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# Microbial Propionic Acid Production

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**Abstract:** Propionic acid (propionate) is a commercially valuable carboxylic acid produced through microbial fermentation. Propionic acid is mainly used in the food industry but has recently found applications in the cosmetic, plastics and pharmaceutical industries. Propionate can be produced via various metabolic pathways, which can be classified into three major groups: fermentative pathways, biosynthetic pathways, and amino acid catabolic pathways. The current review provides an in-depth description of the major metabolic routes for propionate production from an energy optimization perspective. Biological propionate production is limited by high downstream purification costs which can be addressed if the target yield, productivity and titre can be achieved. Genome shuffling combined with high throughput omics and metabolic engineering is providing new opportunities, and biological propionate production is likely to enter the market in the not so distant future. In order to realise the full potential of metabolic engineering and heterologous expression, however, a greater understanding of metabolic capabilities of the native producers, the fittest producers, is required.

**Keywords:** propionic acid; propionibacteria; fermentation

## 1. Introduction

Propionic acid is an FDA approved, generally regarded as safe (GRAS) three-carbon chemical with applications in a wide variety of industries. Propionate is primarily used for its antimicrobial properties with major markets as a food preservative or herbicide [1]. The antimicrobial properties of propionate result in its increasing use in construction and cleaning products as sodium, calcium or potassium salt. Propionate salts are effective suppressors of mould growth on surfaces and, when combined with lactic and acetic acids, can inhibit the growth of *Listeria monocytogenes* [2]. In the plastic industry, it is used in the manufacture of cellulose derived plastics such as textiles, membranes for reverse osmosis, air filters, and as a component of lacquer formulations and moulding plastics [3]. In the pharmaceutical industry, sodium propionate is used primarily in animal therapy for the treatment of wound infections and as a component of conjunctivitis and anti-arthritic drugs [4]. In the cosmetics industry, propionate salts are used as perfume bases together with butyl rubber to improve the consistency and shelf life of products. Propionate finds additional application as a flavour enhancer in the form of citronellyl or geranyl propionate.

Global production of propionic acid is estimated at ~450,000 tonnes per year with a 2.7% annual growth [5] and a price ranging between $2–$3 USD/kg. Four manufacturers supply 90% of the global propionate market: BASF covers approximately 31% of the market with plants in Germany and China; The Dow Chemical

Company supplies 25% of the global market with production in the USA; Eastman Chemical provides 20% of the market with production in the USA; and Perstorp in Sweden supplies 14% of the global market. At present, propionate is industrially synthesized by petrochemical processes, predominantly through the Reppe process, which converts ethylene, carbon monoxide and steam into propionate, and the Larson process, which converts ethanol and carbon monoxide into propionate in the presence of boron trifluoride. Other less common synthesis techniques include oxidation of propionaldehyde, the Fischer-Tropsch process and pyrolysis of wood.

However, recent market needs demand biological propionic acid biosynthesis as a sustainable alternative [6]. First described by Albert Fitz in 1878 [7], *Propionibacterium* species can ferment sugars into propionic acid as their main fermentation product. Later on, Swick and Wood [8] described the set of reactions involved in the process of propionate production currently known as the Wood-Werkman cycle (Figure 1(AIII)). After many years of development, the gap between production costs of propionate via petrochemical processes and by fermentation by propionibacteria is narrowing [6,9]. Fermentation economics of low-value products such as propionate depend on the ability to convert carbon sources at a high yield and productivity to a high titre. Recent advances in metabolic engineering and fermentation have significantly improved the economic viability of the propionic acid fermentation process, however, separation of the various organic acids produced alongside propionic acid remains a problem. This problem is compounded by the fact that most product specifications for propionic acid require >99% purity which is reasonably achievable through the petrochemical manufacturing routes, but very challenging to achieve biologically. To overcome the downstream purification challenges, improvement of the biocatalyst remains the most viable and economical option. As a result of advances in systems and synthetic biology, new strains are being developed that meet the fermentation yield target while maintaining a high productivity; hereby overcoming one of, if not the, most technically challenging hurdles to commercialization of bio-propionic acid synthesis [10].

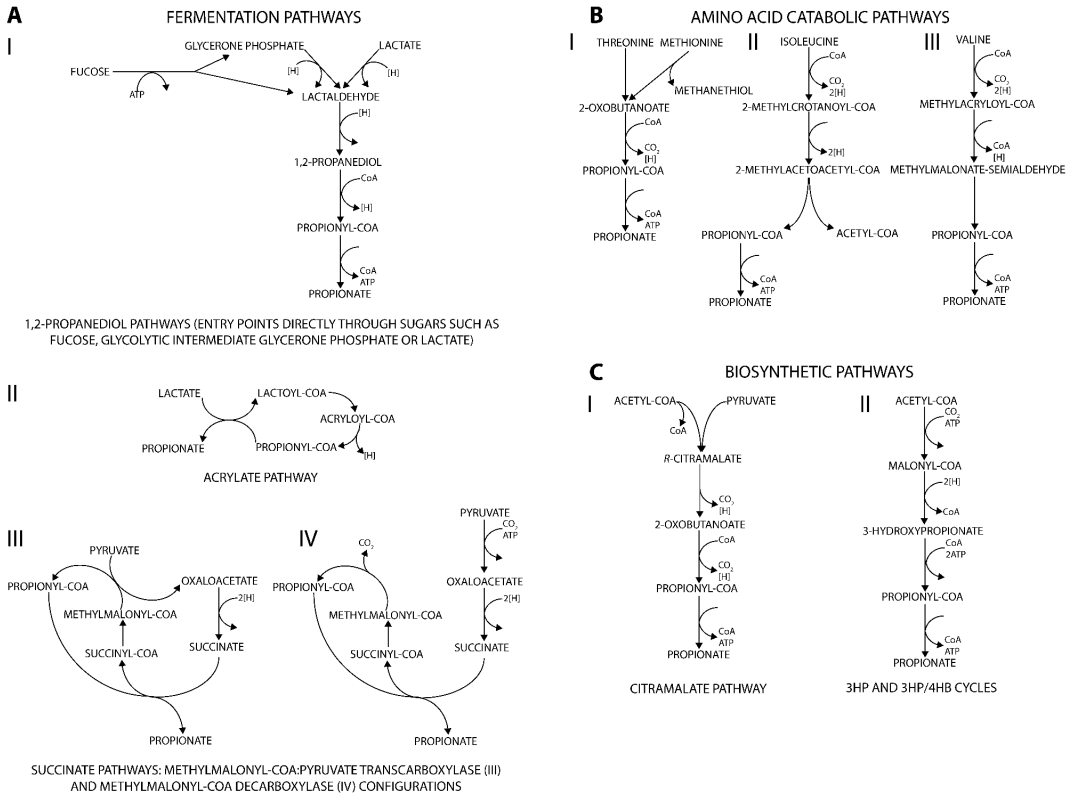


**Figure 1.** Metabolic pathways allowing the synthesis of propionate. **A**: Fermentation pathways (I-propanediol pathways, II-acrylate pathway, III-Wood-Werkman cycle, IV-sodium-pumping); **B**: Amino acid catabolic pathways (I-threonine and methionine catabolism, II-isoleucine catabolism, III-valine catabolism); **C**: Biosynthetic pathways (I-citramalate pathway, II-3-hydroxypropanoate and 3-hydroxypropanoate/4-hydroxybutanoate pathways).

