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# 1 **Process engineering for microbial production of 3-hydroxypropionic acid**

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## 14 **Abstract**

15  
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 **Key-words**

32 3-hydroxypropionic acid, biosynthesis, bioconversion, fermentation, *in situ* product recovery,  
33 multi-step processes, metabolic routes  
34

## 35 **1 Introduction**

36

37 Since the advent of modern microbiology in the 19<sup>th</sup> 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 20<sup>th</sup> 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  $\beta$ -  
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**

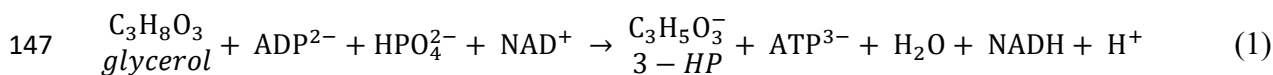
101

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 B<sub>12</sub>)-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; Mavrouniotis, 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 CO<sub>2</sub>.  
 157 Biomass production from glycerol also produces reducing equivalents. Glycerol already being  
 158 a very reduced carbon source (4.67 e<sup>-</sup>/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 B<sub>12</sub>-  
 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



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<sup>-1</sup> in  
206 shake-flasks, with a 0.80 mol<sub>c</sub> mol<sub>c</sub><sup>-1</sup> 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

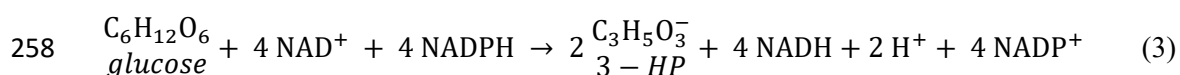
217 Among the various metabolic routes for 3-HP production from sugars, such as glucose, the  
218 malonyl-CoA pathway was the most studied until now (Table 1). The use of this pathway for  
219 3-HP biosynthesis was already extensively reviewed (Liu et al., 2017). Glucose conversion to  
220 3-HP through 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<sup>-1</sup>, 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  $\text{mol}^{-1}$  (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  $48.8 \text{ g L}^{-1}$ , with a  
 256 corresponding yield of  $0.53 \text{ molc molc}^{-1}$  (Lynch et al., 2011).

257



259

#### 260 **2.1.4 $\beta$ -alanine pathway**

261

262 Another strategy for 3-HP production from glucose was proposed in *E. coli* and *S. cerevisiae*,  
 263 through the  $\beta$ -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  $\beta$ -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  $\beta$ -alanine. In turn,  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -alanine pathway. They showed that in batch mode, the  $\beta$ -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  $\beta$ -alanine pathways were also compared in the case of 3-HP production from atmospheric  $\text{CO}_2$  in *Synechococcus elongatus* (Lan et al., 2015). In this case,  $\text{CO}_2$  was first assimilated into phosphoenolpyruvate, which subsequently underwent either one of this pathway. In this study, the 3-HP titre was lower through the  $\beta$ -alanine pathway than through the malonyl-CoA pathway:  $0.186 \text{ g L}^{-1}$  versus  $0.665 \text{ g L}^{-1}$ .

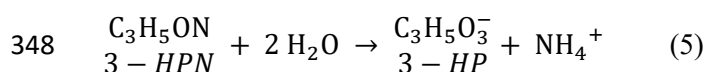
An alternative  $\beta$ -alanine pathway was developed by Song et al. They implemented a route where aspartate is obtained from fumarate instead of oxaloacetate (Fig. 2.D). They obtained higher performances than the oxaloacetate-based pathway. Their final engineered *E. coli* strains reached a  $31.1 \text{ g L}^{-1}$  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  $\beta$ -alanine pathways uses common metabolic intermediates implies that many side-products are synthesised along with 3-HP.  $\beta$ -Alanine is notably a precursor of vitamin B<sub>5</sub>. 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



