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1 Process engineering for microbial production of 3-hydroxypropionic acid

2
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14 15 Abstract

16
17 Due to concerns about the unsustainability and predictable shortage of fossil feedstocks,
18 research efforts are currently being made to develop new processes for production of
19 commodities using alternative feedstocks. 3-Hydroxypropionic acid (CAS 503-66-2) was
20 recognised by the US Department of Energy as one of the most promising value-added
21 chemicals that can be obtained from biomass. This article aims at reviewing the various
22 strategies implemented thus far for 3-hydroxypropionic acid bioproduction. Special attention
23 is given here to process engineering issues. The variety of possible metabolic pathways is also
24 described in order to highlight how process design can be guided by their understanding. The
25 most recent advances are described here in order to draw up a panorama of microbial 3-
26 hydroxypropionic acid production: best performances to date, remaining hurdles and
27 foreseeable developments. Important milestones have been achieved, and process metrics are
28 getting closer to commercial relevance. New strategies are continuously being developed that
29 involve new microbial strains, new technologies, or new carbon sources in order to overcome
30 the various hurdles inherent to the different microbial routes.

31 32 Key-words

33 3-hydroxypropionic acid, biosynthesis, bioconversion, fermentation, *in situ* product recovery,
34 multi-step processes, metabolic routes

35 **1 Introduction**

36

37 Since the advent of modern microbiology in the 19th century, fermentative processes have
38 been continuously improved and better understood. Microbial production of bulk chemicals
39 was first attempted around a century ago (Buchholz and Collins, 2013). Still, development of
40 bioprocesses for bulk chemical production remained limited throughout the 20th century,
41 because they were not competitive enough comparatively to chemical processes based on
42 fossil feedstocks. However, petrochemical resources are bound to become scarce and their use
43 was proven to contribute to global warming (IPCC, 2007). It has thus become a necessity to
44 reduce dependency on non-renewable feedstocks and to start a transition towards a more
45 sustainable, bio-based economy. Therefore, biotechnological processes for bulk chemical
46 production have been gaining great interest over the past decades, and within the wider
47 context of biorefinery (Choi et al., 2015).

48

49 In order to foster research in this field, the US Department of Energy (DoE) published a list of
50 the most promising building-blocks that can be obtained from biomass (Bozell and Petersen,
51 2010; Werpy and Petersen, 2004). Thanks to its two functional groups (carboxyl and β -
52 hydroxyl), 3-hydroxypropionic acid (3-HP, CAS 503-66-2) is a versatile molecule and has
53 great potential for further transformation into useful chemicals. 3-HP was therefore included
54 by the DoE in the top value added chemicals list (Werpy and Petersen, 2004). 3-HP
55 applications can be divided into three categories: (i) direct use, (ii) polymerisation, and (iii)
56 conversion to other value added chemicals. 3-HP can be used directly by the food and feed
57 industry as an additive or as a preservative agent (Gokarn et al., 2002). It can also be
58 polymerised to poly(3-hydroxypropionate) (poly(3-HP)) or to 3-HP containing co-polymers.
59 In particular, poly(3-HP) has very promising properties and may become one alternative to
60 fossil fuel-derived plastics (Andreeßen et al., 2014). Lastly, 3-HP can also be converted to
61 numerous other useful chemicals, among which acrylic acid is the most noteworthy.

62

63 Figure 1 provides an overview of the chronology of research on 3-HP bioproduction. From
64 the early nineties to the early 2000s, research efforts remained low and focused mostly on the
65 bacterial production of the 3-HP-containing polymer, poly(3-hydroxybutyrate-co-3-
66 hydroxypropionate) by *Alcaligenes* sp. (Cao et al., 1998; Nakamura et al., 1991; Wang et al.,
67 2002, 1999). Since the early 2000s, research has been focusing more and more on 3-HP
68 production using recombinant bacterial strains, mainly of *Klebsiella pneumoniae* and

69 *Escherichia coli* (Gokarn et al., 2002; Suthers and Cameron, 2001). By considering the
70 number of references in the Web of Science and the number of patents related to the
71 bioproduction of 3-HP, it seems that the DoE report published in 2004 has indeed triggered
72 research on this topic (see figure 1). For the last few years, approaches to 3-HP production
73 have been diversifying. This is true on different levels: (i) new organisms have been tested
74 and new metabolic pathways have been explored; (ii) different process modes have been
75 implemented; and (iii) new – or emerging – technologies have been applied to the
76 improvement of 3-HP production. The present study aims at reviewing the various process
77 engineering strategies that were implemented for the purpose of 3-HP microbial production.
78 To this end, present understanding of the different possible metabolic pathways is also
79 reviewed in order to highlight how an understanding of the metabolism can help develop
80 more efficient processes. This study also includes a review of available *in situ* product
81 recovery (ISPR) processes and highlights the questions addressed by these methods.

82

83 **2 3-HP as microbial product: various routes with various hurdles**

84

85 One of the essential strategic choices when designing a new process for biobased chemicals
86 production is to select a relevant microbial agent and an efficient metabolic pathway. Ideally
87 the selected microorganism is an easy-to-use, non-pathogenic, genetically-stable microbe, and
88 an efficient producer. In the case of 3-HP production, various approaches have been
89 undertaken in order to develop microbial hosts suitable for such a process. Some of the best 3-
90 HP biosynthesis performances are summed up at Table 1. Here, we aim at providing an
91 overview of the possible metabolic routes and of the main strategies that were implemented,
92 for better understanding the rationale behind the various operational strategies that are being
93 reviewed later in this article. Genetic engineering works for the purpose of 3-HP production
94 were already reviewed extensively several times. For details on these, readers are therefore
95 referred to more comprehensive reviews (David et al., 2017; Jiang et al., 2009; Kumar et al.,
96 2013; Kumar and Park, 2017; Liu et al., 2017; Matsakas et al., 2014).

97

98 **2.1 Metabolic pathways for 3-HP production**

99

100 **2.1.1 Glycerol oxidation through a coenzyme A-dependent pathway**

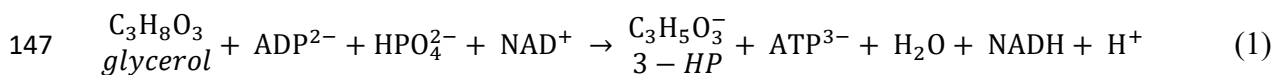
101

102 3-HP is a natural end product of glycerol oxidation in *Lactobacilli*, which are obligate

103 fermentative, Gram-positive bacteria. Glycerol metabolism was notably investigated in detail
104 in *Lactobacillus reuteri*, a heterofermentative species living in human and animal
105 gastrointestinal ecosystems (Kandler et al., 1980). This species cannot grow on glycerol as
106 sole carbon or energy source (Sobolov and Smiley, 1960; Talarico et al., 1990). However,
107 both resting (*i.e.* non-growing) cells and cells growing on other substrates (*e.g.* glucose or
108 lactose) are able to consume glycerol. It is first dehydrated into 3-HPA in metabolosomes by a
109 cobalamin (vitamin B₁₂)-dependent diol dehydratase (Sriramulu et al., 2008; Talarico and
110 Dobrogosz, 1990). Then, in the case of growing cells of *L. reuteri*, 3-HPA is reduced to 1, 3-
111 PDO with consumption of one mole of reducing equivalent per mole of 1,3-PDO produced. 3-
112 HPA is used as electron acceptor for sugar fermentation. It leads to a fermentative shift from
113 ethanol to the more oxidised product, acetate, thus stimulating cell growth from glucose
114 (Talarico et al., 1990). On the other hand, when glycerol is supplied to resting cells, there is
115 no such need for electron dissipation. 3-HPA is mainly accumulated in the medium (Krauter
116 et al., 2012; Sardari et al., 2013; Talarico et al., 1988). However, 3-HP and 1,3-PDO are also
117 found as by-products (Talarico et al., 1988). The reductive pathway (from 3-HPA to 1,3-
118 PDO) remains the same. An oxidative pathway is activated that converts 3-HPA into 3-HP.
119 The aldehyde is converted to 3-HP through a coenzyme A (CoA)-dependent, ATP producing
120 pathway (see Fig. 2.A) (Amin et al., 2013; Dishisha et al., 2014; Sabet-Azad et al., 2015).
121 Sabet-Azad *et al.* successfully introduced this pathway into *Escherichia coli*. When resting
122 cells were used, 3-HP was the only product obtained, while 1,3-PDO was found as co-product
123 when growing cells were used instead (Sabet-Azad et al., 2015). 3-HPA oxidation and
124 reduction are in redox balance, thus implying that the theoretical 3-HP yield from glycerol is
125 limited to $Y_{3\text{-HP}} = 0.5 \text{ mol}_{3\text{-HP}} \text{ mol}_{\text{glycerol}}^{-1}$, with 1,3-PDO as an obligate by-product. In
126 practice, yields are even lower due to the accumulation of 3-HPA. Moreover, this aldehyde
127 accumulation is deleterious to cells. When accumulated, monomeric 3-HPA is in equilibrium
128 with its hydrated form and its cyclic dimeric form; the mixture of these with acrolein is called
129 reuterin (Engels et al., 2016; Talarico and Dobrogosz, 1989). Due to the toxicity of reuterin,
130 bioconversion of glycerol by *L. reuteri* in batch mode stops within a few hours (Burgé et al.,
131 2015).

132
133 The overall free energy liberation during conversion of glycerol into 3-HP is
134 $\Delta rG_{\text{glycerol} \rightarrow 3\text{-HP}}^{\circ} = -50 \text{ kJ mol}^{-1}$ (considering equation 1). Standard free energies were
135 calculated using a group contribution method, according to Jankowski et al., 2008, and for the

136 following conditions: pH = 7, zero ionic strength and a temperature of 298 K. The pathway
 137 thus seems thermodynamically feasible, however, the penultimate step has a positive standard
 138 free energy release ($\Delta rG_{3-HP-CoA \rightarrow 3-HP-P}^{\circ} = 15 \text{ kJ mol}^{-1}$). Therefore, some bottlenecks might
 139 exist and further investigation would be necessary to identify in which conditions this step
 140 becomes favourable (Jiang et al., 2009; Mavrovouniotis, 1996). Despite the ATP formation
 141 accompanying 3-HP production, the metabolic route might not be energy-effective because of
 142 thermodynamic bottlenecks and because of the energetic cost for exporting products outside
 143 the cell (van Maris et al., 2004). This CoA-dependent pathway is also present in *Klebsiella*
 144 *pneumoniae*, along with the CoA-independent pathway (Kumar and Park, 2017). 3-HP
 145 production in *K. pneumoniae* is however mostly considered through the latter (part 2.1.2).



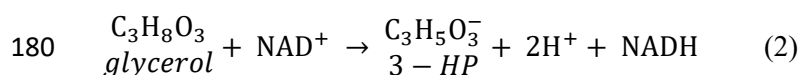
150 2.1.2 Glycerol oxidation through a coenzyme A-independent pathway

151

152 Bacteria from genera *Klebsiella*, *Clostridium*, *Enterobacter* and *Citrobacter* are able to grow
 153 on glycerol during anaerobic fermentation. This property has typically been investigated for
 154 the purpose of 1,3-PDO production (Biebl et al., 1999). During glycerol fermentation,
 155 different metabolic routes are in redox balance with one another. Part of the glycerol is
 156 converted to more oxidised end products, such as lactate, succinate, formate, acetate and CO₂.
 157 Biomass production from glycerol also produces reducing equivalents. Glycerol already being
 158 a very reduced carbon source (4.67 e⁻/C), its fermentation requires efficient routes for the
 159 dissipation of reducing equivalents. Possible end products depend on the microbial strain. For
 160 species like *Klebsiella pneumoniae* and *Citrobacter freundii*, 1,3-PDO is the main product
 161 along with acetate (Clomburg and Gonzalez, 2013), the 1,3-PDO pathway serving as the
 162 reducing equivalent dissipation pathway. 1,3-PDO production from glycerol is a two-step
 163 route (Biebl et al., 1999; Clomburg and Gonzalez, 2013), which makes it a quick way of
 164 dissipating electrons. The intermediary product between glycerol and 1,3-PDO is 3-HPA (Fig.
 165 2.B). In some species, such as *K. pneumoniae*, this first step is catalysed by a vitamin B₁₂-
 166 dependent glycerol dehydratase. Despite 3-HPA being present as an intermediate, no
 167 significant amount of 3-HP is naturally produced by these microorganisms. 3-HP is not
 168 mentioned as an end product of glycerol fermentation (Biebl et al., 1999; Clomburg and

169 Gonzalez, 2013) and, when given, mass balances close around 100 % without any 3-HP
170 (Moscoviz et al., 2016b). Nevertheless, *K. pneumoniae* was shown to possess a NAD⁺-
171 dependent aldehyde dehydrogenase that can use 3-HPA as a substrate and convert it to 3-HP
172 (Raj et al., 2010). Aldehyde dehydrogenase overexpression is however required for 3-HP
173 biosynthesis in significant amounts. The two successive reactions leading to 3-HP from
174 glycerol both have negative free energy releases: $\Delta rG_{\text{glycerol} \rightarrow 3\text{-HPA}}^{\circ} = -38 \text{ kJ mol}^{-1}$ and
175 $\Delta rG_{3\text{-HPA} \rightarrow 3\text{-HP}}^{\circ} = -41 \text{ kJ mol}^{-1}$. The overall free energy release is then $\Delta rG_{\text{glycerol} \rightarrow 3\text{-HP}}^{\circ} = -79$
176 kJ mol^{-1} (see equation 2 for the stoichiometrics), so this pathway is thermodynamically
177 favourable. Despite the standard free energy being negative, it is likely that thermodynamical
178 barriers arise in non-respiratory conditions, due to less efficient NAD⁺ regeneration.