In this review, we look at the current stage, progress and perspective of propionic acid biosynthesis, in particular at the improvements over the past decade as a result of the omics revolution. We present an in-depth analysis of different metabolic pathways for propionic acid production from an energy optimization perspective. This contribution provides a clear path to numerous development opportunities, including metabolic engineering of current native producers as well as future heterologous production via a deep understanding of propionibacteria metabolism. We demonstrate that *Propionibacterium* is the best natural producer of propionate and remains the most suitable candidate for industrial-scale production.

## 2. Overview of Developments in the Fermentation Process

The fermentation process for propionate production has been studied for more than 150 years (Table 1). In recent years, many studies have aimed at improving the fermentation process as a means of improving process economics by enhancing the productivity, yield and final titre.

Native propionate producers, propionibacteria, have been the primary candidates for the development of a biological process due to their unique metabolism. As will be subsequently shown, the Wood-Werkman cycle is energetically the most efficient propionate fermentation route currently known. Propionibacteria are Gram-positive, non-motile, non-spore forming, rod-like, facultative anaerobic bacteria. Dairy species, especially *P. acidipropionici* strains, have been explored as potential propionic acid producers due to their tendency to produce propionate at a higher titre and yield [9]. However, even the best performing native producers are limited by low growth rates and correspondingly low productivities, acid stress and, most importantly, the co-generation of by-products which increase downstream processing costs and limit the economic feasibility of the fermentation process. The benchmark for an economically feasible fermentation process by 2020 from sugar has been set at a yield of 0.6 g/g (1.52 mol propionate/mol glucose) and a productivity of 1 g/L/h [9]. Various fermentation technologies including batch, fed-batch, continuous fermentation and immobilised cells to prevent product inhibition [11–15] have been explored, but still fermentations fall short on simultaneously meeting both criteria (Table 2). While a single study simultaneously met both requirements, an exceptionally high initial biomass of ~56g/L was used and a low final propionate titre of 34.5 g/L was achieved [15]. For economic feasibility, the final titre should be closer to 100 g/L, and the requirement for the high initial biomass may present additional economic hurdles. Anaerobic processes are preferred for the production of propionate as they do not require additional aeration, thereby reducing the costs related to pneumatic power and fermenter scale-up. While biomass immobilisation has been used as a leading strategy to overcome acid stress limitations as previously reviewed [16], the use of these reactors is yet to be realised on an industrial scale due to two key challenges. Firstly, it is necessary to adapt the reactors from a three-dimensional system into a two-dimensional system, which would limit the amount of biomass within the reactors. Secondly, the cleaning process presents a major challenge. In addition, most of the immobilization techniques also increase cost as there is a membrane/cloth/gel bead that wears out after a few fermentation cycles.

**Table 1.** Overall historical development of the propionic acid fermentation process.

| Year | Event |
| --- | --- |
| 1854 | Adolph Strecker observed the formation of propionic acid from sugar in a mixture of calcium carbonate-sugar [1]. |
| 1861–1879 | Pasteur showed that fermentation occurs due to the activity of microbes. |
| 1878 | Fist work on propionic acid production by Propionibacteria. Albert Fitz predicted that 3 moles of lactic acid would lead to the production of 2 moles of propionic acid, 1 mole acetic acid, 1 mole $CO_2$ and 1 mole $H_2O$ [7]. |
| 1906 | 11 species of propionibacteria were identified as propionic acid producers during cheese making [1]. |

**Table 1.** *Cont.*

| Year | Event |
|---|---|
| 1928 | First mention of glycerol as carbon source for propionic acid production [1]. |
| 1937 | First complete study on propionibacteria metabolism during propionic acid fermentation by Wood [1]. |
| 1949 | A complete review of the factors affecting propionic acid fermentation was published [1]. |
| 1920–1953 | 17 patents for propionic acid production by different *Propionibacterium* strains were approved [1]. |
| 1961 | Immobilized cells are first used to reach higher production yields [1]. |
| 1962 | The Wayman process was developed. It consisted of a continuous system with immobilised cells of *P. acidipropionici* [1]. |
| 1960–2010 | Selection of overproducer strains and new production strategies. |
| 2011–2013 | Complete genome of *P. shermanii* [17] and *P. acidipropionici* [18] were sequenced and published. |
| 2013–2014 | Techno economic studies suggest the fermentation of sugar to propionate can be profitable if productivity reaches 1–2 g/L/h, yield reaches 0.6 g/g and final titre reaches ≈100 g/L [9,19]. |

Improving fermentation yield is the most critical step to achieve an economically viable fermentation, given productivity is readily improved by densifying the inoculation medium or by cell recycle [9]. The maximum theoretical yield of 0.7 g propionate/g glucose (1.71 mol/mol) can be achieved through the Wood-Werkman cycle if reduced cofactors are supplemented through an alternative pathway, such as the redirection of carbon from glycolysis to the pentose phosphate pathway, making an apparent yield of 0.6 g propionate/g glucose in complex media achievable. One technique to improve yield is to restrict biomass production [20]. This has been done by restricting nutrient availability [21] and by reducing the pH which also favours propionate production over acetate [22,23].

**Table 2.** Comparison of some propionic acid (PA) fermentation approaches from the literature dating 2010 to 2015.

| Strain | Fermentation Approach | Substrate (s) | Titre (g/L) | PA Yield (g/g) | Productivity (g/L/h) | References |
|---|---|---|---|---|---|---|
| *P. acidipropionici* | Batch | Glucose/Glycerol | 22 | 0.57 | 0.152 | [24] |
| | Fed-batch | Glucose/Glycerol | 30 | 0.54 | 0.152 | [24] |
| | Sequential batch | Glucose | 35 | 0.62 | 1.28 | [15] |
| | Fed-batch | Glucose | 56 | 0.43 | 2.23 | [15] |
| | Continuous | Lactose | 19 | 0.4 | 0.9 | [25] |
| | Fed-batch | Glucose | 71 | - | - | [26] |
| | Fed-batch | Glycerol | 48 | 0.59 | 0.2 | [27] |
| | Batch | Glucose | 45 | 0.45 | 2 | [6] |
| | Batch | Corn mash | 24 | 0.6 | 0.5 | [9] |
| | Sequential batch (with cell recycle) | Glycerol | 27 | 0.78 | 0.22 | [28] |
| | Fed-batch | Xylose | 53 | 0.35 | 0.23 | [29] |
| | Fed-batch | Corncob molasses | 72 | - | 0.28 | [29] |
| | PEI-Poraver bioreactor (Continuous) | Glycerol | 14 | 0.86 | 1.4 | [30] |
| | Fibrous-bed bioreactor (Fed-batch) | Glucose | 51 | 0.43 | 0.71 | [31] |
| | Fibrous-bed bioreactor (Fed-batch) | Sugarcane bagasse hydrolysate | 59 | 0.37 | 0.38 | [31] |
| *P. shermanii* | Fibrous-bed bioreactor (Repeated-batch) | Glucose/Glycerol | 75 | 0.57 | 0.25 | [32] |