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



350

### 350 **2.1.7 Autotrophic CO<sub>2</sub> 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 CO<sub>2</sub> 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 CO<sub>2</sub> 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). CO<sub>2</sub> 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  $\beta$ -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<sup>+</sup>. 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<sup>-1</sup> 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<sup>-1</sup>). 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* (Ansele 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
<b>Glycerol oxidation through a CoA-dependent pathway</b>						
<i>Lactobacillus reuteri</i>	glycerol	Fed-batch, 3 L bioreactor	14 g L <sup>-1</sup>	0.25 g L <sup>-1</sup> h <sup>-1</sup> <sup>b</sup>	0.49 mol <sub>C</sub> mol <sub>C</sub> <sup>-1</sup> <sup>b</sup>	(Dishi sha et al., 2015)
<b>Glycerol oxidation through a CoA-independent pathway</b>						
<i>Escherichia coli</i> <sup>a</sup>	glycerol + glucose	Fed-batch, 5 L bioreactor, vitamin B <sub>12</sub> addition, IPTG induction	71.9 g L <sup>-1</sup>	1.8 g L <sup>-1</sup> h <sup>-1</sup>	–	(Chu et al., 2015)
<i>Klebsiella pneumoniae</i> <sup>a</sup>	glycerol	Fed-batch, 5 L bioreactor, IPTG induction	83.8 g L <sup>-1</sup>	1.16 g L <sup>-1</sup> h <sup>-1</sup> <sup>b</sup>	0.60 mol <sub>C</sub> mol <sub>C</sub> <sup>-1</sup> <sup>b</sup>	(Y. Li et al., 2016)
<i>Corynebacterium glutamicum</i> <sup>a</sup>	xylose + glucose	Fed-batch, 5 L bioreactor, vitamin B <sub>12</sub> addition, IPTG induction	54.8 g L <sup>-1</sup>	0.76 g L <sup>-1</sup> h <sup>-1</sup> <sup>b</sup>	–	(Z. Chen et al., 2017)
<i>Corynebacterium glutamicum</i> <sup>a</sup>	glucose	Fed-batch, 5 L bioreactor, vitamin B <sub>12</sub> addition, IPTG induction	62.6 g L <sup>-1</sup>	0.87 g L <sup>-1</sup> h <sup>-1</sup> <sup>b</sup>	0.51 mol <sub>C</sub> mol <sub>C</sub> <sup>-1</sup>	(Z. Chen et al., 2017)
<b>Malonyl-CoA pathway</b>						
<i>Escherichia coli</i> <sup>a</sup>	glucose	Fed-batch, 1.8 L bioreactor, IPTG induction	48.4 g L <sup>-1</sup>	–	0.53 mol <sub>C</sub> mol <sub>C</sub> <sup>-1</sup>	(Lynch et al., 2011)
<i>Escherichia coli</i> <sup>a</sup>	glucose	Fed-batch, 5 L bioreactor, IPTG	40.6 g L <sup>-1</sup>	0.56 g L <sup>-1</sup> h <sup>-1</sup> <sup>b</sup>	0.19 mol <sub>C</sub> mol <sub>C</sub> <sup>-1</sup>	(C. Liu et