179



181

182 CoA-independent 3-HP production from glycerol in *K. pneumoniae* is one of the most
183 intensively investigated strategies. Works on engineering *K. pneumoniae* for 3-HP synthesis
184 were recently reviewed by Kumar *et al.* (Kumar and Park, 2017). It was identified that the
185 limiting step of 3-HP production, is the conversion of 3-HPA by an aldehyde dehydrogenase
186 (Y. Li et al., 2016). Efforts have therefore been made in order to engineer more efficient
187 aldehyde dehydrogenases and to make this step more favourable. Li *et al.* carried a systematic
188 optimization for 3-HP production by *K. pneumoniae*, taking multiple factors into account,
189 such as promoter strength, metabolic pathway modifications, medium composition and
190 operating parameters. Then the mutant strains were tested in a bioreactor, in fed-batch mode.
191 They obtained the highest 3-HP titre so far: 83.8 g L⁻¹ with a productivity of 1.16 g L⁻¹ h⁻¹ (Y.
192 Li et al., 2016).

193

194 This two-step, CoA-independent pathway has been implemented in other microbial hosts as
195 well. It is predominantly used when 3-HP production from glycerol is considered with *E. coli*
196 (David et al., 2017). This approach in *E. coli* led to one of the highest 3-HP titre so far and the
197 highest obtained in this species (71.9 g L⁻¹, see Table 1). Chu *et al.* transformed *E. coli*
198 W3110 with *gabD4*, a gene from *Cupriavidus necator* encoding a highly active aldehyde
199 dehydrogenase. The enzyme activity was then improved by site-directed and saturation
200 mutagenesis. During fed-batch conditions in a 5 L bioreactor, the most efficient mutant *E. coli*
201 could produce 3-HP up to 71.9 g L⁻¹ with a productivity of 1.8 g L⁻¹ h⁻¹ (Chu et al., 2015).

202 This CoA-independent pathway was also recently implemented in the industrial chassis
203 *Bacillus subtilis* (Kalantari et al., 2017). *B. subtilis* was transformed with the two-step
204 pathway from *K. pneumoniae*. Further optimization of medium composition and strain
205 construction led to a stable genetic construction and a maximum 3-HP titre of 10 g L⁻¹ in
206 shake-flasks, with a 0.80 mol_c mol_c⁻¹ yield.

207

208 Interestingly, this pathway was implemented in *E. coli* and *Corynebacterium glutamicum* for
209 3-HP synthesis from a mix of sugars, using glycerol only as an intermediate. Glucose and
210 xylose were first converted to dihydroxyacetone (DHA) through glycolysis or pentose
211 phosphate pathway, respectively. DHA was subsequently converted to glycerol through a
212 synthetic pathway. 3-HP production was achieved from glycerol through the CoA-
213 independent pathway (Z. Chen et al., 2017; Jung et al., 2015).

214

215 **2.1.3 Malonyl-coenzyme A pathway**

216

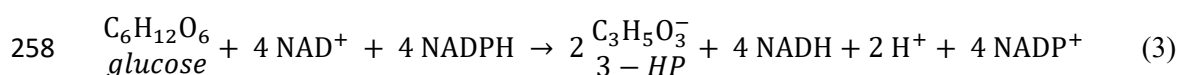
217 Among the various metabolic routes for 3-HP production from sugars, such as glucose, the
218 malonyl-CoA pathway was the most studied until now (Table 1). The use of this pathway for
219 3-HP biosynthesis was already extensively reviewed (Liu et al., 2017). Glucose conversion to
220 3-HP through the malonyl-CoA pathway has typically been investigated in model organisms,
221 such as *E. coli*, *Saccharomyces cerevisiae* (Liu et al., 2017), and more recently in the fission
222 yeast *Schizosaccharomyces pombe* (Suyama et al., 2017). The first part of the pathway, from
223 glucose to two acetyl-CoA (Fig. 2.C), is achieved through endogenous sugar catabolism,
224 comprising glycolysis. Malonyl-CoA is then obtained from acetyl-CoA using an acetyl-CoA
225 decarboxylase. The last two steps, from malonyl-CoA to 3-HP (Fig. 2.C), are both catalyzed
226 by a malonyl-CoA reductase (MCR) from the cyanobacterium *Chloroflexus aurantiacus*. The
227 MCR has two distinct functional domains: the first one is C-terminal and catalyses malonyl-
228 CoA's reduction into malonate semialdehyde (MSA), the second one is N-terminal and
229 catalyses MSA's reduction into 3-HP. It was shown that the overall enzymatic activity was
230 increased when MCR was separated in two fragments, MCR-C and MCR-N. The MCR-C
231 step was however found rate limiting, and one of the highest 3-HP titer for the malonyl-CoA
232 pathway (40.6 g L⁻¹, see Table 1) was achieved thank to balancing both MCR-C and MCR-N
233 enzyme activities.

234

235 The overall transformation of glucose to 3-HP is redox neutral, since both molecules have the

236 same electron equivalent per carbon atom ($4 e^-/C$). Consequently, this pathway does not
 237 require an effective dissipation of excess electrons, like pathways from glycerol do (see parts
 238 2.1.1 and 2.1.2). In addition, the overall transformation of glucose into 3-HP is
 239 thermodynamically favourable, with a negative free energy release $\Delta rG_{\text{glucose} \rightarrow 3\text{-HP}}^{\circ} = -182 \text{ kJ}$
 240 mol^{-1} (see equation 3 for stoichiometrics). A further advantage of the malonyl-CoA pathway is
 241 that it uses common metabolic intermediates, such as pyruvate and acetyl-CoA.
 242 Consequently, a great variety of feedstocks could be considered for 3-HP production. The
 243 malonyl-CoA pathway was notably used for 3-HP biosynthesis from a mix of glucose and
 244 xylose in *Saccharomyces cerevisiae* (Kildegaard et al., 2015), from atmospheric carbon
 245 dioxide in cyanobacteria *Synechocystis* sp. PCC 6803 (Wang et al., 2016b) and
 246 *Synechococcus elongatus* (Lan et al., 2015), and more recently from methanol in
 247 *Methylobacterium extorquens* (Yang et al., 2017). However, a downside of resorting to
 248 common and central metabolic intermediates lies in the fact that there exist many competitive
 249 pathways. It leads to a broad range of by-products, thus limiting the maximal 3-HP yield from
 250 the substrate. Notably, acetate overflow is commonly observed when rapidly growing cells of
 251 *E. coli* are used. Moreover, malonyl-CoA is involved in the synthesis of fatty acids. It is thus
 252 necessary to implement strategies in order to limit the diversion of carbon flux towards other
 253 products. The highest performances obtained through the malonyl-CoA pathway were
 254 achieved by OPX Biotechnologies. Their work aims both at increasing MCR activity and at
 255 decreasing the fatty acid synthesis. They could reach a final 3-HP titre of 48.8 g L^{-1} , with a
 256 corresponding yield of $0.53 \text{ molc molc}^{-1}$ (Lynch et al., 2011).

257



259

260 **2.1.4 β -alanine pathway**

261

262 Another strategy for 3-HP production from glucose was proposed in *E. coli* and *S. cerevisiae*,
 263 through the β -alanine pathway. This was first patented by Cargill in *E. coli* (Liao et al., 2005),
 264 and by Novozymes in *S. cerevisiae* (Jessen et al., 2012). Moreover, Novozymes recently
 265 patented a β -alanine pathway for various microbial hosts, both bacterial and fungic (Barnhart
 266 et al., 2015; Tassone and Diano, 2017). Glucose is first oxidised into pyruvate, which is
 267 subsequently converted to β -alanine. In turn, β -alanine is converted to malonic semi-aldehyde
 268 (MSA). This step also consumes an equivalent of pyruvate and releases an equivalent of L-

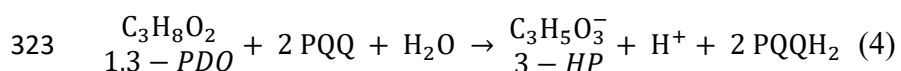
alanine (Fig. 2.D). The L-alanine thus produced can be converted back into β -alanine using a 2,3-alanine aminomutase (Borodina et al., 2015). Finally, MSA is reduced to 3-HP. Borodina et al. used a genome-scale metabolic model and showed that the β -alanine route has a higher maximum theoretical 3-HP yield in comparison to the malonyl-CoA pathway, thus making it a more attractive route for an industrial process (Borodina et al., 2015). The maximal titre achieved in *S. cerevisiae* through this β -alanine pathway was reached in the same study by Borodina et al. They further optimised the expression of the critical enzymes and increased aspartate's synthesis. The *S. cerevisiae* strain they developed was implemented in fed-batch and reach a final titre of $13.7 \pm 0.3 \text{ g L}^{-1}$, with a yield from glucose of $0.14 \pm 0.0 \text{ molC molC}^{-1}$ (Borodina et al., 2015), which remains below the best performances achieved with the malonyl-CoA pathway (Table 1). Like the malonyl-CoA pathway, the β -alanine route uses common metabolic intermediates and can thus be used with other substrates than glucose. Indeed, attempt was made to produce 3-HP from xylose by Kildegaard et al. They compared *S. cerevisiae* strains harbouring either the malonyl-CoA or the β -alanine pathway. They showed that in batch mode, the β -alanine pathway achieved higher titres than the malonyl-CoA pathway: $6.09 \pm 0.33 \text{ g L}^{-1}$ versus $2.30 \pm 0.09 \text{ g L}^{-1}$ (Kildegaard et al., 2015). The malonyl-CoA and β -alanine pathways were also compared in the case of 3-HP production from atmospheric CO_2 in *Synechococcus elongatus* (Lan et al., 2015). In this case, CO_2 was first assimilated into phosphoenolpyruvate, which subsequently underwent either one of this pathway. In this study, the 3-HP titre was lower through the β -alanine pathway than through the malonyl-CoA pathway: 0.186 g L^{-1} versus 0.665 g L^{-1} .

An alternative β -alanine pathway was developed by Song et al. They implemented a route where aspartate is obtained from fumarate instead of oxaloacetate (Fig. 2.D). They obtained higher performances than the oxaloacetate-based pathway. Their final engineered *E. coli* strains reached a 31.1 g L^{-1} 3-HP titre in fed-batch mode, with a $0.423 \text{ molC molC}^{-1}$ yield from glucose (Table 1) (Song et al., 2016). As for the malonyl-CoA pathway, the fact that the β -alanine pathways uses common metabolic intermediates implies that many side-products are synthesised along with 3-HP. β -Alanine is notably a precursor of vitamin B₅. Free energy release is not calculated here due to difficulties associated with the variety of possible routes and with the recycling of some of the intermediates.

2.1.5 Aerobic 1,3-propanediol oxidation

303 Acetic acid bacteria have been known since the beginning of modern microbiology for their
304 ability to transform ethanol into acetic acid during vinegar production (Pasteur, 1864), but
305 they are among the latest actors to have entered the scene of 3-HP production. These bacteria
306 are Gram-negative, obligate aerobes, and are included in the *Acetobacteraceae* family. They
307 possess various membrane-bound dehydrogenases, which gives them the ability to
308 incompletely oxidise a broad range of alcohols and sugars directly in the periplasm
309 (Deppenmeier and Ehrenreich, 2009). Of interest here is that some strains from genera
310 *Acetobacter* and *Gluconobacter* were shown to selectively oxidise 1,3-PDO to 3-HP
311 (Dishisha et al., 2015; J. Li et al., 2016). As first suggested by Dishisha *et al.* (Dishisha et al.,
312 2015), the conversion is likely to occur similarly to that of ethanol into acetate, with two
313 membrane-bound dehydrogenases involved (see Fig. 2.E). First a pyrroquinoline quinone
314 (PQQ)-dependent alcohol dehydrogenase oxidises 1,3-PDO into 3-HPA (Adachi et al., 1978a,
315 1978b; Tkac et al., 2009). The aldehyde is then further oxidised into 3-HP by a PQQ-
316 dependent aldehyde dehydrogenase (Adachi et al., 1980; Ameyama et al., 1981; Tkac et al.,
317 2009). Each step releases two electrons that are transferred to dioxygen through the
318 respiratory chain (Tkac et al., 2009). Thus, the molar yield $Y_{3-HP/1,3-PDO}$ is not redox limited
319 and can theoretically reach $1.0 \text{ mol}_{3-HP} \cdot \text{mol}_{1,3-PDO}^{-1}$ when using resting cells. The overall free
320 energy release is $\Delta rG_{1,3-PDO \rightarrow 3-HP}^{\circ} = -385 \text{ kJ mol}^{-1}$ (considering equation 4), which suggests
321 that 1,3-PDO oxidation is very favourable.