**Table 2.** *Cont.*

| Strain | Fermentation Approach | Substrate (s) | Titre (g/L) | PA Yield (g/g) | Productivity (g/L/h) | References |
|---|---|---|---|---|---|---|
| *P. freudenreichii* | Multi-point fibrous-bed bioreactor (Fed-batch) | Glucose | 67 | 0.43 | 0.14 | [23] |
| | Plant fibrous-bed bioreactor (Fed-batch) | Hydrolysed cane molasses | 92 | 0.46 | 0.36 | [33] |
| | Plant fibrous-bed bioreactor (Fed-batch) | Hydrolysate of cane molasses & waste *Propionibacterium* cells | 80 | 0.4 | 0.26 | [33] |

In these cases, productivity typically suffers and increased stress lowers final titres. Recently, carbon dioxide sparging was identified as a viable alternative, where propionate yield was improved without loss of productivity and a higher titre of both propionate and biomass was obtained through increased substrate catabolism (unpublished data [34]). Inoculating at a higher cell density can also improve yield while enhancing productivity, achieving yields of up to 0.62 g propionate/g glucose [9]. The use of alternative carbon sources has also been explored. Glycerol has been studied in particular, as it has a similar redox state to propionate and consequently can achieve much higher maximum theoretical yields (Table 2). Given the relatively high cost of media compared to propionate, a greater emphasis has been placed on finding cheaper feedstocks. From this perspective, a fermentation using enzymatically treated corn mash has achieved the yield target of 0.6 g propionate/g glucose, where additional nutrients in the mash are responsible for the increase in apparent yield [9].

While progress has been made in optimizing fermentation conditions, Table 2 demonstrates few sugar fermentations are able to exceed the yield targets, particularly in an industrially scalable fermentation. This has resulted in an increasing reliance on genetic engineering to further enhance fermentation yields and restrict by-product fermentation to improve economics of the process.

## 3. Biological Propionic Acid Biosynthesis

Propionic acid is a metabolic by-product of many organisms, ranging from bacteria to humans, although few organisms produce it as a primary fermentation product (Table 3). Metabolic pathways leading to the production of propionic acid can be classified into three classes. The primary fermentation pathways catabolize different carbon sources to propionate and include the well-known acrylate and Wood-Werkman cycle pathways of native propionate producers (Figure 1A). Catabolic pathways can degrade a number of amino acids to propionic acid (Figure 1B). Finally, anabolic pathways associated with the production of biomass precursors from pyruvate or carbon dioxide can be harnessed to produce propionate (Figure 1C). Redox balancing is the main limitation to achieving higher yields from glucose. More favourable yields can be achieved by using more reduced substrates such as glycerol with a similar degree of reduction to propionate, although these are not likely to be as economically favourable as sugars [6]. We have therefore calculated all pathway yields referenced to glucose catabolism.

Pathways leading to propionate are typically linked to substrate level phosphorylation via the promiscuous activity of enzymes associated with acetate metabolism and can act as either electron sinks or sources. Because glycolysis results in the net production of reduced cofactors, pathways that act as electron sinks achieve more favourable maximum yields. In this work, we have analysed the feasibility of propionate production with a focus on energetic optimality; studying two separate scenarios. The first is the maximal propionate yield achievable while metabolism operates to capture as much potential energy in the form of ATP as possible. Energy maximisation is consistent with the evolutionary drive to maximise growth, which can approximate the behavior of microorganisms [35]. The second scenario is the maximum energy yield when propionate is produced at the maximum theoretical yield. This represents maximum energy available to the organism for growth and stress tolerance when metabolism is artificially perturbed away from optimality, such as by gene

overexpression and deletion, to achieve maximum propionate yield. All calculations are performed under anaerobic conditions because this is the most practical approach for propionate production, in terms of both process costs and the ability to growth-couple propionate production; a requirement given the high target yield. An underlying model of central carbon metabolic pathways common to *E. coli* (glycolysis, pentose phosphate pathway, acetate and ethanol metabolism and the dicarboxylic branch of the tricarboxylic acid cycle (TCA) leading to succinate production) was used. For simplicity, all reduced cofactors are treated as NADH. An optimal pathway will not only have a high maximum theoretical yield for propionate, but a high energetic yield; driving the organism to produce propionate as a primary fermentation product while providing sufficient energy to overcome the inhibitory effects of propionate accumulation. We also compare our in silico calculations to performances obtained by these pathways in vivo where data are available.

### *3.1. Fermentation Routes for Propionate Production*

Propionate, as a primary fermentation product, is produced via pathways that contain 1,2-propanediol (PDO) as an intermediate, the acryloyl-CoA pathway and the methylmalonyl-CoA or succinate pathways. Compared to the amino acid degradation and biosynthetic pathways (see Sections 3.2 and 3.3), fermentative pathways provide energy and help consume reduced cofactors that result from the catabolism of sugars. Both their role in energy generation and maintaining a redox balance permits these pathways to be growth coupled.

**Table 3.** Microbial species able to generate propionic acid during fermentation.

| Microorganism | Substrates | Products | Pathway |
|---|---|---|---|
| *Propionibacteria acidipropionici* *P. freudenreichii* [1] *P. shermanii* [2] | Glucose, sucrose, lactate, glycerol | Propionate, acetate, succinate, $CO_2$ | Wood-Werkman cycle (Figure 1(AIII)) |
| *Clostridia propionicum* | Glycerol, lactate, alanine, serine, threonine | Propionate, succinate, formate, acetate, n-propanol | Acrylate pathway (Figure 1(AII)) |
| *Bacteroides fragilis B. ruminicola* | Glucose | Acetate, lactate propionate, succinate, formate, $CO_2$ | Succinate pathway (Figure 1(AIV)) |
| *Veillonella parvula V. alcalescens* | Lactate, succinate | Propionate, acetate, $CO_2$, $H_2$ | Succinate pathway (Figure 1(AIV)) |
| *Propionigenum modestum* | Succinate | Propionate, $CO_2$ | Succinate pathway (Figure 1(AIV)) |
| *Selenomonas ruminantium* *S. sputigena* | Lactate Glucose | Propionate, lactate, acetate, $CO_2$ | Succinate pathway (Figure 1(AIV)) |
| *Megasphaera elsdenii* | Lactate | Acetate, propionate, butyrate | Acrylate pathway (Figure 1(AII)) |
| *Salmonella typhimurium* | Deoxy sugars, glucose, 1,2-propanediol | 1,2-propanediol, propanol, propionate, acetate, formate, lactate, $CO_2$ | 1,2-propanediol pathway (Figure 1(AI)) |