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

411 <sup>a</sup> Engineered microorganisms

412 <sup>b</sup> 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<sup>-1</sup> versus 7.4 g L<sup>-1</sup> 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<sup>-1</sup> was reached (productivity: 1.35 g L<sup>-1</sup> h<sup>-1</sup>; yield: 0.99 mol<sub>3-HP</sub>  
479 mol<sub>glycerol</sub>) (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  $\text{NAD}^+$  as  
490 electron acceptor.  $\text{NAD}^+$  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  $\text{B}_{12}$  synthesis (see part 2.3).  $\text{NAD}^+$  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  $\text{NAD}^+$ -independent aldehyde dehydrogenase  
495 from *Pseudomonas* sp. AIU 362 (Li et al., 2013). This enzyme uses  $\text{O}_2$  as electron acceptor,  
496 so this strategy does not fully resolve the contradiction with anaerobic vitamin  $\text{B}_{12}$  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<sup>-1</sup> (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 B<sub>12</sub> on their own. As explained above,  
573 vitamin B<sub>12</sub> 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 B<sub>12</sub> to the culture medium is required (Table 1). Using the vitamin  
578 B<sub>12</sub>-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 B<sub>12</sub>, 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 B<sub>12</sub> synthesis  
594 and NAD<sup>+</sup> regeneration. Indeed, maximum NAD<sup>+</sup> regeneration is reached under aerobic  
595 conditions, while maximum vitamin B<sub>12</sub> synthesis is achieved anaerobically. Using the  
596 vitamin B<sub>12</sub>-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 B<sub>12</sub> 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<sup>-1</sup> was reached, corresponding to a 0.67 mol<sub>3-HP</sub> mol<sup>-1</sup><sub>glycerol</sub> 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 CO<sub>2</sub>. 3-HP production was already achieved using inorganic carbon. The proof  
609 of concept was provided through an original approaches in archaea *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 CO<sub>2</sub> 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: CO<sub>2</sub> 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<sup>-1</sup> to 25 g L<sup>-1</sup> (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<sup>-1</sup> had detrimental effects on the growth of *K. pneumoniae*.  
661 Maximal tolerated concentrations were estimated at 110 g L<sup>-1</sup> and 133 g L<sup>-1</sup> 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 ( $q_{\text{Gly}}$ ) of 128.3 mg<sub>glycerol</sub> 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<sub>glycerol</sub> h<sup>-1</sup>, which is half the maximum rate given the dry weight of biocatalyst used in  
684 the experiment. This process achieved a 14 g L<sup>-1</sup> 3-HP titre with a 0.25 g L<sup>-1</sup> h<sup>-1</sup> 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 B<sub>12</sub> regeneration and aerobic NAD<sup>+</sup> 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<sup>+</sup> 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<sup>-1</sup> 3-HP titre, with a yield of 0.19 mol<sub>3-HP</sub> mol<sub>glycerol</sub><sup>-1</sup> (Kim et al., 2017). This proof of  
709 concept demonstrates that NAD<sup>+</sup> regeneration can be significantly improved in anaerobic  
710 conditions: it then becomes possible to achieve both high vitamin B<sub>12</sub> and NAD<sup>+</sup> 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<sup>-1</sup>. In these conditions, a maximal 3-HP titre of 20.7 g L<sup>-1</sup>

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  $0.24 \text{ mg L}^{-1}$ ) while 1,3-PDO production was slightly reduced  
737 (from  $0.14$  to  $0.10 \text{ mg L}^{-1}$ ). 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  $\text{NAD}^+$  regeneration while preserving glycerol dehydratase activity and vitamin  $\text{B}_{12}$   
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 B<sub>12</sub>.  
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<sup>-1</sup> 3-HP titre, with a 0.06 g L<sup>-1</sup> h<sup>-1</sup> 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<sup>+</sup> 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<sup>+</sup>-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<sup>-1</sup> 3-HP titre, corresponding to a 0.52 molc molc<sup>-1</sup> yield on glycerol and a  
811 0.94 molc molc<sup>-1</sup> 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<sup>-1</sup> (Dishisha *et al.*, 2015). This  
816 additional bioconversion step allows an increase in the maximal theoretical 3-HP yield to 1  
817 mol<sub>3-HP</sub> mol<sup>-1</sup><sub>glycerol</sub>, 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 60<sup>th</sup> batch with a molar yield ranging from 0.4 to 0.9 mol<sub>3-HP</sub>  
848 mol<sub>3-HPN</sub><sup>-1</sup>, depending on the immobilisation material (Yu et al., 2016). After 5 cycles,  
849 *Acetobacter* sp. CGMCC 8142 still achieved a 0.81 mol<sub>3-HP</sub> mol<sub>1,3-PDO</sub><sup>-1</sup> yield (final titre:  
850 47.8 g L<sup>-1</sup>; productivity: 0.8 g L<sup>-1</sup> h<sup>-1</sup>), while the yield of suspended cells was already below  
851 0.60 mol<sub>3-HP</sub> mol<sub>1,3-PDO</sub><sup>-1</sup> (final titre: 35.5 g L<sup>-1</sup>; productivity: 0.6 g L<sup>-1</sup> h<sup>-1</sup>) (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<sup>-1</sup> h<sup>-1</sup> for the first batch and 7 g L<sup>-1</sup> h<sup>-1</sup> 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<sub>cdw</sub> L<sup>-1</sup>. In  
857 comparison, J. Li *et al.* used a 5 g<sub>cdw</sub> L<sup>-1</sup> cell load. Yu *et al.* reached a 36.9 g L<sup>-1</sup> h<sup>-1</sup>  
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<sup>-1</sup> 3-HP from 70 g  
863 L<sup>-1</sup> 1,3-PDO compared to 50.4 g L<sup>-1</sup> 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<sup>-1</sup> HPN within 24 h while free cells were not  
866 able to cope with more than 320 g L<sup>-1</sup>. 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<sup>-1</sup>  
887 L<sup>-1</sup>, 3.3 g L<sup>-1</sup> 3-HP were produced (productivity: 0.09 g L<sup>-1</sup> h<sup>-1</sup>; molar yield: 0.48 mol<sub>3-HP</sub>  
888 mol<sub>glycerol</sub><sup>-1</sup>) 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<sup>-1</sup> 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 CO<sub>2</sub>.