322



325

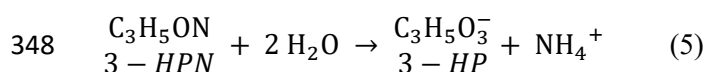
325 **2.1.6 3-hydroxypropionitrile hydrolysis**

326

327 Some bacterial genera, such as *Rhodococcus* and *Comamonas*, are able to hydrolyse aliphatic
328 nitriles into corresponding carboxylic acids, in particular 3-hydroxypropionitrile (3-HPN) to
329 3-HP. The conversion can be carried out in one step using a nitrilase or in two steps by a
330 combination of a nitrile hydratase and an amidase (Fig. 2.F). In the latter case, 3-HPN is first
331 dehydrated by the nitrile hydratase to 3-hydroxypropionamide (3-HPAm), which is
332 subsequently converted into 3-HP by the amidase (Bramucci et al., 2002; Hann et al., 2003).
333 A nitrile-hydrolyzing activity was recently reported for the first time for the *Meyerozyma*
334 genus. A yeast strain, identified as *Meyerozyma guilliermondii*, was isolated from soil and
335 was found to be able to convert 3-HPN to 3-HP (Zhang et al., 2017). As highlighted by

336 Bramucci *et al.*, carboxylic acids are readily obtained from nitriles through various chemical
337 processes, however these chemical processes require strong acid or base use, or high
338 operating temperatures. Therefore, using a biocatalyst instead should allow a greener and less
339 energetically costly process. This biological pathway has several advantages: (i) its
340 selectivity, 3-HP was each time the only reported product during 3-HPN bioconversion
341 (Bramucci *et al.*, 2002; Hann *et al.*, 2003; Zhang *et al.*, 2017), (ii) the fact that no cofactor is
342 required. However, 3-HPN may not be as readily available as other substrates, such as
343 glycerol or glucose. It should also be noted that the overall reaction (see equation 5) has a
344 slightly positive standard free energy $\Delta rG_{3-HPN \rightarrow 3-HP}^{\circ} = 8 \text{ kJ mol}^{-1}$, thus indicating that
345 operating conditions of the process have to be carefully chosen, in order to avoid
346 thermodynamic hurdles.

347



349

350 **2.1.7 Autotrophic CO₂ assimilation through the 3-hydroxypropionate/4-** 351 **hydroxybutyrate cycle and the 3-hydroxypropionate bi-cycle**

352

353 3-HP is also involved in carbon dioxide-assimilating metabolic pathways. There are six
354 different CO₂ fixing pathways, as reviewed by Saini *et al.* and by Fuchs (Fuchs, 2011; Saini *et al.*, 2011). Two of these pathways involve 3-HP as a key intermediary product: the 3-
355 hydroxypropionate/4-hydroxybutyrate cycle and the 3-hydroxypropionate bi-cycle. The latter
356 was discovered almost 30 years ago, while the former was discovered ten years ago (Berg *et al.*, 2007; Holo, 1989). Producing 3-HP from carbon dioxide would be very interesting,
357 because it is a cheap, abundant and environmentally relevant carbon source. This would
358 constitute the main advantage of using this kind of pathway. However, 3-HP is not an end
359 product of these pathways and it is not naturally accumulated, or if so only in small amounts
360 (Holo, 1989). Genetic engineering is therefore required in order to take advantage of these
361 metabolic routes. In fact, research aiming at 3-HP production from CO₂ has already been
362 undertaken, but is still at its early stages (Keller *et al.*, 2013; Lian *et al.*, 2016). In these
363 studies, part of the 3-hydroxypropionate/4-hydroxybutyrate cycle from the archaeon
364 *Metallosphaera sedula* was introduced into the archaeon *Pyrococcus furiosus*. Hence, 3-HP
365 formation was achieved from carbon dioxide as carbon source, dihydrogen as electron donor
366 and maltose as acetyl-CoA and ATP source. At best, the final 3-HP titre reached a few
367
368

369 hundreds of milligrams per litre (Table 1). Enzymes involved in these autotrophic pathways
370 can also be used for the creation of synthetic heterotrophic pathways. In particular, the
371 malonyl-CoA reductase from the 3-hydroxypropionate bi-cycle of *Chloroflexus aurantiacus* is
372 used for 3-HP production by recombinant *Escherichia coli* or *Saccharomyces cerevisiae* from
373 glucose through the malonyl-CoA route (C. Liu et al., 2016) (see part 2.1.3).

374

375 As mentioned above, inorganic carbon fixation into 3-HP was also attempted
376 photosynthetically, using engineered cyanobacteria *Synechocystis* sp. PCC 6803 or
377 *Synechococcus elongatus* PCC 7942 (Lan et al., 2015; Wang et al., 2016b; Y. Wang et al.,
378 2015). CO₂ was assimilated into pyruvate or phosphoenolpyruvate through the ubiquitous
379 Calvin cycle. These intermediates were then further reduced into 3-HP either through the
380 malonyl-CoA pathway (Lan et al., 2015; Wang et al., 2016b) or through the β -alanine
381 pathway (Lan et al., 2015).

382

383 **2.1.8 Other pathways**

384

385 Another metabolic route, the propionyl-CoA pathway, was recently explored for 3-HP
386 production from propionate in *E. coli* (Luo et al., 2016). Propionate is first converted to
387 propionyl-CoA, which is subsequently oxidised into acryloyl-CoA using an equivalent of
388 NADP⁺. Acryloyl-CoA is then hydrated to 3-HP-CoA, whose CoA residue is then transferred
389 to acetate, thus leading to the production of one equivalent of 3-HP and acetyl-CoA. A
390 maximum of 1.60 g L⁻¹ was obtained in shake flasks experiments (Luo et al., 2016).

391 Interestingly, it was observed in the same study that in the absence of glucose, 3-HP
392 production was low (0.06 g L⁻¹). Indeed, when propionate serves as sole energy and carbon
393 source for bacterial growth, the flow through the propionyl-CoA pathway decreases. It was
394 also suggested that the quantity of ATP produced from propionate, which is not a preferential
395 energy source for *E. coli*, could not ensure the synthesis of both growth-related and
396 heterologous proteins. 3-HP production was therefore further limited. Glucose addition could
397 help compensate for this energy deficiency (Luo et al., 2016).

398

399 Lastly, 3-HP was also found to be an end product of uracil or acrylic acid degradation. This
400 was reported for both prokaryotes and eukaryotes: *Escherichia coli* K-12 and *Saccharomyces*
401 *kluveri* were shown to produce 3-HP from uracil (Andersen et al., 2008; Loh et al., 2006);
402 and 3-HP production starting from acrylic acid was observed for fungi (Dave et al., 1996;

403 Takamizawa et al., 1993) and for bacterium *Alcaligenes faecalis* (Ansedè et al., 1999). It was
 404 also reported that 3-HP can be produced – through an unknown pathway – by endophytic
 405 fungi as a nematicidal agent (Schwarz et al., 2004). However, except for acrylic acid
 406 degradation by *Rhodococcus erythropolis* (Lee et al., 2009), these various pathways were not
 407 evaluated as such for 3-HP commercial production.

408

409 **Table 1 – Best 3-HP microbial production performances listed by type of metabolic**
 410 **pathway**

Organism	Carbon source	Operational technique	Titre	Overall process productivity	Yield	Ref
Glycerol oxidation through a CoA-dependent pathway						
<i>Lactobacillus reuteri</i>	glycerol	Fed-batch, 3 L bioreactor	14 g L ⁻¹	0.25 g L ⁻¹ h ⁻¹ ^b	0.49 mol _C mol _C ⁻¹ ^b	(Dishi sha et al., 2015)
Glycerol oxidation through a CoA-independent pathway						
<i>Escherichia coli</i> ^a	glycerol + glucose	Fed-batch, 5 L bioreactor, vitamin B ₁₂ addition, IPTG induction	71.9 g L ⁻¹	1.8 g L ⁻¹ h ⁻¹	–	(Chu et al., 2015)
<i>Klebsiella pneumoniae</i> ^a	glycerol	Fed-batch, 5 L bioreactor, IPTG induction	83.8 g L ⁻¹	1.16 g L ⁻¹ h ⁻¹ ^b	0.60 mol _C mol _C ⁻¹ ^b	(Y. Li et al., 2016)
<i>Corynebacterium glutamicum</i> ^a	xylose + glucose	Fed-batch, 5 L bioreactor, vitamin B ₁₂ addition, IPTG induction	54.8 g L ⁻¹	0.76 g L ⁻¹ h ⁻¹ ^b	–	(Z. Chen et al., 2017)
<i>Corynebacterium glutamicum</i> ^a	glucose	Fed-batch, 5 L bioreactor, vitamin B ₁₂ addition, IPTG induction	62.6 g L ⁻¹	0.87 g L ⁻¹ h ⁻¹ ^b	0.51 mol _C mol _C ⁻¹	(Z. Chen et al., 2017)
Malonyl-CoA pathway						
<i>Escherichia coli</i> ^a	glucose	Fed-batch, 1.8 L bioreactor, IPTG induction	48.4 g L ⁻¹	–	0.53 mol _C mol _C ⁻¹	(Lynch et al., 2011)
<i>Escherichia coli</i> ^a	glucose	Fed-batch, 5 L bioreactor, IPTG	40.6 g L ⁻¹	0.56 g L ⁻¹ h ⁻¹ ^b	0.19 mol _C mol _C ⁻¹	(C. Liu et

		induction				al., 2016)
β-Alanine pathway						
<i>Escherichia coli</i> ^a	glucose	Fed-batch, 6.6 L bioreactor	31.1 g L ⁻¹	0.63 g L ⁻¹ h ⁻¹ ^b	0.423 mol _C mol _C ⁻¹	(Song et al., 2016)
<i>Issatechenkia orientalis</i> ^a	glucose	Fed-batch, 3 L bioreactor	22.8 g L ⁻¹	0.50 g L ⁻¹ h ⁻¹ ^b	–	(Barn hart et al., 2015)
<i>Saccharomyces cerevisiae</i> ^a	glucose	Fed-batch, 1 L bioreactor	13.7 g L ⁻¹	0.17 g L ⁻¹ h ⁻¹	0.14 mol _C mol _C ⁻¹	(Boro dina et al., 2015)
Other heterotrophic routes						
<i>Gluconobacter oxydans</i>	1,3-PDO	Batch, 7 L bioreactor	60.5 g L ⁻¹	2.52 g L ⁻¹ h ⁻¹ ^b	0.94 mol _C mol _C ⁻¹	(Zhao et al., 2015)
<i>Comamonas testosteroni</i>	3-HPN	Batch, 50 mL jacketed reaction vessel	–	23 g L ⁻¹ h ⁻¹	–	(Hann et al., 2003)
Autotrophic routes						
<i>Syneocytis</i> sp. PCC 6803 ^a	NaHCO ₃	Fed-batch, 100 mL Shake-flask, cupric ion induction	0.837 g L ⁻¹	–	–	(Wan g et al., 2016b)
<i>Pyrococcus furiosus</i> ^a	CO ₂	Batch, 1 L bioreactor, heat activation of the recombinant enzymatic pathway	0.366 g L ⁻¹	–	–	(Lian et al., 2016)

411 ^a Engineered microorganisms

412 ^b Value calculated from data given in the article

413 – Value could not be calculated from given data

414

415 2.2 Optimizing 3-HP production through metabolic engineering

416

417 An efficient microbial host is required in order to ensure a commercially relevant process.

418 Therefore, once a metabolic route towards 3-HP is introduced in a non-native producer,

419 further metabolic engineering works are needed to obtain an efficient microbial candidate.
420 This is also true for some native producers, such as *Lactobacillus reuteri*, that do not naturally
421 produce high amounts of 3-HP. Metabolic engineering strategies were already extensively
422 reviewed on several occasions (David et al., 2017; Jiang et al., 2009; Kumar et al., 2013;
423 Kumar and Park, 2017; Liu et al., 2017; Matsakas et al., 2014). The key points to
424 understanding the main challenges are presented here along with the most recent works.