[1] *P. freudenreichii subsp. Freudenreichii;* [2] *P. freudenreichii subsp. Shermanii.*

#### 3.1.1. 1,2-propanediol Associated Pathways

Propionic acid production occurs in the rumen as a net result of the microbial consortia consisting of PDO fermenters and PDO consumers. Some organisms including *Salmonella typhimurium* [36] and *Roseburia inulinivorans* [21] are known to perform both processes, demonstrating a novel fermentative pathway for propionate production. PDO can be generated from the catabolism of deoxy sugars via lactate or from the glycolytic intermediate glycerone phosphate (DHAP) [37]. PDO is catabolized stoichiometrically to propionate yielding one ATP and one reduced cofactor by the combined actions of diol dehydratase and two promiscuous enzymes commonly associated with acetate metabolism; the CoA-dependent aldehyde dehydrogenase phosphotransacylase and acetate kinase (Figure 1(AI)). Table 4 contains the yield calculations for each pathway. While the biological production of PDO is an area of active research, its further conversion to propionic acid production has not been explored because of the higher value of PDO, despite the fact that the conversion of PDO to propionate is energetically favourable. Because of the liberation of an additional reduced cofactor through PDO catabolism; the maximum molar yield of propionate from glucose is 30% higher; accompanied by

a 70% increase in ATP generated as compared to PDO. In the case of the lactate pathway, this additional energy increases the net ATP yield from glucose to 4 and growth couples the production of propionate (Table 4). While the lactate pathway appears extremely promising, almost all reported strategies to produce PDO to date utilise the less efficient DHAP pathway [38]. This focus on the DHAP pathway is due to a lack of biochemical evidence to support the existence of the lactate pathway [39] which has been postulated to exist in just a single source [40]. The high cost for deoxy sugars also limits their potential application since the cheapest sells for over $300/kg [41]. Still, promising progress has been made constructing an artificial pathway for the conversion of lactate to propanediol in *E. coli* which consumes 1 ATP equivalent [39,42]. While this strategy could benefit similarly if PDO were subsequently degraded to propionate, propanediol production from glucose in an engineered mutant only just exceeded 1 g/L [39].

**Table 4.** Estimated yields for propionate and ATP production in propanediol fermentative pathways.

| PDO pathway | Maximum Yields (mol/mol Glc) | | Expected Yields (mol/mol Glc) | |
|---|---|---|---|---|
| | PA | ATP | PA | ATP |
| Deoxy sugar [1] | 1 | 2.5 | 1 | 3 [2] |
| DHAP | 1.71 | 0 | 0 | 3 |
| Lactate | 1.71 | 3.43 | 1.33 | 4 |
| Engineered lactate pathway | 1.71 | 0 [3] | 1 | 3 |
| All | 1.71 | 3.43 | 0 | 3 |

[1] This pathway utilises fucose instead of glucose; [2] Without the pyruvate formate lyase, the pathway is expected to be active producing 1 propionate and 2.5 ATP and also conferring a phenotypic advantage. [3] Based on a cost of 2 ATP consumed to reassimilate acetate, 1.71 ATP can be extracted if the reassimilation cost is reduced to 1 ATP.

### 3.1.2. Acrylate Pathway

The acrylate pathway enables ATP neutral conversion of lactate to propionate with the consumption of NADH. The pathway is found in several distantly related bacteria including *Clostridium propionicum*, *Megasphaera elsdenii* and *Prevotella ruminicola*. Though a variety of substrates can be catabolised to propionate and acetate, including lactate, serine, alanine and ethanol, the fermentation of glucose does not appear to result in the production of propionate in any native producer, presumably because glucose fermentation does not trigger expression of the lactate racemase required to initiate the cycle [43].

The metabolic advantage of the acrylate cycle is not immediately clear since a pyruvate formate lyase (PFL) is present in *C. propionicum*. The combination of these metabolic functionalities enables the energetically equivalent production of either propionate or ethanol with acetate in a 1:1 molar ratio. However, it does enable the consumption of acrylate to propionate [44]. Propionate production could be growth coupled to glucose catabolism if strains were engineered to utilise this pathway. In the presence of glucose, net yields of 3 ATP/glucose and about 0.4 g propionate/g glucose (1 mol propionate/mol glucose) could be achieved, while higher yields can be achieved through the use of the pentose phosphate pathway with a concomitant energetic penalty (Table 5, Table 6). While this pathway has been successfully engineered into *E. coli* [45], initial yields were 2 orders of magnitude lower than the 0.4 g/g that would be expected.

**Table 5.** Maximum propionate yield analysis of all metabolic pathways leading to propionate production.

| Products | Pathway Yields (mol/mol Glc) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Catabolic Pathways | | | Biosynthetic Pathways | | Fermentation Routes | | | | Overall [1] |
| | Val/Iso | Thr | Met | Citramalate | 3HP/4HB | Propanediol | Acrylate | $Na^+$ Pumping | Wood-Werkman | |
| ATP | 2.29 | 0 | 1 | 2.4 | 0 | 3.43 | 1.71 | 2.57 | 3.43 | 3.43 |
| Propionate | 0.29 | 1.33 | 1 | 0.4 | 1.33 | 1.71 | 1.71 | 1.71 | 1.71 | 1.71 |
| Acetate | 0 | 0.67 | 1 | 0 | 0.67 | 0 | 0 | 0 | 0 | 0 |
| Ethanol | 1.43 | 0 | 0 | 1.2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Formate | 1.43 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| $CO_2$ | 0.86 | 0.67 | 0 | 0.4 | 0.67 | 0.86 | 0.86 | 0.86 | 0.86 | 0.86 |

[1] To test whether synergistic interactions could occur between pathways to improve propionate production, all glucose catabolising pathways leading to propionate production were allowed to carry flux, although performance did not improve over the Wood-Werkman cycle.

**Table 6.** Maximum energy yield analysis of all metabolic pathways leading to propionate production.

| Products | Pathway Yields (mol/mol Glc) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Catabolic Pathways | | | Biosynthetic Pathways | | Fermentation Routes | | | | Overall [1] |
| | Val/Iso | Thr | Met | Citramalate | 3HP/4HB | Propanediol | Acrylate | $Na^+$ Pumping | Wood-Werkman | |
| ATP | 3 | 3 | 3 | 3 | 3 | 4 | 3 | 3.25 [2] | 4 | 4 |
| Propionate | 0 | 0 | 0 | 0 | 0 | 1.33 | 1 | 1 [2] | 1.33 | 1.33 |
| Acetate | 1 | 1 | 1 | 1 | 1 | 0.67 | 1 | 1 [2] | 0.67 | 0.67 |
| Ethanol | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Formate | 2 | 2 | 2 | 2 | 2 | 0 | 1 | 1 [2] | 0 | 0 |
| $CO_2$ | 0 | 0 | 0 | 0 | 0 | 0 | 0.67 | 0 [2] | 0 | 0.67 |

[1] To test whether synergistic interactions could occur between pathways to improve energy production, all glucose catabolising pathways leading to propionate production were allowed to carry flux, although performance did not improve over the Wood-Werkman cycle. [2] In the absence of a pyruvate formate lyase, propionate is expected to be produced in a 2:1 ratio with acetate giving a yield of 1.33 propionate, 0.67 acetate and $CO_2$ and 3 ATP per glucose.