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<sup>-1</sup>. 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<sup>-1</sup> h<sup>-1</sup> 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 CO<sub>2</sub> 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**

1102

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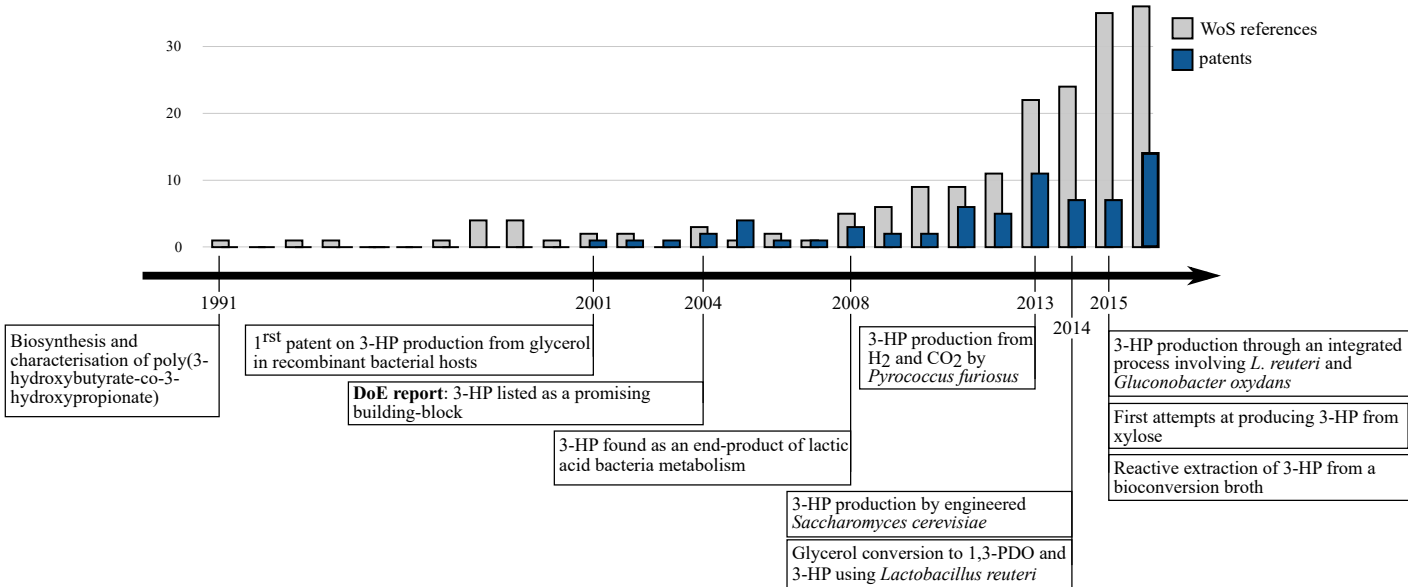
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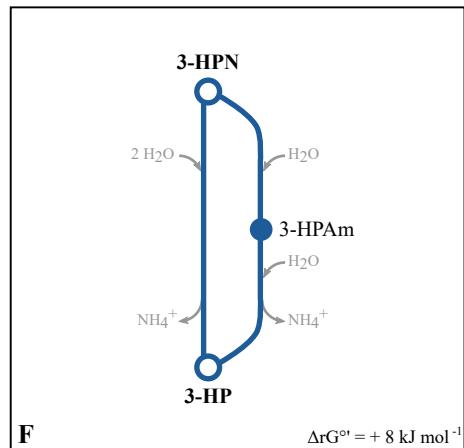
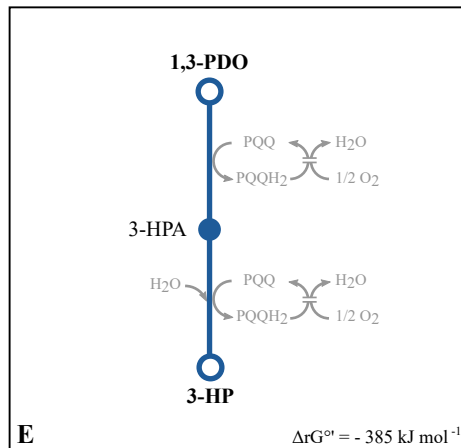
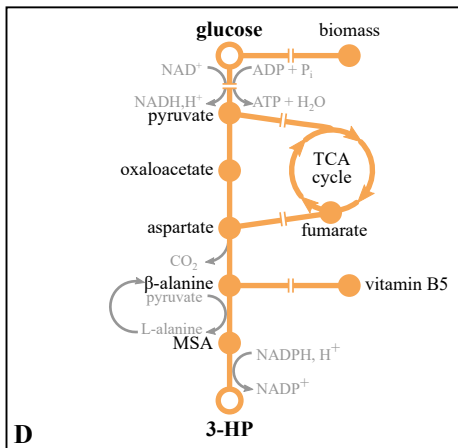
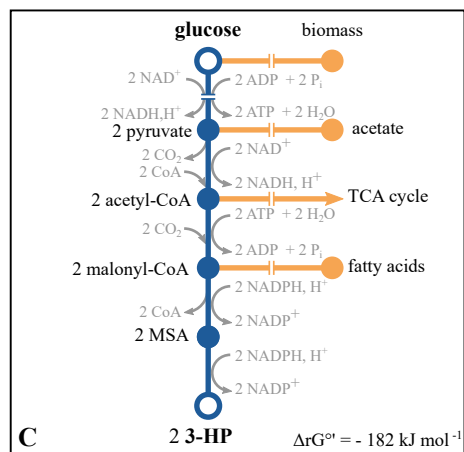