425
426 In order to improve 3-HP yields, it is necessary that the carbon flux from the substrate is
427 sufficiently channelled through the 3-HP producing pathway. Efforts have thus been made in
428 order to reduce the carbon flow in competitive pathways, which mainly include biomass
429 build-up and efficient ATP-generating pathways. Lactate and acetate are major by-products
430 during 3-HP production from glycerol or from sugars. Final titre of lactate was successfully
431 reduced by Chen *et al.* by knocking out the *ldhA* gene encoding a lactate dehydrogenase in
432 *Corynebacterium glutamicum*. In shake-flask cultivation, the resulting strain produced almost
433 no lactate (0.4 g L⁻¹ versus 7.4 g L⁻¹ with the control strain) and had an increase of 19.4 % in
434 3-HP production from glucose (Z. Chen et al., 2017). Knockouts of genes encoding enzymes
435 involved in the production of acetate was also proven efficient during 3-HP production
436 through the CoA-independent glycerol pathway in *C. glutamicum* and *E. coli* by Chen *et al.*
437 and Jung *et al.*, respectively. A decrease of 52.8 % and 97.2 % in acetate production were
438 observed in *C. glutamicum* and *E. coli* respectively, while 3-HP production was improved by
439 25.9 % and 27.3 % respectively (Z. Chen et al., 2017; Jung et al., 2014). Such knockouts had
440 however no significant impact on lactate and acetate production by *K. pneumoniae*. This was
441 attributed to the existence of other lactate- or acetate-producing pathways that were not
442 knocked-out (Y. Li et al., 2016). In the case of acetate, other approaches were also
443 undertaken, such as down-regulating the assimilatory metabolism of glycerol (Kim et al.,
444 2014; Ko et al., 2017; Tokuyama et al., 2014), or deleting a gene encoding a TCA cycle
445 repressor (ArcA), so that acetate overflow is prevented (M. Liu et al., 2016). When
446 considering 3-HP production through the malonyl-CoA pathway, an important issue to
447 address is the competition with the fatty acid biosynthesis pathway (see part 2.1.3).
448 Attenuating the latter pathway leads to higher intracellular levels of malonyl-CoA, thus
449 improving 3-HP production. The fatty acid synthesis was primarily inhibited by using
450 cerulenin (Li et al., 2015; Lynch et al., 2011; Rogers and Church, 2016; Ye et al., 2016). Due
451 to the cost of adding such an inhibitor to a fermentation broth, alternative approaches were
452 proposed through genetic engineering (X. Chen et al., 2017; Lynch et al., 2011; Ye et al.,

453 2016). Although the disruption of genes involved in central metabolism can improve the 3-HP
454 yield, it was also observed that it can hinder cell growth (Ko et al., 2017; Y. Li et al., 2016;
455 Lynch et al., 2011; Tokuyama et al., 2014). A novel approach was recently proposed by
456 Tsuruno *et al.* They developed a metabolic toggle switch in order to perform conditional
457 repression of genes related to central glycerol metabolism, so that 3-HP is produced while
458 avoiding growth defect (Tsuruno et al., 2015). Another promising strategy was proposed by
459 David *et al.*, who achieved a dynamic control of the malonyl-CoA node in *S. cerevisiae*. The
460 expression of a fatty acid synthase was subjected to control by a glucose-sensitive promoter.
461 Hence, fatty acid biosynthesis was down-regulated in glucose-limited conditions only. This
462 can ensure that sufficient biomass is produced, before the carbon flux is channelled towards 3-
463 HP synthesis (David et al., 2016).

464

465 Another major aspect of pathway optimisation is the balancing of the different steps within
466 the 3-HP producing pathway. Imbalanced pathways can lead to the accumulation of metabolic
467 intermediates, thus hindering the process performances. This is particularly true when 3-HP is
468 produced from glycerol: imbalance between the high glycerol dehydratase activity and the
469 low aldehyde dehydrogenase activity results in detrimental accumulation of the toxic
470 intermediate 3-HPA (see parts 2.1.1 and 2.1.2). Until now, this issue was mainly addressed by
471 varying the strength of the promoters, or by varying the number of gene copies on the
472 plasmids. A novel technique was recently applied by Lim *et al.* They used the forward
473 engineering “UTR Designer” software in order to optimise the glycerol dehydratase activity.
474 This software helps predict the translation initiation efficiency of a mRNA sequence, given
475 the 5'-untranslated region (5'-UTR) located upstream. Using this software, Lim *et al.*
476 designed 5'-UTRs with different strength, and showed that the glycerol dehydratase activity
477 was dependent on the 5'-UTR used. Their study resulted in a well-balanced pathway in *E. coli*
478 and a maximum titre of 40.51 g L⁻¹ was reached (productivity: 1.35 g L⁻¹ h⁻¹; yield: 0.99 mol_{3-HP}
479 mol_{glycerol}) (Lim et al., 2016). Pathway balancing was also achieved in *E. coli* by using a
480 bicistronic synthetic cassette architecture. No 3-HPA accumulation was measured when the
481 aldehyde dehydrogenase activity was 8-fold higher than the glycerol dehydratase activity
482 (Sankaranarayanan et al., 2017). As mentioned in section 2.1.3, an imbalance also exists
483 between the two functional sites of the malonyl-CoA reductase (MCR) in the malonyl-CoA
484 pathway. Balance between them was achieved by Liu *et al.* by improving the MCR-C
485 fragment by directed evolution and by decreasing the MCR-N activity (C. Liu et al., 2016).

486

487 Redox cofactors regeneration is a major issue for metabolic pathways optimisation. Indeed, 3-
488 HP generating pathways generally involve at least one redox equilibrium (Fig. 2). For
489 instance, when 3-HP is produced from glycerol, 3-HPA is oxidised to 3-HP using NAD^+ as
490 electron acceptor. NAD^+ regeneration is maximal in aerobic conditions, when the electron
491 transport chain is active and electrons are accepted by oxygen. However, aerobic conditions
492 are incompatible with efficient vitamin B_{12} synthesis (see part 2.3). NAD^+ regeneration has
493 typically been ensured by using a NADH oxidase (Li et al., 2013). An alternative strategy was
494 suggested by Li *et al.*, which consists in using a NAD^+ -independent aldehyde dehydrogenase
495 from *Pseudomonas* sp. AIU 362 (Li et al., 2013). This enzyme uses O_2 as electron acceptor,
496 so this strategy does not fully resolve the contradiction with anaerobic vitamin B_{12} synthesis.
497 In the malonyl-CoA pathway, the reduction of malonyl-CoA to 3-HP necessitates that two
498 electron equivalents are provided by NADPH. Meanwhile, the upstream part of the pathway
499 consists in the oxidation of glucose to pyruvate, thus generating reducing equivalents (Fig.
500 2.C). Chen *et al.* took advantage of this by engineering *S. cerevisiae*, so that the reducing
501 equivalents generated by the upstream steps of the pathway could be generated as NADPH,
502 instead of NADH, thus ensuring the NADPH supply of the last steps of the pathway (Chen et
503 al., 2014).

504

505 Furthermore, cells' transporting functions were shown to have significant impact on 3-HP
506 production. 3-HP production was successfully increased by 8 % in *C. glutamicum* when the
507 native glucose uptake system was replaced by a route involving inositol permeases and
508 glucokinase. By doing so, glucose uptake was no longer subjected to the need for
509 phosphoenolpyruvate (Z. Chen et al., 2017). In *Synechocystis* sp. PCC6803, proteomic and
510 metabolomic approaches led to the identification of 11 genes potentially relevant for 3-HP
511 synthesis. Interestingly, five of them were related to transport functions in cells. Results
512 showed that overexpressing genes putatively involved in cobalt/nickel, manganese and
513 phosphate transports could improve 3-HP production (Wang et al., 2016a).

514

515 Lastly, a major concern when designing a bioprocess is the genetic stability of the constructed
516 microbial agent. Genetic engineering in bacteria is mainly achieved using plasmids as vectors
517 because for many species, efficient methods to transfer a plasmid into a cell are known. Yet,
518 they are subjected to major instabilities, such as structural and segregational instabilities
519 (Wang and Tian, 2017). Attempt has thus been made at developing a plasmid-free host for 3-
520 HP production. The proof of concept was provided by Wang and Tian who engineered a

521 plasmid-free, 3-HP-producing strain of *K. pneumoniae* through homologous recombination
522 (Wang and Tian, 2017).

523

524 **2.3 Microbial agents for 3-HP production**

525

526 As illustrated in the previous part, a variety of both microbial species and carbon sources are
527 being investigated for the purpose of 3-HP production. Figure 3 sums up the different
528 microorganisms/substrates couples already implemented. Classical microbial chassis are
529 interesting candidates because they are well-known and well-characterised. They are
530 generally easy-to-use fast growing organisms. A lot of tools are already available for their
531 metabolic engineering as well as for their scaling-up. In particular, *E. coli* is one of the most
532 widely used microorganisms for 3-HP production so far. *Bacillus subtilis* and
533 *Corynebacterium glutamicum* are further bacterial model organisms for industrial applications
534 (Becker and Wittmann, 2012; Zhang and Zhang, 2010). As reviewed by Zhang and Zhang,
535 2010, *B. subtilis* displays interesting advantages such as the Generally Recognised As Safe
536 (GRAS) status, fast growth, low nutrient requirements, and great tolerance to salts and
537 solvents. In addition, the biomass of *B. subtilis* thus produced may be further utilised as a feed
538 additive (Rychen et al., 2017). Those features are particularly relevant in the case of 3-HP
539 production. Indeed, it is only a low-value added bioproduct so the process needs to be as cost-
540 effective as possible in order for it to be economically viable. What is more, *B. subtilis* is able
541 to naturally uptake a variety of sugars, including xylose. Xylose is an interesting substrate
542 because it is one of the sugars that can be obtained from lignocellulosic feedstocks. Until now,
543 3-HP production from xylose was considered in *E. coli*, *C. glutamicum* and *S. cerevisiae* (Z.
544 Chen et al., 2017; Jung et al., 2015; Kildegaard et al., 2015), but *B. subtilis* may also be a
545 good candidate for second-generation (*i.e.* using non-food feedstocks) 3-HP production.

546

547 From an industrial point of view, yeasts are often preferred over bacteria due to their higher
548 tolerance to low pH values, both facilitating the downstream processes and lowering the
549 overall cost. In addition, *S. cerevisiae* also has the GRAS status (Fig. 3). Only a few studies
550 have yet focused on yeasts in comparison to *E. coli*. Several methods for 3-HP production
551 from glucose in recombinant yeasts were patented in recent years by Novozymes and SK
552 Innovation (Barnhart et al., 2015; Bindel, 2014; Park et al., 2016). Interestingly, some yeast
553 species were chosen over *S. cerevisiae* for their greater tolerance to 3-HP (Barnhart et al.,
554 2015; Suyama et al., 2017). They notably include *Issatechenkia orientalis*, which currently

555 holds the record 3-HP titre in yeast, which is 22.8 g L⁻¹ (Table 1). Recombinant *I. orientalis*
556 expressed an aspartate 1-decarboxylase from the insect *Aedes aegypti*, and was implemented
557 in a 3L-bioreactor with a glucose feed (Barnhart et al., 2015). However, as explained by the
558 authors of this patent, an important drawback of using yeasts is that their metabolic
559 engineering is typically more complicated than for bacteria. This is notably due to the
560 compartmentalisation of eukaryotic cells. This requires that special attention be paid to
561 targeting signals and also to the transportation of intermediates from one cell compartment to
562 another (Barnhart et al., 2015). Nevertheless, efficient tools exist for engineering *S.*
563 *cerevisiae*. For instance, a toolkit was developed for marker-less integration of genes into *S.*
564 *cerevisiae* using CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats
565 (CRISPR) and CRISPR-associated endonuclease (Cas9)). This toolkit was used for 3-HP
566 production by enhancing the malonyl-CoA pathway through three different strategies. The
567 most successful strategy consisted in overexpressing a pyruvate dehydrogenase complex and
568 could increase the 3-HP titre by +23 % when using the industrial strain Ethanol Red (Jessop-
569 Fabre et al., 2016).

570

571 When considering 3-HP production from glycerol a major limitation of using an industrial
572 chassis is that they generally cannot produce vitamin B₁₂ on their own. As explained above,
573 vitamin B₁₂ is a necessary cofactor for glycerol's conversion to 3-HPA, both through the
574 CoA-dependent pathway of *Lactobacilli* sp. and through the CoA-independent pathway of *K.*
575 *pneumoniae* (see parts 2.1.1 and 2.1.2). Therefore, when microbial chassis such as *E. coli*, *B.*
576 *subtilis* or *C. glutamicum* are transformed with a glycerol dehydratase from *K. pneumoniae*,
577 costly addition of vitamin B₁₂ to the culture medium is required (Table 1). Using the vitamin
578 B₁₂-independent glycerol dehydratase from *Clostridium butyricum* (O'Brien et al., 2004)
579 could be a way to work around this issue. Another possibility is to use the native species:
580 *Lactobacillus reuteri* and *K. pneumoniae* are indeed prototroph for vitamin B₁₂, so there is no
581 need to supply it to the medium. These species also have further advantages. *L. reuteri*
582 notably has the GRAS status and was even proven to be a probiotic agent (Casas and
583 Dobrogosz, 2000). As for it, *K. pneumoniae* was the most studied organism for 3-HP
584 production so far and offers the following advantages: (i) glycerol fermentation pathways
585 have been studied extensively: present knowledge of glycerol metabolism in *K. pneumoniae*
586 was recently and extensively reviewed (Kumar and Park, 2017); (ii) it can grow directly on
587 glycerol, so the fermentative process could come down to a single step; (iii) *K. pneumoniae*
588 can grow on glycerol both anaerobically and aerobically; and (iv) it has a great glycerol

589 assimilation rate. However, *K. pneumoniae* is pathogenic to humans (Podschun and Ullmann,
590 1998), making a process based on this species unlikely to reach industrial scale. Yet, a new
591 isolate *Klebsiella* sp. AA405 carrying low levels of virulence factors was recently identified
592 as potential host for 3-HP production (Li et al., 2017). Furthermore, an important limitation to
593 efficient 3-HP production remains: there exists a contradiction between vitamin B₁₂ synthesis
594 and NAD⁺ regeneration. Indeed, maximum NAD⁺ regeneration is reached under aerobic
595 conditions, while maximum vitamin B₁₂ synthesis is achieved anaerobically. Using the
596 vitamin B₁₂-independent glycerol dehydratase from *C. butyricum* would be subjected to the
597 same limitation, since it is active only in strictly anaerobic conditions (O'Brien et al., 2004).
598 Zhou *et al.* proposed an alternative solution by using *Pseudomonas denitrificans*. The strain is
599 able to produce vitamin B₁₂ aerobically, and was transformed with glycerol dehydratase and
600 aldehyde dehydrogenase genes from *K. pneumoniae* (Zhou et al., 2013). A maximum titre of
601 4.92 g L⁻¹ was reached, corresponding to a 0.67 mol_{3-HP} mol⁻¹_{glycerol} yield.