#### 3.1.3. Succinate Pathway

The catabolism of pyruvate to succinate via the dicarboxylic branch of the TCA cycle offers an alternative electron sink to ethanol. While an ATP is typically consumed or lost to fix carbon dioxide and pyruvate or phosphoenolpyruvate into oxaloacetate, this is at least partially compensated by an anaerobic electron transport chain consisting of the NADH dehydrogenase and fumarate reductase. In the absence of the PFL, the cell suffers an energetic penalty; less energy is gained through substrate level phosphorylation via production of succinate and acetate in a 2:1 ratio as compared to the dissimilation of glucose to acetate and ethanol in a 1:1 ratio. Some organisms have evolved energy conservation strategies that allow this fermentation strategy to equal or even better the energy available via the mixed acid fermentation strategy by the further decarboxylation of succinate to propionate. Two separate mechanisms have evolved to facilitate this; the sodium pumping methylmalonyl-CoA decarboxylase (Figure 1(AIV)) and the methylmalonyl-CoA:pyruvate transcarboxylase (Figure 1(AIII)).

The sodium pumping pathway, found in organisms such as *Propionigenium modestum*, couples the decarboxylation of methylmalonyl-CoA derived from succinate to propionyl-CoA with the pumping of two sodium ions across the cell membrane [46]. The mechanism of this reaction is likely to be identical to the well-studied oxaloacetate decarboxylase [47] and is probably linked to the consumption of a periplasmic proton [48], leading to a net energy gain of roughly 0.25 ATP.

While a modest conservation of energy, the 2:1 production of propionate to acetate becomes energetically equivalent to the mixed acid fermentation mode yielding 3 ATP/glucose; although this pathway is typically associated with *Veilonella* and *Parvula* species which do not catabolise sugars. Further energy can be extracted in the presence of the PFL enabling the production of 3.25 ATP/glucose and 1:1 production of propionate and acetate.

The second pathway, the Wood-Werkman cycle, is found predominantly in *Propionibacterium* and produces propionate in a similar way to the sodium pumping pathway, except the decarboxylation step is replaced by the methylmalonyl-CoA:pyruvate transcarboxylase which transfers a carboxyl group from methylmalonyl-CoA to pyruvate to generate propionyl-CoA in an ATP-independent manner (Figure 1(AIII)).

As opposed to the sodium pumping pathway, this bypasses the loss of ATP required to fix carbon dioxide to oxaloacetate and therefore conserves an entire ATP. This additional energy growth couples the 2:1 production of propionate and acetate, regardless of the presence of the PFL, while enabling the generation of 4 ATP per glucose.

While both of these pathways can improve energy yields from catabolising glucose and favour propionate production, the Wood-Werkman cycle is far superior to any other pathway in terms of its ability to promote propionate production corresponding to the metabolic objective of energy maximisation (Table 5, Table 6). Only the hypothetical lactate pathway for PDO production combined with PDO catabolism could equal the Wood-Werkman cycle in terms of energetic efficiency. Given propionibacteria both contain the Wood-Werkman cycle and naturally ferment sugars to achieve high propionate yields; it is of little surprise that they have been the focus of the bulk of the effort to design and scale a biological process for the industrial-scale synthesis of propionate.

### *3.2. Degradation of Amino Acids to Produce Propionate*

The degradation of valine, threonine, isoleucine and methionine (Figure 1(BI-III)) can lead to the production of propionate and ATP via propionyl-CoA. As such, fermentations using complex media can result in the production of propionate in many organisms. However, the low market value of propionate compared to amino acids is restrictive. Alternatively, since pathways for the synthesis and subsequent catabolism of amino acids are present in a broad range of microorganisms, the combination of amino acid anabolic and catabolic pathways can be used to produce propionate from glucose. For example, Table 7 shows the metabolic costs of the synthesis, degradation, and combination of the two pathways for the production of 1 mole of amino acid from pyruvate and subsequent degradation to 1 mole of propionate. The maximum theoretical yields for propionic acid production from amino acid fermentations are shown in Table 8. In all cases, the synthesis and subsequent degradation of amino acids result in a net reduction in the energy yield from glucose. Therefore, these pathways are inconsistent with the metabolic objective of maximizing energy for growth and, consequently, rely on the over-expression of each enzyme.

**Table 7.** Theoretical molar yields [1] for propionic acid production using via amino acids synthesis and degradation.

| Amino Acid | Valine | | | | Isoleucine | | | | Threonine | | | | Methionine | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Substrate** | **Pyr** | **ATP** | **NADH** | **NADPH** | **Pyr** | **ATP** | **NADH** | **NADPH** | **Pyr** | **ATP** | **NADH** | **NADPH** | **Pyr** | **ATP** | **NADH** | **NADPH** |
| Degradation | 0 | 1 | 5 | 0 | 0 | 2 | 3 | 1 | 0 | 1 | 0 | 1(0) | 0 | 1 | 0 | 1(0) |
| Biosynthesis | −2 | 0 | −1 | −1 | −3 | 0 | 1 | −2(−1) | −1 | −3 | −1 | −3 | −2 | −2 | −1 | −2(−1) |
| Combined | −2 | 1 | 4 | −1 | −2 | 1 | 4 | −1(0) | −1 | −2 | −1 | −1(−2) | −2 | −1 | −1 | −1 |

[1] Calculations were performed by lumping ferredoxin and FADH2 with NADPH and NADH, respectively. Values in parenthesis indicate when formate is produced through the pyruvate formate lyase. These calculations consider the costs of regenerating all substrates of the metabolic pathways.

**Table 8.** Estimated yields for propionic acid production via degradation of amino acids.

| Amino Acid Pathway | Maximum Yields (mol/mol Glc) | | Expected Yields [1] (mol/mol Glc) | |
|---|---|---|---|---|
| | **PA** | **ATP** | **PA** | **ATP** |
| Valine/Isoleucine | 0.29 | 2.29 | 0 | 3 |
| Threonine | 1.33 | 0 | 0 | 3 |
| Methionine | 1 [2] | 1 [2] | 0 | 3 |
| All | 1.45 | 0 | 0 | 3 |

[1] Expected yields assume cells operate to maximise energy. [2] The yield for propionate production through the methionine pathway allows a stoichiometric yield of 1.125 propionate and 0 ATP per glucose to be obtained if acetate produced by methionine degradation is allowed to be re-consumed to pyruvate. If the energetic cost of this step can be reduced to 1 ATP, the pathway will perform similarly to the threonine pathway.

