.5 \text{ mg}_{3-\text{HP}} \cdot \text{g}_{\text{CDW}}^{-1} \cdot \text{h}^{-1}$. As the cell concentration 476 at the end of the growth phase $(3.0 \text{ g}_{\text{CDW}} \cdot \text{L}^{-1})$ was similar to that of the reference condition (3.3 477 ± 0.3 g_{CDW}·L⁻¹), this improvement could be explained by a better ability of each cell to produce 478 3-HP from glycerol. This was supported by the higher ratio between 3-HP production to 479 enzymatically-active cells determined by flow cytometry, which increased from 0.98 ± 0.21 g₃-480 HP.gviable cells⁻¹ in the reference condition to 2.05 g₃-HP.gviable cells⁻¹ in the optimized recipe. 481

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In conclusion, the influence of various growth conditions was assessed on the growth efficiency and on the ability of resting cells of *L. reuteri* DSM 17938 to produce 3-HP at a given glycerol feeding rate of 0.5 g·h⁻¹. The implementation of a Plackett and Burman experimental design enabled 11 factors to be tested. The supplementation of MRS medium with glucose and the use
of a higher temperature (i.e. 37 °C) induced a greater cell quantity at the end of the growth
phase. Meanwhile, the addition of phytone peptone, Tween 80, 1,2-PDO, betaine with KCl and
the use of a suboptimal temperature together with an optimal pH, were recognized as options
to improve the bioconversion duration and the 3-HP production yield. The best set of conditions
has been validated as it enhanced the 3-HP titer (+ 25 %), the 3-HP production yield (+ 150 %)
and the 3-HP specific production rate (+ 61 %).

With the aim of further improving the 3-HP bioproduction by *L. reuteri* DSM 17938, the environmental conditions during the bioconversion step will have to be optimized, together with avoiding 3-HPA accumulation. Finally, in order to better understand the cellular modifications that are linked to the improvement of 3-HP bioproduction, a more in-depth analysis of the physiological state of the cells may be done, mainly by using omic methods.

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Experiment					Expe	rimental fa	actors				
code	Additional glucose (g·L ⁻¹)	Additional yeast extract (g·L ⁻¹)	Phytone peptone p (g·L ⁻¹)	1,2- propanediol (g·L ⁻¹)	Cysteine (g·L ⁻¹)	Betaine (g·L ⁻¹) and KCl (g·L ⁻¹)	Tween 80 (g·L ⁻¹)	Vitamin B12 (mg·L ⁻¹)	Temperature (°C)	рН	Base type (and concentration, in mol·L ⁻¹)
T1	20	0	0	0	0	0 and 0	1	0	37	6.0	NH4OH (14.8)
T2	50	0	25	0	0	0 and 0	5	0.1	33	6.0	NaOH (8.75)
Т3	50	25	0	3	0	0 and 0	1	0.1	37	5.5	NH ₄ OH (14.8)
T4	20	25	25	0	1	0 and 0	1	0	37	5.5	NaOH (8.75)
Τ5	50	0	25	3	0	0.234 and 0.745	1	0	33	5.5	NaOH (8.75)
T6	50	25	0	3	1	0 and 0	5	0	33	6.0	NaOH (8.75)
Τ7	50	25	25	0	1	0.234 and 0.745	1	0.1	33	6.0	NH4OH (14.8)
Τ8	20	25	25	3	0	0.234 and 0.745	5	0	37	6.0	NH4OH (14.8)
Т9	20	0	25	3	1	0 and 0	5	0.1	33	5.5	NH4OH (14.8)
T10	20	0	0	3	1	0.234 and 0.745	1	0.1	37	6.0	NaOH (8.75)
T11	50	0	0	0	1	0.234 and 0.745	5	0	37	5.5	NH4OH (14.8)
T12	20	25	0	0	0	0.234 and 0.745	5	0.1	33	5.5	NaOH (8.75)

Table S1. Experimental	factors and their	levels in the Plac	ckett and Burman	experimental of	design
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	Cell concentration (g _{CDW} ·L ⁻¹)	Top 3-HP titer (g·L ⁻¹)	Top 1,3-PDO titer (g·L ⁻¹)	Total 3-HP produced (g)	Total 1,3-PDO produced (g)	3-HP production yield (g _{3-HP} ·g _{CDW} ⁻¹)	Bioconversion duration (h)
T1a	3.4	11.2	9.4	12.4	10.4	0.76	57.7
T1b	3.0	11.9	9.0	13.7	10.4	0.90	53.4
T1c	3.6	12.3	10.0	14.1	11.5	0.68	56.2
T2	3.0	10.9	10.0	12.4	11.3	0.60	51.4
Т3	4.8	9.9	10.4	11.1	11.6	0.42	52.2
T4	3.2	11.0	10.0	13.3	12.1	0.88	63.2
T5	5.1	13.9	14.0	18.9	19.0	0.61	92.3
T6	5.7	12.7	12.8	16.1	16.1	0.50	75.6
T7	4.5	9.9	11.3	11.9	13.5	0.35	64.3
T8	3.0	14.7	10.9	19.6	15.7	1.99	88.6
Т9	4.5	11.7	10.8	14.3	13.2	0.68	58.8
T10	2.4	10.1	8.0	11.0	8.7	0.83	44.1
T11	3.8	12.9	12.7	16.3	16.0	0.63	72.7
T12	3.5	9.8	9.2	11.1	10.4	0.68	50.1

 Table S2. Data characterizing L. reuteri DSM 17938 growth and glycerol bioconversion performances