602
603 Until now, 3-HP production is primarily achieved from glycerol or glucose, which are both
604 relatively cheap and abundant carbon sources. Glycerol is notably a relevant substrate for
605 biorefinery processes: it is a by-product of first-generation biodiesel production. A large
606 glycerol surplus has been formed, making it a cheap and abundant carbon source (Chen and
607 Liu, 2016). An even cheaper and more abundantly available carbon source would be
608 atmospheric CO₂. 3-HP production was already achieved using inorganic carbon. The proof
609 of concept was provided through an original approaches in archea *Pyrococcus furiosus* and
610 *Metallosphaera sedula* (Keller et al., 2013; Lian et al., 2016), as well as in cyanobacteria
611 *Synechocystis elongatus* and *Synechocystis* sp. PCC 6803 (Lan et al., 2015; Wang et al.,
612 2016b; Y. Wang et al., 2015) (see part 2.1.7). In contrast, genetic engineering tools are limited
613 for these species as compared to those available for usual microbial chassis. However,
614 cyanobacteria and *Synechocystis* sp. in particular were recently identified as potential next
615 generation chassis for synthetic biology (Adams, 2016; Branco dos Santos et al., 2014). 3-HP
616 production could therefore benefit from the development of new toolkits for these species,
617 such as CRISPR-Cas systems. Interestingly, a process was recently patented by Verdant
618 Bioproducts Limited for 3-hydroxypropionamide (3-HPAm) production from atmospheric
619 CO₂ and nitrogen, using *Acetobacter lovaniensis* (Finnegan, 2017). The patent only mentions
620 the possibility of subsequently converting 3-HPAm to 3-HP through a chemical process.
621 However, a two-step bioprocess could be envisaged: CO₂ and nitrogen are first assimilated by
622 *A. lovaniensis* into 3-HPAm, which is then converted to 3-HP by amidases from *Comamonas*

623 *testosteroni*, for instance.

624

625 Lastly, the combined use of two complementary microorganisms was recently investigated for
626 3-HP production from glycerol (Dishisha et al., 2015; Sabet-Azad et al., 2015; Zhao et al.,
627 2015) (see part 3.2). In particular, acetic acid bacteria were used to oxidise the 1,3-PDO
628 produced either by *K. pneumoniae* or *L. reuteri* into 3-HP. One great advantage of using
629 acetic acid bacteria is their ability to resist organic acids, and grow under acidic conditions
630 (Raspor and Goranovič, 2008; B. Wang et al., 2015). Furthermore, acetic acid bacteria have
631 long been used in the food industry for vinegar production, so they should be suited well for
632 industrial scale-up.

633

634 **3 Overcoming hurdles to 3-HP commercial production through process engineering**

635

636 **3.1 Operational strategies for 3-HP-producing fermentative processes**

637

638 **3.1.1 Fermentation mode**

639

640 To this day, 3-HP production is primarily achieved through bench-scale, fed-batch
641 fermentations. Almost all studies reviewed for this paper that carried out 3-HP production in
642 bioreactors used smaller ones (from 1 L to 7 L, see below for exceptions) and most of them
643 were operated under fed-batch mode. An important part of the research effort being focused
644 on genetic engineering, fed-batch fermentations in bioreactors are essentially undertaken in
645 order to confirm a novel strain's potential as a 3-HP producer. During a fed-batch, the
646 medium is gradually supplied with substrate, as cell population grows and products
647 accumulate, which allows higher cell densities and product titres, in comparison with batch
648 mode. Fed-batch cultures are advantageous for the production of both growth-associated and
649 non growth-associated metabolites (Liu, 2017). In the former case, a continuous exponential
650 substrate supply can control the microbial growth rate in order to make the exponential phase
651 last longer, thus maximizing the metabolite production (Liu, 2017). This kind of approach,
652 however, requires an in-depth understanding of the strain's physiological needs and does not
653 yet appear to have been applied to 3-HP biosynthesis.

654

655 An important feature of fed-batch techniques is that the substrate concentration is kept low
656 throughout the fermentation. This helps limit deleterious phenomena due to high substrate

657 concentrations or due to detrimental intermediates accumulation. Glycerol concentration is
658 typically kept around a relatively low set value, ranging from 5 g L⁻¹ to 25 g L⁻¹ (Huang et al.,
659 2013; Y. Li et al., 2016; Zhao et al., 2015). Indeed, it was previously reported that glycerol
660 concentrations above 40 g L⁻¹ had detrimental effects on the growth of *K. pneumoniae*.
661 Maximal tolerated concentrations were estimated at 110 g L⁻¹ and 133 g L⁻¹ for aerobic and
662 anaerobic cultures respectively (Cheng et al., 2005). This rules out the possibility of efficient
663 batch strategies for 3-HP production at high titres from glycerol. Moreover, in conjunction
664 with genetic engineering strategies, fed-batch mode also offers a way to control levels of
665 undesired side products in the final broth. During aerobic cultures, fast-growing cells use
666 fermentation instead of the more energy efficient respiratory chain (Basan et al., 2015). This
667 ubiquitous phenomenon is generally referred to as the overflow metabolism. Part of the
668 carbon flux is then diverted from the tricarboxylic acid (TCA) cycle to incompletely oxidised
669 end-products, such as acetate in the case of *E. coli* or ethanol in the case of *S. cerevisiae*. Not
670 only does the production of these metabolites hinder 3-HP yields, but it also has toxic effects
671 on cells. This issue was acknowledged several times during 3-HP production (Kildegaard et
672 al., 2016; M. Liu et al., 2016; Raj et al., 2009; Sankaranarayanan et al., 2014) and was
673 addressed through both fed-batch (Kildegaard et al., 2016) and genetic engineering (M. Liu et
674 al., 2016) strategies. As for wild type *Lactobacillus reuteri*, since it cannot grow on glycerol
675 as sole carbon or energy source, no overflow phenomenon is involved. However, it was
676 demonstrated by Dishisha *et al.* that glycerol dehydration to 3-HPA by resting *L. reuteri* cells
677 is at least 10 times faster than 3-HPA consumption, leading to detrimental 3-HPA
678 accumulation in batch mode (Dishisha et al., 2014). A specific glycerol consumption rate
679 (q_{Gly}) of 128.3 mg_{glycerol} per grams of cell dry weight (cdw) was hence identified as maximum
680 value in order to produce only 1,3-PDO and 3-HP from glycerol, without any 3-HPA
681 accumulation. This allowed Dishisha *et al.* to design a fed-batch bioconversion using wild
682 type *L. reuteri* as a biocatalyst that did not produce 3-HPA: the glycerol feed was maintained
683 at 0.75 g_{glycerol} h⁻¹, which is half the maximum rate given the dry weight of biocatalyst used in
684 the experiment. This process achieved a 14 g L⁻¹ 3-HP titre with a 0.25 g L⁻¹ h⁻¹ productivity
685 (Dishisha et al., 2015). This illustrates how process design can be guided by the understanding
686 of the microbial metabolism and how it can help overcome hurdles related to enzymatic
687 kinetics. As pointed out by Li *et al.*, in *K. pneumoniae* too, the aldehyde dehydrogenase is the
688 velocity-limiting enzyme for glycerol conversion to 3-HP (Y. Li et al., 2016). So, similarly to
689 the work of Dishisha *et al.*, fed-batch strategies should allow better 3-HP production using *K.*
690 *pneumoniae*.

691
692 The contradiction between anaerobic vitamin B₁₂ regeneration and aerobic NAD⁺ regeneration
693 during glycerol conversion to 3-HP (see part 2.3) was recently addressed through electro-
694 fermentation for the first time (Kim et al., 2017). Electro-fermentation is still an emerging
695 technology and was defined in 2016 as a fermentative process in which polarised electrodes
696 are added and can be used both as a driving tool and for fine redox control (Moscoviz et al.,
697 2016a; Schievano et al., 2016). Electro-fermentation relies on the ability some
698 microorganisms have to exchange electrons with solid electrodes, either directly or through
699 redox shuttles. In the case of anodic electro-fermentation, electrons are transferred from
700 microorganisms to the anode. In their study, Kim *et al.* grew a recombinant *K. pneumoniae*
701 overexpressing aldehyde dehydrogenase in the anodic compartment of a bioelectrochemical
702 bioreactor. Electrodes were poised at + 0.5 V vs. Ag/AgCl and 2-hydroxy-1,4-
703 naphthoquinone (HQN) was added in the medium as an electron shuttle between *K.*
704 *pneumoniae* and the anode. During anaerobic fermentation, electrons were transferred by the
705 bacteria to the electrode, resulting in current generation in the external circuit and in lower
706 intracellular NADH/NAD⁺ ratios. The 3-HP to 1,3-PDO ratio thus shifted from 0.84 in
707 fermentative control to 2.26 in electro-fermentative conditions. The process reached a 1.94 g
708 L⁻¹ 3-HP titre, with a yield of 0.19 mol_{3-HP} mol_{glycerol}⁻¹ (Kim et al., 2017). This proof of
709 concept demonstrates that NAD⁺ regeneration can be significantly improved in anaerobic
710 conditions: it then becomes possible to achieve both high vitamin B₁₂ and NAD⁺ regeneration.
711 Electro-fermentation gives rise to promising opportunities for overcoming redox limitations
712 of glycerol conversion to 3-HP.

713
714 As mentioned at the beginning of this section, 3-HP synthesis is primarily achieved in bench-
715 scale bioreactors and to this date, no industrial production of 3-HP is fully implemented. Two
716 notable exceptions can be found in the scientific literature. The archaeon *Pyrococcus furiosus*
717 that was transformed with part of the 3-HP/4-HB cycle in order to autotrophically produce 3-
718 HP was operated in a 20 L bioreactor (Keller et al., 2013). Furthermore, in their 2011 patent,
719 OPX Biotechnologies demonstrated 3-HP production in a 250 L reactor in fed-batch mode.
720 The *E. coli* inoculum was prepared in two-steps: 1 mL of stock culture was first inoculated
721 into a 100 mL shake-flask, which was subsequently used to inoculate a 14 L bioreactor. This
722 latter culture was then used to inoculate the 250 L bioreactor. IPTG was used for the malonyl-
723 CoA pathway induction. The glucose feed rate was adjusted in order to maintain its
724 concentration between 10 and 15 g L⁻¹. In these conditions, a maximal 3-HP titre of 20.7 g L⁻¹

725 was reached, with a $0.43 \text{ g L}^{-1} \text{ h}^{-1}$ productivity (Lynch et al., 2011).

726

727 **3.1.2 Process parameters**

728

729 Influence of operating parameters, such as aeration rate and medium composition, on the
730 microbial production of 3-HP were studied. Yet, those aspects have been the object of less
731 intense attention, in comparison to metabolic engineering issues. As previously mentioned, 3-
732 HP production from glycerol is an oxidative process; consequently the level of aeration has an
733 impact on final metabolite profile. A first study was conducted by Raj *et al.* on an *E. coli*
734 strain expressing the CoA-independent glycerol pathway (Fig. 2.B). By decreasing the liquid-
735 to-flask volume ratio from 0.4 to 0.2 to improve the aeration level, 3-HP titer was enhanced
736 by 2.2-fold (0.54 instead of 0.24 mg L^{-1}) while 1,3-PDO production was slightly reduced
737 (from 0.14 to 0.10 mg L^{-1}). However, a 2.5-fold higher accumulation of acetate and formate
738 was observed under aerobic condition. This was attributed to the changes in aeration
739 conditions and to an undefined metabolic overflow phenomenon (Raj et al., 2009). To
740 understand the response of *K. pneumoniae* to oxygen supply, Huang *et al.* measured the
741 transcription levels of genes coding for enzymes of the CoA-independent glycerol oxidation
742 pathway and of the formate hydrogen lyase pathway during aerobic, micro-aerobic, and
743 anaerobic growth in a 5-L bioreactor (Huang et al., 2016). Aeration led to a decrease in the
744 glycerol dehydratase transcription level and to an increase in that of aldehyde dehydrogenase.
745 Simultaneously, hydrogenase- and formate dehydrogenase-encoding genes were upregulated.
746 Authors hypothesized that excess NADH formed by overexpressed *aldH* is consumed through
747 the pathway comprising these enzymes, while producing dihydrogen. To overcome the issue
748 of NAD^+ regeneration while preserving glycerol dehydratase activity and vitamin B_{12}
749 synthesis, Ko *et al.* worked on aeration conditions during *K. pneumoniae* growth on glycerol
750 (Ko et al., 2017). After disrupting enzymes of competing pathways (see part 2.2.1), they
751 observed that deletion of the *pta-ackA* gene responsible for acetate production led to
752 degradation of cell growth, decrease in glycerol uptake, and reduction of 3-HP production. To
753 reduce acetate production without modifying this gene, various aeration rates were tested in a
754 1-L bioreactor by applying agitation speed between 200 and 600 rotations per minute (rpm),
755 at an air flow rate of 1 vessel volume minute (vvm). Increase in oxygen transfer enhanced cell
756 growth 2.8-fold. 3-HP production yield was improved from 0.18 to $0.38 \text{ mol}_{3\text{-HP}} \text{ mol}_{\text{glycerol}}^{-1}$ at
757 200 and 600 rpm, respectively. In the same time, a drastic decrease in 1,3-PDO yield, from
758 0.36 to $0.01 \text{ mol}_{1,3\text{-PDO}} \text{ mol}_{\text{glycerol}}^{-1}$, was observed. With this strategy, acetate production was

759 minimized at 600 rpm but never totally suppressed (Ko et al., 2017). It is also important to
760 keep in mind that high energy needs linked to high aeration and agitation rates contribute to
761 expand production costs at the industrial scale. Simultaneous 3-HP and 1,3-PDO production
762 allows performing process under low-aeration conditions as the pathway is redox balanced.