The high redox generation associated with propionate production via branched chain amino acids ultimately limits the yield by requiring the generation of an oxidized product such as ethanol. In these cases, targeting propanol instead can improve the maximum theoretical yields by 33%. While both methionine and threonine have a net consumption of reduced cofactors which can be balanced through the use of the pentose phosphate pathway, the threonine pathway is both more energetically efficient and much shorter, making it the most feasible pathway for propionate production. Threonine production and catabolism have been explored extensively in *E. coli* as a possible source of propionyl-CoA for the production of various chemicals including propanol [49,50], erythromycin (6-DB) [51] and 3-hydroxyvalerate [52]. *E. coli* has been engineered to co-produce propanol and butanol previously using just threonine synthesis and catabolism with some success. The final concentration reached was 1 g/L of propanol (apparent yield of 0.09 g/g glucose, 29% of the maximum theoretical yield for the citramalate pathway) in a semi-defined medium [53]. Also, the combination of the valine/isoleucine and threonine pathways are synergistic given the redox and energy balances and can, together, slightly improve the propionate yield.

### *3.3. Biosynthetic Routes via Propionyl-CoA*

Three pathways associated with anabolic metabolism that lead to the synthesis of propionyl-CoA have been explored: the citramalate pathway (Figure 1(CI)) associated with isoleucine biosynthesis, the 3-hydroxypropanoate (3HP) cycle and the 3-hydroxypropanoate/4-hydroxybutanoate (4HB) cycles related to carbon fixation (Figure 1(CII)).

#### 3.3.1. Citramalate Pathway

Many organisms contain the citramalate pathway, which condenses pyruvate and acetyl-CoA to generate 2-oxobutanoate. This compound is a precursor for isoleucine biosynthesis and is also an intermediate of methionine and threonine degradation pathways to propionate. The direct synthesis of 2-oxobutanoate from pyruvate results in the net production of just one reduced cofactor when the PFL is used to generate acetyl-CoA. Overall this pathway can yield 0.4 mol propionate per mol glucose and 2.4 ATP, which is again less than the 3 ATP that would otherwise be extracted from glucose through central carbon metabolism. The potential for the citramalate pathway to supply propionyl-CoA for the production of its derivatives has been analyzed for three separate products. When propanol was targeted, a yield of 0.11 g propanol/g glucose (~0.28 mol/mol) has been achieved [31], about half of the maximum theoretical yield for the citramalate pathway. Additionally, it has been utilised for the production of erythromycin [51] and determined to be the most significant source of propionyl-CoA in the native production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in *Haloferax mediterranei* [54]. The synergistic interaction of the threonine and citramalate pathways improves the maximal propionate yield to 1.6 mol propionate per mol glucose without ATP production; this interaction was observed experimentally when combining these pathways further improved propanol yield to 0.15 g/g glucose in a semi-defined medium [53].

#### 3.3.2. 3HP/4HB Cycles

The 3HP and 4HB cycles are particularly attractive pathways for the production of propionate due to their capacity to fix carbon dioxide as a sole carbon source. These pathways have been identified in the phototrophic *Chloroflexacae* and Archaea, respectively. Both cycles enable carbon fixation for biomass generation through an acetyl-CoA/propionyl-CoA carboxylase [55] and only differ in the final steps; the 3HP cycle fixes carbon dioxide to glyoxylate whereas the 4HB cycle generates acetyl-CoA. Because the shared initial steps of the pathways enable acetyl-CoA to be converted to propionyl-CoA, it could be possible to source propionate from acetyl-CoA resulting from glucose dissimilation (Figure 1(CII)). Indeed, the 3HP cycle has been reconstituted successfully in *E. coli* in sub-pathways including that for the production of propionyl-CoA [56], but to date there are no reports in the literature attempting to utilise this pathway for the production of propionic acid. A key limitation of this strategy is the high ATP requirements of these reactions; consuming a net two ATP and two NADPH per acetyl-CoA consumed, resulting in a maximum theoretical yield of 1.33 mol propionate/mol glucose with no ATP generation (Table 5).

Although energy demanding and highly reducing, these cycles may offer new opportunities to explore the production of propionate from carbon dioxide as a substrate. The 3HP pathway is likely the second-greatest contributor to propionyl-CoA production for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in *Haloferax mediterranei* [54]. The production of propionate through the 4HB cycle is possible with one and a half turns of the cycle; leading to the overall consumption of 8 ATP and 7 reducing equivalents (8 NADPH consumed, 1 NADH generated). Natively, the 4HB pathway operates in aerobic conditions to supply the high ATP requirements and depends on hydrogen gas to feed the reducing equivalents [55]. The 3HP cycle is slightly more energetically demanding; leading to a net consumption of 9 ATP and 7 reducing equivalents (8 NADPH consumed and one quinol produced) in the production of propionate. Despite this, the properties of organisms that contain this pathway are even more unusual, such as the phototrophic *Chloroflexus aurantiacus* that performs anoxygenic photosynthesis with hydrogen or hydrogen sulfide as electron donors [57].

## 4. Genetic Engineering to Overcome the Current Challenges for Propionate Production

Targeted genetic engineering of propionibacteria remains challenging for several reasons: propionibacteria have a high GC content, which complicates genetic manipulation and contributes to poor gene annotations [58]; relatively few closed genomes are available [17,18]; a small number of cloning vectors are available [22,59,60]; the ability of strains to readily develop spontaneous antibiotic resistance; thick cell walls; and the presence of strong restriction modification systems which contribute to the low transformation efficiency of propionibacteria [61]. A number of recent studies have reported the modification of *P. freudenreichii* subsp. *shermanii* (*P. shermanii*) and *P. jensenii* while only a couple of contentious studies have reported modification of the high-producing, genetic modification resistant strain, *P. acidipropionici* (Table 9). The expression of methylation components of restriction modification systems in host organisms has resulted in large improvements in the transformation efficiencies of non-model organisms [62,63] and may be a critical step to improving the transformation efficiency in propionibacteria, particularly *P. acidipropionici*. Despite promising progress in the rational design of *P. shermanii* and *P. jensenii*, these modified strains still fall short of the natively high-producing *P. acidipropionici*. Therefore, a second line of research has focussed on random-mutagenesis strategies to enhance propionic acid production in *P. acidipropionici*.

