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Process engineering for microbial production of 3-hydroxypropionic acid

Florence de Fouchécour^a, Ana-Karen Sánchez-Castañeda^a, Claire Saulou-Bérion^a, Henry Éric Spinnler^{a*}

^a UMR 782 GMPA, AgroParisTech, INRA, Université Paris Saclay, 78850 Thiverval-Grignon, France

florence.de-salivet-de-fouchecour@inra.fr

ana-karen.sanchez@inra.fr

claire.saulou@agroparistech.fr

*corresponding author: spinnler@agroparistech.fr

Abstract

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

Key-words

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

1 Introduction

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

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

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

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

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

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

2.1 Metabolic pathways for 3-HP production

2.1.1 Glycerol oxidation through a coenzyme A-dependent pathway

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

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

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

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

2.1.2 Glycerol oxidation through a coenzyme A-independent pathway

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

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

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

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

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

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

2.1.3 Malonyl-coenzyme A pathway

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

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

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

2.1.4 β-alanine pathway

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

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

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

2.1.5 Aerobic 1,3-propanediol oxidation

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

2.1.6 3-hydroxypropionitrile hydrolysis

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

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

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

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

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

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

2.1.8 Other pathways

Another metabolic route, the propionyl-CoA pathway, was recently explored for 3-HP production from propionate in *E. coli* (Luo et al., 2016). Propionate is first converted to propionyl-CoA, which is subsequently oxidised into acryloyl-CoA using an equivalent of NADP⁺. Acryloyl-CoA is then hydrated to 3-HP-CoA, whose CoA residue is then transferred to acetate, thus leading to the production of one equivalent of 3-HP and acetyl-CoA. A maximum of 1.60 g L⁻¹ was obtained in shake flasks experiments (Luo et al., 2016). Interestingly, it was observed in the same study that in the absence of glucose, 3-HP production was low (0.06 g L⁻¹). Indeed, when propionate serves as sole energy and carbon source for bacterial growth, the flow through the propionyl-CoA pathway decreases. It was also suggested that the quantity of ATP produced from propionate, which is not a preferential energy source for *E. coli*, could not ensure the synthesis of both growth-related and heterologous proteins. 3-HP production was therefore further limited. Glucose addition could help compensate for this energy deficiency (Luo et al., 2016).

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

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

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

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

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

^a Engineered microorganisms

^b Value calculated from data given in the article

– Value could not be calculated from given data

2.2 Optimizing 3-HP production through metabolic engineering

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

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

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

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

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

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

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

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

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

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

2.3 Microbial agents for 3-HP production

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

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

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

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

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

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

testosteroni, for instance.

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

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

3.1 Operational strategies for 3-HP-producing fermentative processes

3.1.1 Fermentation mode

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

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

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

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

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

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

3.1.2 Process parameters

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

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

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

3.2 Multi-step and integrated approaches

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

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

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

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

et al., 2017).

4 Downstream processes

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

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

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

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

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

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

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

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

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

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

5 Conclusions and perspectives

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

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

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

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

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

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

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Declaration of interest

1099

1100 The authors declare that they have no competing interests.

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1614

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

Comamonas testosteroni (Hann et al. 2003)
Meyerozyma guilliermondi (Zhang et al. 2017)

Rhodococcus erythropolis (Lee et al. 2009)

☐⚙️ *Escherichia coli*
 (Chu et al. 2015, Jung et al. 2015,
 Luo et al. 2016, Song et al. 2016,
 Yu et al. 2016)

Acetobacter sp. GCMCC 8142 (J. Li et al. 2016)
Gluconobacter oxydans (Zhao et al. 2015)

⚙️ *Synechococcus elongatus* (Y. Wang et al. 2015)
 ⚙️ *Synechocystis* sp. PCC 6803 (Wang et al. 2016)
 ⚙️ *Pyrococcus furiosus* (Lian et al. 2016)

⚙️ *Methylobacterium extorquens* (Yang et al. 2017)

⚙️ *Schizosaccharomyces pombe* (Suyama et al. 2017)
 ⚙️ *Issatechenkia orientalis* (Barnhart et al. 2015)

☐⚙️ *Corynebacterium glutamicum*
 (Chen et al. 2017)

☐⚙️⚡ *Saccharomyces cerevisiae*
 (Borodina et al. 2015, Kildegaard et al. 2015)

⚡ *Lactobacillus reuteri* (Dishisha et al. 2015)

⚙️ *Klebsiella pneumoniae* (Y. Li et al. 2016)

☐⚡⚙️ *Bacillus subtilis* (Kalantari et al. 2017)

⚙️ *Pseudomonas denitrificans* (Zhou et al. 2013)

Resting cells

Genetically engineered strain

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 β -alanine pathway, free energy release was not calculated for this pathway (see part 2.1.4).

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

Abbreviations:

CoA: coenzyme A

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

3-HPA: 3-hydroxypropionaldehyde

3-HP-CoA: 3-hydroxypropionyl-coenzymeA

3-HP-P: 3-hydroxypropionyl-phosphate

1,3-PDO: 1,3-propanediol

MSA: malonic semialdehyde

TCA: tricarboxylic acid

3-HPN: 3-hydroxypropionitrile

3-HPAm: 3-hydroxypropionamide

Figure 3.

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

Genetically engineered species are indicated with a gear.

Abbreviations:

3-HPA: 3-hydroxypropionaldehyde

3-HPN: 3-hydroxypropionitrile

1,3-PDO: 1,3-propanediol

C_{in}: inorganic carbon

Figure 4.

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

Abbreviations:

1,3-PDO: 1,3-propanediol

3-HPA: 3-hydroxypropionaldehyde