763
764 The influence of the fermentation medium composition was also assessed in several studies.
765 Notably, part of the record-holder study of Li *et al.* was dedicated to this issue (Y. Li et al.,
766 2016). 3-HP production from glycerol by *K. pneumoniae* was tested on different media in
767 shake-flasks. Individual variation of each component of the medium was tested at six different
768 levels: 0 %, 25 %, 50 %, 100 %, 150 %, 200 % (in comparison to their previous study). The
769 optimised medium that was selected led to 1.8-fold increase in 3-HP production. Ten different
770 levels of IPTG concentrations were also tested. Surprisingly, IPTG concentration showed no
771 impact on growth and 3-HP production. pH was also showed to be a critical factor to 3-HP
772 production by *K. pneumoniae*. The adequate pH value that was chosen was 7.0, because it
773 corresponded to the highest glycerol conversion to 3-HP (Y. Li et al., 2016). A study on
774 medium optimisation of growth medium for *Lactobacillus reuteri* was also carried out by
775 Couvreur *et al.* They screened 30 different media, varying by their concentrations of carbon
776 source (sugar beet and wheat processing coproducts), yeast extract, tween 80 and vitamin B₁₂.
777 In the case of *L. reuteri*, bacterial growth is separated from glycerol conversion in two distinct
778 steps (see part 3.2, Fig. 4). Couvreur *et al.* studied the impact of the medium composition of
779 the growth step on the performances of the second step. They showed that the optimal
780 medium could lead to an increase of 70 % in subsequent 3-HP production, with reduced 3-
781 HPA titre (Couvreur et al., 2017).

782

783 **3.2 Multi-step and integrated approaches**

784

785 Recently, process approaches to 3-HP bioproduction have been diversifying. In order to
786 improve the yield and selectivity of the overall process, multiple-step integrated processes are
787 being designed. This is especially true for 3-HP production from glycerol. The first step
788 consists of the conversion of glycerol into either 3-HPA or a mix of 3-HP and 1,3-PDO. The
789 undesired product (3-HPA or 1,3-PDO) is subsequently converted into 3-HP (Figure 4).
790 Sabet-Azad *et al.* implemented a two-step process involving both wild-type and recombinant
791 organisms (Sabet-Azad et al., 2015). Glycerol was first supplied in fed-batch mode to wild-
792 type *Lactobacillus reuteri*, which converted it into 3-HPA mostly (56 % of the amount of

793 products), along with 3-HP (22 %) and 1,3-PDO (22 %). By coupling this step with an *in situ*
794 3-HPA complexation with bisulfite and an extractive module, a 3-HPA solution free of 3-HP
795 and 1,3-PDO, could be obtained. 3-HPA was then converted in fed-batch mode either by
796 growing cells or by resting cells of a recombinant *E. coli*. When using resting cells, this
797 second step reached a 1.1 g L⁻¹ 3-HP titre, with a 0.06 g L⁻¹ h⁻¹ productivity. Interestingly, no
798 by-product was obtained along with 3-HP. This approach provides another way to circumvent
799 the contradiction between the optimisation of cobalamin availability and NAD⁺ regeneration
800 (see part 2.3). By using the 3-HPA accumulating bacterium *L. reuteri*, it is possible to
801 separate the anaerobic, cobalamin-dependent step (from glycerol to 3-HPA) from the aerobic
802 NAD⁺-dependent step (from 3-HPA to 3-HP-CoA). Then, each step can be optimised
803 separately, according to its specificities. Zhao *et al.* and Dishisha *et al.* implemented two step
804 processes during which glycerol was first converted respectively into 1,3-PDO by growing
805 *Klebsiella pneumoniae* in batch mode or into a mix of 1,3-PDO and 3-HP by resting
806 *Lactobacillus reuteri* in fed-batch mode (Dishisha *et al.*, 2015; Zhao *et al.*, 2015). Then,
807 resting cells of acetic acid bacterium *Gluconobacter oxydans* were used to convert 1,3-PDO
808 to 3-HP. In the first study, *G. oxydans* was implemented in the same bioreactor as *K.*
809 *pneumoniae*, after the latter was heat inactivated at 65 °C during 30 min. The overall process
810 reached a 60.5 g L⁻¹ 3-HP titre, corresponding to a 0.52 molc molc⁻¹ yield on glycerol and a
811 0.94 molc molc⁻¹ yield on 1,3-PDO (Zhao *et al.*, 2015). The second process implemented *G.*
812 *oxydans* in a separate batch from *L. reuteri*. Glycerol bioconversion by *L. reuteri* produced an
813 equimolar mix of 1,3-PDO and 3-HP. The supernatant was given as bioconversion medium to
814 *G. oxydans*, which quantitatively and selectively oxidized 1,3-PDO to 3-HP in less than 5
815 hours. The process reached a final 3-HP titre of 23.6 g L⁻¹ (Dishisha *et al.*, 2015). This
816 additional bioconversion step allows an increase in the maximal theoretical 3-HP yield to 1
817 mol_{3-HP} mol⁻¹_{glycerol}, which is not achievable when using *K. pneumoniae* or *L. reuteri* alone
818 (see part 2.2). Despite the attractiveness multi-step processes may have, particularly regarding
819 overall yields, their economic feasibility is questionable. Indeed, their practical
820 implementation is likely to prove too constraining, with higher associated costs, in
821 comparison to a single-step fermentation. Still, these studies open up new perspectives,
822 notably towards integrated continuous processes. Continuous processes have great industrial
823 interest due to reduced operating costs. Moreover, dual-microbe strategies may be seen as a
824 first step towards implementation of both pathways into a single microbial chassis, or towards
825 mixed cultures for 3-HP production.

826

827 An important characteristic of these studies is that at least one step is performed by resting
828 cells (Figure 4). Resting cells are also used for 3-HP production from 3-HPN, which is carried
829 out by *Comamonas testosteroni* or recombinant *E. coli* (Hann et al., 2003; Yu et al., 2016).
830 Generally speaking, when resting cells are used for bioconversions, a preliminary step is
831 required in order to produce the necessary biomass of whole cell biocatalyst. It is thus
832 beneficial to recycle resting cells through several batches: the more bioconversion batches can
833 be carried out from a single biomass production, the more cost-effective the process gets. Cell
834 recycling in the context of 3-HP production was reported in several studies and was achieved
835 by cell immobilisation on alginate beads (Hann et al., 2003; J. Li et al., 2016; Yu et al., 2016),
836 by cryostructured cross-linking (Zaushitsyna et al., 2017), or by microfiltration (Dishisha,
837 2013). Hann *et al.* and Yu *et al.* used *C. testosteroni* and *E. coli* respectively for 3-HPN
838 conversion to 3-HP, while Li *et al.* implemented the acetic acid bacteria *Acetobacter* sp.
839 CGMCC 8142 for 3-HP production from 1,3-PDO. *C. testosteroni* and *E. coli* cells were
840 recycled many times: up to 100 cycles for the former, and up to 74 cycles for the latter. As for
841 it, the study on *Acetobacter* sp. CGMCC 8142 focused on a more restricted number of cycles:
842 5 consecutive batches were performed. In each study, significant enzymatic activities were
843 retained after the successive cycles, for instance *E. coli* retained 70 % of its enzymatic activity
844 after 74 batches. When compared to suspended cells, it was shown that immobilised cells
845 could retain their ability to produce 3-HP over a greater number of cycles. Suspended cells of
846 *E. coli* could not produce 3-HP after the tenth batch, while immobilised cells could still
847 convert 3-HPN to 3-HP after the 60th batch with a molar yield ranging from 0.4 to 0.9 mol_{3-HP}
848 mol_{3-HPN}⁻¹, depending on the immobilisation material (Yu et al., 2016). After 5 cycles,
849 *Acetobacter* sp. CGMCC 8142 still achieved a 0.81 mol_{3-HP} mol_{1,3-PDO}⁻¹ yield (final titre:
850 47.8 g L⁻¹; productivity: 0.8 g L⁻¹ h⁻¹), while the yield of suspended cells was already below
851 0.60 mol_{3-HP} mol_{1,3-PDO}⁻¹ (final titre: 35.5 g L⁻¹; productivity: 0.6 g L⁻¹ h⁻¹) (J. Li et al., 2016).
852 In the Hann *et al.* study, molar yields are not given, but productivities are remarkably high: 23
853 g L⁻¹ h⁻¹ for the first batch and 7 g L⁻¹ h⁻¹ for the last one (Hann et al., 2003). These 3-HP
854 productivities are the highest ones reported so far for a single-step microbial process (Table
855 1). This can be attributed, at least partly, to the high biocatalyst density that was used: given
856 the data available in the paper, the cell load can be estimated around 19 g_{cdw} L⁻¹. In
857 comparison, J. Li *et al.* used a 5 g_{cdw} L⁻¹ cell load. Yu *et al.* reached a 36.9 g L⁻¹ h⁻¹
858 productivity, but this value accounts for 3-HP obtained after isolation and purification of the
859 production of the first 30 cycles. Interestingly, it was also shown that cell immobilisation
860 improved their tolerance to the substrate (1,3-PDO or 3-HPN). Like this, higher 3-HP titres

861 can be reached with immobilised cells, in comparison to suspended cells. For instance,
862 immobilized *Acetobacter* sp. CGMCC 8142 were able to produce 67.0 g L⁻¹ 3-HP from 70 g
863 L⁻¹ 1,3-PDO compared to 50.4 g L⁻¹ 3-HP in the case of suspended cells, which corresponds
864 to a 1.32-fold higher molar yield (J. Li et al., 2016). In the same manner, immobilized *E. coli*
865 cells reached a complete hydrolysis of 497 g L⁻¹ HPN within 24 h while free cells were not
866 able to cope with more than 320 g L⁻¹. Thus, cell immobilisation appears as a suitable solution
867 to one of the issues pointed out by Kumar *et al.* in 2013. Indeed, they identified that
868 metabolite toxicity (3-HPA and 3-HP in particular, but it also applies to substrates) is one of
869 the key issues to address before commercial 3-HP bioproduction can be considered. Another
870 advantage of using cell immobilisation techniques is that they facilitate subsequent
871 downstream processes. Indeed, the filtration step usually needed for cell removal, can be
872 skipped. In case of *in situ* product recovery, cell immobilisation could help reduce the toxic
873 effect of the extractant phase (see part 4). In order to allow a scale up of immobilisation
874 techniques, special attention should be paid to the bioreactor design, so that process
875 performances are not hindered by mass transfer limitations. The issue of mass transfer was
876 recently addressed by Zaushitsyna *et al.* who evaluated a novel immobilisation technique by
877 direct aggregation of recombinant *L. reuteri* cells for the conversion of glycerol into 3-HPA,
878 3-HP and 1,3-PDO (Zaushitsyna et al., 2017). Dry cell pellets were mixed with
879 glutaraldehyde, oxidized dextran or a mix of activated polyethyleneimine(PEI)/modified
880 polyvinyl alcohol (PVA) used as various crosslinkers. After freezing at -12°C for 3 days,
881 these cryogels were characterized in terms of structural and mechanical stability, elastic
882 strength, and porosity. Cell load and retaining, preservation of cell viability, and maintenance
883 of metabolic and catalytic activities were also assessed to compare impacts of crosslinkers.
884 On the basis of these results, the PEI/PVA (0.55/0.35 % w/v) mix was retained for glycerol
885 conversion. After packing into cylindrical glass column, embedded resting cells were used for
886 3-HP production performed in fed-batch mode. At a very low glycerol feeding rate of 3 mg h⁻¹
887 L⁻¹, 3.3 g L⁻¹ 3-HP were produced (productivity: 0.09 g L⁻¹ h⁻¹; molar yield: 0.48 mol_{3-HP}
888 mol_{glycerol}⁻¹) with 1,3-PDO as obligate co-product (Zaushitsyna et al., 2017). These results are
889 three times lower than that reported earlier for free cells (Dishisha et al., 2014). This was
890 explained by adverse conditions encountered by cells during cryostructuration process and
891 conversion step by itself. In spite of these low performances, this study offers interesting
892 perspectives in order to overcome mass transfer limitations in cell immobilisation processes.
893 Several improvements were suggested by the authors: limiting oxygen exposure during
894 cryogel preparation, increasing biocatalyst load, and optimising column packing (Zaushitsyna

895 et al., 2017).