**Table 9.** Genetic engineering strategies performed in propionibacteria to improve propionic acid production [1].

| Aim | Strategy | Strain | Results | Reference |
|---|---|---|---|---|
| Decrease by-products | Genome editing | *P. acidipropionici* ACK-Tet strain | Acetate production reduced ~14%. ~13% improvement of propionate production. | [64] |
| | Genome editing, overexpression | *P. jensenii poxB* or *ldh* knock-out and *ppc* overexpression | Maximum 30% improvement in titre and 24% improvement productivity | [65] |
| Improve acid tolerance | Overexpression | *P. acidipropionici otsA* overexpression strain | Propionic acid yield 11% higher. | [66] |
| | Overexpression | *P. jensenii strains* overexpressing *gadB*, *arcA*, *arc*, *gdh* or *ybaS* | Up to a 1.5-fold increase in yield and 5.4-fold increase in titre, in shake flasks | [67] |
| Increase of metabolic flux towards propionate production | Overexpression | *P. shermanii CoAT* overexpression strain | Increase yield and productivity, maximum 10% and 46%, respectively. | [68] |
| | Overexpression of heterologous enzymes from *P. acidipropionici* | *P. shermanii* overexpressing *mmc*, *pyc* or *mmd* | Strongest phenotype observed with *mmc* overexpressing strain with 14% increase in yield from glucose and 17% increase in productivity from glucose/glycerol co-fermentation. Performed in serum bottles. | [69] |
| | Overexpression of heterologous enzymes from *E. coli* | *P. shermanii* overexpressing *ppc* strain | Improved productivity on glycerol only, no improvement in yield. | [70] |
| | Overexpression of heterologous enzymes from *E. coli* and *Klebsiella pneumoniae* | *P. jensenii* co-expression of *gdh* and *mdh* | Increase in propionate synthesis, but slow growth of the mutant strain. | [71] |

[1] Results correspond to experiments performed in reactors unless otherwise stated.

### *4.1. Empirical Strain Design*

Non-rational engineering approaches, such as random mutagenesis and genome shuffling have been extensively used to optimise *P. acidipropionici* strains [6,9,64,72–74]. Propionate production serves as an electron sink and generates energy through oxidative phosphorylation through the Wood-Werkman cycle, thus from a metabolic engineering perspective it is growth coupled. One option is relying on high-throughput HPLC to identify high producers [6]. Alternatively, growth rate may be used to identify mutants that are more acid tolerant and exhibit improved propionate yields [10,75,76].

Genome-shuffling has by far been the most successful empirical approach. Developed in the late 1970s, genome shuffling is routinely used to improve strains in industry. Compared to classical strain improvement methods such as chemical or UV mutagenesis, genome shuffling accelerates the evolutionary process by using multiple genotypes to provide an initial pool of genetic diversity, which can be refined for genomes that display desirable and diverse phenotypes. Genome shuffling combines the advantages of multi-parental crossing facilitated by DNA exchange, thus allowing the incorporation of foreign DNA [77]. Recursive genomic recombination combines classical breeding (asexual recursive mutagenesis), DNA shuffling (sexual recursive recombination) and screening for the desired phenotype and provides a feasible strategy to improve strains.

Genome shuffling in propionibacteria has been used to improve vitamin B12 production in *P. shermanii* [78] and propionic acid in *P. acidipropionici* [71,75,76]. Recently, multiple propionibacteria species were genome shuffled resulting in a strain which achieved a yield of 0.55 g propionate/g glucose [75,76]. Next-generation sequencing was used to analyse recombination events and identify novel/unique regions from each strain leading to the improved phenotype, including changes linked to acid tolerance mechanisms and possibly to a new transcriptional mechanism through mutation in ribosomal RNAs. Further rounds of genome-shuffling produced a strain that exceeded the 0.6 g propionate/g glucose and 1 g/L/h yield and productivity targets for an economically viable fermentation [10,76].

### *4.2. Rational Strain Engineering*

Research into the rational design of propionibacteria metabolism to enhance propionic acid production remains in its infancy. Necessarily, the strategies implemented to date have been relatively simple; in fact, the first study to co-express two genes in propionibacteria was published in 2015 [79]. Recently, genome-scale models for a number of closed *Propionibacterium* genomes [58] were developed and these were used to design rational engineering strategies in *P. shermanii* [34]. Two strategies were explored to enhance propionate production. The first relied on increasing the availability of reduced cofactors by overexpressing the pentose phosphate pathway to favour propionate production, where a 4-fold improvement in the propionate to acetate ratio was observed at the end of the exponential growth phase. The second strategy introduced an alternative, high-energy linear pathway for propionate production that includes the phosphoenolpyruvate carboxykinase and the sodium pumping methylmalonyl-CoA decarboxylase [34]. This work demonstrated the power of genome-scale models to rationally design propionibacteria metabolism. However, further development of genetic engineering tools is required before more complicated strategies such as those proposed in our work [34] can be tested experimentally or in the higher-producing *P. acidipropionici* strains.

Though modest in scale, genetic engineering of propionibacteria have covered gene knockouts, gene overexpression as well as the expression of heterologous genes (Table 9).

### *4.3. Gene Knockouts*

The first report on *Propionibacterium* genetic engineering aimed to construct a strain unable to produce acetate by knockout of the acetate kinase gene (*ack*) in *P. acidipropionici.* [64]. The *ack* gene interrupted by a tetracycline resistance cassette was electroporated into the wild-type strain of *P. acidipropionici* and ACK-Tet mutant strain was obtained by homologous recombination. While this work set the basis for molecular engineering, the subsequent release of the genome sequence [18]

showed that the *ack* gene was not present in the genome. After careful review of the publication, it is evident that no validation of the knockout was performed, results were not statistically significant and acetate production was only reduced ~14% in the mutant strain compared to the wild-type strain.

Subsequently, homologous recombination was performed for gene inactivation in *P. acidipropionici*, targeting trehalose 6-phosphate synthase (*otsA*) and maltooligosyl trehalose synthase (*treY*) [66]. Single knockout strains Δ*otsA* and Δ*treY*, and a double knockout strain Δ*otsA*Δ*treY* were constructed.

The authors concluded that the OtsA-OtsB pathway is the major route for trehalose synthesis under acid stress in *P. acidipropionici* and overexpressed this pathway to improve propionate production; however, no significant change in the production of propionate or other fermentation end products was observed. Again, no appropriate validation of these knockouts was performed and results should be considered dubious with apparent yields of intracellular trehalose reported with no consideration for extracellular trehalose titre, which we estimate accounts for ~90% of the total trehalose (unpublished data). It has been claimed that Wild type *P. acidipropionici* can produce up to 27 g/L of extracellular trehalose [80] leading one to believe that the relatively small differences in intracellular yield observed by [66] could be insignificant. Furthermore, when trying to use the same strategy in our laboratory we failed to obtain mutant strains and learnt that introduction of a suicide vector to genetically engineer *P. acidipropionici* by homologous recombination, either by electroporation or conjugation with *E. coli*, is not a feasible approach. Our attempts resulted in a great number of false positives that could have led to erroneous results if not correctly validated. In our hands, the introduction of a suicide vector by electroporation or conjugation with *E.coli* has repeatedly failed to inactivate any gene in *P. acidipropionici*.

Acetate and lactate by-products were targeted in a gene knockout study in *P. jensenii* [65]. In this work, knock-out of the lactate dehydrogenase improved the final propionate titre slightly in fed-batch cultures but improvements in the productivity could not be observed. Deletion of the pyruvate oxidase severely reduced acetate production along with biomass generation; overall propionate production was negatively impacted with a much lower titre and productivity. The authors additionally overexpressed the phosphoenolpyruvate carboxylase from *Klebsiella pneumoniae* alone or in combination with the lactate dehydrogenase deletion. While a clear benefit in terms of final propionate titre and productivity was observed, there was no obvious benefit from the lactate dehydrogenase deletion.