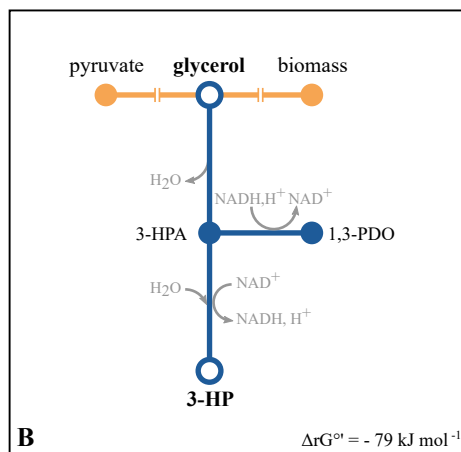
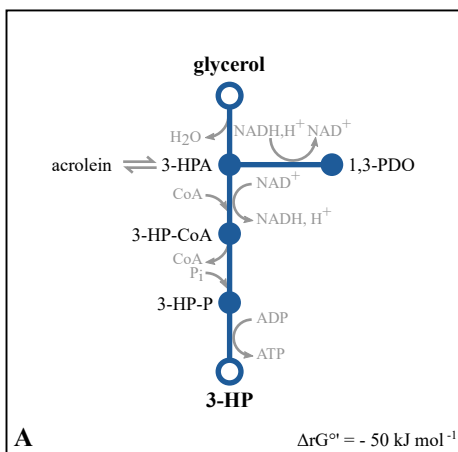
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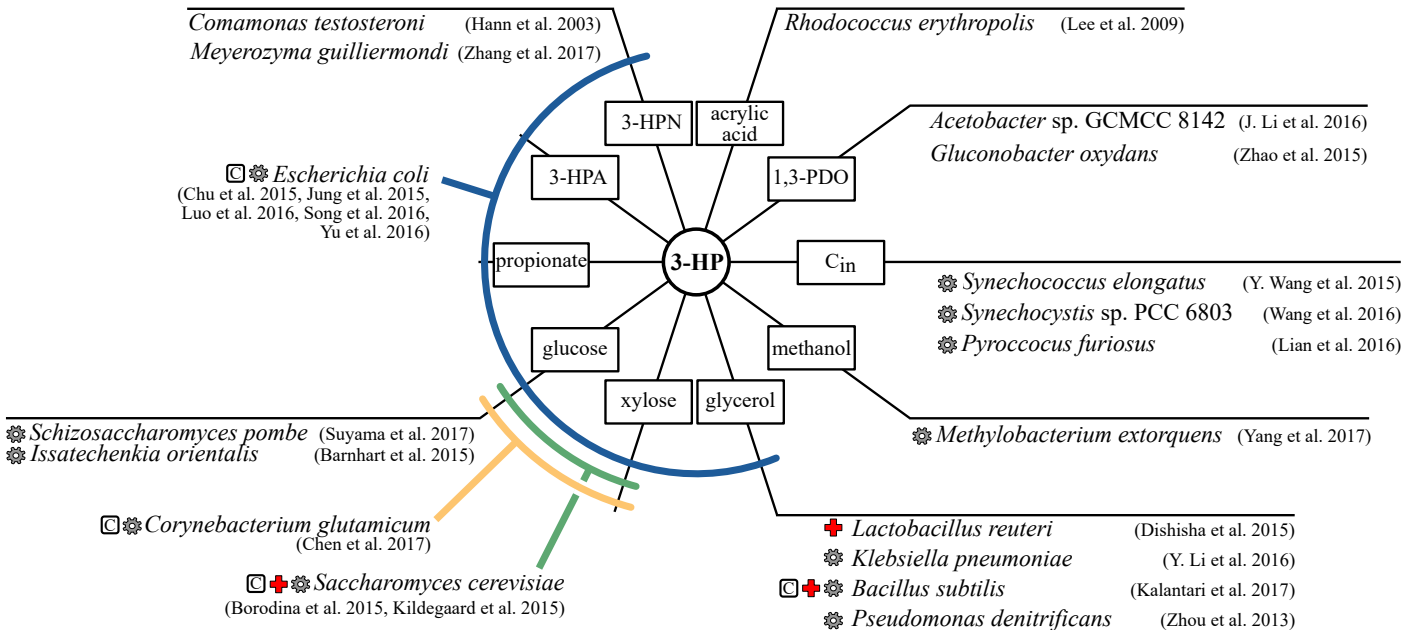
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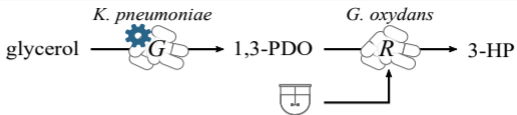




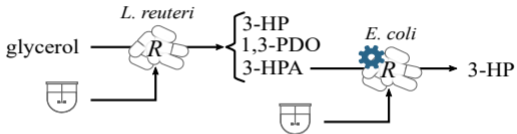
○ substrate / targeted product (3-HP)    — one-step transformation     $\rightleftharpoons$  possibly enzymatic equilibrium  
 ● intermediary or side product    — multiple-step transformation



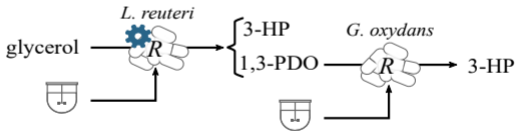
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*et al.*, 2015





Sabet-Azad  
*et al.*, 2015





Dishisha  
*et al.*, 2015



  $\rightarrow$  Resting cells

 Genetically engineered strain

  $\rightarrow$  Growing cells

 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  $\beta$ -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.  $\beta$ -Alanine pathway
- E. Aerobic 1,3-PDO oxidation
- F. 3-HPN hydrolysis

### Abbreviations:

CoA: coenzyme A

P<sub>i</sub>: inorganic phosphate ( $\text{HPO}_4^{2-}$ )

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<sub>in</sub>: 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