896

897 **4 Downstream processes**

898

899 Final product recovery is a critical step for the commercialisation of bio-based compounds,
900 especially in the case of 3-HP. Several aspects make 3-HP recovery in high concentrations
901 and purity, especially challenging: (i) when produced by bioconversion with cells, 3-HP is
902 dissolved in a complex culture medium, at a relatively low concentration; (ii) being a
903 carboxylic acid of low molecular weight, it is very soluble in aqueous media; and (iii)
904 traditional distillation techniques are not effective because 3-HP decomposes at high
905 temperatures and easily polymerises at high concentrations (Hoppe et al., 2016). Because of
906 that, downstream steps can represent an important percentage of total production cost. For
907 carboxylic acids, this percentage can go from 20 % to 60 % (Hoppe et al., 2016; Straathof,
908 2011), so it is not surprising that 3-HP is commercially available as a 30 % aqueous solution
909 and at a relatively high price.

910

911 Attempts have been made in order to overcome these difficulties and decrease recovery and
912 purification costs. Quite complete reviews of carboxylic acid recovery are given by López-
913 Garzón and Straathof (2014) and Murali *et al.* (2017), where the main primary recovery
914 processes described are: adsorption, liquid-liquid extraction, salt precipitation, filtration, and
915 electrodialysis (López-Garzón and Straathof, 2014; Murali et al., 2017). Although the
916 selection of the best process varies in the literature because of the difference in properties of
917 carboxylic acids, *in situ* product recovery (ISPR) by extraction during bioconversion has been
918 the most popular approach. This strategy has been proven to improve productivity and yield
919 of several carboxylic acid production processes.

920

921 Until just recently, there were no studies in the literature focused on ISPR techniques for 3-
922 HP. However, it could be assumed that processes which proved to be effective for the
923 extraction of carboxylic acids of similar molecular structures may also work for 3-HP. For
924 example, 2-hydroxypropionic acid, also known as lactic acid, is a positional isomer of 3-HP.
925 The recovery of this molecule from fermentation broths has been widely studied, and the
926 ISPR approach has proved to enhance the productivity and yield of several lactic acid
927 production processes (Boonmee et al., 2016; Gao et al., 2009; Wasewar et al., 2004). Liquid-
928 liquid reactive extraction stands out from the other approaches studied, because it can

929 selectively remove acids from diluted solutions. Like this, 3-HP is continuously removed
930 during its production and is not accumulated in the fermentation broth. This should help
931 relieve the microorganisms from the acidic stress. This method is performed in two stages: the
932 first consists in solute extraction through an organic acid-base complex formation; and the
933 second stage is the regeneration of the extractant phase by stripping the solute from the
934 organic complex using another aqueous phase. This second step is also known as back
935 extraction (Datta et al., 2015)

936

937 Based on this, Moussa *et al.* made a comparative study of 3-HP and lactic acid behaviour
938 during reactive extraction (Moussa et al., 2015). They used different concentrations of tri-n-
939 octylamine (TOA) and tri-n-octylmethylammonium chloride (Aliquat 336) as extractants,
940 since they have exhibited good extraction performances on lactic acid (Wasewar et al., 2004;
941 Yankov et al., 2004). Both compounds were diluted in n-decanol. Solutions of 1 g L⁻¹ of each
942 acid were extracted with organic phases. pH of these solutions was 2.9 and 3.2 for lactic acid
943 and 3-HP, respectively. Lactic acid was better extracted than 3-HP for all extractant
944 concentrations. This was related to the stronger acidity of lactic acid (pKa = 3.86) compared
945 to that of 3-HP (pKa = 4.51), since the interaction between the acid and the extractant is
946 stronger (Tamada and King, 1990). However, results obtained for 3-HP were encouraging
947 with high distribution coefficients over a wide range of experimental conditions, including 3-
948 HP dissolved in real fermentation broth. In the latter case, the highest performances were
949 achieved using a mix of TOA (10 % vol/vol) and Aliquat 336 (10 % vol/vol): the extraction
950 yield reached 62 % and the selectivity for 3-HP was $\alpha_{3\text{-HP}} = 15.7 \%$ (Moussa et al., 2015).
951 Selectivity ($\alpha_{3\text{-HP}}$) was defined as the ratio of extracted 3-HP to the total amount of extracted
952 molecules (3-HP + 3-HPA + glycerol + 1,3-PDO).

953

954 Afterwards, Burgé *et al.* evaluated the reactive extraction of 3-HP on a hollow fibres
955 membrane contactor, using the organic phases compositions mentioned above (Burgé et al.,
956 2016). This experimental configuration displays advantages that make it a good candidate for
957 extractive fermentation: (i) dispersion between phases is avoided, as well as a subsequent
958 separation step between organic and aqueous phases; (ii) direct contact with microorganisms
959 is significantly reduced, thus enhancing the biocompatibility of the process; and (iii) hollow
960 fibre configuration offers a high interfacial area, which generally improves both the product
961 transfer to the extractant phase and the process compactness (Drioli et al., 2006; Gabelman
962 and Hwang, 1999). They obtained higher distribution coefficients (K_D) and yields in

963 comparison to reactive extraction of the same solutions in separatory funnels: $K_D = 3.05$
964 versus $K_D = 2.44$ and $Y = 75.3\%$ versus $Y = 71\%$ respectively, thus demonstrating the
965 potential of this configuration. Further optimisation studies were reported and consist of a
966 screening of solvents used as extractant phase, and of a detailed study of 3-HP extraction
967 mechanism. It has been found that tertiary amines like TOA, have a better extraction
968 performance when diluted in active solvents like long chain alcohols, since they act as proton
969 donors, which improves the acid-amine complex solvation in the organic phase (Chemarin et
970 al., 2017a). Also, mathematical models that take into account complexation-solvation
971 mechanisms of 3-HP extraction have been developed (Chemarin et al., 2017b). This approach
972 of process optimisation is a promising tool for its use on carboxylic acids extraction.

973
974 Biocompatibility is a key factor for ISPR systems: it is known that for reactive extraction,
975 finding a biocompatible organic phase with good extraction performance is challenging. For
976 example, although Aliquat 336 addition to the extractant phase improves the reaction
977 performance, it has been found to be very toxic to bacteria, even at low concentrations
978 (Kumar et al., 2012). Therefore, Aliquat 336 cannot be considered as a good candidate for
979 extractive bioconversion. Because of the promising results mentioned above, extraction with
980 TOA diluted in decanol, assisted by a hollow fibres membrane contactor, was evaluated for
981 the extraction of 3-HP produced by *Lactobacillus reuteri*. Unfortunately, the ISPR system had
982 a significant inhibition effect over 3-HP bioproduction, resulting in lower titres when
983 compared to bioconversion alone (Burgé et al., 2017). This was attributed to the high toxicity
984 of the organic phase, despite the use of the membrane contactor. This is why it is important to
985 find a compromise between extraction performance and the biocompatible ISPR process used.
986 Some good approaches might be (i) screening for more biocompatible solvents, and (ii)
987 changing the membrane material of the hollow fibres contactor, in order to reduce direct
988 contact of the bioconversion medium with the organic phase.

989
990 Currently, several patents describe purification processes applied to 3-HP (Abraham et al.,
991 2014; Hoppe et al., 2016; Meng et al., 2006; Tengler and DeCoster, 2013). They include an
992 initial clarification of the aqueous phase to remove cells and other solids of the bioconversion
993 medium, so they do not comprise ISPR. 3-HP primary recovery consists of different strategies
994 that combine classical separation processes like salt precipitation and liquid extraction with
995 solvents, and is followed by evaporation and/or distillation. The compositions resulting from
996 the methods used may deliver a highly pure 3-HP, from 70 % up to 99.5 %; and in

997 concentrations that vary from 30 % up to 95 % (weight/volume). However, reaching the
998 maximal concentration and purity represents high energetic costs and makes the economic
999 viability of the overall process questionable. This is why, obtaining an aqueous solution of 3-
1000 HP that already has both high purity and high concentration could significantly reduce the
1001 cost of further purification steps, and *in situ* reactive extraction may play an important role in
1002 delivering this objective. One of the most appealing advantages over traditional processes is
1003 that, with the adequate back extraction strategy, the product can be recovered in the form of
1004 the acid and not as a salt. Studies of other similar organic acids have shown that the
1005 temperature swing, diluent swing, and using a volatile base, such as trimethylamine, in the
1006 stripping phase, enables obtaining the acid form and could be an interesting approach for the
1007 recovery of 3-HP (Keshav and Wasewar, 2010). Further research is needed in order to
1008 integrate the constraints of both microbial production and downstream steps.

1009

1010 **5 Conclusions and perspectives**

1011

1012 In recent years important progress in 3-HP bioproduction has been made, thanks to ever-
1013 increasing research and industrial interest. Research efforts are mainly dedicated to the
1014 metabolic engineering of the various metabolic pathways and microbial hosts. Given the great
1015 variety of possible substrates, metabolic pathways, microbial agents, and process techniques,
1016 it can be expected that the future of 3-HP production is likely to be diverse. Recent studies
1017 have notably benefited from the development of synthetic biology tools, such as metabolite
1018 sensors, CRISPR-Cas systems for genetic editing, dynamic flux control, and omics and *in*
1019 *silico* techniques. Yet, research is now also focusing on process engineering and downstream
1020 processes. Various fermentation strategies are currently under investigation, including one-
1021 step fed-batch, electro-fermentation, multi-step and integrated processes. As for them,
1022 downstream processes are however mainly focused on *in situ* product recovery. These
1023 fermentation and extractive strategies form a complementary approach to metabolic
1024 engineering, towards sustainable and economically viable 3-HP production.

1025

1026 Industrial manufacturers' attention is essentially drawn by the possibility of converting 3-HP
1027 into acrylic acid in one step only. Indeed, acrylic acid is one of the most produced
1028 commodities and its production relies on petro-chemical processes. Bio-based acrylic acid
1029 production through 3-HP is a very promising strategy toward greener and less petrol-
1030 dependent processes. This is in any case the approach that was initiated by the industrial

1031 consortium composed of Novozymes and Cargill. Other companies, such as BASF and P&G,
1032 have recently been patenting catalytic methods for dehydrating 3-HP to acrylic acid, thus
1033 proving their interest for bio-based acrylic acid (Blaschke et al., 2016; Velasquez et al., 2017).
1034 However, it is still unknown to what extent bio-based acrylic acid production is less energy
1035 consuming and less greenhouse gas emitting in comparison to petro-chemically-based acrylic
1036 acid. Still, a study published in 2014 assessed the life-cycle fossil energy consumption and
1037 greenhouse gas emissions of acrylic acid produced from 3-HP in comparison to fossil-based
1038 acrylic acid (Adom et al., 2014). It was estimated that bio-based acrylic acid led to a 53 %
1039 reduction in greenhouse gas emissions and to 58 % less fossil fuel consumption. It can be
1040 expected in the coming years that the production of bio-based chemicals through
1041 biotechnological processes will continue to develop, encouraged by public policies. For
1042 instance, the European Commission has designated bio-based chemical production and
1043 bioprocesses as priority areas for investments (European Commission, 2014). However, when
1044 assessing the commercial feasibility of a new process, one has to take into account the risk of
1045 the investment, which mostly relies on the prices of raw materials. Given the low price of
1046 petrol and the volatility of substrate prices such as glycerol or glucose, it can be expected that
1047 bio-based 3-HP will not be competitive with fossil-based acrylic acid in the next years.
1048 Furthermore, because of the price volatility of carbon substrates it is difficult to make
1049 forecasts, thus investments in biotechnological processes remain risky. These limitations
1050 might hinder the commercial development of 3-HP in the coming years. Nevertheless, the
1051 company Verdant Bioproducts Limited built the first biorefinery plant aiming at producing 3-
1052 HP – among other commodities – from waste CO₂.

1053

1054 Despite this context, significant progress has been made in the production of 3-HP. Due to
1055 some of these improvements, the results of certain process indicators are getting closer to
1056 industrial relevance, notably in terms of final titres, the highest so far being 83.8 g L⁻¹. Since
1057 most studies are essentially proofs of concept for new recombinant strains, processes are not
1058 fully optimized and productivity in particular remains relatively low. In the report published
1059 in 2004 by the US DoE, it was estimated that a minimal productivity of 2.5 g L⁻¹ h⁻¹ is
1060 required for 3-HP production to be economically competitive. To this day and according to
1061 the scientific literature, this threshold has rarely been crossed. The progress made to date has
1062 been possible thanks to intensive efforts in genetic engineering. Physiology of the different
1063 producer strains now needs to be characterised in detail for processes to be further optimised.
1064 As such, modelling works that have already been carried out have been of great help for better

1065 understanding microbial metabolisms for 3-HP production. Process approaches, such as
1066 multi-stepping, are starting to be developed, which should enable improve performances.
1067 Immobilisation technologies may mark a step toward continuous process, and these are often
1068 preferred in industry due to their cost efficiency and their robustness. With the combination of
1069 genetic engineering and process approaches, some of the metabolic hurdles, such as toxicity
1070 of some products or redox limitations, are being overcome. Some approaches are however
1071 still in their infancy: electro-fermentation in particular offers exciting possibilities for
1072 enhancing 3-HP synthesis, but it still requires intensive research efforts before it can be scaled
1073 up.