A study showing clearly beneficial results from a gene knock-out in propionibacteria is yet to be achieved. It is difficult to extrapolate the potential implications of the failed acetate knock-out on glycerol [65] to the ability to use this strategy to overcome the yield challenges facing sugar fermentations. The over-expression of pathways that provide reduced cofactors, such as the pentose phosphate pathway, may be a more promising alternative as shown in our recent work [79], particularly since it may be impossible to eliminate acetate production by gene knock-out due to the promiscuity of the propionyl-CoA: succinate CoA-transferase (CoAT).

### *4.4. Gene Overexpression*

Overexpression of enzymes as a way to increase propionate production has been tested in *P. shermanii* and *P. jensenii.* For example, a recent study upregulated the last step in propionic acid biosynthesis, the CoAT [68]. The native *coAT* gene was cloned into a replicative vector [60] under the control of a strong native promoter and used to transform *P. shermanii* by electroporation [15]. Even though the reaction catalysed by CoAT has been proposed to be the rate limiting step in propionic acid synthesis [73], the overexpression of this enzyme only slightly improved propionate production. The mutant strain showed improved propionic acid yield and productivity compared to the wild type strain, but the effects varied with fermentation conditions. Maximum yield and productivity were obtained during co-fermentation with glycerol and glucose as carbon sources (4:1 ratio). The strain also showed a marked decrease in the by-products succinate and acetate under this condition.

Another approach explored was the overexpression of three carboxylases, namely, pyruvate carboxylase (PYC), methylmalonyl-CoA carboxyltransferase (MMC) and methylmalonyl-CoA decarboxylase (MMD), from the dicarboxylic acid pathway controlling the carbon flux in the Wood-Werkman cycle in

*P. shermanii* [69]. The effects of the overexpression on propionic acid fermentation were studied in serum bottles with glucose, glycerol or co-fermentation of both as substrates. Overall, only mutants overexpressing MMC and MMD showed increased propionic acid production relative to the wild-type strain. Maximal improvements were obtained with the MMC overexpression with a 14% improvement in propionate yield, obtained on glucose, and 17% increase in productivity, obtained on a 2:1 glycerol/glucose mix.

A novel approach for improving propionate production was explored by Guan et al. by over-expressing acid resistance mechanisms in *P. jensenii* [67]. Compared to the strain harbouring an empty plasmid, over-expression of components of the arginine deiminase and glutamate decarboxylase systems typically improved the yield of propionate and the absolute titre reached in a glycerol media in shake-flasks. This study demonstrates the potential importance of incorporating acid tolerance mechanisms in a rational strain design approach.

The introduction of heterologous enzymes has also been used to improve propionate biosynthesis [70,71]. Phosphoenolpyruvate carboxylase (PPC) from *E. coli* catalyses the conversion of phosphoenolpyruvate to oxaloacetate (OAA) by fixing $CO_2$. Not present in any known propionibacteria, expression of PPC might increase carbon flux towards OAA and thus propionate in *P. freudenrenchii*. Although the mutant showed better cell growth and propionate productivity than the wild type strain, propionate yield was not markedly improved under the conditions tested [70]. On glycerol, glycerol dehydrogenase (GDH) and malate dehydrogenase (MDH) are proposed to be rate-limiting steps in propionic acid synthesis from glycerol [71]. While the co-expression of GDH and MDH from *Klebsiella pneumoniae* in *P. jensenii* showed an increase in propionate synthesis, the mutant suffered from slow growth probably due to the extra burden of plasmid replication, transcription and expression [71]. While the GDH/MDH overexpression pair performed the best, the same study also experimented with fumarate hydratase overexpression in combination with GDH or MDH.

### *4.5. Propionic Acid Biosynthesis by Non-Native Producers*

Genetic engineering limitations in propionibacteria have driven researchers to study *E. coli* as a platform for the heterologous production of propionic acid.

The so-called succinate pathway [81], encoded by the *sbm* operon in *E. coli's* genome, is the most thoroughly studied approach for propionate production in *E. coli*. The succinate dissimilation pathway is similar to the methylmalonyl-CoA decarboxylase configuration previously described (Figure 1(AIV)) (see Section 3.1.3). However, this operon lacks a methylmalonyl-CoA epimerase [73] and should not be functional. Nevertheless, several groups have reported propionate production from overexpression of the operon in complex media [82–84]. We suspect that the reported propionate production is a result a ghost-peak interfering with propionate quantification [45].

The acrylate pathway from *Clostridium propionicum* has also been expressed in *E. coli* [45]. This system only produced up to 3.7 mM propionic acid under anaerobic conditions in complex media supplemented with glucose (20 g/L). The authors attributed their results to low enzymatic activity and a possible down-regulation of the *pfl* gene caused by the intermediates of the exogenous pathway. However, because *E. coli* contains the threonine degradation pathway (Figure 1(BI)), the low levels of propionate produced may result from the use of complex media.

Propionate yields and titres for heterologous expression are too low to meaningfully compare with propionibacteria. Further studies are needed to establish if substantial production is feasible and if *E. coli* can be engineered to tolerate propionate.

## 5. Concluding Remarks and Future Directions

Recent techno-economic analyses have set yield and productivity goals for the microbial production of propionate. Several challenges still need to be addressed for an economically feasible fermentation process. *Propionibacterium* remains the best candidate organism for the biological production of propionate at an industrial-scale. This is of little surprise given this species contains novel enzymes encoding the Wood-Werkman cycle that allow the production of propionate at the maximum theoretical yield with

a high energy efficiency. While we identified many pathways that can achieve a similar maximum theoretical yield, none of them can compete with the energy yield obtained by the Wood-Werkman cycle.

Numerous studies have focussed on optimizing the fermentation process in order to improve propionate production, but often these focus on fermenters that have not yet been used on an industrial scale. Attention should be placed on identifying cheap, renewable feedstocks that provide high apparent propionate yields, such as enzymatically treated corn mash, or genetically modifying *P. acidipropionici*. While there have been many successful studies in native producers with lower propionate production such as *P. shermanii* and *P. jensenii*, these are still uncompetitive with wild-type *P. acidipropionici* strains. Rather, these organisms should be viewed as model propionibacteria and used to guide development of strategies ultimately to be implemented in *P. acidipropionici* when the tools become available. To this end, overcoming the transformation barrier by considering restriction modification systems may be a way forward. In the meantime, empirical strain design approaches such as genome-shuffling have proven to be the most successful approach to enhancing the performance of *P. acidipropionici* with new strains emerging that exceed the requirements of an economically viable fermentation.

While the heterologous production of propionate in model organisms remains an interesting possibility, yields remain low and discrepancies within the field, particularly with respect to propionate quantification, need to be resolved for further improvement. Nevertheless, the pathway analysis presented here revealed interesting alternative organisms with a potential for the production of propionate via the fixation of carbon dioxide through the 3HP and 4HB cycles, and via propanediol, a valuable commodity chemical, through the putative lactate pathway.

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