1074
1075 Until now, 3-HP production was mostly attempted from glycerol or glucose as a carbon
1076 source. However, some recent studies also achieved 3-HP production from other carbon
1077 sources of interest, such as CO₂ and xylose, although performances are still low. It should
1078 however be noted that almost no attempt has been made at producing 3-HP from crude
1079 industrial by-products, even though it could be very interesting to develop in the context of
1080 biorefinery. Only a recent study focused on *L. reuteri*'s growth on wheat and sugar beet by-
1081 products (Couvreur et al., 2017). 3-HP production has not yet been attempted using crude
1082 glycerol: it is however an important issue to address before commercial production can be
1083 considered. Indeed, crude glycerol may contain some impurities that are toxic to cells. Finally,
1084 downstream processes for 3-HP extraction and purification will benefit from the experience
1085 gained with other organic acids, such as lactic acid in particular. However, the specificities of
1086 3-HP extraction are still to be addressed. Downstream processes hold a central place of the
1087 overall process because they are indispensable and they account for an important part of the
1088 total cost. In this respect, an integrated approach of *in situ* extraction coupled to the
1089 fermentation or bioconversion step is promising.

1090

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1092

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1097

1098 **Declaration of interest**

1099

1100 The authors declare that they have no competing interests.

1101 **References**

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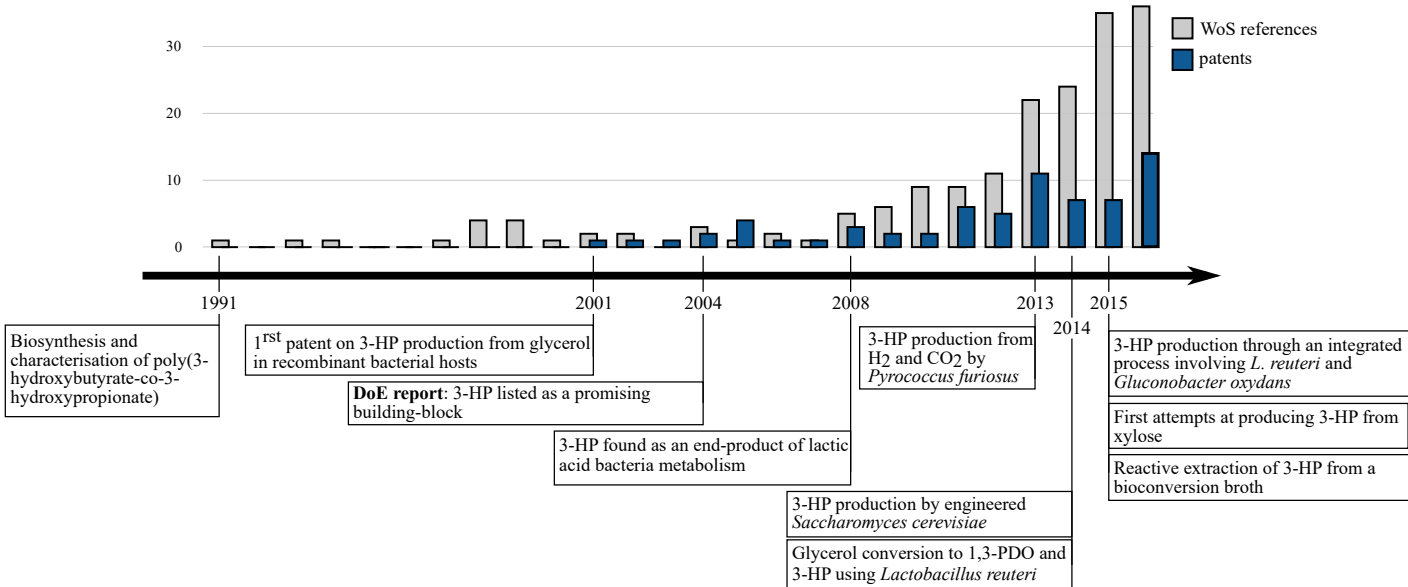
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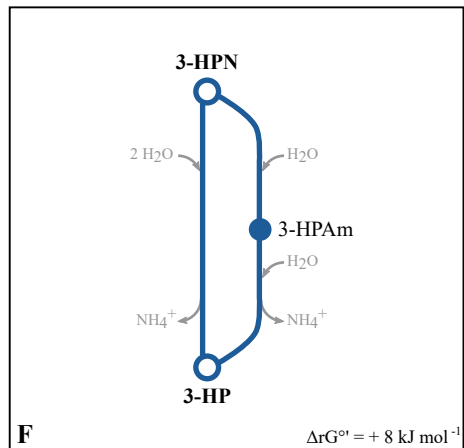
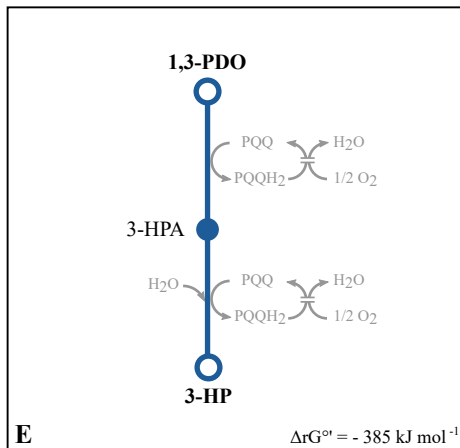
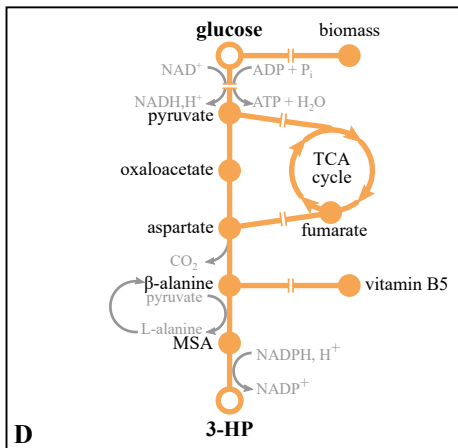
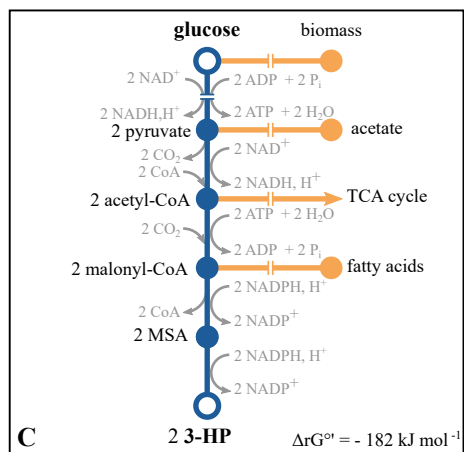
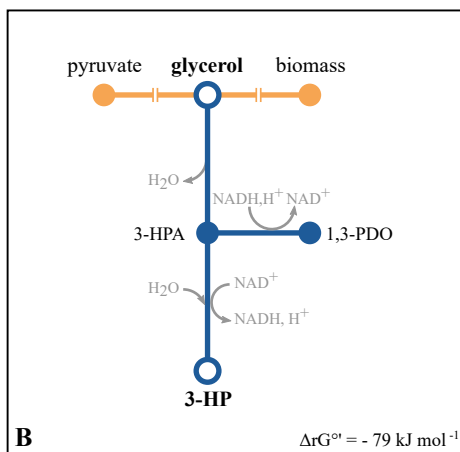
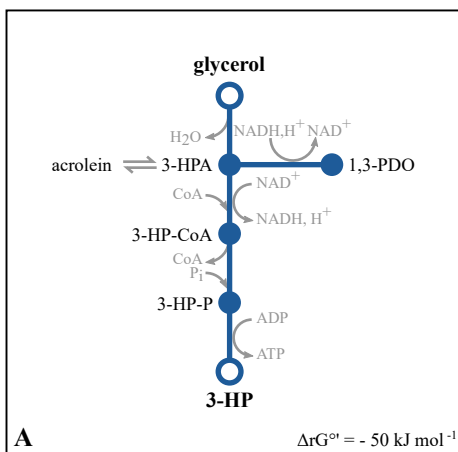
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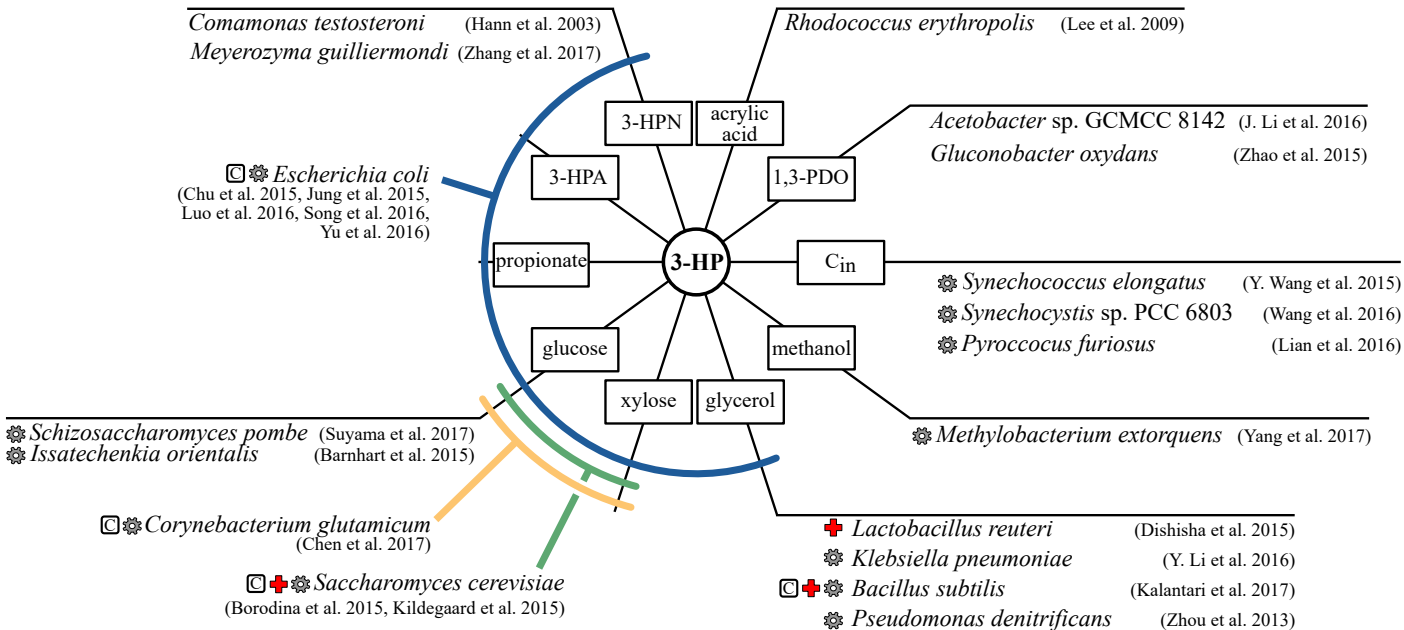
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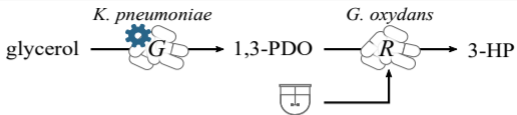




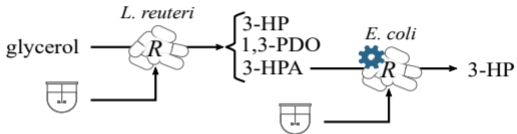
○ substrate / targeted product (3-HP) — one-step transformation \rightleftharpoons possibly enzymatic equilibrium
 ● intermediary or side product — multiple-step transformation



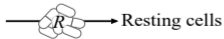
Zhao
et al., 2015




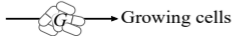
Sabet-Azad
et al., 2015



Dishisha
et al., 2015



 Genetically engineered strain




 Biomass production step

Figure 1.

Evolution over the years of the number of references on the Web of Science (WoS) and the number of patents concerning 3-hydroxypropionic acid bioproduction and overview of the key milestones.

Figure 2.

Main heterotrophic routes for 3-HP synthesis.

Reactions presented with stoichiometrics are displayed in blue. Reactions presented without stoichiometrics (only qualitatively) are displayed in orange. Due to the different possible variants of the β -alanine pathway, free energy release was not calculated for this pathway (see part 2.1.4).

- A. Glycerol oxidation through a CoA-dependent pathway
- B. Glycerol oxidation through a CoA-independent pathway
- C. Malonyl-CoA pathway
- D. β -Alanine pathway
- E. Aerobic 1,3-PDO oxidation
- F. 3-HPN hydrolysis

Abbreviations:

CoA: coenzyme A

P_i: inorganic phosphate (HPO_4^{2-})

3-HPA: 3-hydroxypropionaldehyde

3-HP-CoA: 3-hydroxypropionyl-coenzymeA

3-HP-P: 3-hydroxypropionyl-phosphate

1,3-PDO: 1,3-propanediol

MSA: malonic semialdehyde

TCA: tricarboxylic acid

3-HPN: 3-hydroxypropionitrile

3-HPAm: 3-hydroxypropionamide

Figure 3.

Microbial agents under investigation for 3-HP production and their carbon sources.

Genetically engineered species are indicated with a gear.

Abbreviations:

3-HPA: 3-hydroxypropionaldehyde

3-HPN: 3-hydroxypropionitrile

1,3-PDO: 1,3-propanediol

C_{in}: inorganic carbon

Figure 4.

Multi-step strategies for 3-hydroxypropionic acid (3-HP) production from glycerol.

Abbreviations:

1,3-PDO: 1,3-propanediol

3-HPA: 3-hydroxypropionaldehyde